NITROGEN REPRESSION OF GILVOCARCIN V PRODUCTION IN *STREPTOMYCES ARENAE* 2064

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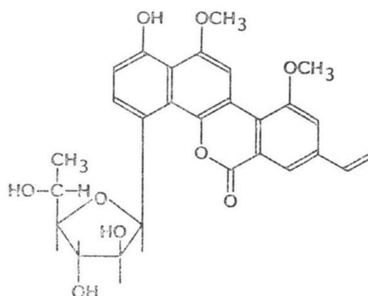
Analysis of gilvocarcin V production by *Streptomyces arenae* in complex and chemically defined media revealed strong nitrogen repression of antibiotic biosynthesis. Nitrogen regulation was first suggested by the observation of a 10-fold increase in gilvocarcin V production when the ammonium ion trapping agent $Mg_3(PO_4)_2 \cdot 8H_2O$ was added to complex medium. In a chemically defined medium, cell mass increased as the initial ammonium sulfate concentrations approached 7.5 mM; however, antibiotic production was strongly repressed at ammonium sulfate concentrations exceeding 1.5 mM. Repression of gilvocarcin V production at 7.5 mM ammonium sulfate was maximally reversed by adding $Mg_3(PO_4)_2 \cdot 8H_2O$ to the medium at 25 mM; specific antibiotic production attained a level 2.5-fold higher than at the nonrepressive ammonium salt concentration of 1.5 mM. Evaluation of the effects of soluble inorganic phosphate concentrations upon gilvocarcin V titers suggested that the relatively insoluble $Mg_3(PO_4)_2 \cdot 8H_2O$ must in fact serve as an ammonium ion-trapping agent, as previously reported in other fermentation systems, not as a supplementary source of phosphate for growth and antibiotic production. These studies also revealed a minor repression of antibiotic synthesis at elevated levels of soluble phosphate. Comparisons of several amino acids as nitrogen sources in a $Mg_3(PO_4)_2 \cdot 8H_2O$-containing medium indicated that L-aspartic acid and glycine promoted the highest yields of gilvocarcin V. Metabolism of these two amino acids into precursors of the polyketide pathway for gilvocarcin V biosynthesis is postulated.

The effects of phosphate and carbon catabolites on the regulation of secondary metabolism are well documented[1,2]. Recently, the biochemical characteristics of nitrogen-regulated biosynthesis have received considerable attention[3~10]. Studies on regulatory mechanisms of antibiotic biosynthesis in streptomycetes are now recognized as necessary companions to fermentation and strain development programs. Although emphasis is still placed on empirical approaches, there is more interest in applying the knowledge gained from studies of streptomycete genetics and biochemistry. The recent demonstration by ŌMURA *et al.*[11,12] of the basic relationship between amino acid catabolism and production of non-nitrogenous precursors to tylosin biosynthesis has furthered our understanding of the role nitrogen catabolites can play in the regulation of secondary metabolite biosynthesis.

The secondary metabolite we chose to study is gilvocarcin V, a new antitumor antibiotic produced by *Streptomyces arenae* 2064 (Fig. 1).

Fig. 1. Structure of gilvocarcin V.



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Gilvocarcin V was discovered recently in numerous laboratories[13~16], and in each case the producing organism was reported to be a different species of *Streptomyces*. Gilvocarcin V and other members of this antibiotic class exhibit several unusual photodynamic properties. Although these compounds are photolabile, visible light is required for their initiation of DNA damage and stimulation of their antimicrobial and cytotoxic activities[17~19].

In this paper we examine some of the regulatory factors affecting gilvocarcin V production in *S. arenae* 2064. We present evidence for nitrogen repression and, to a lesser extent, phosphate repression of antibiotic productivity. Possible mechanisms of regulatory control are postulated.

Materials and Methods

Microorganism

The gilvocarcin V-producing culture was identified as a strain of *S. arenae* by the American Type Culture Collection, Rockville, Maryland. It was subsequently designated *S. arenae* 2064. The culture was one of 2,681 cultures supplied to the Frederick Cancer Research Facility by Smith, Kline and French Laboratories (Philadelphia, Pennsylvania) for fermentation and testing in our natural product antitumor screening program.

Medium and Culture Conditions

Fermentation of *S. arenae* 2064 was initiated by adding 0.1 ml of frozen spores (2×10^9 spores/ml) to a 500-ml baffled flask containing 100 ml of seed medium consisting of soluble starch 3.0%, sucrose 1.0%, dextrose 1.0%, soy peptone 1.5%, corn steep liquor 1.0%, KH_2PO_4 0.3% (22 mM), $CaCO_3$ 0.3%, NaCl 0.1%, and 0.1% of a mineral salt solution. Mineral salts contained $ZnSO_4\cdot7H_2O$ 0.28%, ferric ammonium citrate 0.27%, $CuSO_4\cdot5H_2O$ 0.005%, $MnSO_4\cdot H_2O$ 0.1%, $CoCl_2\cdot6H_2O$ 0.01%, $NaB_4O_7\cdot10H_2O$ 0.009%, and $Na_2MoO_4\cdot H_2O$ 0.0002%. This seed was incubated at 28°C for 48 hours on a rotary shaker operating at 250 rpm. A 2 ml aliquot of seed was used to inoculate a 500-ml baffled flask containing 100 ml of production medium consisting of soluble starch 3.0%, soy peptone 0.1%, KH_2PO_4 0.05% (3.7 mM), $MgSO_4\cdot7H_2O$ 0.01%, and $CaCl_2$ 0.1%, or a defined medium consisting of glucose 3.0%, $(NH_4)_2SO_4$ 0.02% (1.5 mM), KH_2PO_4 0.014% (1.0 mM), $MgSO_4\cdot7H_2O$ 0.01%, $CaCl_2$ 0.1%, and morpholinoethanesulfonic acid (MES) buffer 50 mM. The pH of the medium was adjusted to 6.5 with 1.0 N NaOH before autoclaving. Sterile glucose and ammonium sulfate were added from concentrated solutions after autoclaving. Production cultures were incubated for 120 hours at 28°C on a rotary shaker operating at 250 rpm. $Mg_3(PO_4)_2\cdot8H_2O$ (MgP) was purchased from Pfaltz & Bauer (Stamford, Connecticut), and bactopeptone from Difco (Detroit, Michigan).

Analytical Methods

All determinations represent the average of duplicate flasks. Gilvocarcin V was assayed using a previously described high performance liquid chromatography system[16]. Dry cell weight (DCW) was determined by centrifuging 10 ml of culture broth in tared tubes for 20 minutes at $1,000\times g$, drying overnight at 105°C, and weighing the samples. For cultures containing insoluble MgP, DCWs were corrected by subtracting the weight of this compound, as determined using matching uninoculated flasks. Specific gilvocarcin V production is expressed as weight of antibiotic produced per unit cell mass.

Results

Effect of MgP in Complex Medium and Development of a Chemically Defined Medium

Experiments with *S. arenae* 2064 were undertaken to increase production of gilvocarcin V in complex medium. Initial results indicated that increasing the percentage of peptone in the production medium from 0.1% to 0.5% had negligible effects upon gilvocarcin V production. However, when

the medium was supplemented with MgP at 25 mM and additional peptone, antibiotic production was raised from 1.7 μg/ml to 17.3 μg/ml (Table 1).

Table 1. Effect of Bacto-peptone on gilvocarcin V production in complex medium containing $Mg_3(PO_4)_2 \cdot 8H_2O$ at 25 mM.

Peptone (%)	Gilvocarcin V (μg/ml)
0.1	1.7
0.2	2.0
0.5	17.3
1.0	17.7

The effects of these changes on antibiotic production were further investigated in a chemically defined medium derived from the complex medium. Substitution of glucose for starch at 30 g/liter increased the yield by 74%. The concentrations of KH_2PO_4 and $CaCl_2$ were reduced to 1.0 mM and 0.002%, respectively, with no significant adverse effect on growth or antibiotic productivity. The latter reduction eliminated the formation of $Ca_3(PO_4)_2$ precipitates when soluble phosphates were added to the production medium.

Ammonium Sulfate as Sole Nitrogen Source

The effect of ammonium sulfate concentration upon gilvocarcin V production was investigated in defined medium containing MES buffer to limit changes in medium pH during fermentation (Fig. 2). Under these conditions, maximum growth was observed at 7.5 mM initial ammonium sulfate while specific production of antibiotic was highest at 1.5 mM. At ammonium sulfate concentrations between 1.5 and 4.0 mM, specific antibiotic production sharply decreased by 67%; a slow decline occurred at higher ammonium concentrations. Variations in pH over the range of ammonium sulfate concentrations tested were minimal.

Effect of MgP on Mycelial Growth and Gilvocarcin V Production in Defined Medium

Chemically defined media with increasing concentrations of MgP were prepared with ammonium

Fig. 2. Effect of ammonium sulfate as sole nitrogen source on mycelial growth and gilvocarcin V productivity.
Mycelial growth is indicated by □, volumetric gilvocarcin V titer by ○, specific gilvocarcin V production by △, and pH at the top of the figure by ●. Monobasic potassium phosphate was present in the medium at 1.0 mM.

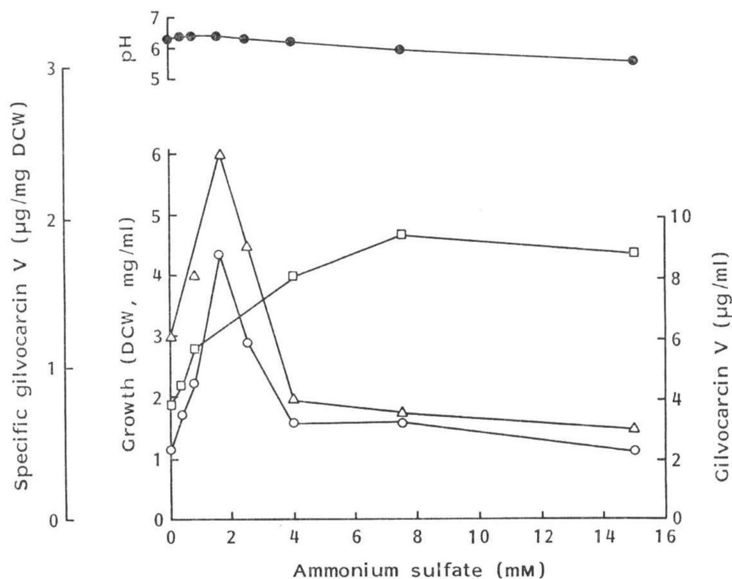
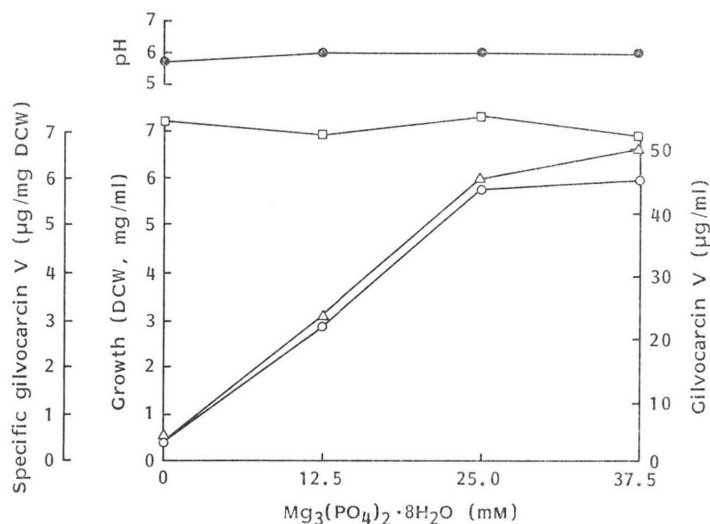


Fig. 3. Effect of $Mg_3(PO_4)_2 \cdot 8H_2O$ on mycelial growth and gilvocarcin V production.

The medium contained a repressive level (7.5 mM) of ammonium sulfate as the major nitrogen source. Mycelial growth is indicated by □, volumetric gilvocarcin V titer by ○, specific gilvocarcin V production by △, and pH by ●.



sulfate fixed at 7.5 mM. This concentration of the ammonium salt was selected because it repressed gilvocarcin V production while favoring maximum cell growth (Fig. 2). The repression of antibiotic production was overcome as the MgP concentration was increased (Fig. 3). The greatest enhancement of antibiotic production (13-fold) was observed as MgP concentrations were elevated to 25 mM. We saw little additional stimulation at higher MgP levels. Specific gilvocarcin V production was actually 2.5-fold higher than the peak level attained at the optimal nonrepressive ammonium sulfate concentration of 1.5 mM in the absence of MgP. Cell mass was unaffected by MgP.

Effects of Soluble Inorganic Phosphate in Defined Medium

Having established that MgP can reverse the repressive effect of ammonium sulfate upon gilvocarcin V production, we undertook an investigation of the susceptibility of antibiotic production to phosphate repression and the role MgP might play as a phosphate source. The effects of soluble phosphate were initially studied using defined medium prepared without MgP and with the ammonium sulfate concentration at 1.5 mM to favor maximum antibiotic production. Soluble phosphate was added from a 2.5 M stock solution of sodium phosphate buffer, pH 6.5. With no exogenous phosphate except that carried over from the seed, specific production was 1.7 μg/mg DCW (Fig. 4). Production increased 41% to a peak level of 2.4 μg/mg DCW when soluble phosphate was elevated to 0.3 mM but decreased by 23% at 3.0 mM phosphate, suggesting a weak repressive phosphate effect. Little further decline was observed at higher phosphate concentrations.

Comparison of Amino Acids as Nitrogen Sources

Several amino acids were evaluated as primary nitrogen sources in defined medium containing MgP at 25 mM (Table 2). Amino acids were added to the medium at concentrations equivalent in nitrogen at 1.0 g/liter. Cultures containing glutamic acid as the nitrogen source had the most abundant growth, but specific production was only 26% of the level observed in controls containing Bacto-peptone.

Fig. 4. Effect of soluble phosphate on mycelial growth and gilvocarcin V production.

Mycelial growth is indicated by □, volumetric gilvocarcin V titer by ○, specific gilvocarcin V production by △, and pH by ●. We used medium in which ammonium sulfate at 1.5 mM was the major nitrogen source. Soluble phosphate was added from a concentrated stock of phosphate buffer, pH 6.5.

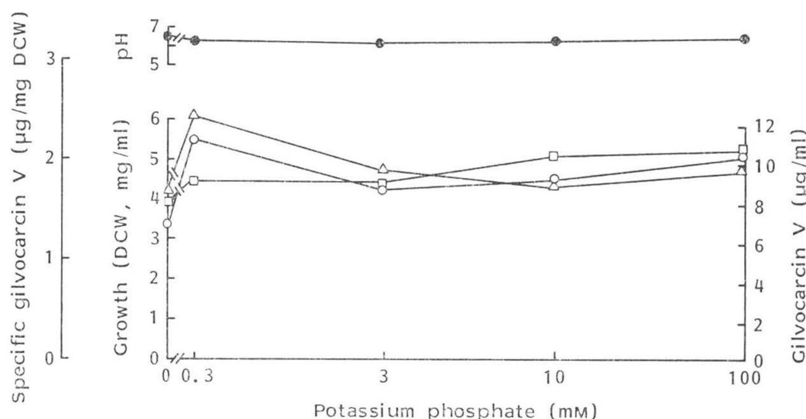


Table 2. Gilvocarcin V production with various nitrogen sources (equivalent in nitrogen at 1.0 g/liter).

Nitrogen source	pH at harvest	Dry cell weight (mg/ml)	Titer (µg/ml)	Specific production (µg/mg dry cell weight)
L-Arginine	6.2	6.9	84	12.2
L-Asparagine	6.3	6.1	103	16.9
L-Aspartic acid	7.9	7.3	197	27.0
L-Glutamic acid	7.3	8.4	74	8.8
L-Glutamine	6.5	6.7	93	13.9
Glycine	7.5	6.7	138	20.6
L-Histidine	7.1	7.6	3	0.4
L-Proline	5.6	5.2	3	0.6
Bacto-peptone	6.4	7.3	247	33.8

On the other hand, cultures with aspartic acid exhibited growth comparable to the controls and specific production averaged 80% of the values observed in the controls. Specific production values for glycine and L-asparagine were at least 50% of the controls. L-Histidine had the second highest mycelial growth but could support only low gilvocarcin V production. L-Proline proved to be a poor substrate for both growth and gilvocarcin V production.

Discussion

Gilvocarcin V production in defined medium was strongly repressed at ammonium sulfate concentrations greater than 1.5 mM, while mycelial growth increased at ammonium sulfate concentrations of up to 7.5 mM. In contrast to other nitrogen-repressed secondary metabolites[4,6,9], production of gilvocarcin V by *S. arenae* 2064 appears to be sensitive to very low concentrations of ammonium ions. A 13-fold increase in specific production occurred when the medium was supplemented with 25 mM MgP, and the pH profile and biomass at time of harvest remained unaffected. This represents a 2.5-fold increase over the maximum specific production obtained when ammonium sulfate was used at the non repressive concentration of 1.5 mM. It would appear that ammonium ions in excess of 1.5 mM, when bound in an insoluble form and slowly liberated into the culture medium, promote higher antibiotic production than batch addition at a lower, nonrepressive concentration.

Studies of the effects of soluble phosphate addition to defined medium suggest that MgP is not a

significant source of phosphate for growth or antibiotic production. Soluble inorganic phosphate supplementation (at least 0.3 mM) was required for high-level antibiotic production in media without MgP (Fig. 4). In addition, MgP at 25 mM stimulated antibiotic production 13-fold in a nitrogen-rich medium already supplemented with 1.0 mM soluble phosphate (Fig. 3). Concentrations of soluble phosphate exceeding 0.3~3.0 mM actually promoted a minor repression of antibiotic synthesis. It would appear that MgP's stimulatory activity in nitrogen-rich media in fact reflects its role as an ammonium ion-trapping agent, as reported in other fermentation systems[5,7~9,20,21]. Even if phosphate is released from MgP, weak phosphate repression allows efficient antibiotic biosynthesis under such nitrogen-depleted conditions.

The complex medium described in this study was used in our screening program when gilvocarcin V production by *S. arenae* 2064 was discovered[16]. It is a simple medium containing a low concentration of both ammonium ions (*ca.* 10 mM) and phosphate (*ca.* 4 mM). Its success as a fermentation medium for producing compounds active in our antitumor prescreens may have resulted from its capacity to support growth while promoting production of secondary metabolites that are subject to either nitrogen or phosphate repression.

The fundamental mechanism of nitrogen regulation in gilvocarcin V biosynthesis is not known. Because the gilvocarcin V molecule contains no nitrogen, the repressive effects of ammonium ions may involve regulation of substrate transport, carbon flow through competing pathways, or more directly the formation of biosynthetic precursors of the antibiotic. Biosynthesis of the gilvocarcin V aglycone occurs through the polyketide pathway with the incorporation of one propionate and nine acetate units[22~24]. It has been established that lower fatty acids can be produced by several organisms through the metabolism of amino acids[25].

Recently, it was reported that valine was the best among several amino acid precursors of the macrolide intermediate protylonolide in tylosin biosynthesis by *Streptomyces fradiae*[12,26]. Valine was metabolized to butyrate and propionate, both precursors of protylonolide. Concentrations of ammonium ions greater than 50 mM strongly suppressed this metabolism, thereby repressing tylosin biosynthesis[11]. In the case of gilvocarcin V production, glycine and L-aspartic acid as the major nitrogen sources led to high gilvocarcin V production in the presence of MgP. Both amino acids are glycogenic and could readily provide acetate units by metabolism to acetyl CoA. Further studies of the relationship between amino acid metabolism and gilvocarcin V production in *S. arenae* 2064 are required to understand the mechanism of nitrogen regulation in this system.

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