REPRESSION OF β-LACTAM PRODUCTION IN *CEPHALOSPORIUM ACREMONIUM* BY NITROGEN SOURCES

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A variety of inorganic and organic nitrogen sources were added to fermentation media to determine their regulatory effects on the production of β-lactam antibiotics by *Cephalosporium acremonium*. $(NH_4)_2SO_4$ at concentrations higher than 100 mM (1.3%) strongly inhibited β-lactam production. L-Asparagine and L-arginine proved to be the best nitrogen sources tested for β-lactam production. The optimum concentration of asparagine was 1.2%. Higher concentrations led to NH_3 accumulation, increase in pH, and lower growth rates. Addition of tribasic magnesium phosphate $[Mg_3(PO_4)_2 \cdot 8H_2O]$ to the $(NH_4)_2SO_4$-containing medium stimulated β-lactam production markedly and ammonium repression of the ring-expansion enzyme was reversed. It appears that the ring-expansion step is a very sensitive part of β-lactam biosynthesis in *C. acremonium* with respect to nitrogen source repression. Other enzymes may also be sensitive in view of the fact that nitrogen source derepression not only led to increases in cephalosporin C but, to a lesser extent, penicillin N and total β-lactam titers.

Regulation of the biosynthesis of secondary metabolites by nitrogen sources has attracted much interest[1~4]. Up to this time, knowledge of nitrogen source control of β-lactam biosynthesis in *Cephalosporium acremonium* is virtually nil. The present study describes the effects of nitrogen sources on the production of β-lactams in *C. acremonium*. We have found that the fermentation is strongly controlled by nitrogen sources such as ammonium salts, and that bypassing such regulation leads to marked increases in antibiotic production.

Materials and Methods

Organism

C. acremonium CW-19 (*Acremonium chrysogenum* ATCC 36225) was used throughout the study.

Medium and Culture Conditions

C. acremonium was maintained on agar slants containing 1/10 strength LEPAGE-CAMPBELL medium[5]. For seed cultures, a medium previously described was used[6]. Fermentations were carried out in 2,800-ml Fernbach flasks containing 400 ml of chemically defined medium[6]. Ten percent inoculum was used. The medium was prepared, adjusted to pH 7.3 with NaOH, and autoclaved without the sugar components. These were sterilized separately and aseptically added before inoculation. Fermentations were carried out at 25°C for 5~6 days on a rotary shaker at 250 rev/minute.

Fermentation Parameters

Growth: Absorbance (Klett units) was measured in a Klett-Summerson colorimeter with a red

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filter. Before reading absorbance, the culture was diluted 40-fold with 0.01 N HCl.

Glucose Concentration: Glucose was determined with a modified ferricyanide method as described by PARK and JOHNSON[7].

Soluble Ammonium Nitrogen Concentration: Soluble ammonium nitrogen was determined by the NESSLER reaction according to HANSON and PHILLIPS[8].

Protein Concentration: Protein in cell-free extracts was measured by the method of BRADFORD[9]. Bovine serum albumin was used as the standard.

Assay of β-Lactam Antibiotics: Production of β-lactam antibiotics during the fermentation was followed by bioassay, using the β-lactam-supersensitive *Pseudomonas aeruginosa* Pss as test strain. Pss was seeded into 1/2 strength Trypticase soy agar with or without 500 units/ml penicillinase (Difco). Penicillin N is considered to constitute the penicillinase-labile portion of the total antibiotic activity, whereas the penicillinase-stable portion includes cephalosporin C, deacetoxycephalosporin C and deacetylcephalosporin C. The standard was authentic cephalosporin C.

Preparation of Cell-free Extracts

Fermentations were carried out in 2,800-ml Fernbach flasks. At several time points 80 ml of fermentation broth was withdrawn and the mycelia were recovered by filtration. After thorough washing with distilled water, 6 g of cells (wet weight) was suspended in 15 ml Tris-salts (0.05 M Tris-HCl pH 8, 0.01 M KCl, 0.01 M $MgSO_4$) and the suspended cells ruptured by sonication as previously described[10]. The extracts were then partially purified by protamine treatment, $(NH_4)_2SO_4$ precipitation and Sephadex G-25 gel filtration. The partially purified preparations were kept at −65°C until use.

Cyclase[10] Assay

The 1-ml final reaction mixture contained 50 mM Tris-HCl pH 7.4, 10 mM KCl, 10 mM $MgSO_4$, 0.2 mM $FeSO_4$, 0.67 mM ascorbic acid, 0.75 mM dithiothreitol (DTT), 0.28 mM LLD-ACV and 100~200 μl enzyme solution (containing 0.17~0.55 mg protein). Before the assay, the bis-[(σ-L-α-aminoadipyl)-L-cysteinyl-D-valine]disulfide (bis-LLD-ACV) was reduced to its monomeric form by preincubation with 3.75 mM DTT in 0.2 ml total volume for 15 minutes at room temperature. The cyclization reaction was started by the addition of the ACV-DTT solution to the mixture containing enzyme and cofactors. Incubation was for 10 minutes at 25°C and 250 rev/minute. The reaction was stopped by the addition of 1 ml methanol. After removal of the precipitated proteins by centrifugation, the supernatant fluid was analyzed for isopenicillin N formation by bioassay against *Micrococcus luteus* ATCC 381[10]; penicillin N served as a standard.

Expandase[10,11] Assay

The 1-ml final reaction mixture contained 50 mM Tris-HCl pH 7.4, 10 mM KCl, 10 mM $MgSO_4$, 0.04 mM $FeSO_4$, 0.67 mM ascorbic acid, 0.8 mM ATP, 0.6 mM α-ketoglutarate, 0.28 mM penicillin N and 400~800 μl enzyme solution (0.68~2.2 mg protein). The reaction was started by the addition of penicillin N and the mixture was incubated at 25°C and 250 rev/minute. After 60 minutes, the reaction was stopped by the addition of 1 ml methanol. After removal of the precipitated proteins, the supernatant fluid was analyzed for deacetoxycephalosporin C formation by bioassay against *P. aeruginosa* Pss, seeded in agar containing 500 units/ml penicillinase.

Definitions of Cyclase and Expandase Activity Units

One unit of activity corresponds to the production of 0.01 μg isopenicillin N or deacetoxycephalosporin C per minute.

Results

Effect of Different Nitrogen Sources on β-Lactam Production by *Cephalosporium acremonium* CW-19

A variety of inorganic and organic nitrogen sources were tested for their effects on the production of β-lactam antibiotics by *C. acremonium* CW-19. Fig. 1 shows that $(NH_4)_2SO_4$, NH_4Cl and urea were

Fig. 1. Antibiotic production with different nitrogen sources.
○ Growth, ● total antibiotics, △ glucose, ▽ cephalosporins, □ penicillin N.

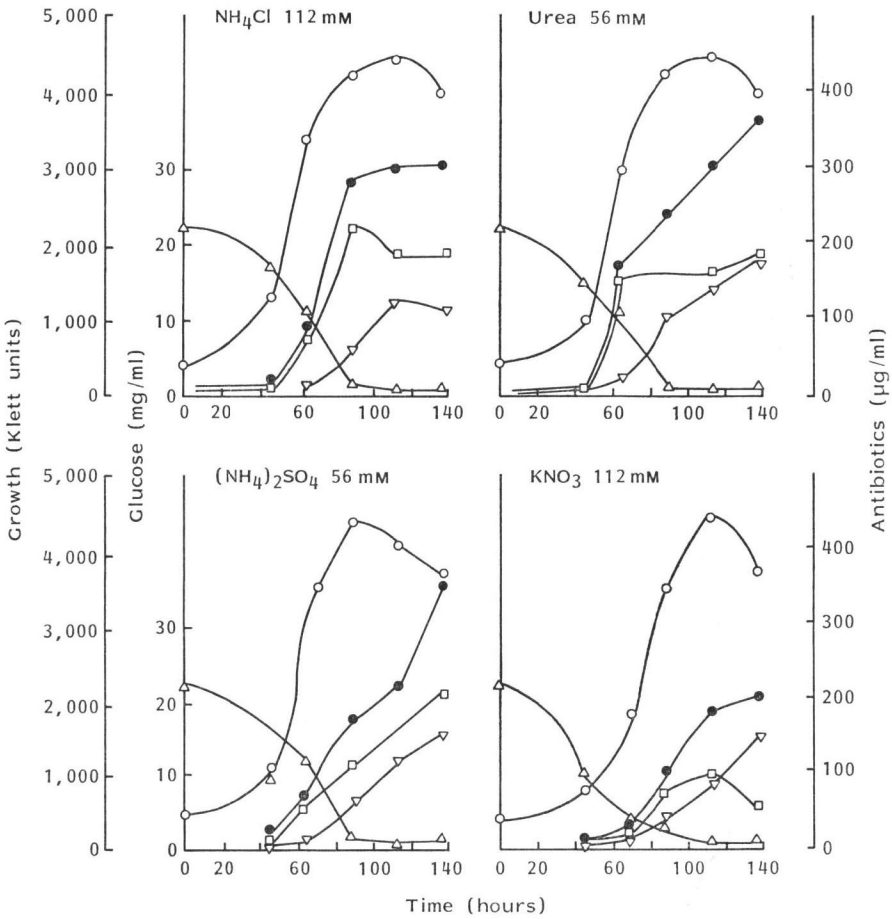


Table 1. Growth and antibiotic formation with various amino acids as major nitrogen source.

Amino acid	Concentration (%)	Maximum (Klett units)	Maximum total β-lactams (μg/ml)	Antibiotic production	
				Cephalosporins (μg/ml)	Penicillin N (μg/ml)
L-Arginine	0.6	4,200	950	730	230
	1.2	5,360	1,050	700	350
L-Asparagine	0.6	4,040	1,020	850	270
	1.2	4,880	1,170	810	360
$(NH_4)_2SO_4$ control	0.75	2,840	300	110	210

better than KNO_3 for production of β-lactam antibiotics. $(NH_4)_2SO_4$ concentrations higher than 100 mM (*ca.* 1.3%) strongly interfered with production (Fig. 2), although the pH patterns and growth were not markedly affected.

Among a number of organic nitrogen compounds tested as sole nitrogen sources, L-asparagine and L-arginine proved to be the best. When combined with the inducer methionine[12], there was a tripling

Fig. 2. Effect of $(NH_4)_2SO_4$ concentration on maximum antibiotic production when used as a major nitrogen source.

Duration was 141 hours.

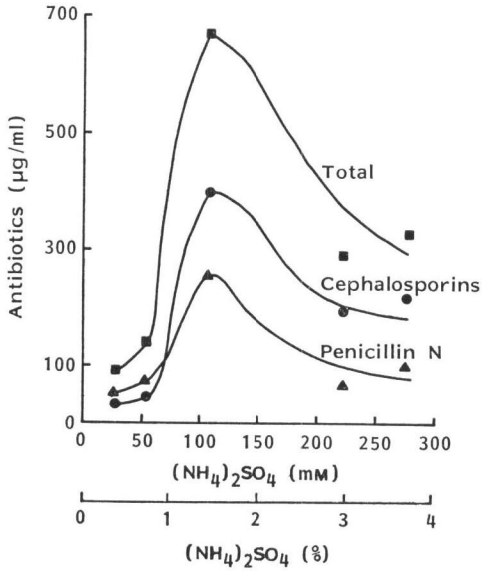


Fig. 3. Effect of L-asparagine concentration on maximum antibiotic production.

Duration was 140 hours.

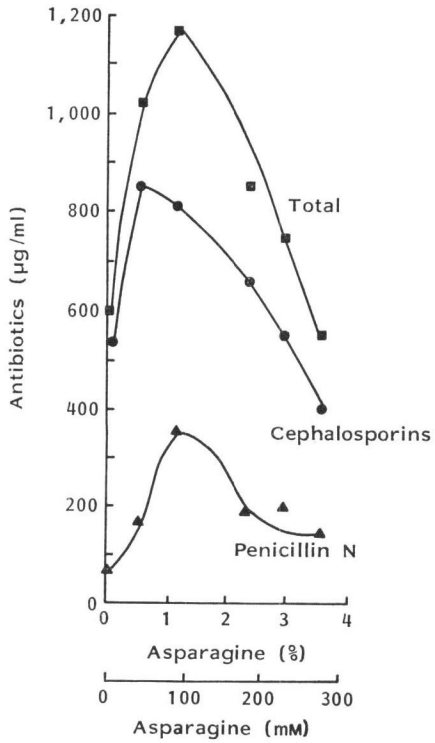
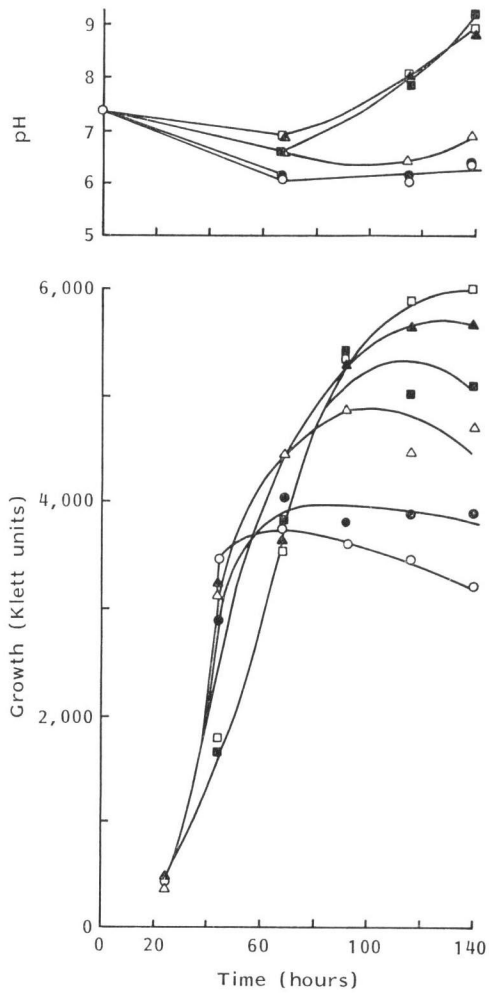


Fig. 4. Effect of L-asparagine concentration on growth and pH.

Data from experiment of Fig. 3.

○ 0.06%, ● 0.6%, △ 1.2%, ■ 2.4%, ▲ 3.0%, □ 3.6%.



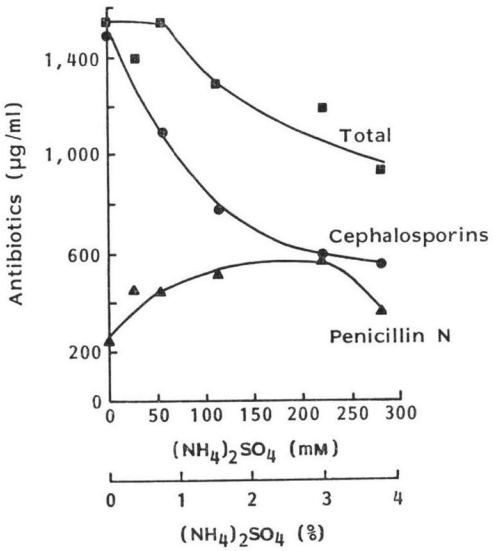
in the volumetric titers of total β-lactam antibiotics as compared to ammonium nitrogen (Table 1). A modified defined fermentation medium was therefore devised in which $(NH_4)_2SO_4$ was replaced by 1.2% L-asparagine.

The effects of L-asparagine concentration on growth, pH and antibiotic production are shown in Figs. 3 and 4. L-Asparagine concentrations higher than the optimum 1.2% decreased antibiotic production (Fig. 3), increased pH and inhibited growth rate (Fig. 4). HPLC analysis

of asparagine (and arginine) fermentations showed that in addition to the predominant cephalosporin C, a considerable amount of deacetylcephalosporin C (an intermediate and breakdown product which can barely be detected in $(NH_4)_2SO_4$ fermentations) was accumulated.

Increasing amounts of $(NH_4)_2SO_4$ added to fermentations with 1.2% L-asparagine markedly interfered with β-lactam production. Fig. 5 shows that the effect was mainly on cephalosporin production whereas there was an increase in penicillin N accumulation.

Fig. 5. Effect of $(NH_4)_2SO_4$ concentration on maximum antibiotic production when added to 1.2% L-asparagine.



Effect of $(NH_4)_2SO_4$ and L-Asparagine as Nitrogen Source for Growth on Cyclase and Expandase Formation

The time course of cyclase and expandase enzyme activities with $(NH_4)_2SO_4$ or asparagine as nitrogen source is shown in Fig. 6. With asparagine, the specific activity of expandase was twice that in the $(NH_4)_2SO_4$ fermentation. In contrast, cyclase activity was slightly lower in the asparagine fermentation. Repression of expandase by NH_4^+ was also seen when 6.3% sucrose was used as major carbon source instead of the standard 2.7% glucose plus 3.6% sucrose (Fig. 7).

Fig. 6. Time-course of cyclase and expandase specific activity with 0.75% $(NH_4)_2SO_4$ or 1.2% asparagine as major nitrogen source in standard medium (2.7% glucose+3.6% sucrose).
○ Growth, ● total antibiotics, □ penicillin N, ■ cyclase, ▽ cephalosporins, ▼ expandase.

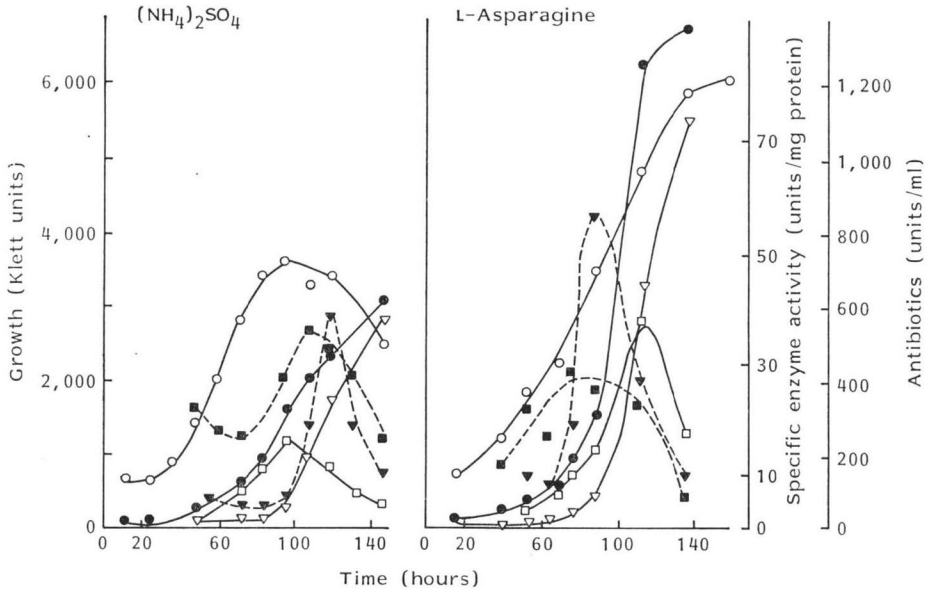
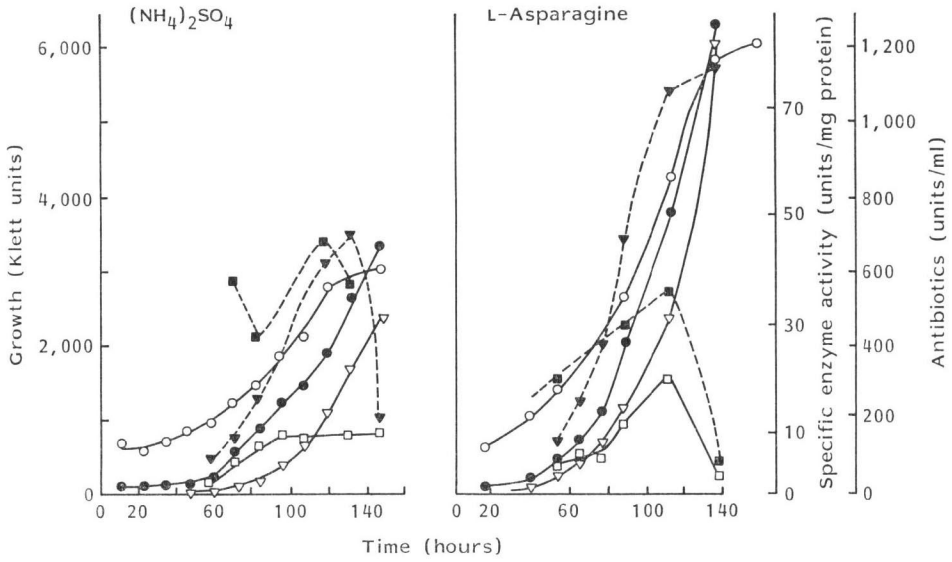


Fig. 7. Time-course of cyclase and expandase specific activity with 0.75% $(NH_4)_2SO_4$ or 1.2% asparagine as major nitrogen source in 6.3% sucrose medium.

○ Growth, ● total antibiotics, □ penicillin N, ■ cyclase, ▽ cephalosporins, ▼ expandase.

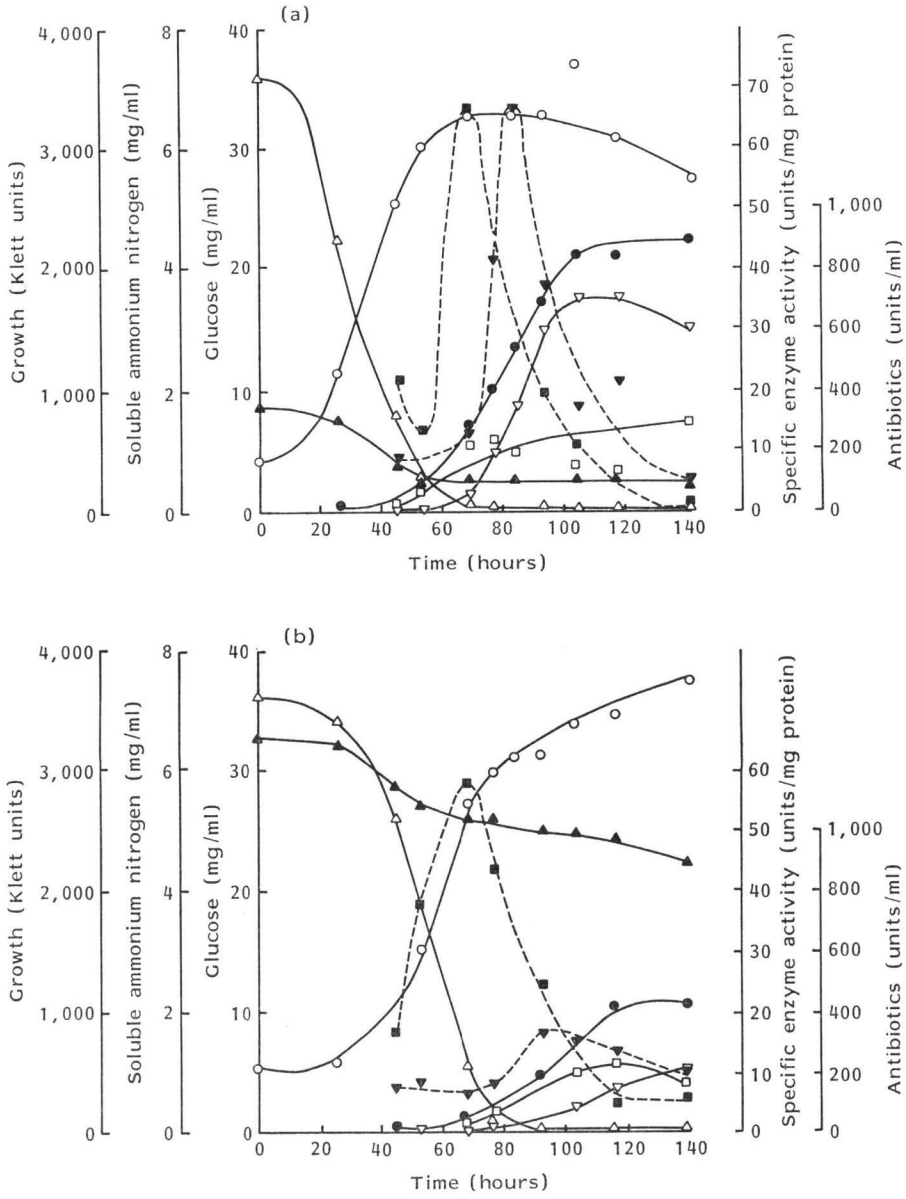


In both Figs. 6 and 7, the volumetric production of β-lactam antibiotics (*i.e.* units/ml) was markedly increased by replacing NH_4^+ with asparagine. However, since asparagine is also used as a carbon source, growth also increased, resulting in little to no positive effect on specific production of antibiotics (units/Klett value). In order to confirm the ammonium repression of expandase in the absence of additional carbon source, fermentations with normal (0.75%) and excess (3.0%) concentrations of NH_4^+ were compared (Fig. 8). As expected[13], penicillin N was produced during growth whereas cephalosporin production did not occur until growth stopped and glucose was consumed. In the normal ammonium fermentation (Fig. 8a), the ammonium nitrogen concentration in the broth reached its minimum at the time of glucose depletion; in the high ammonium fermentation (Fig. 8b), high broth levels of ammonium nitrogen persisted throughout the experiment. A comparison of Figs. 8a and b shows that expandase was markedly repressed by NH_4^+ but cyclase was only slightly repressed. As would be expected from such an effect, production of cephalosporins was markedly repressed but not that of penicillin N.

Reversal of Ammonium Effect by Magnesium Phosphate

If repression of expandase and of cephalosporin production is caused by ammonium, the addition of an ammonium-trapping agent should reverse repression. Such a compound is tribasic magnesium phosphate[14]. We found that the addition of 1% tribasic magnesium phosphate to the 3% $(NH_4)_2SO_4$-containing medium stimulated β-lactam production to a remarkable extent. Indeed, not only was the total β-lactam antibiotic production raised above the 450 μg/ml level seen with excess NH_4^+ (Fig. 8b), it even exceeded the 900 μg/ml titer observed with low NH_4^+ (Fig. 8a) by about 80%. Even more important were the observations (Fig. 9) that the broth ammonium nitrogen level was lowered and the expandase was derepressed; of course, cephalosporin production was also derepressed. Neither the negative effect of a high NH_4^+ concentration (Figs. 8a and b) nor the positive effect of magnesium phos-

Fig. 8. Fermentation time-course with (a) 0.75% or (b) 3% $(NH_4)_2SO_4$ as major nitrogen source.
○ Growth, ● total antibiotics, □ penicillin N, ■ cyclase, ▽ cephalosporins, ▼ expandase, △ glucose, ▲ nitrogen.

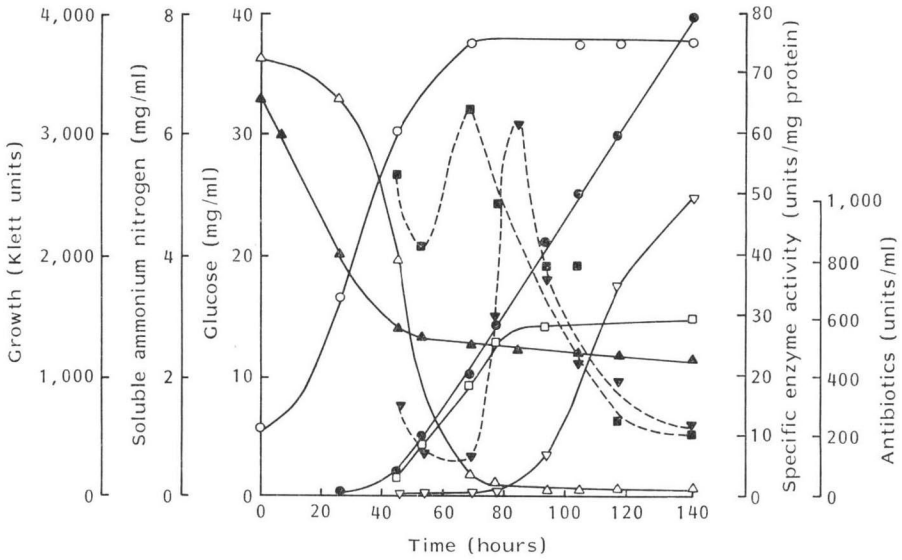


phate (Fig. 9) was due to pH differences. In all cases, the pH dropped from the original pH of 7.3 down to the range of 5.3~5.7. Fig. 10 shows that at all levels of $(NH_4)_2SO_4$ tested except the lowest (30 mM=0.39%), magnesium phosphate markedly increased antibiotic production.

Discussion

The biosynthesis of antibiotics is often repressed by sources of carbon, nitrogen and/or phosphorus

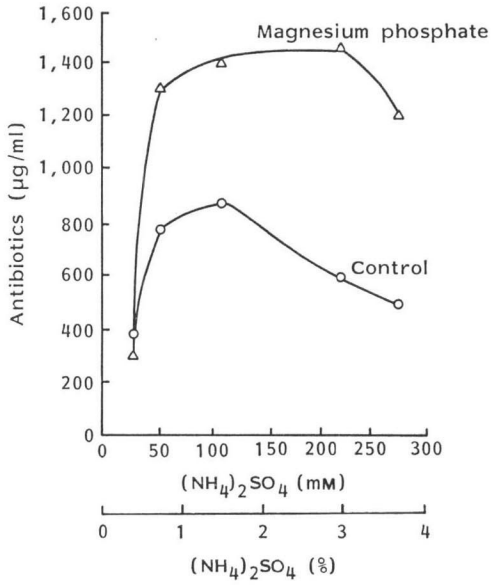
Fig. 9. Fermentation time-course with 3% $(NH_4)_2SO_4$ plus 1% magnesium phosphate.
○ Growth, ● total antibiotics, □ penicillin N, ■ cyclase, ▽ cephalosporins, ▲ expandase, △ glucose, ▲ nitrogen.



used for growth. In these cases, high production of antibiotic might be achieved when the producing organism is cultivated under conditions which favor escape from one or more of such controls. The general technique which has been employed for this purpose is the use of media containing slowly utilized nutrients or continuous feeding of nutrient sources at low rates.

In the present work, we have found that organic nitrogen sources such as asparagine and arginine are superior to ammonium salts for cephalosporin production, apparently because they derepress expandase; cyclase is not markedly affected. The beneficial effects of magnesium phosphate on cephalosporin and expandase titers when added to NH_4-based fermentations strongly indicate that a reduction in the ammonium nitrogen level in the broth leads to derepression of expandase. It is likely that the nitrogen effect also influences earlier reactions of the pathway since in many cases of nitrogen source derepression (*e.g.* compare Figs. 8 and 9), penicillin N and total β-lactam titers are also increased although the major stimulatory effect is on cephalosporin production. Of course, the most important parameter is the intracellular concentration of some nitrogenous effector(s), but the identity of such an effector has not yet been determined.

Fig. 10. Reversal of NH_4 effect on antibiotic production by magnesium phosphate.
Duration was 120 hours.



We previously studied the interference in cephalosporin formation by glucose and found that carbon source repression in *C. acremonium* also acts mainly at the ring-expansion step, not the ring-cyclization step[18,15].

In summary, all of our data point to expandase[6] as a sensitive site of carbon and nitrogen source repression of β-lactam biosynthesis in *C. acremonium*, but the mechanism(s) by which regulation is effected and the identity of the true effector(s) is still unknown.

Aknowledgments

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