EFFECT OF AMMONIUM AS NITROGEN SOURCE ON PRODUCTION OF δ-(L-α-AMINOADIPLY)-L-CYSTEINYL-D-VALINE SYNTHETASE BY *CEPHALOSPORIUM ACREMONIUM* C-10

JINYOU ZHANG†, SAUL WOLFE†† and ARNOLD L. DEMAIN

Fermentation Microbiology Laboratory,
Department of Applied Biological Sciences,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139, U.S.A.
††Department of Chemistry, Queens University,
Kingston, Ontario, Canada K7L 3N6

(Received for publication June 13, 1987)

Cephalosporin production by *Cephalosporium acremonium* strain C-10 was suppressed when the organic nitrogen source (1.2% L-asparagine) was replaced by 1.2% $(NH_4)_2SO_4$. A higher level of $(NH_4)_2SO_4$ (3.5%) led to even greater suppression. Ammonium repression was exerted on formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, together with that of expandase; a lesser effect by ammonium was observed on cyclase production. Inhibition of ACV synthetase activity byammonium was also observed (*ca.* 50% inhibition at 250 mM NH_4^+).

Nitrogen sources which are readily assimilated for growth often interfere with secondary metabolism[1~3]. Such control has been shown to be important in organisms producing β-lactam antibiotics, *i.e.*, *Streptomyces clavuligerus*[3,4], *Nocardia lactamdurans*[5], *Cephalosporium acremonium*[6] and *Penicillium chrysogenum*[7].

In *C. acremonium* strain CW-19, ammonium appeared to interfere primarily with production of the cephalosporins rather than that of the intermediate, penicillin N[8]. It was found that ammonium repressed the formation of deacetoxycephalosporin C synthetase ("expandase") but had only a slight affect on isopenicillin N synthetase ("cyclase") formation. However, higher concentrations of ammonium shut off penicillin N production, indicating additional negative control of an early enzyme of the pathway.

We recently developed a cell-free system for detecting δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase ("ACV synthetase") activity[8]. The present paper describes the effect of ammonium on the formation of this initial enzyme of the cephalosporin biosynthetic pathway as well as expandase and cyclase. For this study, we have used the high producing strain, *C. acremonium* C-10[9].

Materials and Methods

Organisms

C. acremonium C-10 (*Acremonium chrysogenum* ATCC 48272) was used throughout the study. Assay organisms were *Micrococcus luteus* ATCC 381 for cyclase and *Escherichia coli* ESS for expandase.

Medium and Culture Conditions

Slant medium, seed medium (No. 1) and chemically-defined medium were as previously described[10], using the same culture conditions except that 1.5 g oleic acid per liter was added to the chemically-defined medium and the inoculum level was 8%. When required, 1.2% or 3.5% $(NH_4)_2SO_4$

† Permanent address: Sichuan Industrial Institute of Antibiotics, Chengdu, Sichuan, People's Republic of China.

(182 or 530 mM NH_4^+, respectively) was added to replace the 1.2% L-asparagine (182 mM N) as major nitrogen source.

Fermentation Parameters

Growth: Growth was measured by absorbance using the Klett Summerson colorimeter with a red filter. A cell dry weight of 1 mg/ml was found equivalent to 180 Klett units. The broths were diluted 40-fold before measurements were made; absorbance was linearly related to dry cell weight when the former was below 150 Klett units.

Protein Concentration: Protein in cell-free extracts was measured by the method of BRADFORD[11]. Bovine serum albumin was used as standard.

β-Lactam Antibiotics: Production of β-lactam antibiotics during fermentations was measured by the chemical assay of LÜBBE *et al.*[12] using cephalosporin C as standard. Strain C-10 produces predominantly cephalosporin C and lesser amounts of deacetoxycephalosporin C and penicillin N[10,12].

Preparation of Cell-free Extracts and Enzyme Assays

The cells were broken with a French Press in the presence of glycerol, as reported earlier[13]. After desalting the crude cell-free extracts, cyclase and expandase assays were carried out as described before[14], using authentic isopenicillin N and cephalosporin C as standards, respectively. One unit of enzyme activity is that which produces 1 μg of isopenicillin N or cephalosporin C equivalent per minute, respectively. ACV synthetase activity was measured by estimating the amounts of ACV produced in the cell-free reaction, using the previously reported HPLC method[8,13]. One unit of ACV synthetase activity produces 1 pmol of ACV per minute.

Results

Comparison of $(NH_4)_2SO_4$ and L-Asparagine as Nitrogen Sources

It is generally recognized that organic nitrogen sources are superior to ammonium salts for β-lactam production. For *C. acremonium*, L-asparagine is the amino acid of choice[6]. Fig. 1 shows that L-asparagine supported higher growth and higher volumetric production of β-lactams than did $(NH_4)_2SO_4$. 1.2% $(NH_4)_2SO_4$ exerted repression on ACV synthetase, along with the expected repression of expandase and a lesser effect on cyclase[6].

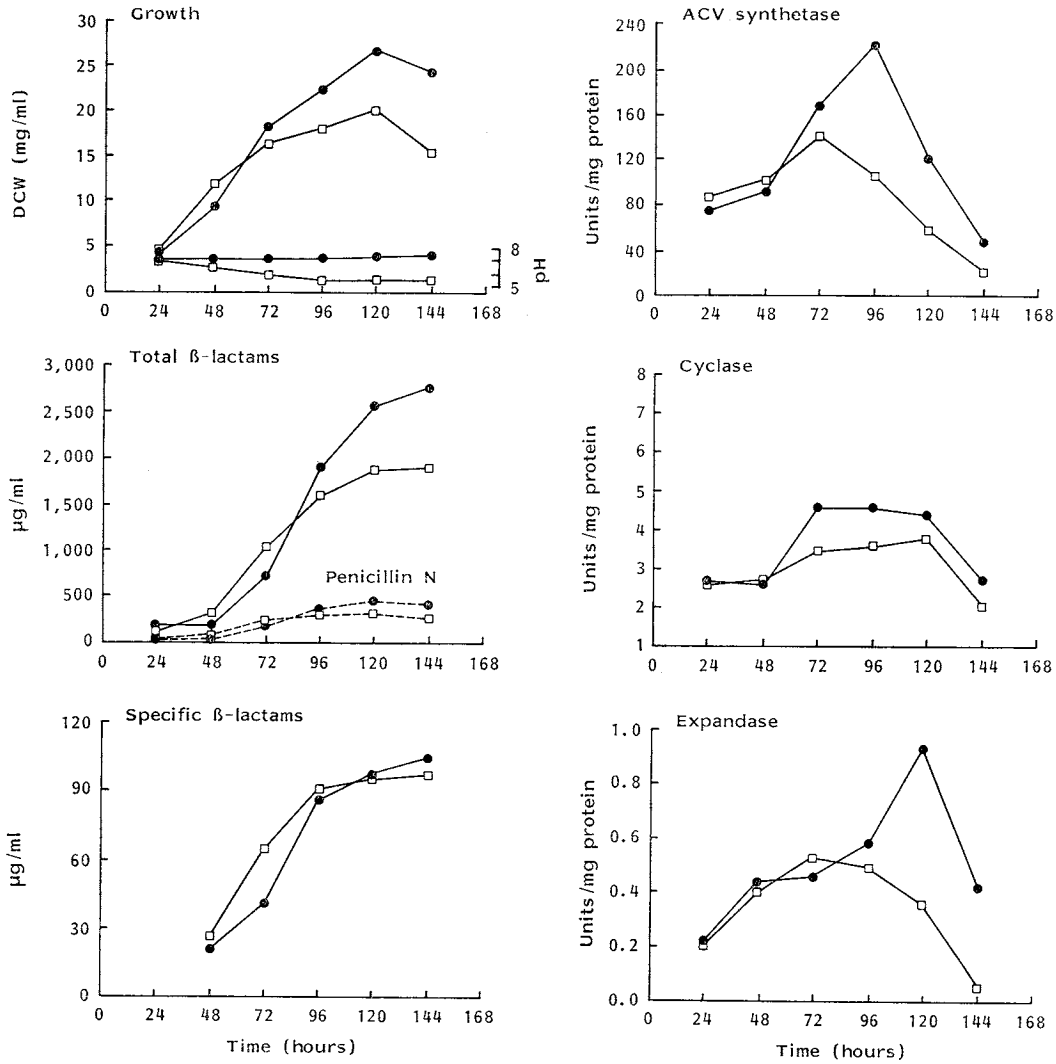
Effect of Ammonium at an Increased Concentration

In Fig. 1, it can be seen that the pH dropped considerably (down to 5.2~5.4) in the fermentation with ammonium sulfate but remained rather stable with asparagine. Furthermore, the cells presumably used asparagine as a carbon source as well as a nitrogen source for growth, thus achieving a higher cell density; no marked effect on specific production of antibiotics by L-asparagine was observed. In order to study ammonium repression in the absence of an additional carbon source, and to rule out the possibility that the pH difference causes the difference in synthetase activities, fermentations with normal (1.2%) and excess (3.5%) concentrations of $(NH_4)_2SO_4$ were compared (Fig. 2). Growth and pH changes were similar in the two fermentations, and there was suppression of antibiotic synthesis on both volumetric and specific bases by the higher concentration of ammonium. The results confirmed the repression of both expandase and ACV synthetase by NH_4^+. Again the effect on cyclase was less pronounced.

Effect of Ammonium on ACV Synthetase Activity

Since the initial enzyme of biosynthetic pathways is often subject to inhibition of enzyme activity, we tested whether NH_4^+ could inhibit ACV synthetase activity. As shown in Table 1, ammonium exerts 50% inhibition at about 250 mM (1.7% $(NH_4)_2SO_4$). Similar effects were observed with NH_4Cl

Fig. 1. Effect of 1.2% $(NH_4)_2SO_4$ (□) and 1.2% L-asparagine (●) on growth, pH and formation of β-lactam antibiotics and cephalosporin synthetases by *Cephalosporium acremonium* C-10.
DCW: Dry cell weight.



(data not shown). CASTRO *et al.*[5] found no inhibition of cyclase, epimerase and expandase by up to 40 mM ammonium in *N. lactamdurans*.

Discussion

ACV synthetase, the first enzyme of the β-lactam biosynthetic pathway, is crucial to both penicillin and cephalosporin biosynthesis. We recently reported that in *C. acremonium* this enzyme is subject to methionine induction[15], leading to an increase in β-lactam production. The data presented in this paper indicate that this enzyme is also the target of ammonium repression, and thus explains the observation that penicillin N production in *C. acremonium* is suppressed by high levels of ammonium[6]. Repression of both expandase and ACV synthetase by NH_4^+ contributes to a decrease in cephalosporin production. We have also observed inhibition of ACV synthetase by ammonium which could also contribute to the interference with β-lactam formation.

Fig. 2. Effect of 1.2% $(NH_4)_2SO_4$ (●) and 3.5% $(NH_4)_2SO_4$ (□) on growth and formation of β-lactam antibiotics and cephalosporin synthetases by *Cephalosporium acremonium* C-10.

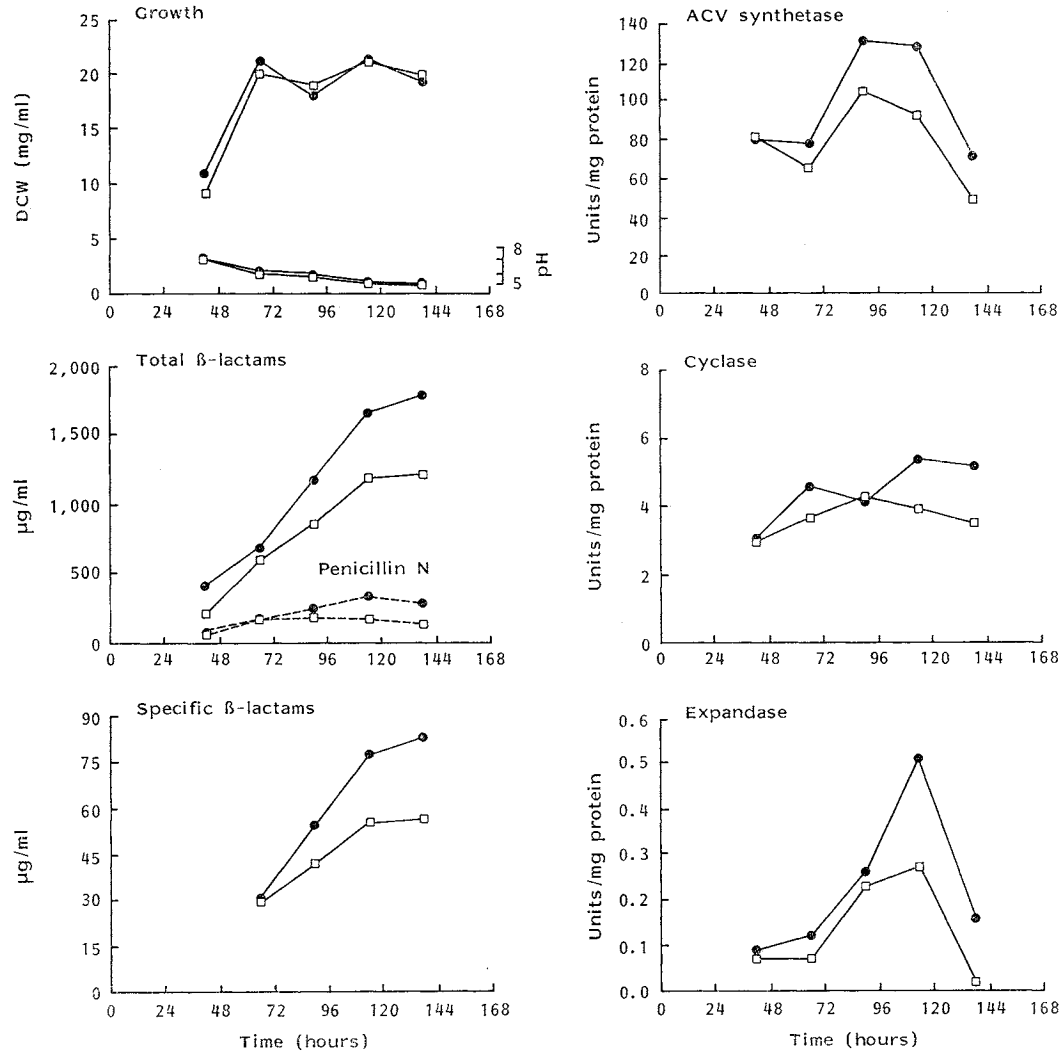


Table 1. Effect of NH_4^+ on ACV synthetase activity[a].

$(NH_4)_2SO_4$ (%)	0	0.08	0.17	0.33	0.66	0.99	1.32	1.98	2.64
NH_4^+ (mM)	0	12.5	25	50	100	150	200	300	400
Activity (%)	100	99.3	91.1	76.4	72.3	63.1	55.4	42.8	36.2

[a] NH_4Cl showed a similar degree of inhibition on a molar basis.

We previously reported that strain C-10 (the high producing strain which was derived from the low producing strain CW-19) was somewhat derepressed in glucose control when compared to CW-19. Here we find that C-10 is still sensitive to ammonium repression and we might expect that screening or selecting for ammonium repression-resistant mutants could provide improved antibiotic producers.

Regulation in other β-lactam producers shows different patterns from that observed in *C. acremonium*. BRAÑA *et al.*[16] compared three enzymes in *S. clavuligerus* and found cyclase to be the most sensitive, expandase moderately sensitive and epimerase inert to NH_4^+ repression. CASTRO *et al.*[5]

found cyclase, epimerase and expandase to be coordinately repressed in *N. lactamdurans*. Although they did not test the effect on ACV synthetase directly, they did report that *in vivo* intracellular accumulation of ACV was decreased by growth in a high concentration of ammonium.

Acknowledgments

The work at Massachusetts Institute of Technology was supported by the National Science Foundation. The Queens University activities were funded by the Natural Science and Engineering Research Council of Canada. J.-Y. ZHANG acknowledges support from the Educational Committee of the People's Republic of China. We thank D. LIBERMAN, M. JERMINI and N. A. SOLOMON for encouragement and advice.

References

1) AHARONOWITZ, Y.: Regulatory interrelationships of nitrogen metabolism and cephalosporin biosynthesis. *In* Genetics of Industrial Microorganisms. *Eds.*, O. K. SEBEK & A. I. LASKIN, pp. 210~217, Amer. Soc. Microb., Washington, D.C., 1979
2) ŌMURA, S.; Y. TANAKA, H. MAMADA & R. MASUMA: Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of tylosin aglycone. J. Antibiotics 37: 494~502, 1984
3) AHARONOWITZ, Y. & A. L. DEMAIN: Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus*. Can. J. Microbiol. 25: 61~67, 1979
4) DEMAIN, A. L. & A. F. BRAÑA: Control of cephamycin formation in *Streptomyces clavuligerus* by nitrogenous compounds. *In* Regulation of Secondary Metabolite Formation. *Ed.*, H. KLEINKAUF *et al.*, pp. 77~88, VCH Verlagsgesellschaft mbH, Weinheim, 1985
5) CASTRO, J. M.; P. LIRAS, J. CORTES & J. F. MARTIN: Regulation of α-aminoadipyl-cysteinyl-valine, isopenicillin N synthetase, isopenicillin N isomerase and deacetoxycephalosporin C synthetase by nitrogen sources in *Streptomyces lactamdurans*. Appl. Microbiol. Biotechnol. 22: 32~40, 1985
6) SHEN, Y.-Q.; J. HEIM, N. A. SOLOMON, S. WOLFE & A. L. DEMAIN: Repression of β-lactam production in *Cephalosporium acremonium* by nitrogen sources. J. Antibiotics 37: 503~511, 1984
7) SANCHEZ, S.; L. PANIAGUA, R. C. MATEOS, F. LARA & J. MORE: Nitrogen regulation of penicillin G biosynthesis in *Penicillium chrysogenum*. *In* Advances in Biotechnology. Vol. III. Fermentation Products. *Eds.*, C. VÉZINA & K. SINGH, pp. 147~154, Pergamon Press, Toronto, 1979
8) BANKO, G.; A. L. DEMAIN & S. WOLFE: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase: A multifunctional enzyme with broad substrate specificity for synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc. 109: 2858~2860, 1987
9) DEMAIN, A. L.: Strain exchange between industry and academia. ASM News 49: 431, 1983
10) SHEN, Y.-Q.; S. WOLFE & A. L. DEMAIN: Levels of isopenicillin N synthetase and deacetoxycephalosporin C synthetase in *Cephalosporium acremonium* producing high and low levels of cephalosporin C. Bio/Technology 4: 61~63, 1986
11) BRADFORD, M. M.: A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. 12: 248~254, 1976
12) LÜBBE, C.; Y.-Q. SHEN & A. L. DEMAIN: A simple and rapid chemical method for the determination of cephalosporins. Appl. Biochem. Biotechnol. 12: 31~35, 1986
13) BANKO, G.; S. WOLFE & A. L. DEMAIN: Cell-free synthesis of δ-(L-α-aminoadipyl)-L-cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. Biochem. Biophys. Res. Commun. 137: 528~535, 1986
14) KUPKA, J.; Y.-Q. SHEN, S. WOLFE & A. L. DEMAIN: Studies on the ring-cyclization and ring-expansion enzymes of β-lactam biosynthesis in *Cephalosporium acremonium*. Can. J. Microbiol. 29: 488~496, 1983
15) ZHANG, J.-Y.; G. BANKO, S. WOLFE & A. L. DEMAIN: Methionine induction of ACV synthetase in *Cephalosporium acremonium*. J. Ind. Microbiol. 2: 251~225, 1987
16) BRAÑA, A. F.; S. WOLFE & A. L. DEMAIN: Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. Can J. Microbiol. 31: 736~743, 1985