EFFECT OF METHIONINE ON CEPHALOSPORIN C AND PENICILLIN N PRODUCTION BY A MUTANT OF *CEPHALOSPORIUM ACREMONIUM*

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A mutant with enhanced potential to utilize sulfate for cephalosporin C production was isolated from a strain of *Cephalosporium acremonium*. The mutant displayed potency levels more than twofold that of the parent in the presence of sulfate but its productivity was severely inhibited by more than 0.5 % of methionine which gave high cephalosporin C production with the parent. In a complex medium norleucine stimulated cephalosporin C production by the mutant in the presence of sulfate, whereas it showed no effect on the parent. In an incubation system with sulfur-starved cells of the mutant, L-methionine, but not the D-isomer, gave lower cephalosporin C production and a delayed production of penicillin N. However, it exhibited a stimulatory effect in the presence of valine or α-aminoadipic acid, the constituent amino acids of the antibiotic. Norleucine showed a similar effect to that of L-methionine in the presence of sulfate. On the basis of these results, characteristics of the mutant are discussed in connection with the effect of methionine.

It has been well known that the production of cephalosporin C (ceph C) by *Cephalosporium acremonium* occurs efficiently in the presence of methionine and less efficiently in the presence of sulfate.[1-3] It was reported that the sulfur for ceph C is derived from methionine sulfur,[3] but another role, which is not yet clear, besides an efficient sulfur donor has been suggested for the mechanism of methionine stimulation.[3-6]

In the course of the studies on strain improvement of *Cephalosporium acremonium* we have isolated a mutant which has an increased ceph C productivity from sulfate. Ceph C production by this mutant was found to be sensitive to methionine as compared with the parental strain. Moreover, it was found that methionine inhibited not only ceph C, but also penicillin N (pen N) production in an incubation system with cell suspensions.

This report describes the characterization of the mutant through studies of the inhibitory effect of methionine on antibiotic production.

Materials and Methods

Strains and Isolation of Mutant

A superior antibiotic-producing mutant of *Cephalosporium acremonium*, designated as N-16, was used as the parental strain for the sulfate-deregulated mutants. After irradiation with ultraviolet light, conidia of N-16 were plated on a minimal medium which is described below. Colonies which appeared on the plate were screened for antibiotic synthesis from sulfate and a strain, designated as IS-5, was isolated as a potent ceph C producer from sulfate.

Media and culture conditions

The seed medium was composed of soybean meal, 1 %; corn steep solid, 1 %; corn

starch, 2 %; $CaCO_3$, 0.3 %; $(NH_4)_2SO_4$, 0.1 %; and methyl oleate, 2 %. The pH of the medium was adjusted to 7.0. The basal complex medium for fermentation was composed of soybean meal, 6 %; beet molasses, 5 %; corn starch, 1 %; NH_4Cl, 0.1 %; $CaCO_3$, 0.5 %; and methyl oleate, 3 %. DL-Methionine or $CaSO_4$ were added to the basal medium as the sulfur source. The defined fermentation medium was composed of sucrose, 2 %; soluble starch, 1 %; $(NH_4)_2SO_4$, 0.7 %; methyl oleate, 2 %; $CaCO_3$, 1 %; and salt solution I, 1 ml per liter. Salt solution I contained, per liter of distilled water: KH_2PO_4 100 g, K_2HPO_4 300 g, $MgSO_4 \cdot 7H_2O$ 90 g, $CaCl_2$ 30 g, $ZnSO_4 \cdot 7H_2O$ 30 g, $FeSO_4$ 20 g, $MnSO_4 \cdot H_2O$ 4 g, and $CuSO_4 \cdot 5H_2O$ 2 g. The medium for sulfur starvation was composed of sucrose, 1 %; glucose, 0.5 %; NH_4Cl, 0.2 %; and salt solution II, 1 ml per liter. Salt solution II was derived from salt solution I by substituting chloride for sulfate of the metal salts. The minimal agar medium was composed of sucrose, 2 %; $(NH_4)_2SO_4$, 0.7 %; agar, 2 %; and salt solution I, 1 ml per liter.

Flasks with 100 ml of the seed medium were inoculated with 5 % of a slant culture, and incubated for 72 hours. The main fermentation was established in flasks containing 40 ml of the complex medium using 5 % inoculum from the seed culture. The preculture for the studies with cell suspensions was grown in flasks containing 40 ml of the defined fermentation medium for 72 hours. For the replacement culture without sulfur starvation, the cells were incubated in flasks with 30 ml of the medium for sulfur starvation supplemented with the substances indicated in the experiment. The sulfur starvation was carried out in flasks with 30 ml of the medium for sulfur starvation supplemented with substances indicated in the experiment. All liquid cultures were incubated in 500-ml Erlenmyer flasks at 25°C on a rotary shaker at 230 rpm.

Antibiotic assays

Culture filtrates were used for antibiotic assays throughout the experiment. Total cephalosporins in the culture broth with the complex medium were determined by a spectrophotometric assay[7] using ceph C as a standard. Ceph C in the incubation mixture with cell suspensions was determined by a bioassay using *Alcaligenes faecalis* ATCC 8750.[8] Pen N was determined by a bioassay using *Sarcina lutea* ATCC 9341.[9] Pen N activity was recorded as units per ml of culture broth. One unit is roughly equal to 1.4 μg of purified pen N.

Methionine assay

Culture filtrates were subjected to paper chromatography using a *n*-buthanol - actate - water (4:1:2) mixture. Methionine on the chromatogram was detected by spraying with ninhydrin, and eluted with 75 % ethanol containing 0.2 mg of $CuSO_4 \cdot 5H_2O$ per 100 ml. Methionine levels were estimated from optical density measurements at 540 nm of the eluted solutions.

Results

Effect of Methionine on Cephalosporin C Production by the Parent and a Mutant Strain

As it is well known, DL-methionine showed a distinct stimulatory effect on ceph C production by a strain, designated as N-16, of *Cephalosporium acremonium* regardless of sulfate supplementation (Fig. 1. A). The optimal concentration of DL-methionine for ceph C production approximately was 0.75 % under the conditions used and antibiotic production was gradually reduced in the presence of more than 1 % of DL-methionine.

In the course of the studies on improvement of N-16, we have isolated a mutant, designated as IS-5, with enhanced potential to utilize sulfate for ceph C production. This mutant displayed potency levels more than twofold that of the parent in the presence of sulfate as the sulfur source (Fig. 1. B). With small amounts of DL-methionine and sulfate, ceph C

Fig. 1. Effect of methionine and sulfate on cephalosporin C production by the mutant.

The fermentation was carried out for 96 hours with the basal complex medium.

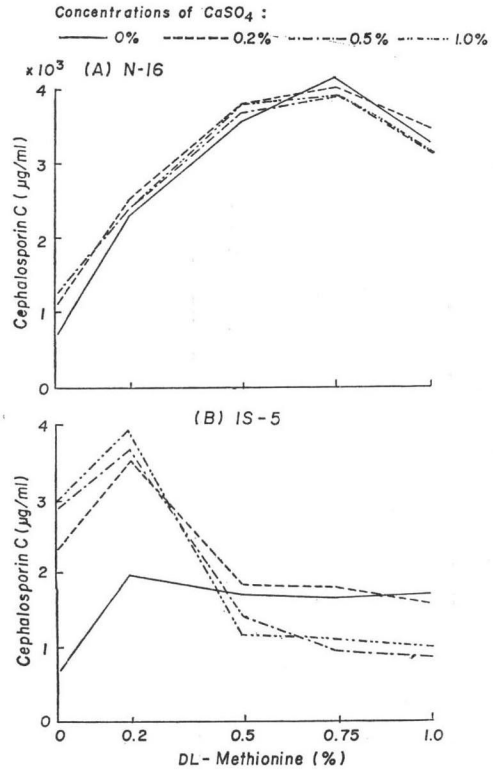
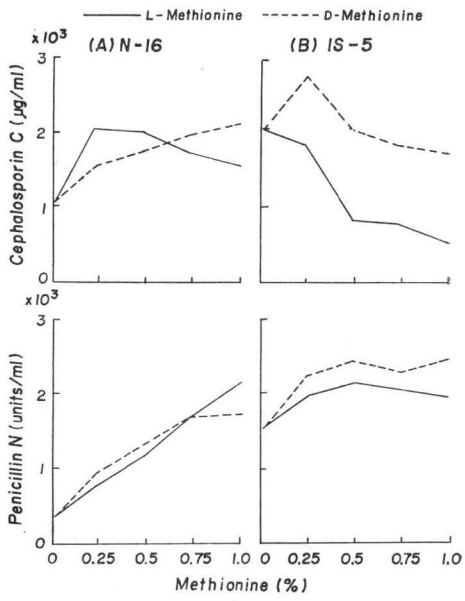


Fig. 2. Effect of stereoisomers of methionine on antibiotic production.

The fermentation was carried out for 72 hours with the basal complex medium supplemented with 0.6% of $CaSO_4$.



production appeared to occur from both sulfur sources. The mutant showed the maximum productivity in the presence of 0.2 % of DL-methionine in addition to 1.0 % of calcium sulfate. These results suggest the presence of the pathway from methionine to ceph C in the mutant. In the presence of sulfate, however, the antibiotic production by the mutant was severely inhibited by more than 0.5 % of DL-methionine, a concentration which gave high ceph C production with the parent.

Effect of Stereoisomers of Methionine on the Antibiotic Production

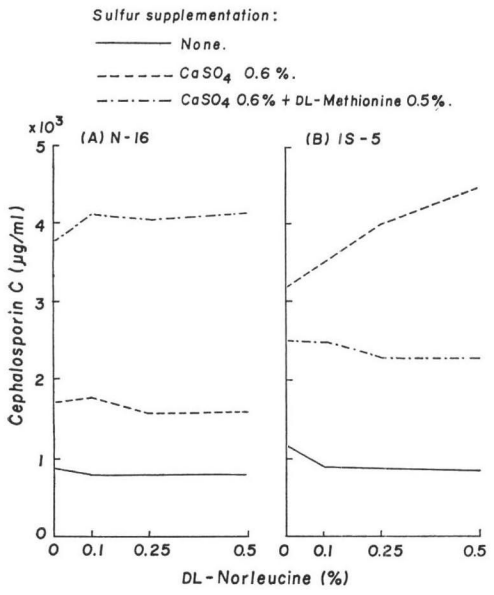
Two isomers of methionine, the D- and L-forms, were added to the complex medium separately, and their effects on the antibiotic production were investigated. As shown in Fig. 2, ceph C production by the parent appeared to be slightly inhibited by L-methionine, but not by the D-isomer, at the early period of fermentation. Pen N production was stimulated with increase in the concentration of either D- or L-methionine. With the mutant, ceph C production was inhibited by L-methionine and, to a lesser extent, D-methionine. Neither isomer exhibited a distinct effect on pen N production by the mutant.

Effect of Norleucine on Ceph C Production

It has been reported that DL-norleucine, the non-sulfur analog of methionine, stimulated ceph C production in chemically defined media,[4] but it could not replace methionine in complex media.[3,5] A study was therefore done to determine if DL-norleucine has any effect on ceph C production by our strains in the complex medium.

Fig. 3. Effect of norleucine.

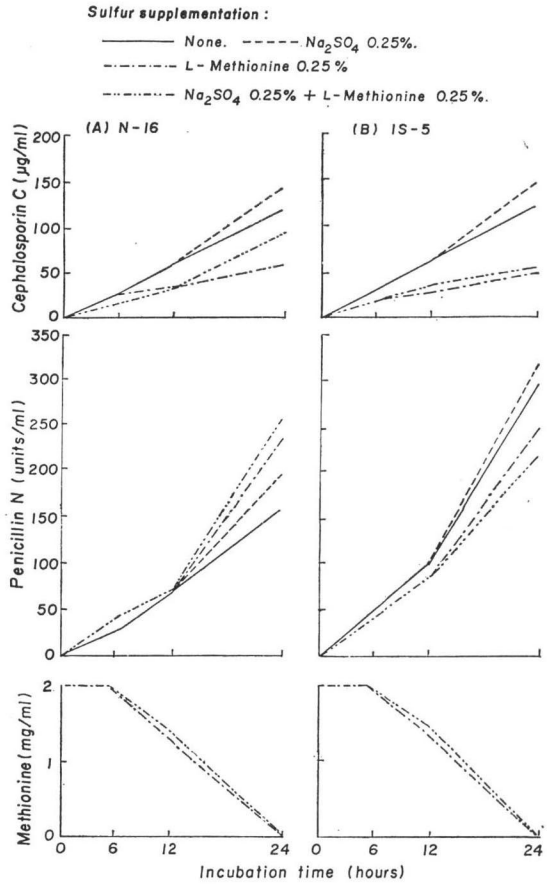
The fermentation was carried out for 120 hours with the basal complex medium.



As shown in Fig. 3, DL-norleucine did not stimulate ceph C production by the parent and this result is consistent with the previously described findings. With the mutant, on the other hand, the analog distinctly stimulated ceph C production in the presence of sulfate. The analog exhibited no effect in the presence of an amount of methionine which causes the inhibition. Pen N was not estimated in this experiment.

Fig. 4. Effect of methionine and sulfate on antibiotic production by cell suspensions without sulfur starvation.

The preculture was grown in the presence of 0.25% of DL-methionine.



Effect of Methionine on the Antibiotic Production by Cell Suspensions

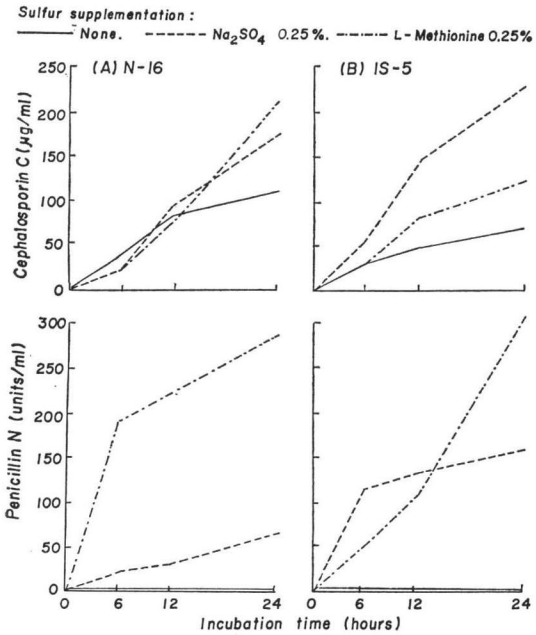
It was thought that the enhanced potential to produce ceph C from sulfate in the mutant was somehow related to its increased sensitivity to methionine. In order to make clear such a relationship antibiotic production was investigated in chemically defined media using the technique of replacement culture. Cells grown in the defined fermentation medium supplemented with 0.25 % of DL-methionine were transferred into the media for replacement culture and the effect of methionine on antibiotic production was examined without the influence of growth.

As shown in Fig. 4, L-methionine severely inhibited ceph C production not only by the mutant but also by the parent. Pen N production by the parent, on the contrary, was rather stimulated by L-methionine. With the mutant, pen N production appeared to be susceptible, to a small extent, to inhibition by L-methionine. There was no significant difference in consumption rate of L-methionine between the two strains. It appeared that a considerable amount of sulfur-containing substances, or precursors of the antibiotics, was stocked in the pool of the cells during the preculture phase, since similar amounts of the antibiotics were produced with or without sulfate supplementation.

Cephalosporin C and Penicillin N Production by Sulfur-starved Cells

As mentioned above, it appeared that the stored sulfur-containing substances, or precursors of the antibiotics in the pool of the cells supported antibiotic production with cell suspensions in the absence of any sulfur sources. An attempt was therefore made to eliminate the influence of the pooled substances by incubating the cells in a medium which contained no sulfur source for 24 hours before the replacement culture. Antibiotic production was again investigated using such sulfur-starved cells.

Fig. 5. Effect of L-methionine and sulfate on antibiotic production by sulfur-starved cells.
The preculture was grown in the presence of 0.25% of DL-methionine.



(1) Effect of L-methionine and sulfate

Antibiotic production by the sulfur-starved cells was somewhat different from that of the cells without sulfur starvation (Fig. 5). With cells of the parent, L-methionine exhibited a stimulatory effect on ceph C and pen N production. The result appeared to correspond well to that obtained in the complex medium. Pen N production was very poor in the presence of sulfate but interestingly ceph C was produced to a level similar to that from methionine. With cells of the mutant, L-methionine showed poor ceph C production and a delayed time course of pen N production. In the presence of sulfate pen N production appeared to stop after 6 hours, but ceph C continued to be produced throughout the incubation period.

As an interesting fact it was found that pen N production did not occur in the absence of any sulfur sources but some ceph C was produced both by the parental and the mutant cells under these conditions. This result shows that the sulfur starvation was not complete, but also suggests that ceph C is preferentially formed when the supply of the tripeptide for the antibiotic is limited. Such a presumption can also be made from the results of antibiotic production from sulfate.

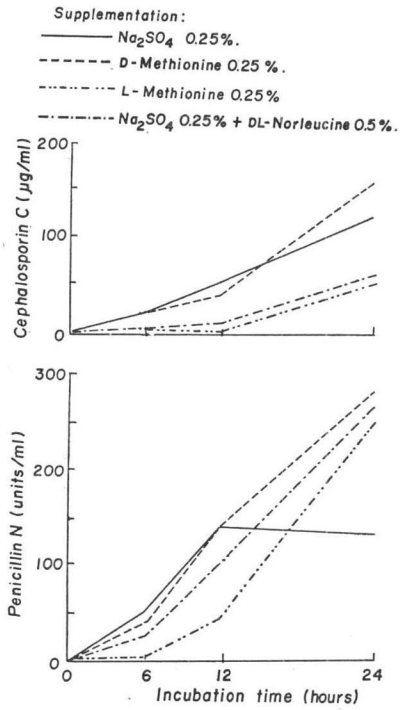
(2) Effect of D-methionine and norleucine

D-Methionine and DL-norleucine were tested in the incubation system with the sulfur-starved cells of mutant IS-5 to study their effects on antibiotic production. The preculture was grown in the absence of methionine in this experiment and neither pen N nor ceph C production occurred with sulfur-starved cells in the absence of a sulfur source.

As shown in Fig. 6, D-methionine appeared to be a somewhat more efficient sulfur donor for both ceph C and pen N production than the L-form. DL-Norleucine behaved like

Fig. 6. Effect of D-methionine and norleucine on antibiotic production by sulfur-starved cells of the mutant.

The preculture was grown in the absence of methionine.



L-methionine on antibiotic production in the presence of sulfate. It inhibited ceph C production severely and pen N production to a lesser extent. DL-Norleucine also exhibited the same effect as L-methionine on antibiotic production by cells of the parent in other experiments.

(3) Effect of amino acids in the presence of L-methionine

With the sulfur-starved cells of IS-5, the effect of several amino acids on the antibiotic production was examined in the presence of L-methionine. As shown in Table 1, valine and α-aminoadipic acid, the constituent amino acids of the antibiotic, showed a stimulatory effect on ceph C and pen N production. The other amino acids examined also stimulated antibiotic production to some extent but their effects were less than those of the two amino acids. In other experiments, valine and α-aminoadipic acid also stimulated antibiotic production from sulfate and reversed the inhibitory effect of DL-norleucine.

Table 1. Effect of amino acids on antibiotic production by sulfur-starved cells of the mutant in the presence of L-methionine.

Incubation time (hrs)		Amino acids						
		None	L-Val	DL-AAA	L-Glu	L-Asp	L-Lys	L-Leu
6	ceph C*	0	<10	0	0	0	0	0
	pen N**	0	11	8	<5	<5	<5	<5
12	ceph C	0	15	<10	0	0	0	0
	pen N	14	48	31	20	20	20	20
25	ceph C	33	150	150	84	90	70	100
	pen N	75	200	190	135	150	130	150

* Cephalosporin C μg/ml. ** Penicillin N units/ml.

Amino acid was added to the medium to give a concentration of 0.1 % in the presence of 0.25 % of L-methionine.

Abbreviations: Val, valine; AAA, α-aminoadipic acid; Glu, glutamic acid; Asp, aspartic acid; Lys, lysine; Leu, leucine.

Discussion

Recently NISS and NASH[10] reported on a mutant with enhanced potential to utilize sulfate for ceph C production, but their mutant has not been described to be 'methionine-sensitive'

like our mutant. We have isolated independently several mutants with increased ceph C productivity from sulfate besides IS-5, and it was found that all such mutants were 'methionine-sensitive'. It is thought, therefore, that increase in productivity from sulfate and increase in methionine sensitivity may be metabolically related and caused by the same mutational event.

In the studies with the sulfur-starved cells, IS-5 appeared to have a low ability to utilize L-methionine for ceph C production as compared with the parent. This low ability of the mutant, however, may not be due to a low efficiency in conversion of methionine to cysteine because of the facts summerized below.

(1) D-Methionine has been thought to be metabolized through the L-form,[11] but it was more efficient than L-methionine for antibiotic production.

(2) L-Methionine appeared to depress pen N production at the early stage of incubation, but produced a large amount of the antibiotic after 24 hours whereas ceph C was still at a low level.

(3) L-Methionine was utilized efficiently for antibiotic production in the presence of one of the constituent amino acids of the tripeptide, namely valine and α-aminoadipic acid.

(4) DL-Norleucine inhibited antibiotic production in the presence of sulfate. This analog exhibited a stimulatory effect on ceph C production by IS-5 in the complex medium with sulfate. DEMAIN and NEWKIRK[1] once postulated that methionine might repress a cysteine-degrading enzyme. Recently DREW and DEMAIN[5] have shown that methionine neither represses nor inhibits cysteine-degrading activity. However, it can be hypothesized from our results that norleucine may have a sparing effect on intracellular cysteine.

On the basis of these results one may assume that the apparent low ability to utilize L-methionine and the inhibitory effect of norleucine in IS-5 are caused by a single mechanism, an inhibition of antibiotic production by the intracellular accumulation of a sulfur-containing substance which can be derived both from methionine and sulfate. This substance may be of a reductive nature, since pen N production was always less susceptible to the inhibition than ceph C production which requires more oxidative steps than the former. IS-5 is presumably a mutant with an improved ability to maintain a high level of the reductive sulfur compound, cysteine for example, and may not be a mutant in the sulfate transport system as reported by NISS and NASH.[10]

In the course of studies with the sulfur-starved cells, we found an interesting fact that ceph C was produced in preference to pen N when the supply of sulfur-containing precursor of the antibiotic was limited by some reasons. A similar result has been reported by GODFREY[12] who found that the preferential synthesis of cephalosporins occurred when an isoleucine-valine mutant of *Streptomyces lipmanii* was supplemented with a limited amount of valine. One can presume from these results that pen N may be an overflow product in the ceph C biosynthetic pathway.

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