SULFUR METABOLISM OF A MUTANT OF *CEPHALOSPORIUM ACREMONIUM* WITH ENHANCED POTENTIAL TO UTILIZE SULFATE FOR CEPHALOSPORIN C PRODUCTION

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Characteristics of a mutant of *Cephalosporium acremonium* with enhanced potential to utilize sulfate for cephalosporin C production were investigated with sulfur-starved cells. DL-Norleucine showed an inhibitory effect on cephalosporin C and penicillin N production by the mutant in the presence of a sulfur source such as sulfate, sulfite, thiosulfate, and L-cystine, but it exhibited no effect when it was added after a certain period of incubation. On the contrary, antibiotic production by the parent was stimulated by norleucine regardless of the addition time. An increase in the intracellular cysteine pool was found when the cells were incubated with L-methionine or norleucine and sulfate. Enzymatic studies revealed that methionine and norleucine stimulated the cysteine desulfhydrase formation, and this effect was significant in the mutant. Finally the mutant was found to have an enhanced L-serine sulfhydrylase activity. The increase in this enzyme activity in the mutant seems responsible for the increase in the sulfate-utilizing ability and the methionine sensitivity by maintaining a high level of the cysteine pool. Accordingly, the effect of methionine and norleucine is assumed to be exerted through cysteine.

There have been many studies on the stimulatory effect of methionine on cephalosporin C (ceph C) and penicillin N (pen N) production by *Cephalosporium acremonium*. A special, but unknown, physiological role other than sulfur donation has been postulated based on some observations,[1~3] although it was clearly shown that ^{35}S-methionine was incorporated into ceph C without dilution.[4] Recently observations which appeared to support such a hypothesis have been reported by DREW and DEMAIN[5~7] using certain blocked mutants on the cysteine and methionine metabolic pathways. However, it is not yet certain whether methionine stimulation is exerted by a single mechanism or the result of multiple effects. It is also obscure whether the true effector is methionine itself or some metabolite of it.

Previously[8] we have isolated a mutant with enhanced ceph C productivity from sulfate in the course of the strain improvement of *Cephalosporium acremonium* and have found that ceph C production by this mutant became sensitive to methionine. It was presumed that the methionine sensitivity might not be caused by methionine itself but by some sulfur-containing compound derived from it. Moreover, norleucine which contains no sulfur depressed antibiotic production with sulfur-starved cells in a similar manner as methionine.

Subsequently we have found that methionine and norleucine increased the level of the intracellular cysteine pool when ceph C production was depressed by them in an incubation system with the sulfur-starved cells. On the basis of this finding, enzyme activities of cysteine metabolism were studied, and it was found that the mutant had an enhanced L-serine sulfhydrylase activity as compared with the parent.

The present paper describes the above findings, and the role of an altered metabolism involving cysteine in the mutant on antibiotic production will be discussed.

Materials and Methods

Strains and Culture Conditions

A superior ceph C-producing strain of *Cephalosporium acremonium*, designated N–16, and the mutant, IS–5, with enhanced ceph C productivity from sulfate were used for the experiments. Cultivation on the defined medium and the subsequent sulfur starvation were conducted as previously described.[8]

Antibiotic Assays

Determination of ceph C and pen N was done as previously described[8].

Analysis of Intracellular Amino Acids

Cells were harvested by centrifugation at 2,000×*g* for 10 minutes, washed twice with cold water, and suspended in water. The amino acid pools were extracted with water at 90°C for 10 minutes. After centrifugation at 6,700×*g* for 10 minutes, the cells were again extracted with cold water and centrifuged. The supernatants obtained by this procedure were combined and freeze-dried. The sample was analyzed on an amino acid analyzer (Yanagimoto LC–5S). The concentration of amino acids was expressed as μmoles per gram of dry cells.

Preparation of Crude Enzyme Extract

Sulfur-starved cells were incubated in a replacement culture system for 6 or 12 hours and then harvested by centrifugation. The collected cells were washed twice with 0.01 M Tris buffer solution (pH 8.0) containing 2 mM EDTA and were resuspended in the buffer containing 1 mM dithiothreitol. The washed cells were disrupted in a homogenizer (B. Braun Melsungen) under cooling with liquid carbon dioxide and then centrifuged at 17,000×*g* for 30 minutes at 5°C. The supernatant fluid was used as a crude enzyme extract for enzymatic studies.

The protein content of the crude enzyme extract was estimated by the method of LOWRY *et al.*[9]

Enzyme Assays

Cysteine desulfhydrase activity was determined by the method of KREDICH[10]. O-Acetyl-L-serine sulfhydrylase and L-serine sulfhydrylase activities were assayed according to the method of PIENIAZEK *et al.*[11].

Results

Effect of Norleucine on Cephalosporin C and Penicillin N Production by Sulfur-starved Cells

As previously described,[8] norleucine showed an inhibitory effect on ceph C and pen N production by sulfur-starved cells of mutant IS–5 in the presence of sulfate. The effect of norleucine was very similar to that of L-methionine. The effect of norleucine was further investigated in the presence of various sulfur-containing compounds on the cysteine biosynthetic pathway from sulfate.

As shown in Fig. 1–A, sulfate and thiosulfate, which might share a common permease system,[12] exhibited similar effects on antibiotic production by mutant IS–5. However, sulfite showed a lag both for ceph C and pen N production presumably because of the reductive nature of this compound. L-Cystine also gave a little different time course of antibiotic production as compared to sulfate, presumably due to the problem of low solubility. When DL-norleucine was added to these systems at the time zero (Fig. 1–B), it depressed ceph C production and also pen N production to a lesser extent regardless of the kind of sulfur source. Norleucine seemed less effective when it was added to the broth later during the course of incubation. As shown in Fig. 2–A, this compound appeared to exhibit no effect at all when it was added at 12 hours in the presence of sulfate. A similar result was obtained in the presence of L-cystine (Fig. 2–B).

As shown in Fig. 3, norleucine stimulated both ceph C and pen N production by the sulfur-starved

Fig. 1. Antibiotic production from various sulfur sources by sulfur-starved cells of mutant IS–5 in the absence (A) or presence (B) of 0.25% of DL-norleucine.
The preculture was grown in the presence of 0.7% of $(NH_4)_2SO_4$ as the sulfur source.

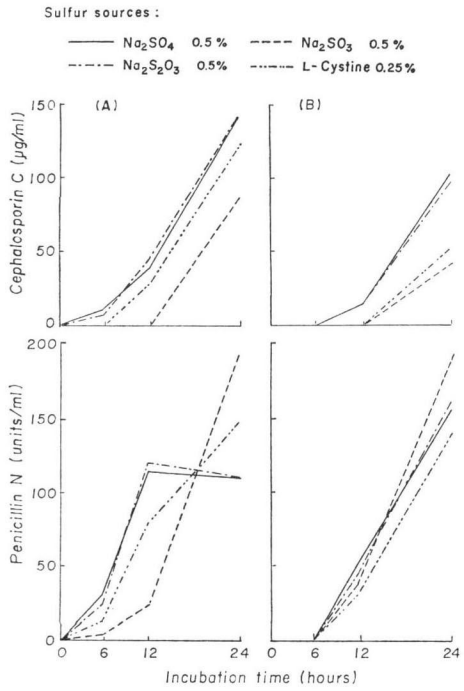
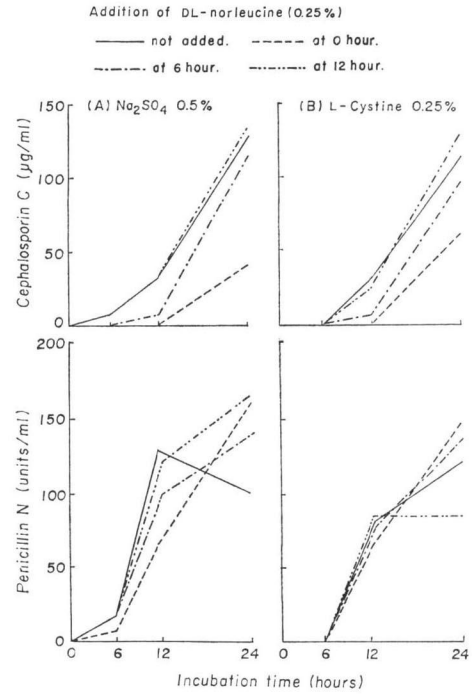


Fig. 2. Effect of DL-norleucine added at various periods on antibiotic production from sulfate (A) or L-cystine (B) by sulfur-starved cells of mutant IS–5.
The preculture was grown in the presence of 0.7% of $(NH_4)_2SO_4$.



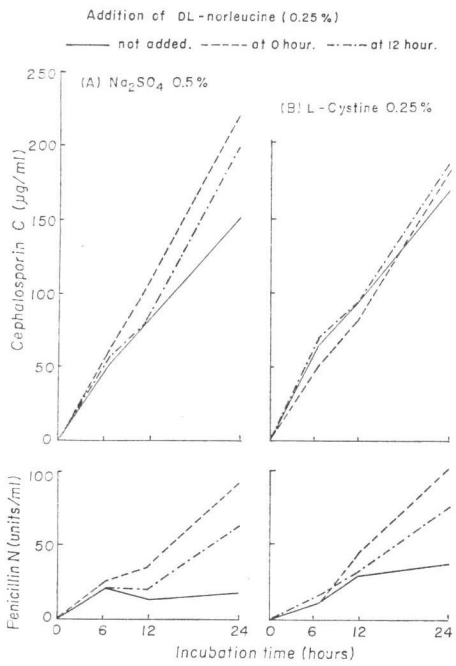
cells of the parent strain N–16 in the presence of sulfate. In contrast to the result with mutant IS–5, this compound was effective even when it was added at 12 hours of incubation. While it stimulated pen N production as well in the presence of L-cystine, ceph C production appeared to be unaffected.

Effect of Methionine and Norleucine on Intracellular Amino Acid Pools

From the results mentioned above and described previously, it was suggested that L-methionine and norleucine might exert the inhibitory effect on antibiotic production through a sulfur-containing compound which can be derived either from sulfate or methionine. For this reason the effect of L-methionine and norleucine on intracellular amino acid pools was examined with the sulfur-starved cells of mutant IS–5. The cells were incubated for 12 hours in the presence of sulfate or methionine as the sulfur source and subjected to the analysis of the intracellular amino acids. The results are given in Table 1.

Cysteine was assayed in the form of cystine, which might be unsuitable for the quantitative analysis in the present procedure. Nevertheless, it was noted that the concentration of cystine increased markedly when the cells were incubated in the presence of norleucine plus sulfate, or in the presence of L-methionine. The level of intracellular methionine was lowered by the addition of norleucine. A significant increase in the concentration of taurine was observed in the cells incubated with L-methionine. This fact supports the previous speculation[8] that the pathway from methionine to cysteine may function

Fig. 3. Effect of DL-norleucine on antibiotic production from sulfate (A) or L-cystine (B) by sulfur-starved cells of the parent strain N–16.
The preculture was grown in the presence of 0.7% of $(NH_4)_2SO_4$ and 0.25% of DL-methionine.



very well in mutant IS–5, because taurine is known to be derived from cysteine. It is also worth noting that the level of proline, which is one of the amino acids present in high concentrations, decreased significantly in the presence of L-methionine or norleucine.

A similar study was made with N–16, and the results are given in Table 2. A cysteine pool was not found in the cells incubated with sulfate alone, but it was found in substantial concentration in the cells incubated with sulfate and norleucine which stimulated antibiotic production by the parent strain. The level of cysteine also was found to increase in the presence of L-methionine. Together with the above findings, the effect of norleucine and L-methionine on the intracellular pools of methionine, taurine, and proline was very similar to the results obtained with mutant IS–5.

Enzyme Activities of Cysteine Metabolism

As it was presumed that L-methionine and norleucine might exert the above-mentioned effects by affecting the metabolism related to cysteine, a few enzyme activities of cysteine metabolism in the parental and the mutant strains were studied in the incubation system using the sulfur-starved cells.

Cysteine desulfhydrase, a catabolic enzyme of cysteine, was at first examined with extracts from the cells incubated in the presence of sulfate or L-methionine. As shown in Table 3, there was no difference in the activity between the parent and the mutant strain when the cells were incubated with sulfate. An increase in the enzyme activity was found when the cells were incubated with L-methionine. However, it was noted that the increase of activity in the mutant was significant as compared with that in the parent. As shown in Table 4, the cysteine desulfhydrase reaction was not inhibited by L-methionine or norleucine. This is consistent with the observation by DREW and DEMAIN.[3]

It was suggested from the above results that cysteine might induce the enzyme formation since L-methionine was found to increase the intracellular cysteine pool. In the further experiments with the mutant cells we examined the effect on the enzyme formation of L-cystine and norleucine, which also increased the cysteine pool. As shown in Table 5, L-cystine as the sulfur source displayed the cysteine desulfhydrase activity almost two-fold over sulfate. Norleucine also appeared to stimulate the enzyme formation, especially in the presence of L-cystine.

From the above results it seems likely that the stimulatory effect of L-methionine and norleucine on the enzyme formation might be exerted through cysteine.

Subsequently the cysteine biosynthetic activity was investigated. Two enzyme reactions have been reported for the biosynthesis of cysteine in microorganisms: O-acetyl-L-serine sulfhydrylase

Table 1. Intracellular amino acids in cells of mutant IS-5 incubated in the presence of sulfate and L-methionine.

The preculture grown in the presence of 0.7% of $(NH_4)_2SO_4$ was subjected to the sulfur starvation for 24 hours and then transferred into a fresh medium for replacement culture. The replacement culture was carried out for 12 hours.

Amino acid	Medium supplemented with		
	Na_2SO_4 0.5%	Na_2SO_4 0.5% & DL-norleucine 0.25%	L-methionine 0.25%
	(μmoles/g dry cells)		
Tryptophan	1.20	0.92	1.38
Lysine	11.3	10.7	16.4
Histidine	2.16	2.78	4.91
Arginine	5.90	8.52	9.23
Taurine	6.63	5.21	30.9
Aspartic acid	4.87	9.52	9.22
Threonine	5.44	—*	7.81
Serine	15.1	30.3	21.2
Glutamic acid	38.1	47.5	42.9
Proline	43.9	34.9	14.7
Glycine	16.4	13.3	18.4
Alanine	32.4	48.1	42.7
Cystine×1/2	tr.**	4.49	6.34
Valine	7.82	7.37	7.22
Methionine	8.54	3.44	92.4
Isoleucine	3.92	3.91	4.09
Leucine	8.18	8.44	9.03
Norleucine	—	16.7	—
Tyrosine	5.92	4.91	8.25
Phenylalanine	5.54	5.06	5.29
(Antibiotic production)			
Ceph C, μg/ml	45	10	0
Pen N, units/ml	130	64	25

* Not resolved from serine.
** Trace amounts.

Table 2. Intracellular amino acids in cells of the parent strain N-16 incubated in the presence of sulfate and L-methionine.

The preculture grown in the presence of 0.7% of $(NH_4)_2SO_4$ and 0.25% of DL-methionine was subjected to the sulfur starvation and replacement culture. The replacement culture was carried out for 12 hours.

Amino acid	Medium supplemented with		
	Na_2SO_4 0.5%	Na_2SO_4 0.5% & DL-norleucine 0.25%	L-methionine 0.25%
	(μmoles/g dry cells)		
Tryptophan	0.34	0.18	0.20
Lysine	4.30	5.16	7.96
Histidine	0.91	1.70	1.61
Arginine	3.03	6.90	6.68
Taurine	1.12	0.90	24.9
Aspartic acid	4.75	9.05	10.3
Threonine	3.84	—*	5.58
Serine	9.03	30.8	15.0
Glutamic acid	36.1	78.3	65.3
Proline	47.0	32.0	17.3
Glycine	12.2	9.02	8.86
Alanine	22.1	47.3	24.7
Cystine×1/2	0	2.19	3.46
Valine	3.90	5.46	6.03
Methionine	7.31	3.03	53.6
Isoleucine	2.14	2.87	3.49
Leucine	3.48	4.30	5.07
Norleucine	—	15.8	—
Tyrosine	1.16	1.45	1.84
Phenylalanine	1.70	2.73	3.02
(Antibiotic production)			
Ceph C, μg/ml	101	132	56
Pen N, units/ml	21	50	74

* Not resolved from serine.

Table 3. Effect of L-methionine on cysteine desulfhydrase formation.
The preculture grown in the presence of 0.7% of $(NH_4)_2SO_4$ and 0.25% of DL-methionine was subjected to sulfur starvation and replacement culture. The replacement culture was carried out for 6 hours in the presence of 0.5% of Na_2SO_4 or 0.25% of L-methionine.

Exp. No.*	Strain	Enzyme activity** of cells incubated with	
		Na_2SO_4	L-methionine
1	N-16	0.373	0.599
	IS-5	0.374	0.873
2	N-16	0.423	0.532
	IS-5	0.414	0.850

* Two experiments were conducted independently from the cultivation of cells up to the enzyme reaction.
** Specific activity (H_2S formed, nmoles/min. mg protein).

(EC 4.2.99.8) and L-serine sulfhydrylase (EC 4.2.1.22). As shown in Table 6, there appeared to be no significant difference in the O-acetyl-L-serine sulfhydrylase activity between the parent and the mutant. On the contrary, the L-serine sulfhydrylase activity in the mutant was found to be about twofold over that of the parent strain. L-Methionine had no effect on the formation of both enzyme activities.

Table 4. Effect of L-methionine and DL-norleucine on cysteine desulfhydrase reaction.
The reaction was conducted with the enzyme extract of IS-5 cells incubated with L-methionine at Exp. No. 1 in Table 3.

Addition to reaction mixture	Specific activity	Relative activity (%)
None	0.850	100
L-Methionine, 10 mM	0.791	93
DL-Norleucine, 20 mM	0.935	110

Table 5. Effect of L-cystine and DL-norleucine on cysteine desulfhydrase formation in mutant IS-5.
The preculture grown in the presence of 0.7% of $(NH_4)_2SO_4$ was subjected to sulfur starvation and replacement culture. The replacement culture was carried out for 12 hours in the presence or absence of 0.25% of DL-norleucine.

Exp. No.*	Sulfur source	Specific activity of cells incubated with	
		no addition	DL-norleucine
1	Na_2SO_4 0.5%	0.440	0.680
	L-Cystine 0.25%	0.780	1.66
2	Na_2SO_4 0.5%	0.373	0.512
	L-Cystine 0.25%	0.839	1.75

* Two experiments were conducted independently from the cultivation of cells up to the enzyme reaction.

Table 6. Cysteine biosynthetic activities of the parent and the mutant strains.
The precultures grown in the presence of 0.7% of $(NH_4)_2SO_4$ and 0.25% of DL-methionine were subjected to sulfur starvation and replacement culture. The replacement cultures were carried out for 12 hours in the presence of 0.5% of Na_2SO_4 or 0.25% of L-methionine.

Exp. No.*	Strain	O-Acetyl-L-serine sulfhydrylase of cells incubated with		L-Serine sulfhydrylase of cells incubated with	
		Na_2SO_4	L-methionine	Na_2SO_4	L-methionine
		Specific activity**			
1	N-16	1.79	2.24	0.59	0.53
	IS-5	2.59	2.88	1.19	1.45
2	N-16	3.08	3.06	0.74	0.63
	IS-5	3.45	3.38	1.42	1.19
3	N-16	2.66	2.58	0.77	0.68
	IS-5	2.77	2.64	1.46	1.65

* Three experiments were conducted independently from the cultivation of cells up to the enzyme reaction.
** Cysteine formed, nmoles/min. mg protein.

Discussion

The effect of norleucine on ceph C and pen N production was not affected by the sulfur sources such as sulfate, sulfite, thiosulfate and L-cystine. This fact might lead to the assumption that norleucine affects uniformly the uptake systems of those compounds. However, such an assumption seems unlikely since norleucine showed a stimulatory effect on antibiotic production by the parent strain, while it was inhibitory in this respect in the mutant in the incubation system with sulfur-starved cells. Norleucine did not exhibit its effect on the mutant when it was added at 12 hours of incubation, whereas it was still effective on the parent. Such a phenomenon may be caused by the difference between the parent and the mutant in the ability to accumulate a compound, presumably cysteine, which is inhibitory to the uptake of norleucine. We have suggested previously[8] that the apparent low ability to utilize L-methionine for ceph C production and the inhibitory effect of norleucine in the mutant might be caused by the intracellular accumulation of a sulfur-containing substance which can be derived both from L-methionine and sulfate. This appears to be supported by the present observation that L-methionine and norleucine raised the level of the cysteine pool, which was assayed in the form of cystine.

Cysteine desulfhydrase was once postulated to be repressed by methionine[13] which might result in the increase of the cysteine level. It was shown in the present study that this was not the case. On the contrary, the enzyme formation was stimulated by L-methionine and norleucine. This enzyme appeared to be induced by cysteine, or cystine, as was reported with *Salmonella typhimurium*.[10] Consequently the stimulatory effect on the enzyme formation appears to be a reflection of the increase in the cysteine pool. Moreover, such an effect of L-methionine was substantially greater with the mutant than that with the parent. This finding seems to support the assumption that the mutant may have an improved ability to maintain a high level of the cysteine pool.

Finally it was found in the study on the cysteine biosynthetic enzymes that the L-serine sulfhydrylase activity in the mutant was about twofold that in the parent. Both the O-acetyl-L-serine sulfhydrylase and L-serine sulfhydrylase activities have been found in fungi,[11,14] but the former has been supposed to be the main enzyme for the anabolic synthesis of cysteine.[11] The latter enzyme has been demonstrated to be identical with cystathionine β-synthase (EC 4.2.1.13) in the rat liver[15] and also in *Aspergillus nidulans*.[11]

There is no evidence to show that L-serine sulfhydrylase actually functions for the biosynthesis of cysteine in *Cephalosporium acremonium*, but it seems likely that our mutant has acquired the enhanced potential to utilize sulfate by increasing the second enzyme activity for the anabolic synthesis of cysteine. Furthermore, the increase in this enzyme activity may facilitate the conversion of methionine to cysteine if the enzyme is identical with cystathionine β-synthase. Thus the mutant seems particularly capable of maintaining a high level of the cysteine pool. The methionine sensitivity of the mutant may be caused by a facilitated conversion of methionine to cysteine which may be accumulated to an inhibitory level for antibiotic production. Such an assumption appears to be supported by the fact that a significant increase in the intracellular concentration of taurine, a degradative product from cysteine, occurred in the presence of L-methionine. It is also likely from our results that the effect of norleucine both on the parent and the mutant may be exerted by maintaining, or increasing the intracellular cysteine pool.

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