Kor. J. Appl. Microbiol. Bioeng.
Vol. 15, No. 5, 361—367 (1987)

Regulation of Sulfur Metabolism in *Cephalosporium acremonium*

Lee, Kyoung, Sang Ho Choi, Jung Joon Lee and Tae Ick Mheen*

Genetic Engineering Center, Korea Advanced Institute of Science and Technology, Seoul 131, Korea

*Cephalosporium acremonium*에서 황화합물 대사의 조절

이 경·최상호·이정준·민태익*

한국과학기술원, 유전공학센타, 미생물공학연구실

A DL-seleno-methionine resistant mutant, *Cephalosporium acremonium* MS-92 showed increased activities of sulfate and L-methionine uptake than the parent strain, and accumulated excess methionine and S-adenosylmethionine (SAM) intracellularly. And the sulfate uptake system was severely inhibited by L-cysteine.

In crude enzyme extracts, the mutant MS-92 showed lower L-serine sulfhydrylase (identical with cystathionine β-synthase) activity than the parent. Also, cysteine desulfhydrylase activity, an index of intracellular L-cysteine concentration, of the mutant MS-92 was decreased by about 50% as compared with that of the parent. Thus, it was supposed that the mutant MS-92 should have a lower level of L-cysteine than the parent.

In *C. acremonium* like *A. nidulans,* the enzymes related to the biosynthesis of methionine might be regulated by L-cysteine, but not by methionine or SAM.

Studies on regulatory mechanisms of L-methionine biosynthesis in *Cephalosporium acremonium* show that exogeneous L-methionine reverses the toxicity of sulfate analogue, selenate(1). And, it was suggested that a regulatory protein interact with a intermediate of sulfur metabolism when the concentration of the intermediate is high enough. The complex formed by this interaction triggers a repression of the synthesis of enzymes involved in the uptake and in the conversion of sulfate to methionine (enzymes 1-5, 7-12 in Fig. 1.) (2-7). It is also suggested that both S-adenosylmethionine (SAM) and methionyl-tRNA be implicated in the regulation of some methionine biosynthetic enzymes in *Saccharomyces cerevisiae* (2-4). Also, in *E. coli* and *Candida petrophilum,* the participation of SAM as a regulatory signal in the biosynthesis of methionine has been reported (5,6). But in *Aspergillus nidulans,* it was found that the enzymes of sulfate assimilation are under cysteine-and/or homocysteine-, but not methionine-or SAM-mediated regulation(7).

In amino acid analogue resistant mutant it is well-known that a target amino acid is overproduced extracellularly or intracellularly due to the insensitivity to the regulatory feedback mechanism. So, it is possible to study the regulatory mechanism of the amino acid biosynthesis by comparison the enzyme

Key words: *Cephalosporium acremonium,* cephalosporin C (CPC), S-adenosylmethionie (SAM), regulation, sulfur metabolism

* Corresponding author

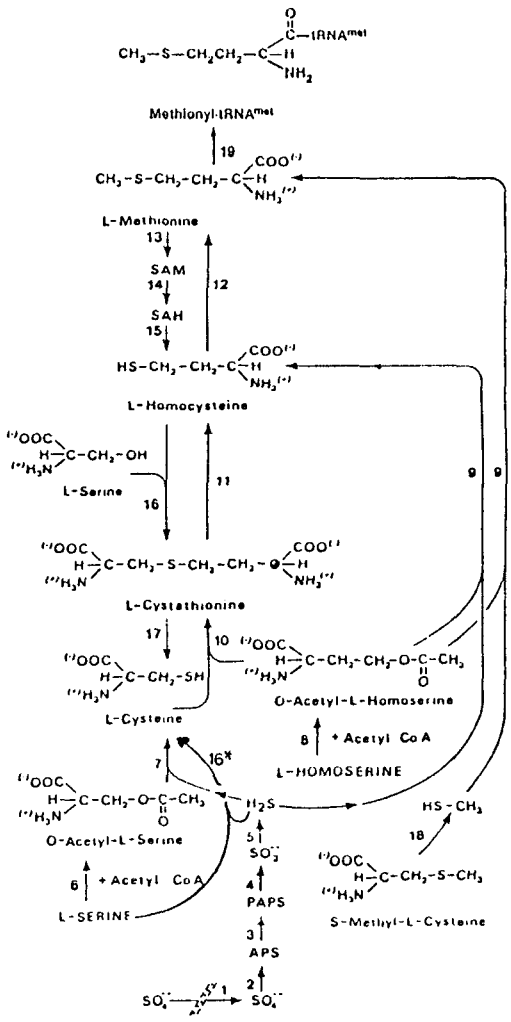

Fig. 1. Sulphur metabolism in *C. acremonium*.
APS = adenosine-5'-phosphosulphate, PAPS = 3'-phosphoadenosine-5'-phosphosulphate. SMA = S-adenosyl-L-methionine. SAH = -adenosyl-L-homocysteine. 1 = sulphate permease, 2 = sulphate adenyltransferase (EC 2.7.7.4), 3 = adenylsulphate kinase (EC 2.7.1.25). 4 = PAPS reductase, 5 = sulphite reductase (EC 1.8.1.2), 6 = serine acetyltransferase (EC 2.3.1.30), 7 = cysteine synthase (EC 4.2.99.8). 8 = homoserine acetyltransferase (EC 2.3.1.31), 9 = methionine synthase (EC 4.2.99.10), 10 = cystathionine γ-synthase (EC 4.2.99.9), 11 = cystathionine β-lyase (EC 4.4.1.8), 12 = homocysteine methyltransferase (EC 2.1.1.10), 13 = methionine adenosyltransferase (EC 2.5.1.6) 14 = methyltransferase (EC 2.1.1.x), 15 = adenosylhomocysteinase (EC 3.3.11), 16 = cystathionine β-synthase (EC 4.2.1.22), 17 = cystathionine γ-lyase (EC 4.4.11.), 18 = S-alkylcysteine lyase (EC 4.4.1.6) 16* = L-serine sulfhydrylase (EC 4.2.1.22)

Table 1. Composition of culture media

Medium	Composition (%)
Minimal agar medium	Glucose 1.0, Asparagine 0.1, KH_2PO_4 0.046, $MgSO_4 \cdot 7H_2O$ 0.05, K_2HPO_4 0.1, Agar 1.5
Seed medium	Soybean meal 1.0, Cornsteep liquor 1.0, Corn starch 2.0, Methyloleate 2.0, $(NH_4)_2SO_4$ 0.1, $CaCO_3$ 0.3
Basal synthetic medium	Sucrose 3.6, Glucose 2.7, Na_2SO_4 0.4, NH_4Cl 0.75, Methyloleate 0.2 (V/V), K_2HPO_4 2.1, KH_2PO_4 1.5, Salt mixture* 10 (V/V)
Basal complex medium	Sucrose 8.0, Soybean meal 6.0, NH_4Cl 0.1, $CaSO_4 \cdot 2H_2O$ 0.4, $CaCO_3$ 0.5

* $MgSO_4 \cdot 7H_2O$ 0.18g, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ 0.15g, $CaCl_2$ 0.06g, $MnSO_4$ 0.3g, $ZnSO_4 \cdot 2H_2O$ 0.03g, $CaSO_4 \cdot 5H_2O$ 0.0075g per 100ml distilled water

activities and the metabolite concentrations of the parent with those of its mutant in the amino acid pathway.

In this paper we describe the characteristics of methionine analogue-resistant mutant which showed an enhanced activity to utilize sulfate for the production of cephalosporin C (CPC). And results presented here suggest that L-cysteine should be a regulator in the biosynthesis of methionine of *Cephalosporium acremonium* like *Aspergillus nidulans*.

Materials and Methods

Strains

A superior antibiotic-producing strain, *Cephalosporium acremonium* N-5-R and its DL-selenomethionine resistant mutant, *C. acremonium* MS-92 were used.

Media and culture conditions

Compositions of the media used are described in Table 1. The seed was propagated in a 100 ml Erlenmeyer flask containing 10 ml of medium for 3 days

at 28 °C on a rotary shaker at 300 rpm. The actively grown cells were inoculated into a 100 m*l* Erlenmeyer flask containing 15 m*l* of the basal medium. The concentration of DL-methionine added is given in the figure legends.

Isolation of analogue-resistant mutants

The culture broth of the basal synthetic medium was filtered through glass wool to separate conidia from mycelia, and the filterate containing conidia was washed twice by centrifugation. The suspension of washed conidia was adjusted to be an order of 10^7 conidia per m*l* of 0.2M potassium phosphate buffer (pH6.5). A condia suspension was treated with 100 μg/m*l* of N-mehtyl-N'-nitro-N-nitrosoguanidine (NTG) to give 90% killing. The treated spores were rapidly filtered and washed with the buffer.

Survivors were spread on minimal agar plates supplemented with 10 μg/m*l* of DL-seleno-methionine and cultivated for 1 week at 28 °C. Colonies that appeared on plates were isolated and then tested for the productivity of CPC.

Assay of CPC

The CPC titer was determined by the agar-diffusion method using a cylinder. *Alcaligenes faecalis* ATCC 8750 was used as a test organism.

Measurement of sulfate uptake rate

Mycelia harvested from the basal synthetic medium were washed twice with potassium phosphate buffer (0.05M, pH6.5) and then suspended in the phosphate buffer to a cell density of 1 mg (dry weight) per m*l*. These resting cells were used for the determination of sulfate uptake rate by the method of Niss *et al.* (8).

And the effect of L-methionine and L-cysteine on sulfate uptake system was investigated as followings. After preincubation for 30 minutes, sulfur-containing amino acid and radioactive sulfate were added simultaneously and the rate of sulfate uptake was measured for 10 minutes.

Measurement of L-methionine uptake rate

Mycelia harvested from the basal synthetic medium supplemented with 0.4% DL-methionine were washed twice with the phosphate buffer and then were suspended in the phosphate buffer to a cell density of 5 mg (dry weight) per m*l*. These resting cells were preincubated for 30 minutes in a rotary shaker at 28 °C and 200 rpm. The uptake was initiated by addition of L-methionine into the cell suspension to a final concentration of 500 μg per m*l*. At time intervals, samples were removed and filtered through membrane filter. The amount of uptaked L-methionine was determined by measuring the residual L-methionine concentration in the filterated broth. L-Methionine concentration was determined by the method of Gehrke *et al.* (9).

Extraction and determination of amino acids and S-adenosylmethionine pools

Cells used for the investigation of internal concentrations of amino acids and SAM were thoroughly washed with distilled water, and extracted with 1.5N perchloric acid for 1 hr in an ice bath. The concentration of SAM was estimated by chromatography on Dowex 50 (Na^+ form, 100-200 mesh) as described by Shapiro and Ehninger (10). Amino acids were analyzed with a fully automatic amino acid analyzer, Bio-tronick LC-500 by the method of Mazumura *et al.* (11).

Preparation of crude enzyme extracts and assay of enzyme activities.

Mycelia harvested from synthetic medium were washed twice with 0.01M Tris buffer solution (pH8.0) containing 2mM EDTA and resuspended in the buffer containing 1mM dithiothreitol (12). The suspension was sonicated with a Fisher dismembrator (model 300) in an ice bath. The protein content of the crude enzyme extracts was estimated by the method of Lowry *et al.* (13). Cysteine desulfhydrylase activity was evaluated according to the method of Kredich (14). And L-serine sulfhydrylase and O-acetyl-L-serine sulfhydrylase activities were measured by the method of Pieniazek *et al.* (15).

Results and Discussion

DL-Seleno-methionine resistant mutant

The minimal concentration of DL-seleno-methionine which had inhibitory effect on the growth of the parent N-5-R conidia was 3μg per m*l*. DL-

Seleno-methionine resistant mutants grown on the minimal medium containing 10 μg per m*l* of the chemical were obtained at a frequency of 3.6×10^{-5} from the parent N-5-R after NTG treatment. A mutant, designated as MS-92, was selected because of its higher productivity of CPC in the sulfate than any other strains. Mutant MS-92 could grow on the minimal medium containing 25 μg/m*l* of DL-seleno-methionine.

Uptake rates of sulfate and L-methionine

In order to illucidate the characteristics of the mutation present in the mutant MS-92, the uptake rates of inorganic sulfate and L-methionine were examined. As shown in Fig. 2 and Fig. 3, the mutant MS-92 exhibited increased activities of sulfate and L-methionine uptake. Although the rate of L-methionine uptake was not remarkably enhanced, the activity of sulfate uptake by the mutant MS-92 exceeded that of its parent by about 50%.

And the enhanced assimilation of sulfate and methionine in the mutant MS-92 was confirmed by tracing ^{35}S-sulfate and by T.L.C. for DL-methionine during the fermentation (16). The specific uptake rates of ^{35}S-sulfate and methionine of the mutant MS-92 were increased during the fermentation in the basal synthetic medium supplemented with 0.4% DL-methionine.

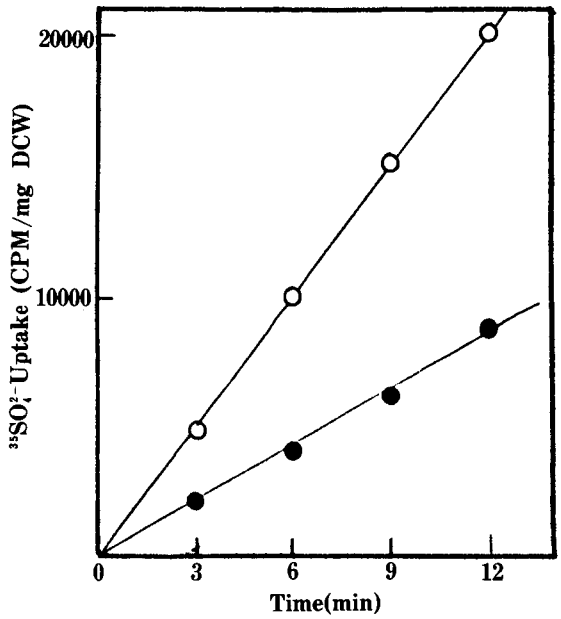

Fig. 2. Uptake of sulfate by the strain N-5-R and the mutant MS-92.
—●—, N-5-R; —○—, MS-92

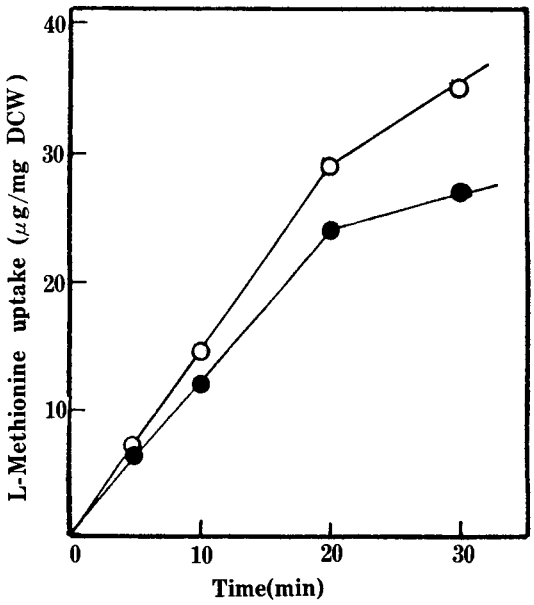

Fig. 3. Uptake of L-methionine by the strain N-5-R and the mutant MS-92.
—●—, N-5-R; —○—, MS-92

Effect of DL-methionine on the production of CPC

The effect of DL-methionine on the production of CPC was compared between the parent N-5-R and the mutant MS-92. Fig. 4 shows that the supplementation of DL-methionine stimulated the production of CPC in both strains. But, in contrast to 0.75% of DL-methionine for the parent N-5-R, the mutant MS-92 required 0.5% of DL-methionine for the maximum production of CPC.

Furthermore, the mutant MS-92 produced more CPC by about 30% than the parent N-5-R in the medium containing sulfate as a sole sulfur source. These different patterns of CPC production might be caused by the difference of the uptake rates of the sulfate and the L-methionine between two strains.

Intracellular pools of amino acids and S-adenosylmethionine

The intracellular amino acids and SAM concen-

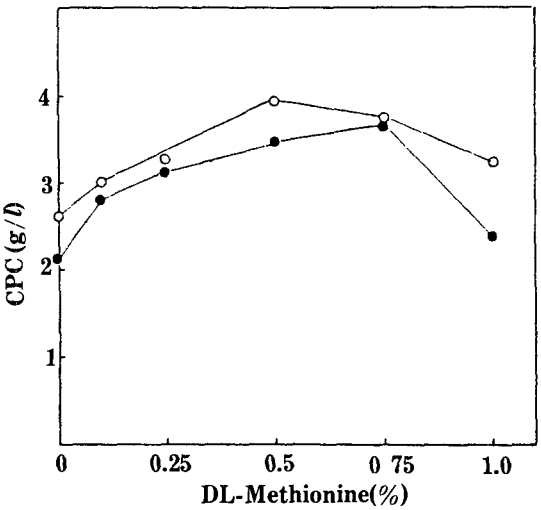

Fig. 4. Effect of DL-methionine on the production of CPC by the strain N-5-R and the mutant MS-92 in the basal complex medium.
—●—, N-5-R; —○—, MS-92

Table 2. Intracellular amino acid concentrations in the cells of the strain N-5-R and the mutant MS-92 grown in the basal synthetic medium*.

A.A. \ Strain	N-5-R	MS-92
Lysine	4.9	4.9
Histidine	2.7	5.5
Arginine	3.0	3.3
Aspartic acid	5.6	4.4
Threonine	8.3	7.1
Serine	5.9	5.7
Glutamic acid	39.2	23.1
Proline	102.9	53.2
Glycine	3.1	2.5
Alanine	17.5	39.5
Valine	1.9	1.6
Methionine**	4.3	58.1
Isoleucine	1.8	0.9
Leucine	1.3	0.4
Tyrosine	0.7	0.9
Phenylalanine	0.9	0.8
Half-cystine	5.2	6.9
Taurine	—	3.7

* Results expressed as μmoles per g cell.
** Not resolved from DL-cystathionine.

trations in the cells of both the parent N-5-R and the mutant MS-92 grown on synthetic medium were compared. As shown in Table 2 and Table 3, the higher intracellular concentrations of methionine and SAM were observed in the mutant MS-92. These results indicated that the mutant MS-92 should more actively synthesize methionine and SAM from sulfate than the parent N-5-R. And that the concentration of SAM of two strains was hardly influenced by the methionine of the media is suggested that methionine should not be a regulator in the biosynthesis of SAM.

Effects of L-methionine and L-cysteine on the sulfate uptake system

A series of experiments had been carried out to investigate the effects of sulfur-containing amino acids on sulfate uptake system and to characaterize its mechanism.

As shown in Table 4, the uptake rate of sulfate of *C. acremonium* N-5-R was influenced by L-methionine very little, if any, but severely inhibited by L-cysteine. This result suggests that L-cysteine might be a extracellular inhibitor on the sul-

Table 3. Intracellular S-adenosylmethionine concentrations of the strain N-5-R and the mutant MS-92

Strain	S-Adenosylmethionine concentration (μmole / g DCW)	
	A[a]	B[b]
N-5-R	0.08	N. D[c]
MS-92	0.55	0.57

a. Basal synthetic medium.
b. Basal synthetic medium supplemented with 0.4% DL-methionine.
c. Not detected.

Table 4. Effect of L-methionine and L-cysteine on the sulfate uptake system of the strain N-5-R

Addition (10μmole/ml)	Relative uptake rate (% of control)
None	100
L-Methionine	> 90
L-Cysteine	< 15

fate uptake system in *C. acremonium*. This amino acid, however, is not extracellular inhibitor in the case of *P. chrysogenum* (17). This different effect of L-cysteine on the sulfate uptake system might represent that sulfur metabolism is regulated in different mode between *C. acremonium* and *P. chrysogenum*. In other words, *P. chrysogenum* can obtain sulfur for antibiotic synthesis very efficiently via the reduction pathway from inorganic sulfate. But *C. acremonium* obtains it from methionine via reverse transsulfuration pathway (from L-methionine to L-cysteine in Fig. 1).

Enzymatic activities of cysteine metabolism

Two enzyme reactions have been reported for the biosynthesis of L-cysteine in microorganism: O-acetyl-L-serine sulfhydrylase (EC 4. 2. 99.8) and L-serine sulfhydrylase (EC 4. 2. 1. 22). Although there was no significant difference in the activity of O-acetyl-L-serine sulfhydrylase between the parent N-5-R and the mutant MS-92, the mutant MS-92 exhibited lower activity of L-serine sulfhydrylase than the parent N-5-R (Table 5).

It was also reported that the activity of O-acetyl-L-serine sulfhydrylase does not influence the production of CPC(18), and sulfate is preferentially utilized via methionine synthase (EC 4. 2. 99. 10) rather than via O-acetyl-L-serine sulfhydlase in *C. acremonium* (19). And L-serine sulfhydrylase had been demonstrated to be identical with cystathionine β-synthase (EC 4. 2. 1. 22) in *A. nidulans* (15). If the two enzymes are also identical in *C. acremonium*, L-serine sulfhydrylase is important in the conversion of sulfate into L-cysteine. Therefore, the mutant should not synthesize L-cysteine from sulfate and methionine as efficiently as the parent N-5-R because of the low activity of L-serine sulfhydrylase.

Table 5. Cysteine biosynthetic activities of the parent N-5-R and the mutant MS-92.

Strain	O-Acetyl-L-serine-sulfhydrylase[a] of cells grown on		L-Serine sulfhydrylaes[a] of cells grown on	
	A[b]	B[c]	A	B
N-5-R	2.40	2.80	1.15	1.00
MS-92	2.34	2.27	0.79	0.72

a. Results expressed as formed L-cysteine, nmole/min mg protein.
b. Basal synthetic medium.
c. Basal synthetic medium supplemented with 0.4% DL-methionine.

Table 6. L-Cysteine desulfhydrylase activities of the strain N-5-R and the mutant MS-92[a]

Strain	Specific activities of cells grown on	
	A[b]	B[c]
N-5-R	40.0	48.7
MS-92	27.0	24.7

a. Results expressed as formed H_2S, nmole/min mg protein.
b. Basal synthetic medium.
c. Basal synthetic medium supplemented with 0.4% DL-methionine.

On the other hand, cysteine desulfhydrylase, a catabolic enzyme of L-cysteine, was different between the parent N-5-R and the mutant MS-92 in its activity. As shown in Table 6, the activity of the enzyme in the mutant MS-92 was decreased by about 50% as compared with that in the parent N-5-R. This enzyme activity is used as an index of intracellular cysteine concentration (12). However, the mutant MS-92 showed slightly higher level of intracellular cysteine than the parent N-5-R. This seemingly contradictionary result might be explained by the relativity of methionine concentration between the parent N-5-R and the mutant MS-92 mentioned later.

In addition, Komazu *et al.* (12) isolated a mutant, *C. acremonium* IS-5 which showed an enhanced activity of L-serine sulfhydrylase. The mutant accumulated an excess amount of L-cysteine from methionine, and then inhibited the production of CPC by an excess amount of DL-methionine. They concluded that methionine should inhibit the production of CPC via L-cysteine.

On the contrary, our mutant MS-92 was less inhibited by methionine for the production of CPC than the parent (Fig. 4). The insensitivity for the production of CPC by the mutant MS-92 from the methionine might reflect a lower activity of L-serine

sulfhydrylase, by which the intracellular concentration of L-cysteine could be maintained relatively lower level even under a high concentration of methionine pool in the mutant MS-92 (Table 2).

If,due to the low intracellular concentration of L-cysteine, the sulfate assimilation of the mutant MS-92 was less inhibited and/or repressed by the amino acid, it could be suggested that L-cysteine should be a regulator in sulfur metabolism of *C. acremonium* like *A. nidulans*. For further studies, employment of ^{35}S-labeled compounds are required to separate sulfur-containing amino acids quantitatively, and then it is possible to confirm L-cysteine as a regulator in the biosynthesis of L-methionine.

요 약

DL-Seleno-methionine에 내성을 갖는 변이주, *Cephalosporium acremonium* MS-92는 무기황과 메치오닌의 uptake rate가 모균보다 우수하였으며, 세포내에 과량의 메치오닌과 S-adenosylmethionine (SAM)을 축적하였다. 또 *C. acremonium*에서 무기황의 uptake rate는 시스테인에 의해 크게 저해되었다. Crude enzymes extracts에서 변이주 MS-92는 cell내에 cysteine을 적게 형성하였다. 즉, L-serine sulfhydrylase 활성이 모균보다 낮았으며 세포내에 시스테인의 양을 간접적으로 알 수 있는 시스테인 분해효소인 cysteine desulfhydrylase 활성이 변이주에는 모균의 약 반이였다.

이상의 결과에서 *C. acremonium*에서 메치오닌을 만드는 효소들의 조절에는 *A. nidulans*와 비슷하게 시스테인이 관여한다는 것을 추측할 수 있었다.

References

1. Queener, S.W., S. Wilkerson, D.R. Tunin, J.P. McDerott, J.L. Charpman, C. Nash, C. Platt and J. Westphening: In Biotechnology of Industrial Antibiotics, ed. E.J. Vandamme, p. 141 (1984) Mercel Dekker Inc., Belgium
2. Surdin-Kerjan, Y.H., Charest and H. Robichon-Szulmajster: *Acta. microbiol. Acad. Sci. Hung.*, **23,** 109 (1976).
3. Cherest, H., Y. Surdin-Kerjan and H. Robichon-Szulmajster: *J. Bacteriol.*, **123,** 428 (1976).
4. Cherest, H., Y. Surdin-Kerjan and H. Robichon-Szulmajster: *J. Bacteriol.*, **106,** 758 (1971).
5. Greene, R.C., J.S.V. Hunter and E.H. Coch: *J. Bacteriol.*, **115,** 57 (1973).
6. Komatsu, K-I., T. Yamada and R. Kodaira: *J. Ferment. Technol.*, **52,** 93 (1974).
7. Paszewski, A and J. Grabski: *Molec. Gen. Genet.*, **132,** 307 (1974).
8. Niss, H.F., and C.H. Nash: *Antimicrob. Ag. Chemother.*, **4,** 474 (1971).
9. Gehrke, C.W., and T.E. Neuner: *JAOAC.*, **57,** 682 (1974).
10. Shapiro, S.K., and Ehninger, D.J.: *Anal. Biochem.*, **15,** 323 (1966).
11. Mazumura, M., T. Yoshida and T. Taguchi: *Eur. J. Appl. Microbiol. Technol.*, **16,** 114 (1982).
12. Komatsu, K-I and R. Kodaira: *J. Antibiot.*, **30,** 226 (1977).
13. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.T. Randall: *J. Biol. Chem.*, **193,** 265 (1951).
14. Kredich, N.M.: *J. Biol. Chem.*, **246,** 3474 (1971).
15. Pieniazek, N.J., P.P. Stepien and A, Paszewski: *Biochem. Biophys. Acta,* **297,** 37 (1973).
16. Lee, K.: M.S. Dissertation, KAIST, (1987).
17. Yamamoto, L.A. and I.H. Segel: *Biochem. Biophys. Acta,* **81,** 158 (1964).
18. Lierseh, M., H.J. Treichler and J. Nüesch: *Experientia,* **36,** 487 (1980).
19. Treichler, H.J., M. Liersch, J. Nüesch, and H. Dobeli: In Genetics of Industrial Microorganisms, ed. O.K. Sebek and A.I. Laskin, p. 97 (1979) ASM, Washington DC.

(Received July 21, 1987)