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Characterization of Methionine Analogue-Resistant Mutant of *Cephalosporium acremonium*

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메치오닌 유사체 내성 *Cephalosporium acremonium* 변이주의 특성

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ABSTRACT: ***Cephalosporium acremonium*** **MAR-80, a strain of methionine analogue-resistant mutant, showed good activity of sulfate utilization as only sulfur source. The effect of methionine on the sulfate uptake system was investigated by using $Na_2^{35}SO_4$ as a tracer in the resting cell system. From this result, it was revealed that sulfate permease of this strain was less repressed and/or less inhibited by methionine than parent type. This deregulation was due to low activity of methionine uptake, which was operated by somewhat simple diffusion. From these studies, it could be anticipated that the improved productivity of cephalosporin C and lower dependence of cephalosporin C production on methionine were related to increased uptake rate of sulfate.**

KEY WORDS □ ***Cephalosporium acremonium*****,cephalosporin C (CPC), DL-seleno-methionine, sulfate uptake, L-methionine uptake.**

A sulfur atom of the antibiotic cephalosporin C (CPC) synthesized by *Cephalosporium acremonium* is derived from L-cysteine. L-Cysteine can be formed from inorganic sulfate via the sulfate reduction pathway or from L-methionine via the reverse transsulfuration pathway (Nüesch *et al.*, 1973). But the sulfur atom of CPC is preferrentially supplied by methionine via the reverse transsulfuration pathway (Caltrider *et al.*, 1966). In fact, the synthesis of CPC occurs more efficiently in the presence of methionine than in the presence of sulfate without methionine (Demain *et al.* 1963).

Thus methionine plays the role of sulfur donor for the synthesis of CPC, and in addition, there is some evidence suggesting that methionine act as a regulator of CPC synthesis (Drew *et al.*, 1975 a; Drew *et al.*, 1975 b; Sawada *et al.*, 1980). Alternatively, Treichler *et al.* (1979) suggested that a direct sulfur donor should be cystathionine, an intermediate in the reverse transsulfuration pathway.

Numerous attempts have been made to obtain specific mutants of *C. acremonium* altered for sulfur metabolism and for their potential to synthesize CPC from sulfate.

Mutant, M8650-sp-1 utilized sulfate as sulfur source for the production of CPC as effectively as they utilized methionine. (Niss *et al.*, 1973).

Another mutant, IS-5, with enhanced potential to use sulfate as the sulfur source for CPC production, had potency levels more than two-fold that of parent. (Komatsu *et al.*, 1975). This mutant required less DL-methionine for the maximal production of CPC, but an excess methionine inhibited the synthesis of the antibiotic (Komatsu *et al.*, 1975, 1977).

A DL-seleno-methionine resistant mutant, SMR-I3 produced three-fold more CPC from sulfate than its parent. Furthermore, the mutant accumulated excess methionine in the amino acid pool and possessed superior activity for sulfate uptake (Mazumura *et al.*, 1982).

Fermentation with methionine produce an odor that is very unpleasant and are costly as well. So, it is highly desirable to obtain a strain capable of using sulfate rather than methionine for the production of CPC. This paper describes the characteristics of DL-seleno-methionine resistant mutant of *C. acremonium* which possesses enhanced productivity of CPC with sulfate. And, the effects of an increased sulfate uptake rate and a reduced methionine uptake rate of strain MAR-80 on the productivity of CPC are revealed.

MATERIALS AND METHODS

Strains

A superior antibiotic-producing strain of *Cephalosporium acremonium* N-5-R was used as a parent strain for the selection of DL-seleno-methionine resistant mutant. A mutant, designated as MAR-80, was selected and used through these experiments.

Media and culture conditions

Microorganisms producing CPC were freeze-dried in vials for storage. Compositions of the media used are described in Table 1.

The seed was propagated in a 100m*l* Erlenmeyer flask containing 10m*l* of medium for 3 days at 28°C on a rotary shaker at 300rpm. The actively grown cells were inoculated into a 100m*l* Erlenmeyer flask containing 15m*l* of the basal synthetic 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(pH 6.5). A conidia suspension was treated with 100μg/m*l* of N-methyl-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 10μg/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.

Table 1. *Composition of culture media*

Medium	Composition(%)
Minimal agar medium	Glucose 1.0, Asparagine 0.1, $MgSO_4$ $7H_2O$ 0.05, KH_2PO_4 0.046, 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)

* $MgSO_4$ 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 100 m*l* distilled water.

ANALYSIS

Cell mass

A 2m*l* portion of the culture broth was filtered through a preweighted whatman filter paper (number 5) and washed twice with distilled water and dried in an oven (90°C) to constant weight.

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 in the resting cell system

Mycelia harvested from the basal synthetic medium were washed twice with potassium phosphate buffer(0.05M, pH6.5) and then suspended in phosphate buffer to a cell density of 1mg(dry weight) per m*l*. Mycelia were preincubated for 30 minutes in a rotary shaker at 28°C

and 200rpm. The measurements of sulfate uptake rate were initiated by adding $Na_2^{35}SO_4$ into cell suspension to a final concentration of 1μCi/m*l*. Samples(1m*l*) were removed, filtered onto membrane filters, and washed successively with 5m*l* of cold phosphate buffer containing 5μg of sodium sulfate per m*l*. Radioactivity was determined by a scintillation counter (Beckman LS 6800). Controls were prepared by treating mycelia with labeled sulfate and by washing them immediately with cold buffer containing sulfate.

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 5mg(dry weight) per m*l*. These resting cells were preincubated for 30 minutes in a rotary shaker at 28°C and 200rpm. The uptake was initiated by addition L-methionine into cell suspension to a final concentration of 500μg per m*l*. At the time intervals, samples were removed and filtered through membrane filter. The amounts of uptaked L-methionine were determined by measuring the residual L-methionine concentration in the filterated broth. L-Methionine concentrations were determined by the method of Gehrke *et al.* (1974).

Measurement of sulfate assimilation during the fermentation in the excess DL-methionine

Initially, 0.5μCi of $Na_2^{35}SO_4$ were added to 15m*l* of the basal synthetic medium supplemented with 0.4% DL-methionine. At the time intervals, cells were harvested and washed with phosphate buffer, and then intracellular radioactivities were checked.

RESULTS AND DISCUSSION

Selection of methionine analogue-resistant mutant

The minimal concentration of DL-seleno-methionine which had an 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 10μg 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 MAR-80, was selected for further studies because of its higher productivity of CPC than any other strains. Mutant MAR-80 showed about four-fold increased minimal inhibitory concentration(MIC) of DL-seleno-methionine than that of the parent(Table 2).

Table 2. *Comparison of the parent N-5-R with the mutant MAR-80 about the minimum inhibitory concentrations (MIC) of DL-seleno-methionine*

Strain	MIC of DL-seleno-methionine[a](μg/m*l*)
N-5-R	3
NAR-80	12

a. Determined in the minimal agar medium.

Effect of methionine on CPC production by mutant MAR-80

The relationship between MIC of methionine analogue and the effect of DL-methionine on the production of CPC by the parent and the mutant MAR-80 was investigated. As shown in Fig. 1A, the mutant MAR-80 produced two-fold more CPC in the abscence of methionine than its parent. Maximal production of CPC by the parent N-5-R was obtained at the concentration of 0.2% of DL-methionine, whereas the mutant MAR-80 did not

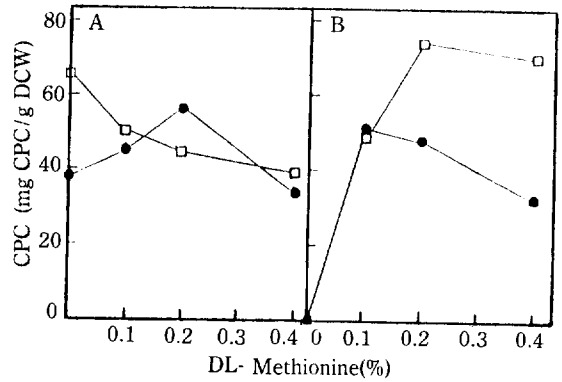

Fig. 1. *Effect of DL-methionine on the productivity of CPC by the strain N-5-R and the mutant MAR-80.*
A; basal synthetic medium
B; basal synthetic medium except 0.4% Na_2SO_4 (●), N-5-R; (□), MAR-80

require DL-methionine for the maximal production. The productivity of CPC of the mutant MAR-80 was not clearly inhibited by an excess amount(0.4%) of DL-methionine as compared with those of the analogous mutants, IS-5 and SMR-I3.

Fig. 1B showed that the mutant MAR-80, however, required higher concentration of DL-methionine for the maximal production of CPC in the media containing DL-methionine as only sulfur source than in the media containing DL-methionine and sulfate. And the mutant MAR-80 produced more CPC from the former media rather than from the media containing both sulfate and DL-methionine.

Comparison of sulfur metabolism between the mutant MAR-80 and the parent

In order to characterize these patterns of CPC production, the activities of sulfate and L-methionine uptake by the mutant MAR-80 were examined. As shown in Fig. 2, the rate of sulfate uptake by the mutant MAR-80 exceeded that of its parent N-5-R by about 50% in the resting cell system. And Fig. 3 showed that the rate of

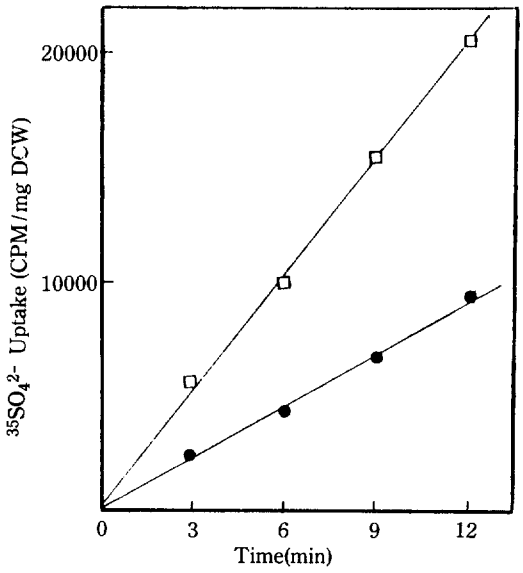

Fig. 2. *Uptake of sulfate by the strain N-5-R and the mutant MAR-80.*
The washed cells grown on the basal synthetic medium were used for sulfate uptake experiments. (●), N-5-R; (□), MAR-80

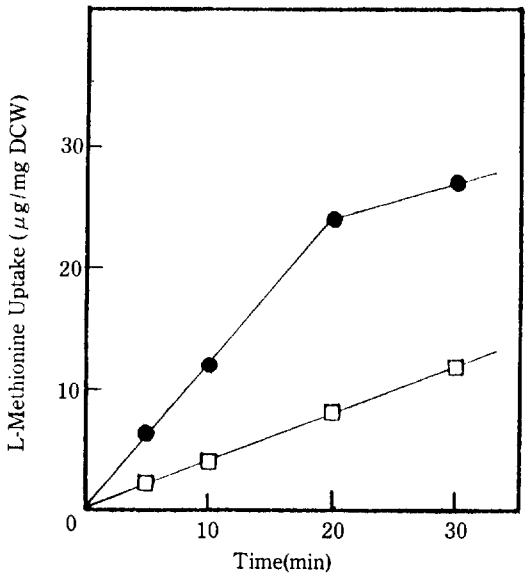

Fig. 3. *Uptake of L-methionine by the strain N-5-R and the mutant MAR-80.*
The washed cells grown on the basal synthetic medium supplemented with 0.4% DL-methionine were used for these experiments.
(●), N-5-R; (□), MAR-80

L-methionine uptake by the mutant MAR-80 was decreased by about 40% as compared with that by the parent N-5-R. Thus, it was suggested that the mutant MAR-80 possessed superior activity of sulfate assimilation and low activity of methionine assimilation.

Above consequence might be related to the production of CPC as shown in Fig. 1. The increased production of CPC by the mutant MAR-80 with sulfate as the sulfur source might be resulted from the superior capacity for sulfate assimilation. On the other hand, the higher productivities in the media containing DL-methionine as only sulfur source are to be related to the low activity of methionine assimilation by the mutant MAR-80. This assumption may be interpreted by the suggestion of Benz *et al.* (1971) and Nüesch *et al.* (1973), which is that D-methionine is more efficient than L-methionine in the production of CPC because the former is much more slowly taken up and metabolized, and therefore, D-methionine ensures a long lasting

pool of free methionine available for CPC synthesis in the cell.

From these results and characteristics of another methionine analogue-resistant mutant, SMR-I3, it could be concluded that the capacity to synthsize CPC with sulfate as only sulfur source is dependent upon the rate of sulfate uptake, and the maximal production of CPC is not always proportional to the rate of L-methionine uptake.

Characterization of L-methionine uptake system

As shown in Fig. 3, the mutant MAR-80 showed a lower activity of L-methionine uptake than the parent N-5-R, and L-methionine permease of the mutant MAR-80 was not saturated, though that of the parent N-5-R was saturated in 30 minutes. Therefore, the mode of permease of L-methionine was to be investigated to determine how it was operated, for example, by an active transport or by a simple diffusion. For this purpose, sodium azide(NaN_3) was selected as a blocking agent of active transport system because it is an inhibitor of oxidative phosphorylation which inibits electron flow between cytochrome($a + a_3$) and O_2.

Table 3 showed that 2mM sodium azide almost completely inhibited L-methionine transport of the parent which suggested that intracellular accumulation of methionine should be dependant on metabolic energy, a characteristic of an active transport. The L-methionine transport of the mutant MAR-80, however, was less inhibited by sodium azide than that of the parent N-5-R. These results indicated that the mode of L-methionine permease was altered from an active transport to a somewhat simple diffusion by mutation. Marzluf (1970) claimed that *N. crassa* has two distinct sulfate transport systems. The transport system (permease I) predominating in the conidial stage is replaced by permease II during the outgrowth into the mycelial phase. Further studies are needed to elucidate whether the L-methionine permease in *C. acremonium* is changeable or alternative like sulfate transport system in *N. crassa*.

Table 3. *Effect of sodium azide on L-methionine uptake by the parent N-5-R and the mutant MAR-80*

Strain	Addition* (2mM)	L-Methionine** uptake (μg/hr·mg DCW)	% of control
N-5-R	None(control)	60.5	100
N-5-R	Sodium azide	3.4	6
MAR-80	None(control)	25.3	100
MAR-80	Sodium azide	5.2	21

* Sodium azide (NaN_3) was added 2 minutes before methionine.

** The washed cells grown on the basal synthetic medium supplemented with 0.4% DL-methionine were used for L-methionine uptake experiment. Uptake of L-methionine was determined for 10 minutes.

Characterization of sulfate uptake system

The mutant MAR-80 possessed superior uptake rate of sulfate to the parent in the resting cell system(Fig. 2). The difference of sulfate uptake rate in the resting cell system was to be confirmed again in the presence of excess amount of DL-methionine between the parent and the mutant MAR-80.

As shown in Fig. 4, despite a presence of an excess DL-methionine, the mutant MAR-80 assi-

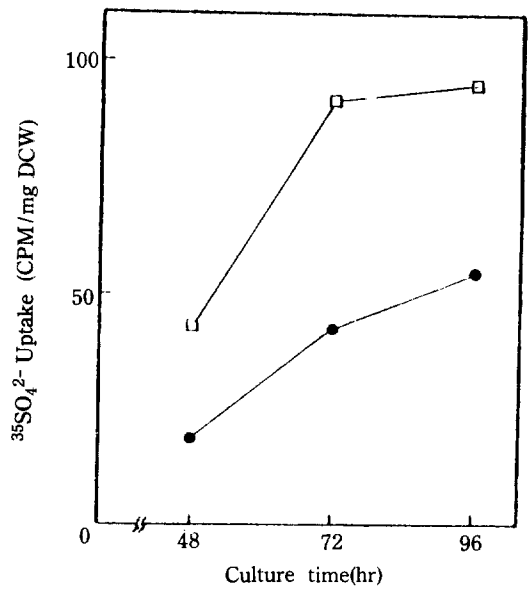

Fig. 4. *Uptake of sulfate during the fermentation of the strain N-5-R and the mutant MAR-80.*
Medium; basal synthetic medium supplemented with 0.4% DL-methionine.
(●), N-5-R; (□), MAR-80

milated two-fold more sulfate than the parent N-5-R. It is assumed that the sulfate permease of the mutant MAR-80 has been less inhibited and/or repressed by the metabolite(s) of methionine as compared with that of the parent N-5-R. This deregulation might be due to low activity of methionine uptake. It is well-known that sulfate utilization(include sulfate permease) is repressed by the metabolite(s) of sulfur-containing end product in other microorganisms(Cherest *et al.*, 1973; Paszewski *et al.*, 1974; Surdin-Kerjan *et al.*, 1976).

In addition, Fig. 4 showed that sulfate was majorly assimilated during the early stationary phase(from 48hr to 72hr) in the culture of basal synthetic medium supplemented with 0.4% DL-methionine. This phenomenon could be interpreted by the followings. Methionine was almost utilized during actively growing phase, and the growth was ceased after 48 hours of cultivation. But, it was not until 48hr cultivation that CPC production was commenced (Lee, 1987). Therefore, it could be suggested that sulfate should be utilized as alternative sulfur source for the CPC production after exhaustion of methionine.

적 요

Cephalosporium acremonium MAR-80은 모균에 비해 메치오닌 유사체(DL-seleno-methionine)에 대한 최소 성장저해 농도(MIC)가 4배 이상 증가된 메치오닌 유사체 내성 변이주이다. 이 변이주는 휴지세포계(resting cell system)에서 모균보다 2배 많이 sulfate를 잘 uptake하였으며, 과량의 메치오닌을 포함한 발효에서도 sulfate를 잘 이용하였다. 이와같이 변이주의 sulfate transport가 모균에 비해 메치오닌에 영향을 더 작게 받은 것은 변이주의 methionine uptake system이 모균에 비해 낮은 활성을 보인 것과 모균의 active transport와 달리 어느 정도 확산(diffusion)에 의해 메치오닌을 이용하였다는 사실로 추정되었다. 이 변이주는 황의 성분으로 sulfate만 있는 배지에서 모균보다 2배 많이 세팔로스포린 C를 생산하였다. 또한 최대 세팔로스포린 C를 만들기 위해 모균은 0.2% DL-메치오닌을 요구하였으나, 이 변이주는 요구하지 않았다. 이것은 변이주의 sulfate uptake system의 활성이 모균에 비해 증가된 것으로 추정될 수 있었다.

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