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Inhibitory Effect of Cephalosporin C on Growth of *Cephalosporium acremonium* M-113

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Cephalosporium acremonium M-113 의 세팔로스포린에 의한 생장억제 효과

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Cephalosporin C (CPC) inhibited the growth of *Cephalosporium acremonium* M-113, a potent CPC producer derived from *C. acremonium* ATCC 20339. Similar inhibitory effects of CPC were also observed in growth of *C. acremonium* ATCC 20339 and ATCC 14553. Minimum inhibitory concentrations (MIC) of CPC on the growth of conidia and hyphae of *C. acremonium* M-113 were 200-500 and 3000-4000 μg/ml respectively in synthetic medium. MIC values were increased in complex media. The inhibitory effect of CPC was due to CPC-exerted inhibition of amino acids uptake by the cells. 3'-Group of CPC might be important in its inhibitory action. In addition, CPC itself could be utilized by the cells as a nitrogen source under nitrogen limited condition.

Generally antibiotic-producing microorganisms are more resistant to the action of their own antibiotics than the species that cannot synthesize them. The antibiotic-producing species manage to avoid suicide by mechanisms that include (i) modification and detoxification of the antibiotic by enzymes formed by the producer, (ii) alteration of the antibiotic target in the producer, and (iii) a decreased inward permeability to the antibiotic after it has been excreted.[1,2] However, it has been reported that some antibiotics such as bacitracin, actinomycin, tetracyciline, novobiocin, streptomycin and nystatin inhibited the growth of their own producers.[1,3-7] Interestingly, we also observed similar inhibitory effect that antibacterial antibiotic, cephalosporin C (CPC) inhibited the growth of its producer, fungus, *Cephalosporium acremonium* M-113. In this paper, we aimed at elucidation of the nature of the inhibitory effects on the cell growth by CPC.

Materials and Methods

Microorganism

Cephalosporium acremonium M-113, a potent CPC-producer, was derived from *C. acremonium* ATCC 20339 through mutagenesis by our research group.

Preparation of resting cells of conidia and hyphae

C. acremonium M-113 was inoculated on the plate of PBNA medium and incubated at 28°C for 1 week to form the lawn of the cells. The composition of the medium was peptone, 1.0%; beef extract, 0.5%; NaCl, 0.25%; and agar, 1.5% with pH7.0. The surface of the cultured PBNA plate was scraped off with 10 ml of sterile saline and conidia were collected by passing the scraped solution through the What-

man No. 1 filter paper with pressure or glass wool filter to separate conidia from hyphae. The filtrate contained almost conidia including unicellular arthrospores. Resuspension of the filtered residue in 10ml of sterile saline solution contained almost hyphae. They were washed 3 times with sterile saline and used as resting cells.

Measurement of minimum inhibitory concentration (MIC) of CPC on cell growth

A synthetic medium was formulated and used to study the inhibitory effects of CPC. It contained glucose, 0.5%; sucrose, 2.0%; DL-methionine, 1.0%; phosphate, 200mM (the ratio in mM of phosphate in KH_2PO_4 to that in K_2HPO_4 was always 1:3); and stock salt solution, 0.1ml per *l* with pH7.0. The stock salt solution was N-free and contained $MgCl_2 \cdot 6H_2O$, 3.0%; $FeCl_2$, 0.4% $MnCl_2 \cdot 4H_2O$, 0.4%; $ZnCl_2$, 0.15%; $CuCl_2$, 0.042%; and $CaCl_2$, 0.6%. Ammonium ion was omitted from the above medium, because *C. acremonium* M-113 could not utilize it as a nitrogen source, which was identified by using an ammonia electrode (Orion Research Inc.). Resting cells of conidia and hyphae were inoculated into the synthetic medium supplemented with filter-sterilized CPC. The culture was incubated under 250 rpm at 28°C. Cell growth was determined after 5 days of culture. Viable cell counting was done to determine the growth of culture that could not be easily observed.

Determination of cell mass

Cell concentration was measured by dry weight of washed cell mass on preweighed the Whatman No. 50 filter paper (washed several times with distilled water and dried over night at 90°C.).

Determination of CPC

CPC was assayed by high performance liquid chromatography (HPLC) using a reverse phase column of μ Bondapak C-18 (Waters Associates).

Table 1. Minimum inhibitory concentration (MIC) of CPC on the growth of *C. acremonium* M-113.

Cell	CFU[a]	Minimum Inhibitory Concentration (μg/ml)
Conidia	10	100-200
	10^4	200-300
	10^6	400-500
Hyphae	10^4	3000-4000

a, Colony-forming units per ml.

Table 2. MIC values on various culture media.

Media	Minimum Inhibitory Concentration (μg/ml)
Synthetic	200-300
Seed[b]	30000-50000
Fermentation[c]	50000-100000

a, Conidia of about 10^4 per ml were used.
b, Corn steep liquor, 0.5%; sucrose, 3.0%; beef extract, 1.5%; $CaCO_3$, 0.15% with pH 7.0.
c, Sucrose, 2.0%; glucose, 0.5%; peanut meal, 3.0%; soybean meal, 3.0%; DL-methionine, 1.0%; $CaCO_3$, 0.15% with pH 7.0.

A varian 5 000 HPLC was run at ambient temperature with pressure of about 100 atm. and flow rate of 1.5 ml/min. For mobile phase, 0.03% potassium dihydrogen phosphate was used.

Determination of glucose and amino acids

Glucose was measured by using PGO enzymes (Sigma Co.). DL-Methionine was determined by ninhydrin reaction as previously reported.[8] Other amino acids including DL-methionine were analyzed with a amino acid analyzer BIOTRONIK LC5000 (WGG, Germany) with column of BTC2710.

Results and Discussion

Minimum inhibitory concentration (MIC) of CPC on growth of *C. acremonium* M-113.

Table 1 shows the MIC of CPC on the growth of conidia and hyphae in synthetic medium, which were two typical morphological types of *C. acremonium* M-113 during growth with differentiation.[9,10] Although the MIC on conidia increased with the increasing number of the cells, MIC was still below 500 μg/ml of CPC concentration. However, hyphae were shown to be more resistant to CPC than conidia at the same colony forming units of 10^4. This might be due to the inherent nature of hyphae rather than the many cells per one hyphal fragment, i.e. one colony forming unit, which will be discussed later. Thus, because one hyphal fragment almost consisted of many cells within a rigid cell wall,[9] conidia including unicellular arthrospores were used in the following experiments, unless otherwise indicated.

The susceptibility of the cells to CPC was markedly influenced by the culture media employed as shown in Table 2. In seed and fermentation media, the MIC increased significantly compared with that in synthetic medium. From

Table 3. Effect of CPC on the growth of other *Cephalosporium* strains[a].

Strain	Minimum Inhibitory Concentration ($\mu g/ml$)
M-113	200-300
ATCC 20339	200-350
ATCC 14553	350-500

a, Conidia of each strain were used at about 10^4 per ml.

this result, it was evident that some nutrient component in these complex media could prevent CPC from exerting its inhibitory effect on cell growth.

In Table 3, MICs of CPC on the the growth of other *Cephalosporium* strains are shown. Both the growth of *C. acremonium* ATCC 14553 and ATCC 20339, a parent strain of *C. acremonium* M-113, were also inhibited by CPC at similar MIC values. Therefore, CPC-exerted inhibition of cell growth was found to be a common phenomenon occured in *C. acremonium* M-113

Effect of CPC addition to growing cultures of *C. acremonium* M-113

When the addition of CPC to a culture of *C. acremonium* M-113 was delayed a few hours after the culture was started, the antibiotic had no effect on the subsequent growth. As shown in Fig. 1, in contrast to the cell growth on the medium without CPC, no significant differences were observed with addition of CPC at 3 hrs after starting of cultivation. If CPC is to be effective in suppressing the cell growth, it should be present at the starting point of culture or shortly thereafter. Since only conidia and their germinations were observed in 3 hours after initiation of the culture, we conclude that the basis for the aquisition of resistance might be due to the slow permeation of CPC into the cells rather than the increse in cell number with culture time.

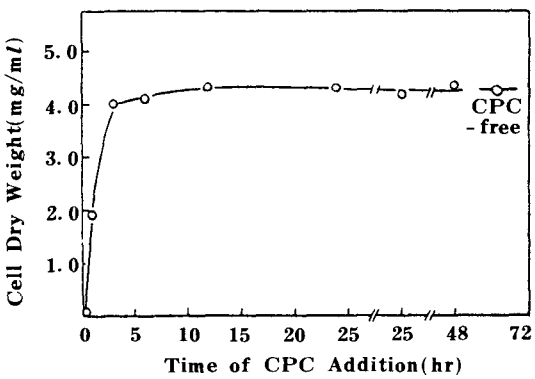


Fig. 1. Effect of CPC addition time on cell growth.
1000$\mu g/ml$ of CPC was added to each flask containing about 10^4 conidia per ml at each time indicated. Cell mass was determined after 5 days of culture.

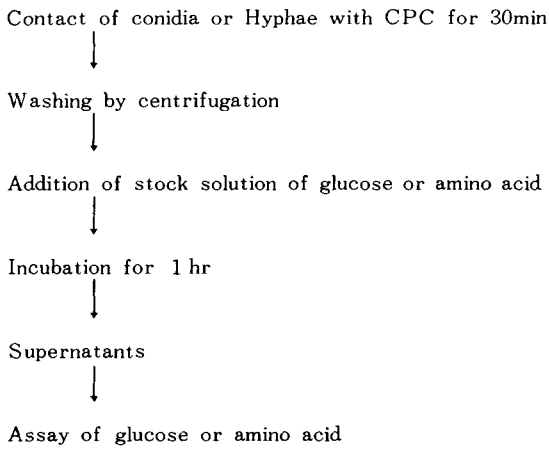


Fig. 2. Overall Procedures of resting cells experiments.

Effect of CPC on carbon and nitrogen by resting cells of *C. acremonium* M-113.

Resting cells were used to elucidate the nature of inhibitory effects of CPC on cell growth. The overall procedures of the resting cells experiments are shown in Fig. 2. To test directly whether uptakes of glucose or DL-methionine by the cells were inhibited by the presence of CPC, various concentrations of CPC were added to the washed conidia or hyphae (ca. 5mg dry wt. of the cells) suspensions. They were shaked with 250rpm at 28°C for 30min for sufficient contact of cells with CPC. Then , CPC was analyzed by HPLC to determine the uptake amount of CPC by the cells. CPC uptake was occurred significantly during 30min and conidia had more ability in CPC uptake than hyphae (Table 4). This indicated that conidia might be more susceptible to the inhibitory effect of CPC than hyphae, which was consistent with the results shown in Table 1. After the removal of CPC, stock

Table 4. The rate of CPC uptake by the cells.

Cell	Rate of CPC-uptake
Conidia	12.4[a]
Hyphae	1.08

a; $\mu g/min/mg$ cell

Table 5. Effect of CPC on glucose uptake.

Cell	CPC ($\mu g/ml$)					
	0	250	500	1000	2000	4000
Conidia	0.091[a]	0.203	0.197	0.206	0.206	N.D.[b]
	(100)[c]	(106.3)	(103.1)	(107.9)	(107.9)	
Hyphae	0.062	N.D.	0.061	0.062	0.061	0.061
	(100)		(98.4)	(100)	(98.4)	(98.4)

a; mg/hr/mg cell
b; not determined
c; %

Table 6. Effect of CPC on DL-methionine uptake.

Cell	CPC ($\mu g/ml$)					
	0	250	500	1000	2000	4000
Conidia	134[a]	119	60	0	0	N.D.[b]
	(100)[c]	(88.8)	(44.8)	(0)	(0)	
Hyphae	55	N.D.	45	30	0	0
	(100)		(81.8)	(54.5)	(0)	(0)

a; $\mu g/hr/mg$ cell
b; not determined
c; %

solution (0.2%) of glucose or DL-methionine was added to the washed conidia or hyphae suspensions. They were again incubated with shaking of 250rpm at 28°C. After 1hr, the supernatants were analyzed for glucose or DL-methionine to determine the amount uptaked by the cells. As shown in Table 5, contact of cells with CPC at various concentrations did not exert any detectable inhibitory effect on glucose uptake by the both of cell types, conidia and hyphae. But DL-methionine uptake was markedly inhibited with the increasing concentration of CPC(Table 6). DL-methionine uptake by conidia and hyphae was completely inhibited at 500-1000 and 1000-2000 μg/ml of CPC respectively. Although the rate of DL-methionine uptake by hyphae was less than that by conidia at CPC-free condition, more amount of CPC was required to inhibit the DL-methionine uptake completely. This indicated that hyphae might have more resistance to CPC than conidia, which was consistent with the results shown in Table 1. The resistance of hyphae might be their inherent nature such as differences in cell wall composition from that of conidia. To test whether uptakes of other amino acids were inhibited by CPC or not, pool of 20 amino acids was added the resting cell suspension. As shown in Table 7, similar in-

Table 7. Effect of CPC on uptake[a] of each of 20 amino acids.

Amino Acids	CPC ($\mu g/ml$)		
	0	500	1000
Met[b]	14.9[c]	14.0	5.9
Ala	12.4	11.4	5.4
Val	23.6	21.7	15.1
Leu	28.0	15.6	7.1
Ile	9.1	8.6	0
Pro	N.D.[d]	N.D.	N.D.
Phe	15.6	17.8	2.2
Trp	N.D.	N.D.	N.D.
Gly	44.7	45.1	45.6
Ser	4.9	3.6	0
Thr	9.5	8.3	3.8
Cys	10.3	8.2	1.2
Tyr	13.1	14.3	0
Asn	N.D.	N.D.	N.D.
Gln	N.D.	N.D.	N.D.
Asp	19.8	15.7	12.1
Glu	27.5	25.1	11.4
Lys	34.7	37.3	23.2
Arg	33.1	39.7	13.8
His	27.2	29.0	17.2

a; Conidia were used.
b; DL-form was used.
c; $\mu g/hr/mg$ cell
d; not determined.

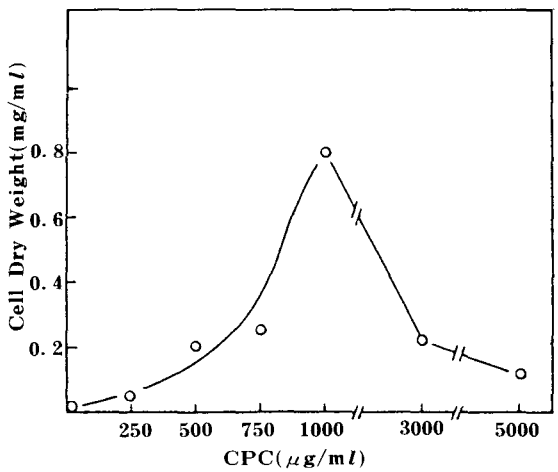


Fig. 3. Cell growth in nitrogen-free synthetic medium containing CPC.

hibitory effects of CPC were observed on the uptakes of most other amino acids. This result revealed that CPC exerted its inhibitory effect through inhibition of amino acid uptake by the cells.

Utilization of CPC as a nitrogen source by *C. acremonium* M-113.

If DL-methionine was omitted from the synthetic medium and, instead, CPC was added to the culture medium as a sole nitrogen compound, cell growth occured (Fig. 3). The extent of cell growth increased with the increasing concentrations of CPC up to 1000μg/ml as if CPC served as a limiting nitrogen compound. But cell growth decreased beyond the concentrations of CPC 1000μg/ml with unknown reason and further study is required. From the above results, it was anticipated that the produced might be reutilized by the cells as a nutrient when the nutrients are starved or limited in batch fermentations. This property of *C. acremonium* M-113 might play a role in disappearance of CPC produced in fermentation broth after CPC production had stopped.[11]

From the results shown above, we conclude that CPC might function as a normal inhibitory substance in cellular metabolism rather than inert secondary metabolite. And we propose the model about the interaction between the cells and CPC as shown in Fig. 4.

Effect of other cephalosporin group antibiotics on growth of *C. acremonium* M-113

Inhibitory effects of other cephalosporin group antibiotics on cell growth were studied to elucidate what portion of CPC was important in exerting its inhibitory effects. As shown in Table 8, other cephalosporin group antibiotics also inhibited cell growth except cephalexin and 7-ADCA. Review on their structures revealed that 3′-group (R′) of CPC might be important in exerting its inhibitory effects rather than 7′-group (R). The relations between the inhibitory effect and 3′-group of CPC should be studied further and now being performed by us. Our long-range goal of this research is to obtain the superproducer of CPC through mutagensis. The increasing resistance either by adaptation to gradually increasing concentaration of CPC or by subjecting mutagen-treated populations of *C. acremonium* M-113 to increase gradients of CPC would be a useful procedure for improving the biosynthetic capacity of the producer, *C. acremonium* M-113.

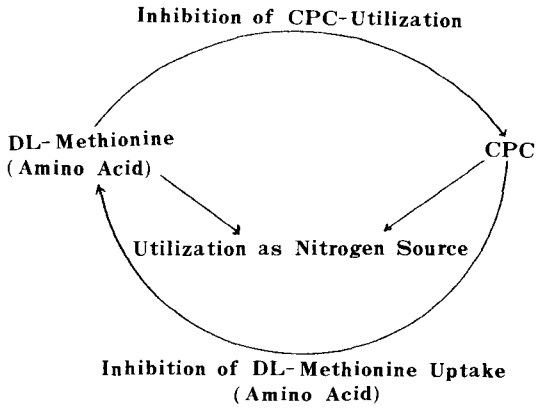
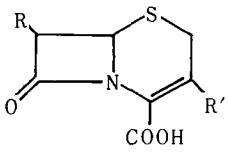
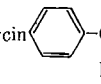
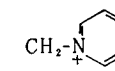


Fig. 4. Model on the effect of CPC on cell metabolism.

Table 8. Effect of other cephalosporin antibiotics on growth of *C. acremonium* M-113[a]



	R	R′	MIC
Cephapirin		CH_2OCOCH_3	< 500
Cephalexin		CH_3	– [b]
Cephaloglycin		CH_2OCOCH_3	< 500
Cephalothin		CH_2OCOCH_3	< 500
7-ADCA	NH_2	CH_3	–
7-ACA	NH_2	CH_2OCOCH_3	< 500
Cephaloridine			< 500
Cephalosporin C	$HOOCCH(CH_2)_3CONH$	CH_2OCOCH_3	< 500

a; Condia of about 10^4 per m*l* were used.
b; not observed inhibition up to 3000μg/m*l* of the antibiotic.

요 약

항세균성 항생제인 세팔로스포린C가 그 자신의 생산균주인 *C. acremonium* M-113의 생장을 저해하였다. 비슷한 저해현상이 *C. acremonium* ATCC 20339와 ATCC 14553에도 관찰되었다. 세팔로스포린C의 최소 생육 저지농도가 분생포자의 경우 200-500 $\mu g/ml$ 이었고 균사의 경우 3,000-4,000 $\mu g/ml$ 이었다. 이 최소 생육 저지농도는 복합배지에서 더욱 상승되었다.

세팔로스포린C는 배양초기에 존재할 경우, 그 생육 저지 효과를 나타내었다. 세팔로스포린C의 생육 저지 효과는 세팔로스포린C가 아미노산의 세포내로의 수송을 방해함으로써 나타나는 것으로 조사되었다. 생육 저지 기저에 세팔로스포린 C의 3′ 기가 중요 역할을 하는 것으로 나타났다. 또한, 세팔로스포린C는 배지에 질소원이 결핍될 때 질소원으로 이용될 수 있는 것으로 나타났다.

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