Regulation of Cell Growth and Tylosin Biosynthesis through Flux Control of Metabolic Intermediate in *Streptomyces fradiae*

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*Streptomyces fradiae*에서 대사중간산물 이용속도에 의한 균체 성장과 Tylosin 생합성의 조절

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ABSTRACT: The aim of the present study was to investigate the effect of glutamate on the biosynthesis of tylosin. Activities of enzymes involved in the metabolic pathway of glutamate to form tylactone, an essential precursor of tylosin, were determined using *Streptomyces fradiae* grown at different concentration of glutamate. As results, it was found that cell growth and tylactone formation was controlled by the metabolic flux of oxaloacetate. It was clear that cell growth was favored by the activities of citrate synthase and aspartate aminotransferase, while the tylactone synthesis was stimulated by the activity of methylmalonyl-CoA carboxyltransferase. Therefore it was concluded that channelling of oxaloacetate was a point for favoring either cell growth or tylosin biosynthesis.
KEY WORDS □ *Streptomyces fradiae*, tylosin, secondary metabolite, tylactone, metabolic regulation.

Tylosin is an antibiotic produced by *Streptomyces fradiae* (McGuire, 1961). The chemical structure of tylosin has been well recognized as a tylactone with mycarose, mycaminose, and mycinose (Morin and Gorman, 1964).

The biosynthetic pathway of tylosin is divided into two steps: One is to synthesize tylactone, the earliest lactonic precursor of the aglycone moiety, and the later step is the conversion of tylactone to tylosin where sugars are attached to the lactone (Corcoran, 1974; Omura, 1977). It was reported that the formation of tylactone was as an essential step to limit the biosynthetic rate of tylosin and that tylactone was built up with propionate, acetate, and butyrate (Omura, 1977). It was thought that methylmalonyl-CoA carboxyltransferase and propionyl-CoA carboxylase were essential en-

zymes for the biosynthesis of tylactone (Vu-trong et al., 1980, Vu-trong and Gray, 1982). Chemical culture conditions, viz. concentration and source of carbon, nitrogen, and phosphate, showed very important roles in the induction and repression of the essential enzymes for the tylactone biosynthesis (Mardy and pape, 1982, Omura et al., 1983, 1984 a, b.). Those results indicated that the availability of low molecules was very important in the formation of secondary metabolites (Demain et al., 1983).

In this study it was attempted firstly to investigate the effects of glutamate on the biosynthesis of tylosin and then the results were compared with the activities of serveral enzymes: NAD-dependent glutamate dehydrogenase (E.C.1.4.1.3), citrate synthase (E.C.4.1.3.7), aspartate amino-

transferase (E.C.2.6.1.2), phosphoenolpyruvate carboxylase (E.C.4.1.1.31) and methymalonyl-CoA carboxyltransferase (E.C.2.1.3.1). The experiments were contemplated to elucidate the roles of glutamate as a donor of the intermediate in TCA cycle, NH_3 and amino group. The major aim of the present study was to examine the metabolic flux of secondary metabolite formation.

MATERIALS AND METHODS

Strain and media used

The current experiments were conducted with *Streptomyces fradiae* NRRL 2702. The strain was stocked in a rich medium and main culture was carried out using a synthetic medium which was slightly modified from the medium reported by Gray and Bhuwapathanapun (1980). The synthetic medium was formulated as follows; glucose 10.0g, sodium glutamate 10.0g, betaine hydrochloride 2.5g, K_2HPO_4 1.15g, $MgSO_4.7H_2O$ 2.5g, NaCl 1.0g, $CoCl_2·6H_2O$ 0.0005g, $ZnSO_4.7H_2O$ 0.0005g, $CaCl_2.2H_2O$ 1.5g, ferric ammonium citrate 0.5g, and methyloleate 12.5g, in 1 liter distilled water.

Culture conditions

Batch culture was conducted in a 1.5 liter fermenter (B. Braun Model M.). Culture pH was maintained at 7.0 by automatic addition of 2N NaOH and 2N HCl. Temperature was controlled to 30°C and dissolved oxygen tension was maintained above 50% of saturation by aeration (0.5-1.2 vvm) and agitation (500-800 rpm).

Chemical analyses

The growth of cell was expressed as dried cell weight after drying at 80°C for 24 hours. For the determination of tylosin titers, culture supernatants were bioassayed using *Sarcina lutea* as a test microorganism. The amount of residual glucose was determined by dinitrosalicylic acid method (Miller, 1959) and the amount of glutamate was measured by the TLC method (Brenner *et al.*, 1973). Ammonium ion concentration was determined by the indophenol methods (Taras *et al.,* 1981).

Preparation of cell extract

The cell extract used for the determination of enzyme activities was prepared as reported by Vutrong *et al.* (1980). The amount of protein was estimated by the method of Lowry *et al.* (1951). One unit of enzyme activity was defined as that amount catalysing transformation of 1 μmol of substrate per minute under the given assay condition.

Assay of citrate synthase (E.C.4.1.3.7)

The activity of citrate synthase was determined according to the method of Shiio *et al.* (1977). The total volume of reaction mixture was 1.5ml containing Tris-HCl buffer (pH8.0) 150 μmol, oxaloacetate 0.3 μmol, acetyl-CoA 0.075 μmol, 5,5-dithio-bis-(2-nitrobenzoic acid) 0.075 μmol, and cell free extract. The reaction was carried out at 25°C and the reaction rate was determined by measuring the absorbance at 410 nm with a spectrophotometer (Gilford).

Assay of methymalonyl-CoA carboxyltransferase (E.C.2.1.3.1)

The activity of the enzyme was measured by determining the amount of pyruvate formed from oxaloacetate (Wood and Stjernholm, 1961). The volume of reaction mixture was 1 ml containing Tris buffer (pH 8.0) 50 μmol, reduced glutathion 0.5 μmol, oxaloacetate 1 μmol, propionyl-CoA 0.25 μmol, and cell free extract. After reaction for 15 min at 30°C, the reaction was stopped by adding 0.5 ml of 10% $HClO_4$. Then the reaction mixture was cooled in an ice bath and neutralized by adding 0.4 ml of 2N KOH. The neutralized supernatants were used for the determination of pyruvate. The amount of pyruvate was estimated by lactate dehydrogenase.

Assay of aspartate aminotransferase (E.C.2.6.1.2)

The activity of aspartate aminotransferase was determined by coupling with the reaction by malate dehydrogenase, measuring the oxidation of NADH (Bergmeyer *et al.,* 1983 a). The volume of reaction was 1.59 ml containing phosphate buffer (pH7.4) 47.3 μmol, aspartate 17.8 μmol, 2-oxoglutarate 3.4 μmol, NADH 0.3 μmol, malate dehydrogenase 3.7 unit, and cell free extract. The reaction

was run at 25°C and the change of absorbance at 340 nm was measured.

Assay of NAD-dependent glutamate dehydrogenase (E.C.1.4.1.3)

The volume of reaction mixture (Bergmeyer *et al.*, 1983 b) was 1.55m*l* containing Triethanolamine buffer 75 μmol, EDTA 3.5 μmol, CH$_3$COONH$_4$ 150 μmol, NADH 0.3 μmol, ADP 1.5 μmol, lactate dehydrogenase 1.5 unit, 2-oxoglutarate and cell free extract. The reaction was run at 25°C and the change of absorbance at 340 nm was measured.

Assay of phosphoenolpyruvate carboxylase (E.C. 4.1.1.31)

The volume of reaction mixture (Bergmeyer *et al.*, 1983 c) was 2 m*l* containing Tris-HCl (pH8.0) 166μmol, KHCO$_3$ 20μmol, PEP, tricyclohexylammonium salt 10 μmol, MgCl$_2$ 13.4 μmol, reduced glutathione 9.8 μmol, NADH 0.4 μmol, malate dehydrogenase 4 unit and cell free extract. The reaction run at 25°C and the change of absorbance at 340 nm was measured.

RESULTS AND DISCUSSION

Batch culture on synthetic media

Data for the growth of S. *fradiae*, tylosin formation, and consumption of glucose and glutamate were shown in Figure 1. By inoculation of seed culture grown on the same media to main culture, a typical lag phase was not observed and an exponential growth phase was continued until the substrates, viz. glucose and glutamate was available. It was evident that both substrates were utilized rapidly and glutamate was more readily uptaken than glucose. The further increase in biomass was not detected after the depletion of the available substrates.

It was apparent that the start-up of tylosin biosynthesis was commenced coincidently when the substrate concentrations were very low. Another interest was that the typical trophophase-idiophase character, which was observed normally when a rich medium was used, was not clear in this culture. This result indicated that the medium used in this experiment allowed the induction of en-
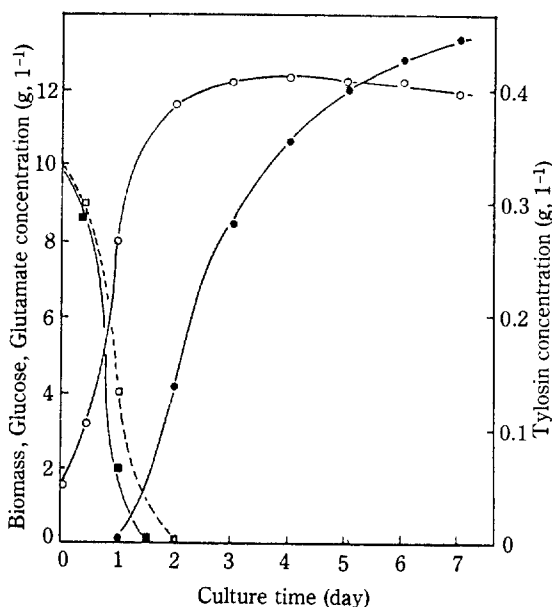

Fig. 1. *Batch culture data for the utilization of glucose (□) and glutamate (■), the increase of biomass (○), and tylosin biosynthesis (●) with S. fradiae.*

zymes for the tylosin biosynthesis.

The activities of three enzymes, viz. methymalony-CoA carboxyltransferase, aspartate aminotransferase and citrate synthase, which are utilizing oxaloacetate as a co-substrate, were estimated through the batch culture. These enzymes were adopted to test, because it was considered that oxaloacetate was an important intermediate to form methylmalonyl-CoA (Raczynska-Bojanowska *et al.*, 1970) and that the formed methylmalony-CoA was also an essential precursor for the tylactone biosynthesis. It was thought that the more formation of methylmalony-CoA from oxaloacetate was the more desirable property for the tylosin over-production. As shown in Figure 2, it was very interesting to note that the activities of citrate synthase and aspartate aminotransferase were decreased coincidently with the specific growth rate (μ) as the culture elapsed. These results showed that these enzyme activities were closely linked with the cell growth rates. In other words, oxaloacetate should be converted to citrate or/and to aspartate for supplying precursors and energy which were demanded to satisfy the rapid
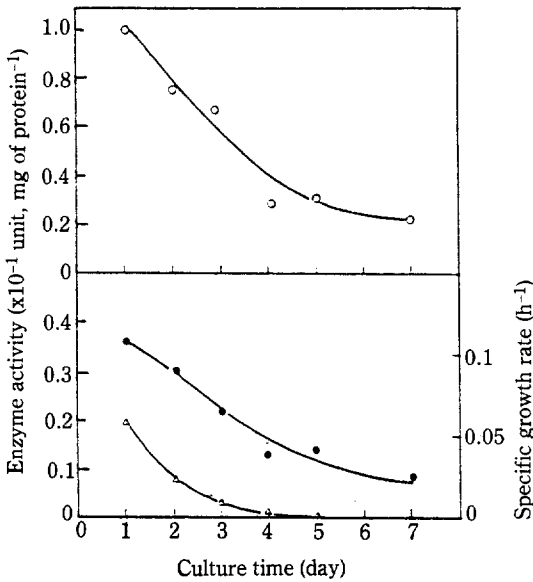
Fig. 2. Effect of culture time on the activities of citrate synthase (○), aspartate aminotransferase (●), and specific growth rate (△) in a batch culture of S. fradiae.

growth of cells. However Figure 3 showed that the specific rate of tylosin formation was coupled very closely with the activity of methylmalonyl-CoA carboxyltransferase. The differences between the data shown in Figure 2 and 3 were that the enzyme activities linked to growth were rapidly reduced through the batch culture, while the
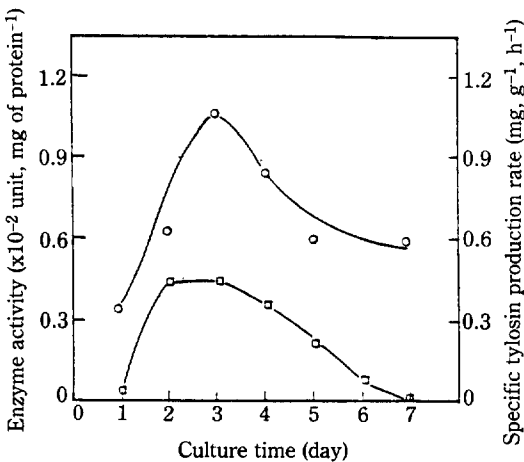

Fig. 3. Effect of culture time on the activities of methylmalonyl-CoA carboxyltransferase (○) and specific tylosin production rate (□) in a batch culture of S. fradiae.

tylosin formation rate and activity of methylmalonyl-CoA carboxyltransferase showed a maximum activity at a certain period of culture (viz. 2-3 days).

From the comparison of the specific activities of the enzymes, it was evident that citrate synthase and aspartate aminotransferase showed 12-20 times higher specific activities than methylmalonyl-CoA carboxyltransferase. The higher activities of citrate synthase and aspartate aminotransferase during the trophophase indicated that the more oxaloacetate was used for the cell growth, therefore methylmalonyl-CoA, which was one of the essential precursors for tylactone, was not supplied sufficiently for the initiation of tylosin biosynthesis. Reduction of the cell growth rate and both activities of citrate synthase and aspartate aminotransferase suggested that the more oxaloacetate could be converted to methylmalonyl-CoA by the higher activity of methylmalonyl-CoA carboxyltransferase, therefore tylactone biosynthesis could be possible.

These results indicate strongly that the channeling of oxaloacetate is a critical point for the cell growth or tylosin biosynthesis. In other sense, cell growth is favored by the activities of citrate synthase and aspartate aminotransferase, while the tylosin biosynthesis is favored by the activity of methylmalonyl-CoA carboxyltransferase.

Roles of glutamate as a donor of α-ketoglutarate, amino group, and NH_3

Batch culture data for the changes in biomass, tylosin and tylosin yield ($Y_{p/x}$) with S. fradiae at initial sodium glutamate concentration of 5, 10, 15, 20, and 25 g/l are shown in Figure 3. It was evident that the biomass concentrations were increased with the sodium glutamate concentrations added. And it was observed that the smaller and less mycelial pellet were formed at the higher concentration of sodium glutamate. However, the final concentration of tylosin was reduced and it was more apparent that the tylosin product yield considering the biomass formed ($Y_{p/x}$) was decreased.

In order to know the mechanism of glutamate utilization and the further effects, various enzyme activities, such as NAD-dependent glutamate
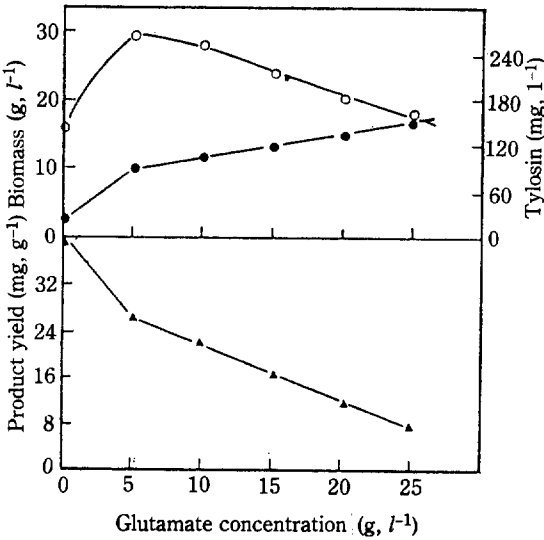
Fig. 4. *Effect of L-glutamate concentration on biomass formation (●), tylosin biosynthesis (○), and tylosin production yield (▲) in 3 day cultured cells of S. fradiae.*
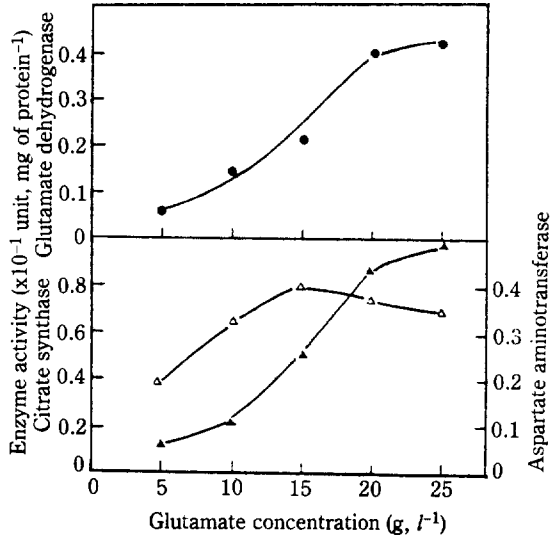

Fig. 5. *Effect of glutamate concentration on the activities of NAD-glutamate dehydrogenase (●), citrate synthase (△) and aspartate aminotransferase (▲) in 3 day cultured cells of S. fradiae.*

dehydrogenase, phosphoenolpyruvate carboxylase, citrate synthase, aspartate aminotransferase, and methylmalonyl-CoA carboxyltransferase, were determined. The experiment was contemplated to investigate the roles of glutamate on the activities of enzymes involved in the uptake of glutamate, the replenishment of intermediate into TCA cycle through anaplerotic reaction, and the flux of oxaloacetate utilization. As shown in Figure 4, it was clear that the activity of NAD-dependent glutamate dehydrogenase which was involved in the incorporation of glutamate into TCA cycle via α-ketoglutarate, was stimulated and it was also evident that the activities of aspartate aminotransferase and citrate synthase were increased by the increases in the concentration of glutamate added to the culture medium. It was clear that an abundant growth was obtained at sufficient glutamate addition, which resulted from the high activities of glutamate dehydrogenase, aspartate aminotransferase, and citrate synthase. However as shown in Figure 5, the activity of methylmalonyl-CoA carboxyltransferase, which was considered as an essential enzyme for the biosynthesis of tylactone, was apparently decreased with

the increase in the concentration of glutamate.

In addition, it was demonstrated that the activity of phosphoenolpyruvate carboxylase, which
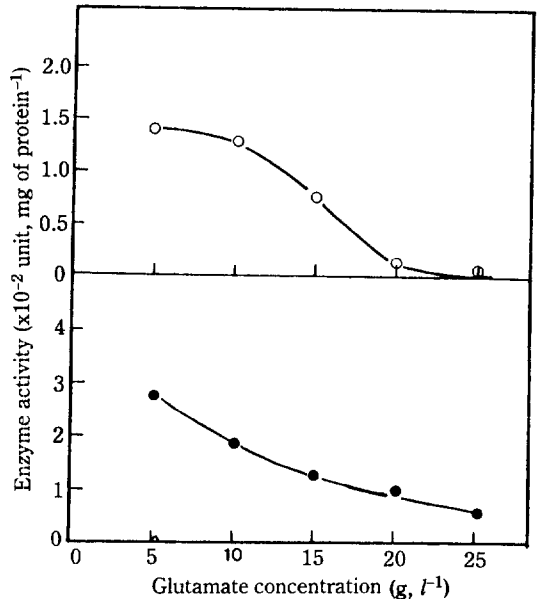

Fig. 6. *Effect of glutamate concentration on the activities of methylmalonyl-CoA carboxyltransferase (○) and PEP carboxylase (●) in 3 day cultured cells of S. fradiae.*

was an essential enzyme to replenish oxaloacetate from phosphoenolpyruvate was decreased as demonstrated in methylmalonyl-CoA carboxyltransferase. The data shown in Figure 4, 5 and 6 indicated that higher rate of cell growth was maintained by the sufficient provision of essential precursors (viz. oxaloacetate, aspartate, and glutamate) and by enough energy generation through higher activity of TCA cycle. In these conditions, it was considered that the formation of essential intermediates for the tylactone biosynthesis such as methylmalony-CoA and acetyl-CoA, which were provided from TCA cycle or related metabolic pathway, were suppressed. Therefore it was concluded again that channelling of oxaloacetate to methylmalony-CoA was one of the critical features to stimulate tylosin biosynthesis.

Figure 7 shows again that glutamate was incorporated into TCA cycle through α-ketoglutarate by NAD-dependent glutamate dehydrogenase, therefore the liberated ammonium were accumulated in the culture broth. It was noted that the amount of acid used for pH control was related to the glutamate utilization (data were not shown). These results indicated that ammonium ions were liberated from glutamate and that glutamate was

uptaken as α-ketoglutarate into the cells. Because many reports pointed that the biosynthesis of antibiotic was controlled by nitrogen catabolite repression (Aharonowitz, 1980, Grafe, 1982), it was worth to note that the liberated ammonium might give rise to repression on a certain essential enzymes(s) in tylosin biosynthesis. Omura *et al.,* (1984 b) reported that the action of ammonium was directed at least toward steps between valin and isobutyrate, and between threonine and propionate, but was not toward steps after formation of the building units for tylactone biosynthesis. They emphasized that amino acid metabolism was important in supplying lower fatty acids which were important building units for the tylosin, and that the metabolism was the target of ammonium regulation.

Effects of α-ketoglutarate

In order to know the other effects of glutamate, glutamate in the medium was replaced with α-ketoglutarate. The experiments were designed to reveal the effect of glutamate as a source of intermediate in TCA cycle and to eliminate some possible effects of glutamate as a nitrogen source. As show in Table 1, it was very interesting to note that biomass formation and tylosin formation were not varied greatly and showed relatively constant values. The activities of aspartate aminotransferase, methylmalonyl-CoA carboxyltransferase and citrate synthase were not greatly affected by the concentration of α-ketoglutarate. However it was evident that the activity of phosphoenolpyruvate carboxylase was reduced apparently by the elevated levels of α-ketoglutarate. These results implied that anaplerotic replenishment of oxaloacetate from phosphoenolpyruvate was inhibited to some extent by the incorporation of α-ketoglutarate. The apparent inhibition of phosphoenolpyruvate carboxylase by α-ketoglutarate as well as by glutamate indicated that the effect of glutamate on TCA cycle activies was induced via α-ketoglutarate.

As conclusion, it was clear that the activity of aspartate aminotransferase was not regulated by α-ketoglutarate, while the enzyme level was very
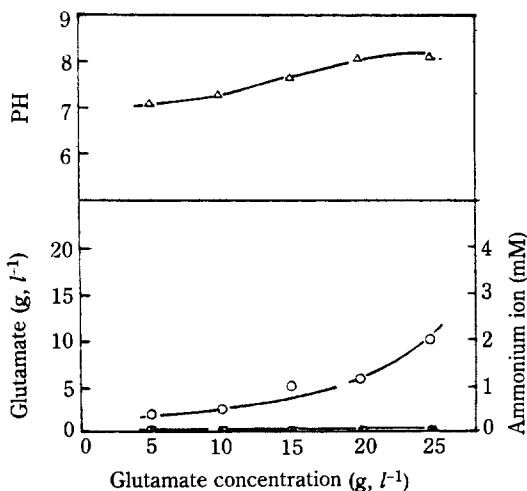

Fig. 7. *Effect of glutamate concentration on the residual concentrations of ammonium (○), glutamate (□), and pH (△) in 3 day cultured broth of S. fradiae.*

Table 1. *Effects of α-ketoglutarate concentration on the biosynthesis of tylosin and on the activities of aspartate aminotransferase, citrate synthase, PEP carboxylase, and methylmalonyl-CoA carboxyltransferase.*

| | Conc. of α-ketoglutarate (g,l^{-1}) | | | |
	5	10	15	20
Tylosin (mg,l^{-1})	155	140	155	180
Biomass (g,l^{-1})	11.0	9.8	11.6	12.3
ASAT (unit, mg^{-1})	0.31	0.28	1.34	0.33
CS (unit, mg^{-1})	1.64	1.66	1.40	1.23
MMCT ($x10^{-2}$ unit, mg^{-1})	0.30	0.36	0.35	0.30
PC ($x10^{-2}$ unit, mg^{-1})	0.26	0.19	0.11	0.08

ASAT : Aspartate aminotransferase
CS   : Citrate synthase,   PC   : PEP carboxylase
MMCT: Methylmalony-CoA carboxytransferase
* Medium Compostion; α-ketoglutarate was added insead of glutamate, and ammonium sulfate was added as a nitrogen source.

closely regulated by glutamate concentration. The results indicated that glutamate exerted a significant effect on the synthesis of aspartate aminotransferase and also on the activity. Therefore it could be concluded that the negative effect of glutamate on the tylosin titer resulted from the over-activity of aspartate aminotransferase, by which more oxaloacetate, an essential intermediate, was converted to aspartate rather than to methylmalonyl-CoA.

From the presenting study, we could be confident that oxaloacetate was an important intermediate to regulate cell growth and tylosin formation. The metabolic flux of oxaloacetate was controlled by citrate synthase, asparte aminotransferase and methylmalonyl-CoA carboxyltransferase. It was apparent that cell growth was favored by the higher activities of citrate synthase and aspartate aminotransferase, while the tylosin biosynthesis was stimulated by the higher activity of methylmalonyl-CoA carboxyltransferase. It was concluded consequently that the channeling of oxaloacetate was a critical point for favoring either cell growth or tylosin biosynthesis. However we could not be more confident that cell growth rate would play any important roles in the regulation of enzyme activities. Another possible explanation might be given as that a certain factor (s) affected firstly or simulatameously on the cell growth to be reduced, therefore the flux of oxaloacetate to citrate and aspartate was not necessary to be excessive. Then the sufficient amount of oxaloacetate could be channelled into methylmalonyl-CoA. The explanation is subjected to next experiment.

적   요

배지성분으로 첨가된 glutamate의 농도가 균의 성장과 tylosin 생합성에 미치는 영향을 조사하였다. 그 결과 oxaloacetate 를 공동기질로 사용하는 효소의 활성에 의하여 균의 성장과 tylosin의 생합성이 조절됨을 알았다. 즉, citrate synthase와 aspartate aminotransferase의 활성은 균의 성장에 아주 긴요하며 methylmalonyl-CoA carboxytransferase의 활성은 tylosin 생합성에 아주 중요한 효소임을 알았다. Glutamate의 농도는 위의 효소의 활성에 직접적으로 영향을 주고 있음을 알았다.

ACKNOWLEDGE

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