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Regulation of Tylosin Biosynthesis by Cell Growth Rate in *Streptomyces fradiae*

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*Streptomyces fradiae*에서 균 성장속도에 의한 Tylosin 생합성 조절

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ABSTRACT: The aim of the present study was to investigate the effects of growth rate on the biosynthesis of tylosin in *Streptomyces fradiae*. In order to elucidate the relation between the growth rate and the tylosin formation rate, the activities of enzymes involved in oxaloacetate metabolism were determined using cells grown at different growth rates in chemostats. As the results, it was found that the specific rate of tylosin formation(q_p) was closely related to the specific cell growth rate and the maximum value of q_p was 1.1mg tylosin, g^{-1} cell, h^{-1} at the growth rate $0.013h^{-1}$. However further increase in the growth rate over $0.013h^{-1}$ resulted in apparent decrease of q_p. The synthesis and activities of citrate synthase, aspartate aminotransferase, and PEP carboxylase were very low at lower growth rate. On the other hand, the activity and synthesis of methylmalonyl-CoA carboxyltransferase was closely related to tylosin formation. Therefore it was concluded that tylosin formation was apparently controlled by the growth rate.

KEY WORDS □ *Streptomyces fradiae*, **tylosin, secondary metabolite, metabolic regulation.**

Tylosin is a commercially important 16-membered macrolide antibiotic produced by *Streptomyces fradiae* (McGuire, 1961). The biosynthetic pathway of tylosin is divided into two steps. The former step is to synthesize tylactone which is derived from two acetates, five propionates and one butyrate (Omura *et al.*, 1977). The latter step is the conversion of tylactone to tylosin where sugars are attached to the lactone ring (Morin and Gorman 1964). It has been thought that the biosynthesis of tylosin was strongly influenced by nutritional conditions, viz. concentration and source of carbon, nitrogen, and phosphate (Mardy and Pape, 1982, Omura *et al.*, 1983, Vu-trong *et al.*, 1980). It was also reported that the formation of tylactone was an essential step to limit the biosynthetic rate of tylosin under those conditions (Omura *et al.*, 1984 a,b).

It is well known that the nutritional conditions exert a strong influence on the expression of secondary metabolism through growth rate which is controlled primarily by nutritional limitations. In general, it is found that the onset of secondary metabolism coincides with the changes in nutritional conditions which limit the growth of cell (Demain *et al.*, 1983).

In previous report (Kang and Lee, 1987), we reported that cell growth and tylactone formation

was controlled by the metabolic flux of oxaloacetate and that channelling of oxaloacetate was a point for favoring either cell growth or tylactone formation. In other words cell growth was favored by the activities of citrate synthase and aspartate aminotransferase, while the tylactone formation was stimulated by methylmalonyl-CoA carboxyltransferase.

In the present experiments, it was contemplated to investigate the effect of specific growth rate on the activities of enzymes involved in oxaloacetate utilization. Further comparisons were also made with the rate of substrate utilization, growth and tylosin 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 formulated as follows; glucose 10.0 g, sodium glutamate 10.0 g, betaine hydrochloride 2.5g, K_2HPO_4 1.15g, $MgSO_4 \cdot 7H_2O$ 2.5g, NaCl 1.0g, $CoCl_2 \cdot 6H_2O$ 0.0005g, $ZnSO_4 \cdot 7H_2O$ 0.005g, $CaCl_2 \cdot 2H_2O$ 1.5g, ferric ammonium citrate 0.5g, and methyloleate 12.5g in 1 liter distilled water.

Culture conditions

Tylosin fermentation 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 oxgen tension was maintained above 50% of saturation by aeration (0.5-1.2 vvm) and agitation (500-800 rpm).

For chemostat operation the medium not containing methyloleate was pumped into the fermenter by a peristaltic pump and methyloleate was separately pumped into the fermenter by another peristaltic pump. The continuous fermentation was operated at the following dilution rates from 0.005, 0.008, 0.013, 0.03, 0.05, to 0.065 h^{-1}.

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).

Assay of enzyme activities

The activities of citrate synthase (E.C.4.1.3.7), aspartate aminotransferase (E.C.2.6.1.2), phosphoenolpyruvate carboxylase (E.C.4.1.1.31), and methylmalonyl-CoA carboxyltransferase (E.C.2.1.3.1) were assayed as previously described (Kang and Lee, 1987). One unit of enzyme activity was defined as that amount catalyzing transformation of 1μmol of substrate per minute under the given assay condition.

Kinetic analyses

Growth yield ($Y_{x/s}$), product yield ($Y_{p/s}$), specific growth rate or dilution rate (μ or D), specific substrate uptake rate (q_s), and specific product formation rate (q_p) in continuous cultures were calculated as suggested by Wang *et al* (1979). All fermentation kinetic parameters were evaluated on the basis of dried cell weight.

RESULT AND DISCUSSION

Steady state data on the concentrations of biomass, tylosin, glucose, and glutamate are shown in Figure 1. As shown in Figure 1, it was apparent that the tylosin concentrations at different dilution rates were reduced greatly with increase in dilution rate. However the biomass concentrations were not varied and showed relatively constant value. The residual amounts of glucose and glutamate were too low to analyze when the dilution rate was below than 0.04 h^{-1}. It was interesting to note that glutamate was more readily uptaken than glucose.

The specific rates of tylosin formation, glucose and glutamate uptake were calculated and shown in Figure 2. The specific rate of tylosin formation ($q_{tylosin}$) increased linearly with the dilution rate until 0.013 h^{-1}, then the maximum value of $q_{tylosin}$ was maintained. However it was apparent that the

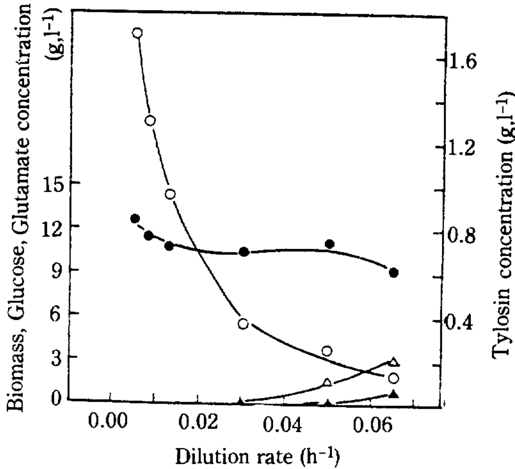

Fig. 1. *Steadystate concentrations of glucose (△), glutamate (▲), biomass (•), and tylosin (○) in a continuous culture of Streptomyces fradiae.*

value of $q_{tylosin}$ decreased when the dilution rate exceeded over 0.05 h^{-1}. The results indicated that $q_{tylosin}$ was associated with the growth rate of cell at low dilution rate. In other words, the biosynthesis of tylosin was closely linked to the anabolic activity of cell at low growth rate (Ryu and Hospodka, 1980). On the other hand, the biosynthesis of tylosin was repressed where the growth of cell was highly activated.

Comparing the specific tylosin formation rate with the specific substrate uptake rates, it was apparent that the specific rates of substrate uptake were more closely linked to the cell growth rate than that of tylosin formation. It was very noticeable that $q_{tylosin}$ decreased where the residual amounts of glucose and glutamate were apparent. It was considered that the growth rate of cell and the specific rate of substrate uptake might play important roles in the biosynthesis of tylosin.

These result indicated that the increases in the inflow rate of substrates at higher dilution rates resulted in the increases in the specific rates of substrate uptake. Then the higher activity of substrate utilization might exert direct effects on the tylosin biosynthetic activity, and additionally the catabolic activity influenced on the cell growth rate. In this sense it was thought that the tylosin biosynthesis might be regulated more closely by the catabolic activity rather than the cell growth rate.

The tylosin production yield considering substrate utilization ($Y_{p/s}$) and biomass formation ($Y_{p/x}$) were calculated from the data of each steady state. The biomass production yield ($Y_{x/s}$) was also estimated and the calculated values were shown in Figure 3. It was very evident that the biomass yield were constant while the tylosin production

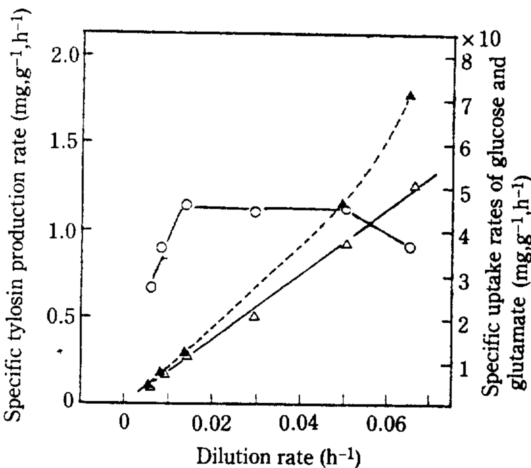

Fig. 2. *Effects of specific growth rate on the specific activities of tylosin production (○) and substrates uptake (▲; glutamate, △; glucose) in a continuous culture of Streptomyces fradiae.*

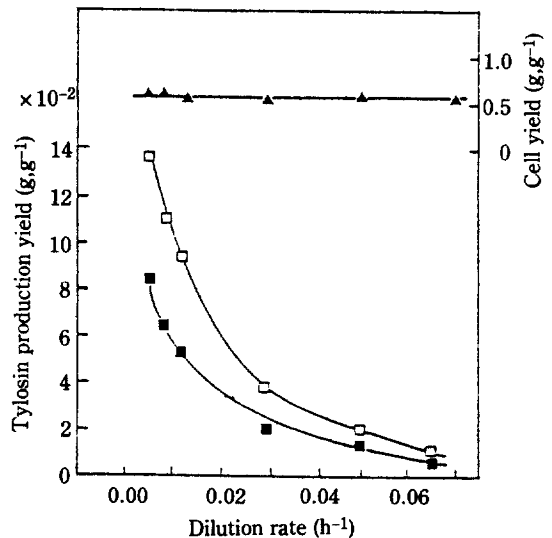

Fig. 3. *Effects of specific growth rate on the tylosin production yield (□; $Y_{p/x}$, ■; $Y_{p/s}$) and cell yield (▲; $Y_{x/s}$) in a continuous culture of Streptomyces fradiae.*

yields were inversely proportional to the growth rate. It was therefore concluded that the optimum growth rate for tylosin production was about 0.013 h^{-1} where showed the maximum production rate of tylosin and high conversion yield of substrate to tylosin. The $q_{tyloson}$ at that dilution rate was 1.1 mg, g^{-1}, h^{-1}, which was much higher than the value of batch culture, 0.47 mg, g^{-1}, h^{-1} (Kang and Lee, 1987).

The activity of citrate synthase, aspartate aminotransferase, PEP carboxylase, and methylmalonyl-CoA carboxyltransferase were determind with the extracts of cells grown at different dilution rates (Figure 4). The specific rates of these enzyme syntheses which were expressed as units, mg^{-1}, h^{-1} were also calculated and compared with the specific formation rate of tylosin (Figure 5).

As shown in Figure 4A and 5A, it was clear

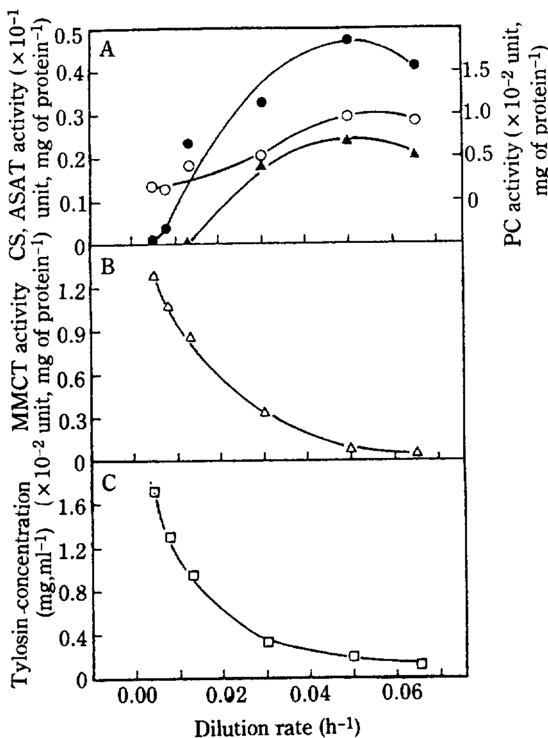

Fig. 4. *Effects of specific growth rate on the activities of citrate synthase (CS,•), aspartate aminotransferase (ASAP,○), phosphoenolpyruvate carboxylase (PC,▲) and methylmalonyl-CoA carboxyltransferase (MMCT,△).* The concentrations of tylosin (□) at the different dilution rates were also shown.

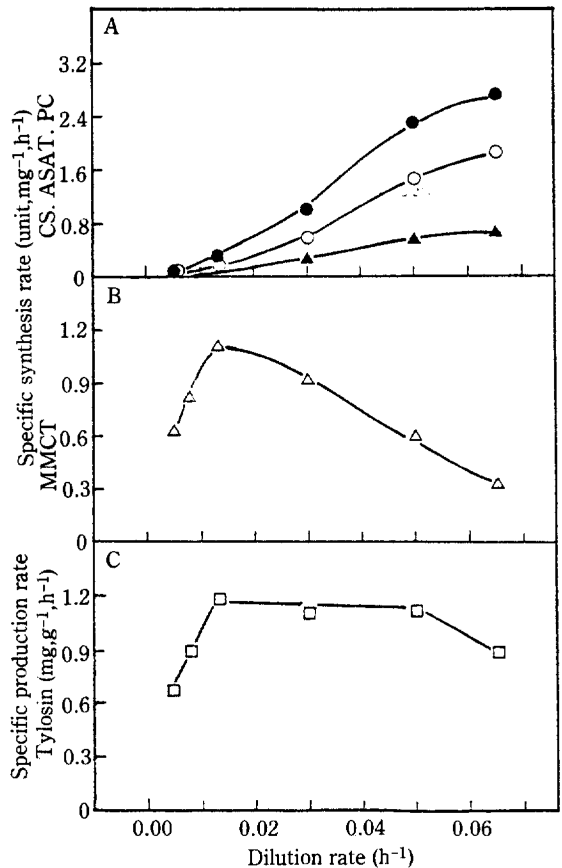

Fig. 5. *Effects of specific growth rate on the specific synthesis rates of citrate synthase (CS,•), aspartate aminotransferase (ASAT,○), PEP carboxylase (PC,▲) and methylmalonyl-CoA carboxyltransferase (MMCT,△).* The specific formation rates of tylosin (□) were also shown.

that the activity and synthesis rate of citrate synthase, an essential enzyme at the entry step of TCA cycle, increased with the increase of growth rate. Furthermore the activities and specific synthesis rates of aspartate aminotransferase and PEP carboxylase were also increased with the growth rate of cell. It was thought that those enzymes were essential for the synthesis of precursors of cell. Therefore the good agreements between the cell growth rate and the enzyme activities were expected to some extent.

It was observed again that the enzyme activities were low when growth rate below 0.013 h^{-1}. It suggested that the energy production and the anabolic activity of the cell were clearly limited by the

substrate limitation at the low dilution rates. Under those conditions, the tylosin biosynthetic activity ($q_{tylosin}$) should be associated with the dilution rate or substrate inflow rate. However when the dilution rate was higher than 0.013 h^{-1}, the substrate fed to the cell was enough to support the cell growth. It indicated that the substrate was more used for the cell growth than for the tylosin formation and hence the formation of tylosin was reduced in terms of synthetic rate ($q_{tylosin}$) and also in tylosin formation yield ($Y_{p/x}$, $Y_{p/s}$).

On the other hand, it was evident that the activity and specific synthesis rate of methylmalonyl-CoA carboxyltransferase was reduced according to the increase of growth rate. Particularly, the specific synthesis rate of this enzyme showed somewhat similar pattern to the specific formation rate of tylosin as shown in Figure 5B and 5C. It was considered that the methylmalonyl-CoA carboxyltransferase was an essential enzyme to form methylmalonyl-CoA which was reported as one of the important precursors of tylosin (Vu-Trong *et al.*, 1980). As discussed in the previous report (Kang and Lee, 1987), oxaloacetate could be used as a cosubstrate of citrate synthase, aspartate aminotransferase, and also methylmalonyl-CoA carboxyltransferase. Therefore the competition for the substrate between these enzymes could be the critical point to favor the cell growth or the tylosin biosynthesis.

It was very interesting that the specific synthesis rate of methylmalonyl-CoA carboxyltransferase and the specific tylosin formation rate of tylosin ($q_{tylosin}$) showed their maximal values at the same dilution rate, 0.013 h^{-1}. It indicated that the specific growth rate would stimulate first on the synthesis of citrate synthase and aspartate aminotransferase. Therefore more oxaloacetate could be used into the synthesis of cell. It might cause the synthesis and activity of methylmalonyl-CoA carboxyltransferase to be repressed and also inhibited at higher growth rate over 0.013 h^{-1}. At present data, we have not any experimental data to support the explanation on what molecule(s) would play to regulate the synthesis and activity of this enzyme.

In chemostat, it was manifest that the increase of dilution caused the increase of substrate concentrations, viz. glucose and glutamate, in each steady state. So the decreased activity of enzyme at higher dilution rate meant that the repression of enzyme might be exerted by substrate or the substrate metabolite (Vu-Trong and Gray, 1982). The observed repression of this enzyme therefore could be related to increasing influx rate of nutrients, especially glucose and glutamate, into the cells. It was reported that this enzyme was subject to carbon catabolite repression by glucose (Vu-Trong *et al*, 1980) and also subject to nitrogen catabolite repression by the rapidly metabolized nitrogen source such as glutamate (Kang and Lee, 1987). In general, high levels of glucose, ammonia (and other readily utilizable nitrogen sources), and phosphate have been described as factors that repress the synthesis of secondary metabolite such as antibiotics (Demain, 1982). Repression of enzymes involved in secondary metabolism during active growth of the organism has been reported in many other secondary metabolite producing systems (Demain and Inamine, 1970, Hurley and Bialerk, 1974, Lilley *et al.*, 1981, Matteo *et al.*, 1976).

The results obtained from this study demonstrated that tylosin biosynthesis must be related to the energy yield steps and also to the activity of intermediate utilization. Under carbon and energy limited conditions, the availability of oxaloacetate and ATP would be crucial and the optimum growth rate for tylosin formation might be determined by the availability. It was thought that more requirement of energy and oxaloacetate for growth might bring about lower provision of energy and oxaloacetate for tylosin formation and hence the tylosin formation was repressed. The repression of tylosin biosynthesis at higher growth rates, where the carbon and energy sources were not limited, could be resulted from the higher substrate uptake rates which in turn exerted catabolite repression. It revealed that the activity and synthesis of methylmalonyl-CoA carboxyltransferase were inhibited and repressed apparently at high growth rates, while citrate synthesis and

aspartate aminotransferase were stimulated.

It could be concluded that the activities of citrate synthase and aspartate aminotransferase were activated by the excessive substrates, from which the growth rate could be increased. Then the growth rate would play an important role in channeling more energy and intermediates, viz. oxaloacetate, into cell formation. Therefore it would not be necessary to over-synthesize methylmalonyl-CoA carboxyltransferase.

적 요

균 성장속도가 tylosin 생합성에 미치는 영향을 조사하기 위하여, 여러 성장속도로 배양한 균체내에서 oxaloacetate 대사에 관여하는 효소들의 활성을 살펴보았다. 그 결과, 비 tylosin 생합성 속도(q_p)는 성장속도 0.013 h^{-1}까지는 성장속도와 함께 증가하지만, 더 높은 성장속도에선 감소됨을 알 수 있었다. Citrate synthase, aspartate aminotransferase와 PEP carboxylase의 활성 및 합성은 0.013 h^{-1}보다 낮은 성장속도에선 매우 낮게 나타났으며, 반면 methylmalonyl-CoA carboxyltransferase의 활성 및 합성은 tylosin 생합성과 마찬가지로 높은 성장속도에선 감소되었다. 따라서 tylosin 생합성은 균 성장속도에 의해 조절됨을 명백히 알 수 있었다.

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