EFFECT OF AMMONIUM ION, INORGANIC PHOSPHATE AND AMINO ACIDS ON THE BIOSYNTHESIS OF PROTYLONOLIDE, A PRECURSOR OF TYLOSIN AGLYCONE†

SATOSHI ŌMURA, YOSHITAKE TANAKA, HIROSHI MAMADA and ROKURŌ MASUMA

The Kitasato Institute and
School of Pharmaceutical Sciences, Kitasato University,
Minato-ku, Tokyo 108, Japan

(Received for publication December 24, 1983)

The production of tylosin by *Streptomyces fradiae* KA-427 in a defined medium was inhibited by ammonium ions and by inorganic phosphate. The production of protylonolide, an early lactonic intermediate of tylosin biosynthesis with the same carbon skeleton as tylosin aglycone, by a mutant of strain KA-427 was also reduced by these two kinds of ions. In contrast, the bioconversion of protylonolide to tylosin by another mutant was less susceptible to ammonium ions but was sensitive to inorganic phosphate. The addition of protylonolide to a culture of *S. fradiae* KA-427 increased the tylosin yield, suggesting that aglycone synthesis is limiting under the conditions used. When L-valine, L-leucine, L-isoleucine, L-threonine, or the corresponding 2-keto acid was added to the culture medium, the protylonolide titer increased. The addition of [^{14}C]valine gave rise to [^{14}C]protylonolide. ^{13}C NMR spectroscopic analysis revealed that *iso*-butyrate, which is a valine metabolite, was incorporated into protylonolide at the carbons known to originate from propionate and *n*-butyrate. Taking account of these findings, the regulation of tylosin biosynthesis in *S. fradiae* by ammonium ion is discussed in relation to amino acid metabolism.

Tylosin, a 16-membered macrolide antibiotic, is produced by strains of *Streptomyces*. The biosynthesis of tylosin in *S. fradiae* is subject to carbon regulation and phosphate regulation. Mechanisms for these regulation processes have been proposed at the enzyme level[1,2]. Tylosin production is also susceptible to inhibition by ammonium ions. However, the mechanism of action of ammonium ions is not well understood.

The biosynthetic pathway to tylosin can be divided into two parts: one is the formation of protylonolide, a precursor of the aglycone (see Fig. 5), and the other involves the synthesis of three sugars (mycaminose, mycinose and mycarose) and their sequential attachment to the aglycone[3]. The aglycone and the sugars are biosynthesized through separate routes, and assembled to form tylosin in a series of reactions[3,4]. Assuming that the biosynthetic steps sensitive to the effect of ammonium ions are involved in nitrogen metabolism, two steps in tylosin biosynthesis are possible targets. One is the biosynthesis (or attachment) of the aminosugar mycaminose. The other lies in the pathway for protylonolide formation. The latter notion came from a recent finding that the production of protylonolide was elevated under low ammonium ion conditions created in the presence of ammonium ion-trapping agents such as natural zeolite[5]. However, nitrogen metabolism was not known to be involved in the biosynthesis of protylonolide, which contains no nitrogen atom.

This paper presents evidence that protylonolide biosynthesis is subject to regulation by ammonium

† Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XXIX. Part XXVIII appeared in ref 5.

ions and by inorganic phosphate in a mutant of *S. fradiae*, and that precursors of protylonolide biosynthesis are supplied by amino acid metabolism. We suggest that the amino acid degradative pathway is one of the possible targets in regulation of tylosin biosynthesis by ammonium ions. A preliminary account of the present study has appeared[6].

Materials and Methods

Microorganisms

Tylosin-producing *S. fradiae* KA-427 (C-373) and two mutant strains, 261 and NP-10, both derived from KA-427, were used. The two mutants co-synthesize tylosin in mixed culture[7].

Method of Cultivation

A loopful of mycelia and spores of strain KA-427, or of the mutant NP-10, was inoculated into a 500-ml Sakaguchi flask containing 100 ml of seed medium (glucose 2%, peptone 0.5%, meat extract 0.5%, dry yeast cells 0.3%, NaCl 0.5%, $CaCO_3$ 0.3%, pH 7.0) and incubated at 27°C for two days with reciprocal shaking (120 rpm). Frozen mycelia of strain 261, obtained from 5 ml of a 2-day culture in the above seed medium and stored at −20°C, were inoculated and grown in a Sakaguchi flask in the same way as KA-427. The seed cultures thus obtained were transferred with an inoculum size of 3% into a 500-ml Sakaguchi flask containing 100 ml of a defined medium. The basal defined medium was composed of: starch 2%, glucose 0.5%, $(NH_4)_2SO_4$ (25 mM NH_4^+) 0.17%, 50% sodium lactate 0.55% (v/v), K_2HPO_4 0.05% (2.9 mM), $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, 3 mg each of trace metals (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}) per liter, presterile pH of 6.8~7.0. When an increase in ammonium or inorganic phosphate concentration was required, a separately sterilized solution of ammonium sulfate or potassium phosphate was added as a supplement. Flasks were incubated at 27°C for 1~7 days with reciprocal shaking (120 rpm).

Preparation of *iso*-[3,3′-$^{13}C_2$]Butyric Acid

Two grams of [^{13}C]CH_3I was refluxed with 930 mg of dimethyl malonate in tetrahydrofuran in the presence of sodium hydride. After addition of ethanol, the reaction mixture was added to water, and the water layer was extracted with ether. The ether layer was concentrated to afford dimethyl [CH_3-$^{13}C_2$]-2,2-dimethylmalonate. The dimethyl ester was hydrolyzed in ethanolic sodium hydroxide, and the resultant acid was extracted with ethyl acetate at an acidic pH. After evaporation, the residue was heated at 100°C for 1.5 hours in pyridine to give *iso*-[3,3′-$^{13}C_2$]butyric acid. It was extracted with acidic ether. Ether was evaporated to obtain 380 mg of *iso*-[3,3′-$^{13}C_2$]butyric acid (62% of theoretical yield).

Preparation and Isolation of ^{13}C-Labeled Protylonolide

Protylonolide-producing strain 261 was grown in six Sakaguchi flasks, each containing 100 ml of defined medium, in the manner described above. On the third day, 40 mg of *iso*-[3,3′-$^{13}C_2$]butyric acid in 1.5 ml of water (pH 7.0) were added to each flask. The flasks were incubated for a total of 7 days. The culture supernatant (500 ml) was extracted three times with an equal volume of benzene. Benzene was evaporated. The residue was dissolved in 4 ml of 50% methanol in 1 N HCl, and was left stand at room temperature for 6 hours. After evaporation of methanol and neutralization with $NaHCO_3$, the labeled protylonolide was extracted with benzene. It was then purified by preparative thin-layer chromatography on silica gel with chloroform - methanol (20: 1) as developing solvent. The substance at Rf 0.3 was extracted with chloroform - methanol (5: 1) to give 13 mg of ^{13}C-labeled protylonolide.

Analytical Method

The amount of tylosin was assayed microbiologically with *Micrococcus luteus* PCI 1001. The test organism was seeded in nutrient agar at pH 8.0. The amount of protylonolide was estimated by thin-layer chromatography, followed by scanning at 283 nm[5]. Mutant strain 261 co-produces protylonolide and a shunt metabolite, mycarosylprotylonolide[8]. The amount of the latter compound, determined at the same time as protylonolide by thin-layer chromatography, was corrected to express its protylonolide content. The total corrected amount of the two compounds is the protylonolide titer described in this paper.

Mycelial growth was expressed either as packed cell volume obtained by centrifugation (2,500 rpm, 5 minutes), or as dried cell weight given after drying at 80°C for 5~7 hours. The amounts of ammonium and of inorganic phosphate ions were assayed as described previously[6].

Chemicals

L-[^{14}C]Valine (250 Ci/mol) was purchased from New England Nuclear (Amsterdam); [^{13}C]methyl iodide (90 atom %) from Merck Sharp & Dohm (Canada). 2-Keto-*iso*-valerate, 2-keto-*iso*-caproate, 2-keto-3-methylvalerate and 2-keto-glutarate were supplied from Kyowa Hakko Kogyo (Tokyo).

Results

Effect of Glucose, Ammonium Sulfate and Potassium Phosphate on Tylosin Production in a Defined Medium

A previous paper[5] showed that when ammonium ion levels of a complex medium were lowered in the presence of natural zeolite, an ammonium ion-trapping agent, tylosin production by *S. fradiae* KA-427 increased about 3-fold. This suggests that natural zeolite caused the release of tylosin biosynthesis from regulation by ammonium ions. The effect of ammonium ions and of other nutrients was studied further in a chemically defined medium. Fig. 1-C shows that ammonium sulfate and potassium phosphate in high concentrations reduced specific production of tylosin. The addition of ammonium sulfate late in the growth phase (on the second day) exhibited a more severe inhibition than an initial addition of the ammonium salt.

It was of interest to determine whether ammonium and inorganic phosphate ions affect the earlier or the later part of tylosin biosynthesis. In one approach, a pair of co-synthetic mutants, strains 261 and NP-10, of *S. fradiae* KA-427 was cultured individually.

Strain 261 is a mycaminose-idiotroph and produces protylonolide. This mutant was grown for 5 days, and the amounts of protylonolide produced in the presence of various effectors were determined. Fig. 1-A shows that an increase in ammonium sulfate or potassium phosphate concentration reduced specific production of protylonolide. Glucose had no inhibitory effect.

Another mutant, strain NP-10, is defective in aglycone biosynthesis and produces no tylosin-related compounds, but is able to convert exogenously supplied protylonolide to tylosin. Strain NP-10 was grown in defined medium with or without a further supplement of glucose, ammonium sulfate or potassium phosphate. On the first day, the cultures received protylonolide and were incubated for an additional two days. As shown in Fig. 1-B, the addition of 1% (75 mM) ammonium sulfate reduced the conversion of protylonolide to tylosin by only 13%. A supplement of 0.3% ammonium sulfate had no effect. Whereas, the addition of potassium phosphate resulted in a decline by about 50%. Again, glucose showed no inhibition.

These results suggest that ammonium ions inhibit tylosin biosynthesis by affecting the steps before, rather than beyond, protylonolide formation, and that inorganic phosphate acts on both steps.

Time Course of Protylonolide Production in the Presence of High Levels of Ammonium Sulfate or Potassium Phosphate

To confirm the above results, a time course of protylonolide production by strain 261 was studied in the presence of varying amounts of ammonium sulfate or potassium phosphate. Fig. 2 shows that in the presence of high initial concentrations of ammonium ion, mycelial growth increased (Fig. 2-A), while specific protylonolide production was reduced (Fig. 2-B), except for the early idiophase. A sup-

Fig. 1. Effect of glucose, ammonium sulfate and potassium phosphate on the production of protylonolide (A) and of tylosin (C), and on conversion of protylonolide to tylosin (B) by *S. fradiae* KA-427 and its mutants in defined medium.

In A, protylonolide-producing mutant strain 261, of *S. fradiae* KA-427 was cultured for 4 days. The control culture (no addition) produced 4.7 μg of protylonolide per mg of dry cells.

In B, mutant strain NP-10 was grown for 3 days. Protylonolide (50 μg/ml) was added at the first day. The control culture produced 430 μg of tylosin from protylonolide per ml of packed mycelial volume.

In C, *S. fradiae* KA-427 was cultivated for 6 days. The control culture produced 89 μg of tylosin per ml of packed cell volume.

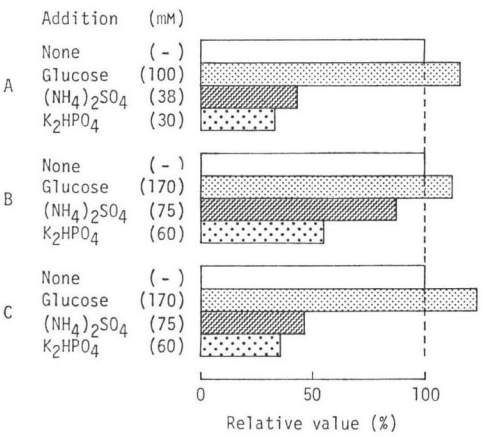
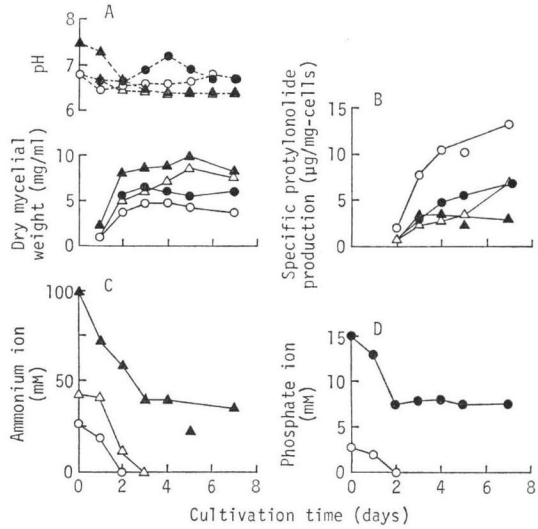


Fig. 2. Course of protylonolide production and fermentation parameters in the presence of varying amounts of ammonium and inorganic phosphate ions.

In A, growth (—) and pH (---); in B, specific protylonolide titers; in C, residual ammonium ion concentrations and in D, residual phosphate ion concentrations are shown.

Protylonolide-producing mutant, strain 261, was grown in defined medium containing initially 25 (○), 50 (△), or 100 mM (▲) ammonium ions with inorganic phosphate fixed at 2.9 mM; and 2.9 (○) or 13 mM (●) of inorganic phosphate ion with ammonium ion fixed at 25 mM.

Mean values of duplicate flasks are shown.



plement of as little as 10 mM potassium phosphate caused about 50% reduction in the protylonolide titer. In the supplemented cultures, both ammonium and phosphate ions remained at higher levels throughout the fermentation, or for a longer period of cultivation (Figs. 2-C and D). In the control culture, the two kinds of ions were consumed by the second day, at which time protylonolide production began.

Increase of Tylosin Production by Exogenously Supplied Protylonolide

The inhibition of protylonolide biosynthesis by ammonium ions described above poses the interesting problem of whether aglycone formation is limiting in tylosin biosynthesis. This can be explored by examining the effect of protylonolide supplements on tylosin production. *S. fradiae* KA-427 was grown in defined medium, to which protylonolide (50 μg/ml) was added on the second day. Fig. 3 shows that tylosin production increased considerably. Growth and pH values were unaffected. A similar result was obtained in a complex medium (data not shown).

Effect of Amino Acids and 2-Keto Acids on Protylonolide Production

Amino acids are incorporated into 16-membered macrolides at acyl side chains[6,10~12]. However, we observed that valine and leucine added to culture media increased production of tylosin, which has

Table 1. Effect of amino acids and 2-keto acids on protylonolide production by *S. fradiae* strain 261 in defined medium.

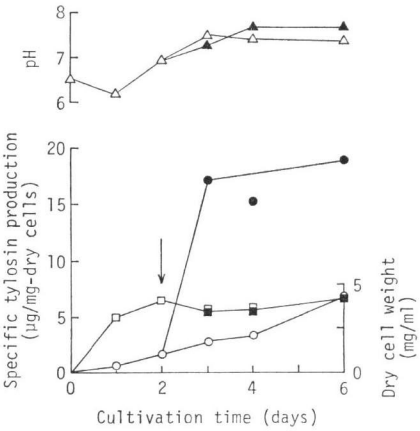
Amino acid added (10 mM)	Protylonolide produced (μg/ml)	Supplement (10 mM)	Specific protylonolide production (μg/mg dry cells)
None (expt 1)*1	18.0	None (expt 2)*2	12.9
L-Leucine	44.3	L-Leucine	17.9
L-Isoleucine	40.1	2-Keto-*iso*-caproate	19.0
L-Threonine	30.1	L-Isoleucine	18.3
L-Valine	29.0	2-Keto-3-methylvalerate	16.6
L-Proline	26.2	L-Threonine	21.5
L-Serine	24.6	2-Keto-butyrate	16.6
L-Alanine	23.9	L-Valine	16.5
L-Phenylalanine	23.8	2-Keto-*iso*-valerate	13.5
L-Tyrosine	23.6		
L-Aspartic acid	20.6		
Glycine	19.4		
L-Glutamic acid	19.0		
L-Glutamine	19.0		
L-Lysine	16.3		
Others*3	<10.0		

*1 Amino acids were added initially. Protylonolide titers in 5-day cultures are shown.
*2 Supplements were added on the third day. The titers in 6-day cultures were determined.
*3 L-Tryptophan, L-methionine, L-arginine, L-histidine and L-cysteine are included.

no acyl side chain (data not shown). This suggests that amino acids serve as precursors or regulators in tylosin biosynthesis. In order to study whether amino acids affect aglycone biosynthesis, naturally occurring amino acids were added to defined medium and the amounts of protylonolide produced were determined. As shown in Table 1 (expt 1), valine, leucine, isoleucine and threonine markedly promoted protylonolide production. Proline, serine and alanine caused smaller increases, but other amino acids including lysine had minor or inhibiting effects. The addition of the 2-keto acids corresponding to valine, leucine, isoleucine and threonine appreciably elevated specific protylonolide titers (Table 1, expt 2). Pyruvate and 2-keto-glutarate had little effect.

Fig. 3. Effect of protylonolide on tylosin production.

A 0.1 ml aliquot of an ethanol solution of protylonolide was added at the time indicated by an arrow to give a final concentration of 50 μg/ml. Ethanol at this concentration had no effect.

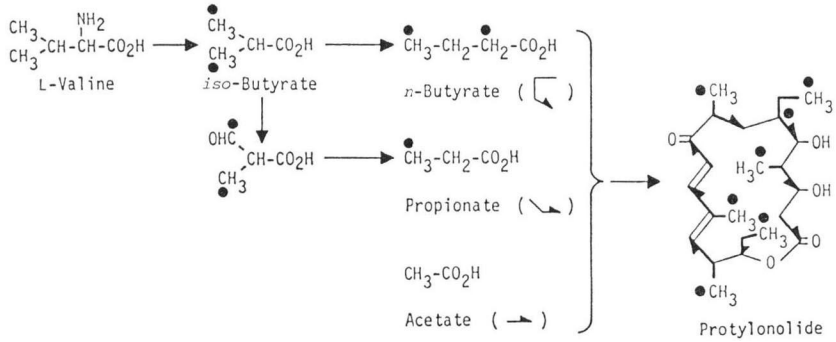


The effect of the amino acids was not due to supplying ammonium nitrogen, thereby increasing cell growth, since the addition of a corresponding amount (10 mM NH_4^+) of ammonium sulfate did not appreciably alter specific protylonolide titers.

Incorporation of [^{14}C]Valine and *Iso*-[^{13}C]butyrate into the Carbon Skeleton of Protylonolide

To study the incorporation of an amino acid into the carbon skeleton of protylonolide, valine was used because it increased protylonolide and tylosin titers reproducibly and because the labeled metabolite

Fig. 4. Incorporation of the carbon atoms of *iso*-[3,3′-$^{13}C_2$]butyrate into protylonolide, and possible routes for its metabolism.



of the amino acid, *iso*-butyrate, was available. [^{14}C]Valine (0.25 μCi/ml) was added to a culture of protylonolide fermentation on the third day. After two hours of shaking, the culture supernatant fluid was extracted with toluene, and the toluene layer was washed once with water. The radioactivity of the toluene layer was 1.14 nCi/ml. On silica gel thin-layer chromatography with $CHCl_3$ - methanol (20: 1), UV-absorbing radioactive substances in the toluene layer migrated the same distances as protylonolide (Rf 0.4) and mycarosylprotylonolide (Rf 0.6). [^{14}C]Lysine, used as a negative control, produced little labeled material under the same conditions. These results demonstrate that valine carbons are incorporated into protylonolide.

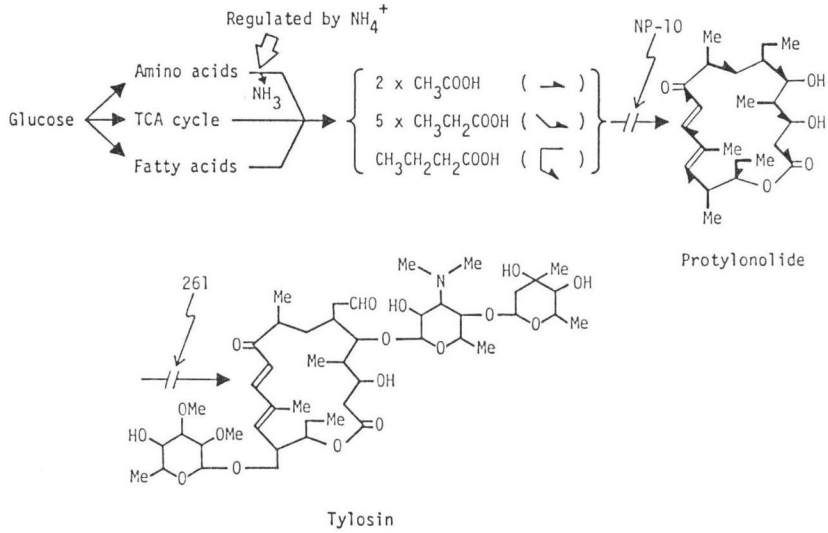
Valine is known to be metabolized to *iso*-butyryl-CoA, which is further converted to propionyl-CoA, one of the direct precursors of protylonolide biosynthesis. To study the incorporation pattern, *iso*-[3,3′-$^{13}C_2$]butyric acid was chemically synthesized, and added to a culture producing protylonolide. ^{13}C-Labeled protylonolide was isolated by solvent extraction and by preparative thin-layer chromatography on silica gel. The ^{13}C NMR spectrum of the labeled protylonolide revealed enrichments not only at carbons of propionate origin, but at carbons known to originate from a butyrate unit (Fig. 4). The enrichment pattern showed that the valine metabolism involved a hitherto unknown isomerization of *iso*-butyrate to *n*-butyrate[6].

Discussion

It is well established that the aglycone of tylosin is built up with two acetate, five propionate and one *n*-butyrate units[18]. These precursors participate in the condensation reaction as CoA esters or CoA esters of their 2-carboxylated derivatives. Although the origin of these lower fatty acids has not been discussed, it has been assumed until now that they are supplied principally by the tricarboxylic acid (TCA) cycle or by diversion from higher fatty acid biosynthesis (Fig. 5). However, the time course of protylonolide production in a defined medium suggested an alternative route, since protylonolide production was more active after cell growth slowed down than in the growth phase (Fig. 2). After cell growth ceases, the activities of the TCA cycle and of higher fatty acid synthesis are generally low, whereas protein, lipids and other storage materials become actively degraded. Amino acid degradation is also active after growth, and produces lower fatty acids.

It would be reasonable to assume that the lower fatty acids thus produced enter the acid pools to be utilized for protylonolide synthesis. As anticipated, the results of experiments with ^{14}C- and ^{13}C-labeled compounds demonstrated the incorporation of valine carbons into protylonolide (Fig. 4). Other amino acids such as leucine and threonine which promoted protylonolide production might have acted in similar ways, or have stimulated valine metabolism, as will be reported elsewhere.

Fig. 5. Proposed pathways supplying precursors of tylosin biosynthesis in *S. fradiae*.
Possible site of regulation by ammonium ions is shown by an open arrow (⇩). Mutant strains 261 and NP-10 are defective at the sites indicated.



The involvement of amino acid metabolism was suggested in tetracycline[14], erythromycin[15] and tetrocarcin[16] biosynthesis. A methionine methyl[2,13] and probably glycine[17] are incorporated into the aglycone of the leucomycin group of 16-membered macrolide antibiotics. Valine enters into milbemycin molecules as an *iso*-butyrate unit[18]. Our data presented in this paper are the first to show that an amino acid is incorporated into all three kinds of building units, *n*-butyrate, propionate and acetate, of a 16-membered lactone. The weak enrichment of acetate-derived carbons was presumably due to dilution by natural acetate, which was derived from lactate added as a basal ingredient of the defined medium employed. Valine metabolism was found to involve a new route from *iso*-butyrate to *n*-butyrate (Fig. 4). The properties and the physiological role of the isomerization enzyme is not known. A similar reaction was recently suggested in monensin biosynthesis by *S. cinnamonensis*[19]. Thus, amino acids are utilized as donors of building units of many polyketide antibiotics. However, the significance of amino acid metabolism for regulation of polyketide biosynthesis has not so far been considered.

Because valine catabolism is involved in protylonolide biosynthesis, the inhibition of protylonolide production by ammonium ions (Fig. 2) is noteworthy. According to MAGASANIK[20] and TYLER[21], the metabolism of amino acids such as histidine, asparagine and proline is subject to regulation by ammonium ions in enteric bacteria. Glutamine synthetase in its unadenylylated form acts as a positive regulator. A similar adenylylation-deadenylylation control of glutamine synthetase has been shown in *S. cattleya*, a thienamycin producer[22,23]. In another actinomycete *Nocardia butanica*, we showed that degradation of glycine was sensitive to ammonium ions, and that glutamine synthetase appeared to be involved[24]. If a similar mechanism operates in amino acid metabolism in *S. fradiae*, the inhibition of protylonolide by ammonium ions can be explained by interference with metabolism of the amino acid which supplies an indispensable precursor of protylonolide biosynthesis.

It is likely that aglycone biosynthesis is limiting in tylosin biosynthesis by *S. fradiae* KA-427 for the following reasons: (1) protylonolide production, like tylosin production, increased under low ammonium ion conditions[5], (2) when a large amount of protylonolide is available, the tylosin yield is elevated (Fig. 3), (3) protylonolide biosynthesis, like tylosin production, is inhibited by high levels of ammonium ions (Figs. 1 and 2). Thus the level of protylonolide available intracellularly appears to determine the rate of tylosin biosynthesis.

In summary, it is suggested that in the presence of high levels of ammonium ions, tylosin production by *S. fradiae* KA-427 is decreased because of the limited supply of the aglycone moiety, which in turn is

caused by the reduced levels of a key precursor derived from certain amino acids, as illustrated in Fig. 5. This hypothesis awaits verification.

Acknowledgments

The authors wish to thank Dr. K. TSUZUKI, The Kitasato Institute, for synthesis of ^{13}C-labeled *iso*-butyrate and for NMR spectroscopy. Thanks are also due to Mr. A. TAKI and Miss K. MATSUDA for helpful assistance.

References

1) VU-TRONG, K.; S. BHUWAPATHANAPUN & P. P. GRAY: Metabolic regulation in tylosin-producing *Streptomyces fradiae*: Regulatory role of adenylate nucleotide pools and enzymes involved in biosynthesis of tylonolide precursors. Antimicrob. Agents Chemother. 17: 519~525, 1980
2) ŌMURA, S. & Y. TANAKA: Macrolides. *In* Biochemistry and Genetic Regulation of Commercially Important Antibitics. *Ed.* L. C. VINING, pp. 179~206, Addison-Wesley Publishing Co., London, 1983
3) ŌMURA, S.; N. SADAKANE & H. MATSUBARA: Biosynthesis of tylosin after protylonolide formation. Chem. Pharm. Bull. 30: 223~229, 1982
4) ŌMURA, S.; H. TANAKA & M. TSUKUI: Biosynthesis of tylosin: Oxidation of 5-*O*-mycaminosylprotylonolide at C-20 and C-23 with a cell-free extract from *Streptomyces fradiae*. Biochem. Biophys. Res. Commun. 107: 554~560, 1982
5) MASUMA, R.; Y. TANAKA & S. ŌMURA: Ammonium ion-depressed fermentation of tylosin by the use of a natural zeolite and its significance in the study on the biosynthetic regulation of the antibiotic. J. Ferment. Technol. 61: 607~614, 1983
6) ŌMURA, S.; K. TSUZUKI, Y. TANAKA, H. SAKAKIBARA, M. AIZAWA & G. LUKACS: Valine as a precursor of *n*-butyrate unit in the biosynthesis of macrolide aglycone. J. Antibiotics 36: 614~616, 1983
7) ŌMURA, S.; H. MATSUBARA, A. NAKAGAWA, A. FURUSAKI & T. MATSUMOTO: X-Ray crystallography of protylonolide and absolute configuration of tylosin. J. Antibiotics 33: 915~917, 1980
8) ŌMURA, S.; N. SADAKANE, C. KITAO, H. MATSUBARA & A. NAKAGAWA: Production of mycarosylprotylonolide by a mycaminose idiotroph from the tylosin-producing strain *Streptomyces fradiae* KA-427. J. Antibiotics 33: 913~914, 1980
9) ŌMURA, S.; Y. TANAKA, C. KITAO, H. TANAKA & Y. IWAI: Stimulation of leucomycin production by magnesium phosphate and its relevance to nitrogen catabolite regulation. Antimicrob. Agents Chemother. 18: 691~695, 1980
10) VEZINA, C.; C. BOLDUC, A. KUDELSKI & P. AUDET: Biosynthesis of kitasamycin (leucomycin) by leucine analog-resistant mutants of *Streptomyces kitasatoensis*. Antimicrob. Agents Chemother. 15: 738~746, 1979
11) FURUMAI, T.; K. TAKEDA & M. SUZUKI: Studies on the biosynthesis of basic 16-membered macrolide antibiotics, platenomycin. IV. Biosynthesis of platenomycins. J. Antibiotics 28: 789~797, 1975
12) MIYAGAWA, K.; M. SUZUKI, E. HIGASHIDE & M. UCHIDA: Effect of aspartic acid family amino acids on production of maridomycin. III. Agric. Biol. Chem. 43: 1103~1106, 1979
13) ŌMURA, S.; H. TAKESHIMA, A. NAKAGAWA, J. MIYAZAWA, F. PIRIOU & G. LUKACS: Studies on the biosynthesis of 16-membered macrolide antibiotics using carbon-13 nuclear magnetic resonance spectroscopy. Biochemistry 16: 2860~2866, 1977
14) BEHAL, V.; Z. HOSTALEK & Z. VANEK: Anhydrotetracycline oxygenase activity and biosynthesis of tetracyclines in *Streptomyces aureofaciens*. Biotechnol. Lett. 1: 177~182, 1979
15) CORCORAN, J. W.: Biochemical mechanisms in the biosynthesis of the erythromycins. *In* Antibiotics. IV. Biosynthesis. *Ed.* J. W. CORCORAN, pp. 132~174, Springer-Verlag, Berlin, Heiderberg, 1981
16) TAMAOKI, T. & F. TOMITA: Biosynthesis and production of tetrocarcin A, a new antitumor antibiotic, in a chemically defined medium. Agric. Biol. Chem. 46: 1021~1026, 1982
17) ŌMURA, S.; K. TSUZUKI, A. NAKAGAWA & G. LUKACS: Biosynthetic origin of carbons 3 and 4 of leucomycin aglycone. J. Antibiotics 36: 611~613, 1983
18) ONO, M.; H. MISHIMA, Y. TAKIGUCHI & M. TERAO: Milbemycins, a new family of macrolide antibiotics. Studies on the biosynthesis of milbemycins α_2, α_4 and D using ^{13}C labeled precursors. J. Antibiotics 36: 991~1000, 1983
19) POSPISIL, S.; P. SEDMERA, M. HAVRANEK, V. KRUMPHANZL & Z. VANEK: Biosynthesis of monensins A and B. J. Antibiotics 36: 617~619, 1983

20) MAGASANIK, B.: Genetic control of nitrogen assimilation in bacteria. Ann. Rev. Genet. 16: 135~168, 1982
21) TYLER, B.: Regulation of the assimilation of nitrogen compounds. Ann. Rev. Biochem. 47: 1127~1162, 1978
22) STREICHER, S. L. & B. TYLER: Regulation of glutamine synthetase activity by adenylylation in the Gram-positive bacterium *Streptomyces cattleya*. Proc. Natl. Acad. Sci. USA 78: 229~233, 1981
23) WAX, R.; L. SYNDER & L. KAPLAN: Inactivation of glutamine synthetase by ammonia shock in the Gram-positive bacterium *Streptomyces cattleya*. Appl. Environ. Microbiol. 44: 1004~1006, 1982
24) TANAKA, Y.; S. ŌMURA, K. ARAKI & K. NAKAYAMA: Derepression of glycine decarboxylase synthesis by magnesium phosphate in *Nocardia butanica*. Agric. Biol. Chem. 45: 2661~2664, 1981