MECHANISM OF NITROGEN REGULATION OF PROTYLONOLIDE BIOSYNTHESIS IN *STREPTOMYCES FRADIAE*†

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A resting cell system was used to study the sites of inhibition by NH_4^+ of protylonolide biosynthesis by a blocked mutant, strain 261, of *Streptomyces fradiae*. With ^{14}C-labeled succinate or valine as an exogenous substrate, labeled protylonolide formation by high-NH_4^+ grown mycelia of strain 261 was lower than by low-NH_4^+ grown mycelia. When ^{14}C-labeled palmitate or acetate + labeled propionate + butyrate were used as the substrates, protylonolide production by mycelia grown under the two NH_4^+ conditions was nearly at the same rates. It is suggested that the metabolism of succinate and valine to lower fatty acid precursors is subject to NH_4^+ regulation, whereas condensation of acid precursors and related steps leading to protylonolide are insensitive to NH_4^+ concentration.

Tylosin, a 16-membered macrolide antibiotic produced by *Streptomyces fradiae*, comprises the aglycone, and the three sugars mycaminose, mycarose and mycinose. The four building blocks are synthesized in separate pathways and assembled in a sequential order[1]. Tylosin biosynthesis is subject to regulation by the nitrogen[2,3]. Recent studies from our laboratories showed that NH_4^+-susceptible step(s) lies within pathways to protylonolide, the earliest lactone precursor of the aglycone[4]. Other routes such as sugar synthesis and attachment involved in tylosin biosynthesis are much less sensitive to NH_4^+. Protylonolide is produced by a blocked mutant of a tylosin-producing *S. fradiae*. Because protylonolide biosynthesis is suppressed by NH_4^+ in this mutant[3,4], the organism provides a good system to study nitrogen regulation of tylosin production.

The lactone protylonolide is constructed with three building units, acetate, propionate, and butyrate. They are condensed by a head-to-tail mechanism, and cyclized, to afford protylonolide. The lower fatty acid precursors are supplied by three metabolic pathways: Those of several amino acids such as valine, leucine, and threonine; organic acids of the tricarboxylic acid (TCA) cycle such as succinic acid; and higher fatty acids like palmitic acid.

Of these three, metabolism of several amino acids was shown[5,6] to be suppressed at the same time by NH_4^+, and their incorporation into protylonolide molecule decreased considerably in the presence of high concentrations of NH_4^+. This mechanism of regulation is reasonable in the sense that the metabolism of nitrogen-containing compounds is controlled by exogenous NH_4^+. In view of the fact that succinate and palmitate are as effective suppliers of lower fatty acid precursors as valine and threonine, it is an interesting question whether or not the metabolism of such non-nitrogenous compounds is susceptible to NH_4^+. In addition, little is known about regulatory properties of the biosynthesis of polyketide condensing enzyme, one of the key enzymes involved in protylonolide formation.

† Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XXXIII. Part XXXII of this series appeared in ref 5.

This paper deals with results of washed mycelia experiments, which suggest that succinate metabolism is strongly repressed by NH_4^+, whereas the condensation step is not so, in protylonolide biosynthesis by a mutant of *S. fradiae*.

Materials and Methods

Microorganism

Protylonolide-producing *S. fradiae* strain 261, derived from the tylosin producer *S. fradiae* KA-427 (C-373), was used throughout this work.

Method of Cultivation

Frozen mycelia of strain 261 were inoculated into 500-ml Sakaguchi flasks containing 100 ml of a complex seed medium[3], and incubated at 27°C for three days. Mycelial pellets obtained from 10 ml of the seed culture by centrifugation (5,000 rpm, 10 minutes at 5°C) were washed twice with sterile water, and resuspended in 10 ml of H_2O. An aliquot (3% in inoculum size) of this vegetative mycelial suspension was used to transfer into 50-ml test tubes (2×20 cm) containing 10 ml of chemically defined media or 500-ml Erlenmeyer flasks containing 50 ml of media. The defined media were composed of: Soluble starch 2%, glucose 0.5%, separately sterilized $(NH_4)_2SO_4$ 0.17~0.66% (25~100 mM NH_4^+), $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, 50% sodium lactate 0.55%, $CaCO_3$ 0.3%, *N*-morpholinopropanesulfonic acid (MOPS) 2.1% (0.1 M), 0.3% of trace metal solution consisting of (each at 1 g/liter) $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 2H_2O$, pH 7.0 before autoclaving. A solution of 10% $(NH_4)_2SO_4$, adjusted to pH 7 with 2 N NaOH and sterilized by filtration through a Tōyō membrane filter (0.45 μm in pore size, TM-2), was added aseptically as required before inoculation. The other conditions were as reported previously[5]. The chemically defined medium containing 25 mM of NH_4^+ will be referred to as the control medium in the text.

[^{14}C]Protylonolide Production from ^{14}C-Labeled Compounds with Washed Mycelia of Strain 261

Strain 261 was grown in chemically defined media with $(NH_4)_2SO_4$ concentration at 25 mM or 100 mM. The mycelia were harvested at daily intervals by centrifugation (5,000 rpm, 10 minutes at 5°C), washed with H_2O, and resuspended in 0.1 M MOPS (pH 7.8) to give a cell concentration normally in the range of 2.8~4.8 mg dry cells/ml. To a 2-ml aliquot of this mycelial suspension were added ^{14}C-labeled substrates (0.2 μCi/ml) and corresponding unlabeled compounds (0.25~2.0 mM), and the suspension was incubated at 27°C with shaking for up to 6 hours. When other buffers or buffers of different pH values were employed, mycelial harvest and washing were carried out in the desired buffers. Reaction mixtures in two test tubes of identical reaction conditions were combined, and the supernatant fluid was used for measuring radioactivity. Determination of radioactivity and other conditions are as described previously[5]. Protylonolide produced under the present conditions is actually a mixture of protylonolide and 5-*O*-mycarosylprotylonolide. The latter compound is a shunt metabolite. The total radioactivity of the two was employed to express the amount of radioactive protylonolide.

Incorporation of ^{14}C-Labeled Compounds into Mycelia of Strain 261

Washed mycelia of strain 261 were incubated with ^{14}C-labeled succinate or valine under the same conditions as for [^{14}C]protylonolide production. At 10 or 20 minutes intervals, the mycelia were collected on a Tōyō membrane filter (0.45 μm, TM-2), washed three times with 5 ml cold water, and dried. The radioactivity of the mycelia was measured with a toluene-based scintillation cocktail.

Determination of Protylonolide by High Performance Liquid Chromatography (HPLC)

The supernatant of a reaction mixture (10 ml) obtained by centrifugation (5,000 rpm, 10 minutes) was extracted twice with an equal volume of benzene. The combined benzene layer was concentrated to dryness *in vacuo*. The residue was dissolved in 100 μl of acetonitrile. Ten μl of this solution was subjected to HPLC, which was developed using LiChrosorb RP-18 as the column material and a mixture of acetonitrile and 10 mM KH_2PO_4 (pH adjusted to 2.5 with H_3PO_4) (6 : 4) as elution solvent. Peak heights of protylonolide (retention time at 4.8 minutes) and of mycarosylprotylonolide (retention time

Fig. 1. Effect of pH on ^{14}C-labeled protylonolide production from ^{14}C-labeled compounds by resting cells of *S. fradiae* strain 261.

S. fradiae strain 261, a protylonolide-producing mutant, was grown in test tubes containing 10 ml of a chemically defined medium with NH_4^+ at 25 mM (control medium).

The mycelia of 2-day cultures were harvested, washed, and re-suspended (3.7 mg dry cells/ml) in the various 0.1 M GOOD's buffers[14], and incubated with [^{14}C]valine (○) or [^{14}C]succinate (●) (each at 2 μCi/ml, 2 mM) at 27°C for 3 hours with shaking (120 strokes/minute).

Buffers used were: Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) for pH 6.0~7.0; MOPS for pH 7.0~7.8; *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid for pH 7.8~9.0; and 3-cyclohexylaminopropanesulfonic acid for pH 9.0~11.0. Relative mean radioactivity of duplicate reaction tubes are shown. Other conditions and the method of radioactive assays were those described previously.

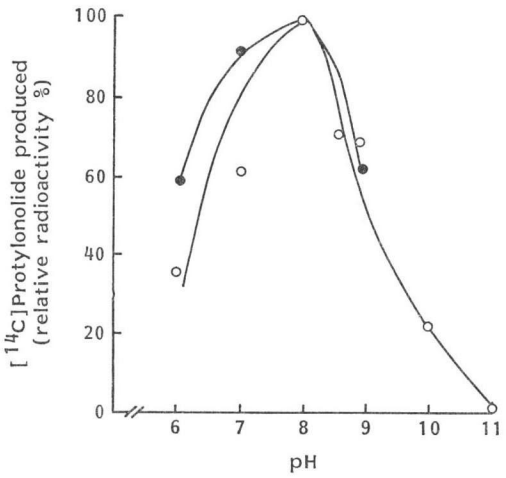
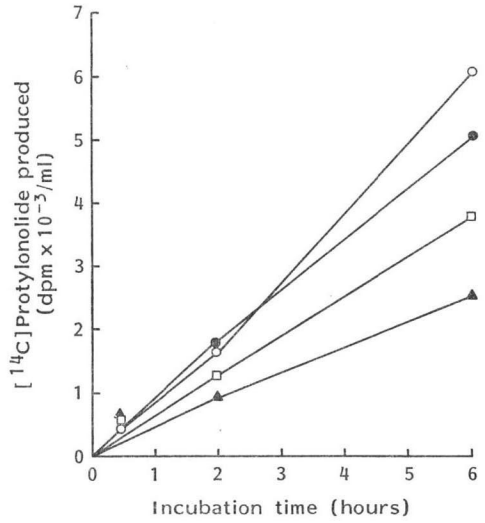


Fig. 2. Time course of labeled protylonolide production from labeled substrates by resting cells of *S. fradiae* strain 261.

Strain 261 was grown in control medium for 2 days. The mycelia, harvested and suspended (3.2 mg dry cells/ml) in 0.1 M MOPS (pH 7.8), were incubated with the indicated ^{14}C-labeled compounds for up to 6 hours.

[^{14}C]Palmitate (□) and [^{14}C]propionate (▲) (each at 2 μCi/ml, 1 mM). Other conditions and symbols were the same as described in Fig. 1.



at 6.2 minutes) were recorded at 283 nm. The amounts of each compound were estimated using calibration curves for peak height vs. known concentration. The amount of mycarosylprotylonolide was corrected to express its protylonolide content. The total corrected amount of the two is the protylonolide titer employed in this paper.

Radiochemicals

Radioactive compounds purchased from New England Nuclear included uniformly ^{14}C-labeled valine and palmitic acid, [3-^{14}C]propionic acid, and [2,3-$^{14}C_2$]succinic acid.

Results

Reaction Conditions for the Formation of [^{14}C]Protylonolide from [^{14}C]Succinate with Washed Mycelia of Protylonolide-producing *S. fradiae* Strain 261

Washed mycelia provides a convenient tool to study regulatory effects of NH_4^+ on protylonolide formation, if an appropriate reaction system is available. Therefore, attempts were made to establish a resting cell system for protylonolide formation with labeled succinate as the substrate. The protylonolide-producing mutant, strain 261, was grown in control chemically defined medium.

Table 1. Specific radioactivity of protylonolide produced by resting cells of *S. fradiae* strain 261.

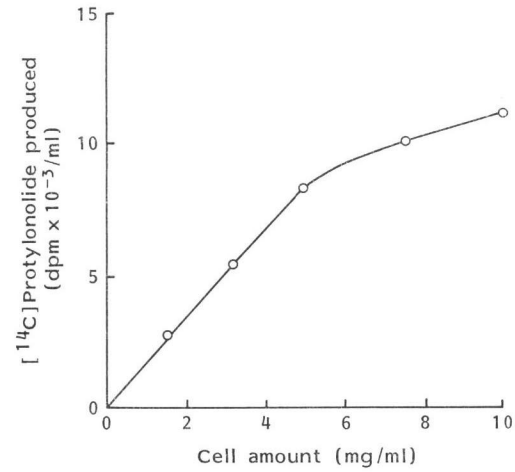
Substrate for protylonolide production (0.2 μCi/ml, 1 mM)	NH_4^+ level for growth (mM)	Specific radioactivity of protylonolide	
		(dpm/μg)	(mCi/mmol)
[^{14}C]Valine	25	1,630	0.27
	75	1,550	0.25
[^{14}C]Succinate	25	4,350	0.71
	75	5,000	0.82

Strain 261 was grown in test tubes containing defined media with NH_4^+ at 25 mM or 75 mM. At day 2 mycelia were harvested, washed, suspended (2.8 mg dry cells/ml) in 0.1 M MOPS (pH 7.8). Two-ml aliquots were incubated with the indicated labeled compounds at 27°C for 3 hours.

Reaction mixtures (12 ml) in 6 reaction tubes were combined, and the supernatant fluid (10 ml) was used to determine protylonolide content by HPLC. Fractions of the HPLC eluate corresponding to protylonolide and mycarosylprotylonolide were collected, evaporated to dryness, and used for the assay of radioactivity.

Fig. 3. Effect of cell concentration on [^{14}C]protylonolide production from [^{14}C]succinate.

The mycelia of strain 261 of 2-day cultures, after washing, were suspended in 0.1 M MOPS (pH 7.8) to give the indicated final cell concentrations, and incubated with [^{14}C]succinate at 27°C for 3 hours. Other conditions are as described for Fig. 1.



At day 2, mycelia were harvested, washed, and resuspended in MOPS buffer, or in other buffers when necessary, and incubated with [^{14}C]succinate for various periods of time.

The optimal pH for [^{14}C]protylonolide formation was 7.8 (Fig. 1). [^{14}C]Protylonolide was produced linearly up to 6 hours at pH 7.8 (Fig. 2). With labeled palmitate or propionate as the substrate, protylonolide production also proceeded linearly. The label of protylonolide produced was proportional to the cell amounts within a range of 0~5 mg/ml (Fig. 3). Fortunately, when [^{14}C]valine was used as an alternative substrate, the optimal reaction conditions for protylonolide formation were identical to those with labeled succinate as the substrate (Figs. 1 and 2). These results allowed the use of valine as a reference substrate, whose metabolism to propionate and butyrate has been demonstrated to be sensitive to NH_4^+ action.

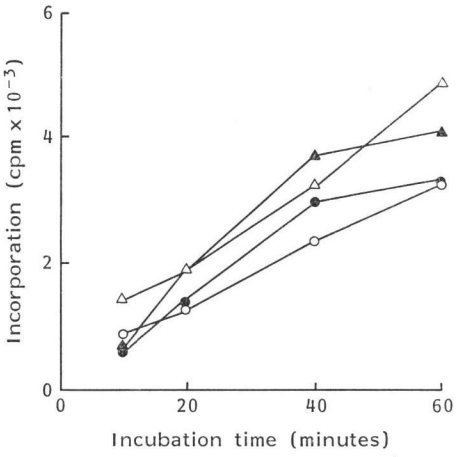
One of the requirements in interpreting the results of label incorporation experiments described below is that the amount of protylonolide produced by the resting cell system is proportional to the radioactivity observed. To examine this, four reaction mixtures obtained with high (75 mM)- and low (25 mM)-NH_4^+ grown mycelia, and with ^{14}C-labeled succinate and valine as substrates were subjected to HPLC. Protylonolide fractions, *i.e.* protylonolide plus mycarosylprotylonolide, were collected, and the amounts and radioactivities were determined. Table 1 shows that the specific radioactivities of protylonolide produced by mycelia previously grown under high- and low-NH_4^+ levels were substantially the same for each substrate employed, although the values obtained with valine were lower than with succinate. It is obvious that, when the substrate for protylonolide production is fixed, the amount of protylonolide can be estimated by the radioactivity, regardless

of NH_4^+ levels under which strain 261 was previously grown.

It is also necessary that the uptake of labeled substrates is not affected by NH_4^+ levels for growth. Strain 261 was grown in the same manner as for protylonolide formation, and washed mycelia obtained in two NH_4^+ levels were measured for their ability to incorporate labeled valine and succinate into mycelia. As shown in Fig. 4, no difference was observed in the incorporation rates of the nitrogen-containing and non-containing substrates. It is concluded that the resting cell system set up here can be used to study regulatory properties of protylonolide biosynthesis in *S. fradiae* strain 261.

Fig. 4. Incorporation of ^{14}C-labeled compounds into resting cells of *S. fradiae* strain 261 previously grown in low and high levels of NH_4^+.

Strain 261 was grown for 3 days in defined medium containing 25 mM (open symbols) or 75 mM (closed symbols) of NH_4^+, harvested, and incubated with [^{14}C]valine (○, ●) (0.2 μCi/ml, 2 mM) or [^{14}C]succinate (△, ▲) (0.2 μCi/ml, 2 mM).



Effect of NH_4^+ on Protylonolide Biosynthesis from Succinate and Palmitate

S. fradiae strain 261 was grown in defined media containing 25 and 100 mM NH_4^+. In the latter medium, protylonolide titers per unit weight of growing mycelia decreased by more than 80%[5]. Washed mycelia obtained at day 2 in the two NH_4^+ levels were compared for their ability to produce [^{14}C]protylonolide from [^{14}C]succinate. Mycelia previously grown in high-NH_4^+ conditions produced a lower amount (18%) of protylonolide from succinate than mycelia grown under low-NH_4^+ conditions. The same type of responses to NH_4^+ was observed when the reference substrate valine was used (data not shown).

To re-examine the above results, mycelia were harvested daily, and used for resting cell experiments. As shown in Fig. 5B, high-NH_4^+ grown mycelia harvested at days 2 through 6 produced lower amounts of protylonolide from succinate than low-NH_4^+ grown mycelia. Again, the same tendency was observed with valine as the substrate (Fig. 5A). These results strongly suggest that NH_4^+ represses succinate metabolism to lower fatty acid precursors.

The effect of NH_4^+ on the metabolism of a higher fatty acid to lower fatty acid precursors was studied under identical conditions. A typical result is shown in Fig. 5C. The results with labeled palmitate fluctuated to some extent. Nevertheless, the two kinds of mycelia appeared to produce protylonolide at nearly the same levels. The fluctuation is probably due to uneven dispersion of this higher fatty acid in the reaction mixture, even though a surfactant was supplemented. Labeled methyl oleate, which was expected to be more dispersive, was not available commercially.

Effect of NH_4^+ on the Condensation Step and Later Processes for Protylonolide Formation

Protylonolide biosynthesis from acetate, propionate and butyrate involves activation of the lower fatty acids by thio-esterification, their condensation, and later steps such as cyclization, forming finally a 16-membered ring system. The present resting cell system was found to produce protylonolide using these three lower fatty acids as exogenous substrates (see Fig. 2). Therefore, it was possible to apply

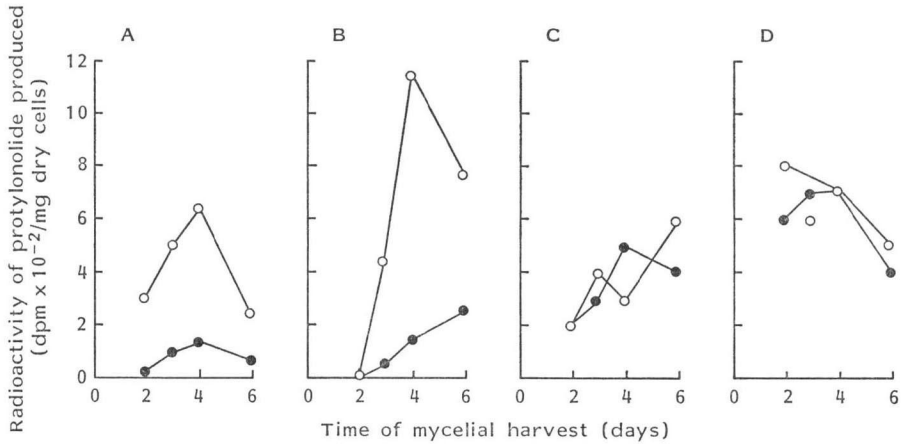
Fig. 5. ^{14}C-Labeled protylonolide production by resting cells of *S. fradiae* strain 261 previously grown under high- and low-NH_4^+ defined media.

S. fradiae strain 261 was grown in 500-ml Erlenmeyer flasks containing 50 ml of a defined medium with an NH_4^+ content of either 25 mM or 100 mM. Two flasks were withdrawn for each NH_4^+ level at the intervals indicated.

An equal volume (5~10 ml) of cultured broths of the two flasks were combined, and subjected to a Teflon homogenizer for 10~20 seconds to disrupt mycelial pellets, followed by centrifugation, washing, resuspension (3.0~4.8 mg dry cells/ml) in 0.1 M MOPS (pH 7.8).

An aliquot (2 ml) of the cell suspension was poured into 10 reaction tubes, to each two of which were added: A) [^{14}C]Valine (0.2 μCi/ml, 2 mM); B) [^{14}C]succinate (0.2 μCi/ml, 2 mM); C) [^{14}C]palmitate (0.2 μCi/ml, 2 mM); and D) acetate (0.5 mM) + [^{14}C]propionate (0.2 μCi/ml, 1.25 mM) + butyrate (0.25 mM).

Incubation and radioactivity determination were done as in Fig. 1. Mean values of duplicate reaction tubes with low-NH_4^+ grown cells (○) and high-NH_4^+ grown cells (●) are shown.



the above radioisotope technique to the study of the NH_4^+ effect on condensation and later steps. High- and low-NH_4^+ grown mycelia of strain 261 were incubated with [^{14}C]propionate together with unlabeled acetate, propionate and butyrate. As shown in Fig. 5D, [^{14}C]protylonolide production by the two kinds of mycelia was nearly at the same levels irrespective of mycelial ages. This indicates that NH_4^+ does not affect protylonolide biosynthesis after the formation of lower fatty acid precursors.

In all the above experiments (Fig. 5), the major products were protylonolide and 5-*O*-mycarosyl-protylonolide with very small amounts of unidentified by-products, as detected by HPLC at 283 nm. Only two radioactive compounds were observed by thin-layer chromatography followed by autoradiography. The Rf values were the same as those of protylonolide and the mycarosyl derivative.

Discussion

The experimental results presented in this paper allow the following conclusions to be drawn as to the mechanism regulating protylonolide biosynthesis:

(1) NH_4^+ suppresses succinate metabolism, as well as valine and other amino acid metabolism, to decrease the supply of lower fatty acid precursors,

(2) palmitate metabolism is probably not subject to NH_4^+ regulation, and

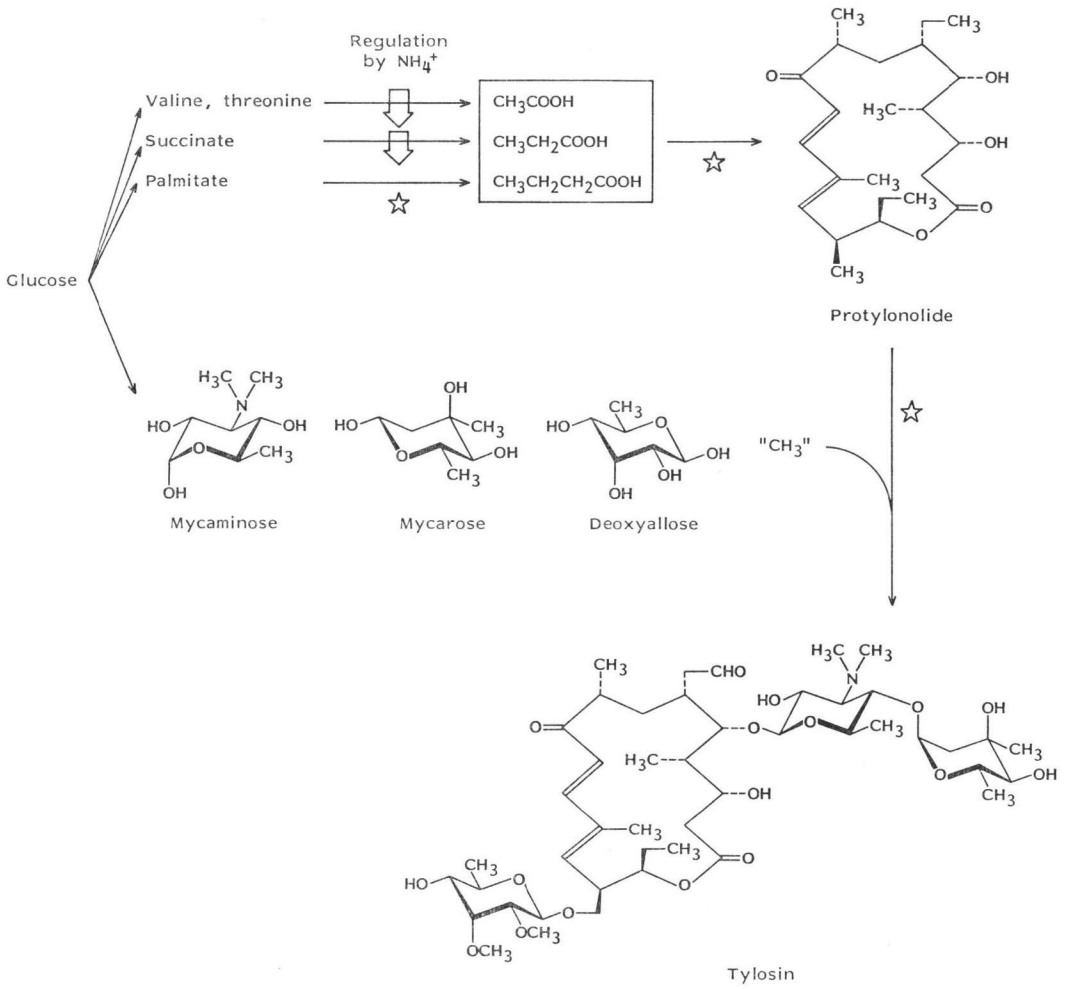
(3) condensation of lower fatty acids and the subsequent steps related to protylonolide formation are not affected by NH_4^+.

Enzymatic basis of this mechanism awaits verification.

Two enzymes of succinate metabolism are assumed to be possible targets of the NH_4^+ action.

Fig. 6. Proposed mechanism of nitrogen regulation of tylosin biosynthesis in *S. fradiae* KA-427.

The steps inhibited by NH_4^+ are shown by open arrows. The steps indicated by asterisks are those insensitive to NH_4^+ action.



These are methylmalonyl-CoA mutase (EC 5.4.99.2), and methylmalonyl-CoA carboxytransferase (EC 2.1.3.1). The two reactions were detected in tylosin-producing *S. fradiae*[7~9]. It is worthy to note that both reactions are not concerned with nitrogen-containing substrates nor products. *m*-Hydroxybenzyl alcohol dehydrogenase, an enzyme involved in patulin synthesis in *Penicillium urticae*, is the first example of an enzyme of secondary metabolism involved in a non-nitrogenous reaction yet subject to nitrogen regulation[10], although a growth-rate dependent mechanism appears also to be related. Succinate metabolism presented here is the second of such examples, in which growth rate regulation is not relevant.

Condensing enzyme is regarded as one of the key enzymes in protylonolide biosynthesis. This is the very enzyme responsible for the formation of a "polyketide" intermediate most characteristic of the polyketide group of antibiotics. Therefore, we had expected that the enzyme synthesis would be suppressed severely by several regulatory effectors including NH_4^+, as are other key enzyme syntheses like that of deacetoxycephalosporin C synthase (ring expandase) in *Cephalosporium acremonium*, which is controlled by carbon and nitrogen sources, and by inorganic phosphate[11,12]. Contrary to our expectation, the experimental results revealed that this is not the case. In fact, protylonolide formation

from acid precursors by resting cells was nearly at the same levels under experimental conditions irrespective of the NH_4^+ levels for cell growth (Fig. 5D). This result is in agreement with our previous observation that growing cells of *S. fradiae* strain 261 produced protylonolide in a high-NH_4^+ medium as efficiently as in a low-NH_4^+ medium, when the former medium was supplemented with the lower fatty acids, acetate, propionate and butyrate[5].

The present results, combined with the previous findings already reported[2~6], suggest the mechanism shown in Fig. 6 for nitrogen regulation of tylosin biosynthesis in *S. fradiae* KA-427. It is concluded that NH_4^+ suppresses the supply of building units to be used for protylonolide formation, resulting in a decrease in the amount of available aglycone. Because NH_4^+ does not affect the other steps[4], the limited supply of the aglycone precursor leads to the decline of tylosin production. A similar mechanism of nitrogen regulation appears to pertain also to the biosynthesis of other 16-membered macrolides such as leucomycin and spiramycin (data not shown). Thus, the general significance of this regulatory mechanism is suggested in macrolide biosynthesis. In this connection, it is of interest to examine the relationship between amino acid and succinate metabolisms and erythromycin biosynthesis, which was recently shown[13] to be sensitive to NH_4^+.

The occurrence of nitrogen regulation of tylosin biosynthesis in *S. fradiae* was first suggested by the present authors using NH_4^+-trapping agents such as zeolite[2,3]. Supporting evidence for this suggestion was obtained later also by the use of zeolite[3] as well as by blocked mutants and enzyme systems[4~6]. The present paper adds additional support for the view that NH_4^+-trapping agents are useful as a new tool for studying regulation of NH_4^+-susceptible antibiotic biosynthesis.

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