Effect of Ferrous Ion on Amino Acid Metabolism in Mildiomycin Production by *Streptoverticillium rimofaciens*

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The physiological features of the mildiomycin production by *Streptoverticillium rimofaciens* were examined in iron-sufficient and -deficient media. Activities of NADP-linked glutamate dehydrogenase (GDH) and aspartate aminotransferase (AAT) were markedly enhanced by the addition of 10 μg/ml of ferrous ion into culture. Ammonium nitrogen assimilation increased with the increase in mildiomycin production. These indicate that ferrous ion contributes the supply of amino acids as a precursor of mildiomycin production. In the iron-sufficient medium, glutamate, aspartate, serine and arginine in cells were 2 to 10-fold to those in the iron-deficient medium. The major amino acid excreted from cells was arginine in the iron-sufficient culture, while in the iron-deficient culture, valine.

Change in the amino acid profile by addition of ferrous ion was useful for mildiomycin biosynthesis, in which ferrous ion played a leading role in amino acid metabolism.

It has been demonstrated that in a variety of microorganisms, ammonium nitrogen is incorporated and metabolized as amino acid compounds such as glutamate,[1] glutamine,[2] alanine[3] and carbamoyl phosphate.[4] In *S. rimofaciens*, mildiomycin production and ammonium nitrogen assimilation were markedly stimulated by addition of ferrous ion into the culture medium.[5] Studies on the metabolic activities for mildiomycin production under iron-sufficient and -deficient conditions revealed that activities of peptidase, protease, phosphatase and the level of intracellular ATP were enhanced by the addition of ferrous ion, and accompanied by an increase in ammonium nitrogen assimilation and mildiomycin production.[5,6] It was suggested that the stimulation of mildiomycin production by ferrous ion was caused by specific changes in amino acid metabolism, relating to the biosynthesis of mildiomycin. In this study, the effect of ferrous ion on mildiomycin biosynthesis and amino acid metabolism is investigated. Moreover we elucidate the relationship between ferrous ion and amino acid metabolism of *S. rimofaciens* in iron-sufficient and -deficient media and in mildiomycin production.

Materials and Methods

Microorganisms

Streptoverticillium rimofaciens C-257,[7] a high-producing mutant derived from strain B-98891,[8] was used throughout this work.

Culture Conditions and Media

Spores grown on a slant medium (YKM) were inoculated into 30 ml of a seed medium in a 250-Erlenmeyer flask and grown at 28°C for 24 hours on a rotary shaker with an agitation rate of 200 rpm. One ml of seed culture was transferred into 10 ml of a production medium in a 250-ml Erlenmeyer flask. Cultures were carried out at 28°C for 8 days on the rotary shaker. YKM contained (g/liter) sucrose 20, soluble starch 5, NH_4NO_3 1.2, KH_2PO_4 2.5, $MgSO_4 \cdot 7H_2O$ 0.5, and $CaCO_3$ 5. The composition (g/liter) of seed medium is glucose 30, corn steep liquor (CSL) 35, $(NH_4)_2SO_4$ 1, $MgSO_4 \cdot 7H_2O$ 0.5, and $CaCO_3$ 5. To investigate effects of the ferrous ion on amino acid metabolism, a basal medium was used with the following composion (g/liter): glucose 100, $(NH_4)_2SO_4$ 10, $MnSO_4 \cdot 4H_2O$ 0.054, casein 5, and $CaCO_3$ 10. Cells were grown in the basal medium with (Fe+) and without (Fe−) addition of 10 μg/ml ferrous ion.

Analytical Methods

Amino acids were determined by the methods described previously[6]. Protein concentration was measured

by the method of LOWRY *et al.*[9].

Enzyme Activities

Cells in the fermentation broth were harvested by centrifugation at 3,000 *g* for 15 minutes, washed twice with 0.85% saline and once with 0.05 M Tris-HCl buffer (pH 7.5). The precipitated cells suspended in the buffer were sonicated at 25,000 *g* for 30 minutes with a Kubota sonifier M-200. The cell debris were removed by centrifugation at 25,000 *g* for 30 minutes and resulting supernatant was stored as the crude enzyme preparation for enzyme assay.

Glutamate dehydrogenase (glutamate: NADP oxidoreductase, deaminating, EC. 1.4.1.4)[10], glutamine synthetase (L-glutamine: ammonia ligase (ATP), EC. 6.3.1.2)[11], alanine dehydrogenase (L-alanine: NAD oxidoreductase, deaminating, EC. 1.4.1.1)[12], and aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC. 2.6.1.1)[13] activities were assayed by the methods of the respective references.

Extraction of Intracellular Amino Acids

The cells were harvested by centrifugation, washed twice with 0.9% saline and once with distilled water, and suspended in distilled water. Intracellular amino acids were extracted by autoclaving the cell suspension at 120°C for 30 minutes.

Results and Discussion

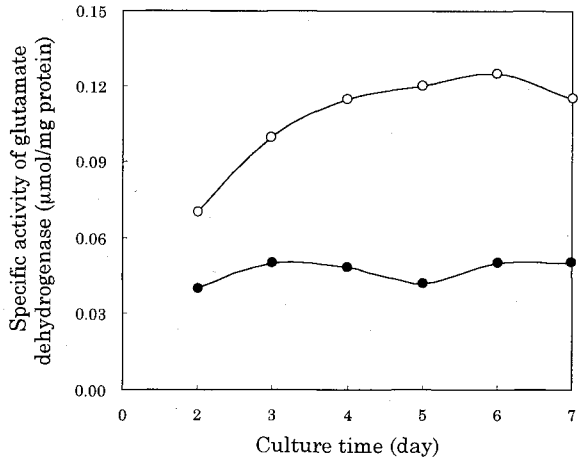
Effect of Ferrous Ion on Glutamate Dehydrogenase and Glutamine Synthetase Activities

In our previous paper[5], we reported that there was a close relationship between iron-dependent ammonia nitrogen assimilation and mildiomycin production. It was of interest to investigate in detail the effect of ferrous ion on ammonia assimilation in iron-sufficient and -deficient cultures.

Fig. 1 shows the effect of ferrous ion on specific activity of glutamate dehydrogenase during the culture with Fe+ and Fe− media. A marked increase in glutamate dehydrogenase activity was observed in the Fe+ medium. This suggests that glutamate dehydrogenase may be one of key enzymes for ammonia assimilation in the iron-sufficient culture. In general, glutamate and ammonium nitrogen are consumed and yield glutamine by the glutamine synthetase in the presence of ATP, while the glutamate is regenerated from glutamine and 2-oxoglutarate by glutamate synthetic reaction. Thus, the specific activities of glutamine synthetase and glutamate dehydrogenase were measured during mildiomycin production in Fe+ and Fe− media. No significant difference in the specific activities between two media was found

Fig. 1. Effect of ferrous ion on specific activity of glutamate dehydrogenase (NADP) in the mildiomycin fermentation.

○ Fe+ medium, ● Fe− medium.



(data not shown). However, as reported in the previous paper[7], since the intracellular ATP concentration under the iron-deficient condition during the culture is much lower than that under the iron-sufficient condition, glutamine synthetase activity catalyzing the formation of glutamine from glutamate and ammonia in the presence of ATP might be weak in cells under the iron-deficient condition.

These suggest that these activities may play direct and important roles for the ammonia nitrogen assimilation in the mildiomycin fermentation.

Effect of Ferrous Ion on Alanine Dehydrogenase Activity

Ammonia is known to incorporate into pyruvic acid and to give alanine by the reaction of alanine dehydrogenase. The specific activity of alanine dehydrogenase during the culture in Fe+ and Fe− media was examined. In the Fe+ medium, it was almost the same level to the Fe− medium (data not shown).

Effect of Ferrous Ion on Aspartate Synthesizing Activities

In view of other routes for ammonium nitrogen assimilation, the activities of aspartate aminotransferase was determined during culture in Fe+ and Fe− media. Fig. 2 shows that the aspartate aminotransferase activity in the Fe+ medium was significantly higher than that under the Fe− medium. This suggests that aspartate aminotransferase activity plays a role for the acceleration of supply of aspartate during mildiomycin biosynthesis.

The above data suggest that the stimulation of

mildiomycin production by the addition of ferrous ion to the culture medium is related to increase in the activities of glutamate dehydrogenase and aspartate aminotransferase.

Alteration of Amino Acid Profile by Ferrous Ion

The stimulation of mildiomycin biosynthesis by ferrous ion may depend upon the stimulation of ammonia assimilation due to increase in the activities of glutamate dehydrogenase and aspartate aminotransferase. It might improve the production of precursor amino acids of mildiomycin such as glutamate, aspartate, serine and arginine.

Fig. 2. Effect of ferrous ion on specific activity of aspartate aminotransferase in the mildiomycin fermentation.

○ Fe+ medium, ● Fe− medium.

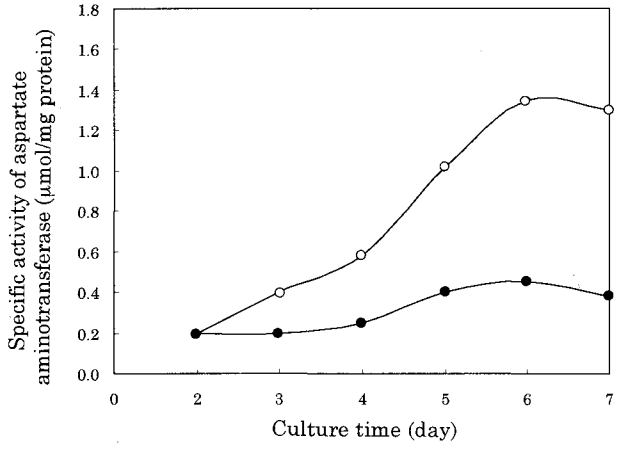


Table 1 shows the intracellular amino acid profile in Fe+ and Fe− media. Total amino acid concentrations in the cells from the Fe+ medium were about twice higher that of the Fe− medium. The major amino acid was glutamic acid which represented about 20% of the total intracellular amino acid concentrations in both cultures. Expectedly, the intracellular concentrations of glutamate and aspartate in the cells grown in the Fe+ medium were 2~3 times those in the Fe− medium.

The extracellular amino acid profile of the culture was also examined in Fe+ and Fe− media. A substantial difference in the profile between the two cultures was found. The concentrations of aspartic acid and arginine in the Fe+ medium were about 5 and 20 times higher than those in the Fe− medium, respectively. The amount of serine also increased markedly in the culture of the Fe+ medium. A striking feature of the culture grown in the Fe− medium was the increase in the extracellular concentrations of valine, leucine and phenylalanine. Thus, the major extracellular amino acids in the Fe+ medium were arginine, aspartate and lysine, but those under the Fe− medium were valine, phenylalanine and leucine. It is reasonable to postulate that the increase in the mildiomycin biosynthesis in the Fe+ medium may be due to an increase in metabolic activities producing arginine and serine. These amino acids are molecular

Table 1. Effect of ferrous ion on amino acid profiles.

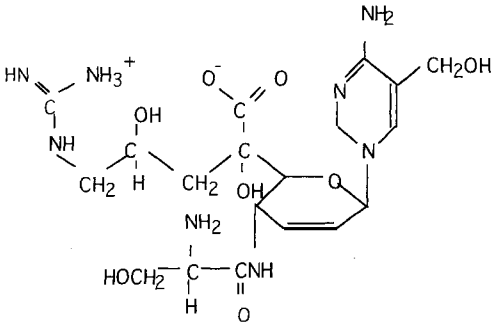
Amino acids	Amino acid concentration			
	Intracellular concentration (mg/g dry cell weight)		Extracellular concentration (μg/ml)	
	Fe+ medium	Fe− medium	Fe+ medium	Fe− medium
Lysine	1.07	0.52	87.7	18.3
Histidine	0.10	0.06	—	—
Arginine	0.27	0.02	504.0	23.2
Tryptophan	0.06	0.03	—	—
Aspartic acid	0.27	0.09	100.0	18.0
Threonine	0.91	0.46	3.1	7.1
Serine	0.21	0.05	10.6	—
Glutamic acid	1.67	0.78	13.0	22.0
Proline	0.18	—	34.5	—
Glycine	0.18	0.10	0.8	1.3
Alanine	0.81	0.70	10.9	10.0
Valine	0.28	0.20	20.0	82.3
Methionine	0.18	0.08	20.1	9.7
Leucine	0.52	0.26	6.1	30.4
Isoleucine	0.16	0.05	2.2	2.4
Tyrosine	0.60	0.16	4.0	0.2
Phenylalanine	0.80	0.37	30.0	45.0
Total	8.27	3.93	847.2	269.9

components of mildiomycin[13] as shown in Fig. 3.

General Aspect of the Role of Ferrous Ion in Mildiomycin Fermentation

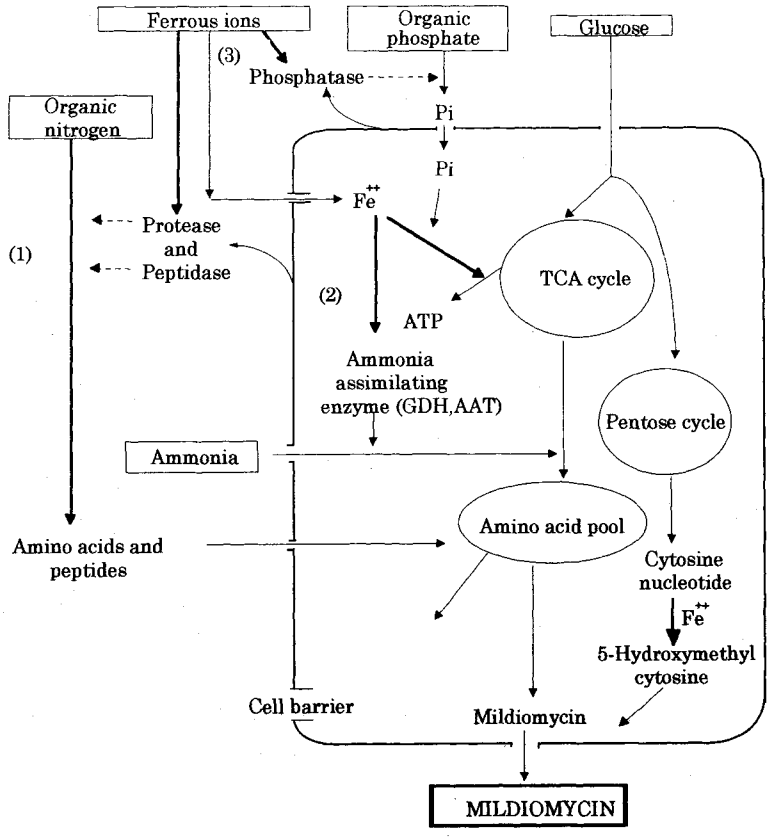
Ferrous ion is essential for high mildiomycin production by *S. rimofaciens*[5] and ammonia nitrogen assimilation. We have tried to explain the effect of the ferrous ion on mildiomycin production from the viewpoint of nitrogen metabolism and mildiomycin biosynthesis. From the results, we propose a scheme for the mechanism of the ferrous ion role in the mildiomycin biosynthesis (Fig. 4).

Fig. 3. Chemical structure of mildiomycin.



Ferrous ion stimulates the formation of: (1) protein-hydrolyzing activities,[5] that digest proteinous substrates into peptides and amino acids; (2) activities that assimilate ammonium ion[5] and convert them into amino acids; (3) and phosphatase activity,[6] that liberates inorganic phosphate from organic materials and increases the ATP level in cells. The increase in protein-hydrolyzing activities (peptidase, protease) by ferrous ion (1) is important to supply amino acids to cells. The stimulation of ammonia-assimilation by ferrous ion (2), is caused by the stimulation of glutamate dehydrogenase (NADP-linked, EC. 1.4.1.4) and aspartate aminotransferase (EC. 2.6.1.1). A large quantity of aspartate and glutamate in cells, as shown in Table 1, probably results from these activities. As illustrated in Fig. 5, amino acid precursors for mildiomycin biosynthesis would arise from glutamate and aspartate as primary products of the ammonia-assimilating enzymes. This is supported by the large serine-arginine pool size in the iron-sufficient conditions (Table 1). The increase in aspartate also could be reasonable for the biosynthesis

Fig. 4. Illustration of the leading role of ferrous ion in the mildiomycin fermentation of *Streptoverticillium rimofaciens*.

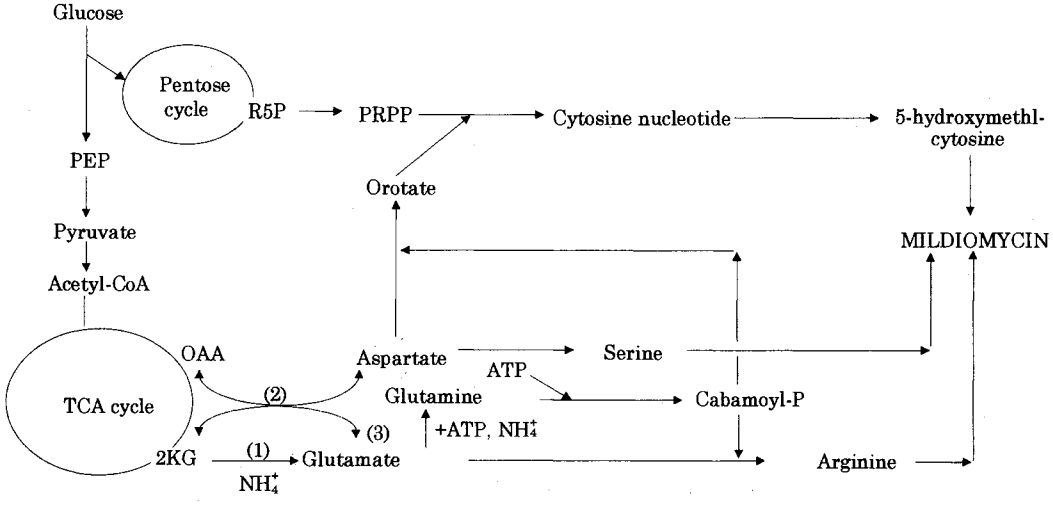


⟶: Increase in enzyme activities, ⟶: metabolic routes ⟶: Enzyme reactions.
GDH: Glutamate dehydrogenase, AAT: aspartate aminotransferase.
▭ : Raw materials in the fermentation medium, ▭: final product.

of the pyrimidine precursor of mildiomycin, as shown in Fig. 5. In addition, excretion of serine and arginine out of cells in the iron-sufficient culture suggests that ferrous ion may act as a switch for a change in amino acid metabolism from valine-leucine producing system *via* pyruvate to serine-arginine producing system *via* glutamate or aspartate, as shown in Fig. 6.

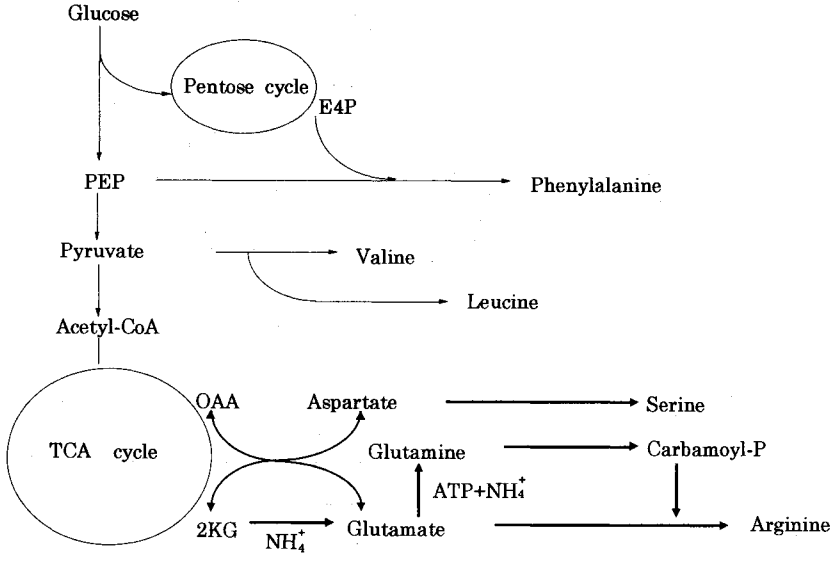
The stimulation of phosphatase activity by ferrous ions (3) accelerates the liberation of inorganic phosphate from organic phosphate compounds which results in an increase of ATP in cells,[6] The intracellular level of adenylates of tylosin-producing *Streptomyces fradiae* was found to be inversely proportional to the rate of the antibiotic biosynthesis.[15] ATP was also suggested to be involved the regulation of tyrothricin[16] and candicidin biosynthesis.[17] In the mildiomycin fermentation, intracellular ATP level was proportional to the rate of mildiomycin biosynthesis (see Table 1). ATP is required for the ammonia-assimilating reactions as the cofactor of glutamine synthesis. In addition, ATP is also required not only for the synthesis of carbamoylphosphate and further reactions of pyrimidine nucleotide synthesis, but

Fig. 5. Possible pathway for biosynthesis of mildiomycin.



PEP: Phosphoenolpyruvate, OAA: oxaloacetate, 2KG: 2-ketoglutarate, R5P: ribose-5-phosphate, PRPP: phosphoribosylpyrophosphate, Carbamoyl-P: carbamoylphosphate.
(1) Glutamate dehydrogenase, (2) aspartate aminotransferase, (3) glutamine synthase.

Fig. 6. Changes in amino acid metabolism caused by ferrous ion in the mildiomycin fermentation.



⟶: Predominantly under Fe− sufficient conditions, ⟶: predominantly under Fe− deficient conditions.
PEP: Phosphoenolpyruvate, OAA: oxaloacetate, E4P: erythrose-4-phosphate, 2-KG: 2-ketoglutarate, Carbamoyl-P: carbamoylphosphate.

also for the energy generation for the maintenance of the cell activity.

SAWADA *et al.*[17] reported that the biosynthesic pathway to 5'-hydroxymethylcytosine (HMC), a molecular constituent of mildiomycin (see Fig. 3), is enzymatically derived from cytosine-5'-monophosphate (CMP) and formaldehyde (or serine) in the presence of tetrahydrofolic acid and that the HMC-forming reaction requires ferrous ion.

These multifaceted actions of ferrous ion mentioned above are thought to be involved in the Fe^{2+}-induced acceleration of mildiomycin production in *S. fimofaciens*.

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