Effect of Phosphate Ion on Mildiomycin Production by *Streptoverticillium rimofaciens*

KATSUMITSU KISHIMOTO[a], YONG SOO PARK[b], MITSUYASU OKABE[b,*] and SHUN-ICHI AKIYAMA[a]

[a]Technology Department Hikari branch Lab., Takeda Chemical Industries, Ltd., 4720 Mitsui, Hikari, Yamaguchi Prefecture 743
[b]Biochemical Eng. Lab., Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan

(Received for publication February 19, 1996)

Effect of inorganic phosphate and ammonium nitrogen on mildiomycin production by *Streptoverticillium rimofaciens* was investigated in culture with and without addition of ferrous ion. In the presence of ferrous ion, the suitable supply of inorganic phosphate increased the intracellular ATP, but that was not observed without ferrous ion addition. The intracellular ATP remarkably affected ammonium nitrogen assimilation and mildiomycin production, and its concentration in the ferrous-sufficient culture was about 2~3 times higher than that in the ferrous-deficient culture. The low concentration of intracellular ATP in the ferrous-deficient culture resulted in the reduction of ammonium nitrogen assimilation and mildiomycin biosynthesis. This phosphate ion effect on the intracellular ATP concentration was demonstrated only when ferrous ion was added into the medium. These suggest that mildiomycin biosynthesis is regulated through the concentration of intracellular ATP related to the ammonium nitrogen assimilation.

Mildiomycin[1,2], produced by *Streptoverticillium rimofaciens*, is effective against powdery mildew diseases in plants. In the previous paper[3], we reported that ferrous ion played a key role in a regulatory mechanism of mildiomycin biosynthesis.

The significance of inorganic salts such as metal compounds[4], phosphate compounds and ammonium compounds[5] in an antibiotic production has been extensively studied. Many inorganic constituents of fermentation media have been used in excess without inhibiting an antibiotic formation. Inorganic phosphate, however, is usually very critical. The inhibition of an antibiotic synthesis by inorganic phosphate has been known in many fermentation[6]. As well as phosphate, the inhibitory effect of ammonium salts on an antibiotic formation has been also reported[7]. However, there are few reports about correlation between inorganic phosphate and ammonium nitrogen in the presence of metal salts.

During the study on mildiomycin fermentation, we found that acid phosphatase activity was markedly stimulated in the culture with the addition of ferrous ion, followed with not only a stimulation of ammonium nitrogen assimilation but also mildiomycin production[3]. This present paper, therefore, is aiming at investigating the effect of phosphate ion on mildiomycin biosynthesis and ammonium nitrogen assimilation with and without the ferrous ion addition.

Materials and Methods

Microorganisms

Streptoverticillium rimofaciens strain C-257[8], a high-producing mutant derived from strain B-98891[1], was used throughout this work.

Culture Conditions and Media

Spores grown on a slant agar medium (YKM) were inoculated into 30 ml of a seed medium in a 250-ml Erlenmeyer flask and grown for 24 hours on a rotary shaker. One milliliter 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 with an agitation rate of 200 rpm. YKM contained (%w/v) sucrose 2, soluble starch 0.5, NH_4NO_3 0.12, KH_2PO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.005, casamino acid 0.00025, yeast extract 0.0025 and agar 2. The composition (%w/v) of seed medium is glucose 3, corn steep liquor (CSL) 3.5, $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, and $CaCO_3$ 0.5. To investigate the effect of phosphate on ammonium nitrogen assimilation, intracellular ATP and mildiomycin production, the basal medium was used as the following composition (%w/v): glucose 10, $(NH_4)_2SO_4$ 0.5, casein 0.3, NaCl 0.5 and $CaCO_3$ 1.

Analytical Methods

Glucose concentration was determined by the method of WASHKO and RICE[9]. Ammonium nitrogen concentration in the culture broth was measured by the method of MCCULLOUGH[10]. DNA extracted from cells by the method of SCHNEIDER[11] was measured by the method

of BURTON[12]. Mildiomycin was assayed by a high performance liquid chromatography (Model 633, Hitachi Co., Tokyo) under the following conditions: column (2.1mm × 40mm) packed Hitachi custom ion exchange resin 2610; solvent, 0.3 M borate buffer (pH 8.8 by lithium hydroxide); flow rate, 0.7 ml/minute; pressure, 120 kg/cm^2 G; detection, 254 nm. Acid phosphatase activity was determined by the method of FISHMAN *et al.*[13]. Phosphate were determined by the methods of NAKAMURA[14].

Intracellular ATP concentration was measured by means of the luciferase reaction[15]. 200 ml of the cell extract was added to 50 ml of a 75 mM potassium phosphate buffer, pH 7.3, 15mM $MgCl_2$. Ten milliliters of cold luciferin-luciferase mixture was added to the reaction cuvette containing 100 ml of assay buffer (40 nM glycyleglycine, 3 nM $MgCl_2$, pH 7.4) at room temperature. The cuvette was placed in the ATP photometer, and 10 ml of the extract was rapidly injected into the mixture. ATP concentration in a range of 2 nM to 10 nM gave a reproducible linear response.

Chemicals

Mixture of luciferin-luciferase was purchased from Sigma Chemical Co.

ATP, phosphoenolpyruvate and pyruvate kinase were purchased from Wako Pure Chemical Ind., Ltd. Adenylate kinase was purchased from Oriental Yeast Co. Other chemicals were of the purest grade on the market.

Results and Discussion

Effect of Phosphate on Mildiomycin Production

Phosphate ions may be supplied from inorganic phosphate in the medium or from organic phosphate compounds by phosphatase. During the development of the fermentation process for the production of mildiomycin, we found that the phosphatase activity was markedly stimulated by ferrous ion, and that the ferrous ion markedly affected both ammonium nitrogen assimilation and mildiomycin production[3]. After culture time of 8 days effect of ferrous ion on the phosphatase activity and mildiomycin production are shown in Fig. 1. Ferrous ion in the range of 8~10 μg/ml gave the maximum phosphatase activity, which was paralleled with mildiomycin production. The phosphatase of mildiomycin producer shows the maximum activity at pH 5 (data not shown).

A typical time course of mildiomycin production with and without addition of the ferrous ion (10 μg/ml) was shown in Fig. 2. In the culture with addition of ferrous ion (Fig. 2a), acid phosphatase activity was remarkably

Fig. 1. Effect of ferrous ion on acid phosphatase activity and mildiomycin production.

△, Acid phosphatase; ○, mildiomycin.

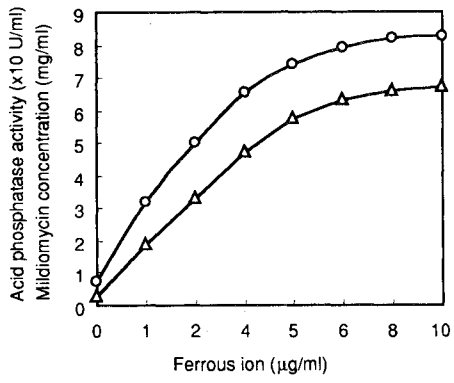
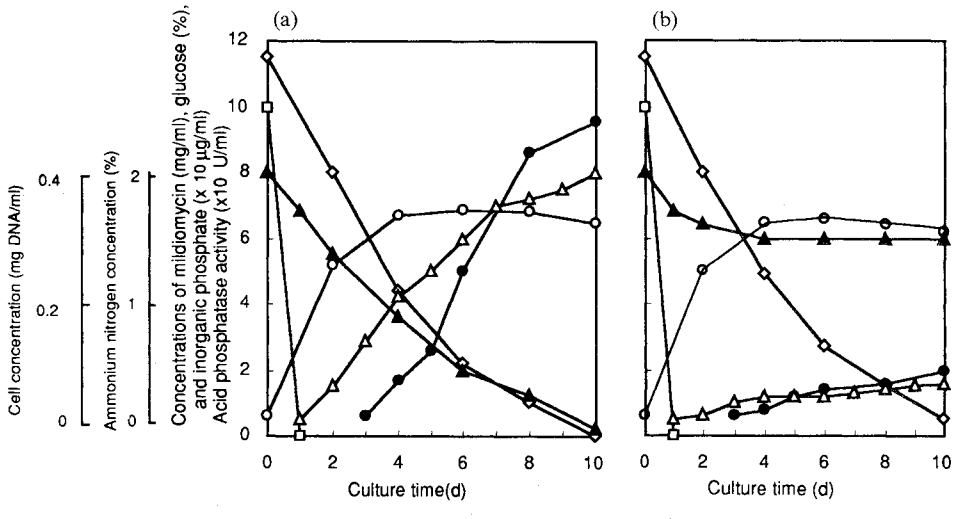


Fig. 2. A time courses of mildiomycin fermentation with (a) and without (b) addition of ferrous ion.

△, Acid phosphatase; ▲, ammonium nitrogen; ○, cell growth; ◇, glucose; ●, mildiomycin, □, inorganic phosphate.



stimulated after inorganic phosphate was exhausted at the 24 hours culture, while in the culture without the ferrous ion addition, low (Fig. 2b). Moreover, the phosphatase enhanced ammonium nitrogen assimilation and mildiomycin production. It may suggest that the ferrous ion in the mildiomycin fermentation has a role to enhance the activity of acid phosphatase. Thus, the phosphatase plays an important role on ammonium nitrogen assimilation and mildiomycin production.

We, therefore, examined the effect of phosphate on mildiomycin fermentation using the medium containing (%w/v) glucose 13, $(NH_4)_2SO_4$ 1, $FeSO_4 \cdot 7H_2O$ 0.005, Proflo (Traders Oil Mill Co. U.S.A.) 4 and $CaCO_3$ 1. The phosphate concentration was varied with potassium dihydrogen phosphate corresponding to 75, 100 and 150 μg/ml phosphorus. The time courses are shown in Fig. 3. The optimal concentration of phosphate was about 100 μg/ml as phosphorus for mildiomycin production. Although glucose consumption (Fig. 3a) and cell growth (Fig. 3c) were hardly affected, the assimilation rate of ammonium nitrogen was higher with the increase in phosphate concentration (Fig. 3b). The ammonium nitrogen assimilation in the culture with 75 μg/ml as phosphorus ceased after 4 day culture, which lead to decrease mildiomycin production.

For the detail analysis of the relationship between phosphate and ammonium nitrogen assimilation, the phosphate was fed in the culture at the 4 day culture as shown in Fig. 4. In the case of without feeding, the inorganic phosphate was completely consumed within 1 day and ammonium nitrogen was not consumed any more (Fig. 4b). However, when potassium dihydrogen phosphate was fed into at the rate of 10 μg/ml/hour as phosphorus at 4 day culture, the ammonium nitrogen assimilation continued (Fig. 4b) and mildiomycin production was markedly increased (Fig. 4a). In these cases, glucose consumption and cell growth were hardly affected by phosphate feeding (Fig. 4c).

This indicates that ammonium nitrogen assimilation and mildiomycin production require the supply of phosphate ion at an adequate concentration.

Effect of Phosphate on Intracellular ATP Level

Inorganic phosphate has been known for many years to affect antibiotic production. An excess amount of inorganic phosphate suppresses the production of such antibiotic as tetracycline, actinomycin and candicidin[16,17]. The regulatory effect of phosphate on candicidin biosynthesis by *Streptomyces griseus* have been reported[18,19], suggesting ATP may be the intracellular effector that controls antibiotic biosynthesis. The possibility of involvement of ATP in controlling an antibiotic biosynthesis was also supported by the data SILAEVA *et al.*[20]. JANGLOVA *et al.* reported that ATP levels are lower in improved antibiotic-producing strains than in their low-producing ancestral strains[21]. In mildiomycin fermentation by *S. rimofaciens* the supply of inorganic phosphate accelerated ammonium nitrogen assimilation, followed with an increase in the

Fig. 3. Effect of phosphate concentration on mildiomycin and sugar (a), ammonium nitrogen and inorganic phosphate (b), and cell growth (c).

Straight and dotted lines in (a) denote mildiomycin and glucose concentrations, respectively; in (b), ammonium nitrogen and inorganic phosphate, respectively. △, 75 mg/ml; ○, 100 mg/ml; □, 150 mg/ml as phosphorus.

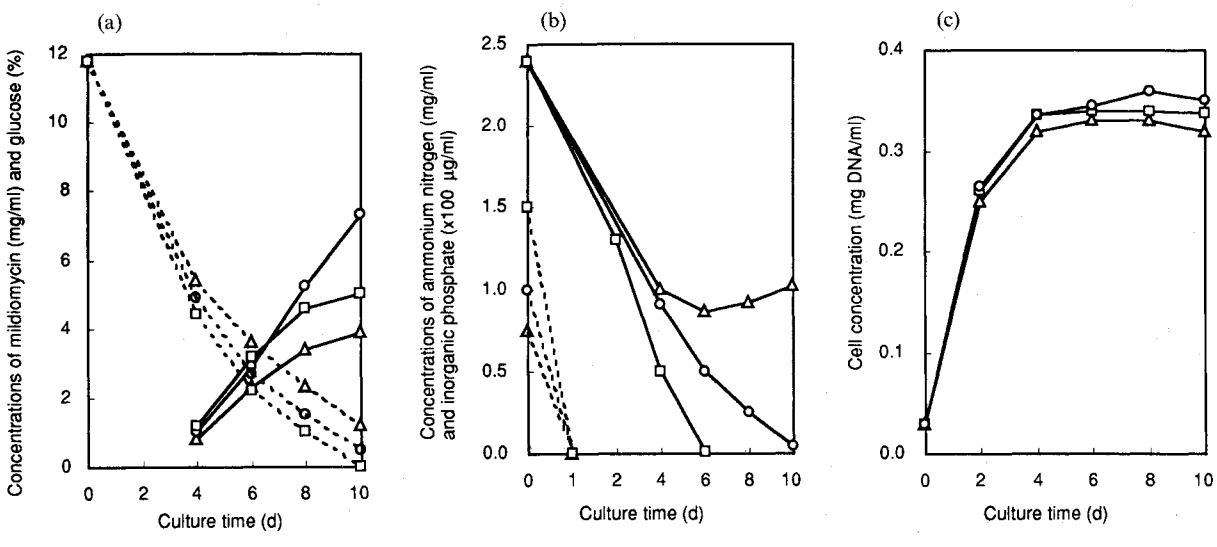


Fig. 4. Effect of phosphate feeding on mildiomycin fermentation.

Potassium dihydrogen phosphate was fed at the time indicated by arrows.
□, Without feeding of phosphate; ○, with feeding of phosphate at the rate of 0.5 μg/ml/hour of KH_2PO_4 (10 μg/ml as phosphorus) at the time indicated arrows. Straight and dotted lines in (a) denote mildiomycin and glucose concentrations, respectively; in (b), ammonium nitrogen and inorganic phosphate, respectively.

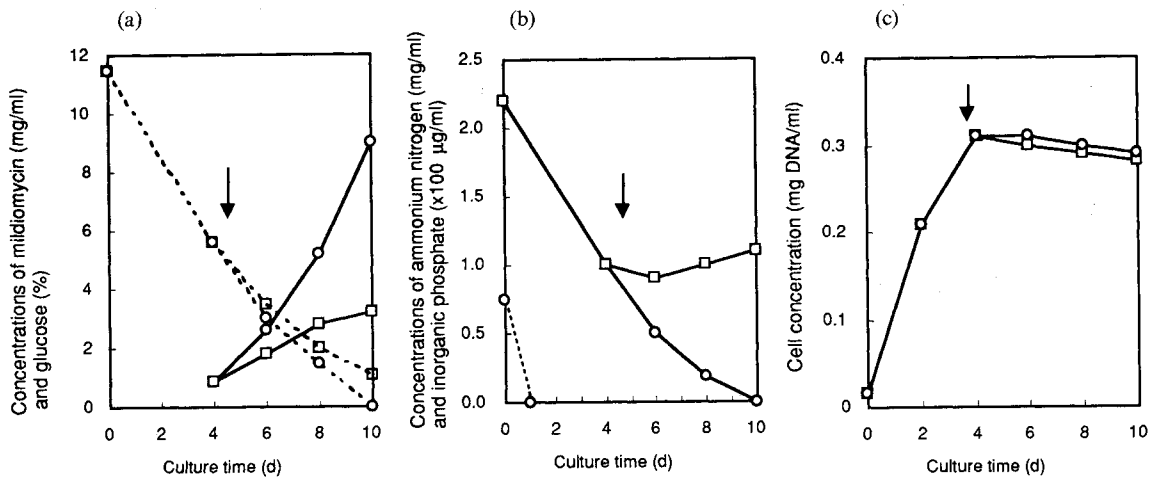
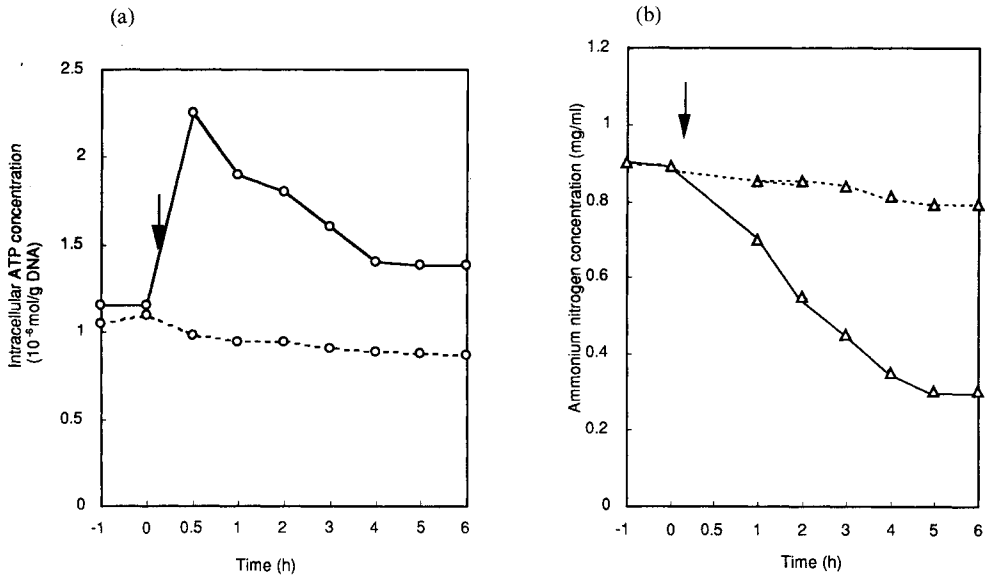


Fig. 5. Changes in the intracellular ATP (a) and ammonium nitrogen (b) concentrations with feeding (straight line) and without (dotted line) inorganic phosphate (10 μg/ml as phosphorus) at the time indicated by arrow.



mildiomycin production. However, whether intracellular orthophosphate is the effector that controls ammonium nitrogen assimilation or not is unclear.

We, therefore, examined the effect of phosphate on intracellular adenylates. Fig. 5 shows the intracellular ATP concentration in the culture containing 75 μg/ml phosphorus with addition to 10 μg/ml phosphorus at the culture time of 4 days. The intracellular ATP level increased rapidly and reached 2-fold after the addition of phosphate, then decreased with culture time and became a plateau (Fig. 5a). The ammonium nitrogen assimilation increased rapidly with the increase in the intracellular ATP level and ceased when intracellular adenylates concentration was constant (Fig. 5b). This suggests that the intracellular ATP may be one of the intracellular effector that stimulate ammonium nitrogen assimilation.

Fig. 6. Changes in the intracellular ATP concentration the iron-sufficient (●) and deficient (○) cultures.

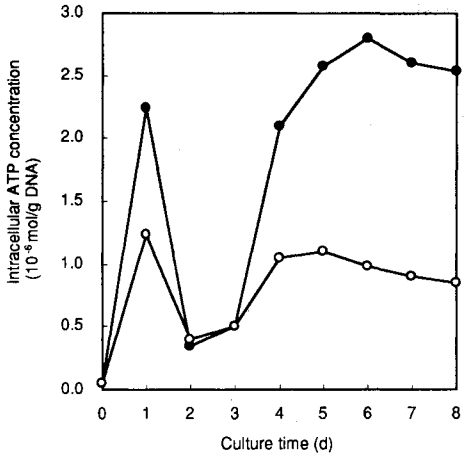
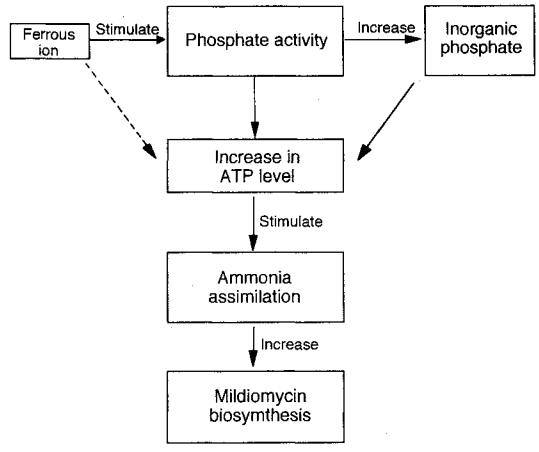


Fig. 7. Summary of the role of ferrous ion in mildiomycin fermentation by *S. rimofaciens*.



Effect of Ferrous Ion on Intracellular ATP

We examined changes in the intracellular ATP concentration in the culture with and without the addition of ferrous ion 10 μg/ml and the result is shown in Fig. 6. In the case of the ferrous ion addition, the ATP concentration was 2~3 times as high as that without. This suggests that the ferrous ion may play one of an important role on the production of intracellular ATP. The high concentration of intracellular ATP (about 2.5×10^{-6} mol/g DNA) promoted the ammonium nitrogen assimilation, but even in the iron-sufficient culture, the low concentration of intracellular ATP (about 1×10^{-6} mol/g DNA) did not promoted the ammonium nitrogen assimilation. This may be the reason that ammonium nitrogen assimilation is depressed in the iron-deficient culture.

We conclude that the cascade effect of ferrous ion on mildiomycin fermentation by *S. rimofaciens* is illustrated in Fig. 7. The ferrous ion stimulates an acid phosphatase activity and then, inorganic phosphate liberated from natural raw materials by the enzyme. This results the increase in intracellular ATP level. A high level of intracellular ATP stimulates uptake of ammonium nitrogen and introduces mildiomycin biosynthesis.

Acknowledgments

We wish to thank Dr. T. ASAI for their valuable discussion and suggestions during this study. Thanks are also due to Messieurs. K. YONETO and M. NAKATSU for their technical assistance.

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