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Phosphate, ammonium, magnesium and iron nutrition of *Streptomyces hygroscopicus* with respect to rapamycin biosynthesis

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SUMMARY

Phosphate, ammonium and magnesium salts interfered with rapamycin production by *Streptomyces hygroscopicus* at concentrations optimal for growth. These observations point to the existence of phosphorus, magnesium and nitrogen-negative regulation mechanisms for rapamycin biosynthesis. On the other hand, Fe^{2+} stimulated rapamycin production at concentrations greater than that required for growth.

INTRODUCTION

Rapamycin was discovered as an antifungal antibiotic in 1975 and later found to be a potent immunosuppressant [5,10,14]. It is an unusual triene macrolide made from acetate, propionate, methionine, pipecolate and shikimate [7,8,9]. Since rapamycin is somewhat more active than the structurally related FK506 [12] and 150 times more active and less toxic than the cyclic peptide cyclosporine A [2], there is great interest now in this compound [10].

There is almost nothing known about the nutritional control of rapamycin biosynthesis. We recently published information on carbon source control [3]. The present study reports the effects of phosphate, ammonium, magnesium and iron on formation of rapamycin by *Streptomyces hygroscopicus.*

MATERIALS AND METHODS

Microorganism

Streptomyces hygroscopicus strain C9, an improved rapamycin producing isolate prepared from strain AY-B1206 [3], was used. Spore suspensions and inocula were prepared as previously described [3].

Fermentation

The chemically-defined medium used for most of this investigation was medium 2 developed in a previous investigation [3]. It contained (per liter) D-fructose, 20 g; D(+)- mannose, 5 g; Na-L-aspartate, 1.5 g; L-arginine, 0.5 g; L-histidine·HCl, 0.5 g; 2-(*N*-morpholino)ethanesulfonic acid buffer (MES), 21.3 g; NaCl, 5 g; K_2HPO_4, 2 g; KH_2PO_4, 2 g; $MgCl_2{\cdot}6H_2O$, 510 mg; Na_2SO_4, 360 mg; $MgSO_4{\cdot}7H_2O$, 256 mg; $FeSO_4{\cdot}7H_2O$, 100 mg; $ZnSO_4{\cdot}7H_2O$, 60 mg; $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$, 18 mg; $MnSO_4{\cdot}H_2O$, 12 mg; $Na_2B_4O_7{\cdot}10H_2O$, 10 mg; $CoCl_2{\cdot}6H_2O$, 10 mg; and $CuCl_2{\cdot}2H_2O$, 1.3 mg. The sugars were autoclaved separately from the rest of the medium. pH was adjusted to 6.0. Production cultures were prepared in duplicate 250-ml baffled flasks containing 25 ml of medium, inoculated and incubated for 8 days at 28 °C on a rotary shaker operating at 200 r.p.m. In one experiment, earlier medium No. 1 [3] was used. This is indicated in the text.

Extraction

A 1.0-ml aliquot of fermented whole broth was centrifuged at 1500 × *g* for 5 min. The supernatant fluid was transferred by pipette into a test tube and the pellet was extracted twice, each time by shaking with 1 ml of methanol for 20 min at room temperature. After centrifugation, the pellets were discarded. The pooled extracts were added to the supernatant phase and assayed as described below. Extraction was necessary since only 15% of rapamycin is found in the supernatant broth.

Determinations

All data shown represent the average of duplicate flasks. Sampling was done on days 6 and 8. The data presented represent the maximum assay of the two samplings since growth and rapamycin production proceeded at different rates in the different media tested. However, maxima were usually not observed prior to 6 days or after 8 days.

Growth as dry cell weight (DCW) was determined by adding 1 ml of whole broth to 1 ml of distilled water and 3 ml of 3 N HCl. The suspension was homogenized by ultrasonic treatment for 90 s with a Branson Sonifier (Cell Disruptor 200) (Ultrasonics Co, Danburg, CT, USA) and turbidity was

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measured using the Klett Summerson Photoelectric Colorimeter with a red filter (Klett Manufacturing Co, New York, NY, USA). Dilutions were made until the Klett readings were in the range between 10 and 100 Klett units. A broth DCW of 1 mg ml^{-1} was found to be equivalent to 286 Klett units.

Bioassay of rapamycin was performed on the pooled supernatant fluid plus extracts described above by paper disc-agar diffusion using *Candida albicans* ATCC 11651 as the assay microorganism. The assay medium consisted of (g L^{-1}) peptone 2 g, glucose 5 g, agar 8 g. It was adjusted to pH 6.0 and was seeded with *C. albicans* before pouring plates. The plates were incubated overnight at 37 °C. Volumetric production refers to mg rapamycin per L of whole fermentation broth. Specific production refers to mg rapamycin per g DCW. Assay variability was less than 10%.

RESULTS

Phosphate nutrition

In earlier media, i.e. medium 1 and medium 2, phosphate was added as K_2HPO_4 (2 g L^{-1}), and KH_2PO_4 (2 g L^{-1}), a total phosphorus concentration of 26.5 mM. To determine whether phosphate exerts control of rapamycin production, we eliminated KH_2PO_4 from medium 1 and varied the concentration of K_2HPO_4 from 0 to 200 mM. Figure 1 shows that growth (DCW) was maximal at 100 mM phosphate but rapamycin production was best at 5 mM (specific) to 10 mM (volumetric). Thus, phosphate levels above 10 mM stimulate growth and interfere with rapamycin production, i.e. phosphate controls rapamycin biosynthesis. A similar effect was observed in medium 2.

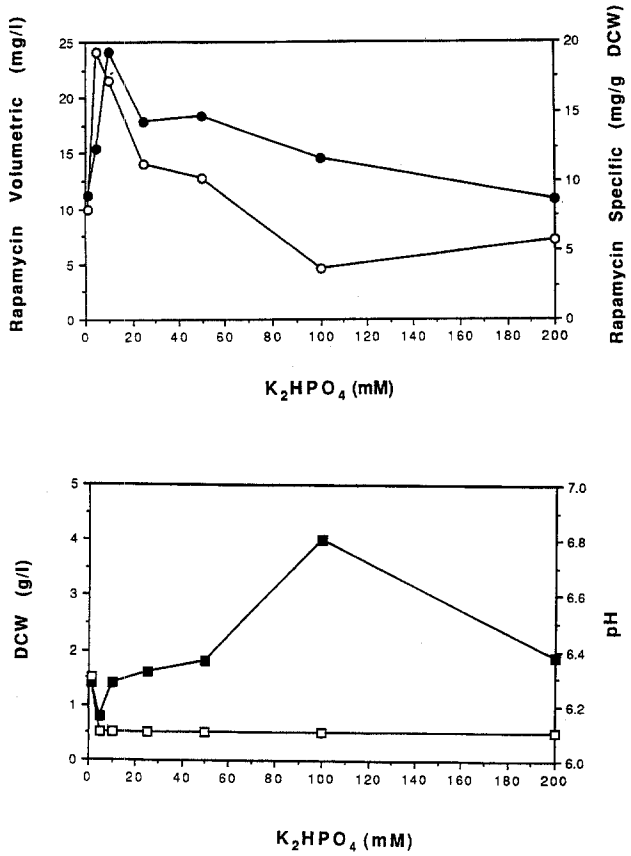

Fig. 1. Effect of K_2HPO_4 concentration on: (●), volumetric rapamycin production (mg L^{-1}); (○), specific rapamycin production (μg mg^{-1} DCW); (■), DCW (g L^{-1}); and (□), pH.

Ammonium nutrition

The effect of ammonium chloride on *S. hygroscopicus* was determined in medium 2 (modified by use of 10 mM K_2HPO_4 and no KH_2PO_4). A negative effect on rapamycin formation was found upon addition of all concentrations of NH_4Cl from 5 mM to 200 mM (Fig. 2). Growth, on the other hand, was stimulated by NH_4Cl up to 25 mM. These were not pH effects since the inclusion of MES buffer in the medium allowed only minor changes in pH.

Magnesium nutrition

Medium 2 contains a very high Mg concentration, added as 256 mg of $MgSO_4 \cdot 7H_2O$ and 510 mg of $MgCl_2 \cdot 6H_2O$ per liter which is a total concentration of 3.5 mM magnesium. To study possible Mg control of rapamycin formation, we eliminated $MgCl_2 \cdot 6H_2O$ from medium 2 (containing 10 mM K_2HPO_4 and no K_2HPO_4) and added increasing concentrations of $MgSO_4 \cdot 7H_2O$. The data are shown in Fig. 3. It is clear that Mg controls rapamycin production. Production (volumetric and specific) was optimal at 0.01 mM $MgSO_4 \cdot 7H_2O$ (2.5 mg L^{-1}), a concentration suboptimal for growth. Further

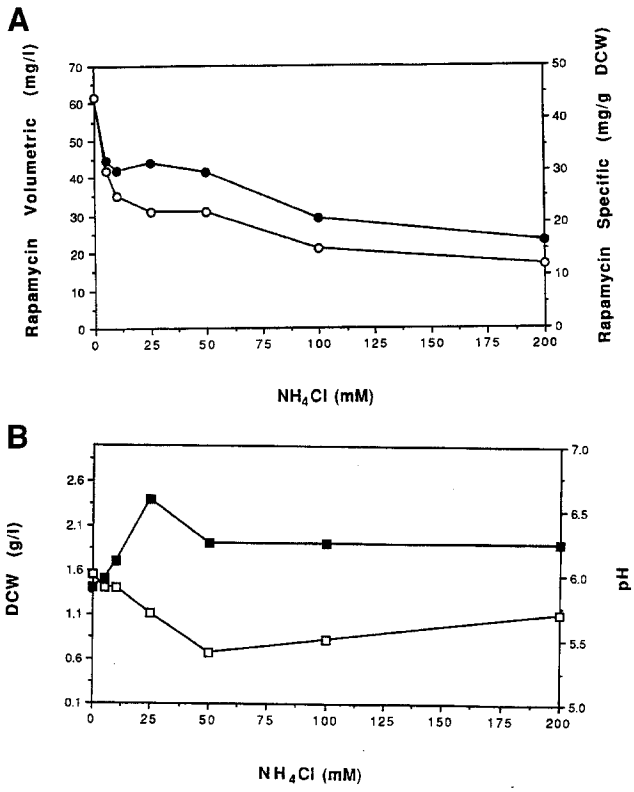

Fig. 2. Effect of NH_4Cl on rapamycin production (A), and growth and pH (B): (●), volumetric rapamycin production (mg L^{-1}); (○), specific rapamycin production (μg mg^{-1} DCW); (■), DCW (g L^{-1}); and (□), pH.

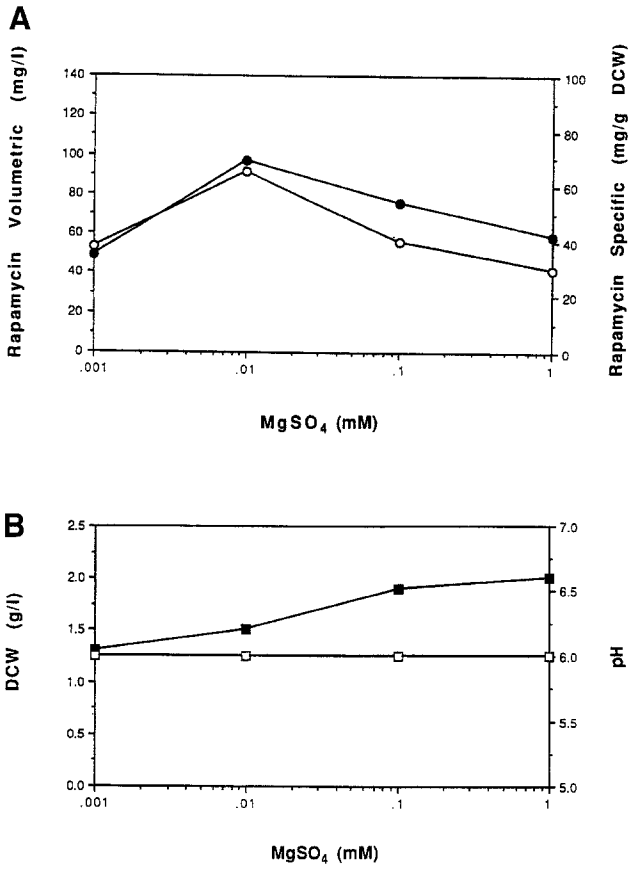

Fig. 3. Effect of $MgSO_4$ on rapamycin production (A), and growth and pH (B): (●), volumetric rapamycin production (mg L^{-1}); (O), specific rapamycin production (μg mg^{-1} DCW); (■), DCW (g L^{-1}); and (□), pH.

TABLE 1

Composition of chemically-defined medium 3

Component	Amount per liter
D-Fructose[a]	20 g
D(+)-Mannose[a]	5 g
Na-L-aspartate	1.5 g
L-arginine	0.5 g
L-histidine·HCl	0.5 g
K_2HPO_4	1.7 g
NaCl	5 g
$ZnSO_4{\cdot}7H_2O$	60 mg
$MgSO_4{\cdot}7H_2O$	2.5 mg
$MnSO_4{\cdot}H_2O$	12 mg
$FeSO_4{\cdot}7H_2O$	100 mg
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	18 mg
$Na_2B_4O_7{\cdot}10H_2O$	10 mg
$CoCl_2{\cdot}6H_2O$	10 mg
$CuCl_2{\cdot}2H_2O$	1.3 mg
Na_2SO_4	360 mg
MES buffer	21.3 g
pH	adjusted to 6.0

[a] Autoclaved but separate from other components.

TABLE 2

Comparison of growth and rapamycin production in chemically-defined media 2 and 3

Parameter	Medium 2	Medium 3
Final pH	6.1	6.0
Maximum DCW (g L^{-1})	1.4	1.5
Volumetric rapamycin (mg L^{-1})	18	97
Specific rapamycin (mg g^{-1} DCW)	13	65

increases with $MgSO_4$ up to 1 mM increased growth but interfered with rapamycin production.

Iron nutrition

$FeSO_4{\cdot}7H_2O$ is present in medium 2 at 100 mg L^{-1} or 0.36 mM. When its concentration was varied between 0 and 0.54 mM, growth and pH were relatively unaffected (Fig. 4). In contrast, rapamycin production was increasingly stimulated as $FeSO_4$ was increased up to 0.36 mM. Thus Fe specifically stimulates rapamycin production at concentrations higher than that required for growth.

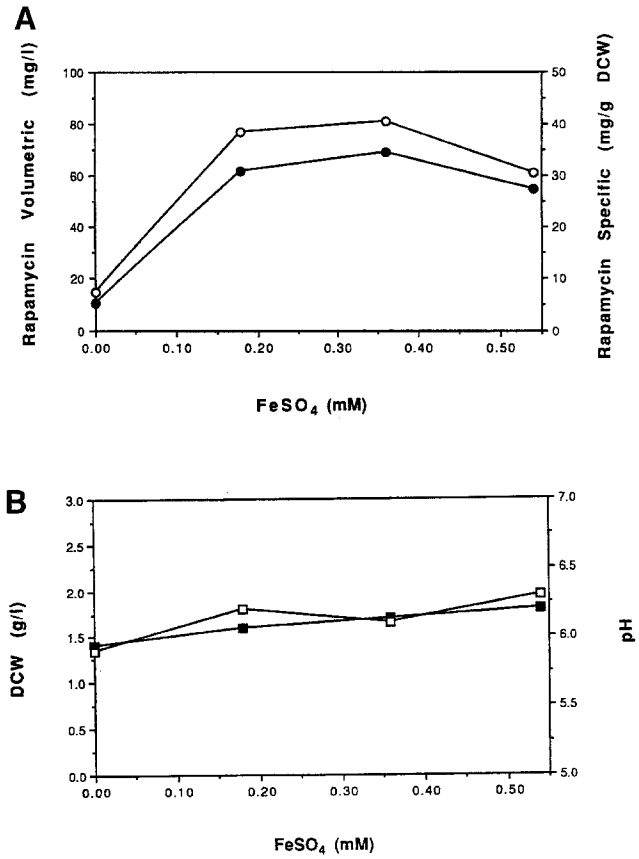

Fig. 4. Effect of $FeSO_4$ on rapamycin production (A), and growth and pH (B): (●), volumetric rapamycin production (mg L^{-1}); (O), specific rapamycin production (μg mg^{-1} DCW); (■), DCW (g L^{-1}); and (□), pH.

DISCUSSION

Rapamycin production was found to be under specific negative control by concentrations of phosphate, ammonium and magnesium salts which were suboptimal for growth. It is not uncommon for production of macrolides to be under nitrogen and phosphate control [1,4,6,11] but magnesium salts only infrequently exert regulatory control of antibiotic biosynthesis [13,15,16] and are usually required at similar concentrations for both primary and secondary metabolism. We observed optimum rapamycin production at the low concentration of 0.01 mM Mg^{2+} whereas growth was highest at 100 times that concentration. In contrast to the above inorganic nutrients, Fe^{2+} stimulated rapamycin production at concentrations which were above the level required for growth, thus showing a specific positive effect on production.

As a result of these studies, an improved chemically-defined medium (medium 3) was developed. Its composition is shown in Table 1. Performance of *S. hygroscopicus* in this medium and previous medium 2 is shown in Table 2.

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