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Effect of nitrogen source on biosynthesis of rapamycin by *Streptomyces hygroscopicus*

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Six non-amino acid nitrogen compounds were examined as nitrogen source for growth of *Streptomyces hygroscopicus* and biosynthesis of rapamycin. Of the nitrogen sources studied, ammonium sulfate was the best with respect to formation of rapamycin, and supported cell growth comparable to the organic nitrogen sources used in the control chemically defined medium, ie, aspartate, arginine plus histidine. In the new chemically defined medium, which is buffered with 200 mM 2-(*N*-morpholino)ethanesulfonic acid to prevent decline of pH during fermentation, an ammonium sulfate concentration of 40 mM was optimal for biosynthesis of rapamycin. Rapamycin production increased by more than 30% on both volumetric and specific bases as compared to the previous medium containing the three amino acids as nitrogen source.

Keywords: nitrogen nutrition; biosynthesis; rapamycin; immunosuppressants; *Streptomyces hygroscopicus*; macrolides; lysine

Introduction

Rapamycin was discovered as an antifungal antibiotic produced by *Streptomyces hygroscopicus* strain AY B994, the organism being isolated from a soil sample collected on Easter Island [16]. In addition to its antifungal activity, rapamycin has antitumor and immunosuppressant activities [1,4,5,8,12]. Its potent activity, unique mode of action and low toxicity have led to a great deal of interest in its potential application in human medicine [13].

Rapamycin is a structurally unusual nitrogen-containing triene macrolide with a very large 31-membered lactone ring [6,15]. Precursors of its biosynthesis are acetate, propionate, methionine, pipecolate and shikimate [9,10,11].

Only little had been published on the nutritional control of rapamycin biosynthesis prior to our studies of carbon source nutrition [7]. We found that the combination of fructose and d-mannose supported good rapamycin production as well as good growth, and that a combination of aspartate, arginine and histidine was an effective mixture as nitrogen source for growth of *S. hygroscopicus* and rapamycin production. Under such conditions of carbon and nitrogen nutrition, we found that further addition of lysine stimulated rapamycin production, presumably as a precursor of pipecolic acid [2]. The concentrations of phosphate and magnesium had to be kept growth-limiting in order to achieve high rapamycin production [3].

In the present study, we examined six non-amino acid nitrogen compounds as nitrogen source to replace the costly amino acid mixture (ie aspartate, arginine, and histidine) in the chemically defined medium.

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Materials and methods

Microorganism

Streptomyces hygroscopicus strain C9, a higher rapamycin-producing spontaneous variant derived from AY B1206 [2], was used throughout the study. Spores were produced in Petri dishes at 28°C for 14 days on oatmeal agar (ISP No. 3) medium [14]. Five milliliters of 20% glycerol were added to each dish to prepare a spore suspension which was stored at −80°C.

Inoculum

A seed culture was initiated by adding 0.4 ml of the thawed spore suspension to a 250-ml baffled Erlenmeyer flask containing 30 ml of medium consisting of (g L^{-1}): glucose 10.0, Bacto-peptone (Difco Laboratories, Detroit, MI, USA) 4.0, yeast extract (Difco) 4.0, casamino acids (Difco) 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, and K_2HPO_4 1.0, pH 7.0–7.3. Incubation was conducted at 30°C for 46 h on a rotary shaker (220 rpm). The resulting culture broth was centrifuged at 4°C for 15 min ($5000 \times g$), and the cells were washed once with 100 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (MES, pH 6.0) containing 0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$. The washed cells were suspended in the same buffer to make a 10-ml cell suspension, and 0.5 ml of the suspension was inoculated into 250-ml baffled Erlenmeyer flasks containing 25 ml of chemically defined fermentation medium for rapamycin production.

Fermentation

For screening of nitrogen sources, we used a chemically defined medium (Medium 3) which had been developed previously [2,3,7], but we modified the lysine·HCl concentration, ie, 5.0 g L^{-1} instead of 10.0 g L^{-1} (= Medium 3A). The detailed composition is described in Table 1. Fermentation was carried out in duplicate 250-ml baffled Erlenmeyer flasks containing 25 ml of medium at 28°C on a rotary

Table 1 Composition of chemically defined media

	Medium 3A (L^{-1})	Medium 4 (L^{-1})
Fructose[a]	20.0 g	20.0 g
Mannose[a]	5.0 g	5.0 g
l-Lysine·HCl	5.0 g	5.0 g
Na-l-Aspartate	1.5 g	–
l-Arginine	0.5 g	–
l-Histidine·HCl	0.5 g	–
$(NH_4)_2SO_4$	–	5.3 g
K_2HPO_4	1.7 g	1.7 g
NaCl	5.0 g	5.0 g
$ZnSO_4 \cdot 7H_2O$	60 mg	60 mg
$MgSO_4 \cdot 7H_2O$	2.56 mg	2.56 mg
$MnSO_4 \cdot H_2O$	12 mg	12 mg
$FeSO_4 \cdot 7H_2O$	100 mg	100 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	18 mg	18 mg
$Na_2B_4O_7 \cdot 10H_2O$	10 mg	10 mg
$CoCl_2 \cdot 6H_2O$	10 mg	10 mg
$CuCl_2 \cdot 2H_2O$	1.3 mg	1.3 mg
Na_2SO_4	360 mg	360 mg
MES buffer	100 mM	200 mM
pH (before autoclaving)	6.0	6.0

[a]The sugar mixture was autoclaved separately from the rest of the medium and added into the medium prior to inoculation.

shaker (220 rpm). Samples were taken at 5 and 7 days for assay. The evaporative losses during autoclaving and fermentation amounted to 27% of the initial 25-ml volume. All assay values of growth and production have been corrected for this volume decrease.

Assays for cell growth and rapamycin production

Fermented whole broth (0.5 ml) was centrifuged at $9000 \times g$ for 10 min. The culture supernatant fluid was transferred into a test tube and the pellet was extracted by shaking with 0.5 ml of methanol for 2 h at 30°C. The pooled extracts were added to the culture supernatant and assayed by the paper disc-agar diffusion method using *Candida albicans* ATCC 11651 as the assay microorganism.

C. albicans was cultured at 30°C for 2 days in YEPD medium consisting of ($g\ L^{-1}$): glucose 20, yeast extract (Difco) 10, and Bacto-peptone (Difco) 20. The resulting culture broth was centrifuged at 4°C for 10 min, and the cells were suspended in the same medium to prepare a cell suspension which was stored at –80°C. The assay medium consisted of ($g\ L^{-1}$): glucose 5.0, Bacto-peptone 2.0 and agar 8.0. Twenty microliters of the *C. albicans* cell suspension were seeded into 100 ml of assay medium before pouring of plates. The plates were incubated after addition of the paper discs saturated with extracts or the rapamycin standard for 16–18 h at 37°C. It should be noted that all the values shown in the Tables and Figure were determined by the *C. albicans* bioassay which are 2–3 times higher than those determined by HPLC. The reason for this discrepancy is unknown but is under active investigation.

Cell growth was measured as dry cell weight (DCW) according to Kojima *et al* [7]. The maximum growth and rapamycin production values of the 5- and 7-day assays are shown in Tables 2 and 3.

Results and discussion

Lysine effect on growth of Streptomyces hygroscopicus and biosynthesis of rapamycin

Medium 3A contained lysine as a precursor of the pipecolate moiety of rapamycin [2] and aspartate, arginine plus histidine as nitrogen source. In order to examine the effect of lysine particularly on growth of *S. hygroscopicus* in Medium 3A, we compared cell growth and rapamycin production using the following three media: Medium 3A, Medium 3A less lysine and Medium 3A less aspartate, arginine and histidine. In the medium without lysine, volumetric and specific rapamycin production were drastically reduced to one fifth and one quarter of that in the Medium 3A, respectively, although the final cell density was much less affected, ie, 70% of that in medium 3A (data not shown). On the other hand, the medium without the three amino acids supported almost no growth of the organism even in the presence of lysine. These results indicate that lysine contributes mainly to biosynthesis of rapamycin due to its role as precursor of pipecolic acid [2], and that lysine alone fails to support good cell growth as nitrogen source in chemically defined medium.

Effect of non-amino acid nitrogen sources on biosynthesis of rapamycin

On the basis of the results described above, we examined a variety of non-amino acid nitrogen sources for cell growth and rapamycin production in the medium lacking the three amino acids. The concentration of each nitrogen source provided 32.4 mM nitrogen which is equivalent to that provided by the three amino acids. The medium also contained 5.0 g L^{-1} of lysine·HCl. Of the nitrogen sources studied, five, ie, ammonium sulfate, ammonium nitrate, ammonium chloride, urea, and potassium nitrate, supported good growth of *S. hygroscopicus*; ammonium citrate failed to support growth (Table 2). With respect to rapamycin production, only ammonium sulfate showed higher production of rapamycin than that observed in Medium 3A on both volumetric and specific bases. The remaining nitrogen sources did not support good production of rapamycin even though cell growth was higher than in Medium 3A.

Effect of ammonium sulfate concentration

Although ammonium sulfate appeared to be a good nitrogen source at 16 mM for cell growth and rapamycin production, its use resulted in a low final pH, considerably lower than normal pH of rapamycin-producing *S. hygroscopicus* broths. To decrease the decline of pH during fermentation, we increased the concentration of MES from 100 mM to 200 mM. As a result, final pH of cultures was maintained near 6 without any negative effect on cell growth and rapamycin production (data not shown). With a 200-mM MES buffered medium, we examined the effect of ammonium sulfate concentration on rapamycin production (Table 3). Maximum rapamycin production occurred at 40 mM, whereas cell growth was highest at 20 mM. Ammonium sulfate concentrations greater than 50 mM inhibited growth of *S. hygroscopicus*.

As a result of these studies, an improved chemically defined medium (medium 4) for rapamycin production was

Table 2 Effect of nitrogen sources[a] on growth of *Streptomyces hygroscopicus* and biosynthesis of rapamycin

Nitrogen source	Final pH	Maximum growth (DCW, g L^{-1})	Maximum rapamycin production	
			Volumetric (mg L^{-1})	Specific (mg g^{-1} DCW)
Control[b]	5.9	2.6	69	26
$(NH_4)_2SO_4$ (16.2 mM)	4.8	3.1	85	27
NH_4NO_3 (16.2 mM)	5.6	3.8	61	16
NH_4Cl (32.4 mM)	4.9	3.2	37	11
NH_4citrate (16.2 mM)	5.8	0	0	0
Urea (16.2 mM)	5.7	3.7	69	19
KNO_3 (32.4 mM)	6.2	2.8	49	17

[a]Concentrations of nitrogen sources were adjusted to make the nitrogen content equal to that of the three amino acids (ie, aspartate, arginine and histidine) on a molar basis. All flasks contained 5.0 g L^{-1} of l-lysine·HCl.
[b]Control medium 3A contained aspartic acid (1.5 g L^{-1}), arginine (0.5 g L^{-1}), and histidine (0.5 g L^{-1}) as nitrogen source and l-lysine·HCl (5.0 g L^{-1}).

Table 3 Effect of ammonium sulfate concentration on growth of *Streptomyces hygroscopicus* and biosynthesis of rapamycin[a]

Concentration (mM)	Final pH	Maximum growth (DCW, g L^{-1})	Maximum rapamycin production	
			Volumetric (mg L^{-1})	Specific (mg g^{-1} DCW)
0	6.1	0.4	18	45
20	5.5	3.4	120	35
30	5.2	3.1	123	40
40	5.2	2.0	147	74
50	5.3	2.2	131	60
80	6.1	0.7	32	46
120	6.1	0.4	<6	<15

[a]All flasks contained 5 g L^{-1} of l-lysine·HCl.

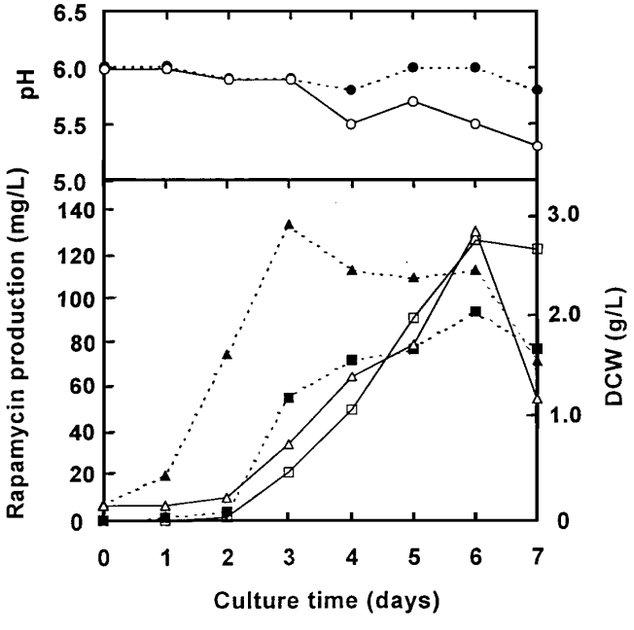

Figure 1 Changes in pH (●,○), growth (▲,△), and rapamycin production (■,□) as a function of time in Medium 3A (closed symbols) and 4 (open symbols).

developed. Its composition is shown in Table 1. The comparative performance between media 3A and 4 with respect to cell growth and rapamycin production is presented in Figure 1. Although cell growth was much slower (by 2–3 days) in Medium 4, maximum rapamycin production was higher than in Medium 3A by more than 30% on both volumetric and specific bases. The cost of the medium was markedly decreased by replacing three amino acids with inorganic nitrogen.

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