CONTROL OF AMMONIUM ION LEVEL FOR EFFICIENT NANAOMYCIN PRODUCTION

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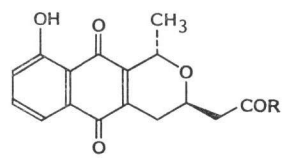
The addition of a small amount of NH_4^+ to a complex medium increased nanaomycin production by *Streptomyces rosa* subsp. *notoensis* OS-3966. The best NH_4^+ donor for nanaomycin production was NH_4^+-saturated natural zeolite, with which the maximum titer of nanaomycin E was 760 μg/ml, about four fold higher than the control titer. In contrast, lowering NH_4^+ levels by adding NH_4^+-trapping agents such as untreated natural zeolite reduced antibiotic production.

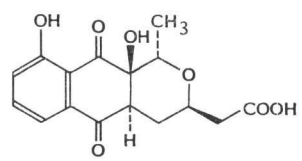
Nanaomycins are a family of benzoisochromane quinone antibiotics active against Gram-positive bacteria and dermatophytes. They were discovered by ŌMURA *et al.* in culture broths of *Streptomyces rosa* subsp. *notoensis* OS-3966.[1] The antibiotic family consists of five components A, B, C, D and E (Fig. 1)[2,3] and the biosynthetic relationships between them have been proposed.[3,4] Nanaomycin A is now being developed as an anti-ringworm agent for cattle. Because it is easily obtained from component E by an alkaline treatment,[3] there are demands for a supply of both nanaomycins A and E. However, neither the efficient production of nanaomycins nor the regulation of nanaomycin biosynthesis has yet been studied.

NH_4^+ and inorganic phosphate levels in fermentation media affect antibiotic production.[5] The present authors reported that the production of leucomycin,[6~8] cerulenin,[9] and tylosin[7,10] was increased by adding natural zeolite, magnesium phosphate or other NH_4^+-trapping agents to the media, and that the increases in titer were associated with decline of the NH_4^+ levels in the media. The condi-

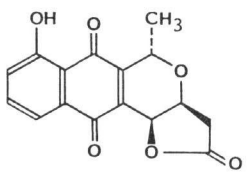
Fig. 1. Structures of nanaomycins produced by *S. rosa* subsp. *notoensis* OS-3966.



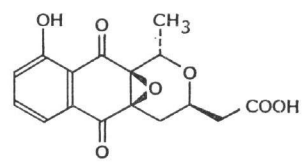
Nanaomycin A R = OH
Nanaomycin C R = NH_2



Nanaomycin B



Nanaomycin D



Nanaomycin E

tions probably favored production of the antibiotics, because they released antibiotic biosynthesis from inhibition by NH_4^+.[11,12] It was expected from these results that nanaomycin production, if it was subject to regulation by NH_4^+, would also be improved by controlling the NH_4^+ concentration in the medium.

The present paper presents evidence that a low level of NH_4^+ favors nanaomycin production. The favorable NH_4^+ level was achieved by the use of NH_4^+-saturated natural zeolite.

Materials and Methods

Microorganism

Streptomyces rosa subsp. *notoensis* OS-3966, a wild-type strain, was used throughout this work.

Method of Cultivation

Spores and mycelia of strain OS-3966 were used to inoculate a 500-ml Sakaguchi flask containing 50 ml of a medium (glucose 2%, meat extract 1%, NaCl 0.5% and $CaCO_3$ 0.3%, pH 6.3~7.0 after autoclaving), and incubated for two days at 37°C with reciprocal shaking (130 strokes/minute). A portion (1.5 ml) of the seed culture thus obtained was transferred into 500-ml Sakaguchi flasks containing 50 ml of the same medium, and incubated as described above.

Estimation of Nanaomycin Titer

A cultured broth adjusted to pH 2 with 2 N HCl was added to an equal volume of EtOAc and the EtOAc layer was used for microbiological assay with *Bacillus subtilis* PCI 219 as test organism. Nanaomycin E was used as standard because it was the main product under the conditions used.

Analytical Methods

The composition of nanaomycin complex produced in a medium was analyzed with a Shimadzu double-beam chromatogram scanner (model GS-910) at 365 nm after thin-layer chromatography on silica gel (0.25 mm, E. Merck, Art 5723) using $CHCl_3$ - MeOH (15: 2) as the developing solvent.[4] The amount of NH_4^+ in a culture supernatant was assayed colorimetrically by the indophenol method,[13] and inorganic phosphate was measured as described previously.[7] For the estimation of mycelial growth, a culture broth acidified to nearly pH 2 with 2 N HCl was centrifuged (2,500 rpm, 10 minutes), and the volume of packed mycelia, expressed as ml/10 ml of culture broth, was taken as the amount of mycelial growth.

Ammonium Ion-trapping Agents

The selection of NH_4^+-trapping agents has been described.[10] Magnesium phosphate [$Mg_3(PO_4)_2 \cdot 8H_2O$, Wako Pure Chemicals Co., Tokyo] and natural zeolite (Fusseki Kako, Tokyo, 40 mesh or smaller in particle size) were used. The natural zeolite consisted of 70% of mordenite and clinoptiolite, 30% of other types of zeolites and clays. It showed a high affinity for NH_4^+.[14] Sodium urate was purchased from Wako Pure Chemicals Co., Tokyo. This compound was assumed to be an NH_4^+-trapping agent, but was proved later to be an NH_4^+-generator.

Preparation of Ammonium Ion-saturated Natural Zeolite

Twenty grams of natural zeolite was suspended in 100 ml of 1 M $(NH_4)_2SO_4$, and the suspension was autoclaved at 121°C for 15 minutes. After decantation, the zeolite was suspended again in 1 M $(NH_4)_2SO_4$, and was autoclaved under the same conditions, and filtered. The residue was washed with H_2O and finally suspended in 40 ml of H_2O.

Results

Effect of Ammonium Ions on Nanaomycin Production

To study the effect of NH_4^+ on nanaomycin production, strain OS-3966 was cultivated in a complex medium supplemented with varying amounts of ammonium sulfate. As shown in Fig. 2, 1 mg/ml

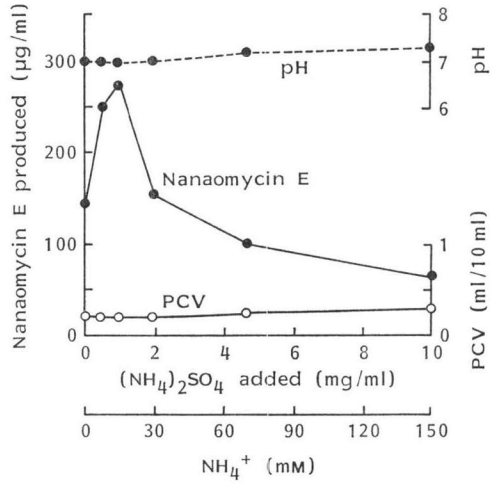
of the ammonium salt (15 mM NH_4^+) added to the medium promoted nanaomycin production with little change in the growth and pH level. Larger amounts of the salt inhibited production to a small extent. This result was unexpected, because it contrasted with that obtained for other antibiotics where inhibition by NH_4^+ was largely proportional to NH_4^+ concentration.

For higher nanaomycin production, control of the NH_4^+ concentration at an appropriately low level by adding ammonium sulfate and other potential NH_4^+-donating substances was attempted. A preparation of NH_4^+-saturated natural zeolite was also tested. As summarized in Table 1, when small amounts of NH_4^+-saturated zeolite were added, nanaomycin titers increased appreciably. Ammonium sulfate, urea and sodium urate also increased nanaomycin production, but were less effective than ammonium salt-treated zeolite preparation. The NH_4^+ concentrations in the supplemented media were higher than that in the control medium, ranging from 6.0 to 33.8 mM.

Fig. 2. Effect of ammonium sulfate on nanaomycin production.

Strain OS-3966 was grown for 36 hours in production medium supplemented with varying amounts of ammonium sulfate as indicated.

PCV: Packed mycelial volume.



Time Course of Nanaomycin Production under Ammonium Ion-controlled Conditions

Fig. 3 illustrates the time courses of nanaomycin production and of NH_4^+ levels in the media supplemented with several NH_4^+ donors. When NH_4^+-saturated zeolite was added, nanaomycin pro-

Table 1. Effect of potential ammonium ion donors on nanaomycin production.

Addition		pH	Packed mycelial volume (ml/10 ml)	Nanaomycin E produced (μg/ml)	NH_4^+ (mM)[b]
Compound	Amount (mg/ml)				
None	—	6.7	0.3	200	2.1
$(NH_4)_2SO_4$	0.5	6.8	0.4	420	7.3
	1.0	6.6	0.4	470	8.7
	2.0	6.5	0.5	300	16.0
NH_4^+-satd	2.0	6.7	0.4	610	6.0
zeolite[a]	5.0	6.7	0.4	640	10.3
	10.0	6.7	0.4	365	12.9
Urea	1.0	7.1	0.2	300	33.0
	2.0	7.1	0.2	435	30.3
	4.0	7.1	0.2	280	33.8
KNO_3	2.0	7.0	0.8	85	7.1
	5.0	7.1	0.7	110	7.7
Sodium urate	5.0	6.4	0.4	420	9.8

Strain OS-3966 was grown at 37°C for 34 hours.

[a] Prepared as described in Materials and Methods.

[b] Concentrations in 34-hour cultures.

Fig. 3. Effect of ammonium ion donors on nanaomycin production.

Strain OS-3966 was grown in media supplemented with none (○), 0.2% ammonium sulfate-treated zeolite (●), 0.1% ammonium sulfate (▲) or 0.1% urea (□). In A, pH (---) and nanaomycin titer (—); and in B, NH_4^+ concentrations are shown.

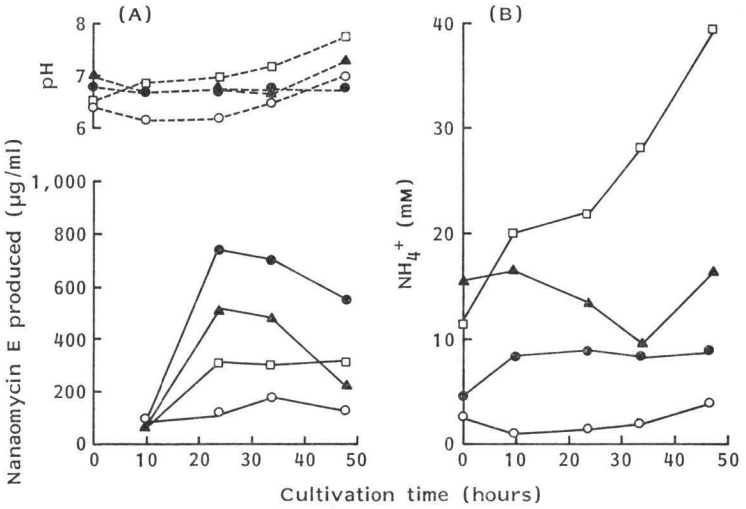
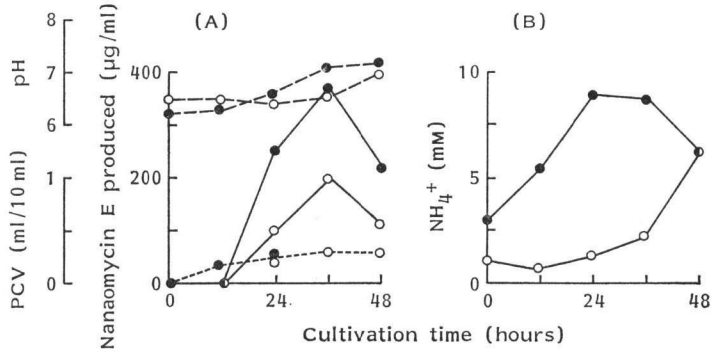


Fig. 4. Effect of sodium urate on nanaomycin fermentation.

Strain OS-3966 was grown at 37°C in the presence (●) or absence (○) of 0.5% sodium urate. At the intervals indicated, a 5-ml portion was withdrawn from each flask, and assayed. In A, pH (---), growth (···) and nanaomycin titer (—), and in B, NH_4^+ concentrations are shown.



duction reached a peak of 760 μg/ml after 24 hours of cultivation. NH_4^+ in the supplemented medium remained at almost a constant level around 10 mM during the fermentation (Fig. 3-B). With urea and ammonium sulfate, NH_4^+ concentrations fluctuated at higher levels, and nanaomycin production was lower.

Fig. 4 shows another example of increased nanaomycin production in the presence of sodium urate. Sodium urate added to the culture medium was most probably degraded by the nanaomycin producer to generate NH_4^+ at a low rate, and promoted antibiotic production. The changes in growth and pH levels were small.

Effect of Ammonium Ion-trapping Agents on Nanaomycin Production

If nanaomycin production is stimulated by a small amount of NH_4^+, the decrease in NH_4^+ con-

centration caused by adding an NH_4^+-trapping agent should suppress antibiotic production. Natural zeolite (not treated with ammonium sulfate) and magnesium phosphate were assumed to act as NH_4^+-trapping agents from the following observation that an acidic solution (or suspension) in water readily formed an ammonium nitrogen-containing complex when neutralized with ammonium hydroxide, but did not form it with sodium hydroxide.[10] In addition, the two materials have been demonstrated to capture NH_4^+ in antibiotic fermentation media.[6,7,9~11]

Table 2. Effect of natural zeolite on nanaomycin production in a complex medium.[a]

Natural zeolite added (mg/ml)	pH	Nanaomycin E produced (μg/ml)	NH_4^+ (mM)[b]
—	6.6	130	3.2
5	6.4	110	2.5
10	6.4	90	1.6
20	6.4	65	0.5

The fermentation was carried out at 37°C for 36 hours.

[a] All cultures contained 0.3~0.4 ml of packed mycelia/10 ml of culture broth.

[b] Concentrations in 36-hour cultures.

When natural zeolite was added to the culture, it reduced both nanaomycin titers and NH_4^+ levels (Table 2). Addition of magnesium phosphate caused a slight reduction of NH_4^+ concentrations but severely inhibited nanaomycin production. This was due to the suppression of antibiotic biosynthesis by inorganic phosphate released from magnesium phosphate (data not shown).

Discussion

The production of leucomycin[7] and cephamycin[15] in defined media, and of cerulenin[9] in a complex medium was suppressed by 3~10 mM of NH_4^+. In contrast, NH_4^+ in the 6~34 mM concentration range promotes nanaomycin production in a complex medium. This amount is not required for adequate growth of the nanaomycin producer, suggesting that the phenomenon is due to regulation. It can be regarded as positive regulation of nanaomycin biosynthesis by NH_4^+ although concentrations higher than 70 mM inhibited nanaomycin production (Fig. 2).

The increases in nanaomycin titers were associated with changes in pH, which differed from control values by up to 1.0 unit. The effect of pH was not studied here, and its role in increasing nanaomycin titers is not ruled out at present. Nevertheless, the results shown in Figs. 2 and 4 implicate NH_4^+ as the major factor promoting nanaomycin biosynthesis. Recently, INOUE *et al.*[16] showed a positive effect of NH_4^+ on streptomycin production by *Streptomyces griseus*.

The mechanism of the stimulation and inhibition of nanaomycin production by NH_4^+ is not known. Because nanaomycin E was nearly the sole component produced under the conditions studied, it is unlikely that NH_4^+ affected the steps after formation of the earliest nanaomycin compound, nanaomycin D.[8] Further studies are now in progress using defined media to clarify the mechanism of action. In addition to being affected by NH_4^+, nanaomycin biosynthesis was inhibited by inorganic phosphate.

Previous papers[6~9] showed that NH_4^+-trapping agents increase production of NH_4^+-susceptible antibiotics by decreasing NH_4^+ levels in the media. In this paper we demonstrate that they are also useful in studying biosynthetic regulation by NH_4^+. Nanaomycin E production was improved significantly by the use of NH_4^+-saturated natural zeolite which appeared to have a buffering activity for the NH_4^+ level. In media supplemented with larger amounts of the zeolite preparation, NH_4^+ concentrations fluctuated less, but were retained at higher levels (not shown). NH_4^+-saturated zeolite as an NH_4^+ carrier is a new use of zeolite for the control of NH_4^+ at low levels with various potential applications in antibiotic fermentation, including screening research.

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