PRODUCTION OF NANAOMYCIN AND OTHER ANTIBIOTICS BY PHOSPHATE-DEPRESSED FERMENTATION USING PHOSPHATE-TRAPPING AGENTS

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(Received for publication May 7, 1986)

Nanaomycin production by *Streptomyces rosa* subsp. *notoensis* in complex media was inhibited by exogenously supplied inorganic phosphate. The inhibition was reversed by phosphate-trapping agents such as allophane and aluminum oxide. Under such condition nanaomycin production increased to the control level, and the phosphate content dropped down to the unsupplemented level. When allophane was added to conventional complex media containing nutrient-derived inorganic phosphate, the production of nanaomycin and several other antibiotics, which are subject to phosphate regulation, was enhanced several fold with the simultaneous reduction of free phosphate. The term "phosphate-depressed fermentation" is proposed for this technique.

In a previous paper[1], we proposed the term "ammonium-depressed fermentation" of antibiotics. This technique yields several fold increases in production of many antibiotics by the use of ammonium-trapping agents such as magnesium phosphate and natural zeolite.

Nanaomycins[2~6], a family of isochromane quinone antibiotics discovered by ŌMURA *et al.* in the culture broth of *Streptomyces rosa* subsp. *notoensis*, has been approved as an anti-ringworm agent. Nanaomycin production was suggested to be inhibited by inorganic phosphate, although it was enhanced by a small amount of ammonium[7]. By analogy with the ammonium-depressed fermentation described above, it was considered that the use of phosphate-trapping agents, if available, might be beneficial for improving nanaomycin production.

The present paper deals with the selection of several inorganic materials with phosphate-trapping potential, and the stimulating effect of allophane, one of these materials, on production of nanaomycin and other antibiotics.

Materials and Methods

Microorganisms

The nanaomycin producer *Streptomyces rosa* subsp. *notoensis* OS-3966[3], a wild-type strain, was used. The other antibiotic producing cultures are listed in Table 3.

Antibiotic Production

Nanaomycin production was carried out as follows. Spores and mycelia of strain OS-3966 grown on an agar slant were used to inoculate a 500-ml Sakaguchi flask containing 100 ml of seed medium (glucose 2%, peptone 0.5%, meat extract 0.5%, dried yeast 0.3%, NaCl 0.5%, $CaCO_3$ 0.3%, pH 7.0). The flask was incubated at 27°C for 2 days with reciprocal shaking (120 strokes/minute).

An aliquot (6 ml) of this seed culture was transferred into 2-liter Sakaguchi flasks containing 300 ml of medium A (glycerol 2%, Bacto-soytone (Difco) 1%, NaCl 0.3%, pH 7.0~7.2) or medium B (glycerol 2%, peptone 0.5%, dried yeast cells 0.3%, meat extract 0.5%, NaCl 0.5% and $CaCO_3$ 0.3%, pH 6.8~7.0), to which test materials were supplemented as indicated in each Table and Fig. The incubation was carried out for 1 to 5 days under the same conditions as the seed culture. Production of other antibiotics was carried out in 500-ml Sakaguchi flasks containing 100 ml of production media, as described in Table 3.

Analytical Methods

Nanaomycin titer was assayed microbiologically with *Bacillus subtilis* PCI 219 as the test organism using nanaomycin E, which was the major component produced under the conditions employed, as the standard. Nanaomycin components were determined after thin-layer chromatography, as described previously[7]. Other antibiotics were also determined microbiologically. Cell growth was estimated by measuring intracellular nucleic acids by the method of SCHNEIDER[8] with yeast RNA (Wako Chemical Co., Tokyo) as standard. Glycerol was determined according to the method of IWAI *et al.*[9], and inorganic phosphate by the method of ALLEN[10]. All the values presented below are averages of duplicate flasks.

Allophane

Sekado KW, supplied by Shinagawa Refractory Co., Tokyo, was used as the source of allophane after finely grinding to 48 mesh or smaller in particle size. This commercial product was purified from Kanuma-tsuchi, a soil found in the vicinity of Kanuma-city and Mōka-city, Tochigi Prefecture, Japan. It consists of Al_2O_3 (*ca.* 50%), SiO_2 (*ca.* 43%) and others (7%), and contains allophane ($Al_2O_3 \cdot SiO_2 \cdot nH_2O$, n=4~6), a hydrated alumina-silica gel, as the major composite, according to the supplier's manual.

Results

Phosphate-trapping Ability of Several Inorganic Materials

Several alumino-silicates are porous, and more or less acidic to litmus. Zeolite, one of them, adsorbs ammonium and other cations[1,7], while others like kaolin[11] and colloidal earths[12] absorb organic materials such as aniline dyes, methylene blue, benzene and pyridine. One of the non-crystalline clays, allophane, adsorbs acetate, fluoride ions and inorganic phosphate[13]. In view of these observations, it seemed worth-while to obtain phosphate-trapping minerals suitable for fermentation technology *e.g.* for improving antibiotic production.

Commercially available kaolin, acid clay, allophane, aluminum oxide and others were employed. These natural minerals and some metal salts were tested for their ability to capture inorganic phosphate under fermentative conditions. The test materials (0.5%) were incubated with KH_2PO_4 (0.74 mM (=100 μg/ml), pH 7.0) at 27°C with shaking, and the residual free phosphate was assayed after 24 hours. The results shown in Table 1 demonstrate that allophane, aluminum oxide, basic magnesium carbonate and calcium carbonate are potent phosphate-trapping agents. Silicon dioxide, aluminum silicate, acid clay, and kaolin showed no trapping ability under the conditions employed. Also no effect was observed with natural zeolite, celite, silicic acid, magnesium sulfate and Amberlite XAD-2, a synthetic porous polymer.

When the suspension mixtures were autoclaved (120°C, 15 minutes) prior to shaking, or when test materials were incubated with 7.4 mM of KH_2PO_4 (pH 7.0), results similar to the above were obtained. The addition of the buffer *N*-morpholinopropanesulfonic acid (MOPS) (0.1 M, pH 7.0) to the reaction mixtures had little influence on the above results. With basic magnesium carbonate, the

Table 1. Phosphate-trapping ability of various inorganic materials.

Test material* (added at 0.5%)	Residual KH_2PO_4 (%)
None	100 (=0.74 mM)
Allophane (Kanuma-tsuchi)	27
Magnesium carbonate, basic	77
Aluminum oxide	39
Silicon dioxide	114
Aluminum silicate	114
Acid clay	100
Kaolin	103
Calcium carbonate	10
Natural zeolite	108

* Test materials were shaken in a KH_2PO_4 solution at 27°C for 1 day.

pH of the reaction mixture without autoclaving rose to above 8 after 24-hour shaking, or did so just after autoclaving. MOPS somewhat suppressed the pH-rise, and the phosphate-trapping ability was enhanced slightly. With other materials, the pH remained neutral with and without MOPS.

Inhibition of Nanaomycin Production by Inorganic Phosphate and Its Reversal by Phosphate-trapping Agents

Table 2 shows that nanaomycin production in a complex medium (medium A) is severely inhibited by inorganic phosphate. When added initially to the medium, 0.37 mM of KH_2PO_4 suppressed antibiotic production by about 50%, and 0.74 mM suppressed it over 80%, while growth increased only slightly. Earlier addition caused stronger inhibition (Fig. 1).

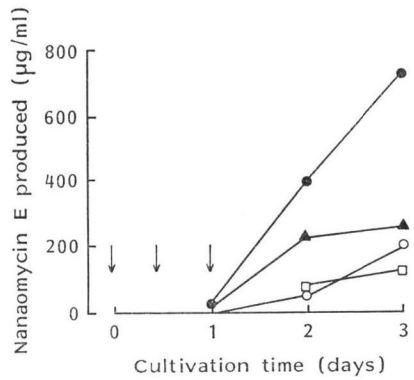
Table 2. Inhibition of nanaomycin production by KH_2PO_4.

KH_2PO_4 added (mM)	Nucleic acid (mg/ml)	pH	Nanaomycin E produced (μg/ml)
0	0.26	7.4	810
0.07	0.27	7.5	800
0.37	0.25	7.2	440
0.74	0.29	7.0	120
7.4	0.32	6.5	—

The fermentations were carried out in Sakaguchi flasks at 27°C for 3 days.

Fig. 1. Effect of the time of KH_2PO_4 addition on nanaomycin production.

To 2-liter Sakaguchi flasks containing medium A KH_2PO_4 solution (pH 7.0) was added in a final concentration of 0.74 mM (=100 μg/ml as KH_2PO_4) at 0 (○), 10th (□), 24th (▲) hour after inoculation, or not added (●), as indicated by arrows (↓). The nanaomycin producer *S. rosa* subsp. *notoensis* OS-3966 was grown in these media for a total of 72 hours. Mean values of nanaomycin titer in duplicate flasks are shown.



It was expected that phosphate inhibition of nanaomycin production would be overcome by adding phosphate-trapping agents to the medium. Allophane and other agents were added to phosphate-supplemented medium A, and nanaomycin titers were assayed after three days of cultivation. As shown in Fig. 2, nanaomycin production was restored to the control level by the addition of allophane, and to an intermediate level by aluminum oxide. Without a trapping agent, nanaomycin titer was at about 1/3 of the control titer due to inhibition by phosphate. Calcium carbonate gave little restoration, although it is a good phosphate-trapping agent in a model system (Table 1). The reason for this is not known.

Confirming the above results, Fig. 3A illustrates time courses of nanaomycin production in the presence of an inhibitory concentration of KH_2PO_4 (0.74 mM) and allophane (0.5%). When phosphate was added, antibiotic production decreased considerably. When allophane was additionally

Fig. 2. Reversal by phosphate-trapping agents of inhibition of nanaomycin production by inorganic phosphate.

Medium A was supplemented with or without 0.74 mM of KH_2PO_4 (pH 7.0). To the phosphate-supplemented flasks were added a 0.5% of the phosphate-trapping agents indicated. Strain OS-3966 was grown in these flasks for 3 days.

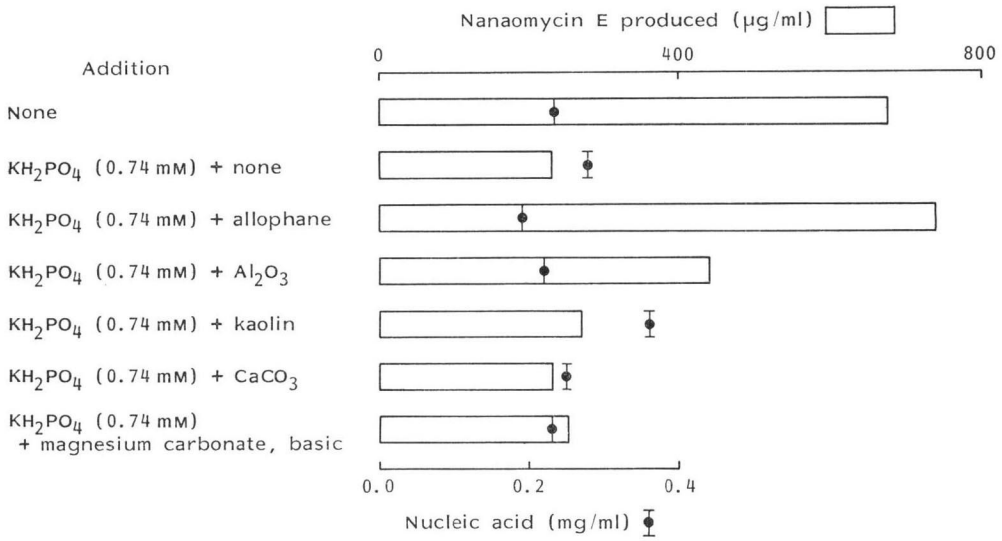
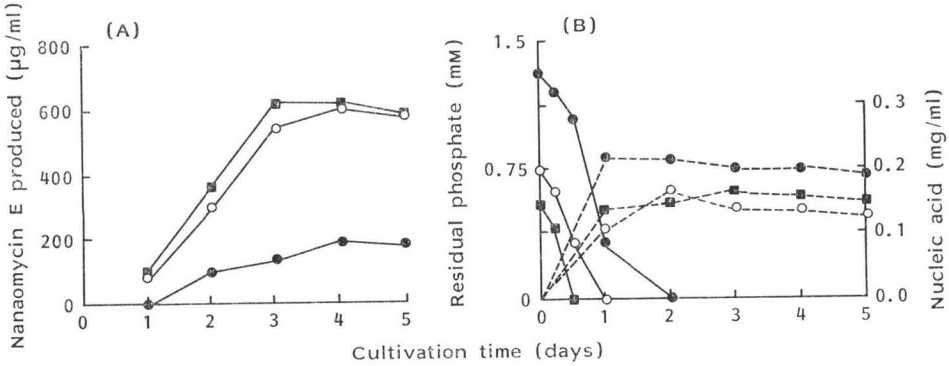


Fig. 3. Time course of nanaomycin production in the presence of added KH_2PO_4 and allophane.

To four Sakaguchi flasks containing medium A an aliquot of KH_2PO_4 solution was added at a final concentration of 0.74 mM. Allophane (0.5%) was further supplemented to two of the four flasks. Another two flasks received neither. Strain OS-3966 was grown in these six flasks for 5 days.

(A) Nanaomycin titers are shown. No addition (○), with KH_2PO_4 (●), and with KH_2PO_4 plus allophane (■).

(B) Phosphate concentrations (——) and amounts of nucleic acid (------) are shown. Symbols are the same as in (A).



supplemented, nanaomycin titer was almost the same or slightly higher than the control titer. The changes in antibiotic titers were inversely correlated with changes in initial phosphate content in the three media (Fig. 3B): Phosphate was higher in the phosphate - supplemented medium, lower in the unsupplemented control medium, and lowest in the medium supplemented with both phosphate and allophane.

Fig. 4. Effect of allophane on nanaomycin production in complex media with low and high phosphate contents.

Strain OS-3966 was grown in media A ((A) low-phosphate medium), and B ((B) high-phosphate medium) with (closed symbols) or without (open symbols) 0.5% allophane. An aliquot (5 ml) was withdrawn from each flask and used for the assays of nanaomycin titers (●, ○), phosphate contents (■, □) and pH (▲, △).

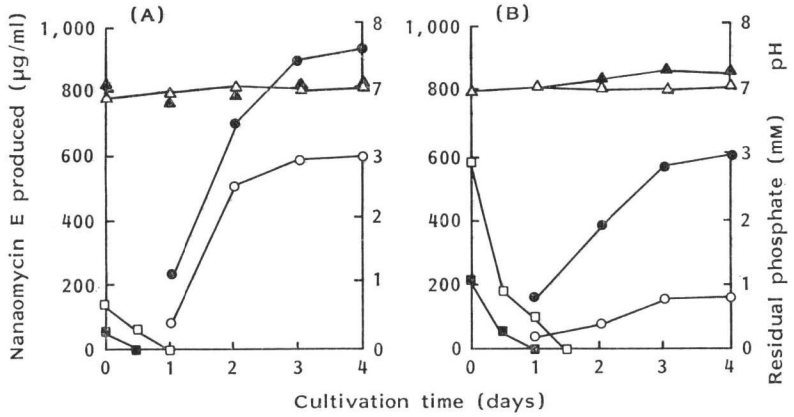


Table 3. Effect of allophane on antibiotic production.

Antibiotic	Producer	Medium*	Initial PO_4^{3-} (mM)	Antibiotic titers (μg/ml)	
				Not added	Allophane added
Nanaomycin	*S. rosa* subsp. *notoensis* KA-301 (ATCC 31135)	A	0.6	600	900
		B	2.8	160	590
Tylosin	*S. fradiae* KA-427 (C-373)	D	1.3	70	140
Erythromycin	*S. erythraeus* KA-1133 (ISP 5517)	C	2.8	29	46
Candicidin	*S. griseus* KA-480 (IMRU 3570)	C	2.8	180	530
Gentamicin	*M. echinospora* KA-566 (ATCC 15837)	E	1.8	3	18
Vancomycin	*S. orientalis* KA-1308 (ISP 5040)	F	0.5	4.5	18.5
Phosalacine	*K. phosalacinea* sp. nov. KA-388 (IFO 14372)	G	0.6	160	23

* A: Glycerol 2%, Bacto-soytone 1%, NaCl 0.3%, pH 7.0.
B: Glycerol 2%, peptone 0.5%, meat extract 0.5%, dried yeast cells 0.3%, NaCl 0.5%, $CaCO_3$ 0.3%, pH 7.0.
C: Glucose 2%, peptone 0.5%, meat extract 0.5%, dried yeast cells 0.3%, NaCl 0.5%, $CaCO_3$ 0.3%, pH 7.0.
D: Starch 2%, glucose 1%, peptone 0.5%, yeast extract 0.5%, L-asparagine 0.3%, $CaCO_3$ 0.4%, pH 7.2.
E: N-Z amine 0.5%, starch 1%, glucose 1%, yeast extract 0.5%, $CaCO_3$ 0.1%, pH 7.0.
F: Glycerol 2%, soybean meal 2%, NaCl 0.3%, pH 7.0.
G: Glucose 0.2%, oatmeal 1.5%, tomato paste 4%, pH 6.0.

Effect of Allophane on Nanaomycin Production in Complex Media with Low and High Phosphate Contents

The above results suggest that phosphate-trapping agents are useful in increasing antibiotic production in media containing nutrient-derived inorganic phosphate at inhibitory levels. This notion led us to examine the effects of allophane on nanaomycin production in two media with low (medium A) and high (medium B) phosphate contents. Medium A, which was also used in the above-

described experiments, contains 0.67 mM of free phosphate, and is a high-production medium selected during improvement of nanaomycin production[7]. Medium B, containing an initial concentration of 2.81 mM free phosphate, was once excluded as unsuited for nanaomycin production. At the time of the above selection, no particular attention was paid to phosphate contents of these media.

Fig. 4 shows nanaomycin production and phsophate levels in the two media with and without allophane. In medium A, the nanaomycin titer reached about 600 μg/ml at day 3. When allophane was added, the antibiotic titer increased to 900 μg/ml at day 3, a level 1.5-fold higher than the control. The initial phosphate level of 0.67 mM decreased to a much lower level (0.22 mM) at time zero, and phosphate disappeared by 12 hours. In medium B, a similar but more stressed tendency was observed in the presence of allophane. That is, nanaomycin titer was 160 μg/ml without allophane. The antibiotic titer increased to 590 μg/ml, which was about 3.7-fold higher than the control. These increases were associated with reduction of free phosphate content down to a level nearly equal to that of medium A. In both media, pH and the rates of glycerol assimilation were not affected substantially by allophane, whereas cell growth decreased somewhat (data not shown).

Enhancement of Antibiotic Production by Allophane

Production of antibiotics is often inhibited by inorganic phosphate when phosphate content exceeds certain thresholds in the media employed. Although the mechanism of inhibition is generally not well understood, accumulated experience shows that the decline of phosphate level is beneficial for efficient production of such antibiotics. Therefore, it was of interest to examine if allophane would increase production of other antibiotics. Laboratory stock cultures of antibiotic producers were grown in conventional complex media, to which 0.5% allophane was added, and antibiotic titers were determined microbiologically.

Table 3 shows that the addition of the phosphate-trapping agent increased production of several antibiotics. These include, in addition to nanaomycin, tylosin, erythromycin, gentamicin, vancomycin and candicidin. Not every antibiotic studied showed increased production in the presence of allophane. One such example is the phosphate-containing antibiotic phosalacine produced by *Kitasatosporia phosalacinea*[14]. The stimulating effect depended on media used for the production of an antibiotic, *e.g.* nanaomycin (Table 3).

Discussion

Nanaomycin production is severely inhibited by exogenously supplied inorganic phosphate. The addition of as little as 0.37 mM of KH_2PO_4 resulted in a 50% reduction of antibiotic titer. The inhibition by phosphate disappeared when phosphate-trapping agents such as allophane were supplemented. The reversal was associated with a decline of phosphate content in the medium.

Allophane, a hydrated alumina-silica gel, captures preferentially free phosphate by its non-crystalline porous structure which is chemically reactive. According to WADA[13], the interaction of phosphate with allophane involves three types of reactions. These are non-specific adsorption by coulombic force due to positive charges on the alumina moiety, specific adsorption referring to the incorporation of this ion as a ligand into the matrix of the alumina moiety, and a reaction which is associated with decomposition of allophane and with the release of hydroxyl ions. If similar reactions occur in antibiotic fermentation broths, a part of adsorbed phosphate is assumed to be liberated back into culture media. Allophane can thus keep phosphate at a very low level and create phosphate-depressed fermentation conditions.

Kanuma-tsuchi, a volcanic soil found in the vicinity of Kanuma-city and Mōka-city, Tochigi

Prefecture, Japan, is an unpurified soil preparation rich in allophane. The addition of Kanuma-tsuchi to a rice field often causes growth retardation of the rice plant due to limitation of phosphate. Kanuma-tsuchi was not very effective in increasing antibiotic production (data not shown). However, Sekado KW (allophane), prepared from Kanuma-tsuchi, was very effective.

Allophane may also adsorb other acids such as acetate and citrate. Nevertheless, phosphate is assumed to be the factor which is most relevant to the events observed with allophane, in view of severe inhibition of nanaomycin production by phosphate.

Complex media usually contain nutrient-derived inorganic and organic phosphates. It is suggested that the phosphate content in medium A, which is referred to as a low-phosphate medium in this paper, is sufficient to cause inhibition of nanaomycin production. The reduction of such phosphate levels to less suppressing levels by allophane releases nanaomycin biosynthesis from inhibition by the ions, thereby favoring antibiotic production. The increase in production of other antibiotics (Table 3) can be assessed on the same basis, because negative phosphate regulation of the biosynthesis of these antibiotics has been demonstrated[15], except for erythromycin.

LIU *et al.*[16] and MÜLLER *et al.*[17] reported that the addition of ferrous or zinc ions decreased phosphate level in a medium, and antibiotic titers increased. This is due to the formation of precipitable metal salts of phosphate. Our result is the first example showing that nonionic natural minerals are also useful as phosphate-trapping agents for antibiotic production. It is emphasized that allophane has almost no effect on metal contents. If other natural and synthetic materials with phosphate-trapping ability are available, they will be potentially useful for the same purpose as allophane. The method presented here is simple and effective in improving antibiotic production. It is interesting that a single addition of allophane to a low-production medium (medium B) resulted in nanaomycin production at nearly the same level as attained in an improved medium (medium A) (Fig. 4). Chemostat and fed-batch culture techniques may also be utilized for maintaining phosphate content at a low level. However, these two techniques require specified apparatus, fermentations in unconventional flasks, and growth-associated production of antibiotics. On the contrary, our technique does not necessitate the above requirements, and is suited for fermentations both on large and small scales in a traditional manner. We call this technique "phosphate-depressed fermentation" of antibiotics.

As ammonium-depressed fermentation proved valuable in basic and applied studies of antibiotic fermentation[18], so are phosphate-trapping agents expected to be useful in screening research for new antibiotics and in studying the mechanism of inhibition of antibiotic biosynthesis by inorganic phosphate.

Acknowledgment

The help of Dr. J. F. MARTÍN, Universidad de Léon, for supplying a culture of *Streptomyces griseus* IMRU-3570 is gratefully acknowleged. The authors are indebted to Shinagawa Refractory Co., Tokyo, for providing us with Sekado KW. Our thanks are also due to Miss T. KONDO for her helpful assistance.

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