# SITE OF REGULATION OF NANAOMYCIN BIOSYNTHESIS BY INORGANIC PHOSPHATE

ROKURŌ MASUMA, DE-ZHONG ZHEN†, YOSHITAKE TANAKA and SATOSHI ŌMURA*

The Kitasato Institute, and School of Pharmaceutical Sciences,
Kitasato University,
Minato-ku, Tokyo 108, Japan

(Received for publication April 20, 1989)

The site of regulation of nanaomycin biosynthesis by inorganic phosphate was studied with washed cells previously grown in a chemically defined medium containing a high- or low-phosphate concentration. The former mycelia produced only about one-tenth the amount of nanaomycin A from acetate as did the latter mycelia. On the other hand, the bioconversions of nanaomycin D to A and nanaomycin A to E were only slightly affected. It is suggested that the site of regulation of nanaomycin biosynthesis by inorganic phosphate lies within steps between acetate and nanaomycin D.

Nanaomycins, a family of quinone antibiotics, are metabolites of *Streptomyces rosa* subsp. *notoensis* KA-301 which was discovered by ŌMURA *et al.*[1,2] In the biosynthesis of nanaomycins, eight acetates are condensed to produce nanaomycin D, which is converted to nanaomycin A and then to E[3,4]. In a previous paper[5], we reported that nanaomycin E production was severely decreased by inorganic phosphate. The decrease was reversed by phosphate-trapping agents such as allophane when added to the phosphate-containing culture medium. The present paper describes the site of regulation of nanaomycin biosynthesis in defined media. The results suggest that the site lies between acetate and nanaomycin D.

## Materials and Methods

### Microorganism

The nanaomycin producer, *S. rosa* subsp. *notoensis* KA-301[5], a wild-type strain, was used.

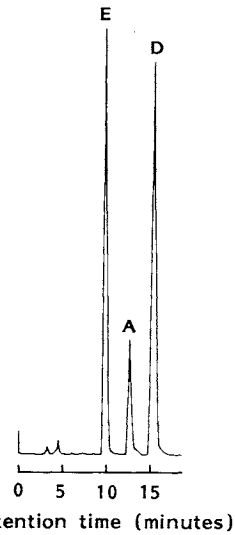
### Method of Cultivation

Nanaomycin production was carried out as follows. Spore suspension of strain KA-301 was inoculated into 500-ml Sakaguchi flasks 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 flasks were incubated at 27°C for 2 days with reciprocal shaking (120 strokes/minute). Two production media were used. Complex medium consisted of glycerol 2%, Bacto-soytone (Difco) 1%, NaCl 0.3%, pH 7.0; and chemically defined medium contained glucose 2%, nitrogen source 0.5%, $KH_2PO_4$ 0~0.67% (50 mM), $MgSO_4 \cdot 7H_2O$ 0.01%, *N*-morpholinopropanesulfonic acid (MOPS) 2.1% (0.1 M), 0.3% of trace metal solution consisting of (each at 1 g/liter) $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 2H_2O$, pH 7.0 before autoclaving. An aliquot (2 ml) of seed culture was transferred into complex medium. Flasks were incubated at 27°C for 3 days. For nanaomycin production in defined medium, mycelia obtained from 100 ml of the seed culture by centrifugation (3,000 rpm, 10 minutes) were washed three times with the defined medium, and resuspended in the same medium (100 ml). An aliquot (3 ml) of this vegetative mycelial suspension was transferred into defined medium (100 ml). The incubation was carried out for 1 to 7 days under the same conditions as the seed culture. When an increase in inorganic phosphate concentration was required,

† Present address: Shenyang College of Pharmacy, 2-7 Wenhua Road, Shenyang, China.

a separately sterilized solution of $KH_2PO_4$ (pH 7.0) was added as a supplement.

Fig. 1. HPLC profile of nanaomycins A, D and E.




Determination of Nanaomycins

Determination of nanaomycins was carried out by two methods as follows: Nanaomycin titer was assayed microbiologically with *Bacillus subtilis* PCI 219 as the test organism using nanaomycin E as standard. The amounts of nanaomycins were also determined with HPLC. Cultured broth (2 ml, adjusted to pH 2.0 by 1 N HCl) was extracted twice with an equal volume of ethyl acetate. The ethyl acetate layer was evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of MeOH. Ten $\mu$l of this solution was subjected to HPLC. The absorbance was measured at 240 nm. HPLC was developed using YMC A-302 (ODS type) as the column material and a mixture of acetonitrile and 10 mM $KH_2PO_4$ (pH adjusted to 3.0 with $H_3PO_4$) (35:65) as elution solvent. Fig. 1 illustrates the elution profile of nanaomycins A, D and E in HPLC under the conditions specified above.

Preparation of Washed Cells of Strain KA-301 and Nanaomycin Production

Strain KA-301 was grown in defined media at 27°C for 2 days. Mycelia were harvested by centrifugation (3,000 rpm, 10 minutes) and washed three times with 2-*N*-(morpholino)ethanesulfonic acid (MES) (pH 6.9) and resuspended in the same buffer to give a cell concentration of 1.5~2.0 mg/ml. Substrate (10 $\mu$l in water for acetic acid or in methanol for nanaomycins) was added to a 2-ml aliquot of this cell suspension and incubated at 27°C, for up to 3 hours with shaking. Methanol at this concentration had no effect on the conversion reactions. The amounts of nanaomycins produced were determinated by HPLC.

Analytical Methods

Dry cell weight was determined after heating at 90°C for 10 hours. Other analytical methods were those described previously[5].

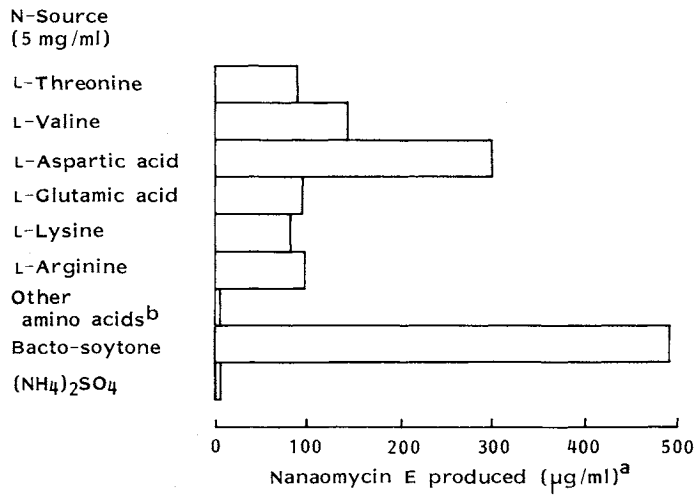
## Results

### Choice of Chemically Defined Medium

Antibiotic biosynthesis and its regulation can be studied better in defined media than in complex media. Attempts were made to construct a defined medium suitable for the study of nanaomycin production and phosphate regulation. The basal medium was chosen based on the results of preliminaly experiments. To select a suitable nitrogen source, the effect of various amino acids on nanaomycin production was studied. Fig. 2 shows that L-aspartic acid was the best sole nitrogen source in defined medium. In this defined medium nanaomycin titer reached 300 $\mu$g/ml at day-5. When valine, threonine or glutamic acid was used as nitrogen source, about 100 $\mu$g/ml of nanaomycin was produced. Other amino acids, *e.g.* leucine or serine, did not support nanaomycin production. Ammonium sulfate at 0.5% supported good growth, while no nanaomycin production occurred. From these results, L-aspartic acid was employed as the nitrogen source in the defined medium. Nanaomycin production was higher with glucose (2%) than with glycerol (2%) as carbon source. MOPS was necessary to reduce fluctuation in pH of the culture medium during fermentations. Without MOPS, growth and nanaomycin production were very poor.

Fig. 2. Effect of amino acids as nitrogen source on nanaomycin E production by *Streptomyces rosa* subsp. *notoensis* KA-301 in defined medium.

[a] Nanaomycin E titers in 5-day cultures are shown.
[b] Fifteen other natural amino acids were included.



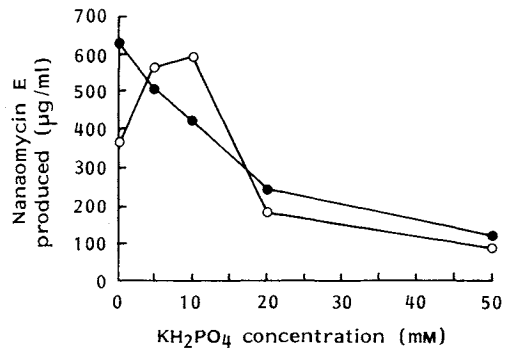

## Effect of Inorganic Phosphate on Nanaomycin Production

Using the defined medium with aspartic acid, the effect of inorganic phosphate on nanaomycin E production was studied. As shown in Fig. 3 nanaomycin production decreased considerably when inorganic phosphate concentration exceeded 10 mM, although it increased moderately with an increase of inorganic phosphate concentration up to 10 mM.

When Bacto-soytone was used as nitrogen source, nanaomycin E production declined steeply with an increase of inorganic phosphate concentration. In all the cases, the pH values of the culture media remained within 6.2~7.2. These results demonstrate that nanaomycin biosynthesis is subject to negative regulation by inorganic phosphate in complex media and in the chemically defined medium employed.

Fig. 3. Effect of inorganic phosphate on nanaomycin E production by *Streptomyces rosa* subsp. *notoensis* KA-301 in defined medium.

○ L-Aspartic acid (0.5%), ● Bacto-soytone (0.5%).




## Bioconversion of Nanaomycin Components by Washed Cells

Previous studies[3,4] showed that the biosynthesis of nanaomycins starts with the condensation of eight acetate units to first form nanaomycin D, and then nanaomycins A and E. To examine the site of phosphate action, bioconversion studies were carried out using washed cells of KA-301, previously grown with low-(5 mM, control) and high-phosphate (50 mM) concentrations.

Washed cells obtained at day-2 with each phosphate level were compared for their ability to convert nanaomycin components. Nanaomycin production was 50 μg/ml and a trace under low- and high-phosphate conditions, respectively, in growing cultures. Fig. 4 summarizes the results of the bioconversion studies.

Fig. 4. Production of nanaomycins from acetic acid, nanaomycins D and A by washed cells previously grown in high- and low-phosphate media.

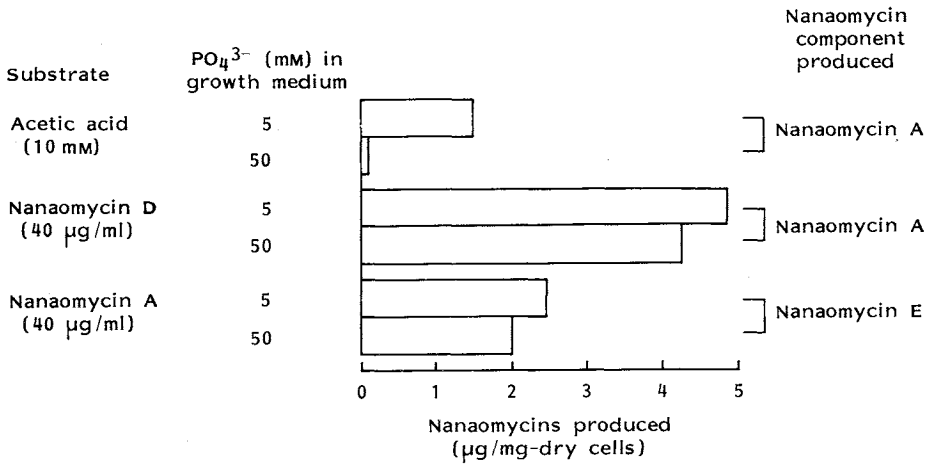
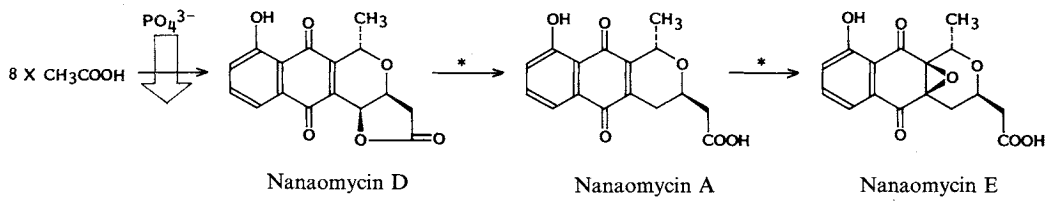



Fig. 5. Site of phosphate regulation of nanaomycin biosynthesis in *Streptomyces rosa* subsp. *notoensis* KA-301.

* Steps insensitive to $PO_4^{3-}$ effect.




When acetate (10 mM) was incubated with washed cells, nanaomycin A, and not nanaomycin D, was the sole product. Nanaomycin A production was 1.5 μg/mg-dry cells by the cells grown under the low-phosphate condition, while it was 0.2 μg/mg-dry cells with the cells grown under high-phosphate condition. When nanaomycins D and A were used as substrates, the bioconversion products were almost solely nanaomycins A and E, respectively. Concentration of phosphate during growth had only a slight negative effect on the bioconversions of nanaomycin D to A and nanaomycin A to E.

## Discussion

Nanaomycin production by *S. rosa* subsp. *notoensis* KA-301 is severely decreased by exogenously supplied inorganic phosphate in both complex and defined media. The addition of as little as 1 mM of $KH_2PO_4$ resulted in a 50%-reduction of antibiotic titer in complex medium.

The site of regulation of nanaomycin biosynthesis by inorganic phosphate was studied with washed cells previously grown with high- and low-phosphate concentrations. The cells previously grown in high-phosphate conditions produced only about one-tenth as much nanaomycin A from acetate as did mycelia grown under low-phosphate conditions. On the other hand, the biocoversions of nanaomycin D to A and nanaomycin A to E were only slightly affected. Accordingly, the principal site of negative regulation of nanaomycin biosynthesis by inorganic phosphate is suggested to lie within steps between acetate and nanaomycin D, as shown in Fig. 5. Further study is needed to confirm the regulation site at the enzyme level.

The results shown in Fig. 4 suggest that the effect of phosphate on nanaomycin biosynthesis is probably of repression type, although the effect on the transport of acetate can not be ruled out at present. The

mycelial growth was the same in the two growth media with high- and low-phosphate concentrations. No appreciable difference was observed microscopically in the morphology of the mycelia. Phosphate does not inhibit the activity of enzymes involved in the conversions of nanaomycin D to A and of nanaomycin A to E[4].

In a previous paper[5], the present authors demonstrated that the addition of allophane, a known phosphate-trapping agent, to a complex medium resulted in a significant increase in nanaomycin production. They also showed that nanaomycin production in complex medium was reduced by exogenously supplied inorganic phosphate, and that the interference by phosphate disappeared when allophane was added. The elimination of the phosphate effect by allophane was also observed in the defined medium used in the present studies (data not shown). All the results described above suggest that the stimulation of nanaomycin production by allophane is due to the release of nanaomycin biosynthesis from regulation by inorganic phosphate ions.

Acknowledgments

This study was supported by a grant from the Waksman Foundation of Japan Inc. The author are grateful to NAOKO OHTA for technical assistance.

## References

1) ŌMURA, S.; H. TANAKA, Y. KOYAMA, R. ŌIWA, M. KATAGIRI, J. AWAYA, T. NAGAI & T. HATA: Nanaomycins A and B, new antibiotics produced by a strain of *Streptomyces*. J. Antibiotics 27: 363~365, 1974
2) TANAKA, H.; Y. KOYAMA, J. AWAYA, H. MARUMO, R. ŌIWA, M. KATAGIRI, T. NAGAI & S. ŌMURA: Nanaomycins, new antibiotics produced by a strain of *Streptomyces*. I. Taxonomy, isolation, characterization and biological properties. J. Antibiotics 28: 860~867, 1975
3) KITAO, C.; H. TANAKA, S. MINAMI & S. ŌMURA: Bioconversion and biosynthesis of nanaomycins using cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses. J. Antibiotics 33: 711~716, 1980
4) ŌMURA, S.; S. MINAMI & H. TANAKA: Biosynthesis of nanaomycin: Syntheses of nanaomycin E from nanaomycin A and of nanaomycin B from nanaomycin E in a cell-free system. J. Biochem. 90: 291~293, 1981
5) MASUMA, R.; Y. TANAKA, H. TANAKA & S. ŌMURA: Production of nanaomycin and other antibiotics by phosphate-depressed fermentation using phosphate-trapping agents. J. Antibiotics 39: 1557~1564, 1986