BAGOUGERAMINES A AND B, NEW NUCLEOSIDE ANTIBIOTICS PRODUCED BY A STRAIN OF *BACILLUS CIRCULANS*

I. TAXONOMY OF THE PRODUCING ORGANISM AND ISOLATION AND BIOLOGICAL PROPERTIES OF THE ANTIBIOTICS

ATSUSHI TAKAHASHI, NORIKO SAITO, KUNIMOTO HOTTA†
YOSHIRO OKAMI and HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
†National Institute of Health, Japan
2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication March 6, 1986)

A bacterial isolate from soil, designated as TB-2125 had a unique pattern of multiple resistance to aminoglycoside antibiotics (AG) and produced new nucleoside antibiotics. Taxonomic properties of this strain fell into those of *Bacillus circulans*, providing unique characteristics such as strict susceptibility to acidic pH, motility of colony as well as multiple AG-resistance. Two new antibiotics which were named bagougeramines A and B had a broad antimicrobial activity and a specific activity against the two spotted spider mite.

We reported previously that naturally occurring actinomycete strains with multiple aminoglycoside antibiotic(AG)-resistance show a high probability of antibiotic production, and that they show individual AG-resistance pattern depending on the type of the antibiotics they produce[1,2]. Based on these facts, we reasoned that bacterial strains with multiple AG-resistance would also have a high probability of antibiotic production. Actually, we isolated numbers of bacterial strains with both multiple AG-resistance and antibiotic productivity (unpublished data). One such strain, designated as TB-2125 was found to have a unique multiple AG-resistance pattern and to produce novel antibiotics which are structurally related to gougerotin[3~5] produced by *Streptomyces gougerotii*. Taxonomically, the producing strain showed some remarkable features such as strict susceptibility to pH lower than 6.9 and colony motility. The antibiotics exhibited an activity against the two spotted spider mite in addition to a broad antimicrobial activity.

This report deals with the taxonomic classification of strain TB-2125 as well as fermentation, isolation and biological properties of the antibiotics.

Materials and Methods

Isolation of Organism

Strain TB-2125 was isolated from a soil collected at Tomioka-shi, Gunma, Japan. The isolation medium consisted of one-fifth strength ISP No. 4 supplemented with yeast extract (Difco) 0.1%, agar 1.5%, kanamycin sulfate 20 μg/ml and two antifungal antibiotics, cyclohexamide and nystatin, 25 μg/ml.

Taxonomic Examination

Morphological, cultural and physiological properties of strain TB-2125 were examined according to the methods described by COWAN[6] and GORDON *et al.*[7] Detailed observation of colony and cell

morphologies was performed with a light microscope (XF-Ph-21, Nikon) and a transmission electron microscope (EM400, Philips) after strain TB-2125 was incubated on nutrient agar or brain heart infusion (Difco) agar medium at 27°C for 1 to 3 days. Susceptibility to 11 different AGs was examined according to the previously described method[1] with the modification that ISP No. 4 medium was supplemented with yeast extract (Difco) 0.1%.

Molar GC percentage of DNA was estimated by the thermal denaturation method of MARMUR and DOTY[8] by using a Beckman DU-8 spectrophotometer. DNA from strain TB-2125 was prepared according to the method described by MARMUR[9]. DNA from *Escherichia coli* B (Sigma) was used as a standard.

The major isoprenoid quinone was isolated by the method described by COLLINS *et al.*[10] and determined by mass spectrometry.

Fermentation

A slant culture of strain TB-2125 on ISP No. 4 medium supplemented with yeast extract 0.1% was inoculated into 100 ml of the medium consisting of dextrin 2.0%, soy-peptone (Bacto-Soytone, Difco) 1.0% and $CaCO_3$ 0.2% (pH 8.5 before autoclaving) and incubated at 27°C for 24 hours on a rotary shaker (180 rpm). For the antibiotic production, 0.1 ml of the culture was transferred to 100 ml of the above medium supplemented with corn-steep liquor (Nisshoku) 1.0% (pH 9.0 before autoclaving) and incubated for 72 hours in the same way as above. The antibiotic activity of the culture was assayed with *Bacillus subtilis* PCI 219 as a test organism.

Isolation of Antibiotics

Antibiotic principles in the filtrate (9,800 ml) were adsorbed on a column of Amberlite IRC-50 (70% Na^+, 500 ml). The column was washed with H_2O (4,000 ml) and developed with 1 N HCl. Active eluates were combined, neutralized with 6 N NaOH and passed through a column of a chromatography grade charcoal (200 ml). After washing the column with H_2O (1,000 ml), antibiotics were eluted with 0.05 N HCl - 50% aq acetone. Active fractions were collected and neutralized with Amberlite IRA-45 (OH^-) after acetone was removed by evaporation. Concentration under reduced pressure of the neutralized solution provided a hygroscopic powder (2.7 g). This crude powder was dissolved in H_2O (10 ml) and then chromatographed on a column of CM-Sephadex C-25 (equilibrated with 0.3 M NaCl, 500 ml) by elution with a linearly increasing concentration of NaCl (0.3 M to 1.0 M). Two active peak fractions were observed. The first peak fraction was collected and desalted by adsorbing on a column of the above active charcoal followed by elution with a linear gradient of aq acetone (0~30%) containing 0.05 N HCl. After removal of acetone by evaporation and neutralization with Amberlite IRA-45 (OH^-), the active eluate containing bagougeramine A was concentrated. The other peak fraction containing bagougeramine B was also subjected to the same procedure as above. Each antibiotic was further purified by repetition of Sephadex LH-20 column chromatography with 50% aq MeOH for elution. The eluate was passed through a column of Amberlite IRA-400 (SO_4^{2-}) and lyophilized. Thus, pure bagougeramine A sesquisulfate (24 mg) and B disulfate (42 mg) were obtained.

Biological Activities of Antibiotics

To determine the minimal inhibitory concentration (MIC) of the purified antibiotics designated as bagougeramines A and B, bacteria were incubated at 37°C for 18 hours in Mueller-Hinton agar (Difco), and yeasts and molds were incubated at 27°C for 18 hours in Nutrient Agar (Difco) supplemented with glucose 1.0%.

The acaricidal effect of bagougeramines against the two spotted spider mite (*Tetranychus urticae*) was examined by a pot test in a green house. Twenty adult mites were inoculated on leaves of kidney bean in the first stage. One day after the inoculation, they were sufficiently sprayed with the diluted antibiotic preparation. The numbers of adult mites surviving on the leaves were determined 14 days after the treatment. The test was carried out by using 3 pots for each concentration of the antibiotic samples. The prevented value (%) was calculated from the following formula: $100 \times [1 - (\text{number of the mites on treated leaves})/(\text{number of the mites on non-treated leaves})]$. Dicofol (1,1-bis(*p*-chloro-

phenyl)-2,2,2-tri-chloroethanol 40% emulsified solution) was used as a control acaricidal drug.

Results

Taxonomic Features of the Strain TB-2125

Taxonomic features of strain TB-2125 were summarized in Tables 1~3. Strain TB-2125 was an aerobic, Gram-variable (mostly negative) and motile rod with peritorichous flagella (Photo. 1). It grew singly or in pairs on agar medium, and formed endospores in sporangia that were definitely swollen. The spores were ellipsoidal to round and located at central or terminal part of the cells. The size of the cells was 0.4~0.8 by 1.7~3.8 μm.

Table 1. Morphological and cultural characteristics of strain TB-2125.

Vegetative cells	Rods, 0.4~0.8×1.7~3.8 μm, with rounded ends, occurring singly or in pairs Gram-variable (mostly negative)
Spores	Ellipsoidal to round (shape); central to terminal (location)
Sporangia	Swollen
Flagella	Peritrichous
Motility	Motile
Nutrient agar (pH 7.5)	Abundant growth, swarming, opaque, viscous appearance, creamy and no soluble pigment
Brain heart infusion agar (pH 7.5)	Abundant growth, swarming, opaque, viscous appearance, creamy and no soluble pigment
Nutrient broth (pH 7.5)	Slight to moderate growth, turbid
Brain heart infusion broth (pH 7.5)	Moderate to abundant growth, turbid
Growth temperature (nutrient agar)	18~35°C (optimum 23~29°C)
Growth pH (nutrient agar)	pH 6.9~8.9 (optimum 7.5~8.0)

Table 2. Physiological properties of strain TB-2125.

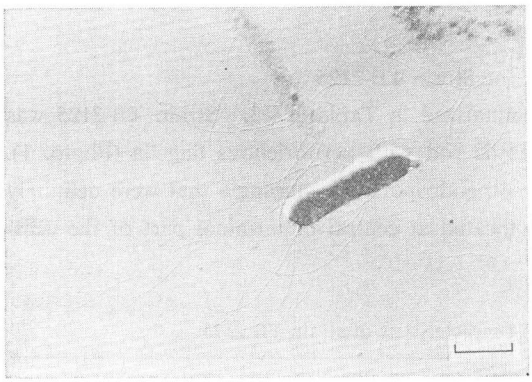
Nitrate reduction	Negative
Gelatin liquefaction	Positive
Indole production	Negative
Starch hydrolysis	Positive
Citrate utilization	Negative
Urease reaction	Negative
Catalase reaction	Positive
Oxydase reaction	Positive
Gas from carbohydrates	Negative
V-P reaction	Negative
(pH in V-P broth)	(>7.5)
NaCl tolerance	0~1%
Susceptibility to 0.001% lysozyme	Resistant
Decomposition of	
casein	Negative
tyrosine	Negative

Table 3. Acid production from carbohydrates.

L-Arabinose	−
D-Xylose	−
L-Rhamnose	−
D-Fructose	−
D-Galactose	−
D-Glucose	+
D-Mannose	−
Cellobiose	+
Lactose	−
Maltose	+
Sucrose	−
Trehalose	−
D-Raffinose	−
Soluble starch	+
Salicin	+
Glycerol	+
D-Mannitol	−
Inositol	−

Medium: Peptone 0.2%, NaCl 0.5%, K_2HPO_4 0.03%, B.T.B. 0.003%, carbohydrate 1.0% and agar 0.3%; pH 8.0 (before autoclaving).

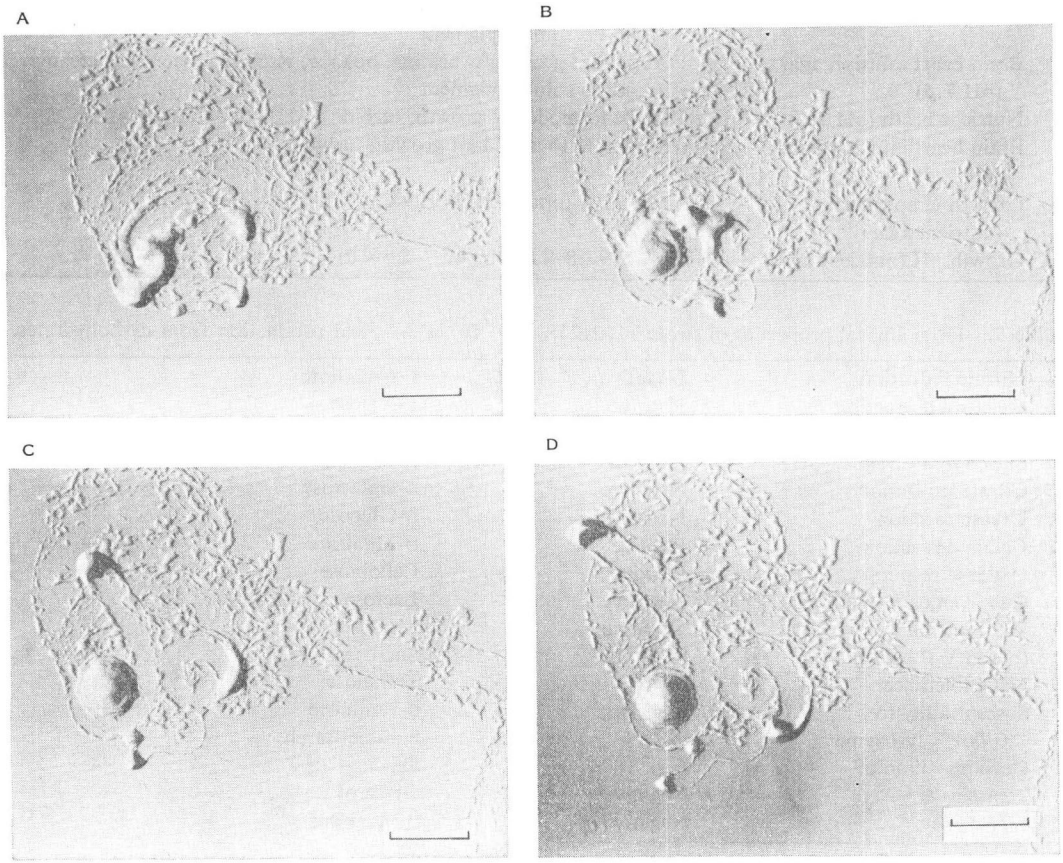
Photo. 1. Morphology of strain TB-2125.
A size mark equals 1 μm.



Strain TB-2125 swarmed on the agar surface. On nutrient agar, motile small transparent colonies with irregular size and shape (mostly bullet shape, ϕ <0.2 mm) were generated from the inoculum. They traveled on the agar plate, leaving a loop-shaped track and forming whirl-shaped colonies (viscous in appearance and irregular in size and shape) as shown in Photo. 2. Based on the photographs which were taken continually, the traveling rate was estimated about 12~36 μm/minutes. The colonies traveled until cells covered the entire surface of the agar medium.

The GC-content of the DNA of the strain TB-2125 was determined to be 50 mol %. The major isoprenoid quinone was unsaturated menaquinone with seven isoprenoid units.

Photo. 2. Swarming of strain TB-2125 on nutrient agar plate.



After strain TB-2125 was incubated at 27°C for 24 hours, photographs were taken at 5 minutes interval in order of A to D.
A size mark equals 0.1 mm.

Strain TB-2125 was positive in catalase and starch hydrolysis and negative in the production of indole, acetoin and citrate utilization. Gas was not formed from carbohydrates. Acid was not detected from any one of the carbohydrates in Hugh-Leifson medium whose pH was adjusted to 7.1 before autoclaving. However, acid production from glucose, cellobiose, maltose, starch, salicin and glycerol was detected when the pH of the medium was adjusted to 8.0 before autoclaving. Carbohydrate metabolism was oxidative in the Hugh-Leifson test. The pH of Voges-Proskauer (V-P) broth was 7.4, 8.0 and 8.7 after 2, 3 and 5-day incubation, respectively. The strain grew in the temperature range of 18~35°C with the optimum being 21~27°C. It also grew at pH 6.9~8.9 on nutrient agar media.

In addition, strain TB-2125 was resistant to 50 μg/ml of each of the following seven AGs: streptomycin, kanamycin, dibekacin, ribostamycin, paromomycin, lividomycin and neamine, and sensitive to istamycin B, gentamicins C complex, butirosin and neomycin B.

The above taxonomic features of strain TB-2125 were thought to fall into those of the genus *Bacillus* with reference to the descriptions of 8th BERGEY'S Manual of Determinative Bacteriology[11], COWAN[6] and GORDON *et al.*[7]. *Bacillus circulans* seemed to be the closest to the strain, given the difference in acid production and pH change of the V-P broth. Strain TB-2125 did not produce acid from arabinose, xylose and mannitol, and the pH of the V-P broth was 7.4~8.7, while *B. circulans* has been known to produce acid from these sugars and pH 4.5~6.6 in the V-P broth. The most remarkable characteristics of strain TB-2125 was the permissive pH range for growth. A pH range lower than 6.9 did not allow the strain to grow.

However, these differences were considered to be insufficient to make a new species or a new subspecies. Therefore, strain TB-2125 was classified and designated as *B. circulans* TB-2125. Strain TB-2125 was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with accession number of FERM P-8518.

Fermentation

A typical time course of bagougeramines production by strain TB-2125 was shown in Fig. 1. The organism reached the stationary phase of growth after a 2-day incubation. Bagougeramine production began after 24 hours, and the maximum accumulation was observed after a 3-day incubation period. The pH of the medium during incubation went down first to 6.7 and then began to rise gradually to 7.2 at the maximum accumulation.

Biological Properties

The MIC of bagougeramines against various

Fig. 1. Time course of the production of bagougeramines.

* Potency of bagougeramines was estimated as bagougeramine B sulfate.

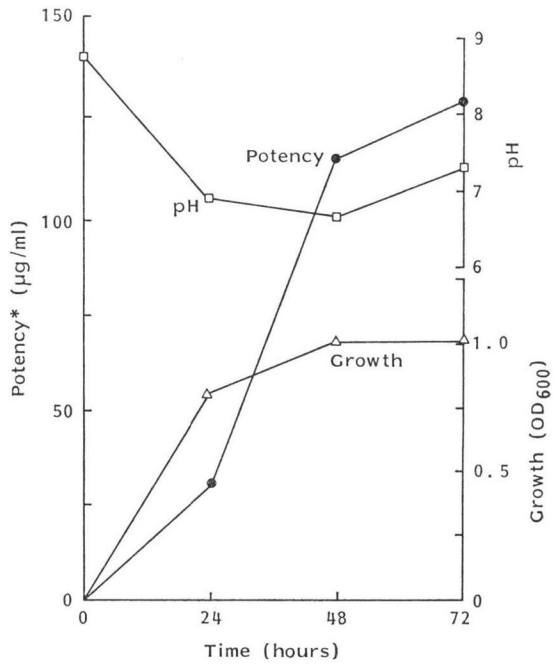


Table 4. Antimicrobial spectra of bagougeramines A and B.

Test organisms	MIC (μg/ml)	
	Bagougeramine A	Bagougeramine B
Staphylococcus aureus FDA 209P	100	50
S. aureus Smith	100	12.5
Micrococcus luteus IFO 3333	25	6.25
Bacillus subtilis PCI 219	50	12.5
Escherichia coli NIHJ	12.5	3.12
E. coli K-12	12.5	6.25
E. coli ML 1629	25	12.5
Salmonella typhi T-63	6.25	3.12
Proteus vulgaris OX19	25	6.25
Serratia marcescens	100	50
Pseudomonas aeruginosa A3	50	100
Klebsiella pneumoniae PCI 602	100	12.5
Mycobacterium smegmatis ATCC 607*	50	12.5
Candida albicans 3147	100	6.25
Saccharomyces cerevisiae F-7	50	6.25
Cryptococcus neoformans F-10	25	6.25
Helminthosporium oryzae	6.25	3.12
Pellicularia filamentosa f. sp. *sasakii*	12.5	6.25
Aspergillus niger	100	50
Trichophyton asteroides 429	100	12.5

* 37°C, 40 hours.

Table 5. Acaricidal effect of bagougeramines in pot test.

Concentration (ppm)	Preventive value (%)*		
	Bagougeramine A	Bagougeramine B	Dicofol**
100	—	—	99.8
30	100	100	63.1
10	99.6	69.2	—
3	99.1	43.1	—

* Average of triplicates.

** Dicofol, (an acaricide: 1,1-bis(*p*-chlorophenyl)-2,2,2-tri-chloroethanol) 40% emulsified solution.

organisms were shown in Table 4. Both bagougeramines A and B inhibited the growth of Gram-positive and Gram-negative bacteria and some fungi. Bagougeramine B showed higher activity than bagougeramine A except against *Pseudomonas aeruginosa* A3.

The activity of bagougeramines against the two spotted spider mite on kidney bean leaves was shown in Table 5. Bagougeramines exhibited higher preventive value against the mite than Dicofol. Bagougeramine A was more active against the mites than bagougeramine B.

Oral administration of bagougeramines A and B at a dose of 100 mg/kg caused no death of mice.

Discussion

As reported previously, we established a new antibiotic screening program consisting of a directed isolation, characterization and screening of actinomycete strains with multiple AG-resistance and/or unique AG-resistance pattern. This approach provided the discovery of dopsisamine from a new subspecies of *Nocardiopsis mutabilis*[12]. In this report, this approach was successfully extended to bacteria, resulting in the discovery of new nucleoside antibiotics (bagougeramines A and B). The

Table 6. Resistance to aminoglycoside antibiotics of strain TB-2125 and a gougerotin producing *Streptomyces* sp. SS-1946.

Strain	Resistance* to 50 μg/ml of										
	IS	SM	KM	DK	GM	RM	BT	NM	PR	LV	NE
Streptomyces sp. SS-1946	●	○	●	●		●	○		●	●	●
TB-2125		●	●	●		●			●	●	●

* Istamycin B sulfate (IS), streptomycin sulfate (SM), kanamycin sulfate (KM), dibekacin (DK), gentamicins C complex sulfate (GM), ribostamycin (RM), butirosin A sulfate (BT), neomycin B sulfate (NM), paromomycin (PR), lividomycin A sulfate (LV) and neamine (NE) were used for resistance test.

** The solid and open circles refer to resistant and weakly resistant, respectively. No indication refers to sensitive.

pattern of multiple AG-resistance of strain TB-2125 was unique in comparison with those previously observed. It was of interest to determine whether a gougerotin producing strain of *Streptomyces* has a resistance pattern similar to that of strain TB-2125. As shown in Table 6, there was a good similarity between two organisms in their AG-resistance patterns. A definite difference was observed in resistance to istamycin B. Our hypothesis that different resistance patterns generally represent the production of different antibiotics was thus supported.

According to the structures which will be described in the accompanying report[13], bagougeramines belong to the group of cytosine nucleoside antibiotics and are closely related to gougerotin. All the antibiotics in this group are produced by streptomycetes except oxamicetin which is produced by an *Arthrobacter* species[14]. Thus, bagougeramines are the first case of this antibiotic group as the product of *Bacillus*. Acaricidal activity was reported for the following two antibiotics in this antibiotics group, moroyamycin[15] and aspiculamycin[16]. However, these antibiotics were found to be identical with gougerotin[14]. Therefore it seems likely that the acaricidal activity of bagougeramines and gougerotin is due to their common structure.

From a taxonomic view point, strain TB-2125 showed a notable property that its colonies traveled counterclockwise or clockwise on agar plate. According to HENRICHSEN[17], this phenomenon of "swarming" was observed in certain species of *Bacillus* such as *B. alvei* and *B. circulans*. In the point of "swarming", strain TB-2125 had very similar mode of translocation as described for *B. alvei*. Difference between them seems to lay in the fact that while colonies of *B. alvei* translocate always counterclockwise, those of the strain TB-2125 traveled not only counterclockwise but also clockwise. The most striking property was the strict susceptibility to acidic pH of strain TB-2125. Examination of the pH range permitting growth on nutrient agar revealed that the strain was able to grow in the pH range of 6.9~8.9 (optimum pH was 7.5).

As described so far, strain TB-2125 belonging to *B. circulans* was unique in antibiotic resistance, antibiotic production, susceptibility to acidic pH and colony motility. These parameters might be useful in obtaining other unique antibiotic-producing bacteria.

Physico-chemical properties and structure determination of bagougeramines A and B will be described in the accompanying paper[13].

References

1) HOTTA, K.; A. TAKAHASHI, N. SAITO, Y. OKAMI & H. UMEZAWA: Multiple resistance to aminoglycoside antibiotics in actinomycetes. J. Antibiotics 36: 1748~1754, 1983

2) HOTTA, K.; A. TAKAHASHI, Y. OKAMI & H. UMEZAWA: Relationship between antibiotic resistance and antibiotic productivity in actinomycetes which produce aminoglycoside antibiotics. J. Antibiotics 36: 1789~1791, 1983

3) KANZAKI, T.; E. HIGASHIDE, H. YAMAMOTO, M. SHIBATA, K. NAKAZAWA, H. IWASAKI, T. TAKEWAKA & A. MIYAKE: Gougerotin, a new antibacterial antibiotic. J. Antibiotics, Ser. A 15: 93~97, 1962

4) FOX, J. J.; Y. KUWADA, K. A. WATANABE, T. UEDA & E. B. WHIPPLE: Nucleosides. XXV. Chemistry of gougerotin. Antimicrob. Agents Chemother. -1964: 518~529, 1965

5) Fox, J. J.; Y. Kuwada & K. A. Watanabe: Nucleosides. LVI. On the structure of the nucleoside antibiotic, gougerotin. Tetrahedron Lett. 1968: 6029~6032, 1968
6) Cowan, S. T. (*Ed.*): Manual for the identification of medical bacteria. Cambridge University Press, Cambridge, 1974
7) Gordon, R. E.; W. C. Haynes & H. C. N. Pang (*Ed.*): The genus *Bacillus*. Handbook No. 427. U.S. Department of Agriculture, Washington D. C., 1973
8) Marmur, J. & P. Doty: Determination of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5: 109~118, 1962
9) Marmur, J.: A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208~218, 1961
10) Collins, M. D.; T. Pirouz, M. Goodfellow & D. E. Minnikin: Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100: 221~230, 1977
11) Buchanan, R. E. & N. E. Gibbons: Bergey's Manual of Determinative Bacteriology. 8th Ed., Williams & Wilkins Co., Baltimore, 1974
12) Takahashi, A.; K. Hotta, N. Saito, M. Morioka, Y. Okami & H. Umezawa: Production of novel antibiotic, dopsisamine, by a new subspecies of *Nocardiopsis mutabilis* with multiple antibiotic resistance. J. Antibiotics 39: 175~183, 1986
13) Takahashi, A.; D. Ikeda, H. Naganawa, Y. Okami & H. Umezawa: Bagougeramines A and B, new nucleoside antibiotics produced by a strain of *Bacillus circulans*. II. Physico-chemical properties and structure determination. J. Antibiotics 39: 1041~1046, 1986
14) Suhadolnik, R. J.: Naturally occurring nucleoside and nucleotide antibiotics. Prog. Nucleic Acid Res. Mol. Biol. 22: 193~200, 1979
15) Sakagami, Y.; R. L. Chang, K. Watanabe, S. Ichikawa & Y. S. Wang: Studies on moroyamycin. Abstracts of Papers 4th Internat. Ferm. Symposium, p. 212, Kyoto, 1972
16) Haneishi, T.; M. Arai, N. Kitano & S. Yamamoto: Aspiculamycin, a new cytosine nucleoside antibiotic. III. Biological activities, *in vitro* and *in vivo*. J. Antibiotics 27: 339~342, 1974
17) Henrichsen, J.: Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. 36: 478~503, 1972