PENITRICIN, A NEW CLASS OF ANTIBIOTIC PRODUCED BY *PENICILLIUM ACULEATUM*

I. TAXONOMY OF THE PRODUCER STRAIN AND FERMENTATION

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A novel antibiotic, penitricin (Ro 09-0804) was discovered in the culture filtrate of a fungal strain NR 5165. Taxonomic studies of the producing organism resulted in its assignment to *Penicillium aculeatum*. Further examination on penitricin production by other strains of this species and related taxa revealed that penitricin was produced by several other strains of *P. aculeatum*, but not by any available strains of the closely similar species, *P. verruculosum*. It was also found that copper ion was essential for production of penitricin.

The antibiotic containing a cyclopropenone ring in the molecule has been hitherto unknown as a microbial product, while several mono- or di-substituted cyclopropenones have been reported from plants[1] and chemically synthesized[2]. In our antibiotic screening from filamentous fungi using *Pseudomonas aeruginosa* as a test organism, we discovered a new class of antibiotic (Ro 09-0804) containing this skeleton, which we named penitricin. The structural characteristics of penitricin will be given in the subsequent papers[3,4]. The producing organism was identified as *Penicillium aculeatum* NR 5165. The genus *Penicillium* is well known to produce various types of antibiotics or mycotoxins[5,6]. However only a few reports have appeared on the metabolites of *P. aculeatum*[7,8], probably because this species has not been so commonly encountered[9]. This report deals with the taxonomy of the producing organism and production condition for this antibiotic.

Materials and Methods

Taxonomic Studies

The taxonomic studies of a fungal isolate NR 5165 producing penitricin were carried out by the methods described in RAPER *et al.*[10] and PITT[9].

Color code numbers used in the description of cultural characteristics were those of the Munsell System[11].

For scanning electron microscopic study, a portion of well sporulating culture was cut off from the agar plate, fixed in 4% OsO_4 solution for 1 hour, dehydrated in graded ethanol series (15 minutes each), followed by rinsing in absolute *iso*-amyl acetate, and critical-point-dried in a critical point drying apparatus (Hitachi Koki Co., HCP-2) using liquid CO_2 as the transitional solvent. The material was then coated with gold-palladium, and examined with a Mini SEM-101 (Hitachi-Akashi) at 30 KV.

Fermentation

Conidial mass from a well sporulating malt extract agar slant of NR 5165 was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of various media. Fermentation was conducted on a rotary

shaker operating at 180 rpm.

Bioassay

Penitricin content in broths was determined by paper disc assay using *P. aeruginosa* A3 as the test organism. Typically in this assay, 100 or 50 μg/ml of penitricin solution absorbed in a 8-mm paper disc produced a 21- or 16-mm inhibition zone, respectively.

Strains Examined

Strain NR 5165 was isolated from soil near Mt. Ozeki, Tsunagi-machi, Ashikita-gun, Kumamoto Prefecture, Japan, in 1971. NR 5166 was isolated from soil in Odawara, Kanagawa Prefecture, Japan in 1981. Other strains were purchased from IFO, ATCC, CMI or CBS.

Results and Discussion

Taxonomic Studies

Cultural Characteristics

On Czapek Solution Agar: Colonies grow rather slowly attaining 11~15 mm in diameter after 7 days, 16~22 mm after 10 days and 30~35 mm after 14 days at 25°C, and appear plane and umbonate with texture velutinous to floccose. Central area is compact to floccose. Conidiogenesis is sparse and dense yellow mycelium is prominent to show orange (7.5YR7/12). If conidiation is moderate, mycelium mixed with conidial mass appears yellowish to greenish (5Y7/8~10Y7/6). The surrounding area is yellow (5Y8/10) predominantly composed of patches of bright yellow mycelium. No exudate is observed. The reverse color is orange to yellow (7.5YR7/12~5Y8/8). Pigment in agar is lacking or if present: bright yellow to orange.

On Malt Extract Agar: Colonies grow rapidly attaining a diameter of 31~37 mm after 7 days, 46~51 mm after 10 days and 51~60 mm after 14 days, with the surface velutinous in appearance. Conidiogenesis is heavy. Central area is grayish olive (10Y5/2~2.5GY6/4). Marginal area is composed of bright yellow mycelium (7.5Y9/8~10Y8/12). No exudate is formed. Reverse is pale orange to yellowish (10YR8/8~2.5Y8/6) with pinkish spot (2.5YR7/6).

On CYA: Colonies grow densely and rapidly as on malt extract agar, attaining 26~31 mm in diameter after 7 days. Surface is velutinous to subfloccose. Conidiogenesis is heavy and bluish to yellow-greenish (2.5G6/2~2.5GY7/6). Conidial area is surrounded by a bright yellow mycelial area (7.5Y9/8~10Y8/12), often mixed with orange or pinkish hyphae. No exudate. Reverse is red brown (5R4/10).

On CYA at 37°C: Colonies grow very slowly reaching 5~7 mm in diameter after 7 days.

On CYA at 5°C: Conidia do not germinate within 7 days.

On G25N: Very slight growth is observed; 1~4 mm in diameter after 7 days.

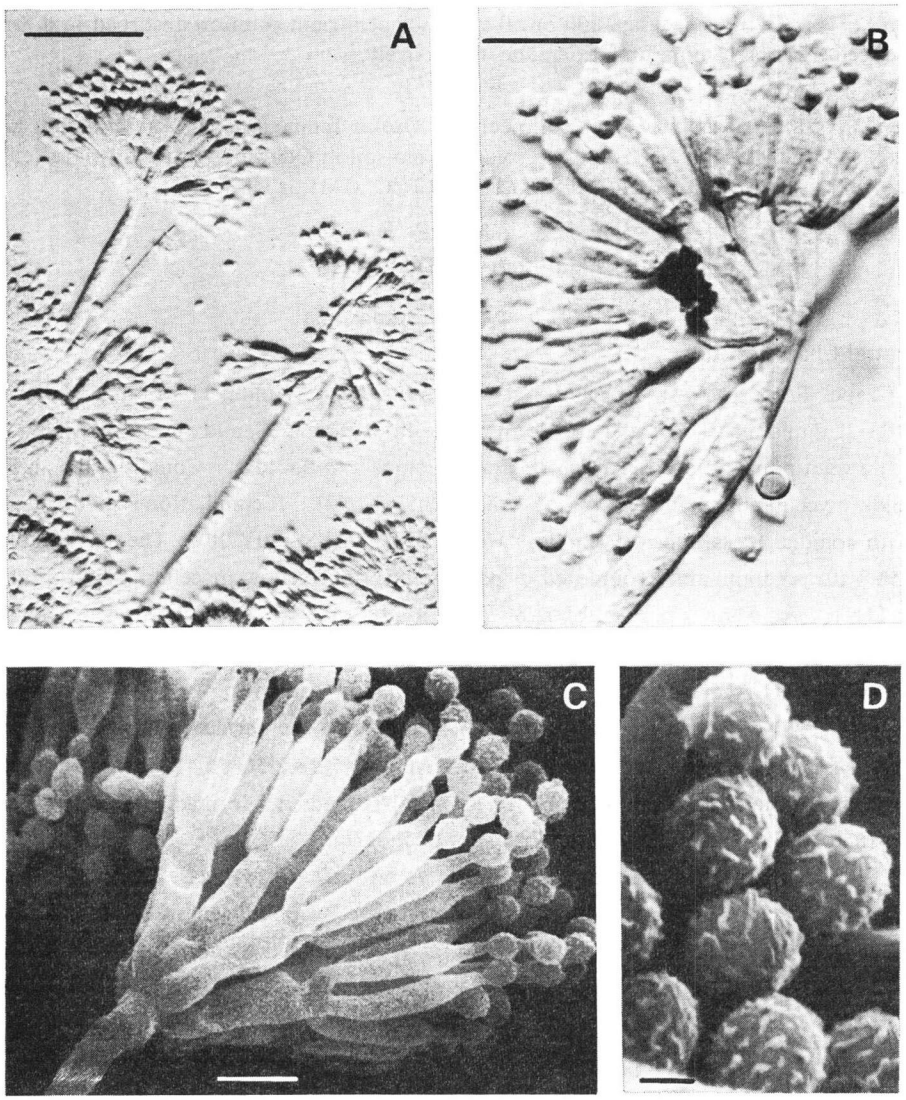
Morphological Characteristics

Conidiophores are borne on a basal felt or directly from the agar, simple or rarely branched, 200~400×3.2~4.3 μm, with a heavy wall. Penicilli are typically biverticillate and symmetrical, comparatively divergent, consisting of a terminal verticil of 4~9 thick metulae, 7.0~12.6×2.5~4.5 μm. Each metula bears 2~6 terminal phialides. Phialides are somewhat thick and ampulliform-acerose, 6.0~11.1×2.0~3.7 μm. Conidia borne in chains, are subglobose to globose, 2.0~3.3 μm in diameter with verrucose to finely roughened walls (Fig. 1).

Identification and Classification

This strain conformed to the general characteristics of the genus *Penicillium*[12,18]. It was readily

Fig. 1. *Penicillium aculeatum* NR 5165.
A: Penicilli, Nomarsky, (bar 25 μm), B: ampulliform-acerose phialides, Nomarsky, (bar 10 μm), C: penicillus, SEM, (bar 5 μm), D: conidia, SEM, (bar 1 μm).



assignable into the Sect. *Simplicium* in the Subgenus *Biverticillium*[6], *i.e.* the Section *Biverticillata-Symmetrica* according to RAPER *et al.*[10], because the penicilli were typically biverticillate and symmetrical with acerose phialides, and because of its growth properties on G25N or at 5°C.

Among these sections, strain NR 5165 was closely related to *Penicillium pinophilum, Penicillium verruculosum* or *Penicillium aculeatum*. Comparison of NR 5165 with these related taxa is shown in Table 1. *Penicillium funiculosum* sensu RAPER *et al.*[10] is now further divided into 3 species, namely *Penicillium minioluteum, P. pinophilum* and *P. funiculosum*[6]. All of these 3 species form long-tapered acerose phialides bearing generally ellipsoidal conidia. Except for *P. minioluteum*, in addition, they grow well at 37°C. *P. verruculosum* produces sulfur yellow mycelium and also grows well at 37°C. On the other hand, *P. aculeatum* forms less yellowish mycelium on CYA and produces verrucose to spinulose conidia.

Table 1. Comparison of NR 5165 with its related taxa according to PITT[9].

	NR 5165	*P. aculeatum*	*P. verruculosum*	*P. funiculosum*	*P. pinophilum*	*P. minioluteum*
Color of mycelium on CYA	Bright yellow ~ pinkish	White ~ yellow (less conspicuous)	White ~ sulfur yellow	White ~ peach	White ~ sulfur yellow	White ~ bright yellow
Colony at 37°C on CYA (7 days, mm in diameter)	5 ~ 7	2 ~ 10	20 ~ 40	30 ~ 45	20 ~ 40	5 ~ 15
Stipes (μm in length)	200 ~ 400	150 ~ 300	150 ~ 250	25 ~ 60	150 ~ 180	150 ~ 200
Phialides	Ampulliform-acerose	Ampulliform-acerose	Ampulliform-acerose	Acerose	Acerose	Acerose
Conidia	Subspherical ~ spherical	Spherical	Spherical ~ subspherical	Cylindrical ~ ellipsoidal	Subspherical ~ ellipsoidal	Narrow ellipsoidal
	Verrucose ~ finely roughened	Verrucose ~ spinulose	Verrucose		Smooth ~ finely roughened	Smooth

On the basis of these growth rates and several microscopic characteristics, NR 5165 resembled *P. verruculosum* or *P. aculeatum* rather than *P. pinophilum*. Although *P. aculeatum* is treated as a synonym of *P. verruculosum* by STOLK and SAMSON[14] we concluded that NR 5165 should belong to *Penicillium aculeatum* Raper and Fennell, primarily based on growth rate at 37°C. We compared these 2 closely related species on the production of penitricin in several kinds of media. Interestingly none of the strains of *P. verruculosum* examined: NR 5166, IFO 5724, IFO 6020, CBS 388.48, CBS 254.56, ATCC 26148 and ATCC 24640, showed any productivity of penitricin. In contrast *P. aculeatum* IFO 5689 and IMI 133243 produced about 100 mg/liter of penitricin in the culture broth. *P. verruculosum* and *P. aculeatum* may be differentiated from each other on the basis of their physiological characteristics, which will be discussed elsewhere.

Fermentation and Production Conditions

Time Course

When fermentation was conducted using the original AS medium consisting of glucose 2.0%, S-3 Meat (Ajinomoto Co.) 1.0%, meat extract (Wako Chemicals) 0.3%, NaCl 0.5% and $CaCO_3$ 1.0% in tap water (pH unadjusted), the penitricin production after 48 ~ 72 hours reached maximum (160 mg/liter). In order to obtain better productivity, medium improvement and production condition studies were carried out as follows.

Nitrogen Sources

Effect of various nitrogen sources was examined. Each component was added to the basal medium ranging in concentration of 0.5 ~ 3%. The basal medium contained glucose 2%, meat extract 0.3%, NaCl 0.5% and $CaCO_3$ 1%. Polypeptone and most materials derived from soybean were effective, whereas inorganic nitrogen or Casamino Acids were less so.

Carbon Sources

The kind of carbon sources to be used was found more important. As shown in Table 2, any carbohydrates tested other than glucose and sucrose showed no productivity. This finding suggests glucose may play important roles in the biosynthesis of penitricin.

When the concentration of glucose increased, maximum production rate was not so affected, but

Table 2. Effect of carbon sources.

C source	%	Penitricin (mg/liter)
Glucose	2	400
	4	210
	6	310
	8	270
	10	350
Sucrose	2	270
Maltose	2	nd
Melibiose	2	nd
Raffinose	2	nd
Glycerol	2	nd

nd: Not detected.

Basal medium contained: Polypeptone 1.0%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CoCl_2 \cdot 6H_2O$ 1 mg/liter, $FeSO_4 \cdot 7H_2O$ 1 mg/liter, $CuSO_4 \cdot 5H_2O$ 7 mg/liter, $MnCl_2 \cdot 4H_2O$ 8 mg/liter and $ZnSO_4 \cdot 7H_2O$ 2 mg/liter in 0.05 M phosphate buffer (pH 5.7).

Table 3. Effect of divalent metals.

Metal	mg/liter	Penitricin (mg/liter)
Metal mixture		230
$MnCl_2 \cdot 4H_2O$	8	40
$CuSO_4 \cdot 5H_2O$	7	600
$ZnSO_4 \cdot 7H_2O$	2	35
$FeSO_4 \cdot 7H_2O$	1	35
$CoCl_2 \cdot 6H_2O$	1	40
No metal		35

Metal mixture contained: $CoCl_2 \cdot 6H_2O$ 1 mg/liter, $FeSO_4 \cdot 7H_2O$ 1 mg/liter, $CuSO_4 \cdot 5H_2O$ 7 mg/liter, $MnCl_2 \cdot 4H_2O$ 8 mg/liter and $ZnSO_4 \cdot 7H_2O$ 2 mg/liter.

Basal medium consisted of glucose 2%, Polypeptone 1% and $MgSO_4 \cdot 7H_2O$ 0.1% in 0.05 M phosphate buffer (pH 5.7).

the time to reach production peak was apparently delayed. Moreover, in the case of high concentration of glucose, the decrease of the antibiotic once produced was rather less distinct than at lower glucose concentration.

Effect of Metals

Among various divalent metals, copper sulfate was essential for production. Other metals such as cobalt, iron, zinc or manganese had no effect (Table 3).

The concentration of copper ion did not have a dramatic effect. Concentrations in the range of 0.005 mM to 0.5 mM were sufficient.

Initial pH and Temperature

Initial pH was adjusted with 0.05 M phosphate buffer. Initial pH greatly affected the production, although the broth pH decreased during production regardless of its initial pH. Optimum pH for production was found to be between 4.2 and 6.3.

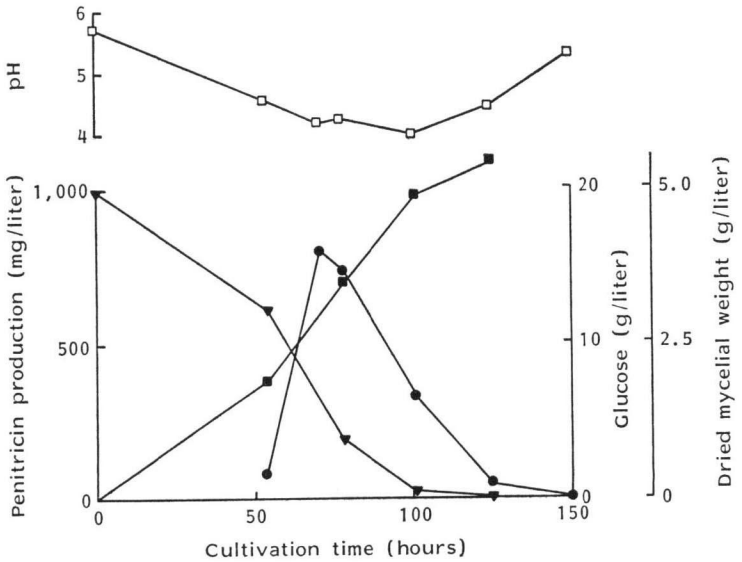
Production of penitricin using AS medium was tested at 24, 27 or 30°C. Among them, fermentation at 24°C was the most suitable for production. Effect of pH or temperature was considered to be partly due to stability of penitricin, that will be discussed in the subsequent paper.

Penitricin Production using PEP Medium under the Suitable Condition

As the results of these findings, productivity of penitricin was improved. Based on them, the better medium (PEP medium) was selected for production. The PEP medium contained glucose 2%, Polypeptone 1%, $MgSO_4 \cdot 7H_2O$ 0.1% and $CuSO_4 \cdot 5H_2O$ 0.01 mM in 0.05 M phosphate buffer (pH 5.7). Fermentation was conducted on a rotary shaker with agitation of 180 rpm at 24°C.

Typical time course of production is shown in Fig. 2. Penitricin production began after 50-hour fermentation and showed a rapid increase until 70 hours. Maximum production rate was about 800 mg/liter, which was 5 times higher than that in the original medium. During this period, glucose in the medium was consumed very rapidly and the mycelial growth rate remained maximal.

Fig. 2. Time course of penitricin production using PEP medium at 24°C.
●: Penitricin production, ▼: glucose concentration, ■: dried mycelial weight, □: pH.



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References

1) BOHLMANN, F.; J. JAKUPOVIC, L. MUELLER & A. SCHUSTER: Naturally occurring cyclopropenone derivatives. Angew. Chem., Int. Ed. Engl. 20: 292~293, 1981
2) BRESLOW, R. & L. J. ALTMAN: Methylcyclopropenone and related compounds. J. Am. Chem. Soc. 88: 504~509, 1966
3) OKUDA, T.; K. YOKOSE, T. FURUMAI & H. B. MARUYAMA: Penitricin, a new class of antibiotic produced by *Penicillium aculeatum*. II. Isolation and characterization. J. Antibiotics 37: 718~722, 1984
4) OKUDA, T.; N. SHIMMA & T. FURUMAI: Penitricin, a new class of antibiotic produced by *Penicillium aculeatum*. III. Structural confirmation by chemical synthesis and biological activity. J. Antibiotics 37: 723~727, 1984
5) TURNER, W. B.: Fungal Metabolites. Academic Press, London, 1971
6) TURNER, W. B. & D. C. ALDRIDGE: Fungal Metabolites. II. Academic Press, London, 1983
7) Centraalbureau voor Schimmelcultures, BAARN (*Ed.*): List of Cultures. p. 181, 1972
8) Commonwealth Mycological Institute, KEW (*Ed.*): Catalogue of the Culture Collection of the CMI. p. 180, 1975
9) PITT, J. I.: The Genus *Penicillium*, and its Teleomorphic States *Eupenicillium* and *Talaromyces*. pp. 1~634, Academic Press, London, 1979
10) RAPER, K. B.; C. THOM & D. I. FENNELL: A Manual of Penicillia. pp. 1~875, The Williams and Wilkins Co., Baltimore, 1949
11) Sogo Bijutsu Kenkyusho, *Ed.*: Munsell System. Color Chart Book Color. Kusakabe Gazai Co., 1977
12) ARX, J. A. VON: The Genera of Fungi Sporulating in Pure Culture. p. 196, J. Cramer, Vaduz, 1974
13) CARMICHAEL, J. W.; W. B. KENDRICK, I. L. CONNERS & L. SIGLER: Genera of Hyphomycetes. p. 142, Univ. of Alberta Press, Edmonton, 1980
14) STOLK, A. C. & R. A. SAMSON: The ascomycete genus *Eupenicillium* and related *Penicillium* anamorphs. Stud. in Mycol., CBS. 23: 1~149, 1983