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Biphenomycin A Production by a Mixed Culture

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Production of biphenomycin A by *Streptomyces griseorubiginosus* 43708 was stimulated by a mixed culture with a partner strain, *Pseudomonas maltophilia* 1928. This stimulatory effect on biphenomycin A accumulation by the mixed culture was caused by the enzyme activity which strain 1928 possessed. It is suggested that in a mixed culture strain 43708 produces a precursor of biphenomycin A in culture broth and that strain 1928 converts the precursor to biphenomycin A.

Biphenomycin A is an antibiotic which was isolated from culture broth of *Streptomyces griseorubiginosus* 43708 in our research laboratories (3, 9, 10). The antibiotic is a cyclic peptide containing a biphenyl moiety included in a 15-member ring (Fig. 1). We found that the production of biphenomycin A by strain 43708 was stimulated by a mixed culture with one strain of bacteria which was isolated from soil samples. In this paper, we describe the taxonomy of the bacterial strain 1928, which was used as a partner strain in the mixed culture, and the mixed culture, the optimum condition for biphenomycin A production. Further, we discuss the role of each microorganism in the mixed culture.

MATERIALS AND METHODS

Microorganisms. *S. griseorubiginosus* 43708 (3) and *Pseudomonas maltophilia* 1928 isolated from soil samples were used for the studies of the mixed culture. The former strain was maintained on yeast extract-malt extract agar slants (ISP2), and the latter strain was maintained on nutrient agar slants.

Taxonomic studies of strain 1928. Taxonomic studies were principally performed according to the routine methods recommended by the Society of American Bacteriologists (5).

Pure culture of strain 43708. A loopful of strain 43708 from a mature slant was inoculated into a 200-ml Erlenmeyer flask containing 50 ml of seed medium I (corn starch [1%], glycerol [1%], glucose [0.5%], Pharmamedia [1%], dried yeast [1%], corn steep liquor [0.5%], $CaCO_3$ [0.2%], pH 6.5) and incubated at 30°C for 3 days on a rotary shaker (250 rpm, 5-cm stroke), which usually provided a 20% packed mycelial volume (PMV) (600 × *g*, 10 min). One milliliter of this seed culture was inoculated into a 200-ml flask containing 50 ml of fermentation medium (soluble starch [4%], Pharmamedia [0.5%], dried yeast [0.5%], gluten meal [1%], $MgSO_4 \cdot 7H_2O$ [0.1%], KH_2PO_4 [2%], $Na_2HPO_4 \cdot 12H_2O$ [1.5%], pH 6.4) and incubated at 30°C on a rotary shaker for 5 days.

Pure culture of strain 1928. A loopful of strain 1928 from a mature slant was inoculated into a 200-ml flask containing 50 ml of seed medium II (nutrient broth [2%], pH 7.0) and incubated at 30°C for 24 h on a rotary shaker, which usually provided CFU of about 10^{10}/ml. One milliliter of this seed culture was inoculated into the fermentation medium and incubated under the same conditions as for strain 43708 described above.

Method of mixed culture. Before the mixed culture was prepared, the seed cultures of strain 43708 and its partner, strain 1928, were prepared independently under the same conditions as for pure cultures. The mixed culture was carried out at 30°C by inoculating a seed culture of strain 1928, the partner strain, into the culture broth of strain 43708 as follows. The seed culture (1 ml) of strain 43708 was inoculated into a 200-ml flask containing 50 ml of fermentation medium and incubated at 30°C on the rotary shaker. Then, the seed culture of strain 1928, which had been incubated for 24 h, was added to the culture of strain 43708, which had been incubated, and the resulting mixed culture was incubated for a few days under the same conditions. Various inoculation volumes and times for the introduction of the partner strain into the culture of strain 43708 were tested. In most cases, one milliliter of the seed culture of strain 1928 was added to the culture of strain 43708 which had been incubated for 48 or 72 h. The cultivation time of the mixed culture was determined as hours from the moment when the seed culture of strain 43708 was inoculated into the fermentation medium.

Analysis of culture broth. A 10-ml volume of culture broth was centrifuged at 600 × *g* for 10 min. The supernatant was used for the determination of biphenomycin A by high-performance liquid chromatography (HPLC) (3). The growth of strain 43708 was determined as PMV by centrifugation as mentioned above. The effect of the mixed culture on the PMV value of strain 43708 could be negligible because of its small mass of strain 1928. The growth of strain 1928 was determined as CFU of culture broth by dilution and spreading on nutrient agar.

Preparation of mycelial suspension and cell extract from *P.*

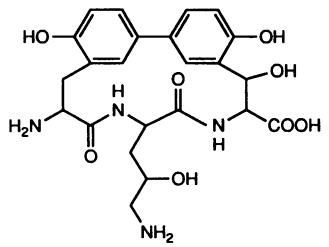

FIG. 1. Structure of biphenomycin A.

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TABLE 1. Characterization of strain 1928

Characteristic	Value or result
Gram stain	Negative
Shape	Rods
Size	0.4–0.5 by 2.4–3.6 μm
Spore	None
Mobility	Motile
Flagella	Polar, mono
Reduction of nitrates	+
Voges-Proskauer test	−
Production of indole	−
Production of hydrogen sulfide	−
Hydrolysis of starch	−
Utilization of citrate	−
Production of pigment	−
Urease	−
Arginine dihydrolase	−
Lysine decarboxylase	− (Weak)
Ornithine decarboxylase	−
Oxidase	−
Catalase	+
Esculin hydrolysis	+
Gelatin liquefaction	+
Milk peptonization	+
Milk coagulation	−
NaCl tolerance	≦7%
Oxygen demand	Aerobic
OF test (Huah and Leison test)[a]	Oxidative
Temperature for growth	17–36°C (optimum, 27°C)
Growth on:	
Azide citrate broth	−
MacConkey agar	+ (Weak)
Acid production from glucose	+
Utilization of sugar	
Positive	Adonitol, lactose, maltose, sucrose, trehalose
Negative	Xylose, starch

[a] OF, oxidation-fermentation.

***maltophilia* 1928.** The mycelia of strain 1928 were harvested from the culture broth (50 ml) of seed medium II, incubated for 24 h, and washed twice with distilled water (5,000 × *g*, 10 min). Then they were suspended in 0.1 M phosphate buffer (pH 6.2, 5 ml), which was used as the mycelial suspension. This suspension was disrupted by ultrasonication. After being centrifuged at 20,000 × *g* for 30 min, the supernatant was collected and passed through a 0.45-μm-pore-size membrane filter (Millipore Corp.). This sample was used as the cell extract.

Biphenomycin A production in cell-free mixture. Two milliliters of a culture filtrate of strain 43708 and 0.1 ml of the cell extract of strain 1928 were poured into a glass tube and incubated at 30°C for 5 h. After the mixture was heated at 80°C for 10 min, biphenomycin A in the mixture was assayed by HPLC. The culture filtrate of strain 43708 was prepared as follows. Culture broth (72 h) of strain 43708 in fermentation medium was passed through a 0.45-μm-pore-size membrane filter (Millipore Corp.). This was used as filtrate I. This filtrate I was further passed through an ultrafilter (Diafilter O1T; Ulvac Service Co.). This was used as filtrate II.

RESULTS

Taxonomic study of strain 1928. Bacterial strain 1928, used as the partner strain in the mixed culture, was isolated from a soil sample collected at Mount Maya, Hyogo, Japan. The morphological, cultural, and physiological properties of this strain are listed in Table 1. Strain 1928 is a gram-negative rod, is motile with a polar flagellum, does not form spores, is oxidative, is strictly aerobic and catalase positive, and forms neither fluorescent nor nonfluorescent pigment. When these characteristics of this strain were compared with those of bacteria described in Bergey's manuals (1, 8), strain 1928 was considered as belonging to the genus *Pseudomonas*. From the precise comparison with the descriptions of *Pseudomonas* species, strain 1928 was identified as *P. maltophilia* (2, 6, 7).

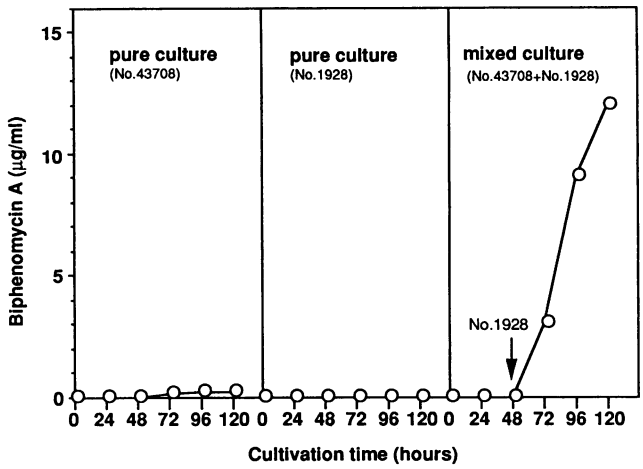

FIG. 2. Biphenomycin A production by mixed culture.

Biphenomycin A production by mixed culture. Strain 43708 produced small amounts of biphenomycin A in its pure culture (0.2 μg/ml) (Fig. 2). Strain 1928 was not able to produce biphenomycin A in its pure culture (Fig. 2). When the culture of strain 1928 was added to the culture of strain 43708, biphenomycin A production was stimulated (Fig. 2). When the seed culture of strain 1928 was added at a 2%

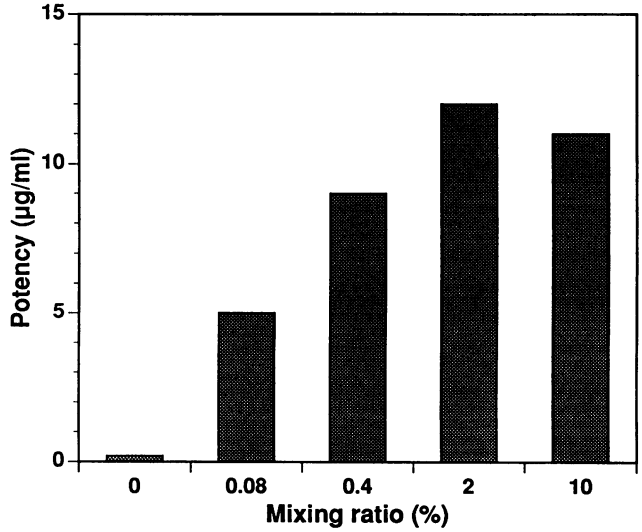

FIG. 3. Effect of mixing ratio on biphenomycin A production. Seed cultures of strain 1928 were inoculated into 48-h cultures of strain 43708 at various mixing ratios.

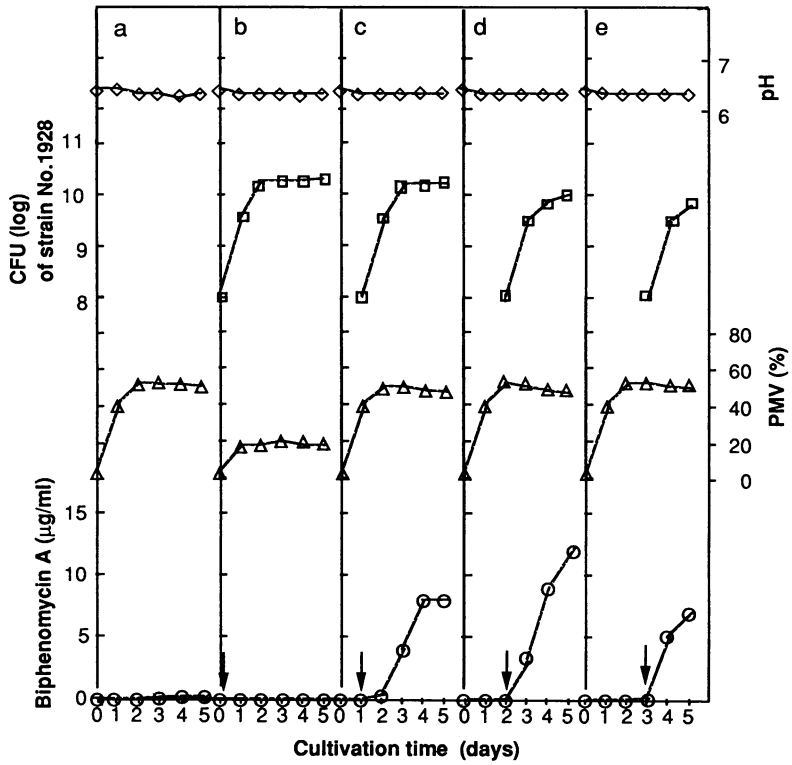

FIG. 4. Effect of mixing times on biphenomycin A production. Shown are the results of the pure culture of strain 43708 (a) and seed cultures of strain 1928 added to a 0-h culture of strain 43708 (b), a 24-h culture of strain 43708 (c), a 48-h culture of strain 43708 (d), and a 72-h culture of strain 43708 (e). Arrows indicate the times of mixing strain 1928 into cultures of strain 43708.

inoculum size to a 48-h culture of strain 43708, biphenomycin A was most abundantly produced in the mixed culture (12 μg/ml) (Fig. 3). As shown in Fig. 4, after strain 1928 was mixed into the culture of strain 43708, CFU of strain 1928 increased rapidly within 24 h. On the other hand, as the PMV of strain 43708 had already reached its maximum, it did not change after strain 1928 was mixed in (Fig. 4).

Biphenomycin A production in cell-free mixture. As shown in Fig. 5, biphenomycin A production was also stimulated by the addition of the mycelial suspension or the cell extract of strain 1928. Furthermore, biphenomycin A was produced in a cell-free mixture in which the culture filtrate of strain 43708 and the cell extract of strain 1928 were mixed (Table 2). Biphenomycin A was produced in this cell-free mixture, even when the culture filtrate of strain 43708 was substituted by filtrate II (passed through an ultrafilter). But biphenomycin A was not produced when the cell extract of strain 1928 was substituted by the cell extract treated with methanol or by heating at a high temperature.

DISCUSSION

Biphenomycin A is produced by *S. griseorubiginosus* 43708, but the amount is small. We found that biphenomycin A production by this strain was stimulated by a mixed culture with *P. maltophilia* 1928 and that the accumulation of biphenomycin A in the mixed culture amounted to about 60-fold that in the pure culture. Strain 1928 is not able to produce biphenomycin A in its pure culture but stimulates its production in a mixed culture with strain 43708 (Fig. 2). The time of the mixing of the two strains has an important effect on biphenomycin A production in a mixed culture. The best yield of biphenomycin A was demonstrated when the seed culture of strain 1928 was mixed into a 48-h culture of strain 43708 with a 2% inoculum size (Fig. 3). The growth of strain 1928 and that of strain 43708 were monitored by determining CFU and PMV, respectively. It was determined that for significant accumulation of biphenomycin A, the growth of strain 43708 must not be suppressed by mixing with strain 1928 (Fig. 4).

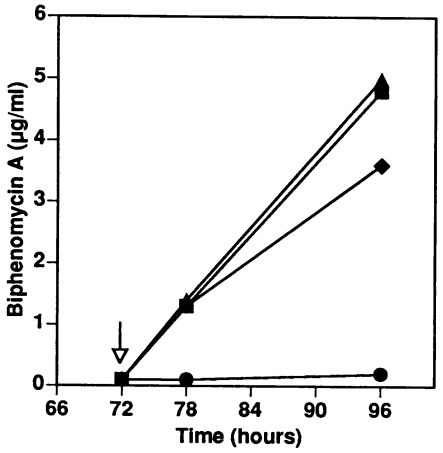

FIG. 5. Additional effects of strain 1928 culture on biphenomycin A production. Pure culture of strain 43708 (●), culture broth of strain 1928 (▲), mycelial suspension of strain 1928 (■), and cell extract of strain 1928 (◆) were added into 72-h cultures of strain 43708.

TABLE 2. Biphenomycin A production in cell-free mixture

Contents of cell-free mixture with the following strain:		Amt of biphenomycin A (μg/ml)
43708	1928	
Filtrate I[a]	None	0.1
Filtrate I	Cell extract	1.4
Filtrate I	Cell extract pretreated with 50% methanol	0.1
Filtrate I	Cell extract pretreated at 80°C for 20 min	0.1
Filtrate II[b]	Cell extract	1.5
None[c]	Cell extract	0

[a] Culture broth passed through a 0.45-μm-pore-size membrane filter. The initial titer, before incubation, was 0.1 μg/ml.
[b] Filtrate I was passed through an ultrafilter (O1T).
[c] A 2-ml volume of distilled water was used.

The stimulating effect of a mixed culture on biphenomycin A production could be achieved by adding a cell extract of strain 1928 into a culture of strain 43708. Furthermore, it was demonstrated that biphenomycin A was produced in a cell-free mixture which included a culture filtrate of strain 43708 and a cell extract of strain 1928. From the results with the cell-free mixture, the following hypothesis was put forward. A precursor of biphenomycin A existed in the culture filtrate of strain 43708, and this precursor was converted to biphenomycin A. The results of the pretreatment of the cell-free mixture suggest that this conversion from the precursor to biphenomycin A was catalyzed by enzyme activity present in the mycelia of strain 1928 (Table 2). We speculate that biphenomycin A, in a mixed culture, might be produced in the manner described above. The difference between the pure culture and the mixed culture might be explained as follows. In the pure culture of strain 43708, the precursor might not be converted, or may be poorly converted, to biphenomycin A, and therefore it does not accumulate. On the other hand, in the mixed culture with strain 1928, the precursor might be extensively converted to biphenomycin A, which does accumulate. Isolation and characterization of the precursor will be shown in a future article (4).

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