STUDIES ON THE BIOSYNTHESIS OF BASIC 16-MEMBERED MACROLIDE ANTIBIOTIC, PLATENOMYCINS. I*

SELECTION OF AND COSYNTHESIS BY NON-PLATENOMYCIN-PRODUCING MUTANTS

TAMOTSU FURUMAI and MAKOTO SUZUKI

Microbial Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd.,
Toda, Saitama, Japan

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In a search for blocked mutants which may produce a biosynthetic intermediate, mutation by N-methyl-N′-nitro-N-nitrosoguanidine treatment and/or ultra-violet irradiation were performed on platenomycin-producing *Streptomyces platensis* subsp. *malvinus* MCRL 0388 (NRRL 3761). Twenty four non-platenomycin-producing stable mutants were thus obtained and tested for cosynthesis ability. Antibiotic cosynthesis with a pair of these mutants made it possible to detect the producer of an intermediate. Among these mutants which were classified into eight groups (A to G and doubtful groups), mutants of groups A and B appeared from their complementation pattern to be the useful producers of biosynthetic intermediates of platenomycin.

A number of intermediates in the biosynthesis of 12- and 14-membered macrolide antibiotics such as methymycin[2], erythromycin[3~5], narbomycin[6] and picromycin[2] have been isolated and identified. However, except for Magnamycin (carbomycin)[7~9] no attempts have been made to determine the intermediates in the biosynthesis of 16-membered macrolide antibiotics. Structures of platenomycins, a family of 16-membered macrolide antibiotics have been elucidated[10~13]. Therefore, it should be possible to elucidate the biosynthetic pathways, if the true biosynthetic intermediates are identified. The present paper is concerned with mutation of *Streptomyces platensis* subsp. *malvinus* MCRL 0388[14], a producer of platenomycins, the detection and ordering the resulting blocked mutants and the presentation of a complementation pattern. The work was conducted by modifying the technique devised by DELIĆ *et al.*[15].

Methods

Organisms

S. platensis subsp. *malvinus* MCRL 0388, a producer of platenomycins, was maintained on an yeast extract-malt extract agar slant (Difco) at 27°C. A well-sporulated culture, 10~14 days old, was used for selection of non-platenomycin-producing mutants (blocked mutants). *Bacillus subtilis* ATCC 6633 was used for the bioassay of platenomycins[14].

Media

The medium for antibiotic cosynthesis (AC medium) was prepared with 10 g of glucose, 20 g of soluble starch, 1 g of yeast extract, 4 g of NaCl, 3 g of $CaCO_3$, 15 g of agar and 1,000 ml of soy bean meal extract prepared as below, pH being adjusted to 6.5 before sterilization at 120°C for 20 minutes in an autoclave. The soy bean meal extract was prepared by boiling 30 g of soy bean meal with 500 ml of water for 20 minutes in an autoclave at 110°C. Aqueous extract obtained by centrifugation at 5,000 r.p.m. for 15 minutes was diluted with water to

* Outlines of the present paper have already been communicated.[1] Platenomycins were previously named YL-704.

1,000 ml. The seed culture medium (SC medium) was prepared with 20 g of glucose, 10 g of peptone, 7.5 g of beef extract, 2.5 g of yeast extract, 3 g of NaCl and 1,000 ml of water, pH being adjusted to 6.5 before sterilization. The platenomycin production medium (PP medium) was prepared with 10 g of glucose, 20 g of soluble starch, 20 g of soy bean meal, 1 g of yeast extract, 3 g of NaCl, 3 g of $CaCO_3$ and 1,000 ml of water (pH was adjusted to 6.5 before sterilization).

Mutation

Ultra-violet (UV) irradiation (UV lamp, Toshiba Co., 3,520 Å) and/or N-methyl-N′-nitro-N-nitrosoguanidine (NTG) treatment were used for mutation according to the following standard procedures.

UV irradiation

The spores on an agar slant were suspended in 10 ml of sterile physiological saline or 0.005% aerozol OT (sodium dioctyl sulfosuccinate, Nakarai Chemicals, Ltd.) solution and filtered through Whatman filter paper (No. 1). Spores were exposed to UV light at intervals of 0~70 seconds. After irradiation, spore suspension was diluted and spread over on the surface of AC medium in a Petri-dish and incubated at 27°C for 4~6 days. Then the agar layer in a dish was vertically cut into a piece with a cork borer (6 mm in diameter) so that each agar cylinder contained a single mycelial colony on its surface. Each cylinder was put on an agar plate embedded with *B. subtilis* ATCC 6633 spores. The dish was incubated at 37°C overnight. A colony on an agar cylinder producing no or a weak inhibition zone was selected and cultivated in PP medium to confirm a lack of production of platenomycin.

NTG treatment

The spore suspension prepared as above was diluted 2 fold with 0.2 M tris buffer (pH 9.0) containing 2 mg/ml of NTG and kept standing for 0~15 minutes at 30°C. Then the spores were collected on the Millipore filter (0.48 μm pore size), washed with 30 ml of sterile physiological saline solution and resuspended in 30 ml of sterile physiological saline solution. The spore suspension thus obtained was spread on the AC medium. Blocked mutants were selected as described above. Blocked mutants designated N or U depending on whether these mutants were induced by NTG or UV respectively. Those mutants designated as NU were obtained by UV irradiation of NTG-induced mutants.

Cosynthesis on an agar medium

Modifying the procedure devised by DELIĆ *et al.*[15], antibiotic cosynthesis by a pair of blocked mutants was examined as follows. Spore suspensions for the test was prepared by suspending the spores of a mutant grown on an agar slant (10~14 days old) in 5 ml of sterile physiological saline solution. The spore suspensions of two blocked mutants to be tested (0.1 ml each) was spread over opposit halves of a plate composing of 20 ml of AC medium, about 1~2 mm apart. The plate was incubated for 5 days at 27°C. An agar strip, 5×60 mm, was cut from the plate at right angles to the line of separation between the two mutants. This strip was placed on the surface of an agar plate embedded with spores of *B. subtilis* ATCC 6633. The plate was incubated overnight at 37°C, and the appearance of an inhibition zone along the agar strip was considered a sign of the production of platenomycins.

Cosynthesis in a mixed fermentation

The positive pairs of mutant in an above test were re-tested in a liquid medium. To verify the production of platenomycins, each of the positive mutants was grown separately in SC medium at 28°C for 48 hours. Two ml of each seed culture were inoculated together into 100 ml of PP medium prepared in a 500-ml flask and cultivated at 24~27°C on a reciprocal shaker. After 5~6 days, the broth was assayed for the production of platenomycins.

Results and Discussion

Among numerous non-platenomycin-producing strains which were induced by NTG and/or UV treatment, 24 stable blocked mutants were used for the cosynthesis test. Antibiotic cosynthesis

Fig. 1. Antibiotic cosynthesis between a group B mutant (U-92) and a group C mutant (N-32).
↓: Line of separation between both strains.

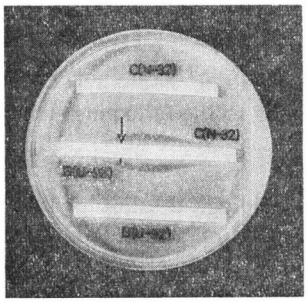

Table 1. Cosynthesis between groups of blocked mutants of *Streptomyces platensis* subsp. *malvinus**.

Groups (Strain No.)	A	B	C	D	E	F	G
A (N-90, N-33, U-253)	—	—	C	D	E	F	—
B (U-92, N-22)		—	C	D	E	—	—
C (N-32, U-21, N-1, N-4, N-5, N-29)			—	—	E	C	—
D (N-9, NU-29)				—	—	D	—
E (N-11)					—	E	—
F (N-17)						—	—
G (N-3, N-15, N-36, N-37)							—
Doubtful (NU-1, NU-5, N-19, N-27, NU-58)							

Complementation pattern

E	C	F	A
D		B	
G			

* —: Indicated no cosynthesis; Cosynthesis was observed on the lettered position, when an inhibition zone appeared on the site of the group described.

was, for example, demonstrated when strains U-92 and N-32 were used (Fig. 1). The upper and lower strips shown on the Petri dish were a pure culture of the strains N-32 and U-92 respectively. In the central strip the right half was inoculated with the strain N-32 and left half was inoculated with the strain U-92. Using this pair of strains, an inhibition zone appeared around the central strip predominantly on the side of strain N-32. This indicates that the strain U-92 was producing an intermediate or intermediates which were converted into an antibiotic substance (platenomycins) by the strain N-32. Therefore, it was concluded that the strains N-32 and U-92 were blocked at a different biosynthetic step, the former being blocked at an earlier step than the latter. Thus, the strain N-32 was a "converter", and the strain U-92 was a "secretor". Based on this sort of antibiotic cosynthesis behavior, 24 non-platenomycin-producing strains were classified into eight groups (A to G and doubtful groups) as shown in Table 1. The complementation pattern drawn from this information was also shown in Table 1. All mutants except those of group G showed the ability of cosynthesis. Mutants of groups A and B showed a similar cosynthesis behavior, but they were differentiated in the behavior against the strain N-17 of group F. A mutant of groups A and B always acted as a secretor. Mutants

Table 2. Production of platenomycins by cosynthesis between blocked mutants in mixed culture*.

Strain No. (Group)	N-90 (A)	U-92 (B)	N-32 (C)	N-9 (D)	N-11 (E)	N-17 (F)	N-3 (G)
N-90 (A)	—	—	100	45	19	11	—
U-92 (B)		—	115	78	11	—	—
N-32 (C)			—	—	14	15	—
N-9 (D)				—	—	35	—
N-11 (E)					—	15	—
N-17 (F)						—	—
N-3 (G)							—

* Production (mcg/ml) was determined with PLM-A_1 as standard.
—: Indicated no antibiotic production.

of groups C, E and F gave positive cosynthetic activity with mutants of the other groups except a pair of groups B-E, C-D and D-F. A mutant of groups C and F acted either as a secretor or a converter, but a mutant of groups D and E always acted as a converter. Other strains which participated in cosynthesis with some strains but where, because of their complicated complementation behavior, it was difficult to define the blocked step were classified as a doubtful group.

The above-mentioned antibiotic cosynthesis observed on an agar medium was further verified in a liquid culture. As shown in Table 2, any pair of blocked mutants which showed cosynthesis on an agar medium showed an antibiotic production in a liquid culture as well.

Mutants strains of groups A, B, C, E and F were arranged in an order by placing a mutant group acting as a secretor after a mutant group acting as a converter. Thus, the order of these five groups could be arranged in the following two sequences: E→C→F→A and E→C→B. Consequently, it was thought that the strains of groups A and B which acted only as a secretor might be a producer of biosynthetic intermediate(s) of platenomycin. Concerning the isolation, characterization and structure of the products (intermediates) produced by mutants of groups A and B will be reported in the succeeding two papers.

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