

## ISOLATION AND CHARACTERIZATION OF FURANOMYCIN NONPRODUCING *Streptomyces threomyceticus* MUTANTS

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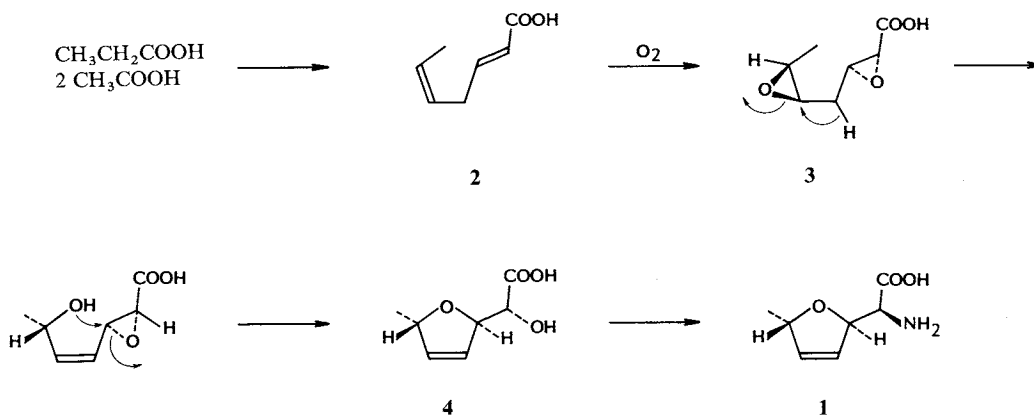
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Stable mutants that are blocked in the production of the polyketide antibiotic furanomycin were generated by treatment of *Streptomyces threomyceticus* (ATCC 15795) with a combination of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ultraviolet light. On the basis of their cosynthetic properties in mixed culture, the mutants were grouped into eleven phenotypic classes. The polarity of the cosynthetic reactions for seven classes of mutants was revealed by filtrate feeding experiments. This allowed them to be arranged in the most probable linear sequence of metabolic blocks. One of the remaining groups of mutants exhibited no cosynthetic behavior and is believed to be blocked in a gene that regulates furanomycin biosynthesis.

In 1967 KATAGIRI *et al.* reported<sup>1</sup> that the fermentation broth of *Streptomyces threomyceticus* (ATCC 15795) contains the novel antibiotic furanomycin (**1**) (Fig. 1). The compound was found to be a competitive antagonist of L-isoleucine and to inhibit the growth of T-even coliphage. Recently, furanomycin has been shown to be charged to isoleucine tRNA by isoleucyl-tRNA synthetase from *Escherichia coli* and to be subsequently incorporated into protein.<sup>2</sup> Previous investigations in our laboratory<sup>3</sup> have demonstrated that **1** is a polyketide derived from two acetate units and one propionate unit, with the latter serving as the starter unit. Furthermore, the ether oxygen atom of furanomycin was found to be derived from molecular oxygen and to be introduced at C-2 of the propionate unit with loss of the 2 *pro-R* hydrogen atom and overall inversion of configuration.<sup>4</sup> These observations and the known stereochemistry of allylic rearrangements catalyzed by  $\beta$ -hydroxydecanoylthioester dehydrase<sup>5</sup> led to the postulate that the biosynthesis of furanomycin proceeds by epoxidation of *trans,cis*-2,3,5,6-heptadienoic acid (**2**) to a diepoxide (**3**) which could then undergo ring-opening of the 5,6-epoxide with subsequent ring-closure to yield the  $\alpha$ -hydroxy acid **4** (Fig. 1). The  $\alpha$ -hydroxyacid could then be converted into furanomycin by oxidation

Fig. 1. The proposed biosynthetic pathway to furanomycin.



of the hydroxyl group to give an  $\alpha$ -keto acid followed by transamination. The mechanism postulated for the formation of furanomycin is of particular interest, since it bears a close resemblance to that suggested for the biosynthesis of the polyether antibiotics.<sup>6</sup> With the eventual goal of gaining additional insight into the biosynthesis of furanomycin, we have therefore prepared and classified furanomycin-nonproducing mutants (Fur<sup>-</sup>) of *S. threomyceticus*.

## Materials and Methods

### Bacterial Strains

The strains of *S. threomyceticus* used in this work are listed in Table 1. *S. threomyceticus* ATCC 15795 was obtained from the American Type Culture Collection, Rockville, MD, and *Bacillus subtilis* UC-28 was a gift from The Upjohn Company, Kalamazoo, MI.

### Growth Media

*S. threomyceticus* was maintained on BENNETT's agar which contained the following components per liter: yeast extract (Difco Laboratories, Detroit, MI), 1 g; beef extract (Difco), 1 g; N-Z-amine type A (Sheffield products, Kraft Inc., Woods Corners, Norwich, NY), 2 g; glucose, 10 g; and Bacto agar (Difco), 15 g. The antibiotic production medium used for the isolation of furanomycin-nonproducing mutants and for cosynthesis experiments contained the following ingredients per liter: starch, 20 g; soybean meal, 10 g; corn steep liquor (Corn Products, Unit of CPC North America, Argo, IL), 5 g; NaCl, 5 g; CaCO<sub>3</sub>, 3.5 g. The medium used in bioassay and bioautography was the glucose-inorganic salts medium described by KATAGIRI *et al.*<sup>1</sup> The Fur<sup>-</sup> mutants were evaluated for furanomycin resistance on the minimal medium (MM) described by HOPWOOD *et al.*<sup>7</sup> All these media were adjusted to pH 7.0 before sterilization. Brain-heart infusion medium (Difco) with 1.5% agar was used for the growth of *B. subtilis* UC-28.

### Isolation of Furanomycin-nonproducing Mutants

For the isolation of furanomycin-nonproducing mutants (Fur<sup>-</sup>), mutagenesis was carried out by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Aldrich Chemical Company, Inc., Milwaukee, WI) according to the method of DELIC *et al.*<sup>8</sup> in combination with UV light from a germicidal mercury lamp. In order to create the mutations, a killing rate from 99.0 to 99.999% was used. Approximately 50% of the colonies surviving mutagenesis were observed to possess normal morphology. Colonies grown from single spores and with normal morphology were transferred to duplicate BENNETT's agar plates, and incubated at 30°C for 8 days. One colony of the pair was then transferred to a 16 × 150 mm tube containing 2.5 ml of antibiotic production medium and the tube shaken at 30°C, 250 rpm for 7 days. If the bioassay of the fermentation filtrate from the tube showed a lack of furanomycin production, then the duplicate colony was used to inoculate a BENNETT's agar slant. After sporulation the organism on the slant was re-evaluated for a lack of furanomycin production in tube fermentation. All of the slants which showed a lack of furanomycin production were then used to prepare spore suspensions in 20% glycerol which were stored at -80°C. The stability of the Fur<sup>-</sup> phenotype was then evaluated for each spore suspension through three serial transfers on BENNETT's agar. Only those mutants that exhibited stability of the Fur<sup>-</sup> phenotype were selected for further study.

### Evaluation of Furanomycin Resistance of Fur<sup>-</sup> Mutants

A 0.10 ml spore suspension of each Fur<sup>-</sup> mutant was applied to one petri dish containing MM medium. A 12.7-mm paper disc impregnated with 100  $\mu$ g of furanomycin was then placed on the surface of the agar and the dish was incubated at 30°C for three days. No inhibition zone appeared with any of the mutants. Three non-furanomycin producing strains of *Streptomyces* (*S. lividans* TK24, *S. ambofaciens* (ATCC 15154), and *S. griseofuscus* (ATCC 23916)) all gave sizeable inhibition zones under these conditions.

### Cosynthesis

(1) Mixed-cultures. A small fragment of mycelium was transferred from slants of two different

mutants into a tube containing 2 ml of production medium, and the tube was incubated as described above. Bioassay for furanomycin production in the mixed culture was carried out as described under "bioassay."

(2) Precursor feeding. *fur* mutant type strains were grown in 2 ml of antibiotic production medium for 2 days. The filtrate (0.50 ml) prepared from a culture of another class of *fur* mutant was added, and the culture to which the filtrate had been administered was then incubated for another 5 days. The culture broth of the fed culture was then bioassayed for furanomycin production. The filtrates were prepared from 30 ml cultures of *fur* mutant type strains which had been grown for 4 days, centrifuged at 10,000 rpm for 20 minutes, and filtered through a 0.2  $\mu$ m sterile filter (Gelman Sciences Inc., Ann Arbor, MI).

#### Paper Chromatography and Bioautography

Strips of Whatman No. 1 paper (0.7 cm  $\times$  20 cm) were used, with the origin being 3 cm from the lower end of the chromatogram. The solvent system used for development was *n*-butanol - acetic acid - water (2:1:1). The fermentation filtrate (20  $\mu$ l) was applied to the origin in 2  $\mu$ l aliquots and the water allowed to evaporate between applications. Development was halted when the solvent front reached a distance of 15 cm from the origin. After drying in air for 30 minutes, the chromatogram was placed on top of a bioassay dish containing 100 ml of glucose - inorganic salts medium seeded with 0.5 ml of a spore suspension of *B. subtilis* UC-28 prepared by washing one brain heart infusion agar slant with 2.5 ml of sterile 0.9% saline. After 30 minutes the paper strip was removed, and the bioassay dish was incubated overnight at 30°C. Compounds possessing antibiotic activity against *B. subtilis* UC-28 were identified by the appearance of an inhibition zone.

#### Bioassay

The fermentation filtrate (20  $\mu$ l) was applied to a 12.7-mm paper disc (Schleicher & Schuell, Inc., Keene, NH) and the solution allowed to dry in air. The paper disc was then heated at 85°C in an oven for 4 hours. After heating, the paper disc was cooled to room temperature and then placed upon the surface of a bioassay dish. The bioassay dish was incubated overnight at 30°C. The presence of furanomycin was indicated by an inhibition zone. Double layered bioassay plates were found to provide more reproducible results since the interior surface of the glass bioassay dish was uneven. When double layered assay plates were used, the bottom layer consisted of 100 ml of 1.3% agar which was allowed to solidify before addition of the top layer which was composed of 100 ml of the bioassay medium described above.

## Results

### Isolation of Furanomycin-nonproducing Mutants

The results of bioautography showed that the wild type strain of *Streptomyces threomyticus* produced two substances in both liquid and solid media which exhibited antibiotic activity against *B. subtilis* UC-28. One of these was furanomycin, while the identity of the other was unknown. Attempts to find a medium which led to the production of only furanomycin were unsuccessful, as were efforts to develop a strain of *B. subtilis* that lacked sensitivity to the unknown antibiotic. Fortunately, it was discovered that the unknown antibiotic is relatively unstable in comparison to furanomycin. Heating the impregnated assay discs for 4 hours at 85°C led to complete loss of the antibiotic activity due to the unknown, while the activity due to furanomycin was unchanged. Therefore, heat treatment was incorporated into the paper disc bioassay procedure used to screen *S. threomyticus* mutants. Utilizing this method 2,222 colonies were screened, and after three serial transfers on BENNETT's agar slants, 19 Fur<sup>-</sup> mutants were recovered that exhibited a stable Fur<sup>-</sup> phenotype (Table 1).

### Classification of Fur<sup>-</sup> Mutants

All 19 Fur<sup>-</sup> mutants were tested for their cosynthetic ability by the mixed culture method, while only the type strains were tested for their biotransformation ability using the precursor feeding method. It was

Table 1. *S. threomyeticus* strains used in this study.

| Strain <sup>a</sup>   | Production of furanomycin | Genotype       | Strain <sup>a</sup>   | Production of furanomycin | Genotype       |
|-----------------------|---------------------------|----------------|-----------------------|---------------------------|----------------|
| ST.4-64 <sup>b</sup>  | no                        | <i>furA</i> 1  | ST.18-67 <sup>b</sup> | no                        | <i>furE</i> 11 |
| ST.4-65               | no                        | <i>furA</i> 2  | ST.29-3 <sup>b</sup>  | leaky                     | <i>furF</i> 12 |
| ST.4-70               | no                        | <i>furA</i> 3  | ST.30-52 <sup>b</sup> | no                        | <i>furG</i> 13 |
| ST.15-65              | no                        | <i>furA</i> 4  | ST.1-26               | no                        | <i>furH</i> 14 |
| ST.22-80              | no                        | <i>furA</i> 5  | ST.2-11               | no                        | <i>furI</i> 15 |
| ST.13-49 <sup>b</sup> | no                        | <i>furB</i> 6  | ST.19-20              | no                        | <i>furI</i> 16 |
| ST.15-63              | no                        | <i>furB</i> 7  | ST.19-46              | no                        | <i>furI</i> 17 |
| ST.15-52 <sup>b</sup> | leaky                     | <i>furC</i> 8  | ST.20-23              | no                        | <i>furJ</i> 18 |
| ST.18-15 <sup>b</sup> | leaky                     | <i>furD</i> 9  | ST.22-13              | no                        | <i>furK</i> 19 |
| ST.24-24              | leaky                     | <i>furD</i> 10 |                       |                           |                |

<sup>a</sup> The parent strain is ST.0.

<sup>b</sup> Type strain used in cosynthesis experiments.

Table 2. Cosynthetic pattern by mixed culture method.

| Mutant class | No. of mutant strains | I | II | III | IV | V | VI | VII | VIII | IX | X | XI |
|--------------|-----------------------|---|----|-----|----|---|----|-----|------|----|---|----|
| I            | 5                     |   | +  | +   | +  | + | +  | +   | +    | +  | - | -  |
| II           | 2                     |   |    | +   | +  | + | +  | +   | -    | -  | - | -  |
| III          | 1                     |   |    |     | +  | + | +  | +   | -    | -  | + | -  |
| IV           | 2                     |   |    |     |    | + | +  | +   | -    | -  | - | -  |
| V            | 1                     |   |    |     |    |   | +  | +   | +    | -  | - | -  |
| VI           | 1                     |   |    |     |    |   |    | +   | -    | -  | + | -  |
| VII          | 1                     |   |    |     |    |   |    |     | -    | -  | - | -  |
| VIII         | 1                     |   |    |     |    |   |    |     |      | -  | - | -  |
| IX           | 3                     |   |    |     |    |   |    |     |      |    | - | -  |
| X            | 1                     |   |    |     |    |   |    |     |      |    |   | -  |
| XI           | 1                     |   |    |     |    |   |    |     |      |    |   |    |

Table 3. Cosynthetic pattern by precursor feeding method (type strains only).

| Converter class | Type strain | Secretor class |     |    |    |    |   |     |
|-----------------|-------------|----------------|-----|----|----|----|---|-----|
|                 |             | I              | III | IV | VI | II | V | VII |
| I               | 4-64        | -              | +   | +  | +  | +  | + | +   |
| III             | 15-52       | -              | -   | +  | +  | +  | + | +   |
| IV              | 18-15       | -              | -   | -  | +  | +  | + | +   |
| VI              | 29-3        | -              | -   | -  | -  | +  | + | +   |
| II              | 13-49       | -              | -   | -  | -  | -  | - | -   |
| V               | 18-67       | -              | -   | -  | -  | -  | - | -   |
| VII             | 30-52       | -              | -   | -  | -  | -  | - | -   |

not possible to perform cosynthetic experiments on solid medium because of the production of the unknown antibiotic. The 19 Fur<sup>-</sup> mutants were classified into eleven different groups on the basis of their cosynthetic behavior and biotransformation capability. The results of the cosynthetic experiments are summarized in Tables 2 and 3. In order to be certain that the antimicrobial activity exhibited in the paper disc diffusion assay was due to furanomycin, all the positive results shown in Table 2 and Table 3 were checked by bioautography. In each case the antimicrobial compound produced by cosynthesis between Fur<sup>-</sup> mutants was found to be chromatographically identical to furanomycin. Each of the cosynthesis experiments

outlined in Tables 2 and 3 gave the same result when the experiment was repeated four or more times. Some cosynthesis experiments yielded less reproducible results. For example, four repetitions of cosynthesis experiments between the following combinations of mutants gave two positive results and two negative results: 15-65 × 13-49, 15-63 × 18-67 and 24-24 × 18-67. Although mutants 15-65, 15-63, 24-24 appear to be in classes, I, II and IV, respectively, they were not selected as type strains because of their erratic behavior, and they were not utilized in precursor feeding experiments. It was observed (Tables 2 and 3) that mutants 13-49 (class II), 18-67 (class V) and 30-52 (class VII) exhibited cosynthesis in mixed culture, but not in precursor feeding experiments.

### Discussion

*S. threomycticus* Fur<sup>-</sup> mutants were isolated at a significant frequency only after the induction of the mutations by treatment with UV light and NTG in pairwise combination. Under these circumstances, the frequency of Fur<sup>-</sup> mutants was about 0.9%. All mutants had normal morphology (only 22-80 exhibited a change in the spore color) and exhibited normal levels of resistance to furanomycin. This indicated that the mutations did not occur in the genes governing the developmental biology or the self-resistance of *S. threomycticus*. All mutants except 22-13 (class XI) cosynthesized furanomycin, a result which shows that these mutants are blocked in the biosynthesis pathway to furanomycin. Since mutant 22-13 did not cosynthesize with any other mutants, the mutation in this strain may lie in a regulatory region. Identical behavior has been observed with the *act* mutants of *Streptomyces coelicolor*.<sup>9)</sup> On the basis of their cosynthetic and biotransformation behavior, the remaining mutants could be classified into ten distinct phenotypic classes. Seven of these classes could be placed in a linear biosynthetic sequence which proceeds as follows:



The largest group of mutants fell into the group which served as a convertor for classes II ~ VII, but not as a secretor for any strain. These mutants were assigned to the earliest steps in the biosynthetic sequence and were designated as class I. This class of mutants is presumably composed of mutations within a polyketide synthase complex which would be involved in the condensation of propionyl CoA with two successive units of malonyl CoA. Similar classes of mutants have been encountered in the analysis of mutants blocked in the biosynthesis of the polyketides actinorhodin,<sup>9)</sup> tetracenomycin C,<sup>10)</sup> and daunomycin.<sup>11)</sup> Classes II, V and VII could be placed at the end of the linear sequence on the basis of the cosynthetic pattern they exhibited in mixed culture. However, a linear relationship between these three classes could not be established, since they only exhibited cosynthesis in precursor feeding experiments with mutant classes I, III, IV and VI (Table 3). A similar result has been observed with some Tcm<sup>-</sup> mutants of *Streptomyces glaucescens*.<sup>10)</sup> This behavior may be due to the decomposition of unstable intermediates or to the timing of the feeding experiments. Attempts to observe cosynthesis between mutants 13-49 (class II) and 18-67 (class V) by varying the timing of the precursor feeding experiments were unsuccessful. The remaining three classes of mutant (VIII ~ X) exhibited cosynthetic behavior which did not allow them to be assigned to any of the other groups nor could they be placed in a linear sequence. The behavior of these three classes of mutant may be due to undetectable levels of cosynthesis, to polar mutations (C. R. HUTCHINSON; private communication.), or to the presence of more than one mutation in the biosynthetic pathway. Similar behavior is exhibited by some *kal* mutants of *Streptomyces tanashiensis* which are blocked in the biosynthetic pathway leading to the polyketide kalafungin.<sup>12)</sup> As some classes of mutants contained only one member, it is possible that other classes of mutants remain to be isolated. The results of cosynthesis experiments involving some mutants were not reproducible. The reasons for this are unclear. Positive cosynthesis experiments involving class I *fur* mutants produced levels of furanomycin production that were similar to that of the wild type strain. Positive cosynthesis between other groups of mutants generally exhibited lower levels of production. Three of the mutants employed in these investigations were leaky and occasionally produced low levels of furanomycin. The production of furanomycin by these mutants was always significantly less than that observed in successful cosynthesis

experiments involving these leaky strains.

The analysis described here of the cosynthetic behavior of the Fur<sup>-</sup> mutants suggests that the biosynthesis of furanomycin proceeds through at least six discrete intermediates after the assembly of the polyketide chain. If the structure of one or more of these intermediates can be elucidated, then this should provide considerable insight into the nature of the steps leading to the formation of the dihydrofuran ring present in furanomycin. Work is in progress to achieve this goal.

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