NEW ANTHRACYCLINE METABOLITES FROM MUTANT STRAINS OF *STREPTOMYCES GALILAEUS* MA144-M1

I. ISOLATION AND CHARACTERIZATION OF VARIOUS BLOCKED MUTANTS

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During genetic study on obtaining high-yield variants of aclacinomycin A-producer, a variety of blocked mutants were isolated from *Streptomyces galilaeus* MA144-M1 and were characterized. The isolated mutants included those which accumulated only specific components of parental glycosides, those which produced new aklavinone glycosides devoid of parental rhodosamine or both rhodinose and cinerulose, those which produced non-glycosidic aglycones, and antibiotic-negative mutants, some of which were able to glycosidate exogenous aklavinone. By biotransformation with the aglycone feeding culture, the precursor activity of new aglycones was also tested. From the results and in relation to the characterization of isolated mutants, the biosynthetic pathway of aclacinomycin A and related antibiotics is discussed.

Streptomyces galilaeus MA144-M1 produced a complex of pair glycosides of aklavinone and ε-pyrromycinone, together with antibiotically inactive non-glycosidic aglycones[1]. Among twenty-one components isolated from the culture[2], aklavinone glycosides, named as aclacinomycin, have been studied in detail, since ε-pyrromycinone glycosides were the same as cinerubins which had been isolated from the culture of *Streptomyces cinereoruber*[3].

Since aclacinomycin A exhibited more favorable activity against experimental tumors and less animal toxicity than cinerubin A, our first attempt for strain improvement was focused on the genetic loss in productivity of counterpart antibiotic cinerubins from the original strain. This was achieved by isolating yellow-pigmented colonies occurred with low frequency during successive mutation of the original strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The yellow variants produced only aklavinone glycosides. We further made this yellow variant mutate to induce variants or blocked mutants being capable of producing abundantly aclacinomycin analogs.

In this course of strain improvement we also obtained new aglycone-producing mutants and antibiotic-negative mutants, some of which are useful for glycosidation of various anthracyclinones to obtain new anthracycline antibiotics. In this paper we describe the isolation of various blocked mutants and the characterization of their products.

Materials and Methods

Microorganisms

The original strain MA144-M1 and mutant strains of *Streptomyces galilaeus* were maintained on

YS slant agar (0.3% yeast extract, 1% soluble starch, 1.5% agar, pH 7.2). Strain MA144-M1 and its products were previously described[1,2,4,5].

Mutation

Spore cells from slant culture grown on YS agar were suspended in 10 ml of saline and dispersed by mild sonication. After filtration of the spore suspension through a defatted cotton-packed glass tube (ϕ 1.5×2 cm), the spore cells were centrifuged and resuspended at $1 \sim 5 \times 10^8$ cells/ml of cell density either in 10 ml of saline in Petri dish to be irradiated by ultraviolet light (UV) or in 5 ml of 0.2 M tris-HCl buffer (pH 8.5) to be exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, final conc. 1 mg/ml). Both mutational treatments were performed to give 5~0.05% survivals followed by colony formation on YS agar. In a mutation step, 200~2000 colonies were tested for their antibiotic production as described below.

Detection of Antibiotic Production

Mutant culture on YS agar slant was inoculated into YS medium (4 ml/test tube) and incubated on a reciprocal shaker at 28°C for 2 days. The subculture was then added to a 250-ml Erlenmeyer flask containing 30 ml of the fermentation medium of the following composition: 1.5% soluble starch, 1% glucose, 3% soybean meal, 0.1% yeast extract, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$, pH 7.4. The fermentation was carried out by cultivation at 28°C for 3 days on a rotary shaker (220 rpm). Five ml of the culture broth were sampled and centrifuged and the antibiotics in mycelial pellet were extracted with 5 ml of acetone. The extract was concentrated *in vacuo* and re-extracted with 2 ml of $CHCl_3$. After evaporation, the pigment residue was chromatographed on silica gel 60 F_{254} plate (E. Merck Co.) using several solvent systems. Anthracycline metabolites were determined by comparison with authentic sample on thin-layer chromatography (TLC). New metabolites were isolated from total 10 liters of mutant culture obtained as mentioned above using 500-ml Erlenmeyer flasks containing 50 ml of medium, and their purification and identification are described in a companion paper[6]. Estimation of metabolites was done by scanning the corresponding spot on TLC by a Shimadzu TLC Scanner Model CS-910 using wave length at 430 nm for aklavinone glycosides and at 490 nm for ε-pyrromycinone glycosides, respectively.

Feeding Cultivation

Antibiotic production by the aglycone feeding cultivation was studied to characterize antibiotic-negative mutants or to examine precursor activity of new aglycones in the aclacinomycin biosynthesis. Feeding culture was made by adding aglycone (final 20 μg/ml) to 24-hour culture grown in the fermentation medium and cultivating for 48 hours. Feeding experiments with aclacinomycin A-producer, strain 6U-21, were performed in the presence or absence of cerulenin, which was added at 20 μg/ml to 24-hour growing culture being immediate prior to the start of antibiotic production and substrate aglycone was also added at the same time. This concentration of cerulenin completely suppressed the production of anthracycline pigments without significant loss of growth. After 48 hours 5 ml of the culture were sampled and antibiotics were determined by TLC after solvent extraction as mentioned above.

Identification of New Products

Purification and structural identification of new anthracycline glycosides, MA144 U5, U6, U7, U8 and U9 and new aglycones 58D, 58C and 58G are described in a companion paper[6].

Results

In the study of genetic improvement for aclacinomycin productivity in *S. galilaeus* MA144-M1 by NTG and UV treatments, we obtained a variety of blocked mutants. The selection process of mutants and anthracycline metabolites obtained are summarized in Fig. 1 and Table 1, respectively. All isolated mutants were characterized by metabolic products as follows.

Fig. 1. Scheme of the mutant selection in *Streptomyces galilaeus* MA144-M1.

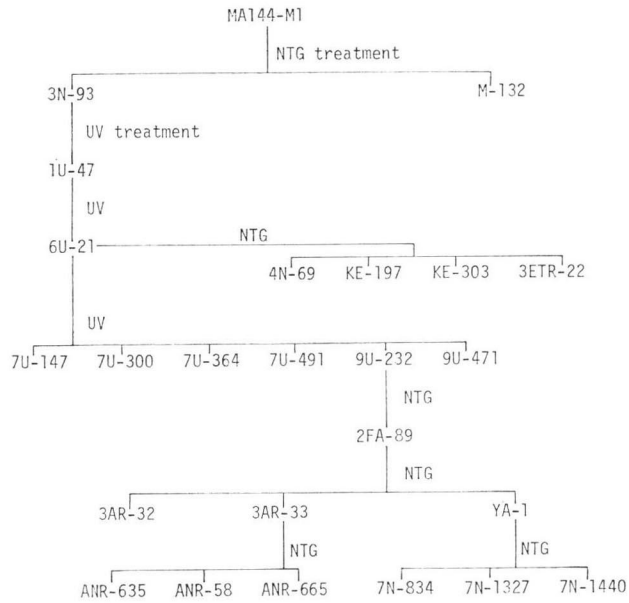


Table 1. Anthracyclines produced by various mutants of *S. galilaeus*.

Group	Strain No.	Major metabolites	Remarks
I	MA144-M1 (original strain)	Aclacinomycins A & B Cinerubins A & B	
	M-132	Cinerubins A & B	
	6U-21	Aclacinomycins A & B	Loss of C-1 oxidation
	9U-232	Aclacinomycin A	
	YA-1	Aclacinomycin A	High-yield variant
	7U-364	Aclacinomycin B	
	KE-197	MA144 N1	Loss of oxidoreductase
	7U-491	MA144 U1 & S1	Loss of rhodinose supply
	7N-1881	MA144 U1 & U9	〃
	9U-653	MA144 U5, U6, U7 & U8	Loss of aminosugar supply
II	3AR-33	Aklavinone	
	ANR-635, ANR-220	Aklavinone	Lverproductive
	ANR-58	2-Hydroxyaklavinone & non-esterified analog	
	ANR-665	Red aglycones (665A & B)	Unidentified
	7U-300	Brownish yellow aglycones (300A & B)	Unidentified
III	KE-303	No pigments	Glycosidation (+)
	3ETR-22	No pigments	〃 (−)

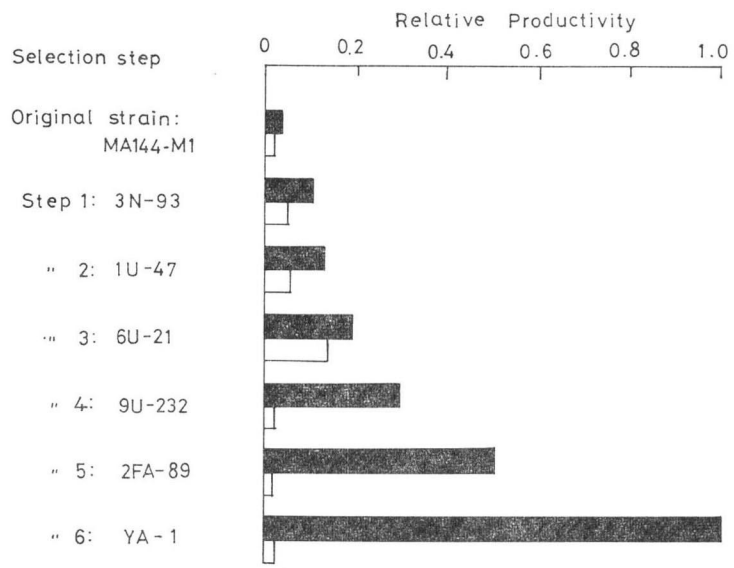
Group I: Anthracycline glycoside-producing strains.
Group II: Aglycone-producing strains.
Group III: Anthracyclinone-non-producing strains.

Yellow and Red Variants

The original strain MA144-M1 of *S. galilaeus* accumulated equivalent levels of yellow aclacinomycin A and orange-red cinerubin A as major products. Thus, colonies grown on YS agar at 28°C for 4 days were usually orange color. Isolation of yellow variants producing only aklavinone glycosides and red variants producing ε-pyrromycinone glycosides was achieved by selection of either yellow-pigmented colonies or red-pigmented colonies which occurred at a low frequency during several mutations with NTG. The loss of productivity of the counterpart glycosides was determined by TLC of the extracted pigments from each variant using a solvent system of acetone - *n*-hexane (90: 10, v/v), and yellow variant 1U-47 and red variant M-132 were obtained. Yellow variant 1U-47 seems to have a permanent genetic loss of productivity of ε-pyrromycinone since no reversion was observed during successive mutations. However, red variant M-132 produced spontaneously parental orange-red colonies.

The high-yield variant of aclacinomycin A was further developed step by step from the strain 1U-47 by sequential mutation with NTG and UV, as shown in Fig. 2. During this process strain 9U-232 showed the reduced productivity of aclacinomycin B. The high-yield variant thus obtained, YA-1 exhibited 30-fold higher productivity of aclacinomycin A than the parent strain.

Fig. 2. Development of high-yield variants for aclacinomycin production.
■: Aclacinomycin A, □: Aclacinomycin B



Specific Glycosidic Metabolite-producing Mutants

Aclacinomycins A, B and concomitant MA144 N1 and S1 were usual yellow fermentation products in *S. galilaeus* and early isolates of yellow variants. Several mutants which permitted specific accumulation of one component of the parent metabolites or which produced new glycosidic metabolites having different sugar moiety were obtained.

Aclacinomycin B-accumulating Mutants

Strain 7U-364 was isolated from strain 6U-21. Aclacinomycin A and any other glycosidic metabolites were not detectable in this culture.

MA144 N1-accumulating Mutants

Strains KE-197 and 7N-1440 accumulated a single metabolite MA144 N1 in which terminal cinerulose of trisaccharide moiety of aclacinomycin A was reduced to rhodinose. It has been suggested that these mutants genetically lost a specific oxidoreductase which catalyzes the conversion of MA144 N1 to aclacinomycins A and Y[7].

MA144 S1-accumulating Mutants

Strain 7U-491 and other four strains were isolated from strains 6U-21 and YA-1, and accumulated specifically MA144 S1 having rhodosamine and 2-deoxyfucose, and a new metabolite MA144 U1 which has rhodosamine and 2 moles of 2-deoxyfucose at equivalent levels. Strain 7N-1881 produced one additional new metabolite MA144 U9 which has only 2 moles of 2-deoxyfucose.

Aminosugar-lacking Metabolite-producing Mutants

Strain 9U-653, 3AR-32 and 3AR-79 exhibited little or no antimicrobial activity against *Bacillus subtilis*, in spite of heavy production of yellow anthracycline pigments. It was found that the metabolites from these mutants did not contain rhodosamine. Major metabolites, designated as MA144 U5, U6, U7 and U8, were isolated from the 9U-653 culture and identified as described in a companion paper[6]. These compounds had 2-deoxyfucose in place of rhodosaminyl residue of parent products.

Aglycone-producing Mutants

Aklavinone-accumulating Mutants

Strains 3AR-33, 4N-69 and 2ETR-8 were isolated from strains 6U-21 and 2FA-89, and produced about 100 μg/ml of aklavinone without any detectable amount of anthracycline glycosides. Further mutation of strain 3AR-33 with NTG induced over productive variants ANR-635 and ANR-220 which yielded around 800 μg/ml of aklavinone.

New Aglycone-producing Mutants

Strains ANR-58 and ANR-665 were obtained from aklavinone producer 3AR-33 by NTG mutation and produced yellow and red aglycones, respectively. The products of mutant ANR-58 were composed of seven components 58A, B, C, D, E, F and G, among which 58D and 58G were major metabolites and structurally determined as described in a companion paper[6]: 58D was 2-hydroxyaklavinone and 58C was 2-hydroxy-7-deoxyaklavinone; 58G was a non-esterified analog of 58D. Mutant ANR-665 as well as mutant 7U-147 obtained from aclacinomycin A producer 6U-21, produced same brown and red unidentified aglycones 665A and 665B which gave Rf values of 0.74 and 0.26 on TLC using a solvent of $CHCl_3$ - methanol (20: 1, v/v). Mutant 7U-300 and two other isolates from strain 6U-21 produced also unidentified brownish yellow aglycones 300A and 300B, which gave Rf values of 0.90 and 0.03 on silica gel TLC with the same solvent.

Antibiotic-negative Mutants

Antibiotic-negative mutants occurred at a relatively high frequency during mutation. These mutants could be selected by a loss of acetone-extractable yellow pigment from mycelia. By feeding cultivation, strain KE-303 catalyzed the glycosidation of exogenous aklavinone to aclacinomycin A, but strain 3ETR-22 had no glycosidation activity. Strain KE-303 also glycosidated various type of rhodomycinones and semisynthetic analogs such as 6-*O*-methylaklavinone, 4-*O*-methylaklavinone *etc.*, and new glycosidic anthracyclines were produced from these aglycones as reported previously[8].

Table 2. Aglycones produced by blocked mutants and their precursor activity in the aclacinomycin biosynthesis.

	Aglycone-producing mutant No.						
	3AR-33	ANR-58		ANR-665		7U-300	
Fermentation products:	Aklavinone (yellow)	58D (yellow)	58G (yellow)	665A (brown)	665B (red)	300A (brown)	300B (brown)
Rf value $CHCl_3$ - MeOH (20: 1)	0.78	0.47	0.10	0.74	0.26	0.90	0.03
Benzene - acetone - formic acid (100: 20: 1)	0.50	0.27	0.16	0.43	0.58	—	—
Conversion by feeding cultivation:							
with strain KE-303	Formed aclacinomycin A	Formed 58D-glycoside	No change	No change	No change	No change	No change
with strain 6U-21	〃	〃	〃	〃	〃	〃	〃

58D and G were identified as 2-hydroxyalklavinone and non-esterified 2-hydroxyaklavinone analog, respectively.

58D-glycoside was identified as 2-hydroxyaclacinomycin A.

Products 665 and 300 have not been identified yet.

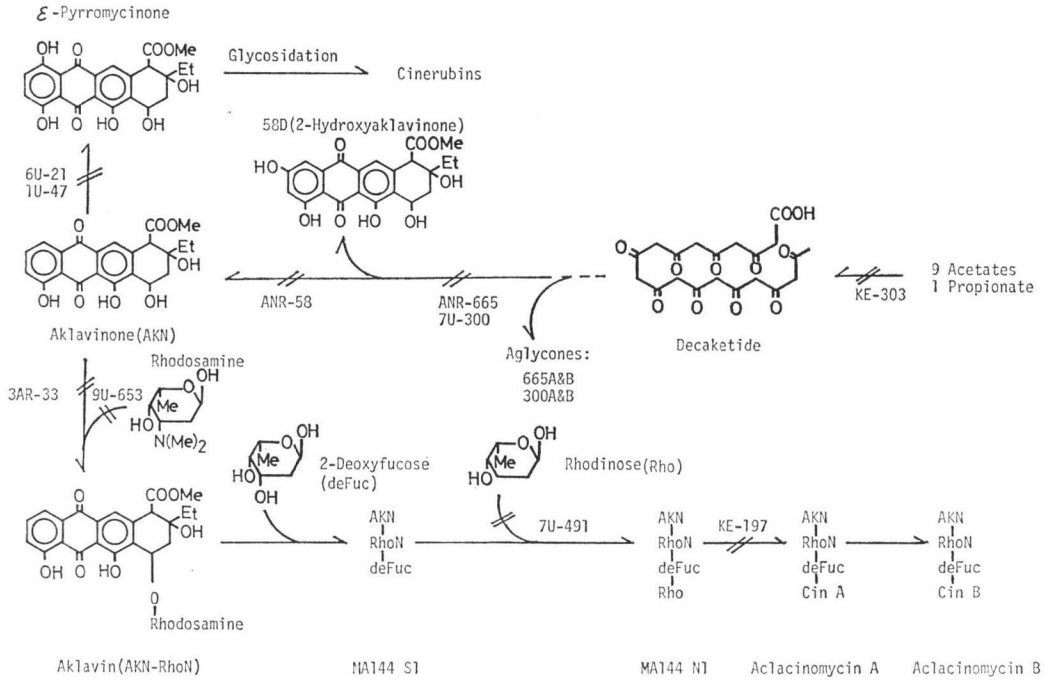
Biotransformation of Aglycones

Precursor activity of new aglycones obtained from mutants in the biosynthesis of aclacinomycin A was tested by feeding cultivation to either aclacinomycin A-producer 6U-21 in the presence or absence of cerulenin or to antibiotic-negative mutant KE-303. The results of biotransformation of aklavinone and new aglycones are summarized in Table 2. Strain KE-303 could glycosidate exogenous aklavinone to aclacinomycin A. Aclacinomycin A-producer 6U-21 also glycosidated aklavinone if *de novo* synthesis of aclacinomycin A was suppressed by cerulenin. It was found that aglycone 58D was transformed to 58D-glycoside, identified as 2-hydroxyaclacinomycin A[9] with or without cerulenin, but not to aclacinomycin A. Aglycone 58G and other aglycones 665A, 665B, 300A and 300B were not changed by feeding cultivation of 6U-21 and KE-303. This suggests that all of these aglycones were not precursors in the biosynthesis of aclacinomycin A and related antibiotics.

Discussion

Yellow variants capable of producing only aklavinone glycosides were obtained from the parent strain MA144-M1 of *S. galilaeus*. The parent strain produced a complex of glycosides of aklavinone and ε-pyrromycinone, but the mutants lacked the ability to oxidize the C-1 position of aklavinone. However strain M-132 produced only ε-pyrromycinone glycosides by the genetic enhancement of C-1 position oxidation. By the sequential mutation of the yellow variants, we developed a high-yield variant YA-1 with 30-fold higher productivity of aclacinomycin A than the parent strain, and also obtained several blocked mutants. Among the blocked mutants, MA144 N1-accumulating mutants had lost the ability to oxidize of terminal rhodinose to cinerulose A[7]. MA144 S1-accumulating mutants had a possible impairment in the biosynthetic supply of rhodinose since they produced rhodinose-lacking metabolites. Mutant 9U-653 and similar isolates had also possible impairment in the biosynthetic supply of aminosugar, rhodosamine. A group of mutants with specific accumulation of aklavinone, for example strain 3AR-33, appears to occur by the mutational damage of either glycosidation enzyme or biosynthetic enzymes of sugar components, while strain KE-303 has been blocked on the formation

Fig. 3. Blocked steps of the isolated mutants in the biosynthesis of anthracycline glycosides in *Streptomyces galilaeus* MA144-M1.



of hypothetical decaketide leading to aklavinone.

New aglycones such as 2-hydroxyaklavinone, 2-hydroxy-7-deoxyaklavinone produced by strains ANR-58, ANR-665 and 7U-300 may not be precursors in the biosynthesis of aclacinomycin A, since they were not converted to aklavinone or aclacinomycin A in the growing culture of aclacinomycin-producers. Thus, 2-hydroxyaklavinone was directly glycosidated to 2-hydroxyaclacinomycin A. These may imply that these new aglycones are by-products in the biosynthesis of aklavinone, and that the formation of aclacinomycin A is not due to the direct glycosidation of aklavinone once produced, but to the glycosidation of pre-aklavinone intermediate followed by modification of the aglycone moiety.

We have shown that the biosynthesis of aclacinomycin A from aklavinone proceeds by step-wise glycosidation[8]. From these results the biosynthesis of anthracycline antibiotics in *S. galilaeus* MA144-M1 is proposed as illustrated in Fig. 3.

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