Tetrahedron Vol. 47, No. 31, pp. 6059-6068, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00

GENETIC STUDIES OF THE BIOSYNTHESIS OF KALAFUNGIN, A BENZOISOCHROMANEQUINONE ANTIBIOTIC

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(*Received in USA* 16 *November* 1990)

Summary; Kalafungin-nonproducing mutants isolated from kalafungin-producing *Streptomyces tanashiensis* strain Kala were classified into seven distinct biosynthetic phenotypes. The transformation system for this strain was established by a combination of a novel restriction-reduced mutant and heat treatment of the protoplasts. By colony hybridization with the genes for polyketide synthase (*act* I and III) as probes, a clone carrying a large DNA fragment (28 kb) was picked up from a genomic library of this strain. In this DNA fragment, the gene cluster for kalafungin biosynthesis containing at least eight genes (*kal* I to VII, and regulatory gene(s)) was identified. Although kalafungin is an intermediate of actinorhodin biosynthesis in *S. coelicolor* A3(2), this gene cluster for kalafungin biosynthesis in *S. tanashiensis* was not identical with that in *S. coelicolor*. This cloned DNA fragment caused *S. tanashiensis* wild-type strain and *kal* mutants to produce a new antimicrobial product named tetrahydrokalafungin.

1. Introduction

Antibiotics often are secondary metabolites which are synthesized *via* multistep pathways utilizing primary metabolites, and their structures and activities are varied. Studies of antibiotic biosynthesis lead to understanding of how antibiotics are formed in producing strains, as well as how to apply the information to stimulate industrial production of antibiotics and the production of new antibiotics.

The benzoisochromanequinone antibiotics, including kalafungin[1,2], actinorhodin[3], and nanaomycin[4], possess antibacterial, antifungal and antimycoplasma activities. Nanaomycin A is used as a chemotherapeutic agent against animal dermatomycosis. These antibiotics are synthesized from eight acetate units *via* a hypothetical intermediate "polyketide" (Fig. 1)[4, 5]. Kalafungin is produced by *S. tanashiensis* strain Kala and also is an intermediate of actinorhodin biosynthesis in *S. coelicolor* A3(2)[6].

Here, we describe kalafungin biosynthesis in *S. tanashiensis* strain Kala.

2. Isolation and characterization of kalafungin-nonproducing mutants[7].

To define the biosynthetic pathway for kalafungin in *S. tanashiensis*, kalafungin-nonproducing (*kal*) mutants were isolated after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and characterized by the cosynthetic procedure[8]. The cosynthetic relationships between two mutants, *i. e.*, whether the mutant acts as a

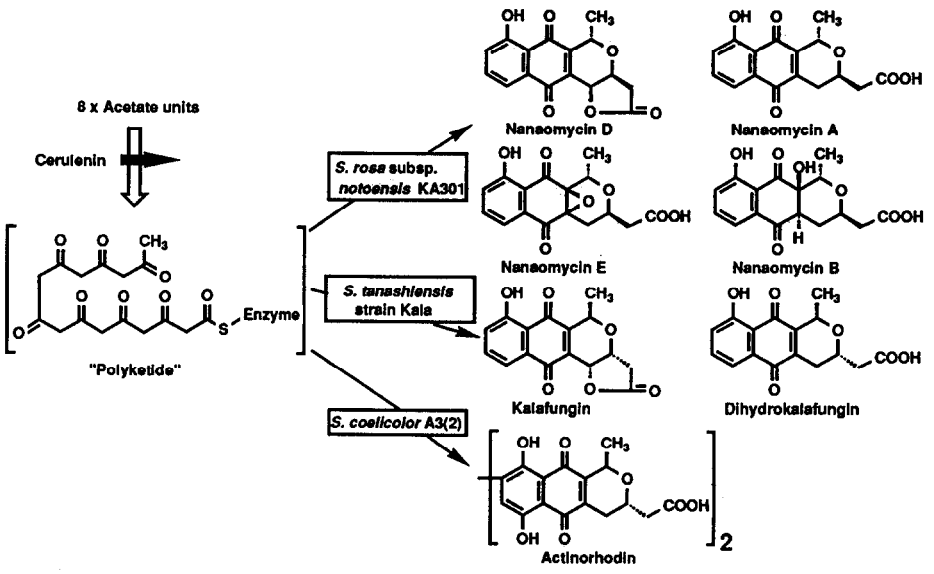


Fig. 1. Biosynthesis of bezoisochromanequinone antibiotics

converter or a secretor strain are summarized in Table 1. From these results, the first nine mutants (*kal* 16 to *kal* 2) were classified into seven distinct phenotypes in the kalafungin biosynthetic pathway as follows; *Kal* I→II→III→IV→V→VI→VII→kalafungin. It was interesting that the *kal*1, 12 or 13 mutants, which acted as converter strains, cosynthesized unidentified antimicrobial compounds that were not detected in the other cosynthetic reactions. These mutants were also able to act as secretors and caused mutants of type I to VI to cosynthesize kalafungin. Therefore, these mutants contain at least two mutations in the biosynthetic pathway. One of the kal mutations affects the step between type VI and VII *kal* mutants because the *kal*1, 12 or 13 caused mutants type I to VI to cosynthesize kalafungin but not type VII mutant. Another mutation would affect in backward type VII mutation, therefore mutants *kal*1, 12 and 13 did not convert an intermediate derived from type VII mutant to kalafungin. The mutants *kal* 4, 9, and 10, which showed no cosynthesis with any other mutants, were considered to be affected in a regulatory region as reported for the *act* II mutant of *S. coelicolor* A3(2)[9]. These results indicate that in *S. tanashiensis* kalafungin is synthesized from acetate units *via* at least seven steps.

Since kalafungin is an intermediate in actinorhodin biosynthesis in *S. coelicolor*[6], it was expected that the biosynthetic process from acetate units to kalafungin in this strain is identical with that in *S. tanashiensis*. To confirm this possibility, cosynthetic experiments between *kal* mutants of *S. tanashiensis* and *act* mutants of *S. coelicolor* were then performed. Four kinds of *kal* mutants (*kal* I to IV), which were affected in the early to middle steps in kalafungin biosynthesis, were found to act as convertor strains and cosynthesize kalafungin with *act* mutants as secretors, whereas *kal* V to VII mutants did not act as convertors. These results suggest that except for the early steps, the biosynthetic pathway for kalafungin in *S. tanashiensis* is not identical with that in *S. coelicolor*.

Table 1. Cosynthetic pattern among *kal* mutants

Converter Class (*kal*)		Secretor (*kal*)[a] 16	14	6	8	5	11	15	3	2	12	13	1	4	9	10	Production of kalafungins[b]
I	16	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
II	14	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+
III	6	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	8	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+
IV	5	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	+
V	11	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+
	15	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+
VI	3	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+
VII	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	12	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
	13	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
	1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] +: Cosynthesis was observed. - : cosynthesis was not observed.
[b] Production of kalafungin and dihydrokalafungin by converter was verified by TLC.
+ : produced; - : not produced.

3. Establishment of transformation system for *S. tanashiensis*.[10]

In cloning the genes for antibiotic biosynthesis, the transformation of antibiotic-nonproducing mutants by the cloned DNA fragment is an important process for functional analysis. However, in preliminary experiments with the wild-type strain no transformants were obtained by transformation of the protoplasts with 10 μg of multi-copy plasmid pIJ702 or single-copy plasmid pKU3[10], and no actinophages with different host ranges (Pal6, R4, φC31 and φKU1[10]) were propagated in this strain. These results indicate that this wild-type strain has a strong restriction system(s).

To isolate restriction-reduced mutants, the protoplasts derived from NTG-treated spores were transformed with 10 μg of the plasmid pIJ702 propagated in *S. lividans* TK24. The transformants (thiostrepton-resistant) obtained were cured and the thiostrepton-sensitive variants (plasmid-cured) were examined for transformation with 2 μg of bifunctional cosmid pKU206[10] propagated in *E. coli*. The mutant R3-5 was selected as a restriction-reduced mutant, but its transformation efficiency was not strong enough for gene cloning.

In the transformation of the mutant R3-5, we found that heat treatment of the protoplasts at 42°C for 15 min stimulated its efficiency. Using heat-treated protoplasts (Table 2), the plasmids propagated in the mutant R3-5 were able to transform both strains of *S. tanashiensis* wild-type and *kal* mutants at the efficiency of 10^3-10^5 transformants/μg of DNA. Further, the high transformation efficiency was obtained without heat treatment when the plasmids propagated in those strains transformed their last hosts.

Table 2. Transformation of *S. tanashiensis* derivatives with the plasmid pKU4[10] propagated in various strains and effect of heat treatment on the transformation

Host strain	Propagated in	Heat treatment [a]	Transformation efficiency (transformants/μg of pKU4)
Wild-type	*S. lividans* TK24	+	0
		-	0
	S. tanashiensis R3-5	+	1.0×10^3
		-	0
	S. tanashiensis wild-type	+	2.1×10^4
		-	1.0×10^5
R3-5	*S. lividans* TK24	+	5.0×10^3
		-	0
	S. tanashiensis R3-5	+	4.2×10^4
		-	1.2×10^5
kal	*S. lividans* TK24	+	0
		-	0
	S. tanashiensis R3-5	+	5.0×10^3 -1.2×10^5
		-	0
	S. tanashiensis kal	+	1.0×10^4 - 2.0×10^5
		-	2.0×10^4 - 4.5×10^5

a: Heat treatment was done at 42°C for 15 min.

4. Cloning of the genes for kalafungin biosynthesis in *S. tanashiensis*.[12]

The genes involved in antibiotic biosynthesis so far reported are clustered[13]. All the genes (*act* I, II, III, IV, VA, VB, VI and VII) involved in actinorhodin biosynthesis in *S. coelicolor* A3(2) have been cloned and shown to be located on about a 20 kb DNA fragment[14]. As both kalafungin and actinorhodin are synthesized *via* a hypothetical "polyketide" intermediate, the genes for polyketide synthesis may be similar in both producing strains. Consequently, we assumed that the genes for kalafungin biosynthesis in *S. tanashiensis* are located in a 20-40 kb DNA fragment containing a region homologous to the polyketide synthase gene (*act* I and III) for actinorhodin biosynthesis.

A genomic library of *S. tanashiensis* was prepared in *E. coli* JM108 using the *Streptomyces-E. coli* bifunctional cosmid pKU205 (10 kb)[12] to clone a large DNA fragment and to introduce a recombinant plasmid directly into this *Streptomyces* for functional analysis. The clone carrying pKU501, in which the 28 kb DNA fragment (Fig. 2) was identified, was picked up by colony hybridization using the polyketide synthase genes (*act* I and III) as probes. The Southern blot hybridization analysis of the cloned DNA fragment shows that this DNA fragment contains the regions hybridized with *act* I, III, VA and VI genes but not with *act* II, IV, VB and VII genes. The arrangement of the four regions that hybridized is similar to that in the gene cluster for actinorhodin biosynthesis (Fig. 2).

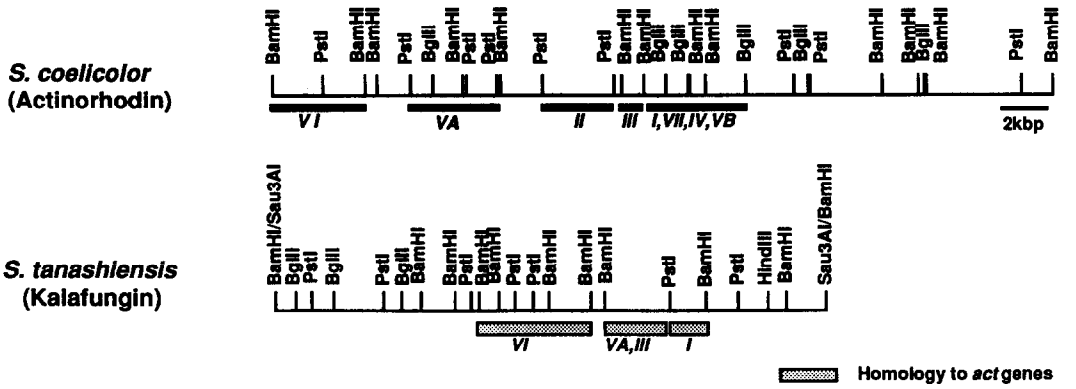


Fig. 2. Restriction maps of cloned DNA involved in bezoisochromanequinone antibiotic biosynthesis.

The restriction map of gene cluster of actinorhodin biosynthesis of *S. coelicolor* was referenced in Ref.14.

To determine whether the cloned DNA fragment contains the genes involved in kalafungin biosynthesis, complementation experiments on *kal* mutants were carried out[12]. Although many transformants of *S. lividans* TK24 were obtained by introducing the recombinant plasmid pKU501 containing large fragment (Fig. 3), the plasmid replicated poorly in *S. lividans* TK24. The missing genes for kalafungin biosynthesis were identified by the following two approaches (Fig. 3).

First, the subclones (pKU507 to pKU522) derived from pKU501 were used. They were constructed in *S. lividans* TK24 using single-copy plasmid pKU4[6], a derivative of SCP2* of *S. coelicolor* A3(2). After their propagation in the restriction-reduced mutant *S. tanashiensis* R3-5, all the *kal* mutants were transformed by each subclone and the production of kalafungin by their transformants was determined. All the transformants tested produced kalafungin only on agar medium. As shown in Fig. 4, the mutants *kal* I to VII classified in the biosynthetic pathway, were restored to kalafungin production by introduction of the fragments. However, the other six mutants (*kal* 1, 12, 13, 4, 9 and 10) were not complemented by any of these subclones.

Second, the whole cloned fragment was introduced into *kal* mutants to determine whether the genes affected in all *kal* mutants were located on it. As shown in Fig. 3, the vector region of pKU501 contains the replication origin of SCP2* but not the stability region, which is important for stable replication in *Streptomyces*[15]. The stability region of SCP2* (5 kb) was introduced into a unique *Eco*RI site (in the region of vector pKU205) of pKU501. All *kal* mutants were transformed by the recombinant plasmid pKU523, and the resulting transformants produced kalafungin not only on an agar medium but also in a liquid medium. All the *kal* mutants shown in Table 1 were restored to kalafungin production by introduction of pKU523.

The DNA fragment cloned from the genomic library of kalafungin-producing *S. tanashiensis* strain Kala was determined to contain at least eight genes (*kal* I to VII, and regulatory gene(s)). As shown in Fig. 5, the regions of the seven genes (*kal* I to VII) which correspond to seven steps in the kalafungin biosynthetic pathway were identified on the cloned DNA fragment. The five genes (*kal* I, II, IV, VI and VII) were located in the region homologous to the genes for actinorhodin biosynthesis, but the other two genes (*kal* III and V) were not. Since

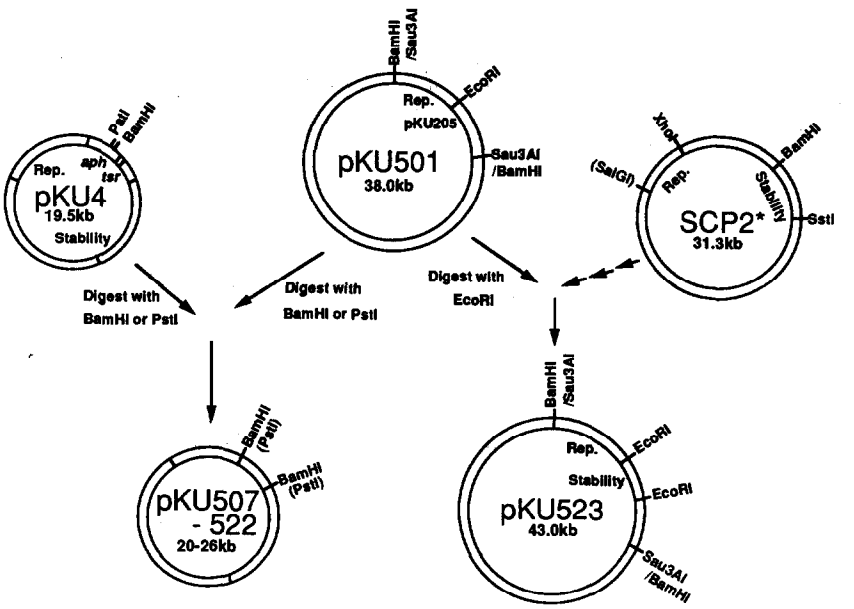


Fig. 3. Construction of subclones and introduction of the stability region of SCP2* into pKU501

Genetic markers are abbreviated as follow; Rep.=the replication region of SCP2* of *S. coelicolor* A3(2), Stability=the stability region of the maintainance of SCP2*, aph=neomycin resistant determinant of *S. fradiae*, tsr=thiostrepton resistant determinant of *S. azureus*. *Bam*HI-*Sst*I fragment of stability region of SCP2* was processed and introduced *Eco*RI site by a synthetic linker.

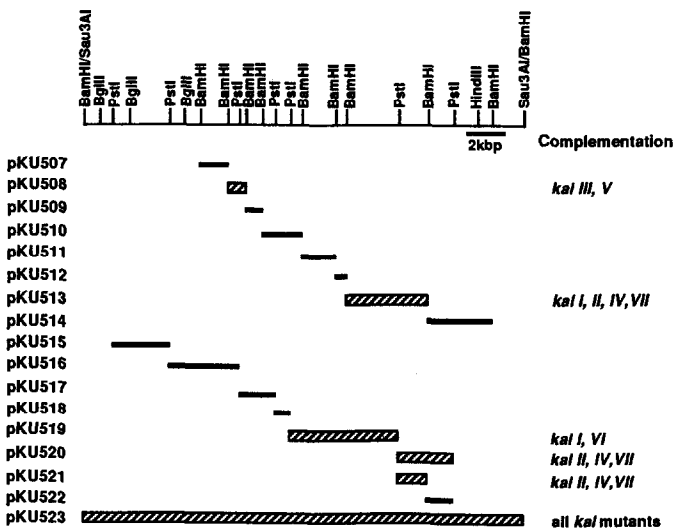


Fig. 4. Analysis of *kal* region by restoration of production to blocked mutants

The complementation analysis was described in Text. The hatched bar means the fragment which restore productivily of kalafungin to blocked mutants.

kalafungin is an intermediate in actinorhodin biosynthesis in *S. coelicolor*, it was assumed that the genes for kalafungin biosynthesis in *S. tanashiensis* are similar in both strains. However, the gene cluster for kalafungin

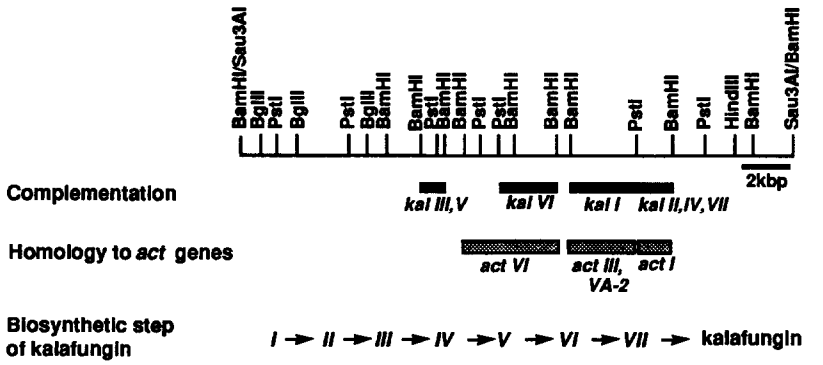


Fig. 5. Gene cluster of kalafungin biosynthesis in *S. tanashiensis*.
Regions which restore productivity to nonproducing mutants representing steps *kal*I to VII were mapped on the results shown in Fig. 4.

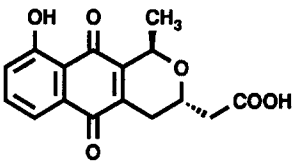
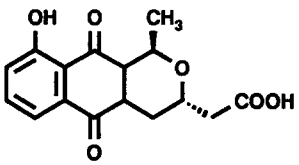
biosynthesis in *S. tanashiensis* is not identical to that in *S. coelicolor*. These results agree with those of cosynthesis experiments between *kal* mutants and *act* mutants described earlier.

5. Production of a new antimicrobial substance

When the recombinant plasmid (pKU523) containing the gene cluster for kalafungin biosynthesis was introduced into *kal* mutants or the wild-type strain, the transformants produced not only kalafungin and dihydrokalafungin but also a new analog of kalafungin, tetrahydrokalafungin. To elucidate the structure of the new compound, we isolated it from a 24-hr culture of the transformants. After the cultured broth was adjusted to pH 2, it was extracted with an equal volume of chloroform. The chloroform extract was concentrated under reduced pressure and then the new antibiotic was purified by preparative silica gel thin layer chromatography using chloroform/methanol(25:2) as a developing solvent. The new antibiotic was labile and easily converted to dihydrokalafungin under neutral or alkaline conditions. From the results of structural analysis of the new antibiotic (UV, SIMS, NMR etc.), the structure has been determined as shown in Table 3.

As shown in Fig. 6, the transformants began to produce tetrahydrokalafungin at 24 hrs of cultivation. At this period, kalafungin and dihydrokalafungin were not produced by transformants. These two antibiotics were produced at 48 hrs of cultivation by the wild type strain or transformants. The production of tetrahydrokalafungin continued until 48 hrs and it disappeared by 72 hrs. We assume that the production of the new antibiotics was caused by the 28 kb DNA introduced into *kal* mutants or the wild type strain.

Table 3. Physico-chemical properties of dihydrokalafungin and tetrahydrokalafugin

	Dihydrokalafungin	Tetrahydrokalafungin
Appearance	yellow	pale yellow
SIMS m/z	303 $(M+1)^+$	305 $(M+1)^+$
Formula	$C_{16}H_{14}O_6$	$C_{16}H_{16}O_6$
λ max(nm)	250, 274, 423 in CH_3OH	241, 256sh, 348 in CH_3OH
	280, 520 in 0.1*N* NaOH-CH_3OH	280, 520 in 0.1*N* NaOH-CH_3OH
Structure		

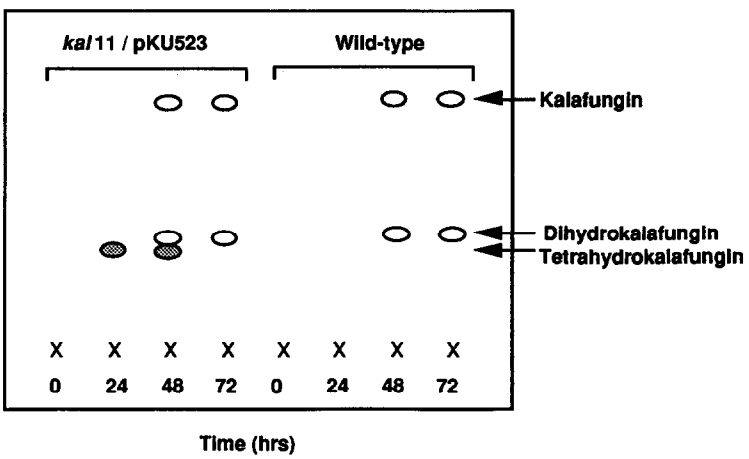


Fig. 6. Time course of production of tetrahydrokalafungin.

Each cultured broth was extracted with equal volume of ethyl acetate and then ethyl acetate layer was evaporated to dryness. The crude extract was dissolved in small volume of chloroform and a portion of the extract was applied to a silica gel thin layer chromatography using chloroform/methanol(25:2) as a developing solvent.

6. Conclusion

The biosynthesis of the benzoisochromanequinone antibiotic kalafungin in *S. tanashiensis* was studied. After cosynthetic experiments between *kal* mutants, we proposed that the kalafungin biosynthetic pathway in this strain involves at least 7 steps (*kal* I to VII).

A transformation system for *S. tanashiensis* was established by using a restriction-reduced mutant and heat treatment of protoplasts. The first report of the use of restriction-reduced mutants in transformation of *Streptomyces* strain has been done by Hunter *et al.*[11]. Also, an effect of heat treatment of protoplasts on transformation has been reported in *S. clavuligerus* by Baily *et al.*[16]. These methods to prevent restriction systems as shown here might be useful in transformation in other streptomycete strains.

The gene cluster for kalafungin biosynthesis, which contains at least eight genes (*kal* I to VII, and regulatory gene(s)), has been cloned as a large DNA fragment from *S. tanashiensis* using a novel bifunctional cosmid pKU205. In comparison with the gene cluster for actinorhodin biosynthesis in *S. coelicolor*, some particular genes, such as polyketide synthase genes (*act*I and III), are similar, but the whole gene cluster for kalafungin biosynthesis is not identical. These results suggest that the gene clusters for kalafungin biosynthesis are specific in individual strains.

A new antibiotic was produced when the clone DNA fragment containing the kalafungin gene cluster was introduced into *S. tanashiensis* wild and its *kal* mutants. The new antibiotic could be an intermediate, a shunt product or a final product in the kalafungin biosynthetic pathway.

7. Experiments

Microbiological techniques.

Tripticase Soy Broth (TSB) and YMS medium[17] were used for cultivation of *Streptomyces* strains. The antibiotic production medium used was YMS (*S. tanashiensis* cultured alone) or CM[18] (*S. coelicolor* and *S. tanashiensis* cultured together), respectively. The production of antibiotic was estimated by bioassay by a synthetic medium seeded with spores of *Bacillus subtilis* PCI 219[7]. R2YE medium[18] and MR0.3S medium[10] were used for regeneration of protoplasts of *S. lividans* and *S. tanashiensis*, respectively. Mutagenesis was done by treatment with NTG as described previously[7]. Cosynthesis experiment was done by the method described by Delic et al.[8] with some modifications[7]. *S. tanashiensis* and *S. lividans* were transformed as described by the present authors[10] and Hopwood *et al.*[18], respectively. *E. coli* was cultured in L-Broth or L-Agar which was L-Broth supplemented with 1.5% agar[21]. Packaging DNA into the lambda head was done as described by Kobayashi and Ikeda[20], using the packaging extract prepared with *E. coli* NS428 and NS433.

Identification of antimicrobial products.

The products in the agar production medium or in the liquid production medium were extracted with chloroform or ethyl acetate and then separated by silica gel TLC. The bioautogram was compared with those of authentic samples of kalafungin and dihydrokalafungin as described previously[7].

DNA manipulations.

Chromosomal DNA was isolated from *S. tanashiensis* strain Kala as described by Chater *et al.*[21] Plasmid DNA was isolated from *Streptomyces* and *E. coli* as described by Kieser[22]. Purification of DNA fragments separated by agarose gel electrophoresis was performed by the method of Chen and Thomas[23]. Nick translation and southern hybridization were done as described by Hopwood *et al.*[18]. A genomic library of *S. tanashiensis* was prepared in *E. coli* JM108 using *Streptomyces-E. coli* bifunctional cosmid pKU205 by an *in vitro* packaging system as described by the present authors[12]. The transductants were replicated onto Colony/Plaque Screen membranes (NEF-978/987A NEN research products). The colony hybridization was done with nick-translated probe (9 kb DNA segment containing *act* I and III genes) as described by the present authors[12].

References

1. Bergy, M. E. *J. Antibiot.* **1968,** 21, 454-457.
2. Hoeksema, H.; Kruger, W. C. *J. Antibiot.* **1976,** 29, 704-709.
3. Brokmann, H.; Zeek, A.; Merwe, K. V. D.; Muller, W. *Justis. Liebigs Ann. Chem.* **1966,** 698, 2909-2929.
4. Tanaka, H.; Koyama, Y.; Nagai, T.; Marumo, H.; Ōmura, S. *J. Antibiot.* **1975,** 28, 868-875.
5. Grost-allman, C. P.; Rudd, B. A. M.; Chang, C.-J.; Floss, H. G. *J. Org. Chem.* **1981,** 46, 4555-456.
6. Cole, S. P.; Rudd, B. A. M.; Hopwood, D. A.; Chang, C.-J.; Floss, H. G. *J. Antibiot.* **1987,** 40, 340-347.
7. Kakinuma, S.; Ikeda, H.; Ōmura, S.; Hopwood, D. A. *J. Antibiot.* **1990,** 43, 391-395.
8. Delic, V.; Pigac, J.; Sermonti, G. *J. Gen. Microbiol.* **1969,** 55, 103-108.
9. Rudd, B. A. M.; Hopwood, D. A. *J. Gen. Microbiol.* **1979,** 114, 35-43.
10. Kakinuma, S.; Ikeda, H.; Tanaka, H.; Ōmura, S. *Agric. Biol. Chem.* **1990,** 54, 2611-2617.
11. Hunter, I. S.; Fried, E. *J. Biochemistry Society transactions.* **1984,** 12, 643-644.
12. Kakinuma, S.; Takada, Y.; Ikeda, H.; Tanaka, H.; Ōmura, S.; Hopwood, D. A. (submitted)
13. Martin, F. J.; Liras, P. *Ann. Rev. Mcrobiol.* **1989,** 43, 173-206.
14. Malpartida, F.; Hopwood, D. A. *Mol. Gen. Genet.* **1986,** 205, 66-73.
15. Lydiate, D. J.; Malpartida, F.; Hopwood, D. A. *Gene* **1985,** 35, 223-235.
16. Baily, R. C.; Winstanly, D. J. *Gen. Microbiol.* 132, 2945-2946.
17. Ikeda, H.; Kotaki, H.; Tanaka, H.; Ōmura, S. *Antimicrob. Agents & Chemother.* **1987,** 32, 282-284.
18. Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kiesrt, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. **1985,** Genetic manipulation of *Streptomyces*, a laboratory manual. The John Innes Foundation, Norwich.
19. Miller, J. **1972,** Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. Kobayashi, I.; Ikeda, H. *Mol. Gen. Genet.* **1977,** 153, 237-245.
21. Chater, K. F.; Hopwood, D. A.; Kieser, T.; Thompson, C. J. *Curr. Topics, Microbiol. Immunol.* **1982,** 96, 69-95.
22. Kieser, T. *Plasmid* **1984,** 12, 19-36.
23. Chen, C.; Thomas Jr, C. A. *Anal. Biochem.* **1980,** 101, 339-341.