# CLONING OF LARGE DNA FRAGMENTS, WHICH HYBRIDIZE WITH ACTINORHODIN BIOSYNTHESIS GENES, FROM KALAFUNGIN AND NANAOMYCIN A METHYL ESTER PRODUCERS AND IDENTIFICATION OF GENES FOR KALAFUNGIN BIOSYNTHESIS OF THE KALAFUNGIN PRODUCER

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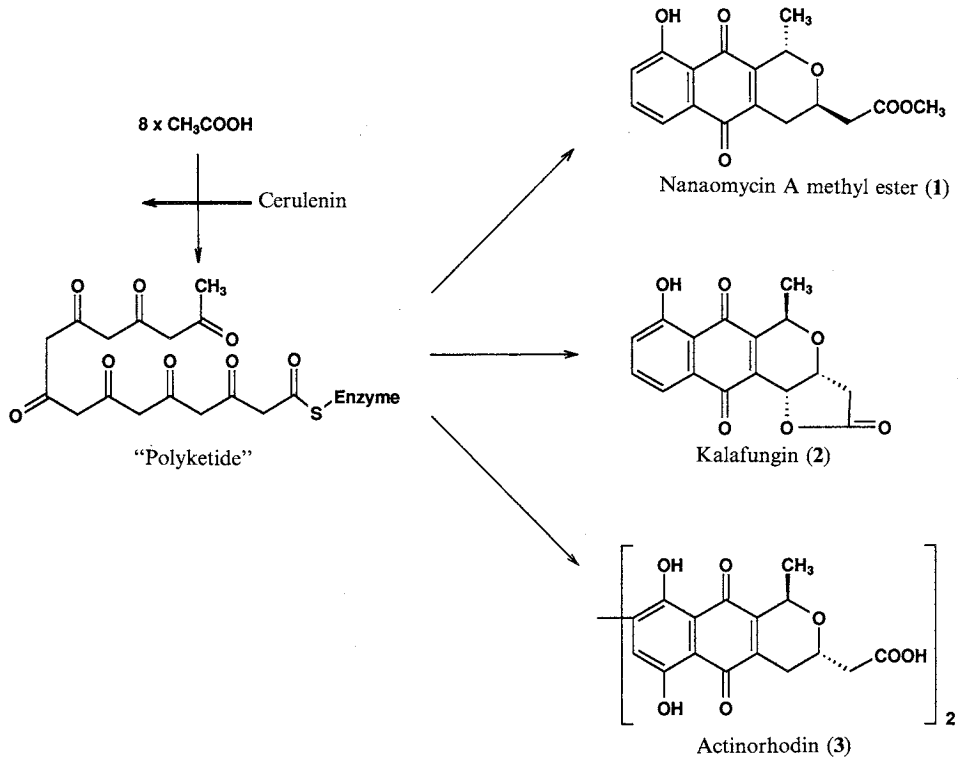
Large *act*I, III-homologous DNA fragments were isolated from genomic libraries of the strains that produce the benzoisochromanequinone antibiotics kalafungin and nanaomycin A methyl ester, *Streptomyces tanashiensis* strain Kala and *Streptomyces* sp. OM-173, respectively. These libraries were prepared in *Escherichia coli* JM108 by using a novel *Streptomyces-E. coli* bifunctional cosmid, pKU205, and screened with polyketide synthase genes (*act*I and III) for actinorhodin biosynthesis from *Streptomyces coelicolor* A3(2) as probes. The cloned DNA fragments (28 and 42 kb) were analyzed by hybridization with DNA containing actinorhodin biosynthetic genes (*act*I, II, III, IV, VA, VB, VI and VII). Both fragments hybridized with the *act*I, III, VA and VI regions, but not with the *act*II, IV, VB and VII regions. The cloned fragment of *S. tanashiensis* DNA was analyzed by complementation tests with kalafungin-nonproducing mutants. Seven genes (*kal*I ~ VII), which correspond to seven steps in kalafungin biosynthesis, were found to be located on a 14 kb continuous DNA fragment. Five of the genes were located on the regions homologous to the genes for actinorhodin biosynthesis, but the other two genes were not. Although kalafungin is an intermediate or shunt product in actinorhodin biosynthesis in *S. coelicolor* A3(2), the genes for kalafungin biosynthesis in *S. tanashiensis* are not identical with those in *S. coelicolor* A3(2).

The benzoisochromanequinone antibiotics, kalafungin (**2**) and nanaomycin A methyl ester (**1**), are produced by *Streptomyces tanashiensis* strain Kala and *Streptomyces* sp. OM-173, respectively[1,2] (Fig. 1). Kalafungin is an enantiomer of nanaomycin D[3]. Actinorhodin (**3**), which is produced by *Streptomyces coelicolor* A3(2)[4], is a dimer of a hypothetical "hydroxylated kalafungin" (Fig. 1).

Biosynthetic studies of benzoisochromanequinone antibiotics have revealed that they are synthesized from eight acetate residues *via* a hypothetical intermediate "polyketide"[5~7] (Fig. 1). Kalafungin is an intermediate (or shunt product) of actinorhodin biosynthesis in *S. coelicolor* A3(2)[8]. The whole gene cluster for actinorhodin biosynthesis has been isolated from *S. coelicolor* A3(2) and introduction of the cluster into different *Streptomyces* species caused the production of actinorhodin[9]. Eight genes (*act*I, II, III, IV, VA, VB, VI and VII) for actinorhodin biosynthesis were found to be located in the cluster[10] (the original class V of *act* mutant was later subdivided by biochemical[8] and genetic evidence into sub-classes A and B). The *act*I and III genes, which are involved in polyketide synthesis, were shown to hybridize

Fig. 1. Proposed biosynthesis of benzoisochromanequinone antibiotics.




with chromosomal DNA from many other polyketide antibiotic-producing *Streptomyces*[11,12].

We are interested in comparing the biosynthetic genes of the benzoisochromanequinone antibiotics kalafungin, nanaomycin and actinorhodin. In this paper, we describe the cloning of large DNA fragments, which hybridize with *act*I,III DNA, from genomic libraries of *S. tanashiensis* strain Kala and *Streptomyces* sp. OM-173 DNA, respectively. We also describe the identification of a series of genes for kalafungin biosynthesis.

## Materials and Methods

### Bacterial Strains and Plasmids

The bacterial strains and plasmids used are listed in Table 1.

### Media and Culture Conditions

*Escherichia coli* was cultured in L-broth or L-agar, which was L-broth supplemented with 1.5% agar[13]. R2YE medium[14] and MR0.3S medium[15] were used for regeneration of protoplasts of *Streptomyces lividans* and *S. tanashiensis*, respectively. Trypticase Soy Broth (TSB) and YMS medium[16] were used for cultivation of *Streptomyces* strains.

### Chemicals and Enzymes

Thiostrepton was provided by Asahi Chemical Industry, Japan. The restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd., Japan, or New England Biolabs, Inc., U.S.A. Conditions for the enzyme reactions used were those recommended by the vendors.

### Transformation and Transduction

*S. tanashiensis* and *S. lividans* were transformed as described by the authors[15] and HOPWOOD *et al.*[14],

Table 1. Bacterial strains and plasmids.

| Designation | Relevant characteristics | References |
|---|---|---|
| Bacterial strains: | | |
| *Escherichia coli* JM108 | F$^-$, *recA* 1, *endA* 1, *gyrA* 96, *thi*-1, *hsdR* 17, *supE* 44, *relA* 1, $\lambda^-$, *Δ(lac-proAB)* | 24 |
| JM109 | *recA* 1, *endA* 1, *gryA* 96, *thi*-1, *hsdR* 17, *supE* 44. *relA* 1, $\lambda^-$, *Δ(lac-proAB)*, [F′, *traD* 36, *proAB*, *lacI$^q$ZΔ*M15] | 24 |
| DH1 | F$^-$, *recA* 1, *endA* 1, *gyrA* 96, *thi*-1, *hsdR* 17, *supE* 44, *relA* 1, $\lambda^-$ | 25 |
| HB101 | F$^-$, *hsdS* 20, *supE* 44, *ara*-14, *galK*-2, *lacY* 1, *proA* 2, *rpsL* 20, *xyl*-5, *mtl*-1, $\lambda^-$, *recA* 13 | 25 |
| SF8 | F$^-$, *supE* 44, *thi*-1, *thr*-1, *leuB* 6, *lacY* 1, *tonA* 21, $\lambda^-$, *hsdR* 17, *recBC*, *lop*-11, *lig*$^+$ | 26 |
| NS428 | N205 *recA* 1 (*λcI*ts857 *Aam*11 *Sam*7b2 *red*3) | 27 |
| NS433 | N205 *recA* 1 (*λcI*ts857 *Eam*4 *Sam*7b2 *red*3) | 27 |
| *Streptomyces lividans* TK24 | Host for plasmid preparation (SLP2$^-$, SLP3$^-$, *str*-6) | 28 |
| *S. tanashiensis* strain Kala | Kalafungin-producing strain | 1 |
| *kal*16 (*kal*I) | Kalafungin-nonproducing mutant | 21 |
| *kal*14 (*kal*II) | Kalafungin-nonproducing mutant | |
| *kal*6,8 (*kal*III) | Kalafungin-nonproducing mutant | |
| *kal*5 (*kal*IV) | Kalafungin-nonproducing mutant | |
| *kal*11,15 (*kal*V) | Kalafungin-nonproducing mutant | |
| *kal*3 (*kal*VI) | Kalafungin-nonproducing mutant | |
| *kal*2 (*kal*VII) | Kalafungin-nonproducing mutant | |
| R3-5 (*sta*A 1) | Restriction-reduced mutant | 15 |
| *Streptomyces* sp. OM173 | Nanaomycin A methyl ester-producing strain | 2 |
| Plasmids: | | |
| pKU 205 | *Streptomyces-E. coli* bifunctional cosmid SCP2* ori, pMB1 ori, *tsr*, *bla*, *cos* | This paper |
| pKU4 | SCP2* ori, SCP2* stability, *tsr*, *aph* | This paper |

respectively. Packaging DNA into the $\lambda$ head was done as described by KOBAYASHI and IKEDA[17], using the packaging extract prepared with *E. coli* NS428 and NS433 (provided by Prof. T. TAKANO of Keio University, Tokyo).

## Isolation of Chromosomal and Plasmid DNA

Chromosomal DNA was isolated from *S. tanashiensis* strain Kala or *Streptomyces* sp. OM-173 as described by CHATER *et al.*[18] except that the growth medium used for *S. tanashiensis* was TSB supplemented with 0.25% glycine and 5 mM $MgCl_2$. Plasmid DNA was isolated from *Streptomyces* and *E. coli* as described by KIESER[19]. For large scale plasmid preparation, the DNA was further purified by cesium chloride ethidium bromide equilibrium centrifugation[14]. Purification of DNA fragments separated by agarose gel electrophoresis was performed by the method of CHEN and THOMAS[20].

## Nick Translation and Southern Hybridization

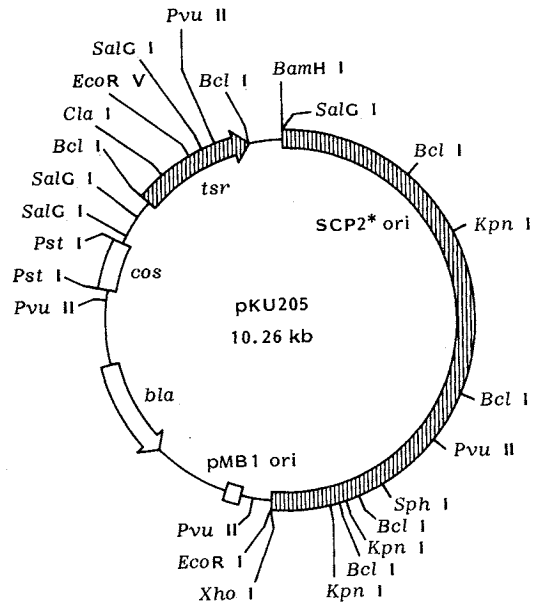
Nick translation and Southern hybridization were done as described by HOPWOOD *et al.*[14].

## Cloning Procedure

Genomic libraries of *S. tanashiensis* strain Kala and *Streptomyces* sp. OM-173 were prepared in *E. coli* using the *Streptomyces-E. coli* bifunctional cosmid pKU205 (Fig. 2) as follows. Vector pKU205 was digested with *Bam*H I completely and the linear molecule formed was treated with calf intestine alkaline phosphatase (Boehringer Mannheim). Chromosomal DNA isolated from *S. tanashiensis* strain Kala or *Streptomyces* sp. OM-173 was partially digested with *Sau* 3 AI under conditions that gave a visible widening of the chromosomal band on a stained agarose gel to yield an average fragment size of 30~40 kilobase pairs (kb). Two $\mu$g of processed vector DNA was ligated overnight with 1 $\mu$g of *Sau* 3 AI-digested 20~40 kb chromosomal DNA in 20 $\mu$l of ligation buffer plus 1 unit of T4 DNA ligase at 8°C. The ligated DNA was packaged in $\lambda$ heads by an *in vitro* packaging system. Maltose-induced *E. coli* was infected with the mature

phages and transductants were selected on L-agar containing 75 $\mu$g of ampicillin per ml. The transductants were replicated onto Colony/Plaque Screen membranes (NEF-978/978A, NEN research products) and colonies on the membranes were lysed and the DNAs were fixed on the membranes by the ordinary method. The membranes were bagged with 10 ml of hybridization buffer (formamide 50%, sodium lauryl sulfate (SDS) 1%, NaCl 1 M, Tris-HCl (pH 7.5) 50 mM, dextran sulfate 10%). Prehybridization was performed at 55°C for 20 hours; then nick-translated probe (a 9 kb DNA fragment containing *act*I and III genes: the *Pst*I fragment between site 12 and 20 on the map in ref 10) was added and the bag was sealed. Hybridization was performed at 55°C for 20 hours. After hybridization, filters were washed twice with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% (w/v) SDS at 45°C for 30 minutes and then twice with 0.1 × SSC at room temperature for 30 minutes.

Fig. 2. Restriction map of bifunctional cosmid pKU205.




Identification of Anitimicrobial Products Obtained in Complementation Experiments

The transformants were transferred onto agar pieces prepared on YMS medium containing 2 $\mu$g of thiostrepton per ml and incubated at 30°C for 3 days. After incubation, the agar pieces were transferred to a bioassay plate containing spores of *Bacillus subtilis*[21] and incubated at 37°C overnight. Transformants which produced an antibacterial substance were detected by appearance of growth inhibition zones against *B. subtilis*. They were streaked on 4 ml of YMS medium containing 2 $\mu$g of thiostrepton per ml in a small plate and incubated at 30°C for 4 days. The products were extracted with ethyl acetate and then identified by silica gel TLC using bioautography and authentic samples of kalafungin and dihydrokalafungin as described previously[21].

## Results

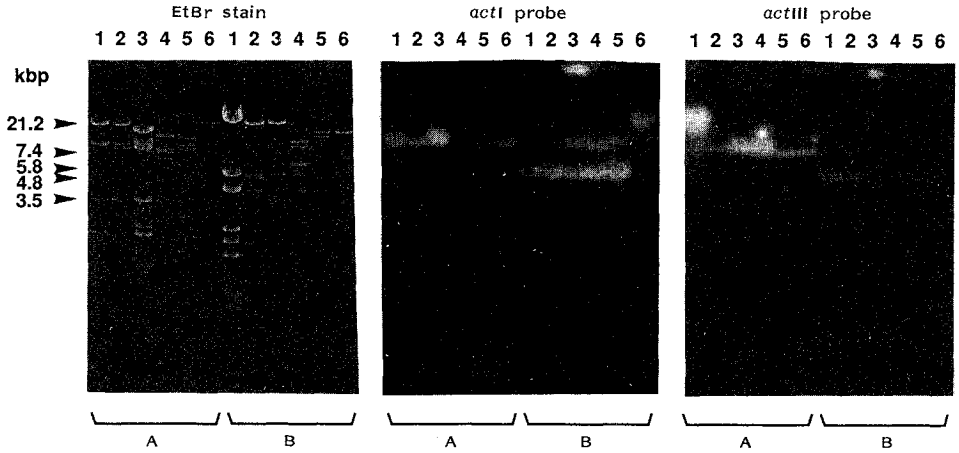
Preparation of Genomic Libraries of *S. tanashiensis* Strain Kala and *Streptomyces* sp. OM-173

To clone large DNA fragments (about 20~40 kb) in *E. coli* and introduce recombinant plasmids into a *Streptomyces* strain directly, we used a *Streptomyces-E. coli* bifunctional cosmid vector pKU205 (10 kb) (Fig. 2). The genomic libraries of *S. tanashiensis* strain Kala and *Streptomyces* sp. OM-173 were constructed by using the above vector and each chromosomal DNA in *E. coli* JM108. About 13,000 ampicillin-resistant transductants were obtained in each library. The sizes of recombinant plasmids isolated from these clones were about 38~52 kb (inserts 28~42 kb). No deletions of recombinant plasmids were observed. However, when *E. coli* HB101, JM109, DH1 or SF8 were used as hosts for preparation of the libraries, few recombinant clones were obtained and deletions of recombinant plasmids were observed.

Cloning of *act*I, III-Homologous DNA Fragments from the Libraries of *S. tanashiensis* Strain Kala and *Streptomyces* sp. OM-173

The libraries prepared from the two *Streptomyces* strains were screened with the *act*I, III DNA fragment as a probe. Six positive clones were selected from each library. The recombinant plasmids (pKU501 to 506 and pKU601 to 606) were isolated from the transductants and analyzed as shown in Fig. 3. Restriction

Fig. 3. Southern hybridization of *act*I and *act*III probes to *Bam*H I-digested cloned DNAs from *Streptomyces tanashiensis* and *Streptomyces* sp. OM-173.




A: Clones of *Streptomyces* sp. OM173, 1; pKU601, 2: pKU602, 3: pKU603, 4: pKU604, 5: pKU605, 6: pKU606, B: clones of *S. tanashiensis*, 1: pKU501, 2: pKU502, 3: pKU503, 4: pKU504, 5: pKU505, 6: pKU506.

enzyme analysis showed that the six clones from each *Streptomyces* strain contained common fragments. Southern hybridization analysis showed that *act*I and *act*III DNA fragments both hybridized with a 4.9 kb *Bam*H I fragment contained in all clones selected from *S. tanashiensis*. In the case of *Streptomyces* sp. OM-173 DNA, the *act*I DNA fragment hybridized with an 8.8 kb *Bam*H I fragment of all clones except pKU504, which did not contain the 8.8 kb *Bam*H I fragment, and the *act*III DNA fragment hybridized with a 7.8 kb *Bam*H I fragment in all the clones. These results indicate that the cloned DNA fragments overlap each other. The clones pKU501 and pKU602 were used for further Southern hybridization analysis.

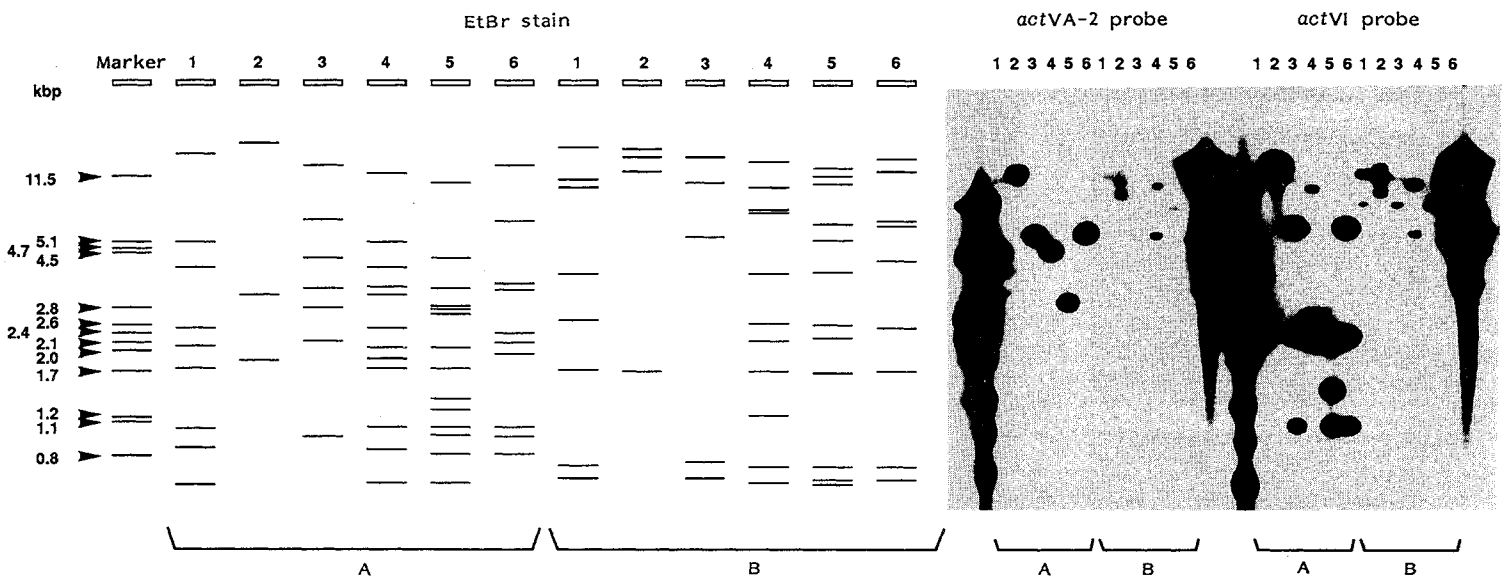
Since the structures of kalafungin and nanaomycin A methyl ester resemble that of actinorhodin (Fig. 1), it was expected that many of the other actinorhodin biosynthetic genes (*act*IV, VA, VB, VI and VII), which are involved in later steps of the actinorhodin biosynthetic pathway, should hybridize with the cloned DNA fragments, which might also contain a homologue of the regulatory *act*II region. This was tested by Southern hybridization using a set of probes carrying various parts of the *act* cluster (see caption to Fig. 5). As shown in Fig. 4, the probes *act*VA-2 and VI hybridized with pKU501 and pKU602; in contrast, the probes carrying *act*II, *act*VII, IV, VB and *act*VA-1 did not hybridize with the clones (data not shown).

From the results of restriction enzyme analysis and Southern hybridization (Figs. 3 and 4), restriction maps of pKU501 and pKU602 were constructed (Fig. 5). As shown in Fig. 5, the arrangements of the regions homologous to the *act*I, III, VA-2 and VI fragments in pKU501 and pKU602 were similar to that in the *act* cluster of *S. coelicolor*, except that the regions homologous to *act*I and III in pKU602 from *Streptomyces* sp. OM-173 were reversed.

## Subcloning of the Kalafungin Biosynthetic Genes of *S. tanashiensis*

To examine whether the *act*I, III-homologous DNA fragments cloned from the two streptomycete strains are involved in biosynthesis of kalafungin and nanaomycin A methyl ester, respecgively, complementation of antibiotic-nonproducing strains with pKU501 and pKU602 was attempted. The

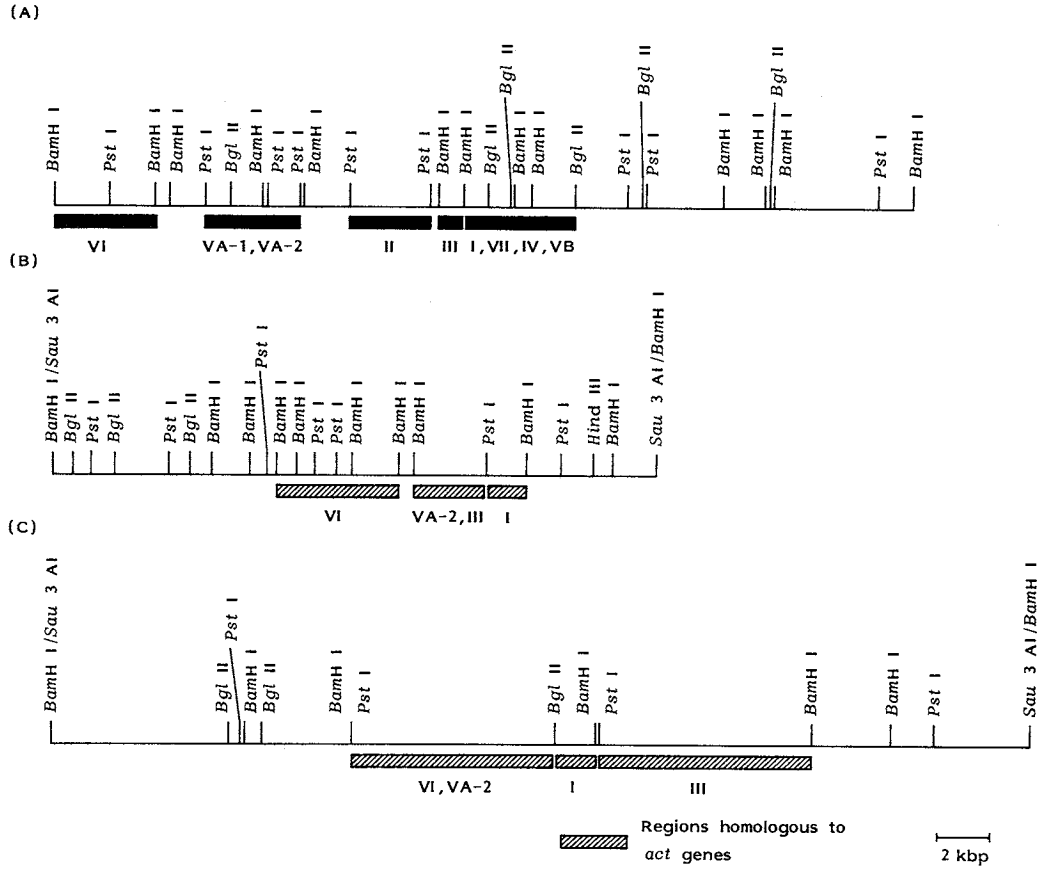
Fig. 4. Southern hybridization of *act* probes to restriction digests of cloned DNA.




A: pKU501 of *Streptomyces tanashiensis*, B: pKU602 of *Streptomyces* sp. OM-173. Samples were digested with 1: *Bam*H I, 2: *Bgl* II, 3: *Pst* I, 4: *Bam*H I + *Bgl* II, 5: *Bam*H I + *Pst* I, 6: *Bgl* II + *Pst* I.

Fig. 5. Restriction maps of cloned DNA fragments in pKU501 and in pKU602 from *Streptomyces tanashiensis* and *Streptomyces* sp. OM-173, respectively.

(A) *S. coelicolor* (actinorhodin), (B) *S. tanashiensis* (pKU501), (C) *Streptomyces* sp. (pKU602).




The regions of *act* probes were shown on the restriction map of actinorhodin cluster of *S. coelicolor* A3(2). The region of *act*VA was divided into two regions, *act*VA-1 and *act*VA-2, as *Pst* I fragments.

recombinant plasmids, pKU501 and pKU602, containing large fragments (28 kb and 42 kb) replicated poorly in *S. lividans*, although the cosmid vector, pKU205, replicated well. To characterize the cloned DNA fragment, subclones of pKU501 were therefore prepared as follows and used for complementation experiments using kalafungin-nonproducing mutants[21]. The pKU602 clone, however, could not be examined in its homologous host because a transformation system for *Streptomyces* sp. OM-173 has not been established.

The single-copy plasmid vector pKU4 (Fig. 6), a derivative of SCP2* of *S. coelicolor* A3(2), was used in a subcloning experiment. The unique *Bam*H I or *Pst* I sites within the neomycin-resistance gene allow cloning and screening by insertional inactivation. The DNA fragments cleaved with *Bam*H I or *Pst* I from the recombinant plasmid pKU501 were purified by agarose gel electrophoresis, and ligated with *Bam*H I- or *Pst* I-cleaved pKU4. Each ligated mixture was introduced into protoplasts of *S. lividans* TK24. After selection of thiostrepton-resistant transformants, clones carrying the inserted fragments were sought by detecting the neomycin-sensitive phenotype. Sixteen kinds of subclones derived from pKU501 were

confirmed by their restriction maps (Fig. 7), and introduced into a restriction-reduced mutant *S. tanashiensis* R3-5 (*sta*A1)[15]. The subclones propagated in this mutant were introduced into protoplasts of kalafungin-nonproducing mutants, which were derived from *S. tanashiensis* strain Kala[21], to look for restoration of kalafungin production.

## Complementation of Kalafungin-nonproducing Strains by Subclones

The *kal* mutants were transformed with the 16 kinds of subclones (Fig. 7). The transformants were examined for their ability to produce antibacterial compounds active against *B. subtilis*, and then the products were identified.

Kalafungin production by the mutants *kal*II,

Fig. 6. Restriction map of pKU4.

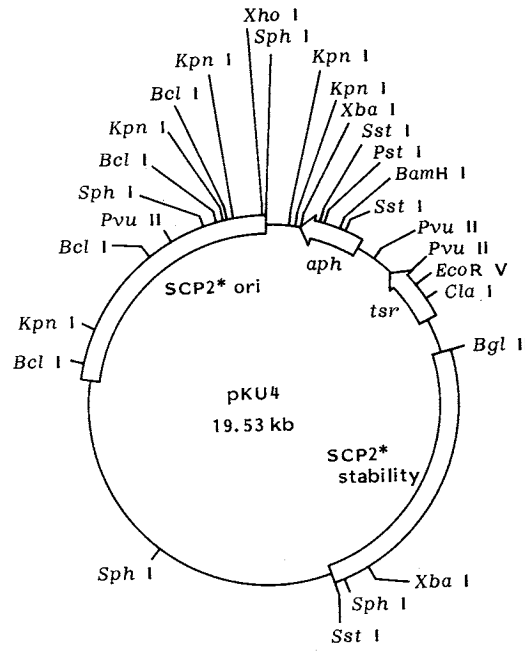



Fig. 7. Analysis of *kal* region by restoration of production to blocked mutants.

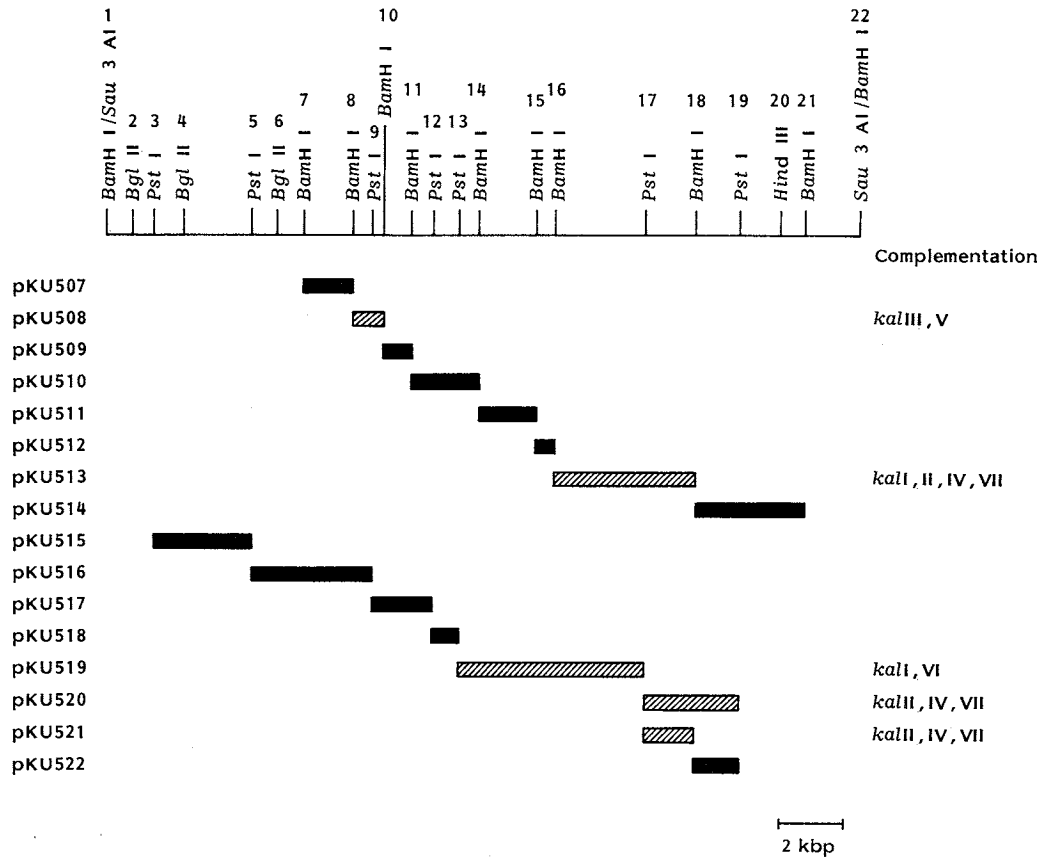
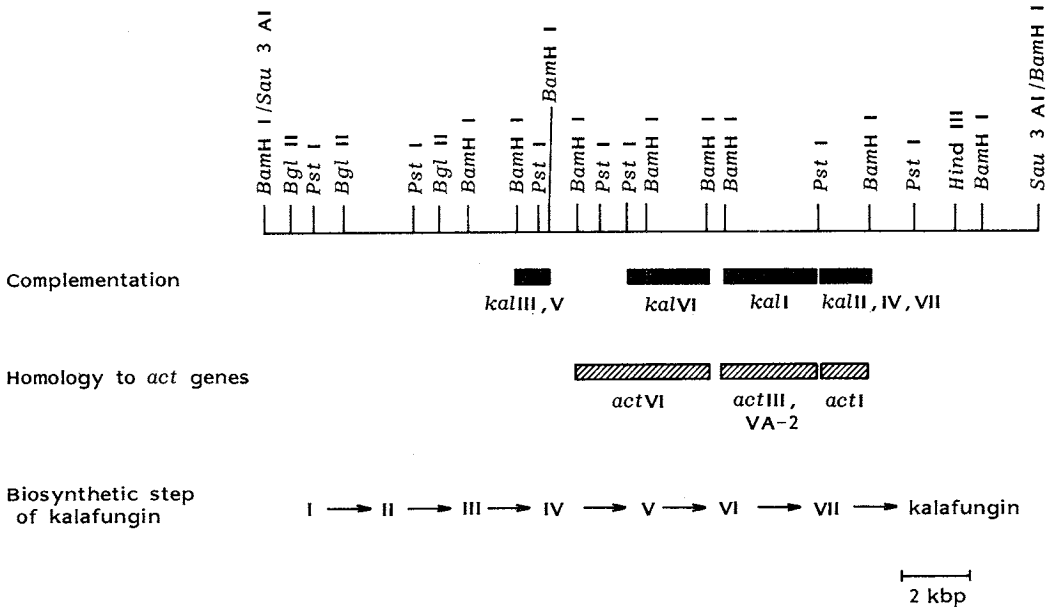


Fig. 8. Structure of gene cluster for kalafungin biosynthesis in *Streptomyces tanashiensis*.




IV and VII was restored with pKU513 or pKU520, indicating that these mutations lie the region which hybridized with the *act*I probe. Since these mutants were also complemented by pKU521, but not by pKU522, their mutations must lie between sites 17~18. The mutant *kal*I was complemented by pKU513 and pKU519, indicating that the *kal*I mutation lies between sites 16~17, in a region which hybridized with the *act*III and VA probes. The mutant *kal*VI was complemented by pKU519 but not by pKU513, placing the *kal*VI mutation between sites 13~16, in a region which hybridized with the *act*VI probe. The mutants *kal*III and V were complemented by pKU508, suggesting that the *kal*III and V mutations lie between sites 7~8, in a region which did not hybridize with any genes for actinorhodin biosynthesis. These results suggest that the genes for kalafungin biosynthesis are not completely identical to those for actinorhodin biosynthesis. The rest of the subclones did not complement any of the *kal* mutants (Fig. 7). A second clone of *kal* mutants (*kal*1, 4, 9, 10, 12 and 13)[21], which showed no evidence of lying on the kalafungin biosynthetic pathway, were not complemented by any of the subclones described above. Overall, seven kinds of *kal* mutations were located on a continuous 14 kb DNA fragment by the complementation results (Fig. 8).

## Discussion

*act*I,III-Homologous DNA fragments were cloned from genomic libraries of kalafungin-producing *S. tanashiensis* and nanaomycin A methyl ester-producing *Streptomyces* sp. OM-173. A cloned fragment of *S. tanashiensis* DNA from the *act*I,III-homologous region contained genes able to complement all the seven classes of *kal* mutants that had been classified as interrupting the kalafungin biosynthetic pathway. The cloned fragments of *Streptomyces* sp. OM-173 DNA could not be examined by homologous complementation experiments, because no transformation system is yet available for this strain.

From the comparison of the biosynthetic genes for the benzoisochromanequinone antibiotics, kalafungin (*kal*) and actinorhodin (*act*), the following points may be made.

(1) The identified *kal* genes lie in a region homologous to four (*act*I, III, VA and VI) out of eight classes of *act* mutations. The order of the genes are similar to each other, but the spacing is not identical.

These results indicate that the enzymes encoded by these genes might also be similar to each other.

(2) However, differences between the two genes sets were also recognized. The isolated *kal* genes do not included regions homologous to *act*II, IV, VII and VB DNA fragments. This was a surprise for *act*IV and VII which act early in the actinorhodin pathway. The *act*II region has been defined as a complex regulatory region for actinorhodin biosynthesis (M. FERNANDEZ-MORENO, J. A. CABALLERO, D. A. HOPWOOD and F. MALPARTIDA; manuscript in preparation), and kalafungin biosynthesis might be controlled by a different type of regulator system. Moreover, the *act*VB region may be required for late steps in actinorhodin synthesis-dimerization and hydroxylation, processes without a counterpart in the kalafungin pathway.

(3) Although kalafungin is an intermediate (or shunt product) of actinorhodin biosynthesis in *S. coelicolor*[8], the *kal* genes in *S. tanashiensis* are not structurally identical with the genes involved in kalafungin biosynthesis in *S. coelicolor*, because the *kal*III and V regions do not hybridize with any *act* genes (Fig. 8). These results coincide with those of cosynthesis experiments between kalafungin-nonproducing mutants and actinorhodin-nonproducing mutants[21].

The cloned DNA fragment isolated from the kalafungin-producing strain by homology with the *act* polyketide synthase (PKS) genes was shown to contain the genes for kalafungin biosynthesis by complementation experiments using subclones of pKU501. This adds a further successful use of the *act* PKS genes to isolate PKS genes for other acetate-derived polyketides to those already reported[12,22,23]. Introduction of the primary fragment (28 kb) into heterologous hosts may be necessary to know whether the cloned fragment contains the whole set of genes for kalafungin biosynthesis. We are modifying the cloned recombinant plasmid pKU501 to allow its stable replication in *Streptomyces* strains to make such tests possible.

Acknowledgments

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