CLONING OF AKLAVINONE BIOSYNTHESIS GENES FROM *Streptomyces galilaeus*

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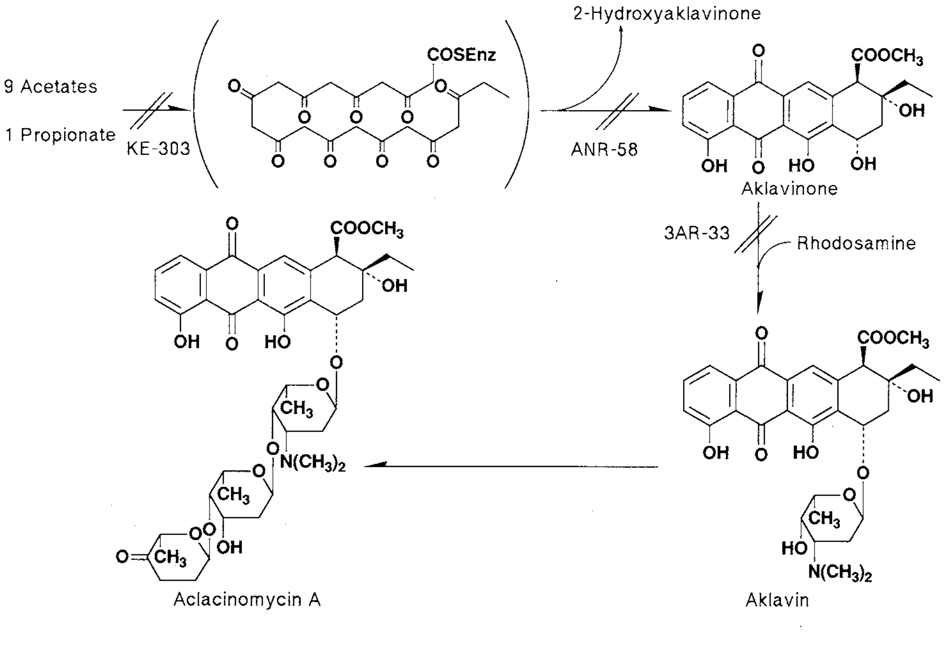
Aklavinone is an aglycone of aclacinomycin A which is an important antitumor drug. Genes for the biosynthesis of aklavinone were cloned from *Streptomyces galilaeus* 3AR-33, an aklavinone-producing mutant, by use of the *act*I and *act*III polyketide synthase gene probes.Restriction mapping and Southern analysis of the DNA cloned in a λ phage vector established that the DNA represented three different regions of the *S. galilaeus* 3AR-33 genome that contained 3.4, 2.5, and 4.1 kb *Bam*H I fragments which hybridized with *act*III. Of those, only the 3.4 kb fragment also hybridized with *act*I. Complementation experiments with specifically blocked mutants confirmed that the cloned 3.4 kb *Bam*H I fragment contains the genes required for the early stage of polyketide synthesis in aklavinone biosynthesis.

Aclacinomycins,[1] which were isolated in 1975 from *Streptomyces galilaeus* MA144-M1, are commercially important anthracycline antibiotics with potent antitumor activity and less cardiotoxicity than doxorubicin and daunorubicin. *S. galilaeus* mutant strain 3AR-33 accumulates aklavinone, an aglycone of aclacinomycins and important biosynthetic intermediate of other anthracycline aglycones. The polyketide origin of aklavinone was confirmed by feeding experiments with labeled acetate.[2]

The so called polyketide synthases are enzymes that catalyze the key steps of polyketide formation,

Fig. 1. Biosynthetic pathway of aclacinomycin in *Streptomyces galilaeus*.



e.g., condensation of C_2 to C_4 units, and cyclization or reduction and dehydration of polyketomethylene intermediates. However, there are only a few examples of well-characterized polyketide synthases, such as 6-methylsalicylic acid synthase[3,4] and chalcone synthase.[5]

On the other hand, molecular genetic studies of polyketide biosynthesis in streptomycetes have led to the isolation of the entire set of genes required for the biosynthesis of actinorhodin,[6] tetracenomycin,[7] and oxytetracycline.[8] These genes were all found as clusters of structural and self-resistant genes, and the products of the early genes of polyketide biosynthesis were determined to exhibit high homology.[9]

Genetic studies of polyketide biosynthesis were undertaken in our laboratory to elucidate the gene organization and molecular properties of enzymes involved in polyketide biosynthesis. We describe here the cloning of aklavinone biosynthesis genes from *S. galilaeus* 3AR-33 by DNA hybridization between streptomycete polyketide synthase genes and complementation of aklavinone biosynthesis mutations.

Materials and Methods

Biochemicals and Chemicals

Thiostrepton (Thio) was obtained from Sigma Chemical Co. 2-Hydroxyaklavinone was obtained from Mercian Corp. (Tokyo, Japan). All other chemicals and biochemicals were obtained from Sigma or Wako. Restriction enzymes and other recombinant DNA materials were purchased from Promega Biotech, Boehringer Mannheim, Takara Shuzo, and Toyobo.

Bacterial Strains and Plasmids

Streptomyces lividans TK24 and plasmid pIJ2345 (pBR329[10] containing *act*I[9,11]), pIJ2346 (pBR329 containing *act*III[11,12]), and pIJ61[13] were kind gifts from David Hopwood (John Innes Institute and AFRC Institute of Plant Science, Norwich, United Kingdom). *Streptomyces galilaeus* 3AR-33, *S. galilaeus* ANR-58 (ATCC 31671), and *S. galilaeus* KE-303 (ATCC 31649) were obtained from Mercian Corp.

Media and Growth Conditions

Cultures for preparation of *Streptomyces lividans* spore stocks were grown on modified R2YE medium.[14] Those of *S. galilaeus* spore stocks were grown on YS agar (0.3% yeast extract, 1% soluble starch, 1.5% agar, pH 7.2) at 28°C for several weeks. *S. lividans* was grown in YEME medium[14] with 5 mM $MgCl_2$ and 0.5% glycine at 28°C for protoplast formation and in TSB medium[14] or modified R2YE medium for plasmid preparation. *S. galilaeus* was grown on YS medium with 5 mM $MgCl_2$ and 0.5% glycine at 28°C for protoplast formation. *Streptomyces* protoplasts were transformed according to Thompson *et al.*[15] Strains containing pIJ61 or derivatives of this plasmid were selected with Thio (20 to 50 μg/ml) neomycin (10 μg/ml). *Escherichia coli* was grown in LB medium at 37°C. For analysis of anthracycline production, subcultures were grown in 5 ml YS medium in 50-ml Falcon tubes for 3 days with rotary shaker (200 rpm at 28°C). The subculture broth (2.5 ml) was inoculated into 50 ml production medium (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) in a 500-ml Erlenmeyer flask and cultured for 3 to 5 days.

Isolation of Chromosomal and Plasmid DNAs

Chromosomal DNA was isolated from *S. galilaeus* 3AR-33 by a modification of the method of Hopwood *et al.*[14] Cells were grown in 5 ml modified R2YE medium for 3 days, then the culture was inoculated into 50 ml modified R2YE medium and grown for 2 days. Cells from 50 ml culture were treated with 5 ml of TSE buffer (25 mM Tris-HCl, pH 8, 0.3 M sucrose, 25 mM EDTA) containing lysozyme (0.6 mg/ml) at 37°C for 30 minutes with gentle shaking. The lysed cells were mixed with 3.6 ml of a solution containing 0.23 M EDTA, 1.2% sodium dodecylsulfate (SDS), pronase (0.46 mg/ml) and incubated at 37°C for 1 hour. The mixture was extracted with 8 ml of chloroform twice, and then treated with RNase A at a final concentration of 75 μg/ml at 37°C for 30 minutes. The mixture was extracted with equal volume of phenol-chloroform and then chloroform, and the DNA was precipitated first with 2-propanol and then with ethanol by standard procedures.

The alkaline lysis method described by MANIATIS *et al.*[16)] was used for large and small scale plasmid preparation from *E. coli*. Plasmid DNA was isolated from streptomycetes by the minilysate procedure of KIESER[17)] or by the large scale preparation described by HOPWOOD *et al.*[14)]

Preparation of a λ Phage Library of *S. galilaeus* 3AR-33 Chromosomal DNA

λ Phage vector λGEM-12 *Xho* I half-site arms were purchased from Promega Biotech. Chromosomal DNA isolated from *S. galilaeus* 3AR-33 was partially digested with *Sau*3 AI and filled in with dATP and dGTP. The vector (1 μg) and chromosomal DNA (0.2 μg) were ligated with 2.8 Weiss units of T4 DNA ligase (Takara) in a reaction volume of 10 μl at 4°C overnight. The ligated DNA was *in vitro* packaged by using the Packagene lambda packaging system (Promega Biotech) as recommended by the manufacturer. The packaged phages were transfected into *E. coli* LE392. The library was screened without amplification.

Southern Hybridization Analysis

The *act*I[9,11)] and *act*III[11,12)] probes were labeled with dig-dUTP by random primed DNA labeling method with the kit from Boehringer Mannheim as recommended by the manufacturer. Southern blot-transfers and plaque lifts were carried out by standard procedures with Hybond-N (Amersham). Hybridization was carried out in a solution containing 2 × SSC, 0.1% SDS, 5% blocking reagent solution, 0.02% sodium *N*-lauroylsarcosine at 60°C overnight. Filters were washed twice with 0.5 ~ 1 × SSC - 0.1% SDS at 60 ~ 70°C for 30 minutes. Enzyme-linked immunodetection using an anti-digoxigenin alkaline phosphatase conjugate with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium salt were carried out as recommended by the manufacturer. Chemiluminescent detection with 3-(2′-spirodamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt (AMPPD) was carried out as recommended by the manufacturer by using Kodak X-Omat AR films.

Analysis of Anthracycline Metabolite Production

Cultures were grown as described above. The culture broth was centrifuged at 1,500 × *g* for 15 minutes and mycelial pellets were extracted with acetone. The extract was evaporated to dryness and dissolved in acetone. Concentrated extracts were first analyzed by oxalic acid impregnated silica gel TLC, which were developed with benzene - acetone, 4 : 1 (v/v). Plates were visualized by their normal pigmentation and fluorescence under UV irradiation at 365 nm. Identification of compounds were also carried out by high performance liquid chromatography (HPLC), using a solvent system of 60% methanol, 35% water, and 5% glacial acetic acid. Elution was monitored at 254 nm.

Acid hydrolysis of concentrated extracts was carried out as follows. Acetone solution (0.5 ml) of concentrated extracts was mixed with 3 ml of 0.4 N HCl and heated in a sealed tube for 40 minutes at 100°C in a sealed tube. Hydrolysates were extracted with ethyl acetate and analyzed by TLC and HPLC as described above.

To isolate aklavinone, the mycelia were extracted with acetone. The extract was evaporated and the residues were extracted with chloroform. This extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residues were chromatographed on an oxalic acid impregnated silica gel column with a benzene - acetone solvent system for elution. Fractions containing aklavinone were pooled and evaporated to dryness. Aklavinone was recrystallized from ethanol.

Instrumental Analysis

Mass spectrum was recorded on a Jeol DX-300 spectrometer. The ^1H and ^{13}C NMR spectra were obtained at 500 MHz and 125 MHz respectively in $CDCl_3$ solution using a Jeol GSX-500 spectrometer.

Results

Isolation and Characterization of the Polyketide Synthase Gene from a *S. galilaeus* 3AR-33 Genomic Library

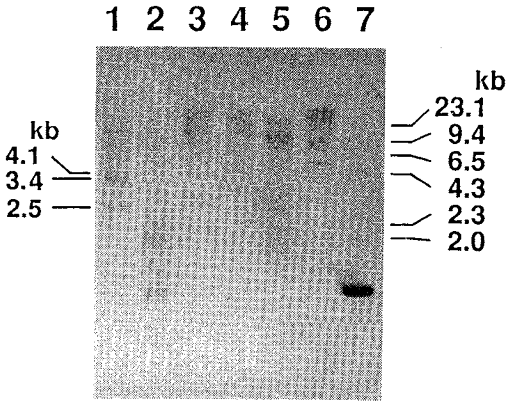
We found three bands (4.1 kb, 3.4 kb, 2.5 kb) hybridizing with *act*III when genomic DNA of

S. galilaeus was digested with *Bam*H I and probed with *act*III (Fig. 2). We cloned these three fragments as follows.

A *S. galilaeus* 3AR-33 genomic library in bacteriophage λ vector λGEM-12 was constructed and screened for the polyketide synthase genes. The *act*III gene was used to screen 2×10^4 phage plaques and a total of 14 plaques hybridized with the probe. Restriction mapping and Southern blot analysis of purified phage DNAs identified the three different *Bam*H I fragments which hybridized with *act*III. One is the 2.5 kb *Bam*H I fragment from 7 clones. The second is the 3.4 kb *Bam*H I fragment which hybridized strongly with *act*III and also hybridized with *act*I. The third is the 4.1 kb *Bam*H I fragment from 4 clones which hybridized weakly with *act*III. Fig. 3 shows the restriction maps of representative phage clones and the fragments which hybridized with *act*III except clones containing the 4.1 kb *Bam*H I fragment. The 2.5 kb *Bam*H I fragment was isolated and ligated into pIJ61 to yield pAKD21. The 3.4 kb *Bam*H I fragment was isolated and ligated into pIJ61 to yield pAKD11F and pAKD11R, in

Fig. 2. Southern blots showing hybridization of the *act*III gene to DNA isolated from *Streptomyces galilaeus* 3AR-33.

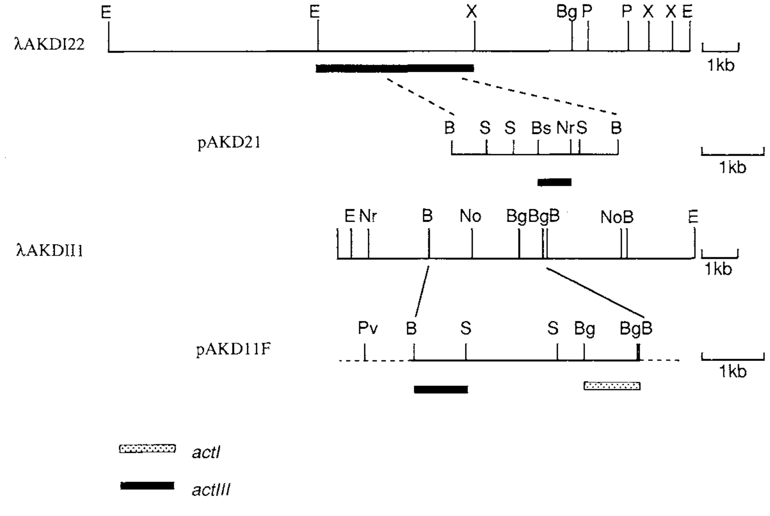
Lanes: 1 to 6, *S. galilaeus* 3AR-33 genomic DNA digested with *Bam*H I, *Sal* I, *Bgl* II, *Pst* I, *Sph* I, *Xho* I, respectively; 7, pIJ2346 digested with *Bam*H I (*act*III gene).



The hybridization were carried out at 65°C overnight using 1 × SSC buffer. The hybrids were detected by enzyme-linked immunoassay and subsequent enzyme-catalyzed color reaction.

Fig. 3. Restriction maps of the two different region of DNA that hybridized to *act*III and *act*I.

Restriction maps were constructed from the results of single and double restriction enzyme digestions. λAKDI22 and λAKDII1 are λGEM-12 clones. The broken line in the map of pAKD11F indicates regions of pIJ61. The location of *act*III-hybridizing DNA is indicated by short, thick lines below the restriction map of each region. The location of *act*I-hybridizing DNA is indicated by hatched line. Abbreviations for restriction endonuclease sites: E, *Eco*R I; X, *Xho* I; Bg, *Bgl* II; P, *Pst* I; S, *Sal* I; Bs, *Bst*E II; Nr, *Nru* I; B, *Bam*H I; No, *Not* I; Pv, *Pvu* II.



which the same 3.4 kb fragment was inserted at the *Bam*H I site of pIJ61 in the opposite direction (Fig. 3).

Complementation of Mutations in *S. galilaeus* ANR-58 and *S. galilaeus* KE-303

Mutants of *S. galilaeus* that produce 2-hydroxy derivatives of aklavinone and related anthracyclines have been isolated and characterized.[18] *S. galilaeus* ANR-58 (ATCC 31671) produces 2-hydroxyaklavinone as its major product. We introduced pAKD11F and pAKD11R into *S. galilaeus* ANR-58 by transformation of protoplasts. The products obtained from suitable transformants were analyzed as described in Materials and Methods. *S. galilaeus* ANR-58 (pAKD11F) and *S. galilaeus* ANR-58 (pAKD11R) both produced aklavinone (Fig. 4), but the *S. galilaeus* ANR-58 (pAKD21) did not. The structure of aklavinone produced

Fig. 4. Detection of aklavinone produced by *S. galilaeus* ANR58 (pAKD11F) and *S. galilaeus* ANR58 (pAKD11R) by high performance liquid chromatography.

Chart A, pAKD11F; B, pAKD11R; C, pIJ61. The peaks of aklavinone are indicated by arrows.

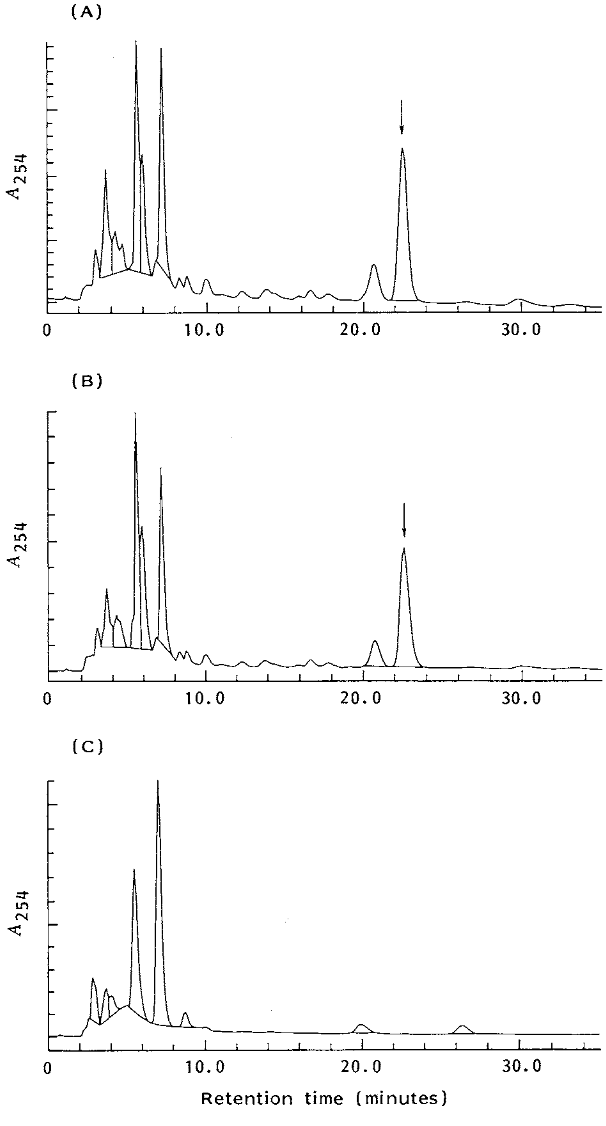
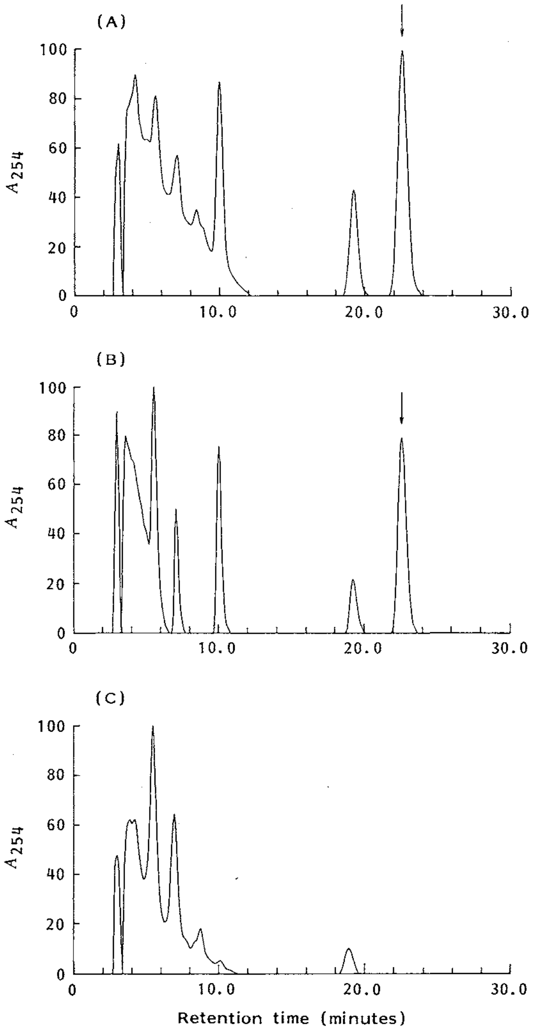


Fig. 5. Detection of aklavinone from acid-hydrolyzed extracts from *S. galilaeus* KE303 (pAKD11F) and *S. galilaeus* KE303 (pAKD11R) by high performance liquid chromatography.

Chart A, pAKD11F; B, pAKD11R; C, pIJ61. The peaks of aklavinone are indicated by arrows.



by *S. galilaeus* ANR-58 (pAKD11R) was confirmed by physico-chemical analysis (mass spectrometry, ^{1}H and ^{13}C NMR). Thus, the cloned 3.4 kb *Bam*H I fragment contains at least the functional reductase gene involved in aklavinone biosynthesis. *S. galilaeus* ANR-58 (pAKD11) produced other yellow pigments that were not produced by *S. galilaeus* ANR-58 carrying pIJ61 only. These compounds are being isolated and characterized.

S. galilaeus KE-303 (ATCC 31649) does not produce any pigments, but has glycosidation ability.[19] Therefore, *S. galilaeus* KE-303 has a mutation of the gene(s) required for the early stage of polyketide synthesis, possibly condensation or cyclization. We introduced pAKD11F and pAKD11R into *S. galilaeus* KE-303. The products obtained from suitable transformants were analyzed as described in Materials and Methods after acid hydrolysis because *S. galilaeus* KE-303 is glycosidation active. Both *S. galilaeus* KE-303

Table 1. Bacterial strains and plasmids used.

Strains or plasmid	Relevant characteristics	Source or reference
Streptomyces strain		
S. galilaeus 3AR-33	Aklavinone-producing strain	T. OKI *et al.*[19)]
S. galilaeus ANR-58	2-Hydroxyaklavinone producer (*act*III-equivalent negative)	T. OKI *et al.*[19)]
S. galilaeus KE-303	Anthracyclinone non-producing strain	T. OKI *et al.*[19)]
Plasmid		
pIJ61	Derivative of SLP1.2; LC; Thio^r Neo^r	D. A. HOPWOOD *et al.*[13)]
pANT45	pIJ61 with 1.1 kb *Bam*H I subclone; contains *act*III locus	H. G. FLOSS *et al.*[20)]
pAKD11F and pAKD11R	pIJ61 with 3.4 kb *Bam*H I subclone from λAKDI11	This work
pAKD21	pIJ61 with 2.5 kb *Bam*H I subclone from λAKDI22	This work

Abbreviations: LC, low-copy-number plasmid; Thio^r, thiostrepton resistance; Neo^r, neomycin resistance; *act*, actinorhodin genetic locus.

In pAKD11F and pAKD11R, 3.4 kb *Bam*H I fragment was inserted into pIJ61 in the opposite direction.

(pAKD11F) and *S. galilaeus* KE-303 (pAKD11R) produced aklavinone mainly as its glycoside, together with small amount of the free form. The aglycone was identified to be aklavinone by TLC and HPLC analysis (Fig. 5). On the other hand, no aklavinone was detected in *S. galilaeus* KE-303 carrying pIJ61 only.

Thus, it was confirmed that the cloned 3.4 kb *Bam*H I fragment contains the genes which code the enzyme(s) in the polyketide synthesis, possibly condensation or cyclization, and the reductase which are involved in aklavinone biosynthesis.

Table 2. Formation of polyketides by nonrecombinant and recombinant strains.

Streptomyces strain	Plasmid	Major polyketide compound formed
S. galilaeus 3AR-33	None	Aklavinone
S. galilaeus ANR-58	pIJ61	2-Hydroxyaklavinone
S. galilaeus ANR-58	pANT45	Aklavinone
S. galilaeus ANR-58	pAKD11F	Aklavinone
S. galilaeus ANR-58	pAKD11R	Aklavinone
S. galilaeus ANR-58	pAKD21	2-Hydroxyaklavinone
S. galilaeus KE-303	pIJ61	Not detected
S. galilaeus KE-303	pAKD11F	Aklavinone glycoside
S. galilaeus KE-303	pAKD11R	Aklavinone glycoside

Discussion

It is evident that the 3.4 kb *Bam*H I fragment cloned as pAKD11F and pAKD11R contains a reductase gene that catalyzes the reduction of the keto group at the ninth carbon from the carboxyl terminus of the assembled polyketide to the corresponding secondary alcohol, which results in the loss of the C-2 hydroxyl group at the time of aromatization. FLOSS *et al.* previously showed that when *S. galilaeus* ANR-58 was transformed with a plasmid carrying only the *act*III gene, aklavinone was produced exclusively.[20)] They also showed that genomic DNAs of *S. galilaeus* MA144-M1 (ATCC 31133), which produces aclacinomycin A, and the strain ANR-58 showed the same restriction digestion patterns and hybridization bands with *act*III, and suggested that the *act*III homologous gene of *S. galilaeus* ANR-58 probably has a point mutation or a series of point mutations.[20)] On the other hand, *Streptomyces glaucescens* ETH 22794, a strain which produces tetracenomycin, an anthracycline antibiotic, and apparently lacks the corresponding reduction step, does not contain DNA fragments that hybridize to the *act*III probe.[9)] Our work has shown that *S. galilaeus* 3AR-33, which produces aklavinone but none of its glycoside, contains a functional *act*III-equivalent gene.

S. galilaeus KE-303 is a mutant obtained by NTG treatment and UV treatment from *S. galilaeus* MA144-M1 and does not produce any pigments, but has glycosidation ability. Hence the strain may have a point mutation in the *act*I-equivalent gene(s). The fact that the cloned 3.4 kb *Bam*H I fragment complements the mutation suggests this fragment also contains a portion of an *act*I-equivalent gene(s). In the actinorhodin biosynthesis gene cluster, *act*I, which is assumed to code the enzyme catalyzing the sequential condensation of seven malonyl-CoA with an initial acetyl-CoA starter unit, is adjacent to the *act*III gene.[11] Therefore, an *act*I-equivalent gene of *S. galilaeus* 3AR-33 was expected to exist close to the *act*III-equivalent. We found that the 3.4 kb *Bam*H I fragment hybridized with both *act*I and *act*III probes (Fig. 3). This indicates that *act*I- and *act*III-equivalent genes are closely linked in the gene cluster of aklavinone biosynthesis.

We found that three regions of DNA from *S. galilaeus* 3AR-33 hybridized to the *act*III gene. HUTCHINSON *et al.* previously reported that four unlinked regions of DNA from *Streptomyces peucetius* contain genes that encode the production of the same or closely related metabolites, some of which are intermediates of the daunorubicin pathway.[21] They believe that one of these four regions is directly associated with daunorubicin biosynthesis and contains all of the genes required for synthesis of ε-rhodomycinone and some (if not all) of the aglycone portion of daunorubicin.[22] Two other regions that we cloned may also encode the genes required for the production of related anthracycline metabolites in *S. galilaeus*.

Acknowledgments

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References

1) OKI, T.; I. KITAMURA, A. YOSHIMOTO, Y. MATSUZAWA, N. SHIBAMOTO, T. OGASAWARA, T. INUI, A. TAKAMATSU, T. TAKEUCHI, T. MASUDA, M. HAMADA, H. SUDA, M. ISHIZUKA, T. SAWA & H. UMEZAWA: Antitumor anthracycline antibiotics, aclacinomycin A and analogues. I. Taxonomy, production, isolation and physicochemical properties. J. Antibiotics 32: 791 ~ 800, 1979

2) KITAMURA, I.; H. TOBE, A. YOSHIMOTO, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthesis of aklavinone and aclacinomycins. J. Antibiotics 34: 1498 ~ 1500, 1981

3) DIMROTH, P.; H. WALTER & F. LYNEN: Biosynthese von 6-Methylsalicylicsäure. Eur. J. Biochem. 13: 98 ~ 110, 1970

4) BECK, J.; S. RIPCA, A. SIEGNER, E. SHILTZ & E. SCHWEIZER: The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulam*: its gene structure relative to that of other polyketide synthases. Eur. J. Biochem. 192: 487 ~ 498, 1990

5) KREUZALER, F. & K. HAHLBROCK: Enzymic synthesis of an aromatic ring from acetate units: partial purification and some properties of flavanone synthase from cell-suspension cultures of *Petroselinum hortense*. Eur. J. Biochem. 56: 205 ~ 213, 1975

6) MALPARTIDA, F. & D. A. HOPWOOD: Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature 309: 462 ~ 464, 1984

7) MOTAMEDI, H. & C. R. HUTCHINSON: Cloning and heterologous expression of a gene cluster for the biosynthesis of tetracenomycin C, the anthracycline antitumor antibiotic of *Streptomyces glaucescens*. Proc. Natl. Acad. Sci. U.S.A. 84: 4445 ~ 4449, 1987

8) BINNIE, C.; M. WARREN & M. J. BUTLER: Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis. J. Bacteriol. 171: 887 ~ 895, 1989

9) MALPARTIDA, F.; S. E. HALLAM, H. M. KIESER, H. MOTAMEDI, C. R. HUTCHINSON, M. J. BUTLER, D. A. SUGDEN, M. WARREN, C. MCKILLOP, C. R. BAILEY, G. O. HUMPHREYS & D. A. HOPWOOD: Homology between *Streptomyces* genes coding for synthesis of different polyketides used to clone antibiotic biosynthetic genes. Nature 325: 818 ~ 821, 1987

10) COVARRUBIAS, L. & F. BOLIVAR: Construction and characterization of new cloning vehicles. VI. Plasmid pBR329, a new derivative of pBR328 lacking the 482-base-pair inverted duplication. Gene 17: 79 ~ 89, 1982

11) MALPARTIDA, F. & D. A. HOPWOOD: Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. 205: 66 ~ 73, 1986

12) HALLAM, S. E.; F. MALPARTIDA & D. A. HOPWOOD: Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene 74: 305 ~ 320, 1988

13) THOMPSON, C. J.; T. KIESER, J. M. WARD & D. A. HOPWOOD: Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. Gene 20: 51 ~ 62, 1982

14) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH,

J. M. WARD & H. SHREMPF: Genetic Manipulation of *Streptomyces*. A Laboratory Manual. The John Innes Foundation, 1985

15) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. 151: 668～677, 1982

16) MANIATIS, T.; E. F. FRITSCH & J. M. SAMBROOK: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, 1989

17) KIESER, T.: Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12: 19～36, 1984

18) MATSUZAWA, Y.; A. YOSHIMOTO, N. SHIBAMOTO, H. TOBE, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. II. Structure of 2-hydroxyaklavinone and new aklavinone glycosides. J. Antibiotics 34: 959～964, 1981

19) YOSHIMOTO, A.; Y. MATSUZAWA, T. OKI, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. I. Isolation and characterization of various blocked mutants. J. Antibiotics 34: 951～958, 1981

20) BARTEL, P. L.; C.-B. ZHU, J. S. LAMPEL, D. C. DOSCH, N. C. CONNORS, W. R. STROHL, J. M. BEALE, Jr. & H. G. FLOSS: Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in streptomycetes: clalification of actinorhodin gene functions. J. Bacteriol. 172: 4816～4826, 1990

21) STUTZMAN-ENGWALL, K. J. & C. R. HUTCHINSON: Multigene families for anthracycline antibiotic production in *Streptomyces peucetius*. Proc. Natl. Acad. Sci. U.S.A. 86: 3135～3139, 1989

22) OTTEN, S. L.; K. L. STUTZMAN-ENGWALL & C. R. HUTCHINSON: Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *S. peucetius* subsp. *caesius*. J. Bacteriol. 172: 3427～3434, 1990