Cloning and Heterologous Expression of Genes from the Kinamycin Biosynthetic Pathway of *Streptomyces murayamaensis*

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The genes for most of the biosynthesis of the kinamycin antibiotics have been cloned and heterologously expressed. Genomic DNA of *Streptomyces murayamaensis* was partially digested with *Mbo*I and a library of ~40 kb fragments in *E. coli* XL1-BlueMR was prepared using the cosmid vector pOJ446. Hybridization with the *actI* probe from the actinorhodin polyketide synthase genes identified two clusters of polyketide genes. After transferal of these clusters to *S. lividans* ZX7, expression of one cluster was established by HPLC with photodiode array detection. Peaks were identified from the *kin* cluster for dehydrorabelomycin, kinobscurinone, and stealthin C, which are known intermediates in kinamycin biosynthesis. Two shunt metabolites, kinafluorenone and seongomycin were also identified. The structure of the latter was determined from a quantity obtained from large-scale fermentation of one of the clones.

Members of the genus *Streptomyces* produce well over half of the known antibiotics of natural origin. Of these, polyketide-derived metabolites are amongst the most numerous and diverse, and include many clinically important members, such as erythromycin, tetracycline, and doxorubicin. Assembly of the skeletons of such compounds by oligomerization of small precursor fatty acid thioesters to form polyketide backbones is carried out by polyketide synthases (PKSs). These are made up of either large multi-functional enzymes (type I PKSs) or multi-enzyme complexes (type II PKSs). Molecular genetic analyses have revealed that the genes for each PKS are clustered in a relatively small region of DNA.¹⁾ The gene clusters for numerous polyketide pathways have been detected by hybridization with probes derived from genes coding for proteins that catalyze equivalent reactions in different pathways.^{2,3)}

We have been studying the biosynthesis of a number of antibiotics derived from benz[*a*]anthraquinones (*i.e.* angucyclinones^{4~7)}). One of these yields the kinamycins, which are broad spectrum antibiotics with modest cytotoxicity that were first reported from *Streptomyces murayamaensis* (*e.g.* kinamycin D, **1**, Figure 1).^{5,8,9)}

Kinamycin biosynthesis proceeds *via* dehydrorabelomycin,⁷⁾ **2**, followed by ring contraction to the recently discovered^{10~12)} benzo[*b*]fluorene skeleton and then by extensive further modifications. We have also identified prekinamycin,^{5,13)} **3**, and ketoanhydrokinamycin,¹⁴⁾ **4**, as likely intermediates in the pathway, have demonstrated kinobscurinone,¹⁵⁾ **5**, stealthin C,¹⁶⁾ **6**, and kinamycins F and E,¹⁴⁾ **7** and **8**, respectively, to be intermediates, and have isolated a large acetyltransferase that sequentially acetylates kinamycin F at C-4 and then at C-2.¹⁷⁾ Two other actinomycetes are known to produce kinamycins.^{18,19)} We now report the cloning and heterologous expression of most of the kinamycin gene cluster from *S. murayamaensis* in *S. lividans* ZX7,²⁰⁾ as evidenced by production of **2**, **5**, and **6** by the recombinant strains.

Materials and Methods

Bacterial Strains and Plasmids Used

Streptomyces murayamaensis was a gift from Professor S. ŌMURA, Kitasato University. *S. lividans* ZX7, used as the recombinant host strain, was obtained from Professor

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Fig. 1. Current understanding of kinamycin D biosynthesis.

DAVID HOPWOOD (John Innes Center, England). The cosmid pOJ446, used for cosmid library construction, was obtained from Lilly Research Laboratories. *E. coli* XL1-Blue MR and pBluescript® II KS⁺ were purchased from Stratagene. *E. coli* DH5 α was purchased from Clontech, and pGEM11zf(+) was purchased from Promega.

Culture Conditions for *S. murayamaensis* and *S. lividans*

S. murayamaensis or *S. lividans* was propagated on ISP4 agar + 0.01% yeast extract²¹⁾ and in R2YE²²⁾ at 29°C for spore preparation, or in YEME liquid media²²⁾ at 27 and at 29°C for genomic DNA preparation.

Genomic DNA Isolation

Genomic DNA was prepared from *S. murayamaensis* as follows: mycelia from a 50 ml culture grown in YEME containing 0.5% glycine were collected by centrifugation, washed twice with 10.3% sucrose, and resuspended in 10 ml of a solution containing 25 mM Tris (pH 8.0), 50 mM glucose, 10 mM EDTA (pH 8.0), 20 μ g/ml RNase, 10 mg/ml lysozyme, and 10 mg/ml achromopeptidase

(Sigma). After 5~10 minutes incubation at room temperature, proteinase K was next added (0.5 mg/ml) and the mixture incubated at 50°C overnight. The mixture was then worked up in standard fashion, and the genomic DNA was dissolved in 3 ml TE buffer.

Preparation of a Cosmid Library of *S. murayamaensis*

Genomic DNA from *S. murayamaensis* was digested with *Bam*HI, *Kpn*I, and *Pst*I, and the digested DNA's were separated by electrophoresis. Southern blots were probed with ³²P-radiolabeled *act*I, the ketoacylsynthase gene from the actinorhodin pathway of *S. coelicolor* A3(2),²³⁾ which was present in pIJ2345, and washed at moderate stringency (2 $\times$ SSC/1% SDS). The probe hybridized moderately strongly with four bands in the *Bam*HI digest (5.6, 4.3, 2.6, and 0.5 kb), and weakly with a few more. The probe hybridized moderately strongly with two large bands in the *Kpn*I and *Pst*I digests.

Genomic DNA from *S. murayamaensis* was partially digested with *Mbo*I. 10~20 μ g of the partially digested DNA fragments in TE buffer were ligated with 4~6 μ g of pOJ446 that had been digested with *Bam*HI and with *Hpa*I, using T4 DNA ligase. The ligated DNA was

packaged *in vitro* using Giga Pack II XL packaging extract (Stratagene) and then used to transfect *E. coli* XL1-BlueMR. The manufacturer's protocol was followed.

Colony Hybridization of the Cosmid Libraries

Approximately 6000 *E. coli* colonies from the *S. murayamaensis* cosmid library were spread on dried nylon membranes (138 mm diameter, Amersham) as described by the manufacturer. After spreading the inoculum, the membrane was transferred onto an Luria-Bertani (LB)-agar plate containing 100 µg/ml apramycin and incubated overnight at 37°C. The cosmid library was replicated to new nylon membranes, lysed, and hybridized as described by the manufacturer, using ³²P-labeled *actI*.

Thirty-eight hybridizing clones were obtained and restriction mapping with *Bam*HI and *Eco*RI showed that they segregated into two non-overlapping sets: 15 clones in one cluster (*kin*) and 23 clones for the other. The first contained three *actI* homologous *Bam*HI fragments (4.3, 2.6, and 0.5 kb), and the second contained a single, 5.6 kb, *actI* homologous *Bam*HI fragment.

Transformation of *S. lividans* ZX7 with Recombinant Plasmids

Plasmid DNAs were isolated according to standard procedures²⁴⁾ from 1.5 ml overnight cultures in LB broth plus 15 µg/ml of apramycin. Correct inserts were confirmed by restriction enzyme analysis. *S. lividans* protoplasts (5×10^8 in 50 µl P-buffer) were mixed with 5 µl of recombinant plasmid (0.01~0.1 µg/µl), followed by 200 µl of T-buffer, and the mixture was then spread on R2YE agar.²²⁾ Transformants were detected by overlaying 2.5 ml of soft nutrient agar (600 µg/ml apramycin) per plate 14~20 hours later.

Fermentation of *S. lividans* and Transformants

Colonies of each organism were inoculated into 5 ml of YEME containing 6 µg/ml of apramycin for transformants. After incubation for 3~4 days at 29°C, 260 rpm, 0.25 ml of the seed culture was used to inoculate 5 ml of three production media, each in 18 ml culture tubes: glucose Proflow-salt (GPS),²⁵⁾ glycerol-asparagine (GA),²⁶⁾ and YEME, each containing 6 µg/ml of apramycin. The rest of the transformant cultures were used for plasmid mini-preps to confirm the presence of the correct plasmids. All cultures were incubated at 29°C, 300 rpm, for both 5 and 9 days.

Analysis of the Metabolites

Cultures were acidified (pH 2.5~3.0) with 0.1 N HCl, extracted with 3 ml of EtOAc, and the extracts dried (SpeedVac centrifuge) and taken up in 100 µl of 10% MeOH/CH₂Cl₂. An aliquot (10 µl) of each extract was analyzed by reverse phase HPLC with a Waters Assoc. 600 E gradient pump. The metabolites were separated on a Waters NovaPak C₁₈ radial compression column (0.8 × 10 cm, 4 µm) using a gradient of 5~95% acetonitrile in water containing 0.1% acetic acid over a period of 30 minutes at 1.5 ml/minute. Detection was by photodiode array using either a Waters 990+ detector or a Waters 996 detector (retention times: seongomycin, 13.39; **6**, 16.26; kinafluorenone, 16.70; **5**, 20.9; **2**, 22.09 minutes).

Preparation of a Physical Map of *kin* Cluster

Four cosmids of the *kin* cluster that produced recognizable *S. murayamaensis* metabolites were digested with combinations of *Xba*I, *Spe*I, and *Eco*RI, both individually and in pairs. The first two are in the pOJ446 polylinker; no sites internal to the inserts were observed. *Eco*RI provided a rough restriction map. The 15.5 kb *Spe*I-*Eco*RI fragment of pSH1590 was subcloned using pBluescript II KS⁺ to generate pSH1590.10. The 6.5 kb and 16.2 kb *Eco*RI fragments of pSH1500, and the 8.7 kb *Eco*RI-*Xba*I fragment of pSH1500 were subcloned using pGEMzf(+) to generate pSH1500.20, pSH1500.30, and pSH1500.40, respectively. Each of these was restriction mapped with *Bam*HI and *Pst*I. Two small *Eco*RI fragments (0.7 and 0.3 kb) were subsequently identified and mapped to positions between pSH1500.30 and pSH1500.40. Southern blots of the digests were probed with ³²P-labeled *actI* and ³²P-labeled *actIII* (the 9-ketoreductase gene of *S. coelicolor* A3(2) contained on pIJ2346).²³⁾

Southern Hybridization

Southern blots of recombinant cosmids were performed using Hybond-N nylon membranes (Amersham), and the blots were hybridized with the *actI* probe, as recommended by the manufacturer.

Other Molecular Biological Techniques

Plasmid DNA isolations from *E. coli* and preparation of *E. coli* competent cells were performed according to standard procedures.²⁴⁾ Restriction enzymes, DNA ligase and calf intestinal alkaline phosphatase were purchased from Gibco BRL, New England Biolabs, United States Biochemical, Promega and Boehringer-

Mannheim, and used according to the manufacturers' instructions. Plasmid isolation and protoplast preparation from *S. lividans* were carried out according to HOPWOOD *et al.*²²⁾

Results

S. murayamaensis proved much less tractable for extraction of genomic DNA than other species, such as *S. lividans*. To overcome this problem, a simple and efficient protocol was developed that has proven to be of general utility. Thus, mycelia were briefly treated with lysozyme and achromopeptidase, followed by complete digestion with proteinase K overnight at 50°C. Genomic DNA from *S. murayamaensis* was then digested with a variety of enzymes as described in the Materials and Methods Section, and Southern blots were probed with ³²P-radiolabeled *actI*, the ketoacylsynthase gene from the actinorhodin pathway of *S. coelicolor* A3(2).²³⁾

Isolation of Two PKS Gene Clusters from *S. murayamaensis*

A *S. murayamaensis* cosmid library containing fragments of ~30~40 kb was constructed using a partial digest of genomic DNA ligated into a cosmid shuttle vector.²⁷⁾ Screening this library with the *actI* probe identified thirty-eight hybridizing clones, and restriction

mapping and Southern hybridization results clearly showed that the clones segregated into two non-overlapping sets.

Expression of the *kin* Genes in *S. lividans* ZX7

All fifteen cosmid clones from the cluster that proved to contain the *kin* genes were used to transform *S. lividans* ZX7,²⁰⁾ derived from *S. lividans* JT46.²⁸⁾ Four of these transformants produced recognizable *S. murayamaensis* metabolites (Table 1), including dehydrorabelomycin. One major metabolite had previously been observed as a very minor metabolite in *S. murayamaensis* mutant MC1,¹¹⁾ a kinamycin-deficient strain that still produces dehydrorabelomycin and kinafluorenone. It was obtained in sufficient quantity from a large-scale fermentation of *S. lividans*/pSH1580 for its characterization as a benzo[*b*]fluorene analogous to stealthin C, but containing an *N*-acetylcysteine residue at C-5, and has been named seongomycin.²⁹⁾ Most importantly, the transformants containing pSH1590 or pSH1610 also produced kinobscurinone, stealthin C, and kinafluorenone, identified by photodiode array-detected HPLC. The first two are intermediates in kinamycin biosynthesis, and the last is a shunt metabolite.

All twenty-three cosmid clones from the other cluster were also used to transform *S. lividans* ZX7. However, no new metabolites were produced by these transformants.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Bacterial strains		
<i>S. murayamaensis</i>	Kinamycin and murayaquinone producer	ŌMURA <i>et al.</i> (1973)
<i>E. coli</i> XL1-BlueMR	$\Delta(mcrA)183$, $\Delta(mcrCB-hsdSMR-mrr)173$, <i>endA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>lac</i>	Stratagene
<i>S. lividans</i> ZX7	Host for expression test (SLP2 ⁻ , SLP3 ⁻)	John Innes Institute
Plasmids		
pOJ446	<i>E. coli</i> - <i>Streptomyces</i> shuttle cosmid	BIERMAN <i>et al.</i> (1992)
pBluescript II KS ⁺		Stratagene
pGEM11zf(+)		Promega
pIJ2345	pBR329 with a 2.2 kb <i>actI</i> insert	John Innes Centre
pIJ2346	pBR329 with a 1.1 kb <i>actIII</i> insert	John Innes Centre
pSH1500	<i>kin</i> + pOJ446, produces dehydrorabelomycin and seongomycin in <i>S. lividans</i>	This work
pSH1580	<i>kin</i> + pOJ446, produces dehydrorabelomycin and seongomycin in <i>S. lividans</i>	This work
pSH1590	<i>kin</i> + pOJ446, produces dehydrorabelomycin, seongomycin, kinafluorenone, kinobscurinone, and stealthin C in <i>S. lividans</i>	This work
pSH1610	<i>kin</i> + pOJ446, produces dehydrorabelomycin, seongomycin, kinafluorenone, kinobscurinone, and stealthin C in <i>S. lividans</i>	This work

Four inserts from the *kin* cosmids producing identifiable metabolites contained the same two 16.2 and 6.5 kb *EcoRI* fragments. The two cosmids that yielded the more advanced metabolites (e.g. **5** and **6**), have *SpeI-EcoRI* fragments approximately 2.0~2.5 kb longer than the other two cosmids. This may indicate that genes for advanced stages of the pathway are at the *SpeI* end of the inserts. Using two of the cosmid clones, four subclones were prepared that spanned the overlapping region that contained the available *kin* genes. A physical map of each was generated from digests with *BamHI* and *PstI*, and the regions containing DNA homologous to *actI* and to *actIII* were identified on pSH1500.30 by hybridization experiments (Figure 2).

Discussion

S. murayamaensis produces two different groups of aromatic decaketides,^{5,9,30)} one of which—the kinamycins—is derived from the angucyclinone dehydrabelomycin. Since typical aromatic polyketide biosynthetic gene clusters encompass approximately 20~35 kb, we constructed a library of approximately 40 kb fragments of *S. murayamaensis* genomic DNA. This approach has previously proven very successful, and we have cloned and heterologously expressed the genes for two other

complete angucyclinone pathways,³¹⁾ those for tetra-nngulol/tetrangomycin³²⁾ and for antibiotic PD116740.³³⁾

The studies reported here have revealed that *S. murayamaensis* contains at least two clusters of PKS genes. Heterologous production of kinamycin biosynthetic intermediates was obtained from only one cluster. It is noteworthy that *S. coelicolor* has two known PKS clusters, one for actinorhodin³⁴⁾ and one for a spore pigment,³⁵⁾ and the genes for the latter product are not expressed during actinorhodin production.³⁶⁾ We have also found two PKS clusters in *S. rimosus* NRRL 3016 and in *S. WP 4669*; in each case heterologous expression was obtained from only one cluster.³¹⁾ The urdamycin producer *S. fradiae* Tü 2717 also contains two.³⁷⁾ It is possible that the unexpressed cluster in each of these organisms may code for a spore pigment. Hybridization probes from the actinorhodin (*act*) pathway were used, and this may have biased the results. Thus, *S. murayamaensis* is unusual in this group of organisms in that it also produces another aromatic decaketide, murayaquinone,³⁰⁾ under normal conditions. Incorporation of isotopically-labeled acetates³⁸⁾ has indicated that its biosynthesis involves ketone reductions and a folding of the acyclic decaketide intermediate in a manner very different from that found in the isochromanquinones (e.g. actinorhodin), the anthracyclines, or the angucyclines.

Fig. 2. Physical map of the *kin* gene cluster.

B = *BamHI*, EI = *EcoRI*, M = *MboI*, P = *PstI*.

Cosmids and subclones used for mapping are shown below the genomic DNA. Regions hybridizing with either *actI* or *actIII* are indicated; the orientation is arbitrarily chosen to conform to that previously observed for other angucyclinone PKS genes.

Fig. 3. Potential relationship of the "cleavase" enzymes in the kinamycin and jadomycin biosynthetic pathways.

Thus, it is possible that the murayaquinone PKS genes would not hybridize with the *act* genes at a useful stringency.

Heterologous production of metabolites representing the bulk of the kinamycin D pathway has now been obtained. Thus, accumulations of the kinamycin intermediates **2**, **5** and **6** were observable, as were the shunt metabolites kinafluorenone and seongomycin. Although other aromatic polyketide gene clusters have been fully encompassed by 40 kb of DNA, this may prove not to be the case for kinamycin D. We have previously isolated an apparently multifunctional protein, or complex of proteins, of >670 KDa from *S. murayamaensis* that catalyzes the sequential acetylations that convert kinamycin F to kinamycin D via kinamycin E. If a single peptide, such a protein would by itself add 15~20 kb of DNA to the cluster.

Although the complete kinamycin D gene cluster is not yet in hand, the portion that is available contains the gene required for cleaving the C-ring of the angucyclinone skeleton, as well as the gene(s) for generating the benzo[*b*]fluorene skeleton. VINING *et al.* has reported³⁹⁾ disruption of the gene for the C-ring cleavage

enzyme in the jadomycin (*e.g.* jadomycin B, **9**) pathway, with concomitant accumulation of rabelomycin, **10**. They have suggested that this is the cleavage substrate, which would require dehydration and aromatization of the D-ring at a later stage. With the availability of the "cleavase" gene for the kinamycin pathway, it will be possible to determine the relationship of these two pathways (Figure 3).

The cloned DNA also contains the gene(s) for introduction of the first nitrogen of the eventual diazo group that characterizes all of the kinamycins. Although kinobscurinone has been detected in *S. murayamaensis* fermentations and, in labeled form, has been incorporated into kinamycin D,¹⁵⁾ the actual amination substrate cannot yet be precisely identified. The compounds in this group can exist in the hydroquinone, quinone, and semi-quinone forms under ambient conditions. With such small differences in redox potentials, the amination substrate could be kinobscurinone, in which case a glutamine- or ammonia-dependent amido-transferase might be considered more likely, or it could be the hydroquinone, in which case a pyridoxamine phosphate-dependent transaminase would be more likely

Fig. 4. Potential involvement of either an aminotransferase or amidotransferase in the formation of stealthin C.

a: Transaminase, b: amidotransferase; c: tautomerase, d: oxidase, e: hydrolase, f: [1,2]reductase, g: [1,6]reductase.

(Figure 4). The availability of the gene(s) for this portion of the pathway bodes well for obtaining the relevant enzymes to resolve this issue.

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