Cloning and Characterization of Actinorhodin Biosynthetic Gene Clusters from Streptomyces lividans TK24

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Actinorhodin antibiotics produced by Streptomyces lividans TK24 are blue pigments with a weak antibiotic activity, derived from one acetyl-CoA and 15 malonyl-CoA units via a typical ployketide pathway. In an attempt to clone polyketide biosynthetic genes of S. lividans TK24, hybridizing fragments in the genomic DNA of S. lividans TK24 were detected by use of actI and actIII polyketide synthase gene probes. Since typical aromatic polyketide biosynthetic gene clusters are roughly 22-34 Kb long, we constructed in E. coli XL-Blue MR using the Streptomyces-E. coli bifunctional shuttle cosmid vector (pOJ446). Then, about 5,000 individual E. coli colonies were thoroughly screened with actI-ORFI and actIII probes. From these cosmid libraries, 12 positive clones were identified. Restriction analysis and southern hybridization showed two polyketide biosynthetic gene clusters in this organism. These cosmid clones can be transformed into Streptomyces parvulus 12434 for expression test that identify product of actinorhodin biosynthetic genes by heterologous expression. Thus, heterologous expression of a derivative compound of a actinorhodin biosynthetic intermediate was obtained in pKE2430. Expression of these compounds by the transformants was detected by photodiode array HPLC analysis of crude extracts.

Streptomycetes are Gram-positive, pseudo-fungal prokaryotes that produce diverse secondary metabolites including antibiotics, chemotherapeutic agents, ionophores and immuno-modulators. Among the nearly 6,000 antibiotics of natural origin that have been characterized, more than 60% are produced by members of the genus Streptomyces (Berdy, 1974). Interestingly, most Streptomyces species have more than one secondary metabolite biosynthetic pathway and produce a variety of secondary metabolites through the pathway.

The polyketides are examples of secondary metabolites. Typical examples of polyketides are the majority of antibiotics applied in human and veterinary medicine and agriculture as well as anti-parasitic agents, herbicides, and pharmacological metabolites. The term polyketide defines a class of molecules produced through the successive condensation of small carboxylic acids (Hopwood and Sherman, 1990). Natural polyketide products representing a large class of complex organic molecules produced by bacteria, fungi, and plants are a group of organic molecules produced through the successive condensation of small

carboxylic acids such as acetate, propionate or butyrate (O'Hagan, 1993).

The actinorhodin biosynthetic gene cluster is the first cloned aromatic polyketide biosynthetic gene cluster from Streptomyces coelicolor A3(2) and became the model polyketide biosynthetic gene cluster (Fernandez-Moreno et al., 1992). The polyketide synthases (PKSs) responsible for the biosynthesis of aromatic polyketide antibiotics in Streptomycetes bacteria are analogous to bacterial and plant Type II fatty acid synthase (FAS) and are called Type II PKSs (Berdy, 1974). These are quite distinct from Type I PKSs. Type I PKSs are described as multifunctional enzyme complexes like Type I FAS, while Type II PKSs are responsible for the production of aromatic polyketides (Berdy, 1974). Aromatic PKSs are determined by four to six genes encoding mono- or bifunctional enzymes; one PKS complex is used for all synthesis steps (Berdy, 1974). All of the genes necessary for biosynthesis of the polyketide antibiotic actinorhodin are together, clustered on the same stretch of genomic DNA (Fernandez-Moreno et al., 1992).

Streptomyces lividans TK24 has been a model host system for expression of various foreign genes including genes for secondary metabolites because of its high transformation efficiency and well-defined genetic characteristics (Hopwood et al., 1985). However, the

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actinorhodin biosynthetic gene cluster of *S. lividans* TK24 produces various hybrid compounds and its innate high expression profiling pattern makes the analysis of heterologous expression difficult. The purpose of our study was to clone the biosynthetic gene cluster of actinorhodin from *S. lividans* TK24 for future use in homologous recombination for creation of *S. lividans* TK24 lacking the actinorhodin biosynthetic gene cluster.

Materials and Methods

Bacterial strains and plasmids

Streptomyces lividans TK24 and *Streptomyces parvulus* were obtained from David Hopwood (John Innes Center, Norwich, England). The cosmid pOJ446, used for cosmid library construction, was obtained from Lilly Research Laboratories. *Escherichia coli* XL1-Blue MR was purchased from Stratagene Co.

Culture conditions and general molecular biological techniques

S. lividans TK24 and *S. parvulus* culture condition, protoplast preparation from *S. parvulus*, and transformation of *S. pavulus* were carried out according to the method of Hopwood et al. (1985). Genomic DNA isolation from *S. lividans* was performed according to the method of Hong et al. (1997). General molecular biological techniques such as plasmid DNA isolations from *E. coli*, preparation of *E. coli* competent cells, restriction enzyme analysis, DNA ligation, and southern hybridization were performed according to standard procedures (Sambrook et al., 1989).

Preparation of S. lividans cosmid library

4,000-5,000 *E. coli* colonies of *S. lividans* TK24 cosmid library in LB broth were spread by pipetting on several dried nylon membranes as described by the manufacturer. After spreading the inoculum, membranes were transferred onto LB-agar plate containing 100 µg/mL of apramycin and incubated at 37°C overnight. After colonies were grown, the membrane was replicated onto several other membranes by pressing down the original membrane to new membranes on a sterile glass plate with another glass plate.

The replica membranes were grown again on LB-apramycin plates overnight. One of the replica membranes was placed colonies side up on a pad of Whatman 3 MM absorbent filter paper soaked in denaturing solution (1.5 M NaCl/0.5 M NaOH) for 7 min. The membrane was then transferred colonies side up on a pad of 3 MM absorbent filter paper soaked in neutralizing solution (1.5 M NaCl/0.5 M Tris HCl, pH 7.2/1 mM EDTA) for 3 min. The membranes were rinsed briefly in 2× SSC, dried in air and the DNA was then fixed by microwave at 256 nm for optimal cross-link.

Colony hybridization of cosmid libraries

The fixed membrane was prehybridized with 30 mL of prehybridization solution (5× SSPE/5× Denhardt's solution/0.5% SDS/20 µg per mL of denatured salmon sperm DNA) in a 150 mm glass dish at 65°C overnight. After hybridization, the filters were washed 1) twice with 2× SSPE/0.1% SDS at room temperature for 10 min, and 2) once in 1× SSPE/0.1% SDS at 65°C, followed by autoradiography. After identification of positive clones, they were picked from the original membrane and incubated onto LB-apramycin (100 µg/mL) agar plates to isolate single colony. After overnight incubation of the plate at 37°C, the individual single colony was used to inoculate 2 mL of LB containing 50 µg/mL of apramycin and incubated at 37°C. Twenty microliters of the overnight culture were spotted onto a dried nylon membrane for dot-blotting and performed Southern hybridization to identify positive clones.

Metabolite analysis of S. parvulus transformants

A *S. parvulus* spore suspension (0.1 mL) was spreaded on R2YE containing 25 µg/mL of apramycin for transformants containing pOJ446-derived recombinant plasmids and was incubated for 10-12 d at 30°C. The spore collections were acidified (pH 2.5-3.0) with 0.1 HCl extracted with EtOAc, and the extracts were dried using a SpeedVac centrifuge. The dried extracts were taken up in 100 µL of 10% MeOH/CH$_2$Cl$_2$. An aliquot (10 µL) of each extract was analyzed on a Waters NovaPak C$_{18}$ radial compression column (0.8 × 10 cm) using a gradient of 5-95% acetonitrile in water over a period of 40 min at 1 mL/min. Detection was done by photodiode array (SPD-M10AVP, SHIMADZU).

Results

Southern hybridization of S. lividans TK24 genomic DNA with actI and actIII

Genomic DNA from *S. lividans* TK24 was digested with a variety of enzymes as described in Materials and Methods, and Southern blots were probed with the actI-ORFI (ketosynthase) and actIII (ketoreductase) genes from the actinorhodin pathway of *S. coelicolor* A3(2) (Fernandez-Moreno et al., 1992) (Fig. 1). Upon hybridization with actI-ORFI DNA fragment, the BamHI digested genomic DNA gave signals for fragments of 6.5 Kb and 2.3 Kb. Hybridization against KpnI digested genomic DNA showed three bands of 8.8 Kb, 6.3 Kb and 2.0 Kb and PstI digested genomic DNA showed two bands of 9.2 Kb and 3.5 Kb. The pattern of SalI digested DNA showed many SalI sites. Upon Southern hybridization with actIII DNA fragment, the BamHI digested genomic DNA gave signals for fragments
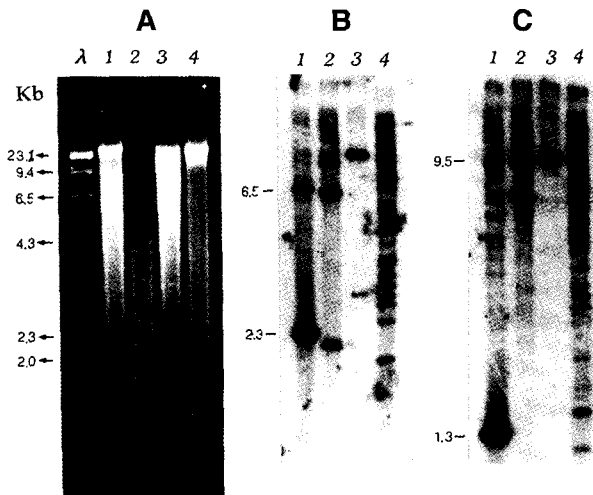
A B C

Fig. 1. Hybridization of *actI*-ORFI and *actIII* probes to total genomic DNAs from polyketide-producing *S. lividans* TK24. A, Ethidium bromide stained agarose gel (0.75%) of restriction-digested total DNAs: lane 1, *Bam*H I -digested DNAs; lane 2, *Kpn* I -digested DNAs; lane 3, *Pst* I - digested DNAs; lane 4, *Sal* I -digested DNAs. B, Hybridization of the DNAs in (A) with the α-^{32}P-labelled *actI*-ORFI (2.2 Kb) fragment. C, Hybridization of the genomic DNAs of *S. lividans* TK24 with *actIII* probe (1.1 Kb).

9.5 Kb and 1.3 Kb. *Kpn*I or *Pst*I digested genomic DNA showed 8.8 Kb and 6.3 Kb or 9.2 Kb. Compared with band pattern of *act* III homologous DNA, *S. lividans* TK24 contained two PKS biosynthetic gene clusters.

Isolation of actI and actIII homologous clones from a S. lividans TK24 cosmid library

About 5,000 individual colonies were thoroughly screened with the *actI*-ORFI and *actIII* probes. This led to the identification of 12 positive clones. Based on restriction digestion followed by southern hybridization, it was found that the 12 clones could be grouped into two clusters. Of these, 2 clones were grouped into Cluster I and 10 clones into Cluster II (Table 1).

DNA of Cluster I contains the 6.5 Kb and 2.3 Kb *Bam*H I fragments that hybridized with *actI*-ORFI and 1.3 Kb *actIII*-hybridized *Bam*H I DNA fragment. DNA of Cluster II contains the 9.5 Kb *Bam*H I DNA fragment that hybridized with *actIII*. Fig. 1 shows Southern hybridization with *Bam*H I digested DNA from one cosmid

clone from each of the two clusters. This clearly demonstrated that the two PKS gene clusters of *S. lividans* TK24 were successfully cloned. The large cloned DNA fragments (35-40 Kb) can increase the chance of expressing a recognizable product from cloned PKS genes. Also, the larger cosmid inserts of genomic DNA made it easier to correctly group PKS clones. The weak Southern hybridization signals from these cosmid clones may have come from non-specific binding of the probe to genes which are slightly homologous to PKS genes, such as those for fatty acid synthesis.

Heterologous expression of a putative actinorhodin biosynthetic gene

Two cosmid clones from Cluster I and 10 from Cluster II were introduced into *S. parvulus* by transformation to identify product of PKS genes by heterologous expression (Table 1). Transformation efficiency of *S. parvulus* with either Clusters was about 100 times lower than that with the cosmid itself. Also, the transformants showed typical phenotype of *S. parvulus* growing in a stress condition. It was possible that both types of recombinant plasmids led to heterologous expression of secondary metabolites potentially harmful to the host strain. *S. parvulus* transformants with the clone (pKE2430) of Cluster I produced a red pigment in R2YE and grew especially slowly. *S. parvulus* sometimes eliminates foreign DNA inserts by intramolecular recombination.

Because of this possibility, multiple colonies were inoculated into liquid media instead of single colonies. After growing the transformants on R2YE agar for 7-10 d multiple colonies were scraped off from the plates to inoculate 5 mL of YEME media. A large quantity of cells was used for inoculation to reduce the chance of intramolecular recombination, since fewer cell divisions would be required to reach log-phase. It is obvious that fewer cell divisions are much better for the expression test. After growing the seed culture for 3-4 d, depending on growth of the transformants, 0.3 mL of the seed culture was used to inoculate 5 mL each of GPS and YEME for production and these were incubated for 9 d. The cultures were extracted with ethyl

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

Transformant	Relevant characteristics	Sources
pOJ446	Stress condition in *S. parvulus*	Oregon university
pKE2430	ClusterI+pOJ446, produce ACT compound in *S. parvulus*	This work
pKE2438	ClusterI+pOJ446, produce ACT compound in *S. parvulus*	This work
pKE2210	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2810	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2850	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2510	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2520	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2525	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2530	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2545	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2550	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work
pKE2555	ClusterII+pOJ446, didn't produce ACT compound in *S. parvulus*	This work

A S. parvulus 12434 (Wild type)

S. parvulus 12434/ pOJ446

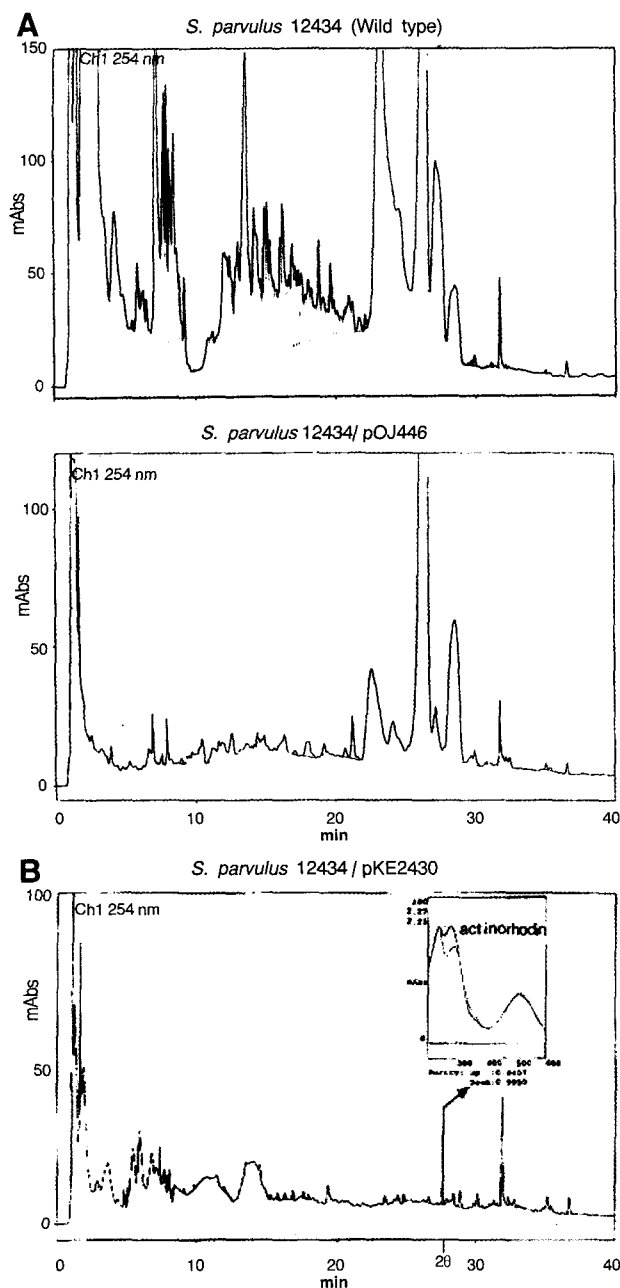**B** S. parvulus 12434 / pKE2430

Fig. 2. A, HPLC trace of *S. parvulus* 12434 and *S. parvulus*/pOJ446. The metabolites were extracted from a 9-d culture in GPS media. B, HPLC trace of *S. parvulus* 12434/pKE2430. The metabolites were extracted from a 9-d culture in GPS media. Actinorhodin is indicated by an arrow.

acetate. The extracts were analyzed using photodiode array HPLC. The HPLC analysis showed that the *Cluster* II transformants did not produce any new metabolites in *S. parvulus*. The cosmids still had an intact insert. Restriction analysis of the cosmid DNA showed that all clones had lost more than half of their insert, presumably by a intramolecular recombination process. As shown in Fig. 1, the aromatic PKS gene

has an unusually large number of *Sal* I site, indicating that the region may be a repetitive area. Repetitive areas very easily losing the PKS gene cluster may explain why there was no expression of PKS genes in *S. parvulus*.

The pKE2430 of Cluster I produced actinorhodin metabolites in *S. parvulus*. Fig. 2 shows the HPLC traces of *S. parvulus* wild type and *S. parvulus* with just pOJ446 and with the recombinant cosmid pKE2430. UV-visible absorption spectra was obtained by photodiode array detection. Actinorhodin started to be detected at the late stage of fermentation (usually after 5 d in GPS). In YEME, *S. parvulus* that had recombinant cosmid pKE2430 did not produce secondary metabolites, actinorhodin. UV-visible spectrum and retention time (28 min) of actinorhodin exactly matched one of metabolites previously observed to be produced by *S. lividans* TK24.

Discussion

Almost all of structurally diverse natural chemical products arise from secondary metabolite biosynthetic pathway of bacteria, fungi and plants in nature. Today, understanding of secondary metabolite biosynthetic pathway has reached a point where we can now contemplate the idea of adapting the system to our own ends (Walsh, 2002; Hutchinson and McDaniel, 2001; Tsoi and Khosla, 1995; Kao et al., 1994). The prospect of genetically manipulating secondary metabolite biosynthetic pathway by combinatorial biosynthesis or rational design to generate libraries of 'unnatural' natural products is no longer within the realm of fantasy (Tsoi and Khosla, 1995). As a first step toward generating libraries of unnatural natural product, we must understand how the biosynthetic gene cluster for secondary metabolite achieve the structural diversity of natural products. It is the essential and prerequisite step to understand the catalytic functions and specificities of the genes for secondary metabolite biosynthesis. However, the innate secondary metabolite biosynthetic genes of host makes analysis of foreign secondary metabolite gene very difficult. To overcome this problem, Khosla et al. developed a *Streptomyces* host system that is *S. coelicolor* CH999 constructed by deleting, through a homologous recombination, the entire actinorhodin biosynthetic gene cluster from the chromosome (McDaniel et al., 1993). However, low transformation efficiency of *S. coelicolor* CH999 has been a problem in manuplating heterologous genes.

The study reported here revealed that *S. lividans* TK24 contain two clusters of PKS genes. *S. coelicolor* has PKS gene cluster for actinorhodin (Malpartida and Hopwood, 1986) and for a spore pigment (Davis and Chater, 1990). The genes for the latter product are not expressed during actinorhodin production. It is possible that the unexpressed clusters from *S. lividans* TK24 may code for spore pigments. Detailed functions of

individual enzyme of a number of polyketides have been deciphered by expressing combination of different genes. This has demonstrated the possibility of rationally designed new polyketide by genetic engineering (McDaniel et al., 1993; Kao et al., 1994). However, this requires a proper *Streptomyces* strain lacking secondary metabolic pathways and still having high transformation efficiency. The actinorhodin biosynthetic gene cluster reported here can be used to delete the actinorhodin biosynthetic gene cluster in *S. lividans*. The strain will be an ideal host expression system having high transformation efficiency and lacking secondary metabolic pathways because *S. lividans* itself has high transformation efficiency.

Acknowledgments

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