(Syngene), Hybaid Hybridization Oven and hybridization bottle (Thermo electron corporation), Microcentrifuge, Model Mikro 12-24 (Hettick), Orbital shaker, Model Innova ™2100 (New Brunswick scientific Co. Inc), Thermocycler model Gene Amp PCR system 2400 (Perkin elmer), and model Master cycler gradient (Eppendorf). Rocker platform (Bellco biotechnology), UV spectrophotometer (Jenway), Water bath shaker, model G-76D (New Brunswick Scientific Co., Inc., U.S.A.), and X-ray film (Kodak, U.S.A.)

Methods

Bacterial Cultivation

All *Streptomyces* species were grown in nonsporulating medium at room temperature for 48-72 hr for production of vegetative mycelium . For spore production, the *Streptomyces* species were cultured on sporulating medium, and incubated as mentioned.

Isolation of Genomic DNA from *Streptomyces*

Thirty milligrams of harvested mycelia of *Streptomyces* were used for genomic DNA extraction. The genomic DNA isolation was performed by the method of Kutchma *et al.* (Kutchma *et al* 1998). *Streptomyces* mycelia were suspended in 500 µl of TE and mixed for 20 sec. Then, the suspension was centrifuged at 12,000xg for 4 min and supernatant was removed. The pellet was resuspended in 1 ml of ice-cold acetone, mixed and incubated on ice for 5 min. After centrifugation at 12,000xg for 4 min, acetone was removed and the pellet was dried. The mycelium cells were resuspended in 500 µl of TE containing 1mg/ml lysozyme and incubated at 37 °C for 2 hr. The suspension was mixed with 75 µl of 10% SDS and 125 µl of 5 M NaCl. The mixture was frozen in liquid nitrogen and thawed at 65 °C. The freeze/thaw cycle

was repeated 3 times. The mixture was further incubated on ice for 10 min, then centrifuged at 12,000xg for 5 min. Supernatant was transferred to a fresh microcentrifuge tube and added Ribonuclease A to a final concentration of 200 μg/ml. After incubation at 37 °C for 1 hr, Proteinase K was added to a final concentration of 50 μg/ml and incubated at 37 °C for 30 min. The mixture was extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). The aqueous phase of the mixture was collected and DNA was precipitated by an addition of 2 volumes of absolute ethanol. DNA was washed by 70% ethanol. Finally, genomic DNA was dissolved in 100 μl of TE and stored at -20 °C. For quantitative and qualitative analysis, DNA was subject to agarose gel electrophoresis and measurement at wavelength of 260 and 280 nanometer.

Dot Blot Hybridization

Preparation and labeling of *picK* gene probe

Plasmid, pMC-*picK*, in *Escherichia coli* was isolated and purified by the method of PEG preparation (Ausubel *et al* 2002). The *picK* gene was removed from pMC-*picK* by restriction digestion with *Hin*dIII and *Nde*I. The *picK* fragment was resolved on agarose gel electrophoresis and purified by the QIAquick gel extraction. The purified *picK* gene was labeled with Digoxygenin (DIG) using DIG- High prime Labeling and Detection Starter Kit. One microgram of purified *picK* DNA in 16 μl of water was denatured in waterbath for 10 min and quickly chilled in ice. The *picK* DNA was labeled by incubating with 4 μl of DIG-High Prime at 37 °C overnight. The reaction was stopped by addition of 2 μl of 0.2 M EDTA pH 8.0.

Preparation of samples

Five hundred nanograms of individual genomic DNA of *Streptomyces* in a final volume of 10 µl were denatured for 10 min, then dotted on a possitively charged nylon membrane. A various concentration of the negative control DNA (100, 500 and 1000 ng of genomic DNA of *E.coli*) and the positive control DNA (50, 500, 1000 of genomic DNA of *Streptomyces venezuelae* and *picK* gene) were also included in the dot blot membrane. Finally, DNA samples on the membrane were UV crosslinked and stored at room temperature for the next experiment (Ausubel *et al* 2002).

Hybridization

Nylon membrane was prewarmed at 42 °C in standard hybridization solution for 1 hr with gently agitation. Then, the hybridization solution was poured off. The prehybridized nylon membrane was added with the same hybridization solution containing denatured DIG-labeled probe (250 ng) and incubated at 42 °C for 18 hr.

After hybridization, the membrane was washed twice with 2XSSC (0.1% SDS) at room temperature for 5 min and washed twice with 0.5XSSC (0.1% SDS) at 68 °C for 15 min.

The washed membrane was treated with blocking solution for 30 min prior to incubation with anti-DIGconjugated with alkaline phosphatase (75 mU/ml) for 30 min. Then, the antibody solution was drained off and the membrane was washed in washing buffer for 15 min. The membrane was equilibrated in detection buffer for 5 min, then placed in a hybridization bag. Approximately one millilitre of CDP-star was added into the bag and the membrane was incubated at room temperature for 10 min. Finally, the membrane was exposed to x-ray film.

Polymerase Chain Reaction (PCR)

The degenerated primers were designed based on known sequences of polyketide cytochrome P-450 hydroxylase from *Streptomyces*, which were retrieved from GenBank (Altschul *et al* 1990). The primers were synthesized by Bioservice unit, Thailand, and the primer sequences are as followed :

MCF1 : 5' (AG)(CG)C C(GA)G CTG CGC A(AG)G CTG GTG 3'

MCR2 : 5' CGC C(GC)A (TG)GC AG(AT) (AG)GT G(GC)A (TC)GC 3'

The reaction mixture of PCR contains 1X *Taq* DNA polymerase buffer, 0.2 mM dNTP mix, 1 mM of each primer, 1.5 mM $MgCl_2$, 5% Dimethylsulfoxide (DMSO) (Chakrabarti *et al* 2002; Sun *et al* 1993), 0.1 unit of *Taq* DNA polymerase, and 100 ng of genomic DNA. The annealing temperatures for PCR were optimized by gradient PCR at 62, 65, 67, 68 °C. The PCR condition was carried out as following : preheating at 95 °C for 30 sec, denaturation at 94 °C for 30 sec, annealing at various temperature for 30 sec, and extension at 72 °C for 2 min. The condition was repeated for 35 rounds. When the amplification completed, PCR product was detected by agarose gel electrophoresis.

Cloning of PCR Product for DNA Sequencing

The PCR products were cloned in PCR4 TOPO by the method of TOPO TA cloning® kit for sequencing as described by the manufacturer. The cloning reaction was composed of 100 ng of PCR product, 1.2 μM and 0.06 μM $MgCl_2$ of salt solution, 10 ng of TOPO® vector and steriled water and was incubated for 5 min at room temperature. Two microlitres of the cloning reaction was mixed to *E.coli* TOP10 competent cell, incubated on ice for 30 min. Then, the cells were subject to heat shock for 30 sec at 42 °C and quickly chilled on ice. The 250 μl of S.O.C. medium was immediately added and the cell suspension was shaked the tube at 37 °C for 1 hr.

Fifty microlitres of transformation reaction was spreaded on a selective medium plate and incubated at 37 °C for 18 hrs.

For screening, each clone was inoculated into LB broth containing 50 μg /ml ampicillin and incubated at 37 °C for 18 hrs. The recombinant plasmid was isolated by miniprep method and the plasmid was digested with *EcoR*I. The selected clones carrying the PCR fragment were sent to BSU for DNA sequencing-using M13 forward and M13 reverse primers.

Sequence Analysis

The nucleotide sequences obtained from previous experiment were used as a query for the BLAST search of the GenBank (Altschul *et al* 1990) in order to identify their homology to other organisms. The predicted amino acid sequences from their nucleotide sequences were also used as a query for protein database search. The sequence homologs obtained from the BLAST search were aligned using the ClustalX program (Thompson *et al* 1994). Genetic relatedness among the polyketide cytochrome P-450s was constructed by PHYLIP version 3.5 program using maximum parsimony (Felsenstein 1995).