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NOTE

An Efficient Approach for Cloning P450 Hydroxylase Genes from Actinomycetes

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Abstract Oligonucleotide primers were designed and successfully applied to amplify DNA fragments of P450 hydroxylase genes from actinomycetes which produce a large variety of medically important metabolites. Primers were designed based on several regions of strong similarities in amino acid sequence of P450 hydroxylases from a variety of actinomycetes, primarily in the regions of an oxygen binding site and a heme ligand pocket. These primers were used to amplify DNA fragments from seven different actinomycetes species producing a variety of different compounds. The deduced amino acid sequences of the isolated fragments revealed significant similarities to known P450 hydroxylase including the product of the *suaC* or *subC* genes from *Streptomyces griseolus* that is capable of metabolizing a number of sulfonylurea herbicides, and to the product of the $P450_{sca2}$ from *S. carbophilus* that produces a specific HMG-CoA reductase inhibitor. This method should help researchers in cloning the P450 hydroxylase genes involved in the biosynthesis of useful compounds.

Key words: Actinomycetes, P450 hydroxylase, PCR cloning

The P450 hydroxylases constitute an extremely large family ('superfamily') of haemoproteins that catalyse the oxidation of a wide range of physiological and non-physiological compounds. A remarkable feature of the P-450s is their capacity to manipulate the same basic structure and chemistry to achieve an enormous range of functions in organisms as diverse as bacteria and man. P450 hydroxylases require two other proteins for electron transport from NADPH to the heme prosthetic group *in vitro*. Amino acid sequences of P450 hydroxylases from a variety of actinomycetes contain several regions of strong similarity, primarily in the regions of an oxygen binding site and a heme ligand pocket which are responsible for attachment and coordination of the heme prosthetic group [14].

Several P450 hydroxylases have been reported in different species of the industrially important genus, actinomycetes. Some of these have been reported to participate in secondary metabolism or biodegradation [4, 6, 7, 15, 19, 20].

EryF and EryK, two of the erythromycin biosynthetic enzymes, catalyses the hydroxylation of 6-deoxyerythronolide B to generate erythronolide B and the hydroxylation of carbon 12 of the erythromycin D (which is a precursor of this macrolide antibiotic), respectively [1, 12]. Recent advances have been the isolation of genes encoding P450 involved in mycinamicin biosynthesis from *Micromonospora griseorubida* [6], oleandomycin antibotic synthesis from *Streptomyces antibioticus* [16], and immunosuppressant biosynthesis from FK506 and FK520 [13]. Also, bacterial P450s are certain to remain a focus of attention as tools for biodegradation of organic pollutants. For example, two herbicide inducible P450 hydroxylases from *S. griseolus*, designated *suaC* ($P\text{-}450_{SU1}$) and *subC* ($P\text{-}450_{SU2}$), are capable of metabolizing a number of sulfonylurea herbicides to compounds that often exhibit reduced phytotoxicity [15]. P450-type hydroxylases, including the product of the *choP* gene from *Streptomyces* sp. strain SA-COO, participate in cholesterol oxidation and the product of the P450*sca2* from *S. carbophilus* produces a specific HMG-CoA reductase inhibitor [4, 20].

In this paper, we report a PCR method which can be used for the rapid amplification of DNA fragments from the genes for P450 hydroxylase from a wide range of actinomycetes strains. This method should help researchers in cloning the genes for P450 hydroxylase homologues involved in the biosynthesis of useful compounds.

Design of PCR Primers for the Amplification of the Genes for P450 Hydroxylase

The application of PCR has proven to be a valuable tool for the identification of actinomycetes genes. Due to the

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biased codon usage of actinomycetes, oligonucleotide primers could be designed rather unambiguously even from short consensus sequences determined by amino acid comparison [10]. The comparison of the sequences of known P450 hydroxylases from *S. carbophilus* [20], *S. griseus* [19], *S. griseolus* [15], and *Amycolata autotrophica* [7] revealed high similarity in the regions for an oxygen binding site and a heme ligand pocket. We prepared primers from amino acid consensus sequences found within the known P450 hydroxylase genes (Fig. 1). On the basis of the consensus sequences of P450 hydroxylase from Amy (D26543), SuaC (M32238), SubC (M32239), Sca2 (D30815), and Soy (X63601), and the codon bias of *Streptomyces* genes, the DNA primers were designed as 5'-TXCTXCTXATCGCXGGXCACGAGAC-3', which corresponds to the conserved region of the O_2 binding site, and 5'-GCXAGGTTCTGXCCXAGGCACTGGTG-3', which corresponds to the complementary sequence of a conserved region in the heme ligand pocket site (X=G+C). These primers were used to amplify DNA fragments from seven different actinomycetes species producing a variety of different compounds (Fig. 2).

The PCR mixture contained 10× PCR buffer (100 mM Tris-HCl [pH8.3], 500 mM KCl, 15 mM $MgCl_2$) supplied by Takara Co. Ltd (Japan). Taq polymerase (2U, Takara) and approximately 100 ng target DNA were added in a final reaction volume of 50 μl. Amplification was performed in a thermal cycler (model 480, Perkin-Elmer Cetus, CT) by denaturing the samples at 94°C for 4 min, subjecting them to 30 cycles of denaturing (98°C, 20s), annealing (67°C, 1 min), and then by elongating at 72°C for 10 min. The 350-bp PCR products were recovered using 1.5% agarose gel electrophoresis and ligated into pT7Blue(R).

	O_2 binding site	Heme ligand pocket
Amy	SDAMLLLIAGHAT	HLAFGFGRHQCLGQNLAR
SuaC	STAMLLLIAGHET	HLAFGFGRHQCLGQNLAR
SubC	TMGRLLLVAGHET	HVAFGFGVHQCLGQPLAR
Sca2	STALLLLVAGHET	HLSFGYGVHQCLGQNLAR
Soy	AFAVILLIAGHET	HLAFGFGVHQCLGQNLAR

Fig. 1. Design of the PCR primers for the amplification of the genes for P450 hydroxylases.
Homologous regions in the amino acid sequence of the O_2-binding site and heme-ligand pocket of P450 hydroxylases from *S. carbophilus* (Sca_2), *S. griseus* (Soy), *S. griseolus* (SuaC and SubC), and *A. autotrophica* (Amy) were aligned. The consensus sequences of the amino acid sequences located in the boxed regions were used to design oligonucleotide primers for the amplification of P450 hydroxyase genes from actinomycete species.

Sequence Analysis of the Subcloned Fragments

PCR fragments obtained from various strains and listed in Table 1 were sequenced. The nucleotide sequences of both strands were determined using an ABI model 373 DNA Sequencer (Applied Biosystems, Inc., Foster City, U.S.A.). The deduced amino acid sequences of the isolated fragments revealed remarkable similarities to each other and to the P450 hydroxylases isolated from *S. carbophilus, S. griseus, S. griseolus*, and *A. autotrophica*, which are known to be involved in secondary metabolism or herbicide degradation (Table 2). The PCR product of *S. albus* (pHCG72) was found to share 60.0% and 59.2% identities with the PCR product of *M. inyoensis* and *A. autotrophica*, respectively. *S. griseus* ATCC10137 showed an amino acid sequence identity of 84.9% to SoyC of *S. griseus*, which encodes a protein involved in xenobiotic

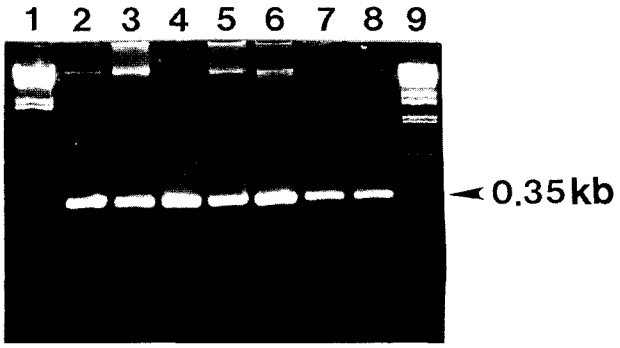

Fig. 2. Analysis of PCR products by agarose gel electrophoresis.
Lanes: 1 and 9, lambda DNA treated with *Hin*dIII or *Bst*PI; 2~8, a 350-bp amplified product of *S. albus, S. glaucescens, S. griseus, M. inyoensis, Stall. hindustanus, A. autotrophica, and S. sclerutialus*, respectively.

Table 1. Strains and plasmids used in this study.

Strains	Plasmids	Description	Characteristics
Streptomyces albus ATCC21838	pHCG72	Partial P450 gene on pT7blue	Salinomycin producer
Streptomyces glaucescens GLA.O	pHCG73	Partial P450 gene on pT7blue	Tetracenomycin producer
Streptomyces griseus ATCC10137	pHCG74	Partial P450 gene on pT7blue	Streptomycin producer
Micromonospora inyoensis ATCC27600	pHCG75	Partial P450 gene on pT7blue	Sisomicin producer
Streptoalloteichus hindustanus ATCC31219	pHCG80	Partial P450 gene on pT7blue	Nebramycin producer
Amycolata autotrophica IFO12743	pJM1	Partial P450 gene on pT7blue	This strain can convert vitamin D3 to 1α, 25-dihydro vitamin D3
Streptomyces sclerutialus ATCC15721	pJM2	Partial P450 gene on pT7blue	This strain can convert vitamin D3 to 1α, 25-dihydro vitamin D3

Table 2. Comparison between the deduced amino acid sequences of PCR products with that of other P450 hydroxylases.

	HCG72	HCG73	HCG74	HCG75	HCG80	JM1	JM2
HCG72		49.2	50.8	60.0	48.7	59.2	53.3
HCG73	49.2		49.2	56.7	51.3	57.5	59.2
HCG74	50.8	49.2		56.7	53.8	55.8	68.9
HCG75	60.0	56.7	56.7		58.0	99.2	61.7
HCG80	48.7	51.3	53.8	58.0		57.1	53.8
JM1	59.2	57.5	55.8	99.2	57.1		60.8
JM2	53.3	59.2	68.9	61.7	53.8	60.8	
Amy	35.7	44.2	45.4	43.3	43.7	44.2	49.6
ChoP	45.8	50.0	49.2	55.0	62.9	54.2	50.8
EryF	36.7	40.8	37.0	43.3	44.8	42.5	42.9
EryK	35.2	33.6	33.9	33.6	36.4	32.8	36.4
FkbD	40.0	40.0	40.8	41.7	46.1	40.8	42.5
MycG	36.4	46.7	38.8	38.8	46.2	39.7	39.7
OleP	39.7	46.7	40.5	43.8	42.7	43.8	47.1
RapJ	42.5	47.5	43.3	46.7	47.8	45.8	48.3
SoyC	52.5	47.5	84.9	56.7	56.3	55.8	67.2
SuaC	52.5	55.0	63.3	60.0	58.8	59.2	69.2
SubC	46.7	49.2	54.2	55.0	56.8	55.0	60.0
Sca2	51.7	54.2	58.3	60.8	59.7	60.0	64.2

BESTFIT [2] analysis was used for comparisons. The amino acid sequences were taken from the following sources: Amy (D26543), ChoP (M31939), EryF (M54943), EryK (L05776), FkbD (U65940), MycG (D16098), OleP (L37200), RapJ (X86780), SoyC (X63601), SuaC (M32238), SubC (M32239), Sca2 (D30815), HCG72 (AF071143), HCG73 (AF071144), HCG74 (AF071145), HCG75 (AF071146), HCG80 (AF071147), JM1 (AF071148), and JM2 (AF071149). Arabic numerals indicate identities (%).

transformation. Comparison of nucleotide and predicted amino acids sequences revealed strong identity (99%) between the PCR products of *M. inyoensis* (pHCG75) and *A. autotrophica* (pJM1); it could be expected that these genes may have the same function and/or evolution process. The PCR product of *Stall. hindustanus* (pHCG80) shared 62.9% identity with ChoP that participates in cholesterol oxidation.

A multi-alignment of the derived amino acids sequences of the cloned fragments of the genes for P450 hydroxylase is given in Fig. 3. Because the >40% homology between the streptomycetes proteins places them all in the same gene family [19], we speculate that these fragments occupy the same gene subfamily and that some of them may possess the same functions. Analysis of DNA fragments amplified in this study suggests that these genes encode P450 hydroxylases and share more similarities to the genes for Sca2, ChoP, SoyC, and SuaC than to antibiotic biosynthetic genes. A phylogenetic tree based on genetic similarities indicates that P450 hydroxylases from actinomycetes were more closely related to each other than to the P450 hydroxylases from the species of other orders (data not shown).

Future Prospects

Actinomycetes produce antimicrobial agents, other pharmacologically active molecules, herbicides, and a variety of extracellular enzymes [9, 11]. Furthermore, those bacteria are used in bioconversion reactions [5]. For example, Trenin *et al.* reported that among 110 strains of actinomycetes possessing antifungal activity, 31 (28%) cultures inhibited cholesterol synthesis. A substantial percentage of sterol inhibitors (43%) interfere with early stages of cholesterol synthesis (including the HMG-CoA reductase step), something that has been proven by mevalonate reversion. The rest of them (57%) alter the later stages (Trenin *et al.* 1994. Abstr. 9th *Int. Symp. Biol. Act.*, Russia, Moscow, p. 171).

We have developed a general and efficient method for cloning P450 type hydroxylase genes from actinomycetes. Sequence analysis indicates that the PCR fragments of P450

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HCG72   LLIAGHETTANNIGLGVVTLLSHP-QWAGDE-------RAVEELLRLHSVADMVALRVAVDDVEIAGQVIRKGEGIVPLLAAANHDTEVFGCPHAFDPERSERRHVAFGYGVHQCLGQNL
HCG73   LLIAGHETTTSMIALSTLLLLDRPELPAELRNDPDLMPAAVDELLRVLSVADSIPLRVAAEDIELSGRTVPADDGVIALLAGANHDPEQFDDPERVDFHRTDNHHVAFGYGMHQCLGQNL
HCG74   LLIAGHETTANMISLGTFTLLNHPEQLEALRSGRTTTAAVVEELLRFLSIAEGLQ-RLATEDIEVAGTTIREGEGVFFSTSLINRDTEVYENPETLDWDRPSRHHLAFGFGVHQCLGQNL
HCG75   LLIAGHETTSHMISLGVTALLEHPDQLAALQNDLTLLPEAVEELLRYLSIADYVPSRVALEDVVIGGTVIRAGEGVVPLLAAADWDPKVFDNPGTLDIHRGNRRHVAFGYGVHQCLGQNL
HCG80   LLIAGHETTANMLALGAFALLEHPEQLAELRANPDLMPGAVEELMRYLSIVHIGPVRTAVADVEIEGQLIRAGESVTVSVPAANWDPAKFPEPERLDLTRRTSGHLAFGHGVHQCLRQNL
PJM1    LLIAGHETTSHMISLGVTALLERPDQLAALQNDLTLLPEAVEELLRYLSIADYVPSRVALEDVVIGGTVIRAGEGVVPLLAAADWDPKVFDNPGTLDIHRGNRRHVAFGYGVHQCLGQNL
PJM2    LLIAGHETTANMISLGTYTLLRHPDRLAELRADPGLAPAAVEELLRMLSIADGML-RVATEDIEAGGTVIRAGDGVIFGTSVINRDETVYTDPDALDWHRPARHHVAFGFGIHQCLGQNL
Amy     LLIAGHATDANLTSMSLYALADRSEQYAALRADRSLVPGAVEDPLRYVAIADIA-----TEDVPIRGRTIAAGNTQIAMGSSANLDHEYYEDPDALTARVEDLVHLAFGFGRHQCLGQNL
ChoP    LLVAGHGTTAHQIALGAFLLLEHPDQLAALRADPALTESAVEELLRHLSVVHHGPTRAALQDADIEGTPVKAGEVVVVSLGAANRDPARFERPDAVDVTREDTGHLAFGHGMHQCLGRQL
Sca2    LLVAGHETTASMTSLSVITLLEHPDQHAALRADPSLVPGAVEELLRVLAIADIAGGRIATADIEIDGQLIRAGEGVIVTNSIANRDSSVFENPDRLDVHRSARHHLSFGYGVHQCLGQNL
SoyC    LLIAGHETTANMISLGTFTLLSHPEQLAALRAGGTSTAVVVEELLRFLSIAEGLQ-RLATEDMEVDGATIRKGEGVVFSTSLINRDADVFPRAETLDWDRPARHHLAFGFGVHQCLGQNL
SuaC    LLIAGHETTASMTSLSVITLLDHPEQYAALRADRSLVPGAVEELLRYLAIADIAGGRVATADIEVEGHLIRAGEGVIVVNSIANRDGTVYEDPDALDIHRSARHHLAFGFGVHQCLGQNL
SubC    LLVAGHETTANMTALSTLVLLRNPDQLARLRAEPALVKGAVEELLRYLTIVHNGVPRIATEDVLIGGRTIAAGEGVLCMISSANRDAEVFPGGDDLDVARDARRHVAFGFGVHQCLGQPL

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Fig. 3. Alignment of the amino acid sequences of PCR products and other P450 enzymes.

The amino acid sequences of ChoP [4], SuaC or SubC [15], Soy [19], Sca2 [20], and Amy [7] were initially matched with PCR products using the multiple sequence alignment CLUSTAL program, after which the resulting comparisons were refined manually [3].

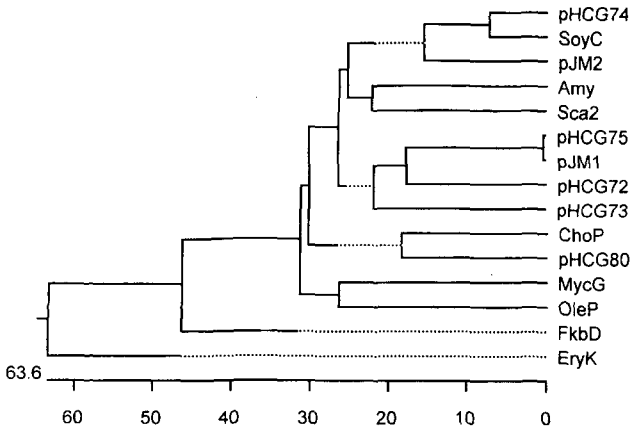

Fig. 4. Phylogenetic tree based on genetic similarities.
The amino acid sequences were taken from the following sources: Amy (D26543), ChoP (M31939), EryK (L05776), FkbD (U65940), MycG (D16098), OleP (L37200), SoyC (X63601), and Sca2 (D30815). The sequences of all other sources were determined in this study. Phylogenetic tree was constructed using the MegAlign program in the DNASTAR sequence analysis program. This analysis indicated that the PCR fragments of P450 hydroxylase genes, isolated from actinomycetes in this study, might be more closely related to cholesterol metabolism or biodegradation than to antibiotic formation.

hydroxylase genes, isolated from actinomycetes in this study, may be more closely related to cholesterol metabolism or biodegradation than to antibiotic formation (Fig. 4).

Since P450 hydroxylases typically do not have stringent substrate specificities, the genes encoding P450 hydroxylases may take advantage of a biotransformation. Therefore, the determination of the P450 primary sequence may be an important step towards our goal of identifying the capacity that P450 enzymes have to bind and transform such a various range of xenobiotic substrates. Although greater insight into the role of cytochrome P450 in the biosynthesis of antibiotics, sterol metabolism, or biodegradation in actinomycetes must await the outcome of further genetic and biochemical studies, we have isolated genes containing the P450 hydroxylase and ferredoxin regions from *A. autotrophica* in order to identify these possibilities [8]. This organism can convert vitamin D3 to 1α, 25-dihydroxyvitamin D3 [7, 17, 18]. Based on sequence homology between our P450 gene and the *choP* [4] or P450*sca2* genes, and structural similarities of vitamin D3 and cholesterol [20], we speculate that this gene may be responsible for 1α-hydroxylation of vitamin D3, which exerts a most potent effect on the mobilization of calcium in the process of bone absorption and formation or cholesterol oxidation.

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