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Detection of the Mevalonate Pathway in *Streptomyces* Species Using the 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Gene

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Isoprenoids found in all living organisms comprise essential components of biological systems such as sterols, carotenoids, dolichols, ubiquinones and prenylated proteins. These compounds are synthesized by consecutive condensations of the five-carbon precursors, isopentenyl diphosphate (IPP), to its isomer, dimethylallyl diphosphate (DMAPP). Two distinct pathways for these precursors are known. One is the classical mevalonate pathway that operates in eucaryotes, archaebacteria and the cytosol of higher plants[1]. The other is a recently discovered pathway, the nonmevalonate pathway, which is used by many eubacteria including actinomycetes, green algae and the chloroplasts of higher plants[1]. To date, five reaction steps and the corresponding enzymes for this new pathway have been identified and characterized[2].

These findings raised an interesting question with regard to the biosynthesis of isoprenoids by the genus *Streptomyces* which belongs to the actinomycetes, since some of the isoprenoid metabolites such as pentalenolactone[3], carquinostatin[4], longestin (KS-505a)[5], novobiocin[6,7], moenomycin[8,9] and teleocidin[10] were biosynthesized through the nonmevalonate pathway, while metabolites such as naphterpin[11,12], furaquinocin[13], napyradiomycin[14] and terpentecin[15] were proved to be of mevalonate pathway origin (Fig. 1). In addition, we have also shown that *Streptomyces* sp. strain CL190 (a naphterpin producer) utilizes the nonmevalonate pathway for the production of the primary metabolite, menaquinone, and that this organism switched to the mevalonate pathway for the biosynthesis of the secondary metabolite, naphterpin, at a later stage of fermentation[12]. To date, there seems to be no taxonomic criteria to distinguish *Streptomyces* species with the mevalonate pathway from those with only the nonmevalonate pathway. It should be noted that all the results cited above were obtained by laborious biosynthetic studies using expensive ^{13}C-labeled precursors.

To pursue this interesting phenomenon more easily and efficiently, we attempted to analyze the distribution of the mevalonate pathway among *Streptomyces* species by assaying the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme of the mevalonate pathway. At first we attempted to detect the enzyme activity in crude extracts prepared from four *Streptomyces* strains that produce naphterpin, furaquinocin, napyradiomycin or terpentecin through the mevalonate pathway. However, the enzymatic activity could not be detected in a reproducible manner in these *Streptomyces* strains, because of the weak activity of the enzyme. Furthermore, the enzyme activity was dependent on the growth stage of the organisms and the procedures to optimize cultivation conditions for expression of the enzyme activity were somewhat tedious. An alternative method to detect the mevalonate pathway in given organisms may be to use Southern hybridization and the HMG-CoA reductase gene as the DNA probe. Since the reductase is a rate-limiting enzyme in the mevalonate pathway in eucaryotes[16], the corresponding gene may be most appropriate as a probe to detect the mevalonate pathway in microorganisms.

We previously succeeded in purifying the HMG-CoA reductase from the naphterpin-producing *Streptomyces* sp. strain CL190 and cloning the corresponding gene (*hmgr*)[17]. The amino acid sequence of the *Streptomyces* HMG-CoA reductase showed significant homology with those of several eucaryote HMG-CoA reductases, and the enzymatic properties of the *Streptomyces* HMG-CoA reductase resembled those of the eucaryote enzymes. Consequently, we decided to use the *Streptomyces hmgr* gene as the DNA probe to detect the mevalonate pathway in *Streptomyces*.

To confirm the reliability of our strategy of using the *hmgr* gene as the DNA probe for detecting the mevalonate pathway, we tested 13 *Streptomyces* strains and one *Actinoplanes* strain listed in Table 1. All of the strains,

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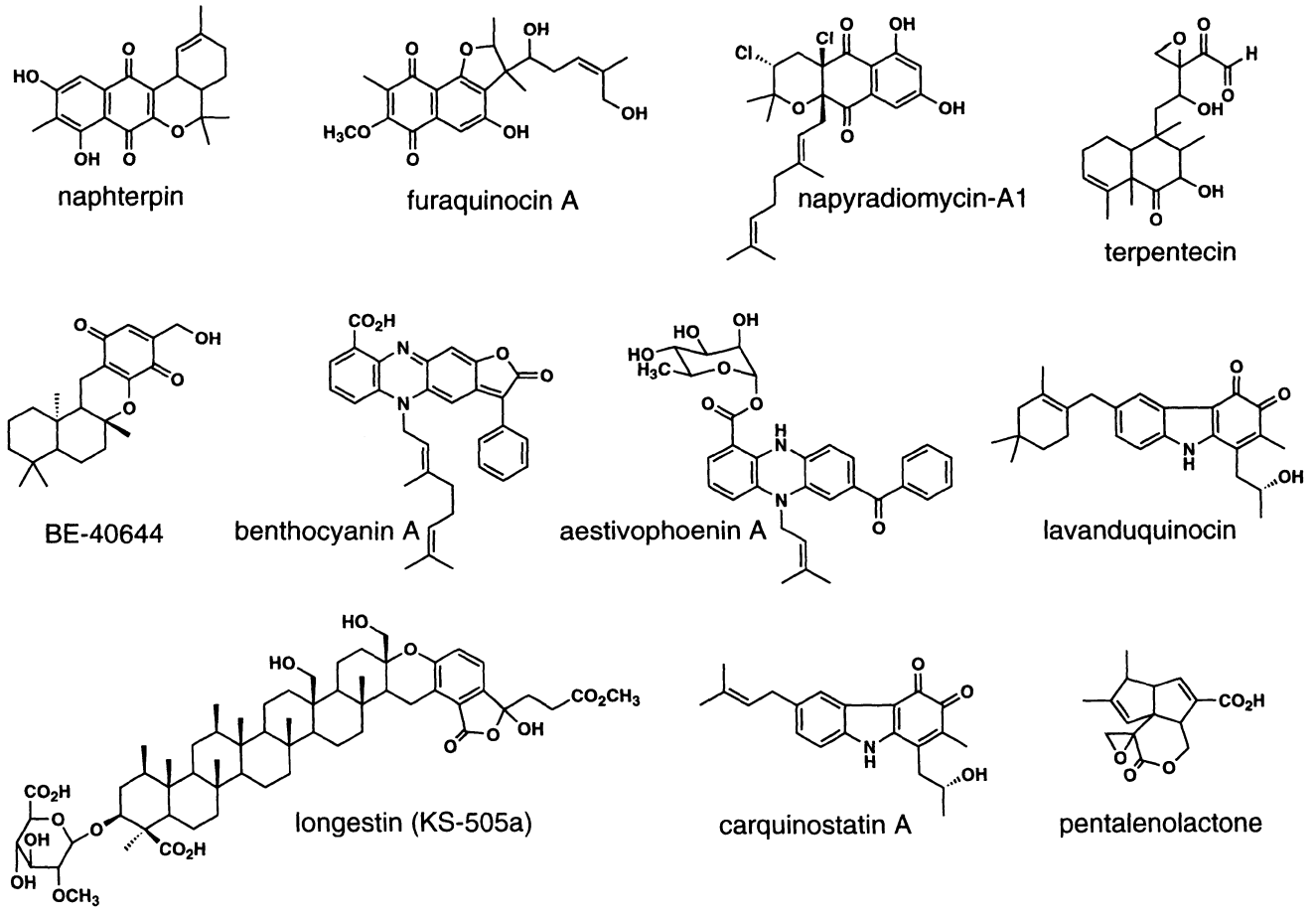
Fig. 1. Structures of isoprenoids produced by the strains listed in Table 1.



except for *S. griseus* subsp. *griseus* ATCC3478 and *S. lividans* 66, produce isoprenoids as secondary metabolites. Experimental results with ^{13}C-labeled precursors had shown that strains 1 to 5[11~15,18] and strains 9 to 12[3~5,19] produce their isoprenoids through the mevalonate and nonmevalonate pathways, respectively. Therefore, only strains 1 to 5 should possess the *hmgr* gene in their genome. On the other hand, nothing is known regarding the biosynthesis of benthocyanin[20], aestivophoenin[21] and lavanduquinocin[22], because the low productivity of these metabolites prevented biosynthetic studies being carried out.

Total DNA from each strain was extracted and prepared as described previously[23]. One μg of each DNA sample was digested with *Bam*HI (TaKaRa Shuzo), separated by 1.0% agarose gel (Invitrogen) electrophoresis (Fig. 2A), and then transferred to Hybond-N^+, a positively charged nylon membrane (Amersham Biosciences). Southern hybridization[24] was carried out using an ECL direct nucleic acid labeling and detection system (Amersham Biosciences) according to the protocol of the supplier. To amplify the *hmgr* gene from *Streptomyces* sp. strain CL190[17], two oligonucleotide primers, 5′-ATGACGGAAACGCACGCCATCGCC-3′ (5′ end of the *hmgr* gene) and 5′-CTATGCACCAACCTTTGCGGTC-3′ (3′ end of the *hmgr* gene), were synthesized (Amersham Biosciences). PCR was carried out in a 20-μl total volume of PCR buffer (Roche Diagnostics) containing 50 ng of total DNA from CL190, 0.2 mM of each dNTP, 2.5 pmol of each primer and 1.8 units of *Taq* polymerase (Roche Diagnostics) using 25 cycles (0.5 minutes at 95°C; 0.5 minutes at 60°C; 1 minute at 72°C). A single DNA fragment of 1.0 kb was amplified and used as the DNA probe.

As shown in Fig. 2B, the *hmgr* gene was detected in the DNA fragments from three *Streptomyces* strains (lanes 2 to 4) and one *Actinoplanes* strain (lane 5) that had been shown to use the mevalonate pathway for isoprenoid biosynthesis.

Table 1. Distribution of the mevalonate pathway in terpenoid-producing *Streptomyces* and *Actinoplanes* species.

No.[a]	strains	isoprenoids[b]	mevalonate pathway[c]	sources or references
1.	*Streptomyces* sp. strain CL190	naphterpin	+	11,12
2.	*Streptomyces* sp. strain KO-3988	furaquinocin	+	13
3.	*S. ruber* MG802-AF1	napyradiomycin	+	14
4.	*S. griseolosporeus* MF730-N6[d]	terpentecin	+	15
5.	*Actinoplanes* sp. strain A40644	BE-40644	+	18
6.	*S. prunicolor* 1884-SVT2	benthocyanin	nt	20
7.	*S. purpeofuscus* 2887-SVS2	aestivophoenin	nt	21
8.	*S. viridochromogenes* 2942-SVS3	lavanduquinocin	nt	22
9.	*S. argenteolus* A-2	longestin (KS-505a)	-	5
10.	*S. exfoliatus* 2419-SVT2	carquinostatin	-	4
11.	*S. exfoliatus* UC5319	pentalenolactone	-	3
12.	*S. chromofuscus* 8403MC$_1$	pentalenolactone	-	19
13.	*S. griseus* subsp. *griseus* ATCC3478	unknown	nt	ATCC
14.	*S. lividans* 66	unknown	nt	John Innes Institute, UK

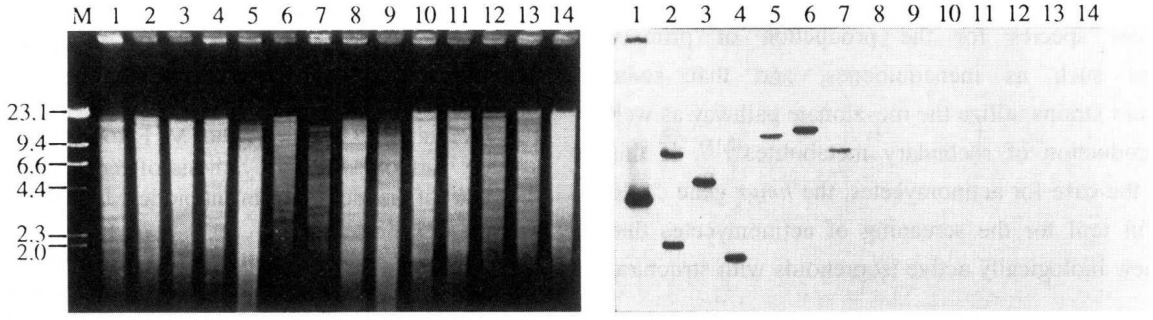
a) These numbers correspond to the lane Nos. in Fig. 2.

b) Only the secondary metabolites are listed here. The structures of these isoprenoids are shown in Fig. 1.

c) Existence of the mevalonate pathway in these strains was revealed by feeding experiments. nt, not tested; ATCC, American Type Culture Collection.

d) This strain is at present classified into the genus *Kitasatospora*. (26,27)

Fig. 2. Southern hybridization between *Bam*HI-digested total DNA and the *hmgr* gene from CL190.



Ethidium bromide staining (left). Molecular sizes in kb are shown on the left. The lane numbers correspond to those in Table 1. Autoradiogram (right).

Among these *Streptomyces* strains, we cloned the *hmgr* gene homologs from the furaquinocin and terpentecin producers and demonstrated that the nucleotide sequences of these homologs showed more than 86% identity with that of the CL190 *hmgr* gene[25]. These results indicate that the *hmgr* gene is highly conserved among *Streptomyces* species. Additionally, since an unambiguous band was detected in *Actinoplanes* sp. strain A40644 (lane 5), the *hmgr* gene may be conserved not only in *Streptomyces* but also in other actinomycete genera. On the other hand, no signals were detected in the DNA fragments from four *Streptomyces* strains (lanes 9 to 12) that use only the

nonmevalonate pathway for isoprenoid biosynthesis. These results were fully consistent with those from the ^{13}C-labeled precursor feeding experiments. Therefore, it was confirmed that Southern hybridization using the HMG-CoA reductase gene as the DNA probe is applicable to the detection of the mevalonate pathway in *Streptomyces*. It should be emphasized that the hybridization blot demonstrated the existence of the *hmgr* gene homologs in the benthocyanin and aestivophoenin producers (lanes 6 and 7) for which biosynthetic studies were impossible due to the low productivity of the isoprenoids. This indicates that hybridization using the *hmgr* gene is a more sensitive and useful method for detecting the mevalonate pathway than feeding experiments. This technique also revealed the absence of *hmgr* gene sequences in the lavanduquinocin producer (lane 8), *S. griseus* subsp. *griseus* ATCC3478 (lane 13) and *S. lividans* 66 (lane 14).

As mentioned above, very little is known about the distribution of the mevalonate pathway in *Streptomyces*. This was due to the lack of reliable and convenient analytical methods to detect the mevalonate pathway. With a useful method in hand, it has now become possible to efficiently classify *Streptomyces* into two groups; one using the mevalonate pathway and the other using only the nonmevalonate pathway. This method is now being used to determine the relationship between the ability of *Streptomyces* species to use the mevalonate pathway and their taxonomic properties.

Our data on the isoprenoid biosynthesis in *Streptomyces* suggest that the nonmevalonate pathway is used by all *Streptomyces* species for the production of primary metabolites such as menaquinones, and that some *Streptomyces* strains utilize the mevalonate pathway as well for the production of secondary metabolites[12,18]. If this were also the case for actinomycetes, the *hmgr* gene could be a useful tool for the screening of actinomycetes that produce new biologically active isoprenoids with structural diversity.

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