Overexpression of a Gene Cluster Encoding a Chalcone Synthase-like Protein Confers Redbrown Pigment Production in *Streptomyces griseus*

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A 7.0-kb DNA fragment that conferred redbrown pigment production on *Streptomyces griseus* was shotgun-cloned with a multicopy vector pIJ486 from this microorganism. By restriction endonuclease mapping and subcloning, a 1.5-kb fragment which is essential for the production of redbrown pigment was determined. The nucleotide sequence of this region revealed the presence of two open reading frames, ORF1 with 109 amino acids (named RppA) and ORF2 with 262 amino acids (RppB), in addition to a truncated ORF3. The termination codon of *rppA* and the initiation codon of *rppB* overlapped, sharing one common nucleotide, which strongly suggests that these two genes are cotranscribed. Both *rppA* and *rppB* were essentially required for the pigmentation. The RppB protein showed great similarity in amino acid sequence to a chalcone synthase, a key enzyme of central importance in the biosynthetic pathway of all classes of flavonoids in plants. Part of RppA showed sequence similarity to the 33 kDa phosphoprotein of adenovirus. Nucleotide sequences homologous to *rppA* and *rppB* were widely distributed in *Streptomyces* species, as determined by Southern hybridization. Further nucleotide sequencing of the entire *orf-3* gene showed that ORF3 with 403 amino acids was a cytochrome P-450 (named P-450$_{RPP}$). These data suggested that the cloned fragment contained part of a gene cluster for the biosynthesis of a certain metabolite. Introduction of the subcloned 1.5-kb fragment into *Streptomyces lividans* as well as *Escherichia coli* also caused production of redbrown pigment, suggesting that RppA and RppB are capable of synthesizing the redbrown pigment from metabolites commonly present in bacteria.

The bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites including antibiotics. The recombinant DNA techniques established in *Streptomyces* have revealed that a contiguous stretch of DNA contains structural biosynthetic genes as well as regulatory and self-resistance determinants, forming a gene cluster for each secondary metabolite. In addition to the members in the gene cluster, several global regulatory genes not closely linked to the biosynthetic genes also control antibiotic production. For example, *afsR*[1~3], *afsS*[4], *afsQ1*/*afs Q2*[5], *absA*[6], *absB*[7], and *abaA*[8] control globally and pleiotropically the secondary metabolite formation in *Streptomyces coelicolor* A3(2). Among these, *afsR*, *afsS*, and *afsQ* were identified as genes that cause production of the pigmented antibiotic, actinorhodin, in a closely related species, *S. lividans*, when introduced on a plasmid.

These observations prompted us to identify and clone regulatory genes for secondary metabolite formation by a similar strategy in other *Streptomyces* species. We chose streptomycin-producing *Streptomyces griseus* as a host and shotgun-cloned its chromosomal DNA on a high copy number plasmid, on the assumption that overexpression of regulatory genes would lead to overproduction of streptomycin and some other secondary metabolites. A shotgun-cloning experiment yielded a transformant that produced a redbrown pigment in a large amount. This paper describes the cloning, nucleotide sequence, and characterization of the genes responsible for pigmentation. One of the proteins encoded by the cloned DNA fragment showed great sequence similarity to chalcone synthases unique to plants. The cloned genes also conferred pigment production on *S. lividans* and *Escherichia coli*. The organization of the genes including a gene encoding a cytochrome P-450-like protein within the cloned fragment suggested that it was part of the biosynthetic gene cluster for a certain metabolite.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used are listed in

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This article is a special contribution in honour of Professor SATOSHI ŌMURA's 60th birthday.

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Relevant characteristics	Source of reference
S. griseus IFO 13350	Wild-type	IFO[a]
S. griseus HH1	A-Factor-deficient mutant strain derived from IFO13350 by incubation at 37°C	33)
S. lividans TK21	Wild-type	D. A. HOPWOOD
S. parvulus IFO 3388	Wild-type	IFO[a]
pTMA1	Thiostrepton resistance; copy number, 1~2	34)
pIJ486	Thiostrepton resistance; copy number, 40~100	15)
pUC19	Ampicillin resistance	11)

[a] Institute of Fermentation, Osaka, Japan.

Table 1. *S. griseus* strains were grown in YMPD medium (pH 7.2) containing the following in grams per liter: yeast extract (Difco Laboratories) 2, Bacto Peptone (Difco) 4, meat extract (Wako Pure Chemicals) 2, NaCl 5, $MgSO_4 \cdot 7H_2O$ 2, glucose 10 and glycine 12.5. *S. lividans* was grown in Bennett-glucose medium (pH 7.2) containing the following in grams per liter: yeast extract 1, meat extract 1, N. Z. amine (Wako Pure Chemicals) 2, and glucose 10. Thiostrepton, provided by Asahi Chemical Industry, Shizuoka, was added at 20 μg/ml, when necessary. *E. coli* strains were grown in Luria broth[9]. Ampicillin was added at 50 μg/ml, when necessary.

General Recombinant DNA Techniques

Restriction endonucleases, T4 DNA ligase, Klenow fragment, and synthetic oligonucleotide linkers were purchased from Takara Shuzo, Co., Ltd. (Kyoto, Japan). [α-^{32}P]dCTP at 400 Ci/mmol for nucleotide sequencing by the M13-dideoxynucleotide method[10] with M13mp 18 and M13mp19[11] and at 3,000 Ci/mmol for the Takara DNA labeling system was purchased from Amersham International. DNA manipulations in *E. coli* were as described by MANIATIS *et al.*[9], and those in *Streptomyces* strains were as described by HOPWOOD *et al.*[12].

Subcloning of the Cloned Fragment

The originally cloned 7.0-kb *Bam*HI fragment was at the *Bam*HI site of pIJ486 (see Fig. 2). For construction of pIJ486-RB41 and -RB43, the *Mlu*I and *Apa*L1 sites in the 7.0-kb fragment were changed into an *Eco*RI site by using an 8-mer *Eco*RI linker. Deletion of the 5-kb and 1.5-kb *Eco*RI fragments thus generated in pIJ486-RB4 yielded pIJ486-RB41 and -RB43, respectively. For construction of pIJ486-RB42 and -RB44, the *Mlu*I and *Apa*L1 sites were changed into a *Hin*dIII site by using an 8-mer *Hin*dIII linker. Similar deletion of the 2.2-kb and 5.5-kb *Hin*dIII fragment from pIJ486-RB4 resulted in pIJ486-RB42 and -RB44, respectively. A frame shift mutation in *rppA* and *rppB* (see Fig. 2) was generated by inserting an 8-mer *Bgl*II linker in the respective coding regions by the standard method. For expression of the genes in the cloned fragment in *E. coli* JM109, the *Hin*dIII-*Bam*HI fragment excised from pIJ486-RB44 was inserted between the *Hin*dIII and *Bam*HI sites of the multilinker of pUC19.

DNA Blotting and Hybridization

Chromosomal DNAs from *Streptomyces* species were prepared by the lysozyme-sodium dodecyl sulfate (SDS)-EDTA method described previously[13]. *Bam*HI fragments separated by 1% agarose gel electrophoresis were alkali denatured and then neutralized. The DNA was transferred and fixed to a nitrocellulose paper by the method of Southern[14]. For making ^{32}P-probe, the 700-bp *Nco*I fragment (nucleotide position, 1584-2317 in Fig. 3) containing parts of *rppA* and *rppB* was purified from agarose gel slices by using the GeneClean kit (Bio 101, Inc.) and labeled with [α-^{32}P]dCTP and the Takara DNA labeling kit. The ^{32}P labeled DNA denatured at 100°C for 3 minutes was hybridized with the nitrocellulose blot in 10 ml of 5×SSC (1×SSC contained 0.15 M NaCl and 0.015 M sodium citrate)-50% formamide-0.1% SDS in a heat-sealed plastic bag at 42°C overnight. The nitrocellulose sheet was washed twice in 2×SSC-0.5% SDS at room temperature and then 0.1% SSC-0.5% SDS at 65°C. After being dried, the hybridized blot was placed against a Kodak XRP film for autoradiography.

Results

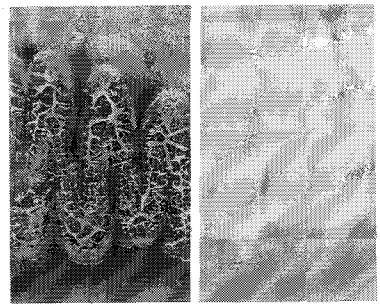
Cloning of a DNA Fragment Conferring Pigment Production on *S. griseus*

We constructed a bank of *Bam*HI-digested fragments of the *S. griseus* chromosomal DNA, with a multicopy plasmid pIJ486 with a copy number of 40 to 100 as the cloning vector, in the same strain. Among about 2,000 thiostrepton resistant transformants grown on Bennett agar medium, a colony producing a redbrown pigment was found (Fig. 1). The transformant harbored a plasmid, named pIJ486-RB4, containing a 7.0-kb insert at the *Bam*HI site of pIJ486. The restriction map of this insert is shown in Fig. 2. Subcloning experiments with the same vector pIJ486 showed that a 1.5-kb *Apa*L1-*Bam*HI fragment on pIJ486-RB44 still conferred pigment production on *S. griseus*. The amounts of pigment produced by *S. griseus* containing pIJ486-RB44 and -RB42 were apparently larger than that produced by the same strain containing pIJ486-RB4. Although the reason for this is not clear, it may be due to the difference in stability of these plasmids.

In pIJ486-RB44, the 1.5-kb fragment was inserted

Fig. 1. Redbrown pigment production by *S. griseus* containing pIJ486-RB44.

The recombinant *S. griseus* (Photo. 1) and the wild-type strain (Photo. 2) were grown on YMPD medium at 30°C for 5 days.

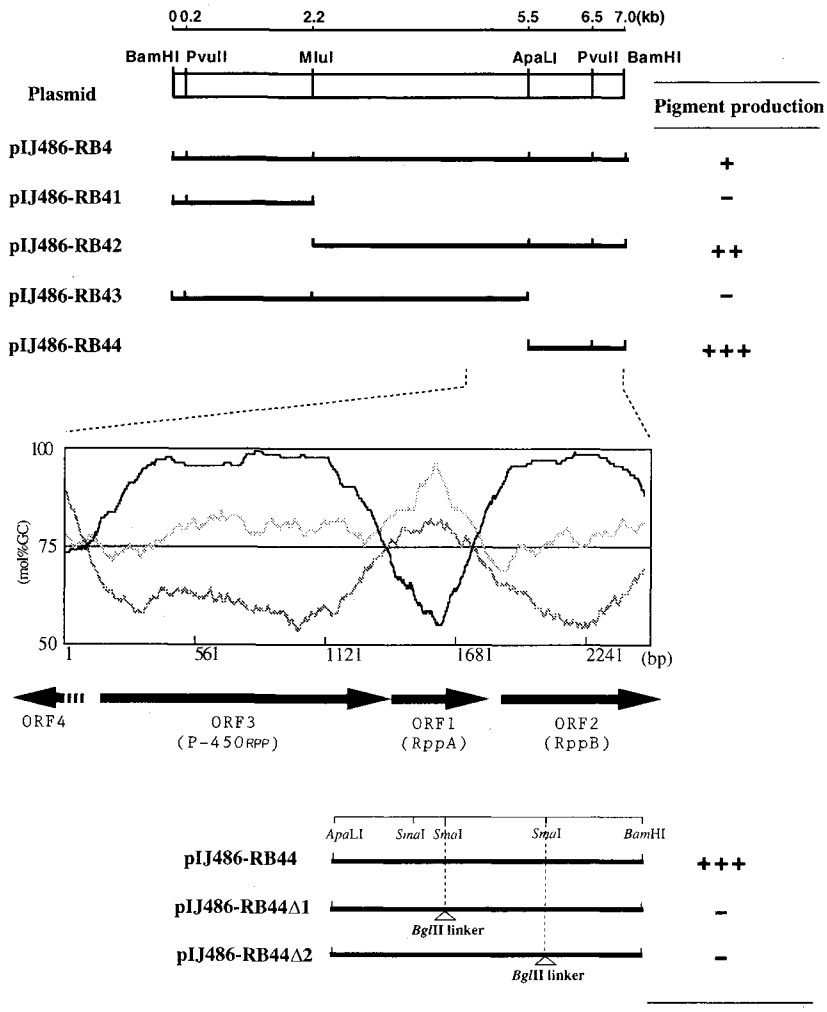


1 2

downstream of the strong transcriptional terminator derived from *E. coli* phage fd. As described below, the fragment was inserted in such an orientation with respect to the terminator that no transcriptional read-through from the vector sequence into the two genes contained in this fragment (see below) ocurred. Since the fd terminator was shown to prevent significant read-through from vector promoters[15], the 1.5-kb fragment contained a promoter of the genes responsible for pigmentation.

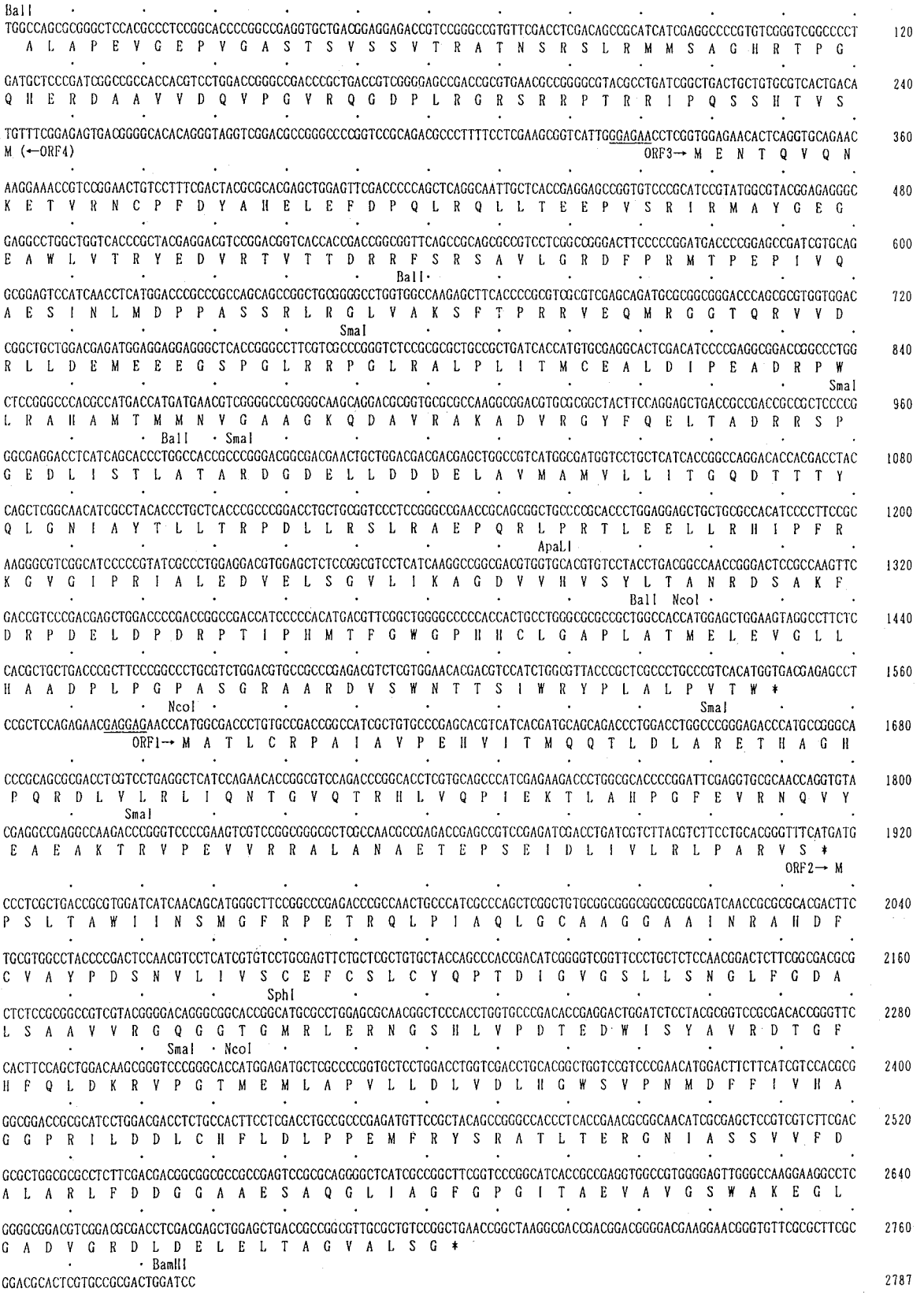
To examine the gene dosage effect of the 1.5-kb fragment on pigment production, we placed the fragment downstream of the same fd terminator on pTMA1 with its copy number of 1 to 2 per genome. The amount of

Fig. 2. Restriction map of the cloned fragment (top), the FRAME analysis of the nucleotide sequence of 2787-bp containing three complete open reading frames (RppA, RppB, and P-450$_{RPP}$), and a truncated ORF4 (middle), and the location of a frame shift mutation in the *rpp* genes (bottom).



The originally cloned 7.0-kb *Bam*HI fragment was subcloned with pIJ486 as described in Materials and Methods. Pigment production by *S. griseus* containing each of the plasmids was examined on YMPD medium. The nucleotide sequence was analyzed by the FRAME analysis[16] with a sliding window of 80 codons. The arrows indicate the extent and direction of the open reading frames. For generating a frame shift mutation in *rppA* and *rppB*, an 8-mer *Bgl*II linker was inserted at the indicated *Sma*I sites. To test pigment production directed by the mutated genes in *E. coli*, the indicated fragments were transferred to the multilinker of pUC19 in such an orientation that the *rpp* genes were under the control of the *lac* promoter in pUC19.

Fig. 3. Nucleotide sequence of the 2787-bp *Bal*I-*Bam*HI fragment and the deduced amino acid sequences of ORF3 ($P\text{-}450_{RPP}$), ORF1 (RppA), and ORF2 (RppB), in addition to a truncated ORF4.



A probable ribosome-binding sequence for each ORF is underlined. The nucleotide sequence has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D45916.

redbrown pigment produced by *S. griseus* containing the 1.5-kb fragment on this low copy number plasmid was apparently smaller than that produced by the same strain containing pIJ486-RB44. This implies that pigment production is a result of overexpression of the genes in the 1.5-kb fragment.

Nucleotide Sequence of the Subcloned Fragment

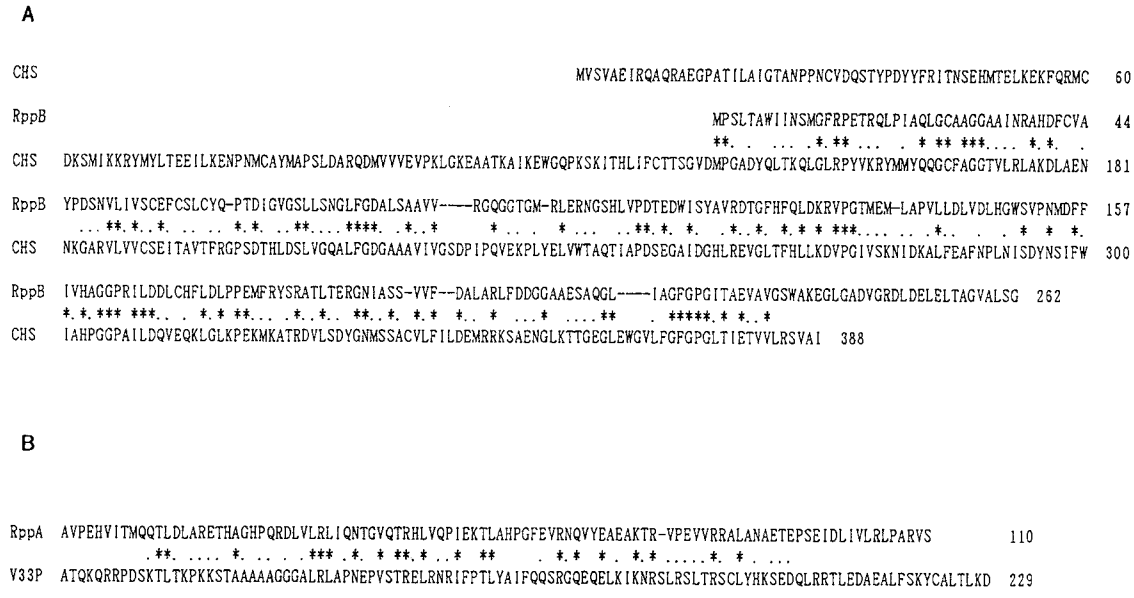
From the data of the subcloning experiments, we determined the nucleotide sequence of the 1.5-kb *Apa*L1-*Bam*HI fragment essentially required for pigment production in *S. griseus* (Fig. 3). There are two open reading frames, ORF1 with 109 amino acids and ORF2 with 262 amino acids, whose codon usage patterns are in good agreement with that of *Streptomyces* genes with an extremely high G+C content, as determined by the FRAME analysis developed by BIBB *et al.*[16] ORF1 is preceded by a possible ribosome-binding sequence, GAGGAG, locating 5 nucleotides upstream of the putative translational start codon, ATG. The termination codon, TGA, of *orf-1* overlapped the initiation codon, ATG, of *orf-2*, sharing the common A residue. This type of overlap strongly suggests that *orf-1* and *orf-2* are cotranscribed. As described below, these two open reading frames are required for pigment production in both *Streptomyces* strains and *E. coli*. We therefore designated ORF1 and ORF2 as RppA (*r*edbrown *p*igment *p*roduction) and RppB, respectively.

Sequence Similarity of RppB to Chalcone Synthase of Plants

A computer-aided search revealed that RppB resembles the chalcone synthases of plants (Fig. 4A), the key enzyme in the biosynthesis of the flavonoids in plants[17]. This enzyme catalyzes the condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA. The product, naringenine-chalcone, is a precursor for the synthesis of a variety of compounds like antocyanines, flavones, flavonols, and isoflavonoids as flower pigments. The 164th cysteine of the chalcone synthase is located at the active site and essential for enzyme activity[18]. The amino acid sequences of this region, including the cysteine residue, of the chalcone synthase and RppB are well-conserved. Because of the end-to-end similarity between these two proteins, we assume that RppB has the same enzymatic activity as the chalcone synthase or catalyzes the condensation between very similar substrates to those of the chalcone synthase.

A similar computer-aided search showed that the COOH-terminal part of RppA, probably cotranscribed with RppB, shows homology in amino acid sequence to the NH_2-terminal portion of the 33 kDa phosphoprotein of adenovirus (Fig. 4B). The phosphoprotein is involved in the morphogenesis of the virion[19]. The function of RppA is yet unclear, although it is essentially required for pigmentation, as described below.

Fig. 4. Alignment of amino acid sequences between the chalcone synthase (CHS) of *Pueraria lobata*[32] and RppB (A) and between the phosphoprotein of adenovirus and RppA (B).



Dashes indicate gaps introduced for alignment. Identical and similar amino acids are marked by asterisks and dots, respectively.

Requirement of both *rppA* and *rppB* for Pigmentation in *S. griseus*

In order to determine whether *rppA* or *rppB*, or both, are required for pigmentation, a frame shift mutation was introduced in each coding sequence of the two genes on pIJ486-RB44 by using an 8-mer *Bgl*II linker as shown in Fig. 2. Neither pIJ486-RB44*Δ*1 with a frame shift in *rppA* nor pIJ486-RB44*Δ*2 with a frame shift in *rppB* caused pigmentation in *S. griseus*. These data clearly show that both *rppA* and *rppB* are essentially required for the accumulation of the redbrown pigment in *S. griseus*.

Pigment Production Induced by the *rpp* Genes in Other *Streptomyces* Strains and *E. coli*

Plasmid pIJ486-RB44 also caused redbrown pigment in *S. lividans* and *S. parvulus*. The color and the diffusibility of the pigment into agar medium produced in these *Streptomyces* strains were the same as those produced in *S. griseus*, which suggests that the *rpp* genes directed the synthesis of the same redbrown metabolite in the two strains.

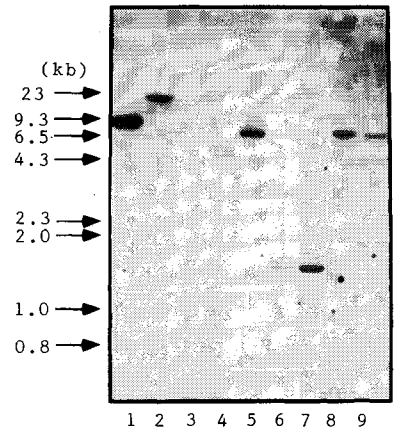
We next placed the *rpp* genes under the control of the *lac* promoter in pUC19 to examine pigment production in *E. coli*. Upon induction of the *lac* promoter with isopropyl-*β*-D-thiogalactopyranoside (IPTG), a similar redbrown pigment was produced both on solid medium and in liquid medium. Pigment production to a less extent was also observed without the induction by IPTG. Similar pUC19-derived plasmids containing the above-described frame shift mutation in either *rppA* or *rppB* failed to confer pigment produciton even on induction with IPTG, indicating that both genes are essential for pigmentation in *E. coli*, as was observed in *S. griseus*.

All these observations suggest that the *rpp* genes encode proteins catalyzing the formation of the redbrown pigment from a metabolite(s) commonly present in bacteria, probably as a primary metabolite(s).

Distribution of the *rpp* Sequences among Actinomycetes

We examined the distribution of sequences homologous to *rpp* among actinomycetes by Southern hybridization under relatively high stringent conditions. The 700-bp *Nco*I fragment containing parts of *rppA* and *rppB* was used as ^{32}P-labeled probe, and chromosomal DNAs digested with *Bam*HI were used as targets. As shown in

Fig. 5. Distribution of DNA sequences homologous to the *rpp* genes among actinomycetes.



The conditions for hybridization between the 700-bp *Nco*I fragment and the chromosomal DNAs from various actinomycetes were relatively stringent, as described in Materials and Methods. The actinomycete strains examined are: *S. griseus* HH1, as a control (lane 1); *S. flaveolus* IFO 3408 (lane 2); *S. viridochromogenes* IFO 12338 (lane 3); *S. fradiae* ATCC 21096 (lane 4); *S. antibioticus* IFO 12652 (lane 5); *S. albus* IFO 12861 (lane 7); *S. lividans* HH21 (lane 8); and *S. coelicolor* A3(2) IFO 3114 (lane 9).

Fig. 6. Alignment of amino acid sequences of a cytochrome P-450 (ChoP) and ORF4 (P-450$_{RPP}$).

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Rpp   MENTQVQNKETVRNCPFDYAHELEFDPQLRQLLTEEPVSRIRMAYGEG-EAWLVTRYEDVRTVTTDRRFSRSAVLGRDFPRMTPEPIVQAESINLMDPPASSRLRGLVAKSFTPRRVEQM  119
ChoP        MTQAAPVTFSTVRENYFGPPAEMQALRHKAPVTRTAFADGRPGWLVTGYSAARAVLSDSRFTARG--EREHPAVPRAATLEDE---------RCRRLIAGQFTARRMRQL   99

Rpp   RGGTQRVVDRLLDEMEEEGSGLRRPGLRALPL--ITMCEALDIPEADRPWLRAHAMTMMNVGAAGKQDAVRAKADVRGYFQELTADRRS-PGEDLISTLATARDGDELLDDDELAVMAMV  236
ChoP  TGRTERIVREHLDAMEHMGSPADLVEHFALPVPSLVIAELLGVPPPDREHFQHDTLRWGGPG-RSTEEVTEAFVSLGGQLQRLVRLKRTEPGDDLLSGLIAA---DPALTDEELASIAFL  215

      O2-binding site                                                                                    Heme ligand pocket
Rpp   LLI TGQDTTT YQLGNIAYTLLTRPDLLRSLRAEPQRLPRTLEELLRHIPFRKGVGIPRIALEDVELSGVLIKAGDVVHVSYLTANRDSAKFDRPDELDPDRPTIPHMT FGWGPHHCL  353
ChoP  LLV AGHGTTA HQIALGAFLLLEHPDQLAALRADPALTESAVEELLRHLSVVH-HGPTRAALQDADIEGTPVKAGEVVVVSLGAANRDPARFERPDAVDVTREDTGHLA FGHGMHQCL  331

Rpp   GAP LATMELEVGLLHAADPLPGPASGRAARDVSWNTTSIWRYPLA-LPVTW  403
ChoP  GRQ LARIELRVALTALLERFPHLRLACPAAEIPLR-HDMQVYGADRLPVAW  381
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Dashes indicate gaps for alignment. Identical and similar amino acids are indicated by asterisks and dots, respectively. The heme ligand pocket and the oxygen-binding site are boxed. The heme cysteine ligand is indicated by a solid triangle. The hydrogen bond donors to the heme propionates, which are proposed for P-450$_{CAM}$, are indicated by open triangles.

Fig. 5, nucleotide sequences homologous to the probe were found in almost all of the actinomycetes examined. The strong intensities of the hybridization signals indicate a high degree of similarity. These data suggest a wide distribution of the *rpp* genes in a variety of actinomycetes.

A Cytochrome P-450 Gene Upstream of the *rppA-rppB* Genes

We found that the amino acid sequence of the truncated ORF3 contained in the subcloned 1.5-kb fragment was similar to those of cytochrome P-450s from procaryotic and eucaryotic origins. We further determined the nucleotide sequence of the region covering the entire open reading frame (Fig. 3). A probable ribosome-binding sequence, GGGAG, is present 7 nucleotides upstream of the translational initiation codon. Figure 6 shows the alignment between ChoP (a cytochrome P-450 from a *Streptomyces* strain)[20] and ORF3. These two proteins show sequence similarity over the entire sequences. The cysteine residue serving as the thiolate-proximal ligand of the heme and the residues around it are strongly conserved. The sequence, Gly/Ala-Gly-X-Asp/Glu-Thr (X is a nonconserved amino acid), that is strongly conserved as the residues forming the oxygen-binding site[21] is highly conserved in ORF3 as Thr-Gly-Gln-Asp-Thr. In addition, three (Arg-104, Arg-294, and His-350) of the five residues serving as the hydrogen bond donors to the heme propionates in $P\text{-}450_{CAM}$[22] are conserved at the identical positions in the aligned sequence. The strong similarity of ORF3 to other cytochrome P-450s suggests that it is a member of the cytochmome P-450 family. We therefore tentatively designated ORF3 as $P\text{-}450_{RPP}$.

Discussion

Our shotgun cloning with *S. griseus* as the host by the strategy to identify possible regulatory genes for secondary metabolite formation has led to the isolation of genes which we assume is part of the biosynthetic gene cluster for a certain metabolite. Because of the great end-to-end similarity of RppB to the chalcone synthases of plants, we suppose that the metabolite is related to a family of flavonoids and isoflavonoids. This is not very surprising since *S. griseus* strains ATCC 13273 and TU6 were found to be capable of removing the glucose moiety from genistein and daidzein to produce their free isoflavonoids[23,24]. In addition, isoflavone compounds were produced by fermentation in media containing plant nitrogen sources such as soybean meal, cotton seed meal and corn steep liquor[25]. These observations indicate that some *Streptomyces* strains including *S. griseus* contain an enzyme system that catalyzes the formation, or at least, a modification of flavonoids and isoflavonoids. Speculatively, such an enzyme system is widely distributed among a variety of actinomycetes, as suggested by the Southern hybridization experiment with the *rpp* genes as the probe.

Our preliminary assay for the enzyme activity[26] directed by the *rpp* genes with [^{14}C]malonyl-CoA and 4-coumaroyl-CoA and cell-lysates from the recombinant *S. griseus* and *E. coli* cells containing pIJ486-RB44 and pUC19-RB44, respectively, showed that the extracts contained an enzyme activity that used [^{14}C]malonyl-CoA as the substrate (data not shown). After incubation of [^{14}C]malonyl-CoA and 4-coumaroyl-CoA with the cell-extract of the recombinant *S. griseus*, the autoradiogram of the reaction mixture showed a ^{14}C spot with its Rf value of 0.22, which was not produced by incubation with an extract from *S. griseus* without the plasmid. However, the authentic sample, naringenin, had an Rf value of 0.32 in the same solvent system (T. AKIYAMA and U. SANKAWA, personal communication). The *E. coli* cell-extract yielded a ^{14}C spot of its Rf value of 0.19. These data showed that the proteins encoded by the *rpp* genes showed little or no activity to catalyze the condensation between the two exogenously added compounds, but showed an activity to modify [^{14}C]-malonyl-CoA with some metabolite present in the cell-extracts of *S. griseus* and *E. coli*. The difference in Rf of the newly appeared spots with the cell-extracts from *S. griseus* and *E. coli* suggests that the substrate to be added to malonyl-CoA is different between the two strains. Anyway, the definitive enzyme activity of the Rpp proteins should be determined by further detailed work, including determination of the structure of the [^{14}C]product.

The properties of the redbrown pigment hampered the purification and determination of its structure. The pigment was water-soluble and it was not extracted with ethyl acetate or hexane. In addition, it was ethanol-precipitable. Because of the similarity of RppB to the chalcone synthase and its ability to modify malonyl-CoA, it is tempting to speculate that the redbrown pigment is a polymerized form of naringenin chalcone.

Both RppA and RppB are required for pigment production in *S. griseus* and *E. coli*. In the *E. coli* system, the *rpp* genes were placed under the control of the *lac* promoter and their expression appeared to be dependent on the IPTG-inducible promoter. This suggests that RppB is concerned with the enzyme activity in conjunction with the chalcone synthase-like protein, RppB, or with the enzyme activity catalyzing the reaction before or after the step of RppB for accumulating the pigment, rather than a regulatory protein for the expression of the *rpp* genes. It is unclear whether RppA and RppB catalyze a certain reaction as a heteromultimer or catalyze a different reaction individually.

Cytochrome P-450s usually catalyze numerous transformation with multifunctional xenobiotics of industrial, environmental, and medical importance. In addition,

several P-450s have also been reported to be a member of the biosynthetic enzymes for antibiotics. For example, EryF is a P-450 hydroxylase in the biosynthesis of erythromycin in *Saccharopolyspora erythraea*[27], and MycG is a P-450 enzyme probably catalyzing both hydroxylation and epoxidation in the mycimamicin biosynthetic pathway in *Micromonospora griseorubida*[28]. Since the *orf-3* gene locating closely to the *rpp* genes seems to constitute a gene cluster, we speculate that the tentatively named P-450$_{RPP}$ is involved in transformation of some compounds related to flavonoids or isoflavonoids. In relation to this, P-450$_{RPP}$ may be the same as the cytochrome P-450 (45 kDa) that is inducibly produced in response to either the flavonoid genistein or soybean flour[23]. The molecular size of P-450$_{RPP}$ calculated from its amino acid sequence is 45.2 kDa. If the P-450$_{RPP}$ gene is expressed inducibly by flavonoid compounds, the induced transcript would read through the *rppA-rppB* genes because of the very close location of the two genes. Thus, the *rpp* genes may be transcribed by two different promoters, the long, flavonoids-inducible transcript and the short transcript initiating the above-described promoter locating just in front of the *rpp* genes.

Finally, we would like to point out that a TTA codon for Leu, which is confined to a very limited number of *Streptomyces* genes engaged in the regulation of morphogenesis and secondary metabolism[29], is present at Leu-104 in the RppB coding sequence. In *S. coelicolor* A3(2), *bldA* controls the expression of TTA-containing genes at the translational level[29,30]. This suggests that, in addition to the above-described induction by flavonoid compounds, the expression of *rpp* is also regulated at the translational level, since the *bldA* gene appears to control morphogenesis in *S. griseus*[31], as in *S. coelicolor* A3(2).

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