Enhanced Heterologous Expression of Two *Streptomyces griseolus* Cytochrome P450s and *Streptomyces coelicolor* Ferredoxin Reductase as Potentially Efficient Hydroxylation Catalysts

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The herbicide-inducible, soluble cytochrome P450s CYP105A1 and CYP105B1 and their adjacent ferredoxins, Fd1 and Fd2, of *Streptomyces griseolus* were expressed in *Escherichia coli* to high levels. Conditions for high-level expression of active enzyme able to catalyze hydroxylation have been developed. Analysis of the expression levels of the P450 proteins in several different *E. coli* expression hosts identified *E. coli* BL21 Star(DE3)pLysS as the optimal host cell to express CYP105B1 as judged by CO difference spectra. Examination of the codons used in the CYP1051A1 sequence indicated that it contains a number of codons corresponding to rare *E. coli* tRNA species. The level of its expression was improved in the modified forms of *E. coli* BL21(DE3), which contain extra copies of rare codon *E. coli* tRNA genes. The activity of correctly folded cytochrome P450s was further enhanced by cloning a ferredoxin reductase from *Streptomyces coelicolor* downstream of CYP105A1 and CYP105B1 and their adjacent ferredoxins. Expression of CYP105A1 and CYP105B1 was also achieved in *Streptomyces lividans* 1326 by cloning the P450 genes and their ferredoxins into the expression vector pBW160. *S. lividans* 1326 cells containing CYP105A1 or CYP105B1 were able sufficiently to dealkylate 7-ethoxycoumarin.

Cytochrome P450 monoxygenase systems are encoded by a gene superfamily and play critical roles in the bioactivation and detoxification of a wide variety of xenobiotics. In humans they are a major class of biocatalyst involved in the oxidative metabolism of exogenous as well as endogenous compounds, including drugs, xenobiotics, fatty acids, bile acids, and steroids. These enzymes have considerable importance for determining the pharmacokinetic and toxicokinetic characteristics of drugs. It is sometimes difficult to chemically synthesize the same hydroxylated products that would occur in vivo in sufficient quantities to carry out toxicological studies on these drug derivatives. A biocatalytic approach to synthesizing large amounts of such hydroxylated compounds is thus attractive. *Streptomyces* species P450 enzymes are soluble, are not membrane bound like their human counterparts, and often have a broad range of substrate specificities. Thus recombinant *Streptomyces* enzymes could be used to prepare drug metabolites in quantity to assess their toxicological effects.

The soil bacterium *Streptomyces griseolus* ATCC 11796 expresses two distinct cytochrome P450 monoxygenases, designated cytochrome P450SU1 (CYP105A1) and cytochrome P450SU2 (CYP105B1) (37). These two enzymes are able to metabolize sulfonyleurea herbicides such as chlorimuron-ethyl and xenobiotics such as phenobarbital (8, 30, 33). These two systems each consist of an inducible cytochrome P450, a ferredoxin, and a poorly characterized NAD(P)H:ferredoxin reductase (27). The two cytochrome P450-encoding genes and their adjacent ferredoxin-encoding genes have been cloned and sequenced (27, 31). The *S. griseolus* P450 and adjacent ferredoxin can be expressed both in bacteria and higher plants, but cells containing the P450 enzymes alone are unable to metabolize herbicides (29, 30). It has been found that genes for both P450s are cotranscribed with those for their ferredoxins, Fd1 and Fd2, demonstrating that there is a well-defined relationship between the P450 and its cognate ferredoxin and that these ferredoxins are necessary for P450 activity (30). The level of NAD(P)H:ferredoxin reductase in cell extracts is very low, and no reductase gene has yet been isolated from *S. griseolus* (28). However, in vitro studies show that the absence of significant *S. griseolus* reductase activity can be overcome by reconstitution with ferredoxin NADP reductase from spinach chloroplasts or putidaredoxin reductase from the *Pseudomonas putida* P450_cam system (27, 30). It has been shown by O’Keefe et al. (30) that there is a direct correlation between the P450/ferredoxin content and the in vivo metabolism rates, and these workers suggested that the overall limiting parameters in vivo are the levels of P450 and/or ferredoxin but not of the reductase. In contrast their in vitro results showed low levels of reductase activity in crude soluble protein extracts, and this implied that whole-cell P450 activity might be limited by reductase availability.

To study the structural and functional aspects of P450 enzymes, large amounts of purified protein are necessary, more than can generally be purified from wild-type bacteria or animal tissue (particularly that of humans) (15). Heterologous expression of cytochrome P450 in *Escherichia coli* has become a very useful tool in biomedical research and is widely used in...
the study of many mammalian cytochrome P450 monooxygenases. In this paper, *S. griseolus* cytochromes P450SU1 and P450SU2 were expressed in *E. coli* to high levels to study the activity of these proteins toward heterologous compounds as well as to investigate their application in biocatalysis. We show that catalytically active P450 levels in vivo are enhanced by cloning a ferredoxin reductase of *Streptomyces coelicolor* and expressing this alongside the P450 and ferredoxin in *E. coli*. We also show the expression of these proteins in *Streptomyces lividans* and efficient biotransformation with the recombinant *S. lividans*.

**MATERIALS AND METHODS**

Strains, plasmids, and culture conditions. *E. coli* DH5α and TOP10 were used as hosts for transformation (Table 1). *E. coli* strains carrying plasmids were grown in Luria-Bertani medium at 37°C. Other standard microbial and recombinant techniques used throughout this work are as described by Sambrook et al. (39). Media were supplemented with 100 μg of ampicillin/ml and 50 μg of kanamycin/ml when required. Plasmid DNA was isolated from bacteria with the use of a Qiagen plasmid isolation kit. The *E. coli* strains and plasmids described in this paper are listed in Table 1.
Qiagen kits. DNA fragments for subcloning were isolated from agarose gels with a QIAEX II gel extraction kit (Qiagen). PCR products were cloned into the pCR II TOPO vector by using the TOPO TA cloning kit (Invitrogen). Several E. coli host strains that are compatible with the pET expression systems from Novagen were used. B. Rudd and J. Stragante were used to overexpress cytochrome P450s from Serratia marcescens.

Standard methods of culture for S. griseolus ATCC 17196, S. coelicolor A3 (2), and S. lividans 1326 and conditions for genomic DNA and plasmid DNA isolation and conjugation were as described previously (18). Thiostrepton, apramycin, and nalidixic acid were used at final concentrations of 10 μg/ml, 1 mg/ml, and 0.5 mg/ml, respectively. Streptomyces strains were grown on complete medium containing 13.5% yeast extract and 1.5% yeast extract or SFM containing 2% maltotriose, 2% soy flour, and 1.6% agar.

Construction of expression vectors in E. coli. The entire coding regions of the genes encoding cytochrome P450s from S. griseolus, S. lividans, and S. coelicolor were PCR-amplified with primers that added an NcoI site at the 5′ end of the coding sequence and have a NdeI site at the 3′ end of the coding sequence. The primers were designed to add an NcoI site at the 5′ end of the coding sequence and have a NdeI site at the 3′ end of the coding sequence. The PCR products were cloned into the pCR II TOPO vector by using the TOPO TA cloning kit (Invitrogen). Several E. coli host strains that are compatible with the pET expression systems from Novagen were used. B. Rudd and J. Stragante were used to overexpress cytochrome P450s from Serratia marcescens.

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For quantitation of recombinant cytochrome P450, cells were harvested, washed by centrifugation in 100,000 × g for 60 min (4°C), and the suspension was left on ice for 30 min. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM, and the mixture was sonicated on ice with an MSE Soniprep cell disrupter by using 10 pulses for 10 s with 30-s cooling intervals. Cell debris was removed by centrifugation, and the cytosol was isolated by centrifugation at 100,000 × g for 60 min. The soluble fraction was then used to measure the activity of recombinant cytochrome P450. The entire coding regions of the genes encoding cytochrome P450s from S. griseolus, S. lividans, and S. coelicolor were PCR-amplified with primers that added an NcoI site at the 5′ end of the coding sequence and have a NdeI site at the 3′ end of the coding sequence. The PCR products were cloned into the pCR II TOPO vector by using the TOPO TA cloning kit (Invitrogen). Several E. coli host strains that are compatible with the pET expression systems from Novagen were used. B. Rudd and J. Stragante were used to overexpress cytochrome P450s from Serratia marcescens.

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AGACGTTGC-3' and reverse primer 5'-GACTGACGTCCGCGTCCTC CG-3'. The PCR conditions were as described above. The 1.2-kb PCR product of the SC4B10 gene and the 1.3-kb PCR product of the SCF15A gene were first cloned into pCRII-TOPO vector to produce pQR294 (for SC4B10) and pQR295 (for SCF15A). The DNA sequence of the PCR-amplified fragment was determined at this stage. The resulting PCR products were then digested with NcoI and PstI and subcloned into those sites in the reconstructed pALTER-E2x expression vector (pQR293). This allowed construction of pQR293 derivatives containing the complete ferredoxin reductase-coding region under the control of the T7 promoter, producing pQR296 (for SC4B10) and pQR297 (for SCF15A). The DNA sequence of the PCR-amplified fragment was determined at this stage. The resulting PCR products were then digested with NcoI and PstI and subcloned into those sites in the reconstructed pALTER-E2x expression vector (pQR293). These new constructs (pQR367 and pQR368) were then cloned downstream of EcoRI digestion, giving the gene order P450-ferredoxin-ferredoxin reductase. These new constructs (pQR367 and pQR368) were then cloned downstream of EcoRI digestion, giving the gene order P450-ferredoxin-ferredoxin reductase. These new constructs were then cloned into the expression vector pET21a derivatives containing the P450SU1(pQR273) and P450SU2(pQR274) genes and their adjacent ferredoxins. These constructs were thereafter transferred into E. coli BL21 Star(DE3) containing plasmids pQR273 (P450SU1) or pQR274 (P450SU2). The additional of tetracycline at the normal concentration (12 μg per ml) to the medium to select for the pQR293 derivatives led to slow cell growth and reduced expression of P450 in E. coli. Therefore, the level of expression was enhanced by reducing the concentration of tetracycline to 5 μg per ml.

In addition to the above constructs, we also constructed operons containing the cytochrome P450, ferredoxin, and ferredoxin reductase genes, which are transcribed from the T7 promoter in one plasmid. The ferredoxin NADP reductase SCF15A gene was subcloned from the palTER-E2x vector derivative, pQR297, into the expression vector pET21a derivatives containing the P450SU1 (pQR273) and P450SU2 (pQR274) genes and their adjacent ferredoxins. This was carried out by first digesting pQR296 and pQR297 with NcoI and blunt ending with Klenow fragments. The plasmids were then digested with EcoRI to isolate fragments that contain the ribosome binding site, the T7 promoter, and the ferredoxin reductase gene. These fragments were then cloned downstream of each of the P450 genes and the associated ferredoxin genes in pQR273 and pQR274, which had been digested with HindIII and blunt ended with Klenow fragments, followed by EcoRI digestion, giving the gene order P450-ferredoxin-ferredoxin reductase. These new constructs (pQR367 and pQR368) were then transformed into E. coli BL21 Star(DE3)pLysS.

RESULTS

Expression of S. griseolus cytochrome P450s in E. coli. The P450 genes and the genes encoding their ferredoxins were amplified from S. griseolus ATCC 11796 genomic DNA by PCR using primers from the DNA sequence of the P450SU1 (CYP105A1) and P450SU2 (CYP105B1) genes and cloned into the pCRII-TOPO vector. The sequences of both DNA strands were determined, and this revealed the expected DNA sequence of the genes encoding P450SU1 and P450SU2 and their ferredoxins, Fd1 and Fd2. The genes encoding P450 and their ferredoxins were then cloned into the pET21a vector and transformed into E. coli BL21(DE3)pLysS (in order to express active P450) and BL21 Star(DE3)pLysS with maximal CO-reduced measurable cytochrome P450 protein for Terrri c broth supplemented with 1 mM ALA, and 125 nmol per g of protein for Terrri broth supplemented with 1 mM ALA and 0.5 mM FeCl3. Thus supplementation with ALA and FeCl3 produced almost a 10-fold increase in active P450. In contrast the yield of active P450SU1 was not enhanced by the addition of ALA and FeCl3 to the growth medium.

Optimizing expression of P450 using different host strains and growth conditions. Different conditions were used in an attempt to optimize the amount of active cytochrome P450SU1 and P450SU2 in E. coli. Initial studies were carried out with E. coli BL21(DE3)pLysS and were aimed at determining the optimal IPTG concentration for induction of P450SU1 and P450SU2. The optimal level of IPTG was 1 mM, and there was no difference between addition of IPTG at the beginning of growth and addition at mid-log phase (optical density at 600 nm, 0.8).

Time course studies revealed that the maximum production of P450SU2, as determined by evaluation of CO difference spectra, was obtained after 24 to 35 h of induction at 25°C, and this amount was reduced by one-half after 60 h. Accordingly all subsequent expression studies were carried out with an induction period of 35 h and growth at 25°C.

Although the BL21(DE3)pLysS E. coli strain is a convenient and effective host for protein expression, the production of the recombinant P450SU1 was limited. Only P450SU2 was successfully expressed in this E. coli strain. In an attempt to increase the levels of P450SU1 and P450SU2 expression in E. coli, P450SU1 and P450SU2 with their ferredoxins (encoded by pQR273 and pQR274, respectively) were transformed into various E. coli expression strains (Table 1). The cells were grown, induced, and harvested 24 and 35 h after induction. SDS-PAGE revealed slight increases in soluble-protein profiles in BL21 Star(DE3) and BL21 Star(DE3)pLysS. E. coli strains transformed with pQR273 and pQR274. The P450SU1 construct was expressed at a low level and yielded a readily measurable cytochrome P450 absorption peak (450 nm) when evaluated on the basis of CO difference spectra for seven E. coli host strains (six of which contain the pLysS plasmid). Five of the strains failed to produce a detectable cytochrome P450 signal. The highest expression of P450SU1, determined by CO-reduced spectra, was obtained with BL21 Star(DE3)pLysS, with maximal CO-reduced measurable cytochrome P450 production of 35 nmol per g of protein (Fig. 2). The expression of active P450SU2 was always higher than that of P450SU1. The most efficient expression was obtained with BL21 Star(DE3) pLysS. The yield of active P450SU2 expressed in BL21 Star(DE3) pLysS was 256 nmol per g of protein.

The side chain cleavage of 7-ethoxycoumarin by recombinant cytochrome P450SU1 and P450SU2 in these strains was tested. 7-Ethoxycoumarin was added to the cultures after induction with IPTG. TLC analysis showed an increase in bioconversion of 7-ethoxycoumarin to 7-hydroxycoumarin when the P450s were expressed in BL21 Star(DE3)pLysS, with maximal CO-reduced measurable cytochrome P450 production of 35 nmol per g of protein (Fig. 2). The expression of active P450SU2 was always higher than that of P450SU1. The most efficient expression was obtained with BL21 Star(DE3) pLysS. The yield of active P450SU2 expressed in BL21 Star(DE3) pLysS was 256 nmol per g of protein.

Optimizing expression of P450SU1 in various E. coli host strains containing rare-codon tRNAs. Examination of the codons present in the genes encoding cytochrome P450SU1 and its ferredoxin indicated that the coding sequences contain a large number of the least-used codons in E. coli, in particular CCC and CCT (proline), GGA (glycine), and CGG and CGA (arginine), which are very rarely used in E. coli genes. This may...
be the reason for the low-level protein expression of cytochrome P450SU1 and low-level expression of either of the cloned ferredoxins as assessed by SDS-PAGE. Comparison of the codon usage of P450SU1 and P450SU2 gene sequences indicated that the P450SU2 gene uses the more-prevalent *E. coli* codons. The frequencies of rare codons in *E. coli* genes are as follows: CCC (Pro), 0.5%; CCT (Pro), 0.7%; GGA (Gly), 0.8%, CGG (Arg) 0.3%, CGA (Arg), 0.5% (23). In the P450SU1 gene the frequencies of these codons are as follows: CCC, 1.5%; CCT, 2.3%; GGA, 2.4%; CGG, 2.4%, CGA, 3%.

Table 2 shows the use of modified BL21(DE3) *E. coli* cells, namely, BL21-CodonPlus(DE3)-RP, BL21-CodonPlus(DE3)-RIL cells, or Rosetta(DE3) and Rosetta(DE3)pLysS. These expression hosts have been constructed to contain extra copies of the *E. coli* tRNA genes with rare codons for the expression of genes from organisms with codon usage different from that of *E. coli*. The most efficient expression of P450SU1 in these hosts was obtained with BL21-CodonPlus(DE3)-RP and BL21-CodonPlus(DE3)-RIL. P450SU1 represented about 18% of the total protein as seen on Coomassie blue-stained SDS-PAGE gel (the value for the expressed protein band in a du-

FIG. 1. (A) SDS-PAGE of different *E. coli* host strains containing extra copies of rare codons expressing P450SU1. Lane 1, molecular mass markers as shown on the left; lane 2, BL21 cells; lane 3, pQR273 in BL21 Star(DE3)pLysS; lane 4, pQR273 in Rosetta(DE3); lane 5, pQR273 in Rosetta(DE3)pLysS; lane 6, pQR273 in BL21-CodonPlus(DE3)-RP; lane 7, pQR273 in BL21-CodonPlus(DE3)-RIL. (B) pQR274 (P450SU2) in BL21 Star(DE3)pLysS. (C) CO difference spectra of P450SU2 expressed in *E. coli* BL21 Star(DE3)pLysS.
plicate induction experiment was within 0.2%). None of the ferredoxins (molecular mass, 7 kDa) were visible on SDS-PAGE gel when stained with Coomassie brilliant blue but were visible when Tricine SDS gels were used and were stained with silver when expressed in all of the expression strains used here (data not shown). The highest activity of P450SU1 was seen when BL21-CodonPlus(DE3)-RIL was used as the expression host. The yield for active cytochrome expressed from P450SU1 was 50 nmol per g of protein (Table 2).

**Optimizing expression of P450 in the presence of ferredoxin reductase.** The genes encoding the native S. griseolus ferredoxin reductases have not yet been cloned or characterized. Instead two of the ferredoxin reductase genes, which have been identified in the genomic DNA sequence of S. coelicolor A3 (2), were amplified and expressed alongside the recombinant P450SU1 and P450SU2 genes. Two forms of construct were made. The first used a coexpression system produced by cloning the ferredoxin reductase genes separately into the expression vector pQR293 (Table 1). Plasmids were then introduced into an E. coli expression host containing genes encoding P450SU1 or P450SU2 and their ferredoxins on a pET vector. This two-plasmid system allows expression of two different genes cloned under the control of T7 promoters and allowed different combinations of cloned genes to be rapidly set up by transforming one of the expression plasmids into a strain with an existing expression plasmid. These two plasmids contain compatible replication origins, which should yield approximately equal copy numbers of both vectors. The proteins were then expressed together in E. coli BL21 Star(DE3), and the CO-reduced activity was measured (Fig. 3A). This shows that for both P450SU1 and P450SU2 only the ferredoxin reductase SCF15A gives an increase in active P450.

The second type of construct was an operon consisting of three genes in one expression plasmid, made by cloning the ferredoxin reductase SCF15A gene downstream of the genes encoding the P450s and their ferredoxins. The new constructs pQR367 and pQR368 were then expressed in E. coli BL21

### TABLE 2. Expression of active S. griseolus cytochrome P450SU1 in different E. coli host strains containing extra copies of E. coli rare-codon tRNA genes

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Protein concn. (g/liter)</th>
<th>Level of active P450&lt;sup&gt;c&lt;/sup&gt; in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450SU1 (pQR273) in BL21 Star(DE3)pLysS</td>
<td>1.55</td>
<td>60 39 4.8</td>
</tr>
<tr>
<td>P450SU1 (pQR273) in Rosetta(DE3)</td>
<td>1.45</td>
<td>27 19 2.5</td>
</tr>
<tr>
<td>P450SU1 (pQR273) in Rosetta(DE3)pLysS</td>
<td>1.25</td>
<td>26 21 2.9</td>
</tr>
<tr>
<td>P450SU1 (pQR273) in BL21-CodonPlus(DE3)-RP</td>
<td>1.45</td>
<td>43 30 4.2</td>
</tr>
<tr>
<td>P450SU1 (pQR273) in BL21-CodonPlus(DE3)-RIL</td>
<td>1.50</td>
<td>75 50 6.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> This is the total protein concentration in the cell extract after differential centrifugation.

<sup>b</sup> Active P450 is calculated from the reduced-CO absorbance as described in Materials and Methods. The data are the means of two different expression experiments for each construct combination. The two values were between 4 and 7% of each other.
FIG. 3. Expression of *S. griseolus* cytochrome P450s in different *E. coli* host strains containing different ferredoxin reductases. (A) Reduced-CO activity. Each column is an average of three experiments. (B) Reduced-CO difference spectrum of P450SU1 expressed in *E. coli* without ferredoxin reductase (i) and with ferredoxin reductase (ii). (C) TLC analysis showing the bioconversion of 7-ethoxycoumarin to 7-hydroxycoumarin. Lanes: 1, pQR273 in BL21 Star(DE3)pLysS; 2, pQR273 and pQR296 in BL21 Star(DE3); 3, pQR273 and pQR297 in BL21 Star(DE3); 4, pQR367 in BL21 Star(DE3)pLysS; 5, pQR274 in BL21 Star(DE3)pLysS; 6, pQR274 and pQR296 in BL21 Star(DE3); 7, pQR274 and pQR297 in BL21 Star(DE3); 8, pQR368 in BL21 Star(DE3)pLysS.
The highest activity was seen with the constructs (pQR367 and pQR368) that contain a P450 operon and that express ferredoxin and ferredoxin NADP reductase SCF15A, with maximum levels of spectrally measurable cytochrome P450 production of 55 nmol per g of protein for P450SU1 and 413 nmol per g of protein for P450SU2 (Fig. 3A). Figure 3B shows the increased amount of the absorbing form of the cytochrome P450, P450SU1, when a ferredoxin reductase is coexpressed in the same cell. There is a shift in the ratio of P420 to P450 in the spectrum shown in Fig. 3B, ii, for the strain containing the ferredoxin reductase SCF15A from S. coelicolor.

The hydroxylation of 7-ethoxycoumarin by recombinant P450SU1 and P450SU2 in several E. coli strains was tested. TLC analysis of extracts shows a qualitative increase in bioconversion of 7-ethoxycoumarin to 7-hydroxycoumarin by the construct that contains P450SU1 or P450SU2 with their ferredoxins and the ferredoxin reductase (Fig. 3C).

**Expression of S. griseolus P450s in S. lividans 1326.** Our first attempt to introduce P450SU1 and P450SU2 into S. lividans used plasmids (pQR291 and pQR292) in which the P450SU1 and P450SU2 genes were positioned downstream of the thiostrepton-inducible tipA promoter. Reduced-carbon monoxide difference spectra of the soluble extract of S. lividans carrying one copy of P450SU1 or -SU2 following the addition of ALA to GY medium, with growth at 28°C after induction of the tipA promoter, were investigated. Identical parallel cultures of S. lividans were used as the negative control. The induced cultures show a distinctive peak at 420 nm for the inactive form of P450 in the strains with the inserted P450SU1 and -SU2 but no 450-nm peak. The effect of addition of FeCl₃ to the medium was investigated, and again only the 420-nm absorbance was found.

The S. griseolus P450 genes were then cloned into the low-copy-number conjugative Streptomyces E. coli shuttle expression vector pBW160, in which the genes encoding P450SU1 and P450SU2 with their ferredoxins were positioned downstream of the actI promoter. The resulting E. coli clones, pQR355 and pQR356, were conjugated into S. lividans 1326. S. lividans carrying recombinant plasmids pQR355, pQR356, and pBW160 as the negative control was grown in GY medium at 28°C following the addition of ALA. Cells were grown for 2, 3, and 4 days, and the cell extract was analyzed for the presence of P450SU1 and P450SU2 as measured by their CO difference spectra. S. lividans 1326 containing pQR355 or pQR356 showed the 450-nm absorbance characteristic of P450s, and this was not observed in extracts of the control culture, S. lividans 1326(pBW160), in spite of the probable presence—because we assume that the S. lividans and S. coelicolor genomes have the same structural and genetic organizations—of 20 cytochrome P450 genes in the genomic DNA of S. coelicolor (S. coelicolor genome project [http://www.sanger.ac.uk/Projects/S_coe/](http://www.sanger.ac.uk/Projects/S_coe/)) (20).

Reduced-carbon monoxide difference spectra of the soluble fractions of S. lividans 1326 strains containing pQR355 (P450SU1) or pQR356 (P450SU2) showed a peak absorbance at 450 nm, which was not observed in extracts of the control culture, S. lividans 1326(pBW160). The yield for active cytochrome expressed from P450SU1 was 27 nmol per g of protein; for P450SU2 the yield was 28 nmol per g of soluble protein.

The catalytic activities of P450SU1 and P450SU2 were investigated with the model substrate 7-ethoxycoumarin following growth at 28°C on GY medium containing ALA for 1, 2, 3, or 4 days. TLC analysis of extracts from cells containing P450SU1 and P450SU2 revealed the o-dealkylation of 7-ethoxycoumarin to 7-hydroxycoumarin (Fig. 4), not observed in extract of the control culture, S. lividans 1326(pBW160). The TLC analysis showed a greater o-dealkylation of 7-ethoxycoumarin by P450SU1 than by P450SU2.

**DISCUSSION**

*S. griseolus* ATCC 11796 contains two cytochrome P450 proteins, P450SU1 and -SU2, which can hydroxylate a variety of sulfonurea herbicides (33). These are distinct gene products with different spectroscopic, chromatographic, and antigenic properties (25, 26, 31). The cytochrome P450SU1 and P450SU2 genes have been expressed previously in *E. coli*, but the amount of measurable cytochrome P450 production was low as calculated from the CO difference spectrum (31). Our first expression constructs of P450SU1 and -SU2 show a P450 band of about 13% of total cell protein for P450SU1 and 25% of total cell protein for P450SU2 on SDS-PAGE gel, but this is not matched by the amount of active P450 as calculated from the CO-reduced absorbance. We have, therefore, attempted to develop an efficient expression system for P450SU1 and P450SU2 to allow production of active, correctly folded enzyme.

A series of modifications were required to improve expression of P450SU1 and P450SU2 in *E. coli*. The changes required were in growth conditions and the choice of *E. coli* host strain. Adding ALA and FeCl₃ to the growth medium increased the amount of spectrally measurable cytochrome P450SU2 expression. In other systems, the addition of ALA to the culture medium stimulates heme biosynthesis to match the increase in P450 polypeptide synthesis (15). It has been observed previously that such an increase of P450 suggests that the rate-limiting step in the synthesis of the hemoprotein lies in the synthesis of the heme prosthetic group (16, 35). This fact, together with the increases above in the amount of CO-re-
duced P450, suggests that it is not only the protein expression level but also the final formation of correctly folded holoenzyme with the heme group in its proper protein environment which increase when ALA and FeCl₃ are added. Significantly, the difference spectra of P450SU2 showed no absorbance at 420 nm, indicating no conversion of the cytochrome to the inactive, low-spin, P420 form when ALA and FeCl₃ were added. The amount of spectrally measurable cytochrome P450SU1 expression was not enhanced by the addition of ALA and FeCl₃ to the growth medium. The observation of P450-to-P420 conversion was first described by Omura and Sato (32), and subsequently this transition has been induced by using various conditions and chemicals (2, 12, 13). Healy et al. (10) explained that the reason that they were unable to observe the 420-nm reduced-CO difference spectrum with cytochrome P450 TtxC may be improper protein folding or improper incorporation of the heme group into the apoenzyme in E. coli.

Although E. coli has a remarkable capacity to produce a large quantity of protein, there are limits when the codon usage in the mRNA for the recombinant gene differs from that of the E. coli host cells (5, 17). It is known that an excess of any of these rare codons creates problems during translation, leading to a reduction in the quantity of the protein synthesized. Moreover, the cellular levels of certain tRNAs may fluctuate with growth conditions (4, 14). This was demonstrated previously by Kleber-Janke and Becker (19), who increased expression of several recombinant peanut allergens in E. coli. The expression of the P450SU1 protein was enhanced from 13% ± 0.2% to 18% ± 0.2% of the total protein (from densitometry of stained bands on SDS-PAGE gel) with these modified cells, in particular BL21-CodonPlus(DE3)-RP and BL21-CodonPlus(DE3)-RIL. The amount of spectrally measurable cytochrome P450SU1 was also increased by the same ratio, from 35 to 50 nmol per g of protein. The levels of active P450SU1 are not as high as those of P450SU2; this is probably due to the presence of other rare codons in the P450SU1 gene which are not provided by these CodonPlus strains. These two strains have extra copies of only the tRNAs for argU (AGA and AGG) and proL (CCC) in BL21-CodonPlus(DE3)-RP and BL21-CodonPlus(DE3)-RIL but do not have cloned tRNA genes with CCT (Pro), GGA (Gly), and CGG (Arg) and CGA codons, which are present in the P450SU1 gene.

Prokaryotic cytochrome P450 systems are usually multicompartment systems and require the presence of a ferredoxin reductase and a ferredoxin to couple electron flow from NAD(P)H to the terminal cytochrome P450 component. Microbial cytochrome P450s can be one of two different classes: class I (or B class) P450, receiving electrons from a two-component reductase system, ferredoxin, and NADH ferredoxin reductase, such as in P450CAM (22), or class II (or E class) P450, receiving electrons from NADPH-cytochrome P450 reductase fused to the P450 in one continuous polypeptide, such as in P450bmt (24) and P450Rhf (36). Ferredoxin reductase is an essential component of the cytochrome P450 monooxygenase complex. Only a few ferredoxin reductases of prokaryotic P450 systems have been purified and characterized because of their unstable nature and relatively low levels of expression (1, 34).

The active level of cytochrome P450SU2 was improved, and that of P450SU1 was slightly improved, in the strains with the rare-codon tRNA as calculated on the basis of the reduced-CO spectrum, so we tested an additional way of improving activity. In E. coli the yield of correctly folded P450 was further improved by cloning the S. coelicolor ferredoxin reductases SCHF15A and SC4B10 and coexpressing these ferredoxin reductases in the same cell from a recombinant plasmid (Fig. 3A and B) and cloning the gene encoding ferredoxin reductase SCHF15A downstream of the genes encoding P450 and their ferredoxins. This shifts the ratio of P420 to P450 in the direction of P450. It appears that simply coexpressing the ferredoxin reductase alongside the P450 and ferredoxin stabilizes the folded, active form of the P450, as shown by the reduced-CO spectrum when no substrate is present and no electron flow is needed. It may be that this indicates an in vivo association of these proteins which stabilizes the P450. Because of this increased active P450 form, a greater whole-cell biotransformation is seen, and this may be further enhanced by the ferredoxin reductase, which provides the correct electron transfer pathway. This shows that the P450-ferredoxin pair functions in vivo, that S. coelicolor ferredoxins can accept electrons from these two S. coelicolor ferredoxin reductases, and that S. coelicolor ferredoxin reductase has a broad specificity to donate electrons to nonnative ferredoxins.

The initial failure to enhance the amount of spectrally measurable cytochrome P450 production of P450SU1 in E. coli prompted us to look at a suitable alternative host. S. lividans was selected because it is the closest relative to S. griseolus for which well-developed expression vectors exist and hence the most homologous expression background that was readily available for expressing recombinant S. griseolus P450 genes. Expression of another cytochrome P450 in S. lividans has been successfully achieved previously (6, 11) although this was done to investigate the role of P450 in antibiotic biosynthesis. An SCP2-based plasmid expression system was used to express P450SU1 and P450SU2 in S. lividans. The level of recombinant P450SU1 and P450SU2 activity obtained in S. lividans was lower than the activity obtained from the E. coli strain. This is probably because of the lower-copy-number expression vector, pBW160, in S. lividans and the use of the actI promoter, which is presumably weaker than the T7 promoter. The level of active P450 might be further enhanced in S. lividans by the expression of the ferredoxin reductase SCHF15A gene of S. coelicolor cloned downstream of the P450 genes, similar what has been achieved in E. coli to enhance expression of P450SU1 and P450SU2 monooxygenases. These activities will probably also be enhanced once the indigenous electron transport component ferredoxin reductase of the S. griseolus cytochrome P450 system is cloned. However, TLC analysis showed that the biocconversion of 7-ethoxy coumarin to 7-hydroxycoumarin by the recombinant P450SU1 and P450SU2 in S. lividans was two- to threefold higher than that obtained from the recombinant E. coli strain. This indicates that the Streptomycetes host might be more robust in the face of prolonged contact with the biotransformation substrate than E. coli.

The strategy for increasing the activity of these two bacterial cytochrome P450s, namely, the inclusion of ferredoxin reduc-
tase to both stabilize the P450 and then provide an optimal electron transfer system, may be a general phenomenon which could be applied to the enhancement of other cloned P450 enzymes.

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