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Characterization of *Saccharopolyspora erythraea* Cytochrome P-450 Genes and Enzymes, Including 6-Deoxyerythronolide B Hydroxylase

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Previous studies of erythromycin biosynthesis have indicated that a cytochrome P-450 monooxygenase system is responsible for hydroxylation of 6-deoxyerythronolide B to erythronolide B as part of erythromycin biosynthesis in *Saccharopolyspora erythraea* (A. Shafiee and C. R. Hutchinson, Biochemistry 26:6204–6210 1987). The enzyme was previously purified to apparent homogeneity and found to have a catalytic turnover number of approximately 10^{-3} min^{-1}. More recently, disruption of a P-450-encoding sequence (*eryF*) in the region of *ermE*, the erythromycin resistance gene of *S. erythraea*, produced a 6-deoxyerythronolide B hydroxylation-deficient mutant (J. M. Weber, J. O. Leung, S. J. Swanson, K. B. Idler, and J. B. McAlpine, Science 252:114–116, 1991). In this study we purified the catalytically active cytochrome P-450 fraction from *S. erythraea* and found by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis that it consists of a major and a minor P-450 species. The gene encoding the major species (*orf405*) was cloned from genomic DNA and found to be distinct from *eryF*. Both the *orf405* and *eryF* genes were expressed in *Escherichia coli*, and the properties of the proteins were compared. Heterologously expressed EryF and Orf405 both reacted with antisera prepared against the 6-deoxyerythronolide B hydroxylase described by Shafiee and Hutchinson (1987), and the EryF polypeptide comigrated with the minor P-450 species from *S. erythraea* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. In comparisons of enzymatic activity, EryF hydroxylated a substrate with a turnover number of 53 min^{-1}, whereas Orf405 showed no detectable activity with a 6-deoxyerythronolide B analog. Both enzymes showed weak activity in the *O*-dealkylation of 7-ethoxycoumarin. We conclude that the previously isolated 6-deoxyerythronolide B hydroxylase was a mixture of two P-450 enzymes and that only the minor form shows 6-deoxyerythronolide B hydroxylase activity.

Before the attachment of two sugar units to form erythromycin D, the macrolide lactone 6-deoxyerythronolide B (Fig. 1, structure 1) is hydroxylated at the 6 position to form erythronolide B (Fig. 1, structure 2). It was originally reported that the enzyme responsible for this hydroxylation was found in a cytochrome P-450 monooxygenase-rich fraction of *Saccharopolyspora erythraea* cellular extract (5, 6). This activity was later purified to apparent homogeneity and shown to be a cytochrome P-450 enzyme that regiospecifically hydroxylated 6-deoxyerythronolide B and its 9-deoxy-9-hydroxy derivative in vitro (27). However, the catalytic activity of the enzyme was quite low, making it less certain that it was the true hydroxylating enzyme in vivo (6, 27).

Recently, Weber et al. (34, 35) created mutations in the region of the erythromycin resistance gene *ermE* of *S. erythraea* by insertional inactivation. One mutant phenotype was characterized by the formation of 6-deoxyerythromycin A and the absence of erythromycin A, suggesting that the mutation was located at the 6-hydroxylase locus, referred to as *eryF* (35). Sequence analysis of this region revealed the existence of an apparent cytochrome P-450-encoding sequence. In the work presented here, the cytochrome P-450 enzyme previously isolated from cellular extracts (27) was reexamined. We show that this cytochrome P-450 preparation actually contains two proteins, one of which is present in large excess. The gene for this major P-450 was cloned and found to be distinct from *eryF*, although the products of the two genes are quite similar. The genes for the two enzymes were expressed in *Escherichia coli*, and the enzymatic activities of both P-450 proteins on 6-deoxyerythronolide B substrates were compared. The results indicate that the minor P-450 species is the *eryF* gene product and the major species has a function that is not related to erythromycin metabolism.

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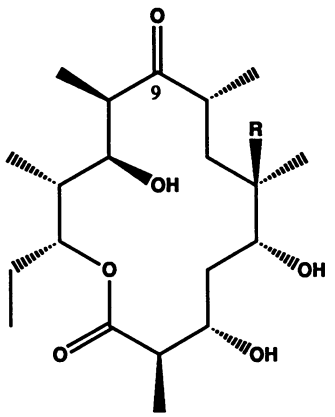
MATERIALS AND METHODS

Bacterial strains, plasmids, and other materials. The *S. erythraea* CA340 strain, which produces levels of erythromycins that are somewhat higher than those of the wild-type strain NRRL 2338 (UW22), was obtained from Abbott Laboratories (North Chicago, Ill.). Both *S. erythraea* strains were grown on R2T solid medium (36) or in MI-102 broth (4). *E. coli* DH5α and JM105 were grown on LB agar or broth at 37°C as described by Sambrook et al. (25). Plasmids pGEM-7zf(+) and pGEM-3zf(−) were obtained from Promega Biotech (Madison, Wis.), and pUC18 was obtained from GIBCO-BRL (Gaithersburg, Md.). Replicative-form M13mp18 and M13mp19 were obtained from New England BioLabs (Beverly, Mass.). The cosmid vector pKC505 (24) was obtained from Richard Baltz, Eli Lilly Co. (Indianapolis, Ind.), and the expression plasmid pTrc99c was obtained from Egon Amann, Behringwerke AG (Marburg, Germany) (1). The construction of the plasmids used in this study is described in Table 1.

The restriction enzymes and other materials for recombinant DNA procedures were obtained from U.S. Biochemical



1) R = H
2) R = OH

FIG. 1. Structures of 6-deoxyerythronolide B (structure 1) and erythronolide B (structure 2).

(Cleveland, Ohio), Promega Biotech, New England Bio-Labs, and GIBCO-BRL. Materials for enzyme purification, analysis, and assays were obtained from Sigma (St. Louis, Mo.), unless otherwise specified. Materials labeled with ^{32}P and ^{35}S were obtained from Amersham (Arlington Heights, Ill.).

Methods for protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels by the method of Laemmli (13), and proteins were visualized by staining with Coomassie brilliant blue R. Western blotting was done with a Bio-Rad (Richmond, Calif.) electroblotting apparatus, and proteins were transferred to polyvinylidine difluoride (Immobilon P; Millipore, Bedford, Mass.) membranes. The immunostaining method was similar to that described by Sambrook et al. (25) and employed goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase. The protein content was determined by using a modification of the Bradford method (30), and the cytochrome P-450 content was quantified by analysis of the optical difference spectra of the oxidized and dithionite-reduced, carbon monoxide-bound enzyme complex (22). Fast protein liquid chromatography was performed with a Pharmacia (Piscataway, N.J.) system, and high-performance liquid chromatography (HPLC) was done with a Waters (Milford, Mass.) model 501 pump system with a Waters 484 variable wavelength absorbance detector.

Hydroxylase assays. The assay for hydroxylation of 6-deoxyerythronolide B utilized the substrate analog (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6-deoxyerythronolide B labeled at the 9 position by reduction of 6-deoxyerythronolide B with NaB^3H_4 (27). For purified proteins or crude preparations from *E. coli*, the assay mixture consisted of 20 μM spinach ferredoxin, 0.25 U of spinach ferredoxin-$NADP^+$ oxidoreductase (EC 1.18.1.2), 0.8 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1.4 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 4 pmol to 10 nmol of cytochrome P-450, and 55 μM substrate (150,000 cpm) in a final volume of 0.5 ml. The assay mixture was incubated for 1 min at 30°C before the addition of substrate to allow the generation of NADPH, and then the mixture was incubated for 5 min at 30°C. The reactions were stopped by adding 0.5 ml of ethyl acetate and vortexing rapidly. The reaction products were extracted with ethyl acetate and purified by silica gel thin-layer chromatography (TLC) as described previously (27). For crude *S. erythraea* extracts, the assays were run in the same manner, with the reaction times increased to 1 h. For measurement of the *O*-dealkylation of 7-ethoxycoumarin, the same assay mixture was used with a substrate concentration of 0.5 mM and a reaction time of 15 min. The reaction products were extracted with ethyl acetate and analyzed by TLC with a solvent system of benzene-ethanol (9:1, vol/vol) (27).

Enzyme purification procedure. Erlenmeyer flasks containing 500 ml of MI-102 broth were inoculated with 10 ml of a starter culture that had been inoculated with 15 μl of a dense spore suspension of the UW22 or CA340 strain, prepared by standard procedures (10, 36), and grown for 48 h at 30°C. The flasks were shaken at 30°C for 48 h, at which time they were found by TLC analysis of the culture broth to accumulate erythromycin A (36). The contents were centrifuged at 6,000 × *g*, and the resulting pellets were washed twice with 100 mM $KHPO_4$ (pH 7.3)–1 mM EDTA. After the second wash, the cells were suspended in 100 mM $KHPO_4$ (pH 7.3)–1 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride–0.2 mM dithiothreitol–1 mM benzamidine–10% glycerol (buffer A), and lysozyme was added to a concentration of 2 mg/ml. The resulting cell suspension was stirred overnight at 4°C, streptomycin sulfate (1 mg/ml) was added, and the suspension was stirred for 1 h. The suspension was centrifuged at 39,000 × *g*, and the precipitate was discarded. Solid ammonium sulfate was added to the supernatant with stirring to 30% saturation. After centrifugation (39,000 × *g* for 20 min), the precipitate was discarded and ammonium sulfate was added to the supernatant to 80% of saturation; the preparation was then centrifuged (39,000 × *g* for 20 min). The 30 to 80% ammonium sulfate precipitate was taken up in buffer A and dialyzed against two changes of buffer A at 4°C. The resulting protein solution was applied to a Q-Sepharose

TABLE 1. Plasmids used in this study with details of their construction

Plasmid	Cloning vector	Digest	Insert[a] (sites)
pWHM800	pGEM7zf	*Bam*HI	2.0-kb genomic *Bam*HI fragment (4–16)
pWHM801	pGEM3zf	*Sal*I	2.2-kb *Sal*I fragment (1–7)
pWHM802	pGEM3zf	*Sst*I	1.5-kb *Sst*I fragment (14–18).
pWHM803	pGEM3zf	*Pst*I, *Sal*I	0.7-kb *Pst*I-*Sal*I fragment (8–12)
pWHM804	pGEM3zf	*Sma*I	0.6-kb PCR amplification product (10–12)
pWHM805	pUC18	*Bam*HI, *Sst*I	1.2-kb *Bam*HI-*Sst*I fragment (9–16)
pWHM806	pTrc99c	*Nco*I, *Bam*HI	0.4-kb *Nco*I-*Sst*II fragment (10–11), *Sst*II–*Bam*HI fragment (11–16)
pWHM807	pTrc99c	*Bam*HI, *Pst*I	2.1-kb *Bam*HI–*Pst*I fragment from *eryF* (19–22)
pWHM808	pWHM807	*Nco*I, *Kpn*I	Synthetic *Nco*I-*Kpn*I double-stranded linker

[a] *S. erythraea* DNA used in the construction of each plasmid. All fragments listed in this column were ligated with the vector digests listed in the third column to produce the plasmids listed in the first column. The restriction site designations given within parentheses refer to the restriction maps shown in Fig. 3.

(Pharmacia) anion-exchange column that had been equilibrated with buffer A, and the column was eluted stepwise with buffer A containing 100, 200, and 650 mM KCl. Only the 200 mM KCl fraction contained P-450, as determined by optical difference spectra. It was concentrated by ultrafiltration through a filter (5,000-molecular-weight cutoff) and dialyzed against 50 mM Tris-HCl (pH 7.5)–0.2 mM dithiothreitol (buffer B). The resulting solution was applied to a Mono-Q (Pharmacia) anion-exchange fast protein liquid chromatography column and eluted with a linear gradient from 0 to 500 mM KCl in buffer B over a period of 30 min. Fractions were screened for the characteristic 416-nm Soret absorbance of P-450 proteins (27), and a single peak was seen. The P-450-containing fractions were pooled and diluted twofold with buffer A containing 3 M ammonium sulfate. This material was applied to an Alkyl Superose (Pharmacia) hydrophobic interaction column, and the proteins were eluted with a linear gradient from 2 to 0 M ammonium sulfate in buffer A (minus glycerol) over a period of 30 min. The cytochrome P-450-containing fractions were pooled and injected on to a Vydac (Hesperia, Calif.) C_4 reversed-phase HPLC column and eluted with a linear gradient from 0 to 90% acetonitrile in 0.1% trifluoroacetic acid. A sample of this protein was digested to completion with trypsin, and the resulting oligopeptides were purified by reversed-phase HPLC with an acetonitrile-water gradient. The N-terminal amino acid sequences of selected peptides were obtained by microscale Edman degradation (the proteolysis, oligopeptide isolation and microsequencing were performed at the Harvard University Microchemical Laboratory, Cambridge, Mass.).

Cellular extracts of *E. coli* were made in a manner similar to that described above for *S. erythraea*. Cells were obtained from 500-ml cultures of *E. coli* DH5α transformants that had been grown overnight at 37°C in LB containing 1 mM isopropyl-β-D-thiogalactopyranoside and 100 μg of ampicillin per ml. After centrifugation at 6,000 × *g*, the cells were washed with 50 mM Tris-HCl (pH 7.5)–1 mM EDTA. After pelleting, the cells were suspended in 10 ml of buffer B containing phenylmethylsulfonyl fluoride (0.2 mM) to which lysozyme (2 mg/ml) was added, and the cell suspension was incubated at room temperature for 30 min. Triton X-100 (0.1%) was added, along with KCl (300 mM), $MgCl_2$ (25 mM), and a small amount of DNase I, to facilitate lysis. The mixture was incubated at room temperature for approximately 5 min and then centrifuged at 39,000 × *g*. Polyethyleneimine (0.1%) was added to the supernatant, and the mixture was centrifuged at 39,000 × *g*. Proteins were precipitated from the resulting supernatant with ammonium sulfate at 30 and 80% saturation as described above, and the 30 to 80% pellet was retained, dissolved, and dialyzed against buffer B.

DNA manipulations. Nick translations were performed as described by Sambrook et al. (25) with [α-^{32}P]dCTP. Oligodeoxynucleotides were 3′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Agarose gel electrophoresis, restriction enzyme digestion, ligation, transformation, and plasmid preparation from *E. coli* were performed by established methods (25). DNA was purified from agarose gels by adsorption onto DEAE paper (Schleicher and Schuell, Keene, N.H.) and subsequent elution with a high-salt buffer as directed by the manufacturer. Genomic DNA was isolated from *S. erythraea* by the procedure of Hopwood et al. (10). Southern blot transfers (25) were made from 0.75% agarose gels to Hybond N nylon membranes (Amersham) as suggested by the manufacturer. Hybridizations were performed in 20 mM NaH_2PO_4–2× Denhardt solution (25)–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% salmon sperm DNA–10% dextran sulfate–50% formamide at 42°C for homologous nick-translated probes and at 48°C for oligodeoxynucleotide probes. Washes were carried out in 2× SSC–0.1% SDS at 48°C for oligodeoxynucleotide probes and in 0.1× SSC–0.1% SDS at 70°C for homologous nick-translated probes. Polymerase chain reactions (PCR) were carried out with a Coy model 50 tempcycler (Coy Research Products Inc., Ann Arbor, Mich.), with *Taq* polymerase and the buffer supplied by the manufacturer (Promega Biotech). Amplification of the high-GC-content template was obtained with a mixture of 7-deaza dGTP–dGTP (3:1) in place of dGTP as suggested by Innis (12). The PCR temperature program was as follows: 6 min at 95°C, 5 min at 55°C; 30 cycles of 1 min at 75°C, 1 min at 95°C, and 1 min at 55°C; and then 7 min at 75°C. The portion of the amplified region used for later experiments was sequenced to verify that the product had the correct nucleotide sequence.

DNA sequencing. Nested deletions were constructed with the Erase-a-Base system (Promega Biotec) according to the manufacturer's instructions. Derivatives of pGEM-7zf(+) containing *S. erythraea* DNA were digested with *Hin*dIII and *Kpn*I before exonuclease III treatment. Single- or double-stranded templates were prepared by established methods (25) and sequenced with Sequenase 2.0 (U.S. Biochemical) or *Taq* polymerase by following the manufacturer's instructions with ^{35}S-dCTP as the labeled nucleotide and substituting 7-deaza-dGTP for dGTP in sequencing reactions. Labeled DNA was separated on 6% polyacrylamide wedge gels containing 10% formamide. Sequence analyses were performed with the University of Wisconsin Genetics Computer Group software, version 6.2 (8).

Nucleotide sequence accession number. The DNA sequence data described in this paper have been deposited at EMBL and GenBank with the accession number M83110.

RESULTS

Purification of the major cytochrome P-450 from *S. erythraea*. Cell extracts and ammonium sulfate precipitates from *S. erythraea* showed levels of 6-deoxyerythronolide B hydroxylase activity (< 0.1 nmol of product formed per nmol of P-450 per min) that were similar to those found in previous studies (5, 6, 27). Immunostaining of Western blots with antisera prepared against the original hydroxylase (27) revealed, as expected, a band at approximately 44,000 Da (27), and optical difference spectra of the crude extracts and ammonium sulfate precipitates showed the presence of a cytochrome P-450. Further purification by anion-exchange and hydrophobic interaction chromatography resulted in an apparently homogeneous preparation of cytochrome P-450 that still possessed low activity when assayed in the presence of spinach ferredoxin and ferredoxin-$NADP^+$ oxidoreductase to provide the requisite electron transport from NADPH. Chromatography on a reversed-phase HPLC column followed by SDS-PAGE showed the presence of a single major protein and small amounts of a second component (Fig. 2A). This minor protein was eluted at the leading edge of the HPLC peak containing the major component and could not be completely separated from it (Fig. 2A). Immunostaining of Western blots containing the HPLC fractions indicated that both the major and minor bands were immunoreactive (Fig. 2B). Denaturation of the cytochrome P-450 during the course of reversed-phase HPLC was evident by the presence of a peak of nonprotein material corresponding

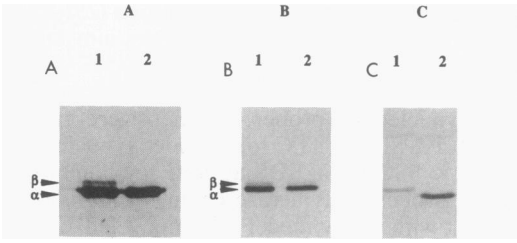


FIG. 2. Cytochrome P-450 enzymes from *S. erythraea* and *E. coli* (A) SDS-PAGE of consecutive fractions from the C_4 reversed-phase HPLC column showing the major (α) and minor (β) protein species in the cytochrome P-450-containing material obtained from *S. erythraea* CA340 after purification on Alkyl Superose. The gel was stained with Coomassie blue. Lane 1 represents the leading edge of the P-450-containing peak, and lane 2 represents the trailing edge. (B) Western blot of consecutive fractions (lanes 1 and 2) from the C_4 reversed-phase HPLC column incubated with antiserum prepared against 6-deoxyerythronolide B hydroxylase (27). Both the major (α) and minor (β) forms of cytochrome P-450 react with the antiserum. (C) Western blot showing expression of EryF (lane 1) and Orf405 (lane 2) in *E. coli*. The blot was incubated with the same antiserum used in panel B.

in retention time to heme. For this reason, the HPLC-purified material could not be assayed for hydroxylase activity.

Cloning of the sequence coding for the major cytochrome P-450. To determine whether the major cytochrome P-450 from *S. erythraea* was responsible for the 6-deoxyerythronolide B hydroxylase activity, its gene was cloned, with the eventual goal of obtaining this protein by expressing its gene in *E. coli*. A preparation of the major polypeptide obtained by HPLC was digested to completion with trypsin, and the resulting peptide fragments were separated by reversed-phase HPLC. Edman degradation of two of these peptides produced the amino acid sequences shown in Table 2. Best-guess oligodeoxynucleotide probes PT1 and PT2 (Table 2) with inosine replacement at highly degenerate codon positions (17) based on the sequences of the two peptides were synthesized. By hybridization with blots from *S. erythraea* NRRL 2338 genomic DNA digested to completion with *Bam*HI, a single 2.0-kb fragment was detected with probe PT1. Probe PT2 hybridized with the same band, but somewhat less specifically. *Bam*HI-digested *S. erythraea* genomic DNA was electrophoresed in an agarose gel, and the size fraction from approximately 1.8 to 2.3 kb was collected on DEAE paper. The eluted DNA was ligated with *Bam*HI-digested pGEM-7zf(+), and *E. coli* DH5α was transformed with the ligation mixture. Plasmids were prepared from 96 Ap^r colonies and screened, after digestion with *Bam*HI, by Southern blot hybridization with probe PT1. A single hybridizing clone (pWHM800), which also hybridized with probe PT2, was detected. The 2.0-kb *Bam*HI fragment from plasmid pWHM800 was used to screen a cosmid library of NRRL 2338 genomic DNA (33) by colony hybridization at high stringency to obtain cloned DNA encompassing the region shown in Fig. 3. Hybridization experiments were also performed with clones from the cosmid library that were known to contain approximately 40 kb surrounding *ermE* (33), including the putative hydroxylase-encoding sequence *eryF* (34, 35). No hybridization was observed between the pWHM800 insert and any of these cosmid DNAs under conditions of moderate to high stringency, indicating that the sequence hybridizing with the synthetic probes was not identical to the *eryF* coding sequence.

Sequencing of the region containing the major cytochrome P-450. By sequencing double- and single-stranded templates, 3.078 kb of sequence in the region encoding the major cytochrome P-450 of *S. erythraea* was obtained (Fig. 4). Analysis of the sequence with the CODONPREFERENCE program (8) revealed three potential open reading frames (Fig. 5). The two oligodeoxynucleotide probes were derived from a sequence in the center open reading frame, *orf405*. This open reading frame is preceded by a potential ribosome binding site (GGAAGG) (Fig. 4) that shows a reasonable degree of complementarity to the 5′ end of the *Streptomyces lividans* 16S rRNA (2), suggesting that the *orf405* coding region begins at ATG-951. Translation of this open reading frame would result in a polypeptide with 405 amino acid residues, a molecular weight of 45,238, and a calculated isoelectric point of 4.81.

Sequence comparisons with the TFASTA and GAP programs (8) indicated that the *orf405* polypeptide bears strong similarity to many other cytochromes P-450, particularly those derived from other actinomycetes (Fig. 6) (11, 21). The similarity is especially strong in the region surrounding Cys-352 (Fig. 6), which corresponds to the residue forming

TABLE 2. Oligodeoxynucleotides used for cloning of *orf405* and construction of cytochrome P-450 expression vectors

Oligonucleotide	Sequence
Cloning of *orf405*[a]	
PT1	TGG ACC AAC GTI CTI GTI GAC GGC TCI CAG CCI GAG GCI CAG GCI CAG GC
	W T N V L V D G S Q P E A Q A Q A
PT2	TTC GGC GAC GTI GTI ATC CCI GAG GGC GAG CTI GTI TGG GTI GCI CTI GGC
	F G D V V I P E G E L V W V A L G
Construction of pWHM808[b]	
NcoI-KpnI linker	CATG ACC ACC GTT CCG GAT CTG GAA TCC GAC TCC TTC CAC GTT GAC TGG TAC
	TGG TGG CAA GGC CTA GAC CTT AGG CTG AGG AAG GTG CAA CTG AC
Wild-type *eryF*	ATG ACG ACC GTT CCC GAT CTC GAA AGC GAC TCC TTC CAC GTC GAC TGG TAC
	M T T V P D L E S D S F H V D W Y
PCR amplification of *orf405*[c] for the construction of pWHM806 forward primer	GATATCATATGA CC ATG GCC ACC GGT CAA GTG CCC G

[a] Inosine replacement probes used in cloning *orf405* based on the amino acid sequence of the major cytochrome P-450 isolated from *S. erythraea*.
[b] Synthetic linker used to clone *eryF* into pTrc99c. Codon usage has been changed to reflect that seen in highly expressed proteins of *E. coli* (7). The Wild-type sequence of the 5′ end of *eryF* is shown for comparison.
[c] Primer used to mutagenize the 5′ end of *orf405* to create an *Nco*I site (underlined) for ligation into pTrc99c.

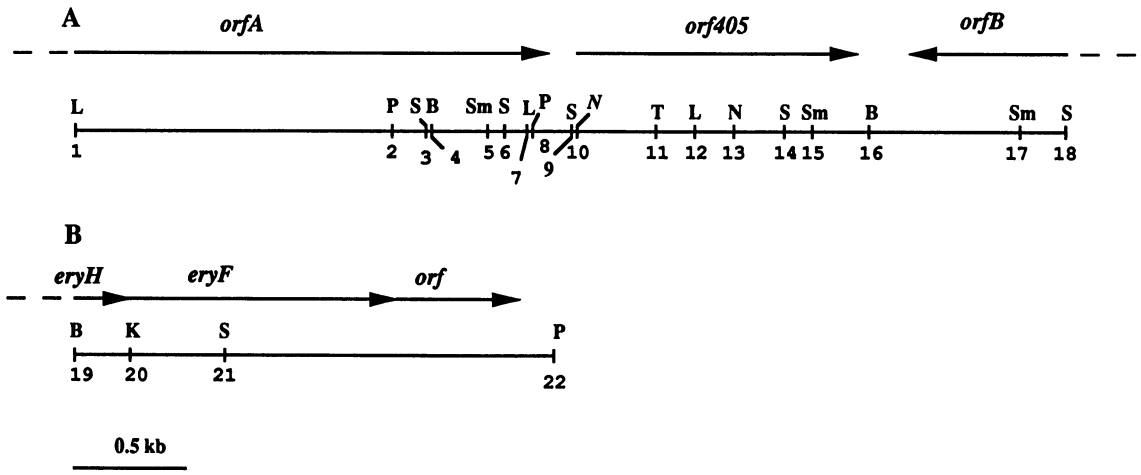


FIG. 3. Map of the *orf405* and *eryF* regions from *S. erythraea* showing selected restriction sites. (A) *orf405* region with arrows indicating the direction and extent of the three potential open reading frames. *N* indicates the *Nco*I site created by PCR mutagenesis that is not present in the wild-type sequence. (B) *eryF* region used for construction of pWHM808. The symbols *eryH* and *orf* indicate, respectively, a portion of *eryH*, and an open reading frame that potentially encodes a thioesterase (35). Restriction site abbreviations: B, *Bam*HI; K, *Kpn*I; L, *Sal*I; N, *Nco*I; P, *Pst*I; S, *Sst*I; Sm, *Sma*I; T, *Sst*II.

the axial iron ligand in other cytochrome P-450 enzymes (23). Among the actinomycete cytochrome P-450 sequences, that of EryF, a polypeptide of 404 amino acid residues with a calculated molecular weight of 45,082 and an isoelectric point of 4.82, shows the highest degree of similarity to Orf405 (47.5% identity by GAP analysis). According to the systematic nomenclature of Nebert et al. (16), *orf405* and *eryF* would be classified in the same cytochrome P-450 family but in different subfamilies. *eryF* and *orf405* would then be referred to as *CYP107A1* (35) and *CYP107B1*, respectively, and the two proteins as P450CVIIA1 and P450CVIIB1, respectively.

A potentially significant feature of the Orf405 sequence is the presence of a TTA leucine codon at position 9. This codon is very rare in *S. lividans* and *Streptomyces coelicolor* and is believed to be important in the regulation of late developmental events (15). Strains with mutations in the cognate tRNA for this codon are known to have deficiencies in sporulation and secondary metabolism (15), suggesting that the regulatory system operates by coupling the translation of particular genes to the presence of the rare $tRNA_{UUA}$. Whether a similar system operates in *S. erythraea* is not presently known.

Analyses of sequences upstream and downstream of *orf405* were performed to determine whether open reading frames encoding electron transport proteins essential to cytochrome P-450 function were present in this region. In *Streptomyces griseolus*, ferredoxin genes immediately downstream of *suaC* and *subC*, the genes that encode two cytochrome P-450 enzymes that are inducible by sulfonyl urea herbicides, have been discovered (18). No sequences showing similarity to ferredoxins or pyridine nucleotide-dependent ferredoxin reductases were found immediately surrounding *orf405*. Directly upstream of *orf405* lies the 3′ end of a potential open reading frame (*orfA*) that would encode a protein whose N terminus lies outside of the sequenced region (Fig. 4). A search of the GenBank data base with the TFASTA program (8) revealed that OrfA bears a strong similarity to the product of the *dnaE* genes of *E. coli* and *Salmonella typhimurium* (14, 31) (Fig. 7), which encode the large α subunit of the DNA polymerase III holoenzyme. Since the region of alignment occurs at the C terminus of both protein products, the sequenced portion of *orfA* is most likely homologous to the corresponding region of *dnaE* (Fig. 7). A single-stranded sequence extending approximately 300 bp in the direction of *orf405* from the terminal *Sal*I site (site 1 in Fig. 3) showed that additional similarity to *dnaE* occurs in the vicinity of this site (data not shown). This suggests that the sequenced portion of *orfA* shown in Fig. 5 forms the 3′ end of a large open reading frame with extensive similarity to *dnaE*. The *orfA* sequence has its terminus at TGA-797, leaving 125 nucleotides of noncoding DNA between it and the start of *orf405*. Notable structural features in this region include a perfect 11-bp direct repeat lying just upstream of the putative *orf405* start codon (Fig. 4).

Downstream of the Orf405 coding sequence lies another potential open reading frame, *orfB*, which extends past the right end of the map shown in Fig. 3. Analysis of codon usage indicated that *orfB* runs in the direction convergent to *orf405*, ending at TGA-2364. Searches of the GenBank data base (as of 14 March 1991) revealed no significant matches with the translated *orfB* protein sequence. A potentially significant feature of this region is a 16-bp perfect inverted repeat in the C-terminal region of *orfB* extending from nucleotides 2395 to 2426 (Fig. 4).

Heterologous expression of *orf405* and *eryF*. Since *orf405* and *eryF* were found to be distinct loci, it was of interest to know whether both Orf405 and EryF actually possessed 6-deoxyerythronolide B hydroxylase activity. The two genes were expressed in *E. coli* by using the pTrc99c vector (1), and the activity of cellular extracts from each of the expression strains was determined in 6-deoxyerythronolide B hydroxylase assays. pTrc99C contains $lacI^q$ plus the inducible *trc* promoter fused with an *E. coli* ribosome binding site and a polylinker containing an *Nco*I restriction site. Cloning into the *Nco*I site allows high-level expression from the *trc* promoter without translational fusion to vector coding sequences. The PCR with a site-specific modified oligonucleotide was used to create an *Nco*I site at the N terminus of *orf405* to facilitate cloning into pTrc99c. The plasmid pWHM803 (sites 8 to 12, Table 1) was amplified with the oligonucleotide shown in Table 2 as the forward primer and

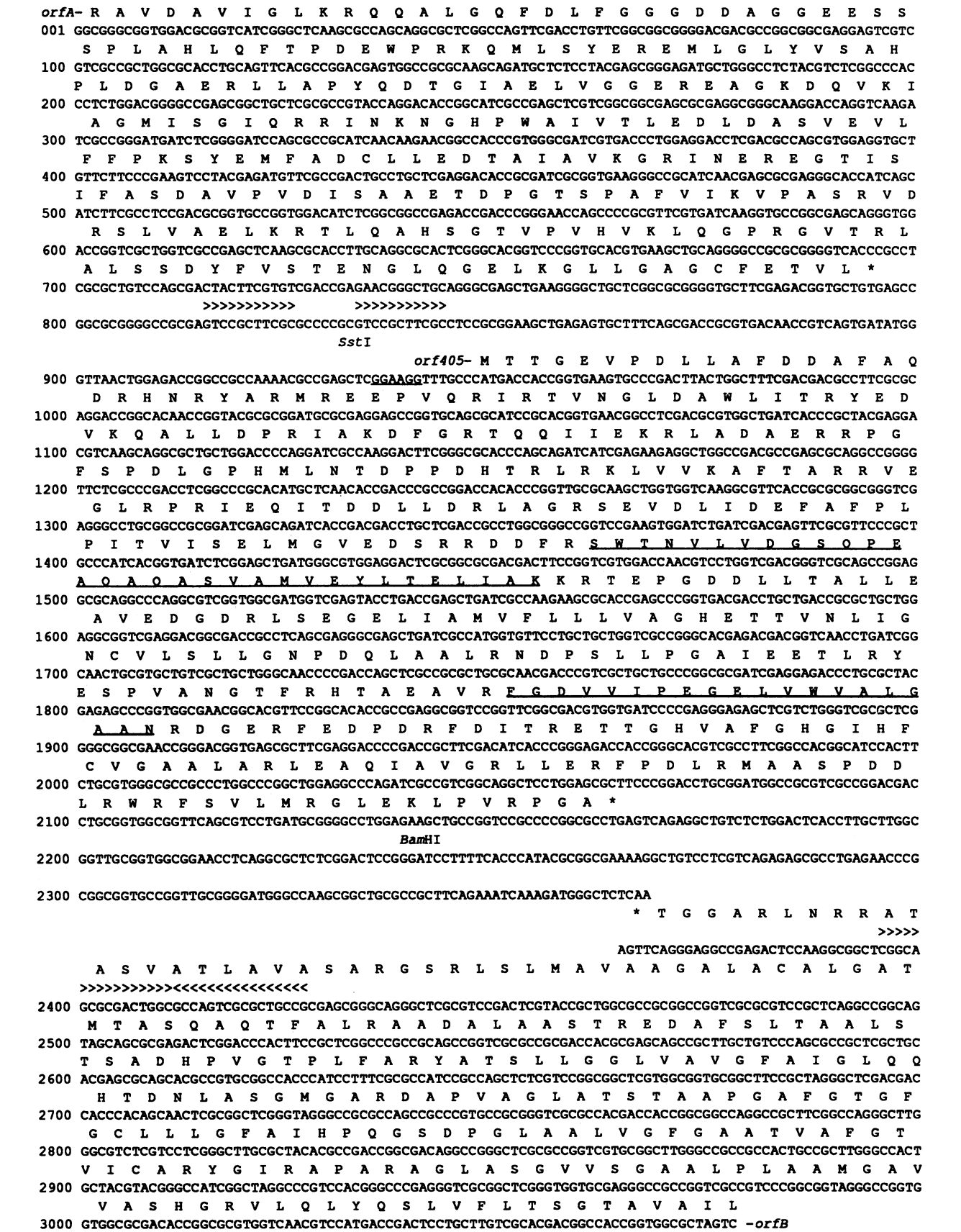


FIG. 4. Nucleotide sequence of the *orf405* region with the single-letter amino acid sequences of the three potential open reading frames. The occurrence and direction of repeat sequences are indicated by < or >. The most likely ribosome binding site for *orf405* and the peptide sequences obtained by Edman degradation are underlined.

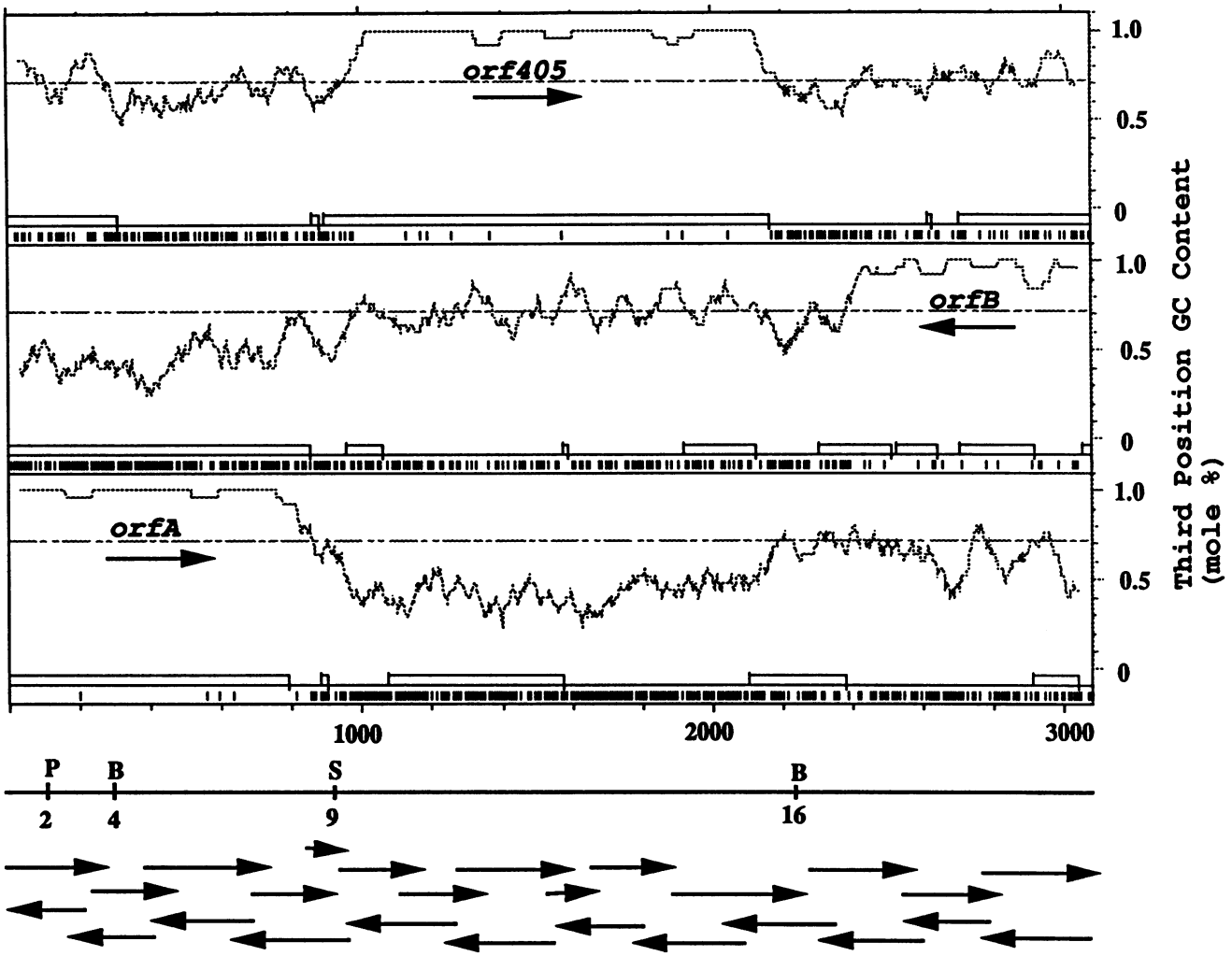


FIG. 5. CODONPREFERENCE (8) plot of the third-position G+C content of each reading frame in the *orf405* region (window size, 25). Potential open reading frames are labeled as in the text, with arrows indicating the direction of transcription. Hatch marks at the bottom of each plot indicate rare codons based on codon usage in *Streptomyces* spp. (3). The restriction sites shown below the plots are labeled and numbered as in Fig. 3A, and the sequencing strategy is shown as the series of arrows below the restriction map. The identity of *orfB* as an open reading frame was determined from a CODONPREFERENCE run in the reverse direction of that shown here (data not shown).

an oligonucleotide homologous to the T7 promoter in pGEM-3zf(−) as the reverse primer. The forward primer contained the sequence CCATGG in place of the wild-type sequence (CCATGA), thereby creating an *Nco*I site at the 5′ end of *orf405*. This mutation also resulted in the substitution of alanine for threonine at position 2 of the translated Orf405 protein. The 604-bp PCR product was ligated into the *Sma*I site of pGEM-3zf(−), and the resulting plasmid (pWHM804) was digested with *Nco*I and *Sst*II (sites 10 and 11; Fig. 3) to produce a 369-bp fragment. This fragment was ligated with an *Nco*I-*Sst*II digest of pTrc99c and the *Sst*II-*Bam*HI fragment (sites 11 to 16, Fig. 3) from pWHM805 (Table 1) to give the expression plasmid pWHM806 (Table 1).

To construct the *eryF* expression vector pWHM808, a 55-bp linker (Table 2) that contained the coding sequence for the N-terminal region of the EryF protein up to a *Kpn*I site lying downstream of the start codon was synthesized (site 20, Fig. 3). The wild-type *eryF* sequence contained in the linker was changed to more closely reflect the optimal codon usage for highly expressed proteins in *E. coli* (7). This linker was ligated with *Nco*I-*Kpn*I-digested pWHM807 (Table 1) to give pWHM808.

Orf405 and EryF were detected in *E. coli* DH5α transformants containing pWHM806 and pWHM808, respectively, by immunoblot analysis of crude cell extracts after the induction of *orf405* or *eryF* expression by isopropyl-β-D-thiogalactopyranoside. The band in extracts of *E. coli*(pWHM806) was identical in electrophoretic mobility to the major cytochrome P-450 isolated from *S. erythraea*, whereas the *eryF* gene product from *E. coli*(pWHM808) exhibited a slightly lower mobility that was identical to that of the minor immunoreactive band in cytochrome P-450 preparations from *S. erythraea* (Fig. 2C). Quantification of the cytochrome P-450 content in extracts of the expression strains by analysis of the optical difference spectra indicated that *E. coli*(pWHM806) and *E. coli*(pWHM808) produced approximately 5 and 0.5 mg, respectively, of native enzyme per liter of culture.

The enzymatic assay of cell extracts from the same transformants showed that the *eryF* gene product was active

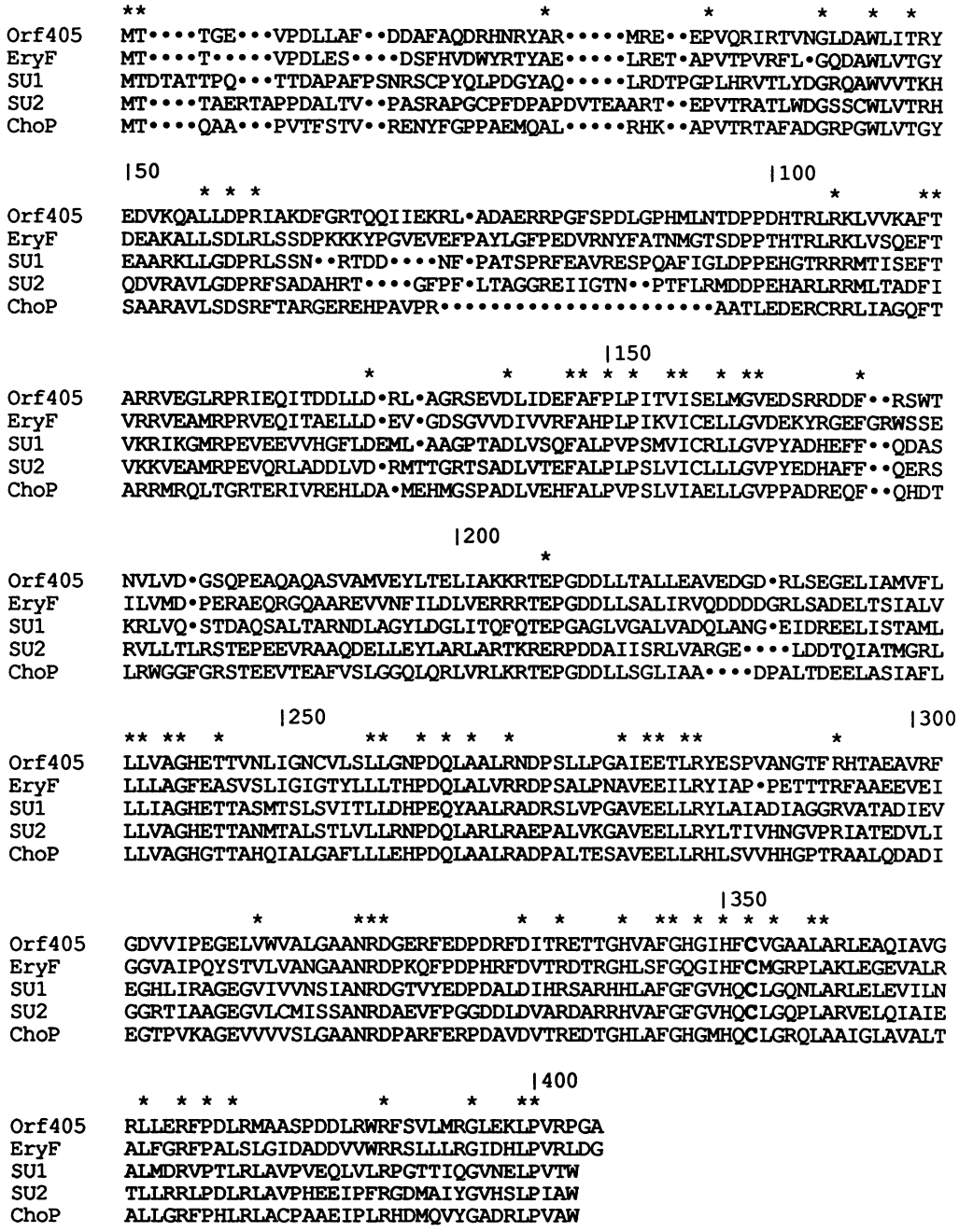


FIG. 6. Amino acid sequence comparisons of Orf405 with four other cytochrome P-450 sequences: EryF from *S. erythraea* (35), SU1 and SU2 from *S. griseolus* (21), and ChoP from *Streptomyces* sp. strain SA-COO (11). Alignments were made manually after pairwise comparisons with GAP analysis (8). The numbers above the sequences indicate the residue numbers of the Orf405 proteins. Identities across all five sequences are indicated by asterisks. The region surrounding Cys-352 of Orf405 forms the putative heme binding pocket (23) and is highly conserved among the five sequences.

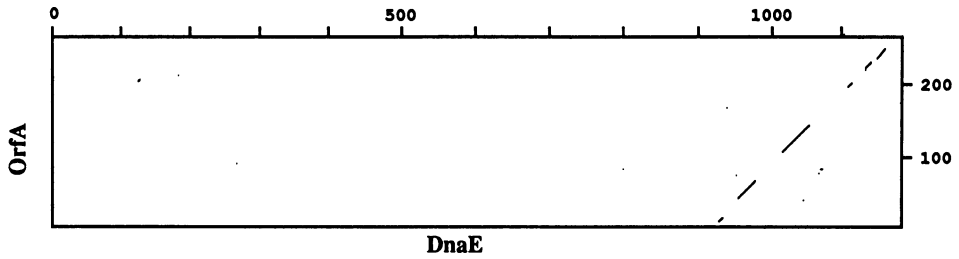


FIG. 7. Amino acid sequence comparison of OrfA from *S. erythraea* with the α-subunit of DNA polymerase I from *S. typhimurium* (14). Sequences were first analyzed with the COMPARE program (8) with a window size of 30 and a stringency of 17; then the results were plotted by using the DOTPLOT program (8). Numbering on the horizontal axis indicates the amino acid residue position in the *dnaE* gene product, and that on the vertical axis indicates the position in OrfA.

in vitro in the conversion of the radiolabeled 6-deoxyerythronolide B analog (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6-deoxyerythronolide B to its 6-hydroxy derivative. Incubation of this substrate with cell extracts from *E. coli*(pWHM806) did not result in the formation of a detectable product, even when the reaction time was extended to 1 h (Table 3). Extracts of *E. coli* DH5α containing only pTrc99c showed no detectable cytochrome P-450 and did not hydroxylate the radiolabeled substrate when supplemented with electron transport proteins and NADPH. Furthermore, the complete electron transport system was required for conversion by the expression strain (Table 3). Both cytochrome P-450 enzymes exhibited an ability to de-ethylate 7-ethoxycoumarin at a low rate as estimated by TLC. This activity was also dependent on the presence of ferredoxin and ferredoxin-NADP$^+$ oxidoreductase, indicating that the Orf405 enzyme produced by *E. coli*(pWHM806) was functional in a typical P-450-mediated reaction.

Quantification of the cellular levels of 6-deoxyerythronolide B hydroxylase in *S. erythraea*. The values obtained from catalytic studies of the EryF hydroxylase enzyme, obtained from *E. coli*, were used to quantify the levels of the *eryF* gene product in the CA340 strain (Table 4). Cell extracts of *S. erythraea* obtained after 24, 36, and 48 h of cell growth were assayed for hydroxylase activity with the 9-deoxy-9-hydroxy substrate. Comparisons with the total cytochrome P-450 levels obtained from optical difference spectra indicated that the 6-deoxyerythronolide B hydroxylase makes up to 3 to 4% of the total P-450 content at the three time points, with the remainder presumably being made up by the Orf405 protein. Low levels of 6-deoxyerythronolide B hydroxylation were seen without the addition of spinach reductase or ferredoxin (data not shown), which is likely to be due to the presence of endogenous electron transport proteins.

TABLE 3. Enzymatic hydroxylation of (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6-deoxyerythronolide B by cellular extracts of the expression strains *E. coli*(pWHM806) and *E. coli*(pWHM808)[a]

Assay system[b]	Sp act (nmol of product formed per nmol of P-450 per min)
E. coli(pWHM806) complete[c]	<0.001
E. coli(pWHM806) minus ferredoxin	<0.001
E. coli(pWHM808) complete	53.2
E. coli(pWHM808) minus ferredoxin and reductase	<0.001

[a] All assays with EryF-producing strains were run for 5 min at 30°C as described in Materials and Methods. Assays wih Orf405-producing strains were run for 1 h.
[b] Strain designations indicate ammonium sulfate precipitates of *E. coli* extracts of transformants prepared as described in Materials and Methods.
[c] The complete assay system described in Materials and Methods.

DISCUSSION

Previous protein purification work by Corcoran (5), Corcoran and Vygantas (6), and Shafiee and Hutchinson (27) has shown that an enzymatic activity can be isolated from *S. erythraea* that results in the hydroxylation of 6-deoxyerythronolide B or its 9-deoxy-9-hydroxy derivative at the 6 position. This activity was associated with a cytochrome P-450-containing fraction but was quite low. The hydroxylation enzyme was purified to apparent homogeneity, yet it still had a low specific activity (27). Nevertheless, from these results, it was concluded that this purified cytochrome P-450 monooxygenase was the 6-deoxyerythronolide B hydroxylase.

More recently, the *eryF* (*CYP107A1*) gene encoding the putative 6-deoxyerythronolide B hydroxylase function was cloned by Weber et al. (35) from the region of the *S. erythraea* chromosome approximately 5.5 kb downstream of *ermE*, the erythromycin resistance gene. Analysis of the erythromycin metabolites produced by recombinant clones containing an insertionally inactivated *eryF* gene resulted in a strain that produced 6-deoxyerythromycin A and not erythromycin A. Sequence analysis indicated that the *eryF* gene encoded a cytochrome P-450 monooxygenase (35).

In our study, the major cytochrome P-450 protein of *S. erythraea* NRRL 2338 was purified and its gene *orf405* (*CYP107B1*) was cloned and sequenced. Initial hybridization studies showed that the gene was not located in the erythromycin biosynthetic gene cluster, so it and *eryF* could not be identical. The results of DNA sequence analysis verified this but nevertheless showed that the two protein products to have a high degree of similarity (Fig. 6). In fact, they are nearly identical in size, charge, and isoelectric point. Expression of their genes in *E. coli* has allowed enzymatic comparisons between the two proteins to be made. The *eryF* gene product shows a much higher level of activity in the hydroxylation of an analog of 6-deoxyerythronolide B than previously reported (27), whereas the product of *orf405* shows no detectable activity against the same substrate. It is unlikely that the single difference in the N-terminal amino acid sequence between the heterologously expressed *orf405* gene product and the wild-type form would result in the abolition of activity, since the crystal structure of P-450$_{cam}$, a paradigm for bacterial enzymes of this type, does not suggest a significant role for the N terminus in binding or catalysis (23).

It appears, then, that the 6-deoxyerythronolide B hydrox-

TABLE 4. Quantification of 6-deoxyerythronolide B hydroxylase and total cytochrome P-450 in cell extracts of *S. erythraea* at three time points after inoculation

Time after inoculation[a] (h)	Cell mass[b] (g)	Hydroxylase content[c] (μg)	Total protein content[d] (mg)	Specific content[e] of hydroxylase (μg/mg)	P-450 content[f] (μg)	% of total P-450 as hydroxylase
24	2.0	5.1	28.2	0.181	146.1	3.5
36	4.0	5.0	52.6	0.095	144.0	3.5
48	5.8	7.1	46.0	0.154	235.6	3.0

[a] Culture (50 ml) inoculated with 1.0 ml of 72-h culture started from spores.
[b] Wet weight of cell pellet after centrifugation.
[c] Hydroxylase content based on a specific activity of 53.2 nmol of product formed per nmol of enzyme per min at a (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6-deoxyerythronolide B concentration of 55 μM.
[d] Measured by a modification (30) of the Bradford method.
[e] Calculated quantity of hydroxylase per milligram of protein.
[f] Measured by using optical difference spectra (22).

ylase preparation originally purified from *S. erythraea* was actually a mixture of two cytochrome P-450 enzymes and that the major species is present in very large excess but has essentially no 6-deoxyerythronolide B-hydroxylating activity. This interpretation is consistent with assay data from cell extracts of *S. erythraea* which indicate that the *eryF* gene product makes up only 3 to 4% of the total soluble cytochrome P-450 content (Table 4) at a time when erythronolide B formation can be detected. The previous reports of low specific activity with regard to the total cytochrome P-450 content thus can be explained by the presence of a 25-fold excess of enzymatically inactive Orf405 protein in the original EryF preparation. The observed differences in relative electrophoretic mobility between the two proteins indicate that the faint immunoreactive band that is only partially separable from the major polypeptide by reversed-phase HPLC (Fig. 2) is in fact the *eryF* gene product. Immunological reactivity to both proteins is very likely to be the result of the preparation of antiserum from a mixture of the two rather than of true cross-reactivity due to structural similarity. Purification studies with both heterologously expressed cytochrome P-450 enzymes have shown them to have nearly identical chromatographic behavior on several media, indicating that the two enzymes would likely copurify when present in *S. erythraea* cell extracts.

The function of Orf405, the major P-450 enzyme of *S. erythraea*, is unknown, although the knowledge that this organism can utilize octane and guaiacol as sole carbon sources suggests a possible function in a catabolic pathway (19). This protein is present over a broad period of culture growth, and its production does not appear to require induction by exogenous factors, as is the case with some of the other actinomycete enzymes of this type (19, 20, 32). No close physical linkage of *orf405* or *eryF* (35) with electron transport protein-encoding sequences is seen, suggesting that such linkage is a characteristic only of inducible cytochrome P-450 systems in bacteria. Mammalian cytochrome P-450 genes and their electron transport protein genes also lack close physical linkage (29). Proteins serving electron transport functions have been isolated from *S. erythraea* cell extracts (28), and efforts are underway to clone their genes (9). The significance of the linkage of the *S. erythraea dnaE* homolog with *orf405* is not known, although linkage (and cotranscription) of *dnaE* with apparently unrelated functions is seen in both *E. coli* and *S. typhimurium* (14, 31).

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REFERENCES

1. **Amann, E., B. Ochs, and K.-J. Abel.** 1988. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69**:301–315.
2. **Bibb, M. J., and S. N. Cohen.** 1982. Gene expression in *Streptomyces*: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. **187**:265–277.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein coding sequences. Gene **30**:157–166.
4. **Corcoran, J. W.** 1975. S-Adenosylmethionine:erythromycin C *O*-methyltransferase. Methods Enzymol. **43**:487–498.
5. **Corcoran, J. W.** 1981. Biochemical mechanisms in the biosynthesis of the erythromycins, p. 132–174. *In* J. W. Corcoran (ed.), Antibiotics IV: biosynthesis. Springer-Verlag, New York.
6. **Corcoran, J. W., and A. M. Vygantas.** 1982. Accumulation of 6-deoxyerythronolide B in a normal strain of *Streptomyces erythreus* and hydroxylation at carbon 6 of the erythranolide ring system by a soluble noninduced cell-free enzyme system. Biochemistry **21**:263–269.
7. **de Boer, H. A., and R. A. Kastelein.** 1986. Biased codon usage: an exploration of its role in optimization of translation, p. 225–285. *In* W. Reznikoff and L. Gold (ed.), Maximizing gene expression. Butterworth Publishers, Stoneham, Mass.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
9. **Donadio, S., and C. R. Hutchinson.** 1991. Cloning and characterization of the *Saccharopolyspora erythraea fdxA* gene encoding ferredoxin. Gene **100**:231–235.
10. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Institute, Norwich, United Kingdom.

11. **Horii, M., T. Ishizaki, S.-Y. Paik, T. Manome, and Y. Murooka.** 1990. An operon containing the genes for cholesterol oxidase and a cytochrome P-450-like protein from a *Streptomyces* sp. J. Bacteriol. **172**:3644–3653.
12. **Innis, M. A.** 1990. PCR with 7-deaza-2'-deoxyguanosine triphosphate, p. 54–59. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. A guide to methods and applications. Academic Press, San Diego, Calif.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lancy, E. D., M. R. Lifsics, P. Munson, and R. Maurer.** 1989. Nucleotide sequences of *dnaE*, the gene for the polymerase subunit of DNA polymerase III in *Salmonella typhimurium*, and a variant that facilitates growth in the absence of another subunit. J. Bacteriol. **171**:5581–5586.
15. **Lawlor, E. L., H. A. Baylis, and K. F. Chater.** 1987. Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor*. Genes Dev. **1**:1305–1310.
16. **Nebert, D. W., D. R. Nelson, M. Adesnid, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, R. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman.** 1989. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. DNA **8**:1–13.
17. **Ohtsuka, E., S. Matsuki, M. Ikehara, Y. Takahoshi, and K. Matsubara.** 1985. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. J. Biol. Chem. **260**:2605–2608.
18. **O'Keefe, D. P., K. J. Gibson, M. H. Emptage, R. Lenstra, J. A. Romesser, P. J. Litle, and C. A. Omer.** 1991. Ferredoxins from two sulfonylurea herbicide monooxygenase systems in *Streptomyces griseolus*. Biochemistry **30**:447–455.
19. **O'Keefe, D. P., and P. A. Harder.** 1991. Occurrence and biological function of cytochrome P450 monooxygenases in the actinomycetes. Mol. Microbiol. **5**:2099–2105.
20. **O'Keefe, D. P., J. A. Romesser, and K. J. Leto.** 1988. Identification of constitutive and herbicide inducible cytochromes P-450 in *Streptomyces griseolus*. Arch. Microbiol. **149**:406–412.
21. **Omer, C. A., R. Lenstra, P. J. Litle, C. Dean, J. M. Tepperman, K. J. Leto, J. A. Romesser, and D. P. O'Keefe.** 1990. Genes for two herbicide-inducible cytochromes P-450 from *Streptomyces griseolus*. J. Bacteriol. **172**:3335–3345.
22. **Omura, T., and R. Sato.** 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. **239**:2379–2385.
23. **Poulos, T. L., B. C. Finzel, and A. J. Howard.** 1987. High resolution crystal structure of cytochrome P-450$_{cam}$. J. Mol. Biol. **195**:687–700.
24. **Richardson, M. A., S. Kuhstoss, P. Solenberg, N. A. Schaus, and R. N. Rao.** 1987. A new shuttle cosmid vector, pKC505, for streptomycetes: its use in the cloning of three spiramycin-resistance genes from a *Streptomyces ambofaciens* library. Gene **61**:231–241.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sariaslani, F. S., and J. P. Rosazza.** 1983. Novel biotransformations of 7-ethoxycoumarin by *Streptomyces griseus*. Appl. Environ. Microbiol. **46**:468–474.
27. **Shafiee, A., and C. R. Hutchinson.** 1987. Macrolide antibiotic biosynthesis: isolation and properties of two forms of 6-deoxyerythronolide B hydroxylase from *Saccharopolyspora erythraea*. Biochemistry **26**:6204–6210.
28. **Shafiee, A., and C. R. Hutchinson.** 1988. Purification and reconstitution of the electron transport components for 6-deoxyerythronolide B hydroxylase, a cytochrome P-450 enzyme of macrolide antibiotic (erythromycin) biosynthesis. J. Bacteriol. **170**: 1548–1553.
29. **Simmons, D. L., P. A. Lalley, and C. B. Kasper.** 1985. Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. J. Biol. Chem. **260**:515–521.
30. **Spector, T.** 1978. Refinement of the Coomassie blue method of protein quantitation. Anal. Biochem. **86**:142–146.
31. **Tomasiewicz, H. G., and C. S. McHenry.** 1987. Sequence analysis of the *Escherichia coli dnaE* gene. J. Bacteriol. **169**: 5735–5744.
32. **Trower, M. K., F. S. Sariaslani, and D. P. O'Keefe.** 1989. Purification and characterization of a soybean flour-induced cytochrome P-450 from *Streptomyces griseus*. J. Bacteriol. **171**:1781–1787.
33. **Vara, J., M. Lewandowska-Skarbek, Y.-G. Wang, S. Donadio, and C. R. Hutchinson.** 1989. Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). J. Bacteriol. **171**:5872–5881.
34. **Weber, J. M., J. O. Leung, G. T. Maine, R. H. B. Potenz, T. J. Paulus, and J. P. DeWitt.** 1990. Organization of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. J. Bacteriol. **172**:2372–2383.
35. **Weber, J. M., J. O. Leung, S. J. Swanson, K. B. Idler, and J. B. McAlpine.** 1991. An erythromycin derivative produced by targeted gene disruption in *Saccharopolyspora erythraea*. Science **252**:114–116.
36. **Weber, J. M., C. K. Wierman, and C. R. Hutchinson.** 1985. Genetic analysis of erythromycin production in *Streptomyces erythreus*. J. Bacteriol. **164**:425–433.