

Biocatalytic Conversion of Avermectin to 4''-Oxo-Avermectin: Heterologous Expression of the *ema1* Cytochrome P450 Monooxygenase

István Molnár,^{1*} D. Steven Hill,^{1†} Ross Zirkle,^{1‡} Philip E. Hammer,^{1§} Frank Gross,^{1¶}
Thomas G. Buckel,^{2||} Volker Jungmann,^{2*} Johannes Paul Pachlatko,^{2††}
and James M. Ligon^{1†}

*Syngenta Biotechnology, Inc., 3054 Cornwallis Rd., Research Triangle Park, NC 27709,¹ and
Syngenta Crop Protection AG, Schwarzwaldallee 215, CH-4002 Basel, Switzerland²*

Received 3 February 2005/Accepted 15 July 2005

The cytochrome P450 monooxygenase Ema1 from *Streptomyces tubercidicus* R-922 and its homologs from closely related *Streptomyces* strains are able to catalyze the regioselective oxidation of avermectin into 4''-oxo-avermectin, a key intermediate in the manufacture of the agriculturally important insecticide emamectin benzoate (V. Jungmann, I. Molnár, P. E. Hammer, D. S. Hill, R. Zirkle, T. G. Buckel, D. Buckel, J. M. Ligon, and J. P. Pachlatko, *Appl. Environ. Microbiol.* 71:6968–6976, 2005). The gene for Ema1 has been expressed in *Streptomyces lividans*, *Streptomyces avermitilis*, and solvent-tolerant *Pseudomonas putida* strains using different promoters and vectors to provide biocatalytically competent cells. Replacing the extremely rare TTA codon with the more frequent CTG codon to encode Leu4 in Ema1 increased the biocatalytic activities of *S. lividans* strains producing this enzyme. Ferredoxins and ferredoxin reductases were also cloned from *Streptomyces coelicolor* and biocatalytic *Streptomyces* strains and tested in *ema1* coexpression systems to optimize the electron transport towards Ema1.

Emamectin benzoate (compound MK-244) is an extremely potent insecticide that controls many agriculturally important pests (7). The widespread commercial exploitation of emamectin benzoate has, however, been hindered by the high cost of its chemical synthesis from its natural product precursor, avermectin. The most costly step of this process is the oxidation of the 4''-carbinol group of avermectin to the corresponding ketone, a demanding step due to the presence of two other hydroxyl groups on the substrate molecule that must be chemically protected before and deprotected after oxidation.

We are developing a cost- and time-effective method for the regioselective oxidation of the 4''-carbinol group of avermectin that involves the utilization of resting microbial cells as a biocatalyst. The accompanying article (12) describes the identification of *Streptomyces* strains and the isolation and characterization of cytochrome P450 monooxygenase (CYP) enzymes

from these strains that are able to carry out the regioselective oxidation of avermectin at the 4'' position, as well as the cloning and sequencing of the corresponding genes (*ema1* to *ema17*). As biocatalysts, CYPs are proficient in the oxidative catabolism of a wide range of xenobiotics; they can also be used to carry out highly enantio- and regiospecific biosynthetic reactions (23, 35). CYPs from or expressed in *Streptomyces* have been shown to catalyze the regiospecific oxidation of secondary metabolites (34) and steroids (5) and the activation and catabolism of xenobiotics (22, 31). Investigations of *Pseudomonas*-derived CYPs and pseudomonads as whole-cell biocatalysts have also been described for the bioremediation of xenobiotics (8) and for the biotransformation of disparate chemicals including natural products (27).

Class II CYPs that are associated with the endoplasmic reticulum in eukaryotes use NADH-cytochrome P450 reductase as an electron donor. Soluble class I CYPs of bacteria receive electrons via the small iron-sulfur protein ferredoxin (FD) and the flavin adenine dinucleotide (FAD)-containing ferredoxin reductase (FRE). Specific CYPs have higher activity when they interact with specific FDs (21, 23). Similar to the case with FDs and CYPs, specific FREs are also known to be better electron donors for certain FDs than for others (16).

The electrons channeled to CYPs by their redox partners are ultimately derived from NAD(P)H; thus, the biotransformation reactions catalyzed by these enzymes require cofactor regeneration. Although cofactor regeneration can be achieved in vitro enzymatically (28, 32), directly (3), or bypassed altogether (10), metabolically active cells could provide an easier and more cost-effective solution. Thus, the present article describes engineered *Streptomyces lividans* and *Pseudomonas putida* strains that express the CYP enzyme Ema1 of *Strepto-*

* Corresponding author. Present address for István Molnár: Natural Products Center, Office of Arid Land Studies, University of Arizona, 250 E. Valencia Rd., Tucson, AZ 85706. Phone: (520) 741-1691. Fax: (520) 741-0113. E-mail: imolnar@cals.arizona.edu. Mailing address for Volker Jungmann: Syngenta Crop Protection AG, Schwarzwaldallee 215, CH-4002 Basel, Switzerland. Phone: 41 61 323 5254. Fax: 41 61 323 4044. E-mail: volker.jungmann@syngenta.com.

† Present address: BASF Agricultural Products, 26 Davis Dr., Research Triangle Park, NC 27709.

‡ Present address: Martek Biosciences, 4909 Nautilus Ct. North, Boulder, CO 80301.

§ Present address: Athenix Corp., 2202 Ellis Rd., Suite B, Durham, NC 27702.

¶ Present address: Gene Bridges GmbH, Tatzberg 47-51, 01307 Dresden, Germany.

|| Present address: F. Hoffmann-La Roche, Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland.

†† Present address: Bölchenstrasse 21, CH-4411 Seltisberg, Switzerland.

TABLE 1. Bacterial strains used in this study^a

Species	Strain	Note	Source
<i>S. lividans</i>	ZX7	Expression host	JIC
<i>S. coelicolor</i>	M145	FRE donor	JIC
<i>S. avermitilis</i>	MOS-0001	Expression host, avermectin producer	SYN
<i>S. tubercidicus</i>	R-922	Ema1 producer	SYN
<i>S. tubercidicus</i>	I-1529	Ema2 producer	SYN
<i>P. putida</i>	17453	Solvent-tolerant expression host	ATCC
<i>P. putida</i>	700801	Solvent-tolerant expression host	ATCC
<i>E. coli</i>	DH10B	Routine cloning host	INV

^a Sources: JIC, John Innes Institute (Norwich, United Kingdom); SYN, Syngenta in-house strain collection (Basel, Switzerland); ATCC, American Type Culture Collection (Manassas, VA); INV, Invitrogen Corporation (Carlsbad, CA).

myces tubercidicus R-922 for the oxidation of the 4''-carbinol of avermectins. We also cloned FD and FRE proteins that support an increased level of biocatalytic activity upon coexpression with Ema1 in these strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and cloning and expression vectors used in this study are listed in Tables 1 and 2, respectively. The *Streptomyces* integrative expression vectors pTBK and pEA are based on the *Escherichia coli* cloning vector pNEB193 (New England Biolabs, Beverly, MA) and the ϕ C31-derived integrase (14) and were developed in-house (37; I. Molnár, unpublished data) from published vector components (Fig. 1). The broad-host-range, transmissible plasmid pRK290 (9) was used for *Pseudomonas* with the alkane-inducible Palk promoter-*alkS*-positive regulator expression cassette (24) or the Ptac promoter derived from pUK21 (33) which is constitutive in *pseudomonads* (Fig. 1).

Culture conditions and molecular genetic procedures. *E. coli* and *Streptomyces* cultivation, transformation, recombinant DNA procedures, and DNA sequence analysis followed standard protocols (13, 26) as detailed in the accompanying article (12). *Streptomyces avermitilis* protoplasts were transformed as previously described (30). *P. putida* strains were cultured in TB broth (26) and transformed by interspecies conjugation from *E. coli* (9).

Agar-based biocatalytic assay. Transformed *Streptomyces* strains were grown on ISP-2 agar plates supplemented with 50 μ g/ml thiostrepton for selection (pEA derivatives) or 50 μ g/ml kanamycin for selection and 5 μ g/ml thiostrepton for induction of the PtipA promoter (pTBK derivatives). The ISP-2 agar media were also supplemented with 1 g/liter (final concentration) of technical-grade abamectin (stock solution, 20 g/liter abamectin in dimethyl sulfoxide:Tween 40 = 1:1. Abamectin is the natural mixture of 85% avermectin B1a and 15% avermectin B1b as produced by *Streptomyces avermitilis*). The cultures were incubated at 28°C for 7 days, two agar cylinders of 5-mm diameter each were cut out and extracted with 1 ml acetonitrile, and the extracts were analyzed as described in the accompanying article (12).

Resting-cell assays. Transformed *S. lividans* and *S. avermitilis* strains were cultivated at 28°C with shaking at 200 rpm for 2 days in 50 ml peptone-yeast-

glucose (PYG) medium, supplemented with 5 μ g/ml thiostrepton (pEA-derived plasmids) or 10- μ g/ml kanamycin (pTBK derivatives); 5 ml of this preculture was transferred to a 500-ml Erlenmeyer flask containing 100 ml PYG medium supplemented with the same antibiotics. Thiostrepton at 5 μ g/ml was used to induce the PtipA promoter in the cultures that had been transformed with pTBK-derived plasmids. The main cultures were incubated at 28°C with shaking at 200 rpm for 2 days. Mycelia were harvested by centrifugation and washed once with 50 mM potassium phosphate buffer, pH 7.0. A total of 500 mg of the mycelium (wet cell weight) was transferred into a 50-ml Erlenmeyer flask and resuspended in 10 ml of 50 mM potassium phosphate buffer, pH 7.0. After the addition of 15 μ l of a solution of avermectin B1a in isopropanol (30 mg/ml), the reaction proceeded at 28°C for 16 h with shaking at 160 rpm. Bioconversion rates measured in *Streptomyces* resting-cell assays followed the same tendencies but were substantially higher than those obtained in the agar-based biocatalytic assays.

Transformed *P. putida* strains were grown overnight in 3 ml of TB medium supplemented with 20 μ g/ml tetracycline at 28°C with shaking at 200 rpm. A total of 0.1 ml of these cultures was used to inoculate 100 ml TB supplemented with 20 μ g/ml tetracycline and incubated overnight at 28°C with shaking at 200 rpm. Heptane at 0.1% (vol/vol) was used in the final cultures (but not in the starter cultures) to induce the Palk promoter for strains carrying pRK-Palk expression vectors. Cells were harvested by centrifugation and washed in 50 mM potassium phosphate buffer, pH 7.0. A total of 500 mg cells (wet weight) were transferred into a 50-ml Erlenmeyer flask and resuspended in 10 ml of 50 mM potassium phosphate buffer, pH 7.0. After the addition of 200 μ l of 20% glucose and 15 μ l of a solution of avermectin B1a in isopropanol (30 mg/ml), the mixture was incubated at 28°C for 16 h with shaking at 160 rpm.

To produce solvent-conditioned cells, transformed *P. putida* strains were grown overnight in 3 ml of TB medium supplemented with 20 μ g/ml tetracycline at 28°C with shaking at 200 rpm. One milliliter of these cultures was used to inoculate 25 ml TB supplemented with 20 μ g/ml tetracycline and incubated for 3 h at 28°C with shaking at 200 rpm. The cultures were supplemented at this point with 0.5% (vol/vol) dioctyl phthalate and the cultivation was continued for 2 days at 28°C with shaking at 200 rpm. To condition the cells to higher solvent concentrations, 1 ml of this culture was used to inoculate 25 ml TB, incubated for 3 h at 28°C with shaking at 200 rpm, supplemented with 2.5%, 5.0%, or 10% dioctyl phthalate; the incubation was then continued for 2 days as described above. One milliliter of the culture with 5.0% solvent was used to inoculate 25 ml TB supplemented with 25%, 37.5%, or 50% dioctyl phthalate and incubated for 3 h as before; the incubation was continued for 2 days. Tetracycline selection (20 μ g/ml) was maintained throughout the fermentation train. Heptane at 0.1% (vol/vol) was used to induce the Palk promoter in the cultures that were used directly for resting-cell assays (but not in the starter or intermediate cultures) in strains carrying pRK-Palk expression vectors. Cells from solvent-conditioned and -induced cultures were collected by centrifugation and washed in 50 mM potassium phosphate buffer, pH 7.0, and 500 mg (wet weight) of cells was used for the biotransformation reaction as described above.

Resting-cell assay mixtures were extracted with 30 ml methyl-*t*-butyl-ether (MTBE). The ether phase was collected and evaporated in vacuo, and the residue was redissolved in 1.2 ml acetonitrile. Formation of 4''-oxo-avermectin was followed by high-performance liquid chromatography as described in the accompanying article (12).

Cloning *ema1* and *ema2* into expression vectors. The *ema1* coding sequence was amplified by PCR using *Pfu* DNA Polymerase (Stratagene, La Jolla, CA)

TABLE 2. Vectors used in this study^a

Vector	Use	Promoter	Selectable marker(s)	Source or reference
pNEB193	<i>E. coli</i> cloning vector	NA	<i>bla</i>	NEB
pTBK	<i>Streptomyces</i> expression vector	PtipA	<i>tsr</i> , <i>aphII</i> , <i>bla</i>	This work and reference 37
pEA	<i>Streptomyces</i> expression vector	PerME*	<i>tsr</i> , <i>bla</i>	This work
pRK290	Broad-host-range-transmissible cloning vector	NA	<i>tet</i>	9
pUK21	<i>E. coli</i> cloning vector	Ptac	<i>aphII</i>	33
pSPZ2Not	<i>Pseudomonas</i> expression vector	Palk	<i>bla</i>	24
pRK-Ptac	<i>Pseudomonas</i> expression vector	Ptac	<i>tet</i>	This work
pRK-Palk	<i>Pseudomonas</i> expression vector	Palk	<i>tet</i>	This work

^a Promoters: PtipA, thiostrepton-inducible promoter for *Streptomyces* (19); PerME*, constitutive promoter for *Streptomyces* (6); Palk, alkane-inducible promoter for *Pseudomonas* (24); Ptac, constitutive promoter in *Pseudomonas*, derived from pUK21 (33); NA, not applicable. Selectable markers: *bla*, β -lactamase gene for ampicillin resistance; *aphII*, aminoglycoside phosphotransferase gene for kanamycin resistance; *tsr*, 23S rRNA methyltransferase gene for thiostrepton resistance; *tet*, tetracycline efflux-type resistance gene. NEB, New England Biolabs (Beverly, MA).

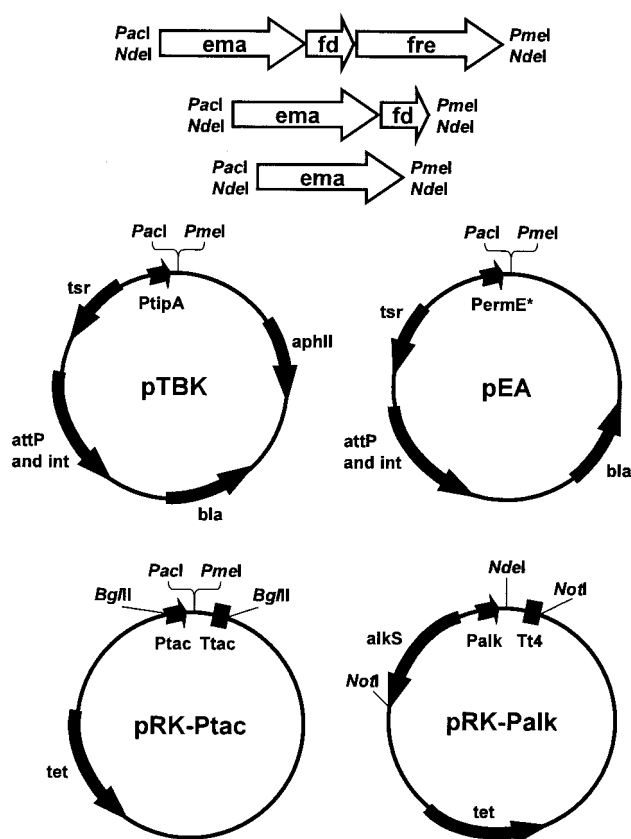


FIG. 1. Schematic representation of expression vectors and artificial operon cassettes used in this study. Details of the cloning procedures are provided in Materials and Methods. attP and int, ϕ C31-derived integrase (14); see Table 2 for explanations of the other gene name abbreviations. The *ema*, *ema-fd*, and *ema-fd-fre* artificial operon cassettes were cloned either as *PacI*-*PmeI* or *NdeI* fragments.

with the following primers: 5'-AGATTAATTAATGTCGGAATTAATGAACTGTCGGTT-3' (where the underlined sequence is a *PacI* recognition sequence and the sequence in boldface type is the start of the coding sequence of *ema1*), and 5'-AAACTCACCCACCCGACCGCAGCGAGTTC-3' (where the underlined sequence is half of a *PmeI* recognition sequence; the boldface type sequence is the reverse complement of the *ema1* translation stop codon followed by the 3' end of the *ema1* gene). The amplified *ema1* gene was cloned into the *Streptomyces* expression vectors as a *PacI*-*PmeI* fragment (Fig. 1).

The *ema2* coding sequence was cloned into the *Streptomyces* expression vectors using a similar strategy, using the primers 5'-AGATTAATTAATGTCGGCATTATCCAGCTCTCC-3' and 5'-AAACTCACCCAGCCGCAACGGCAGGG AAT-3' (see the paragraph above for an explanation of the typefaces used).

For expression in *Pseudomonas* hosts using the *Ptac* promoter, the *ema* genes were cloned as *PacI*-*PmeI* fragments into the plasmid pUK21 (33) between the *Ptac* promoter and *Ttac* terminator. The *Ptac-ema-Ttac* expression cassettes were excised from pUK21 as *BglII* fragments and cloned into the broad-host-range transmissible plasmid, pRK290 (9). For expression in *Pseudomonas* hosts using the *Palk* promoter, the *ema* genes were PCR amplified as *NdeI* fragments and cloned into the *NdeI* site of plasmid pSPZ2Not (24) between the *Palk* promoter and the *T4* transcriptional terminator *Tt4*. The *alkS-Palk-ema-Tt4* expression cassettes were then excised from the resulting plasmids by *NotI* and cloned into the *NotI* site of a pRK290 derivative where the unique *BglII* site was replaced by a *BamHI*-*NotI*-*BamHI* adapter.

Cloning of ferredoxins. The FD-encoding genes *fd233* and *fdEA* (from *S. tubercidicus* I-1529; GenBank accession no. AY549200 and AY549197, respectively), and *fd230* (from *S. tubercidicus* R-922; GenBank accession no. AY549204), were found to be clustered with the CYP-encoding genes *cyp233*, *cypEA*, and *cyp230* cloned from the corresponding strains (12). The FD-encoding gene *fd232* (GenBank accession no. AY552101) was identified using the *fd233*

gene as a hybridization probe against a cosmid library of strain *S. tubercidicus* R-922 prepared in SuperCos I (Stratagene, La Jolla, CA). A strongly hybridizing cosmid, pPEH232, was identified, and the hybridizing DNA was subcloned and sequenced. To test the biological activity of each of these ferredoxins in combination with the *Ema* CYPs, each individual FD-encoding gene was amplified by PCR to produce a gene fragment that included a blunt 5' end, the native ribosome-binding site, the FD coding sequence, and a *PmeI* restriction site on the 3' end. Each such FD gene fragment was cloned into the *PmeI* site located 3' to the *ema* genes that had already been inserted into the *Streptomyces* expression plasmids. In this way, artificial operons consisting of one of the *ema* genes and one of the FD-encoding genes functionally linked to the same promoter were created (Fig. 1). The artificial operons consisting of one of the *ema* genes and one of the FD-encoding genes were also subcloned into the *Pseudomonas* expression vectors as described above for the *ema* genes.

Cloning of ferredoxin reductases. Bacterial FRE protein sequences (GenBank accession no. 9929802, 3059213, 3059191, 9885215, 7619822, 3411185, 3251295, 1813616, and 146002) were retrieved from GenBank and aligned with the program CLUSTALW. Two conserved regions, approximately 266 amino acid residues apart, were used to design degenerate oligonucleotides for PCR. The FRE forward primer 5'-CGSCCSCSCTSWSSAAS (where S is C or G and W is A or G) and the FRE reverse primer 5'-SASSGCSTTBSBCCARTGYTC (where S is C or G; B is C, G, or T; R is A or G; and Y is C or T) were used to amplify 800-bp products from both *S. tubercidicus* R-922 and I-1529. These amplicon pools were cloned into TOPO TA cloning vectors (Invitrogen, Inc., Carlsbad, CA), and 20 clones (each) from strains R-922 and I-1529 were sequenced. Sequencing revealed that four unique *fre* gene fragments were isolated: three from R-922 (*fre3*, *fre12*, and *fre14*; GenBank accession numbers AY549205, AY549206, and AY549207), and one from I-1529 (*fre16*; GenBank accession number AY549208). The *fre3*, *fre12*, *fre14*, and *fre16* gene fragments were used as probes to identify full-length FRE genes from cosmid libraries of strains R-922 and I-1529, both prepared in SuperCos I (Stratagene, La Jolla, CA).

The gene *freEA* (AY549197), encoding a FRE-homologous protein, was found to be clustered with the CYP-encoding gene *cypEA*, cloned from *S. tubercidicus* I-1529 (12). The FRE genes for SCO 7117, SCO 0681, and the oxidoreductase/FRE-homologous genes for SCO 0158 and SCO 4595 were identified in the *Streptomyces coelicolor* genome database (<http://streptomyces.org.uk/S.coelicolor/index.html>). All FRE-encoding genes were amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), using the appropriate cosmids as templates for *freEA*, *fre3*, *fre12*, *fre14*, and *fre16* or *S. coelicolor* M145 genomic DNA as the template for the SCO-encoding genes. Each amplicon included a blunt 5' end, the native ribosome-binding site, the FRE coding sequence, and a *PmeI* restriction site on the 3' end. Each FRE gene fragment was cloned into the *PmeI* site located 3' to the *ema-fd233* operons that had already been inserted into the *Streptomyces* expression plasmids (Fig. 1). In this way, artificial operons consisting of one of the *ema* genes, the ferredoxin *fd233*, and one of the FRE genes functionally linked to the same promoter were created. Some of these artificial operons were also subcloned into the *Pseudomonas* expression vectors as described above for the *ema* genes.

RESULTS

Expression of *ema1* and *ema2* in *S. lividans* and *P. putida*.

The accompanying article (12) describes CYP enzymes that are able to catalyze the bioconversion of avermectin B1a and B1b to 4"-oxo-avermectin B1a and B1b, respectively. The genes encoding these enzymes were isolated, sequenced, and expressed in *Escherichia coli* to produce active enzymes for further biochemical characterization (12). To facilitate the genetic manipulation of the biocatalytic enzymes and their electron supply partners, we set out to develop heterologous expression systems for the *Ema* proteins. We chose the CYPs *Ema1* and *Ema2* as our initial targets based on their favorable enzymatic and expression characteristics in their native hosts (12). For primary expression hosts, we chose *S. lividans* based on its ease of manipulation and widespread use as a *Streptomyces* host for gene expression and later *P. putida* based on its extensive use in industrial biocatalysis and relative ease of manipulation. Both of these host strains were tested and found

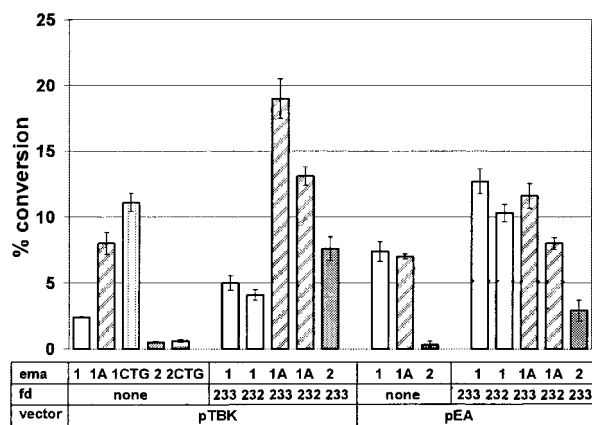


FIG. 2. Agar-based biocatalytic conversion assays with *S. lividans* ZX7 strains. Avermectin to 4'-oxo-avermectin end-point bioconversion assays were performed in triplicate with strains growing on avermectin-containing agar plates as described in Materials and Methods. *S. lividans* ZX7 carried different expression vector-*ema* gene-*fd* (ferredoxin) gene combinations as shown below the bars. Gene versions for *ema*: 1, *ema1*; 1A, *ema1A*; 1CTG, *ema1CTG*; 2, *ema2*; 2CTG, *ema2CTG*. Fd-encoding genes: 232, *fd232*; 233, *fd233*; none, no Fd-encoding gene was included in the construct.

to be unable to carry out the biocatalytic reaction in the absence of the cloned *ema* genes.

The coding sequence of *ema1* was cloned into *Streptomyces* expression vectors harboring the inducible PtipA (19) or the constitutive PermE* (6) promoters (Table 2; Fig. 1) as described in Materials and Methods. These constructs were transformed into *S. lividans* ZX7, where they integrated into the host chromosome via the ϕ C31-derived integrase (14). Formation of 4'-oxo-avermectins was detected with cultures grown on avermectin-containing agar medium as described in Materials and Methods. The biocatalytic conversion was found to be more effective with the expression vector pEA that harbored the constitutive PermE* promoter (Fig. 2).

In a similar experiment, the coding sequence of *ema2* was also subcloned into the same expression vectors and tested in the bioconversion reaction in *S. lividans* ZX7 as a host. Unexpectedly, these strains displayed barely detectable biocatalytic activities (Fig. 2).

Next, the *ema1* gene was cloned into the broad-host-range, transmissible plasmid pRK290 (9) and introduced by conjugal transfer from *E. coli* into *P. putida* ATCC 700801 and ATCC 17453. The alkane-inducible Palk promoter (24) and the Ptac promoter (33), which is constitutive in pseudomonads, were used to drive the expression of *ema1*. *P. putida* ATCC 700801 and ATCC 17453 containing plasmids pRK-Ptac-*ema1* and pRK-Palk-*ema1* were tested for their ability to catalyze the oxidation of avermectin in resting-cell assays as described in Materials and Methods. Biocatalytic conversion of avermectin to 4'-oxo-avermectin was detected with each recombinant strain (Fig. 3), reaching 15% with hexane-induced *P. putida* ATCC 17453 (pRK-Palk-*ema1*). In a similar manner, a pRK-Ptac-*ema2* construct was created and expressed in *P. putida* ATCC 700801. The biocatalytic conversion rate of *P. putida* ATCC 700801 expressing Ema2 was similar to that of the strain carrying Ema1 (results not shown).

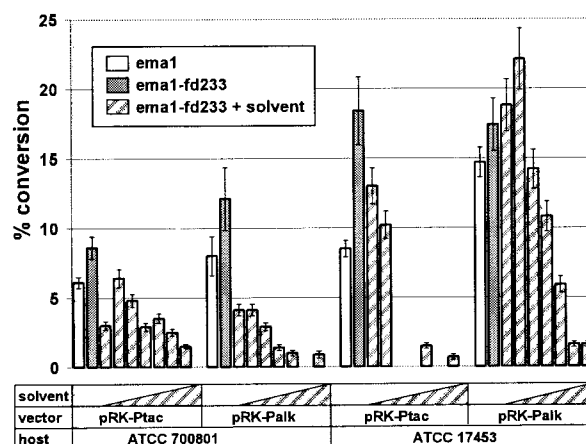


FIG. 3. Biocatalytic conversion with resting cells of *P. putida* strains. Avermectin to 4'-oxo-avermectin conversion assays with resting cells of *P. putida* strains ATCC 700801 or ATCC 17453, carrying different expression vector-*ema* gene-ferredoxin gene combinations were performed in triplicate as described in Materials and Methods. White bars, *ema1*-expressing strains grown with 0% solvent; gray bars, *ema1-fd233*-expressing strains grown in the presence of 0% solvent; bars with diagonal stripes, *ema1-fd233*-expressing strains grown in the presence of increasing concentrations of solvent (0.5%, 2.5%, 5.0%, 10%, 25%, 37.5%, and 50% dioctyl phthalate, respectively). Missing bars in the solvent concentration series indicate that experiments were not done.

Expression of altered *ema1* and *ema2* genes in *S. lividans*.

Both *ema1* and *ema2* contain the leucine codon TTA, encoding Leu4 in the corresponding CYPs. The TTA(Leu) codon is extremely rare in *Streptomyces* and has been implicated as a posttranscriptional regulatory device (17, 18). During the cloning of *ema1* into expression vectors, we identified a clone where this CYP gene was serendipitously truncated with a new start at Met5 (Ema1, MSELMNS. . .; Ema1A, MNS. . .) (the new start is indicated by boldface type), thereby omitting the TTA-encoded Leu4. We subcloned *ema1A* into the expression vectors pTBK and pEA, introduced these constructs into *S. lividans* ZX7, and tested the resulting strains for biocatalytic activity upon growth on avermectin-containing agar medium. A significant increase in the biocatalytic activity of the strain carrying *ema1A* was seen in the pTBK, but not in the pEA, background compared to similar strains carrying *ema1* (Fig. 2).

To further investigate the effect of the presence of the TTA(Leu) codon in Ema1, we changed this codon to CTG(Leu) by site-directed mutagenesis and measured the biocatalytic activity of *S. lividans* ZX7 (pTBK-*ema1CTG*). The strain carrying this mutated gene converted 4.6 times more avermectin to 4'-oxo-avermectin than the strain with pTBK-*ema1* and 1.4 times more than the strain with pTBK-*ema1A* (Fig. 2).

Since Leu4 is also encoded by a TTA codon in the *ema2* gene, we were interested to see whether a similar change would improve the biocatalytic capacity of *S. lividans* strains expressing this gene. The TTA(Leu4) codon was changed to CTG(Leu4) via site-directed mutagenesis, and *S. lividans* ZX7 strains carrying this *ema2CTG* gene were assayed on agar medium. No improvement in the bioconversion was seen compared to *S. lividans* ZX7 carrying *ema2* (Fig. 2).

A

		10 (13) 16		54
Fd233	(1)	MRITITDIDICISAGCCALTAPGVFTODDDGFSALLPGREDGAG-DPL		REAAARACFVCFISVTDD-----
Fd232	(1)	MRITITDIDICISAGCCALTAPGVFTODDDGFSALLPGREDGAG-DPL		REAAARACFVCFISVTDD-----
Fd_ave	(17)	MRITITDRDRICISGCCAMTAPGVFTODDDALVALVPGHEDGAG-DPR		HDRVPMACFVCFVAIFED-----
Fd_coe	(1)	MHIGIDKDTICISAGCCALTAPGVFTODDDGYSTLRPGREDGGG-SAL		REAAARACFVCFITVSEVRG-----
Fd_liv	(1)	MHIGIDKDTICISAGCCALTAPGVFTODDDGYSTLRPGREDGGG-SAL		REAAARACFVCFITVSEVRG-----
Fd_GrhO4	(1)	MHLSVTSDRICISAGCCAVTVPSVFEQDEDGLSTVPGREDGGG-EPM		REAVRACFVCFIVLKETEGV-----
Fd_NysM	(1)	MRIIVDPGRVCGAGCCVLTAPDLFDODDDGLVTVLAGAADAAD-PGD		RDAAALCFSGEISVAAD-----
Fd_SoyB	(1)	MGVQVVKERCVCAGMCALTAPDVFQDDDLSEVLPGREATSGTHPI		GEAVRACFVCFVVLSSD-----
Fd230	(1)	MRIIVDEDKVCAGCCVLSAAEVFDQDEDGIVALLDATPADGLRAF		EEAAARCFALIEVLP-----
FdEA	(1)	MRIAVLNLRCQSYACAYLAHKDFRLTQGEALTYEFPNDERR--LQ		ERAAAACFVCFIVIDRLDGAKGSATS

B

		FAD ADP binding GxGxxGxxxAXxxxxxG		NAD (ADP) binding GxGxxGxxxAXxxxxxGxxxxxxE		FAD ribytil binding TxxxxxxAXGD
Fre12	(53)	AVIGASLAGLYAARSLRSQFDG	(194)	VVIGGGFIGAEVASSCAALGHDTVVEAAP	(314)	VTPLPAVVAAGDVA
Fre14	(8)	IIAGAGLAGAKAAETLRAEGFGG	(150)	VVIGGGWIGLETTAAARAAGVEVTVLESAP	(268)	CSSHPDIYAAGDVA
Fre16	(9)	VIVGGGLAGAKAAETLRAEGFTG	(155)	VIACAGWIGLEVAAAARSYGAETVVEAAP	(278)	RTSDPYIYAAGDVA
Fre3	(9)	VIVGGGLAGAKAAETLRAEGFTG	(155)	VIACAGWIGLEVAAAARSYGAETVVEAAP	(278)	RTSDPYIYAAGDVA
FreEA	(21)	VIVGASLAGLRAAEALRQEGFTG	(162)	VVICAGFIGSEVTSVCRELDLPVTVVERGS	(288)	GVVTDSIYVAGDVA
SCO 7117	(8)	VIVGGGLAAGKAAEELREHGHDG	(150)	VVCGGWIGLETTAAARAAGAEVTVLARGE	(268)	RTSAGVHAAGDVA
SCO 4595	(167)	PLTTLTVQALGFEFLSRKEAERS	(325)	AALCAAFGGALAVTTTSGPG-VALKSETIG	(456)	QFAQGNHTLDDGS
SCO 0681	(8)	AIVGSGPAGIYAADALLKSEVAA	(150)	AVLCVGNVALDIARVLAKTADLELPTIIPP	(295)	LDGTGNVKGTGEFK
SCO 0158	(6)	VVLGAGYAGAYVAGTLARLSRQ	(155)	LIVCVLTIETATEIAESRPLSVTLIAR	(270)	SVSHPNVYAAGDSA
SCO 2469	(9)	VIVGGGLAGAKAAETLRTEGFTG	(156)	VIACAGWIGLEVAAAAREYGAETVVEPAP	(279)	RTSDPDIYAAGDVA
SCO 2106	(2)	VVVGAGMAGVQTAVALREGFTG	(138)	VVVCAGWIGAEFATAAREAGCAVTVVEAAD	(259)	RTSAPDVHAAGDCA

FIG. 4. Sequence alignments of ferredoxins and ferredoxin reductases. (A) Multiple sequence alignment of the ferredoxins described in this study with their nearest homologs from the databanks. Residues conserved in all 10 proteins are shown in white letters on a black background. Residues conserved in at least 7 of 10 proteins are highlighted in light gray. Numbers above the sequences denote the position of the cysteine residues that coordinate the irons of the iron-sulfur clusters in FDs. Fd_ave, ferredoxin from *S. avermitilis* (GenBank accession number 5824147); Fd_coe, ferredoxin from *S. coelicolor* (GenBank accession number 21219296); Fd_liv, ferredoxin from *S. lividans* (GenBank accession number 3293541); Fd_GrhO4, ferredoxin GrhO4 from the griseorhodin biosynthetic gene cluster of *Streptomyces* sp. JP95 (GenBank accession number 21039506); Fd_NysM, ferredoxin NysM from the nystatin biosynthetic gene cluster of *S. noursei* (GenBank accession number 8050844); Fd_SoyB, soy flour-inducible ferredoxin SoyB from *S. griseus* (GenBank accession number 119970). (B) Multiple sequence alignment of conserved regions in ferredoxin reductases. Consensus sequences for the FAD ADP-binding motifs, the NAD ADP-binding motifs, and the FAD ribytil-binding motifs (2) are shown above the alignment. Fre12, Fre14, Fre16, Fre3, and FreEA are the ferredoxin reductases described in this study. Three FREs (SCO 7117, SCO 0681, and SCO 2469) and three probable oxidoreductases that are homologous to FRE enzymes (SCO 0158, SCO 2106, and SCO 4595) are from the completely sequenced genome of *S. coelicolor* A3 (2,4).

Identification of ferredoxins that are active with Ema1.

None of the 17 *ema* genes described in the accompanying article (12) was found to be clustered with FD- or FRE-encoding genes. During our initial attempts to clone the biocatalytic CYP, however, we isolated and sequenced several additional CYP-encoding genes from strains R-922 and I-1529. Although these CYPs did not have a role in the biocatalytic reaction, some of them were clustered with genes encoding FDs. Thus, we have identified FD-encoding genes *fd230* from strain R-922 and *fd233* and *fdEA* from strain I-1529. To test the biological activity of each of these FDs in combination with Ema1, artificial operons consisting of the *ema1A* gene and one of the *fd* genes were created and functionally linked to the PtipA promoter in the *Streptomyces* expression vector pTBK (see Materials and Methods). Fd233, but not the other two FDs, provided for a significantly increased biocatalytic activity in *S. lividans* ZX7, compared to that of *ema1A* alone in the same plasmid and host background (Fig. 2 and results not shown).

Since *fd233* is derived from strain I-1529 and *ema1* is from strain R-922, the proteins encoded by the two genes cannot interact with each other in nature. Thus, we cloned an *fd233*-homologous gene from strain R-922, as described in Materials

and Methods. Comparison of the deduced amino acid sequences of Fd233 (from strain I-1529) and its R-922 homolog, Fd232, revealed that they differ in only a single amino acid (S in Fd232 versus T in Fd233 at position 60). Fd233 and Fd232 were tested in pTBK- and pEA-based coexpression systems with Ema1A and separately with full-length Ema1 in *S. lividans* ZX7. Both FDs increased the biocatalytic conversion rate in both expression vectors carrying *ema1A* or *ema1*, with Fd232 being slightly less effective than Fd233 (Fig. 2).

In a similar manner, an artificial operon was created from *ema2* and *fd233*, cloned into pTBK and pEA, and assayed in *S. lividans* ZX7 as the host for the biocatalytic reaction. Dramatic increases in the biocatalytic ability of these strains were recorded compared to the strains carrying *ema2* in the same vector background (Fig. 2).

The *ema1-fd233* artificial operon cassettes were also subcloned into the *Pseudomonas* vectors pRK-Ptac and pRK-Palk and introduced into *P. putida* strains ATCC 700801 and ATCC 17453. The resulting strains showed elevated levels of biocatalytic activity in resting-cell assays, compared to strains harboring the *ema1* gene alone in the corresponding plasmids (Fig. 3).

A BLAST analysis of the amino acid sequence of Fd233 revealed that the closest match in the databanks was to a

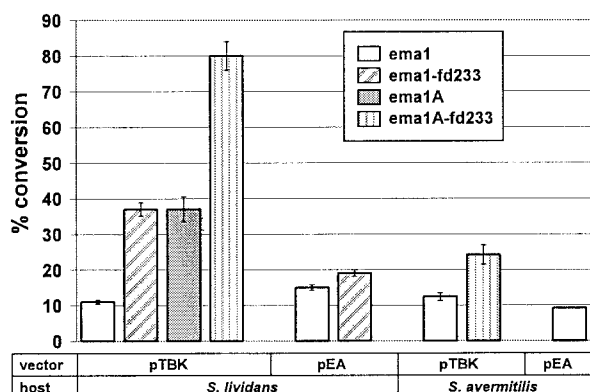


FIG. 5. Biocatalytic conversion with resting cells of *Streptomyces* strains. *S. lividans* ZX7 and *S. avermitilis* MOS-0001 strains carrying different expression vector-*ema* gene-*fd* gene combinations were cultivated in triplicate in liquid media, and the cells were harvested and used in resting-cell bioconversion assays as described in Materials and Methods.

ferredoxin from *S. coelicolor* (GenBank accession number 21219296; 79% identity at the amino acid level). All FDs described here belong to the low-potential monocluster 3Fe-4S ferredoxin group (29) where the iron-sulfur cluster is covalently attached to the protein at Cys10, Cys16, and Cys54 (Fd233 numbering). The Cys that would coordinate the fourth iron in 4Fe-4S clusters was replaced by Ala13 in Fd233 (Fig. 4A).

Bioconversion with *Streptomyces* resting cells. Selected *S. lividans* ZX7 transformants were cultivated in liquid PYG medium, and the mycelia were used in resting-cell bioconversion assays as described in Materials and Methods. Resting cells of *S. lividans* ZX7 transformants were more effective biocatalysts than agar surface cultures but converted avermectin to 4''-oxo-avermectin with the biotransformation rates following the same trend as was seen in the agar-based bioconversion assays (*ema1A-fd233* > *ema1A* ~ *ema1-fd233* > *ema1*). The highest level of conversion was attained with *S. lividans* ZX7 (pTBK-*ema1A-fd233*), with the product yield exceeding 80% after 16 h of incubation (Fig. 5).

In addition, some of the constructs were introduced into *Streptomyces avermitilis* MOS-0001 by protoplast-mediated transformation (30), and the resulting strains were evaluated for the bioconversion reaction by the resting-cell assay as described in Materials and Methods. *S. avermitilis* MOS-0001 carrying the cloned *ema1* gene was able to transform avermectin to 4''-oxo-avermectin, with 24% product yield in a 16-h incubation in the case of pTBK-*ema1A-fd233* (Fig. 5). *S. avermitilis* MOS-0001 is a producer of avermectin, so we also evaluated the transformed strains in avermectin fermentations for the direct production of 4''-oxo-avermectin. No formation of 4''-oxo-avermectin was detected (results not shown). Sampling the producer cultures on each day of the fermentation followed by resting-cell bioconversion assays revealed that the biocatalytic activity of the MOS-0001 transformants declined rapidly during the fermentation, with no biocatalytic activity measurable after day 5, while avermectin production starts around day 8 (results not shown).

Resting-cell assays with *P. putida* transformants grown in the presence of solvents. Since avermectins are barely soluble in water, we were interested in delivering higher concentrations of substrate to the biocatalyst by employing a biphasic reaction system. The *P. putida* strains used in this study are described in the literature as resistant to different solvents (36). We tested the growth of *P. putida* strains ATCC 17453 and ATCC 700801 in different organic solvents that were also able to solubilize an excess of 50 g/liter avermectin in water-saturated biphasic systems. Dioctyl phthalate was found to be well tolerated by both *Pseudomonas* strains used and served as an excellent solvent for avermectins (solubility, 300 g/liter). We grew *P. putida* ATCC 17453 and ATCC 700801, carrying one of the plasmids pRK-Ptac-*ema1-fd233* or pRK-Palk-*ema1-fd233*, in the presence of various concentrations of dioctyl phthalate and used these solvent-conditioned cells in resting-cell assays as described in Materials and Methods. As shown in Fig. 3, the biocatalytic performance of the biocatalyst rapidly declined with increasing concentrations of solvent, possibly as a result of a decreased expression of the *ema1-fd233* expression cassette (results not shown).

Identification and cloning of genes encoding ferredoxin reductases. The electron transport chain that supports class I CYPs includes an FRE that channels electrons from NAD(P)H to FD. To further optimize the biocatalytic activity of our engineered strains, a number of FRE genes from *Streptomyces* strains were cloned and evaluated for their impacts on the biocatalytic potential of the recombinant strains. To do this, degenerate oligonucleotides were designed for two conserved regions in bacterial FRE sequences and were used for PCR against *S. tubercidicus* R-922 and I-1529 genomic DNA. Unique PCR products were used as probes to identify full-length FREs from R-922 and I-1529 genomic clone banks, as described in Materials and Methods. Three unique FRE genes from R-922 (*fre3*, *fre12*, and *fre14*), and one from I-1529 (*fre16*) were cloned and sequenced in this manner.

Three FRE genes (genes for SCO 7117, SCO 0681, and SCO 2469) have been identified in the completely sequenced genome of *S. coelicolor* A3 (2, 15). Two additional probable oxidoreductases were also annotated in the *S. coelicolor* genome project as close homologs of FRE enzymes (SCO 0158 and SCO 2106), while another oxidoreductase, SCO 4595, has a Pfam match to flavodoxin/ferredoxin oxidoreductases (4). We amplified the genes encoding SCO 7117, SCO 0681, SCO 0158, and SCO 4595 from the genomic DNA of *S. coelicolor* M145 (13), as described in Materials and Methods. Finally, an additional FRE gene, *freEA*, was found adjacent to the *fdEA* gene on the chromosome of *S. tubercidicus* I-1529.

To assess the potential of the cloned FREs in increasing the biocatalytic activity of Ema1, each gene was amplified by PCR and used to create artificial operons consisting of *ema1*, *fd233*, and the individual FRE-homologous genes. Each of these artificial operon cassettes was subcloned into the *Streptomyces* expression vectors pTBK and pEA and transformed into *S. lividans* ZX7, and the resulting strains were evaluated by the agar-based bioconversion assay. Significant increases in 4''-oxo-avermectin formation were seen with some of the FRE-homologous genes tested (Fig. 6). The improvements provided by the different FRE-homologous genes, however, varied with the vector background: the oxidoreductase/FRE-homologous SCO 4595, for example,

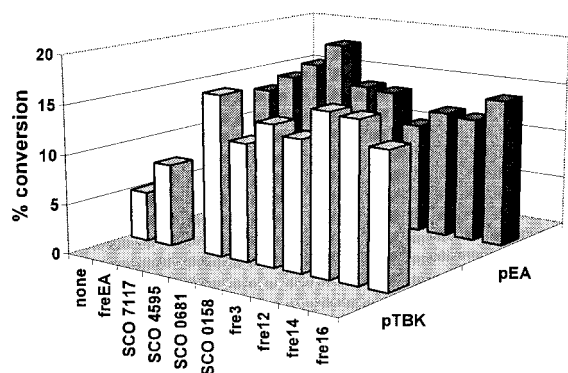


FIG. 6. Agar-based biocatalytic conversion assays with *S. lividans* ZX7 strains carrying different *ema1-fd233-fre* operons. Avermectin to 4'-oxo-avermectin end-point bioconversion assays were performed in triplicate with strains growing on avermectin-containing agar plates as described in Materials and Methods. *S. lividans* ZX7 carried different *ema1-fd233-fre* (ferredoxin reductase) artificial operons in pTBK (white bars) or pEA (gray bars). None, the expression cassette contained *ema1* and *fd233* but no *fre* genes.

yielded a threefold-higher rate of conversion in the pTBK background compared with the 1.5-fold enhancement in the pEA background.

The *S. coelicolor* ferredoxin reductases SCO 7117, SCO 4595, and SCO 0158 and the I-1529-derived FreEA were also used to create artificial operons with *ema1A-fd233* in the pTBK and pEA vectors. The FRE genes, however, did not provide significant increases in the bioconversion yields in the agar-based bioassays (results not shown).

In a similar approach, the R-922 and I-1529-derived *fre* genes were inserted into the *Pseudomonas* expression plasmid pRK-Ptac-*ema1-fd233*. These constructs were introduced into *P. putida* strains ATCC 17453 and ATCC 700801, and the transformants were analyzed for their biocatalytic activity by the resting-cell assay. The effect of the FRE genes were found to be host strain dependent (results not shown): while the FREs did not increase the biocatalytic activity of ATCC 700801-derived strains, Fre12 and Fre16 provided for a nearly twofold-higher 4'-oxo-avermectin yield in *P. putida* ATCC 17453.

The FRE from *S. tubercidicus* R-922 and I-1529 all contained the three conserved regions implicated in FAD and NAD(P)H binding. Thus, the putative ADP-binding site of NAD(P)H (GXGX₂GX₃AX₆GX₆E), and the ADP-binding site (GXGX₂GX₃AX₆G) and ribityl-binding site (TX₆AXGD) of FAD (2) are well conserved (Fig. 4B). Fre3 (from strain R-922) and Fre16 (from I-1529) are 95.5% identical at the amino acid level, while FreEA shows <35% identity with the other four FRE proteins identified in this study. Fre3, Fre14, and Fre16 were most similar to FRE from *S. coelicolor* (SCO 7117 and SCO 2469), with identities in the 50 to 65% range. Fre12 and FreEA showed the highest identities (57% and 59%, respectively) to FRE from *S. avermitilis* (GenBank accession number 29833498) and *Myxococcus xanthus* (GenBank accession number 4633137), respectively.

DISCUSSION

In this work, we expressed CYPs Ema1 and Ema2, derived from *S. tubercidicus* R-922 and I-1529, respectively, in *Pseudo-*

monas and *Streptomyces* host strains and cloned ferredoxins and ferredoxin reductases from different *Streptomyces* strains that were able to increase the biocatalytic activity of these enzymes when coexpressed in the same host strains.

The gene for Ema1 was expressed from different promoters to provide easily measurable biocatalytic activities in the *S. lividans* host in the agar-based test system and in the *P. putida* hosts in resting-cell assays. The successful expression of *ema1* in *Streptomyces* and *Pseudomonas* strains to produce biocatalytically active cells indicates that Ema1 is able to partner with FD and FRE electron transport proteins from these strains. CYP enzymes do not require exclusive FD partners but are able to work with a range of FDs, and this relative promiscuity seems to apply to the FD-FRE partners too (15). Thus, 18 CYPs have been identified in the *S. coelicolor* genome project, but these enzymes apparently rely on six FDs and three to five FREs for electron transport (4). *S. avermitilis* has been reported to command 33 CYPs, served by six FDs and nine FRE enzymes (11). The daunorubicin-producer *S. peuceitius* ATCC 27952 harbors 19 CYPs supported by only two FDs and four FRE (25). The completely sequenced genome of *P. putida* KT2440 contains two CYP-encoding genes, with five deduced proteins each annotated as FD and FRE (20). Some heterologous CYP enzymes expressed in surrogate *Streptomyces* hosts were able to function well (34), while others were nonfunctional unless a suitable FD was coexpressed (21). In this study, we have cloned four different 3Fe-4S FD genes from *S. tubercidicus* strains R-922 and I-1529 and tested these by creating artificial operons with *ema1* for expression in *S. lividans*. Coexpression of Fd233 with Ema1 led to an approximately twofold increase in the bioconversion rate in agar-based biocatalytic assays (Fig. 2), while FdEA and Fd230 did not support improvements in this system. In spite of the near identity of Fd232 (derived from R-922) and Fd233 (derived from I-1529), Fd232 was found to be a less effective electron donor partner for Ema1 (itself derived from R-922) in the *S. lividans* surrogate host. Improved biocatalytic performances with *ema1-fd233* or *ema1A-fd233* operons (compared to *ema1* or *ema1A* alone) were also recorded in resting-cell assays in the *S. lividans* host (Fig. 5). The increase in the biocatalytic activity with *ema1-fd233* artificial operons, compared to *ema1* alone, was also manifested in other hosts like *P. putida* ATCC 17453, but this was less evident with *P. putida* ATCC 700801 (Fig. 3). Further testing of FDs from strain R-922, possibly those with 2Fe-2S or 4Fe-4S clusters, in coexpression systems with Ema1 can lead to the identification of the cognate FD partner for this CYP and might further improve the efficiency of the recombinant biocatalyst.

In contrast to *ema1*, *S. lividans* ZX7 strains carrying *ema2* displayed barely detectable biocatalytic activities. Indeed, our initial attempts to clone the biocatalytic enzyme from strain I-1529 that had relied on expression libraries constructed in *S. lividans* ZX7 have proven futile (12). On the other hand, *S. tubercidicus* I-1529, the native producer of Ema2, shows vigorous biocatalytic activity, and purified Ema2 enzyme from strain I-1529 or recombinant Ema2 from *E. coli* has been shown to be competent in biocatalysis, with K_m and V_{max} values similar to those of Ema1 (12). When expressed in *P. putida* host strains, Ema2 provided for a similar biocatalytic performance, as did Ema1 (results not shown). The biocatalytic

activity of Ema2 in *S. lividans* could not be improved by site-directed mutagenesis that replaced the rare TTA codon by CTG encoding Leu4. Creating artificial operons of *ema2* with *fd233*, however, dramatically improved the biocatalytic performance in *S. lividans* ZX7 strains (Fig. 2), although the biocatalytic conversion still did not reach the same level as with the *ema1-fd233* cassette in the same strain-vector combinations. We speculate that the CYP Ema2 is not receptive to the FD and FRE partners found in *S. lividans*, but the electron flow and concomitant activity of the enzyme are restored with a cloned FD mediator that is acceptable to both the cloned Ema2 and the native FRE from this host.

Both *ema1* and *ema2* contain a TTA codon near their 5' end, encoding Leu4 in the corresponding CYP proteins. This codon is extremely rare in *Streptomyces*, featured in only 260 out of 7,825 genes in the completely sequenced *S. coelicolor* genome (4). TTA codons are translated by the *bldA*-encoded Leu tRNA that becomes available only in the late phases of growth, thereby providing for a growth phase-dependent regulation of protein expression at the posttranscriptional level (17, 18). Releasing Ema1 from *bldA* regulation by either truncating the *ema1* gene or mutating the TTA codon to CTG supported significant increases in the biocatalytic activities of *S. lividans* strains carrying these genes functionally linked to the inducible PtipA promoter but not with strains where the *ema* genes were under the control of the constitutive PerME* promoter (Fig. 2). In these experiments, the PtipA promoter was induced with thiostrepton from the moment of inoculation onto the avermectin-containing agar medium. In detailed investigations of the PtipA promoter, maximum levels of expression were achieved when *S. lividans* cultures were induced at the early stages of growth, with the expression level peaking at the exponential phase and declining rapidly in later stages (1). Inducibility also seemed to be restricted to the early growth phase, with little induction (and overall expression) achieved when thiostrepton was added to the cultures in the stationary phase (1). We hypothesize that the initial absence of the *bldA*-encoded tRNA at the early stages of growth equally restricted the translation of the *ema1*-derived mRNA with both PtipA and PerME* as promoters. By the time the *bldA*-encoded tRNA became available in the stationary phase, the activity of the PtipA promoter had already declined, while the PerME* promoter still actively transcribed the *ema1* gene. Since the translation of the *ema1A* or the *ema1CTG* mRNA does not require the *bldA*-encoded tRNA, the strains expressing these genes can utilize the high early stage activity levels of the PtipA promoter, leading to a significant increase in the biocatalytic performance of these strains compared with those strains that express *ema1*.

To further optimize the biocatalyst, we cloned three FREs from *S. tubercidicus* R-922, two from *S. tubercidicus* I-1529, and four FRE homologs from *S. coelicolor* and used these to create artificial operons with *ema1-fd233* or *ema1A-fd233*. These operons were cloned into expression vectors and tested with *S. lividans* and *P. putida*. Fre12 and Fre16 provided for a nearly twofold-higher 4"-oxo-avermectin yield in *P. putida* ATCC 17453, but none of the FRE tested increased the biocatalytic activity of the *P. putida* ATCC 700801-derived strains (results not shown). In *S. lividans*, significant increases in the biocatalytic performance were seen when FRE-encoding genes were

coexpressed with Ema1 and Fd233 with the PtipA promoter (Fig. 6), while the improvements were less pronounced with the PerME* promoter and absent when Ema1A was used instead of Ema1, irrespective of the promoter choice (results not shown). We propose that FRE availability might have been the limiting factor in *S. lividans* only in those cases where the translation of the CYP-encoding mRNA was reduced in the early stages of the culture by the absence of the *bldA*-encoded tRNA. This limitation was accentuated by the expected reduction in the expression of CYP from the PtipA promoter in the later stages of growth. Indeed, while the expression of the *S. coelicolor* CYP complement could be detected even in early stage cultures, FRE expression was found to be restricted to older cultures, representing a novel mechanism for the regulation of CYP activity in this close relative of the *S. lividans* host used in our experiments (16). It is possible, however, that none of the R-922-derived FREs cloned in this study represented the cognate FRE for the Ema1 CYP, and identification of the physiological FRE partner, in conjunction with the cognate FD, would yield more substantial improvements in the performance of our biocatalysts.

We have planned to use solvent-water biphasic systems for the biocatalytic reaction to facilitate the conversion of larger amounts of the water-immiscible substrate; to this end, we cultivated solvent-tolerant *P. putida* strains ATCC 17453 and ATCC 700801, carrying different Ema expression constructs, in the presence of dioctyl phthalate. In spite of acceptable growth rates, bioconversion activities of cells grown in the presence of higher concentrations of solvent declined rapidly (Fig. 3), apparently due to a rapid decrease of the expression of the Ema1 protein (results not shown).

We also attempted to create a bacterial strain that would produce 4"-oxo-avermectins de novo. To this end, the *ema1* gene or the *ema1A-fd233* operon was engineered into *S. avermitilis* MOS-0001, a producer of avermectins. Although young mycelia from the transformed cultures were able to carry out the biocatalytic conversion (Fig. 5), stationary-phase cultures were not biocatalytically active, and no direct production of 4"-oxo-avermectin was observed (results not shown). Optimization of the expression pattern of *ema1*, using promoters that are active in the stationary phase, might help to harmonize avermectin production and biocatalytic enzyme expression.

In conclusion, we engineered *S. lividans*, *S. avermitilis*, and *P. putida* strains to express the CYP Ema1 and improved the biocatalytic performance of these strains by using different vectors and promoters, replacing an expression-limiting codon and coexpressing newly identified ferredoxins and ferredoxin reductases. Further development of these biocatalysts by fermentation optimization could lead to the replacement of the costly chemical process of avermectin 4" oxidation with a more environmentally friendly and cheaper biological route for the crop protection industry.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Amber Gaudreau, Sandy Emhart, and Keri Cavanaugh; the analytical support provided by Belle Abrera, James Pomes, Alex Frank, and Mario Jörg; and DNA sequencing by Ann Hu and Makoto Ono.

This work was supported by Syngenta Biotechnology, Inc. (Research Triangle Park, NC), and Syngenta Crop Protection AG (Basel, Switzerland).

REFERENCES

- Ali, N., P. R. Herron, M. C. Evans, and P. J. Dyson. 2002. Osmotic regulation of the *Streptomyces lividans* thioesteron-inducible promoter, ptpA. *Microbiology* **148**:381–390.
- Asturias, J. A., E. Diaz, and K. N. Timmis. 1995. The evolutionary relationship of biphenyl dioxygenase from gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from gram-negative bacteria. *Gene* **156**: 11–18.
- Baik, S. H., C. Kang, C. Jeon II, and S. E. Yun. 1999. Direct electrochemical regeneration of NADH from NAD⁺ using cholesterol-modified gold amalgam electrode. *Biotechnol. Tech.* **13**:1–5.
- Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabinowitz, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**:141–147.
- Berrie, J. R., R. A. Williams, and K. E. Smith. 1999. Microbial transformations of steroids. XI. Progesterone transformation by *Streptomyces roseochromogenes*—purification and characterisation of the 16 α -hydroxylase system. *J. Steroid Biochem. Mol. Biol.* **71**:153–165.
- Bibb, M. J., G. R. Janssen, and J. M. Ward. 1985. Cloning and analysis of the promoter region of the erythromycin-resistance gene (*ermE*) of *Streptomyces erythraeus*. *Gene* **38**:E357–E368.
- Cvetovich, R. J., D. H. Kelly, L. M. DiMichele, R. F. Shuman, and E. J. J. Grabowski. 1994. Syntheses of 4'-epi-amino-4"-deoxyavermectins B1. *J. Org. Chem.* **59**:7704–7708.
- Dejonghe, W., N. Boon, D. Seghers, E. M. Top, and W. Verstraete. 2001. Bioaugmentation of soils by increasing microbial richness: missing links. *Environ. Microbiol.* **3**:649–657.
- Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* **77**:7347–7351.
- Faulkner, K. M., M. S. Shet, C. W. Fisher, and R. W. Estabrook. 1995. Electrocatalytically driven omega-hydroxylation of fatty acids using cytochrome P450 4A1. *Proc. Natl. Acad. Sci. USA* **92**:7705–7709.
- Ikedo, H., J. Ishikawa, A. Hanamoto, M. Shinose, H. Kikuchi, T. Shiba, Y. Sakaki, M. Hattori, and S. Omura. 2003. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* **21**:526–531.
- Jungmann, V., I. Molnár, P. E. Hammer, D. S. Hill, R. Zirkle, T. G. Buckel, J. M. Ligon, and J. P. Pachlatko. 2005. Biocatalytic conversion of avermectin to 4"-oxo-avermectin: characterization of biocatalytically active bacterial strains and of cytochrome P450 monooxygenase enzymes and their genes. *Appl. Environ. Microbiol.* **71**:6968–6976.
- Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. *Practical Streptomyces genetics*. The John Innes Foundation, Norwich, United Kingdom.
- Kuhstoss, S., and R. N. Rao. 1991. Analysis of the integration function of the streptomycete bacteriophage ϕ C31. *J. Mol. Biol.* **222**:897–908.
- Lamb, D. C., T. Skaug, H.-L. Song, C. J. Jackson, L. M. Podust, M. R. Waterman, D. B. Kell, D. E. Kelly, and S. L. Kelly. 2002. The cytochrome P450 complement (CYPome) of *Streptomyces coelicolor* A3(2). *J. Biol. Chem.* **277**:24000–24005.
- Lei, L., M. R. Waterman, A. J. Fulco, S. L. Kelly, and D. C. Lamb. 2004. Availability of specific reductases controls the temporal activity of the cytochrome P450 complement of *Streptomyces coelicolor* A3(2). *Proc. Natl. Acad. Sci. USA* **101**:494–499.
- Leskiw, B. K., M. J. Bibb, and K. F. Chater. 1991. The use of a rare codon specifically during development? *Mol. Microbiol.* **5**:2861–2867.
- Leskiw, B. K., E. J. Lawlor, J. M. Fernandez-Abalos, and K. F. Chater. 1991. TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants. *Proc. Natl. Acad. Sci. USA* **88**:2461–2465.
- Murakami, T., T. G. Holt, and C. J. Thompson. 1989. Thioesteron-induced gene expression in *Streptomyces lividans*. *J. Bacteriol.* **171**:1459–1466.
- Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapfle, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummier, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:799–808.
- O'Keefe, D. P., K. J. Gibson, M. H. Emptage, R. Lenstra, J. A. Romesser, P. J. Little, and C. A. Omer. 1991. Ferredoxins from two sulfonyleurea herbicide monooxygenase systems in *Streptomyces griseolus*. *Biochemistry* **30**:447–455.
- Omer, C. A., R. Lenstra, P. J. Little, 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.
- Ortiz de Montellano, P. R. 1995. *Cytochrome P450: structure, mechanism, and biochemistry*, 2nd ed. Plenum Press, New York, N.Y.
- Panke, S., A. Meyer, C. M. Huber, B. Witholt, and M. G. Wubbolts. 1999. An alkane-responsive expression system for the production of fine chemicals. *Appl. Environ. Microbiol.* **65**:2324–2332.
- Parajuli, N., D. B. Basnet, H. C. Lee, J. K. Sohng, and K. Liou. 2004. Genome analyses of *Streptomyces peucetius* ATCC 27952 for the identification and comparison of cytochrome P450 complement with other *Streptomyces*. *Arch. Biochem. Biophys.* **425**:233–241.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schmid, A., J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, and B. Witholt. 2001. Industrial biocatalysis today and tomorrow. *Nature* **409**:258–268.
- Seelbach, K., B. Riebel, W. Hummel, M.-R. Kula, V. I. Tishkov, A. M. Egorov, C. Wandrey, and U. Kragl. 1996. A novel, efficient regenerating method of NADPH using a new formate dehydrogenase. *Tetrahedron Lett.* **37**:1377–1380.
- Sticht, H. 1999. The structure of small electron-transfer proteins. *Recent Res. Dev. Biochem.* **1**:1–27.
- Stutzman-Engwall, K., Y. Katoh, and H. McArthur. 1999. *Streptomyces avermitilis* gene directing the ratio of B2:B1 avermectins. WO patent 99/41389.
- Taylor, M., D. C. Lamb, R. Cannell, M. Dawson, and S. L. Kelly. 1999. Cytochrome P450105D1 (CYP105D1) from *Streptomyces griseus*: heterologous expression, activity, and activation effects of multiple xenobiotics. *Biochem. Biophys. Res. Commun.* **263**:838–842.
- Taylor, M., D. C. Lamb, R. J. P. Cannell, M. J. Dawson, and S. L. Kelly. 2000. Cofactor recycling with immobilized heterologous cytochrome P450 105D1 (CYP105D1). *Biochem. Biophys. Res. Commun.* **279**:708–711.
- Vieira, J., and J. Messing. 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**: 189–194.
- Walczak, R. J., J. V. Hines, W. R. Strohl, and N. D. Priestley. 2001. Bioconversion of the anthracycline analogue desacetylcladriamycin by recombinant DoxA, a P450-monoxygenase from *Streptomyces* sp. strain C5. *Org. Lett.* **3**:2277–2279.
- Waterman, M. R., and E. F. Johnson (ed.). 1991. *Methods in enzymology*, vol. 206. Academic Press, Inc., San Diego, CA.
- Weber, F. J., L. P. Ooijkaas, R. M. W. Schemen, S. Hartmans, and J. A. M. de Bont. 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* **59**:3502–3504.
- Zirkle, R., J. M. Ligon, and I. Molnár. 2004. Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. *Microbiology* **150**:2761–2774.