Biocatalytic Conversion of Avermectin to 4"-Oxo-Avermectin: Improvement of Cytochrome P450 Monooxygenase Specificity by Directed Evolution^{∇†}

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Discovery of the CYP107Z subfamily of cytochrome P450 oxidases (CYPs) led to an alternative biocatalytic synthesis of 4"-oxo-avermectin, a key intermediate for the commercial production of the semisynthetic insecticide emamectin. However, under industrial process conditions, these wild-type CYPs showed lower yields due to side product formation. Molecular evolution employing GeneReassembly was used to improve the regio-specificity of these enzymes by a combination of random mutagenesis, protein structure-guided site-directed mutagenesis, and recombination of multiple natural and synthetic CYP107Z gene fragments. To assess the specificity of CYP mutants, a miniaturized, whole-cell biocatalytic reaction system that allowed high-throughput screening of large numbers of variants was developed. In an iterative process consisting of four successive rounds of GeneReassembly evolution, enzyme variants with significantly improved specificity for the production of 4"-oxo-avermectin were identified; these variants could be employed for a more economical industrial biocatalytic process to manufacture emamectin.

Emamectin benzoate is a potent semisynthetic insecticide used to control many agriculturally important pests. However, wider application of emamectin has been hindered by the high cost of its chemical synthesis, which involves regioselective oxidation of the 4" alcohol of the natural product avermectin, followed by the reductive amination of the resulting key intermediate 4"-oxo-avermectin (see Fig. 1). Achievement of high regioselectivity in the oxidation reaction requires the costly protection and deprotection of the

very reactive allylic hydroxyl function at C-5 of the avermectin molecule.

As an alternative to chemical synthesis, microbial conversion has been used successfully in other regioselective oxidation reactions to bypass complex protection and deprotection strategies. Many microbial oxidative bioconversion reactions are carried out by dehydrogenases (4) or oxidases (3, 11) while some use cytochrome P450 monooxygenases (CYPs) (12a, 12b, 15–17). The cytochrome P450 family is a large class of enzymes that catalyze hydroxylation, epoxidation, and demethylation reactions. Class II CYPs from eukaryotes accept electrons from NADPH via a NADPH-cytochrome P450 reductase, whereas class I CYPs in prokaryotes use distinct ferredoxins and ferredoxin reductases for electron transfer from NADH. Biotransformations using CYPs have to regenerate the cofactor, which can be achieved cost-effectively on an industrial scale by using metabolically active whole cells as biocatalysts. Examples of CYP-catalyzed biotransformations include the 11 β -hydroxylation of Reichstein S to hydrocortisone (2) and the microbial hydroxylation of zofimarin, a sordarin-related antibiotic (32).

Recently, we have reported the isolation and characterization of 17 CYP enzymes, collectively named the Ema CYPs, capable of regioselectively oxidizing avermectin to 4"-oxo-avermectin (13, 19, 20). The Ema CYPs were isolated from closely related *Streptomyces* species, show a high degree of sequence similarity, and form a distinct CYP subfamily (CYP107Z). It was demonstrated that resting cells expressing the Ema CYPs could be used as biocatalysts in a one-step biosynthetic route to

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the key emamectin intermediate 4"-oxo-avermectin. For these biocatalysts to be commercially successful, they must achieve high substrate conversion (>80%) and low yield loss (<10% side product formation). However, follow-up studies showed that all the Ema CYPs are somewhat promiscuous, generating noticeable amounts of side products at high substrate conversion levels (see Fig. 1). These products reduce the overall yield and purity of 4"-oxo-avermectin and subsequently the final product emamectin benzoate. Consequently, improvements in the regiospecificity of the Ema CYP-catalyzed reaction were expected to increase the product yield and purity of the industrial bioconversion process.

The natural substrate(s) of the Ema CYP enzymes and their physiological roles in their native producer strains remain unclear. The fortuitous substrate avermectin might display poor docking in the enzyme active site, leading to the relatively low turnover observed (13), and contribute to the relative lack of specificity observed at high conversion levels in this biotransformation.

Protein engineering via site-directed mutagenesis and, more recently, molecular evolution have been successfully employed to improve enzymatic properties in industrial applications. Characteristics such as thermostability (10), solvent tolerance (25), enantioselectivity (7), substrate specificity (8), and catalytic efficiency (9) have all been altered to better adapt enzymes for specific purposes. Several examples applying molecular evolution to CYP enzymes have also been described in the literature, including the modification of the substrate specificity of the CYP BM3 from *Bacillus megaterium* (18, 33). The wild-type CYP BM3 hydroxylates long-chain fatty acids predominantly in the ω -2 position. Site-directed mutagenesis provided mutants able to hydroxylate shorter-chain fatty acids and cyclic hydrocarbons. Multiple rounds of molecular evolution were necessary to adapt the enzyme to catalyze the direct hydroxylation of first octane, then propane, and finally ethane as a substrate (18).

Since its inception, many different methods for molecular evolution have been described and applied to improve enzyme characteristics. These methods utilize molecular diversity in parent molecules that are recombined and then screened or selected for the desired properties. Molecular evolution methods can be roughly divided into two classes: (i) those that use mutagenesis to create a limited number of substitutions within a single parental sequence before recombination and (ii) those that use natural diversity found in distinct parent genes and recombine these into chimeras (6).

This work describes generation of chimeric Ema CYPs with improved substrate regiospecificity. The approach taken utilizes high-throughput screening and GeneReassembly, a proprietary molecular evolution technique, to identify optimized CYPs by blending different parent genes and artificial gene variants that differ in their substrate binding pockets. Several rounds of optimization using a combination of random mutagenesis, protein structure-guided site-directed mutagenesis, and recombination of multiple natural and synthetic parent gene fragments to fine-tune the substrate binding pocket for 4"-oxo-avermectin production resulted in enzymes which fulfill the specificity requirements for an improved commercial process. A yield of 70% 4"-oxo-avermectin (equivalent to conversion of >80% of avermectin with <10% side product formation) was considered necessary to

deliver the increased efficiency sought for the commercial manufacture of emamectin.

MATERIALS AND METHODS

Bacterial strains, plasmids, cultivation, and molecular genetic manipulations.

Escherichia coli TOP10 (Invitrogen Corp., Carlsbad, CA) was used for maintenance of plasmids, routine cloning, and generation of libraries. *E. coli* MV10dlac/pRK2526 (27) was used as helper for conjugative plasmid transfer to *Streptomyces diversus* (Diversa Corp., San Diego, CA). *Pseudomonas putida* ATCC 700801, *Streptomyces diversus*, and *Streptomyces tubercidicus* R922 (13) were used as hosts for screening and expression of Ema CYPs. Routine cultivation and molecular biological procedures were carried out as described previously (*E. coli* [24], *Streptomyces* [14], and *Pseudomonas putida* ATCC strain 700801 [20]). pRT803 is based on pRT801 (12) and contains the *ermEp** promoter and the *fd* terminator for expression of target genes in *Streptomyces*. pSET Δ ema1 was used to replace the *ema1* gene on the *S. tubercidicus* R922 chromosome. It is based on a derivative of pSET152 (5) in which the Φ C31 integrase was inactivated by the deletion of two small HindIII fragments. Inserted into the NotI site, it contains two DNA fragments covering the *S. tubercidicus* R922 chromosomal regions immediately upstream (2,978 bp) and downstream (1,751 bp) of the *ema1* coding sequence, separated by a unique SmaI site for the insertion of genes of interest. Gene replacement in *S. tubercidicus* R922 was performed as described previously (14). Gene replacements were confirmed by Southern hybridizations, PCR, and direct sequencing of PCR products.

Biotransformation, extraction, and high-pressure liquid chromatography (HPLC) analysis. Biotransformation of avermectin with resting cells was performed as described previously (20) using a standardized starting substrate concentration (45 mg/liter). Growth conditions were adapted to 96-well format by optimizing shaking conditions (500 rpm) and incubation times (16 to 18 h). Reaction products in 96-well format assays were extracted by adding acetonitrile to a final concentration of 70% (vol/vol).

S. tubercidicus R922 strains were cultivated in a TF₃₀ fermentor (Infors HT, Bottmingen, Switzerland) for large-scale bioconversion reactions. Twenty liters of PHG medium (10 g/liter peptone, 10 g/liter yeast extract, 5 g/liter glycerol, 2 g/liter NaCl, 0.15 g/liter MgSO₄, pH 7.3) was inoculated with 1 liter of a 3-day-old starter culture and incubated at 28°C for 20 to 24 h with stirring at 500 rpm, airflow at 35 liters/min at 0.7 bar, and the pH kept constant at 7.2 $\pm$ 0.1. The mycelia were collected by filtration, washed with 70 mM phosphate buffer, and stored at -20°C. Laboratory-scale bioconversion reactions (500 mg mycelium, 45 mg/liter substrate; 10-ml final volume; bioconversion performed as described in reference [20]) were extracted with methyl-*t*-butyl ether, concentrated in vacuo, and resuspended in acetonitrile.

HPLC analysis was carried out on Agilent 1100 HPLC instruments using acetonitrile-water solvent systems on either a 100-Å, 5- μ m, 125- by 4-mm Kromasil C₁₈ column (Macherey-Nagel Inc., Easton, PA) or a 50- by 4.6-mm Chromolith SpeedROD RP-18e column (Merck, Darmstadt, Germany) and detected at 243 nm. A 3-min isocratic method (70% acetonitrile in H₂O; flow rate of 2 ml/min; Chromolith column) was used for high-throughput analysis in the screening process. Under these conditions, 4"-oxo-avermectin is in equilibrium between the 4" keto form and the geminal diol 4"-hydroxy-avermectin (the hydrated form of the ketone, hereupon referred to as the ketohydrate), which elute at 2.2 min and 1.0 min, respectively. The avermectin substrate elutes at 1.4 min, while side products elute at various times (1.6 min and 0.8 min), including a significant peak that is not resolved from the 4"-oxo-hydrate peak at 1.0 min. A 60-min gradient method (Kromasil column; flow rate, 1.5 ml/min; gradient profile, 30% CH₃CN from 0 to 10 min, 30% to 53% CH₃CN from 10 to 23 min, 53% CH₃CN from 23 to 33 min, 53% to 87% CH₃CN from 33 to 53 min, 87% to 100% CH₃CN from 53 to 54 min, 100% CH₃CN from 54 to 57 min, and 100% to 30% CH₃CN from 57 to 60 min) was used to resolve all products and side products, including the 4"-oxo-avermectin keto and ketohydrate products from side product peaks. Side products were recognized as having UV spectra identical to that of the avermectin substrate. The elution times follow: 41.2 min for the avermectin substrate; 34.3 min for the ketohydrate form of 4"-oxo-avermectin and 47.5 min for the keto form of 4"-oxo-avermectin; 33.1 min for major side product 4'-desoleandrosyl-avermectin and 43.7 min for major side product 4"-oxo-3'-desmethyl-avermectin.

Screening and calculation of specificity. Biocatalytic reactions with resting cells were performed during the screening program in 96-well format with eight negative-control reactions (host strain carrying the relevant expression vector with no *ema* gene) and eight positive-control reactions (host strain expressing the parental *ema* gene of the respective library) per screening plate. Analysis of the reaction products was performed with the 3-min HPLC method (see above).

FIG. 1. (Bio)synthetic conversions of avermectin. The white block arrows show the synthetic route for emamectin via 4''-oxo-avermectin. The black block arrow show the biocatalytic reaction catalyzed by the Ema enzymes. The hatched block arrows show side product formation catalyzed by the Ema enzymes. The gray block arrows show spontaneous chemical reactions. The broken-line arrows show uncharacterized degradation steps leading to the isolated side product 4'-desoleandrosyl-avermectin and more advanced, uncharacterized side products. Me, methyl.

Percent substrate conversion (SC%) and side product formation (SP%) were calculated as follows:

$$SC\% = 100 \times (S_o - S_t)/S_o$$

$$SP\% = 100 \times (S_o - S_t - P_t)/S_o$$

where S_o is the amount of substrate at the start of the reaction, represented by the average peak area of the avermectin substrate at 1.4 min in the negative-control reactions; S_t is the amount of unreacted substrate after conversion, represented by the peak area of the avermectin substrate at 1.4 min in a test reaction; and P_t is the amount of 4''-oxo-avermectin product generated. Under standard chromatographic conditions, the keto and ketohydrate forms of 4''-oxo-avermectin are in equilibrium at a 1:1 ratio, as verified by the addition of pure standard to cells followed by extraction and analysis (results not shown). Therefore, P_t is represented in a test reaction by twice the area of the peak for the keto form of 4''-oxo-avermectin at 1.4 min.

Strains with decreased side product formation in biocatalytic reactions were selected and subjected to a secondary screen for confirmation of specificity improvement. In the secondary screen, cells of the selected strains were replicated into multiple wells of a 96-well plate. Reaction mixtures with decreased side product formation were subsequently analyzed using the 60-min HPLC method to recalculate their SP%. For this calculation, areas of all peaks with a

UV spectrum indicative of avermectin were combined to arrive at S_o , while the combined area of the chromatographically resolved keto and ketohydrate peaks was used as P_t .

The amount of side products generated by Ema1 increases exponentially as the reaction proceeds (data not shown). To directly compare the specificity of different biocatalysts, an SC%-independent measure of specificity is required. SP% data points recorded at various substrate conversion levels (SC% of 5 to 90%) from *in vitro* reactions (13) with the purified Ema1 enzyme fit an exponential curve with the formula:

$$ESP\%_{Ema1} = 0.9315 \times e^{(0.045 \times SC\%)}$$

where $ESP\%_{Ema1}$ is the expected side product formation of the Ema1 enzyme (R^2 of 0.9989; Microsoft Excel curve-fitting software). The relative specificity (RS) of a biocatalyst expressing an Ema enzyme variant thus can be defined as follows: $RS = ESP\%_{Ema1}/SP\%_{Mutant}$, where $SP\%_{Mutant}$ is the observed SP% of the mutant enzyme and $ESP\%_{Ema1}$ is calculated as described above for the same SC% that was measured in the reaction with the mutant enzyme.

Library construction. Molecular evolution libraries were constructed by Gene-Reassembly (23, 26). Detailed description of the construction of these libraries can be found in Fig. S1 to S4 and Experimental Procedures in the supplemental material. Briefly, reassembly gene fragments between preselected crossover

FIG. 2. Examples of screening data from GeneReassembly library 2. Percent side product formation was plotted against percent avermectin substrate conversion. (A) Data from a typical primary screening plate. Biocatalytic reactions were conducted with resting cells of the *P. putida* host expressing the positive-control Ema-V1 enzyme (green circles) or chimeric Ema enzymes from library 2 (blue diamonds) or not expressing Ema enzymes (negative controls [red triangles]). Two chimeras selected from this plate are highlighted as yellow squares. (B) Data from a secondary screening plate. Eight biocatalytic reactions per strain were analyzed with *P. putida* strains expressing the positive-control Ema-V1 enzyme (green circles), no Ema enzymes (negative controls [red triangles]), the wild-type Ema1 enzyme as a further control (blue squares), and chimeric Ema enzymes from library 2 preselected in primary screens (other symbols). Yellow squares show biocatalytic reactions with *P. putida* expressing Ema-V2, the best-performing chimera originating from library 2. The black trend lines show the extrapolated side product formation in biocatalytic reactions with *P. putida* expressing Ema1 (broken line) or Ema-V1 (solid line), based on an exponential relationship between SC% and SP%. The broken-line triangle indicates the area where data points for enzyme variants with improved specificity would fall.

points from each parent gene were generated by PCR or using synthetic DNA fragments. Compatible overhangs of one to four bases were introduced at both ends of the fragments. Homologous fragments were pooled in equimolar ratios. DNA fragment pools were ligated in solution or on solid-phase support. Ligation products were gel purified and isolated. Full-length products were cloned into the appropriate expression vector and transformed into *E. coli* TOP10. Ema gene libraries were isolated as mixed plasmids from pooled transformants. For libraries 1 and 2, these plasmid DNA pools were electroporated into *Pseudomonas putida* 700801 for expression. For libraries 3 and 4, the *ema* gene libraries were directly transferred from *E. coli* to *S. diversus* using a triparental mating protocol. Individual clones from each library were arrayed into 96-well format.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes encoding Ema-V1, Ema-V2, Ema-V3, Ema-V4a, Ema-V5a, and Ema-V5b variants discussed here were deposited in GenBank and given accession numbers EF577160, EF577161, EF577162, EF577163, EF577158, and EF577159, respectively.

RESULTS

Side product formation in biocatalytic reactions. Biotransformation reactions run to high substrate conversions (SC%) with *S. tubercidicus* R922, the producer strain of Ema1 (CYP107Z12 [GenBank accession no. AY545995]), generated numerous avermectin-derived side products. The two major side products were 4"-oxo-3"-desmethyl-avermectin and 4'-desoleandrosyl-avermectin, as determined by HPLC-mass spectrometry measurements and comparison with reference compounds (results not shown). These, and probably the other unidentified minor side products are derived by the stepwise oxidative degradation of avermectin and the primary 4"-oxo-avermectin product (Fig. 1). The formation of (at least some of these) side products is catalyzed by the Ema1 enzyme itself, as no similar degradation products are formed when incubating avermectin or 4"-oxo-avermectin with *S. tubercidicus* R922 strains carrying an inactivated *ema1* gene (results not shown). The amount of side products formed by the native Ema1 enzyme increases exponentially as the reaction proceeds to a

higher conversion (see Materials and Methods), depleting the desired 4"-oxo-avermectin product and leading to a loss in the yield. Similar results were observed with the other Ema enzymes.

While understanding the exact mechanisms of side product formation could lead to rational approaches to improve the specificity of the Ema enzymes, a more empirical alternative approach employing molecular evolution was selected to develop an improved commercial biocatalyst.

Development of a high-throughput screening protocol. To allow analysis of tens of thousands of chimeras generated by a molecular evolution program, a fast and reproducible screening procedure for assessing the regiospecificity of bioconversions was required. *Pseudomonas* and *Streptomyces* strains were found to be both amenable to high-throughput biotransformation reactions. A rapid 3-min isocratic HPLC method that allowed separation of avermectin and the keto form of 4"-oxo-avermectin was also developed; however, a number of side products coeluted with the ketohydrate form of 4"-oxo-avermectin.

To visualize the differences in the specificity of different biocatalysts, side product formation (SP%) was plotted against substrate conversion (SC%), as described in Materials and Methods. Representative data plots of SP% versus SC% for a primary screening plate and a secondary screening plate are shown in Fig. 2A and B. Due to well-to-well variations in culture growth and expression levels, SC% in the individual reactions can vary significantly. SP% data points from reactions using the same biocatalyst fall on an exponential curve with increasing SC% (Fig. 2B). For a biocatalyst expressing an improved Ema variant, this curve would appear shifted down compared to that of the wild-type enzyme. This quantitative graphical analysis can detect even small changes in reaction

FIG. 3. Molecular evolution of Ema enzymes. A schematic representation of the wild-type Ema1 enzyme (blue bar) with conserved regions for substrate recognition and heme and O₂ binding (black boxes I to VI) is shown at the top of the figure. Color-coordinated bars represent the best chimeric enzymes from libraries 1 to 4 and the minilibrary from libraries 3 and 4 as follows: Ema1 (blue), Ema6 (red), Ema12 (green), Ema16 (purple), Ema8 (cyan), and artificial consensus sequence (yellow) (see Results and see Fig. S2 in the supplemental material for explanation), and Ema13 (violet). Crossover sites for libraries 1 to 3 and target amino acids for site-directed combinatorial mutagenesis in library 4 and libraries 3 and 4 are given as amino acid positions over the bars relative to Ema1. For sequence alignments and detailed descriptions of library construction, see Fig. S1 to S4 and Experimental Procedures in the supplemental material. Fractions under the bars for Ema-V1 and Ema-V2 show the frequencies at which the indicated parent fragments are represented in the selected best chimeras from that library. Fractions under the names of the libraries indicate the theoretical complexity of each library versus the number of clones screened from that library. Stretches A to D indicate regions of amino acids close to the reactive heme iron center that were mutated in a combinatorial fashion in library 4. Amino acids in bold red under the bars for Ema-V4a and -V4b and Ema-V5a and -V5b indicate preferred residues identified in the screening process.

specificity that would be nearly impossible to discern by directly comparing HPLC chromatograms.

For selected biocatalysts, SP% was precisely measured by completely resolving the desired product from the side products and remaining substrate, and RS was calculated (see Materials and Methods). The conversion level-independent RS measure enables the direct comparison of the regiospecificity of biocatalysts expressing different enzyme variants across different experiments and at different conversion levels.

The RS for the purified Ema1 enzyme from *in vitro* biocatalytic reactions is 1.0 by definition (see Materials and Methods). Since the specificity of these reactions is influenced by the microbial expression host (see below), the observed RS with resting cells of *S. tubercidicus* R922 expressing Ema1 is actually 0.75 ± 0.06 . Thus, RS values are treated as a property of the whole-cell biocatalyst and best used when comparing mutant enzymes expressed in the same host.

Molecular evolution. (i) GeneReassembly library 1. After surveying the bioconversion specificities of the Ema enzymes isolated in the previous study (13), Ema1, Ema6 (CYP107Z5v2 [GenBank ac-

cession no. AY549185]), Ema12 (CYP107Z9 [GenBank accession no. AY549191]), and Ema16 (CYP107Z4 [GenBank accession no. AY549195]) were chosen as the parent genes for GeneReassembly library construction. Based on amino acid sequence alignments of these Ema enzymes with other CYPs, six conserved regions potentially involved in substrate recognition, O₂ binding, and heme binding were identified (34) (Fig. 3). Crossover sites for reassembly were chosen on the *ema* genes to fall between sequences encoding each of these conserved regions in order to maintain their integrity (see Fig. S1 in the supplemental material). To increase diversity, a gene fragment encoding the N-terminal region of Ema8 (CYP107Z2v1 [GenBank accession no. AY549187]) which differs significantly from the other Ema CYPs was also included (see Fig. S1 in the supplemental material). A library of chimeras expressed in *P. putida* as a host was created, and a total of 25,000 clones from this library were analyzed. Sixteen chimeras with increased regiospecificity were identified and further characterized by detailed analysis of their avermectin bioconversion reaction products. Nucleotide sequencing of their

FIG. 4. Relative specificities of Ema CYP variants. Ema1 and the chimeras indicated were expressed in *P. putida* (white bars), *S. diversus* (hatched bars), or *S. tuberculosis* R922 (black bars), and the RSs of the resulting biocatalysts were calculated as described in Materials and Methods.

genes showed a pronounced preference for certain parents in some (but not all) of the reassembly fragments, as shown in Fig. 3. Comparison of the product profiles of conversions carried out with resting *P. putida* cells expressing these enzymes identified the variant Ema-V1 (the best-performing Ema variant from library 1) as having the highest specificity with an RS value of 1.75 ± 0.25 (Fig. 4). While this enzyme displayed a significant improvement in regiospecificity over the wild-type Ema1, which shows an RS of 0.91 ± 0.23 in this host, further improvements were deemed necessary for an improved commercial biocatalytic process.

(ii) GeneReassembly library 2. In library 1, the whole *ema* gene had been reassembled from fragments that kept the predicted substrate recognition regions of the four individual parent enzymes intact. To obtain further improvements, the next library was constructed using the best chimera from library 1, Ema-V1, as a parent gene and focusing on blending smaller portions within and outside the substrate recognition regions of Ema1, Ema6, Ema12, and Ema16. Eight fragments representing maximum sequence diversity among the four parents were thus identified (see Fig. S2 in the supplemental material). Further, all of the Ema CYPs were surveyed to identify additional sequence diversity not captured by the previously chosen parents. Consensus sequences representing this additional Ema sequence diversity were designed for five of the eight regions targeted for reassembly, and the genes encoding their fragments were included as a fifth parent (see Fig. S2 in the supplemental material). After the resulting library was transferred to *P. putida*, 50,000 clones were screened. Seven chimeras with improved specificities were identified. Sequence analysis showed clear preferences for a parent in four out of the eight reassembly areas (Fig. 3). The best of these seven variant biocatalysts, Ema-V2 expressed in *P. putida*, had an RS of 2.35 ± 0.21 .

(iii) GeneReassembly libraries 3 and 4. To further improve the regiospecificity of the biocatalyst, two new reassembly libraries were designed taking into account the crystal structure of Ema1 (unpublished data; see Fig. S5 in the supplemental material). These libraries focused on the substrate binding cleft regions of the Ema CYPs. It was considered that achiev-

ing a better fit of the substrate in the active site was crucial for higher enzyme specificity and reduction of side product formation. Two parallel evolution approaches were undertaken to optimize the substrate binding pocket. Library 3 sampled the loops of the Ema CYPs that form the wide entrance to the substrate binding pocket, while library 4 focused on specific residues deep in the cleft. For both libraries, Ema-V2, the best chimera from library 2, was chosen as the parent gene for blending regions of interest, and enzyme variants were expressed in *S. diversus* for screening (see below).

In library 3, the outer loops of the substrate binding pocket were reassembled using fragments of the previously used parents Ema1, Ema6, Ema12, and Ema16, as well as those of Ema13 (CYP107Z8 [GenBank accession no. AY549192]). Ema13 was added because of its high catalytic activity and significant sequence diversity compared to the other parents. Redundant sequences were excluded, resulting in two to five parent fragments in each reassembled region (see Fig. S3 in the supplemental material). Sixteen thousand eight hundred clones from this library were screened in *S. diversus* (see below). Three clones with significant improvements were identified, including the best-performing variant from library 3, Ema-V3 (Fig. 3), which displayed 16% side product formation at a substrate conversion of 85% with an RS of 2.68 ± 0.22 in *S. diversus* (Fig. 4).

Based on the crystal structure of Ema1 and the superposition of the homologous structure of CYP154A1 from *Streptomyces coelicolor* A3(2) (22), 13 amino acids were expected to interact with the disaccharide moiety of avermectin. CYP154A1 was crystallized with a P450 substrate analog 4-phenylimidazole which provides empirical data for fitting a two-ring compound near the heme (22). While not chemically analogous, the 4-phenylimidazole substrate approximates the position of the distal two sugar rings of avermectin and consequently, the corresponding putative binding residues. These residues are located in four stretches (stretches A to D in Fig. 3; see Fig. S4 in the supplemental material) at the base of the substrate binding cleft, just above the Fe^{2+} center. Interestingly, the Ema CYPs show little natural diversity in these positions. Library 4 was designed to randomize these positions to introduce diversity. A full combinatorial evaluation of these 13 residues would have required constructing a library of $20^{13} = 8.2 \times 10^{16}$ clones. To limit the library size, the Ema CYPs were aligned with closely related CYP homologs from actinomycetes found in public databases. The most common residues present in the 13 selected positions were chosen as likely natural solutions to both function in this fold and subtly alter substrate specificity. This hypothesis results in a more experimentally tractable number of two to six possibilities at each amino acid position (see Fig. S4 in the supplemental material). To further reduce the number of combinations, stretches A+B+D, A+C+D, and B+C were reassembled, but not all four simultaneously (see Fig. S4 in the supplemental material for detailed description). Forty-eight thousand clones from this library were screened in *S. diversus* as a host. Fifty-three chimeras showed improved biocatalytic performance over Ema-V2. Comparison of the sequences of the improved enzymes identified four positions with preferences for certain amino acid residues (114T, 266Q, 419M/V, and 421S/G [Fig. 3]). When mapped to the structure of Ema1, these variants reveal

a subtle but concerted change at more than one contact surface in the binding pocket for the substrate, validating our focused combinatorial approach targeting discontinuous stretches of protein sequence (see Fig. S5 in the supplemental material). The best chimeras were Ema-V4a ($RS = 3.55 \pm 0.49$ in *S. diversa* [Fig. 4]) and Ema-V4b, both of which showed less than 10% side product formation at 80% avermectin conversion.

Combining libraries 3 and 4. The four mutations identified in library 4 as beneficial towards specificity were introduced into Ema-V3, the best chimera from library 3, in a combinatorial fashion. Eleven gene variants were generated by site-directed mutagenesis as described in Materials and Methods and expressed in *S. diversa*. Two chimeras, Ema-V5a and Ema-V5b, displayed further improvements over Ema-V3 and Ema-V4a and -V4b with 7% side product formation at 85% conversion. Ema-V5a ($RS = 4.91 \pm 0.98$ in *S. diversa*) harbors the mutations 266Q, 419M, and 421G, while Ema-V5b ($RS = 5.50 \pm 0.31$ in *S. diversa*) has an additional 114T mutation (Fig. 3 and 4).

Influence of the host on observed specificity. The chimera Ema-V2 from library 2 had shown a significant improvement of biocatalytic specificity when expressed in the screening host *P. putida* compared to Ema-V1, the best chimera from library 1, or the parental Ema1 enzyme, expressed in the same host. As it became clear that the performance of *Pseudomonas* biocatalysts dramatically declines in the presence of solvents (20), these hosts were no longer considered for the final industrial process. Based on its favorable growth characteristics, Ema enzyme expression, moderate solvent tolerance, and performance as a biocatalyst, *S. tubercidicus* R922, the native producer of Ema1, became our preferred candidate as expression host for the biocatalytic enzymes. The improved *ema* genes could be expressed in *S. tubercidicus* by performing markerless gene replacements of the *ema1* coding sequence with the optimized gene variants. Unexpectedly, the RS of Ema-V2 expressed in *S. tubercidicus* R922 was found to be significantly lower ($RS = 1.14 \pm 0.16$) than in the *P. putida* host ($RS = 2.35 \pm 0.21$ [Fig. 4]). A similar reduction in the observed RS value (compared to the *Pseudomonas* host) was also found in the expression host *S. diversa* ($RS = 1.56 \pm 0.32$). Thus, while the evolved Ema variants discovered in *P. putida* display improved specificity in these *Streptomyces* strains compared to the native Ema1 enzyme, the host used for the biotransformation significantly influences the amount of side products formed during the biocatalytic reactions with these resting cells. While the observed RS value for any given enzyme was lower in *S. tubercidicus* R922 or *S. diversa* than in *P. putida*, mutations that increased the specificity in one host also increased the specificity in the other hosts (Fig. 4 and results not shown). Since the transformation and gene replacement efficiencies of *S. tubercidicus* R922 are low, direct screening of gene reassembly libraries in this host was not feasible. Thus, the closely related actinomycete, *S. diversa*, was chosen as the host for the screening program starting with library 3, and the most promising enzyme variants were also expressed in *S. tubercidicus* R922.

Expression of optimized Ema enzymes in strain R922. The gene for the best chimera Ema-V5b was transferred to *S. tubercidicus* R922 using markerless gene replacement of *ema1*. The resulting strain was fermented at a 20-liter scale. Laboratory-scale bioconversions were carried out with resting cells as

FIG. 5. HPLC analysis of biocatalytic reactions. Chromatograms show the product profiles of biocatalytic reactions with *S. tubercidicus* R922 expressing Ema1 (A) or Ema-V5b (B), analyzed using the 60-min HPLC method (see Materials and Methods). Peak 1 is geminal diol 4"-hydroxy-avermectin ("ketohydrate"), peak 2 is unreacted avermectin substrate, and peak 3 is 4"-oxo-avermectin. SC% and SP% were calculated as described in Materials and Methods.

described previously (13) (see Materials and Methods). Detailed characterization of the reaction products showed <10% side product formation at >80% avermectin conversion with Ema-V5b expressed in strain R922. As before, the apparent RS of this enzyme, as measured in the R922 background is lower than that of the same enzyme in *S. diversa* as a host ($RS = 4.09 \pm 0.69$ versus 5.50 ± 0.31 [Fig. 4]). However, the R922 strain producing the optimized enzyme still provides a biocatalyst with a highly improved specificity compared to the wild-type Ema1 expressed in the same strain, as illustrated by the substantially reduced SP% measured at nearly identical SC% during the chromatographic analysis of the reaction products (Fig. 5).

DISCUSSION

The present work describes the successful directed evolution of CYPs for the improved regiospecific biocatalytic conversion of avermectin to 4"-oxo-avermectin, a key intermediate in the synthesis of the commercial insecticide emamectin benzoate. Multiple rounds of molecular evolution provided a series of novel Ema CYPs that show increasingly specific production of the desired 4"-oxo-avermectin product with decreasing production of side products formed at high (>80%) avermectin conversion levels. The most advanced enzyme of this program has an RS more than fivefold higher than that of the best wild-type Ema CYP (Ema1) expressed in the biocatalytic strain of choice, *S. tubercidicus* R922.

Molecular evolution has been widely applied to a large number of enzymes from different classes, and a number of methods for the creation of recombinant daughter genes from a family of distinct parents have been reported. Most of these methods rely on the extension of annealed random DNA fragments from multiple parents by enzymatic DNA synthesis (28). Therefore, significant sequence identity is required for the DNA fragments to anneal and create crossover points, and the position of these crossover points cannot be controlled easily. Because the stability of DNA hybrids and their fitness to prime DNA synthesis are determined by the extent of sequence homology, these methods also lead to the frequent re-creation of wild-type parents and the generation of frameshift mutations

and thus inactive variants. Overall, these limitations result in a diminished structural diversity and a preference towards certain regions for the crossovers within libraries that were generated by relying on preexisting sequence homology.

To optimize the Ema CYPs, we have utilized GeneReassembly, a proprietary recombination procedure (26). In contrast to the homology-based reassembly methods, GeneReassembly is a ligation-based process that generates chimeras from designed DNA fragments. These fragments can be generated by PCR, oligonucleotide synthesis, or a combination of both. Using this method, the parent fragments can be constructed to provide crossover points at any given position of a gene, irrespective of sequence homology among the parent genes. It also allows inclusion of various numbers of parents to reassemble different regions of the gene in a single library and is easily adaptable to the incorporation of artificial sequences. Since the reassembly fragments are provided in defined molar ratios and crossover events do not rely on sequence homology, re-creation of wild-type sequences is not favored over formation of chimeric descendants. This approach also results in high-quality libraries with a large proportion of full-size chimeric open reading frames that do not contain frameshift mutations.

Direct measurement of reaction products by HPLC was chosen as the screening method for this program because of the complex nature of the conversion and the nonlinear correlations among substrate conversion and formation of product and side products. Higher throughput in the screening program could have been achieved by utilizing substrate analogs or derivatives allowing for colorimetric or similar rapid analysis methods (1, 21, 29, 33). We reasoned, however, that enzymes evolved using these surrogate substrates might not show increased specificity for the industrially relevant avermectin substrate. While the developed HPLC-based method allowed the screening of tens of thousands of variants in each round of evolution, the complexities of each of the libraries had to be limited to allow analysis of a significant portion of clones (compared to the theoretical size of the given library) in each step. Even with this limitation, over 130,000 clones were evaluated in small-scale bioconversion reactions analyzed by quantitative HPLC during this program.

As demonstrated in this process, it was possible to further refine the designs of each subsequent Ema libraries based on results from the previous rounds of molecular evolution. Thus, we have incorporated information gained from sequence alignments and from the crystal structure of Ema1 both to tailor libraries to the natural diversity found in the Ema CYPs and to generate further diversity in these regions. With the consecutive rounds of reassembly of the Ema CYPs, we focused on regions of particular interest in the protein and introduced diversity not present in the parents.

The evolution program described here yielded several Ema CYP variants with improved specificity for the direct biocatalytic conversion of avermectin to 4"-oxo-avermectin. After four consecutive rounds of directed enzyme evolution, the best-performing Ema CYPs were complex mosaics incorporating sequence variations from three different sources: (i) fragments from the wild-type Ema1, -6, -12, -13 and -16 CYP enzymes; (ii) designed artificial "consensus" sequences representing further sequence diversity from other Ema CYPs; and (iii) point mutations from selective combinatorial mutagenesis of tar-

geted positions in the substrate binding pocket and its immediate surroundings. Residues chosen for combinatorial mutagenesis were limited to the natural sequence space for the selected positions found in actinomyces CYP enzymes from the databanks. Thus, all three sources of diversity used for the molecular evolution of the Ema enzymes preserved natural amino acid solutions that might have evolved to facilitate the folding and functionality of enzymes in the CYP protein family. Consequently, the sizes of the resulting libraries were manageable, as opposed to a random, combinatorial mutagenesis approach intrinsic to other molecular evolution methods (28).

The increased yield and purity of 4"-oxo-avermectin simplify the elimination of the contaminating side products from biocatalytic reactions that have similar chemical properties to 4"-oxo-avermectin during the final synthesis step of emamectin benzoate. The *S. tubercidicus* R922 strain expressing the most advanced enzyme, Ema-V5b, has repeatedly demonstrated a 4"-oxo-avermectin yield of >70% at a substrate concentration of 45 mg/liter, without the costly protection/deprotection of the highly reactive C-5 allyl alcohol used in the chemical process. This is a significant improvement that might provide significant cost savings when implemented in an industrial biocatalytic process. The potential influence of the starting substrate concentration and other process parameters on the efficiency and specificity of the biocatalyst will be addressed in future studies.

The improved Ema CYPs were expressed in both *Pseudomonas* and *Streptomyces* hosts to yield highly active whole-cell biocatalysts. However, the apparent substrate specificity of the improved enzymes showed an unexpected dependence on the expression host during the biocatalytic reaction (Fig. 4). Thus, the apparent specificity of the same enzyme was highest when expressed in *P. putida*, followed by *S. diversa* and *S. tubercidicus* R922. *S. tubercidicus* R922 in particular was found to also catalyze the reverse conversion of 4"-oxo-avermectin to avermectin. This reaction is assumed to be catalyzed by an uncharacterized oxidoreductase enzyme of the host, as *S. tubercidicus* resting cells lacking an *ema* gene that would express an active Ema CYP enzyme still catalyze this reduction (results not shown). This reaction results in a futile cycle during biocatalysis, from which only side products escape. Especially at high conversion levels, this cycle leads to increased side product generation and reduced apparent specificity in strain R922 compared to a host that lacks this uncharacterized reductive activity, such as *S. diversa*. Elimination of this unproductive enzymatic activity from *S. tubercidicus* R922 is an important goal in the further optimization of the biocatalyst, as other biocatalytic parameters of this strain make it the most desirable host for Ema CYP expression. The unforeseen and only partially understood influence of the surrogate screening hosts on the performance of this biocatalyst also emphasizes the need for using a relevant screening system as close to the final application as possible during molecular evolution programs (30, 31) and reinforces the notion that in a complex whole-cell bioconversion, the entire system has to be analyzed and optimized.

In summary, a CYP enzyme with highly improved substrate regiospecificity that can be employed for the biocatalytic conversion of avermectin to 4"-oxo-avermectin has been developed. The flexibility of the GeneReassembly evolution method allowed us to specifically focus on regions of interest in the

Ema CYPs and keep improving enzymatic specificity in each successive molecular evolution cycle. Further optimization of the host strain *S. tubercidicus* R922 as well as the biocatalytic process parameters will lead to a more economical biocatalytic alternative to a costly chemical synthesis step during the commercial production of the insecticide emamectin benzoate.

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