A high-throughput digital imaging screen for the discovery and directed evolution of oxygenases

Hyun Joo, Akira Arisawa, Zhanglin Lin and Frances H Arnold

Background: Oxygenases catalyze the hydroxylation of a wide variety of organic substrates. An ability to alter oxygenase substrate specificities and improve their activities and stabilities using recombinant DNA techniques would expand their use in processes such as chemical synthesis and bioremediation. Discovery and directed evolution of oxygenases require efficient screens that are sensitive to the activities of interest and can be applied to large numbers of crude enzyme samples.

Results: Horseradish peroxidase (HRP) couples the phenolic products of hydroxylation of aromatic substrates to generate colored and/or fluorescent compounds that are easily detected spectroscopically in high-throughput screening. Coexpression of the coupling enzyme with a functional mono- or dioxygenase creates a pathway for the conversion of aromatic substrates into fluorescent compounds *in vivo*. We used this approach for detecting the products of the toluene-dioxygenase-catalyzed hydroxylation of chlorobenzene and to screen large mutant libraries of *Pseudomonas putida* cytochrome $P450_{cam}$ by fluorescence digital imaging. Colors generated by the HRP coupling reaction are sensitive to the site of oxygenase-catalyzed hydroxylation, allowing the screen to be used to identify catalysts with new or altered regiospecificities.

Conclusions: The coupled oxygenase–peroxidase reaction system is well suited for screening oxygenase libraries to identify mutants with desired features, including higher activity or stability and altered reaction specificity. This approach should also be useful for screening expressed DNA libraries and combinatorial chemical libraries for hydroxylation catalysts and for optimizing oxygenase reaction conditions.

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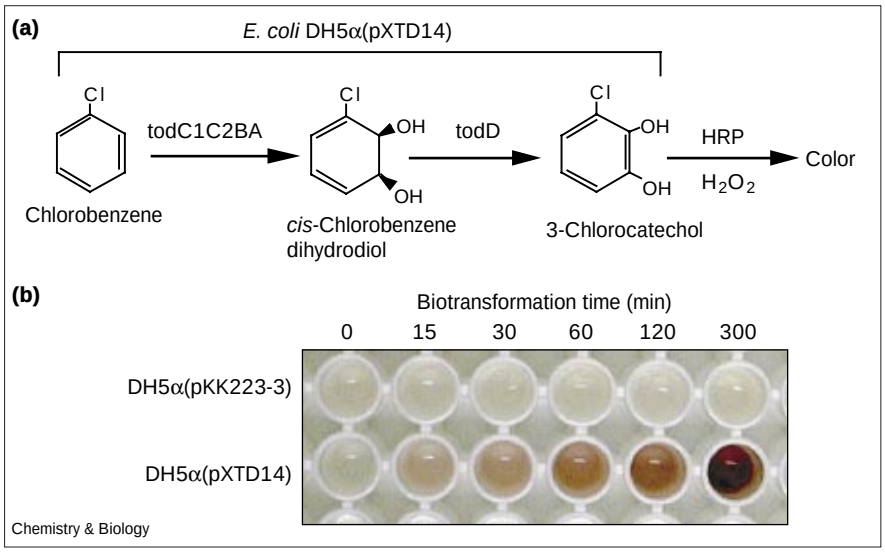
Introduction

The ability of oxygenase enzymes to catalyze the hydroxylation of organic substrates affords many potential applications in synthetic chemistry, environmental remediation, toxicology and gene therapy [1]. The oxygenases (including the di-iron enzymes such as methane or toluene monooxygenases, the ubiquitous cytochrome P450 heme monooxygenases, as well as a large number of dioxygenases [2] involved in degradation of xenobiotics) nearly always function as large, multimeric protein complexes that are only marginally stable and are often difficult to express in recombinant form. Furthermore, they are often poorly active in nonnatural environments or towards particular substrates of interest. These properties render them unsuitable for many applications. The discovery of new oxygenases, for example by high-throughput screening of expressed libraries of DNA isolated from environmental samples [3,4] and their improvement by directed evolution [5,6] will greatly accelerate the recruitment of oxygenases for biotechnology and organic synthesis.

Identifying new or improved enzymes by these routes requires a sensitive assay that can be carried out on large numbers of samples using crude enzyme preparations (e.g. cell lysates or even whole cells). The commonly used spectrophotometric oxygenase assays [7,8] require multiple manipulations and/or are highly specific to a particular substrate and reaction chosen for ease of use rather than any biological or technological significance. Measuring consumption of NAD(P)H [9] is not suitable for crude samples that contain other NAD(P)H-utilizing enzymes and furthermore may not reflect oxygenated product formation, because of uncoupling of substrate oxidation and turnover of NAD(P)H [9,10]. Because an improvement in enzyme activity on one substrate by directed evolution is often accompanied by a decrease in activity towards other substrates not included in the screen [11], it is particularly important that the assay include substrates (and reactions) that are as close as possible to the desired ones [12].

Here we describe a new method for very high-throughput screening of catalysts for aromatic hydroxylation based on the long-recognized ability of horseradish peroxidase (HRP) to oxidatively couple phenols and catechols to form colored and/or fluorescent compounds [13,14]. Combining the oxygenase-catalyzed hydroxylation reaction

Figure 1

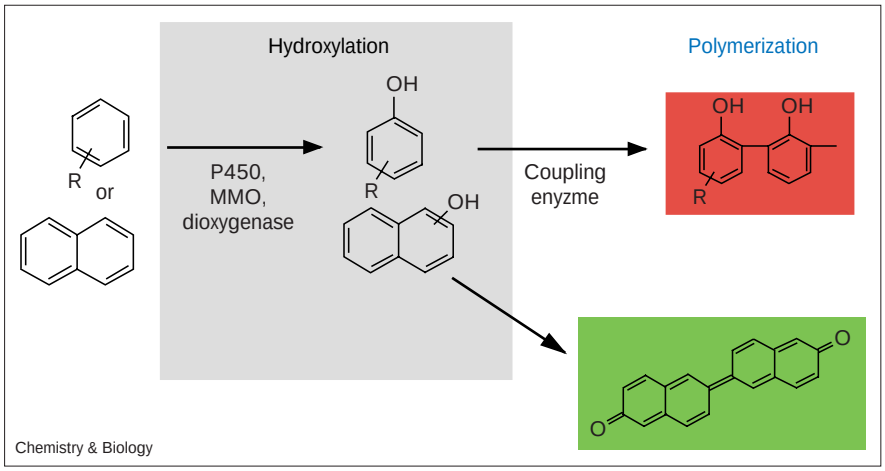


Screen for dioxygenase activity using horseradish peroxidase (HRP). **(a)** Aromatic substrates are converted to catechols by toluene dioxygenase (TDO) and dihydrodiol dehydrogenase. Catechol coupling with HRP generates a red-brown color (500 nm) when the substrate is chlorobenzene. Catechol formation is catalyzed by *E. coli* DH5α(pXTD14), which expresses genes *todC1C2BA* (for toluene dioxygenase) and *todD* (for cis-toluene dihydrodiol dehydrogenase). **(b)** Development of color upon HRP-catalyzed coupling in a 96-well plate of the products formed after various incubation times with TDO-expressing cells (DH5α(pXTD14)) and control cells (DH5α(pKK223-3)).

with oxidative coupling (illustrated in Figures 1, 2) generates compounds that are readily detected and quantified spectroscopically, allowing high-throughput screening of oxygenase libraries using plate readers, digital imaging or fluorescence-activated cell sorting (FACS).

For rapid screening, the coupling enzyme may be added to the medium containing the products of the oxygenase-catalyzed reaction. HRP can, therefore, be used with a plate reader, for example, to assay the phenolic products of aromatic monooxygenation or the catechol products from biological dioxygenation reaction (e.g. cells containing active dioxygenase and dihydrodiol dehydrogenase). Alternatively, the coupling enzyme can be coexpressed with the oxygenase to create an *in vivo* pathway for the formation of the colored and/or fluorescent compounds. We recently reported the functional expression in *Escherichia coli* of a variant of HRP [15], which was available previously from *E. coli* only by refolding polypeptide recovered from inclusion bodies (the native HRP is highly glycosylated and has four disulfide bridges). We also showed that cells expressing a functional oxygenase and HRP become brightly fluorescent in the presence of low levels of H_2O_2 and suitable aromatic substrates, and used this fact to identify oxygenase mutants with improved activity by fluorescence digital imaging [16]. We describe here the general features of this coupled-enzyme screening approach and how it can be implemented to screen mutant libraries of a dioxygenase and a cytochrome P450 monooxygenase.

Figure 2



Combining the oxygenase with a coupling enzyme (e.g. HRP) creates a pathway for the conversion of aromatic substrates into fluorescent or colored compounds useful for screening libraries for oxygenase activity. When the two enzymes are coexpressed in the same cell, the products remain associated with the cell and allow screening by digital imaging or FACS.

Results and discussion

Detection of catechols formed by toluene dioxygenase (TDO)-catalyzed dioxygenation of chlorobenzene

The catechol products of TDO-catalyzed dioxygenation followed by dehydrogenation can be detected using the HRP coupled enzyme assay. Catechol formation is catalyzed by *E. coli* DH5α(pXTD14), which expresses genes *todC1C2BA* (for toluene dioxygenase) and *todD* (for cis-toluene dihydrodiol dehydrogenase; Figure 1a) [17]. After the cells were incubated with chlorobenzene, the supernatant from the cell suspension was assayed in a 96-well microtiter plate for catechol formation by addition of HRP and H_2O_2. As shown in Figure 1b, the supernatant of cells expressing the dioxygenase (DH5α(pXTD14)) generate a strong red color, whereas controls cells with no dioxygenase (DH5α(pKK223-3)) show no red color. The colored product generated by the peroxidase coupling reaction has two distinct absorption maxima, at 310 and 500 nm, either of which can be used to screen libraries of TDO mutants for enhanced stability or improved activity towards chlorobenzene (A.A., unpublished observations).

Detecting the products of cytochrome P450 monooxygenase-catalyzed hydroxylation by coexpressing the oxygenase with HRP

If the coupling enzyme is expressed in the same cell as the oxygenase, the colored or fluorescent coupling products of aromatic hydroxylation accumulate inside the cell [16]. This approach can be adapted to high-throughput solid-phase screening by digital imaging or solution-phase screening, for example by FACS. To demonstrate this we coexpressed HRP and the heme monooxygenase cytochrome $P450_{cam}$ from *Pseudomonas putida* in *E. coli*. $P450_{cam}$ can hydroxylate aromatic substrates using H_2O_2, via the 'peroxide shunt' pathway [18,19]. In the experiments described here, neither of the proteins that transfer electrons from NADH, reductase or putidaredoxin, are added and H_2O_2 provides the oxygen.

$P450_{cam}$ and HRP variant HRP1A6 [15] were coexpressed in *E. coli* on separate plasmids (Table 1). Although the functionally expressed HRP variant (HRP1A6) is produced at low levels (~100 μg/l when produced alone in *E. coli*), the rate of the HRP coupling reaction is generally much (10^{6-8} times) greater than the rate of the P450-catalyzed oxygenation [9,20]. The overall rate of fluorescence generation will, therefore, reflect the (total) activity of the oxygenase (see below). Bacteria expressing the monooxygenase and HRP convert naphthalene and other aromatic substrates into fluorogenic products that can be detected quantitatively, often at a wavelength not characteristic of the substrate [21].

Table 1

Plasmids used for coexpression of $P450_{cam}$ with HRP in *E. coli*.

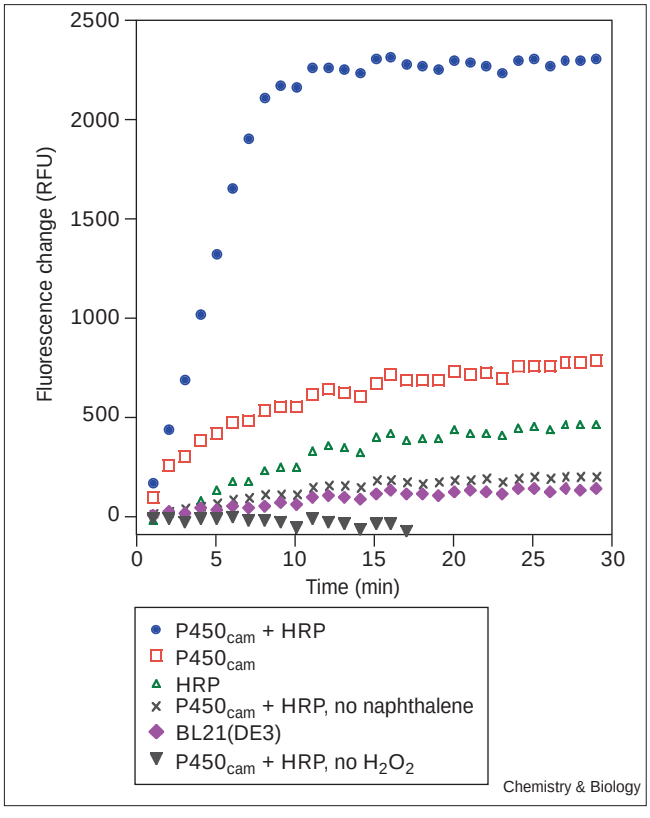
Gene insert; vector	Promoter	Replication origin	Antibiotic marker
$P450_{cam}$ from *P. putida* (ATCC17453); pCWori+	P*tac* P*tac*	pBR322	Ampicillin
HRP1A6 [15]; pET26b(+)*	T7	pBR322	Kanamycin

* contains pelB signal.

Detecting the products of $P450_{cam}$-catalyzed hydroxylation in liquid media

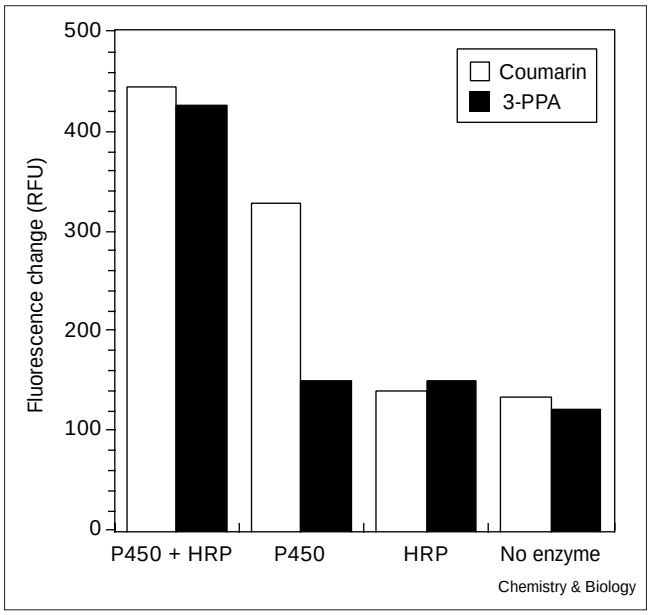
E. coli cells expressing the monooxygenase and the coupling enzyme were tested for their ability to generate fluorescence using three different aromatic substrates, naphthalene, 3-phenylpropionate (3-PPA) and coumarin. Fluorescence was monitored and compared with that from cells expressing only $P450_{cam}$ or HRP, cells expressing neither enzyme, and $P450_{cam}$ + HRP cells without the aromatic substrate or H_2O_2. The results for naphthalene are shown in Figure 3. After a 30 minute reaction, the fluorescence generated by cells coexpressing both enzymes is more than three times that of any of the other cells. For 3-PPA, cells expressing both enzymes also generated

Figure 3



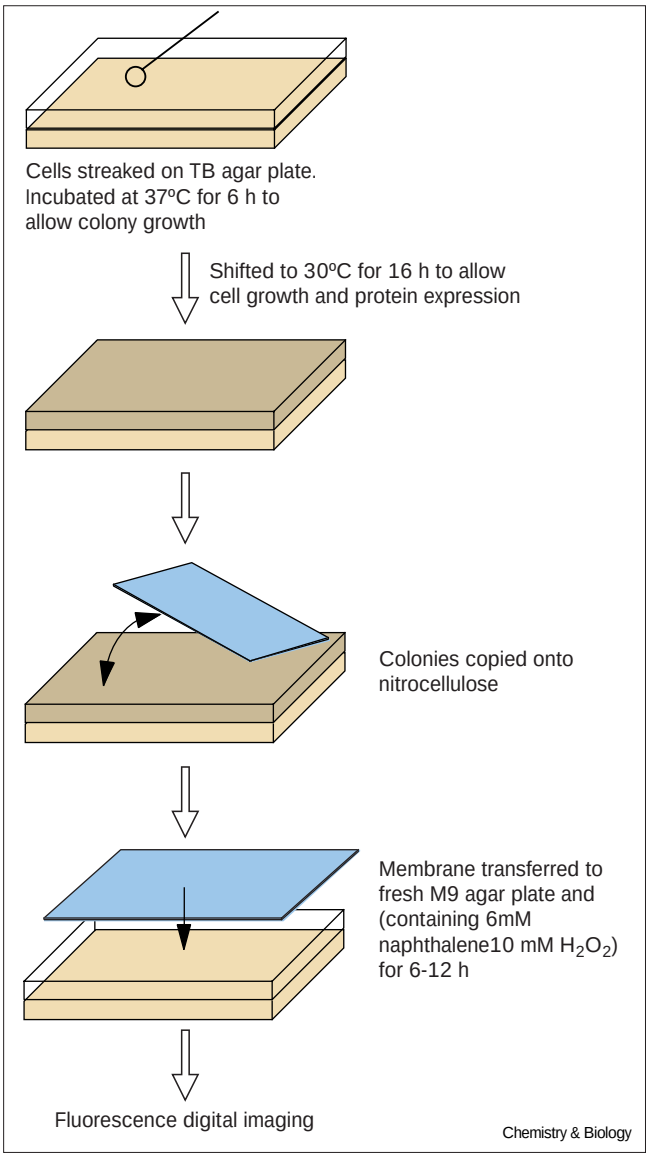
Change in fluorescence observed during $P450_{cam}$-catalyzed hydroxylation of naphthalene in a solution-phase whole-cell assay. Cells expressing $P450_{cam}$ and HRP generate significantly higher fluorescence than control cells expressing only one or no enzyme or without substrates. RFU, relative fluorescence units.

Figure 4



Change in fluorescence observed during $P450_{cam}$-catalyzed hydroxylation of coumarin and 3-PPA in a solution-phase whole-cell assay. Cells expressing $P450_{cam}$ and HRP generate significantly higher fluorescence than control cells expressing only one or no enzyme.

Figure 5



General procedure for screening oxygenase mutant library by fluorescence digital imaging.

300% higher fluorescence than the controls ($P450_{cam}$ only, HRP only and cells expressing neither; Figure 4).

Co-expression with HRP does not increase the fluorescence for all aromatic substrates. Coumarin, for example, is already quite fluorescent at pH 9, and only a modest intensification of the fluorescence was observed for cells coexpressing both enzymes (35% higher than with the $P450_{cam}$ expression alone; Figure 4). At pH 9, 4- and 7-hydroxycoumarin show a characteristic blue–green fluorescence (emission at 450–495 nm; excitation at 350 nm). This fluorescence is barely detectable in the whole-cell system, however, because of the high background fluorescence from coumarin itself.

Detecting the products of $P450_{cam}$-catalyzed naphthalene hydroxylation by fluorescence digital imaging

This coexpression system can be readily adapted to screening in the solid phase by digital imaging. We recently reported the results of directed evolution of $P450_{cam}$ for peroxide-mediated hydroxylation, using fluorescence digital imaging to screen large numbers of clones. Mutants showing up to 20-fold increases in activity towards naphthalene were identified after random mutagenesis and recombination of several improved mutants [16].

The procedure used for solid-phase screening is outlined in Figure 5. *E. coli* cultures were spread onto plates and grown at 37°C for 6 hours, at which point the incubation temperature was lowered to 30°C . This temperature shift helps the colonies grow to a uniform, small size (<0.8 mm), which greatly facilitates image analysis. After cell growth and protein expression, the colonies were replicated onto a nitrocellulose membrane (21×21 cm^2) and transferred to a fresh plate containing naphthalene and H_2O_2.

Scanned images were further processed by configuring overall thresholding, geometry recognition, intensity quantification, global and local segmentation, and cutting edges to obtain individual colony fluorescence values. With zero as the lower threshold intensity value and 255 as the highest, most of the acquired fluorescent colony

intensities could be linearized and analyzed without any intensity thresholding. (This configuration can be modified as appropriate.)

A main goal of the processing was to separate overlapping colonies in the two-dimensional fluorescence image. The original scanned fluorescence image was rather complex and involved many unclear edge cuts (Figure 6a). By imposing the sequential combinations of Boolean bit-map digitization and by passing through a uniform luminance enhancement algorithm (provided by the OPTIMAS software package), the images could be fine-tuned for further evaluation (mainly cutting edge by volume downsizing, boundary deletion, and dividing; Figure 6b–d). After three-dimensional luminance volume downsizing (the original fluorescence intensities remain unchanged during this processing) [22], the images were subjected to a contour recalculation to separate the newly segmented colonies (Figure 6c). Figure 6d shows the fluorescence images after several cycles of processing.

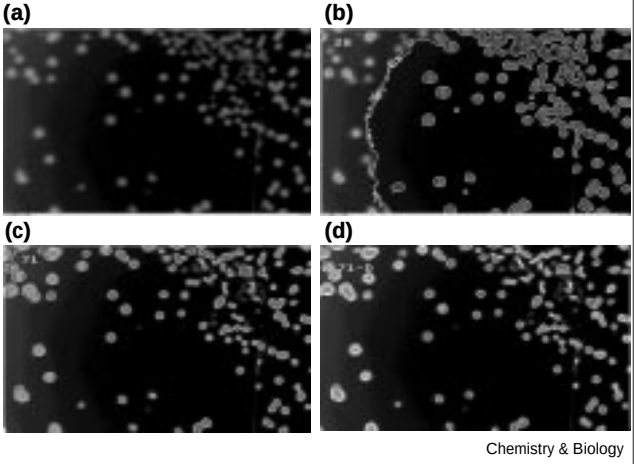
Fluorescence intensities were compared for the $P450_{cam}$ + HRP cells and the same controls as in the solution experiments of Figure 3 (data not shown). Coexpression of $P450_{cam}$ and HRP gave the highest fluorescence intensity among the cells tested. A threefold increase in the absolute fluorescence level was observed for $P450_{cam}$ + HRP cells, as compared with cells expressing $P450_{cam}$ alone. Fluorescence intensity analysis of 912 $P450_{cam}$ + HRP colonies generated an average fluorescence (RFU) of 356 with a standard deviation of 40.3. Background fluorescence levels for the other controls (HRP, host strain, and in the presence or absence of naphthalene and H_2O_2) were even lower than for cells expressing $P450_{cam}$ alone and clearly distinguishable from the fluorescence generated by the coexpression system. Fluorescence intensities of these control cases almost all fell between 0 and 5 (compared to 356 for $P450_{cam}$ + HRP clones). These image analysis results are fully consistent with the results of assays carried out in 96-well plates (Figure 3).

Small colonies are best for the fluorescence image analysis. For colony diameters of ~0.4–0.8 mm, standard deviations of ~9–17% of the average fluorescence of $P450_{cam}$ + HRP clones were observed for the four plates tested. We observed lower fluorescence values and larger variations for larger colonies, where, because of mass transfer limitations, the reactions are catalyzed only by cells close to the membrane.

Identification of improved mutants of $P450_{cam}$

A random mutant library of cytochrome $P450_{cam}$ generated by error-prone polymerase chain reaction (PCR) and expressed in *E. coli* containing the HRP expression vector (pETpelBHRP1A6Kan) was prepared on agar plates containing naphthalene and H_2O_2 for analysis by fluorescence digital imaging. Approximately 200,000 colonies were scanned, and the fluorescence values of ~32,000 of the colonies are plotted in descending order in Figure 7 [16]. A large number (~20%) show activity roughly comparable to or higher than wild-type $P450_{cam}$ activity. Adjusting the threshold level to the point where the wild-type fluorescence is near or lower than the detection limit allows one to see (count) only the colonies expressing $P450_{cam}$ activity comparable to or greater than wild-type levels. As indicated in Figure 7, the variation in colony fluorescence for this mutant library is large compared with the colony-to-colony variation observed for clones expressing wild-type $P450_{cam}$.

Figure 6

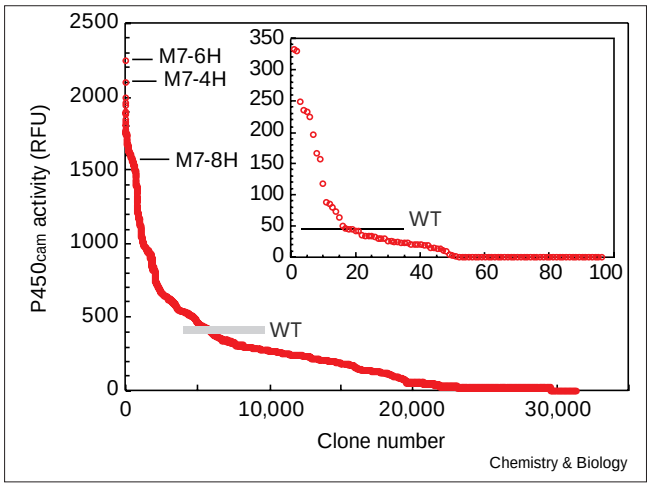


Digital image analysis. **(a)** Scanned image before processing, showing numerous overlapping colonies. **(b)** Automated contour detection of the original image. **(c)** Separation of touching colonies by volume downsizing. **(d)** Final processed image.

For comparison, 96 randomly selected clones from a mutant library were assayed in a 96-well plate with a fluorescence microplate reader. As shown in Figure 7 (inset), the profile of fluorescence values is very similar to that obtained by image analysis; a little less than 20% of the randomly selected clones exhibited improved naphthalene hydroxylation activity. The solid-phase assay with image analysis, however, is much faster, requires less handling and smaller amounts of reagents and is therefore better suited to very high-throughput screening (hundreds of thousands of colonies).

In this experiment there was no evidence of an upper ceiling limiting fluorescence intensity, which indicates that the HRP coupling activity does not limit the generation of fluorescence. This system is highly sensitive: we estimate that we can observe the napthalene hydroxylation activity of 0.2 nmole of wild-type $P450_{cam}$, or the conversion of ~1 μmole of naphthalene, in a single colony.

Figure 7



Results of screening *E. coli* library expressing HRP and mutants of $P450_{cam}$ for hydroxylation of naphthalene, using fluorescence digital imaging. Activities, as measured by the total fluorescence generated at the end of a fixed incubation period, are plotted in descending order. Range of total fluorescence of clones expressing wild-type $P450_{cam}$ is indicated with gray bar (average intensity = 356, std dev. = 40.3). This variation is small compared to the variation from the mutant library (average = 270). Inset: results of the same assay performed on 96 randomly picked clones, measured in solution in a 96-well plate.

Verification of improved activity mutants

Five positive $P450_{cam}$ variants (designated M7-4H, M7-6H, M7-8H, M7-9H and M7-2R) were selected from among ~200,000 colonies on nine plates screened by fluorescence image analysis (Figure 7). Three clones with fluorescence values near the threshold (wild-type activity) were also selected for comparison (M7-1, M7-2 and M7-3). These were grown and analyzed for activity towards naphthalene, 3-PPA and coumarin. One clone, M7-2R, proved to be a false positive and was not analyzed further. The results are summarized in Table 2.

Four of the P450 positives showed highly improved activity towards both naphthalene and 3-PPA. Variant M7-6H showed a >9-fold increase in naphthalene hydroxylation activity compared with wild-type $P450_{cam}$, with increases of 5–9-fold for the other positive mutants. These mutants also show 2–3-fold increases in activity towards 3-PPA. Activities towards coumarin were only slightly increased. The activities of the control colonies are all similar to wild type. Further characterization of one purified enzyme (from M7–8H) revealed that the increased hydroxylation activity reflected an increase in the specific activity of the enzyme rather than increased expression, and DNA sequencing of the modified genes has identified mutations responsible for the observed functional changes [16].

The screen is sensitive to the regiospecificity of hydroxylation

The UV–visible absorption and fluorescence properties of the products of the HRP reaction can be quite distinct for different hydroxylated products, offering the potential to distinguish oxygenases with different regiospecificities. Bacteria expressing wild-type $P450_{cam}$ generate only blue fluorescence (460 nm), corresponding to the conversion of naphthalene to 1- or 2-naphthol. Bacteria expressing the $P450_{cam}$ mutants, however, generate a range of colors, indicating that the regiospecificity of the $P450_{cam}$-catalyzed hydroxylation is altered by mutations in the enzyme. Analysis of the reaction products from one clone exhibiting pink fluorescence (designated P-67) confirmed a shift in product profile from mostly 1-naphthol for wild-type to mostly 1,3-dihydroxynaphthalene for P-67 [16].

The screen is also highly selective for one of two possible hydroxylated isomers of 3-PPA. Although an oxygenase can potentially hydroxylate different positions on the aromatic backbone of 3-PPA, only the product hydroxylated at the 4-position, 3-(4-hydroxyphenyl) propionate, generates strong blue fluorescence (emission at 465 nm, 350 nm excitation) when coupled using HRP [23]. HRP does not generate any detectable fluorescence with 3-(2-hydroxyphenyl) propionate as the substrate in an *in vitro* assay.

Generality of the assay approach

HRP's very broad substrate range for oxidative coupling includes a large number of phenols, catechols [20] and substituted anilines [24,25]. This approach should, therefore, be broadly useful for screening oxygenases that act on aromatic substrates. Other peroxidases or even a laccase may be used for this coupling reaction. Laccases catalyze similar

Table 2

Relative rates for P450cam variants towards 3-PPA, coumarin and napthalene.

		Positive colonies				Control colonies		
Substrate	Wild type	M7-4H	M7-6H	M7-8H	M7-9H	M7-1	M7-2	M7-3
3-PPA	13.8	42.8	43.6	35.4	33.0	7.8	9.0	16.2
Coumarin	8.2	11.5	14.1	12.8	9.3	2.6	1.2	3.1
Naphthalene	9.2	84.1	86.7	53.1	82.9	5.4	6.7	11.4

Rates were measured in a 96-well plate assay using whole cells. The fluorescence was measured as a function of time, and the relative rates presented are the slopes of that measurement (RFU/min). Each entry is the average of five measurements, and errors are ± 3%.

oxidative coupling reactions, also on a wide range of aromatic substrates, accompanied by reduction of dioxygen rather than H_2O_2 [26].

Significance

Cytochrome P450s, other monooxygenases and dioxygenases hydroxylate a wide variety of organic substrates [27,28]. The ability to tailor the substrate specificities and to improve the activities, stabilities and expression of these enzymes using recombinant DNA techniques will greatly expand their utility in chemical synthesis, bioremediation, drug discovery and medicine. Generating new enzymes using directed evolution requires high-throughput screening methods that can be applied to crude preparations, ideally whole cells. The simple screening approach described here does not require cell disruption or other separation steps (e.g. extraction) and can be used with existing imaging technology for high-throughput screening of oxygenases. The use of digital imaging also obviates many manipulations required to grow cells and screen in microtiter plates. The greater the signal amplification provided by the use of the coupling enzyme, the lower the oxygenase activity that can be identified and quantified and the fewer false positives that will be generated. Because the fluorescence is generated internally, and remains with the cells, it should also be possible to screen libraries of mutant oxygenases using fluorescence-activated cell sorting (FACS). A disposable, microfabricated FACS device has recently been developed for this purpose [29]. To generate practically useful biocatalysts, genes encoding improved oxygenases can be recombined or further mutated in additional cycles of directed evolution [16,30].

Our method uses horseradish peroxidase (HRP), a highly versatile peroxidase that can oxidatively couple a wide array of hydroxylated aromatic compounds. Furthermore, HRP retains this activity in a number of organic solvents. This approach should, therefore, also be useful for high-throughput screening of genetic libraries isolated from the environment for aromatic hydroxylation catalysts, for optimizing reaction conditions, and for screening combinatorial libraries of chemical catalysts.

Materials and methods

Horseradish peroxidase (type II, E.C. 1.11.1.7, oxidoreductase), coumarin, 7-hydroxycoumarin, 4-hydroxycoumarin, ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid], 30% H_2O_2, thiamine, glycerol and δ-aminolevulinic acid (δ-ALA) were purchased from Sigma (St. Louis, MO). Naphthalene, 1-naphthol, 2-naphthol, 3-PPA (hydrocinnamic acid), 3-(2-hydroxyphenyl) propionate, 3-(4-hydroxyphenyl) propionate, chlorobenzene and 3-chlorocatechol were purchased from Sigma and Aldrich (Milwaukee, WI). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from ICN Biomedicals, Inc. (Aurora, OH). *E. coli* DH5αMCR and BL2I(DE3) were obtained from GIBCO BRL (Life Technologies, Grand Island, NY) and Stratagene (La Jolla, CA). PROTRAN nitrocellulose filters were purchased from Schleicher & Schuell (Keene, NH). Trace element stock: 1 l HCl solution (90% v/v distilled water: concentrated HCl) containing 0.5 g $MgCl_2$, 30 g $FeCl_2$ $6H_2O$, 1 g $ZnCl_2$ $4H_2O$, 0.2 g $CoCl_2$ $6H_2O$, 1 g Na_2MoO_4 $2H_2O$, 0.5 g $CaCl_2$ $2H_2O$, 1 g $CuCl_2$, and 0.2 g H_2BO_3.

Toluene-dioxygenase-catalyzed conversion of chlorobenzene

E. coli DH5α MCR cells transformed with plasmid pXTD14 (containing *todC1C2BAD* genes [17] (from pDTG602, kindly provided by D. Gibson, U. Iowa; A.A *et al.*, unpublished results)) were grown in LB-amp (37°C) until the OD_{600} reached 0.7 and induced with 1 mM IPTG for 3 h at 30°C, then harvested by centrifugation at 4°C, washed once with 50 mM sodium phosphate buffer (pH 7.4) containing 0.2% glucose and resuspended in the same buffer. For biotransformation, 1.25 µl of 1 M chlorobenzene in ethanol was added to 0.25 ml of the cell suspension in 15 ml tubes, which were shaken at 30°C for 0–300 min. The mixture was transferred to a 1.5 ml microfuge tube and spun at 14,000 rpm for 1 min. For the HRP-based assay, 100 µl of the supernatant was mixed with 5 µl of 1 M H_2O_2 and 5 µl of 45.5 µM HRP solution in the wells of a 96-well microtiter plate.

$P450_{cam}$-catalyzed hydroxylation in cells coexpressing $P450_{cam}$ and HRP

P. putida cytochrome $P450_{cam}$ and HRP were coexpressed in *E. coli* BL21(DE3) cells using vectors $P450_{cam}$_pCWori(+) (kindly provided by Paul Ortiz de Montellano, University of California, San Francisco) and pETpelBHRP1A6Kan, as described previously [16]. For detection of fluorescence in solution, cells were grown in 10 ml terrific broth (TB) supplemented with 100 µg/ml ampicillin, 30 µg/ml kanamycin containing 0.2 mM thiamine, 1 mM δ-ALA, and 20 µl trace element stock solution per ml growth medium. Cells were harvested by centrifugation at 3350 rpm (Beckman CS SR) and resuspended in 1 ml dibasic sodium phosphate buffer (pH 9.0, 100 mM). After the addition of 10 µl naphthalene stock (32 mM naphthalene in pure ethanol), 10 µl ethanol, and 10 µl H_2O_2 solution (stock 100 mM) to the 170 µl cell suspension (total 200 µl reaction volume), the fluorescence was measured in a 96-well microplate fluorescence reader (emission 465 nm with excitation at 350 nm; Perkin Elmer HTS 7000). A 96-well white microplate (C96 White Maxisorp Fluoronunc plate, Nalge Nunc International, Naperville, IL) was used to reduce the background fluorescence of the reaction chamber during the detection and integration time (20 ms). Assays on 3-PPA and coumarin were carried out with 0.5 mM 3-PPA or 0.021 mM coumarin at the same excitation and emission range.

For solid-phase screening, *E. coli* containing $P450_{cam}$_pCWori(+) and pETpelBHRP1A6Kan were spread on TB agar plates (Falcon, #1007 or Q-tray #6021, Genetix, UK) supplemented with 100 µg/ml ampicillin, 30 µg/ml kanamycin, 100 µl/50 ml trace element stock solution, 0.25 mM thiamine, 1 mM δ-ALA and 0.5 mM IPTG, and were grown at 37°C for 6 h, at which point the incubation temperature was lowered to 30°C to obtain small and even colony size distribution (<0.8 mm diameter). After 16 h, colonies were replicated on a nitrocellulose membrane, which was transferred to a fresh M9 agar/10% (w/v) glucose/5% (v/v) ethanol plate containing 6 mM naphthalene and 10 mM H_2O_2. Plates incubated for 12 h at 30°C were subjected to fluorescence image analysis.

High-throughput screening of $P450_{cam}$-catalyzed hydroxylation by fluorescence digital imaging

Fluorescence images were obtained digitally using a cooled charge-coupled device (CCD) fluorescence image analyzer (Eagle Eye II, Stratagene, La Jolla, CA) with a top-mounted 350 nm ultraviolet illuminator. Fluorescent signals were acquired using a blue band-pass filter (430–470 nm range), a 4× lens zoom level, and a 1/10 s CCD exposure time. Automated colony detection and individual fluorescence intensity measurements were performed using OPTIMAS 5.0 image analysis software (Optimas Corp., WA), with a weighted score of 27,000 monochrome (blue) fluorescence scale. Fluorescence intensities were calculated based on the 27,000-grade scale. The fully automated image segmentation algorithm (pattern recognition and back-propagation algorithm) for colony recognition and size measurement was adopted to avoid time-consuming and subjective manual tracing of colony contours. Individual colony size measurement and

automatic single isolated colony detection were derived from computer-determined colony boundaries and fluorescence differences with different sets of threshold levels (0–255).

Random mutagenesis

Random mutagenesis was performed as described previously [31] with some modifications. A 100 µl reaction included: 10 µl 10× PCR buffer (100 mM Tris/HCl, 500 mM KCl, pH 8.3 at 20°C), 7 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 2 mM dCTP (100 mM), 1 mM dTTP, 0.7 mM $MnCl_2$, 14 pmoles forward and reverse primers, 5 units *Taq* DNA polymerase (Boehringer Mannheim), 0.01% gelatin, 20 fmoles template $P450_{cam}$–pCWori(+), 42 µl ddH_2O. These conditions are expected to give an error rate of ~0.5%. Mutagenic PCR was performed in a programmable thermocycler (PTC200, MJ Research) for 30 cycles (denaturation 94°C, 30 s; annealing 30 s; elongation 72°C, 2 min). Forward (24-mers) and reverse primer (25-mers) sequences: 5′-CATCGATGCTTAGGAGGTCATATG-3′, 5′-TCATGTTTGACAGCTTATCATCGAT-3′. The sequence to be amplified between the two primers is 1.4 kb.

PCR product purification, cloning and transformation

The Qiaex II kit (Qiagen, Germany) was used for PCR product purification. Purified PCR product was redissolved in a TE buffer (10 mM Tris-HCl, pH 8.0) and was subjected to electrophoresis on preparative 1% agarose gels to separate the template plasmid. After digestion with *Nde*I (10 units) and *Hind* III (10 units) for 2 h at 37°C, the *Nde*I–*Hind* III cut fragment was purified again by gel extraction and was inserted into pCWori+ vector. The ligation was carried out at 16°C for 9 h with 200 units of T4 DNA ligase (Boehringer Mannheim). The ligation mixture was then used to transform *E. coli* BL21 (DE3) cells, which contain the pETpelBHRP1A6Kan vector.

Chemical transformation using $CaCl_2$ (60 mM) and heat shock (45 s at 42°C) was used to introduce pETpelBHRP1A6 (Kan^r) into *E. coli* BL21(DE3). Successful transformants were identified by selection on LB/kan (6–30 µg/ml) agar plates. Positive clones were then made chemically competent and transformed with the ligation product. Identification of the *E. coli* BL21(DE3) clones containing both genes were identified by growth on LB/kan (30 µg/ml)/amp (100 µg/ml) plates.

Whole-cell microtiter plate assay for $P450_{cam}$ activity

Cells grown in 4 ml TB/amp (100 µg/ml) were centrifuged for 10 min at 4°C. After the supernatant was removed, the harvested cells were carefully resuspended in 1 ml buffer solution (dibasic phosphate, 100 mM, pH 9.0) and 20 µl aliquots were transferred to a Nunc fluorescence microplate. The total 180 µl reaction mixture was made up of 100 µl dibasic sodium phosphate buffer (100 mM, pH 9.0), 20 µl ethanol, 10 µl substrate stock (4.5 mM coumarin in 10% ethanol, or 2 mM 3-PPA in 10% ethanol, 2 mM naphthalene in pure ethanol), and 10 µl H_2O_2 stock solution (50 mM).

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