Mutations in Distant Residues Moderately Increase the Enantioselectivity of *Pseudomonas fluorescens* Esterase towards Methyl 3-Bromo-2-methylpropanoate and Ethyl 3-Phenylbutyrate

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Abstract: Directed evolution combined with saturation mutagenesis identified six different point mutations that each moderately increases the enantioselectivity of an esterase from *Pseudomonas fluorescens* (PFE) towards either of two chiral synthons. Directed evolution identified a Thr230Ile mutation that increased the enantioselectivity from 12 to 19 towards methyl (*S*)-3-bromo-2-methylpropanoate. Saturation mutagenesis at Thr230 identified another mutant, Thr230Pro, with higher-than-wild-type enantioselectivity ($E = 17$). Previous directed evolution identified mutants Asp158Asn and Leu181Gln that increased the enantioselectivity from 3.5 to 5.8 and 6.6, respectively, towards ethyl (*R*)-3-phenylbutyrate. In this work, saturation mutagenesis identified other mutations that further increase the enantioselectivity to 12 (Asp158Leu) and 10 (Leu181Ser). A homology model of PFE indicates that all mutations lie outside the active site, 12–14 Å from the substrate and suggests how the distant mutations might indirectly change the substrate-binding site. Since proteins contain many more residues far from the active site than close to the active site, random mutagenesis is strongly biased in favor of distant mutations. Directed evolution rarely screens all mutations, so it usually finds the distant mutations because they are more common, but probably not the most effective.

Keywords: directed evolution · enantioselectivity · hydrolases · mutagenesis · screening

Introduction

Enantioselectivity is an important, but incompletely understood, characteristic of enzymes. Enantioselective enzymes, especially hydrolases, are useful catalysts to make pure enantiomers needed for pharmaceuticals, agrochemicals and materials. However, the molecular details of enantioselectivity remain incomplete and sometimes contradictory. For example, directed evolution experiments suggest that changes in distant residues are most effective at increasing enantioselectivity, while rational design experiments suggest that residues in contact with the substrate are most effective.

Recently, several groups used directed evolution[1] (recursive mutagenesis and screening for improved variants) to increase, or even to reverse, the enantioselectivity of hydrolases. Surprisingly, many of the mutations revealed by directed evolution to change the enantioselectivity lie far from the substrate-binding site. For example, Reetz, Jaeger and coworkers increased the enantioselectivity of a *Pseudomonas aeruginosa* lipase (PAL) from 1.1 to 26 using four rounds of directed evolution.[2] The resulting enzyme contained five mutations all outside, and none within, the substrate-binding site. These results, as well as others,[3] suggest that the mutations far from the active site control the enantioselectivity, although the precise reason for the increase remains uncertain.

On the other hand, rational approaches to increase enantioselectivity focus on residues close to the active site. For example, a single mutation doubled the enantioselectivity of *Candida antarctica* lipase B (CAL-B)[4] and another mutation increased it from 1.2 to 22.[5] However, these mutations also drastically decreased the reaction rate. Rational design is difficult because it requires one to predict changes that not only increase enantioselectivity, but also will not degrade the activity or stability of the enzyme.[6]

In this paper, we use directed evolution to moderately increase the enantioselectivity of *Pseudomonas fluorescens* esterase (PFE). Like others, we found distant mutations that increase enantioselectivity. However, we suggest that random

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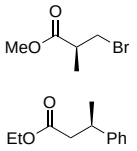
mutagenesis biases directed evolution to mutations far from the active site. We use a homology model of the enzyme to propose a possible molecular basis for the increases in enantioselectivity based on indirect changes to the substrate-binding site. We propose that focusing mutations into the active site would be a more effective strategy for increasing enantioselectivity.

A key to success in directed evolution is a screen or selection method to identify improved mutants.[7] We used the Quick E screen, a colorimetric screen for enantioselectivity.[8] The advantages of Quick E are firstly, that it measures the true enantioselectivity, not an estimated value, and secondly, that it uses the true substrate, not a chromogenic analogue. Although we previously used Quick E to screen commercial enzymes for high stereoselectivity, this is the first use of Quick E to screen hydrolases in whole cells.

Our target enzyme, PFE, is easily expressed in *E. coli*,[9] is stable over a wide pH and temperature range,[10] and has broad substrate specificity.[11] Initial substrate mapping identified several target substrates for directed evolution, Scheme 1. The esterase from *Pseudomonas fluorescens* catalyzes the hydrolysis of these esters (Scheme 1) with low-to-moderate enantioselectivity, where the structures show the absolute configuration of the fast-reacting enantiomer.



Scheme 1. Chiral synthetic intermediates: methyl (*S*)-3-bromo-2-methylpropanoate and ethyl (*R*)-3-phenylbutanoate.

One substrate, methyl 3-bromo-2-methylpropanoate (MBMP), is a useful chiral synthon for the preparation of captopril (a high blood pressure treatment),[12] retroviral protease inhibitors,[13] unnatural amino acids,[14] cyclooxygenase inhibitors,[15] and alkaloids.[16] Other enzymes catalyzed hydrolysis of this ester,[12] but the enantioselectivities were only moderate. Indeed, MBMP represents a difficult resolution, as the enzyme must distinguish between two stereocenter substituents of relatively similar size: methyl versus bromo-methyl. PFE catalyzed hydrolysis of MBMP with moderate enantioselectivity ($E = 12$) in favor of the *S*-enantiomer. Increasing the enantioselectivity of PFE towards MBMP would provide a good synthetic route to this useful synthon.

A second substrate is ethyl 3-phenylbutanoate (EPB), a chiral synthon for malyngolide,[17] an antibiotic discovered in algae, β-methyl phenylalanine and β-methyl tyrosine,[18] and several drug candidates.[19] Pure enantiomers of 3-phenylbutyric acid are accessible from classical resolutions,[20] lipase-catalyzed resolution,[21] or stereoselective synthesis,[22] but an esterase-catalyzed route could be a useful alternative. Wild-type PFE showed low enantioselectivity ($E = 3.5$ favoring the *R*-enantiomer) towards esters of 3-phenylbutyric acid and previous directed evolution experiments employing a fluorescence-based screening assay identified three improved mutants (Leu181Gln, Asp158Asn, and Ala209Gly) with enantioselectivities of 5 – 7.[3b]

To enhance the enantioselectivity of PFE towards hydrolysis of MBMP, we used directed evolution by random mutagenesis, saturation mutagenesis, and Quick E screening. To further increase the enantioselectivity of the previous mutants towards EPB, we used saturation mutagenesis at positions 158 and 181 and fluorescence-based screening.

Results

Thr230Ile mutant: To increase the enantioselectivity of PFE towards hydrolysis of MBMP ($E_{WT} = 12$ favoring the *S* enantiomer), we used random mutagenesis by an *E. coli* mutator strain followed by screening of 288 crude cell lysates with Quick E (Figure 1).[23] Most of the mutants had enantioselectivities near that of the wild type (Quick E = 12), but one mutant, MS6-31, showed significantly higher enantioselectivity (Quick E = 21). Figure 1a shows the data of 96 colonies,

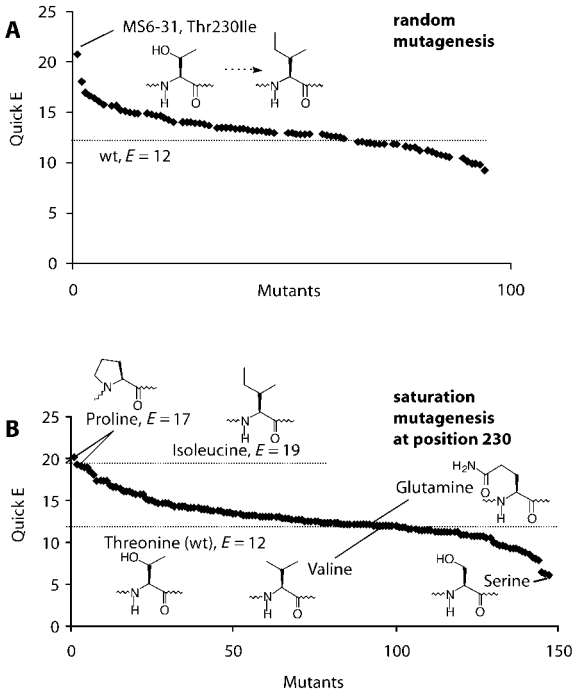


Figure 1. Enantioselectivity of PFE mutants towards MBMP. A) Mutants generated by random mutagenesis of the entire protein and ordered from highest-to-lowest enantioselectivity. B) Saturation mutagenesis at amino acid residue 230.

although 288 colonies were screened in this experiment. The flat central part of curve a) represents colonies where there was little or no change in enantioselectivity because they either contained no mutations or mutations that did not affect enantioselectivity. The best mutant showed $E = 21$ by Quick E and $E = 19$ in a scale-up reaction. DNA sequencing revealed that Thr230 changed to Ile in this mutant as mentioned above. DNA sequencing revealed two single nucleotide substitutions. The first was a $G_{303}A$ transition, which still codes for the same amino acid, valine. Although the new codon (GTA) occurs approximately half as frequently in *E. coli* as the original codon (GTG),[24] we saw no change in the level of protein expression (data not shown). The second mutation was a $C_{689}T$ transition, which changes Thr230 to isoleucine.

We confirmed the increased enantioselectivity of the Thr230Ile mutant after purification by the endpoint method of Chen et al.,[25] Table 1. The endpoint method on purified enzyme using gas chromatography to measure enantiomeric purity yielded an E value of 19 ± 2 (error represents standard error), which is significantly higher than wild type ($E = 12 \pm 1$) at the 99.5% confidence level. These values were in good agreement with Quick E from the original screen. The differences between Quick E and the endpoint method are within experimental error even though we measured Quick E values on cell lysates, but measured endpoint method values on purified enzyme. Although we did not carefully measure kinetics, a comparison showed that the rate of reaction of the mutant with MBMP was within 40% of the wild type.

Table 1. Enantioselectivities of selected PFE mutants towards methyl 3-bromo-2-methylpropanoate (MBMP).

Mutation	Quick E[a]	Endpoint E[b]
Wild type	12	12 ± 1
Thr230Ile	21	19 ± 2
Thr230Pro	19	17
Thr230Gln	12	nd[c]
Thr230Val	12	nd[c]
Thr230Ser	6	nd[c]

[a] Colorimetric measurement in microplates using *p*-nitrophenol as the pH indicator and resorufin acetate as the reference compound. Wild type and all mutants favored the *S*-enantiomer. [b] Small scale reactions (50 μmol) where the enantiomeric purity of the products and starting materials were measured by gas chromatography on a chiral stationary phase. Errors shown are standard errors. [c] nd = not determined.

Saturation mutagenesis at position 230: Random mutagenesis at low mutation rates may not identify the optimal amino acid. Low mutation rates are likely to change only one nucleotide per codon, but some amino acid substitutions require two nucleotide changes per codon. Changing only a single nucleotide per codon gives on average only 5.7 instead of 19 possible amino acid substitutions.[26] To test all 19 substitutions, researchers use saturation mutagenesis.

Saturation mutagenesis inserts the degenerate codon NNK at the desired position and generates a mixture of 32 possible mutants, coding for all twenty amino acids at least once.[27] We screened 175 colonies to ensure with >99% probability that we tested each of the 32 mutants at least once.[28] None of the mutants had higher enantioselectivity than the Thr230Ile mutant, Figure 1b. Of 175 colonies screened towards MBMP, 28 clones or 16% had no activity. DNA sequencing of the two best mutants (Quick E = 20, 19) revealed a proline substitution, while the worst mutant (Quick E = 6) contained a serine substitution. Two mutants showing enantioselectivity similar to wild type (Quick E = 12) contained a valine or glutamine substitution. A single endpoint determination of enantioselectivity for the best mutant (Thr230Pro) indicated an E value of 17, which is better than wild type, but not better than the Thr230Ile mutant.

Saturation mutagenesis at positions 158 and 181: Previous directed evolution of PFE increased its enantioselectivity towards EPB from 3.5 (wild-type) to 5.8 (Asp158Asn) and 6.6 (Leu181Gln).[3b] To further increase this enantioselectivity, we tried saturation mutagenesis at each position. We screened the mutants using a fluorogenic analogue of the EPB, 3-phenylbutyric acid resorufin ester, and identified several improved mutants, Table 2. Endpoint determinations of enantioselectivity using gas chromatography to measure enantiomeric purity confirmed that several mutants showed increased enantioselectivity towards EPB. The best mutants at position 158 were Asp158Leu and Asp158Phe, which had enantioselectivities of 12 and 9, respectively. The best mutants at position 181 were Leu181Ser and Leu181Thr, which had enantioselectivities of 10 and 9, respectively. Double mutants combining the two best 158 mutants and the two best 181 mutants did not improve the enantioselectivity above that of the best single mutant.

Table 2. Enantioselectivities of selected PFE mutants towards ethyl 3-phenylbutanoate (EPB).

Mutation	Estimated E[a]	True E[b]
Wild type	3.7	3.5
Asp158Asn	6.1	5.8
Asp158Glu	6	8
Asp158Leu	10	12
Asp158Cys	7	8
Asp158Phe	11	9
Leu181Gln	6.7	6.6
Leu181Trp	5	8
Leu181Ser	9	10
Leu181Thr	12	9
Asp158Phe, Leu181Ser	nd	9
Asp158Phe, Leu181Thr	nd	10
Asp158Leu, Leu181Ser	nd	12
Asp158Leu, Leu181Thr	nd	9

[a] Ratio of initial rates of reaction of (*R*)-3-phenylbutyrate resorufin ester and (*S*)-3-phenylbutyrate resorufin ester. nd = not determined. Wild type and all mutants favored the *R*-enantiomer. [b] Enantioselectivity towards ethyl 3-phenylbutyrate measured by endpoint method where the enantiomeric purity of the products and starting materials was measured by gas chromatography on a chiral stationary phase.

Homology model of PFE-containing reaction intermediate: To identify the possible molecular basis for the enantioselectivity increases, we created models of the key intermediates in the hydrolysis. Since the X-ray structure of PFE is not solved, we first built a homology model of wild-type PFE using the automated homology modeler at SWISS-MODEL.[29] The templates for the model were non-heme haloperoxidases, which show 46–51% amino acid sequence identity with PFE. Although haloperoxidases catalyze oxidations, their protein fold and catalytic mechanism resembles esterases. They contain the α/β-hydrolase fold and the catalytic triad characteristic of hydrolases.[30, 31] Haloperoxidases catalyze oxidations by catalyzing formation of a peroxycarboxylic acid from free carboxylic acid and hydrogen peroxide using an esterase-like mechanism. The peroxycarboxylic acid then oxidizes the halide, probably independently of the enzyme.[32, 33] Non-heme haloperoxidases have low esterase activity and PFE has low bromoperoxidase activity. Antibodies raised against a haloperoxidase also react with PFE. Thus, in spite of the different enzyme class, haloperoxidases are a good template

for a model of PFE. Homology models generated by SWISS-MODEL with this level of sequence identity are usually reliable. When researchers compared predictions with known structures, 63% of the predicted models were within 3 Å of the known structure.

The first step in hydrolysis is nucleophilic attack of the Ser95 on the ester carbonyl carbon yielding a tetrahedral intermediate. We built models of these tetrahedral intermediates for the slow-reacting enantiomers of MBMP and EPB into the homology model of PFE, Figure 2. In both cases, mutations that increase enantioselectivity occurred far from the substrate, but on opposite sides of the substrate. The mutated amino acid does not contact the tetrahedral intermediate. Thr230 lies ≈14 Å to the left of the MBMP tetrahedral intermediate (distance from Cα of Thr230 to the stereocenter in MBMP). Similarly, Leu181 lies ≈12 Å to the right of the EPB tetrahedral intermediate and Asp158 lies ≈13 Å to the right.

Mutation of Thr230 could indirectly affect the substrate-binding site, but the details are speculative at this point. Thr230 lies between a helix and a loop. This loop (221–228) protrudes into the acyl-binding region of the substrate-binding site. In particular, residues Ile225 and Val226 (green in Figure 2a) on this loop contact the MBMP tetrahedral intermediate. The OH of Thr230 may donate a hydrogen bond to the backbone carbonyl of Gln134. A mutation that retained this OH (Thr230Ser) caused a decrease in enantioselectivity, while the mutations that increased enantioselectivity (Thr230Ile, Thr230Pro) disrupted this hydrogen bond. This disruption may move the 221–228 loop including Ile225 and Val226 thereby changing the substrate-binding site and increasing the enantioselectivity.

Mutations of Asp158 or Leu181 could also indirectly affect the substrate-binding site, but again the details are speculative. Both of these residues are located in the variable D′ helices which cover the active site and are thought responsible for the differences in substrate specificity among α/β hydrolases.[30] Mutations on these helices may propagate to the substrate-binding site via Trp29 and thereby increase the enantioselectivity.

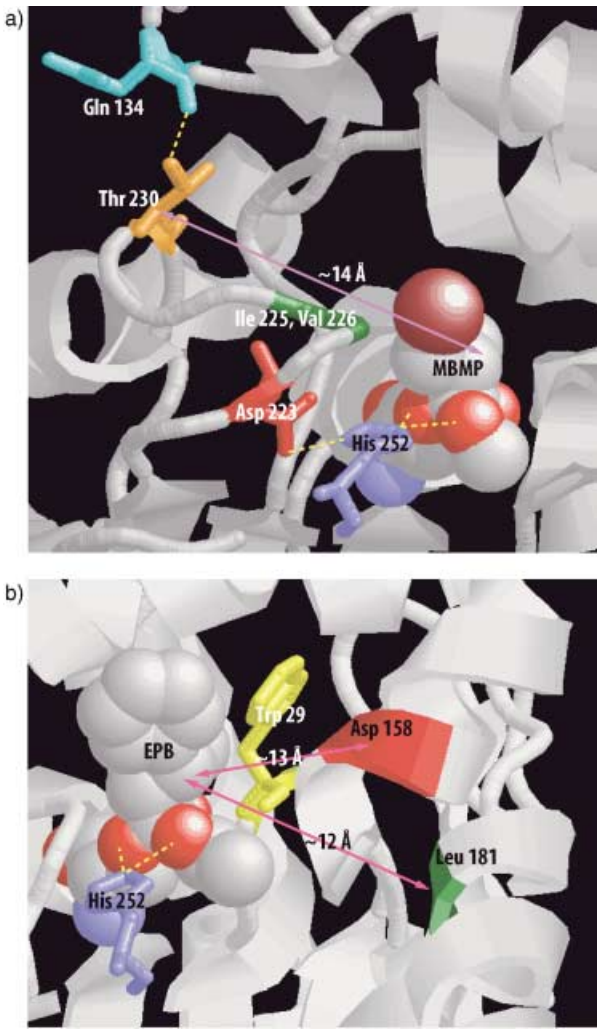


Figure 2. A homology model of PFE containing the first tetrahedral intermediate for hydrolysis of the slow-reacting a) *R*-enantiomer of MBMP and b) *S*-enantiomer of EPB, both in space-filling representation. The catalytic His252 forms hydrogen bonds (dotted yellow lines) to the Oγ of the catalytic Ser95, to the oxygen of the leaving group methanol (a), and ethanol and to the catalytic Asp223 (red sticks) (b). a) Thr230 (orange sticks), where mutations caused changes in enantioselectivity, lies approximately 14 Å from MBMP (distance from Cα of Thr230 to the stereocenter in MBMP). The OH of Thr230 may donate a hydrogen bond to the backbone carbonyl of Gln134 (cyan sticks). Mutations at position 230 may move the 221–228 loop including Ile225 and Val226 (green section), which contact the substrate. This loop also contains the catalytic Asp223. b) The two residues where mutations caused increased enantioselectivity (Asp158 and Leu181) lie on helices approximately 13 and 12 Å, respectively, from EPB (distance from Cα of amino acid to the stereocenter in EPB). A loop containing Trp29 (yellow sticks) lies between these helices and the substrate. Structural changes caused by mutations at Asp158 and Leu181 may propagate to the substrate via Trp29. For clarity, hydrogen atoms are not shown and the loop containing residues 161–174, which lies in front lower right of this picture, is also not shown.

Discussion

One round of mutagenesis by an *E. coli* mutator strain followed by Quick E screening identified a Thr230Ile mutant with *S*-enantioselectivity improved from $E = 12$ to 19 towards MBMP. Since the low frequency mutagenesis used in this experiment is unlikely to substitute all 19 possible amino acids at a given position, we used saturation mutagenesis to test all 19 amino acids at the positions where we found the initial mutations. Saturation mutagenesis at position 230 did not yield a further increase in enantioselectivity towards MBMP, but did yield another mutant Thr230Pro, whose enantioselectivity was similar to Thr230Ile, $E = 17$. This experiment also identified two mutations at 230 that showed unchanged enantioselectivity (Val, Gln) and one with lower enantioselectivity (Ser, $E \approx 6$).

In contrast, saturation mutagenesis at sites 158 and 181 did yield further increases in enantioselectivity towards EPB. An Asn158Leu mutation doubled enantioselectivity from 5.8 (for Asp158Asn) to 12 towards EPB (wild-type Asp158, Leu181 enantioselectivity is 3.5). A Gln181Ser mutation increased enantioselectivity from 6.7 (for Leu181Gln) to 10. Double mutants did not improve the enantioselectivity, showing that the individual benefits of each mutation are not additive.

Directed evolution experiments, including experiments in this paper, consistently identify residues outside the active site as key residues for enantioselectivity as well as other properties. At first glance these results suggest the surprising conclusion that distant residues influence properties like enantioselectivity more than residues close to the active site. However, if one corrects for the numbers of close residues versus distant residues, one comes to the opposite conclusion, that residues close to the active site are more important than those far from the active site. For PFE, directed evolution identified three locations (positions 158, 181, and 230) where mutations can moderately increase enantioselectivity. These mutations lie approximately 13, 12, and 14 Å, respectively, from the tetrahedral intermediate for hydrolysis of (*R*)-MBMP or (*S*)-EPB. Although these mutations are outside the active site, they lie much closer than one would expect from random mutations, Table 3. Only seven Cα atoms lie within 7 Å of the stereocenter carbon of the tetrahedral intermediate for hydrolysis of (*R*)-MBMP. These seven Cα atoms correspond to only 2.6% of the total 270 Cα atoms in this protein. Similarly, only twenty-three Cα atoms lie within 10 Å (8.5%) and only ninety-seven within 15 Å (36%). Thus, only about one third of random mutations involve an amino acid whose Cα lies within 15 Å of the stereocenter carbon. Random mutagenesis is strongly biased towards mutations far from the active site simply because there are more residues far from the active site than there are close to it. In spite of this bias, all three mutations identified to increase enantioselectivity in this paper lie closer to the active site than expected by random mutation. These results suggest that mutations within the substrate-binding site would be even more effective in increasing enantioselectivity than random mutations throughout the protein. Indeed, we have confirmed this hypothesis (manuscript in preparation).

That amino acid residues distant from the active site can influence catalysis has been observed previously. The reasons for these effects are sometimes explained by large conformational changes (for example, open and closed conformations),[34] subunit interface mutations affecting quaternary structure,[35] or protein folding problems.[36] Often, however, no major structural change is detected, and more subtle effects such as long-range electrostatics,[37] disorder in essential loops,[38] or disruption of hydrogen-bonding networks[39] explain the effect of the distant residue. Many reports do not present a clear rationale for the effect beyond suggesting that subtle structural changes from a mutation are somehow propagated through the protein to the distant active site.[40] For example, Chen et al.[40b] suggested that isocitrate dehydrogenase activity was reduced by transmission of long-range conformational changes made by mutations in a loop some 14 Å from the active site. In another example, a triple mutant of a lipase inverted enantioselectivity.[41] Structural data indicates that one of the residues, Phe221, is 20 Å from the active site.[42] However, the individual contribution of this residue to enantioselectivity is unclear.

Table 3. Number of Cα atoms in PFE within different distances from the stereocenter in hydrolysis of (*R*)-MBMP.

Distance[a]	Number of Cα atoms[b]	Fraction of all Cα atoms in protein [%]
within 7 Å	7[c]	2.6
within 10 Å	23	8.5
within 15 Å	97	35.9
within 17.5 Å	136	50.4
all	270	100

[a] Distance from the Cα of the amino acid to the stereocenter (C-2 of 3-bromo-2-methylpropanoic acid) of the tetrahedral intermediate. [b] Does not include the Cα of Ser95 or Met1, which was omitted from the homology model. [c] Gly28, Trp29, Met96, Val122, Phe199, Ile225, Val226.

Experimental Section

General: Chemicals, buffers, and lysozyme were purchased from Sigma Aldrich. LB media were obtained from Difco. RNase A was purchased from USB and DNase I was purchased from Gibco BRL. The Sheldon Biotechnology Centre (McGill University, Montréal, Canada) provided primers and performed dideoxy termination sequencing.

Cloning of PFE and generation of random mutants: The esterase gene was amplified from the plasmid pUE1251 and inserted into a His_6-tagged derivative (pJOE2775) of the rhamnose-inducible expression vector pJOE2702[43] as described previously[11] to yield the vector pJOE2792. Random mutants were generated as previously described[44–46] by transforming the plasmid containing the PFE wild-type gene into the mutator strain *E. coli* XL-1 Red (Stratagene, La Jolla, USA) according to the manufacturer's instructions, and growing the culture in LB medium (50 mL; $MgCl_2$, 20 mM; glucose, 20 mM) supplemented with ampicillin (100 $\mu g\,mL^{-1}$) overnight at 37 °C. Subsequent repetitions of mutagenesis were performed by addition of overnight culture (0.5 mL) to LB medium (20 mL; ampicillin, 100 $\mu g\,mL^{-1}$) and further growth. Plasmid was isolated from aliquots of culture (2 mL) from mutagenic repetitions 5, 6 and 7, and transformed into *E. coli* DH5α. Colonies were transferred from LB agar plates containing ampicillin (100 $\mu g\,mL^{-1}$) to sterile 96-well microplates, and grown overnight at 37 °C in LB medium (100 µL; ampicillin, 100 $\mu g\,mL^{-1}$). Overnight culture was used fresh for further experiments, and remaining culture was stored in 50% (*v*/*v*) glycerol at −20 °C.

Saturation mutagenesis: The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) was used according to the manufacturer's instructions with complementary primers T230 (5′-C CAG ATC GTG CCG TTC GAG NNK ACC GGC AAA GTG GCG GCG G-3′) and cT230 (5′-C CGC CGC CAC TTT GCC GGT MNN CTC GAA CGG CAC GAT CTG G-3′). The mutant plasmids were transformed into supercompetent XL1-Blue cells, of which non transformed controls showed no hydrolysis of methyl-3-bromo-2-methylpropanoate by Quick E screening. Transformant colonies (175) were transferred from LB agar plates containing ampicillin (100 $\mu g\,mL^{-1}$) to 96-well microplates and grown overnight at 37 °C in LB medium (100 µL; ampicillin, 100 $\mu g\,mL^{-1}$). Overnight culture was used fresh for further experiments, and remaining culture was stored in 50% (*v*/*v*) glycerol at −20 °C. Similarly, saturation mutagenesis at D158 used primers D158-V (5′-GCG CAG TTC ATC AGC NNN TTC AAC GCA CCG TTC-3′) and D158-R (5′-GAA CGG TGC GTT GAA NNN GCT GAT GAA CTG CGC-3′) and mutagenesis at L181 used primers L181-V (5′-GTG CAG ACC CAG ACC NNN CAA ATC GCC CTG CTG-3′) and L181-R (5′-CAG CAG GGC GAT TTG NNN GGT CTG GGT CTG CAC-3′). Mutants (330 for D158x and 298 for L181X) were screened towards 3-phenylbutyric acid resorufin ester using fluorescence detection as in previous work.[3b]

Enzyme production in 96-well format: To hasten the process of protein expression and isolation and thus facilitate the screening of large numbers of enzymes, bacteria were cultured in 96-well assay blocks (Costar, Cambridge, MA, USA), where each well had a total available volume of 2 mL. Fresh overnight culture from microplates (10 µL) was added to each well containing LB medium (1 mL; ampicillin, 100 $\mu g\,mL^{-1}$), and the assay block was incubated at 37 °C and 325 rpm for approximately 3 h, after which time the OD_{600} was estimated to be 0.5. Protein expression was then induced by the addition of sterile rhamnose solution to each well (50 µL;

4% *w*/*v*), followed by incubation as above for 6 h. The assay blocks were centrifuged (10 min, 2700 × g, 4 °C), and the supernatant was removed. The cell pellet was resuspended in BES buffer (400 μL; 5 mM, pH 7.2; lysozyme, 0.4 mg mL^{-1}) and incubated at 37 °C and 325 rpm for 45 min followed by freezing at −20 °C. After thawing at room temperature, the lysed cells were treated with nucleases (RNase A, 10 μg mL^{-1}; DNase I, 1.7 μg mL^{-1}) for 15 min at 37 °C and 325 rpm, and then centrifuged (30 min, 2700 × g, 4 °C). The supernatant was used for further experiments. Quick E control experiments ensured that no detectable hydrolysis of methyl 3-bromo-2-methylpropanoate occurred due to the nucleases, lysozyme, or cell-free extracts of non-transformed DH5α.

Quick E screening: Enzymatic hydrolyses of pure enantiomers of esters were monitored colorimetrically in the presence of a reference compound as previously described.[8] Rates of hydrolysis of substrate (11 mM *R*-enantiomer or 1.1 mM *S*-enantiomer) and resorufin acetate (0.11 mM) in buffer solution (100 μL; BES, 5 mM, pH 7.2; Triton X-100, 0.33 mM; acetonitrile, 8% *v*/*v*; enzyme solution, 10% *v*/*v*) were determined from the change in absorbance at 404 and 574 nm as a function of time using a microplate reader.

Confirmation of enantioselectivity by endpoint method: Enantioselectivity of hydrolysis of (±)-methyl 3-bromo-2-methylpropanoate was calculated using the method of Chen et al.[25] from the enantiomeric excesses of both starting ester and esterified acid product determined by gas chromatography as previously described.[11c]

Homology models containing tetrahedral intermediate in active site: The primary amino acid sequence (excluding the His_6-tag) of the esterase from *Pseudomonas fluorescens* was submitted to the SWISS-MODEL automated homology modeling server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/swissmod/SM_FIRST.html)[29] and the results returned by e-mail. The homology model was based on 46–51% amino acid identity with non-heme haloperoxidases,[31] which have the α/β-hydrolase fold and exhibit low esterase activity. The quality of the model was judged to be acceptable based on the low energy of the backbone and side chain residues for the entire protein except for a few loops distant from the active site. The tetrahedral intermediate formed after nucleophilic attack by the catalytic serine (Ser95) on the carbonyl carbon of either methyl 3-bromo-2-methylpropanonate or ethyl 3-phenylbutyrate was built into the homology model using Sybyl version 6.6 (Tripos Software). The substrate was positioned such that the oxyanion was stabilized by two hydrogen bonds: one to the backbone N–H of Met96, and one to the backbone N–H of Trp29. The protonated catalytic His252 formed hydrogen bonds with both the Ser95 oxygen and the alcohol-leaving group of the substrate.

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