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# A Novel Genetic Selection System for Improved Enantioselectivity of *Bacillus subtilis* Lipase A

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*In directed evolution experiments, success often depends on the efficacy of screening or selection methods. Genetic selections have proven to be extremely valuable for evolving enzymes with improved catalytic activity, improved stability, or with altered substrate specificity. In contrast, enantioselectivity is a difficult parameter to select for. In this study, we present a successful strategy that not only selects for catalytic activity, but for the first time also for enantioselectivity, as demonstrated by the selection of Bacillus subtilis lipase A variants with inverted and improved enantioselectivity. A lipase mutant library in an aspartate auxotroph Escherichia coli was plated on minimal medium that was supplemented with the aspartate ester of the desired enantiomer (S)-(+)-1,2-O-isopropylidene-sn-glycerol. To inhibit growth of less*

*enantioselective variants, a covalently binding phosphonate ester of the opposite (R)-(–)-1,2-O-isopropylidene-sn-glycerol enantiomer was added as well. After three selection rounds in which the selection pressure was increased by raising the phosphonate ester concentration, a mutant was selected with an improved enantioselectivity increased from an ee of –29.6% (conversion 23.4%) to an ee of +73.1% (conversion 28.9%) towards the (S)-(+)-enantiomer. Interestingly, its amino acid sequence showed that the acid of the catalytic triad had migrated to a position further along the loop that connects  $\beta 7$  and  $\alpha E$ ; this shows that the position of the catalytic acid is not necessarily conserved in this lipase.*

## Introduction

Enzymes are powerful tools for the industrial production of pharmaceuticals and their intermediates. They are capable of performing highly regioselective and/or enantioselective reactions, and can accelerate reaction rates by orders of magnitude.<sup>[1,2]</sup> However, most applications of enzymes do not rely on the natural reactions they catalyze, but use non-natural substrates. Wild-type enzymes are often not optimal catalysts for these applications because of poor substrate specificity, low stability or insufficient (enantio-)selectivity for the cost-effective production of a particular product. In this respect, directed evolution has emerged as an important method to make enzymes more suitable as industrial biocatalysts.<sup>[3]</sup>

In directed evolution experiments the successful identification of improved variants strongly depends on the availability of effective screening or selection methods.<sup>[4]</sup> Screening is widely applied as a first selection tool; however, the probing of the enantioselectivity of lipases and esterases depends on time-consuming assays.<sup>[5]</sup> As an alternative, in vivo selection strategies can be used that exploit conditions that favor the exclusive survival of desired variants. Consequently, uninteresting variants are never seen, and larger mutant libraries can easily be evaluated, even up to sizes of  $10^{10}$  variants.<sup>[6,7]</sup>

A powerful selection method is based on linking cell survival to the desired enzymatic activity. The general strategy for such a selection system involves the introduction into the host cells of a stringent metabolic requirement for the desired activity. Plasmids that encode a mutant library of the protein of interest are introduced into the selection host. Next, selective growth conditions are imposed in such a way that only those cells

that express variants of the protein of interest with the desired activity are viable.<sup>[3,7,8]</sup>

Many examples of genetic selections for improved activity are available.<sup>[7–12]</sup> Often substrate mimics are used in the design of an in vivo selection method for a specific reaction to fulfill the metabolic requirement.<sup>[13]</sup> These mimics should be soluble in water, and they and their products should not interfere with the cellular environment.

Our aim is to develop an enantioselective lipase for the production of enantiopure 1,2-O-isopropylidene-*sn*-glycerol (IPG), a precursor in the synthesis of  $\beta$ -adrenoceptor antagonists. Previously, we have shown that lipase A (LipA) of *Bacillus subtilis* 168 is capable of hydrolyzing butyrate esters of IPG, although hardly any enantiopreference was observed (ee value of –20.3% for (R)-(–)-IPG).<sup>[14]</sup> Since the desired enantiomer is (S)-(+)-IPG, we decided to apply directed evolution to develop

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a LipA variant with a high (inverted) enantioselectivity towards (S)-(+)-IPG. The three-dimensional structure of LipA<sup>[15]</sup> suggested that residues 132 to 136 would be good targets for a directed evolution approach even though Asp133 is one of the catalytic triad residues. These residues were mutated and the mutant library was transformed to *E. coli* K-12 PA340/T6, a strain in which both pathways for aspartate synthesis have been knocked out.<sup>[16]</sup> The *E. coli* knock-out was plated onto selective minimal medium plates, supplemented with enantiopure (S)-(+)-IPG that had been esterified to L-aspartate. Only those LipA variants expressed in the periplasm that could hydrolyze this aspartate ester were able to provide the aspartate that was needed for bacterial growth. To avoid growth of bacteria that express less enantioselective variants, the stringency of the selection was increased by adding a phosphonate ester of the undesired IPG enantiomer to covalently inhibit variants with some activity against the (R)-(-)-IPG enantiomer. Mutants with an improved enantioselectivity towards (S)-(+)-IPG were selected. This selection strategy for improved enantioselectivity might be generally applicable as well to other hydrolases that are expressed in the periplasm.

## Results and Discussion

### Construction of the mutant library

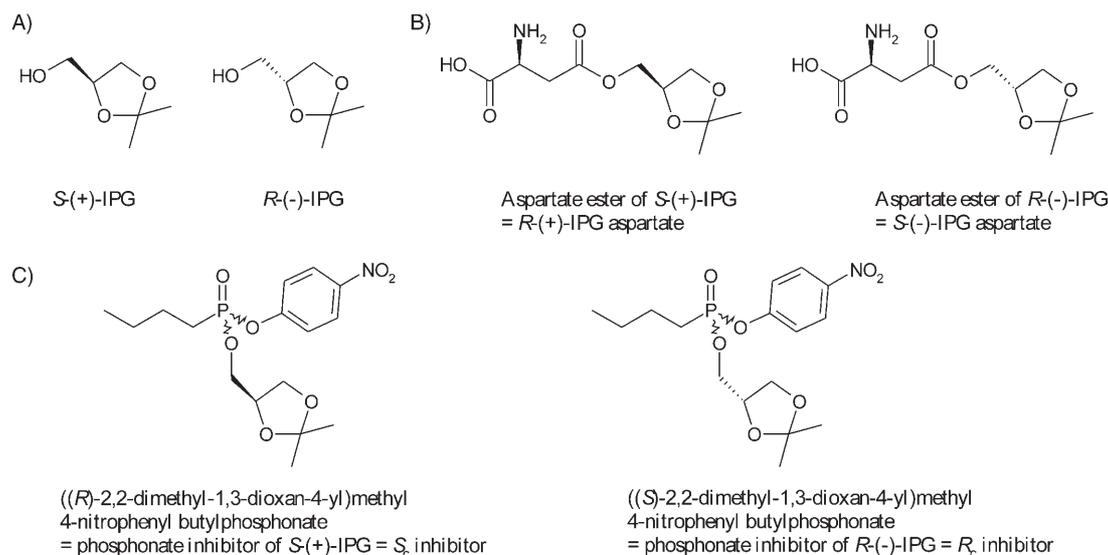
Cassette mutagenesis was applied to construct a saturated mutant library directed towards amino acids 132 to 136 of mature LipA. The primer sequences resembled the *lipA* sequence by approximately 80%. Sequence analysis of 13 clones revealed that the percentage of both single and double-mutated amino acids was 31%. Two clones showed deletions, while two other clones had no amino acid mutation. After transformation of *E. coli* TG-1, a mutant library that consisted of  $3.5 \times 10^4$  colonies was obtained. This should be more than sufficient

to obtain all possible single mutations at the amino acid level. This library was used for the transformation of the aspartate auxotroph *E. coli* K-12 PA340/T6.

### Dual selection

To examine the functionality of the IPG aspartate substrate mimic, its hydrolysis by purified wild-type LipA was determined in the IPG assay and compared with the hydrolysis of the substrate of interest IPG butyrate. The enzyme converts both substrates to a similar degree: 29.4% for IPG butyrate versus 21.8% for the aspartate ester. Wild-type LipA hydrolyzes the commonly used substrate IPG butyrate with an enantiomeric excess (*ee*) of  $-12.9\% \pm 1.6$  of the (R)-(-)-enantiomer. IPG aspartate is also hydrolyzed, with a somewhat lower enantiomeric excess of  $-5.5 \pm 1.1\%$  towards the (R)-(-)-enantiomer. No statistically significant difference was observed in enantioselectivity towards the substrates ( $p < 0.001$ ). These results indicate that IPG aspartate can be used as a mimic substrate.

To introduce a dual selection step, soluble IPG phosphonate inhibitors were used (Scheme 1). To assess the inhibitory effect of the IPG phosphonate esters, an inhibition assay was performed by using purified wild-type LipA according to ref. [17]. The  $t_{50}$  of inhibition was 10 min for both enantiomers. Furthermore, transformed wild-type LipA was plated on minimal medium supplemented with the IPG phosphonate ester and on LB agar plates. The latter showed approximately 2500 colonies after overnight incubation, while only a few small colonies appeared on the phosphonate plates after 5 days of incubation. These results indicate that the phosphonate inhibitor limits rather than completely abolishes growth. This allows the use of phosphonate inhibitors in directed evolution experiments.



**Scheme 1.** A) Chemical structure of 1,2-O-isopropylidene-*sn*-glycerol (IPG). B) Chemical structure of the aspartate ester of IPG. C) Chemical structure of the soluble phosphonate inhibitors. Note that the inhibitor is enantiopure at the chiral atom of the IPG molecule and racemic at the phosphorous atom. The absolute configuration of IPG changes upon attachment to the phosphorous atom or upon ester bond formation.

### Selection on selective minimal medium

*E. coli* K-12 PA340/T6-competent cells were transformed with the mutant library and, as a control, with wild-type LipA. Approximately 2500 colonies appeared per plate after overnight incubation on LB agar plates. On plates supplemented with either aspartate or the aspartate ester, approximately 1500 colonies appeared after two days. On plates supplemented with the phosphonate inhibitor in addition to the aspartate ester, colonies also appeared after two days, although their number was reduced to approximately 1000 colonies. Negative control plates showed approximately 100 small colonies after 5 to 10 days of incubation. After the second round of selection, approximately 750 colonies appeared on plates supplemented with both the inhibitor and the aspartate ester, while after the third round approximately 400 colonies were found.

At the end of each round after two days of growth, the 50 largest colonies were chosen from the plates supplemented with both the aspartate ester and the phosphonate inhibitor, and their LipA proteins were isolated from the periplasmic fraction. Table 1 shows the catalytic activities, enantioselectivities (expressed as enantiomeric excess *ee* and *E* value) and the mutations that were introduced into the variants.

The first round of selection was designed in such a way that conditions were not too stringent. All selected variants showed an (inverted) enantioselectivity towards (S)-(+)-IPG. However, under these mild conditions not many variants with a significantly improved enantioselectivity (*ee* > +30%) were found. Ultimately, two more improved variants were sequenced; one variant had only one mutation, I135F, while the other variant had mutations A132P/M134Q.

The enantioselectivity of the best variant (A132P/M134Q) increased in subsequent selection rounds with higher selection pressure, from an *ee* value of +34% after the first round, to an *ee* value of +73% after the third selection round. Sequence analysis revealed that all 5 residues in the region of 132 to 136 were prone to mutation, and residue 134 showed the highest mutation frequency.

Some colonies were picked from the plates that were supplemented with (S)-(–)-IPG aspartate ester as well; these had activities and enantioselectivities that were comparable to those of wild-type LipA (conversion approximately 20%, *ee* approximately –25% towards (R)-(–)-IPG).

Interestingly, the catalytic residue Asp133 was prone to mutation as well, without apparent effect on the viability of the clones. Therefore, the mutant found in the third selection round, D133A/V136D, was subjected to further analysis.

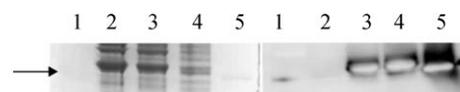
**Table 1.** Enantioselective hydrolysis of racemic esters of IPG butyrate by periplasmic fractions of selected LipA variants (*n* = 3).

Variant	Conversion (%)	<i>ee</i> (%)	<i>E</i> <sup>[a]</sup>	Enantiomer formed in excess	Mutation
WT LipA	23.4 ± 1.1	–29.6 ± 0.5	1.8	(S)-(–)-IPG	
1	36.6 ± 1.0	+34.4 ± 0.7	2.4	(S)-(+)-IPG	A132P, M134Q
2	6.7 ± 0.6	+43.2 ± 0.8	2.6	(S)-(+)-IPG	I135F
3	7.2 ± 2.5	+68.7 ± 0.9	5.7	(S)-(+)-IPG	D133N
4	15.2 ± 1.2	+62.5 ± 1.2	4.9	(S)-(+)-IPG	A132T, M134T
5	21.2 ± 0.9	+55.7 ± 1.0	4.1	(S)-(+)-IPG	D133E, M134R
6	19.7 ± 0.8	82.5 ± 2.5	12	(S)-(+)-IPG	D133G, M134L, I135N
7	23.4 ± 1.3	61.5 ± 1.8	5.1	(S)-(+)-IPG	D133Q, M134L, I135T
8	28.9 ± 0.5	73.1 ± 0.7	8.5	(S)-(+)-IPG	D133A, V136D

[a] *E* values were calculated by using the programme *Selectivity* by K. Faber and H. Hoenig, <http://www.orgc.tugraz.at/>

### Characterization of the D133A/V136D mutant

The expression of variant D133A/V136D in the periplasm was examined by SDS-PAGE and western blot (Figure 1), and was found to be similar to wild-type LipA. D133A/V136D had a



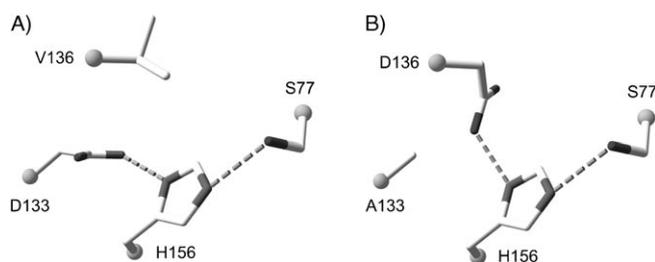
**Figure 1.** Determination of the expression levels of wild-type LipA and variant D133A/V136D. Left panel: Expression of wild-type LipA (lane 3) and variant D133A/V136D (lane 4) in the periplasm of *E. coli* HB2151 on SDS-PAGE (12%). An empty *E. coli* HB2151 strain (lane 2) was taken as a control, as well as purified lipase (lane 5). The arrow indicates lipase; note that lipase in the periplasmic fractions runs slightly higher on the gel due to a (His)<sub>6</sub>-tag. The marker is shown in lane 1. Protein (25 µg) was loaded onto the gel. Right panel: Expression of wild-type LipA (lane 3) and variant D133A/V136D (lane 4) in the periplasm of *E. coli* HB2151 on western blot (detection by using αLipA). An empty *E. coli* HB2151 strain (lane 2) was taken as a control, as well as purified lipase (lane 5). The marker is shown in lane 1.

catalytic activity that was comparable to that of the wild-type enzyme (conversion of 28.9% versus 23.4% of wild-type LipA). In contrast, its *K<sub>M</sub>* for (S)-(+)-IPG was significantly lowered, and its *K<sub>M</sub>* for (R)-(–)-IPG was significantly increased (Table 2, *p* < 0.05); this resulted in an improved enantioselectivity with an *ee* of 73.1% ± 0.7 towards (S)-(+)-IPG (*E* value of 8.5).

An intriguing question is why the mutation of Asp133, one of the catalytic triad residues, to an alanine does not abolish catalytic activity. Inspection of the 3D structure of LipA,<sup>[15]</sup>

**Table 2.** *K<sub>M</sub>* determination of variant D133A/V136D (*n* = 3)

Enzyme	<i>K<sub>M</sub></i> [mM] butyrate ester of	
	(–)-IPG	(+)-IPG
WT LipA	0.53 ± 0.33	1.05 ± 0.10
LipA D133A/V136D	1.91 ± 0.41	0.36 ± 0.22
Statistical significance of differences: <i>p</i> < 0.05.		



**Figure 2.** Model of wild-type LipA compared to mutant D133A/V136D. A) Positioning of catalytic residues in wild-type LipA. B) Positioning of catalytic residues in variant D133A/V136D, in which the acid is repositioned.

shows that the role of Asp133 might be taken over by the aspartate introduced at position 136 in the D133A/V136D variant (Figure 2).

To test this hypothesis, the double mutant D133AV136A was constructed and expressed in the periplasm of *E. coli* HB2151. The mutant appeared to have no activity on IPG butyrate. In contrast, changing residue 136 into an aspartate, as in the D133A/V136D double mutant, restores activity; this demonstrates that Asp136 can take over the role of Asp133. In the case of mutant D133A/V136D, the alanine at position 133 is unable to stabilize the positive charge of His156. Since the double mutant does show appreciable activity, it is likely that the aspartate residue that was introduced at position 136 takes over the role of Asp133. Modeling shows that indeed an aspartate residue at position 136 can interact with His156 in such a way that the His156 side chain can also still interact with the Ser77 side chain (Figure 2B).

## Conclusions

In the past, genetic selections such as presented here have been successful in obtaining enzymes with improved catalytic activity, increased stability, or altered substrate specificity. Yet, enantioselectivity has proven to be a difficult property to select for. Some indirect screening methods for enantioselectivity based on bacterial growth have been described.<sup>[18–20]</sup> However, these screening methods involve the individual examination of variants, and are therefore cumbersome and often time consuming. In contrast, selection strategies encompass the generation of a large number of variants of which only the best ones are retained, thus the amount of work involved in the characterization of the obtained variants is reduced. To be able to select directly for enantioselectivity in addition to catalytic activity, we have developed a novel genetic selection system that allows the analysis of larger libraries compared to the previously described indirect screening methods. Moreover, the addition of a phosphonate ester of the undesired enantiomer, which covalently inhibits the enzyme reduces the number of uninteresting variants.

Our selection system is based on the growth of an aspartate auxotroph *E. coli* strain; the imposed selection pressure is increased by raising the concentration of the phosphonate inhibitor. After the third round, no further improvement of the enantioselectivity was observed, possibly because of the small

library of only five randomized amino acids. The variant with the highest enantioselectivity was found in the second selection round (D133G/M134L/I135N). It showed an *ee* of +82.1% (*E* value of 12), but its activity was less than that of wild-type LipA (conversion of 19.7% versus 23.4% for LipA), and, therefore, it was not further analyzed. The best mutant from the third selection round was variant D133A/V136D. It combines a 20–25% enhanced conversion rate with an enantioselectivity that is improved from an *ee* value of –29.6% for the wild-type enzyme, to an *ee* of +73.1% for the desired substrate. It is likely that by using larger libraries or by applying DNA shuffling<sup>[21]</sup> or CASTing<sup>[22]</sup> variants with even higher enantioselectivity might be generated.

A surprising result of our work was the finding that the catalytic triad residue Asp133 became mutated as well (to Asn or Glu), without completely destroying the catalytic activity of the enzyme. Nevertheless, these mutations are not unique. In phospholipase A<sub>2</sub> it has also been shown that an Asn residue can take over the role of the Asp as a catalytic acid,<sup>[23]</sup> and a Glu instead of an Asp is the catalytic acid in some other  $\alpha/\beta$  hydrolases.<sup>[24]</sup> Thus some variability is possible with respect to the nature of the acidic catalytic residue without a drastic effect on the activity of the enzyme.

However, not only the nature of the acidic catalytic residue can vary in the  $\alpha/\beta$  hydrolase fold enzymes, but also its topological location. While the nucleophile and the general base histidine are located after  $\beta$ -strand 5 and  $\beta$ -strand 8, respectively, the acidic catalytic residue has in some cases been found after strand  $\beta$ 6 instead of after  $\beta$ 7.<sup>[25]</sup> In this work, two variants were obtained with another topological variation of the catalytic acid, which moved by two or three sequence positions. The significant activity of mutants D133G/M134L/I135N and D133A/V136D suggests that loop 133–136 has sufficient plasticity to stabilize a productive conformation of the His156 side chain. In the D133A/V136D mutant, the absence of a large side chain at position 133 allows an Asp at position 136 to easily assume a side-chain rotamer conformation that brings it within hydrogen-bonding distance of the His156 side chain. No major conformational changes are required, although a slight readjustment of the His156 side chain to improve its interaction with Asp136 cannot be excluded. In the D133G/M134L/I135N mutant, a productive interaction with the His side chain can also be modeled, although the required conformational changes are somewhat larger. So far, this positional variation seems only to occur in LipA; a one-to-all fit<sup>[26]</sup> with D133A/V136D as a search model against other  $\alpha/\beta$  hydrolase fold enzymes did not reveal any other enzymes with such a repositioned catalytic acid as in D133A/V136D.

Both variants have an improved enantioselectivity towards (S)-(+)-IPG. It is, however, difficult to find a possible molecular explanation for this improved enantioselectivity. From the 3D structure of wild-type *B. subtilis* LipA, it is clear that the active-site cleft is relatively open, and both IPG enantiomers can be accommodated equally well.<sup>[14]</sup> Mutation of residues 133–136 might lead to some adaptation of the His156 side-chain orientation, but the exact effect on the binding of the two IPG enantiomers remains hard to assess in the absence of detailed

structural and dynamic information on the mutants and their interaction with the IPG enantiomers.

The genetic-selection system as presented here has led to the successful identification of improved LipA variants. A disadvantage of the system could be the required translocation of the protein to the periplasm of the *E. coli* auxotroph, which is required in order to convert the substrate mimic. Theoretically, this might limit a broad applicability of this method. We have, however, recently demonstrated that even an intracellular carboxyl esterase (CesA) can be translocated to the periplasm solely by inserting a specific signal sequence.<sup>[27]</sup> CesA is also active on IPG-aspartate esters, and thus the same selection strategy as described here might be applied to CesA as well. In summary, the presented growth selection system might be generally applicable in the selection of more enantioselective hydrolase variants that can be translocated to the periplasmic space.

In conclusion, a novel bacterial growth system was developed that is suitable for the enantioselective selection of hydrolytic enzymes. The applicability of this strategy was demonstrated by the selection of lipase variants with an inverted and improved enantioselectivity. Since this system can be applied for the enantioselective selection of variants of other hydrolases as well, we believe we have established a strategy that is generally applicable, and that offers a new perspective for the evolution of enzyme enantioselectivity.

## Experimental Section

**Plasmids, bacterial strains and media:** *E. coli* K-12 PA340/T6 (thr-1, leuB6(Am), fhuA2, lacY1, glnV44(AS), gal-6,  $\lambda^-$ , gdhA1, hisG1(Fs), rfbD1, galP63,  $\Delta$ (gltB-gltF)500, rpsL9, malT1( $\lambda^R$ ), xylA7, mtlA2,  $\Delta$ argH1, thi-1) was kindly provided by the *E. coli* Genetic Stock Center (Yale University, New Haven, USA). *E. coli* HB2151 (K12  $\Delta$ (lac-pro), ara, nalr, thi/F', proAB, laqI, lacZ $\Delta$ -M15) and pCANTAB 5E were purchased from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden). 2xTY medium, which contained Bactotrypton (1.6% v/w), Bacto yeast extract (1% v/w) and NaCl (0.5% v/w) was used. As antibiotic agents ampicillin (100  $\mu$ g mL<sup>-1</sup>) and streptomycin (100  $\mu$ g mL<sup>-1</sup>); Duchefa Biochemie, Haarlem, The Netherlands) were used. M8 minimal medium, which contained Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (4 g L<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (15 g L<sup>-1</sup>) and NaCl (2.5 g L<sup>-1</sup>) was used.

**Chemicals:** The aspartate and butylphosphonate esters of both IPG enantiomers were synthesized by Syncom BV (Groningen, The Netherlands; Scheme 1). Butyrate esters of both IPG enantiomers were kindly provided by Prof. M. T. Reetz (Max-Planck Institut für Kohlenforschung, Mülheim, Germany). Supplemental amino acids (Thr, Arg, Leu, His, Asp), thiamine, MgSO<sub>4</sub> and CaCl<sub>2</sub> were purchased from Sigma–Aldrich.

**Construction of the mutant library:** The LipA-encoding gene was cloned into the phagemid pCANTAB 5E, downstream of a modified g3p signal sequence, as described previously.<sup>[17]</sup> To introduce a XbaI restriction site, a silent mutation was introduced at base pair position 423 of the *lipA* gene by using LipXbaIFor (Life Technologies, Paisley, UK): 5'-TTACTTATCTAGATTAGATGGTGCTGA-3' and LipXbaIRev: 5'-CTAGCACCATCTAATCTAGATAAGTAA-3' as primers. The XbaI restriction site is indicated in bold italics. To construct the mutant library in the region of residues 132 to 136, the following primers were used: CanHindFor (Life Technologies, Paisley, UK) 5'-

CCATGATTACGCCAAGCTTTGGAGCC-3' (HindIII restriction site in bold italics) and 00B470Rev (Eurogentec, Groningen, The Netherlands) 5'-TAATCTAGATAAGTAATTCAT867665765657887ACTGCTGTAATGGA-3' (XbaI restriction site in bold italics; 5=80% T; 6=80% A; 7=80% C; 8=80% G; the remaining 20% is an equal mixture of the other three bases). Recombinant DNA procedures were carried out as described by Sambrook et al.<sup>[28]</sup> Plasmid DNA was prepared by using the Qiaspin miniprep kit (Qiagen) and DNA purification was performed by using the Qiaquick gel extraction kit (Qiagen). The library was amplified from *B. subtilis* 168 chromosomal DNA as a template by using *Pfu* polymerase (Stratagene). The gene fragment was digested with HindIII and XbaI (New England Biolabs, Ipswich, MA, USA), and cloned in *E. coli* TG-1 into the HindIII and XbaI sites of the pCANTABLip-CH plasmid.<sup>[17]</sup> The obtained mutant library was sequenced to assess the mutation ratio. The LipA variant D133A/V136A was constructed by using Quikchange® PCR (Stratagene) with the following primers: LipDAVAFor 5'-CATTACAGCAGTCCGCAATGATTGCCATGAATTACTTATCCAGA-3' (Operon, Cologne, Germany) and LipDAVARev 5'-TCTGGATAAGTAATTCATGGCAATCATTGCGGCACTGCTGTAATG-3'.

**Selection on selective minimal medium:** *E. coli* K-12 PA340/T6 cells were made chemically competent and transformed with mutant plasmid DNA and wild-type LipA (50 ng).<sup>[28]</sup> A negative control was investigated as well. Transformation mixtures were starved by incubation in 0.9% NaCl for 2 h at 37 °C, and plated onto selective M8 minimal medium agar (1.6%) plates. The medium was supplemented with MgSO<sub>4</sub> (1 mM), CaCl<sub>2</sub> (0.1 mM), essential amino acids (Thr, His, Arg, Leu; 20  $\mu$ g mL<sup>-1</sup>) and thiamine (1  $\mu$ g mL<sup>-1</sup>). The aspartate ester of (*R*)-(-)-IPG or (*S*)-(+)-IPG (1.5 mM) was added to the agar plates as the sole aspartate source. To select for enantioselective variants, butylphosphonate ester of the undesired IPG enantiomer (1.5 mM) was added to the medium as well to eliminate nonselective variants or variants that were selective for the opposite enantiomer. Approximately 2500 viable *E. coli* K-12 PA340/T6 cells that contained the mutant plasmids were plated onto each plate. We used LB, Asp (20  $\mu$ g mL<sup>-1</sup>; Sigma) and Min (no aspartate or esters present) as control plates. Plates were incubated at 30 °C. Upon appearance 50 colonies were picked, and their periplasmic fraction was isolated and used in the 1,2-*O*-isopropylidene-*sn*-glycol ester assay as described below. The remaining colonies were harvested and plated again onto selective minimal medium with an increased concentration of phosphonate ester (3 mM) for a second round of selection. For a third round of selection, the phosphonate ester concentration was increased to 7.5 mM. The plasmid DNA was isolated from bacteria that express a lipase variant with an inverted enantioselectivity, and sequenced to examine the mutation(s).

**Isolation of the periplasmic fraction:** *E. coli* K-12 PA340/T6 was grown in 50-mL tubes that contained 2xTY medium (10 mL), ampicillin and isopropyl- $\beta$ -D-galactopyranoside (IPTG, 1 mM). The tubes were incubated at 37 °C at 250 rpm for 16 h. The OD<sub>600</sub> was recorded and the cells were harvested and resuspended in Tris–HCl buffer (10 mM, pH 7.4). After centrifugation, the cells were resuspended in buffer (200  $\mu$ L) that contained Tris–HCl (10 mM, pH 8.0), 25% (v/w) sucrose, ethylene diamine tetraacetic acid (EDTA, 2 mM) and lysozyme (0.5 mg mL<sup>-1</sup>, Sigma–Aldrich). After incubation on ice for 20 min, buffer (50  $\mu$ L) that contained Tris–HCl (10 mM, pH 8.0), 20% (v/w) sucrose and MgCl<sub>2</sub> (125 mM) was added. The suspension was centrifuged and the supernatant that contained the periplasmic fraction was isolated and used as the enzyme solution in the IPG ester assay. The protein content of this fraction was determined by using the Bradford assay in triplicate with bovine serum albumin (BSA) as a standard (Pierce, Rockford, Illinois, USA).

**1,2-O-Isopropylidene-sn-glycerol ester assay:** Periplasmic fractions were diluted with 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (0.07 M, pH 7.5), that contained 0.2% (*v/w*) BSA, to a final volume of 100  $\mu$ L. References were diluted correspondingly, but contained no enzyme solution. The esters of IPG (1 mM) were dissolved in MOPS buffer (10 mL, 0.07 M, pH 7.5), that contained Tween 80 (14.3% *v/w*), and diluted to 50 mL with MOPS buffer. Substrate solution (500  $\mu$ L) was added to the enzyme solution and the final mixture was incubated in a water bath at 32 °C. After 16 h incubation, a saturated NaCl solution (400  $\mu$ L) and internal standard solution (10  $\mu$ L, racemic hex-3-en-1-ol, 5 mg mL<sup>-1</sup> in assay buffer) were added to the sample solution and the aqueous solution was extracted twice with ethyl acetate (1 mL). GC analysis was performed as described by Dröge et al.<sup>[29]</sup> Enantiomeric excesses, *ee*, and the optical purities of the product fractions were calculated according to Chen et al.<sup>[30]</sup> All data were expressed as mean  $\pm$  sd. Enantioselectivities *E* were calculated by using the program Selectivity (K. Faber, H. Hoenig. <http://www.orgc.TUGraz.at>). The statistical significance of the differences in enantiomeric preference was tested at a significance level of  $p < 0.05$  by using a two-tailed Student's *t*-test.

**Determination of  $K_M$ :** Because of higher expression levels, *E. coli* HB2151 was used in this case for overexpression and was grown for 16 h at 37 °C in 2xTY medium (1 L) that was supplemented with ampicillin (100  $\mu$ g mL<sup>-1</sup>) and 1 mM IPTG. The periplasmic fraction was isolated as described above. The total protein content was determined by performing a Bradford assay. To determine the expression level of LipA and variant D133A/V136D, SDS-PAGE was performed on a 12% separating and a 3% stacking gel (Invitrogen, Groningen, The Netherlands). Molecular mass markers were purchased from Invitrogen. After electrophoresis, proteins were blotted to nitrocellulose and immunostained with a rabbit antiserum against LipA. Detection of the antibody was performed with horse radish peroxidase (HRP)-conjugated antibodies against rabbits. To obtain a  $K_M$  value, the IPG assay was performed in triplicate. The  $K_M$  value for each enantiomer was obtained by fitting the experimental data from Eadie-Hofstee plots. The mean and sd of three measurements were calculated.

**Structural alignment of variant D133A/V136D with other  $\alpha/\beta$  hydrolase fold enzymes:** In variant D133A/V136D, the position of the catalytic acid was shifted towards the end of the loop that connects  $\beta$ 7 and  $\alpha$ E. To examine whether this was a unique feature within the family of  $\alpha/\beta$  hydrolases, a one-to-all fit was made by using TOPOFIT.<sup>[26]</sup> This method analyzed the similarity in protein structure by using 3D Delaunay triangulation patterns that were derived from a backbone representation.<sup>[31]</sup> A structural alignment of the repositioned catalytic site was made by using the SwissProt PDB viewer. The structures were superimposed by using the catalytic His residue and the C $\alpha$  and C $\beta$  atoms of the other two catalytic residues; the final overlay figure was constructed by using PyMOL version 0.99 (DeLano Scientific, Palo Alto, CA, USA).

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