A NOVEL SELECTION SYSTEM FOR ENANTIOSELECTIVITY OF *Bacillus subtilis* Lipase A based on Bacterial Growth

Ykeliën L. Boersma, Melloney J. Dröge, Almer M. van der Sloot, Tjaard Pijning, Bauke W. Dijkstra & Wim J. Quax

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A GROWTH SELECTION SYSTEM FOR \textit{B. subtilis} LIPASE A

A novel selection system for enantioselectivity of \textit{Bacillus subtilis} Lipase A based on bacterial growth

In directed evolution experiments, success strongly depends on the availability of screening or selection methods. Genetic selections have been rated extremely valuable for evolving enzymes with improved catalytic activity, improved stability, and altered substrate specificity. However, enantioselectivity is a difficult parameter to select for. In this study, we present a novel selection strategy which not only selects for catalytic activity, but for the first time for enantioselectivity as well. An \textit{Escherichia coli} aspartate auxotroph was transformed with a mutant library of \textit{Bacillus subtilis} lipase A and plated onto selective minimal medium, supplemented with an ester of aspartate coupled to the desired enantiomer of interest \textit{S-(+)-1,2-O-isopropylidene-sn-glycerol} (IPG). To develop a dual selection tool, a phosphonate ester of the opposite IPG enantiomer was added to the selective minimal medium as well to inhibit growth of less enantioselective variants. Three rounds of selection were imposed, in which selection pressure was increased by raising the phosphonate concentration. Mutants with an inverted and improved enantioselectivity towards \textit{S-(+)-IPG} were selected. One variant, D133AV136D, was of particular interest, as the catalytic acid had migrated to a position further along the loop connecting \textit{β7} and \textit{αE}. This variant was further characterised.

Introduction

Biocatalysis has emerged as a powerful tool for the industrial synthesis of pharmaceuticals and their intermediates.\textsuperscript{246} Enzymes are capable of performing complex regioselective and/or enantioselective reactions and can accelerate reaction rates by enormous factors. High enzymatic selectivity also results in efficient reactions with few by-products, thus making enzymes an environmentally friendly alternative to conventional chemical catalysis\textsuperscript{10,13}. To date, successful applications of biocatalysts in industrial processes have been largely confined to hydrolytic enzymes such as lipases and esterases\textsuperscript{15,16}.

Most applications of enzymes do not rely on the natural reaction catalysed by them, but rather concern non-natural substrates. However, wild type enzymes often show poorly compatible substrate specificity, poor stability or insufficient (enantio-) selectivity for the cost-effective production of a particular product. In this respect, directed evolution has emerged as an important means for the improvement of nature’s catalysts to make them more suitable as industrial biocatalysts. This technique is essentially composed of two steps: first, mutagenesis of the gene(s) encoding the enzyme(s) and, second, identification of desired biocatalyst variants within these mutant libraries by screening or selection\textsuperscript{246}. Thus, the success of directed evolution experiments often depends on the choice of diversity-generation methods and the availability of screening or selection methods\textsuperscript{247}.

The identification of improved variants by high-throughput screening or selection requires high-quality substrates and assays as the selectivity obtained with a surrogate substrate can...
differ significantly from that towards the real substrate, leading to false-positive variants\textsuperscript{248}. For many enzymes however, such as lipases and esterases, no high-throughput screening methods are available. Especially the probing of the enantioselectivity of lipases and carboxyl esterases depends on time-consuming assays\textsuperscript{249}. A major improvement can be obtained if selection instead of screening can be introduced. The main advantage of selection over screening is that many more variants in the library can be analysed simultaneously. Selection strategies exploit conditions favouring the exclusive survival of desired variants; consequently, uninteresting variants are never seen. Thus, selecting enzyme variants is much faster and can be carried out with higher throughput: these strategies allow for the evaluation of larger libraries of mutants, even as large as $10^{10}$\textsuperscript{11,250}.

A powerful method for selection is based on catalytic potency reflected in bacterial growth. \textit{In vivo} selection links cell survival to enzymatic activity. The general strategy for this genetic selection involves the introduction of a metabolic requirement for the desired activity into the host cells. Plasmids, encoding for a mutant library of the protein of interest, are introduced into a suitable host for selection, preferably a mutant strain of a well-characterised bacterium, such as \textit{Escherichia coli}. Selective conditions for the target function of the protein encoded by the plasmid are imposed in such a way that only those cells expressing variants with the desired phenotype are viable (figure 1)\textsuperscript{74,246,250}.

![Figure 1: General strategy for selection based on bacterial growth. A compound is taken up by the bacterium and converted by the expressed enzyme to the essential nutrient.](image)

Often, mimic substrates supplemented to the minimal medium are required to allow an \textit{in vivo} selection to be designed for a specific reaction. This approach might be especially useful to generalise the use of \textit{in vivo} selections to most chemical reactions and to the reactions yielding non-natural products\textsuperscript{251}. These substrates should be soluble in aqueous solutions as cell growth is generally not possible in mixtures of organic solvents with aqueous solutions. Interference of substrates and products with the cellular environment should also be avoided as much as possible. Many examples of genetic selections have been described previously\textsuperscript{74,81,92,250,252,253}. 


Our aim was to develop a novel growth selection system in which variants were not only selected on the basis of their catalytic potency, but for the first time on their enantioselectivity as well. We chose to study lipase A (LipA) of Bacillus subtilis 168, a hydrolytic enzyme that was characterised in detail. LipA can be expressed in the periplasm of the auxotroph E. coli K-12 PA340/T6, a bacterium in which both pathways for aspartate synthesis have been knocked out. It was previously established that LipA is capable of hydrolysing acetate, butyrate and caprylate esters of IPG, a precursor in the synthesis of β-adrenoceptor antagonists, although hardly any enantioselective preference was observed (ee ranging from 20.3% to 73.2% for (-)-IPG). The ultimate goal was to improve and moreover invert the enantioselectivity towards the wanted enantiomer S-(+)-IPG. Therefore, we mutated residues 132 to 136; D133, one of the amino acids of the catalytic triad, is within this region and therefore this stretch of amino acids might be of particular interest for the catalytic activity and enantioselective properties of the enzyme. We transformed the mutant library to the E. coli aspartate knock-out and plated it onto selective minimal medium plates, supplemented with an ester of enantiopure IPG coupled to aspartate (scheme 1). The expressed LipA variant had to hydrolyse this aspartate ester to release aspartate for uptake and subsequent bacterial growth. To avoid growth of bacteria expressing less enantioselective variants, a dual selection step was introduced, in which a phosphonate ester of the undesired IPG enantiomer was added to the minimal medium. These phosphonates bind covalently to the active site serine of the lipase, thus mimicking the transition state in ester hydrolysis. By using the crystal structure of wild type LipA, models were made to provide insight in the structural rearrangements explaining the altered enantioselectivity of LipA mutants.

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 lipase inhibitors. Note that the inhibitor is enantiopure at the chiral atom of the IPG molecule (Sc or Rc inhibitor), and racemic at the phosphorous atom (mixture of Rp and Sp inhibitor). The absolute configuration of IPG changes upon attachment to the phosphorous atom or upon ester bond formation.
Experimental procedures

Plasmids, bacterial strains and media

E. coli K-12 PA340/T6 (thr-1, leuB6(Am), lacY1, glnV44(AS), gal-6, λ-, gdhA1, hisG1(Fs), rfbD1, galP63, Δ(gltB-gltF)500, rpsL9, malT1(λR), xylA7, mtlA2, ΔargH1, thi-1) was kindly provided by the E. coli Genetic Stock Center (Yale University, New Haven, USA). pCANTAB 5E was purchased from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden). 2xTY medium contained: Bactotrypton (1.6% w/v), Bacto yeast extract (1% w/v) and sodium chloride (0.5% w/v). As antibiotic agents ampicillin (100 µg.mL⁻¹) and streptomycin (100 µg.mL⁻¹) (Duchefa Biochemie, Haarlem, The Netherlands) were used. M9 minimal medium contained Na₂HPO₄.7H₂O (4 g. L⁻¹), KH₂PO₄ (15 g.L⁻¹), and sodium chloride (2.5 g.L⁻¹).

Chemicals

Both enantiomers of the aspartate esters of IPG as well as both enantiomers of the butyl phosphonate esters of IPG were synthesised by Syncom BV (Groningen, The Netherlands). Butyrate esters of both enantiomers of 1,2-O-ispropylidene-sn-glycerol (IPG) were kindly provided by Prof. M.T. Reetz (Max-Planck Institut für Kohlenforschung, Mülheim, Germany). p-Nitrophenyl caprylate was purchased from Sigma Chem. Co. (Axel, The Netherlands). Supplemental amino acids (Thr, Arg, Leu, His, Asp), thiamine, MgSO₄ and CaCl₂ were purchased from Sigma-Aldrich (Steinheim, Germany).

Construction of the mutant library

The LipA encoding gene was cloned in the phagemid pCANTAB 5E, downstream of a modified g3p signal sequence, as described previously 103. To introduce an XbaI restriction site in the lipA gene, a silent mutation at base pair position 423 of the lipA gene was introduced using primers LipXbaIFor (Life Technologies, UK):

5' – TTACTTA\textsc{tctaga}TTAGATGGTGCTGA – 3' and LipXbaIRev:
5' – CTAGCACCATCTAA\textsc{tctaga}TAAGTAA – 3'. The XbaI restriction site is indicated in bold italics. To construct the mutant library on the region of amino acid 132 to 136, the following primers were used: CanHindFor (Life Technologies, UK) and 00B470Rev (Eurogentec, Groningen, The Netherlands):
5' – CCATGATACGCG\textsc{aagc}TTGGAGCC – 3' (HindIII restriction site in bold italics) and 00B470Rev (Eurogentec, Groningen, The Netherlands)
5' – TAAT\textsc{tctaga}AAGTATCTAT8676657657887ACTGCTGTAAATGGA – 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 176. Plasmid DNA was prepared using the Qiaspin miniprep kit (Qiagen, Hilden, Germany) and DNA purification was performed using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). The library was amplified from chromosomal DNA of B. subtilis 168 using Pfu polymerase (Stratagene, La Jolla, CA, USA). 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 plasmid pCANTABLip-CH 103. The obtained mutant library was sequenced to assess the mutation ratio. The LipA variant D133AV136A was constructed using Quikchange® PCR (Stratagene, La Jolla, USA) with the following primers (Operon, Cologne, Germany): LipDAVAFor
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5’ – CATTTACAGCAGTGCCGCAATGATTGCCATGAATTACTTATCCAGA – 3’
and LipDAVARev
5’ – TCTGGATAAGTAATTCATGGCAATCATTGCGGACTGCTGTAATG -3’.
The obtained constructs were sequenced to verify the base pair order.

**Selection on selective minimal medium**

*E. coli* K-12 PA340/T6 cells were made chemically competent and transformed with 50 ng of mutant plasmid DNA. They were starved by incubation in 0.9% w/v NaCl for 2 h at 37°C and plated onto selective M9 minimal medium agar (1.6% w/v) plates. The medium was supplemented with MgSO4 (1 mM), CaCl2 (0.1 mM), essential amino acids (Thr, His, Arg, Leu; 20 µg.mL⁻¹) and thiamine (1 µg.mL⁻¹). As a sole aspartate source, an aspartate ester of R-(−)-IPG or S-(+)-IPG (1.5 mM) was added to the agar plates. To select for enantioselective variants, a butyl phosphonate ester of the undesired IPG enantiomer (1.5 mM) was added to the medium, to eliminate non-selective variants or variants selective for the opposite enantiomer. Approximately 2500 viable *E. coli* K-12 PA340/T6 containing the mutant plasmids were plated onto each plate. As control plates were used: LB, aspartate (20 µg.mL⁻¹; Sigma) and Min (no aspartate or esters present). Plates were incubated at 30°C. Upon appearance colonies were picked, the periplasmic fraction was isolated and used in the 1,2-O-isopropylidene-\(sn\)-glycol ester assay as described below. 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, colonies were plated onto selective minimal medium with a concentration of 7.5 mM of phosphonate ester, selected and the enantioselectivity was determined using the 1,2-O-isopropylidene-\(sn\)-glycerol ester assay. From bacteria expressing a lipase variant with an inverted enantioselectivity the plasmid DNA was isolated and sequenced to examine the mutation(s).

**Isolation of the periplasmic fraction**

*E. coli* K-12 PA340/T6 was grown in 50 mL tubes containing 10 mL 2xTY medium, ampicillin and isopropyl-\(\beta\)-D-galactopyranoside (IPTG, 1 mM). The tubes were incubated at 37°C at 250 rpm for 16 h. The OD600 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 200 µL buffer containing Tris HCl (10 mM, pH 8.0), sucrose (25% w/v), EDTA (2 mM) and lysozyme (0.5 mg.mL⁻¹, Sigma-Aldrich, Steinheim, Germany). After incubation on ice for 20 min, 50 µL buffer containing Tris HCl (10 mM, pH 8.0), sucrose (20% w/v) and MgCl2 (125 mM) was added. The suspension was centrifuged and the supernatant, containing the periplasmic fraction, was isolated and used as enzyme solution in the IPG ester assay. The protein content of this fraction was determined by performing a Bradford assay in triplicate using bovine serum albumin (BSA) as a standard (Pierce, Rockford, Illinois, USA).

**1,2-O-isopropylidene-\(sn\)-glycerol ester assay**

Periplasmic fractions were diluted with MOPS buffer (0.07 M, pH 7.5), containing BSA (0.2% w/v), to a final volume of 100 µL. References were diluted correspondingly but contained no enzyme solution. The esters of IPG (1 mM) were dissolved in 10 mL MOPS
buffer (0.07 M, pH 7.5), containing Tween 80 (14.3% w/v), and diluted to 50 mL with MOPS buffer (0.07 M, pH 7.5). 500 µL substrate solution was added to the enzyme solution and the final mixture was incubated in a water bath at 32°C. After incubation, 400 µL of a saturated NaCl solution and 10 µL internal standard solution (racemic 3-hexene-1-ol, 5 mg.mL⁻¹ assay buffer) were added to the sample solution and the aqueous solution was extracted twice with 1 mL ethyl acetate. GC analysis was performed as described by Dröge et al. One unit (U) is defined as the amount of enzyme that hydrolyses 1 µmol IPG ester per minute. Enantiomeric excesses, ee, were calculated according to Chen et al and were defined as the ability of the enzyme to distinguish between enantiomers. All data were expressed as mean ± SEM. The statistical significance of differences was tested at a significance level of p < 0.05 using a two-tailed Student's t-test.

Modelling of variant D133AV136D
In variant D133AV136D, the position of the catalytic acid was shifted towards the end of the loop connecting β7 and αE. A model of the selected variant was constructed with Swiss-PDBViewer version 3.7 using wild type LipA (PDB code 1I6W) while mutating positions 133 and 136. To examine whether the repositioning of the acid was a unique feature within the family of α/β hydrolases or this same topology was shared, a one-2-all fit was made using TOPOFIT. This method analyses the similarity in protein structure by making use of 3D Delaunay triangulation patterns derived from backbone representation.

Results
Construction of the mutant library
The LipA encoding gene (Genbank accession number M74010) was cloned in phagemid pCANTAB 5E as described previously. An XbaI site was introduced at base pair 423 by a silent mutation. Cassette mutagenesis was applied to construct a saturated mutant library directed towards amino acids 132 to 136 of mature LipA. These oligonucleotides resembled the lipA sequence for approximately 80%. In theory, this would yield a mutant library composed mostly of single and double mutations. Sequence analysis of 13 clones revealed that 15% of the mutants had no amino acid mutation while the percentage of single and double mutated amino acids was for both 31% each. Two clones showed deletions. After transformation of E. coli TG-1, a mutant library consisting of 3.5 × 10⁴ colonies was obtained. This should be more than sufficient to saturate 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
In order to develop a growth selection system based on bacterial growth for LipA variants with improved enantioselectivity, aspartate esters of enantiopure IPG were synthesised. To examine the functional character of the mimic substrate IPG aspartate, the hydrolysis of the mimic substrate by purified WT LipA was determined in the IPG assay and compared with the hydrolysis of the real substrate IPG butyrate. The enantiomeric excess ee of the hydrolysis of IPG butyrate was 12.9% ± 1.6 towards the (-) enantiomer. The enantiomeric
excess ee of the hydrolysis of IPG aspartate was 5.5% ± 1.1 towards the (-) enantiomer. No statistically significant difference was observed in catalytic activity towards the substrates (p < 0.001).

To introduce a dual selection step, soluble IPG phosphonate inhibitors showing similarity to the aspartate esters were synthesised. This type of inhibitor was previously used in the selection of phagebound lipase, then coupled to SIRAN beads. The soluble inhibitors contained the enantiopure substrate analogue IPG, a leaving group, p-nitrophenol and a butyryl side chain connected to the racemic phosphonate. To assess the inhibitory effect of the IPG phosphonate esters, an inhibition assay was performed using purified wild type LipA according to 103. The t50 of inhibition was 10 minutes for both enantiomers.

Furthermore, cells expressing wild type LipA were plated on minimal medium supplemented with the IPG phosphonate ester and incubated at 30 degrees. LB agar plates, taken as a control, showed approximately 2500 colonies after overnight incubation, while on the minimal medium plates supplemented only with the phosphonate a few small colonies appeared only after 5 days of incubation. These results indicate that the used phosphonate inhibitor limits rather than abolishes growth.

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. A negative control (untransformed E. coli) was taken into account as well. All transformation mixtures were plated on selective minimal medium, for which M9 was prepared without ammonium chloride to exclude a nitrogen source for the auxotroph, thereby limiting growth. As a positive control for growth, all transformation mixtures were plated on LB agar plates; approximately 2500 colonies appeared after overnight incubation. On all selective minimal medium 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, though their number was reduced to approximately 1000 colonies. Negative control plates showed small colonies after 5 to 10 days of incubation. After the second round of selection, approximately 750 colonies would appear on plates supplemented with both the inhibitor and the aspartate ester, while after the third round approximately 400 colonies were found.

After two days of growth, 50 colonies were randomly chosen from the plates supplemented with both aspartate ester and the phosphonate inhibitor to assess their activity and enantioselectivity compared to wild type LipA. As (+)-IPG is the enantiomer of interest, colonies selected from plates supplemented with the R-(+)-IPG aspartate ester and the S-(-)-IPG phosphonate inhibitor were of greater interest. The remaining colonies were harvested and plated onto new selective plates. The periplasm of the selected colonies was isolated and analysed using the IPG assay. Only the best LipA variants were sequenced to verify the base pair order. The catalytic activities, enantioselectivities and the mutations are shown in table I.

Most selected colonies from the S-(−)-IPG aspartate ester had activities and enantioselectivities comparable to those of wild type LipA (conversion approximately 20%, ee approximately 25% towards R-(−)-IPG). However, mutants selected from plates supplemented with the R-(+)-IPG aspartate ester showed an inverted and improved
Table I: Enantioselective hydrolysis of racemic esters of IPG butyrate by periplasmic fractions of selected LipA variants (n=3). 
*R* = R-(+)-IPG aspartate; *S* = S-(−)-IPG aspartate.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Conversion (%)</th>
<th><em>E</em> (%)</th>
<th>Enantiomer formed in excess</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LipA</td>
<td>23.4 ± 1.1</td>
<td>-29.6 ± 0.5</td>
<td>(-)-IPG</td>
<td></td>
</tr>
<tr>
<td><strong>1st round</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>36.6 ± 1.0</td>
<td>+34.4 ± 0.7</td>
<td>(+)-IPG</td>
<td>A132P, M134Q</td>
</tr>
<tr>
<td>R2</td>
<td>6.7 ± 0.6</td>
<td>+43.2 ± 0.8</td>
<td>(+)-IPG</td>
<td>I135F</td>
</tr>
<tr>
<td>S1</td>
<td>53.6 ± 0.8</td>
<td>-10.4 ± 1.2</td>
<td>(-)-IPG</td>
<td>V136F</td>
</tr>
<tr>
<td><strong>2nd round</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>7.2 ± 2.5</td>
<td>+68.7 ± 0.9</td>
<td>(+)-IPG</td>
<td>D133N</td>
</tr>
<tr>
<td>R4</td>
<td>15.2 ± 1.2</td>
<td>+62.5 ± 1.2</td>
<td>(+)-IPG</td>
<td>A132T, M134T</td>
</tr>
<tr>
<td>R5</td>
<td>21.2 ± 0.9</td>
<td>+55.7 ± 1.0</td>
<td>(+)-IPG</td>
<td>D133E, M134R</td>
</tr>
<tr>
<td>R6</td>
<td>19.7 ± 0.8</td>
<td>+82 ± 2.5</td>
<td>(+)-IPG</td>
<td>D133G, M134L, I135N</td>
</tr>
<tr>
<td>R7</td>
<td>23.4 ± 1.3</td>
<td>+61.5 ± 1.8</td>
<td>(+)-IPG</td>
<td>D133Q, M134L, I135T</td>
</tr>
<tr>
<td><strong>3rd round</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R8</td>
<td>28.9 ± 0.5</td>
<td>+73.1 ± 0.7</td>
<td>(+)-IPG</td>
<td>D133A, V136D</td>
</tr>
</tbody>
</table>

Enantioselectivity. The catalytic activity remained comparable to that of the wild type. The enantioselectivity increased in subsequent rounds of selection with higher selection pressure, from an *ee* value of 34% in the first round of selection, to an *ee* value of 73% in the third round of selection. Sequence analysis revealed that mutations were distributed throughout the whole region of amino acids 132 to 136, where mutation of position M134 was most frequently found. However, a mechanistic explanation for this inversion and increase in enantioselectivity is hard to find, since the active site cleft of *B. subtilis* LipA is relatively open and accommodates both IPG enantiomers equally well, and both enantiomers have equal binding modes. Thus, small local rearrangements will most probably take place, the exact outcome of which is hard to predict.

Interestingly enough, position D133 was prone to mutations as well. This aspartate residue is part of the catalytic triad of the enzyme, and therefore, special attention was paid to mutations at this position. The mutant found in the third round of selection, D133AV136D, was subjected to further analysis.

**Characterisation of the D133AV136D mutant**

After three rounds of selection, mutant D133AV136D was obtained. This mutant showed an inverted and improved enantioselectivity compared to wild type LipA. Its specific activity was comparable to the wild type (conversion of 23.4% for wild type LipA versus 28.9% for mutant D133AV136D). The most interesting feature of this mutant lies in the fact that the D133 residue of the catalytic triad is replaced by the catalytically inactive alanine residue. As the 3D structure of LipA has been solved, the crystal structure was used to examine this mutation in more detail. It was speculated that the mutated aspartate residue at position 136 might take over the role of D133 as the catalytic acid.
During substrate hydrolysis, the hydroxyl group of the serine at position 77 is activated by transferring its proton to the H156 side chain, which becomes positively charged. The \( \text{O}^- \) ion of S77 performs a nucleophilic attack on the substrate, which results in the formation of a negatively charged tetrahedral intermediate. This intermediate is stabilised by two neighbouring peptide NH-groups. The proton transfer from the S77 hydroxyl group to H156 is facilitated by D133, which properly orients the imidazole ring of H156 and ensures that H156 is in the right tautomeric form to accept a proton from S77. In addition, the positive charge on the histidine residue is stabilised by the negatively charged carboxyl moiety of D133 (figure 2A)\(^{25,27,29}\). The ester bond is cleaved and, upon protonation by H156 the alcohol moiety of the substrate is released, but the acid part of the substrate remains covalently bound to the enzyme (the acyl-enzyme). In the second step of the reaction the acyl enzyme is hydrolysed by a water molecule which is activated by the uncharged H156. Finally, H156 donates the proton, acquired from the hydrolytic water molecule, to S77 and the enzyme is ready for a new round of catalysis. In the case of mutant D133AV136D however, the alanine at position 133 is unable to stabilise the positive charge of H156. Since the double mutant does show appreciable activity, it is likely that, the aspartate residue introduced at position 136 takes over the role of D133. Modelling shows that indeed an aspartate residue at position 136 can interact with H156 in such a way that the H156 side chain can also still interact with the S77 side chain (figure 2B).

To test the hypothesis that D136 takes over the role of D133 the double mutant D133AV136A was constructed. D133AV136A was expressed in the periplasm of \( E.\ coli \) HB2151, which was then isolated and used in the IPG assay. Compared to the blanks, there was no conversion of IPG butyrate by the variant. Thus, in the D133AV136D double mutant D136 is essential for activity, demonstrating that D136 can take over the role of D133.

Mutant D133AV136D shows an inverted and improved enantioselectivity compared to wild type LipA. Since the active site cleft of \( B.\ subtilis \) LipA is relatively open and accommodates both IPG enantiomers equally well, it is difficult to explain the inverted and
improved enantioselectivity. Most likely, a somewhat altered orientation of H156 as a result of its interaction with D136 instead of D133 causes subtle differences in the interaction between the enzyme and the substrate, which may lead to the observed differences in enantioselectivity.

**Discussion**

In the past, genetic selections such as presented in this study have had their impact on the evolution of enzymes with improved catalytic activity, improved stability, and altered substrate specificity. However, enantioselectivity is a difficult property to select for, though some indirect screening methods based on bacterial growth have been described\(^ {93,258}\). In these methods, the enantiopure substrate of interest was coupled to a compound, which, upon release, was toxic to the bacterium. Individual variants could be identified by measuring growth rates of cells in liquid media as cells showing hydrolytic activity were unable to grow due to released toxic compound. A high-throughput screening method can thus be established for enhanced enantioselectivity of hydrolytic enzymes as these methods are based on growth rate. However, screening involves the individual examination of variants, and is therefore cumbersome. By applying a selection strategy, multiple variants can be examined at the same time. Here, we have reported a novel genetic selection system which selects for enantioselectivity in addition to catalytic activity.

An aspartate auxotrophic *E. coli* strain was used for selection based on bacterial growth. The bacterium was transformed with a library directed towards a small loop region around the active site of LipA and grown on selective minimal medium agar plates. By supplementing an aspartate ester of enantiopure IPG to the minimal medium, lipase variants expressed in the periplasm could be selected not only for catalytic activity, but also for enantioselectivity. The enantioselectivity of wild type LipA towards this mimic substrate IPG aspartate and towards its real substrate IPG butyrate did not differ in a statistically significant manner, implying that the mimic compound was a suitable substrate in our selection system. After each round of selection, colonies were picked, the periplasmic fraction was isolated and assayed, and the more enantioselective variants were sequenced in order to verify the base pair order. Ultimately, three rounds of selection were performed, in which the imposed selection pressure was increased by raising the concentration of the phosphonate inhibitor. After the third round, a further improvement of the enantioselectivity was not observed. Most likely, this can be explained by the small library size, which is only comprised of five randomised amino acids. Upon using bigger libraries, it would be expedient to apply DNA shuffling\(^ {60}\) or CASTing\(^ {269}\) in order to select variants with even higher enantioselectivities.

A dual selection strategy using phosphonate suicide substrates was introduced here to decrease the total number of colonies and reduce the frequency of selecting less enantioselective variants. This inhibitor reduces rather than completely inhibits growth, which might be due to the poor enantioselectivity of the wild type LipA towards the substrate of interest, IPG. The ultimate goal was to select only the more enantioselective variants. By using the phosphonate suicide inhibitor, one could argue that a discrepancy between \(k_{cat}\) and \(K_m\) is introduced: the expressed variant could show a high value of \(k_{cat}\) towards the phosphonate while the \(K_m\) value towards the aspartate ester can be equally low.
Thus, due to binding to the phosphonate inhibitor, interesting variants could be missed. Nevertheless, in our case the selection strategy yielded lipase variants with an inverted and improved enantioselectivity.

In the past, genetic selections have been useful in the elucidation of structure-function relationships in enzymes. Hence, important and often overlooked roles played by multiple subtle interactions between active-site residues could be examined. In our work, variants were selected from a library around the catalytic acid D133. It was tempting to speculate on the mechanism of action of variants with a mutated D133 residue. As demonstrated by the sequences of the selected variants, the aspartate residue was not conserved but prone to mutation as well. Similar to other α/β hydrolase fold enzymes, the catalytic triad of *B. subtilis* LipA has a preserved arrangement. The topological positions of the nucleophile and the general base histidine after β-strand 5 and 8, respectively, are fully conserved within this family, while the location of the catalytic acid is not. In most α/β hydrolase family members, the catalytic acid residue aspartate or glutamate is located following strand β7 (figure 3A); in mutant D133AV136D, the catalytic acid has slightly migrated to a position near the end of the loop connecting β7 and αE (figure 3B).

![Figure 3: A) Canonical α/β hydrolase fold, with the nucleophile after β5, the acid after β7, and the histidine residue after β8. B) Topology of variant D133AV136D, in which the acid has shifted towards the end of the loop connecting β7 and αE. C) Topology of HPL, which contains a catalytic acid after β6. An aspartate residue after β7 is still present, though it is not active.](image)

To investigate the existence of other α/β hydrolase fold enzymes with a repositioned catalytic acid similar to mutant D133AV136D, a one-2-all fit with D133AV136D as a search model was made. No other enzymes with a similar topology as in D133AV136D were found. In contrast, the enzymatically active human pancreatic lipase shows an equivalent catalytic acid which has been repositioned in the opposite direction compared to the acid in variant D133AV136D to a position following strand β6 (figure 3C).
Double mutants of *Geotrichum candidum* lipase\(^{261}\) and of haloalkane dehalogenase from *Xanthobacter auxotrophicus*\(^ {262}\), both constructed to resemble the topology of human pancreatic lipase, were found to be enzymatically active as well.

Mutant D133AV136D showed an inversion and improvement of the enantioselectivity as well. From the 3D structure, it is clear that the active site cleft of *B. subtilis* LipA is relatively open. Thus, both IPG enantiomers are accommodated equally well; it is therefore difficult to explain the inverted and improved enantioselectivity. The side chain of D133 is replaced with a shorter alanine side chain, thereby creating space for the side chain of D136. Small local rearrangements will most probably take place due to a different orientation of H156; this is likely to affect the interaction with the substrate. However, the exact effect on the binding situation of the desired IPG enantiomer remains hard to predict.

The main prerequisite of a growth-based selection system such as presented here is that the mutants must be expressed in the periplasm of the *E. coli* auxotroph in order to convert the mimic substrate, and, theoretically, this might limit a broad application of this method. We have, however, recently demonstrated that even an intracellular enzyme can be translocated to the periplasm solely by inserting a specific signal sequence. The intracellular carboxylesterase A (CesA) from *B. subtilis* 168 was successfully translocated to the periplasm using a Sec-dependent signal sequence\(^ {108}\) and preliminary results show that wild type CesA transformed to the *E. coli* aspartate auxotroph and plated on selective minimal medium plates, displayed more growth on plates supplemented with S-(-)-IPG aspartate. Upon hydrolysis, the absolute conformation of IPG changes to R-(-)-IPG. The enantiopreference of CesA towards S-(-)-IPG is therefore in accordance with its enantioselectivity towards the real substrate IPG butyrate\(^ {45}\), which is directed towards R-(-)-IPG. The developed selection system can therefore in the future be applied in the selection of CesA mutants. In summary, the presented growth selection system can be generally applied in the selection of hydrolase variants, as long as they are translocated to the periplasmic space.

**Conclusion**

In this study, a novel bacterial growth system has been 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 other hydrolase variants as well, we believe we have established a strategy that is generally applicable and will provide new perspectives in the evolution of enzyme enantioselectivity.

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