Abschlußbericht AZ 13198-32

Förderschwerpunkt Biotechnologie: **"Entwicklung eines innovativen Verfahrens zur kombinierten Selektion und *in vivo* Evolution von Esterasen mit dem Ziel der Veränderung ihrer Enantioselektivität am Beispiel der rekombinant in *E. coli* vorliegenden Esterase aus *Pseudomonas fluorescens* SIKW1 (PFE-I)"**

Kurztitel: **"Selektive *in vivo* Evolution"**

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Zusammenfassung

Im Rahmen dieses Projektes sollte ein *in vivo* Selektionssystems etabliert und dessen Validierung durchgeführt werden. Die neuartige Methode wurde am Beispiel der Selektion von Esterasevarianten mit erhöhter Enantioselektivität gegenüber 3-Phenylbuttersäureester getestet. Geeignete Positiv- und Negativkontrollen in Form von enantioselektiven und nicht-enantioselektiven Esterasevarianten (*Pyrobaculum calidifontis* Esterase pEST vs. *Bacillus subtilis* Esterase BS2) konnten identifiziert und in dem bevorzugten *E. coli* Stamm (*E. coli* JM109(DE3)) exprimiert werden. Diese ausgewählten Kontrollen wurden anschließend zur Validierung der Methode eingesetzt.

Zu Beginn des Projektes wurden strukturell ähnliche Selektions-Moleküle ausgewählt, also chirale Moleküle, die einen Selektionsdruck auf die Bakterien ausüben. Der so genannte „Positivimpuls“ ist die Kohlenstoffquelle (C-Quelle) Glycerol, und den so genannte „Negativimpuls“ stellen bromierte Glycerolderivate dar, die in einem Vorscreening mit verschiedenen halogenierten Glycerolderivaten getestet und wegen ihrer geringen Minimalen Hemm-Konzentration (*Minimal Inhibitory Concentration*, MIC) von 5-10 mM für *E. coli* JM109(DE3) ausgewählt wurden. Die Synthese der Selektionssubstrate war erfolgreich und die neuen Substanzen wurden anschließend charakterisiert. Nach Veresterung der (*R*)- und (*S*)-Enantiomere von 3-Phenylbuttersäure an Positiv- und Negativ-Impuls erhielt man die „Selektions-Ester“, bei deren enzymatischer Hydrolyse entweder eine C-Quelle oder ein Toxin freigesetzt wird. Weiterhin wurden die Kultivierungsbedingungen angepasst, um ein wässriges System zu erhalten, in dem die Bakterien in Gegenwart der Selektionssubstrate leben und sich vermehren können, was eine Interaktion mit den Zellen erlaubt.

Als die Grundlagen für das System etabliert waren und das Know-how für das Durchfluss-zytometer entwickelt war, konnte der nächste Selektionsschritt durchgeführt werden, die Anwendung der Methode für das Hochdurchsatzscreening, das finale Ziel des Projektes.

Hierbei wurden verschiedene Systeme zur Detektion von Bakterien und der Unterscheidung ihres metabolischen Zustandes im Durchflusszytometer erprobt und für die Unterscheidung ihrer verschiedenen physiologischen Zustände hinsichtlich ihrer Effektivität überprüft. Die Kombination der DNA Farbstoffe Syto9 und Propidiumiodid wurde als am besten geeignet festgestellt. Im Folgenden wurden Positiv- und Negativkontrolle in dem Selektionssystem separat kultiviert, und mit dem Durchflusszytometer analysiert. Man konnte dabei beobachten, dass das gewünschte unterschiedliche Verhalten mit der Enantioselektivität der jeweils exprimierten Esterase korreliert. Anschließend wurde eine Mischung von Positiv-und Negativkontrolle zusammen in selektivem Medium kultiviert und nach der Analyse mit dem Durchflusszytometer konnten die metabolisch aktivsten Klone sortiert und analysiert werden. Dabei konnte erfreulicherweise eine Anreicherung der Positivkontrolle (pEST) gegenüber der Negativkontrolle (BS2) festgestellt werden, was den ersten Schritt für die erfolgreiche Validierung der Methode darstellt.

1 Summary

The establishment of an *in vivo* selection system and the beginning of its validation has been performed in this project. The method has been adapted for the selection of esterase variants with increased enantioselectivity towards 3-phenylbutyric esters. Suitable positive and negative controls in the form of enantioselective and non-enantioselective esterase variants have been identified and expressed in the *E. coli* strain of interest (*E. coli* JM109(DE3)). These selected controls have been used for the method validation.

To start with, structurally similar suitable selection molecules have been selected. The positive impulse is the carbon source glycerol, and as negative impulse a bromated derivate has been chosen, due to its low MIC (5-10 mM) for *E. coli* JM109(DE3) identified in a pre-screening with different halogenated glycerol derivates. The synthesis of the selection substrates has been successfully performed and the new substances have been characterized. *R* and *S* enantiomers of 3-phenylbutyric acid have been coupled to both positive and negative impulses resulting the selection esters whose hydrolysis will release either a carbon source or a toxin. Furthermore, the cultivation conditions have been adjusted in order to get an aqueous system where the bacteria can live and proliferate while the selection substrates can be in a suspension which allows their interaction with the cells.

Once the basic elements of the system had been established, a know how of the flow cytometer has been developed in order to perform a further selection step with this device, allowing the application of this method for high-throughput screening, which is the final aim of the project. In this way, different systems for the detection of bacteria in the cell sorter, and for the differentiation of their different physiological statements have been performed, and their effectiveness has been checked. The combination of the DNA dyes Syto9 and propidium iodide was chosen to be the best system for our purpose. Thus, positive and negative controls were cultured separately in the selection system and analyzed in the cell sorter, observing the desired different behaviour associated to the enantioselectivity of the esterase expressed in each case. Afterwards, mixtures of positive and negative control were cultured in selection media and after flow cytometer analysis the fittest clones were sorted and analyzed. An enrichment of the positive control over the negative one was observed, being in this way successful the first step in the validation of the method.

2 Aim of the project

The objective of this project is the creation of a method for simultaneous *in vivo* evolution and selection to improve the enantioselectivity of esterases. The enzyme chosen to start with in a first moment was the esterase I from *Pseudomonas fluorescens* (PFE-I, 1va4) because its expression and handling has been largely investigated, but the use of this enzyme is not compulsory.

The generation of improved clones can be achieved via *in vitro* or *in vivo* evolution of the gene of interest. In the first case, the classical asexual and sexual methods of directed evolution, such as error prone PCR (epPCR), site directed mutagenesis or DNA shuffling can be applied, and after generation of a mutants library, the clones will be subjected to a selective environment, that will entail an enrichment of the improved mutants over the non-enantioselective or inactive ones.

The selective environment as well as the selection method for the enantioselective variants has to be developed as well. The basic concept of this selection consists on binding one enantiomer of the substrate of interest to a carbon source, and the other enantiomer to a toxin. In this case, both inactive and non-enantioselective clones will die either of starvation or of toxicity, while the variants with the desired enantiopreference will get only the carbon source and in consequence will grow. This selection can be applied for both *(R)* and *(S)* enantiopreference, just by inverting the chirality of the selection substrates.

The concept of *in vivo* selection appears with the requirement of creating libraries of better quality, by applying a selection pressure at the same time with the mutation process. For this aim, a number of clones carrying the wild type esterase gene should be cultivated in minimal medium in the presence of a mutagen and the selection substrates mentioned above. The clones with the desired mutations will present an evolutionary advantage, and in consequence, will survive and generate a larger progeny.

The few improved variants will be further selected after their enrichment with the help of a cell sorter. Flow cytometry techniques are nowadays a hot topic, and beside its broad use for analysis of eukaryotics, its application in differentiation and characterization of bacterial populations is gaining importance. In this project flow cytometry and sorting will be applied as final procedure for the screening of mutant libraries.

3 List of abbreviations

[α]: specific rotation

eq.: equivalent

epPCR: Error-prone polymerase chain reaction

GC-FID: Gas chromatography with flame ionization detector

GC-MS: Gas chromatography with mass spectrometry detector

GFP: Green fluorescent protein

HPLC-UV: High pressure liquid chromatography with ultraviolet detector

IPTG: Isopropyl-beta-D-thiogalactopyranoside

LB media: Luria-Bertani media

MIC: Minimum inhibitory concentration

MS: Mass Spectrometry

NMR: Nuclear Magnetic Resonance

PCR: Polymerase chain reaction

*p*NPA: *para*-nitrophenylacetate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

RT: Room temperature

THF: Tetrahydrofurane

TEA: Triethylamine

TLC: Thin layer chromatography

TMS-diazomethane: Trimethylsilyl diazomethane

4 Methods

4.1 Organic synthesis

Azeotropic esterification under Dean-Stark conditions

Acid activation (acid chloride) and esterification with different alcohols under inert atmosphere (N_2)

Different selective acetal deprotection methods [1]

TLC

Vacuum distillation

Column chromatography

Evaluation of the synthesized substrates: ^{1}H-NMR, ^{13}C-NMR, MS, [α]

4.2 Biocatalysis and analytic methods

Enzymatic hydrolysis of esters of chiral acids in analytic and preparative scale

HPLC-UV, GC-FID, GC-MS

Derivatisation of chiral acids to the correspondent methyl esters using TMS-diazomethane solution

4.3 Molecular biology and protein analysis

Gene subcloning by digestion and ligation

Generation of mutant library by: epPCR, digestion and ligation or by epPCR followed by plasmid PCR (“MegaWhop”)

Colony PCR

Protein expression with different induction systems: L-rhamnose and IPTG

Cell lysis (with lysis buffer or by sonication) protein analysis by BC Assay and SDS-PAGE

Determination of enzymatic activity by: *p*NPA hydrolysis, esterase/ amidase overlay agar assay, esterase activity staining of SDS gel

4.4 Microbiology

Cultivation in LB media, minimal media M9

Cell analysis and sorting using the flow cytometer: Partec CyFlow® Space

5 Results and discussion

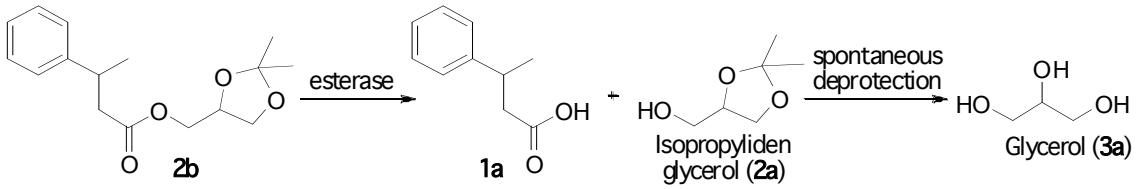
5.1 Selection substrates

5.1.1 Selective molecules: selection and synthesis

In order to establish an effective selection procedure the substances which will determine the positive and the negative effect have to be carefully selected. These compounds have to be subsequently chemically coupled to the model chiral substrate - in this project 3-phenylbutyric acid (**1a**) (Scheme 1). Thus the prerequisite of the selection molecules is the ability to form an ester bond with the acid **1a**.

5.1.1.1 Positive impulse

A positive impulse for bacteria is a source of carbon and energy. Glucose is the optimal carbon source, but the synthesis of its ester with 3-phenylbutyric acid (**1a**) would require the use of an activated analogue of glucose which is very expensive. A suitable alternative to glucose is glycerol. In a first moment the idea of using an ester of isopropyliden glycerol (**2b**) was considered [2], because its synthesis would be less demanding than the synthesis of the glycerol ester. This idea was based in the hypothesis that after the ester hydrolysis occurs, a spontaneous cleavage of the acetal group would take place resulting in the release of glycerol (**3a**) as final product (Scheme 1).



Scheme 1. General scheme for biocatalysis of **2b** resulting in formation of glycerol (**3a**).

Prior to growth experiments, the hypothesis of the spontaneous acetal cleavage had to be confirmed. For this aim, cultures were grown in minimal media supplemented with isopropyliden glycerol (**2a**) as carbon source (5-300 mM). As positive controls for bacterial growth, glucose (5-30 mM) and glycerol (5-30 mM) were used. Bacterial growth was monitored by OD measurement at 600 nm at different time intervals (Figure 1). Unfortunately, it was confirmed that no spontaneous cleavage of the acetal protection group of **2a** occurred and thus isopropylidene glycerol is not suitable as a carbon source. Hence the next efforts were put on the synthesis of the glycerol ester.

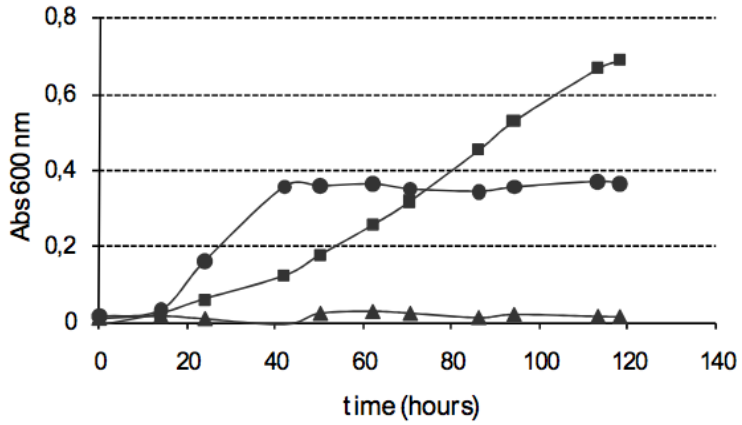


Figure 1. Growth curves with different substrates as carbon source. Circles: 30 mM glycerol; squares: 30 mM glucose; triangles: 30 mM isopropyliden glycerol.

In order to synthesize the ester of free glycerol and 3-phenyl butyric acid, different strategies have been considered:

a) Esterification with acid chloride (**1b**) and isopropyliden glycerol (**2a**) followed by chemical deprotection of the acetal group [1] (Scheme 2).

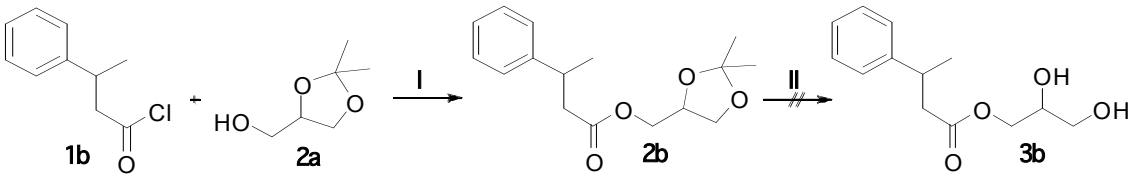
The first step was carried out successfully and the acetonide of 2',3'-dihydroxypropyl-3-phenylbutyrate (**2b**) was isolated in good yield. Unfortunately, none of the tested acetal deprotection strategies resulted in formation of the diol (**3b**). An alternative synthetic route was suggested instead of further optimization trials.

Acetal deprotection strategies:

75% trifluoroacetic acid in water, RT

1 eq. Pyridimium-*p*-toluenesulfonate in methanol, RT

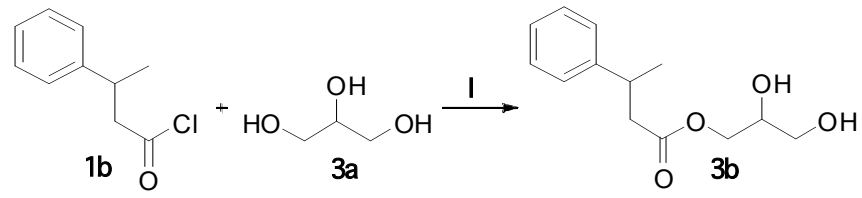
1 eq. *p*-toluenesulfonic acid in acetone, RT



Scheme 2. General procedure for the synthesis of 2',3'-dihydroxypropyl-3-phenylbutyrate by strategy **a**. **I**: THF as solvent, 10 eq. TEA, inert atmosphere. **II**: different deprotection techniques.

b) Esterification with acid chloride **1b** and excess of glycerol (Scheme 3)

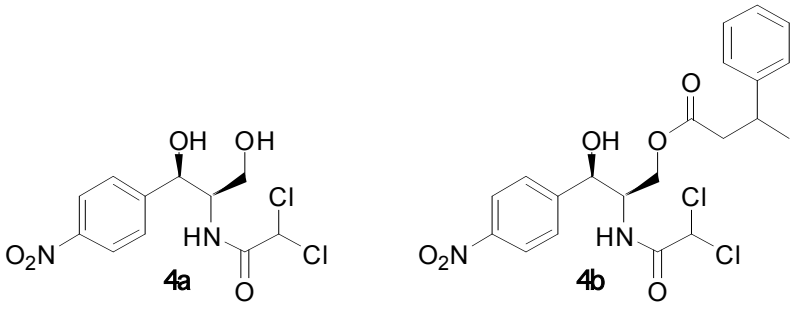
Obstacles with the deprotection step could be avoided by the direct esterification of glycerol (**3a**) with 3-phenylbutyric acid chloride (**1b**). Based on the fact that primary alcohols are more reactive than secondary ones (due to their reduced steric hinderance), a selective monoesterification of glycerol can be achieved by slow addition of the acid chloride to a stirring mixture of glycerol. The alcohol muss remain in excess to prevent diesterification, but this is not a problem in this case as glycerol is cheap and can be easily removed from the reaction mixture by extraction. Following this strategy **3b** was obtained in good yields (5 g product, 21 mmol, 50% yield).



Scheme 3. General procedure for the synthesis of 2',3'-dihydroxypropyl-3-phenylbutyrate by strategy **b**. **I**: THF as solvent, 10 eq. TEA, inert atmosphere.

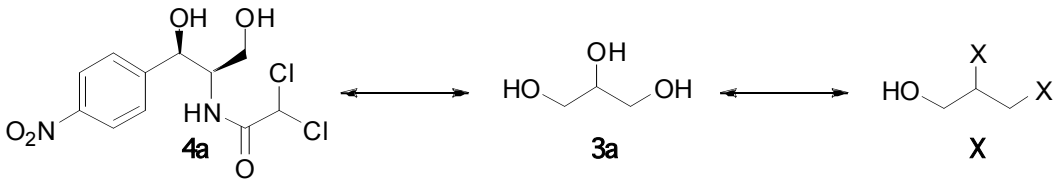
5.1.1.2 Negative impulse

When this project was planed the use of an antibiotic [3] as the toxin was considered. Amongst all the different antibiotics considered, chloramphenicol (**4a**, Scheme 4) was selected, because it possesses one primary alcohol moiety in its structure, which makes the esterification with any carboxylic acid specific and simple. The MIC of chloramphenicol for *E. coli* DH5α was found to be ≤ 3 µM, while the MIC of its 3-phenylbutyric ester (**4b**) was ≤ 1mM. No growth inhibition was observed in presence of 0.5 mM of **4b**, so this concentration was used for the first experiments carried out in presence of esterase activity (see 5.3.1.1).



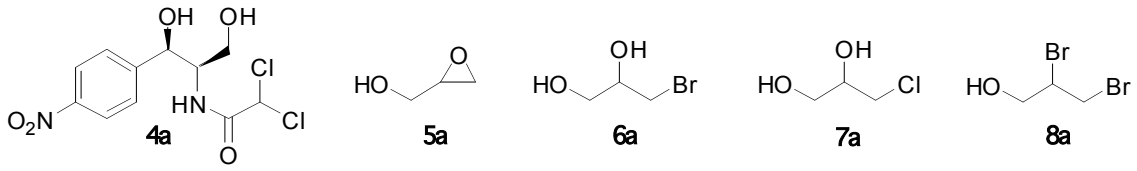
Scheme 4. Chemical structure of Chloramphenicol **4a** and its ester **4b**.

A disadvantage with the application of chloramphenicol is the low structural similarity between glycerol and the antibiotic. With the aim of developing a high throughput selection assay for enantioselectivity, the selection molecules should ideally differ minimally in the enantiomeric centre to which selection is directed. Thus, alternative substances were considered to be used as negative impulse (Scheme 5). Based on the structural similarity to glycerol, halogenated derivates as well as an epoxide derivate were investigated as toxins.



Scheme 5. The structurally different chlorampenicol (**4a**) and structural analogs of glycerol (**X**).

A requirement for the suitable negative impulse is that the released molecule is toxic for *E. coli* cells while the ester does not influence growth in the concentrations used for the selection. Thus, toxicity of both forms has to be checked. First of all, the toxicity of different glycerol derivates was tested (Scheme 6 and Table 1) and those with the lowest MIC were selected for preparation of esters with 3-phenylbutyric acid (**1a**). The toxicity study of the corresponding ester was carried out subsequently (Scheme 7).

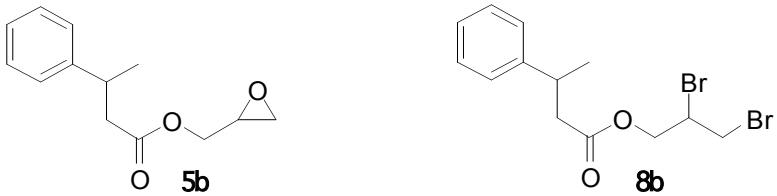


Scheme 6. Chemical structure of the substrates which have been tested as toxins for *E. coli.* Apart from chloramphenicol (**4a**) the rest of the molecules tend to be glycerol analogues. **4a:** chloramphenicol, **5a:** glycidol, **6a:** epibromhydrin, **7a:** epichlorhydrin, **8a:** 2,3-dibromopropanol.

Table 1. The MIC of the substances checked as possible toxins for *E. coli* BL21.

Substance	**4a**	**5a**	**6a**	**7a**	**8a**
MIC (mM)	≤0.003	≤13.5	≤57	≤240	≤11.5

Glycidol (**5a**) and 2,3-dibromopropanol (**8a**) were the most toxic substances for the bacteria, so esters with 3-phenylbutyric acid were synthesized (Scheme 7) and the toxicity tested. Both substances did not inhibit bacterial growth at concentrations of 20 mM, the use of higher concentrations was not possible due to their low solubility in the aqueous media. 2,3-dibromopropanol (**8a**) was selected as the substance of choice because of the slightly higher toxicity compared to glycidol (**5a**). The second reason is that the high autohydrolysis rate of glycidol into glycerol would involve additional complications in the evaluation of the selection system.



Scheme 7. 5b: oxiran-2'-yl-3-phenylbutanoate, **8b:** 2',3'-dibromopropyl-3-phenylbutanoate.

The synthesis of all 3-phenylbutyric esters was performed by esterification of the acid chloride **1b** under inert atmosphere, followed by product purification via chromatography on

silica gel. As all substances possess only one alcohol functionality, no special precautions were required to avoid the formation of side products. Both, **5b** and **8b** were obtained in good yields (**5b**: 780 mg product, 3.5 mmol, 32% yield; **8b**: 3 g product, 8.2 mmol, 70% yield).

5.2 Target enzymes

Once the selection substrates had been selected and the 3-phenylbutyric acid derivates had been synthesized, our effort was focused on the search of model catalysts to prove the concept. The esterases used in this assay must be able to hydrolyze the selection substrates so that the selection process can take place. To develop and validate the method, positive and negative controls are necessary. As a negative control a non-enantioselective esterase is needed and the positive control has to be obviously an enantioselective esterase.

5.2.1 Negative control

The negative control for the development of the assay will be the target enzyme for the future evolution process. First of all, effectiveness of the assay has to be proved and afterwards mutants of this non-enantioselective enzyme can be generated. The improved clones will be selected employing the established *in vivo* selection method.
First, the esterase I from *Pseudomonas fluorescens*, also named PFEI (pdb: 1va4) was selected as model enzyme for the evolution process. Unfortunately the preliminary experiments to check the selection hypothesis were not successful. The expected growth inhibition of clones expressing active PFEI in LB media supplemented with **4b** was weak and slow (

Figure 2) and no difference in bacterial growth was observed in M9 media supplemented with **3b**, where clones with or without esterase activity were cultured (

Figure 3). Surprisingly, in this second case bacterial growth was observed in all cases, although no bacterial growth would be to expect in clones which can not release glycerol (**3a**) from its ester (**3b**). Before planning the design and synthesis of new selection molecules the rest of the system components were checked.

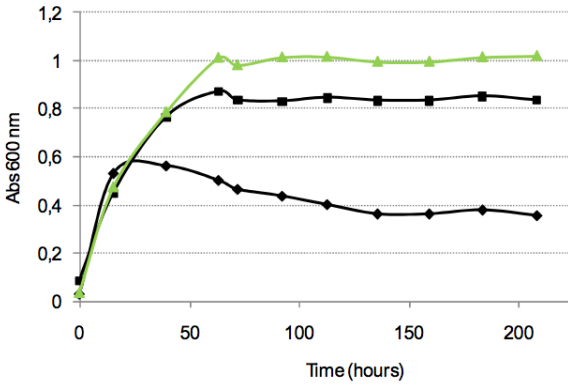


Figure 2. Growth curves for the negative selection experiment with *E. coli* DH5α expressing active or inactive PFEI. Green curve: positive control, bacteria grown in LB-Ampicilin media supplemented with 0.2% rhamnose. Black curves: bacteria cultivated in LB-Ampicilin supplemented with 0.2% rhamnose and 0.5 mM **4b**. Diamonds: bacteria expressing the esterase PFEI; squares: bacteria expressing an inactive variant of PFEI. The inactive variant of PFEI was generated by partial digestion of the gene.

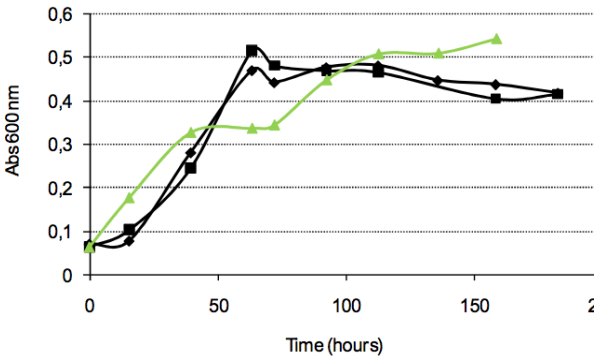


Figure 3. Growth curves for the positive selection experiment with *E. coli* DH5α expressing active or inactive PFEI. Green curve: positive control, bacteria grown in M9-Ampicilin media with 0.2% rhamnose. Black curves: bacteria cultivated in M9-Ampicilin with 0.2% rhamnose supplemented with 5 mM **3b**. Diamonds: bacteria expressing the esterase PFEI; squares: bacteria expressing an inactive variant of PFEI.

The ability of PFEI to hydrolyze the selection substrates was tested setting a simple biocatalysis experiment with conversion monitored by TLC. We observed that **4b** was hydrolyzed at very slow rate by the esterase; while no conversion of **3b** could be observed by the employed detection method. This fact partially explains the failure of the preliminary experiments, as the not complete growth inhibition in the negative selection (

Figure 2) might be due to the slow hydrolysis of 4b**. However, the extremely low hydrolytic activity of PFEI towards** 3b **does not explain why in the positive selection (**

Figure 3) no difference between tests and controls was observed. Thus, the analysis of other components in the selection system was performed.

The answer to the issue surrounding the positive selection was found in the expression system. PFEI was cloned in the pGaston vector, which is a rhamnose inducible vector. According to general procedure, 0.2% rhamnose is added to the culture media to start the protein expression, but rhamnose can also be utilized by the bacteria as a carbon source. Therefore it was necessary to change the induction system to enable the positive selection to work.

In conclusion, a new active esterase towards the selection substrates had to be found and cloned in a different expression system which did not use a carbon source as inductor. We selected two esterases which could be used as negative controls and simultaneously as target enzymes for the evolution process: *p*-nitrobenzyl esterase from *Bacillus subtilis* (BS2) and a variant from PFEI named PFEQ [4] (aminoacid substitutions: F125L F143L F158L I224L) where the entrance channel to the active site has been widened by site directed mutagenesis (Figure 4). Both esterases were cloned into pET28a and expressed in active form (Figure 5). The inducer of the protein expression for this vector is IPTG, added to the media in a final concentration of 0.1 µM which can not be used as carbon source for the bacteria. With the new established system further experiments were performed.

a) b)

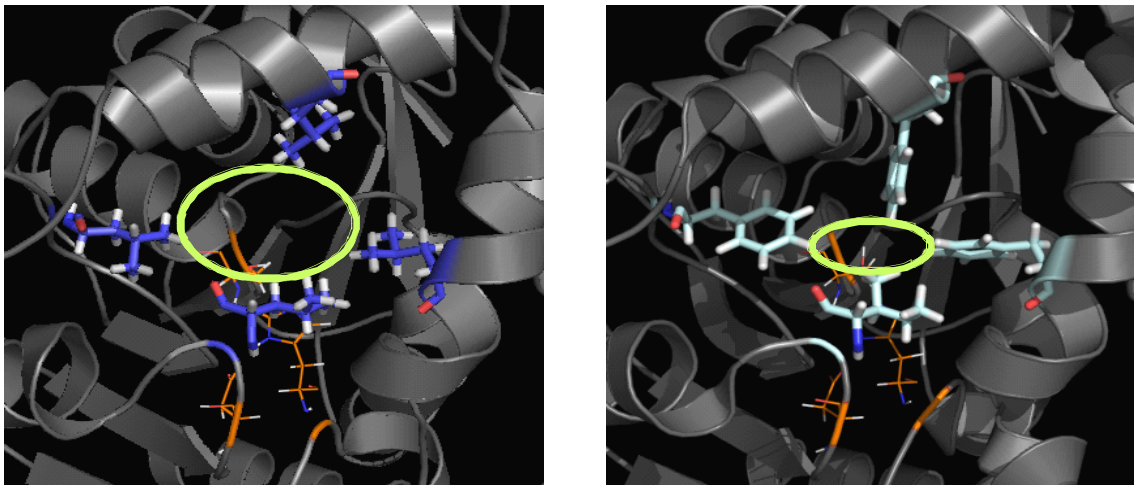


Figure 4. Comparison between (a)) the homology model of the mutant PEFQ and (b)) the crystal structure of PFEI. The catalytic triad in the active site is represented in orange. The residues subjected to mutation are represented in dark blue in the mutant and light blue in the wild type. The width of the entrance channel is indicated with a green circle.

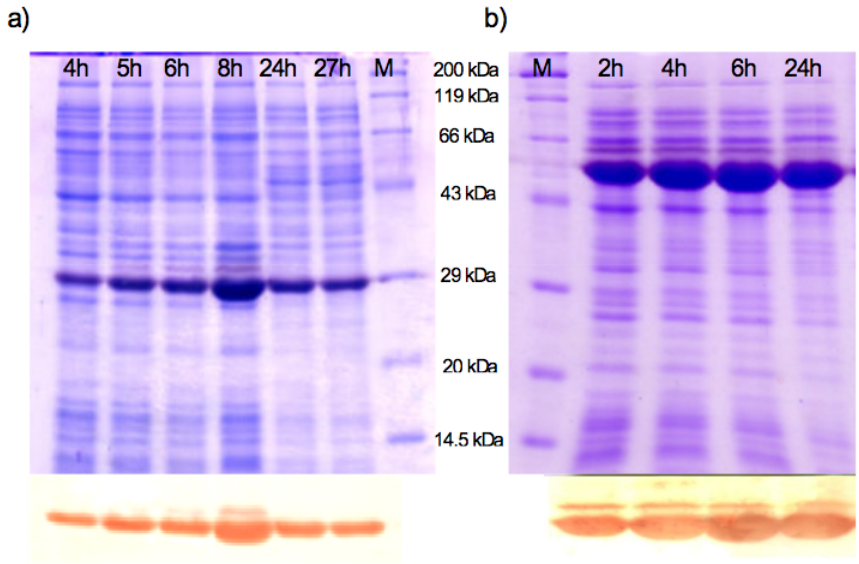


Figure 5. Protein analysis by SDS-PAGE of PFEQ (a)) and BS2 (b)). The protein expression was performed at 30˚C and samples were taken after the above indicated time periods. The size of the protein marker (M) standards are indicated in kDa. The overexpression bands have, in both cases, approximately the expected size: 27 kDa for PFEQ and 54 KDa for BS2. The esterase activity of the overexpressed protein was confirmed by esterase activity staining, whose result is showed below the gel stained with Coomassie.

5.2.2 Positive control

Without a positive control, the validation of the *in vivo* selection method is not possible. A positive control should be an esterase which is easy to express in the *E. coli* strain used and possess high enantioselectivity towards 3-phenylbutyrates. Unfortunately, no esterases with high enantioselectivity towards these substrates, expressed in recombinant form, have yet been described. Hence, a screen for a suitable esterase was necessary. In this screen a library of esterases derived from the metagenome as well as the collection of esterases available in our laboratories were analyzed for their enantioselectivity. In the first step, an activity test based on a pH assay was used [5] followed by biocatalysis in analytical scale to detect the best candidates. In this way, two enantioselective catalysts towards the ethyl-3-phenylbutyrate were found: pEstA and Est CL1.

PEstA is the name for the thermostable esterase of *Pyrobaculum calidifontis* [6] and belongs to the collection of esterases available in our laboratories. Est CL1 [7] was found in the metagenome library made available to us by the company BRAIN AG, who also kindly provided the esterase encoding gene. Both esterases were cloned into pET vectors and expressed in *E. coli* JM109(DE3) in active form (Figure 6). *E. coli* JM109(DE3) is the strain chosen for the selection process (see 5.3.1.2).

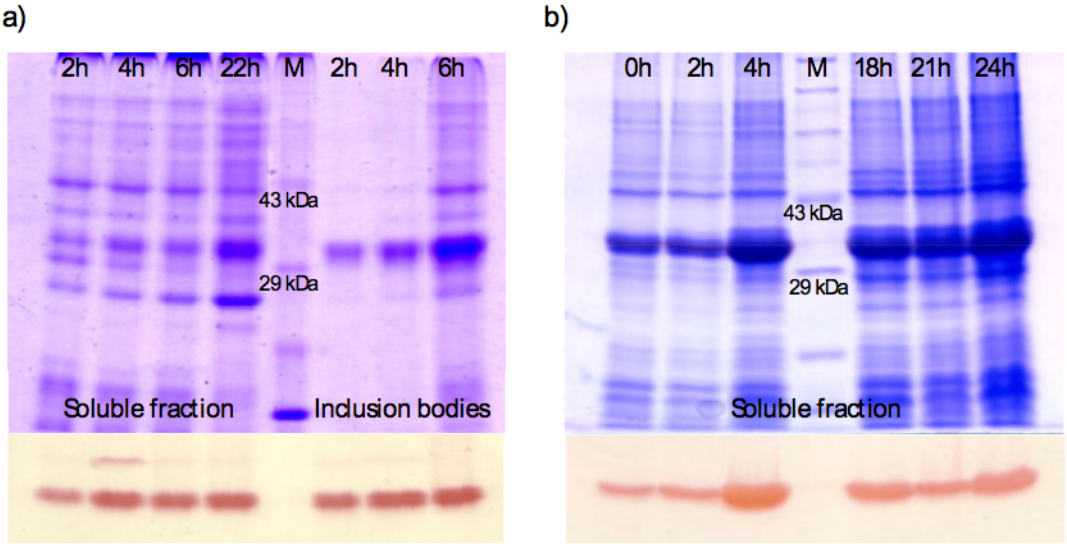


Figure 6. Protein analysis by SDS-PAGE and activity staining of esterases pEstA (a)) and CL1 (b)). Both proteins could be successfully expressed in *E. coli* JM109(DE3). The esterase activity of the overexpressed protein was confirmed in both cases by activity staining as shown. The expected molecular masses are: 34 kDa for pEstA and 33 kDa for CL1 [8].

5.3 Selection system

5.3.1 Cell counting

5.3.1.1 Chloramphenicol/glycerol

Before changing the negative impulse, the first assays that confirm this selection hypothesis were carried out. At this point, it had been already determined that the esterase I from *Pseudomonas fluorescens* (PFEI) was not suitable as model esterase for this project, and BS2 had been selected as negative control. The E-value of BS2 towards ethyl-3-phenylbutanoate is 1. At that time of this work, no positive control to mimic the real system was available as there was no esterase described in the litera-

ture which was enantioselective towards the target substrate. Thus, an alternative way to emulate the selection system had to be implemented. This challenge was overcome by studying the effects of both selection molecules separately in bacteria expressing active esterase or not.

In the case of the glycerol ester, the bacteria were cultivated in M9 media supplemented with different concentrations of 2',3'-dihydroxypropyl-3-phenylbutyrate as carbon source. If the selection hypothesis is valid, only the clones expressing active esterase would be able to obtain glycerol and subsequently grow, while the clones without esterase activity would starve. As control for this assay, the bacteria were cultivated in M9 media supplemented with different concentrations of glycerol. From this experiment two important conclusions can be drawn:

- The selection hypothesis was confirmed, given that only the clones expressing active esterase can grow using **3b** as the only source of carbon and energy (Figure 7).
- Surprisingly, **3b** is toxic for *E. coli*. The mechanism of bacterial growth inhibition has not been clarified yet, but a MIC ≤ 7.5 mM for *E. coli* BL21 has been determined for this substance. This fact was discovered because in media supplemented with **3b** in a concentration higher than 7.5 mM no bacterial growth took place, both in the presence and absence of esterase activity. A culture supplemented with **3b** and glycerol also did not grow. This confirmed our belief that **3b** has an inhibitory effect on bacterial growth.
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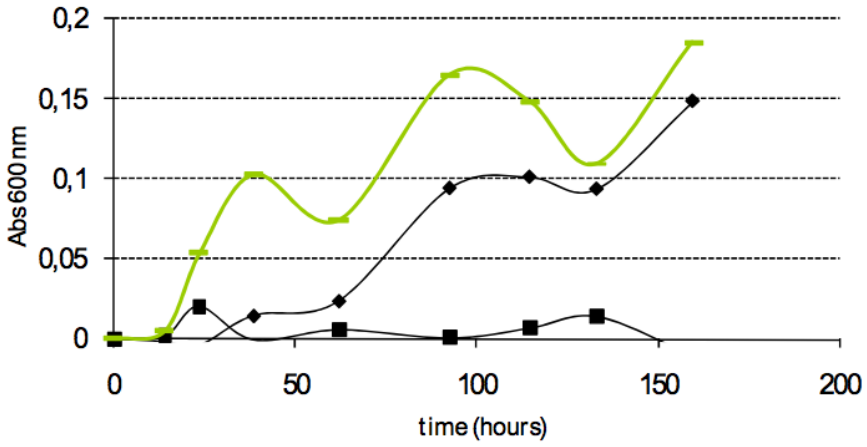


Figure 7. Selection hypothesis confirmed for the positive selection with **3b**. Green curve: positive control, M9 supplemented with 5 mM glycerol. Black curves: M9 supplemented with 5 mM **3b**. Squares: *E. coli* without esterase activity (a plasmid encoding the GFP gene was transformed instead). Diamonds: *E. coli* expressing the esterase BS2.

In the case of the control of negative selection, the bacteria were cultivated in M9 media supplemented with 10 mM glycerol and **4b**. As a control, a parallel culture with 10 mM glycerol in the absence of **4b** was used. In this system the clones expressing active esterase will catalyze ester hydrolysis and chloramphenicol will be released which causes inhibition of bacterial growth. In contrast, the inactive clones will grow because the toxin can not be released. The active clones would mimic in this case the non enantioselective mutants, because they would hydrolyze both enantiomers, releasing both glycerol and chloramphenicol. The inactive clones represent the mutants with

the desired enantioselectivity, as they would only hydrolyse the enantiomer which is esterified with glycerol, releasing in this way only carbon source and no toxin. To our delight, the bacterial growth was in fact inhibited by the clones expressing esterase, while the inactive clones and the control reached similar ODs (Figure 8). While the control of positive selection was performed with 5 mM substrate concentration (**3b**), 0.5 mM chloramphenicol ester (**4b**) was used in this case. This is due to toxicity and solubility problems and also because the low MIC of chloramphenicol would make growth inhibition possible already by a hydrolysis rate of ≤ 1% (MIC ≤ 3 µM).

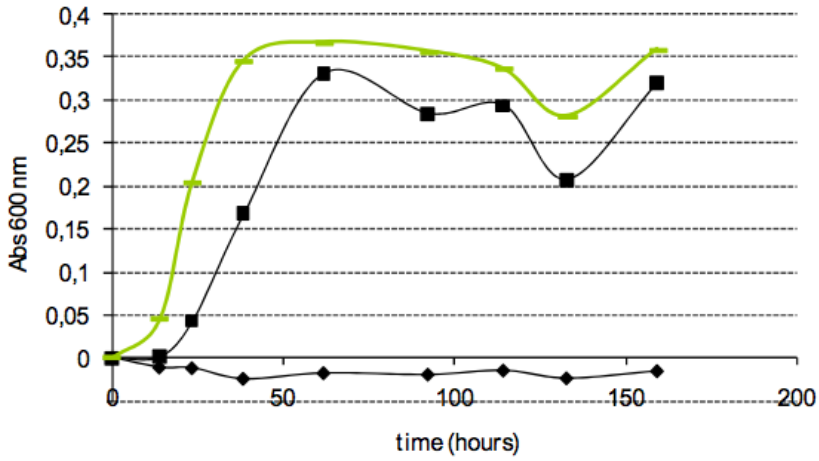


Figure 8. Selection hypothesis confirmed for the negative selection. Green curve: control, M9 media supplemented with 10 mM glycerol without **4b**. Black curves: M9 with 10 mM glycerol and 0.5 mM **4b**. Squares: clones expressing GFP (no esterase activity). Diamonds: clones expressing BS2 (esterase activity).

Although the positive and negative selection models worked well separately, biocatalysis using cell lyophilisate and **3b** and **4b**, resulted in very different enzyme activity towards the different substrates. We attempted to find a second pair of substrates more suitable for the selection, possessing greater structural similarity and thus similar enzymatic hydrolysis rates. As mentioned above (see 5.1.1.2), **8a** was elected as the new toxin and the simulation of negative selection was repeated in media supplemented with **8b**.

5.3.1.2 Substrates *2,3-dibromopropanol/glycerol*

The advantage of using **8a** instead of **4a** is the similar enzyme activity towards both selection substrates **3b** and **8b** attributed to the greater structural similarity; the main disadvantage is the decreased power of **8a** as inhibitor of bacterial growth compared to **4a**. The MIC of **8a** and **3b** for *E. coli* BL21 were very similar (Figure 9 and Table 2), so a small screen among the *E. coli* strains available in our laboratories was performed. *E. coli* JM109(DE3) exhibited a better sensibility profile for our goals (Figure 9 and Table 2). Thus we changed the *E. coli* strain accordingly.

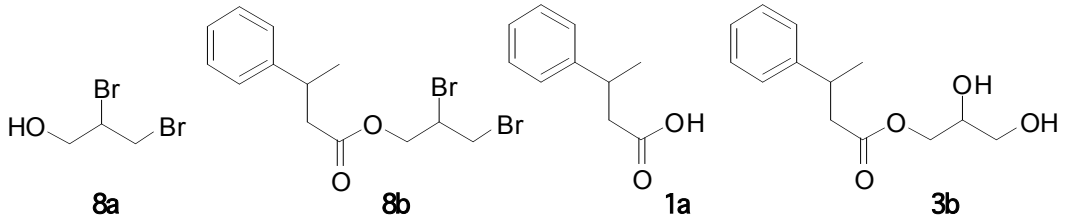


Figure 9. Chemical structures of the substrates used for the strain screening.

Table 2. The most important results obtained from the strain screening are summarized below. The sensitivity profiles of the *E. coli* strain used in the beginning are compared with the strain that showed the best sensitivity profile. The sensitivity towards the relevant substrates which will be present in different concentrations in the selection system was determined.

***E. coli* strain**	**MIC 8a (mM)**	**MIC 8b (mM)**	**MIC 1a (mM)**	**MIC 3b (mM)**
BL21	> 15	> 15	≈5	≈5
JM109(DE3)	> 15	5-10	≈15	≈ 15

An additional drawback of the change of toxin is the low solubility in water of 2,3-dibromopropanol (**8a**) and its 3-phenylbutyric ester (**8b**) at the required concentrations, making it necessary to adapt the cultivation system in order to get the substrates dispersed in the media allowing substrate- bacteria interactions to take place. Water miscible organic solvents are commonly used to increase the solubility of a variety of substrates in water, but the final concentration of the solvent in the system must be compatible with bacterial life. For our system we could not find any organic solvent which was tolerated at the concentration required to disperse the selection esters. We tried many alternative co-solvents/ additives and β-cyclodextrin was selected as the best option. This cyclic oligosaccharide is added to the culture media at a final concentration of 5 mg/ml, which facilitates to disperse the 3-phenylbutyric esters in the culture media. Additionally, the bacterial growth in M9 media supplemented exclusively with 5 mg/ml β-cyclodextrin was monitored in order to confirm that the substance can not be used by *E. coli* as carbon source.

As a consequence of the substrate dispersion the culture media became cloudy, resulting in the determination of bacterial growth through OD measurement impossible. This problem was overcome by use of the cell sorter, which can follow the bacterial growth by determining the cell concentration of samples. With this system, the MIC of **8a** was corroborated (Figure 10) and selection experiments with **3b** and **8b** were performed using the cell sorter to determine cell growth.

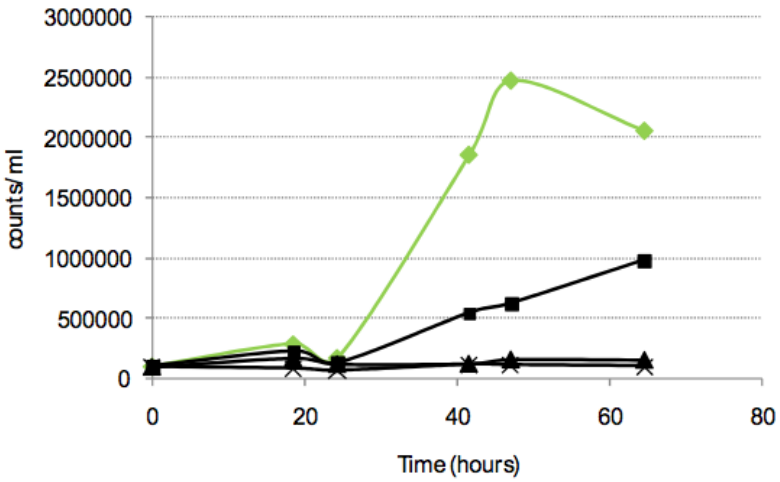


Figure 10. Growth curves in the presence of **8a**. Green curve: control, M9 media with 10 mM glycerol. Black curves: M9 media with 10 mM glycerol and different concentrations of **8a**; squares: 5 mM, triangles: 10 mM, crosses: 15 mM.

Initially, the growth of cells exhibiting esterase activity in presence of **3b** as only carbon source was checked again (Figure 11). The absence of cell growth when both esters are present was also verified (Figure 11). As positive and negative controls, cultures supplemented with 10 mM glycerol or in absence of any carbon source were used (Figure 11). The control without any carbon source is to demonstrate that no other element of the system (inductor, co-solvents, etc) facilitates bacterial growth. Having confirmed that the new system was working, the next step of the research was to use pure enantiomers and esterases with different enantioselectivities towards the substrates.

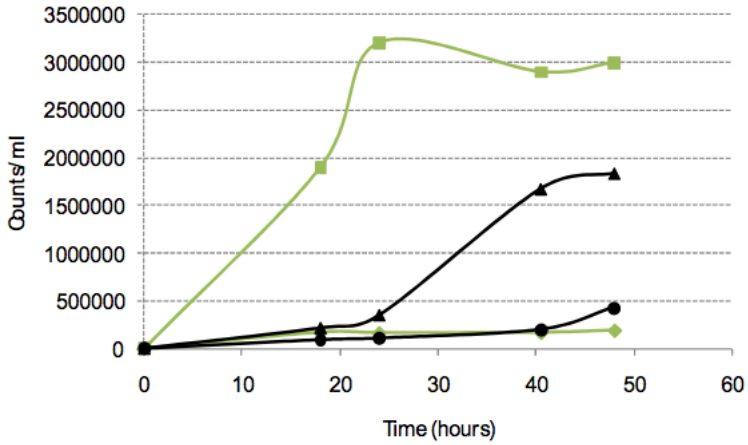


Figure 11. Bacterial growth of *E. coli* JM109(DE3) expressing BS2 in the different media described above. Green curves: controls; squares: M9 media supplemented with 10 mM glycerol; diamonds: M9 media without any carbon source supplement. Black curves: growth in clones expressing active esterase; triangles: M9 media supplemented with 10 mM **3b**; circles: M9 media supplemented with 10 mM **3b** and 20 mM **8b**.

5.3.1.3 Positive and negative controls

As mentioned above (see 5.2.2), the esterases CL1 and pEstA were identified as high enantioselective biocatalysts towards the ethyl-3-phenylbutyrate. Thus, its use as positive control for the *in vivo* selection was evaluated.

We had to validate the selection system and so developed a set of criteria. For a successful validation, our objective was to use a combination of negative and positive controls (BS2 and PFEQ, and CL1 and pEstA respectively) in differing proportions (1:1, 1:10, 1:100, etc). The method will be deemed successful if, in all cases, a clear enrichment of the positive control over the negative control is observed.

The validation was started by cultivating BS2 and pEstA separately in minimal media supplemented with (3*R*)-**3b** and (3*S*)-**8b**. The opposite enantiomers of each substrate were added to a second culture media (this means, (3*S*)-**3b** and (3*R*)-**8b**). As pEstA is *R*-selective, bacteria expressing this enzyme should be able to grow much better than bacteria expressing, for example, BS2 in the media supplemented with *R*-glycerol ester ((3*R*)-**3b**) and (*S*)-dibromopropanol ester ((3*S*)-**8b**). This is due to the fact that pEstA should only hydrolyze the glycerol ester while BS2 would hydrolyze both – the carbon source and toxin will be released. In media supplemented with the opposite enantiomers of each substrate, neither of the clones should grow as both would hydrolyze **8b**, releasing the toxin in the media. This experiment was successful as (Figure 12) in the four different culture media we observed a clear bacterial growth only in the media supplemented with (3*R*)-**3b** and (3*S*)-**8b** inoculated with *E. coli* expressing pEstA. This means that both the selection system developed and the selected controls are adequate.

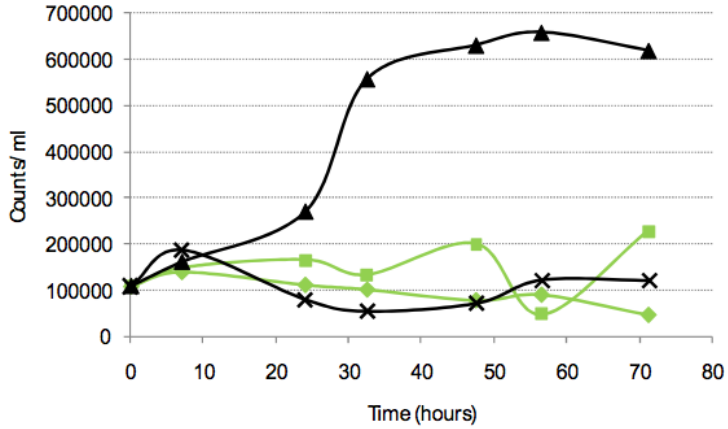


Figure 12. Growing curve with negative and positive controls (*E. coli* cells expressing BS2 and pEstA respectively) in the above described selection medias. Green curves: negative control; black curves: positive control. Triangles and diamonds: M9 media supplemented with 5 mM (3*R*)-**3b** and 20 mM (3*S*)-**8b**. Squares and crosses: M9 media supplemented with 5 mM (3*S*)-**3b** and 20 mM (3*R*)-**8b**.

5.3.2 Differentiation between E. coli's physiological status

The system reliability had been verified by quantifying cell growth for the positive and negative controls separately in the selection system. Thus, an enrichment of the positive control over the negative one, when both are cultured together in the selection system, should be expected. Nevertheless, when screening a mutant library the task is often described as being as difficult as trying to find a needle in a haystack [9]. In order to accelerate this typically laborious task, we attempted to establish a method to differentiate cell populations based on their different physiological status. In this way, only the fittest clones would be sorted out and cultivated, selected and re-sorted again in an iterative manner, until the desired enrichment has been achieved.

The application of cell sorters for the differentiation of bacterial physiological status is increasing, and as consequence in the last decade many protocols to perform this task have been published. Unfortunately, these techniques are adaptations of the methods developed originally for eukaryotes, and due to the smaller size and different composition and permeability of cell wall of prokaryotes, the finding of a suitable technique for the microorganism of interest and the pertinent toxin becomes a difficult challenge [10].

5.3.2.1 GFP/propidium iodide

Due to the small size of *E. coli* cells (0.5 x 1-3 µm) it is easier to distinguish them from the background when the cells emit fluorescence, rather than relying solely on forward and side scatter, which indicate the size and granularity of the analyzed particle [11]. Thus, we proposed that co-expression of GFP with the esterases of interest would result in all cells emitting a basal green fluorescence [12] aiding detection. The differentiation of the physiological status would be performed by using a nucleic acid stain like propidium iodide. This dye penetrates much better in cells with permeabilized cell membranes than in cells with intact membranes. Measurement of the intensity of the red fluorescence would offer us an indication of the physiological condition of cells.

The method was tested in cells expressing only GFP, and the results were acceptable (Figure 13). The GFP expression took place in LB media, afterwards these cells were further cultivated in M9 media supplemented with 10 mM glycerol, and presence (a)) and absence (b)) of 2,3-dibromopropanol (**8a**). In Figure 13 it can be observed how both cultures emit green fluorescence in similar intensity, while the intensity of the red fluorescence is significantly higher in the culture supplemented with toxin. Thus, the representation of green fluorescence (FL1) against red fluorescence (FL2) in a two parameter histogram would result in the separation of two cell populations with different physiological status. R3 and R4 are living cells and R2 and R5 are death cells; however, a better separation of the different populations would be able in the future. These results were unfortunately not reproducible when BS2 and GFP were co-expressed (Figure 14). Protein analysis of the four different populations analyzed with the cell sorter showed that the likely reason for the failure lies in the drastic reduction of the GFP expression when the protein is co-expressed with BS2, resulting in a reduction of the green fluorescence emitted.

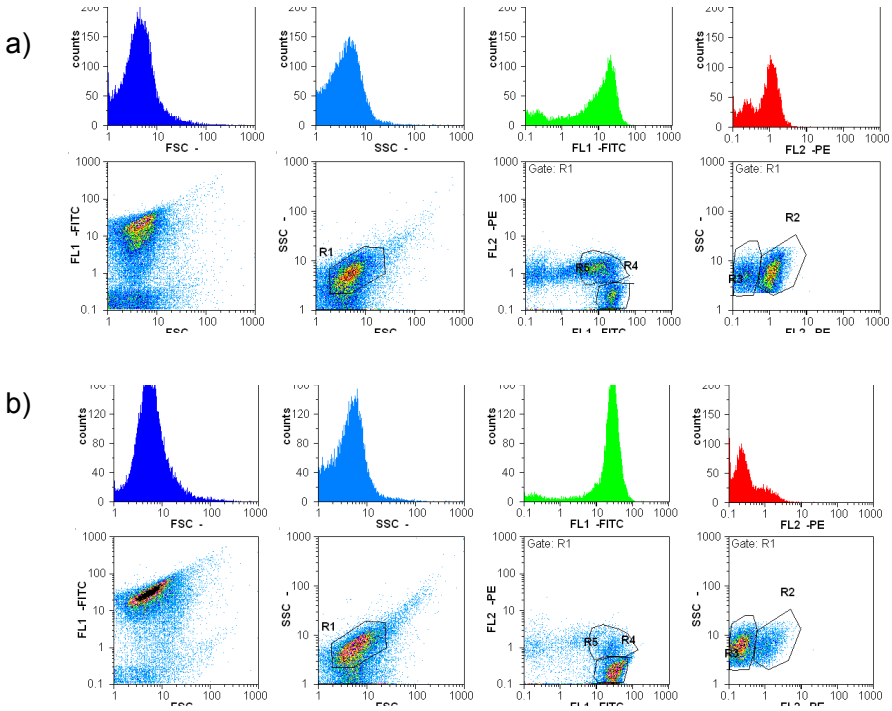


Figure 13. Flow cytometry analysis of *E. coli* JM109(DE3) expressing GFP cultivated in M9 media with 10 mM glycerol supplemented or not with **8a**. a) 15 mM **8a**, b) 0 mM **8a**. FSC: forward scatter, SSC: side scatter, FL1: green fluorescence, corresponding in this case to the GFP emission; FL2: red fluorescence, corresponding to the emission of propidium iodide bound to DNA.

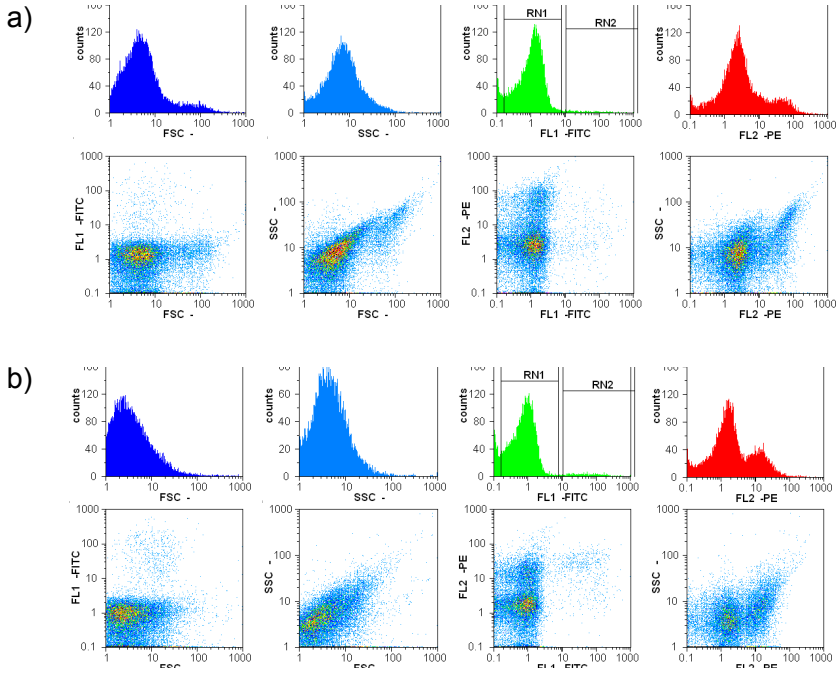


Figure 14. Flow cytometer analysis of *E. coli* JM109(DE3) expressing GFP and BS2 cultivated in M9 with 10 mM glycerol supplemented or not with **8a**. a) 15 mM **8a**, b) 0 mM **8a**. In this case there is no observable difference between the two populations.

The co-expression of GFP and BS2 (Figure 15, a)) had been performed as a first attempt of cloning each gene in a different vector, using pET28a for BS2 (kanamycine resistance) and pET11a for GFP (ampicilin resistance). After some research we discovered that vectors with identical replication origin (type ColE1 in our case) are not the optimal system for co-expression, and so tried various vectors with different replication origin: BS2 cloned in pET28a and GFP cloned in pALTER CDC42 (pACYC plasmid with p15A ori) [13]. The co-expression of GFP with BS2 was considerably better in this new system, but the expression level of BS2 was decreased. Considering the difficulty of maintaining the GFP expression level constant when co-expressed with different esterases, it was decided to substitute the green fluorescence of GFP by a two-dye system, with the aim of having a better control of the fluorescence levels and to have as much expression of the esterase as possible.

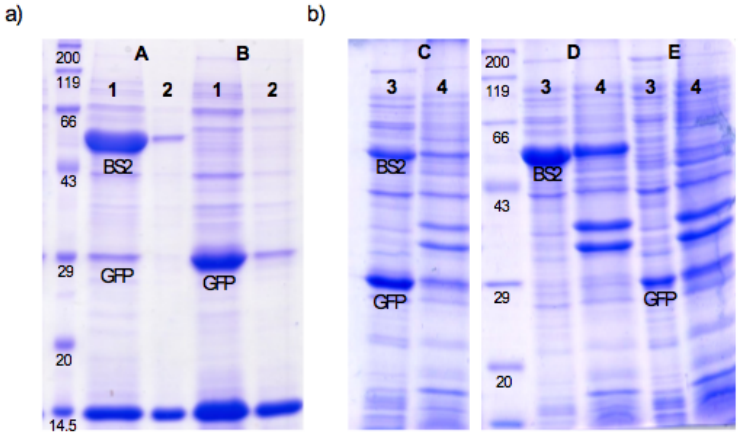


Figure 15. Protein analysis of the different expression systems by SDS-PAGE. **a)** Analysis of the four populations studied with the flow cytometer. A: *E. coli* JM109(DE3) expressing BS2 and GFP cloned in pET28 and pET11; B: *E. coli* JM109(DE3) expressing GFP in pET11. 1: bacteria cultivated in M9 media supplemented with 10 mM glycerol. 2: bacteria cultivated in M9 media supplemented with 10 mM glycerol and 15 mM **8a**. **b)** Protein analysis of *E. coli* JM109 expressing BS2 and GFP cloned in vectors presenting compatible replication origins. These cultivations were carried out in LB media because the aim was to check the relative expression of each protein. C: coexpression of BS2 and GFP; D: expression of BS2; E: expression of GFP. 3: soluble fraction, 4: pellet. In both cases the size of the standards of the protein marker are indicated in KDa.

5.3.2.2 Two-dye system

Two combinations of DNA dyes have been tested for the differentiation of *E. coli* JM109(DE3) cells affected, or not by 2,3-dibromopropanol (**8a**). These combinations are DIBAC/propidium iodide and Syto9/propidium iodide [10]. It has been described in the literature [14] that ethidium bromide in combination with Syto9 should be more sensitive than propidium iodide for the differentiation of intermediate cellular status between 100% life and 100% death. In our case we did not observe significant differences by use of propidium iodide or ethidium bromide and thus decided to use propidium iodide because of its lower toxicity.

The combination Syto9/propidium iodide was selected, because the results obtained were more easily interpreted. To establish fluorescence emission in the different physiological status, positive and negative controls were dyed with the above mentioned combinations and analyzed using the cell cytometer. As control for living cells,

E. coli JM109(DE3) cells cultivated in minimal media supplemented with 5 mM glycerol were used. The control for death cells was made by permeabilizing cells from the same culture by heating them at 95°C for 10 min. This is the standard procedure used in flow cytometry to obtain controls for death cells, after this treatment normal *E. coli* cells are death. The results obtained with the combination Syto9/propidium iodide are shown in Figure 16. Prior to the obtaining of these results some refinement of the measurement protocol has to be carried out. The intensity of the fluorescence emitted has to be adjusted so that all the different signals can be detected is the selected scale; this can be performed by selecting an adequate gain value for each parameter [15]. Afterwards, crosstalk compensation of the different fluorescence signals is performed, in order to minimize the crossed contribution between them. If these compensation values are adequately set, the different populations will be optimally differentiated in the two parameter histograms.

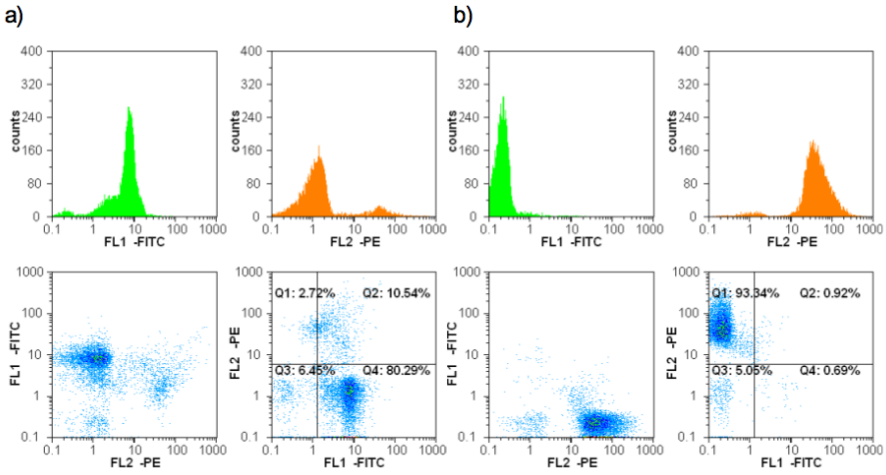


Figure 16. Flow cytometric analysis of control *E. coli* JM109(DE3) populations. The dye combination Syto9 and propidium iodide was used in both cases. a) control for living cells, bacteria cultivated in M9 supplemented with 5 mM glycerol; b) control for death cells, bacteria cultivated in M9 supplemented with 5 mM glycerol and heated at 95°C-10 min.

The control esterases (BS2, CL1, pEstA and PFEQ) were expressed overnight at 30°C in *E. coli* JM109(DE3) and these cultures were used for inoculation with an initial OD of 0.1, minimal media supplemented with 5 mM glycerol and presence or absence of 20 mM 2,3-dibromopropanol (**8a**). The effect of the toxin on cells expressing different proteins was analyzed with the established system: in comparison to the controls mentioned above the different populations appearing in two parameter histograms representing green fluorescence towards red fluorescence can be identified as following (Figure 16):

Q1: death cells or those with very low recuperation rate (<1%) after sorting out and plating on LB agar. This population emits low green fluorescence and high red fluorescence.

Q2: the status of these cells has not been completely defined until now. The recuperation rate is very low (<1%) and this is a clear sign of cell damage, probably due to membrane permeabilization. Still, the difference between Q1 and Q2 population is not apparent.

Q3: background. This is the noise due to the sheath fluid, which is used to transfer the sample through the analysis cuvette.

Q4: living cells, with a recuperation rate of circa 50% after sorting and plating on LB agar. This population emits high green fluorescence and low red fluorescence.

Thus, the counts of populations Q1, Q2 and Q4 were added up to quantify the total number of cells analyzed in each sample. In order to compare the different samples the amount of the different populations was expressed as a percentage over the total amount of cells analyzed in each individual sample (Figure 17).

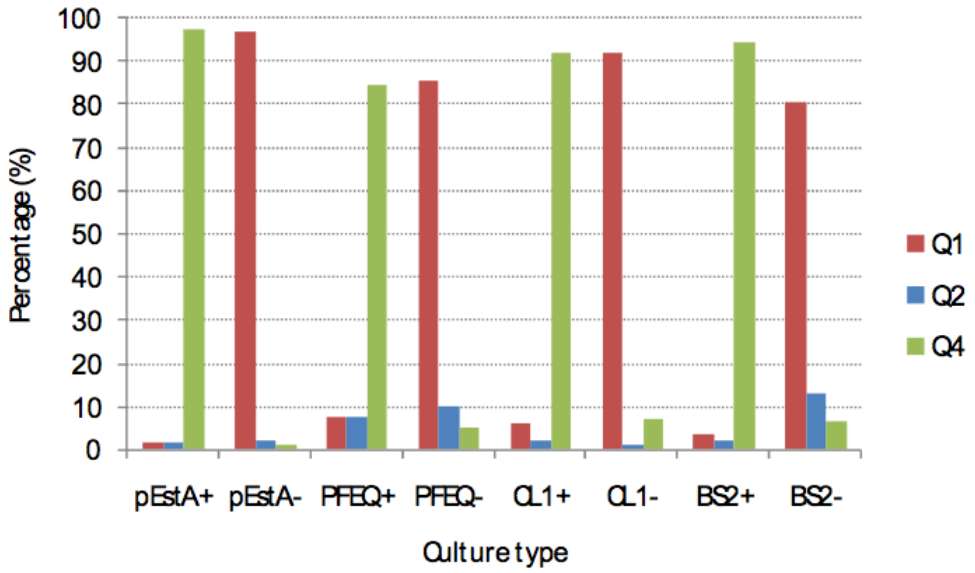


Figure 17. Graphical representation of the population distribution in the different cultures set as control. Q1 corresponds to death cells; Q2 to damaged/death cells and Q4 to living cells. All cultures expressing different proteins respond to the toxin in a similar way. Each culture expressing a different esterase is indicated with the name of the protein expressed; +: positive controls, cultures supplemented with 5 mM glycerol. -: negative controls, cultures supplemented with 5 mM glycerol and 20 mM **8a**.

5.3.3 Determination of the effect of the selection system in bacterial physiological status

A difference in the physiological status of bacteria should be faster to detect with the help of the DNA dye system, than a difference in the cell count (see 5.3.1). Moreover, when sorting mixtures of clones, this physiological difference must be detectable so that only the fittest clones are sorted out. In this way, a double enrichment will take place. The fittest clones will generate a larger progeny than the clones which are getting higher toxin concentrations, while only these fitter clones will be sorted out and recovered for a further enrichment cycle.

The effect of the selection system in the different cultures is currently under determination. Beside the toxin concentrations, a large number of factors influence the physiological status of cells, such as enzyme expression, hydrolytic activity towards the selection substrates of the different esterases, culture maturity, etc.

It has been already proofed that pEstA and BS2 do not show a similar growing behaviour in the selection system. While BS2 does not grow in M9 supplemented with either 5 mM *(R)*-**3b** and 20 mM *(S)*-**8b** or 5 mM *(S)*-**3b** and 20 mM *(R)*-**8b**; pEstA, the positive

control with (*R*) enantiopreference, grows in M9 supplemented with 5 mM *(R)*-**3b** and 20 mM *(S)*-**8b** but not in the presence of the opposite enantiomers *(S)*-**3b** and *(R)*-**8b** (see Figure 12). Similar results are obtained when these populations are analyzed with the DNA dye system after shorter times (Figure 18); while a high difference between pEstA cultured in the different selection systems is appreciable, both BS2 cultures are very similar. The fact that the toxic effect is not as clear in the case of BS2 as in pEstA, might be probably due to the different activity and enantioselectivity of both enzymes. PEstA hydrolyzes mainly the (*R*) enantiomer of 3-phenylbutyric acid, so in each system either glycerol or toxin (**8a**) is hydrolyzed. On the contrary, BS2 hydrolyzes (*R*) and (*S*) enantiomers with a similar kinetic, so a similar release of both, glycerol and **8a** occurs in the two opposite systems.

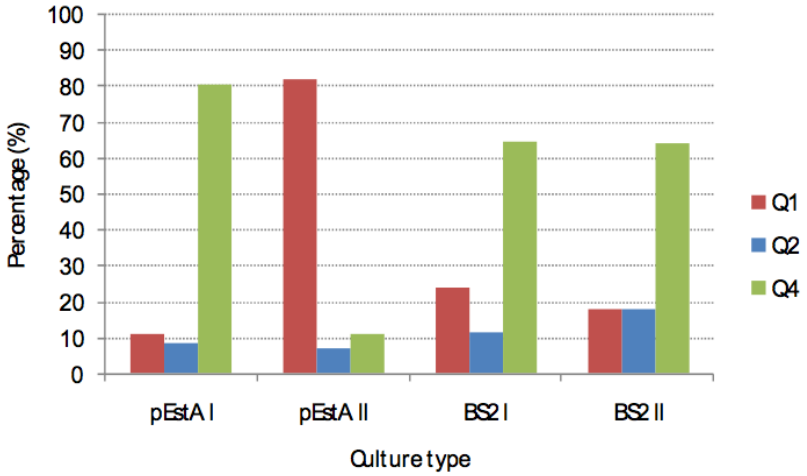


Figure 18. Graphical representation of the distribution of populations in the different cultures described above. pEstA and BS2 refer to the esterase expressed in each culture. I: cultures supplemented with 5 mM *(R)*-**3b** and 20 mM *(S)*-**8b**, II: cultures supplemented with 5 mM *(S)*-**3b** and 20 mM *(R)*-**8b**. The cultures were analyzed after 23 hours cultivation.

5.3.4 Validation of the selection system

It has been already proved that the designed selection substrates have an effect on bacterial growth when they are hydrolyzed. Moreover, this effect can be detected by flow cytometry with the help of differential dyes. All these assays until this point had been performed with single-clone cultures, but for the validation of the system, tests with mixtures of clones have to be carried out. An enrichment of the clones expressing enantioselective esterases (positive controls) over the non-enantioselective variants (negative controls) is desired. If a successful enrichment of a positive control over the negative one takes place, it is anticipated the same fact will take place when a mutant library is screened, with sorting out and further cultivation of only the more enantioselective variants.

The protocol followed in this *in vivo* selection system is represented schematically in Figure 19. The cultivation in the selection system influences the vitality of the clones and these different cells are stained with the help of differential dyes and analyzed using the flow cytometer. The fittest clones are sorted out and plated on agar, afterwards the controls can be identified and enrichment factors can be calculated.

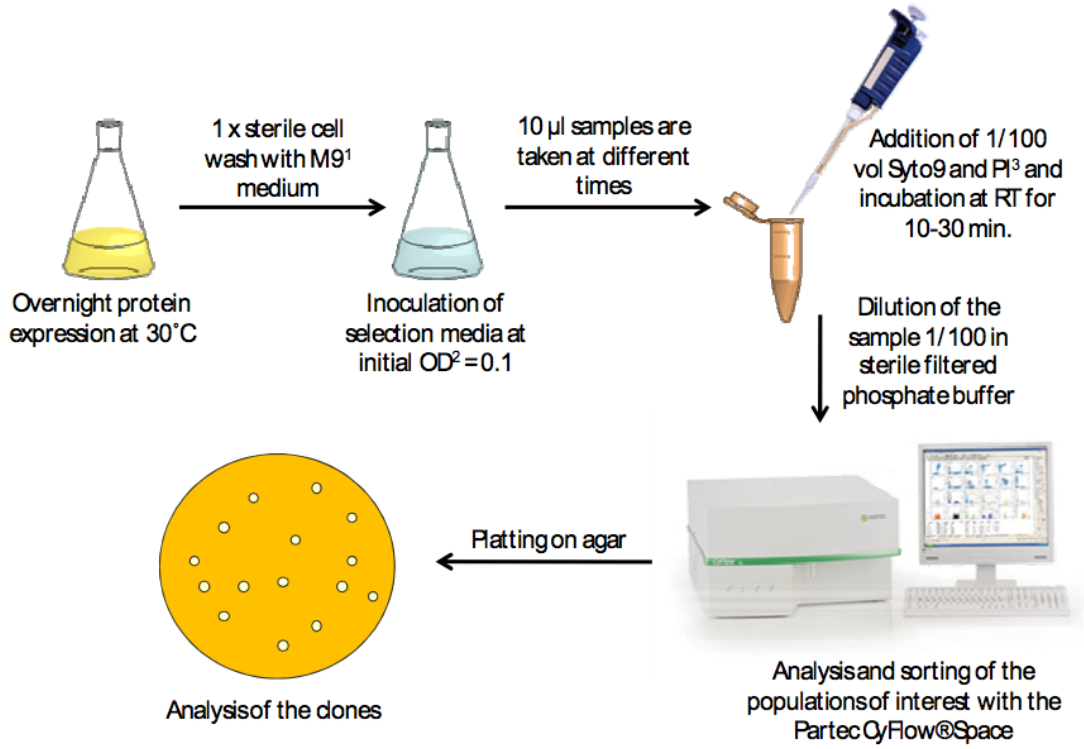


Figure 19. Scheme representing the general procedure of the *in vivo* selection system. [1]: M9 minimal media; [2]: optical density; [3]: propidium iodide.

5.3.4.1 Identification of the clones

Different methods have been selected for the identification of the different clones. Beside gene sequencing, alternative techniques can be performed which are cheaper and enable the analysis of a much higher number of clones, obtaining statistically more representative results.

5.3.4.1.1 Colony PCR

As the work was initially conducted using mixtures of BS2 (negative control) and pEstA (positive control), it was decided to analyze the clones by colony PCR. This technique relies on the amplification of the insert contained in a specific vector and is commonly used to check the success of cloning processes. The gene coding for BS2 consists of 1470 nucleotides [16], while the PEstA gene consists of 939 bp [6], allowing for the relative abundance of each population. After selection and sorting out, gene size can be estimated via electrophoresis in an agarose gel. A mixture of BS2 and pEstA in a 1:1 proportion was selected and sorted as described in Figure 19, 44 clones were analyzed by colony PCR. From the recognizable bands, 3% had the estimated size of BS2 and 97% the size of pEstA, so we can conclude that there had been enrichment in a factor of 2.

These results were very encouraging for prompting further selecting and sorting out of mixtures of positive and negative control in different proportions: 1:1, 1:10, 1:100, etc. These experiments are under way.

When creating a mutant library, the different variants will present different expression levels and characteristics. The relative overexpression rate of each protein, as well as

the structure and properties of each one, may influence cell growth manifesting as variability in the viability of the different clones in the selection system. The aim of our research is to link cell survival only to the enantioselectivity of the esterase expressed by each clone, so the ability to demonstrate the effectiveness of the system with various esterases would allow a better validation of the method.

As mentioned in section 5.2, for the development of our selection system we count with two esterases which can be used as positive control (pEstA and CL1) and two esterases for the negative control (BS2 and PFEQ). Unfortunately, the coding gene for pEstA, CL1 and PFEQ have a very similar size (around 1 kb) making the differentiation of each clone very difficult when observing the size of the amplified fragment by colony PCR. This problem will be easily overcame using a single gene specific primer for the colony PCR in addition to the pair of vector specific primers. In this way, the effectiveness of the amplification process as well as the presence of the different clones can be confirmed.

5.3.4.1.2 Overlay agar assay for differentiation of BS2 from the positive controls pEstA

The method of colony PCR for the analysis of the clones, although valid, is laborious and time consuming if many different plates need to be analyzed. When CL1 or pEstA are cultured in combination with PFEQ, the use of this method with gene specific primers is the only alternative, nevertheless when BS2 is used as the negative control a special feature of this enzyme makes it possible to perform the clone-screening in a faster and simpler manner.

BS2 has high amidase activity which makes it possible to differentiate it from the other esterases by an overlay agar assay for amidase activity (Figure 20). Replica plates with IPTG (inducer of the protein expression) are made with the cultured clones after the selection and sorting process. After incubation overnight, all the clones of the plate can be analyzed in 15 minutes the following day. This alternative method can be very useful when mixtures of positive and negative controls in different proportions are selected and sorted, as it enables the fast analysis of a high number of plates.

Figure 20. Picture of the overlay agar assay for amidase activity. In this replica plate, in the presence of inductor, an esterase without amidase activity (PFEI, left side) and BS2 (right side) have been cultivated. The clear difference in colour after the reagents addition makes it very easy to differentiate BS2 from the other esterases used in this project.

In a first attempt, mixtures in different proportions of BS2 and pEstA (1:1, 1:10 and 1:100) were cultured in M9 media supplemented with 5 mM *(R)*-**3b** and 20 mM *(S)*-**8b**, and after analysis in the flow cytometer (see 5.3.2.2) the quadrant Q4 was sorted out and plated on agar. Replica plates containing IPTG were done and the overlay agar assay for amidase activity was carried out. An example of the overlay agar assay with the replica plates is shown in Figure 21.

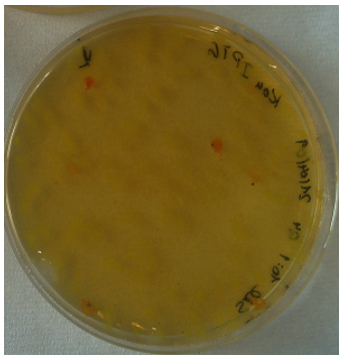


Figure 21. Overlay agar assay for amidase activity of the clones sorted out after *in vivo* selection and sorting with the Partec CyFlow® Space. From the 136 colonies, only six of them show a great amidase activity.

In order to confirm these results, when successful enrichment of pEstA expressing cells over BS2 expressing cells was observed, the same experiment was repeated again. Also the "proof of concept" experiment with opposite enantiomers of **3b** and **8b** was repeated. Living cells were sorted out after 24 hours of cultivation and plated on agar. Quality of colonies was analyzed via overlay assay while amidase activity was detected.

Quantity and quality of colonies, when BS2- and pEstA-expressing cells were mixed in various ratios, is summarized below in Table 3 and Table 4. If R-positive impulse and S-negative impulse were employed, an enrichment of pEstA- over BS2-expressing culture was clearly seen. Total number of colonies decreased with increasing ratio of BS2 over pEstA as the amount of theoretically "surviving" cells was lowered.

The results obtained from this experiment fulfil our expectations. When selecting and sorting mixtures of BS2 and pEstA in 1:1 and 10:1 proportions the maximum enrichment possible takes place. Moreover, the results obtained in a previous experiment analyzed by colony PCR (see 5.3.4.1.1) agree with the enrichment factor obtained this time, so the system seems to be reproducible. In the culture where BS2 and pEstA were mixed in a proportion 100 to 1, an enrichment of 88 over the 100 possible took place.

Table 3. Enrichment factors based on the relative initial and final abundance of pEstA in the different culture mixtures using (*R*)-**3b** and (*S*)-**8b**.

BS2/pEstA	Total number of colonies	pEstA (%)	BS2 (%)	Enrichment factor
1:1	186	179 (96%)	7 (4%)	2
10:1	136	130 (96%)	6 (4%)	10
100:1	41	36 (88%)	5 (12%)	88

In the case when opposite enantiomers of positive (S-enantiomer) and negative impulse (R-enantiomer) were utilized (Table 4), only few colonies were found on agar plates. This result is in agreement with the theory as both culture should die due to released 2,3-dibromopropanol. Interestingly, all of formed colonies were pEstA-expressing cells.

Table 4. Enrichment factors based on the relative initial and final abundance of pEstA in the different culture mixtures using (*S*)-**3b** and (*R*)-**8b**.

BS2/pEstA	Total number of colonies	pEstA (%)	BS2 (%)	Enrichment factor
1:1	4	100%	0%	n.d.
10:1	8	100%	0%	n.d.
100:1	11	100%	0%	n.d.

The same experiments as above were also performed with the newly synthesized fluorinated ester **9b** due to their better solubility. The same trend as with dibromopropanol ester was observed – total amount of colonies decreased with increasing ratio of BS2-expressing cells if R-positive impulse and S-negative impulse were employed (Table 5). In general, much lower amount of colonies was obtained than in the case of dibromopropanol ester (*S*)-**8b**. Due to this fact the calculation of enrichment factor was not possible in some cases.

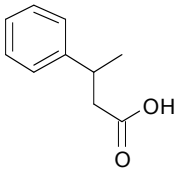
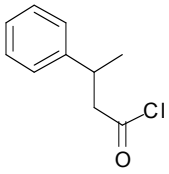
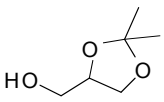
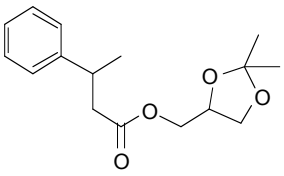
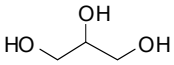
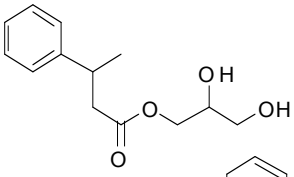
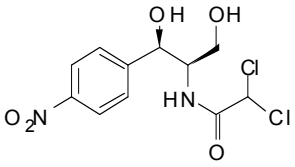
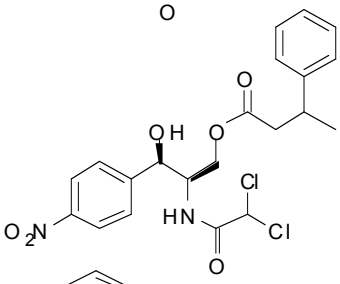
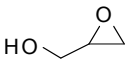
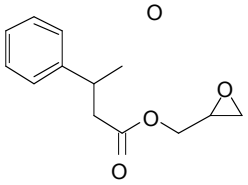
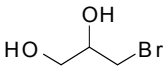
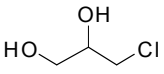
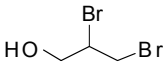
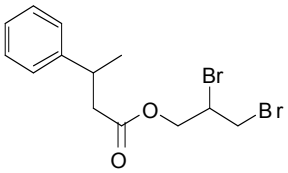
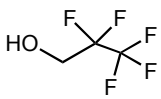
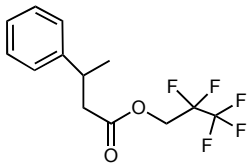
If opposite enantiomers (positive impulse (*S*)-**3b** and negative impulse (*R*)-**9b**) were utilized, no colonies were obtained at all.

Table 5. Enrichment factors based on the relative initial and final abundance of pEstA in the different culture mixtures using (*R*)-**3b** and (*S*)-**9b**.

BS2/pEstA	Total number of colonies	pEstA (%)	BS2 (%)	Enrichment factor
1:1	45	77%	23%	1.5
10:1	35	72%	28%	7.2
100:1	23	66%	33%	66

But despite these promising results these esters could not by used as selective substrates because already the ester itself showed higher toxicity than the brominated counterpart.

6 List of compounds

Formula	Nr.	Formula	Nr.
	1a		1b
	2a		2b
	3a		3b
	4a		4b
	5a		5a
	6a		7a
	8a		8b
	9a		9b

7 Literature

1. Kocienski, P.J. (2005). Protecting groups, 3rd Edition (Georg Thieme Verlag, Stuttgart).
2. Böttcher, D. (2002). Entwicklung eines in vivo-Evolutionssystems zur Veränderung der Substratspezifität von Esterasen. Diploma thesis, Greifswald University, Greifswald.
3. Hwang, B.Y., Oh, J.M., Kim, J., and Kim, B.G. (2006). Pro-antibiotic substrates for the identification of enantioselective hydrolases. Biotechnology Letters *28*, 1181-1185.
4. Hidalgo, A. Unpublished data.
5. Baumann, M., Hauer, B.H., and Bornscheuer, U.T. (2000). Rapid screening of hydrolases for the enantioselective conversion of 'difficult-to-resolve' substrates. Tetrahedron: Asymmetry *11*, 4781-4790.
6. Hotta, Y., Ezaki, S., Atomi, H., and Imanaka, T. (2002). Extremely stable and versatile carboxylesterase from a hyperthermophilic archaeon. Applied and Environmental Microbiology *68*, 3925-3931.
7. Schmeisser, C., Stockigt, C., Raasch, C., Wingender, J., Timmis, K.N., Wenderoth, D.F., Flemming, H.C., Liesegang, H., Schmitz, R.A., Jaeger, K.E., and Streit, W.R. (2003). Metagenome survey of biofilms in drinking-water networks. Applied and Environmental Microbiology *69*, 7298-7309.
8. Fernández-Álvaro, E., Kourist, R., Winter, J., Böttcher, D., Liebeton, K., Naumer, C., Eck, J., Leggewie, C., Jaeger, K.E., Streit, W.R., and Bornscheuer, U.T. (2009). Enantioselective kinetic resolution of phenylalkyl carboxylic acids using metagenome-derived esterases. Microbial Biotechnology *accepted*.
9. Böttcher, D., and Bornscheuer, U.T. (2006). High-throughput screening of activity and enantioselectivity of esterases. Nature Protocols *1*, 2340-2343.
10. Hawley, T.S., and Hawley, R.G. Methods in Molecular Biology: Flow Cytometry Protocols, 2nd Edition (Humana Press Inc., Totowa, NJ).
11. Shapiro, H.H. (2000). Microbial analysis at the single-cell level: tasks and techniques. Journal of Microbiological Methods *42*, 3-16.
12. Aharoni, A., Amitai, G., Bernath, K., Magdassi, S., and Tawfik, D.S. (2005). High-throughput screening of enzyme libraries: Thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. Chemistry & Biology *12*, 1281-1289.
13. Brakmann, S., and Grzeszik, S. (2001). An Error-Prone T7 RNA Polymerase Mutant Generated by Directed Evolution. ChemBioChem *2*, 212-219.
14. Looser, V., Hammes, F., Keller, M., Berney, M., Kovar, K., and Egli, T. (2005). Flow-cytometric detection of changes in the physiological state of *E. coli* expressing a heterologous membrane protein during carbon-limited fedbatch cultivation. Biotechnology and Bioengineering *92*, 69-78.
15. Shapiro, H.M. (2003). Practical Flow Cytometry, 4th Edition (Wiley & Sons, New York).
16. Schmidt, M., Henke, E., Heinze, B., Kourist, R., Hidalgo, A., and Bornscheuer, U.T. (2006). A versatile esterase from *Bacillus subtilis*: Cloning, expression and characterization, and its application in biocatalysis. Biotechnology Journal *2*, 249-253.