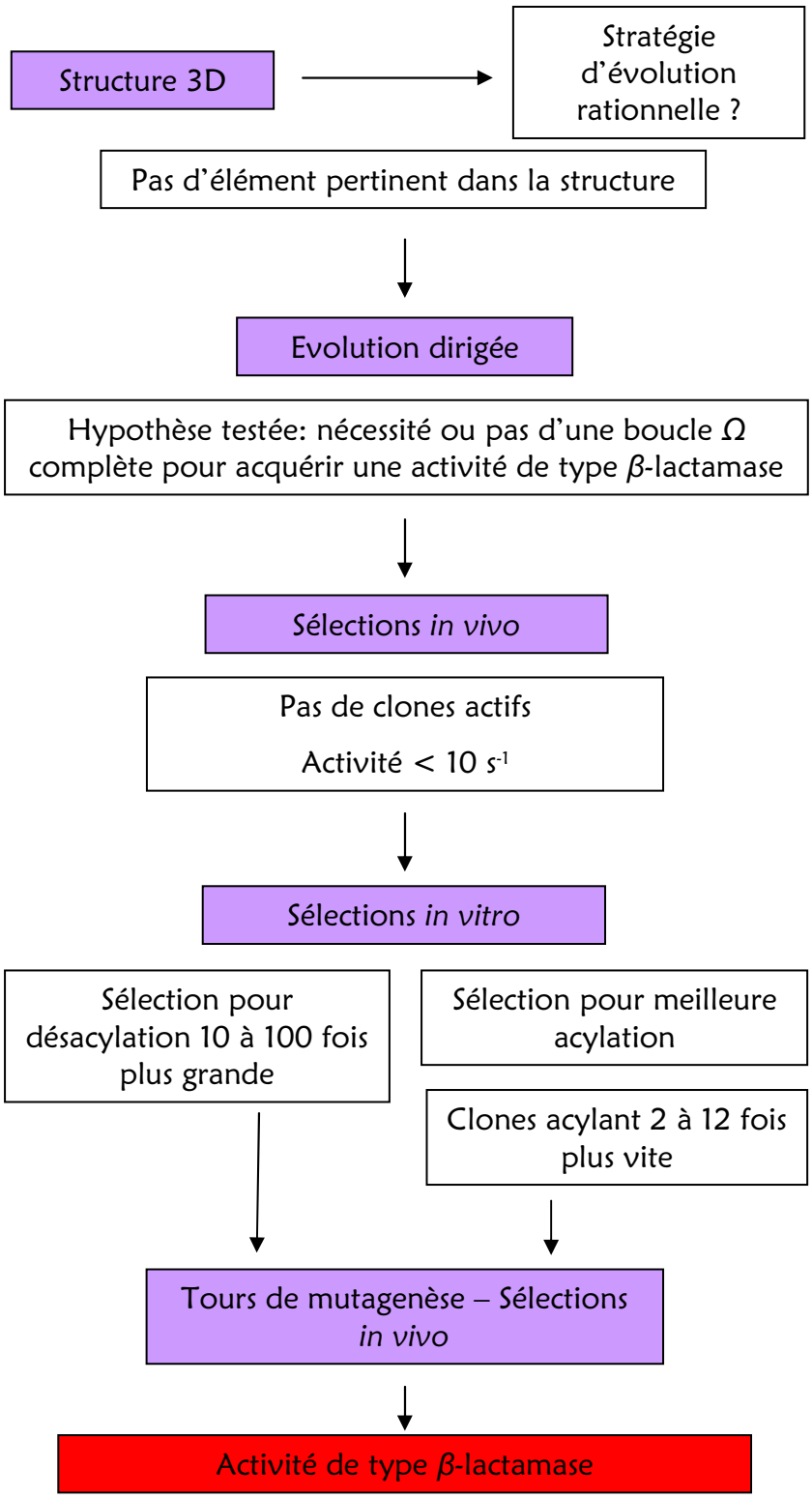
Tentatives d'évolution de PBP-A en ß-lactamase. Différentes stratégies associant mutagenèse et sélections

Les études cristallographiques de PBP-A et PBP-A-L_{158}E montrent qu'une stratégie d'évolution rationnelle est limitée puisqu'il est difficile d'établir un lien entre la structure du site actif de PBP-A et sa faible activité d'hydrolyse des β-lactames. Ainsi, nous avons établi deux stratégies pour la construction de banques de mutants de PBP-A avec pour finalité la sélection d'enzymes ayant acquis une activité de type β-lactamase.

La taille et certains résidus de la boucle Ω sont des éléments très conservés chez les β-lactamases de classe A, et les alignements de séquence de PBP-A avec ces β-lactamases montrent qu'en plus de la délétion de six acides aminés, les résidus sont peu conservés au niveau de cette boucle. La première stratégie est donc la construction d'une banque de mutants avec une boucle Ω dégénérée, plus longue de 6 acides aminés.

D'autre part, les résultats encourageants obtenus par la seule mutation du résidu L_{158} nous ont amené à élaborer une deuxième stratégie. Si la taille de la boucle Ω complète n'est pas un facteur essentiel, quelques mutations réalisées par PCR mutagénéisante suffiraient peut-être à créer une activité β-lactamase.

Deux banques ont donc été crées, et des sélections *in vivo* ont été réalisées dans un premier temps. Ces premières sélections n'ont pas permis de mettre en évidence de clones ayant des propriétés recherchées. Deux nouvelles stratégies ont alors été mises en place. La première stratégie consiste à sélectionner les enzymes acylant mieux la pénicilline par phage display, en utilisant une pénicilline biotinylée pour la capture. En effet, la vitesse d'acylation d'une β-lactamase est 10^4 fois plus élevée que celle de PBP-A, il pourrait donc être important d'isoler, lors d'une première série de sélections, des mutants capables de mieux acyler la pénicilline. A partir des clones sélectionnés, nous pourrions créer une banque de deuxième génération par shuffling et/ou PCR mutagénéisante suivi d'une sélection *in vivo* pour activité β-lactamase. Plusieurs tours de mutations et sélections seraient éventuellement envisagés. Un protocole utilisant des incubations des clones des banques sur phages avec de la pénicilline biotinylée


Structure 3D
Stratégie d'évolution rationnelle ?
Pas d'élément pertinent dans la structure
Evolution dirigée
Hypothèse testée: nécessité ou pas d'une boucle Ω complète pour acquérir une activité de type β-lactamase
Sélections in vivo
Pas de clones actifs
Activité < 10 s-1
Sélections in vitro
Sélection pour désacylation 10 à 100 fois plus grande
Sélection pour meilleure acylation
Clones acylant 2 à 12 fois plus vite
Tours de mutagenèse – Sélections in vivo
Activité de type β-lactamase

pendant des temps courts nous a permis de sélectionner des clones acylant aussi bien voire mieux que la protéine sauvage. Deux clones issus de chaque type de banque ont été exprimés et purifiés, confirmant l'augmentation de la vitesse d'acylation et nous permettant de constater que la vitesse de déacylation basale de l'enzyme n'est pas altérée dans le cas des mutants de la boucle Ω.

La deuxième stratégie est la sélection de clones ayant une activité intermédiaire qui se situerait entre celle de PBP-A et celle d'une β-lactamase. En effet, on estime que pour pouvoir conférer une résistance à une pénicilline *in vivo*, l'activité de l'enzyme doit être supérieure à environ 10 s^{-1}. L'activité de PBP-A-L_{158}E est de $1{,}42.10^{-2}$ s^{-1}, nous pensons trouver dans les banques des clones possédant une activité 10 à 100 fois plus élevée que l'enzyme mutante, ne pouvant pas être sélectionnés *in vivo*. Afin de sélectionner ces clones, C. Claeys Bouuaert a testé dans le cadre de son mémoire, une méthode de sélection *in vitro* utilisant le phage display, et préalablement mise au point par F. Chang-Pi-Hin. Il a initialement validé la méthode en montrant la sélection du mutant L_{158}E dans un mélange de phages PBP-A/PBP-A-L_{158}E dans des proportions 90/10. Il a ensuite testé la méthode de sélection sur les banques de mutants de PBP-A, mais n'a pas pu sélectionner d'enzymes améliorées. Les résultats suggèrent que le bruit de fond dû à l'accrochage non spécifique des phages sur le module de capture utilisé est trop grand par rapport aux phages qui seraient réellement sélectionnés. La méthode nécessite d'être affinée afin de diminuer ce bruit de fond. Ces résultats sont également présentés dans ce chapitre.

Abstract

PBP-A from *Termosynechococcus elongatus* is a probable DD-carboxypeptidase which belongs to a family of proteins from cyanobacteria highly homologous to class A *β*-lactamases. The main characteristic of this family is a deletion of 6 amino acids in the structurally and catalytically important *Ω*-loop of class A *β*-lactamases. Moreover, the essential Glu_{166} is missing in this shorter *Ω*-loop. On the basis of kinetic and crystallographic studies of the wild-type and $L_{158}E$ mutant that deacylates the acyl-enzyme formed with benzylpenicillin 90 times faster, it was suggested that this family of proteins share a common ancestor to class A *β*-lactamases, and may be a good starting point for *in vitro* evolution of lactamase activity.

Since this protein scaffold seems versatile enough for attempting evolution into a *β*-lactamase, two libraries of mutants were created on phagemids, one by introducing random mutations along the entire $L_{158}E$ mutant (P library), and another by inserting a degenerated cassette of full-length *Ω*-loop in place of the shorter one (L library).

Facing the failure of *in vivo* selections for ampicillin resistance, less ambitious strategy was investigated. Besides improving the deacylation rate, one step further lactamase evolution is increasing acylation rate. This was achieved by *in vitro* selections using a biotinylated penicillin substrate. Both library populations were enriched in mutants capable of acylating benzylpenicillin at very low concentration. Expression and purification of 4 mutants allowed determining kinetic parameters. Penicillin acylation rate was generally increased for all mutants, and activity towards thiolesters, that are typical DD-peptidases substrates, showed activity decreases and specificity changes. A detailed analysis of the results is shedding some light on a possible evolution path between PBP-A and *β*-lactamases.

Understanding the emergence of novel proteic functions is nowadays a great challenge, and engineering proteins that compete with natural ones has encountered only limited successes. Though the evolutionary adaptability of enzymes through the Darwinian process of mutation and selection naturally gave rise to proficient and robust enzymes, reproduction of this entire evolution process in a laboratory remains a very hard task [1].

One of the best illustrations of this problem is the difficulty to find an evolutionary path between two phylogenetically linked families of enzymes, PBPs and *β*-lactamases. PBPs or DD-peptidases are membrane bound enzymes involved in bacterial peptidoglycan synthesis. They are inhibited by *β*-lactam antibiotics by forming a stable covalent acyl-enzyme with these molecules. One of the most efficient defence mechanisms that bacteria developed for fighting these antibiotics is the emergence of *β*-lactamases, which, through formation of an acyl-enzyme that is hydrolysed extremely rapidly, are able to destroy *β*-lactams. On one hand, there are two classes of DD-peptidases of high and low molecular weight (H/LMW) each subdivided into three sub-classes on basis of the sequence alignments. On the other hand, three classes of serine *β*-lactamases A, C and D (class B *β*-lactamases are metallo-enzymes) are sharing with DD-peptidases a catalytic serine in their active site and a similar mechanism of action [2]. This functional similarity, together with structural homology highlighted by a similar fold, and conservation of three consensus sequences (SXXK, S/YXN and KS/TG), indicate that these enzymes are phylogenetically linked, and that they have probably evolved from a common DD-peptidase ancestor. In a more detailed manner, it was proposed by *Massova & Mobashery* that the PBP ancestor gave rise to different PBPs that evolved in a divergent way, and some subclasses of PBPs may be related to one class of *β*-lactamases [3]. Although catalytic residues are conserved, the main difference resides in the absence of a residue that may catalyse hydrolysis of the penicilloyl-enzyme in PBPs. The first reported increase in a PBP deacylation was the result of a point mutation in R61 DD-peptidase which is homologous to class C *β*-lactamases. Mutant $W_{233}L$ deacylated penicillin G 300 times faster (k_{cat}) than the wild-type protein, however, catalytic efficiency (k_{cat}/K_m) was decreased by 200 times. This mutant could not provide *in vivo* resistance to ampicillin [4]. One of the well studied *β*-lactamases TEM-1, belongs to the most widespread class A *β*-lactamases. This class of enzymes is characterised by an *Ω*-loop of highly conserved length containing a glutamic

acid residue (Glu_{166}), strictly conserved and essential for the catalytic mechanism, particularly in the deacylation mechanism. Introducing an aspartate in place of Phe_{450} of PBP2x, spatially equivalent to Glu_{166} in TEM-1, allowed increasing deacylation rate by 110 times [5]. On the basis of this work, *Peimbert & Segovia* introduced further semi-random mutations and could select clones for *in vivo* cefotaxime resistance. Among selected clones, MutE contains only three mutations and has a deacylation rate increased for both cephalosporin and penicillin. Though k_{cat} was in the same range for both antibiotics, catalytic efficiency was 10 times lower for ampicillin, and this clone could not confer *in vivo* resistance to this antibiotic [6]. Hence, these results show the difficulty to evolve a PBP into a *β*-lactamase, especially when selecting for penicillin resistance.

Recently, a new family of PBPs closely related to class A *β*-lactamases but containing a six amino acids deletion in the *Ω*-loop and no equivalent residue to Glu_{166} has been identified. Of these proteins, PBP-A from *Termosynechococcus elongatus* was characterised. Activity towards the peptidic substrate Ac_2-L-Lys-D-Ala-D-Ala was undetectable but it was very active against thiolester substrates, suggesting that PBP-A is probably a DD-carboxypeptidase. Activity towards *β*-lactams showed PBP-A to be one of the fastest among PBPs for the deacylation reaction with $k_3 = 2{,}3.10^{-3}\ s^{-1}$ at 55°C (optimal *Termosynechococcus elongatus* growth temperature) and $1{,}55.10^{-4}\ s^{-1}$ at room temperature. PBP-A-L_{158}E mutant was generated by introducing a glutamate in place of leucine 158 which is aligned with Glu_{166} of class A *β*-lactamases. This mutant showed a 90 times increase in k_3 and seven fold decrease in acylation rate. PBP-A and mutant crystallisation showed the enzymes to adopt the DD-peptidase family overall fold, and an organisation of the catalytic site similar to class A *β*-lactamases.

This information suggests that PBP-A is a good candidate for evolution experiment towards *β*-lactamase activity. For this purpose, two strategies were investigated for creating mutants libraries from which PBP-A having acquired increased activity might be selected *in vivo*. A random mutagenesis strategy was investigated by creating an error-prone PCR library along the whole gene of PBP-A-L_{158}E. The second strategy was based on the assessment that natural catalytically active *β*-lactamases contain a 19 residues *Ω*-loop that forms a side of the active site, and is a critical element for *β*-lactamases activity. This *Ω*-loop that can be defined from residues 161-179 in class A *β*-lactamases has been extensively studied [7, 8]. Three invariable electrostatic interactions, a salt bridge between residues Arg_{164} and Asp_{179}, hydrogen bonds between main chain carbonyl and amine groups of Asn_{136}, and main chain

amine and carbonyl groups of Glu_{166} respectively, and interactions between Glu_{166} and Lys_{73}, maintain the structural integrity of the *Ω*-loop, giving the good orientation to Glu_{166} for activating a water molecule in the hydrolytic deacylation step. Another important residue is Asn_{170} which maintains the hydrolytic water molecule in the right position. To a less extent, residue at position 169 is a hydrophobic anchor, containing a conserved hydrophobic residue. Besides those highly conserved amino acids, the loop length seems an important element for the substrate selectivity, since engineered lactamases with longer *Ω*-loops or a natural SHV-16 containing a pentapeptide duplication in the *Ω*-loop are enzymes with extended spectrum lactamase activity [9, 10]. On the other hand, only the C-terminal part could support deletions. Particularly, region 171-177 could afford complete deletion while maintaining level of ampicillin resistance only five times lower than wild-type TEM-1 [8]. Thus, our idea for creating the second library was to engineer an *Ω*-loop of full-length in place of the shorter one in PBP-A, introducing variability at each position such that the main represented amino acids in class A *β*-lactamases is present as well as the wild-type PBP-A sequence.

Construction of both libraries in phagemid allowed either *in vivo* or *in vitro* selections. *In vivo* selections for ampicillin resistance on both libraries were unsuccessful; and it was decided to rather mimic natural evolution by evolving PBP-A in two steps. Class A *β*-lactamases are characterised by both high acylation and deacylation rates. Though reasonable, PBP-A acylation rate is 3 to 4 orders of magnitude lower than in TEM-1, we thus investigated the kinetic propensity of some mutants to acylate penicillin G faster.

Phage display is a method that relies upon the ability of an entity (protein, peptide, or antibody fragment) to be displayed on the surface of a phage particle, creating a physical link between the entity and its encoding gene. This technology was first described by *Smith* in 1985 [11], and since then, numerous applications in selecting enzymes of high interest for a desired binding property, or other characteristics have proven efficient [12]. Previous work on a library of mutants of a *β*-lactamase mutated in the *Ω*-loop allowed selection of highly reactive PBPs: mutants of TEM-1 *β*-lactamase displayed on phages were exposed to a penicillin substrate conjugated to a biotin module that allowed phages capture on streptavidin coated beads. Controlling incubation times and substrate concentration allowed selecting PBPs with acylation rates approaching 10^6 $M^{-1}s^{-1}$ that is only two orders of magnitude lower than *β*-lactamases [13]. In this work, PBP-A mutants displayed on pIII phagemid particles were selected for fast penicillin acylation, using the same strategy. After several rounds of panning, the reactivity of randomly picked clones was analysed by phage-ELISA. Expression

and purification of two clones from each library permitted to measure kinetic parameters on both benzylpenicillin and thiolester substrates, giving new insights for the evolution of PBP-A into a β-lactamases.

Display of PBP-A and PBP-A-L$_{158}$E enzymes on phage

The efficient display of functional wild-type and mutated enzymes is a prerequisite for the *in vitro* selection of enzymatic activities. In this study, we first displayed both enzymes PBP-A or PBP-A-L$_{158}$E in fusion to the C-terminal portion of pIII (amino acids 249-406) on filamentous phage. For this purpose, genes of interest were cloned into the pHDi.Ex.Kan^R vector, a kanamycin resistant version of the pHDi.Ex vector [14]. The resulting fusion gene encodes sequentially for the signal sequence OmpT, PBP-A or PBP-A-L$_{158}$E, a (His)$_6$ tag, a suppressible amber stop codon and the truncated pIII protein. This construct is under the control of two promoters, a strong promoter pT7 which can be used for protein overexpression, and a promoter pLac, for basal protein expression necessary for phage display. From these vectors, phages were produced using standard procedures, and high titres were obtained (> 10^{10} t.u. per mL in bacterial supernatants).

The display of PBP-A and PBP-A-L$_{158}$E on phage was tentatively analysed by western blotting using an anti-pIII monoclonal antibody [15]. A band of the size of pIII was clearly detectable, but not of the fusion protein, certainly due to the low display of the protein on phages (results not shown).

However, the display and functionality of PBP-A and PBP-A-L$_{158}$E was confirmed by phage-ELISA, using a biotinylated penicillin derivative (Pen-Biot, Figure 53) [16] and streptavidin coated microtitre plates.

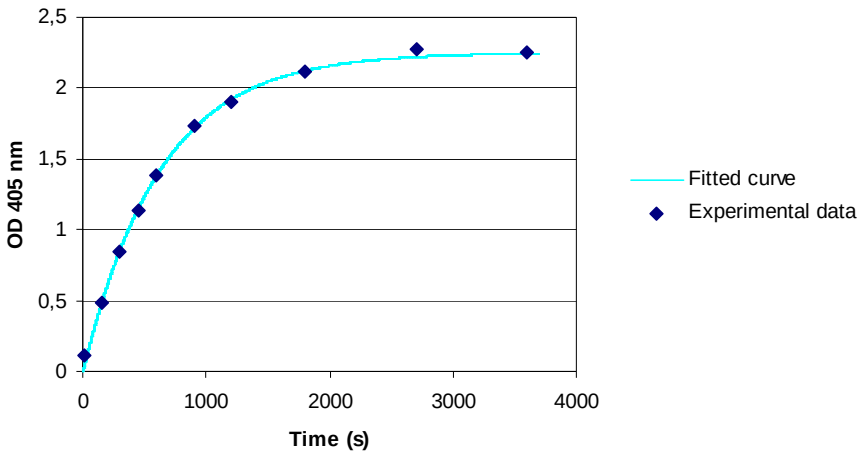


Figure 45 : kinetic of acylation of PBP-A: fitted curve (cyan line) vs experimental data (♦) obtained by ELISA (using Pen-Biot).

10^9 PBP-A infectious phages were incubated with 10^{-7} M Pen-Biot, reaction was stopped at different times by adding β-lactamase, and aliquots were submitted to ELISA. After incubation of biotinylated phages in the streptavidin coated wells, a peroxidase coupled to anti-M13 antibody allowed relative quantification of bound phages at 405 nm. From the equation $d[ES] = (k_2/Ks).[S]_0.d[E].dt$ an exponential curve dependent on k_2/Ks can be fitted with experimental values obtained by ELISA (Figure 45).
In this way, k_2/Ks obtained for PBP-A was $1{,}6.10^4$ $M^{-1}.s^{-1}$ which is only 1,5 times higher than the value found for acylation rate of PBP-A with benzylpenicillin (Table 17).

	k_2/K_s (M^{-1} s^{-1})	k_3 (s^{-1})	k_{cat} (s^{-1})
PBP-A	$1{,}07.10^4$	$1{,}55.10^{-4}$	$2{,}7.10^{-4}$
PBP-A-L_{158}E	$1{,}56.10^3$	$1{,}44.10^{-2}$	$1{,}42.10^{-2}$
TEM-1	$8{,}4.10^7$	1500	ND
L-b	9.10^4	$1{,}64.10^{-4}$	$1{,}8.10^{-4}$
L-t	$1{,}33.10^5$	$1{,}4.10^{-4}$	ND
$P_{2\text{-}a}$	$2{,}3.10^4$	8.10^{-5}	ND
$P_{9\text{-}17}$	$> 1.10^5$	$6{,}1.10^{-5}$	ND

Table 17 : kinetic parameters for hydrolysis of benzylpenicillin at 20°C for PBP-A and PBP-A-L_{158}E mutant (see page 105) and four mutants selected for better acylation. k_2/Ks and k_3 were determined indirectely using [^{14}C]-Penicillin G and k_{cat} was determined directly following benzylpenicillin hydrolysis at 232 nm. Constants for TEM-1 are from [16].

This shows that PBP-A is displayed on phage and has the same behaviour with Pen-Biot than soluble PBP-A with Penicillin G. ELISA performed in the same way for PBP-A-L_{158}E showed no phages binding on streptavidin coated beads (results not shown). This is not surprising as at low benzylpenicillin concentrations, the higher deacylation rate of this mutant on phage (concentration probably around 2.10^{-11} M) allows only 1% transient phage-enzyme formation. Hence, the ELISAs experiments clearly show that enzymes of activity comparable to that of PBP-A are present on phages, getting out any doubt on the protein display. For PBP-A-L_{158}E, we assumed that it follows the same behaviour as the wild-type.

Libraries design and construction.

We chose PBP-A-$L_{158}E$ as the template for the construction of a random whole gene mutagenesis scheme with the hope that a few mutations increase deacylation activity. Thus, error-prone PCR (*Cadwell & Joyce*, 1992) allowed creating two libraries P_1 and P_2 with a diversity of respectively $3{,}2.10^5$ and $8{,}65.10^6$ clones with an average of 5 effective mutations per gene for P_1, and 3 effective mutations per gene for P_2, of which around 35 % were transitions. Randomly picked clones were sequenced and showed the expected large variability, and a good repartition along the sequence.

The most striking difference between PBP-A and β-lactamases is the size of the Ω-loop which is strictly conserved in class A β-lactamases, and is shorter in PBP-A. Following the hypothesis that restoring a full size Ω-loop would help conferring lactamase activity to PBP-A, a full size loop was introduced by cassette mutagenesis.

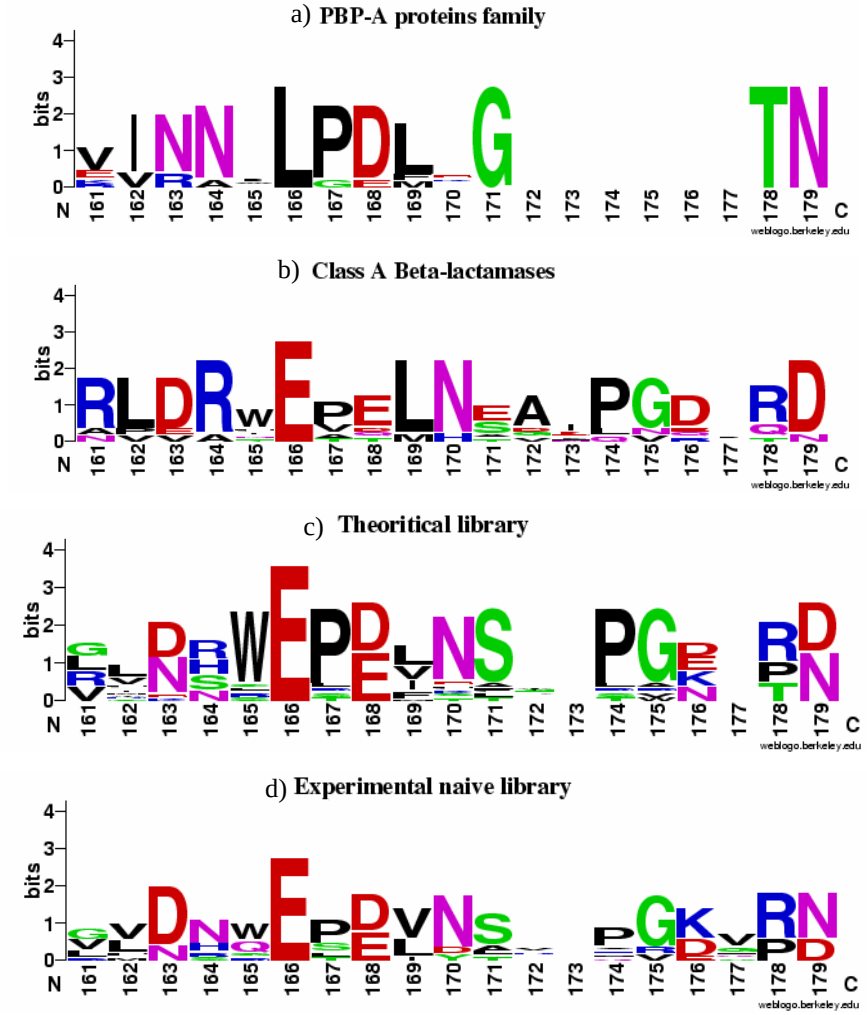


Figure 46 : logo representation of the amino acids variations in the region of the Ω-loop in PBP-A proteins family (a), in class A β-lactamases (b), and in the theoretical (c) and naive (d) libraries achieved at weblogo website http://weblogo.berkeley.edu/ [17]. Numerotation follows Ambler numbering scheme [18].

The cassette was designed such that the proportion of wild-type and class A β-lactamases sequence is equal except in the region of the deletion where the insertion follows class A β-lactamases sequence, taking into account the constraints of the genetic code (Figure 46). Exception was made at position 166 where a glutamate was imposed. Residues 153 to 165 in PBP-A (161 to 179 in class A β-lactamases) were replaced by a degenerated loop of 19 residues, giving rise to the L library of $3{,}1.10^6$ clones. Among sixteen sequenced clones, seven had a deletion inducing a frame shift and the remaining well constructed ones were used to construct the logo presented in Figure 46-d, showing a sample of amino acids represented at each position. The sequences observed are in agreement with the theoretical sequence, indicating no important bias in the construction of the library.

P_1 and L libraries were amplified without addition of glucose, allowing expression of the mutated protein at a basal level. Plating colonies on agar plates indicated a heterogeneous population with big and small colonies. Sequencing of big colonies in P_1 library indicated the presence of mutations in the active site, and sequencing in L library showed introduction of stop codons at a higher level than theoretically expected. However, these stops could more often be suppressed to Gln. These observations suggest a certain level of toxicity of the protein, certainly due to its activity towards peptidoglycan. However, this toxicity is not sufficient to prevent periplasmic overexpression of wild-type protein in BL21(DE3) cells, suggesting that the specificity of the protein is poorly adapted to *E. coli* peptidoglycan.

In vivo selections.

At first, we assessed the presence of *in vivo* selectable clones for ampicillin resistance in the libraries. Two bacterial systems were used, the BL21(DE3) system which allows proteins overexpression under the T7 promoter control; and the JM109 system which has the SupE44 phenotype, allowing the proteins to be expressed in the phage display format, and under the control of pLac promoter. Selections in BL21(DE3) under overexpression conditions may increase the chances to select clones having increased deacylation rate. However, it did not give any positive clones at concentrations of ampicillin as low as 5 µg/mL. Selections in JM109 cells permitted to select six clones with MICs ranging from 2 to 5 µg/mL (MIC of JM109 expressing PBP-A-L_{158}E mutant protein is around 1 µg/mL) in L library. The absence of selected clones in BL21(DE3) may be due to some toxicity inherent to the plasmid and/or protein overexpression. It has been shown that BL21(DE3) cells growth is sensitive to pET vectors and the effects are more or less severe in function of the protein overexpressed [19]. Selected clones in JM109 host strain were retransformed and analysed. Sequencing of the best

clone (MIC = 5 µg/mL) showed a single deletion in the sequence, resulting in a frame shift giving rise to a truncated protein missing the KTG motif. This kind of observation has already been made, where polypeptidic fragments lacking essential elements of the catalytic machinery are able to confer some ampicillin resistance. Since ampicillin is susceptible to hydrolysis by simple acid or base catalysis, it was proposed that polypeptides displaying appropriate groups may promote ampicillin hydrolysis at low but detectable rates. In this case, selections for lactamase activity may have to be performed at ampicillin concentration as high as 10 µg/mL to prevent this type of false positives [20]. Two other clones with MICs of 2 and 4 µg/mL containing a well constructed longer *Ω*-loop were overexpressed and purified. Catalytic activity was measured (see Annex 4), but surprisingly, there was no increase in the enzymatic activity of these mutants compared to the wild-type protein. This confirms the previous hypothesis that clones below MICs of 10 µg/mL may be false positives. Another conclusion from these selections is that both libraries do not contain clones featuring k_{cat} around 1-10 s^{-1} or above, since this is the minimal rate necessary for providing *in vivo* resistance (information from the lab).

In vitro selections-Model experiment

Previous studies on PBP-A show higher sequence and structural homologies to class A *β*-lactamases than other PBPs. Hence, it was hoped that one round of mutagenesis may be sufficient for raising *β*-lactamase activity on PBP-A scaffold. However, after the failure of *in vivo* selections, the necessity to go through this evolution by smaller steps of activity improvment was obvious. Therefore, we decided to test an *in vitro* selection strategy which theoretically allows the selection of weaker catalysts than the *in vivo* approach. When comparing PBP-A kinetic parameters with those of the best studied class A *β*-lactamase TEM-1, it is important to note that not only the deacylation efficiency is low, but acylation is also 4 orders of magnitude lower (Table 17). In nature, it can be assumed that to evolve to *β*-lactamases and following duplication, PBPs may have initially acquired features favouring acylation and probably overexpression, and then they may have evolved towards better deacylation. Hence, the selection of better acylating enzymes could be a prerequisite step for the evolution of a *β*-lactamase from a PBP.

For this purpose, we established a simple selection protocol allowing the capture of the faster acylating phage-enzymes by rapidly incubating the enzymes with a biotinylated penicillin at low concentrations, and capturing the labelled phages with streptavidin coated beads. A model experiment was initially performed: 10^9 PBP-A or PBP-A-L_{158}E phages were

incubated with 10^{-7} M Pen-Biot. After 10, 100, 400, 1000 and 2000 seconds, the reaction was stopped and panning of biotinylated phages on streptavidin coated magnetic beads allowed their capture. After extensive washing of the phages adsorbed by non-specific interactions, the selected phages were eluted by reduction of Pen-Biot disulfide bond. They were finally titrated by infection of an *E. coli* TG1 culture in exponential phase. The results are shown in Figure 47, though the binding efficiency of the phages is low, due most certainly to the low display of protein, the evolution of binding as a function of time is in quite good agreement with what is expected.

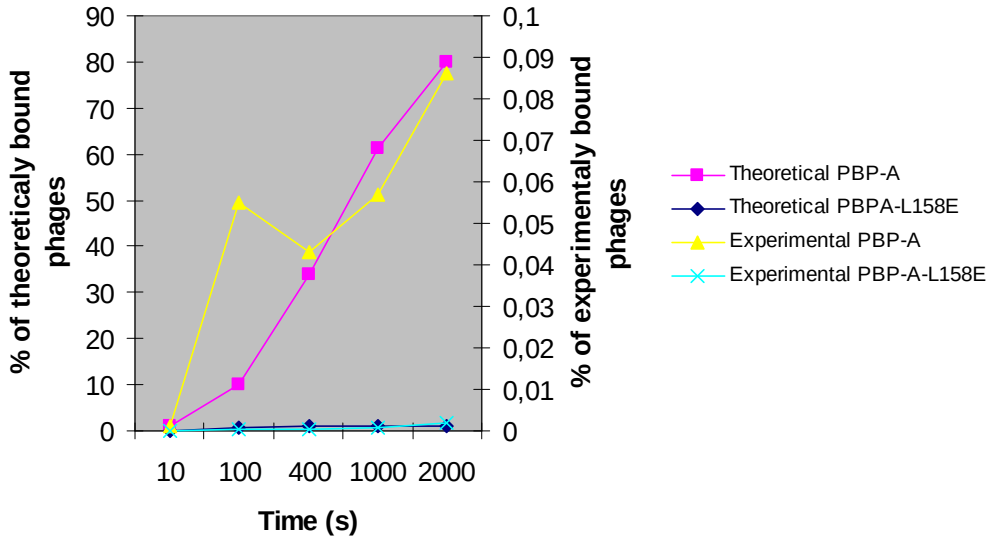


Figure 47 : model experiment for the capture of phage-enzymes over a 2000 s period of time. Theoritical values were calculated for 1.10^9 phages and 10^{-7} M Pen-Biot, experimental values were determined in the same conditions, by counting the number of colonies infected with phages from elution from streptavidin coated beads with DTT.

Wild-type phage is progressively acylated and subsequently captured, at a level much higher than $L_{158}E$ mutant. The restrictions of this selection protocol reside in two aspects. First, phage-enzymes that acylate and deacylate rapidly are going to be discarded, as illustrated by PBP-A-$L_{158}E$. In our case, *in vivo* selections showed that high deacylating enzymes do not exist in the libraries; hence, the objective is to maintain the basal enzyme deacylation rate while increasing the acylation rate. The second restriction comes from technical limits. It is impossible to manually mix phages and Pen-Biot and then add *β*-
lactamase for stopping the reaction in less than 10 seconds. Hence, it will be a hard task to increase the selection pressure over 10 seconds incubation with Pen-Biot.

Though P libraries were constructed for obtaining a majority of clones that deacylate through E_{158}, the rate of mutations and library size allows us to expect ~ 6.10^4 clones and ~10^5 clones

having E_{158} mutation reverted for P1 and P2 libraries, respectively. This is still rather high diversity for exploring libraries acylation rate. In the same way for L library, though E_{158} is imposed, we expect that the loop can adopt many conformations, some of which allow acylation but not deacylation.

In vitro selections

Following the model experiment, five rounds of panning were performed in parallel with the three libraries, incubating 1.10^{10} phages with 10^{-7} M Pen-Biot, and decreasing the incubation times from 1000 to 100 s during two first rounds of selection, followed by 3 rounds of panning with 10 s incubations. A control selection consisting of an experiment without increasing the selection pressure was achieved with P_2 library ($P_{2\text{-}C}$). A negative control was performed during the 5th panning, incubating phages with benzylpenicillin instead of Pen-Biot indicated that the capture of non specific eluted phages was very low. Thereafter, selected clones from $P_{1\text{-}5}$ and $P_{2\text{-}5}$ were mixed and this P_1-$P_{2\text{-}5}$ pool (P) was subjected, as L-5, to four other rounds of selection, decreasing the concentration of biotinylated penicillin to 10^{-8} M, with 10 s incubation times. Two parameters were indicators of the selections effectiveness.

First, enrichment in total phages after each round of panning was estimated using the elution yield (ratio of infectious phages recovered on elution over infectious phages committed). If a selection is working, it is expected that enrichment in total phages increases over selection rounds. Enrichment in phages binding is clearly observed after the 5th round and less obviously after the 9th round of panning (Figure 48), suggesting that the selection was efficient. Second, the sequencing of clones at the two main steps of the selection indicated a profile of efficient selection with recurrent clones in the different selected libraries after the 5th and 9th round of panning (Annex 5).

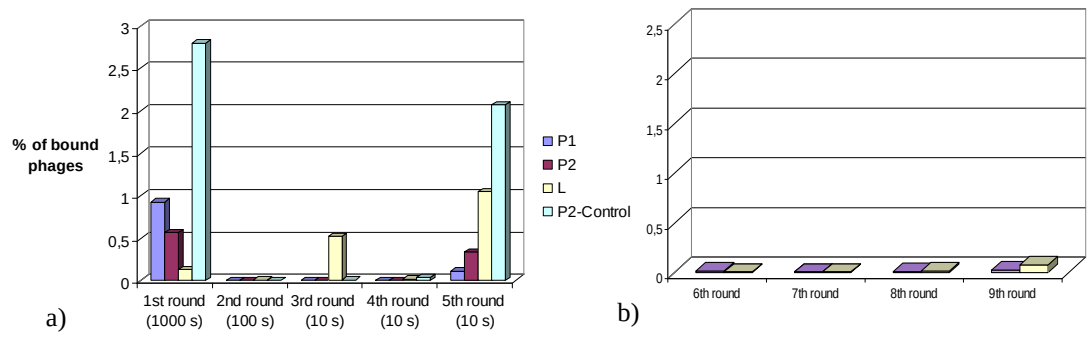


Figure 48 : phages binding rate after each round of selection, a) at 10^{-7} M in Pen-Biot and b) at 10^{-8} M in Pen-Biot. After 5th round, P1 and P2 are mixed in one fraction named P and represented in purple in graphic b.

Each clone that gave a readable sequence was tested in phage-ELISA, over a 2000 s period of time and compared to PBP-A acylation. Six clones from each non selected library were also randomly picked and tested in phage-ELISA. The kind of results that could be observed is represented in Figure 49, where acylation curves of different clones were superimposed to that of PBP-A indicating a relative rate of acylation. This test could help us roughly determining acylation rates as it is quite sensitive to the number of phages used during the reaction (Annex 6).

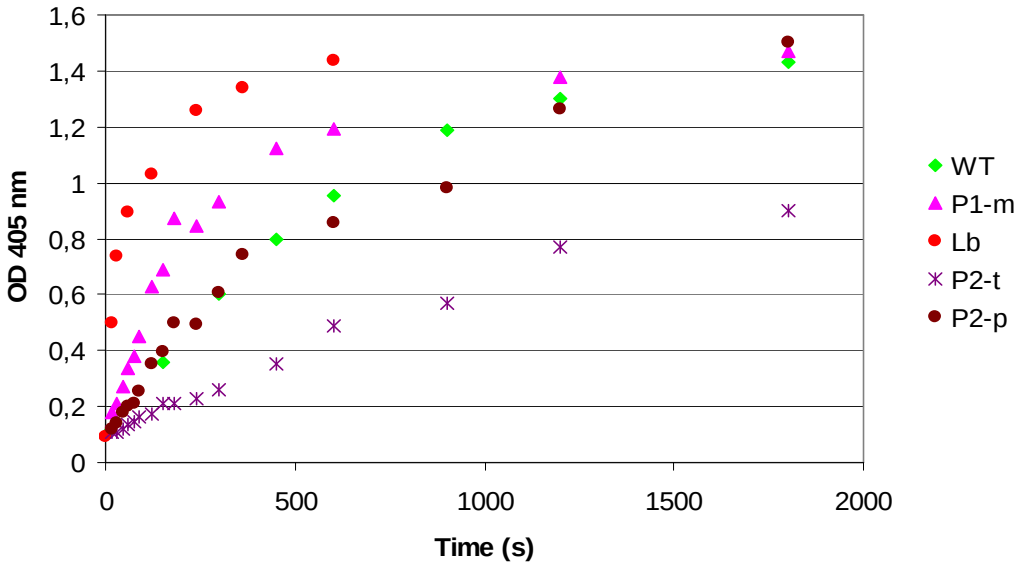


Figure 49 : typical results of a phage-ELISA. OD at 405 nm represents the concentration of acylated phages, that allows following acylation kinetics.

Clones were classified into three categories: non acylating clone, low rate acylating clone (i.e rate inferior to wild-type), and clones with acylating rate equal or superior to wild-type. The results shown in Table 18 are in good agreement with the enrichment rate and sequences that predicted of a successful selection.

Concerning control $P_{2\text{-}C}$, one would expect that keeping an incubation time with Pen-Biot constant over five rounds of selection, the number of eluted phages is constant. However, it is not the case, and binding rate falls after the first round, and higher binding is only observed after five rounds of panning. It has been observed that in many selections, phages binding rate decreases after the first round of panning, due to high non specific binding (information from the lab). Also, the higher binding rate for $P_{2\text{-}C}$ after the 5^{th} round suggests that it has been enriched in phages able to acylate, and in P_2, better acylating clones may be under represented. The analysis of clones by ELISA shows that four clones over seven are acylating

as well as or better than the wild-type enzyme, which is the same proportion for P2 after five rounds with increasing selection pressure. Hence, further rounds of selection may be needed for better representation of interesting clones

Number of tested phages		*No acylation*	*Acylation*	
			< WT	*≥ WT*
Native P1	6	6	-	-
P1-5	16	-	2	14
Native P2	6	6	-	-
P2-5	19	-	8	11
P2-C	7	-	3	4
P-9	14	-	-	14
Native L	6	2	4	-
L-5	16	-	-	16
L-9	17	-	-	17

Table 18 : results of the phage-ELISA tests. Results are given for the three native libraries (P1, P2 and L), for P1, P2 and L libraries after 5 rounds of selections (P1-5, P2-5 and L-5), for the control on P2 over 5 rounds of selection (P2-C), for the mix of P1 and P2 libraries and L library after 9 rounds of selection (P-9 and L-9).

Sequences of selected clones in P libraries

When analysing randomly picked clones by phage-ELISA in the native P libraries, none of them was capable of acylating, as expected regarding the libraries construction design. The template used for these libraries was PBP-A-L_{158}E mutant which features a high deacylation rate and is therefore not captured efficiently during the selections. After five rounds of panning 100 % phages acylation could be followed and most of them seemed to acylate as rapidly or faster as the wild-type. After nine rounds of selection, all clones were acylating at least as efficiently as PBP-A. Regarding the sequences, it was difficult to extract a consensus, however, some mutations were found several times and are listed in Table 19. All sequenced clones can be found in Annex 5.

Interestingly, 8 different mutants still feature E_{158}, and have mutations either in the *Ω*-loop or at positions were residues interact with E_{158} (N_{128}S and I_{67}F) (Annex 5, A). This suggests that these mutations modify E_{158} conformation, that is probably not able to promote deacylation anymore, and explaining the selection of these clones. Not surprisingly, many sequences (16/24) have Glu_{158} mutated to either Gly, Val, Ala, Gln or */Gln, since E_{158} is deacylating 90 times faster than wild-type and is therefore poorly captured (Figure 47). However, the reverse mutation was often associated with one or several mutations, repeated among the different sequences, suggesting a functional selection for enzymes with increased acylation, stability,

or ability to be displayed on phages. Those mutations repeated at least twice are analysed in details and their location on the structure is shown in Figure 50.

	Acylation		$P_{2\text{-}a}$	$P_{9\text{-}17}$
	< WT	≥ WT		
Ser_{9}			Leu	
Ser_{29}		Asn		
Gln_{31}		Leu	Leu	
Phe_{40}			Val	
Val_{75}	Glu	Glu		
Glu_{96}	Gly	Gly/Val		Val
Gln_{103}			Lys	
Asn_{106}	Tyr	Tyr		
Ile_{119}	Ser/Thr	Ser		
Asp_{123}	-	Val		
Asn_{128}	Ile	Ser		
Phe_{144}	Leu	Leu/Ser		
Asn_{156}	-	Tyr	Tyr	
Glu_{158}	Gly/Val	Gly/Ala/Val/Gln		Gln
Asp_{160}	Val	Ala		
Met_{161}		Val/Thr		
Lys_{162}		Met	Met	
Gly_{163}			Val	
Thr_{215}	Ser	Ser		
Asp_{222}	Gly	Gly		
Asn_{252}			Lys	
Val_{267}				Ala

Table 19 : mutations found at least twice in the selected clones from P libraries. Sequences of $P_{2\text{-}a}$ and $P_{9\text{-}17}$ overexpressed, purified and characterised clones.

Ser_{29} is found mutated into Asn twice, and in both cases, it is associated with a same set of mutations: Glu_{96}Val, Glu_{158}*/Gln and Met_{161}Val. This position is not conserved among PBP-A proteins family, and it seems spatially equivalent to Asp_{38} of TEM-1 which is nor conserved among class A β-lactamases. This suggests that this residue is not directly implicated in the protein activity.

Gln_{31} is a residue of medium conservation in PBP-A proteins family, suggesting that the role played by this amino acid is not of high importance for protein activity or structure. Mutation Gln_{31}Leu, located in a coil between helix α_{-1} and strand β_{-1}, is spatially equivalent to Gln_{39} in TEM-1. In this latter enzyme, Gln_{39} side chain is orientated towards Asp_{35} and they are hydrogen bonded. A variant of TEM, named TEM-2 contains a Gln_{39}Lys mutation that results in an enhanced K_m for penicillin when compared to that of TEM-1. It was suggested that Lys_{39} forms a salt bridge with Glu_{281}, therefore influencing protein stability and hence activity [21]. In our case, the only contact of Gln_{31} with the rest of the protein is a hydrogen bond formed with its side chain and main chain carbonyl of Leu_{27}. Mutating Gln_{31} into Leu may not influence that much the protein stability, and this mutation alone may not trigger the enzyme

acylation. In both cases where this mutation is found, it is associated with $Asn_{156}Tyr$ mutation. However, this residue is located in the *Ω*-loop, far from Gln_{31}, and there is no obvious link between these two mutations.

$Val_{75}Glu$ mutation is repeated twice among the different sequences, and it is carried by clone $P_{2\text{-}t}$ that was found 6 times over the 5th round of selection. It is located at the C-terminal end of helix *α*-2 that carries the catalytic serine, and is highly conserved in PBP-A proteins family. Its spatial equivalent in TEM-1 is Val_{84}, a position which is not conserved within class A *β*-lactamases, however, a negatively charged residue is never observed at this place. It has been suggested that acylation in *Stretomyces* K15 DD-peptidase occurs by activation of the catalytic serine by Lys_{38} (equivalent of Lys_{73}) with assistance of the dipole formed by helix α-2 and the polarized carbonyl carbon group of the substrate [22]. This mechanism is fully applicable to PBP-A, and introducing a glutamate at the extremity of this helix may change somewhat helix dipole, influencing acylation.

A glutamate at position 96 was mutated nine times into Gly or Val, and once into */Gln (suppressible stops are traduced into Gln). It forms part of the 91-106 loop equivalent to the TEM-1 100-115 loop and discussed previously (page 159). It was proposed that Glu_{96} in PBP-A is spatially equivalent to Glu_{104} in TEM-1 though the hydrogen bonding network of this residue was supported by Ala_{97}. The 100-115 loop in class A *β*-lactamases contains highly conserved residues, except at position 104, where it is a hydrophobic residue in 4 *β*-lactamases over 18 [23], and an aromatic residue within carbapenemases [24]. Mutation studies at this position showed that activity towards expanded spectrum *β*-lactams is enhanced, and that breaking the H bond between Glu_{104} and Asn_{132} induces a displacement of the SDN loop, favouring the entry of cephalosporins in the active site [25]. Another observation was that hydrophobic residues at this place increase the enzyme K_m for all substrates, and is often associated with multiple mutations, particularly at position 164 and residues situated downstream the KTG motif (Gly_{238}, Glu_{240}, Thr_{264}) [23-26]. In PBP-A, each time Glu_{96} is mutated to a hydrophobic residue, it is associated with mutations in the *Ω*-loop, suggesting that it plays a similar role as that played by Glu_{104} in TEM-1. When mutated to */Gln, there is no amino acid mutated in the *Ω*-loop, suggesting that introducing a polar residue at this position is sufficient for restoring acylation, may be by changing the electrostatic field in the region of the *Ω*-loop.

$Asn_{106}Tyr$ was found twice. This residue equivalent to Thr_{114} of TEM-1 is not conserved as well in class A *β*-lactamases as in PBP-A proteins family and is situated at the C-terminal end

of the 91-106 (100-115) loop. Its main chain nitrogen atom forms H bond with main chain carbonyl oxygen of Met_{88}. There is no obvious consequence of changing this amino acid to Tyr, and this mutation does not seem to play any role.

Mutation at Ile_{119}, a residue situated on helix α-3 preceding the SDN loop and equivalent to TEM-1 Ile_{127}, was found three times and was replaced by Serine or Threonine. A hydrophobic residue at this position is highly conserved in both PBP-A and class A *β*-lactamases families, however, the role played by this residue and its neighbours is versatile from one *β*-lactamase to another. Random mutagenesis on TEM-1 showed Ala_{125} to do not tolerate mutations and Ile_{127} could be mutated to Leu, Met, Ser or Val [27]. Pse-4 lactamase had a different behaviour in tolerating mutations at position 125, but not at 127 where Methionine was suggested to play a structural role in maintaining the helix integrity and the SDN motif [28]. Finally, the same kind of SDN disordering was suggested for explaining differences in catalytic parameters of two *β*-lactamases from *Staphylococcus aureus* differing in one amino acid at position 128 [29]. Whereas in TEM, Ile_{127} has only one contact with one residue of the preceding α-helix, Ile_{119} in PBP-A makes two hydrogen bonds through its main chain nitrogen and main chain carbonyls of Glu_{116} and Leu_{117}, the latter being also hydrogen bonded to Asp_{123}. Hence, Ile_{119} may play a different role than in TEM-1, and may be involved in stabilising the SDN loop. Its mutation might therefore influence acylation.

Asp_{123}Val mutation is found twice, each time associated with Glu_{158}Val mutation. This residue is part of the highly conserved motif SDN that forms a wall of the catalytic cavity. In *β*-lactamases, Ser_{130} and Asn_{132} side chains point towards the active site and are implicated in maintenance of the active site structure and the catalytic process respectively. Asp_{131} side chain points in the opposite direction and is involved in protein stability [30]. Its role in protein stability is played through hydrogen bonds with main chain amino groups of several amino acids at positions 108, 109, 132, 133 and 134 [31]. Introduction of Glycine instead of Aspartate introduced some floppiness in the SDN loop, affecting the protein stability. In PBP-A, Asp_{123} is also at hydrogen bond distance of main chain amino groups of residues 99, 100, 124, 125 and 126. However, mutation into Valine does not seem to destabilise the protein, but it may induce slight movements in the SDN loop, favouring acylation.

Asn_{128} is equivalent to Asn_{136} in TEM-1. This residue is conserved in all class A *β*-lactamases except in Per-1, and is higly conserved in PBP-A proteins family. Through hydrogen bonds formed with main chain atoms of Glu_{166}, Asn_{136} was suggested to maintain the cis conformation of the peptidic link between amino acids 166 and 167, thus ensuring a good orientation of Glu_{166} for deacylation [7, 32]. The same type of contacts between Asn_{128} and

Leu/Glu$_{158}$ have been identified, introducing Serine instead of Asparagine may break this contact thus changing the Ω-loop conformation and may be PBP-A acylation efficiency.

In place of Phe$_{144}$ on helix α-5, a leucine is found twice. This is a highly conserved residue within PBP-A proteins family, and corresponds to Leu$_{152}$ in TEM-1, though not conserved within class A β-lactamases, a hydrophobic residue is always found. This residue is situated 13 Å far from the active site, and its potential role in affecting acylation efficiency remains unclear.

Asn$_{156}$ is spatially equivalent to Arg$_{164}$ in TEM-1, it is highly conserved in class A β-lactamases Ω-loop [3] and, as shown by crystallographic studies [33, 34], it forms a salt bridge with Asp$_{179}$ through its side chain. This contact has been shown by many studies to be critical for the loop conformation and hence for the protein substrate selectivity [35, 36]. Asn$_{156}$ is a highly conserved residue in the family of proteins of PBP-A, although it points towards the solvent and forms only two hydrogen bonds through its amide and carbonyl main chain atoms with Asn$_{165}$ carbonyl and amine side chain groups, respectively. Its mutation into Tyr is found three times, including in a clone isolated 9 times after the 5th round of selection and five times after the 9th round of selection. However, on the basis of the structure its role in protein activity is not clear and it may influence more the stability. In the same way three other residues in the Ω-loop, Asp$_{160}$, Met$_{161}$ and Lys$_{162}$ are mutated to Ala/Val, Val/Thr and Met respectively. The effect of these mutations is most probably linked to the modification of the Ω-loop structure.

Thr$_{215}$, located at the N terminal end of strand β-3 is not conserved in PBP-A proteins family and its equivalent Phe$_{230}$ in TEM-1 is not conserved in class A β-lactamases. It is directed towards the solvent and mutated into the equivalent residue Ser in terms of polarity, charge and chemistry, suggesting that this mutation is not of high importance.

Finally, Asp$_{222}$ was mutated to Glycine in two clones. This residue equivalent to Ala$_{236}$ in TEM-1 is adjacent to the KTG motif and is involved in formation of the oxyanion hole, as discussed previously (see page 159). It was noted that Asp$_{222}$ main chain nitrogen formed only a weak hydrogen bond with O$_8$ carbonyl oxygen atom of penicillin whereas this link in class A β-lactamases is strong. Asp$_{222}$ forms a hydrogen bond with guanidinium side chain of Arg$_{249}$ situated on the following strand, pulling down strand β-3, and increasing the distance between the polypeptidic chain and penicillin. Breaking the H bond between Asp$_{222}$ and Arg$_{249}$ by mutating Asp$_{222}$ to Gly may help pulling up the strand, rendering the interaction

with penicillin stronger. Moreover, the presence of a carboxylate may be deleterious for the interaction with penicillins by destabilising the oxyanion hole.

As a general manner, the mutations identified were localised at putative hot spots for the protein activity.

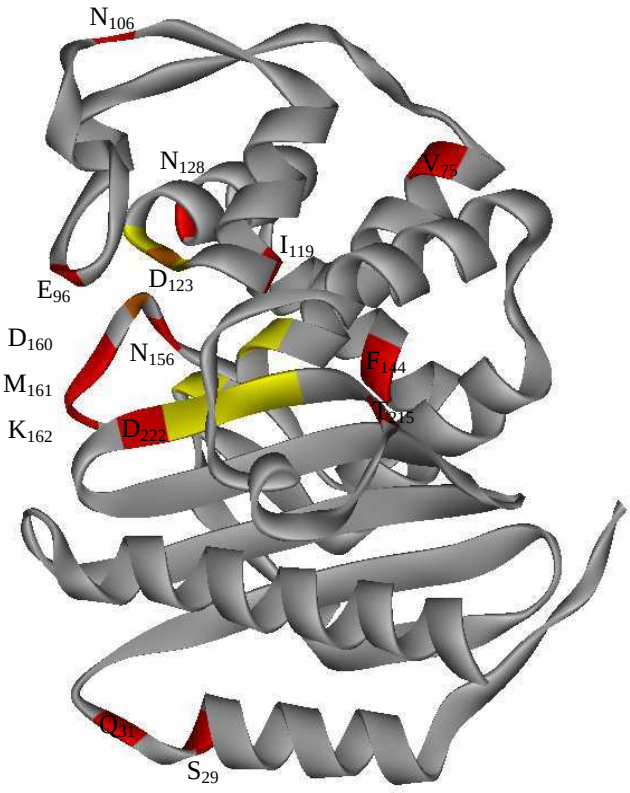


Figure 50 : repartition of the mutations along PBP-A structure. Residues of the catalytic site are indicated in yellow and mutated amino acids are indicated in red. Mutated residues important for catalysis are in orange.

Sequence of clones in L library

Concerning selections in L library, randomly picked clones analysed by phage-ELISA showed that two third of the initial library was still able to acylate penicillin, though at a rate inferior to the wild-type protein. After 5 or 9 rounds of selections, 100% of the clones were acylating penicillin at least as fast as PBP-A (Table 18). Logos created from sequences of the selected clones after rounds 5 and 9 indicate conservation of residues at each position along the Ω-loop (Figure 51), for easier understanding, Ambler numbering scheme is used.

The first observation is that there are few evolutions in the sequences between rounds 5 and 9. Also, the sequence along the Ω-loop follows globally the constraints imposed by the cassette composition. Only two variable residues in the initial library are strictly conserved, i.e Arg_{178}

and Asn_{179}. A counter selection for leucine and the selection for many glycines seem to have occurred at position 161. In the same way, many serines are found at position 164.

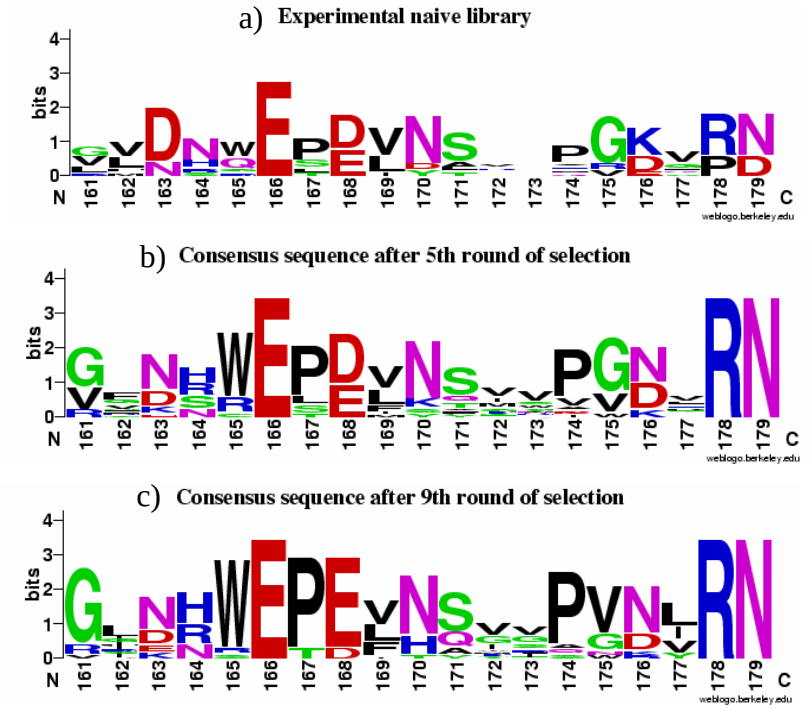


Figure 51 : logos created from sequences of clones from naïve library (a), library after five rounds of selection (b) and nine rounds of selection (c).

In class A *β*-lactamases four residues are highly conserved, Arg_{164}, Glu_{166}, Asn_{170} and Asp_{179}. One highly conserved interaction among class A *β*-lactamases *Ω*-loop is the salt bridge between Arg_{164} and Asp_{179} that confers its conformation to the loop for efficient penicillin hydrolysis [31]. Mutations at Arg_{164} or Asp_{179} that break the salt bridge induce loop disordering that helps the enzyme accepting more bulky substrates, changing its specificity profile in TEM-1 [36] and reducing activity in PC1 lactamase from *S. aureus* [35]. However, breaking this bond in TEM-1 is still compatible with efficient acylation of penicillin [16]. The exception is Per-1 *β*-lactamase for which Asn_{179} does not form a salt bridge but only hydrogen bonds with main chain nitrogen atoms of residues 163 and 164 and main chain oxygen atom of residue 164 [37]. In PBP-A *Ω*-loops, Asn_{179}, is strictly conserved, as in the natural context of this enzyme. In the wild-type protein, this residue is the C-terminal anchor of the loop and makes several hydrogen bonds with the opposite peptidic chain, suggesting an important role for loop conformation. A similar role might be played in the mutants. Conservation of Arg_{178} might be due to interactions possible with conserved residues loop E/D_{168} or E_{166}.

Though much conserved, Asn_{170} which is important for maintaining the catalytic water in the right position for deacylation is not strictly conserved in PBP-A sequences. This is not much surprising as the clones were selected here for acylation that does not require the presence of a water molecule.

Overall, these observations suggest that the cassette has been well designed for creating a structured loop, and many residue combinations render the loop capable of adopting a suitable conformation for acylation provided that Arg and Asn are present at positions 178 and 179.

Mutants expression, purification and kinetics.

In order to characterize some of the selected mutants, 4 genes from clones susceptible to acylate faster than PBP-A were subcloned into pBAD-Tet vector, allowing proteins cytoplasmic expression, and appending a hexahistidine tag at the C-terminus. Two clones from P library, $P_{2\text{-}a}$ which was found nine times and five times at the 5^{th} and 9^{th} rounds of panning respectively, and $P_{9\text{-}17}$ that only contained three mutations were kinetically characterised for benzylpenicillin and thiolesters hydrolysis. The same parameters were measured for L-b and L-t, two clones from the L library that kept selected after 9^{th} round of panning. Intriguingly, their sequences are quite close with 14 over 19 amino acids in common.

	161									170								179
L-b	G	G	K	R	W	E	P	E	L	N	S	V	V	P	G	N	L	R	N
L-t	G	R	D	H	W	E	P	E	L	N	A	V	V	P	V	N	L	R	N

Expression levels were quite similar to that of PBP-A for all clones. Kinetics parameters with benzylpenicillin are exposed in Table 17. Acylation was increased by 2 and more than 10 times for $P_{2\text{-}a}$ and $P_{9\text{-}17}$, respectively, whereas deacylation was slightly decreased. Acylation was also enhanced for L-b and L-t clones by 8 and 12 times, respectively. In the case of mutants of the Ω-loop, no decrease in, deacylation was observed.

Kinetic parameters with thiolesters were also measured and are listed in Table 20, substrate S2a is a benzoyl-Gly-Thioglycolate, S2c is a benzoyl-Gly-LD-Thiolactate, and S2d is a benzoyl-DL-Ala-Thioglycolate. Results were different between P clones and L clones. It was striking to observe that for both L clones, catalytic efficiency for S2a substrate was not affected due to compensation between large decrease in both k_{cat} and K_m. As evidenced by kinetics with S2c substrate, activity was impaired on the preferred enantiomer but was not affected on the other, suggesting a slightly decreased specificity on the last residue. In the

same way, specificity on the penultimate residue was lost as catalytic efficiency was decreased for D enantiomer and some activity appeared for L enantiomer.

		S2a	*S2c*		*S2d*	
			X	*Y*	*X*	*Y*
PBP-A	k_{cat} (s^{-1})	≥ 7,7	≥ 6,6	≥ 0,21	≥ 52	-
	K_m (mM)	≥ 3	≥ 1,5	≥ 1,5	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$2,58.10^3$	$4,38.10^3$	$1,4.10^2$	$3,47.10^4$	-
L-b	k_{cat} (s^{-1})	0,173	≥ 0,42	≥ 0,26	0,367	0,0074
	K_m (mM)	0,08	≥ 1,5	≥ 1,5	0,85	0,85
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$2,16.10^3$ (1,2)	$2,79.10^2$ (20)	$1,71.10^2$ (0,7)	**$4,32.10^2$ (100)**	8,71
L-t	k_{cat} (s^{-1})	0,252	≥ 0,47	≥ 0,28	0,444	0,009
	K_m (mM)	0,12	≥ 1,5	≥ 1,5	0,76	0,76
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$2,1.10^3$(1,2)	$3,15.10^2$ (15)	$1,85.10^2$ (0,6)	**$5,84.10^2$ (70)**	12
P_{2-a}	k_{cat} (s^{-1})	0,033	≥ 0,014	≥ 0,013	≥ 0,24	-
	K_m (mM)	0,32	≥ 1,5	≥ 1,5	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$1,02.10^2$ (25)	**9,24 (400)**	9,06 (15)	**$1,6.10^2$ (200)**	-
P_{9-17}	k_{cat} (s^{-1})	0,223	≥ 0,45	0,048	≥ 0,17	-
	K_m (mM)	0,754	≥ 1,5	0,521	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$2,96.10^2$ (9)	3.10^2 (14)	91,4 (1,5)	**$1,13.10^2$ (300)**	-

Table 20 : kinetic parameters of selected clones at 37°C determined at 250 nm. Note that for clone P_{9-17} with substrate S2d, an initial deviation was observed that did not correspond to hydrolysis of one enantiomer, and fitting was made ignoring this deviation. Also, activity of P_{2-a} with S2c was very slow and ½ arbitrary. Finally, values obtained for Lb and Lt with substrate S2c were fitted over 6 parameters and may be imprecise. Decrease in catalytic efficiency compared to wild-type is indicated for each clone and substrate in parenthesis. More deleterious effect is indicated in bold.

P_{2-a} and P_{9-17} had decreased catalytic efficiency with substrate S2a, due to a large decrease in k_{cat} that was not compensated by the decrease in K_m, P_{9-17} being more affected. With S2c, P_{2-a} catalytic efficiency was even more decreased for X enantiomer and slightly for Y enantiomer, resulting in altered specificity. Clone P_{9-17} catalytic efficiency was also decreased for S2c X enantiomer, however, specificity change was not so obvious. Finally, catalytic efficiency for both clones was decreased for S2d hydrolysis and specificity maintained. Globally, substrate specificity was changed and stereospecificity was relaxed for all clones.

Finally, it was interesting to assess the propensity of the protein to support mutations without disturbing its stability. PBP-A is a thermostable protein as indicated by the midpoint for denaturation in GuHCl, measured by loss of fluorescence at 20°C on a fluorimeter, that is around 2 M. This is quite high compared to the first transition of TEM-1 (0,8 M) for its denaturation [38]. Both P_{2-a} and P_{9-17} mutants were as stable as PBP-A ($[GuHCl]_{50}$ = 2 M),

whereas clones Lb and Lt had largely decreased stability with $[GuHCl]_{50}$ of 1 M and 1,1 M, respectively.

Structure activity relationship of selected clones with penicillin

Two libraries were created by error-prone PCR, using PBP-A-L_{158}E as a template (P library) or by insertion of a degenerated cassette of 19 amino acids in place of PBP-A 16 amino acids *Ω*-loop (L library) in order to generate higher rate of penicillin deacylation on PBP-A scaffold. Preliminary *in vivo* selections were unsuccessful and a strategy in two steps where acylation is improved before acylation was started. After five and nine rounds of phages panning at increasing levels of selection pressure, clones were sequenced, activity was assessed by phage-ELISA, and four of them were expressed and kinetically characterised. On the basis of phage-ELISA results, clones could be grouped into two categories: those acylating slower than PBP-A (negative clones) and those acylating as rapidly or faster (positive clones). After nine rounds, selected clones were 100 % positives.

In P libraries, numerous residues were mutated in both categories of clones: Val_{75}, Glu_{96}, Asn_{106}, Ile_{119}, Asn_{123}, Phe_{144}, Asp_{160}, Thr_{215} and Asp_{222}. Two types of reasons may explain the differences in activity though the same kind of mutations is found. First, experiments were only performed once for each clone and one clone for each set of repeated clones was tested, false negatives may have appeared. Second, the type of mutation is sometimes different between negative and positive clones. This is the case for three positions, Glu_{96}Val, Phe_{144}Ser and Glu_{158}Ala/Gln are mutations found only in positive clones. Finally, mutations are often synergistic and it is not the mutation itself but a set of combined mutations that may provide the selected functionality.

Two P-clones were chosen for expression and more detailed kinetic studies among positive clones that seemed to acylate faster than PBP-A upon phage-ELISA. We chose to assess the real activity of clone $P_{2\text{-}a}$ that contained three mutations in the *Ω*-loop while conserving Glu_{158} combined to Gln_{31} mutation. We also wanted to test the effects of a small number of mutations, $P_{9\text{-}17}$ harbouring only three mutations of which the repeated Glu_{96}Val and the interesting Glu_{158}Gln was the second candidate. $P_{9\text{-}17}$ acylated penicillin twice faster than PBP-A but deacylated twice slower. The constants are not greatly changed and we suggest that besides the acylation rate, other factors like an effect of protein stabilisation, or higher level of expression enhancing the protein display might be the reasons why the protein was

found repeated several times at each round of selection. Mutations around the Ω-loop may revert the effect of Glu_{158} and restore a slow deacylation. Which of the other mutations is responsible for $P_{2\text{-}a}$ selection remains unclear and would necessitate directed mutations of the residues. On the other way, $P_{9\text{-}17}$ acylated penicillin G more than 10 times faster and deacylated 2,5 times slower. These parameters are more interesting as it can be suggested that Glu_{158} mutation into Gln is responsible for restoring a low deacylation rate, and higher acylation may be due to the lone $E_{96}V$ mutation. Hence, it may be interesting to study the effects of each $Glu_{96}Val$ and $Leu_{158}Gln$ mutations on acylation to check whether Gln_{158}, Val_{96} or both are responsible for the high rate of acylation.

L library was constructed on the assessment that the Ω-loop in class A β-lactamases contains residues involved in catalysis and maintenance of the topology of the active site. Whereas penicillins fast deacylation is only possible in a sequence frame compatible with a certain loop configuration, it was shown that fast acylation is possible when some of these interactions are broken [13]. Deletion of PBP-A Ω-loop showed to decrease slightly acylation rate and increase by three times deacylation (see page 124). Hence, the loop in its wild-type form was not an essential feature for penicillin reactivity, and when introducing a longer degenerated Ω-loop, it was expected that conformation of some new loops could induce changes in the penicillin hydrolysis mechanism. However, *in vivo* selections could not provide efficient clones, and instead we selected for clones acylating faster. It is interesting to note that the naïve library contains a great proportion of clones still able to acylate penicillin, and five rounds of panning were sufficient to enrich the phages population in mutants acylating as fast or faster than PBP-A. Sequencing those clones revealed that several residue combinations are amenable for efficient pencillin acylation. Two of them were expressed and purified, and kinetic parameters determination showed a large increase in acylation, while deacylation remained at wild-type level. A salt bridge may exist in this loop, between the strictly conserved Arg_{178} and one other conserved loop residue, E/D_{168} or E_{166}. Indeed, in the case of mutants selected for acylation E_{166} can adopt conformation where it is not required to play a catalytic role. Asn_{179} is also strictly conserved, and this may be related to its role of C-terminal anchor for the Ω-loop. It may be interesting to determine kinetic parameters of clones with sequences different from L-b and L-t. Another interesting element would be obtaining crystals of one of the two clones for analysing the loop conformation and the consequences on the protein catalytic site.

Structure activity relationship of selected clones with thiolesters

All four purified clones show different reactivity towards thiolesters than PBP-A. *Ω*-loop of clones from L library seemed to adopt a conformation that altered specificity on both D-alanine. The effect was more pronounced for the penultimate D-Ala as catalytic efficiency was more decreased, though an increase in K_m could be observed. Moreover, these two clones become able to hydrolyse the L-enantiomer of S2d, witnessing a relaxed specificity.

As a general manner, catalytic efficiency for clones from P library was more affected for all substrates, and specificity relaxing was observed for S2c substrate but not for S2d. Decrease in catalytic efficiency was particularly marked for $P_{9\text{-}17}$ with substrate S2d. As stated earlier, this clone contains only three residues mutated, two of which being close to the active site. Gln_{158} mutation probably affects the catalytic mechanism of hydrolysis by bringing an uncharged polar side chain in the active site, however, there is no apparent link with the residue in penultimate position. Simulating the $E_{96}V$ mutation with Swiss-PdbViewer 3.7 indicates a putative hydrophobic and steric obstacle that could be the origin of the marked effect on the penultimate residue of thiolester, exhibiting a D-methyl group (Figure 52).

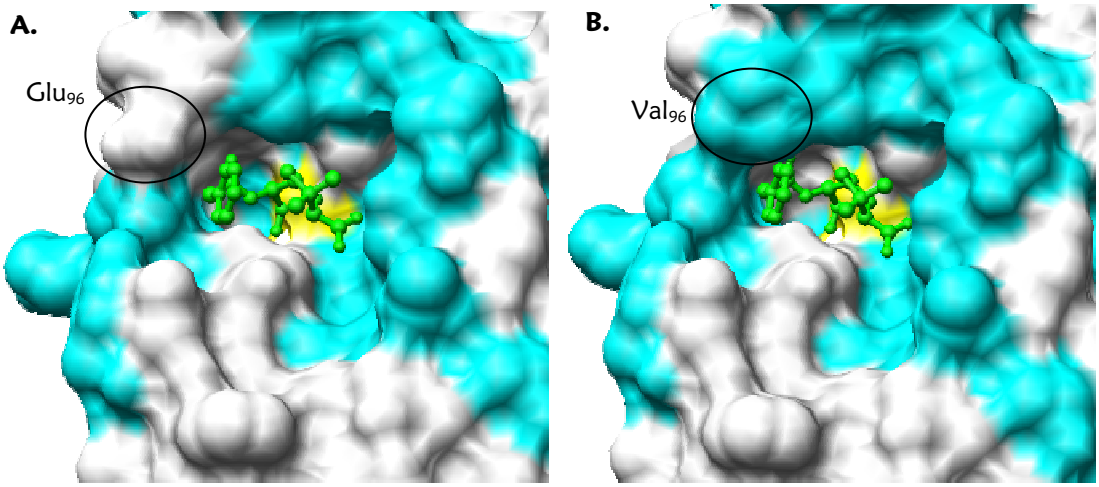


Figure 52 : view of the catalytic site surface of PBP-A acylated with benzylpenicillin (green), hydrophobic residues are represented cyan, and catalytic serine yellow. A. PBP-A catalytic site with Glu96 closing the cavity. B. simulation of the mutation E96V, the hydrophobic residue prompts a steric clash with benzylpenicillin.

However, this is only a simulation that can give an idea of what is occurring, as it does not take into account possible movements of other residues that could be induced by this mutation. Further crystallisation and structure studies may shed some light on the real effect of this mutation on the active site, as well for interaction with thiolesters than with *β*-lactamases.

Implications for PBP-A evolution to β-lactamase

Two libraries were tested for the *in vivo* selection of mutants having acquired *β*-lactamase activity. Both failed, and *in vitro* selection for better acylating mutants was investigated. Though the libraries were originally designed for deacylating rapidely, it was possible to find interesting clones.

Conservation of the *Ω*-loop is an important functional feature for class A *β*-lactamases. For PBP-A, two ideas were contradictory. On one hand, study of a mutant of PBP-A with deletion of the *Ω*-loop showed no great impact on PBP-A activity. On the other hand, structural alignments with *β*-lactamases showed a conserved conformation of the shorter loop corresponding to the N-terminal part of class A *β*-lactamases loop and shaping one side of the active site. Hence, it was interesting to assess whether changes in the *Ω*-loop of PBP-A, in length and/or amino acids composition could modulate the protein reactivity towards *β*-lactams. This was achieved selecting better acylating clones in the L library, indicating that the loop can actually be an element for evolution of PBP-A to *β*-lactamases. Both mutants expressed and purified showed decreased stability. Engineering of *Staphylococcus aureus* PC1 lactamase with a deleted *Ω*-loop had an impact on residues 236-244, the following loop and *β*-strand. The structural *β*-loop-*β* unit was perturbed to the extent that Arg_{244}, a residue important for substrate binding, was not oriented anymore towards the active site. Finally, it was suggested that the *Ω*-loop restricts the position of the *β*-strand, preventing it from occupying the space needed for substrate binding [39]. Hence, the region downstream the KTG seems closely dependent on the conformation of the *Ω*-loop. Introducing a larger *Ω*-loop in PBP-A may have an impact over this region of the protein. Further evolution experiment may thus rely on introducing random mutations along this region.

Massova & Mobashery proposed that for PBPs to evolve to *β*-lactamases, PBPs may have acquired some penicillins hydrolysis capability while maintaining their original peptidase activity, and then they may have lost this ability to optimise their lactamase function [3]. Here, we propose that after duplication of one PBP, one copy loses its function related to peptidoglycane maintenance, while increasing acylation towards *β*-lactams. Hence, this protein will capture the antibiotic that would be less available for inhibiting DD-peptidases. This view of evolution is strengthened by the properties of all four expressed clones that feature higher loss of activity on substrates that imitate better the peptidoglycane in that they contain a D-methyl group in the penultimate position. $P_{9\text{-}17}$ clone moreover contains $E_{96}V$ mutation. It has been argued that one of the structural elements for evolution of PBPs to *β*-

lactamases is the loss of ability to accept the methyl group of penultimate D-alanine, concomitant with loss of capability to bind peptidoglycane [40]. $P_{9\text{-}17}$ clone adopts this type of scheme with largely increased acylation rate for benzylpenicillin while decreased catalytic efficiency for thiolester substrate, particularly when D-alanine is the penultimate residue. The hypothesis based on simulation of $E_{96}V$ mutant (Figure 52) is that this lone mutation is responsible for these activity changes and could be an important mutation towards β-lactamase evolution. These hypotheses remain to be confirmed by study of site-directed mutagenesis mutants $L_{158}Q$ and $E_{96}V$.

Chemicals and antibiotics. Penicillin G and [^{14}C]-Penicillin G were purchased from Sigma. Ampicillin was purchased from Applichem, and kanamycin was from Invitrogen. Biotinylated penicillin (Pen-Biot, Figure 53) was prepared as described [16].

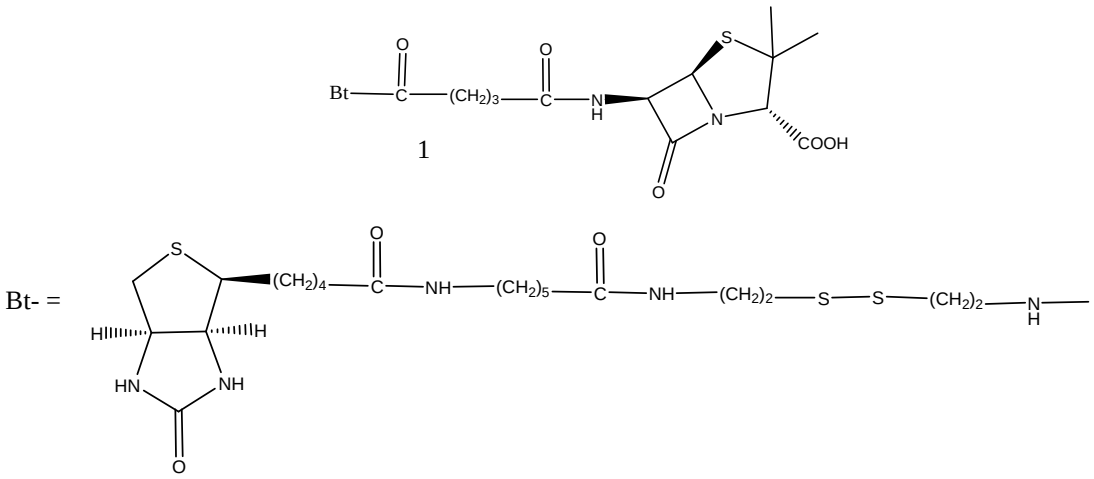


Figure 53 : substrate used for the selections. 1 = penicilline module, and Bt = biotin module containing a disulfide bridge.

Oligonucleotides were from Eurogentec, with “up” for sense primer and “rp” for reverse primer:

PhDiex-up: 5’TTGTGTGGAATTGTGAGC3’

PhDiex-rp: 5’CCCCTTATTAGCGTTTGC3’

PBP-A cassette:

5’CACAGTTTCAGGAATGAAGACTTTACGSKNN9KRA9MRT988GAG77KGAKNTN6

6997KRNNNNK77K88KRANNNKMSRRATACCACCGTCTTCGG3’

Primer Cassette: 5’CTCGTAGTCGCAACCGAAGACGGTGGT3’

PBP-A SalI: 5’GCGTCGACACCCGCTCCTGAGGCAC3’

PBP-A HindIII: 5’GAAGCTTATAGGCGGCGAAAGTTT3’

PBP-A BbsI-5’-up: 5’GGCTTAGAAAATACGGTGTCTTCAATCCGCTGCCGG3’

PBP-A BbsI-5’-rp: 5’CCGGCAGCGGATTGAAGACACCGTATTTTCTAAGCC3’

PBP-A BbsI-3’-up: 5’CCGGATATGAAGGGAAGACACACCACCAGTCCC3’

PBP-A BbsI-3’-rp: 5’GGGACTGGTGGTGTGTCTTCCCTTCATATCCGG3’

PBP-A-up: 5’TCGCCATGGCCGCTCCTGAGGCACC3’

PBP-A-rp: 5’CGTTCTAGAGGCGGCGAAAGTTTC3’

With: R = (A, G) – S = (G, C) – K = (G, T) – M = (A, C) – B = (T, C, G) – D = (A, T, G) – H = (A, T, C) – V = (G, A, C) - N = (A, G, C, T) – 6 = (90% A, 10% B) – 7 = (90% C, 10% D) – 8 = (90% G, 10% H) – 9 = (90% T, 10% V).

Bacterial strains. A total genome extract of *Thermosynechococcus elongatus* BP-1 was used as a source of DNA for PCR amplification of the tll2115 gene. *E. coli* TG1 was used for libraries construction as well as phage display selections. *E. coli* BL21(DE3) was used for *in vivo* selections and mutants periplasmic expression. Top 10 (Invitrogen) was used for standard subcloning procedures as well as for mutants cytoplasmic expression.

Construction of vectors. To construct our library, we used a derivative of the pHDi.Ex phagemid vector (a kind gift from Pr P. Minard, Université de Paris-Sud, France) where the ampicillin resistance gene was replaced by the kanamycin resistance gene [14]. This was achieved by cutting pHDi.Ex and pET-26b vectors by *DraIII* and *BspLU11I*, ligating the 1603 bp fragment from PHDi.Ex with the 1903 bp fragment from pET-26b, transforming in Top 10 cells and selecting for clones resistant to kanamycin, giving rise to pHDi.Ex.Kan^R vector. We also introduced an OmpT leader sequence and a *SalI* cloning site for the gene of interest. For this purpose, pHDi.Ex.Kan^R and pET-12a were digested with *NdeI* and *SalI*, the 3498 bp fragment from pHDi.Ex.KanR was ligated to digested pET-12a, and transformed in Top 10 cells. Clones able to be cut by *AvrII* integrated the OmpT sequence and were named pHDi.Ex.Kan^R.OmpT. Hence, cloning between *SalI* and *HindIII* allowed protein periplasmic expression with a histidine tag.

PBP-A-pHDi.Ex and PBP-A-L_{158}E- pHDi.Ex were constructed by cloning PBP-A and PBP-A-L_{158}E genes in the phagemid vector. PCR was performed using primers PBP-A SalI and PBP-A HindIII and the purified *T. elongatus* chromosome or PBP-A-L_{158}E/pBAD as templates. In this way, *SalI* and *HindIII* restriction sites were added at the 5' and 3' termini of PBP-A or PBP-A-L_{158}E genes. The amplified fragments were then cloned in the pGEM-T Easy cloning vector (Promega). DNA sequencing confirmed the correct nucleotide sequence of the PCR product. In the final construct, PBP-A and PBP-A-L_{158}E genes were cloned in the pHDi.Ex.Kan^R.OmpT vector.

PBP-A-BbsI was created by inserting in a sequential manner *BbsI* restriction sites in both sides of the *Ω*-loop encoding sequence using the Quick Change® Site-Directed Mutagenesis kit (Stratagene). PBP-A-pHDi.Ex was used as a template with PBP-A BbsI-5'-up and PBP-A BbsI-5'-rp primers for introducing mutation at the 5' end of the *Ω*-loop and PBP-A BbsI-3'-up and PBP-A BbsI-3'-rp primers for introducing mutation at the 3' end of the *Ω*-loop.

Some interesting clones were difficult to express with pHDi.Ex vector and were cloned in pBAD-Tet vector as explained in Chapter 1 under Materials and Methods.

Construction of libraries by error-prone PCR. A random mutagenesis library was constructed by error-prone PCR using $MnCl_2$ and $MgCl_2$ essentially as described [41]. 30 samples with 10 mM dCTP and dTTP, and 2 mM dATP and dGTP were prepared in water and mixed with 10 mM Tris, 50 mM KCl pH 8,3, 7 mM $MgCl_2$ and 500 nM $MnCl_2$. 150 pmole of primers PhDiex-up and PhDiex-rp, 150 ng of PBP-A-L_{158}E-pHDi.Ex, and 5 units of Taq Polymerase (Roche) were added in a total reaction volume of 50 µL. Samples were subjected to 1 cycle of 4 min at 94°C, followed by 30 cycles at 94°C, 1 min; at 50°C, 1 min; at 72°C, 1 min, followed by a final extension of 10 min at 72°C. PCR fragments were purified using a High Pure PCR Purification Kit (Roche) and recovered in a final volume of 750 µL. DNA was cut with SalI and HindIII, purified with Qiaquick PCR purification kit and cloned into pHDi.Ex.Kan^R.OmpT vector cut with the same enzymes. 100 µL of ligation were electroporated by aliquots of 10 µL in 100 µL TG1 electrocompetent cells and suspended in 900 µL SOC. After 1h at 37°C, 180 rpm, 9 mL of LB medium supplemented with 50 µg/mL kanamycin were added to each sample and bacteria were grown ON at 37°C, 180 rpm. Bacteria were centrifuged and resuspended in 10 mL LB supplemented with 50 µg/mL Kanamycin. 1,2 mL were used for preparation of plasmids using High Pure Plasmid Purification Kit (Roche), the remaining bacteria were frozen at -80°C in 20% glycerol.

Constructing this library, a bias was introduced as during bacteria growth, proteins expression was not repressed, and counter-selection for inactive proteins may have occurred. Hence a second library was prepared as described above, glucose 1 % was added for overnight bacterial growth, repressing protein expression under pLac promotor. The first library was named P_1 and the second P_2.

Construction of a library by insertion of a cassette. For annealing the single stranded cassette to its primer, 12 µg of PBP-A cassette were mixed with 38 µg of primer cassette in 20 mM Tris pH 7.8, 2 mM $MgCl_2$ and 50 mM NaCl, and subjected to heating 10 min at 78°C, cooled below 37°C and put on ice. For polymerisation, annealing reaction product was mixed to 1,25 mM each dNTP in 1 mM DTT, 8,8 mM $MgCl_2$ and 20 mM NaCl. 6 units of T4 DNA Polymerase (Roche) were added and sample was subjected to the following cycle: 10 min at 4°C, 10 min at 20°C, 40 min at 37°C and reaction was stopped by cooling to 4°C. Double stranded cassette was purified on 15% acrylamide gel, eluted in sterile mQ water and subjected to phenol chloroform purification and ethanol precipitation. DNA was cut with *BbsI* (Roche), digested fragment was purified on 15% acrylamide gel, eluted in sterile mQ water,

and precipitated with butanol and washed with Ethanol 70 %. It was cloned into PBP-A-BbsI vector cut with the same enzyme, and electroporation was performed as described above for P_2.

Phage preparation. Phages libraries were prepared from 10^{10} TG1 cells from the libraries glycerol stock and grown to exponential phase in 100 mL LB supplemented with 50 µg/mL Kanamycin and glucose 0,1 %. At this time, bacteria were infected by 20 R408 helper phage per bacteria, incubated 30 min at 30°C and another 30 min at 37°C, 80 rpm. Infected bacteria were centrifuged 5 min at 4500 x g. Pellet was resuspended in 500 mL LB with 50 µg/mL Kanamycin and incubated overnight at 37°C, 180 rpm. To recover the phage particles, bacteria were centrifuged 10 min, 4000 x g, 4°C. Supernatant was transferred to a sterile tube and phages were precipitated 1h at 4°C with 1/5° volume of PEG/NaCl (20% PEG 6000, 2.5 M NaCl). Phages were centrifuged 15 min, 14000 x g, 4°C, resuspended in 20 mL TBS (130 mM Tris, 140 mM NaCl pH 7.5), and filtered through 0,45 µM. Phages were subjected to another PEG/NaCl precipitation and suspended in a final volume of 1 mL TBS. The number of infective phage particles was determined by incubating phages with an excess of exponentially growing *E. coli* TG1 cells for 30 min at 37°C and plating them immediately on LB agar containing 50 µg/mL Kanamycin. The concentration of physical phage particles was measured by absorption spectroscopy [42].

Wild type phage and selected phages from the libraries were prepared as above, one isolated colony was used to inoculate 10 mL LB. After infection, bacteria were transferred to 200 mL LB for an overnight culture at 30°C.

Western blotting. A total of 10^8 or 10^9 infective phages were denatured by heating 10 minutes in presence of 15 µL of reducing SDS-sample buffer. Samples were loaded on a 10% (w/v) polyacrylamide gel, separated by electrophoresis and transferred on to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with TBS buffer containing 5% w/v milk powder and proteins were detected with an anti-pIII murine monoclonal antibody (MoBiTech) followed by a secondary anti-mouse HRP-goat IgG antibody (Sigma). The peroxydase activity was detected by electrochemiluminescence using an ECL kit from Bio-Rad.

Phage-ELISA. $1{,}2.10^{10}$ PBP-A phages were suspended in 1200 µL PBS containing 10^{-7} M Pen-Biot. Aliquots of 100 µL containing 10^9 phages were removed at different times. 5 µL of soluble *β*-lactamase from *Bacillus cereus* (Sigma) were added and samples were frozen on a mix of ethanol and dry ice. At the end of the sampling, aliquots were transferred to a streptavidin coated microplate (Roche) first blocked 1 h at RT with PBS containing 2% w/v

milk. After 15 min incubation RT, bound phages were revealed with a horseradish peroxidase conjugated anti-M13 monoclonal antibody (MoBiTech), and detected at 405 nm using ABTS as a substrate (Sigma). All steps were interspersed by three washes with PBS containing 0.05% Tween-20. For the negative controls, phages or biotinylated penicillin were not added.

In vivo selections. An amount of plasmid prepared from the libraries and representing 10 times more than the size of the library was used to transform BL21(DE3) allowing proteins overexpression and JM109 cells allowing proteins basal expression. BL21(DE3) transformants were plated on agar plates containing 5 or 10 µg/mL ampicillin supplemented with 50 µg/mL Kanamycin and 1 mM IPTG for protein overexpression. JM109 transformants were plated on agar plates containing 2.5, 5 or 10 µg/mL ampicillin supplemented with 50 µg/mL Kanamycin. The minimum inhibitory concentrations (MIC) for the selected colonies were determined by a dilution method on LB agar plates at 37°C, with inocula of 10-30 colony forming units dispensed in 7 µL drops [43]. The plates contained ampicillin at different concentrations plus kanamycin at 50 µg/mL.

Model selection experiment for better acylating enzyme. 10^9 to 10^{10} PBP-A or PBP-A-L_{158}E phages were incubated with 10^{-7} M biotinylated penicillin in M-TBS 1% (TBS supplemented with 1% of milk) in a final volume of 1,5 mL. Aliquots of 250 µL were taken at 10, 100, 400, 1000 and 2000 s, and reaction was stopped by addition of 5 µL β-lactamase from *Bacillus cereus* (Sigma) at 10 U/µL, such that the remaining free penicillin is hydrolysed in a few seconds, and frozen on a mix of ethanol and dry ice. 20 µL of streptavidin coated beads (Dynabeads M-280, Dynal) were presaturated 1h with M-TBS 2%. Samples were thawed at room temperature, and incubated on beads 30 min on a wheel. Beads were washed 4 times with T-TBS 0,05 % (TBS supplement with 0,05 % of Tween 20), and once with TBS. Beads were resuspended in 1 mL TBS and phages were eluted with 50 mM DTT. The number of phage particles was determined by infection.

In vitro selection for better acylating enzymes in the libraries. For each round of selection, 2.10^{10} infectious phages were incubated with biotinylated penicillin 10^{-7} M in a final volume of 500 µL M-TBS 1%. After 1000 s for the first round, 100 s for the second round and 10 s for three other rounds of selection, reaction was stopped and selection was accomplished as described in the model selection. Eluted fraction was used to infect 9 mL *E. coli* TG1, 100 µL were used for titrating and the remaining bacteria were plated on LB agar supplemented with 50 µg/mL Kanamycine and glucose 0,1 %, and incubated overnight at 30°C. The colonies were pooled by washing the large plate (23 x 23 cm) with 5 mL of LB supplemented with 50 µg/mL kanamycin and 15 % glycerol. 500 µL of bacteria were used for plasmid extraction,

the remaining cells were stored at -80°C. Phages were produced for the further round of selection as described above.

Expression and purification of selected clones. 4 clones from selection for better acylating enzymes were cloned in pBAD vector and were expressed and purified exactly as described under Material and Methods in chapter 1.

Kinetics. Determination of kinetic parameters for penicillin G and thiolesters was as described under Material and Methods in chapter 1.

Denaturation study. To a 2 mL solution of purified protein at a concentration around 3 µM in Hepes 20 mM, EDTA 1 mM, pH 7,4 were added progressively 100 µL drops of GuHCl 6M. Fluorescence spectroscopy was performed on a CARY Eclipse fluorescence spectrometer (VARIAN). Upon fluorescence signal stabilisation with emission measured at 340 nm (280 nm excitation), one drop was added. That allowed plotting fluorescence intensity versus GuHCl concentration. The midpoint of the curve determined graphicaly was the concentration of GuHCl needed for half denaturation of the protein.

Complementary study: selection for phages acylating 100 to 1000 times faster

C. Claeys Bouuaert

Introduction

Evolving new enzymes catalysts requires creation of large mutants' libraries, and robust techniques that allow capturing the mutant with the desired characteristics. Selection of enzymes for their catalytic properties is often a hard task. Unless the enzyme possesses an activity that gives the bacteria growth advantage or other types of phenotypes easily detectable by *in vivo* methods, researchers need robust *in vitro* techniques that may help selecting the desired enzymes [44]. Many researchers attempt to circumvent this problem by creating new technologies applicable to every kind of enzyme. One of the most promising technique is based on *in vitro* compartmentalisation system where all the machinery for proteic expression of one mutant is contained in a droplet. A fluorogenic substrate of the enzyme is also introduced in the droplet, allowing selection of the most active enzymes by FACS [45]. The major drawback of this technique is that it supposes the use of a flow cytometer associated to a FACS, that is expensive and non standard laboratory equipment.

One famous technique used for enzymes selection is phage display [46]. Though it is easier to select mutants that bind a specific ligand, mutants that are catalytically active on a specific substrate are still difficult to isolate by this technique. F. Chang-Pi-Hin developed a technique applicable to catalytic machineries that involve a covalent intermediate enzyme-substrate. In the case of β-lactamases for example, inactive enzymes are initially blocked by the natural substrate of the enzyme, the acyl-enzyme of the active ones are then trapped by adding a biotinylated substrate and rapidly denaturing the enzyme using GuHCl. Biotinylated phages are then captured on a streptavidin coated support. Such a kind of technique was applied on a model system comprising the R61 DD-peptidase and the mutant $W_{233}S$ known to deacylate 80 times faster. This selection method based on counter-selection of phage-enzymes deacylating slowly by penicillin G and trapping of phage-enzymes that are capable of turning-over was assessed on a mix of R61/$W_{233}S$ mutant in proportions 90/10. Acylation rate on phage was not tested, however, it was assumed that it was close to the values for the free enzyme, k_2/K_s = 13 000 $M^{-1}.s^{-1}$ for R61 and k_2/K_s = 30 $M^{-1}.s^{-1}$ for $W_{233}S$ [4]. Using the rate constants for acylation and deacylation, it is possible to simulate numerically the evolution of acyl-enzyme

and biotinylated acyl-enzyme of each protein, depending on the concentrations of penicillin G and biotinylated penicillin used, and the times of incubation. This allowed determining the appropriate concentration of penicillin and associated incubation times required to capture preferentially the most active mutant. One round of selection was sufficient to enrich significatively phage population in mutant enzyme.

To confirm the efficiency of the method, selection of PBP-A-L_{158}E was tested on a mix of phagemids containing PBP-A and PBP-A-L_{158}E in 90:10 proportions. The selection could then be used for selecting better deacylating mutants in P and L libraries.

Results

Model selection experiment for better deacylating enzyme.

Numeric simulations were achieved in order to determine the appropriate parameters for capturing PBP-A-L_{158}E phagemid in a mix of mutant and wild-type PBP-A phages. In the protocol used, 10^{10} phages were incubated with 10^{-6} M of penicillin G for 400 s, allowing counter selection of wild-type enzyme, followed by incubation with 10^{-4} M biotinylated penicillin during 60 s. Reaction was stopped by addition of 4 M GuHCl, previously shown to denaturate completely PBP-A and mutant at concentrations as low as 3 M (page 216). Phages were then submitted to a PD10 desalting column to remove the excess of Pen-Biot, captured on streptavidin coated magnetic beads and eluted. With this protocol, the theoretical enrichment factor is calculated to be 30.

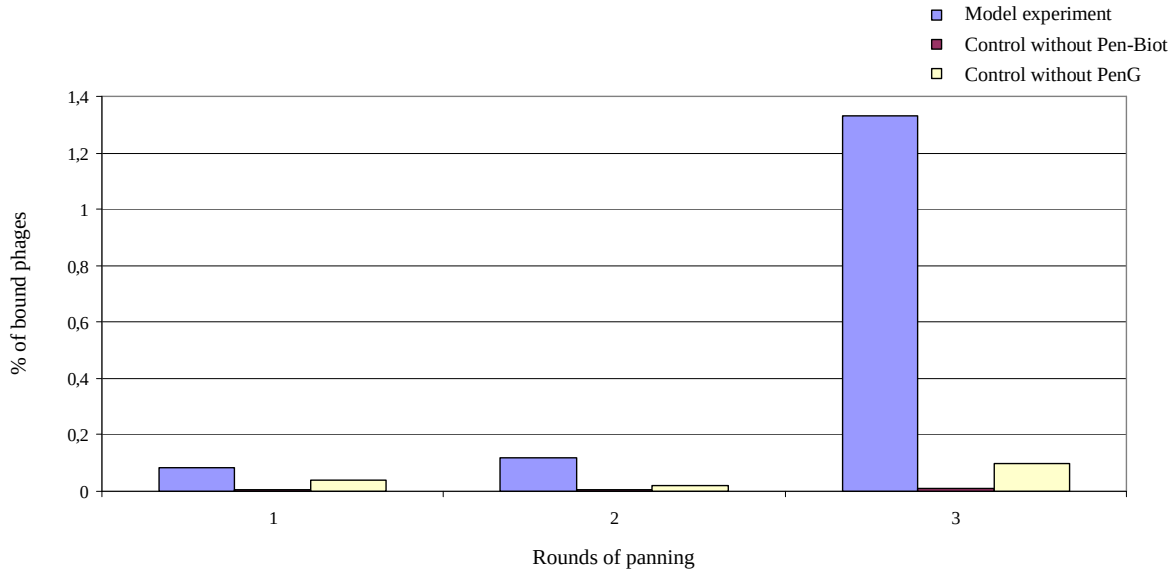


Figure 54 : histogram representing the pourcentage of bound phages determined by titration of the number of phages committed and eluted and calculation of the ratio of phages eluted over phages committed after each round of selection, using 10^{-6} M penicillin G, 10^{-4} M biotinylated penicillin and 4 M GuHCl.

Three successive rounds of panning were achieved in order to enrich the population of phages in mutant. To determine the binding specificity, phages committed (IN) and eluted (OUT) were titrated. The proportion of bound phages was calculated thanks to the ratio OUT/IN. For each round of panning, controls were achieved without biotinylated penicillin or penicillin G. The results presented in Figure 54 show the evolution of the number of bound phages over the different rounds of panning. After the 3rd round, the proportion of phages increased up to 1%, as expected if the population is enriched in PBP-A-L_{158}E. Controls without Pen-Biot were each time lower than the experiment, and represent the binding background, which is quite low. Finally, controls without PenG were supposed to give each time a higher yield of binding. However, it is always slightly inferior to the experiment, giving some uncertainty about the efficacy of the selection. The second information was the analysis of plasmidic DNA proportion from PBP-A and PBP-A-L_{158}E. After each round of panning, bacteria infected with the eluted phages were grown and used for plasmid extraction. Restriction of the plasmids by *EcoRI* and *BspEI* that cut both vectors and only PhDiex-PBP-A-L_{158}E, respectively, was used as a control to evaluate the quantity of each plasmid and hence of each phage at the end of the panning. Analysing the intensity of bands at 4000 (wild-type) and 3000 bp (Mutant) allow to observe that the proportion of PBP-a-L_{158}E phages increases after each round, and after the 3rd round, the population contains mainly mutants (Figure 55).

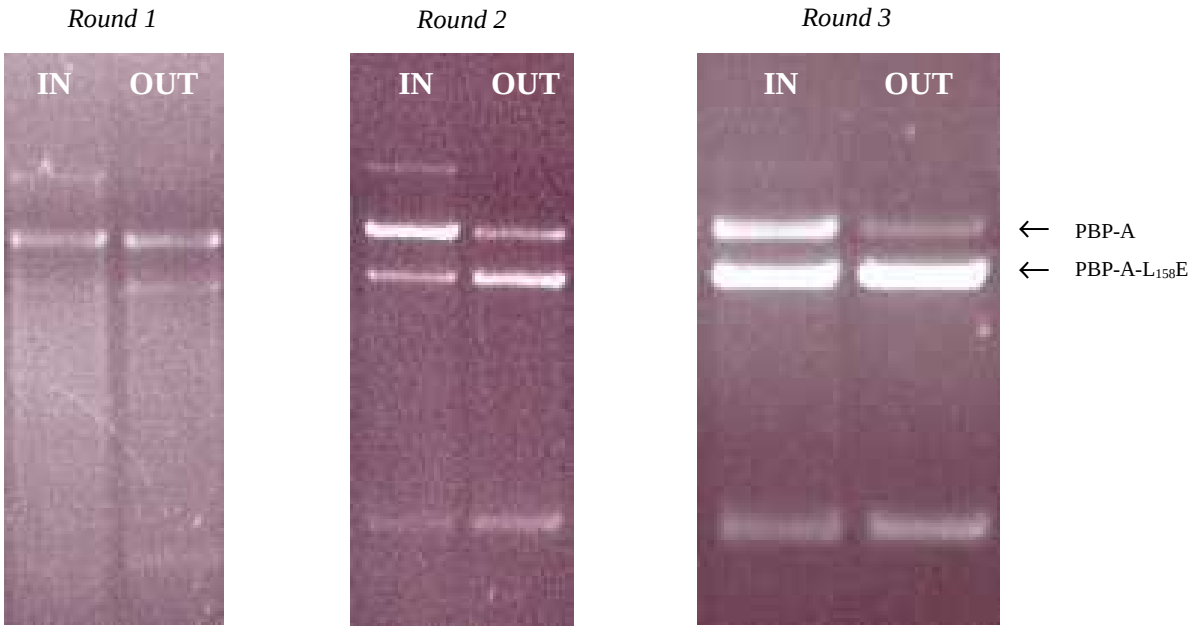


Figure 55 : 1% agarose gel analysis of the mix of phagemids DNA before panning (IN) and after panning (OUT). Infected bacteria were amplified ON, plasmids were extracted and digested with *EcoRI* and *BspEI*. PBP-A DNA was only cut by *EcoRI* giving one band at ~4000 bp whereas PBP-A-L_{158}E was cut by *EcoRI* and *BspEI* giving two bands at ~3000 and 900 bp. Proportion of PBP-A over PBP-A-L_{158}E is decreased after each round of panning.

For each round, the enrichment factor was estimated around three. Though positive control for phages binding did not work as expected, composition analysis of the population of phage is a good proof of the effectiveness of the method. Theoretically, the population may have been enriched much better after each round of panning. This may be due to fact that phages are sticky.

Selection for better deacylating enzymes in the libraries.

Selections within P (a mix of P1 and P2 in equal quantity) and L libraries consisted in two rounds of panning using the same conditions as used for the model selection, allowing the population to be enriched in enzymes deacylating as fast or faster as PBP-A-L_{158}E, and three rounds of panning increasing the counter selection by using higher penicillin G concentration (10^{-5} M) in order to enrich better the population in phages deacylating faster than PBP-A-L_{158}E mutant.

After the 5th round of panning, an enrichment is observed in both libraries (Figure 56), however when a control of binding was assessed without biotinylated penicillin, the ratio between specific binding over non specific binding was only 10 for P library and 5 for L library. This suggested that the number of really active clones within the library is mixed with unspecific clones. Few clones from both libraries were sequenced to assess whether a consensus could emerge.

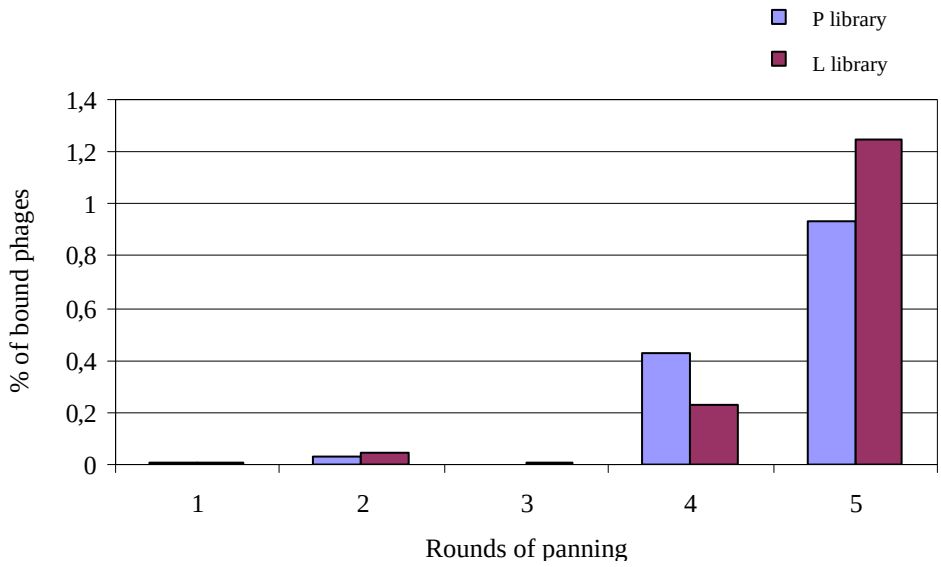


Figure 56 : evolution of phages binding on streptavidin beads over the five rounds of panning for phages from P and L libraries. Counter selection was increased at 3rd round of panning.

Twelve clones from P libraries were sequenced (Table 21). The average mutation rate found in the selected clones is 2 per gene, a bit lower that what is observed in the library (5 for P_1 and 3 for P_2).

Clone	*Mutations*
P_1 = L_{158}E	-
P_2	V_{80}A / Q_{103}R / N_{165}S / F_{271}S
P_3	Q_{23}H / E_{113}G
P_4	T_{99}S / F_{271}S
P_5	Q_{26}L / T_{81}A / V_{114}M / R_{240}H
P_6	L_{149}F
P_8	S_9P / K_{178}* (UAG)
P_9	Q_{103}H / F_{271}S
P_{11}	K_{178}I / E_{258}K
P_{13}	L_{185}H
P_{14}	G_{46}S / S_{189}R
P_{15}	A_3H / E_{96}V / L_{177}R

Table 21 : mutations found in 12 clones randomly picked after 5th round of panning in P library.

No consensus mutations were found and wild-type PBP-A-L_{158}E sequence was found once. 21 different mutations were found, 9 mutated positions are common to mutations found in clones selected for better acylation, and three are identical mutations, Q_{23}H, E_{96}V and N_{165}S. When looking at the repartition of the mutations along the three-dimensional structure (Figure 57), it is striking to observe that only two mutations at positions 96 and 165 approach the active site.

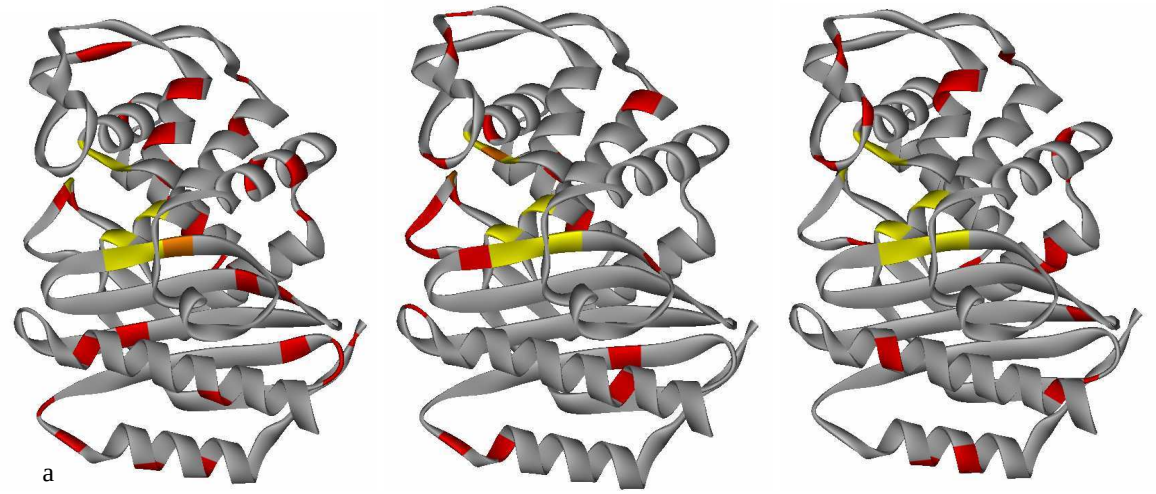


Figure 57 : repartition of the mutations along PBP-A structure in the native P2 library (a), in better acylating clone (b) and in selected clones for better deacylation (c). Note that for b, only mutations at least repeated twice are indicated Residues indicated in yellow are conserved residues of the active site, orange ones are mutated residues of the active site, and red ones are mutated positions.

Hence, clones selected are those bearing few mutations that do not seem to affect the enzyme activity. To try understanding this phenomenon, modelisations studies were made considering a mix of enzymes containing PBP-A, PBP-A- $L_{158}E$ and a virtual mutant deacylating 10 times faster than PBP-A. With the enrichment obtained in the selection model, nine rounds of panning would be necessary to enrich the population in virtual phage. This information, taken with the sequenced clones suggest that the population of clones after 5 rounds of panning contains essentially phage-enzymes having the same type of activity than PBP-A-$L_{158}E$. P_{15} and P_2 were the only mutant containing mutations close to the active site, P_2 was overexpressed and purified. The estimated k_{cat} was $3{,}38.10^{-3}$ s^{-1}, which is twelve times higher than PBP-A and only four times lower than PBP-A-$L_{158}E$. This straightens the hypothesis that clones have activities close to that of the mutant.

Analysing nine sequences after the 5th round (Table 22) of selection on L library allowed to create a logo for the sequence in the *Ω*-loop (Figure 58).

	161									**170**								**179**
L_3	G	*/Q	N	S	**C**	E	P	D	F	N	S	S	P	P	G	E	A	R	N
L_5	V	L	D	R	W	E	*/Q	D	L	N	S	G	K	P	G	E	V	P	N
L_6	V	V	N	S	W	E	P	D	V	N	S	R	V	P	G	N	L	R	D
L_7	G	L	N	R	W	E	Q	D	V	N	S	E	V	P	G	K	S	R	D
L_9	V	L	D	S	L	E	P	D	L	N	S	R	W	P	G	K	V	R	D
L_{10}	V	F	D	S	**C**	E	P	E	V	N	S	G	**C**	R	G	K	F	T	N
L_{11}	G	L	D	R	**C**	E	P	D	V	N	S	G	V	P	G	D	I	R	D
L_{13}	G	**C**	K	R	G	E	T	D	I	N	S	V	G	P	G	E	V	R	N
L_{15}	V	V	E	S	W	E	L	E	L	N	S	V	R	P	G	N	R	T	D

Table 22 : sequences of 9 clones selected for better deacylation.

No sequence was recurrently found, however, the logo allowed identifying besides Glu_{166}, three strictly conserved positions, Asn_{170}, Ser_{171} and Gly_{175}. Other positions showed marked preferences for particular residues, Val and Gly at position 161, Arg and Ser at position 164, Asp at position 168, Pro at position 174 and Arg at position 178. The high conservation of the residues suggests that a functional selection occurred.

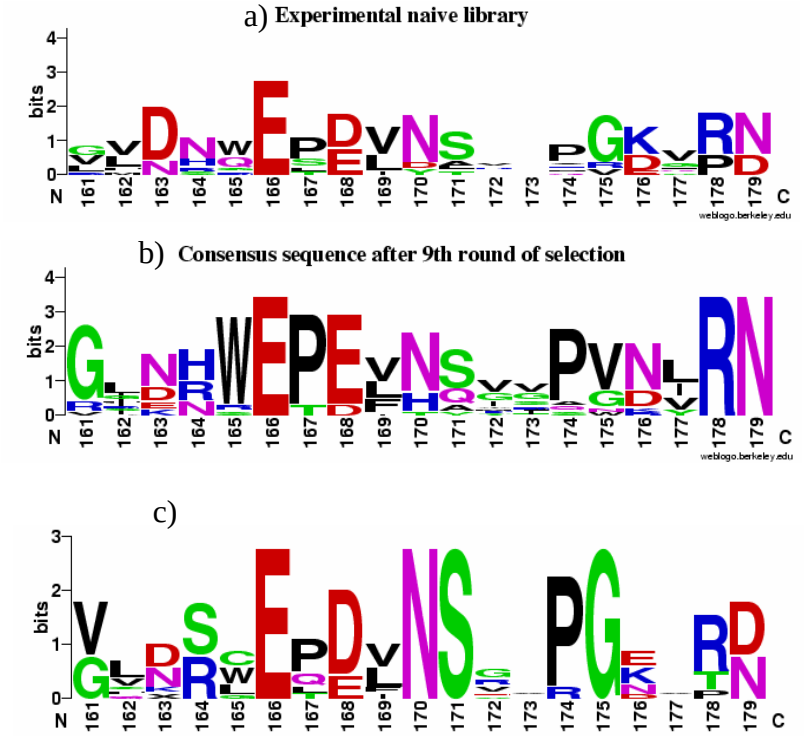


Figure 58 : sequences of nine clones in the *Ω*-loop after five rounds of panning, and logos from naïve library (a), clones selected for better acylation after 9th round (b) and clones selected for deacylation (c).

Three clones were expressed and purified. Clones L_6 and L_{15} present sequences where all favoured residues are present. Their catalytic activity was roughly determined to be as slow or even slower than PBP-A. Another intriguing observation in the sequences was the presence of cysteins in four clones, of which L_{10} that contained two cysteins. This clone was the third expressed, and k_{cat} was roughly estimated to $1{,}4.10^{-3}$ s^{-1} which is five times faster than PBP-A and ten times slower than PBP-A-L_{158}E. Other clones containing cysteins were not produced; however, their presence is interesting because the phage proteins are exported to the bacteria periplasm which is an oxidant environment unfavourable to free cysteins. Moreover, clone L_{11} contains a sequence very close to one that was found in a library of *β*-lactamase clones mutated in the *Ω*-loop and selected with a biotinylated suicide inhibitor with a similar spacer arm (Figure 53). This clone had the following sequence:

	163							170	
Clone from [13]	D	R	C	E	P	D	V	N	V
L_{11}	D	R	C	E	P	D	V	N	S

The clone isolated by *Vanwestwinkel & al.* had catalytic activity of 34 s^{-1}. Though free cysteins are generally not tolerated in the periplasmic space, selection of catalytic antibodies

with a reactive cystein was possible by phage display in two cases, and it was suggested that the the cystein was buried to prevent oxidation [47, 48]. This suggests that clones containing free cysteins may have been selected for reacting with the disulfide of biotinylated penicillin by thiol exchange, as would have occurred for the lactamase isolated with the suicide inhibitor-biotinylated substrate. However, no cysteins were found in P libraries selections for better acylating enzymes, though similar substrate was used.

Discussion

This work aimed at answering two questions: Is the method based on counter-selection by natural substrate followed by selection with a biotinylated substrate and denaturation applicable to another system than R61-phages? Is it possible to apply this technique to a library of mutant and target a range of enzymes having a determined activity?

Using the system selection for selecting PBP-A-L_{158}E in a mix of wild-type and mutant enzymes allowed answering the first question. Though the selection method was not as efficient as expected since enrichment was lower, it was capable of selecting the most active mutant. Further step to prove the efficacy would be to apply the technique to a completely different system from PBPs were formation of a covalent complex enzyme-substrate occurs, such as an epoxide hydrolase system that involves formation of a hydroxyl alkyl-enzyme intermediate [49].

To answer the second question, the selection protocol was applied to two libraries of PBP-A mutants generated by error-prone PCR (P library) and by insertion of a degenerated cassette in place of PBP-A Ω-loop (L library). Enrichment was observed after five rounds of panning.

Sequencing of twelve clones from P library indicated that PBP-A-L_{158}E selected proteins contained less mutations than that observed in the native library and were far from the active site. Kinetic analysis of one clone containing mutations close to the active site showed an activity slightly smaller than PBP-A-L_{158}E. This suggests that the population of enzymes is constituted mainly by clones having similar activity as PBP-A-L_{158}E. Further expression and characterisation of some clones may be necessary to ascertain this hypothesis. Moreover, simulations studies based on the model experiment showed that more rounds of panning may be necessary for active phages enrichment.

Concerning L library, though sequencing showed conservation of several residues in the Ω-loop, the low difference rate between specific and unspecific binding and presence of many cysteins suggest that the selection might have occurred for non specific binding to Pen-Biot or to the streptavidine coated magnetic beads. Expression and characterisation of three clones

indicated one clone to have 10 times enhanced activity than PBP-A, but no catalytic activity was better than PBP-A-L_{158}E mutant. Finding a clone containing a cystein and which sequence is very close to one found in another selection involving a suicide inhibitor with the same Bt module raises the hypothesis that some clones have been unspecifically selected. To assess this hypothesis, it would be necessary to check the binding of phages using only the biotin module of Pen-Biot. If the results showed high binding in these conditions, an issue for efficient selections would be the use of another penicillin substrate, in alternation with Pen-Biot. One clone showed 10 fold higher deacylation rate than PBP-A, which was encouraging as L library is most probably composed of high number of PBPs (see page 205).

Regarding evolution of a PBP with greatly enhanced capacity of hydrolysing penicillin G, nothing can be concluded at this time of the experiment, as other rounds of panning with protocol adjustments are necessary.

Material and methods

Model selection experiment for better deacylating enzyme. A mix of 90:10 PBP-A and PBP-A-L_{158}E phages for a total number of 10^{10} infectious phages were incubated with 1 µM Penicillin G in TBS in a final volume of 100 µL. After 400s, an aliquot of 50 µL is taken to which is added biotinylated penicillin 10^{-4} M to a final volume of 100 µL. After 60 s, reaction was stopped by addition of TBS, GuHCl 4M to a final volume of 500 µL. Sample was subjected to desalting on PD 10 column (Amersham) preequilibrated with 25 mL TBS, GuHCl 4M. 20 µL of streptavidin coated beads (Dynabeads M-280, Dynal) were presaturated 1h with M-TBS 2%. 1 mL fraction from the desalting column, containing the phages was incubated with the beads 30 min on a wheel. Beads were washed 4 times with T-TBS 0,05 %, GuHCl 4M, and once with TBS, GuHCl 4 M. Beads were resuspended in 1 mL TBS and phages were eluted with 50 mM DTT. Eluted fraction was used to infect 9 mL *E. coli* TG1, 100 µL were used for titrating and the remaining infected bacteria were grown at 30°C overnight in 100 mL LB supplemented with 50 µg/mL Kanamycine. Phages were produced for the further round of panning as described in the previous section .

The proportion of wild-type versus mutant phages could be assessed by preparing plasmids from 10 mL of the overnight amplification of bacteria infected with the eluted fraction using plasmid purification kit (Roche), and analysing the restriction pattern obtained with *EcoRI* and *BspEI* enzymes.

Selection for better deacylating enzymes in the libraries. Biopanning experiments on the libraries were performed essentially as described in the previous section. 10^9 to 10^{11} phages were submitted to selection in each round of panning in the conditions determined in the model experiment. After three rounds of panning, counter-pressure selection was increased by using higher penicillin G concentration (10^{-5} M). Infected TG1 cells were plated on agar plates supplemented with 50 µg/mL kanamycine. The colonies were pooled and used to produce new phages.

Expression and purification of selected clones.

4 clones from the selection for better deacylation enzyme were expressed in the periplasmic space of *E. coli* BL21(DE3). Clones of interest were grown in 1 litre of Luria-Bertani broth medium supplemented with kanamycin 50 µg/mL at 37°C until the A_{600} reaches approximately 0,6. The temperature was lowered to 30°C and protein production was induced by addition of IPTG 1 mM for 2h. The culture was centrifuged at 4,400 × g for 10 min, the harvested cells were resuspended in 33 mL 20% sucrose, 30 mM Tris-HCl pH 8 supplemented with 1 mM EDTA. After 10 min incubation time at room temperature with shaking, cells were centrifuged 10 min, 4400 x g, 4°C. Pellet was resuspended in 50 mL of 5 mM cold $MgSO_4$ and incubated 10 min at 4°C with shaking. The supernatant obtained by centrifugation at 15,000 × g for 10 min at 4°C was the periplasmic fraction. Protein was purified by IMAC as described under Material and Methods in chapter 1, with 20 mM Hepes instead of phosphate in buffer A.

Annex 4**:** In vivo selections

In vivo selected clones sequences are indicated with their related MIC:

	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	MIC µg/mL
L_1-	V	L	N	S	W	E	A	D	F	N	*/Q	V	C	S	A	D	M	R	D	4
L_3-	G	L	N	S	W	E	P	E	L	N	*/Q	D	G	P	G	N	T	R	N	2
L_5-	G	V	D	H	G	E	P	D	L	K	S	V	C	P	G	D	I	R	D	2
L_6-	G	I	N	S	W	E	P	D	V	N	L	I	G	R	G	K	G	R	D	2
L_8-	G	V	D	N	W	E	L	D	F	N	K	I	A	P	G	N	V	R	D	2
L_9-	G	I	N	N	W	E	P	E	L	I	L	L	F	L	V	M	F	R		5

L_9 clone contained a deletion resulting in a sequence frame shift. L_1 163 position where a suppressible stop is present was mutated into Gln in order to allow expression in a non suppressive strain and both clones were cloned in the pBAD vector for cytoplasmic expression. Hence, L_1-*$_{163}$Q and L_8 were overexpressed and purified. Kinetic parameters were measured directly at 232 nm for determination of the k_{cat} and indirectly by incubation with 2 µM [^{14}C]-benzylpenicillin for determination of k_2/Ks and 10 µM for k_3.

	k_2/Ks ($M^{-1}.s^{-1}$)	k_3 (s^{-1})	k_{cat} (s^{-1})
L_1-*$_{163}$Q	ND	$1{,}8.10^{-4}$	$3{,}7.10^{-4}$
L_8	$8{,}9.10^{2}$	$3{,}5.10^{-5}$	ND

Explanation for selection of clones with same k_{cat} as PBP-A-L_{158}E may have come from the level of enzyme saturation if the enzymes have different k_2/Ks. Indeed, if we consider k_3 of $1{,}4.10^{-2}$ s^{-1} for both enzymes, and k_2/Ks of $1{,}56.10^{3}$ $M^{-1}.s^{-1}$ and 10^{5} $M^{-1}.s^{-1}$ for PBP-A-L_{158}E and a putative clone, respectively. At concentration of antibiotic of 2 µg/mL, the putative clone will be 97,6 % saturated whereas PBP-A-L_{158}E will be 38 % saturated, hence for the same k_{cat}, the putative enzyme hydrolyses penicillin 2,6 times faster. Even at ampicillin concentration of 4 µg/mL, PBP-A-L_{158}E is only 52 % saturated and the putative clone 98,6 %, thus twice faster. However in our case, both analysed clones show k_3 as low as wild-type PBP-A, suggesting these clones to be false positives.

The following two primers were from eurogentec.

PBP-A L_1-*$_{163}$Q-up: 5'GCTGATTTCAACCAGGTCTGTTCTGCGG5'

PBP-A L_1-*$_{163}$Q-rp: 5'CCGCAGAACAGACCTGGTTGAAATCAGC3'

L1 clone was subjected to site directed mutagenesis using PBP-A L_1-*$_{163}$Q-up and PBP-A L_1-*$_{163}$Q-rp primers, and Quick Change® Site-Directed Mutagenesis kit (Stratagene). Then L_1-*$_{163}$Q and L8 were cloned in pBAD vector and were expressed and purified exactly as described under Material and Methods in chapter 1.

Annex 5 : sequences of clones from panning after rounds 5 and 9

Sequences in L library

Sequences of clones after 5th round of panning using 10^{-7} M biotinylated penicillin are shown in Table 23.

	161									*170*								*179*
a	V	F	N	S	W	E	L	D	I	N	*	I	N	P	V	D	P	R	N
b	**G**	**G**	**K**	**R**	**W**	**E**	**P**	**E**	**L**	**N**	**S**	**V**	**V**	**P**	**G**	**N**	**L**	**R**	**N**
c	**G**	**V**	**N**	**H**	**W**	**E**	**P**	**D**	**V**	**N**	**S**	**T**	**V**	**D**	**G**	**D**	**T**	**R**	**N**
d	R	M	N	N	W	E	S	E	F	N	S	I	V	P	G	N	M	R	N
e	V	L	N	N	R	E	S	E	F	N	F	S	M	P	W	K	H	R	N
g	R	G	N	S	R	E	L	D	V	N	S	G	G	P	G	D	V	R	N
i	**V**	**F**	**N**	**S**	**W**	**E**	**P**	**D**	**L**	**N**	**T**	**V**	**S**	**P**	**V**	**D**	**V**	**R**	**N**
j	G	S	E	N	W	E	T	E	V	N	S	M	R	A	G	N	V	R	N
k	G	C	D	H	C	E	P	D	L	N	S	M	W	P	G	N	V	R	N
l	G	F	N	R	W	E	P	D	L	K	S	I	V	V	G	D	W	R	N
m	G	G	D	R	W	E	P	D	V	N	S	V	V	P	G	N	L	R	N
n^2	**R**	**L**	**D**	**L**	**G**	**S**	**R**	**R**	**L**	**I**	**L**	**W**	**F**	**L**	**G**	**I**	**W**	**N**	
o	G	V	N	H	W	E	P	E	V	S	S	G	S	P	V	N	P	R	N
q	**V**	**V**	**K**	**H**	**W**	**E**	**P**	**D**	**V**	**Y**	*****	**R**	**Y**	**P**	**G**	**K**	**H**	**R**	**N**
r	**V**	**F**	**D**	**R**	**R**	**E**	**P**	**D**	**V**	**K**	**Y**	**V**	**W**	**I**	**G**	**N**	**F**	**R**	**N**
t	**G**	**R**	**D**	**H**	**W**	**E**	**P**	**E**	**L**	**N**	**A**	**V**	**V**	**P**	**V**	**N**	**L**	**R**	**N**

Table 23 : sequences of clones in L library after 5th round of panning.

Coloured sequences are those that show better acylation, sequence Ln containing a one nucleotide deletion is found twice.

Sequences of clones after 9th round of panning using 10^{-8} M biotinylated penicillin are in. Some sequences are the same than found at L-5, and the same name was used in L-9. Sequence $L_{5\text{-}10}$ is found four times and sequence L-t is found twice.

In each case, stops are suppressible ones.

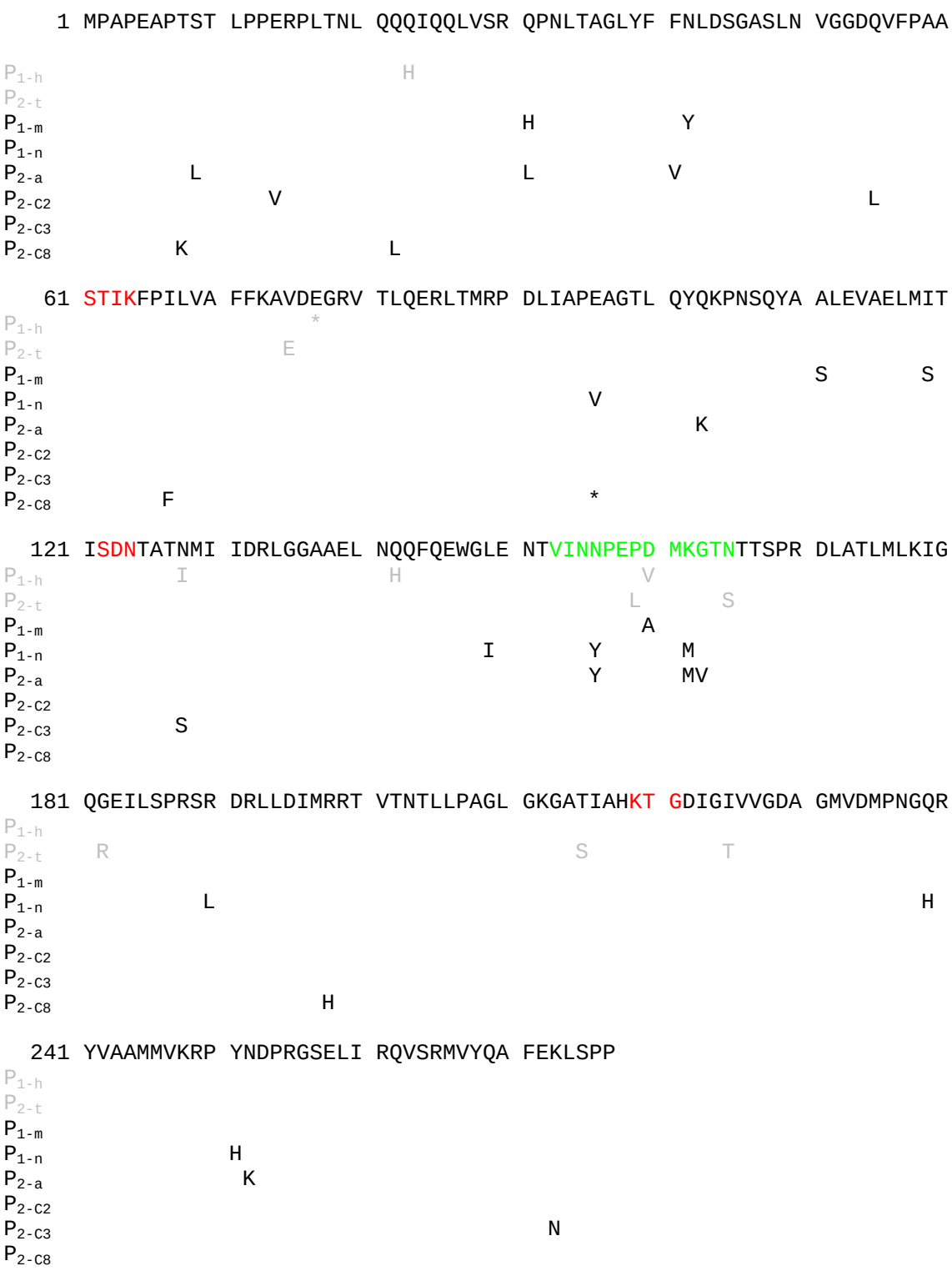
	161									*170*								*179*
b	**G**	**G**	**K**	**R**	**W**	**E**	**P**	**E**	**L**	**N**	**S**	**V**	**V**	**P**	**G**	**N**	**L**	**R**	**N**
J	G	S	E	N	W	E	T	E	V	N	S	M	R	A	G	N	V	R	N
3	**G**	**S**	**D**	**R**	**W**	**E**	**P**	**E**	**L**	**N**	**S**	**S**	**V**	**P**	**V**	**N**	**L**	**R**	**N**
5	G	V	N	N	W	E	P	D	F	N	S	V	S	P	V	D	V	R	N
6	R	F	N	H	S	E	P	E	V	T	S	R	R	P	W	D	V	R	N
8	G	G	D	R	W	E	P	E	L	N	Y	I	V	P	G	N	L	R	N
9	**G**	**G**	**N**	**R**	**W**	**E**	**P**	**D**	**F**	**N**	**S**	**V**	**T**	**P**	**N**	**R**	**L**	**R**	**N**
10^4	**G**	**L**	**N**	**H**	**W**	**E**	**P**	**E**	**V**	**H**	*****	**G**	**G**	**P**	**V**	**N**	**I**	**R**	**N**
11	V	L	K	R	W	E	P	E	F	N	S	I	F	Q	G	K	V	R	N
t^2	**G**	**R**	**D**	**H**	**W**	**E**	**P**	**E**	**L**	**N**	**A**	**V**	**V**	**P**	**V**	**N**	**L**	**R**	**N**
17	**G**	**L**	**E**	**N**	**R**	**E**	**T**	**E**	**I**	**N**	**S**	**V**	**I**	**S**	**V**	**N**	**L**	**R**	**N**
18	G	I	N	N	W	E	P	E	V	N	S	A	S	P	V	D	Y	R	N
20	**R**	**F**	**N**	**H**	**W**	**E**	**P**	**E**	**F**	**N**	**S**	**I**	**S**	**P**	**V**	**D**	**Y**	**R**	**N**

Table 24 : sequences of clones in L library after 9th round of panning.

Sequences in P libraries

Consensus motifs are in red, and residues from the *Ω*-loop are green. Grey sequences are for clones presenting low rate deacylation. Clones from 5th round have simple names: P_{1-} or P_{2-}, selection control is P_{2-C} and clones from 9th round of selection are P_{9-}. Clones P_{1-t} was found 6 times at round 5 and then disappeared, P_{1-b} was found 8 times at round 5 and 4 times at round 9, and P_{2-a} was found 9 and 5 times, respectively. Clones are classified as clones without reversion of E_{158} (A), clones with E_{96} and E_{158} mutated (B), and the others (C). Stops are suppressible ones, that give Gln.

A.


1 MPAPEAPTST LPPERPLTNL QQQIQQLVSR QPNLTAGLYF FNLDSGASLN VGGDQVFPAA
61 STIKFPILVA FFKAVDEGRV TLQERLTMRP DLIAPEAGTL QYQKPNSQYA ALEVAELMIT
121 ISDNTATNMI IDRLGGAAEL NQQFQEWGLE NTVINNPEPD MKGTNTTSPR DLATLMLKIG
181 QGEILSPRSR DRLLDIMRRT VTNTLLPAGL GKGATIAHKT GDIGIVVGDA GMVDMPNGQR
241 YVAAMMVKRP YNDPRGSELI RQVSRMVYQA FEKLSPP
P1-h
P2-t
P1-m
P1-n
P2-a
P2-C2
P2-C3
P2-C8

B.

```
    1 MPAPEAPTST LPPERPLTNL QQQIQQLVSR QPNLTAGLYF FNLDSGASLN VGGDQVFPAA
P2-q
P2-c4
P1-a
P1-b                     P           Q N
P1-g                                   N
P2-C7                 L
P9-17

   61 STIKFPILVA FFKAVDEGRV TLQERLTMRP DLIAPEAGTL QYQKPNSQYA ALEVAELMIT
P2-q                                            G
P2-c4                                           G           Y
P1-a                                            G                       *
P1-b                                            V
P1-g                                            V
P2-C7                                           G
P9-17                                           V

  121 ISDNTATNMI IDRLGGAAEL NQQFQEWGLE NTVINNPEPD MKGTNTTSPR DLATLMLKIG
P2-q                               R         A    G
P2-c4                                      I      G
P1-a                               L               G
P1-b                                               *    V
P1-g                                               *    V
P2-C7                              S               G    T
P9-17                                              *

  181 QGEILSPRSR DRLLDIMRRT VTNTLLPAGL GKGATIAHKT GDIGIVVGDA GMVDMPNGQR
P2-q
P2-c4
P1-a                                       E  S
P1-b
P1-g
P2-C7
P9-17

  241 YVAAMMVKRP YNDPRGSELI RQVSRMVYQA FEKLSPP
P2-q
P2-c4       I
P1-a
P1-b
P1-g
P2-C7                     V
P9-17                               A
```

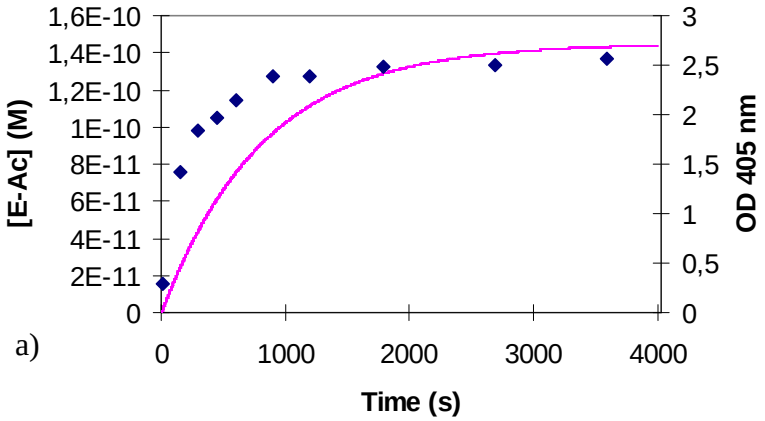
C.

```
    1 MPAPEAPTST LPPERPLTNL QQQIQQLVSR QPNLTAGLYF FNLDSGASLN VGGDQVFPAA
P1-p
P2-o
P2-C5
P1-d
P1-l
P2-l                                    L
P2-p
P9-7                T
P9-13                                                           E

   61 STIKFPILVA FFKAVDEGRV TLQERLTMRP DLIAPEAGTL QYQKPNSQYA ALEVAELMIT
P1-p
P2-o                                                                   S
P2-C5                                                                  T
P1-d                       Q                            Y
P1-l
P2-l
P2-p                    E
P9-7                                    H
P9-13

  121 ISDNTATNMI IDRLGGAAEL NQQFQEWGLE NTVINNPEPD MKGTNTTSPR DLATLMLKIG
P1-p                                            V
P2-o                                            G           R
P2-C5                           L             I V
P1-d                                    S T     A
P1-l                                            G
P2-l                                        Y   G
P2-p           S                                A
P9-7      V                                     V
P9-13     V                                     V

  181 QGEILSPRSR DRLLDIMRRT VTNTLLPAGL GKGATIAHKT GDIGIVVGDA GMVDMPNGQR
P1-p
P2-o                                                G
P2-C5            G        V
P1-d           S
P1-l                                                G
P2-l
P2-p
P9-7
P9-13

  241 YVAAMMVKRP YNDPRGSELI RQVSRMVYQA FEKLSPP
P1-p
P2-o
P2-C5
P1-d
P1-l
P2-l
P2-p
P9-7
P9-13
```

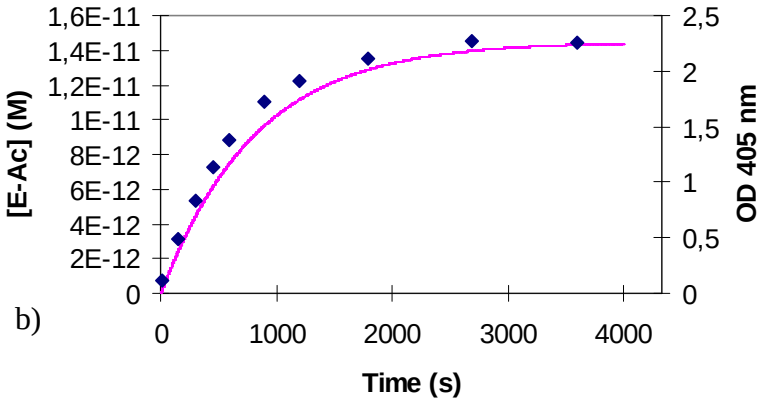
Annex 6 : effects of the number of phages in phage-ELISA

Phage-ELISA was tested at different concentrations in PBP-A phages: 10^{10} phages (a), 10^{9} phages (b) and 10^{8} phages (c). When using 10^{10} phages, the signal saturation induces an apparent effect of higher acylation rate as shown on the graphs were modelisation is in pink and experimental data are blue diamonds.

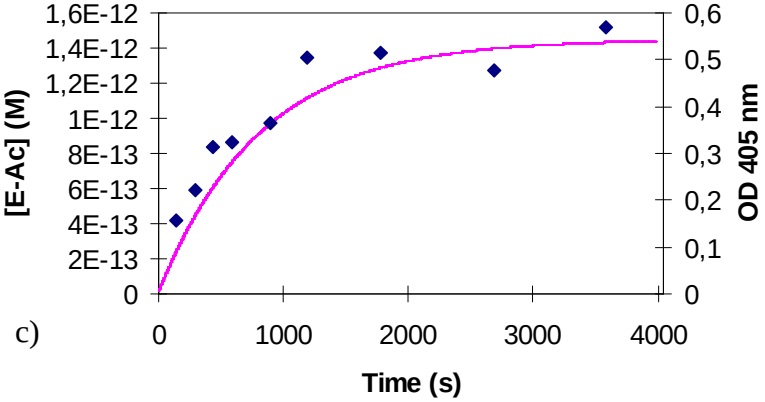
a)



b)



c)



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