Identification d'une nouvelle famille de PBPs chez des cyanobactéries, une famille de protéines homologues aux β-lactamases de classe A :

Sur base de leur similarité de structure et de la présence d'éléments conservés dans leur site actif, les PBPs et les *β*-lactamases de classe A sont des enzymes phylogénétiquement liées, et partageraient un ancêtre commun. Cependant, un chemin évolutif possible menant d'une faible activité d'hydrolyse de *β*-lactames, caractéristique des PBPs, à une activité d'hydrolyse 10^8 fois plus rapide caractéristique d'une *β*-lactamase est difficile à réaliser. Une raison de cette difficulté réside très probablement dans la divergence importante en termes de séquence entre ces deux types d'enzymes. L'objectif de cette première partie est la caractérisation d'une PBP, la plus similaire aux *β*-lactamases de classe A et ce, afin de mieux comprendre les schémas évolutifs menant d'une activité à une autre.

Une série de recherches de type Blast nous a permis d'identifier dans des génomes récemment séquencés différentes familles de protéines phylogénétiquement plus proches des *β*-lactamases de classe A que les PBPs connues. Parmi ces familles de protéines, nous nous sommes particulièrement intéressés à une famille de protéines exprimées chez les cyanobactéries et comportant une délétion de 6 acides aminés dans une région de la protéine, importante pour l'activité des *β*-lactamases de classe A, et généralement appelée boucle *Ω*. De plus, cette boucle ne comporte pas de résidu équivalent au Glu_{166} des *β*-lactamases de classe A, une base catalytique essentielle. Alors que la plupart des protéines de cette famille ont été annotées "putative *β*-lactamase" sur base des recherches de type Blast couramment utilisées lors de séquençages de génomes pour attribuer une fonction à un gène, les deux différences notées dans la boucle *Ω* suggèrent plutôt que ces protéines sont des PBPs. Après une étude d'alignements de séquences, nous avons choisi d'étudier les caractéristiques biochimiques d'une de ces protéines, la PBP-A de *Thermosynechococcus elongatus*. Un des critères de choix pour cette PBP est sa thermostabilité qui en fait un candidat plus apte à tolérer les mutations et donc plus apte à des expériences d'évolution dirigée (1).

L'expression recombinante et la purification de PBP-A nous ont permis de réaliser une série de tests biochimiques mettant en évidence l'acylation de l'enzyme par la pénicilline et la

faible activité pénicilline hydrolase de PBP-A, typique d'une PBP. Bien qu'une activité d'hydrolyse de substrats de type peptidique n'ait pu être observée, la capacité de PBP-A à hydrolyser des substrats thiolesters synthétiques couramment utilisés pour caractériser des DD-peptidases suggère qu'il s'agit d'une DD-carboxypeptidase très spécifique. L'étude de mutants ponctuels de la protéine a permis de mieux caractériser la protéine, nous renseignant sur les éléments impliqués ou non dans son activité pénicillinase.

Enfin, l'introduction d'un glutamate dans la boucle Ω, à la place du résidu aligné avec le Glu_{166} des β-lactamases de classe A a permis d'augmenter la vitesse de désacylation de l'acyl-enzyme formé avec la benzylpénicilline d'un facteur 90. Les paramètres cinétiques de ce mutant ont été étudiés en détails.

L'ensemble des résultats obtenus a permis de mettre en évidence une nouvelle famille de DD-peptidases, la plus proche des β-lactamases de classe A connue à ce jour. Cette proximité phylogénétique devrait permettre de réaliser plus aisément l'évolution dirigée d'une de ces enzymes vers une β-lactamase.

A new family of penicillin binding proteins in cyanobacteria, a family of proteins homologous to class A β-lactamases

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Abstract

It is largely accepted that serine β-lactamases evolved from some ancestral DD-peptidases involved in the biosynthesis and maintenance of the bacterial peptidoglycan. DD-peptidases are also called penicillin binding proteins (PBPs) since they form stable acyl-enzymes with β-lactam antibiotics such as penicillins. On the other hand, β-lactamases react similarly with these antibiotics but the acyl-enzymes are unstable and rapidly hydrolysed. Besides this mechanistic difference, all known PBPs and β-lactamases share very low sequence similarity, thus rendering it difficult to evolve a PBP into a β-lactamase by directed or random mutagenesis. In this study, we identified a new family of cyanobacterial PBPs featuring high sequence similarity with the most widespread class A β-lactamases. Interestingly, the Ω-loop, which, in the β-lactamases, carries an essential glutamate involved in the deacylation process, is six amino acids shorter and does not contain any glutamate residue. From this new family of proteins, we characterised PBP-A from *Thermosynechococcus elongatus* and discovered hydrolytic activity with synthetic thiolester that are usually good substrates of DD-peptidases. Penicillin acylation and deacylation rates that are characteristic of PBPs were also measured. In a first attempt to generate β-lactamase activity, a 90 fold increase in deacylation rate was obtained by introducing a glutamate in the shorter Ω-loop.

D-alanyl-D-alanine peptidases are enzymes involved in the synthesis of the peptidoglycan, the bacterial cell wall constituent which is responsible for the cell shape and resistance to osmotic pressure (2). These enzymes catalyse transpeptidation and carboxypeptidation reactions, thus controlling the peptidoglycan synthesis and crosslinking. As a result of the acylation of their catalytic serine, these enzymes form stable acyl-enzymes with β-lactam antibiotics such as penicillin, the stability of these complexes being at the origin of the antibiotic effect. Hence, DD-peptidases are also called "Penicillin-Binding Proteins" or PBPs (3,4). PBPs are divided into two main groups: the low-Mr PBPs are monofunctional catalytic entities (5) and the high-Mr PBPs comprising an additional N-terminal domain (6). To counteract β-lactam antibiotics, some bacteria produce β-lactamases (7-9), which are enzymes able to hydrolyse penicillins up to 10^8 times faster than PBPs. According to their primary structure, serine β-lactamases can be divided into three classes: A, C and D (class B constituting a group of metallo-enzymes). The penicillin-binding module of DD-peptidases and β-lactamases share a similar three-dimensional structure composed of two domains, an all-α domain and an α/β-domain, the catalytic site being at the interface (8). The three common essential motifs of the active site are, following Ambler numbering (10), S_{70}XXK containing the active nucleophile serine, S_{130}(Y)XN on a loop in the all-α domain and K_{234}(R)S(T)G on a β-sheet forming the opposite wall of the catalytic cavity. Regarding β-lactamases, the rapid hydrolysis of the penicilloyl-enzyme is performed by a water molecule activated by an additional enzymatic element. In class A β-lactamases, this role is carried out by a strictly conserved Glu_{166} (11) situated on a 16-residues loop (Arg_{164}-Asp_{179}) usually referred as the Ω-loop (12,13).

On the basis of sequence similarities, it was proposed that each class of PBP is subdivided into three subclasses, some of which are related to specific classes of β-lactamases (3). Sequence alignments and comparative analysis of the structures and the reaction mechanisms indicate that these enzymes are phylogenetically linked (4,14,15), and that each class of β-lactamases evolved most probably from an ancestral PBP, bringing to the active site an efficient catalysis mechanism for the deacylation reaction. The low-Mr class C PBPs,

including PBP4 from *E. coli* and *Actinomadura* R39 are believed to be the phylogenetically closest PBPs to class A β-lactamases even though sequence similarities are almost undetectable (14). Hence, despite a similar organisation of the catalytic sites, the evolutionary pathway has disappeared and the precise structural and chemical elements that are required in the different reactivities towards β-lactams are still misunderstood.

In this work, we identified a new family of PBPs in cyanobacterial genomes featuring the highest sequence similarity to class A β-lactamases ever reported. The protein of the thermophilic *Thermosynechococcus elongatus* was chosen for biochemical characterization. After cloning, expression and purification, the protein was examined for hydrolytic and aminolytic activity towards various substrates, and for β-lactam reactivity. Based on the sequence alignment with class A β-lactamases, a few mutations were introduced with the aim of increasing the β-lactamase activity. The single mutation $L_{158}E$ in the shorter Ω-loop afforded a 90-fold improved deacylation of the penicilloyl-enzyme.

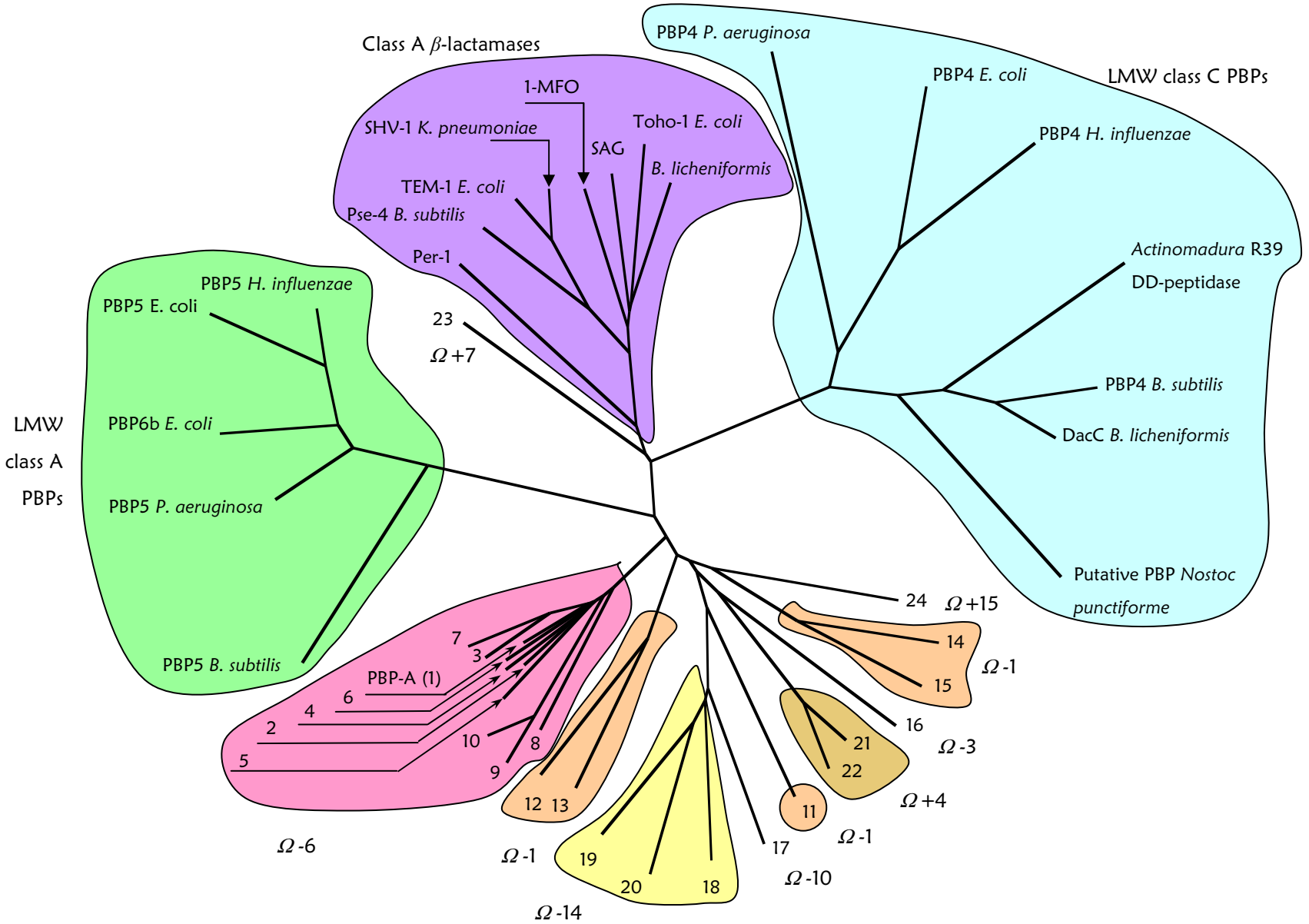


Figure 28: phylogenetic tree representing the families of proteins with variations in the Ω-loop size, LMW class A and C PBPs, and class A β-lactamases. Alignment was generated using T-coffee alignment program. Phylogenetic tree was then constructed using the neighbour-joining method. For proteins with variations in the Ω-loop size, corresponding numbering can be found in Table 1**,** Annex 1.

Comparison of PBP-A with β-lactamases.

β-lactamases evolved from DD-peptidases by acquiring the machinery for deacylation of the penicilloyl-enzyme. Besides the three conserved consensus motifs, class A β-lactamases have an Ω-loop of strictly conserved size containing a glutamate in position 166 that is also strictly conserved. This residue acts as a general base for serine activation through a water molecule for acylation leading to formation of an acyl-enzyme intermediate. Glu_{166} then activates another water molecule that attacks the carbonyl of the penicilloyl-enzyme leading to enzyme deacylation (12). Moreover, amino acids mutations in the Ω-loop have a marked impact on substrate specificity (16-18). While substitutions and insertions can occur naturally giving rise to extended spectrum lactamases (19,20), deletions were only genetically engineered until now (21,22). One main difference between β-lactamases and DD-peptidases is the absence of such conserved loop and consequently of a residue such as Glu_{166} for catalysing hydrolysis. Another difference is that all known DD-peptidases do not show significant sequence similarity to β-lactamases.

For understanding the evolutionary pathway leading from PBPs to β-lactamases, we searched for homologous sequences for which the Ω-loop was not as well conserved as in β-lactamases. In this way, we identified several putative lactamases, most of them from recently sequenced genomes, with variations in the Ω-loop length, but all of them having the three other motifs SXXK, SXN and KS/TG conserved. Multiple alignment with these proteins, some LMW class A and C PBPs, and class A β-lactamases were generated using T-coffee, and a phylogenetic tree was created (Figure 28). For this purpose, raw protein sequences were manipulated. The putative Ω-loop of each protein was removed in order to prevent alignment upon the Ω-loop size, that could introduce a bias. Putative sequence signal, transmembrane segments or insertions at the extremities of the proteins, possibly introducing noise in the alignment, were removed. Finally, large insertions from LMW class C were removed on basis of structure comparison with class A β-lactamases. It is important to note that LMW PBPs could only be aligned correctly when all the manipulations were done, whereas all other proteins aligned well with class A β-lactamases using the raw sequences.

Several groups are formed on basis of sequences similarities (indicated by coloured leaves on Figure 28), and the average distance from one group to another can be evaluated by

measuring the distance between families divergence points. A whole family of ten putative class A β-lactamases from cyanobacteria with a 6 amino-acids deletion in the region of the Ω-loop emerged. Since all ten proteins were missing the essential Glu_{166}, and this shorter loop, these proteins were suggested to be PBPs. The average distance of this protein to class A β-lactamases is lower than the distance from the closest PBP homologs like *E. coli* PBP4 or *Actinomadura* R39 peptidase. A multiple alignment is shown in Figure 29 with cyanobacteria proteins family, and some class A β-lactamases of known structure.

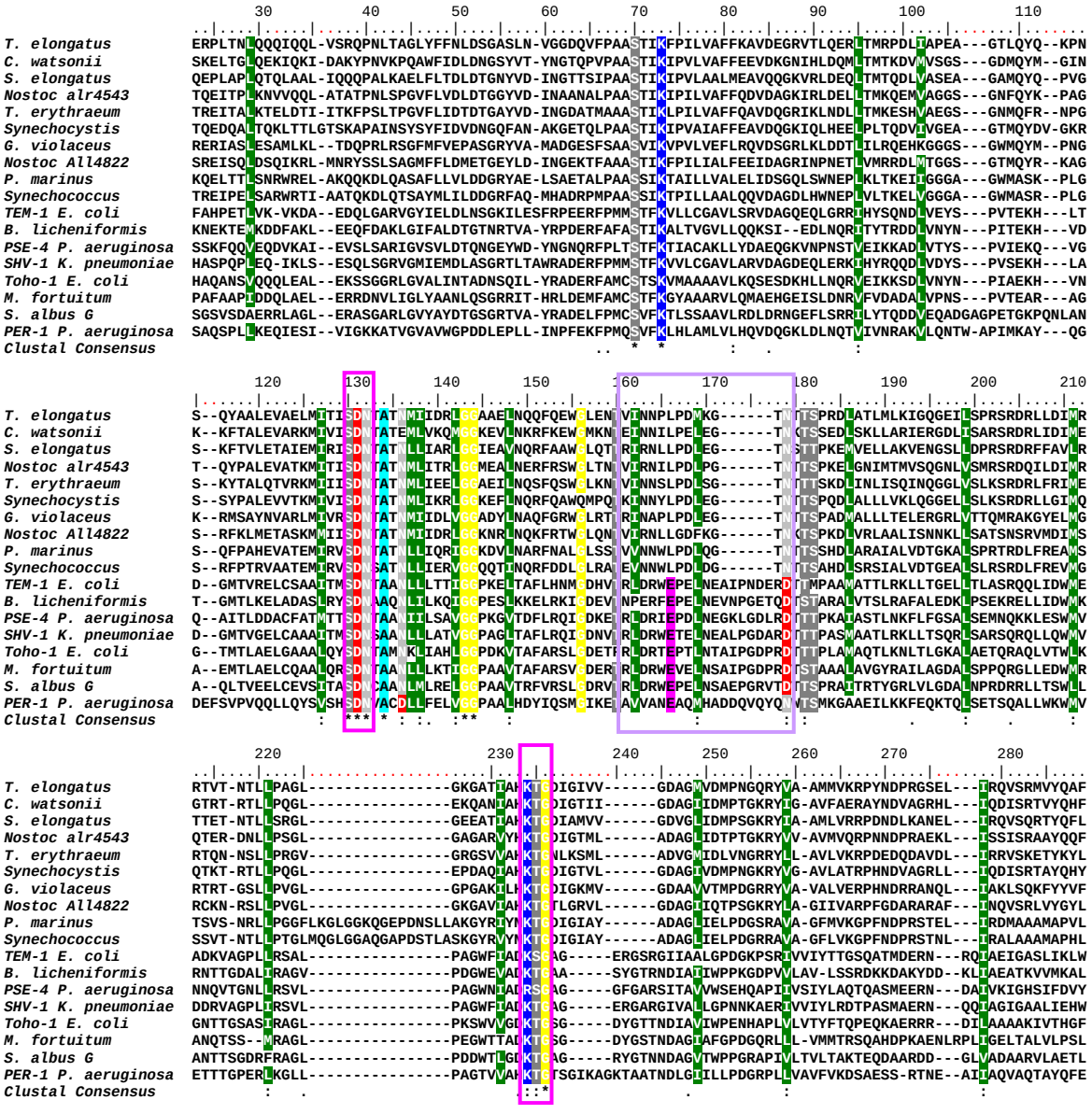


Figure 29 : Sequence alignment of the Ω-6 family proteins and some β-lactamases of known structure. Residues identical or similar, according to BLOSUM 62 matrix, and over a threshold of 90%are coloured. Conserved motifs are in magenta boxes in the following order : SXXK, SXN, and KT/SG and Ω-loop is in purple box. Numbering corresponds to that of TEM-1. Glu_{166} is highlighted in magenta.

The N-terminal part corresponding to putative sequence signals of the Ω-6 family and few residues of the C-terminal end were removed on the picture. Whereas with usual PBPs this kind of alignment is difficult to achieve because of sequence insertions in PBPs, in this case the proteins are well aligned. There is high residue conservation inside the family itself, and between the family and class A β-lactamases. Particularly, three regions corresponding to the three essential motifs are conserved, the differences lying in the Ω-loop deletion, and in one residue deletion on either side of the KT(S)G motif. Hence we suggest that this new family of PBPs is the most homologous to class A β-lactamases ever studied. Preliminary studies (Annex 2) allowed us to focus on the putative lactamase of *Thermosynechococcus elongatus* BP-1. The polypeptide deduced from the nucleotide sequence of PBP-A consists of 368 amino acids. Global alignments of PBP-A penicillin binding (PB) domain (roughly corresponding to amino acids 93 to 368) were achieved at http://www.ch.embnet.org/software/LALIGN_form.html. The only PBP that could be more or less correctly aligned with PBP-A entire PB domain was *P. aeruginosa* PBP5 with 17,8 % identity, however, alignment after KTG was very bad, suggesting that the identity is even lower. Global alignments with *E. coli* TEM-1 and *P. aeruginosa* Per-1 class A β-lactamases were better with 24,1 and 24,9 % identity, and the three motifs were very well aligned. It is interesting to note that a global alignment with *P. aeruginosa* PBP5 and *E. coli* TEM-1 gave 17,7 % identity over the whole proteins and was badly aligned at the C-terminal end, as with PBP-A. This suggested that PBP-A was phylogenetically closer to class A β-lactamases than to PBPs.

Cloning and expression of PBP-A protein and mutants.

Our initial attempts to clone the full-length PBP-A into pBAD-myc-HisB-Tet succeeded; however, protein expression in *E. coli* Top 10 was undetectable by western-blot. Depending whether the option $Gram^+$ or $Gram^-$ was chosen, a signal sequence with cleavage site between Ala-66↓Ala-67 or Ala-92↓Pro-93 respectively was predicted with a low score by SignalP. Proteomics studies of Synechocystis sp PCC 6803 showed that in some cases, start codons for periplasmic proteins were badly assigned by CyanoBase (23). There are several methionines in the N-terminal sequence of PBP-A, suggesting that it may be the case for this protein. We tried to express the protein, starting at the third methionine M28 (for which the signal sequence score was higher), but protein expression was undetectable too. We then submitted the protein sequence to Toppred transmembrane segments prediction program. A

transmembrane segment from residues 58 to 78 was predicted with high certainty, allowing us to suppose that the protein is membrane anchored and is active in the periplasmic space (Figure 30).

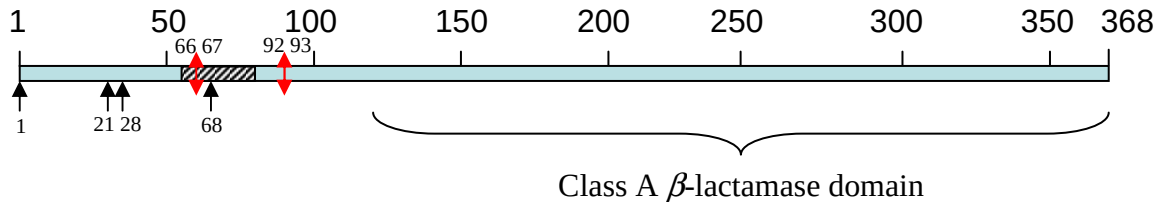


Figure 30: schematic representation of PBP-A protein. Putative starts are indicated by a black arrow. Putative transmembrane domain is in dashed zone and probable peptidase cleavage sites are indicated by red arrows.

Thus the coding sequence from Pro-93 to the C-terminus was cloned in frame to a myc-tag and a $(His)_6$-tag. Soluble expression of the tagged protein in the cytoplasm was possible and PBP-A was purified as described in Experimental Procedures with a final yield of 0,4 mg of protein per litre of culture. The protein migrated in SDS-PAGE under 35 kD molecular weight marker (Fermentas) in agreement with the calculated molecular weight of 32770,4 Da. All mutants were expressed under the same conditions, and were obtained as pure proteins.

DD-Peptidase and D-aminopeptidase activity of PBP-A.

DD-carboxypeptidase activity was assessed by incubating PBP-A with Ac_2-Lys-D-Ala-D-Ala and D-Ala-D-Ala substrates. Using an assay for the detection of D-Alanine, no hydrolysis of these compounds was observed at room temperature. Catalytic hydrolysis of Ac_2-Lys-D-Ala-D-Ala was absent at 55°C, the optimal growth temperature of *T. elongatus*. The same negative results were obtained with D-Ala-paranitroanilide, a substrate used for the D-aminopeptidase activity of *Ochrobacterium anthropi*, one member of the serine PRE (Penicillin Recognising Enzymes) family (24,25). Finally, after 30 min preincubation with Ac_2-Lys-D-Ala-D-Ala or D-Ala-D-Ala 0,5 mM, PBP-A retained its full reactivity towards penicillin G, indicating that in these conditions, the protein does not form any stable covalent complex with these D-Ala substrates.

Hydrolysis of thiolester substrates at 37°C was observed for both thioglycolates and thiolactates, with a preference for substrates with alanine instead of a glycine (Table 5). PhacATl is a diastereoisomer mixture of Phac-D-Alanyl-Thio-L-Lactate and Phac-D-Alanyl-Thio-D-Lactate. The results show that PBP-A is specific for the D-Alanyl group and has a marked preference for one thiolactic stereoisomer. PBP-A was also found to be very active on

one enantiomer of the racemic S2d substrate, most certainly the D-Alanyl-Thioglycolate given the previous result on PhacAtl. No activity was detected on the L-Alanyl-Thioglycolate isomer indicating a strict D-stereospecificity on that carbon. A lower specificity observed with the racemic Glycyl-Thio-(D,L)-Lactate S2c substrate indicates a more tolerant recognition on the leaving group side. Hydrolysis efficiency of thiolester substrates is very variable among PBPs, hydrolysis rates and k_{cat}/K_m were lower for *Streptomyces* K15 DD-peptidase, which is homologous to class A *β*-lactamases and catalytic activity was in the same range for *Streptomyces* R61, but the K_m was smaller (26,27).

		PhacATl		*S2c*		*S2a*	*S2d X*
		D-X	*D-Y*	*X*	*Y*		
WT	**k_{cat} (s^{-1})**	≥ 47,7	≥ 0,1	≥ 6,6	≥ 0,21	≥ 7,7	≥ 52
	K_m (mM)	≥ 1,5	≥ 1,5	≥ 1,5	≥ 1,5	≥ 3	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$3{,}18.10^4$	66,6	$4{,}38.10^3$	$1{,}4.10^2$	$2{,}58.10^3$	$3{,}47.10^4$
PBP-A-Δ	**k_{cat} (s^{-1})**	ND	ND	~ 0	~ 0	~ 0	0,01
	K_m (mM)	ND	ND	ND	ND	ND	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	ND	ND	~ 0	~ 0	~ 0	10,2
$Leu_{158}Glu$	**k_{cat} (s^{-1})**	≥ 8,43	≥ 0,23	0,35	0,024	0,35	≥ 5,8
	K_m (mM)	≥ 1,5	≥ 1,5	1,3	1,3	2,2	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$5{,}62.10^3$	$1{,}53.10^2$	$2{,}68.10^2$	18,7	$1{,}6.10^2$	$3{,}89.10^3$
***Streptomyces* K15[a]**	**k_{cat} (s^{-1})**	0,6		0,017		> 0,024	0,11
	K_m (mM)	1,2		0,22		> 3	1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$4{,}6.10^2$		75		8	75
***Streptomyces* R61[b]**	**k_{cat} (s^{-1})**	ND		5		5	70
	K_m (µM)	ND		50		50	100
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	ND		1.10^5		1.10^5	7.10^5

Table 5 : interaction of thiolester substrates with PBP-A and PBP-A-L_{158}E at 37°C. X and Y are the two possible racemic forms of the substrates. Values for *Streptomyces* K15 and R61 are from (26)and (27) respectively. No catalytic activity could be determined for the Y-stereoismer of S2d substrate. ND means Not Determined.

Hence, these kinetic values for PBP-A are typical of DD-peptidases suggesting that this protein is a DD-carboxypeptidase. Aminolysis in the presence of D-Alanine acceptor could not be determined, due to the high K_m observed with all substrates, hence transpeptidase activity could not be evidenced. The limiting step for PBP-A with thiolester seemed to be acylation, and may be an explanation for the absence of PBP-A reactivity with peptidic D-Ala-D-Ala substrates in the range of concentrations used.

β-lactamase activity of purified PBP-A.

PBPs incubated with a radioactive β-lactam form a covalent complex that can be visualized following SDS-PAGE and autoradiography (28). PBP-A could be visualised as a large band by autoradiography, indicating that PBP-A gene encodes a PBP. PBP-A was also able to slowly hydrolyze Penicillin G with $k_{cat} = 2{,}7.10^{-4}$ s^{-1} at room temperature, and $3{,}8.10^{-3}$ s^{-1} at 55°C. The kinetic parameters of [^{14}C]-Penicillin G hydrolysis by PBP-A were also determined and compared to kinetic constants of TEM-1 and some low-Mr PBPs (Table 6).

	Cluster	*Mr (kDa)*	*k_2/Ks (M^{-1} s^{-1})*	*k_3 (s^{-1})*
PBP-A	?	40	-	-
Truncated PBP-A[a]		30	$1{,}7.10^{4}$	$1{,}55.10^{-4}$
TEM-1	Class A β-lactamase	31,5	$2{,}4.10^{7}$	1500
PBP4 (*E. coli*)	Low-Mr class C PBP	49	7.10^{3}	4.10^{-4}
R39 (*Actinomadura*)	Low-Mr class C PBP	50	3.10^{5}	3.10^{-6}
PBP5 (*E. coli*)	Low-Mr class A PBP	34	390	$7{,}8.10^{-3}$

Table 6: molecular weight and constants of acylation (k_2/Ks) and deacylation (k_3) of Penicillin G for PBP-A at RT, TEM-1 (29), PBP4 from *E. coli* and *Actinomadura* R39 (30) and PBP5 from *E. coli* (31) at 20°C. a - The molecular weight given for PBP-A are the theoretical molecular weight of the full-length protein and the molecular weight of the truncated protein used in this study.

The key element in PBPs neutralisation by β-lactam antibiotics is the high acylation rate followed by a slow deacylation rate making the acyl-enzyme stable and allowing fast inactivation. Large variations in the kinetic constants can be observed, the highest k_2/Ks value of 3.10^5 $M^{-1}.s^{-1}$ observed in *Actinomadura* R39 can be 2 orders of magnitude lower in some PBPs. In the same way, k_3 can range between 10^{-3} and 10^{-6} s^{-1} (32,33). By comparison, one of the best β-lactams catalysts, TEM-1 from *E. coli*, has an acylation rate 10^2 to 6.10^4 higher and the deacylation rate is 10^5 to 10^8 times faster. The kinetic values of PBP-A are far from being in that range and with such a molecular weight, this protein can be clustered in the low-Mr PBPs family.

To determine the β-lactams sensivity profile of PBP-A, we spectrophotometrically followed hydrolysis of different lactams at 55°C, at the appropriate wavelength (Table 7). The protein shows a higher hydrolytic activity with penicillins than cephalosporins, comparable to the substrate profile of most class A β-lactamases (17).

Antibiotic substrate	k_{cat} (s^{-1})	
	PBP-A	*PBP-A-$L_{158}E$*
Penicillins		
Penicillin G	$3{,}8.10^{-3}$	$92{,}9.10^{-3}$
Ampicillin	$1{,}8.10^{-3}$	$58{,}7.10^{-3}$
Amoxicillin	$1{,}3.10^{-4}$	2.10^{-4}
Cephalosporins		
Cephalotin	$< 10^{-5}$	$4{,}3.10^{-4}$
Cefotaxime	$< 10^{-5}$	$5{,}4.10^{-4}$
Cefoxitin	$< 10^{-5}$	$< 10^{-5}$
Nitrocefin *	4.10^{-5}	2.10^{-3}

Table 7: Substrate profiles of PBP-A and PBP-A-L_{158}E mutant at 55°C. * Nitrocefin experiments were run at 25°C because of nitrocefin sensitivity to heating.

The penicillin degradation products released by PBP-A were also examined. It has been shown that benzylpenicillin fragmentation in PBPs can occur at the C7-N4 or C5-C6 bonds, releasing penicilloic acid or phenylacetylglycine and N-formyl-D-penicillamine respectively, whereas the only product from *β*-lactamases is penicilloic acid (34-38). In the experiments carried out with *Streptomyces* R61 DD-peptidase, phenylacetylglycine may be a direct breakdown product of the acyl-enzyme and N-formyl-D-penicillamine may be a secondary product from hydrolysis of D-5,5-dimethyl-Δ_2-thiazoline-4-carboxylic acid (Annex 3) (34,38). Release of D-5,5-dimethyl-Δ_2-thiazoline-4-carboxylic acid can be followed at 257 nm and appearance of free thiol groups from N-formyl-D-penicillamine can be kinetically followed by using Ellman's reagent (39). For PBP-A, incubation with benzylpenicillin monitored at 257 nm showed a first phase with an increase of the absorbance and a second phase where the absorbance slowly decreased. These observations suggest the formation of a product during the first phase followed by its degradation, similarly to the transient accumulation of D-5,5-dimethyl-Δ2-thiazoline-4-carboxylic acid observed for some PBPs. Apparition of free thiols was also evidenced using Ellman's reagent. A time dependent first order increase was preceded by a short lag phase, suggesting the release of N-formyl-D-penicillamine. When extrapolated to the time required for complete penicillin degradation, the free thiols represented 10% of the starting benzylpenicillin concentration. Thus, penicillin degradation in PBP-A seems to occur via the two pathways, hydrolysis of the C7-N4 bond being favoured.

PBP-A-D_{160}A PBP-A-S_{61}A and PBP-A-Δ mutants.

An aspartic acid in position 160 is the only well conserved acidic residue in the shorter Ω-loop of the PBP-A family. For testing its possible involvement in the protein activity, we expressed the mutant Asp_{160}Ala, and spectrophotometrically measured the penicillin degradation activity. There was no loss of activity, suggesting that Asp_{160} is not involved in the penicillin degradation mechanism (Table 8).

	PenG Binding*	k_2/K_s (k_{ac}) $M^{-1}.s^{-1}$	k_3 s^{-1}	k_{cat} s^{-1}		k_{cat} s^{-1}
				20°C	*55°C*	*Nitrocefin*
PBP-A	Yes	$1,07.10^4$	$1,55.10^{-4}$	$2,7.10^{-4}$	$3,8.10^{-3}$	4.10^{-5}
PBP-A-D_{160}A	Yes	ND	ND	ND	$3,4.10^{-3}$	ND
PBP-A-S_{61}A	No	0	ND	0	ND	ND
PBP-A-Δ	Yes	$2,8.10^3$	$4,25.10^{-4}$	ND	ND	$5,93.10^{-5}$
PBP-A-L_{158}E	Yes	$1,56.10^3$	$1,44.10^{-2}$	$1,42.10^{-2}$	$92,9.10^{-3}$	2.10^{-3}
PBP-A-L_{158}D	Yes	ND	ND	ND	$3,4.10^{-3}$	ND
S-PBP2x	ND	$1\text{-}2.10^5$	$3,3.10^{-5}$	ND	ND	ND
S-PBP2x Phe_{450}Asp	ND	$2,5.10^4$	$3,68.10^{-3}$	ND	ND	ND
TEM-1	ND	$8,4.10^7$	1500	ND	ND	ND
TEM-1 E_{166}N	ND	$4,5.10^5$	$5,2.10^{-6}$	ND	ND	ND

Table 8: characteristics and kinetic parameters of PBP-A and PBP-A mutants for hydrolysis of benzylpenicillin and nitocefin at RT. k_2/K_s and k_3 constants were calculated from time courses of acylation and deacylation of [^{14}C]-Penicillin G, and k_{cat} was measured spectrophotometrically with Penicillin G at 232 nm or nitrocefin at 482 nm. Constants for PBP2x were taken from (40), k_2/Ks is given for pH 7 and k_3 is given for pH 8, and TEM-1 from (41). ND = not determined

We assessed in the same way the roles of S_{61} and the shorter Ω-loop. For this purpose the S_{61}A mutant was generated by site directed mutagenesis, and an Ω-loop deletion mutant from N_{155} to T_{164} was created by an overlap PCR (PBP-A-Δ). As expected, PBP-A-S_{61}A could not hydrolyse thiolester substrates or bind [^{14}C]-Penicillin G, and no activity could be detected spectrophotometrically with benzylpenicillin as a substrate (Table 8). As in all enzymes of the Active Site Serine Penicillin Recognizing Enzyme (ASPRE) family (25), the serine 61 of the SXXK motif is confirmed to be the catalytic residue forming the acyl-enzyme with the penicillin and thiolester substrates.

The Ω-loop deletion mutant thiolesterase activity was nearly undetectable, even with best substrate S2d (Table 5), but surprisingly, it was still capable to bind and slowly hydrolyse penicillin. In class A β-lactamases, the Ω-loop is an essential structure for catalytic activity

and specificity towards β-lactam antibiotics. *Banerjee & al.* studied the effects of the Ω-loop deletion in *Staphylococcus aureus* PC1 β-lactamase. The deletion from amino acids 163 to 178 had a severe impact on the protein structure such as the exposure of internal residues to the solvent and the enlargement of the active site. By implying steric restrictions, the Ω-loop is also involved in the formation of the oxyanion hole, an essential structural element formed in the active site of serine enzymes for catalysis. The loop deletion thus induced the loss of the oxyanion hole. As a consequence, this β-lactamase mutant shows a decreased activity against nitrocefin, is still able to bind cephalosporin type substrates but has completely lost the ability to bind penicillins. For PBP-A-Δ, acylation rate for benzylpenicillin is decreased by a factor 4 but deacylation is increased by a factor 3, and activity towards nitrocefin is conserved (Table 8). Thus the Ω-loop influences somewhat the protein reactivity with penicillin, but do not seem directly involved in its catalysis considering the slight activity variations.

PBP-A-L_{158}D and PBP-A-L_{158}E mutants.

Glu_{166} which is strictly conserved in class A β-lactamases plays a critical role in both acylation an deacylation as shown by mutations of TEM-1 severely impairing protein activity (11,41). It was first shown by *Adachi & al.* that mutating Glu_{166} into Gln, Asn or Ala abolished penicillin hydrolysis, but mutants were still able to acylate the substrates. A more quantitative study of E_{166}N mutant by *Guillaume & al*. showed that deacylation rate is decreased by 8 orders of magnitude for one of the best substrates benzylpenicillin, whereas the acylation rate is also decreased but by only 2 orders of magnitude.

Chesnel & al. (40) showed that mutating Phe_{450} of PBP2x, which occupies the same spatial position as Glu_{166} in β-lactamases, into an aspartic acid residue increased the deacylation rate 110 times. In the same way, L_{158} which is aligned with E_{166} was mutated in either Asp or Glu. Both mutants were still acylated by benzylpenicillin. Whereas no significant difference in penicillin G hydrolysis rate was observed for PBP-A-L_{158}D, the k_{cat} of PBP-A-L_{158}E was increased by 24 times at 55°C and 50 times at room temperature, the acylation rate being decreased by one order magnitude while deacylation rate is increased by ~ 90 times (Table 8). A similar effect is observed in PBP-A and PBP2x when a carboxylic residue susceptible to play the role of Glu_{166} is introduced in the appropriate position. If there was a doubt in the place of the 6 amino acids deletion in the putative PBP-A Ω-loop, this experiment shows that Leu_{158} occupies most certainly a similar position as Glu_{166} in the class A β-lactamases.

Concerning the hydrolysis of thiolester substrates, the k_{cat} was decreased by 5 to 16 times depending on the substrate. This mutant was also D-setereospecific, and activity with the L-stereoisomers was not affected by the mutation.

When looking at the fragmentation of benzylpenicillin at 257 nm, $L_{158}E$ mutant showed a decrease in the absorbance until reaching a plateau, corresponding to the normal penicillin hydrolysis, and suggesting that no D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid was produced. This was confirmed by thiol titration where there was no reaction with Ellman's reagent, reflecting no release of free thiols and thus of N-formyl-D-penicillamine. Introduction of $L_{158}E$ mutation favours the *β*-lactamases mechanism, which, with the increase in catalytic efficiency and the loss of activity towards thiolesters, constitutes a first step in PBP-A evolution into a *β*-lactamase. Similar observations have been previously observed in *Streptomyces* R61 DD-peptidase when mutations were introduced at residues of the catalytic site, evidencing the close relationship of PBPs and *β*-lactamases, see Annex 3 (27,42,43).

Catalytic activity in a pH range from 5 to 10 was assessed in both PBP-A and PBP-A-$L_{158}E$ proteins. Catalytic parameters were determined only for the mutant protein since complete penicillin hydrolysis time courses could only be spectrophotometrically followed for this protein (Table 9).

		pH 5	*pH 6*	*pH 7*	*pH 7.4*	*pH 8*	*pH 9*	*pH 10*
PBP-A	$k_{cat}.10^{-4}$ (s^{-1})	8,2	7,2	7,4	7,1	5,8	2,6	2,6
PBP-A-$L_{158}E$	$k_{cat}.10^{-4}$ (s^{-1})	258	277	179	133	88,1	49,6	40,9
	K_m (µM)	17,38	< 10	< 10	< 10	< 10	29,9	112,7
	$k_{cat}/K_m.10^3$ ($M^{-1}.s^{-1}$)	1,49	> 2,77	> 1,79	> 1,33	> 0,881	0,166	0,00363

Table 9 : variations of k_{cat} as a function of pH for PBP-A, and constant parameters of penicillin G hydrolysis measured at different pH for PBP-A-$L_{158}E$ at RT and determined by fitting complete time courses of benzylpenicillin hydrolysis at 232 nm.

The discrepancies in k_{cat} observed for PBP-A at Table 8 and Table 9 may be due to higher room temperature, and in the inaccuracy of measuring very low variations of absorbance. No variations of activity were observed for both proteins at acidic pH. When placed in such an environment the carboxylic function of E_{158} tends to remain protonated but at pH 5, only 10% of the protein may be protonated considering pKa of 4 for the carboxylic acid function, hence, the results are concordant. At basic pH however, the hydrolysis rate of penicillin G is decreased in both proteins. K_m of PBP-A-$L_{158}E$ protein was even more affected at basic pH, hence inducing a decrease in k_{cat}/K_m, showing a perturbation in the protein acylation efficiency. Two residues in the catalytic site have ionisable side chains that titrate at basic pH,

Lys_{64} (Lys_{73}) and Lys $_{219}$ (Lys_{234}). Without mutant studies of these residues, their implication with the general mechanism of catalysis remains difficult to elucidate.

We wanted to assess whether $Leu_{158}Glu$ mutation was concomitant with a change in substrate specificity. As had been done for the wild type protein, hydrolysis of different lactams at 55°C, was spectrophotometrically followed (Table 3). The profile was exactly the same with a preference for penicillins.

Finally, we measured the k_{cat} of the proteins at increasing temperatures and traced the Arrhenius plot from which the activation energy for both proteins was calculated (Figure 31).

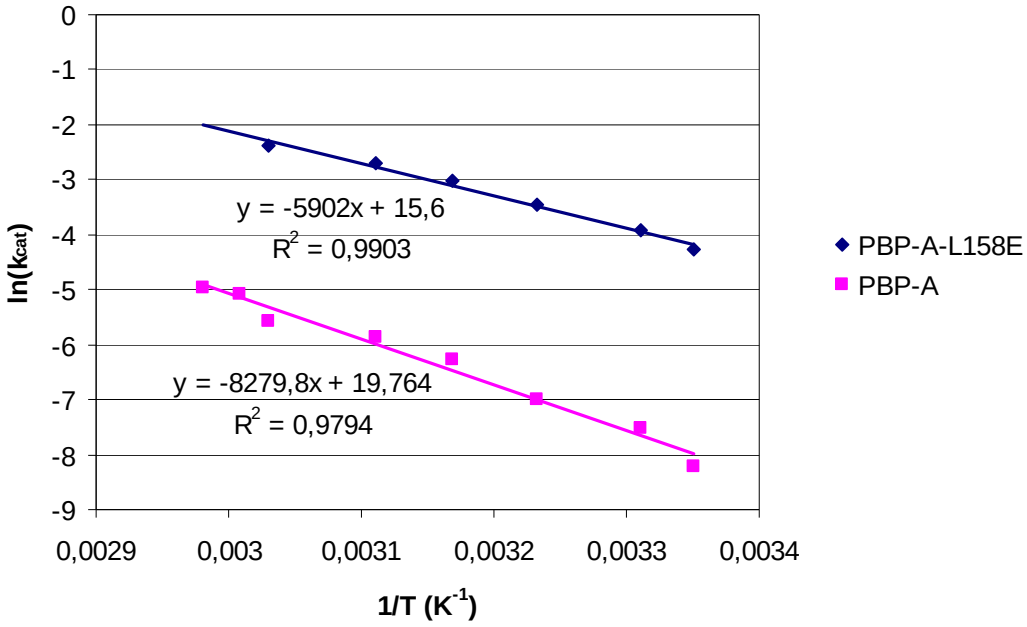


Figure 31 : Arrhenius plot could be constructed measuring PBP-A and PBP-A-L_{158} k_{cat} at increasing temperatures, from 25,5 to 62,5°C for PBP-A and 25,5 to 57°C for PBP-A-L_{158}E. If variation of $ln(k_{cat})$ as a function of 1/T is linear, the slope of the line equal Ea/R where Ea is the activation energy and R is the perfect gaz constant).

We found 68,8 $kJ.mol^{-1}$ for the wild type and 49 $kJ.mol^{-1}$ for the mutant. This decrease of the activation energy is consistent with the increase in the reaction catalysis.

Physiological role of PBP-A.

High-Mr PBPs catalyse transglycolase and transpeptidation reactions whereas low-Mr PBPs do not synthesise peptidoglycan and are involved in its modification by removing the terminal D-Ala from pentapeptide side chains (carboxypeptidase activity) or by cleaving the peptide crosslinks that hold the glycan chains together. Considering the molecular weight, its probable exportation to the periplasm, its ability to hydrolyse thiolesters with good efficiency and its activity towards β-lactams, PBP-A can be considered as a low-Mr weight DD-carboxypeptidase. Inability to hydrolyse Ac_2-L-Lys-D-Ala-D-Ala and D-Ala-D-Ala only reflects that these substrates may not be appropriate, or that the protein may be active only under certain conditions (see below).

Cyanobacteria cell wall shows an overall Gram negative structure, the peptidoglycan is thicker, the degree of cross-linking between the peptidoglycan chains is higher (more similar to the Gram positives'), teichoic acids characteristic of Gram positive walls are absent and carotenoids are found, unlike Gram negative membranes (44). Although the cell wall structure seems to be different from Gram positive or Gram negative bacteria, its chemical composition has been shown to have no striking difference compared to other bacteria (45), eliminating the hypothesis of a very different substrate. Pentapeptides involved in cross-linking have shown to contain only *m*-DAP (44), and that may explain the absence of activity on L-Lys substrate.

Moreover, it has been shown that *in vitro* DD-peptidase activity of purified enzymes is never as high as it should be on the basis of the proteins physiological function, and optimal activity for these enzymes may be obtained in a specific membrane environment or specific multienzyme complexes (46). The soluble forms of PBP5 and 6 from *E. coli* were also reported to be inactive with Ac_2-L-Lys-D-Ala-D-Ala substrate (47,48). Finally, the variability in activity towards Ac_2-L-Lys-D-Ala-D-Ala substrate in PBPs has been studied in relation with Ω-loop like structures. It was shown that some PBPs have substrate specificity on the third position of the peptide, and proteins with a shorter Ω-loop structure like PBP5 may be preferentially active if diaminopimelate is in the third position whereas proteins with much longer Ω-loop like PBP3 from *Streptococcus pneumoniae* only recognize peptides with a Lys instead of a diaminopimelic acid (49).

Without PBP-A, only four HMW PBPs and one LMW PBP have been predicted in the genome of *Termosynechococcus elongatus* (50), and this may be sufficient for bacteria survival. However, LMW PBPs that give the bacteria shape are underrepresented since bacteria like *E. coli* have seven LMW PBPs (51). Homologous proteins to PBP-A are found in all sequenced cyanobacteria. Their neighbouring genes are variable from one genome to another and do not give clues about the function. Moreover, no mobile genetic elements are found in the gene vicinity and this indicates a quite ancient origin. Altogether, these observations suggest that PBP-A may be an important DD-carboxypeptidase with specificity for peptides with diaminopimelate in third position of the peptide. The high catalytic efficiency of the protein with thiolesters enforces the idea that the protein may have a role in peptidoglycan maintenance.

Place of PBP-A in the PRPs

From a wide point of view, is it possible to assign PBP-A to a specific PBP cluster ? The biochemical characterisation of PBP-A and the preliminary studies on Nostoc (Annex 2) both indicate that these two proteins are LMW PBPs, as stated earlier. However, low-Mr PBPs are usually membrane anchored since their C-terminal part, and high-Mr PBPs are linked to the membrane by their N-terminal part (6,52). PBP-A subcellular location predictions (TopPred) suggested this protein would be with a highest probability membrane anchored through its N-terminal end. Subsequent analysis on all the proteins family gave the same results (data not shown). Hence, we propose that we identified a new family of low-Mr PBPs.

When searching for homologous proteins in GenBank, a few proteins of unknown functions and the class A β-lactamases are the first hits with much better scores than any known LMW PBP. Interestingly, in the multiple alignment the proteins of unknown functions display Ω-loops of various lengths. The phylogenetic tree in Figure 28 represents the different proteins with LMW PBPs, PBPs with variations in the Ω-loop size and class A β-lactamases. In the case of proteins with 6 amino acids deleted compared to class A β-lactamases, they all belong to cyanobacteria. As two of them have shown to be PBPs, one can extend this characteristic to the whole group and design these proteins as a new family of LMW PBPs. Surprisingly, the average distance from this family to class A or C LMW PBPs is higher than the distance to class A β-lactamases. Hence, this family is the closest to the family of class A β-lactamases known today.

β-lactamases are suggested to have evolved from PBPs but an evolutionary process that occurred in the PREs family is the emergence of penicillin-receptors involved in the

regulation of β-lactamases expression. This kind of receptors has been identified in *Bacillus licheniformis* and *Staphylococcus aureus* for the induction of class A β-lactamases (53,54). They are composed of a transmembrane domain and a penicillin-sensor domain which shares high sequence similarity with class D β-lactamases (53). For this reason, it is proposed that these receptors have evolved from a β-lactamase. The penicillin sensor domain of the BlaR penicillin receptor of *Bacillus licheniformis* has been characterised and shows high rate of acylation ($8{,}7.10^{6}$ $M^{-1}.s^{-1}$) and a low rate of deacylation ($1{,}4.10^{-5}$ s^{-1}) for benzylpenicillin, implying a very rapid formation of acyl-enzyme. These proteins have no activity on D-Ala-D-Ala containing substrates or thiolester analogs (55). In the case of PBP-A, the acylation rate is much slower (4 orders of magnitude), which is against a function of sensor. Moreover, PBP-A proteins have been identified in the genome of all sequenced cyanobacteria. Finally, these kind of receptors emerged for the regulation of β-lactamases. In the case of *T. elongatus* where no lactamase is clearly annotated in the genome, such a receptor would be an energy waste. Hence, we suggest that PBP-A is not a product of evolution of a β-lactamase.

According to Massova & Mobashery, evolution is not a linear process but several PBPs would have evolved separately from a primordial PBP into different classes of β-lactamases in a parallel and independent process. In a first step, PBPs would have acquired the ability to catalyse deacylation of the acyl-enzyme. Then, the acylation and deacylation processes would have been improved by substitutions, insertions or deletions to give rise to the today β-lactamases, some of them being almost perfect enzymes. Another requirement in this evolution process is the loss of PBP original activity to gain efficiency in the new function, by incorporating structural features that disfavour interaction with peptidoglycan. PBP5 from *E. coli* contains all the features of a PBP but also displays an Ω-loop like structure. On basis of the protein crystal structure study, it was suggested that class A β-lactamases evolved directly from a carboxypeptidase PBP5-like by incorporating two amino acids in the Ω-loop that could catalyse deacylation (like the couple Glu_{166}/Asn_{170}), and replacing some residues that are essential for DD-carboxypeptidase activity (56). For example, two hydrophobic residues on PBP5 Ω-loop like are involved in accepting the methyl group of the penultimate D-Ala of peptidic substrates and may be replaced for steric hindrance of a peptidic substrate (56,57).

The characterisation of PBP-A allowed us to determine the essential roles of S_{61} and shows a comparable penicillin hydrolysis rate as PBP5 (k_{cat} value at 55°C). The effects of the $L_{158}E$ mutation is representative at a lower scale of how evolution leads from one PBP to a β-lactamase: deacylation rate of the penicilloyl-enzyme is increased by 90 times, activity toward

thiolesters (representative of interactions with peptidoglycan) is decreased and the penicillin hydrolysis path has changed to mimic that of β-lactamases (only penicilloic acid seems to be released). Based on the sequence alignment, the family of the Ω-6 proteins contains all the specific features of class A β-lactamases excepted 6 amino acids in the Ω-loop, the essential Glu_{166} and two residues flanking the KT(S)G motif. In conclusion, PBP-A is most probably a DD-carboxypeptidase that belongs to a whole family of proteins with the same signature sequence, all these proteins are expressed in the early branched bacterial species of cyanobacteria, a single mutation tends to give the protein lactamases properties, and the phylogenetic analysis clearly reveals a close proximity to the family of class A β-lactamases. Altogether, these evidences indicate that those two families have a common ancestor which was a missing link in the evolution of class A β-lactamases.

Materials and methods

Chemicals and antibiotics. All β-lactam antibiotics, D-Ala-D-Ala and Ac_2-Lys-D-Ala-D-Ala substrates were purchased from Sigma. D-Ala paranitroanilide substrate was from Bachem. Thiolesters substrates were a kind gift from M. Galleni Professor at ULG, in the Département des sciences de la vie / Macromolécules biologiques. Oligonucleotides were from Eurogentec, with "fp" and "rp" standing respectively for forward and reverse primer:

PBP-A-fp 5'TCGCCATGGCCGCTCCTGAGGCACC3'

PBP-A-rp 5'CGTTCTAGAGGCGGCGAAAGTTTC3'

D_{160}A-fp 5'ATCCGCTGCCGCCTATGAAGGGCACC3'

D_{160}A-rp 5'GGTGCCCTTCATAGCCGGCAGCGGAT3'

L_{158}D-fp 5'AAATACGGTGATTAACAATCCGGATCCGGATATGAAGGGCACCAA3'

L_{158}D-rp 5'TTGGTGCCCTTCATATCCGGATCCGGATTGTTAATCACCGTATTT3'

L_{158}E-fp 5'CGGTGATTAACAATCCGGAGCCGGATATGAAGGGC3'

L_{158}E-rp 5'GCCCTTCATATCCGGCTCCGGATTGTTAATCACCG3'

S_{61}A-fp 5'GTTTTTCCTGCCGCGGCTACGATTAAGTTTC3'

S_{61}A-rp 5'GAAACTTATCGTAGCCGCGGCAGGAAAAAC3'

PBP-A-Δ1 5'AATACGGTGATTAACACCACCAGTCCCCGG3'

PBP-A-Δ2 5'ACTGGTGGTGTTAATCACCGTATTTTCTAAGC3'

Alignment. The amino acid sequences for this study were recovered from the ncbi database. Multiple alignments were achieved with the program T-Coffee (58), and edited using BioEdit version 7.0.3 (59). Phylogenetic tree was constructed by the neighbour joining method and edited using Treeview.

Bacterial strains. *Thermosynechococcus elongatus* BP-1 was used as a source of DNA for PCR amplification of the tll2115 gene, a total genome extract of the cyanobacteria was kindly given by M. Sugiura, Assistant Professor at the Department of Applied Biological Chemistry, Osaka Prefecture University. *E. coli* Top 10, F^- (Invitrogen) was used for standard subcloning procedures as well as for protein expression.

Cloning of PBP-A gene and mutants. PBP-A gene was identified in the complete genome of *Thermosynechococcus elongatus*, available in CyanoBase, the genome database for cyanobacteria; http://www.kazusa.or.jp/cyano/cyano.html (60,61). Since probable signal peptide and/or transmembrane segments were predicted by SignalP and TopPred online

softwares between amino acids 1 to 92 (Figure 1), the gene encoding the PBP domain of PBP-A protein, i.e. truncated from its 92 first N-terminal residues, was recovered by PCR using primers PBP-A-fp and PBP-A-rp and the purified *T. elongatus* chromosome as a template. In this way, *NcoI* and *XbaI* restriction sites were added at the 5' and 3' termini of PBP-A gene. The amplified 853-bp fragment was then cloned in the pGEM-T Easy cloning vector (Promega). After sequencing confirmation, PBP-A gene was cloned in the pBAD/myc-HisB-Tet vector in fusion with a myc-tag and a $(His)_6$-tag. This expression vector is a derivative of pBAD/myc-HisB [Invitrogen] but the ampicillin resistance marker has been replaced by a tetracycline resistance gene. In the final construct, a methionine was added to the N-terminal end of the truncated protein, giving the numbering Met_1Pro_2…, which is used throughout the report. The expression vector for the *Ω*-loop deletion mutant PBP-A-*Δ* was constructed similarly but the initial PCR fragment was generated by running an overlap extension of two PCR fragments obtained by amplification with the PBP-A-*Δ*1 / PBP-A-rp and PBP-A-*Δ*2 / PBP-A-fp pairs of primers. $D_{160}A$, $L_{158}D$, $L_{158}E$ and $S_{61}A$ mutations were generated with the Quick Change® Site-Directed Mutagenesis kit (Stratagene) using the respective pairs of fp and rp primers and the wt expression vector as a template.

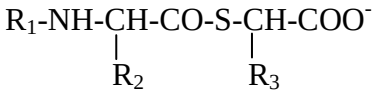
Culture and PBP-A production. Transformed *E. coli* Top 10 were grown in 1 litre of Luria-Bertani broth medium supplemented with tetracycline (7.5 µg/mL) at 37°C until the A_{600} reaches approximately 0,6. The temperature was lowered to 30°C and protein production was induced by addition of L-arabinose at a final concentration of 0.2 % (wt/vol) for 3-4 h. The culture was centrifuged at 4,400 × g for 10 min, the harvested cells were resuspended in 25 mL PBS, and stored overnight at -20°C. Cells were thawed to 37°C and centrifuged at 5,400 × g for 10 min. Supernatant (S1) was stored at 4°C and pellet was resupended in 8 mL 50 mM tris-HCl pH 8, 1 mM EDTA, and 100 mM NaCl supplemented with 100 µL egg white lysozyme (10 mg/mL). After 20-min incubation time at room temperature followed by 45-min incubation time at 37°C, 200 µL DNase (1 mg/mL) was added and lysate was incubated 15 minutes at room temperature. Supernatant obtained after centrifugation at 21,000 × g for 15 min at 4°C was mixed with S1, providing the cytoplasmic extract.

PBP-A purification. The cytoplasmic extract was dialyzed against 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 10 mM imidazole (buffer A) using a Spectra por membrane (Mr cut-off, 10,000, Spectrum Laboratories) overnight, at 4°C. Following passage through a 0.22 µM filter, the protein mixture was applied to a Ni^{2+}-chelating column (HiTrap chelating, Amerscham Biosciences), washed with 10 column volumes of buffer A, and finally eluted

with a 10-500 mM imidazole gradient in buffer A. Fractions were analysed by SDS-PAGE, those containing PBP-A protein were pooled and submitted to steric exclusion chromatography on a HiLoad 26/60 Superdex 200 Prep grade column (Amerscham Biosciences), and using HEPES 20 mM, EDTA 1 mM, pH 7.4 as elution buffer. Protein fractions were analysed by 10% polyacrylamide gel electrophoresis (PAGE) and either stained with Coomassie blue R250 or submitted to western blot using an anti-myc antibody (Invitrogen). Protein concentration in pure fractions was determined by measuring the absorbance at 280 nm using an extinction coefficient of 13370 M^{-1} cm^{-1}.

Enzymatic assays. DD-peptidase activity was tested by incubating PBP-A 3 µM with Ac_2-L-Lys-D-Ala-D-Ala (1 mM) or D-Ala-D-Ala (1mM) in 20 mM Tris-HCl buffer, pH 8, in presence or not of a glycine acceptor (1 mM), at RT. The release of D-Ala was estimated continuously using the D-amino acid oxidase method (62). Incubation with Ac_2-L-Lys-D-Ala-D-Ala was also performed at 55°C, aliquots were taken at different times, reaction was stopped by addition of 1 mM penicillin G and the release of D-Ala was estimated by the same D-amino acid oxidase method. D-amino peptidase activity was also assayed on the chromogenic substrate D-Ala paranitroanilide (63) at RT and 55°C with concentrations varying from 5 to 18 mM in Hepes 20 mM, EDTA 1 mM, pH 7.4.

Hydrolysis of 0.3 mM S2a, racemic S2c and S2d, and diastereoisomeric PhacATl thiolester substrates (Table 10) were performed at 37°C and monitored spectrophotometrically at 250 nm (26,64,65). The general formula for thiolesters is given by:



Substrate	R1-	R2	R3
S2a	C_6H_5-CO-	H	H
S2c	C_6H_5-CO-	H	CH_3
S2d	C_6H_5-CO-	CH_3	H
PhacATl	C_6H_5-CH_2-CO-	CH_3 (D)	CH_3

Table 10 : Structures of the substrates

Kinetic parameters were extracted by numerical simulation of the progression curves using the following equations :

$$d[S_1] + d[S_2] = (((k_{cat1}[E] + k_0)[S_1]/(K_{m1} + [S_1])) + ((k_{cat2}[E] + k_0)[S_2]/(K_{m2} + [S_2]))) dt$$

$Abs = \varepsilon_S ([S_1] + [S_2]) + \varepsilon_P ([P_1] + [P_2])$

where ε_S and ε_P are the extinction coefficients of the substrate and the product, and the 1 and 2 subscripts stand for the two enantiomers or diastereoisomers. k_0 is the constant for spontaneous thiolester hydrolysis and was measured independently. ε_S, ε_P, k_{cat1}, K_{m1}, k_{cat2} and K_{m2} were optimised together during the simulations. This is possible because each parameter is affecting a different region of the curve.

Penicillin binding assay. Enzyme around 3 µM was incubated 2 min with 10^{-5} M [^{14}C]-Penicillin G (Sigma) in Hepes 20 mM, EDTA 1mM, pH 7,4 in a final volume of 15 µL. The reaction was stopped by addition of ½ volume of SDS-PAGE denaturing buffer (Tris-HCl 0.5 M pH 6.8, DTT 200 mM, SDS 4%, Bromophenol Blue 0.2% and glycerol 20%), boiling for 10 min and the reaction mixture was subjected to SDS-PAGE. Gels were dried and exposed 4 days to a Kodak Biomax MR film in a hypercassette™ (Amersham life science).

Determination of Acylation and Deacylation rate Constants for β-lactam antibiotics. The kinetic scheme for the interaction of a PBP with a β-lactam antibiotic is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} E \bullet S \underset{k_2}{\rightarrow} E\text{–}S \underset{k_3}{\rightarrow} E + P$$

where E • S is the non covalent Michaelis complex, E–S is the covalent acyl-enzyme complex, and P is the released product. The deacylation rate constant, k_3, was derived from the first order decay rates of the [^{14}C]penicilloyl-PBP complex. PBP-A protein around 3 µM was incubated for 2 min with 10 µM [^{14}C]-Penicillin G in Hepes 20 mM, EDTA 1 mM, pH 7.4. 1 µL of penicillinase from *Bacillus cereus* (Sigma) was added at a concentration such that the remaining free penicillin is hydrolysed in a few seconds. At various times, 15 µL aliquots were removed and the deacylation was stopped by addition of ½ volume of SDS-PAGE denaturing buffer and immediate freezing at -80°C. Reaction mixtures were boiled for 10 min and were subjected to SDS-PAGE, gels were dried and exposed 4 days to the CS screen of a phosphorimager (GS 525 Molecular imager system, Bio-Rad), the quantity of radioactive protein was measured with the Molecular Analyst software, version 1.4 (Bio-Rad). The optimised k_3 constants were determined by fitting an exponential to the experimental points.

Acylation reactions were performed by mixing 2 µM [^{14}C]-Penicillin G with 3 µM of protein in Hepes 20 mM, EDTA 1 mM, pH 7.4. Every 10 seconds, 15 µL aliquots were removed and treated using the same protocol described for deacylation. Since Ks ($k_{-1}+k_2/k_1$) is most probably much higher than the experimental concentrations of enzyme and substrate, the acylation proceed via a second order reaction with a rate constant $k_{ac} = k_2/Ks\ k_1k_2/(k_{-1}+k_2)$.

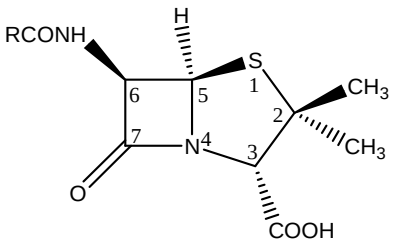
The optimised constant was extracted from the data by numerical simulation using the following differential equation:

$d[E\text{-}Ac] = (k_{ac}[E][S] - k_3[E\text{-}Ac])\,dt$

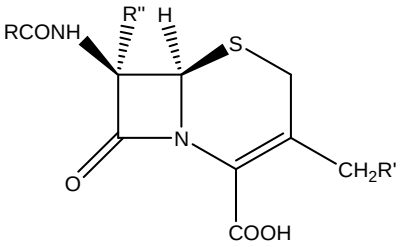
where k_3 is known from the previous deacylation experiment.

β-lactam antibiotics hydrolysis. All the spectrophotometric measurements were performed with the help of a UVIKON 933 (KONTRON instruments) or a CARY 3 Bio (VARIAN analytical instruments) interfaced to microcomputers and to a thermoblock Ecoline 003 (LAUDA). The time courses of the hydrolysis of the β-lactam compounds (Table 11) were performed in Hepes 20 mM, EDTA 1 mM, pH 7.4, with an enzyme concentration around 3 µM and penicillin antibiotics at 0.5 mM or cehalosporins at 0.25 mM.

Penam



Cephem



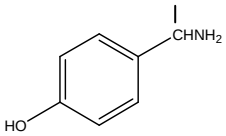
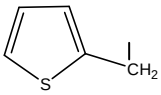
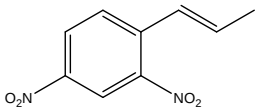
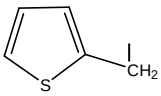
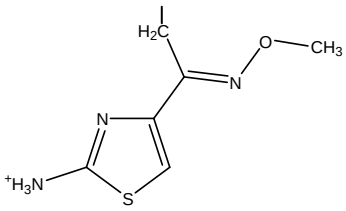
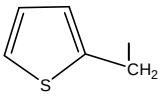
Substrate	R	R'	R''
Benzylpenicillin	C_6H_5-CH_2		
Ampicillin	C_6H_5-$CHNH_2$		
Amoxicillin			
Nitrocefin			H
Cephalotin		CH_3-COO-	H
Cefotaxime		CH_3-COO-	H
Cefoxitin		NH_2-COO-	-OCH_3

Table 11 : Structure of the antibiotics

Absorbance was followed at the appropriate wavelength (Penicillin G $\Delta\varepsilon_{232}$ = -1010 $M^{-1}.cm^{-1}$, Ampicillin $\Delta\varepsilon_{235}$ = -670 $M^{-1}.cm^{-}1$, Amoxicillin $\Delta\varepsilon_{235}$ = -780 $M^{-1}.cm^{-}1$, Cephalotin $\Delta\varepsilon_{265}$ = -8790 $M^{-1}.cm^{-1}$, Cefotaxime $\Delta\varepsilon_{265}$ = -6260 $M^{-1}.cm^{-1}$, Cefoxitin $\Delta\varepsilon_{265}$ = -7380 $M^{-1}.cm^{-1}$, Nitrocefin $\Delta\varepsilon_{482}$ = 15000 $M^{-1}.cm^{-1}$).

Annexes

Annex 1: table containing proteins used for construction of phylogenetic tree in Figure 28
gi number references a protein code on ncbi server http://www.ncbi.nlm.nih.gov.

Microorganism	***gi number***	***Ω-loop variations***
1 *Thermosynechococcus elongatus* BP-1	22295842	- 6 amino acids
2 *Nostoc* sp. PCC 7120 alr4543	17232035	
3 *Crocosphaera watsonii* WH8501	67923291	
4 *Trichodesmium erythraeum* IMS101	113477326	
5 *Nostoc* sp. PCC 7120 All4822	17133959	
6 *Synechococcus elongatus* PCC 7942	81300412	
7 *Synechocystis* sp. PCC 6803	1001780	
8 *Gloeobacter violaceus* PCC 7421	35212955	
9 *Prochlorococcus marinus* str. MIT 9313	33640853	
10 *Synechococcus* sp. WH 8102	33632421	
11 *Rubrobacter xylanophilus* DSM 9941	108804547	- 1 amino acid
12 *Alkaliphilus metalliredigenes* QYMF	77683558	
13 *Fusobacterium nucleatum* ATCC 25586	19712798	
14 *Pediococcus pentosaceus* ATCC 25745	48870359	
15 *Lactobacillus plantarum* WCFS1	28271757	
16 *Symbiobacterium thermophilum* IAM 14863	51855742	- 3 amino acids
17 *Symbiobacterium thermophilum* IAM 14863	51855807	- 10 amino acids
18 *Moorella thermoacetica* ATCC 39073	83590885	- 14 amino acids
19 *Thermoanaerobacter ethanolicus* ATCC 33223	76796525	
20 *Clostridium thermocellum* ATCC 27405	67876449	
21 *Deinococcus radiodurans* R1	6458117	+ 4 amino acids
22 *Deinococcus geothermalis* DSM 11300	94984424	
23 *Erythrobacter litoralis* HTCC2594	85375695	+ 7 amino acids
24 *Solibacter usitatus* Ellin6076	67934387	+ 15 amino acids

Annex 2: Preliminary studies

At the time we started to study families of proteins containing *Ω*-loops of variable size, we retained three putative PBP candidates: two proteins containing 6 amino acids deletion, from *Thermosynechococcus elongatus* (PBP-A) and *Nostoc* PCC 7120 (All4822 named PBP-N), and one protein containing one amino acid deletion from *Fusobacterium nucleatum* (PBP-F). It was attempted to produce these three proteins in order to choose one protein candidate for evolution studies.

The genes encoding PBP-A, PBP-N and PBP-F were cloned in the expression vector pBAD. PBP-F could be expressed under its native form (Figure 32) in the cytoplasm of *E. coli* and purified.

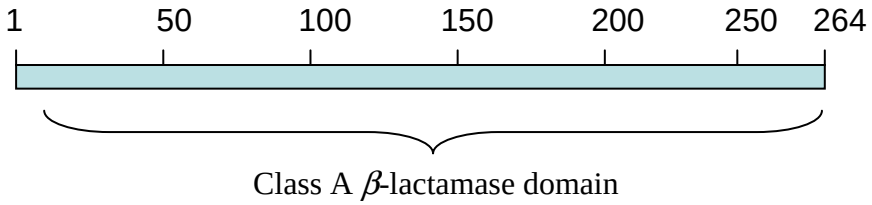


Figure 32 : schematic representation of *Fusobacterium nucleatum* coding sequence.

For PBP-N, three forms were attempted to be produced: as observed for PBP-A, the native protein (PBP-N_1) could be cloned but not expressed in *E. coli*, presumably because of a signal peptide (predicted with high certainty by SignalP). The portion of the protein aligned with class A *β*-lactamases (PBP-N_2) was expressed in the cytoplasm, but the protein seemed to precipitate when concentrated. The final version of the protein (PBP-N_3) was produced in the cytoplasm without 20 N-terminal amino-acids corresponding to a putative signal peptide (Figure 33). The protein formed inclusion bodies that were solubilised, allowing purification of the protein. For the details on PBP-A, see the main work.

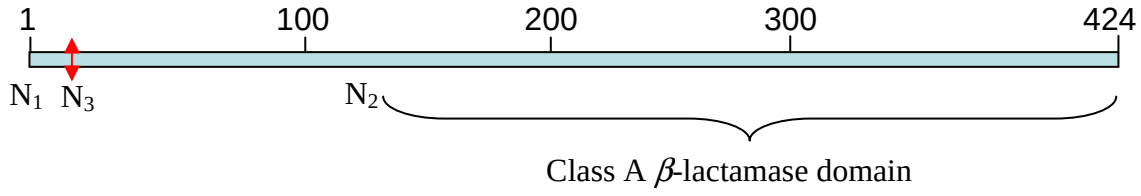


Figure 33 : schematic representation of All4822 from Nostoc spPCC 7120 coding sequence. The red arrow represents the cleavage site predicted with high certainty by SignalP.

Hydrolysis of penicillin G was followed at 232 nm and no significant hydrolysis could be determined within 60 min. Radio-labelling with [^{14}C]-Penicillin G was assessed, PBP-F did

not bind penicillin, whereas PBP-A, PBP-N_2 and PBP-N_3 where able to form a covalent link with pencillin G. No DD-peptidase activity for PBP-A, PBP-N3 and PBP-F could be detected with the substrate Ac_2-L-Lys-D-Ala-D-Ala at a 1 mM concentration.

This preliminary studies allowed us to choose a protein candidate for our evolution investigations: as PBP-A and PBP-N had been shown to be PBPs but PBP-A was easier to purify and thermostable, we chose to study more in details PBP-A. However, interpretations about the families of proteins with variable *Ω*-loop sizes are limited. Surprisingly, PBP-F is a cytoplasmic protein and do not bind penicillin or hydrolyse it. Hence, a putative role for this protein is difficult to predict. Nevertheless, other proteins with one amino-acid deletion may have a different behaviour in presence of penicillin as topological predictions suggest that three of them (11, 12 and 13) are cytoplasmic, two of them (14 and 15) are membrane-anchored trough their N-terminal part and the other two (16 and 17) are membrane-anchored through their C-terminal part. Thus, this family of proteins is quite heterogeneous, and further investigations on the different members of this family would be interesting.

It is striking to observe that in the family of proteins with 6 amino acids deletion in the *Ω*-loop, two proteins from Nostoc spPCC 7120, Alr 4543 and All4822 share 47 % identity (over 261 amino acids). While Alr4543 protein is predicted to be membrane anchored by its N-terminal part (as PBP-A), the presence of a signal peptide in All4822 means its exportation to the periplasm as a free protein. Hence, it may also be interesting to investigate the role of these proteins and the relationships that they may share.

Oligonucletides were from Eurogentec:

PBP-F-up 5’CGGCCATGGAAAAATATACAGAATG3’

PBP-F-rp 5’GGACTAGTCCTTCAAGACTTTCCTCCATT3’

PBP-N_1-up 5’CGGTCATGAAGTCACCTTTTCTCTTACTC3’

PBP-N_2-up 5’CGGCCATGGTAAGTAATAATTTAGAATTT3’

PBP-N_3-up 5’TCGCCATGGCTCGTTTAGAATCATGG3’

PBP-N-rp 5’GCCCTAGGCCAAAGTTTCTCGCTGATGTTG3’

Cloning, protein production and purification were performed as above. Changing in the purification protocol for PBP-N_3 was as follows: bacteria were lysed as seen for PBP-A, but the pellet was resuspended in 9 volumes of lysis buffer supplemented with 0,5% triton X-100 and EDTA 10 mM pH 8. It was incubated 5 min at room temperature, centrifuged 15 min, 21000x g, 4°C. Pellet was resuspended in 10 mL PBS. This suspension containing inclusion bodies was mixed to 40 mL GuHCl 6 M, Tris 100 mM pH 8 and 10 mM imidazole (Buffer

A). It was centrifuged 15 min, 21000 x g, 4°C and supernatant was filtered through 0,45 µM. Purification was then processed, Hitrap column was washed with 5 volumes of PBS pH 7,4 and equilibrated with 5 volumes of Buffer A. Sample was loaded on the column. Column was washed with 5 volumes of Buffer A and 5 volumes of renaturing buffer 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 10 mM imidazole (Buffer B). Proteins were let to renature for 15 min and were then eluted with 5 volumes of Buffer B containing 250 mM imidazole.

Annex 3 : possible fragmentation scheme for reaction of penicillin G with PBP-A

Reaction of benzylpenicillin (1) with DD-peptidases leads to formation of a covalent intermediate (2) and release of a complex mix of products among which the normal hydrolysis product, penicilloic acid (4), but also phenylacetyl-glycine (7), and N-formyl-D-penicillamine (8). Figure 34 proposes a mechanism explaining the apparition of these two last products. Deacylation may occur by two mechanisms: direct hydrolysis, or an intramolecular attack generating an oxazolone (3), which would subsequently fragment into D-5,5-dimethyl-Δ2-thiazoline-4-carboxylic acid (6) and oxazolinone (5) (blue arrows). Late hydrolysis of these fragments may release products (7) and (8) (34,38).

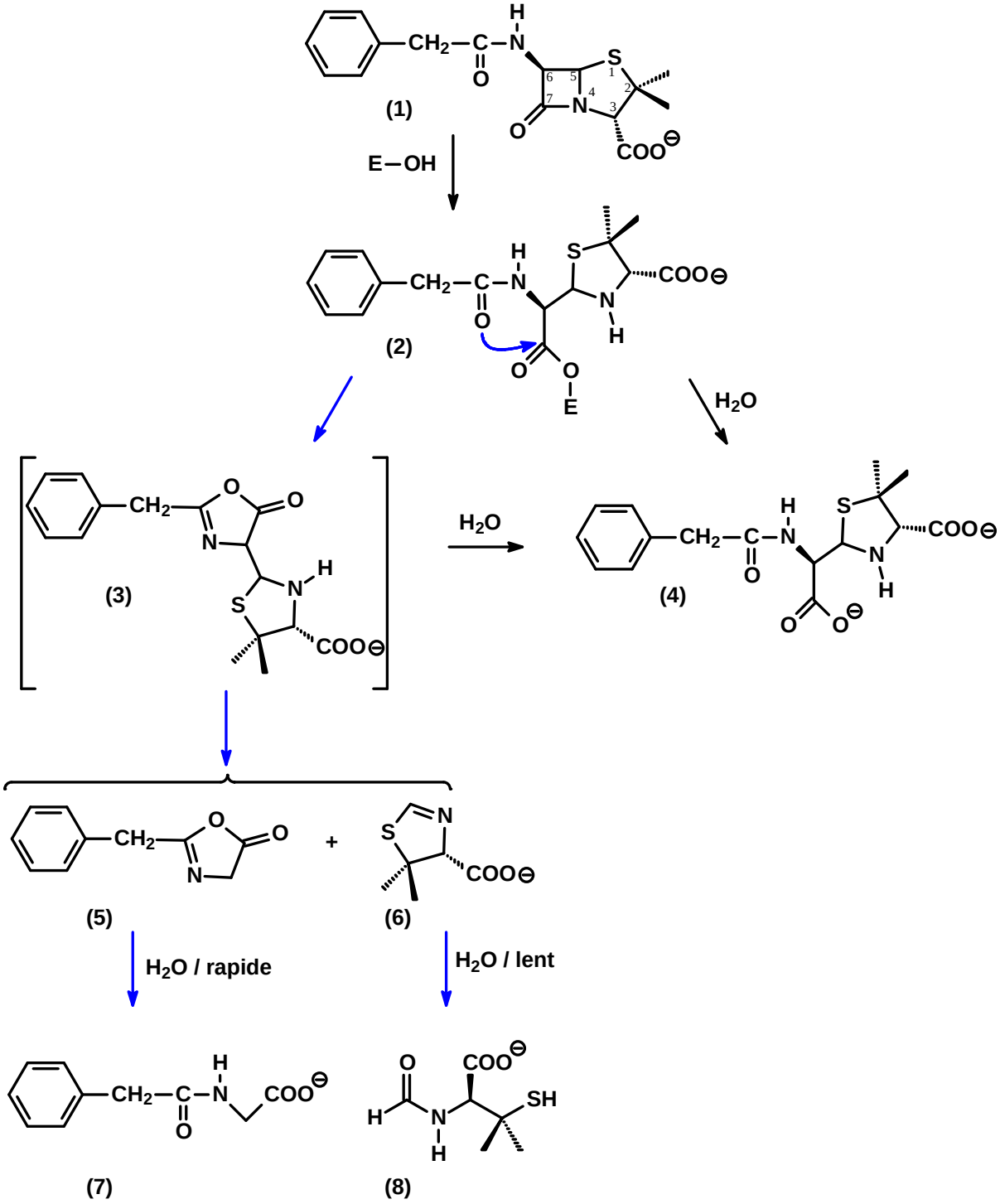


Figure 34 : fragmentation of benzylpenicillin catalysed by DD-peptidases.

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