A short tandem duplication in the Omega-loop of class A beta-lactamase : a key event in the emergence of catalytic activity ?

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Introduction

In nature, proteins evolve through substitutions, insertions and deletions. Most directed evolution studies are based on substitutional mutagenesis, although a substitution may not be the more potent mutational event for creating a new property, especially in the case of the emergence of a new catalytic mechanism. This is because insertional and deletional mutagenesis are practically much more challenging. Hence, the evolutionary potential of proteins through insertions and deletions is not well documented. In this project, we will evaluate the functional impact of an ancestral insertion that may have been a key event in the evolutionary birth of class A beta-lactamases.

Background

It is widely accepted that beta-lactamases evolved from D-alanyl-D-alanyl peptidases, also called penicillin-binding proteins (PBPs), by acquiring a new hydrolytic machinery in their active site. In cyanobacteria, we have discovered a familly of PBPs presenting all the sequence features of class A beta-lactamases but having a six amino-acid deletion in the conserved Omega-loop and lacking the essential Glu166 in this loop. In our laboratory, we are trying to evolve such a PBP into a beta-lactamase by substitutional mutagenesis but only modest activity improvements have been obtained so far. We suspect that an insertional event in the omega-loop may be required for generating a fully active beta-lactamase. Bioinformatic analysis also reveals the trace of an ancestral tandem duplication of 7 residues in the Omega-loop of class A beta-lactamases.

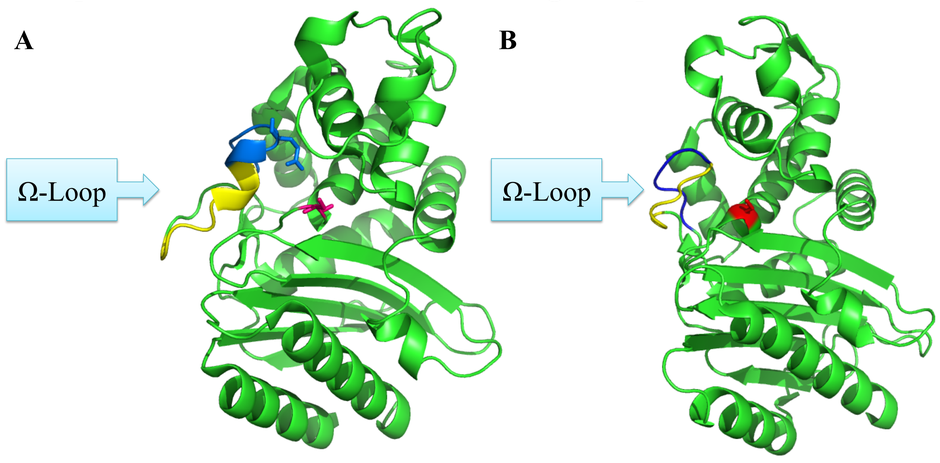


Figure 1 : A) 3D structural representation of TEM-1 beta-lactamase from *E. coli* (PDB : 1ZG4). The position correponding to the hypothetical duplication is indicated in blue (encoding Glu166) and yellow. The main active site residue (Ser70) is represented in red. B) 3D structural representation of PBP-A from Thermosynechococcus elongatus (PDB : 12J8Y). The shorter omega loop is indicated also in blue (lacking the essential Glu166) and yellow. The main active site residue (Ser70) is represented in red.

TEM-1_Escherichia_coli	G	D	H	V	T	R	L	D	R	W	E	P	E	L	N	E	A	I	P	N	D	E	R	D	T	T	M	P
SHV-1_Klebsiella_pneumoniae	G	D	N	V	T	R	L	D	R	W	E	T	E	L	N	E	A	L	P	G	D	A	R	D	T	T	T	P
Class_A_beta-lactamase_Escheriachia_hermannii	G	D	H	V	T	R	L	D	R	T	E	P	T	L	N	E	A	T	P	G	D	A	R	D	T	S	S	P
Beta-lactamase_Streptomyces_griseus	G	D	E	V	T	R	M	V	R	R	E	T	E	L	N	E	W	T	P	G	A	T	R	D	T	S	T	P
Beta-lactamase_Streptomyces_clavuligerus	G	D	S	V	S	R	M	D	Q	Y	E	P	E	L	N	H	D	P	P	H	D	P	R	D	T	T	T	P
beta-lactamase_Streptomyces_sp_C	G	D	P	V	T	R	L	D	R	W	E	P	E	L	N	S	A	E	P	E	R	V	T	D	V	T	T	P
Beta-lactamase_Streptosporangium_roseum	K	D	P	V	S	R	L	D	R	W	E	T	E	L	N	N	W	S	P	K	E	K	R	D	T	T	T	P
CTX-M-1_Escherichia_coli	G	D	E	T	F	R	L	D	R	T	E	P	T	L	N	T	A	I	P	G	D	P	R	D	T	T	S	P
Beta-lactamase_Citrobacter_koseri	G	D	T	T	F	R	L	D	R	K	E	P	E	L	N	T	A	I	P	G	D	E	R	D	T	T	C	P

Figure 2 : Several Omega-loop sequences show the traces of an ancestral tandem duplication. The presence of a rare tryptophan either in the blue part, the yellow part or in the both colors, is a strong indicator for the duplication event.

Strategy

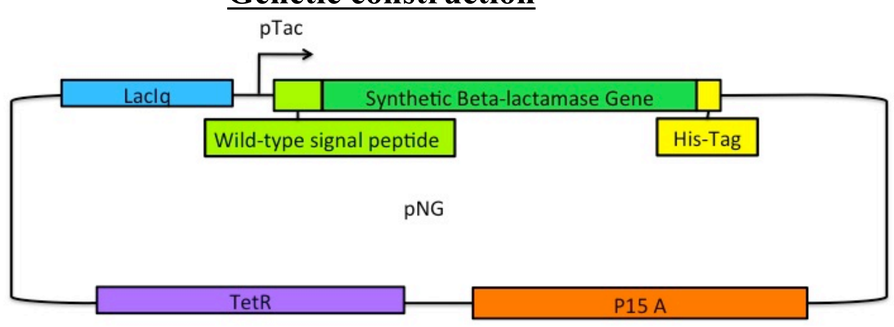
I. Recreate a perfect tandem duplication (P.T.D.) into the Omega-loop of a class A beta-lactamase : We will study the catalytic properties of this mutant.
II. Delete one of the two hepta-peptide from the P.T.D enzyme : We will study the catalytic properties of this probable ancestral mutant.
III. Directed evolution of both engineered proteins : we will create mutants libraries and select mutants for antibiotic hydrolysis.

The rational reason for the choice of amino acid substitution creating a perfect tandem duplication LNRWE^{166}PELNRWEPE are :

- L 162/169 : L is the most frequent residue found in multiple protein alignment
- N 163/170 : N 170 plays an important role in the beta-lactamase catalysis
- R 164/171 : R is the most frequent residue in the position 164 and sometimes observed at the position 171
- W 165/172 : W is a rare amino acid which is often observed in position 165 and/or, in a few cases, in position 172
- E 166/173 : E166 is a very important residue involved in the catalysis (hydrolysis step); E is sometimes observed in 173
- P 167/174 : P is the most frequent residue found in multiple protein alignment at both positions; a cis-peptide bond is found between E166 and P167.
- E 168/175 : E is the most frequent residue found in multiple protein alignment at the position 168 and is sometimes observed in 175

Results

Genetic construction



Protein characterization

Strain and gene	Omega-loop sequence	MIC on ampicillin	Penicillin-G hydrolysis in periplasmic extract	Detection by SDS-PAGE gel	Detection by Western Blotting
Top 10 + TEM-1 Wild Type (W.T.)	HVTRLDRWEPELNEAIPNDERDT	> 5000 µg/ml	3,2*10^{-2} U/µl	Yes	Periplasmic and insoluble fraction
Top10 + TEM-1 tandem duplication (P.T.D.)	HVTRLNRWEPELNRWEPEDERD	450-500 µg/ml	6,5*10^{-5} U/µl	No	insoluble fraction
Top10 + TEM-1 Deletant (Δ7)	HVTRLNRWEPE - - - - - - - DERD	2-4 µg/ml	Not detected	No	insoluble fraction
Top10	x	2-4 µg/ml	Not detected	No	No

Mutant Libraries and selection for activity

TEM-1 P.T.D

Found	*Ampicilline concentration*	*Non synonymous mutations*	*Silent mutations*
1x	700[a] µg/ml	N163D	0
1x	700[a] µg/ml	N163D	4
1x	700[a] µg/ml	N163D – Q205K	4
4x	700[b] µg/ml	K111R – R120K - N163D	1
1x	800[b] µg/ml	L30M - D38E	2
2x	800[b] µg/ml	Q88R -Q206L -N276D	4
1x	800[b] µg/ml	I47V	1
1x	800[b] µg/ml	D115V - R120S - N276D	6
1x	700[b] µg/ml	F60Y	3
4x	700[b] µg/ml	F60Y	4

TEM-1 Δ7

Found	*Ampicilline concentration*	*Non synonymous mutations*	*Silent mutations*
3x	20[b] µg/ml	I47V – Q90V - E168V	1
4x	20[b] µg/ml	I47V – E89D – T149N - E168V	0
15x	20[b] µg/ml	I47V – E89D – T149N - E168V	1
1x	5[b] µg/ml	M182T	1

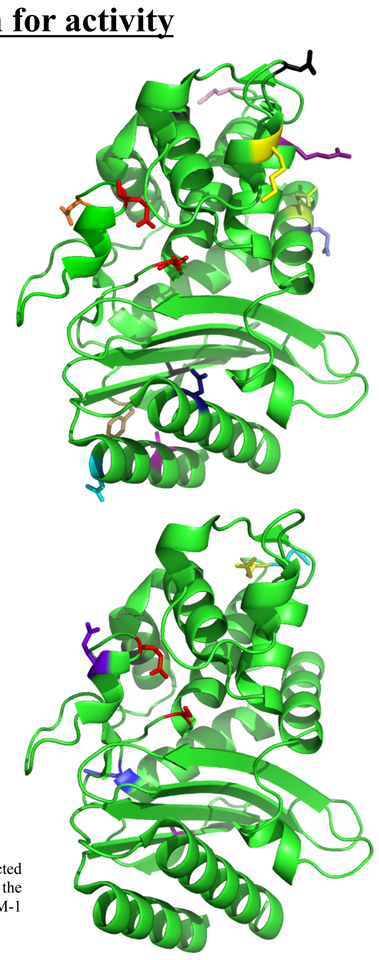


Figure 3 : This experiement was performed with induction of IPTG (a) or without induction of IPTG (b). Selected mutations are represented in the 3D structural representation beside the table. The M182T mutation found in the deletant enzyme is known to act at the level of folding but does not affect the thermodynamic stability of TEM-1 (Sideraki *et al.*, 2001)

Conclusions and perspectives

A perfect tandem duplication introduced in the omega-loop of TEM-1 results in a substantial decrease of the beta-lactamase activity but it is still sufficient for conferring resistance to ampicillin. The mutant with a deletion of one of the two hepta-peptide is not active anymore. Selections on TEM-1 P.T.D. mutants libraries lead to back mutation (N163D) or mutations which could reshape the active site. Selections on TEM-1 Δ7 libraries allow the emergence of ampicillin resistant clones. In this case, some mutations are direcly link with the active site (in or at the hinge of the Omega-loop). We will caracterize theses mutants and will try to evolve so far as possible mutants with the shorter Omega-loop.

References

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Acknowledgements

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