

## Implementation, achievements and opportunities

### WP6

#### A mutagenesis study aiming to establish C<sub>α</sub> hydroxylation activity at the active site of a pseudo enzyme

Ba3943 is a pseudo polysaccharide deacetylase (like YlxY and Ba0150) which displays no enzymatic and no hydroxylation activity. Basic properties of Ba3943:

- Hypothetical protein according to pubmed.com
- Belongs to the CE4 family and has a NodB domain according to Pfam
- Exhibits a 57% identity with YlxY from *B.subtilis*, they main conserved motifs of PDAs are only partially conserved in Ba3943.

BA3943	113	VG	LTIN	V	AWG	166	VG	NHSY	T	HPN	203	VR	WFAP	P	S	GS	231	WT	VDTI	D	WK	259	IV	L	M	H	P	T	S																
YlxY	132	VA	FLIN	V	AWG	185	IG	NHSY	N	HPD	222	PK	WFAP	P	S	GS	250	WT	VDTI	D	WQ	278	MI	L	M	H	P	T	D																
BA0150	59	VA	FTFD	I	SWG	113	IG	SMGY	N	YTS	150	IK	LLRP	P	S	GD	178	WS	NNSD	W	DK	206	IV	L	L	H	A	S	D																
BC0361	200	IF	V	TFDD	G	GMK	261	VQ	SHTA	T	HAD	296	VI	A	I	A	P	F	G	H	324	TK	PG	Q	F	I	T	338	LL	K	M	K	R	V	R										
BA0424	70	IY	L	TFD	N	CYE	123	IG	NHS	W	SHPD	161	VK	Y	V	R	P	R	G	V	189	WS	L	A	F	L	D	W	K	220	IL	L	L	H	A	I	S								
BsPdaA	68	IY	L	TFD	N	CYE	121	IG	NHS	F	HHPD	159	NL	Y	L	R	P	P	R	G	V	187	MS	V	A	F	L	D	W	K	218	IY	L	L	H	T	V	S							
BC1960	83	VA	L	TFD	D	CPD	136	IG	N	R	T	Y	S	H	P	N	173	PK	F	I	R	P	P	Y	G	E	201	WS	V	D	T	V	D	W	K	229	VI	L	Q	H	S	T	P		
BC1974	71	AY	L	TFD	D	C	P	G	123	VG	M	R	S	M	T	H	N	F	160	PK	L	R	P	P	Y	G	E	191	WT	I	D	S	L	D	W	R	226	VI	L	M	H	D	I	H	
SpPgdA	270	VA	L	TFD	D	C	P	N	323	VG	N	H	S	W	S	H	P	I	360	SK	L	M	R	P	P	Y	G	E	385	WD	V	D	S	L	D	W	K	413	IV	L	M	H	D	I	H
ErPgd	285	IY	L	TFD	D	C	P	G	336	VA	I	H	S	A	S	H	K	Y	374	AS	I	R	F	P	G	E	413	WN	V	S	S	G	D	A	N	445	VV	L	Q	H	D	I	K		
SmPgdA	109	VF	L	TFD	D	G	V	D	163	LG	I	H	S	F	S	H	V	Y	207	TG	V	W	R	Y	R	G	G	H	239	WN	A	A	V	G	D	A	E	277	VV	L	M	H	D	I	S

Sequence alignment of the NodB domain (all 5 conserved motifs) of BA3943 with YlxY, BA0150 (4M1B), BC0361 (4HD5), BA0424 (2J13), BsPdaA (1W17), BC1960 (4L1G), BC1974 (5N1J), SpPgdA (2C1G), ErPgd (5JMU) and SmPgdA (2W3Z). The PDB id codes are in parentheses. Inactive PDAs are depicted in black, BC0361 (unknown substrate, PG binding) in orange, MurNAc PDAs in red and GlcNAc PDAs in blue.

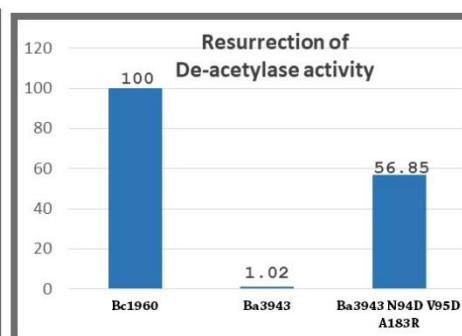
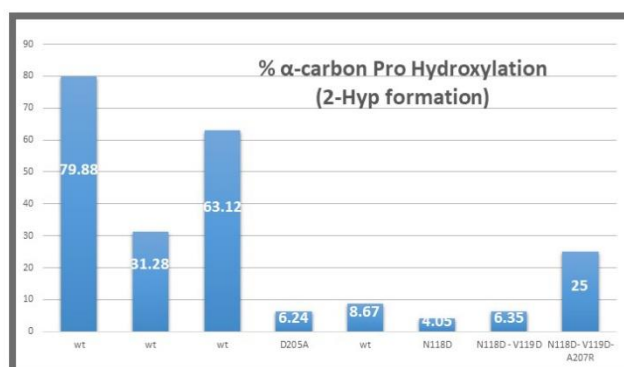
- BA3943 and BA0150 structurally resemble to MurNAc deacetylases
- Similarities: BA3943 retains the catalytic histidine (MT5), the metal-binding histidines (MT2) and the conserved proline (MT3)
- Differences: BA3943 does not retain the catalytic aspartic acid which is replaced by asparagine (MT1), the metal-binding aspartic acid which is replaced by valine (MT1) and the, very significant for both deacetylation and hydroxylation activities, arginine which replaced by alanine (MT3)
- Inactive against glycol-chitin, chito oligomers and PG isolated from *E.coli*

On the basis of sequence-structure-activity relationships established in the project, a number of mutants were engineered and tested. The aim was to restore the essential motifs associated with enzymatic and hydroxylation activity. The following mutants of Ba3943 were engineered as steps towards restoration of sequence motifs and establishment of hydroxylation/ catalytic activity: Ba3943 N94D, Ba3943 N94D V95D, Ba3943 N94D V95D A183R. Several other mutants were also produced to examine specific structural or hydroxylation aspects of the Ba3943 pseudoenzyme.

ba3943 and the mutant genes, were expressed in *E.coli* and the recombinant protein was purified to near homogeneity. Heavy aggregation was a common property of

purified proteins. Hydroxylation activity was examined through the detection of 2-Hyp by MS or by X-ray crystallography. Enzymatic activity was examined through incubation with commonly used deacetylase substrates. BA3943, Ba3943 N94D and Ba3943 N94D V95D were enzymatically inactive against radio labeled glycol chitin, chito-oligomers and peptidoglycan isolated from *E.coli*, when tested in a wide range of pH and in the presence or absence of the divalent cations  $\text{Co}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Ni}^{+2}$  and  $\text{Mn}^{+2}$ . A 2-Hyp residue could not be detected either. On the other hand, the triple mutant Ba3943 N94D V95D A183R exhibits a significant enzymatic and hydroxylation activity.

Ba3943 (wt protein) inactive
Ba3943 N94D inactive
Ba3943 N94D V95D inactive
Ba3943 N94D V95D A183R active

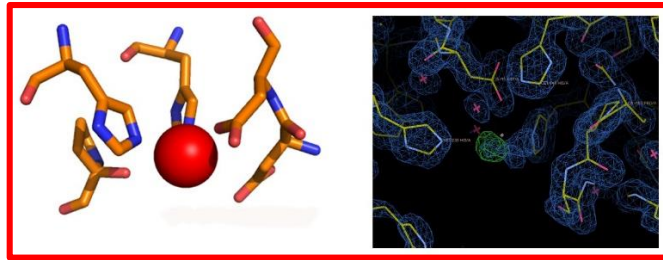


BC1960 wt	77	LTFDDG	128	IGNHTYSHP	165	PKLIRPXYG
BC1974 wt	73	LTFDDG	123	VGMHSMTHN	160	PKLIRPPYG
BA0330 wt	202	VTFDDG	261	MQSHTATHA	296	VIAVAYXFG
BA0330 D205A	202	VTFADG	261	MQSHTATHA	296	VIAVAYXFG
BA3943 wt	91	LTINVA	141	VGNHSYTHP	178	VRWFAPPSG
BA3943 N94D	91	LTIDVA	141	VGNHSYTHP	178	VRWFAPPSG
BA3943 N94D V199D	91	LTIDDA	141	VGNHSYTHP	178	VRWFAPPSG
BA3943 N94D V199D A183R	91	LTIDDA	141	VGNHSYTHP	178	VRWFAPPSG

Motif 1
Motif 2
Motif 3

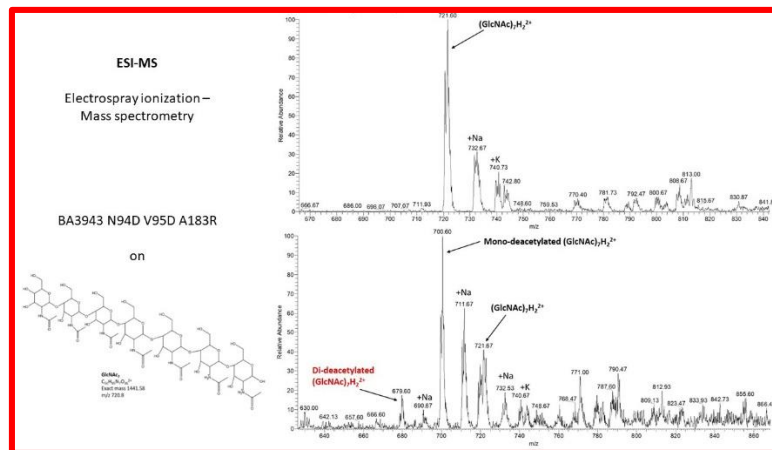
The catalytic activity of Ba3943 N94D V95D A183R in the presence of metals exhibits a maximum value for  $\text{Co}^{+2}$ , a common property of PDAs, despite the fact that the natural catalytic metal for these enzymes is  $\text{Zn}^{+2}$ .

With the exception of Ba3943 N94D V95D A183R, no metal could be detected from electron density maps in Ba3943 and its mutants. However, even for the activated mutant Ba3943 N94D V95D A183R, the  $\text{Zn}^{+2}$  occupation of the metal binding site is low.

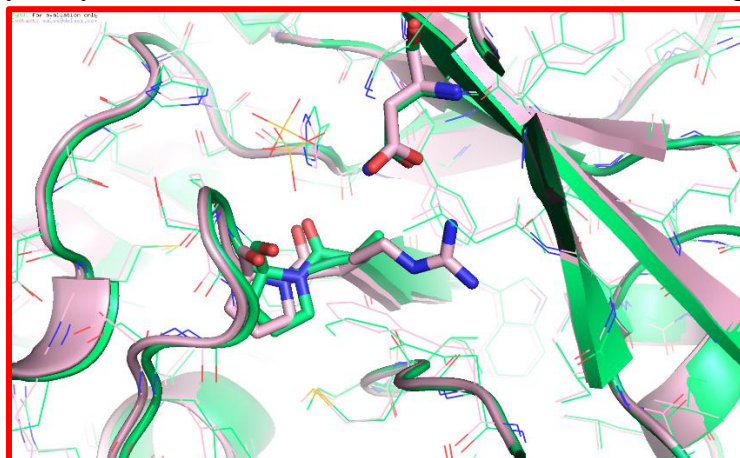


Right: Electron density maps of Ba3943 N94D V95D A183R calculated in the region corresponding to the catalytic/ hydroxylation site of PDAs (blue:  $2F_o-F_c$ , green:  $F_o-F_c$  maps, contoured at  $2\sigma$  and  $4\sigma$  respectively). Left: The probable  $Zn^{+2}$  (red) binding site of Ba3943 N94D V95D A183R, modelled according to the electron density.

Catalytic activity of the Ba3943 N94D V95D A183R mutant was detected using electrospray ionization-mass spectrometry:

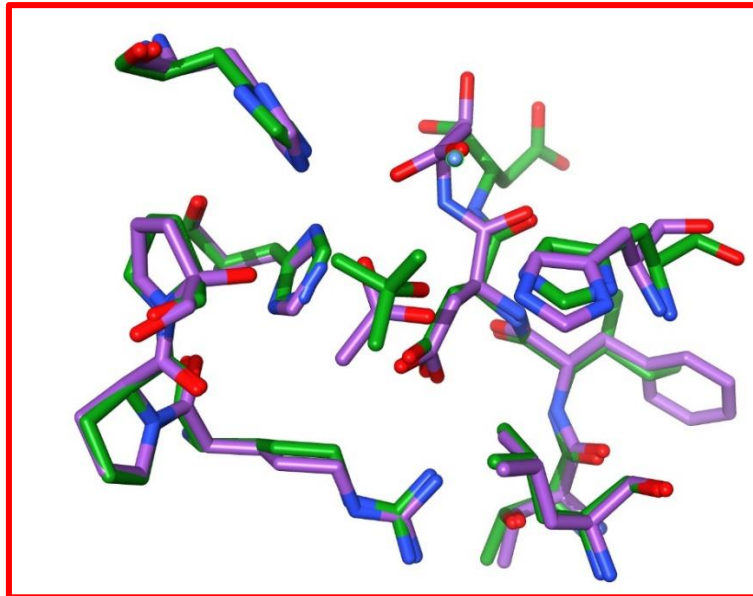


All structural differences between Ba3943 and Ba3943 N94D V95D A183R, cluster in the active/hydroxylation site of the mutant, as shown in the following figure:



Superposition of the active/ hydroxylation site of Ba3943 N94D V95D A183R with that of Ba3943 (green)

The crystal structure of the “resurrected” Ba3943 N94D V95D A183R, reveals a striking structural similarity in the potential hydroxylation/ catalytic site with corresponding site of fully active PDAs, e.g. Bc1960:



*Superposition of the active/ hydroxylation site of Bc1960 with the engineered site of Ba3943 N94D V95D A183R.*