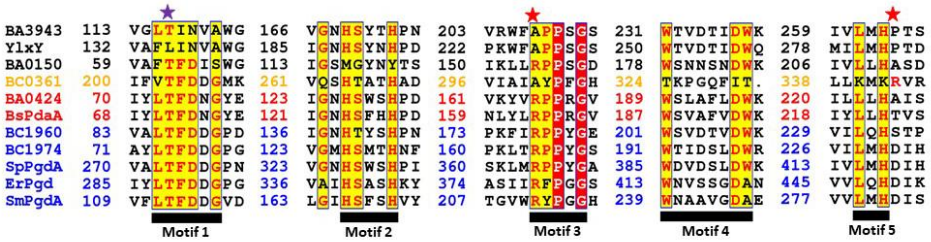
Implementation, achievements and opportunities

WP6

A mutagenesis study aiming to establish C_α hydroxylation activity at the active site of a pseudo enzyme

*Ba*3943 is a pseudo polysaccharide deacetylase (like YlxY and Ba0150) which displays no enzymatic and no hydroxylation activity. Basic properties of B*a*3943:

- 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 B*a*3943.



*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.*

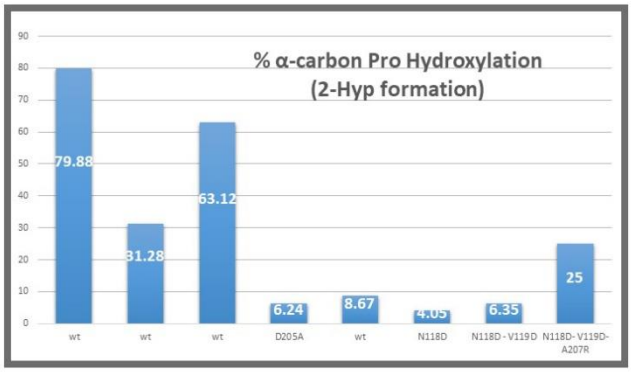
- BA3943 and BA0150 structurally resemble to MurNAc deacetylases
- Similarities: BA3943 retains the catalytic histidine (MT5), the metal-binding histidines (MT2) and the conserved proline (MT3)
- Differences: BA3943 does not retain the catalytic aspartic acid which is replaced by asparagine (MT1), the 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, chitooligomers and PG isolated from *E.coli*

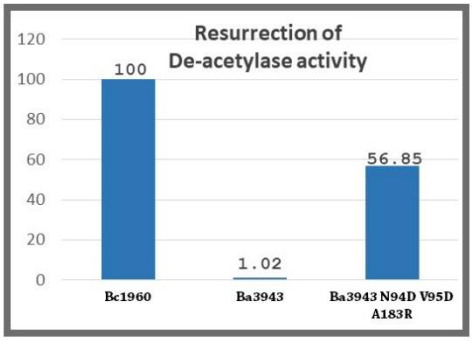
On the basis of sequence-structure-activity relationships established in the project, a number of mutants were engineered and tested. The aim was to restore the essential motifs associated with enzymatic and hydroxylation activity. The following mutants of B*a*3943 were engineerd as steps towards restoration of sequence motifs and establishment of hydroxylation/ catalytic activity: B*a*3943 N94D, B*a*3943 N94D V95D, B*a*3943 N94D V95D A183R. Several other mutants were also produced to examine specific structural or hydroxylation aspects of the B*a*3943 pseudoenzyme.

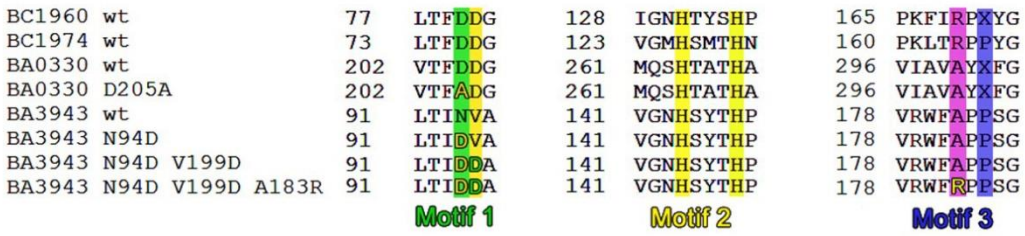
ba3943 and the mutant genes, were expressed in E.*coli* and the recombinant protein was purified to near homogeneity. Heavy aggregation was a common property of

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, B*a*3943 N94D and B*a*3943 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 Co^{+2}, Zn^{+2}, Ni^{+2} and Mn^{+2} . A 2-Hyp residue could not be detected either. On the other hand, the triple mutant B*a*3943 N94D V95D A183R exhibits a significant enzymatic and hydroxylation activity.

B*a*3943 (wt protein) inactive
B*a*3943 N94D inactive
B*a*3943 N94D V95D inactive
B*a*3943 N94D V95D A183R active

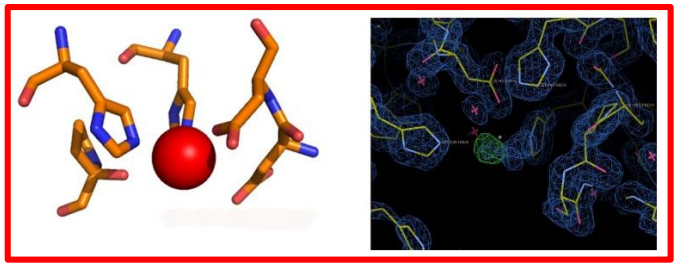






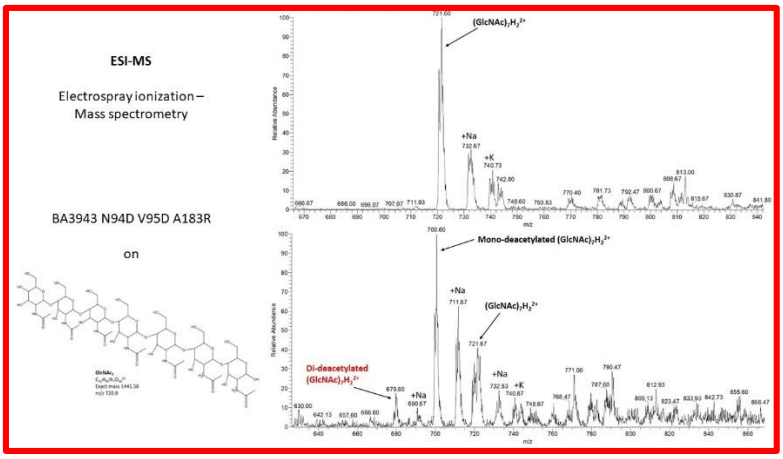
The catalytic activity of B*a*3943 N94D V95D A183R in the presence of metals exhibits a maximum value for Co^{+2}, a common property of PDAs, despite the fact that the natural catalytic metal for these enzymes is Zn^{+2}.

With the exception of B*a*3943 N94D V95D A183R, no metal could be detected from electron density maps in B*a*3943 and its mutants. However, even for the activated mutant B*a*3943 N94D V95D A183R, the Zn^{+2} occupation of the metal binding site is low.

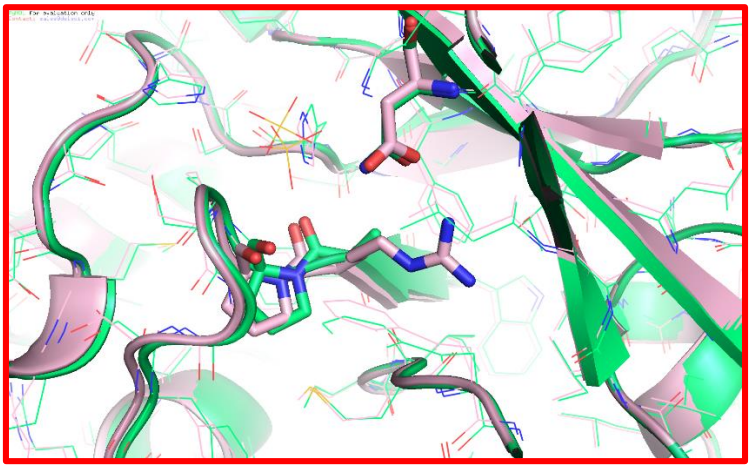


Right: Electron density maps of Ba3943 N94D V95D A183R calculated in the region corresponding to the catalytic/ hydroxylation site of PDAs (blue: $2F_o$-F_c, green: F_o-F_c maps, contoured at 2σ and 4σ respectively). Left: The probable Zn^{+2} (red) binding site of Ba3943 N94D V95D A183R, modelled according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

Catalytic activity of the B*a*3943 N94D V95D A183R mutant was detected using electrospray ionization-mass spectrometry:

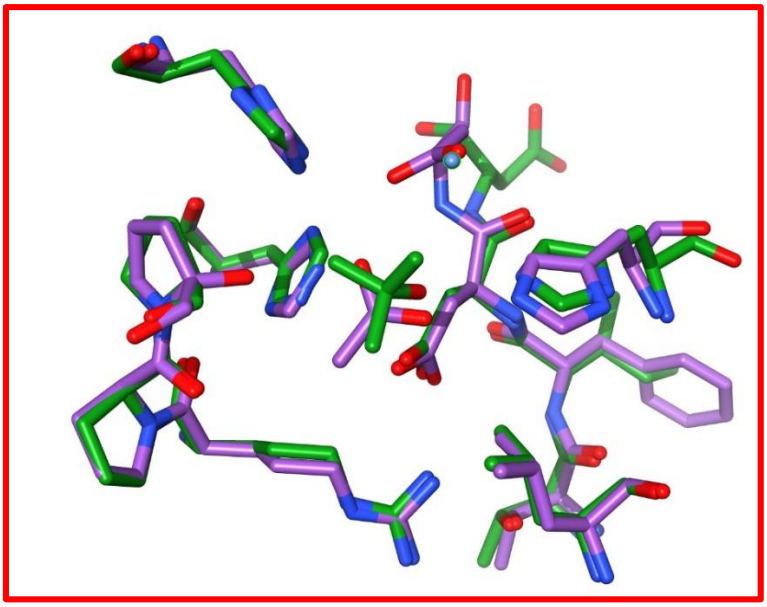


All structural differences between B*a*3943 and B*a*3943 N94D V95D A183R, cluster in the active/hydroxylation site of the mutant, as shown in the following figure:



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

The crystal structure of the “resurrected” B*a*3943 N94D V95D A183R, reveals a striking structural similarity in the potential hydroxylation/ catalytic site with corresponding site of fully active PDAs, e.g. B*c*1960:



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