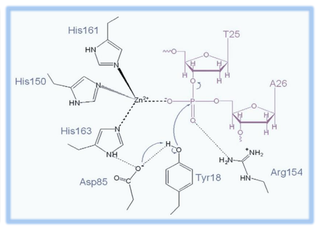
Identification of residues indirectly involved in cation coordination by HUH endonucleases

Lorena González-Montes and Gabriel Moncalián
Molecular Biology Department and
Institute of Biomedicine and Biotechnology of Cantabria, University of Cantabria, Spain

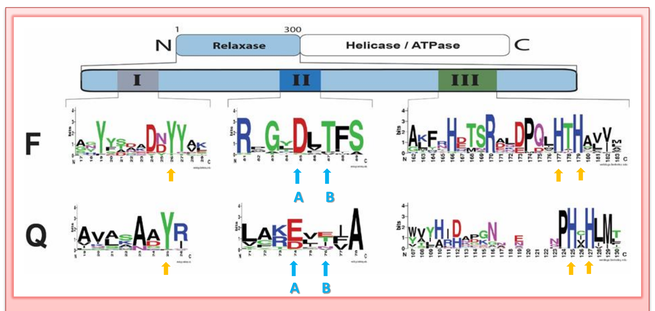
Background

HUH endonucleases rely on a divalent metal ion to perform different site-specific DNA processing reactions in biological processes like plasmid replication, transposition or bacterial conjugation. They all have an HUH motif (U=hydrophobic residue) and a Y motif (one or two catalytic Tyr). A catalytic Tyr creates a covalent 5'-phosphotyrosine intermediate and a free 3'OH at the cleavage site. This 3'-OH primes replication or acts as nucleophile for strand transfer. The metal is coordinated by the two HUH His and a third polar residue (Glu, Asp, His or Gln) being Mg^{+2} and Mn^{+2} the physiological cofactors. TrwC is one of the most studied HUH endonucleases at a biochemical and structural level. Its function is to transfer a single-stranded DNA (ssDNA) plasmid copy from one cell to another at conjugation process by nicking at the *nic* site of the plasmid origin of transfer *(oriT),* guiding the copy to the recipient cell and catalyzing there the recircularization of the transferred ssDNA plasmid.

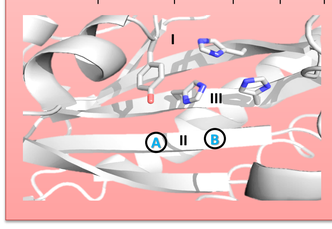


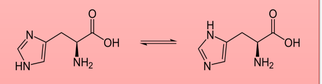
Hypothesis

All HUH endonucleases superfamily members have conserved HUH and Y catalytic domains (orange arrows). However, they have different metal specificities. We propose that metal affinity depends on the different character of the residues surrounding the amino acids directly involved in metal coordination at the active site, which are conserved within each relaxase family (blue arrows). Polar residues are found in the relaxases binding Mg^{+2} or Mn^{+2} such as TrwC and MobA. Hydrophobic residues are present in relaxases only binding Mn^{+2} such as NES. These secondary residues could modify cation specificity by affecting histidine tautomerization. In this work we have changed the protein metal specificity by mutating one of these polar amino acids (T87I in TrwC) by a non polar residue.



Relaxase	Plasmid	Family	(A)	(B)	Metals used	PDB	Reference
MobA	R1162	Q	E74	E76	Mg+2/Mn+2	2NS6	(Monzingo *et al.*, 2007)
NES	pLW1043	Q	E86	I88	Mn+2	4HT4	(Edwards *et al.*, 2013)
TrwC	R388	F	D87	T87	Mg+2/Mn+2	1OMH	(Boer *et al.*, 2006)

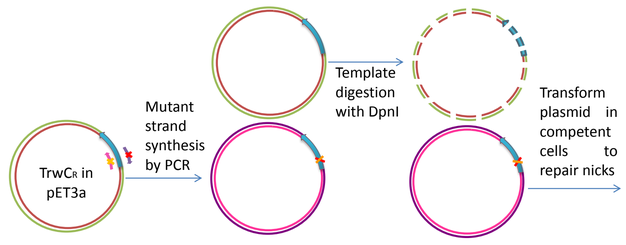




Histidine tautomerization

Methodology

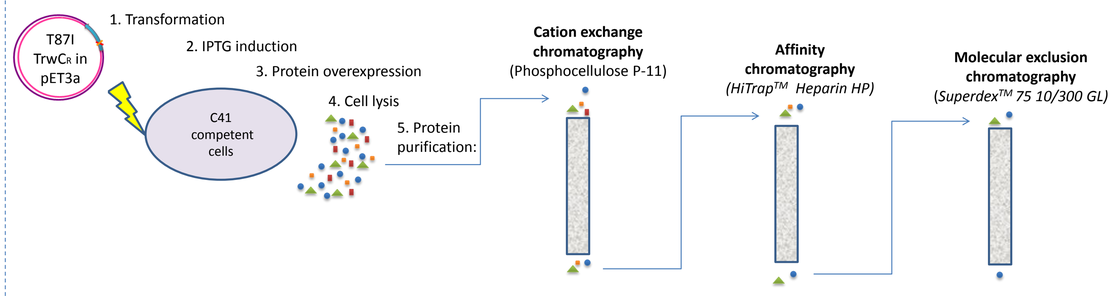
Site-directed mutagenesis



Thr codon changed to Ile at position 87 of $TrwC_R$ coding sequence

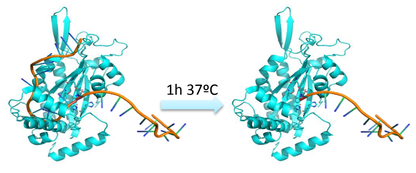
3'-ctcgcgtagccggaactggagtAtaagtcgcgcgggttctcgcat-5'
…cagcaaagagcgcatcggccttgacctcacattcagcgcgcccaagagcgtatcgcttc…
…gtcgtttctcgcgtagccggaactggagtgtaagtcgcgcgggttctcgcatagcgaag…
5'-gagcgcatcggccttgacctcaTattcagcgcgcccaagagcgta-3'

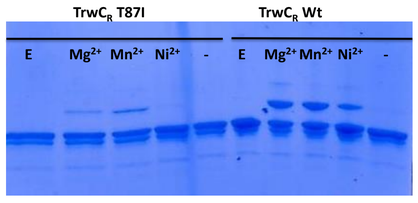
Protein overexpression and purification



Results and Discussion

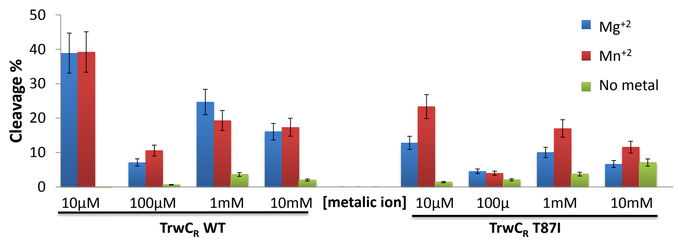
Mg^{+2} does not allow TrwC T87I cleavage of *nic*-containing oligonucleotides





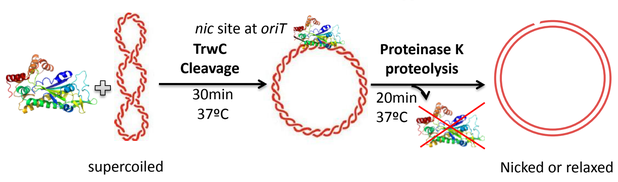
Different 12+18 oligonucleotide cleavage yield by $TrwC_R$ WT *vs.* $TrwC_R$ T87I with different metal cofactors at 10µM, EDTA (E) or without adding any metal (-).

Reaction
12+18 oligonucleotide (TGCGTATTGTCT/ATAGCCCAGATTTAAGGA) 15 µM + $TrwC_R$ WT or $TrwC_R$ T87I at 6 µM in Tris-HCl pH 7.5 10mM and EDTA (5mM) or metals at 0 (-), 10µM, 100µM, 1mM or 10mM. Complex formation was observed by differential migration at SDS-PAGE 12% gel ($TrwC_R$-DNA complex : upper band).

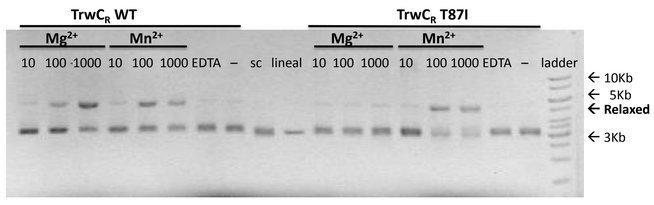


Effect of different cations in the oligonucleotide cleavage by $TrwC_R$ WT and $TrwC_R$ T87I at different cofactor concentrations. Mean and standard deviation of three independent experiments.

Mg^{+2} does not allow $TrwC_R$ T87I relaxation of *nic*-containing sc plasmids



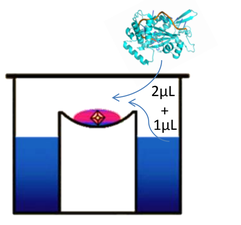
Reaction
$TrwC_R$ 500nM and pSU1186 supercoiled plasmid 10nM in Tris-HCl pH 7.5 10 mM and metals at 0 (-), 10 µM, 100 µM, 1000 µM or EDTA 5 mM.



Effect of different metal ions in the cleavage of a supercoiled (sc) plasmid containing the *oriT* (lower band) by $TrwC_R$ WT or $TrwC_R$ T87I mutant at different cofactor concentrations (expressed in µM).

While $TrwC_R$ WT is active both with Mg^{+2} and with Mn^{+2} as cofactors, TrwC T87I works *in vitro* significantly better in the presence of Mn^{+2}.

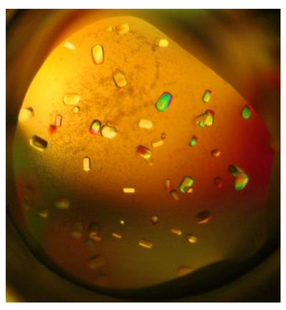
$TrwC_R$ T87I structure shows a slight change in the H163 orientation



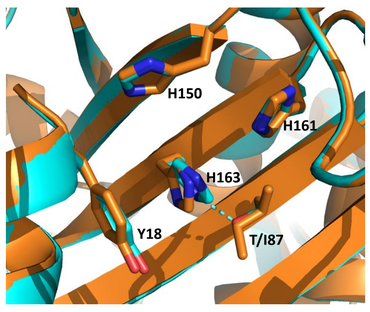
$TrwC_R$ WT or $TrwC_R$ T87I at a 1 : 1.5 protein : 23+0 oligonucleotide molar ratio were incubated with precipitant solution until rising equilibrium at 22ºC.

Crystals were soaked in 100mM Mn^{+2} solution and frozen for X ray diffraction and data collection at synchrotron.

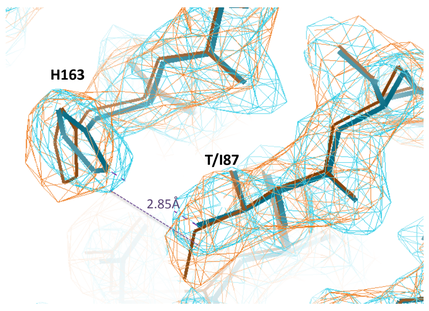
It is proposed that T87 by hydrogen bond formation plays an important role in the orientation of one of the histidines (His163) that coordinate the divalent cation.



$TrwC_R$ T87I – 23+0 crystals. Precipitant: Phosphate Ammonium 2M, TrisHCl pH=8.5 0.05M and Mn^{+2} 5mM.
Space group $P6_5$; a=b=149Å; c=78Å.

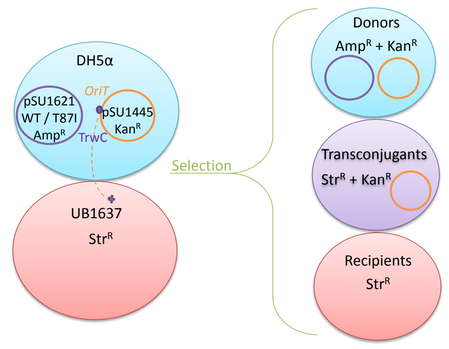


Superposed models of $TrwC_R$ active site showing the mutation and its consequence on His163 orientation. $TrwC_R$ WT is shown in blue and $TrwC_R$ T87I in orange. Hydrogen bond shown in dashed line.

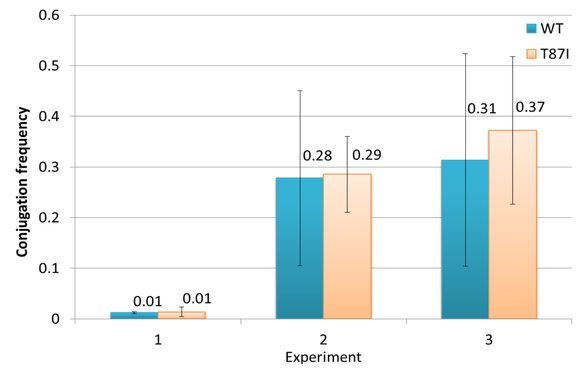


Electronic density map of residues H163 and T/I87 of $TrwC_R$ WT (blue) and T87I mutant (orange). Resolution: 2.2 Å and 1.6 Å, respectively. Hydrogen bond distance between His163 and Thr87 in WT protein is shown in purple.

T87I full length TrwC is able to efficiently transfer DNA *in vivo.*



Donor and receptor cells at stationary state were mixed at 1:1 proportion and plated on a LB plate for 1hour at 37ºC.



The experiment was done by triplicate three independent days. Conjugation frequency=transconjugants/donor colony. Cells containing pSU1445 complemented with pET3a showed a conjugation frequency lower than 10^{-6}.

$TrwC_R$ threonine 87 is not essential *in vivo*. TrwC T87I is able to drive plasmid DNA transfer at the same efficiency than TrwC WT. Thus, TrwC T87I is active using the Mn^{+2} at the low concentration present in the culture media, and TrwC could have evolved to be active using any of the available metals (mainly Mg^{+2} or Mn^{+2}).

Conclusions and Future Research

We have proved that a charged amino acid not directly interacting with the metal cofactor is involved in the orientation of the catalytic histidines in TrwC. This residue is therefore indirectly involved in the metal coordination and specificity. Thus, we think that by mutating the equivalent hydrophobic amino acid for a polar residue on an HUH endonuclease only able to bind Mn^{+2}, the protein could recover the ability to use Mg^{+2} too. But this hypothesis has still to be proven.
These findings open a new path to protein engineering for cofactor specificity modification and *de novo* protein design for different purposes.

References

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Acknowledgments

We thank Sandra Sagredo and Fernando de la Cruz for sharing their knowledge and advice. We also thank the European Commission for supporting EvoTAR project to FdC and the *Ministerio de Cultura y Deporte* for the collaboration grant given to LG-M.