Supporting Information

Constrained evolution of a bispecific enzyme: lessons for biocatalyst design

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Table 1 Impact of individual mutations on the thermostability and kinetic parameters of TrzN with atrazine and ametryn when compared to the *Nocardioides* sp. strain AN3 background.

Mutation	Background			Fold-change[a]		
				Atrazine hydrolysis	Ametryn hydrolysis[b]	Absolute change in T_m (°C)
Tyr67	-	-	-	-2	N/A[c]	4.7
	Pro214	-	-		†	
	-	Tyr215		-1	N/A	4.4
	-	-	Glu241		†	
	Pro214	Tyr215	-	-1	N/A	2.1
	Pro214	-	Glu241		†	
	-	Tyr215	Glu241	-3	0	5.6
	Pro214	Tyr215	Glu241	-5	0	6.5
Pro214	-	-	-	-2	N/A	-1.6
	Tyr67	-	-		†	
	-	Tyr215	-	0	N/A	1.7
	-	-	Glu241		†	
	Tyr67	Tyr215	-	0	N/A	0.2
	Tyr67	-	Glu241		†	
	-	Tyr215	Glu241	1	2	-1.2
	Tyr67	Tyr215	Glu241	0	2	-0.3
Tyr215	-	-	-	-2	N/A	-2.3
	Tyr67	-	-	0	N/A	-2.6
	-	Pro214	-	0	N/A	2.6
	-	-	Glu241	-6	2	-1.5
	Tyr67	Pro214	-		†	
	Tyr67	-	Glu241		†	
	-	Pro214	Glu241		†	
	Tyr67	Pro214	Glu241		†	
Glu241	-	-	-	7	38000	-7.1
	Tyr67	-	-		†	
	-	Pro214	-		†	
	-	-	Tyr215	3	57000	-6.3
	Tyr67	Pro214	-		†	
	Tyr67	-	Tyr215	1	53000	-5.1
	-	Pro214	Tyr215	5	89000	-10.0
	Tyr67	Pro214	Tyr215	1	91000	-5.6

†One of the proteins does not express

[a]Fold-change when compared to the *Nocardioides* sp. strain AN3 background. Grey indicates negative change, yellow neutral, and green positive.

[b]Fold-change in activity when compared to the assay detection limit

[c]Not applicable

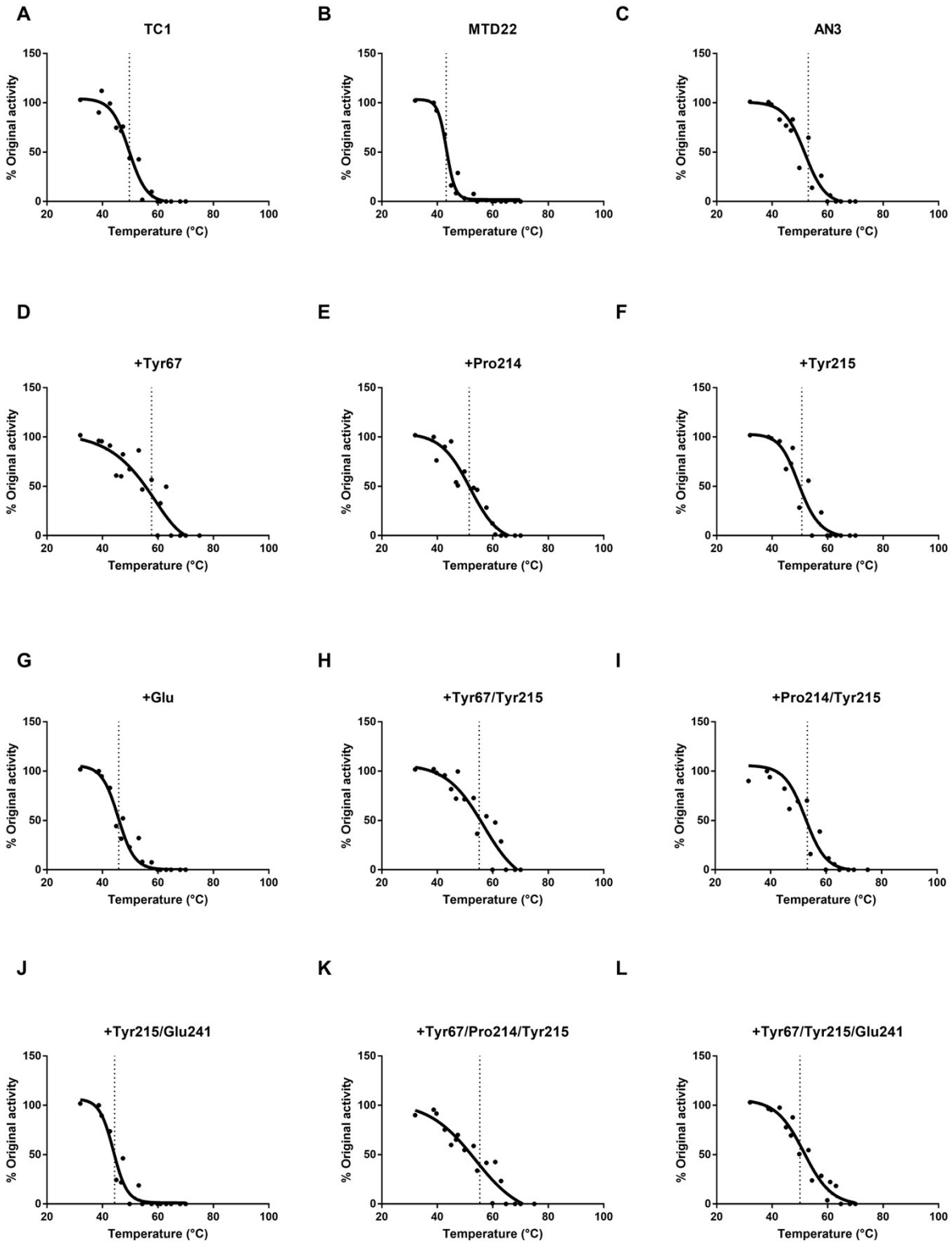

Figure 1 Representative replicates of thermostability assay curves used to calculate the T_m of variants. *Arthrobacter aurescens* strain TC1 TrzN, *Nocardioides* sp. strains MTD22 TrzN, and AN3 TrzN are shown from A-C, the remainder of soluble variants from D-L. The T_m (°C) calculated from three independent replicates is indicated with a dotted line.

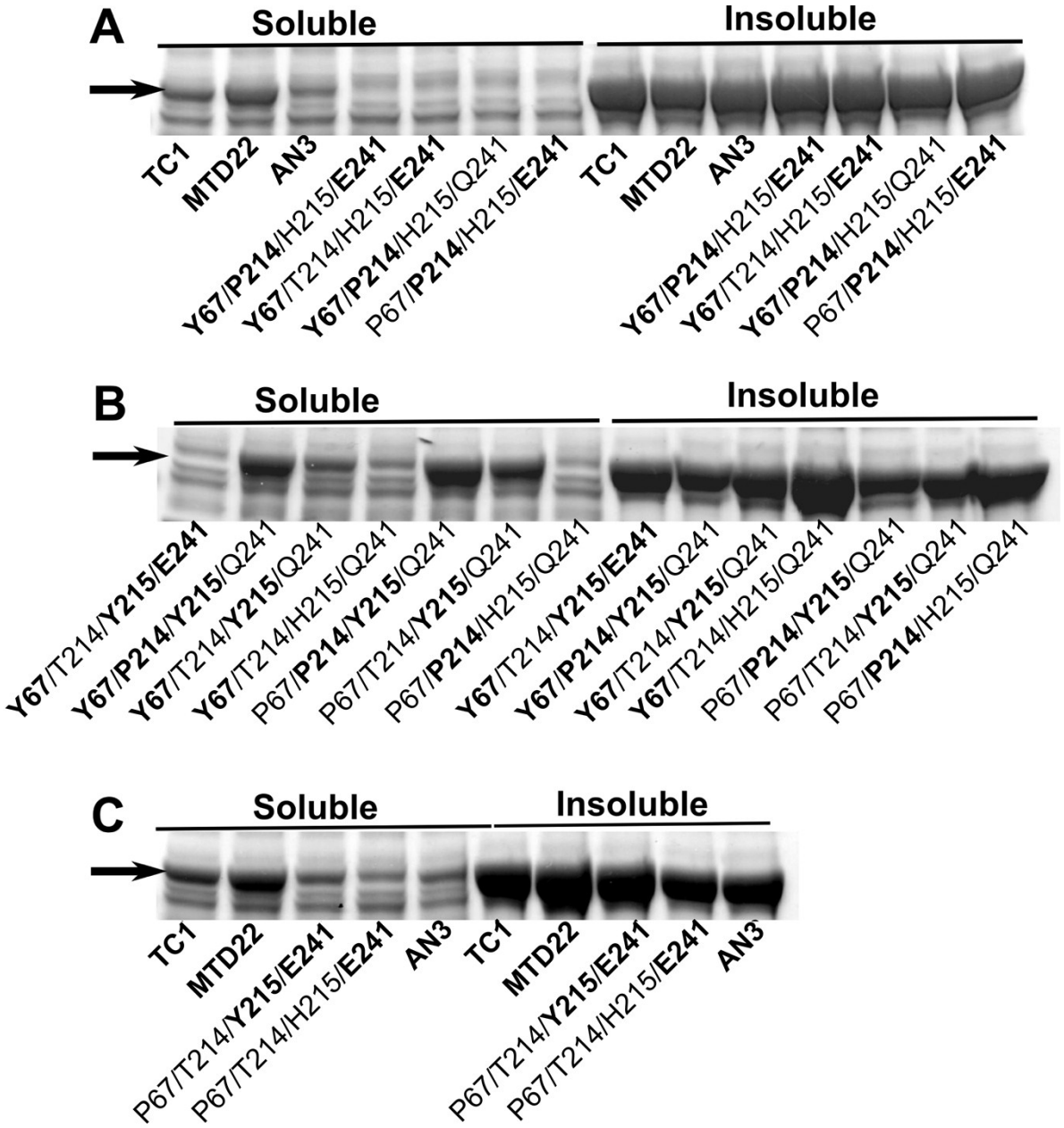

Figure 2 (A) Four variants were unable to be solubly expressed compared to *Arthrobacter aurescens* strain TC1 TrzN, *Nocardioides* sp. strains MTD22 TrzN, and AN3 TrzN, which exhibit soluble expression. **Tyr67**/**Pro214**/His215/**Glu241 TrzN, Tyr67**/Thr214/His215/**Glu241, Tyr67**/**Pro214**/His215/Gln241, and Phe67/**Pro214**/His215/**Glu241** show no soluble expression but substantial insoluble expression. (**B**) Soluble expression is much higher in the presence of Gln241. (**C**) Addition of Glu241 is observed to decrease expression.

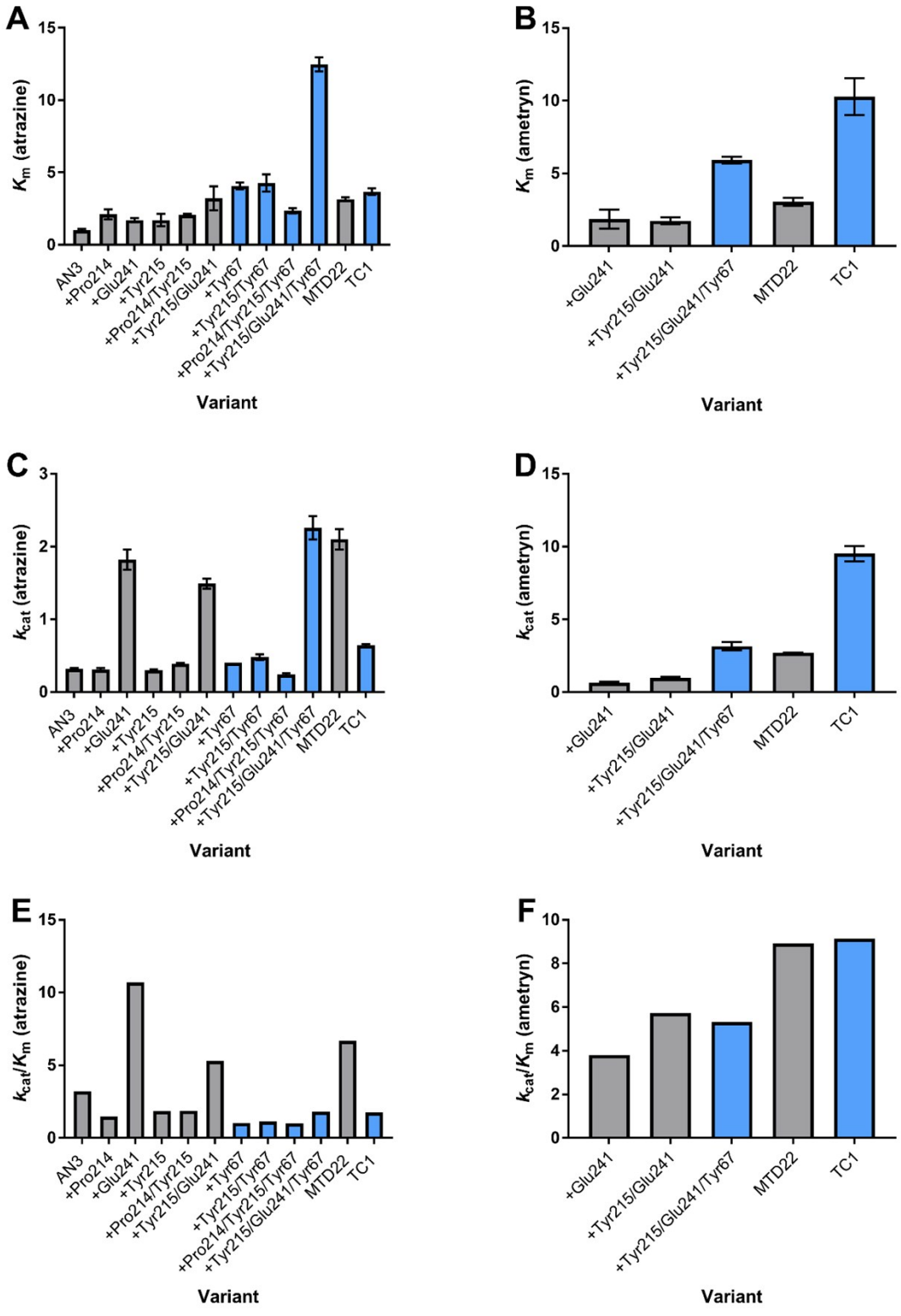

Figure 3 Modification of Phe67 to Tyr67 is observed to change the kinetic parameters of all TrzN variants. Modification of Phe67 to Tyr67 (blue bars) increases the K_m with both atrazine (**A**) and ametryn (**B**) independent of pre-existing mutations. Addition of Tyr67 is also observed to increase the k_{cat} with atrazine (**C**) and ametryn (**D**) in some genetic backgrounds. Overall, the k_{cat}/K_m with atrazine is observed to be reduced in Tyr67 containing variants (**E**) whereas the k_{cat}/K_m with ametryn is observed to be generally unchanged (**F**)

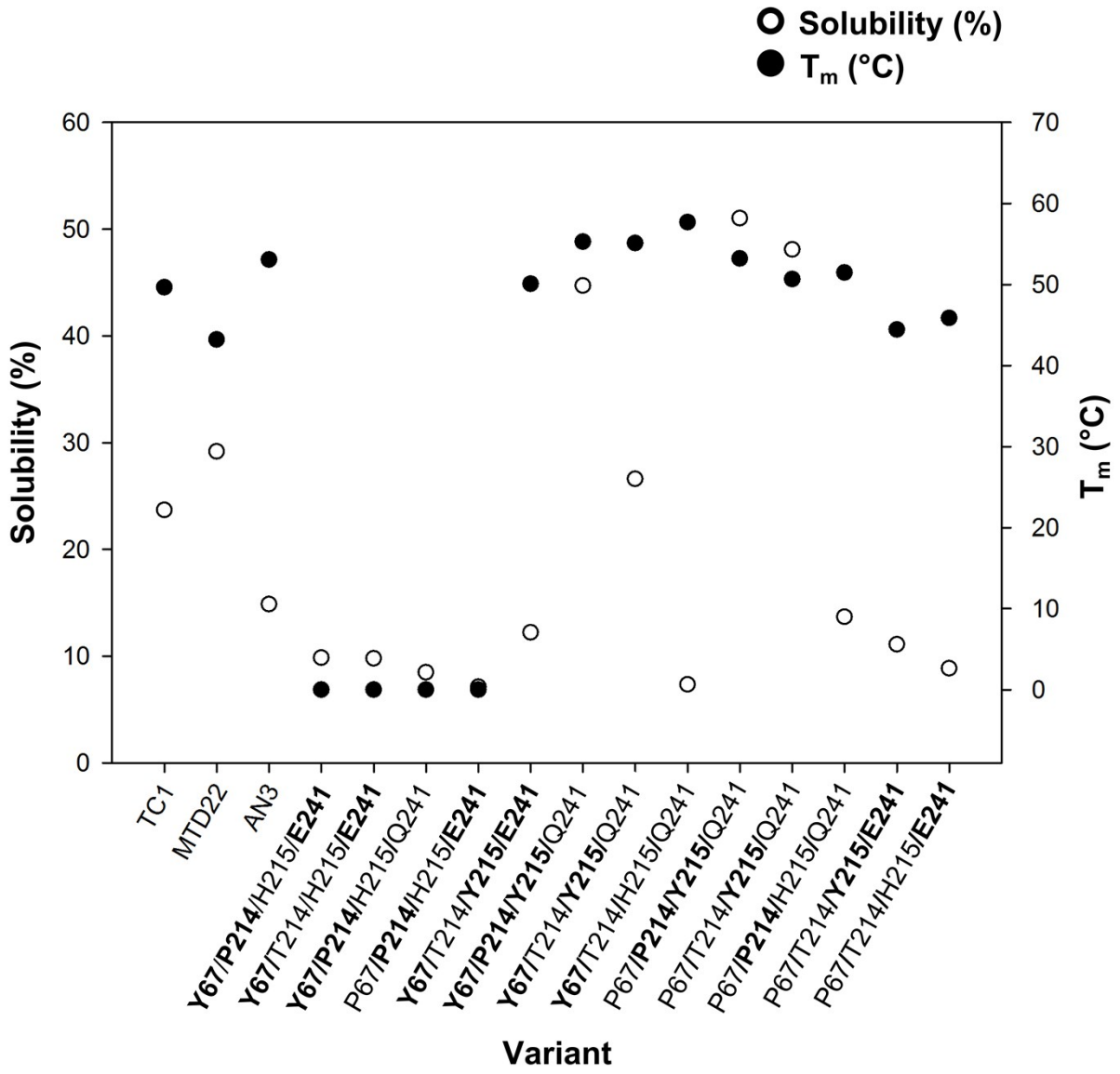

Figure 4 The soluble expression (○) and thermostability (●) of different TrzN variants.