

# Systematic Analysis of Pyrazinamide-Resistant Spontaneous Mutants and Clinical Isolates of *Mycobacterium tuberculosis*.

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**Supplementary Table 1:** Selection of spontaneous mutants (CDC1551) on 7H11 plates, pH6.0 and 100 or 500 µg/ml PZA from 39 (40-1 excluded) independent culture flasks.

culture number	PZA 500 µg/ml			PZA 100 µg/ml			difference in # colonies (100-500µg) 10 <sup>7</sup> ***	difference in # colonies (100-500µg) 10 <sup>5</sup> **
	Number of bacteria plated			Number of bacteria plated				
	10 <sup>7</sup>	10 <sup>5</sup>	10 <sup>3</sup>	10 <sup>7</sup>	10 <sup>5</sup>	10 <sup>3</sup>		
1	110	0	0	123	0	0	13	0
2	87	0	0	200	2	0	113	2
3	84	1	0	105	4	0	21	3
4	191	0	0	200	3	0	9	3
5	75	0	0	200	0	0	125	0
6	37	0	0	105	10	0	68	10
7	78	1	0	96	1	0	18	0
8	83	0	0	82	3	0	-1	3
9	184	1	0	71	1	0	-113	0
10	94	1	0	133	0	0	39	-1
11	130	2	0	167	4	0	37	2
12	113	1	0	200	1	0	87	0
13	101	0	0	108	0	0	7	0
14	101	0	0	123	0	0	22	0
15	117	0	0	105	0	0	-12	0
16	119	1	0	158	0	0	39	-1
17	95	0	0	106	0	0	11	0
18	34	2	0	84	0	0	50	-2
19	106	0	0	94	1	0	-12	1
20	149	1	0	191	3	0	42	2
21	106	0	0	200	1	0	94	1
22	92	2	0	181	3	0	89	1
23	184	4	0	200	8	0	16	4
24	100	0	0	102	2	0	2	2
25	134	1	0	139	2	0	5	1
26	95	0	0	123	2	0	28	2
27	87	0	0	141	1	0	54	1
28	61	0	0	98	1	0	37	1
29	98	0	0	180	1	0	82	1
30	94	2	0	220	3	0	126	1
31	107	0	0	115	0	0	8	0
33	115	1	0	133	0	0	18	-1
34	118	0	0	200	2	0	82	2
35	113	0	0	135	1	1	22	1
36	107	0	0	150	0	0	43	0
37	99	0	0	150	1	0	51	1
38	109	1	0	152	5	0	43	4
39	64	1	0	163	0	0	99	-1
40	84	0	0	96	1	0	12	1
Total	4055	23	0	5529	67	1		
Average	<b>103,97</b>	<b>0,59</b>	<b>0,00</b>	<b>141,77</b>	<b>1,72</b>	<b>0,03</b>	<b>38</b>	<b>1</b>
Median	101	0	0	135	1	0	37	1

\*\*\* p=6.08E-06 (Statistically significant difference, paired Student's T Test)

\*\* p=0.00114 (Statistically significant difference, paired Student's T Test)

**CONCLUSION**

~100 mutants /  $10^7$  bacteria = ~1 mutant /  $10^5$  bacteria

- mutation frequency of either  $1.5/10^5$  or  $1/10^5$  when selected at 100 or 500  $\mu\text{g/ml}$  respectively.

**Count of colonies on the petri dishes with 7H11+OADC supplement + PZA 100 or 500  $\mu\text{g/ml}$  (pH6.0)**

Inoculation : 100  $\mu\text{l}$  on 7H11 + 100 or 500  $\mu\text{g/ml}$  PZA , pH 6.0

(14Jan2010)

colony count : after 4 weeks

(11Feb2010)

**Supplementary Table 2:** Structural analysis of *Mycobacterium tuberculosis* PncA protein in relation to identified mutations in clinical isolates and PZA<sup>R</sup> spontaneous mutant strains.

A positive or negative  $\Delta\Delta\text{G}$  indicates whether the mutation is respectively thermodynamically stabilizing or destabilizing while the magnitude of  $\Delta\Delta\text{G}$  indicates the extent of the alteration.

Selection <sup>a</sup>	Nucleotide mutation	Amino acid mutation	sol vacc <sup>b</sup>	$\Delta\Delta\text{G}$ (CS) <sup>c</sup>	$\Delta\Delta\text{G}$ (PM) <sup>d</sup>	Comments
500	T-7C	NA	NA	NA	NA	Nucleotide change in promoter region
500	G-6C	NA	NA	NA	NA	Nucleotide change in promoter region
500	del A-4	NA	NA	NA	NA	Nucleotide deletion in promoter region
500 (2)	T2C	Met1Thr	42.5%	+0.31	-0.31	N-terminal translation initiator Met1
100 (3) 500 (10)	G3C*	Met1Ile	42.5%	-0.17	+0.01	N-terminal translation initiator Met1
100	del G7*	Ala3 fs*	0.0%	NA	NA	Frameshift
100	C8A	Ala3Glu	0.0%	-0.74	-2.86	buried residue; destabilizing mutation
500	del T17*	Ile6 fs*	0.5%	NA	NA	Frameshift
500/CI	G22A	Asp8Asn	3.5%	+0.97	-0.01	Asp8 is a catalytic residue
500/CI	G22T	Asp8Tyr	3.5%	+0.95	-0.28	Asp8 is a catalytic residue
CI	A23C*	Asp8Ala*	3.5%	+1.73	-1.62	Asp8 is a catalytic residue
500	del G22 +del C24	Asp8 fs	3.5%	NA	NA	Frameshift
500 (4)	C24A	Asp8Glu	3.5%	+1.51	1.57	Asp8 is a catalytic residue
100	T26G	Val9Gly	0.6%	-1.35	-2.64	buried residue; Gly substitution destabilizing
CI	A29C	Gln10Pro	0.0%	-2.13	-1.75	buried residue; Pro substitution destabilizing
CI	C28T	Gln10Stop	0.0%	NA	NA	early termination codon
100	G34A	Asp12Asn	16.0%	-2.68	-0.56	buried residue; Asn substitution destabilizing
CI	C39G*	Phe13Leu*	12.5%	-0.84	-1.41	buried residue; Leu substitution destabilizing; Phe13 is part of PZA binding site (Unissa <i>et al</i> Bioinformatics 2009, 4(1):24-29)
CI (1 PZA <sup>R</sup> , 3 PZA <sup>S</sup> )	T40G	Cys14Gly	1.4%	+0.17	-2.06	buried residue; Gly substitution destabilizing
500	C42A	Cys14Stop	1.4%	NA	NA	early termination codon
500	ins G48*	Gly16 fs	100.0%	NA	NA	Frameshift
500	ins A49*	Gly17 fs	21.6%	NA	NA	Frameshift
500	G70C*	Gly24Arg*	0.0%	-5.21	-0.98	buried residue; destabilizing mutation
CI	G71A	Gly24Asp	0.0%	-0.34	-1.40	buried residue; destabilizing mutation
500	T80C	Leu27Pro	6.0%	-6.60	-2.96	buried residue; destabilizing mutation
500	ins 86AA*	Arg29 fs	62.4%	NA	NA	Frameshift
CI	T92G	Ile31Ser	0.0%	-3.63	-3.07	buried residue; destabilizing mutation
100/500	C102A	Tyr34Stop	13.6%	NA	NA	early termination codon
100	ins C104	Leu35 fs	19.1%	NA	NA	Frameshift
CI	T104C	Leu35Pro	19.1%	-0.57	-1.96	Partially buried residue; destabilizing mutation
100	C123G	Tyr41Stop	16.4%	NA	NA	early termination codon

CI	A128C	His43Pro	22.0%	-1.96	-2.91	partially buried residue; mutation destabilizing
500	T131A*	Val44Asp*	8.5%	-2.60	-2.71	buried residue; destabilizing mutation
100/CI	C137T	Ala46Val	0.0%	+2.28	-0.13	buried residue; mutation rather stabilizing
500	G145A	Asp49Asn	4.9%	-3.29	-0.33	Fe-chelating residue
500/CI	A146C	Asp49Ala	4.9%	+1.41	-0.50	Fe-chelating residue
100	C151T	His51Tyr	0.5%	+1.56	-0.33	Fe-chelating residue
CI	A152G	His51Arg	0.5%	-1.57	-1.07	Fe-chelating residue
CI	C161A	Pro54Gln	5.6%	-2.12	-0.64	buried residue; destabilizing mutation
500	del C161	Pro54 fs	5.6%	NA	NA	Frameshift
100/CI	del G164*	Gly55 fs	48.3%	NA	NA	Frameshift
100 (2)	C169A*	His57Asn*	7.1%	-1.89	-1.49	Fe-chelating residue
500	A170G	His57Arg	7.1%	-0.71	-1.04	Fe-chelating residue
500	ins A172*	Phe58 fs	21.9%	NA	NA	Frameshift
100 (2) 500(1)/CI	T175C	Ser59Pro	32.4%	-2.60	-1.57	partially buried residue; destabilizing
500	del T175*	Ser59 fs*	32.4%	NA	NA	Frameshift
CI	A188C	Asp63Ala	59.7%	-0.59	-0.57	solvent accessible residue; destabilizing
CI	A188G	Asp63Gly	59.7%	-0.45	-1.16	solvent accessible residue; destabilizing
CI (PZA <sup>S</sup> )	A191C*	Tyr64Ser*	62.2%	+1.81	-0.74	solvent accessible residue; rather stabilizing
500 (3)	ins A193	Ser65 fs	81.1%	NA	NA	Frameshift
500	T199C	Ser67Pro	12.8%	-0.9	-3.18	partially buried residue; destabilizing mutation
500	T202C	Trp68Arg	18.3%	-1.84	-1.68	partially buried residue; destabilizing; Trp68 is part of PZA binding site (Unissa <i>et al</i> Bioinformatics 2009, 4(1):24-29) (Petrella <i>et al</i> PlosOne 2011:6(1):e15785)
500	del G204*	Trp68 fs*	18.3%	NA	NA	Frameshift
500/CI	C211T	His71Tyr	2.7%	-2.94	-0.64	Fe-chelating residue
CI	C211A	His71Asn	2.7%	+0.96	-1.87	Fe-chelating residue
CI	G215A	Cys72Tyr	0.0%	-9.28	-0.84	buried residue; destabilizing mutation
CI	A226C	Thr76Pro	39.6%	-1.01	-2.28	solvent accessible residue; destabilizing mutation
CI	C244G	His82Asp	17.0%	+0.34	-0.80	partially buried residue; rather neutral
CI	A245G	His82Arg	17.0%	+0.04	-0.95	partially buried residue; rather neutral
CI	ins CA 246*	His82 fs	17.0%	NA	NA	Frameshift
CI	T254G	Leu85Arg	4.9%	-6.06	-1.40	buried residue; destabilizing mutation
CI	C260T	Thr87Met	60.6%	+0.45	-0.28	solvent accessible residue; neutral
500	del G306*	Ala102 fs	10.0%	NA	NA	Frameshift
500	T307C	Tyr103His	66.4%	-0.74	+0.08	solvent accessible residue; neutral
500	C309A	Tyr103Stop	66.4%	NA	NA	early termination codon
CI	C309G	Tyr103Stop	66.4%	NA	NA	early termination codon
500/100	C312A*	Ser104Arg	0.0%	-0.95	-1.95	buried residue; destabilizing; found to significantly reduce activity (Lemaitre <i>et al</i> Biochem J 2001; 535:453-458)
CI	G319T*	Glu107Stop	50.4%	NA	NA	early termination codon
500	ins T322*	Gly108 fs*	0.0%	NA	NA	Frameshift
100	del C344	Pro115 fs	36.6%	NA	NA	Frameshift
CI	T347C	Leu116Pro	0.0%	+1.77	-2.11	buried residue; neutral mutation
500	del C367*	Arg123 fs*	42.4%	NA	NA	Frameshift
100	ins G372*	Gly124 fs*	45.7%	NA	NA	Frameshift
100	Del 385-401*	Asp129 fs	0.0%	NA	NA	Frameshift
CI	Ins GG391	Val131 fs	0.0%	NA	NA	Frameshift
500 (2)	G395T	Gly132Val	0.0%	-2.54	-0.85	buried residue; destabilizing; active site region
500	C401T	Ala134Val	8.2%	+1.07	-0.40	buried residue; part of catalytic site (Petrella <i>et al</i> PlosOne 2011:6(1):e15785)
500	del C402	Ala134 fs	8.2%	NA	NA	Frameshift
CI (PZA <sup>S</sup> )	A403C	Thr135Pro	2.9%	+1.05	-0.96	buried residue; neutral mutation
500/100	C404A*	Thr135Asn*	2.9%	-1.36	-1.23	buried residue; destabilizing mutation
CI	A407G	Asp136Gly	4.2%	-1.46	-2.07	buried residue; destabilizing mutation
100	G413A	Cys138Tyr	8.5%	-3.18	-0.93	catalytic residue (Zhang <i>et al</i> FEBS J

						2008:275:753-762) (Lemaitre <i>et al</i> Biochem J 2001; 535:453-458) (Petrella <i>et al</i> PlosOne 2011:6(1):e15785)
500	G415T*	Val139Leu	0.0%	+0.59	-0.92	buried residue; located near the active site pocket
500/CI	T416C	Val139Ala	0.0%	-4.93	-2.64	buried residue; located near the active site pocket
CI	C418G	Arg140Gly	26.6%	+0.41	-2.26	buried residue; rather destabilizing mutation
100	C421T	Gln141Stop	24.1%	NA	NA	early termination codon
CI	A422C	Gln141Pro	24.1%	-1.48	-1.93	rather solvent accessible residue; destabilizing
100/CI	C425T	Thr142Met	0.0%	+3.32	+0.42	buried residue; stabilizing; active site pocket
100	C425A	Thr142Lys	0.0%	-6.25	-1.36	buried residue; destabilizing mutation
CI	G427C	Ala143Pro*	0.0%	-4.46	-2.27	buried residue; destabilizing mutation
CI (PZA <sup>S</sup> )	G427A	Ala143Thr	0.0%	-4.45	-1.10	buried residue; destabilizing mutation
CI	G436A	Ala146Thr	0.0%	-3.09	-1.21	buried residue; destabilizing mutation
500	del 456-565*	Ala152 fs*	45.4%	NA	NA	Frameshift
100	G461T*	Arg154Met*	27.1%	-2.67	-0.10	partially buried residue; rather destabilizing
CI	T464G	Val155Gly	0.0%	-3.29	-3.91	buried residue; destabilizing
CI	ins T465	Val155 fs	0.0%	NA	NA	Frameshift
500	ins G466*	Leu156 fs	0.5%	NA	NA	Frameshift
500 (2)	ins T468*	Leu156 fs	0.5%	NA	NA	Frameshift
500	T476C	Leu159Pro	3.3%	-3.68	-2.70	buried residue; destabilizing mutation
500	C512A	Ala171Glu	5.4%	-1.00	-1.04	buried residue; destabilizing mutation
500	del CG512-513*	Ala171 fs	5.4%	NA	NA	Frameshift
500 (2)	T515C	Leu172Pro	27.8%	-0.53	-3.06	partially buried residue; destabilizing
CI	T515G*	Leu172Arg*	27.8%	-2.67	-1.24	partially buried residue; destabilizing
CI (PZA <sup>R</sup> +PZA <sup>S</sup> )	G525A	Met175Ile	0.0%	+0.37	-0.80	buried residue; neutral
CI	A523G	Met175Val	0.0%	+0.33	-1.19	buried residue; rather destabilizing
CI	del C530	Thr177 fs	90.1%	NA	NA	Frameshift
CI	T545C	Leu182Ser	26.8%	+2.82	-1.99	Solvent accessible residue; neutral
100 (14)	WT	WT	NA	NA	NA	MIC PZA >400µg/ml (MGIT960 system)
CI (14 PZA <sup>R</sup> , 71 PZA <sup>S</sup> )	WT	WT	NA	NA	NA	

<sup>a</sup> CI: mutation found on clinical isolate. 100 or 500: Selection of spontaneous mutant on either 100 or 500 µg/ml. If the mutation was found in more than one isolate, the number of isolates is indicated.

<sup>b</sup> relative solvent accessibility of the wild type residue computed from CUPSAT program

<sup>c</sup> predicted protein thermal stability change ( $\Delta\Delta G$  in kcal/mol) of mutation from CUPSAT program

<sup>d</sup> predicted protein stability change ( $\Delta\Delta G$  in kcal/mol) of mutation from PoPMuSiC program

NA= Not Applicable

\* previously not reported