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1 **Introducing RpsA Point Mutation Δ438A or D123A into the Chromosome of *M.***
2 ***tuberculosis* Confirms its Role in Causing Resistance to Pyrazinamide**

3

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15 Running title: *rpsA* Mutation Causing Pyrazinamide Resistance

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23 **ABSTRACT**

24 Pyrazinamide (PZA) is a unique frontiline drug for shortening tuberculosis treatment, but its
25 mechanisms of action are elusive. We previously identified RpsA as a target of PZA and found
26 an alanine deletion at position 438 (Δ438A) in RpsA associated with PZA resistance, but its
27 role in PZA resistance is controversial. Here, we introduced RpsA mutation Δ438A or D123A
28 into *M. tuberculosis* chromosome and demonstrated that the introduced RspA mutations are
29 indeed responsible for PZA resistance.

30

31 **TEXT**

32 PZA is an important first-line drug that plays a unique role in shortening TB therapy from 9-
33 12 months to 6 months due to its unique sterilizing activity in killing persisters that are not
34 killed by other TB drugs (7, 29). Despite its high activity in vivo, PZA is a peculiar drug that
35 has virtually no activity in vitro under normal culture conditions (19, 29), but is active at acid
36 pH (6, 31). Recent studies of new drugs such as bedaquiline, PA-824, and moxifloxacin have
37 highlighted the essentiality of PZA as these new agents mainly work in conjunction with PZA
38 but cannot replace it (20, 21). Thus, PZA is a key drug that will likely play a major role in new
39 drug regimens for treatment of TB and drug-resistant TB.

40

41 It is well established that mutations in the *pncA* gene encoding nicotinamidase/pyrazinamidase
42 (PZase) involved in conversion of PZA to active form pyrazinoic acid (POA) are the major
43 mechanism of PZA resistance (11, 32), accounting for85% of all PZA resistance (32). However,
44 some PZA-resistant strains without *pncA* mutations may have mutations in potential targets of

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45 PZA, including ribosomal protein S1 (RpsA) involved in trans-translation (5, 9, 13, 17, 24),
46 aspartate decarboxylase (PanD) (12, 27) involved in synthesis of β-alanine - a precursor for
47 pantothenate and CoA biosynthesis, and ATP-dependent protease ClpC1(26, 28). We have
48 previously identified a 3–base pair "GCC" deletion resulting in loss of an Alanine at amino
49 acid 438 in RpsA in a low-level PZA-resistant clinical isolate DHM444 (MIC = 200 to 300
50 μg/ml PZA compared with 100 μg/ml in susceptible *M. tuberculosis*) without *pncA* mutation
51 (13). *rpsA* mutations are subsequently shown to be associated with PZA resistance in clinical
52 strains (3, 14, 18). However, certain nonsynonymous mutations in RpsA protein (A364G) seem
53 to occur in some PZA susceptible strains (1). In addition, a recent study by Dillon et al. claims
54 that RpsA is not a target of PZA and that the RpsA Δ438A mutation does not cause PZA
55 resistance (2). These discordant results raise doubts about RpsA being a target of PZA and the
56 role of RpsA Δ438A mutation in PZA resistance.
57
58 In this study, to more convincingly address the role of the RpsA Δ438A mutation in PZA
59 resistance, we transferred this mutation into the genome of *M. tuberculosis* H37Rv by
60 homologous recombination. It is worth noting that the same strategy has been successfully used
61 to demonstrate *inhA* and certain *embB* point mutations being responsible for INH and
62 ethambutol resistance, respectively (10, 22). The RpsA Δ438A mutation was created by a two-
63 step allelic exchange method as described (8). Briefly, a 3,440-bp fragment spanning the *rpsA*
64 Δ438A deletion mutation was amplified by PCR with DHM444 genomic DNA using primers
65 containing HindIII and PacI restriction sites (underlined) in the forward primer (FrpsA 5'-
66 CGGAAGCTTCCACACCACGTTCAACCAGAC-3' and reverse primer RrpsA 5'-

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67 GCTTAATTAAGCACGCGCTTGTGCCACAGAG-3'), respectively. The PCR fragment was
68 then cloned into the p2NIL vector followed by insertion of a PacI cassette containing the *sacB*
69 and *lacZ*. The recombinant plasmid was transformed in *M. tuberculosis* H37Rv as described
70 (12). The desired sucrose-resistant but kanamycin-susceptible transformants were analyzed by
71 PCR and sequenced to confirm that the transformed *M. tuberculosis* has the correct RpsA
72 Δ438A mutation (Fig. 1). We determined the PZA MICs for the RpsA Δ438A mutant and the
73 parent strain *M. tuberculosis* H37Rv using the proportion method and found them to be 200
74 μg/ml and 100 μg/ml (pH5.8), respectively. In addition, we measured the PZA and POA MICs
75 for the RpsA Δ438A mutant in liquid 7H9 medium (pH5.8) using microdilution method. The
76 PZA MIC for the *M. tuberculosis* RpsA Δ438A mutant strain was 300 μg/ml while the parent
77 strain H37Rv was susceptible at this concentration (Fig. 2A). The POA MIC for the *M.*
78 *tuberculosis* RpsA Δ438A mutant strain was 50 μg/ml compared with 25 μg/ml for the parent
79 strain H37Rv (Fig. 2B). On the other hand, both strains had the same MIC for the control drugs
80 isoniazid (INH) (0.03 μg/ml) and rifampin (RIF) (0.12 μg/ml) (Fig. 2C, 2D). These results
81 clearly demonstrate that transfer of the RpsA Δ438A mutation into the genome of *M.*
82 *tuberculosis* caused resistance to PZA and POA specifically, but not to INH and RIF. This
83 provides conclusive evidence that the RpsA Δ438A mutation is indeed responsible for the PZA
84 resistance in the strain DHM444.
85
86 In addition, in our previous study we found another low level PZA-resistant clinical isolate that
87 has two *rpsA* mutations RpsA T5S and D123A with wild type *pncA* gene (13). To provide
88 further proof that RpsA point mutations can cause PZA resistance, we constructed another

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89 RpsA mutation D123A into the genome of *M. tuberculosis* H37Rv. Site-directed mutagenesis
90 of *rpsA* was performed with primers S1D123AF 5'-
91 GCTCAAGGAGAAGGCCGAGGCCGTCAAGG-3'and S1D123AR 5'-
92 CCTTGACGGCCTCGGCCTTCTCCTTGAGC-3'using the QuikChange™ site-directed
93 mutagenesis kit as described by the manufacturer (Agilent Technologies). Then the point
94 mutation RpsA D123A was transferred to the parental strain *M. tuberculosis* H37Rv as decribed
95 as above. We found that indeed the PZA MIC for the *M. tuberculosis* RpsA D123A mutant was
96 increased by 2 fold (200 μg/ml PZA) compared with the parental strain *M. tuberculosis* H37Rv
97 (100 μg/ml PZA) on 7H11 agar plate at pH5.8.
98
99 It is worth discussing the possible causes for the discrepant results between the Dillon et al.
100 study (2) and ours. One possibility is the unusual and not accepted method used by that group
101 to determine the PZA susceptibility by using OD_{600} and MIC_{90}. PZA susceptibility testing is
102 notoriously difficult because of the acid pH requirement for drug activity and many factors
103 affect the susceptibility results (30). The inability to observe PZA or POA resistance in their
104 *rpsA* overexpression or point mutation strains could be attributed to the questionable method
105 used in that study. Another argument used by Dillon et al. against the RpsA Δ438A mutation
106 conferring PZA resistance (2) is that DHM444's PZA resistance could be due to defective
107 PZase activity reported in a previous study (16). To address this question, we tested PZase
108 activity for DHM444 as described (27) and found that contrary to the previous report (2), The
109 PZA-resistant strain DHM444 still has PZase activity (Data not shown). Thus, the low level
110 PZA resistance in DHM444 could not be attributed to defective PZase activity, and this study

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111 provides clear evidence that its PZA resistance is most likely due to the RpsA Δ438A mutation
112 (Fig. 2A, 2B).
113
114 That RpsA is a target of PZA (13) is supported by a number of observations including
115 association of RpsA mutations and PZA resistance in clinical strains (3, 5, 9, 14, 18, 23), *rpsA*
116 overexpression causing PZA resistance (13), as well as biochemical and structural studies
117 showing the binding of the drug to the RpsA (4, 15, 25). Despite these supporting studies, and
118 in view of the question about the role of RpsAΔ438A mutation in PZA resistance, point
119 mutation construction into the chromosome, though challenging, is by far the most convincing
120 method to prove if a specific mutation is the cause for drug resistance. Our findings that the
121 RpsAΔ438A mutation and the D123A mutation when introduced into the *M.*
122 *tuberculosis* chromosome are indeed responsible for PZA resistance provide further proof that
123 RpsA is a real target of PZA. Future studies are needed to address the role of certain *rpsA*
124 mutations in some seemingly "PZA-susceptible" strains by constructing more isogenic strains
125 with *rpsA* mutations to determine whether they are involved in PZA resistance while others
126 may not or may confer only borderline resistance that is easily mischaracterized as PZA
127 susceptible due to insensitive PZA DST.
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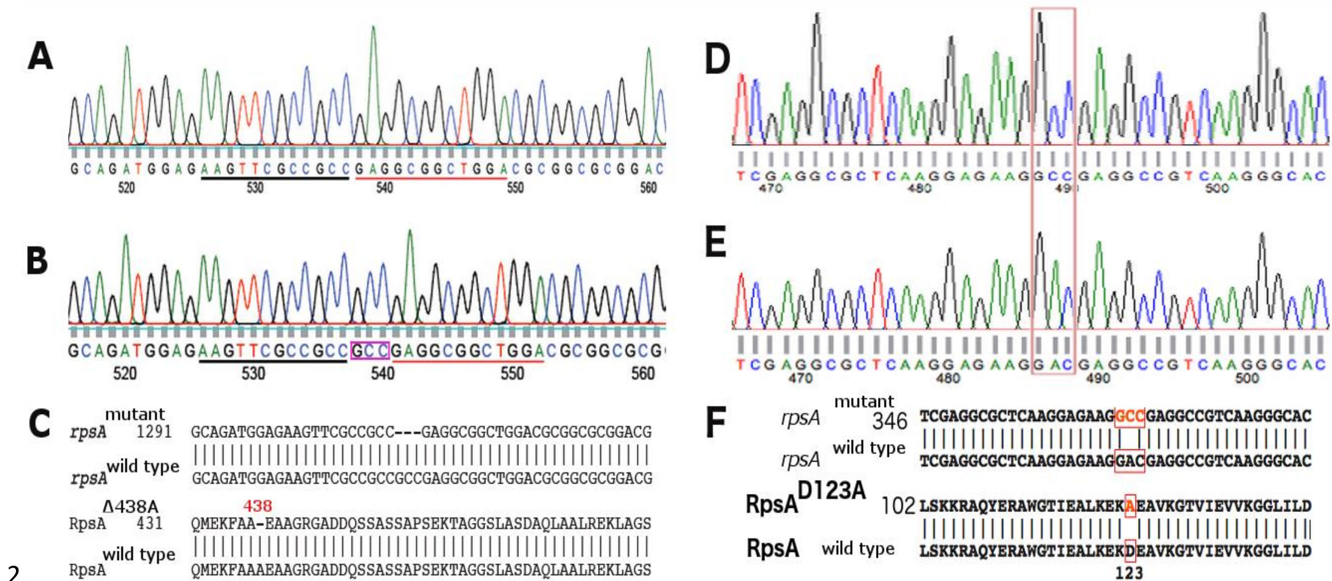

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263
264 Fig. 1. Confirmation of RpsA Δ438A and D123A point mutation construction by Sanger sequencing.
265 **A.** Chromatogram of partial *rpsA* sequence showing the "GCC" (coding for Alanine, A) deletion
266 in the constructed RpsAΔ438A mutant from PZA-resistant strain DHM444. **B**. Chromatogram
267 of partial *rpsA* sequence showing the presence of "GCC" (Alanine) in the *M. tuberculosis*
268 H37Rv parent strain. **C**. Alignments of partial *rpsA* sequence from *M. tuberculosis* H37Rv wild
269 type and the constructed RpsA Δ438A mutant at the nucleotide level (upper panel) and amino
270 acid level (lower panel). D and E. Chromatogram of partial *rpsA* sequence showing "GAC"
271 (wild type) change to "GCC" which is contained in one PZA-resistant clinical isolate. **F**.
272 Alignments of partial *rpsA* sequence from *M. tuberculosis* H37Rv wild type and the constructed
273 RpsA D123A mutant at the nucleotide level (upper panel) and amino acid level (lower panel).
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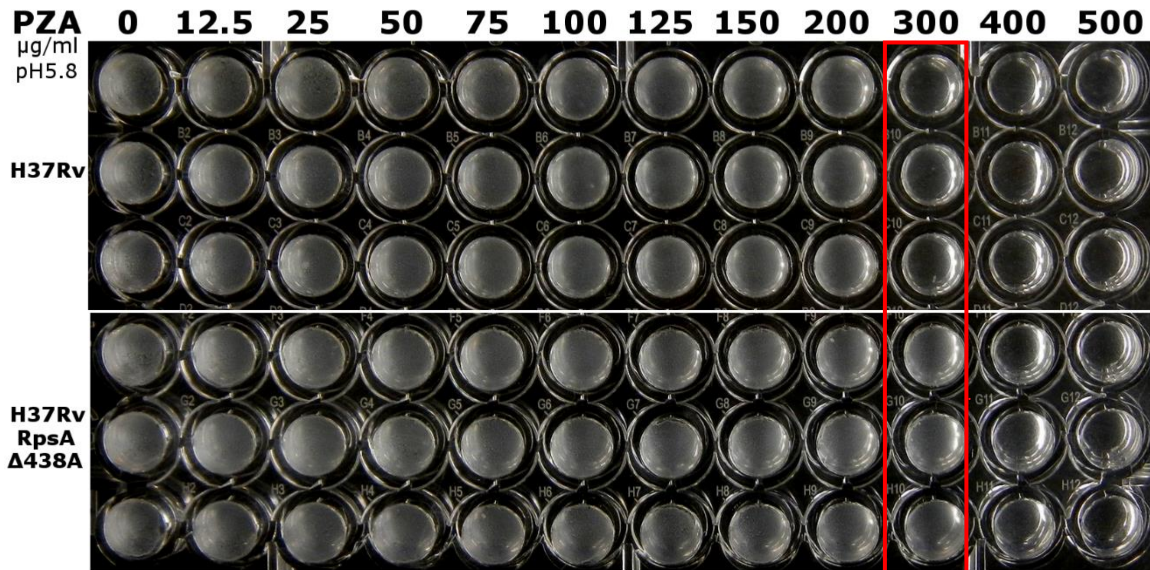
279 **A.**



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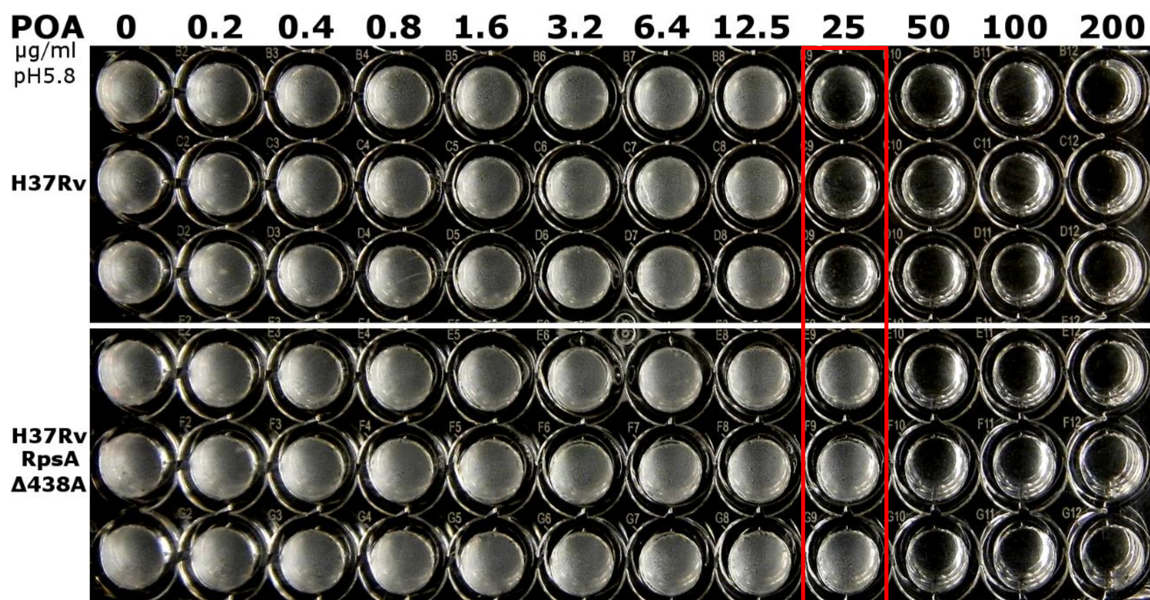

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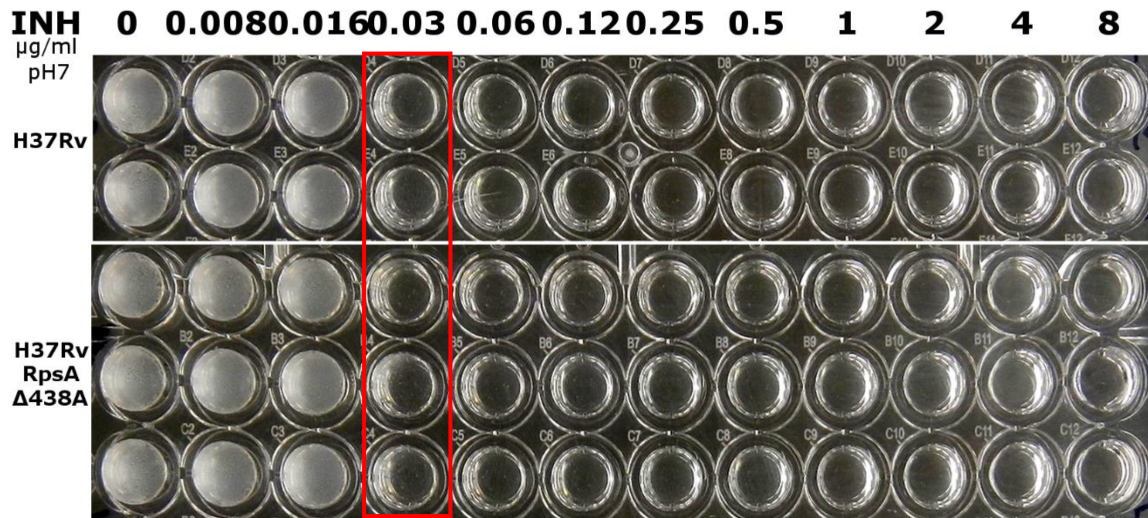
289 **C.**



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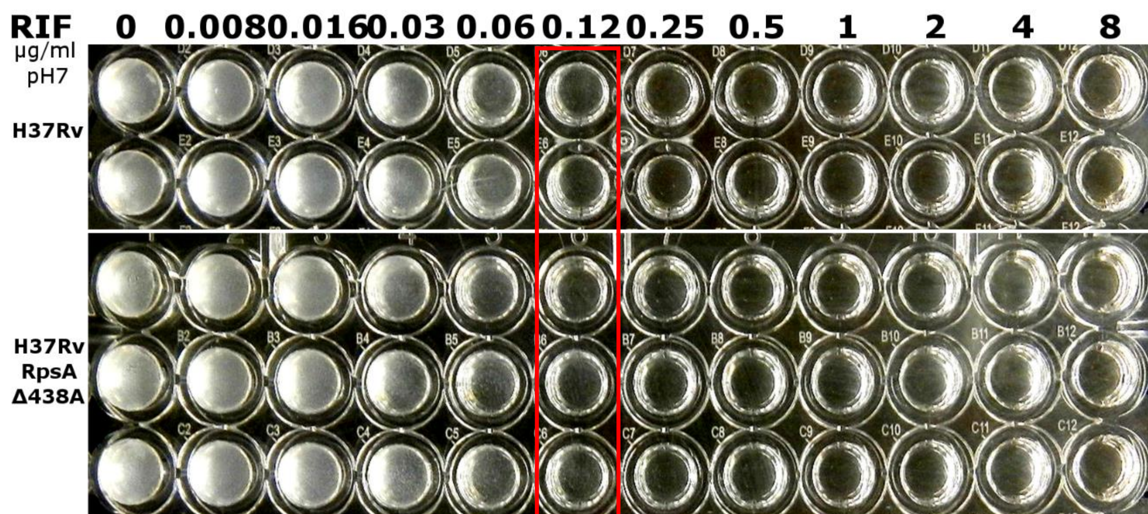
291 **D.**



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293 Fig. 2. Drug susceptibility testing (DST) results of the constructed RpsA Δ438A mutant strain
294 compared with the control strain H37Rv. The drug susceptibility testing was performed in
295 7H9/ADC broth using microdilution method with pH5.8 for PZA and POA DST, and pH 7.0
296 for INH and RIF DST. The constructed RpsA Δ438A mutant strain was more resistant to PZA
297 (A) and POA (B) than the control strain H37Rv but was as susceptible to INH (C) and RIF (D)
298 as the control strain H37Rv. **A.** The PZA MIC of the *M. tuberculosis* H37Rv control strain was
299 300 μg/ml while the *M. tuberculosis* RpsA Δ438A mutant strain was resistant at this

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300 concentration. **B.** The POA MIC of *M. tuberculosis* H37Rv parent strain was 25 μg/ml while
301 the POA MIC for the *M. tuberculosis* H37Rv RpsA Δ438A mutant strain was 50 μg/ml.
302 **C.** Isoniazid (INH) MICs for the parent strain *M. tuberculosis* H37Rv and the *M. tuberculosis*
303 H37Rv RpsA Δ438A mutant were the same (0.03 μg/ml). **D.** The RIF MICs for the parent
304 strain *M. tuberculosis* H37Rv and the *M. tuberculosis* H37Rv RpsA Δ438A mutant were the
305 same (0.12 μg/ml). The red boxes are used to highlight the difference in PZA and POA
306 susceptibility between the RpsA Δ438A mutant strain and the parent strain H37Rv.
307
308