Large-scale Identification of Compensatory Mutations in the RNA Polymerase of *M. tuberculosis*

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The case for investigating compensatory mutations

Resistance mutations in *M. tuberculosis* to various antibiotics often introduce a fitness cost for the bacteria. Accordingly, resistance to the first-line drug rifampicin, arising predominantly through mutations within the β *(rpoB)* subunit of the RNA polymerase, leads to lower fitness compared to susceptible bacteria [1]. This fitness cost can be partially alleviated by compensatory mutations in other regions of the polymerase, most often in the β' *(rpoC)* subunit. Compensatory mutations are of particular interest clinically, since they are suspected to lead to fixation of resistance mutations. Fixation then encourages the spread of resistant strains worldwide.

Objective 1: Identify further compensatory mutations in the RNA polymerase of *M. tuberculosis*

Dataset: 70,000 *M. tuberculosis* genomes collected and sequenced by the CRyPTIC project [2]. Enables use of powerful statistical tests (see Methods) to investigate association of potential compensatory mutations with known resistance mutations. We identified 51 high-confidence compensatory mutations.

We mapped all hits onto the available protein structure (Figure 1).

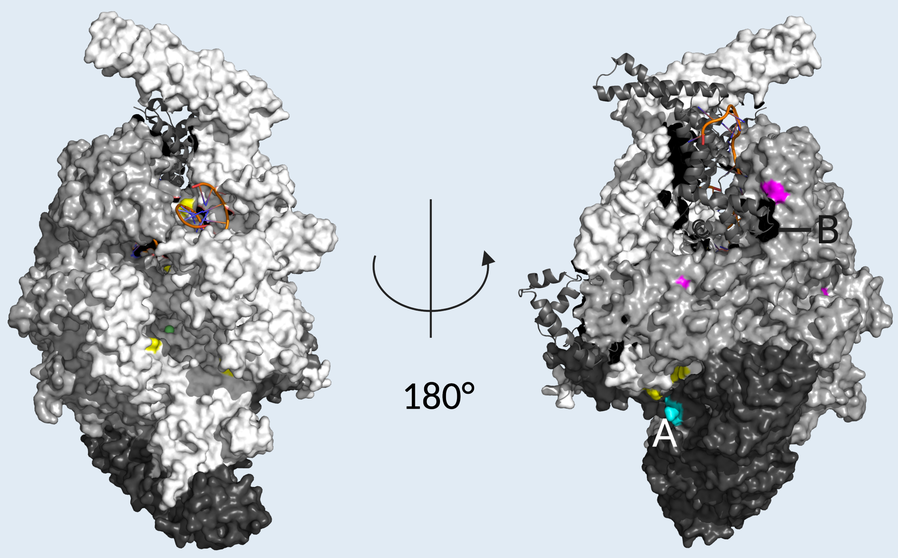


Figure 1: Putative compensatory mutations mapped onto the *M. tuberculosis* RNA polymerase structure (PDB: 5UHB). Subunits represented in grey shades, mapped hits highlighted in color. A and B indicate regions with clusters of compensatory mutations.

Methods: Dealing with Linkage Disequilibrium and inflated p-values

Fisher's exact test was used to interrogate pairwise associations between resistance mutations and other co-occurring mutations in the RNA polymerase.

Challenge: Linkage disequilibrium resulted in inflated p-values for random associations.
Approach: Use of a distribution-informed cut-off (Figure 2) and homoplasy as necessary criteria for putative hits.

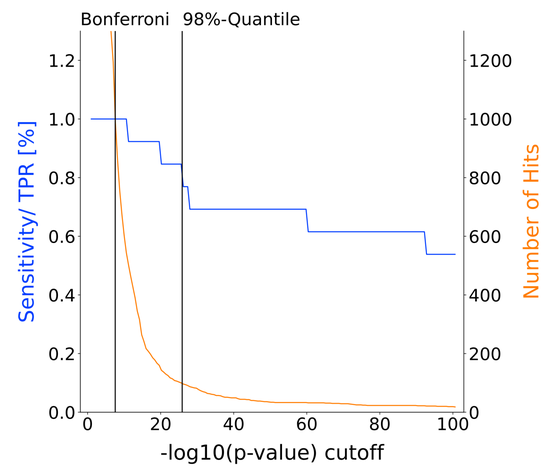
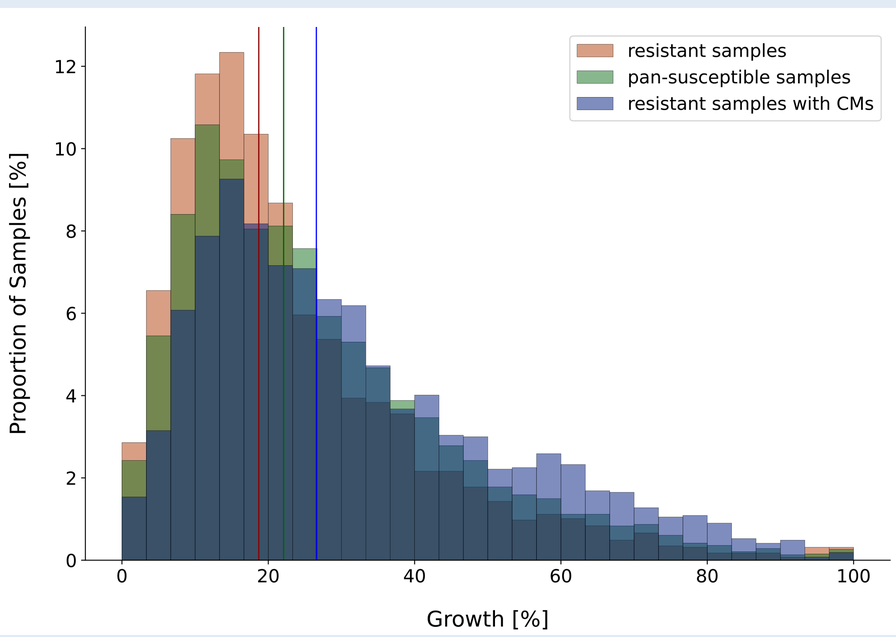


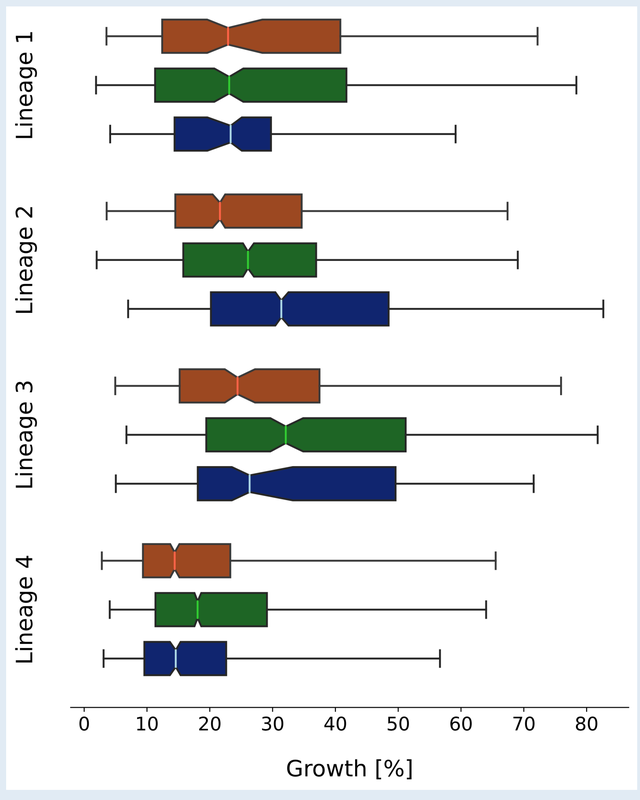
Figure 2: Sensitivity and number of significant hits (putative compensatory mutations) depending on p-value cut-off.

Objective 2: Test the association of compensatory mutations with growth



Dataset: Growth data from 96-well growth experiments [3].
In the overall dataset, resistant samples with compensatory mutations (CM) exhibit higher growth densities than samples with only resistance, and surprisingly even better than susceptible samples (above).

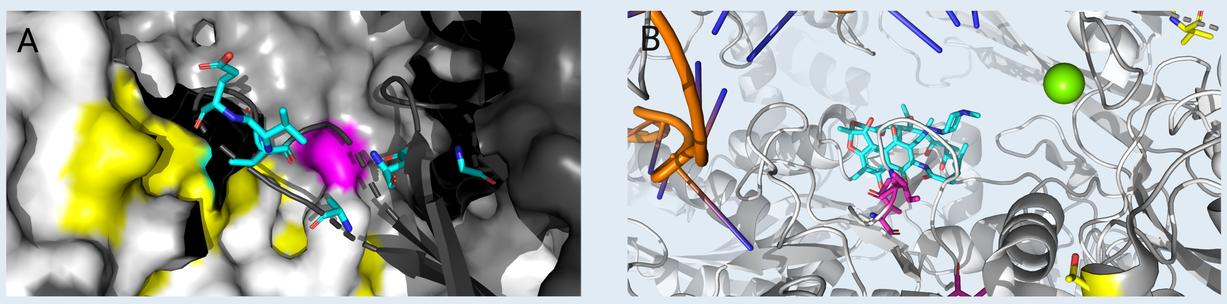
A significant growth advantage can only be shown for Lineage 2, but a trend towards higher growth in presence of CMs can be seen in Lineages 3 and 4 as well (right).



Objective 3: Propose hypotheses for the mechanism of action of compensatory mutations

Most compensatory mutations cluster close to subunit interfaces (A), which suggests that they change the overall conformation and stability of the protein complex. Several hits are located on the β subunit around the active center where rifampicin binds (B). These hits might affect the reactivity of the enzyme.

Overall this indicates that there are different strategies for compensation.



Conclusion

- Compensatory mutations can be identified through their statistical association with resistance. We confirmed 51 hits, 39 previously described and 12 novel.
- In *M. tuberculosis* Lineage 2, compensatory mutations are significantly associated with growth densities higher than wild-type levels.
- Mapping identified compensatory mutations on the RNA polymerase structure suggests that most of them change the subunit interfacial regions, but there might be different strategies for compensation.

The above findings increase the urgency for accurate, cheap and widely accessible diagnostics to prevent the emergence of more virulent strains through compensation.

References

[1] Gagneux *et al*, *Science* (2006) **312,** 5782, 1944-1946
[2] CRyPTIC Consortium, *The New England Journal of Medicine* (2018) **379,** 15, 1403-1415
[3] Fowler *et al*, *Microbiology* (2018) **164,** 12, 1522-1530

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