Moradigaravand, D; Grandjean, L; Martinez, E; Li, H; Zheng, J; Coronel, J; Moore, D; Trk, ME; Sintchenko, V; Huang, H; Javid, B; Parkhill, J; Peacock, SJ; Kser, CU (2016) DfrA-thyA double deletion in para-aminosalicylic acid resistant Mycobacterium tuberculosis Beijing strains. Antimicrobial agents and chemotherapy, 60 (6). pp. 3864-7. ISSN 0066-4804 DOI: https://doi.org/10.1128/AAC.00253-16

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DOI: 10.1128/AAC.00253-16

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Para-Aminosalicylic acid (PAS) is a group 4 antituberculosis agent (1). It targets folate metabolism as shown in Fig. S1 in the supplemental material, which also summarizes the known mechanisms of resistance to this produg (2). Recently, we reported a multidrug-resistant (MDR) Mycobacterium tuberculosis Beijing strain harboring a deletion of both dfrA and thyA from Australia (Fig. 1A and Table S1) (3). Since then, we have found deletions affecting both genes in five further MDR Beijing strains (two isolated in Australia and three from Peru) and one extensively drug-resistant (XDR) Beijing strain from China. The Australian MDR strains were recovered from three patients with no apparent epidemiological links who were likely infected in their country of origin (Table S1). The three Peruvian isolates were closely related and consequently shared the same deletion, whereas the remaining strains were distantly related and had deletions that differed in size (Fig. 1A). Consequently, these five distinct deletions were acquired independently, which can be a signal for positive selection of resistance mechanisms. In line with this hypothesis, the strains from Australia and China were found to be PAS resistant when tested with the Bactec MGIT 960 system and on Löwenstein-Jensen medium, respectively (see Supplemental methods). Two out of the three Peruvian deletion mutants were also found to be PAS resistant on 7H10 medium at 8 μg/ml, whereas the two closely related ancestral wild-type strains were found to be susceptible (Fig. 1B). We were unable to retest the strains at 2 μg/ml, the critical concentration recommended by the Clinical and Laboratory Standards Institute and the World Health Organization, which would have clarified whether the result for the third deletion mutant as susceptible was an artifact (1, 4).

The observation that dfrA could be deleted was remarkable in light of our current understanding of folate metabolism in M. tuberculosis. Two studies suggested that dfrA is essential in vitro in the H37Rv laboratory strain (5, 6). More recently, it was shown that dfrA is conditionally essential and can be knocked out in M. tuberculosis with inactive dihydrofolate reductase activity compared to what of DfrA (5, 7, 8). Our in silico analysis of the seven dfrA thyA double deletion mutants did not reveal any known Rv2671 mutations (Table S1), such as the G-to-A upstream mutation at position –12 that results in its overexpression and consequently confers PAS resistance (this mutation was incorrectly referred to as affecting position –11 in two of our prior studies [7, 9]). Assuming that no other pertinent differences that are specific to the Beijing genotype relative to H37Rv exist or that a yet-unknown acquired mutation elsewhere in the genome that resulted in the overexpression of Rv2671 was present, we propose that this apparent contradiction can be reconciled if the essentiality of dfrA was dependent not only on the expression level of Rv2671 but also on the presence of wild-type thyA. The fact that thyA was deleted in all seven dfrA mutants meant that only the second thymidylate synthase, encoded by the essential thyX gene, was active in these strains (Fig. S1). Contrary to ThyA, ThyX generates tetrahydrofolate rather than dihydrofolate upon catalysis and therefore does not require high dihydrofolate reductase activity to provide sufficient levels of tetrahydrofolate (2). This is in line with the fact that dfrA is not required in bacterial species that lack thyX (10). Consequently, Rv2671 appeared to be sufficient to sustain growth, even without being overexpressed in these deletion mutants. It should therefore be possible to knock out dfrA in strains of M. tuberculosis with inactive thyX. Moreover, the adjacent locations of thyX and dfrA in the genome should make their simultaneous deletion possible (Fig. 1A).

Interestingly, all but one of the deletion mutants also convergently acquired mutations upstream of thyX compared to what was observed for the two closely related Peruvian control strains.
In fact, the cluster of three Peruvian strains and two of the unrelated Australian strains shared the same C-to-T upstream mutation at position H11002 that has previously been found to be associated with resistance to several drugs and experimentally shown to result in the overexpression of thyX (12). It is therefore plausible that these changes compensated for the reduced expression levels and enzymatic activity of ThyX compared to those of ThyA (11, 13). Based on our data, however, it was not possible to deduce whether the thyX mutations were acquired after the deletions of thyA and dfrA in each strain, as would be expected with compensatory mutations (11).

In summary, these data demonstrated that the folate metabolism and the genetic basis of PAS resistance are more complex than previously appreciated, which is relevant for the development of novel DfrA and ThyX inhibitors and potentially the use of trimethoprim-sulfamethoxazole to treat drug-resistant tuberculosis (Fig. S1) (14–25). Although deletions are often excluded from large-scale whole-genome studies, owing to the limited read lengths.
lengths of next-generation sequencers and the fact that algorithms are optimized for single-nucleotide polymorphism (SNP) calling, this study highlighted that deletions can no longer be ignored (3, 26).

ACKNOWLEDGMENTS

We thank L. Camacho for helpful comments regarding this topic.

M. E. Török, J. Parkhill, S. J. Peacock, and C. U. Köser have collaborated with Illumina, Inc., on a number of scientific projects. M. E. Török and S. J. Peacock have received funding for travel and accommodation from Illumina, Inc. J. Parkhill has received funding for travel and accommodation from Pacific Biosciences, Inc., and Illumina, Inc. B. Javid is a Tsinghua-Janssen Scholar. C. U. Köser is a consultant for the Foundation for Innovative New Diagnostics and was a technical advisor for the Tuberculosis Guideline Development Group of the World Health Organization. The Bill & Melinda Gates Foundation and Janssen Pharmaceutica covered C. U. Köser’s travel and accommodation to present at meetings. The European Society of Mycobacteriology awarded C. U. Köser the Gertrud Meissner Award, which is sponsored by Hain LifeScience. All other authors have no conflicts of interest to declare.

FUNDING INFORMATION

This publication presents independent research supported by the Health Innovation Challenge Fund (HICF-T5-342 and WT098600), a parallel funding initiative. This publication is covered by a Creative Commons Attribution (CC BY) licence, which allows non-commercial reuse provided appropriate citation is made to the original work.

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