Short-form paper

Role of alanine racemase mutations in *Mycobacterium tuberculosis* D-cycloserine resistance

Running title: alanine racemase and D-cycloserine

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Abstract

Screening of more than 1,500 drug-resistant strains of *Mycobacterium tuberculosis* revealed evolutionary patterns characteristic of positive selection for three alanine racemase (Alr) mutations. We investigated these mutations using molecular modeling, *in vitro* MIC testing, as well as direct measurements of enzymatic activity, which demonstrated that these mutations likely confer resistance to D-cycloserine.

Manuscript

In 2015, the Global Drug Facility declared that the cost of D-cycloserine (DCS), a group C drug to treat tuberculosis (TB), would be cut by more than half to as little as $0.19 per capsule to support the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, which represent a major threat to public health (1). In light of this announcement, a better understanding of the resistance mechanisms to this drug is

required to facilitate phenotypic as well as genotypic drug-susceptibility testing (DST), both in the context of surveillance and individual patient treatment to avoid the severe side-effects of this drug (2, 3).

Studies of the mode of action of DCS in mycobacteria have produced contradictory results, with some studies pointing to alanine racemase (Alr) as the primary target and others supporting D-alanine-D-alanine ligase (DdlA) (4-9). However, molecular data from *Mycobacterium tuberculosis* complex (MTBC) have only implicated the former gene in DCS resistance, which can also be conferred by mutations in alanine dehydrogenase (*ald*) or a permease (*cycA*) (10, 11). Using molecular modeling, we had predicted that the *alr* M319T mutation observed in an XDR strain would likely confer resistance to DCS, which was subsequently confirmed by Desjardins et al. using the unrelated strain TKK_04_0105 (Table S1 (2, 11)). Desjardins et al. described a number of additional *alr* mutations in strains with elevated DCS MICs, including a C to T nucleotide change 8 base pairs upstream of the experimentally confirmed start codon of *alr* (strain TKK_02_0050 in Table S1 (11, 12)). This was notable as Merker et al. had previously reported that, compared with the susceptible, parental *alr* wild-type strain, the acquisition of this mutation during treatment with DCS correlated with DCS resistance, which suggested that *alr* mutations might be both necessary and sufficient to confer DCS resistance (13).

To gain further insights into the impact of *alr* mutations, we first confirmed that the aforementioned *alr* C-8T promoter mutant that evolved during treatment correlated in MICs above the current World Health Organization (WHO)-endorsed critical concentration (CC) of 30 µg/ml using the 1% proportion method on Löwenstein-Jensen (LJ) (strain PBm0 and PBm14 in Table S1; Desjardins et al. and Merker et al. had used 10% as the critical proportion and therefore had not adhered to the current WHO recommendations (11, 13, 14)). Using the same method, we also showed that two strains with *alr* M319T or Y364D mutations from XDR TB patients with a treatment history with DCS had MICs above the CC (Table S1). Moreover, we observed the M319T mutation in three XDR strains (PT1, PT2 and PT5) from Lisbon, Portugal (15). Although no CC exists for MGIT 960, this mutation correlated in an MIC increase from 16 to 64 µg/ml compared with three closely related wild-type control strains (PT3, PT6 and PT7) and one more distantly related control strain (PT4), which supported the role of this mutation in DCS resistance (Figure 1A and Table S1). By contrast, no or minimal MIC increases were recorded when testing these Portuguese strains using Sensititre MycoTB plates (Table S1) (16). Finally, a pre-XDR *alr* R373L mutant from a patient with DCS exposure, which also harbored a deletion in *ald*, tested resistant on LJ using the 1% proportion method (Tables S1 and S2).

To study the importance of the C-8T, M319T, Y364D and R373L mutations from an evolutionary perspective, we screened previously published and unpublished genomes of more than 1,500 MDR strains (mostly from Germany, Eastern Europe, and Swaziland), which identified eight additional strains with mutations at these *alr* positions or codons (Table S1). Interrogating the genomes of these 17 strains in the context of a phylogenetically diverse reference collection that included all major MTBC lineages and species showed that the mutations had either been acquired multiple times independently and/or that different amino acid changes were present at the same codons (Figure 1B). These mutation patterns are typically a signal of positive selection, which could have occurred in response to DCS exposure.

Molecular modeling of these coding mutations supported this hypothesis. Alr functions as a homodimer, aided by the co-factor pyridoxal 5'-phosphate (PLP) to which it is covalently bound. DCS inhibits Alr irreversibly by covalently bonding to PLP (4). We generated and analyzed a model of the complex between the *M. tuberculosis* Alr and DCS (Alr_{Mtb}-DCS) (Figure S1) (4, 17). Amino acid residues 319 and 364 were located directly in the active site (Figure S1B). M319T was positioned close enough to allow interaction with the DCS moiety, which, given the large change of the character of the side chain, could strongly affect DCS reactivity (Figure S1C). Y364 is involved in the positioning of the phosphate moiety of PLP and thus represents a prominent active site residue in the conserved inner layer of the substrate entrance corridor of Alr (Figure S1B) (17). Mutation to aspartic acid introduced a shorter and negatively charged side chain, which could potentially affect PLP orientation in the active site (Figure S1C). Moreover, it could influence DCS uptake through alteration of the entrance corridor. Interestingly, M319 is located near Y364 and, as a result, it is possible that the M319T mutation could alter the interaction with Y364, thereby affecting DCS inhibition. In contrast, the R373L mutation was not directly located within the active site but near the dimer interface and close to residues M319 and D320, which play an important role in the makeup of the active site (Figure S1B). Consequently, the replacement of arginine with the short and hydrophobic side chain of leucine might disrupt molecular interactions at the dimer interface as well as destabilize the DCS binding site (Figure S1C).

To test these predictions experimentally, we expressed and purified the aforementioned Alr_{Mtb} coding mutants, along with wild-type Alr_{Mtb}, and determined their half maximal inhibitory concentration (IC_{50}) to measure the effectiveness of inhibition by DCS (Figure 2). The IC_{50} for wild-type Alr_{Mtb} was 26.4 ± 1.7 µM, which was in the range previously reported for this compound (18, 19). From our structure-based analysis, we expected the two mutations located in the active site to show the greatest effect on DCS inhibition. Indeed, the

M319T mutant enzyme showed minimal inhibition by DCS, even at 1000 µM (Figure 2). Thus, the IC_{50} of this mutant could not be determined. The IC_{50} of the Y364D mutant showed a 50-fold increase to 1328.0 ± 340.0 µM. The R373L mutation, which was not located directly within the active site, also showed a significant increase in resistance to DCS with an IC_{50} of 712.0 ± 138.5 µM (27-fold increase).

Taken together, these data suggested that *alr* mutations likely confer DCS resistance, although allelic exchange experiments are required to formally prove this (particularly for R373L, which coincided with a deletion in *ald* and, consequently, may not be sufficient to confer resistance on its own). Although the relationship between MICs and IC_{50}s can be complex, the observation that MICs increased by only 4-16 fold vs. at least 25-fold increases for IC_{50}s supported the notion that DCS inhibits multiple targets, as noted earlier. This study should be complemented with extensive MIC testing of phylogenetically diverse, pan-susceptible MTBC strains to define the epidemiological cut-off value given that it is unclear based on which evidence the current WHO CC on LJ has been set (3, 14, 20, 21). Moreover, further MIC testing of likely DCS-resistant strains is needed to investigate whether the Sensititre system is less reliable at detecting DCS resistance compared with LJ and MGIT. Finally, the impact of *alr* mutations on resistance on terizidone remains to be investigated.

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Conflicts of interest

K. L. K., Y. N. and H. K. O. R have received funding for alanine racemase related projects from L2 Diagnostics LLC, New Haven, Conn. J. P., S. J. P. and C. U. K. have collaborated with Illumina Inc. on a number of scientific projects. J. P. has received funding for travel and accommodation from Pacific Biosciences Inc. and Illumina Inc. S. J. P. has received funding for travel and accommodation from Illumina Inc. C. U. K. is a consultant for the Foundation for Innovative New Diagnostics. The Bill & Melinda Gates Foundation and Janssen Pharmaceutica covered C. U. K.'s travel and accommodation to present at meetings. The European Society of Mycobacteriology awarded C. U. K. and M. M. the Gertrud Meissner Award, which is sponsored by Hain Lifescience.

Figure 1.

Maximum likelihood tree based on a concatenated sequence alignment of 45,740 variable sites (1,000 resamplings, GTR nucleotide substitution model) showing the *alr* mutants from Table S1 in the context of a globally representative reference collection of 287 MTBC strains. Inset A, a zoomed-in part of the overall tree B, shows the phylogenetic relationship between the three Portuguese M319T mutants (PT1, PT5 and PT2) and the control strains (PT7, PT3, PT6 and PT4) tested in MGIT and Sensititre. The three Indian M319T, R364D and R373G mutants that were tested with the 1% proportion LJ method in this study are underlined. The T-8C, M319T and R364D mutations were homoplastic (i.e. they were acquired multiple times independently) and two different amino acid changes were observed at codon 373 (i.e. R373L, and. R373G). Thus, all mutations show evolutionary patterns of positive selection.

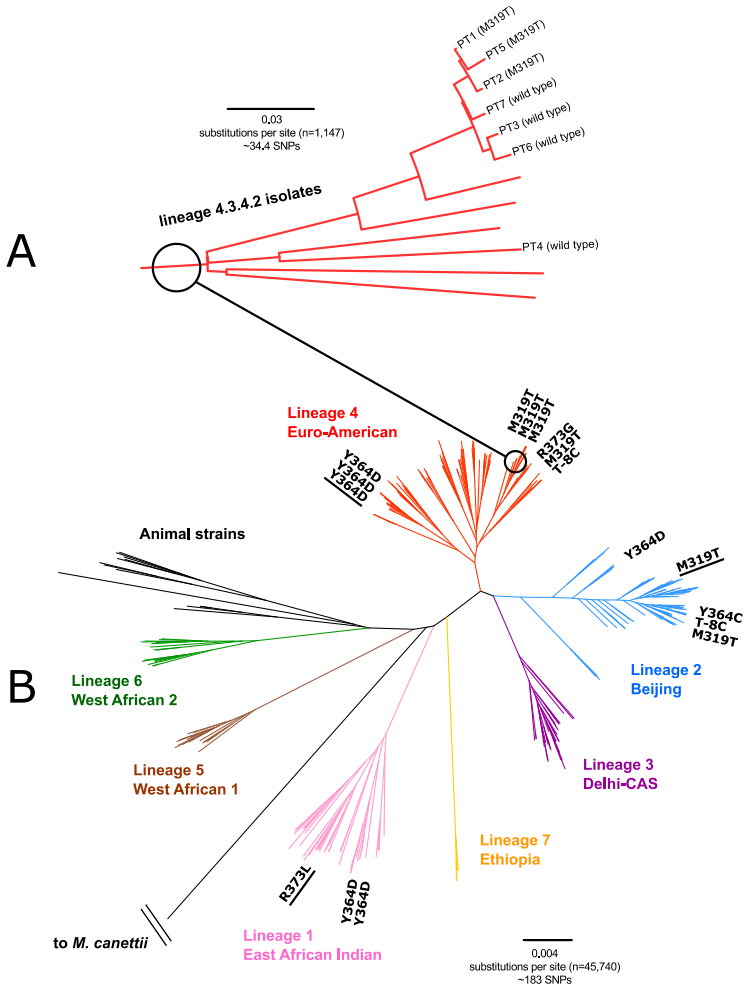
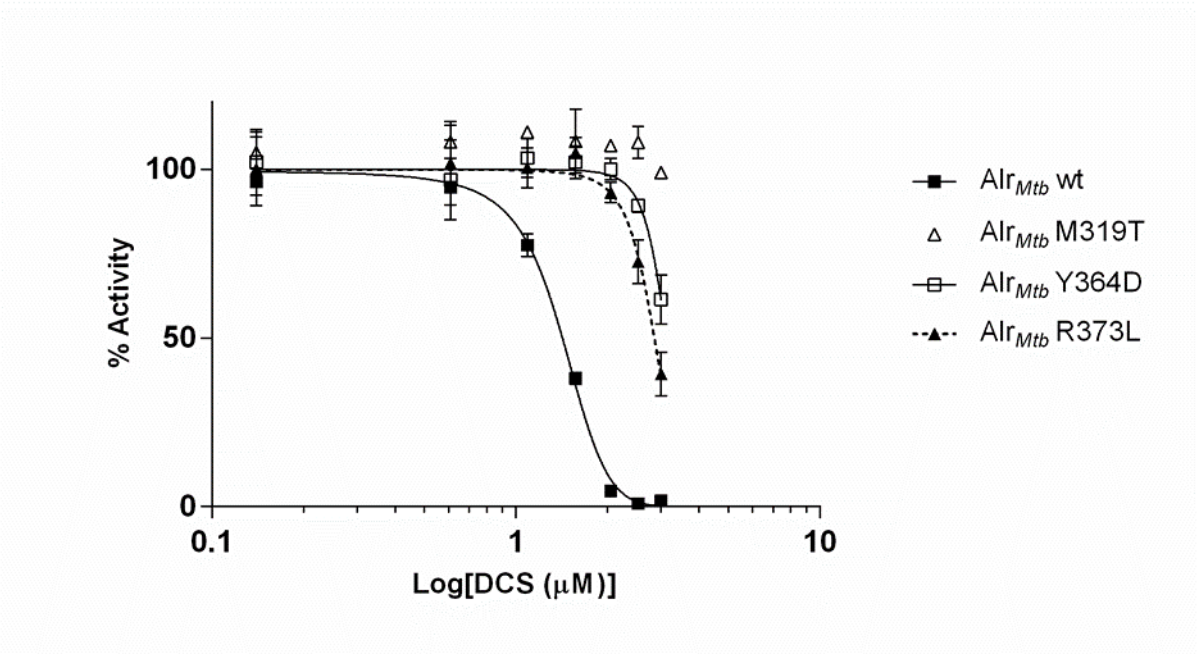


Figure 2. Determination of DCS IC_{50} for wild-type (wt) Alr_{Mtb} and the M319T, Y364D and R373L mutants. The activity was normalized against a control with no DCS present in the assay mix. The activity assay at each concentration was performed in triplicate, resulting in the error bars, which represent 95% CI. A variable slope model was fitted to determine the IC_{50} values, which were 26.4 ± 1.7, 1328.0 ± 340.0, 712.0 ± 138.5 μM for the wild-type, Y364D, and R373L enzymes, respectively. The inhibition of M319T was too weak to allow for IC_{50} determination.



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