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Identification of novel mutations associated with cycloserine resistance in *Mycobacterium tuberculosis*

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Objectives: D-Cycloserine is an important second-line drug used to treat MDR- and XDR-TB. However, the mechanisms of resistance to D-cycloserine are not well understood. Here we investigated the molecular basis of D-cycloserine resistance using *in vitro*-isolated resistant mutants.

Methods: *Mycobacterium tuberculosis* H37Rv was subjected to mutant selection on 7H11 agar plates containing varying concentrations of D-cycloserine. A total of 18 D-cycloserine-resistant mutants were isolated and subjected to WGS. The identified mutations associated with D-cycloserine resistance were confirmed by PCR and Sanger sequencing.

Results: We identified mutations in 16 genes that are associated with D-cycloserine resistance. Interestingly, we found mutations only in *alr* (*rv3423c*) encoding alanine racemase, but not in other known D-cycloserine resistance-associated genes such as *ddl*, *cycA* or *ald*. Instead, we identified 13 new genes [*rv0059*, *betP* (*rv0917*), *rv0221*, *rv1403c*, *rv1683*, *rv1726*, *gabD2* (*rv1731*), *rv2749*, *sugI* (*rv3331*), *hisC2* (*rv3772*), the 5′ intergenic region of *rv3345c* and *rv1435c*, and the 3′ region of *rv0759c*] that had solo mutations associated with D-cycloserine resistance. Our findings indicate that the mechanisms of D-cycloserine resistance are more complex than previously thought and involve genes participating in different cellular functions such as lipid metabolism, methyltransferase, the stress response and transport systems.

Conclusions: New mutations in diverse genes associated with D-cycloserine resistance have been identified that shed new light on the mechanisms of action and resistance of D-cycloserine. Future studies are needed to verify these findings in clinical strains so that molecular detection of D-cycloserine resistance for improved treatment of MDR-TB can be developed.

Introduction

D-Cycloserine is a cyclic analogue of D-alanine and is a broad-spectrum antibiotic that inhibits the growth of Gram-positive and Gram-negative bacteria. Although D-cycloserine can elicit adverse psychiatric and nervous system reactions, it displays no cross-resistance with any other known antitubercular drugs.[1] D-Cycloserine is an important second-line drug for the treatment of MDR- and XDR-TB,[1] and is currently classified as a Group C agent for the treatment of MDR-TB by WHO (http://www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/resources/en/).

Since D-cycloserine is a structural analogue of D-alanine, enzymes with substrates of D-alanine are the drug targets of D-cycloserine in mycobacteria.[2–4] These enzymes include D-alanine racemase (Alr) and D-alanine:D-alanine ligase (Ddl), which are required for the synthesis of peptidoglycan in the mycobacterial cell wall. Overexpression of *alr* and *ddl* has been shown to cause resistance to D-cycloserine in *Mycobacterium smegmatis*.[5,6] Moreover, SNPs in these genes were also found in resistant *Mycobacterium tuberculosis*.[7–9] Consistent with the cell-wall peptidoglycan being a target of D-cycloserine, previous studies have shown that D-cycloserine competitively inhibits both Alr and Ddl.[5,10] However, a more recent metabolomic study showed that Ddl is a primary target of D-cycloserine and is preferentially inhibited over Alr in *M. tuberculosis*.[11] In addition, CycA is a transporter protein of D-alanine, D-serine and glycine,[12] and its SNP may partially contribute to the natural resistance to D-cycloserine in *Mycobacterium bovis* bacillus Calmette–Guérin (BCG).[9,13] *ald* (*Rv2780*), encoding L-alanine dehydrogenase, was the fourth gene in *M. tuberculosis*, and its mutation was found in D-cycloserine-resistant clinical isolates.[9] However, mutations in *cycA* and *ald* only contribute to very

low-level resistance and the *ddl* mutation was rarely found in studies without known phenotype in MDR/XDR-TB strains.[9]

Despite the above progress, and significant advancements in general about the molecular understanding of drug resistance mechanisms in *M. tuberculosis*,[14] the distribution and characterization of D-cycloserine resistance in clinical strains are still unclear. This is partly due to the technical difficulties with D-cycloserine susceptibility testing such that routine phenotypic testing is not performed in clinical laboratories,[15,16] as well as the poor understanding of the molecular basis of resistance to this drug. To better understand the mechanisms of D-cycloserine resistance and to develop more rapid molecular tests for detection of its resistance, we characterized 18 D-cycloserine-resistant mutants isolated *in vitro* from *M. tuberculosis* H37Rv and discovered a panel of new mutations associated with D-cycloserine resistance that have not previously been reported.

Materials and methods

D-Cycloserine-resistant mutant isolation

M. tuberculosis H37Rv cultures, 1 month old, grown in 7H9 liquid medium supplemented with 0.05% Tween 80 and 10% BSA/dextrose/catalase (ADC) enrichment were plated on 7H11 plates containing 20, 40, 80, 160 and 320 mg/L cycloserine. After incubation at 37 °C for 4 weeks, the mutant colonies were picked to confirm the drug resistance phenotype by transferring the mutants onto new plates containing different concentrations of D-cycloserine (20, 40, 80 and 160 mg/L).

Drug susceptibility testing

Cultures (2–3 weeks old) of *M. tuberculosis* H37Rv included as a drug-susceptible control and BCG included as a resistant control were plated at 1:100 dilution on 7H11 agar plates containing 0, 5, 10, 20, 30 and 40 mg/L D-cycloserine. The plates were incubated at 37 °C for 4 weeks. Drug susceptibility testing of the D-cycloserine-resistant mutants of *M. tuberculosis* was performed on 7H11 agar plates containing 0, 20, 40, 80 and 160 mg/L D-cycloserine. The mutants that were consistently resistant to D-cycloserine were further analysed by WGS or PCR DNA sequencing, as described below.

WGS analysis

Genomic DNA from 18 D-cycloserine-resistant mutants, as well as the parent strain *M. tuberculosis* H37Rv, was sequenced using MiSeq (Illumina, Inc.) as described previously,[17] except that paired-end sequencing libraries were constructed using Nextera XT DNA Sample Preparation kits (Illumina, Inc.) following manufacturer's instruction. For each isolate, 500 M to 1.5 G base (110-fold to 350-fold genome coverage) sequences were generated after barcodes were trimmed. Single-nucleotide variants (SNVs) and insertions and deletions (InDels) ranging from 1 to 5 bp were sorted and called with more than 4 reads using *M. tuberculosis* H37Rv genome (NC_018143.1) as a reference. Mutations in proline-glutamic acid (PE)/proline-proline-glutamic acid (PPE) family genes and regions having repetition sequences were excluded from the analysis. Mutations in the parent strain *M. tuberculosis* H37Rv compared with the genome online (NC_018143.1) were also excluded from the analysis.

PCR and DNA sequencing

The genomic DNA from D-cycloserine-resistant mutants isolated *in vitro* was then subjected to PCR amplification using primers described in Table 1. The PCR products were obtained using the amplification parameters and sequenced by the Sanger method to confirm the mutations in these genes in selected mutants.

Table 1. Primers and PCR conditions used to verify gene mutations in D-cycloserine-resistant mutants

Gene	Primer	Sequence (5′–3′)
alr	alr_F2	GAAAATAAAAGACACGCCTACTTTCGCTCCA
	alr_R2+300bp	GACATCCATCGCCATGGCAATACCCTT
rv0759c	Rv0759c-For	AAAGCCGAAATCACTGAGGCTGCGGG
	Rv0759c-Rev	TAAGGAGGCCGTCGGCGCCTTCTTC
rv1403c	Rv1403c-For	AATTCGCCGGCGCTAAACGGGAGG
	Rv1403c-Rev	ATCAGTTCGGCGCCGACCAACCG
rv1435c	Rv1435c-For	AGCCGCCGCGTCCGGGCTTAA
	Rv1435c-Rev	AGGCCCGGTAGAAGTTGCGTCCGAT
rv1726	Rv1726-F	AGTACTGGCCGTACGGCTGCA
	Rv1726-R	GAGGAGTAGCTGTCGGCTTCTATCG
rv1731	Rv1731-For	GGCCGGTTCTTCGTGGTAACGTGCC
	Rv1731-Rev	CTGTGCCTTACGGGGCTTCAGCAGG
rv2831	Rv2831-F	GCCACTTCATCAAGCAAGGAGAG
	Rv2831-R	CTCTTCAACAGCCGCACCGAG
rv3331	Rv3331-For	ACACTGATCGAACCCGACCCGTCGC
	Rv3331-Rev	CCGTGGTGGTCAGCAACTCCTGTTCTC
rv3690	Rv3690-F	GTATTGCAGACCGGCTTAGAAGCC
	Rv3690-R	GTCAAATAGGTGCCGATCGAGG
rv3772	Rv3772-For	TGAGCTACGGGCGCTTGTCTTCAGTTG
	Rv3772-Rev	GTGTGGTCGGTCAACGGCACCTGGA

Conditions: 95 °C for 5 min; 35 turns of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 90 s; and finally 72 °C for 5 min.

Results and discussion

Isolation of *M. tuberculosis* H37Rv mutants resistant to D-cycloserine

The D-cycloserine MIC for the susceptible *M. tuberculosis* H37Rv parent strain was found to be 10–20 mg/L, while the naturally resistant *M. bovis* BCG was resistant to 40 mg/L but susceptible to 80 mg/L D-cycloserine. To isolate mutants resistant to D-cycloserine, about 10^8 *M. tuberculosis* bacteria were plated on 7H11 plates containing different concentrations of D-cycloserine (20, 40, 80 and 160 mg/L). After 4 weeks of incubation, no mutants grew on plates containing D-cycloserine at 80 mg/L or higher. We found that only two mutants (DT61-1 and DT69-1) grew on plates containing 40 mg/L D-cycloserine and 16 mutants were low-level resistant and were obtained on plates containing 20 mg/L D-cycloserine. The mutation frequency of resistant mutants to 20 mg/L D-cycloserine was found to be about 2×10^{-8}.

Mutations identified in D-cycloserine-resistant mutants by WGS

WGS of the 18 D-cycloserine-resistant mutants showed that 16 isolates had only one mutation (SNV or InDels) and 2 isolates had two mutations. In total, 16 different gene mutations were identified in the 18 mutants (Table 2) and it is of interest to note that none of these mutations was dominant. Non-synonymous mutations in

Table 2. Mutations identified in D-cycloserine-resistant mutants of *M. tuberculosis* by WGS analysis

Strain ID	MIC of cycloserine (mg/L)	Mutation type	Locus_tag	Nucleotide mutation	Amino acid change	Gene product
DT69-1	40	non-synonymous	*alr* (*rv3423c*)	C1030T	D344N	Alr
DT61-1	40	intergenic SNV	*PE_PGRS50* (*rv3345c*)	G(−52)T	—	PE family
		non-synonymous	*alr* (*rv3423c*)	C1030T	D344N	Alr
DT16-2	20	non-synonymous	*rv0059*	C278T	A93V	hypothetical protein (heterogeneous)
DT63-1	20	non-synonymous	*rv0221*	C166T	P56S	acyltransferase, wax ester synthase, diacylglycerol acyltransferase and monoacylglycerol acyltransferase (WS/DGAT/MGAT) (heterogeneous)
DT72-1	20	intergenic insertion	*rv0759c*	(−27) Ins C	—	hypothetical protein
DT71-1	20	non-synonymous	*betP* (*rv0917*)	T941G	L314R	betaine–carnitine–choline transporter (BCCT) family transporter
DT1-1	20	non-synonymous	*rv1403c*	T19C	T7A	methyltransferase
DT76-1	20	intergenic SNV	*rv1435c*	T(−150)C	—	hypothetical protein
DT82-4	20	intergenic SNV	*rv1435c*	T(−150)C	—	hypothetical protein
DT15-2	20	non-synonymous	*rv1683*	G1126A	A376T	long-chain acyl-CoA synthase
DT81-2-1	20	non-synonymous	*rv1726*	T440C	V147A	oxidoreductase
DT81-2	20	non-synonymous	*rv1726*	T440C	V147A	oxidoreductase
DT79-6	20	non-synonymous	*gabD2* (*rv1731*)	A92C	E31A	succinate-semialdehyde dehydrogenase
DT9-1	20	non-synonymous	*rv2749*	C4T	P2S	hypothetical protein (heterogeneous)
DT21-5	20	non-synonymous	*sugI* (*rv3331*)	C16T	Q6stop	sugar-transport integral membrane protein, SugI, MFS transporter
DT6-6	20	intergenic SNV	*PE_PGRS50* (*rv3345c*)	G(−52)T	—	PE family
DT3-2	20	non-synonymous	*rv3690*	G103A	D35N	hypothetical protein
		non-synonymous	*echA16* (*rv2831*)	G253T	A85S	enoyl-CoA hydratase
DT12-2	20	non-synonymous	*hisC2* (*rv3772*)	T110C	L37P	histidinol-phosphate aminotransferase (heterogeneous)

alr (*rv3423c*), *rv0059*, *betP* (*rv0917*), *rv0221*, *rv1403c*, *rv1683*, *rv1726*, *gabD2* (*rv1731*), *rv2749*, *sugI* (*rv3331*) and *hisC2* (*rv3772*), a single mutation in the 5′ intergenic region of *rv3345c* and *rv1435c*, and an insertion in the 3′ region of *rv0759c* were identified as solo mutations in their respective cycloserine-resistant mutants, suggesting these mutations associated with cycloserine resistance are highly diverse. In addition, one mutant (DT61-1) had two mutations in *alr* (*rv3423c*), as well as a −52 G→T change in *rv3345c* (hypothetical protein), while the other mutant (DT3-2) had double mutations in both *rv2831* and *rv3690* (Table 2). Ten mutated genes (*rv3690*, *rv0739c*, *rv1403c*, *rv1435c*, *rv2831*, *rv1726*, *rv1731*, *rv3331*, *rv3772* and *alr*) were verified by the Sanger sequencing method using primers and PCR conditions as described in Table 1.

The two mutants DT61-1 and DT69-1 that had higher resistance to D-cycloserine (40 mg/L) had the same non-synonymous mutation in the *alr* gene (nucleotide change C1030T, causing amino acid change D344N). Except for *alr*, which is known to be involved in D-cycloserine resistance,[5,6] the remaining 15 are novel and have not been reported previously. These 15 mutations include three lipid metabolism proteins (Rv0221, Rv1683, Rv2831), two transport proteins (BetP, SugI), one toxin/antitoxin (Rv0059), three proteins involved in intermediary metabolism and respiration (Rv1726, GabD2, HisC2), one methyltransferase (Rv1403c), one PE family protein (PE_PGRS50) and four unknown hypothetical proteins (Rv0759c, Rv1435c, Rv2749 and Rv3690). *rv0059* is the toxin of a toxin/antitoxin module (Rv0059 and Rv0060).[18]

One mutant (DT21-5) had an N-terminal stop codon mutation in the *sugI* gene, which resulted in complete loss of its protein function (Table 2). The *sugI* gene encodes a probable sugar-transport integral membrane protein in *M. tuberculosis*.[19] Since the cycloserine structure is similar to natural furanose and the *sugI* mutation is the only mutation detected in the genome, D-cycloserine could use SugI as the transporter for intake into the cell. The loss-of-function mutation in *sugI* could result in a lower uptake of cycloserine inside the cell, therefore leading to higher resistance to D-cycloserine. This is consistent with the previous observation that a transport protein involved in alanine and serine uptake was implicated in the uptake and resistance of D-cycloserine.[4] BetP was the other transport protein whose SNV was detected in a different mutant DT71-1. BetP transports molecules with a quaternary ammonium group such as betaine, carnitine and choline. Whether mutations in SugI and BetP could cause resistance through altering the transport of D-cycloserine remains a matter of investigation for future studies.

Previous studies have attempted to identify the molecular basis of D-cycloserine resistance based on the analysis of only a few isolated D-cycloserine-resistant mutants.[4,13] This study analysed the largest number of D-cycloserine-resistant mutants so far. It is worth noting that among the known D-cycloserine resistance-associated genes, we found mutations in *alr* in only 2 of 18 D-cycloserine-resistant mutants (11%), but in none of the other known D-cycloserine resistance-associated genes (*ddl*, *cycA* or *ald*).

Instead, we found mutations in 16 novel genes that are associated with D-cycloserine resistance. Our finding of *alr* mutation in D-cycloserine-resistant mutants is consistent with a previous study that demonstrated Alr to be the primary target of D-cycloserine,[7] but is in contrast to a recent study suggesting that Ddl is a target of D-cycloserine.[11,20] However, this could be a reflection of the relatively small number of strains (18 mutants) being analysed. Future investigations on more strains are required to better determine the frequency of the mutations in *alr* and *ddl*, as well as the new genes we identified in this work in D-cycloserine-resistant clinical strains. In addition, the role of the 16 novel genes in D-cycloserine resistance should be addressed in the future by molecular studies, such as overexpression studies, as well as by point mutation constructions and biochemical studies.

Although identification of mutations associated with resistance is generally useful for rapid molecular detection of drug resistance, the finding of diverse genes and mutations without significant clustering or conservation of resistance mechanisms associated with D-cycloserine resistance indicate it would be a challenge to develop a rapid molecular test to identify D-cycloserine resistance based on detection of mutations in candidate genes. This may partly explain why there are hardly any studies that find a good correlation between D-cycloserine resistance in MDR/XDR-TB strains and mutations in the genes *alr* and *ddl* known to be involved in D-cycloserine resistance. Future studies are needed to determine if D-cycloserine-resistant MDR/XDR-TB strains harbour any of the mutations associated with D-cycloserine resistance identified in this study. It is worth noting that the D-cycloserine-resistant mutants we identified in this study are of low-level resistance (MIC = 20–40 mg/L), given the MIC for the parent strain H37Rv is about 10–20 mg/L. The clinical relevance of the low-level D-cycloserine resistance is unclear as the achievable serum concentration of D-cycloserine in patients is 20–35 mg/L. This will raise issues about a suitable cut-off for resistance. Additional MIC testing may be necessary in clinical strains to determine an adequate epidemiological cut-off (ECOFF) that separates susceptible and resistant populations or microbiological breakpoints, and an adequate resistant population cut-off (RCOFF) that correlates with clinical breakpoints and outcome. Future studies are needed to address the clinical significance of the low-level resistant strains in animals and humans and to establish an appropriate cut-off for resistance.

Conclusions

In conclusion, we identified novel mutations associated with D-cycloserine resistance in *M. tuberculosis*. Our findings indicate that the mechanisms of D-cycloserine resistance are quite complex in *M. tuberculosis* and involve genes participating in different cellular functions, such as lipid metabolism, methyltransferase, the stress response and transport systems. This study provides useful information for improved understanding of the molecular basis of D-cycloserine resistance and mechanisms of action and for molecular detection of D-cycloserine resistance for improved treatment of MDR-TB. Future studies are needed to validate our findings in clinical isolates with D-cycloserine resistance and to address the role of the newly identified mutations in causing D-cycloserine resistance in *M. tuberculosis*.

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Transparency declarations

None to declare.

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