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

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Abstract

Objectives D-cycloserine (DCS) is an important second-line drug used to treat multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis. However, the mechanisms of resistance to DCS are not well understood. Here we investigated the molecular basis of DCS resistance using in vitro isolated resistant mutants of *Mycobacterium tuberculosis*.

Methods *M. tuberculosis* H37Rv was subjected to mutant selection on 7H11 agar plates containing varying concentrations of DCS. A total of 35 DCS-resistant mutants were isolated and 18 mutants were subjected to whole genome sequencing. The identified mutations associated with DCS resistance were confirmed by PCR-Sanger sequencing.

Results We identified mutations in 17 genes that are associated with DCS resistance. Except mutations in *alr (rv3423c)* which is known to be involved in DCS resistance, 16 new genes *rv0059*, *betP* (*rv0917*), *rv0221*, *rv1403c*, *rv1683*, *rv1726*, *gabD2* (*rv1731*), *rv2749*, *sugI* (*rv3331*), *hisC2* (*rv3772*), single mutation in 5' intergenic region of *rv3345c* and *rv1435c*, and insertion in 3' region of *rv0759c* were identified as solo mutations in their respective DCS-resistant mutants. Our findings indicate that the mechanisms of DCS resistance are more complex than previously thought and involve genes participating in different cellular functions such as lipid metabolism, methyltransferase, stress response, and transport proteins.

Conclusions New mutations in diverse genes associated with DCS are identified, which shed new light on the mechanisms of action and resistance of DCS. Future studies are needed to verify these findings in clinical strains so that molecular detection of DCS resistance for

43 improved treatment of MDR-TB can be developed.

44

45 **Introduction**

46 D-Cycloserine (DCS) is a cyclic analog of D-alanine and is a broad-spectrum antibiotic that
47 inhibits the growth of Gram-positive and Gram-negative bacteria. Although DCS has
48 psychiatric and nervous system adverse reactions, it displays no cross-resistance with any
49 other known antitubercular drugs [1]. DCS is an important second-line drug for the treatment of
50 multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis [1], and is
51 currently classified as a Group C agent for the treatment of MDR-TB treatment by WHO [2].

52

53 Since DCS is a structural analog of D-alanine, enzymes whose substrates are D-alanine are
54 the drug targets in mycobacteria [3-5]. These enzymes include D-alanine racemase (Alr) and d-
55 alanine:d-alanine ligase (Ddl), which are required for the synthesis of peptidoglycan in the
56 mycobacterial cell wall. Overexpression of *alr* and *ddl* has been shown to cause resistance to
57 DCS in *M. smegmatis* [6, 7]. Moreover, single nucleotide polymorphisms in these genes were
58 also found in resistant *M. tuberculosis* [8-10]. Consistent with the cell wall peptidoglycan being
59 a target of DCS, previous studies have shown that DCS competitively inhibits both Alanine
60 racemase (Alr) and D-alanine-D-alanine ligase (Ddl) [6, 11]. However, more recent metabolomic
61 study showed that Ddl is a primary target of DCS that is preferentially inhibited over alanine
62 racemase (Alr) in *M. tuberculosis* [12]. In addition, CycA is a transporter protein of D-alanine,
63 D-serine and glycine D-serine/alanine/glycine [13], and its single nucleotide polymorphism

(SNP) may partially contribute to the natural resistance to D-cycloserine in BCG [10, 14]. *ald* (*Rv2780*), encoding L-alanine dehydrogenase, was the fourth gene in *M. tuberculosis*, whose mutation was found in DCS resistant clinical isolates [10]. However, mutations in *cycA* and *ald* only contribute to very low level resistance, and Ddl mutation was rarely found in studies without known phenotype in MDR/XDR-TB strains [10].

Despite the above progress and the significant advancements in general about the molecular understanding of drug resistance mechnanisms in *M. tuberculosis* [15], the distribution and characterization of DCS resistance in clinical strains are still vague. This is partly due to the technical difficulties with DCS susceptibility testing such that routine phenotypic assay is not performed in clinical labs [16, 17], as well as poor understanding of the molecular basis of resistance to this drug.

To better understanding the mechanisms of DCS resistance and to develop more rapid molecular tests for detection of its resistance, we characterized 35 DCS-resistant mutants isolated in vitro from *M. tuberculosis* H37Rv and discovered a panel of new unique mutations that are associated with DCS resistance that have not previously been reported.

Materials and methods

DCS resistant mutant isolation

85 One-month-old *M. tuberculosis* H37Rv cultures grown in 7H9 liquid medium supplemented
86 with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment
87 were plated on 7H11 plates containing 20, 40, 80, 160, 320 mg/L cycloserine. After
88 incubation at 37°C for 4 weeks, the mutant colonies were picked to confirm the drug
89 resistance phenotype by transferring the mutants onto new plates containing different
90 concentrations of DCS (20, 40, 80, 160 mg/L).
91
92 **Drug susceptibility testing**
93 Drug susceptibility testing of the DCS-resistant mutants was performed on 7H11 agar plates
94 containing 0, 20, 40, 80, 160 mg/L cycloserine *M. tuberculosis* H37Rv was included as a
95 drug-susceptible control and BCG was included as a resistant control. The susceptible strain
96 H37Rv did not grow on DCS-containing plates and only mutants that were consistently
97 resistant to DCS were further analyzed by PCR and whole genome sequencing (WGS), as
98 described below.
99
100 **Whole genome sequencing analysis**
101 Genomic DNA from 18 cycloserine-resistant mutants, as well as the parent strain *M.*
102 *tuberculosis* H37Rv, was sequenced using MiSeq (Illumina, Inc.) as described previously [18],
103 except that paired-end sequencing libraries were constructed using Nextera XT DNA Sample
104 Preparation kits (Illumina, USA) following manufacturer's instruction. For each isolate, 500
105 M to 1.5 G bases (110-fold to 350-fold genome coverage) sequences were generated after

106 barcodes were trimmed. Single-nucleotide variants (SNVs) and insertions and deletions
107 (InDels) ranging from 1 to 5 bp were sorted and called at a minimum coverage of 4 reads
108 using *M. tuberculosis* H37Rv genome (NC_018143.1) as a reference. Mutations in PE/PPE
109 family genes and regions having repetition sequences were excluded from the analysis.
110 Mutations in the parent strain *M. tuberculosis* H37Rv comparing with the genome online
111 (NC_018143.1) were also excluded from the analysis.
112
113 **PCR and DNA sequencing**
114 The genomic DNA from DCS-resistant mutants isolated in vitro was then subjected to PCR
115 amplification using primers listed in Table 1. The PCR products were obtained using the
116 amplification parameters listed in Table 1 and sequenced by Sanger method to confirm the
117 mutations in these genes in selected mutants.
118
119 ## Results and discussion
120
121 **Isolation of *M. tuberculosis* H37Rv mutants resistant to DCS**
122 The cycloserine MIC for the sensitive *M. tuberculosis* H37Rv parent strain was found to be
123 below 20 mg/L. To isolate mutants resistant to DCS, about 10^8 *M. tuberculosis* bacteria were
124 plated on 7H11 plates containing different concentrations of DCS (20, 40, 80, 160, 320
125 mg/L). After 4 weeks of incubation, no mutants grew on plates containing DCS higher than
126 80 mg/L. We found that only two mutants DT61-1 and DT69-1 grew on plates containing 40

mg/L cycloserine, and 35 mutants were obtained on plates containing 20 mg/L cycloserine. The mutation frequency of resistant mutants to 20 mg/L cycloserine was found to be about 2 x 10^{-8}.

Mutations identified in DCS resistant mutants by WGS

WGS of 18 DCS resistant mutants showed that 16 isolates had only 1 mutation (SNV or InDels) and 2 isolates had 2 mutations. Totally 17 different gene mutations were identified in 18 mutants (Table 2), and it is of interest to note that none of these mutations were dominant. Nonsynonymous mutations in *alr (rv3423c)*, *rv0059*, *betP* (*rv0917*), *rv0221*, *rv1403c*, *rv1683*, *rv1726*, *gabD2* (*rv1731)*, *rv2749*, *sugI* (*rv3331)*, *hisC2* (*rv3772)*, single mutation in 5' intergenic region of *rv3345c* and *rv1435c*, and insertion in 3' region of *rv0759c* were identified as the solo mutation 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 mutation 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 which had higher resistance to DCS (40 mg/L) had the same nonsynonymous mutation in alanine racemase gene *alr*, (nucleotide change C1030T,

148 causing amino acid change of D344N). Except for *alr*, which is known to be involved in DCS
149 resistance [6, 7], the remaining 16 are novel and have not been reported previously. These 16
150 mutations include 3 lipid metabolism (Rv0221, Rv1683, Rv2831), 2 transport proteins (BetP,
151 SugI), 1 toxin/antitoxin (Rv0059), 3 intermediary metabolism and respiration (Rv1726, gabD2,
152 HisC2), 1 methyltransferase (Rv1403c), 1 PE family protein (PE_PGRS50), and 5 unknown
153 hypothetical proteins (Rv0759c, Rv1435c, Rv2749, Rv3345c, Rv3690). *rv0059* is toxin of a
154 TA-module (Rv0059 and Rv0060) [19].
155
156 One mutant (DT21-5) had an N-terminal stop codon mutation in the *sugI* gene, which resulted
157 in complete loss of its protein function (Table 2). The *sugI* encodes a probable sugar-transport
158 integral membrane protein in *M. tuberculosis* [20]. Since the cycloserine structure is similar to
159 natural furanose and *sugI* is the solo mutation detected in the genome, DCS could use SugI as
160 the transporter for intake into the cell. The loss of function mutation in *sugI* could result in a
161 lower uptake of cycloserine inside the cell and therefore leading to higher resistance to DCS.
162 This is consistent with the previous observation that a transport protein involved in alanine and
163 serine uptake was implicated in the uptake and resistance of DCS [5]. *betP* was the other transport
164 protein whose SNV was detected in a different mutant DT71-1. BetP transports molecules with
165 a quaternary ammonium group like betaine, carnitine and choline. Whether mutations in SugI
166 and BetP could cause resistance through alterating the transport of DCS remains further
167 investigation in future studies.
168

169 Five genes encoding hypothetical proteins were identified in the mutants, including intergenic
170 SNVs of *rv3345c*, *rv0759c* and *rv1435c*, and non-synonymous SNVs of *rv3690* and *rv2749*.
171 Rv3345c seems to be a stress response protein, as it is regulated by *sigD* in strain H37Rv [21].
172 The mutation is located in the promoter region of *rv3345c*, which may alter its expression.
173 Rv3690 is a probable conserved membrane protein, and its expression was found to be elevated
174 in MDR-TB strains [22]. Although the role of *rv0759c* is not known, *rv0759c* may be an important
175 gene in stress response. The intergenic region of this gene was predicted to be directly bound
176 by SigF, SigC and SigK [23], and the control by SigF and SigH in heat stress were verified by Q-
177 PCR [24, 25]. Future studies are needed to address the role of the 5 intergenic regions in genes
178 involved in stress response and membrane ptoteints in causing DCS resistance.
179
180 Rv1403c together with Rv1405c are important methyltransferase in *M. tuberculosis*. Their
181 expression was very low under *in vitro* non-stressed and *ex vivo* conditions, but highly
182 upregulated under *in vivo* and stressed conditions *in vitro* [26]. They are both tightly regulated by
183 the product of the *Rv1404* gene, encoding a member of the MarR family of transcriptional
184 regulators and the stress condition upregulated their expression included acid shock, antibiotics
185 like thioridazine, detergent like sodium dodecyl sulfate, hypoxic conditions and macrophage
186 stress [26-30]. Mutation in *rv1403c* impaired its growth phenotype in acidified 7H9 medium
187 (pH5.5) compared with the wild type strain [30]. *rv1726* encodes an FAD-binding dehydrogenase
188 and its mutation was identified in 2 of 16 resistant mutants (DT81-2 and DT81-2-1) (Table 2).
189 This oxidoreductase is structurally similar to 6-hydroxy-D-nicotine oxidase (6-HDNO) of

190 *Arthrobacter oxidans* (29.5% identity), which oxidizes 6-hydroxy-D-nicotine to 6-hydroxy-N-
191 methylmyosmine.
192
193 *rv3772* encodes a histidinol-phosphate aminotransferase (HisC2), involved in the histidine-
194 biosynthetic pathway. The pathway leads to enzymatic synthesis of histidine from 5-
195 phosphoribosyl-1-pyrophosphate in ten steps, which is among the essential pathways required
196 for optimal growth of *M. tuberculosis* and is conserved in archaea, bacteria, fungi and plants
197 but not in mammals [31, 32]. The gene was shown to be involved in the adaptation to stress
198 response and host cell environment, as it is regulated by FurA, an oxidative stress sensing
199 regulator, and upregulated by rhodanine agent (D157070) in *M.*
200 *avium* ssp. *paratuberculosis*[33, 34]. *rv1731* encodes a succinate-semialdehyde dehydrogenase
201 (GabD2), which catalyzes the $NAD(P)^+$-coupled oxidation of succinic semialdehyde to
202 succinate, the last step of the γ-aminobutyrate (GABA) shunt [35].
203
204 Three non-synonymous mutations in 3 lipid metabolism genes, including *rv0221* encoding a
205 verified triacylglycerol synthase [36], *rv1683* encoding a long chain acyl-CoA synthase and
206 *rv2831* encoding an enoyl-CoA hydratase were identified in 3 different mutants. *rv0221*
207 belongs to the RD10 of *M. bovis* BCG and is located near LipC (*Rv0220*), lipW (*Rv0217c*),
208 acyl-CoA synthetase (*Rv0214*), acyl-CoA dehydrogenase (*Rv0215c*), and an integral
209 membrane acyltransferase (*Rv0228*). These genes may be cotranscribed under specific stimuli
210 and may release fatty acid from triacylglycerol, carry out the transport of fatty acids and

211 catalyze the resynthesis of triacylglycerols in the pathogen [36, 37]. *rv0221* was demonstrated
212 up-regulated in intraphagosomal lesions and under multiple stresses [38, 39]. The acyl-CoA
213 synthase *Rv1683* is suspected to be essential for triacylglycerol hydrolysis and growth [37, 40],
214 and was shown to be a functional esterase in all active, dormant and reactivation culture
215 conditions [41]. Rv2831 encodes an enoyl-CoA hydratase/isomerase family protein (EchA16),
216 which is up-regulated in clinical MDR-TB strains and under exposure to mefloquine [22, 42]. As
217 the triacylglycerol storage is important for the bacteria to transform into the dormancy-like
218 state *in vitro* and survive during starvation, these three genes could be involved in bacterial
219 dormancy and prolonged survival under stress. In this study, the mutations in these lipid
220 metabolism genes may alter their enzyme activity, turn the bacteria into dormancy and
221 therefore increase the survival against cycloserine.
222
223 Previous studies have reported only a few isolated DCS mutants for identifcation of
224 molecular basis of DCS resistance [5, 14]. This study analyzed the largest number of DCS
225 resistant mutants so far. It is worth noting that among the known DCS resistance associated
226 genes, we only found a mutation in *alr* in 2 of 18 DCS-resistant mutants (11%) but in none of
227 the other known DCS associated resistance genes *ddl,* or *cycA* or *ald.* Instead, we found
228 mutations in 16 novel genes that are associated with DCS resistance. Our finding of *alr*
229 mutation in DCS resistant mutants is consistent with a previous study that demonstrated Alr
230 being a primary target of DCS [8] but is in contrast to a recent study suggesting Ddl being a
231 target of DCS [12, 43]. However, this could be a reflection of the relatively small number of

strains (18 mutants) being analyzed. Future investigations on more strains are required to better determine the frequency of the mutations in *alr*, *ddl*, as well as the new genes we identified in this work in DCS resistant clinical strains. In addition, the role of the 16 novel genes in DCS resistance should be addressed by molecular studies such as overexpression as well as point mutation constructions in future studies.

Conclusions

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

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253 **Transparency declarations**

254 None to declare.

255

256 **References**

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372 **Table 1. Primers and PCR conditions used to verify gene mutations in DCS resistant**

373 **mutants**

Genes	**primers**	sequence (5'-3')	condition
alr	**alr_F2**	GAAAATAAAAGACACGCCTACTTTCGCTCCA	95°C for 5min; 35 turns of 94°C for 30s, 62°C for 30s, 72°C for 90s; and finally 72°C for 5min
	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

374

375

376 **Table 2. Mutations identified in cycloserine resistant mutants of *M. tuberculosis* by**

377 **whole genome sequencing analysis**

Number	Strain ID	MIC to cycloserine (mg/L)	Mutation type	Locus_tag	Nucleotides mutation	Amino acid change	Gene product
DT69-1	DT69-1	40	Nonsyn	*alr* (*rv3423c*)	C1030T	D344N	Alanine racemase
DT61-1	DT61-1	40	Intergenic SNV	*rv3345c*	G(-52)T	-	Hypothetical protein
			Nonsyn	*alr* (*rv3423c*)	C1030T	D344N	alanine racemase
No.18	DT16-2	20	Nonsyn	*rv0059*	C278T	A93V	Hypothetical protein (heterogeneous)
No.9	DT63-1	20	Nonsyn	*rv0221*	C166T	P56S	Acyltransferase, WS/DGAT/MGAT (heterogeneous)
No.12	DT72-1	20	Intergenic insertion	Rv0759c	(-27) Ins C	-	Hypothetical protein
DT71-1	DT71-1	20	Nonsyn	*betP* (*rv0917*)	T941G	L314R	Betaine carnitine choline transporter BCCT family transporter
No.8	DT1-1	20	Nonsyn	*rv1403c*	T19C	T7A	Methyltransferase
No.15	DT76-1	20	Intergenic SNV	*rv1435c*	T(-150)C	-	Hypothetical protein
No.19	DT82-4	20	Intergenic SNV	*rv1435c*	T(-150)C	-	Hypothetical protein

DT15-2	DT15-2	20	Nonsyn	*rv1683*	G1126A	A376T	Long chain acyl-CoA synthase
No.1	DT81-2-1	20	Nonsyn	*rv1726*	T440C	V147A	Oxidoreductase
DT81-2	DT81-2	20	Nonsyn	*rv1726*	T440C	V147A	Oxidoreductase
No.16	DT79-6	20	Nonsyn	*gabD2* (*rv1731*)	A92C	E31A	Succinate-semialdehyde dehydrogenase
No.4	DT9-1	20	Nonsyn	*rv2749*	C4T	P2S	Hypothetical protein (heterogeneous)
No.13	DT21-5	20	Nonsyn	*sugI* (*rv3331*)	C16T	Q6stop	Sugar-transport integral membrane protein, sugI, MFS transporter
DT6-6	DT6-6	20	Intergenic SNV	*PE_PGRS50* (*rv3345c*)	G(-52)T	-	PE family
DT3-2	DT3-2	20	Nonsyn	*rv3690*	G103A	D35N	Hypothetical protein
			Nonsyn	*echA16* (*rv2831*)	G253T	A85S	Enoyl-CoA hydratase
No.5	DT12-2	20	Nonsyn	*HisC2* (*rv3772*)	T110C	L37P	Histidinol-phosphate aminotransferase (heterogeneous)

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