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1 **A point mutation in *cycA* partially contributes to the D-cycloserine resistance trait of**
2 ***Mycobacterium bovis* BCG vaccine strains.**

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13

14 **Abstract**

15 In mycobacteria, CycA a D-serine, L- and D-alanine, and glycine transporter also functions in
16 the uptake of D-cycloserine, an important second-line anti-tubercular drug. A single nucleotide
17 polymorphism identified in the *cycA* gene of BCG was hypothesized to contribute to the
18 increased resistance of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) to D-cycloserine
19 compared to wild-type *Mycobacterium tuberculosis* or *Mycobacterium bovis*. Working along
20 these lines, a merodiploid strain of BCG expressing *Mycobacterium tuberculosis* CycA was
21 generated and found to exhibit increased susceptibility to D-cycloserine albeit not to the same
22 extent as wild-type *Mycobacterium tuberculosis* or *Mycobacterium bovis*. In addition,
23 recombinant *Mycobacterium smegmatis* strains expressing either *Mycobacterium tuberculosis*
24 or *Mycobacterium bovis* CycA but not BCG CycA were rendered more susceptible to D-
25 cycloserine. These findings support the notion that CycA-mediated uptake in BCG is impaired
26 as a result of a single nucleotide polymorphism; however, the partial contribution of this
27 impairment to D-cycloserine resistance suggests the involvement of additional genetic lesions in
28 this phenotype.

29

30 **Introduction**

31 *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), is a live vaccine originally derived
32 from a virulent isolate of *Mycobacterium bovis*, and has been used to immunize more than three
33 billion people against tuberculosis [1]. Despite widespread use, the protective efficacy imparted
34 by BCG wanes significantly with time, and the molecular mechanism for this is poorly
35 understood [1,2]. Additionally, the mechanisms underlying the derivation of attenuated BCG
36 from virulent *M. bovis* also remain incompletely understood [1,2]. To further complicate matters,
37 13 strains of BCG with documented differences in attenuation and protective efficacy currently
38 exist, and several of these are in use in various parts of the world [3,4]. Clearly, the basic
39 biology of BCG is far from being fully understood. The availability and simultaneous analyses of
40 complete genome sequences of several *M. bovis* and BCG strains however have generated
41 fresh insights into the biology of BCG [1,4-6]. Indeed, the demonstration that loss of the RD1
42 locus by BCG was a key event leading to its attenuation, originated from such comparative
43 genome studies [7].

44 D-cycloserine (DCS), a cyclic analog of D-alanine, is an important second-line antibiotic
45 used to treat multi-drug- and extensively drug-resistant *Mycobacterium tuberculosis* infections
46 [8,9]. Compared to wild-type *M. tuberculosis* and *M. bovis*, BCG has always been found to be
47 more resistant to DCS [6,10]. Although the molecular basis for this phenotype is unknown, this
48 feature is used to differentiate the vaccine strain from *M. bovis* and *M. tuberculosis* strains [10].
49 *In vitro*, DCS inhibits *M. tuberculosis* alanine racemase (Alr) which converts L-alanine to D-
50 alanine, and D-alanine:D-alanine ligase (Ddl) which synthesizes D-alanine pentapeptides
51 [8,11,12]. Both enzymes are required for the synthesis of peptidoglycan in the cell wall of
52 mycobacteria [8,11,12]. Overproduction of Alr and Ddl in BCG and *Mycobacterium smegmatis*
53 confers increased DCS resistance [13,14], while their genetic inactivation in *M. smegmatis*
54 increases susceptibility to the drug [15,16]. The resistance determinants and the cellular targets

55 of DCS in *M. tuberculosis* and *M. bovis* are presumed to be Alr and Ddl, both of which are
56 essential in *M. tuberculosis* [17]. However, the nucleotide sequences and expression patterns of
57 *alr* and *ddl* in *M. bovis* and BCG appear to be identical and as such, might not contribute to the
58 DCS resistance of the vaccine strain [1,6].

59 A non-synonymous single nucleotide polymorphism (nsSNP) in the *cycA* gene of all
60 BCG strains was recently identified and predicted to result in a glycine 122 to serine substitution
61 (G122S) in the transporter [6]. CycA is a bacterial D-serine/L- and D-alanine/glycine/D-
62 cycloserine: proton symporter, comprising 12 helical trans-membrane domains, of the amino
63 acid transporter (AAT) family (TCID: 2.A.3.1.7) [18]. In the early 1970's, David isolated and
64 characterised two low-level and equally DCS-resistant forms of *M. tuberculosis* mutants - one
65 that was also D-serine, L- and D-alanine, and glycine uptake defective, and the other uptake
66 competent [19]. Point mutations resulting in transitions and transversions, as well as small
67 deletions and duplications within the *cycA* gene of *Escherichia coli*, have been found to confer
68 resistance to DCS [20]. Inspired by these reports, it was hypothesized that the G122S change
69 functionally impairs BCG CycA and contributes to the vaccine strain's characteristic resistance
70 to DCS [6].

71 A bioinformatics analysis shows that the G122S change in BCG CycA lies in a
72 presumably critical extracellular loop between the conserved third and fourth helical trans-
73 membrane domains. Taking a genetic approach we show that BCG CycA is indeed impaired for
74 DCS uptake, however the G122S change only partially contributes to DCS resistance in BCG,
75 thus implicating additional mutations in this phenotype.

76

77 **Results**

78 ***In silico* analyses and helical trans-membrane domain modeling predicts a functionally**
79 **important extracellular loop in CycA.**

80 Comparison of CycA orthologues from six different mycobacteria revealed 72-99 %
81 identity at the amino acid sequence level. Multi-species protein sequence alignment shows that
82 CycA from all mycobacterial strains, except BCG possess a conserved glycine residue in
83 position 122 (Figure 1A). Helical trans-membrane (HTM) domain modeling of the *M. bovis* AF
84 2122/97 CycA based on HMMTOP prediction [21], shows the transporter to be a cell membrane
85 protein with 12 HTM domains (Figure 1B). The G122S change in BCG CycA is predicted to lie in
86 an extracellular loop between the third and fourth trans-membrane helices (Figure 1B). Similar
87 modeling of the HTM domains of *M. tuberculosis* H37Rv, *M. marinum* M and *M. smegmatis*
88 mc²155 CycA also place the conserved glycine residue at the beginning of the extracellular loop
89 between the third and fourth trans-membrane helices. Single point mutations in the *cycA* gene
90 have been identified in DCS-resistant *E. coli* mutants [20]. Therefore *E. coli* CycA was also
91 modeled and DCS-resistance-associated single point mutations were mapped to the model.
92 Like the mycobacterial CycA proteins, *E. coli* CycA was predicted to form a membrane protein
93 with 12 HTM domains. Of twenty previously identified point mutations, we were able to place a
94 threonine 114 to proline substitution (from an adenine to cytosine transversion) [20], in the
95 extracellular loop between the third and fourth trans-membrane helices of *E. coli* CycA
96 (Supplemental figure S1).

97 Our *in silico* analyses suggest that the extracellular loop between the third and fourth
98 trans-membrane helices of CycA may be sensitive to modifications and is functionally important
99 for transport activity.

100

101 **Merodiploid expression of *M. tuberculosis* CycA in BCG partially restores susceptibility**
102 **to D-cycloserine.**

103 A merodiploid strain of *M. bovis* BCG-Pasteur was generated by transformation with an
104 integrating cosmid (I425) containing the *M. tuberculosis* *cycA* allele. Sequencing from the 5'-end
105 of the *cycA* PCR product amplified from the genomic DNA of a Pasteur::I425 knock-in clone
106 showed both GGC (encoding Gly) and AGC (encoding Ser) sequences, indicating the
107 successful integration of the cosmid into the chromosome (Figure 2A). In contrast, a
108 Pasteur::pYUB412 control showed only the AGC (encoding Ser) sequence (Figure 2A). 3'-end
109 sequencing of *cycA* PCR products from both clones confirmed these findings.

110 The susceptibility of the Pasteur::I425 knock-in strain to DCS was assessed and
111 compared to that of the Pasteur::pYUB412 control strain. In 7H9 broth cultures containing
112 increasing concentrations of DCS (0, 2, 4, 8, 16 and 32 µg/mL), the Pasteur::pYUB412 strain
113 showed complete growth inhibition at > 32 µg/mL of the antibiotic, which is consistent with
114 published observations [10,13,22]. The Pasteur::I425 strain however, appeared to be more
115 susceptible to DCS, exhibiting significant growth inhibition at 16 and 8 µg/mL DCS compared to
116 the control strain – the colony forming units of the Pasteur::I425 strain recovered was almost 1-
117 log less at these concentrations of DCS than that of the control strain (Figure 2B). The
118 difference in susceptibility to DCS between the two strains was even more pronounced in
119 experiments measuring growth inhibition over time upon exposure to 32 µg/mL of the antibiotic.
120 Pasteur::I425 showed more rapid inhibition of growth by DCS compared to the
121 Pasteur::pYUB412 strain as the colony forming units of the knock-in strain recovered at all time-
122 points post-exposure was consistently 1-log lower than the control strain (Figure 2C). However,
123 we did not observe in the Pasteur::I425 strain, the restoration of wild-type *M. tuberculosis* or *M.*
124 *bovis* levels of DCS susceptibility, which is reportedly between 4 and 5 µg/mL of DCS [10,19].

125 We next wanted to verify that the higher susceptibility of the Pasteur::l425 strain to DCS
126 was not due to the integration of an extra copy of the *cycA* gene. Although *M. smegmatis*
127 mc²155 has a higher MIC for DCS [13,14] compared to members of the *M. tuberculosis*
128 complex, we transformed this strain with l425 and pYUB412 to test if we could obtain a similar
129 fold increase in susceptibility to DCS as seen with BCG. Using *M. tuberculosis cycA*-specific
130 primers we confirmed the successful integration of the cosmid prior to testing with DCS (data
131 not shown). Growth of wild-type *M. smegmatis* mc²155 is inhibited by DCS at ≥ 75 $\mu\text{g/mL}$
132 [13,14], and consistent with this, the *M. smegmatis*::pYUB412 strain exhibited complete growth
133 inhibition at ≥ 80 $\mu\text{g/mL}$ DCS (Figure 2D). The growth of the *M. smegmatis*::l425 knock-in strain
134 was inhibited by DCS to the same extent as the *M. smegmatis*::pYUB412 control strain (Figure
135 2D). Likewise, time-course experiments measuring growth inhibition by DCS also did not show
136 significant differences between the two strains (Figure 2E). This shows that having one extra
137 copy of *M. tuberculosis cycA* does not appreciably increase DCS susceptibility in *M. smegmatis*.
138 All of these observations taken together indicate that the increased DCS susceptibility of
139 Pasteur::l425 results from the production of a functional CycA in a genetic background
140 expressing either a non-functional or impaired CycA.

141

142 ***M. smegmatis* overexpressing BCG CycA does not become more susceptible to DCS.**

143 Heterologous expression of *M. tuberculosis* proteins involved in efflux and drug
144 resistance in *M. smegmatis* has been an effective approach to study their function [23-25]. We
145 hypothesized that if *M. bovis* BCG CycA is defective, then *M. smegmatis* strains overexpressing
146 functional *M. tuberculosis* and *M. bovis* CycA but not BCG CycA, would become more
147 susceptible to DCS-mediated growth inhibition. Working along these lines, *M. tuberculosis*, *M.*
148 *bovis* and BCG *cycA* alleles and their promoter regions were individually cloned into the pMD31
149 plasmid for multi-copy ectopic expression in *M. smegmatis* mc²155. Colony PCR was used to

150 discriminate *bona fide* transformants harbouring plasmids containing *M. tuberculosis*, *M. bovis*
151 or BCG *cycA* alleles from those harbouring the empty pMD31 vector alone (data not shown).

152 The DCS sensitivities of these recombinant *M. smegmatis* strains were then tested in
153 7H9 broth containing increasing concentrations of DCS (0, 40, 80 or 160 µg/mL). *M. smegmatis*
154 strains producing *M. tuberculosis* and *M. bovis* CycA exhibited significantly increased
155 susceptibility to DCS-mediated growth inhibition compared to the vector-only control strain
156 (Figure 3A). In contrast, the susceptibility of *M. smegmatis* producing BCG CycA was similar to
157 that of the vector-only control strain (Figure 3A). Consistent with this observation, in the
158 presence of 200 µg/mL DCS, only *M. smegmatis* strains producing *M. tuberculosis* and *M. bovis*
159 CycA but not BCG CycA exhibited significantly faster growth inhibition compared to the vector-
160 only control (Figure 3B). These results indicate that raising the gene-dosage of *cycA* readily
161 increases DCS susceptibility in *M. smegmatis*. More importantly it shows that BCG CycA is
162 defective because even in the *M. smegmatis* genetic background, raising the gene-dosage of
163 BCG *cycA* does not affect its susceptibility to DCS.

164

165 **Extra copies of *cycA* alleles in mycobacteria do not confer an in vitro growth advantage.**

166 Since CycA is a D-serine, L- and D-alanine, and glycine transporter, we wanted to verify
167 that the differences in DCS susceptibilities observed were not due to changes in the growth
168 rates of the strains tested. Accordingly growth measurements were made for all of the strains in
169 7H9 broth. No difference in growth between the merodiploid Pasteur::l425 and the
170 Pasteur::pYUB412 control strain were found (data not shown). Similarly no growth differences
171 between *M. smegmatis*::l425 and *M. smegmatis*::pYUB412 were observed (data not shown).
172 Consistent with the above results, *M. smegmatis* strains producing *M. tuberculosis*, *M. bovis*
173 and BCG CycA, also grew at rates similar to the control strain in 7H9 media (data not shown).

174 These findings indicate that expression of extra copies of *cycA* do not confer a growth
175 advantage to the host strain in the culture media used to test for susceptibility to DCS.

176

177

178

179 **Discussion**

180 We employed bioinformatics as well as two complementary genetic approaches to
181 investigate the involvement of the nsSNP in BCG *cycA* in DCS resistance. Evidence is provided
182 that suggests this nsSNP causes a defect in the BCG CycA transporter but only partially
183 contributes to DCS resistance in the vaccine strain.

184 Trans-membrane helical domain modeling places the BCG G122S mutation in an
185 extracellular loop between the conserved third and fourth helical trans-membrane domains of
186 CycA. This finding hints at possible substrate recognition or binding defects at the structural
187 level in BCG CycA and warrants further study. Consistent with the notion that BCG CycA is
188 defective for DCS uptake, heterologous overexpression of *CycA* from *M. tuberculosis* and *M.*
189 *bovis* but not from BCG in *M. smegmatis* results in increased susceptibility to DCS. This effect
190 of increased *cycA* gene-dosage on DCS susceptibility in *M. smegmatis* raises the question of
191 whether a decrease in CycA expression in BCG might result in increased resistance. However,
192 the identical expression patterns of *cycA* in *M. bovis* and BCG rule out this possibility [1,6]. More
193 importantly, the G122S mutation in BCG CycA appears to be a minor contributor as we were
194 unable to restore *M. tuberculosis* and *M. bovis* levels of DCS susceptibility to the merodiploid
195 Pasteur::I425 strain. This is not surprising as the isolation of two equally DCS-resistant mutants
196 of *M. tuberculosis*, one of which is transport defective and the other transport competent, has
197 been reported [19]. This strongly suggests there are other genetic lesions besides the nsSNP in
198 *cycA* that also contribute to DCS resistance in BCG. Indeed an *M. smegmatis* isolate, resistant
199 to both DCS and vancomycin was found to have no mutations in the *alr*, *ddl* or *cycA* genes [26].
200 Only multi-copy transformation of a gene encoding a putative penicillin-binding protein (Pbp4)
201 homolog restored DCS and vancomycin susceptibility to this strain [26]. Our analyses of *pbp*
202 genes in BCG however did not reveal any nsSNPs that might explain the BCG phenotype.
203 Moreover, a recent metabolomic study of *M. smegmatis* revealed that there may potentially be

204 alternative routes to D-alanine synthesis in mycobacteria that could circumvent DCS inactivation
205 of Alr and Ddl [12]. This notion is supported by the observation that *alr* mutants of *M. smegmatis*
206 are not complete D-alanine auxotrophs [12,16]. As such, genetic mutations in these alternate
207 alanine metabolic pathways could potentially augment CycA-mediated DCS resistance in BCG.

208 BCG underwent *in vitro* evolution during its initial derivation from a virulent *M. bovis*
209 progenitor strain as well as during its subsequent global dissemination [1,2]. A consequence of
210 this evolution was the acquisition of mutations causing defects in L-serine and L-alanine
211 catabolism, dysregulated nitrogen metabolic pathways and *in vitro* growth defects [27]. CycA is
212 the sole transporter for D-serine, L- and D-alanine, and glycine in mycobacteria, and their
213 uptake by the transporter is induced by L-alanine [19]. Although in this study BCG and *M.*
214 *smegmatis* strains bearing extra *cycA* alleles and their control strains grew similarly in 7H9
215 broth, growth differences may yet appear in L-serine and/or L-alanine-replete conditions not
216 found in 7H9 broth. A link between defective L-serine and L-alanine catabolism and the
217 acquisition of the CycA G122S mutation impairing D-serine, L- and D-alanine and DCS uptake
218 is intriguing and merits additional investigation as this will add to our understanding of BCG
219 nitrogen metabolism.

220 In conclusion, this study reveals the involvement of CycA in the resistance of BCG to
221 DCS and provides fresh insights into BCG biology as well as clues for the improvement of DCS
222 as an anti-mycobacterial agent.

223

224 **Materials and Methods**

225 **Reagents**

226 Restriction and DNA modifying enzymes were purchased from New England Biolabs (Ipswich,
227 MA, USA). High fidelity *Pfu* polymerase for PCR was purchased from Promega (Madison, WI,
228 USA). Custom oligonucleotides were synthesized by Microsynth (Balgach, Switzerland).
229 Middlebrook 7H9, 7H11 media, albumin-dextrose-catalase (ADC) and oleic acid-albumin-
230 dextrose-catalase (OADC) were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA).
231 All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

232

233 **Bacterial strains and growth conditions**

234 *M. tuberculosis* H37Rv, *M. bovis* BCG Pasteur 1173P2 and *M. smegmatis* mc²155 were
235 routinely cultured in 7H9 broth supplemented with ADC and 0.05% Tween-80 or on 7H11 agar
236 plates supplemented with OADC. For the selection of relevant mycobacterial clones,
237 hygromycin at 50 µg/mL and kanamycin at 25 µg/mL were used.

238

239 **Genetic manipulations and genotyping**

240 An integrating cosmid (I425) [28], bearing the *M. tuberculosis cycA* allele (*rv1704c*) was
241 transformed into *M. bovis* BCG-Pasteur 1173P2 and *M. smegmatis* mc²155 following standard
242 electroporation procedures [29]. pYUB412, the cosmid backbone, was electroporated to obtain
243 parental control strains. Genomic DNA was extracted from several *M. bovis* BCG colonies
244 obtained after selection on 7H11 agar plates containing hygromycin. Positive BCG-
245 Pasteur::I425 clones were verified by PCR amplification of the genomic DNA using *cycA*-
246 specific internal primers (*cycA*-int-Forward: agctgctgctgacactg and *cycA*-int-Reverse:
247 gttgggaagaaccgttgc) and sequencing of the PCR products. *M. smegmatis*::I425 clones were
248 obtained and identified by PCR amplification using *M. tuberculosis cycA*-specific primers

249 described above. *M. smegmatis*::pYUB412 served as control. Table 1 describes all strains
250 generated in this study.

251

252 **Plasmid constructions, generation and identification of recombinant *M. smegmatis*** 253 **strains over-expressing *M. tuberculosis*, *M. bovis* and BCG *CycA***

254 Multi-copy episomal vectors containing *cycA* alleles from *M. tuberculosis*, *M. bovis* and *M. bovis*
255 BCG were constructed as follows: *cycA* gene including 267 bp upstream of the start site was
256 amplified from *M. tuberculosis* H37Rv, *M. bovis* AF2122/97 and *M. bovis* BCG-Pasteur 1173P2
257 genomic DNA using high fidelity *Pfu* polymerase and primers *cycA-HindIII-Forward*:
258 *cagaagctt*atcgggtccgcccaact and *cycA-PstI-Reverse*: cgc*ctgcag*cgtgggcgaggacatag introducing
259 5'-*HindIII* (bold italics) and 3'-*PstI* (bold italics) restriction sites. The PCR fragments obtained
260 were digested with *HindIII* and *PstI*, purified and then ligated into *HindIII*- and *PstI*- linearized
261 pMD31 [30]. Plasmid clones containing *cycA* alleles were verified by sequencing before
262 electroporation into *M. smegmatis* mc²155. Positive transformants were identified by colony
263 PCR using the primers *cycA-HindIII-Forward* and *cycA-PstI-Reverse* described above.

264

265 **D-cycloserine susceptibility testing**

266 DCS is unstable in aqueous solutions buffered at pH 7 including Middlebrook 7H9 broth [8,31]
267 therefore working solutions of the antibiotic were prepared freshly in sodium phosphate buffer
268 (pH 9.0) just before use. Two-fold serial dilutions of DCS covering the required range of
269 concentrations were prepared in 7H9 broth (supplemented with ADC and 0.05% Tween-80) for
270 all experiments. For assessing DCS-sensitivity over time, the antibiotic was added to 7H9 broth
271 (supplemented with ADC and 0.05% Tween-80) at approximately 2X the reported MIC per strain
272 for every experiment. Colony forming units at different time points were determined by 7H11
273 agar plate spreading of serially diluted aliquots of cells.

274 **Statistics**

275 The unpaired, two-tailed *t* test was used to assess the statistical significance in differences
276 when comparing experimental groups using the GraphPad Prism program
277 (<http://www.graphpad.com>).

278

279 **CycA amino acid sequence analyses and helical trans-membrane domain modeling**

280 Alignments of CycA amino acid sequences of different mycobacteria obtained from TubercuList,
281 MarinoList and SmegmaList genome browsers (<http://mycobrowser.epfl.ch/>) [32] were done
282 using the MultAlin server (<http://multalin.toulouse.inra.fr/multalin/multalin.html>) [33]. Secondary
283 structure prediction was done using the PSIPRED protein structure prediction server
284 (<http://bioinf.cs.ucl.ac.uk/psipred/>) [34]. Prediction of trans-membrane domains in CycA amino
285 acid sequences was done using the HMMTOP (version 2) algorithm [21]. Two-dimensional
286 graphical representations of the trans-membrane topologies of CycA sequences were made
287 using the TMRPres2D program (<http://bioinformatics.biol.uoa.gr/TMRPres2D/index.jsp>) [35].

288

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292

293

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401 **Table 1. DNA and bacterial strains used in this work.**

Plasmid/Cosmid	Description	Reference
pMD31	Episomal, multicopy, Kan ^R , <i>oriE</i> , <i>oriM</i>	[30]
pMDcycA_{Mtb}	Episomal, multicopy, <i>M. tb cycA</i> , Kan ^R , <i>oriE</i> , <i>oriM</i>	This study
pMDcycA_{Mbov}	Episomal, multicopy, <i>M. bovis cycA</i> , Kan ^R , <i>oriE</i> , <i>oriM</i>	This study
pMDcycA_{BCG}	Episomal, multicopy, BCG <i>cycA</i> , Kan ^R , <i>oriE</i> , <i>oriM</i>	This study
pYUB412	<i>attB</i> site integrative cosmid backbone, Hyg ^R , Amp ^R , <i>oriE</i>	[28]
I425	<i>attB</i> site integrative cosmid with <i>rv1701</i> to <i>rv1733c</i> fragment, Hyg ^R , Amp ^R , <i>oriE</i>	[28]
Strains	Description	Reference
<i>M. tb</i> H37Rv	Wild-type	[36]
<i>M. smegmatis</i> mc²155	Wild-type	[28]
<i>M. bovis</i> BCG-Pasteur 1173P2	Vaccine strain	[1]
Pasteur::pYUB412	BCG-Pasteur::pYUB412, Hyg ^R	This study
Pasteur::I425	BCG-Pasteur::I425, Hyg ^R	This study
mc²155::pYUB412	<i>M. smegmatis</i> mc ² 155::pYUB412, Hyg ^R	This study
mc²155::I425	<i>M. smegmatis</i> mc ² 155::I425, Hyg ^R	This study
mc²155/pMD31	<i>M. smegmatis</i> mc ² 155+ pMD31, Kan ^R	This study
mc²155/pMDcycA_{Mtb}	<i>M. smegmatis</i> mc ² 155+ pMDcycA _{Mtb} , Kan ^R	This study
mc²155/pMDcycA_{Mbov}	<i>M. smegmatis</i> mc ² 155+ pMDcycA _{Mbov} , Kan ^R	This study
mc²155/pMDcycA_{BCG}	<i>M. smegmatis</i> mc ² 155+ pMDcycA _{BCG} , Kan ^R	This study

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403

404 **Figure legends.**

405 **Figure 1. Analyses of CycA amino acid sequences. (A)** Partial CycA amino acid sequence
406 alignment of various mycobacteria (the conserved G122 residue is indicated by the arrow). **(B)**
407 2-dimensional topological representation of *M. bovis* CycA with the G122S mutation found in all
408 BCG (circled) in the extracellular loop (boxed) between the 3rd and 4th trans-membrane helices
409 from the amino (N) terminus.

410
411 **Figure 2. Characterization of *cycA* merodiploid BCG and *M. smegmatis* strains. (A)**
412 Sequence of the SNP containing region in *cycA* from BCG-Pasteur::l425 and BCG-
413 Pasteur::pYUB412 clones from the 5' end. **(B)** CFUs of BCG-Pasteur::l425 (white bars) and
414 BCG-Pasteur::pYUB412 (black bars) 4 days after treatment with indicated concentrations of
415 DCS. **(C)** CFUs of BCG::l425 (solid squares) and BCG::pYUB412 (solid diamonds) obtained
416 over time upon exposure to 32 µg/mL DCS. **(D)** CFUs of *M. smegmatis*::l425 (white bars) and
417 *M. smegmatis*::pYUB412 (black bars) 24 hours after treatment with indicated concentrations of
418 DCS. **(E)** CFUs of *M. smegmatis*::l425 (solid squares) and *M. smegmatis*::pYUB412 (solid
419 diamonds) obtained over time upon exposure to 200 µg/mL DCS. Data points and error bars are
420 means ± standard deviations. Statistically significant differences are denoted by asterisks (*, P <
421 0.05; **, P < 0.01). Representative of 3 independent experiments performed in duplicate.

422
423 **Figure 3. Susceptibilities of *M. smegmatis* strains overexpressing *cycA* alleles to DCS.**
424 **(A)** CFUs of recombinant *M. smegmatis* strains after 24 hours of treatment with indicated
425 concentrations of DCS. **(C)** CFUs of recombinant *M. smegmatis* strains obtained over time upon
426 exposure 200 µg/mL DCS. *M. smegmatis* harbouring pMD31 (black bars), pMDcycA_{Mtb} (white
427 bars), pMDcycA_{Mbov} (light-gray bars) and pMDcycA_{BCG} (dark-gray bars). Data points and error

428 bars are means \pm standard deviations. Statistically significant differences are denoted by
429 asterisks (*, $P < 0.05$). Representative of three independent experiments performed in duplicate.

430

431 **Supporting information.**

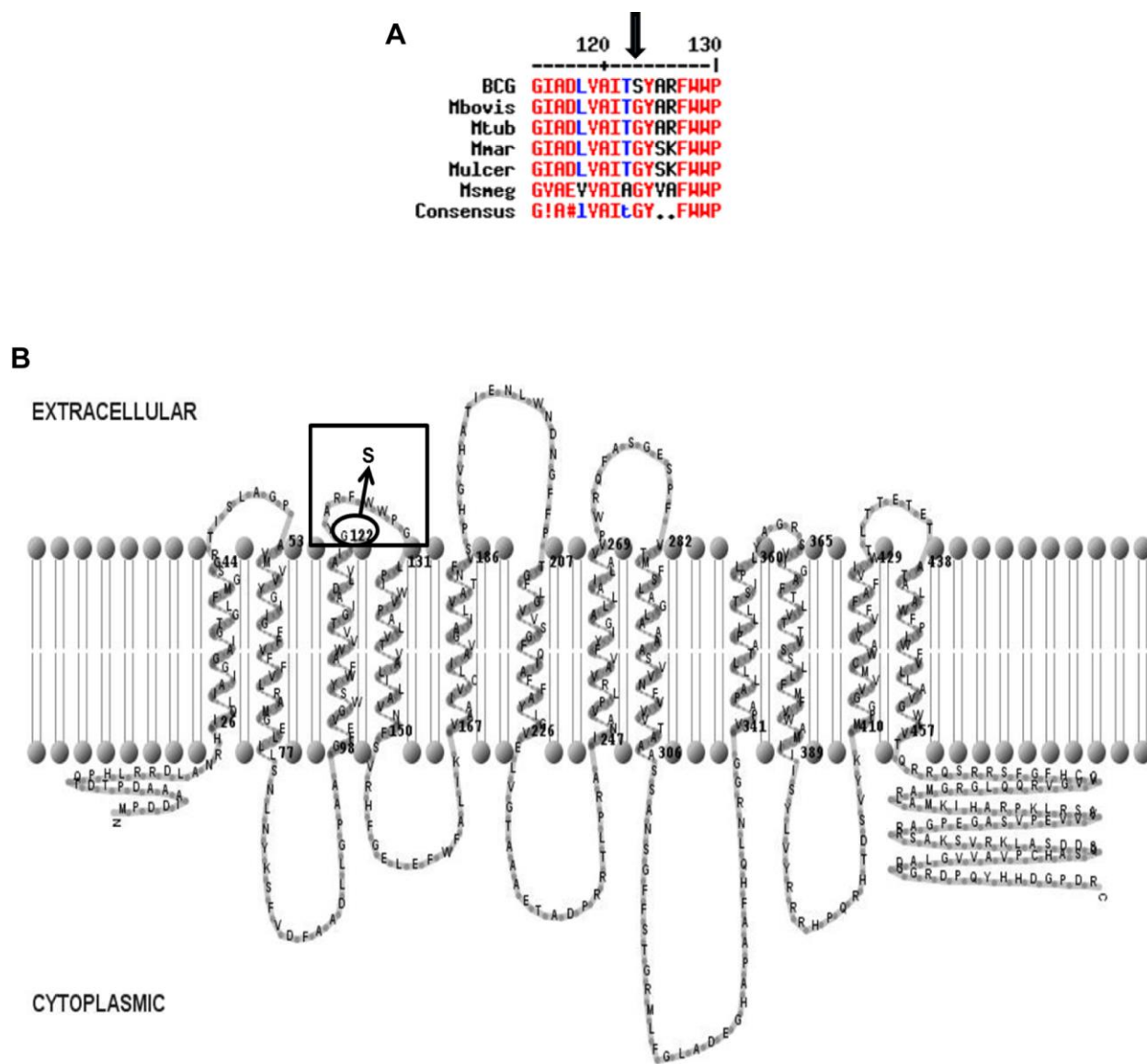
432 **Supplemental figure S1.** 2-dimensional topological representation of *E. coli* CycA with a DCS-
433 resistance associated T114P mutation (circled) in the extracellular loop (boxed) between the 3rd
434 and 4th trans-membrane helices from the amino terminus.

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438 Figure 1

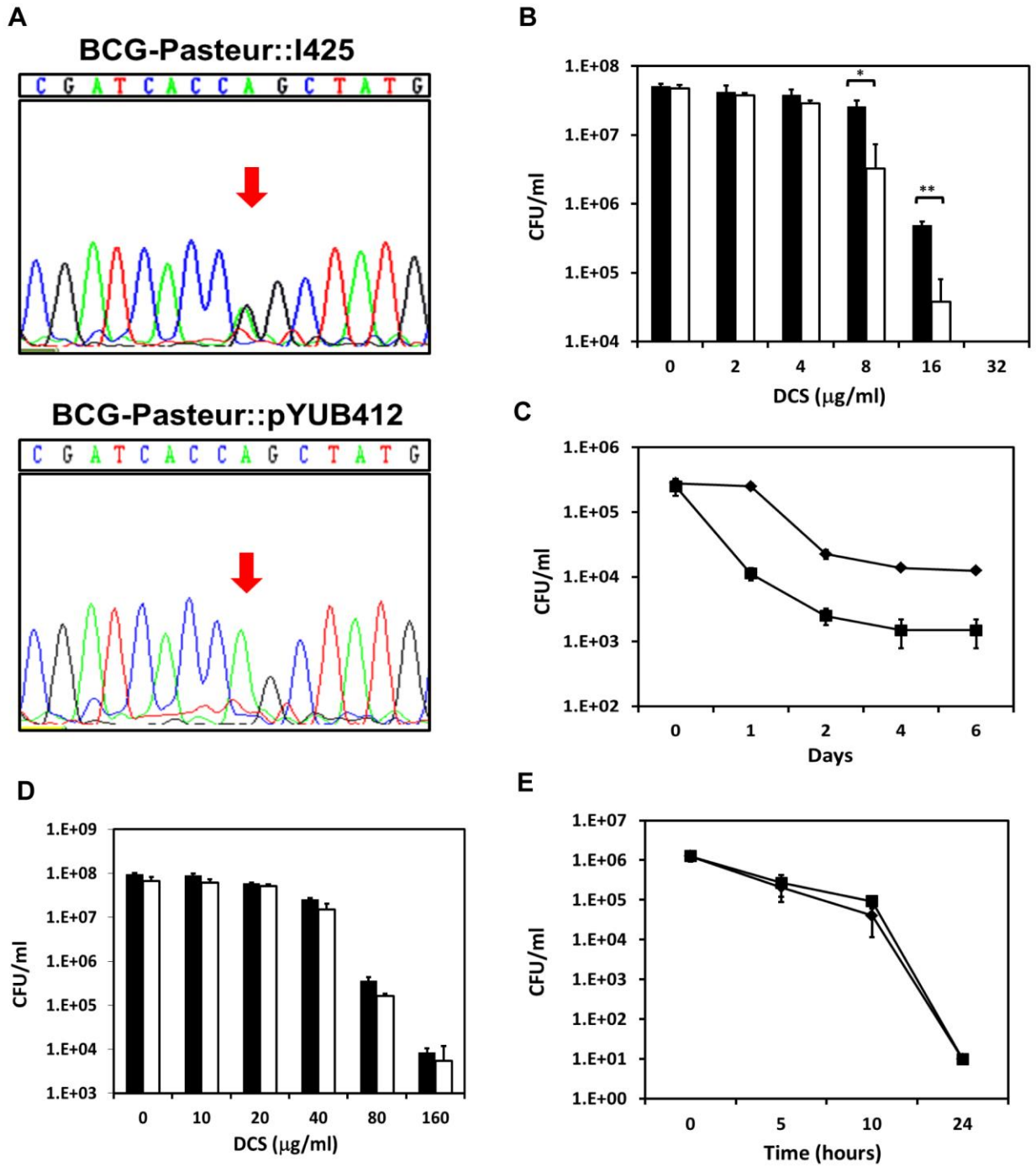


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442 Figure 2



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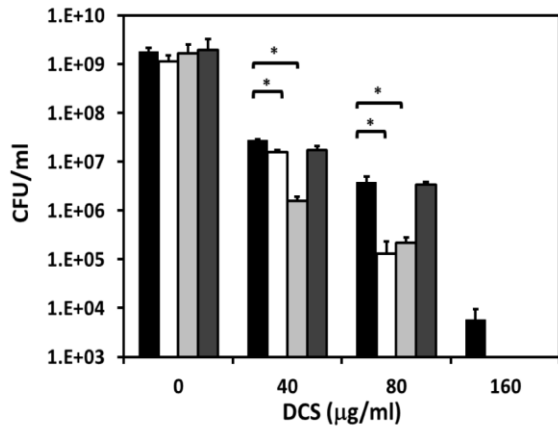
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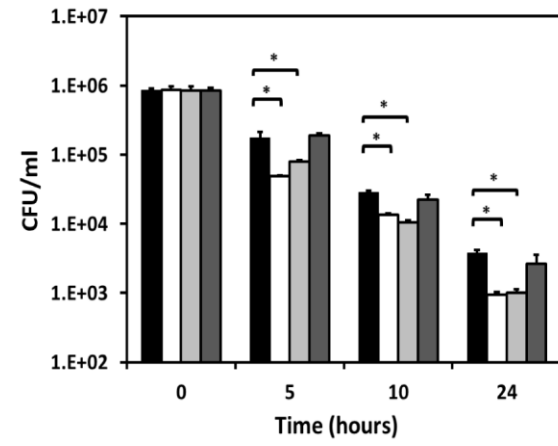
447 Figure 3

448

A



B



449

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