

1 **Determination of Cas9/dCas9 associated toxicity in microbes**

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23 **Abstract**

24 The CRISPR-Cas9 system has been used extensively in eukaryotic and prokaryotic systems for
25 various applications. In case of the latter, a couple of previous studies had shown Cas9 protein
26 expression associated toxicity. We studied the same in five microbes, viz *Escherichia coli*,
27 *Salmonella typhimurium*, *Mycobacterium smegmatis*, *Xanthomonas campestris* and
28 *Deinococcus radiodurans*. Transformation efficiency of plasmids carrying genes coding for
29 Cas9 or dCas9 was used to gauge toxicity associated with Cas9 protein expression. Results
30 showed differential levels of Cas9 toxicity among the bacteria and lower transformation
31 efficiency for *cas9/dcas9* bearing plasmids compared to controls in general. This indicated
32 lethal effect of Cas9/dCas9 expression. While *E. coli* and *S. typhimurium* seemed to tolerate
33 Cas9/dCas9 fairly well, in GC rich microbes, *M. smegmatis*, *X. campestris* and *D. radiodurans*,
34 Cas9/dCas9 associated toxicity was acute.

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41 Keywords: CRISPR, Cas9, dCas9, toxicity, microbes.

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45 **Introduction:**

46 Recombinant DNA technology together with High Throughput Sequencing in recent
47 times, has allowed us to harvest a large amount of genetic information from the microbial
48 world. The technologies have been used extensively to find out which genes determine how
49 microbes, grow, travel, starve, cause diseases, ward of predators and even die. This information
50 is especially important for studying pathogenic bacteria, bacteria of industrial importance and
51 ones with special stress tolerance abilities. Perturbing the normal functioning of the genome
52 has emerged as the best method to probe function and dynamics of individual genes.

53 Discovery of the CRISPR -Cas viral defence systems opened up another novel and
54 efficient tool box for genome editing, gene silencing, targeted gene methylation, etc. in all
55 kinds of organisms from bacteria to humans [1][2][3][4]. The ease and efficiency of the system
56 has made it an extremely popular *go-to* system for various applications. The system has further
57 had widespread applications in metabolic engineering of bacteria as it allows easy
58 programming and multiplexing [5][6]. Among the CRISPR-Cas systems, the Cas9 system from
59 *Streptomyces pyogenes* has gained popularity on account of being one of the earliest systems to
60 be discovered and its simplicity of usage [7][8][9]. The system comprises a single protein, Cas9
61 and the sgRNA , which together can be easily employed to bring about a host of desired changes
62 inside the cell of virtually any living being [10]. While the nucleoprotein, Cas9 itself has been
63 extensively used for genome editing [7][8][11][9], its nuclease deficient variant, dCas9 has
64 been useful for regulation of gene expression [3][1]. Systems employing the Cas9 variants have
65 shown great promise for use in eukaryotic, particularly mammalian systems [11]. Attempts to
66 use them in microbes have met with mixed success.

67 The Cas9/dCas9 and also Cas9 nickase systems were used successfully to probe gene
68 function and cell dynamics in several microbes [7][2]. With the increasing use of Cas9, toxicity

69 associated with Cas9 was noticed in certain microbes [12][13][14]. Further, toxicity was
70 reported not only for the Cas9 protein, where non-specific nuclease activity would be expected
71 to cause cell killing but also with the dCas9 protein, further compounding the problem. In a
72 few cases, this problem could be circumvented by placing the *cas9/dcas9* genes under tight
73 inducible control [12][14]. In a few microbes, even complete removal of promoter could not
74 solve the toxicity [14]. The results indicated that different microbes have different threshold
75 for tolerance towards *cas9/dcas9* expression which needs to be addressed for making the
76 CRISPR-Cas9 system useful in such organisms.

77 In this study, we used appropriate plasmid systems to report *cas9/dCas9* mediated
78 toxicity in five microbes, viz. *Escherichia coli*, *Salmonella typhimurium*, *Mycobacterium*
79 *smegmatis*, *Xanthomonas campestris* and *Deinococcus radiodurans*. We investigated possible
80 effect of methylation status of the host genome upon toxicity of *cas9/dcas9*. The study has also
81 enabled comparisons of levels of toxicity imparted by *cas9* or *dCas9* in each organism and thus
82 provides a comprehensive differential toxicity analysis in different groups of microbes.

83

84 **Materials and methods:**

85 **Bacterial strains, plasmids and growth conditions:**

86 *E. coli* and *S. typhimurium* cultures were grown in Luria Bertani medium (Tryptone Yeast
87 extract and sodium chloride) at 37°C. *M. smegmatis* was grown in Middlebrook 7H9 with
88 Tween 80 at 0.1% for with glycerol at 37°C. *D. radiodurans* was grown in Tryptone Glucose
89 Yeast extract (TGY) broth at 32°C. *X. campestris* was grown in Luria Bertani medium at 28°C.
90 All liquid cultures were grown with aeration at 180rpm, orbital shaking, The media were
91 supplemented with Kanamycin, (50µg/ml for *E. coli* and 10 µg/ml for *M. smegmatis*)
92 Carbenicillin (100 µg/ml for *E. coli*), Chloramphenicol (33 µg/ml for *E. coli* and 3 µg/ml for

93 *D. radiodurans*), or Gentamycin (25 µg/ml), when necessary. Wherever required
94 Anhydrotetracyclin (Atc) was added at a concentration of 1µM. The bacterial strains used are
95 described in Table 1. The plasmid vectors used in this study are listed in Table 2.

96

97 **Construction of the *cas9* – *dCas9* expressing plasmids for use in *E. coli*, *S. typhimurium*,**
98 ***M. smegmatis*, *X. campestris* and *D. radiodurans*:**

99 This study uses a host of plasmids that were either procured or constructed in various shuttle
100 vectors under different promoters to suit their application in different microbes (Table 2). Of
101 these, for *S. typhimurium* and *E. coli*, the wild type *cas9* from *Streptomyces pyogenes* cloned in
102 the pUC19 vector under the inducible promoter, PLtetO along with *dcas9* cloned in pACYduet
103 under a similar inducible promoter were employed. These were procured as shown in Table 2.

104 *Mycobacterium smegmatis* – The gene for Cas9 was codon optimized for use in
105 *Mycobacterium*. This was synthesized as a single fragment under P_{myc1tetO}control while the
106 Cas9 handle was placed under P_{smyc}. The entire fragment was cloned into the KpnI -HindIII
107 site of the multicopy vector, pSTKT to generate pST-cas9. Further the nuclease deficient
108 mutant for Cas9 was generated by Gibson cloning [15] by generating the mutations D10A and
109 H840A[16] The list of oligo primers used is given in Supplementary table1. The dCas9 thus
110 generated, was similarly cloned into pSTKT to generate, pST-dcas9.

111 Results from the Graphical Codon Analyzer showed that the codon frequency in the *M.*
112 *smegmatis*-optimized *cas9/dcass9* sequence matched well with codon usages in *D. radiodurans*
113 and *X. campestris* (Supplementary Figures 1 & 2) .

114 *Xanthomonas campestris*- The *cas9/das9* genes, optimized for *M. smegmatis* expression along
115 with the promoters and sgRNA was cut out as a KpnI-HindIII cassette from pST-cas9 or pST-
116 dcas9 and cloned into pBBR1MCS5 to generate, pBB-Cas9 and pBB-dCas9 respectively.

117 Though the *cas9/dcas9* was under the inducible promoter, $P_{myc}tetO$ in *X. campestris*, due to
118 absence of a Tet repressor, one would expect constitutive expression of the genes in this
119 organism.

120 *Deinococcus radiodurans* –The open reading frames coding for Cas9/dCas9 optimized for *M.*
121 *smegmatis* was cut out from pST-cas9 or pST-dcas9 using NdeI-BamHI restriction digestion
122 and cloned into pRAD1 under control of the P_{groESL} to generate, pRA-Cas9 and pRA-dCas9
123 respectively. To generate suitable controls for transformation efficiency, the plasmids were
124 also cloned without any promoter, pRA-Cas9P- and pRA-dCas9P-.

125

126 **Transformation of plasmid**

127 *E. coli* and *D. radiodurans* were transformed into cells made competent using $CaCl_2$ as
128 described before [17]. *S. typhimurium*, *M. smegmatis* and *X. campestris* were transformed by
129 electroporation. The details for electroporation have been given in Table 3.

130

131 **Expression of Cas9 and dCas9 in *E. coli***

132 Expression of Cas9 and dCas9 proteins was determined by separation of protein extracts of *E.*
133 *coli* strains bearing the pRAD1, or pRA-cas9 and pRA-dcas9 by electrophoresis on a 10%
134 denaturing polyacrylamide gel. The proteins were stained using Commassie Brilliant blue for
135 visualization. The proteins were transferred to a PVDF membrane followed by incubation with
136 Anti-Cas9 antibody conjugated to FITC (Sigma Aldrich). Anti-mouse secondary antibody
137 conjugated to Alkaline phosphatase was used to develop the blot with NBT-BCIP (nitro-blue
138 tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate).

139

140 **Results:**

141 **Transformation efficiencies (TE) of plasmids bearing *cas9* in enterobacteria:**

142 In *S. typhimurium*, number of transformants recovered with pwtCas9 on Atc selection plates
143 was half the number recovered in the absence of Atc. In Novablue strain, no transformants
144 could be recovered on induction with Atc (Fig. 1a). In *E. coli* DH5 alpha, TE remained
145 unaffected on Atc induction. However, Atc induction did result in smaller colony size of
146 transformants in *E. coli* DH5alpha as well as *S. typhimurium* compared to uninduced culture
147 carrying pwtCas9 but not pUC19 (Fig. 1c). This indicated that upon induction, toxicity also
148 manifested as reduction in growth. Cas9 associated toxicity was therefore observed in *S.*
149 *typhimurium* as well as *E. coli* Novablue, with a very pronounced effect in the latter.

150

151 **Transformation efficiencies of plasmids bearing *dcas9* in enterobacteria:**

152 In *S. typhimurium*, number of colonies recovered with pdCas9 on Atc induction was
153 comparable to that in the absence of induction. However, as in case of pwtCas9, the size of the
154 colonies was smaller on Atc induction of dCas9 expression (Fig. 1c). In Novablue, TE with
155 pdCas9 decreased around three fold on Atc induction, while it was unaffected when
156 pACYCDuet-1 was used (Fig. 1b). In *E. coli* DH5 alpha, TE was unaffected on Atc induction.
157 The results indicate that dCas9 mediated toxicity was highest in *E. coli* Novablue followed
158 by *S. typhimurium*, while *E. coli* DH5 alpha tolerated dCas9 expression well.

159

160 **Transformation efficiencies of plasmids bearing *cas9/dcass9* in *M. smegmatis* and *X.***
161 ***campestris*:**

162 In *X. campestris*, no transformants for pBB-cas9 or pBB-dcas9 could be recovered, while on
163 average, a TE of 1.5×10^3 CFU/ μ g of DNA could be obtained with the vector control (Fig. 2a).
164 Rarely a few transformants were recovered only in the case of dCas9 which did not grow
165 subsequently in liquid medium.

166 In *M. smegmatis* also, transformation of pST-cas9 and pST-dcas9 plasmids yielded no
167 transformants even when no Atc was added to the selection plates, while in empty vector
168 control, a TE of 7.6×10^3 CFU/ μ g DNA was obtained (Fig. 2b). Here, too, rarely small colonies
169 of transformants could be recovered on selection plates that failed to grow in liquid culture.
170 Both these organisms, therefore displayed acute toxicity to both Cas9 as well as dCas9.

171

172 **Transformation efficiencies of plasmids bearing *cas9/dcas9* in *D. radiodurans*:**

173 On transforming *D. radiodurans* with pRA-Cas9 and pRA-dCas9 plasmids, no colonies could
174 be recovered, while with control plasmids, an average TE of 1.5×10^3 CFU/ μ g DNA could be
175 obtained (Fig. 3a). Even when *cas9/dcas9* plasmids without the P_{groESL} promoter were used,
176 transformants could not be recovered. To increase the overall transformation efficiency in this
177 organism, the plasmids were passaged through a dam^-/dcm^- strain, *E. coli* JM110. Plasmids
178 isolated from this *E. coli* strain when transformed into *D. radiodurans* resulted in a higher
179 transformation efficiency of control plasmid, pRAD1 (average of 2.3×10^4 CFU/ μ g DNA) (Fig.
180 3b & c). Importantly, at this transformation efficiency, *cas9/dcas9* bearing transformants could
181 be recovered at an efficiency of 1.7×10^2 and 2.9×10^2 CFU/ μ g DNA respectively which was
182 100 fold lower than that for pRAD1 (Fig. 3c). Transformants were also recovered at an
183 efficiency of 2×10^3 and 2.5×10^3 CFU/ μ g DNA with *cas9* and *dcas9* bearing plasmids in the
184 absence of a promoter (Fig. 3b & c). The results indicate that toxicity of Cas9/dCas9 in *D.*
185 *radiodurans* is moderate.

186

187 **Effect of DNA methylation on Cas9/dCas9 toxicities**

188 As Cas9 is a nuclease and dCas9 retains the DNA binding property, the effect of methylation
189 of genomic DNA on Cas9/dCas9 mediated toxicity was analysed. Two strains of *E. coli* that
190 were dam^+/dcm^+ , DH5alpha and JM109 and two strains that were dam^-/dcm^- , JM110 and
191 GM2163 were employed. Plasmids, pRA-cas9 and pRA-dcas9 where Cas9/dCas9 is expressed
192 from a strong constitutive promoter P_{groESL} were transformed into each of these *E. coli* strains.
193 To incorporate size control for such plasmids, pRA-cas9P- or pRA-dcas9P- were also
194 transformed into all *E. coli* strains. Results did not show a marked effect for DNA methylation
195 in determining Cas9/dCas9 based toxicity (Fig. 4a). However, the fraction of transformants
196 obtained with *cas9/dcas9* bearing plasmids compared to promoterless plasmids was marginally
197 fewer in dam^-/dcm^- strains compared to dam^+/dcm^+ strains (Fig. 4a). To determine expression
198 of Cas9/dCas9 in each strain of *E. coli*, cell extracts were separated on a SDS-PAGE gel by
199 electrophoresis and visualized by Coomassie staining and Western blot. Cas9/dCas9
200 expression was seen in all the *E. coli* strains carrying *cas9/dcas9* bearing plasmids as a 160
201 kDa band that was absent in empty vector control (Fig. 4b &c). The levels of Cas9/dCas9
202 expression was higher in dam^+/dcm^+ strains compared to that in dam^-/dcm^- strains, with the
203 highest expression in DH5alpha and lowest in GM2163 strain (Fig. 4b &c). The results indicate
204 marginally higher toxicity of Cas9/dCas9 dam^-/dcm^- strains compared to dam^+/dcm^+ strains
205 despite lower protein expression.

206

207 **Discussion:**

208 Cas9/dCas9 CRISPR system has enormous potential to be applied in a wide variety of
209 organisms but the challenge in utilizing its full potential is the toxicity associated with it.

210 Cas9/dCas9 associated toxicities have been found in many microbes such as *Synechococcus*
211 *elongates* UTEX [14], *E. coli* [18][19], *M. smegmatis* [12], *Chlamydomonas reinhardtii* [20]
212 *Corynebacterium glutamicum* [6] etc. The reason for toxicity of Cas9/dCas9 in microbes has
213 been alternating between the obvious and the mysterious ever since development of this
214 technology. Early reports put down toxicity of the Cas9 protein to possible non-specific
215 nuclease activity. But similar toxicity with dCas9 required the theory to be revised. In *M.*
216 *smegmatis* it was reported that dCas9 causes proteotoxicity that sensitizes the bacteria to stress
217 [12]. The last two years have provided more insights on *cas9/dcas9* toxicity. Overall, it
218 emerged that by keeping the expression levels low or only transiently expressing these proteins,
219 toxicity could be avoided [14][12]. The determinants of Cas9 toxicity however remained
220 elusive.

221 In this study, we bring forth toxicity data for Cas9/dCas9 in five different bacteria, two
222 of which (*M. smegmatis* and *E. coli*) are standard model organisms, two are pathogens, (*X.*
223 *campestris* and *S. typhimurium*) and one shows phenomenal stress tolerance to radiation (*D.*
224 *radiodurans*). Comparison of transformation efficiencies obtained with *cas9/dcas9* bearing
225 plasmids against empty vector or promoter-less controls, or on induction of protein expression
226 have been interpreted to reflect Cas9/dCas9 mediated toxicity. In all cases, the choice of
227 plasmids and promoters was guided by well-established expression systems for the respective
228 organism. Therefore, though toxicities of Cas9/dCas9 are discussed across the five microbes
229 in this study, they are not directly comparable in absence of a way to normalize expression
230 levels. Wild type versions of *cas9/dcas9* were employed in experiments involving *E. coli* and
231 *S. typhimurium* under an inducible promoter, while for *M. smegmatis*, *X. campestris*, *D.*
232 *radiodurans*, and also *E. coli* for certain experiments, the genes were codon optimized for use
233 in *M. smegmatis* that also fulfilled codon usages for *X. campestris* and *D. radiodurans*.

234 In all the organisms tested, generally, lower transformation efficiencies were obtained with
235 *cas9/dcas9* bearing plasmids compared to empty vector control or the promoter-less versions
236 of plasmids or in absence of inducer for Cas9/dCas9 expression. Acute Cas9 mediated toxicity
237 was observed in *X. campestris*, *M. smegmatis* while *D. radiodurans* exhibited moderate
238 toxicity. In *M. smegmatis*, toxicity was severe even in the absence of induction, while in others,
239 observed toxicities were due to *cas9/dcas9* expression that was driven constitutively. All earlier
240 studies where dCas9 systems were used in *M. smegmatis* involved use of integrative plasmids
241 with tight control on expression [21][22][12] due to problems associated with dCas9 toxicity
242 in *M. smegmatis*. A direct comparison between Cas9 and dCas9 toxicity was possible only in
243 the systems where the same plasmid and promoter systems were utilized (*D. radiodurans*, *X.*
244 *campestris*, *M. smegmatis* and *E. coli* where pRAD1 based systems were employed) and the
245 results showed that the toxicity due to Cas9 and dCas9 was comparable.

246 Several studies showed that Cas9/dCas9 toxicity become evident at high levels of
247 expression of the proteins even in *E. coli* [18][13]. Further a bad seed effect describing effect
248 of certain sgRNAs known to not target essential genes was also described in *E. coli* [18]. One
249 study reported changes in expression levels of several genes and cell morphology in *E. coli* at
250 high levels of dCas9 expression [13]. In our study too, using either the wild type version or the
251 codon optimized *cas9/dcas9* caused a certain degree of toxicity with both inducible and
252 constitutive systems in *E. coli*. Surprisingly, the different strains employed, showed large
253 variations in their response to Cas9/dCas9 expression. DH5alpha seemed to be the most robust
254 strain that remained minimally affected with both versions of the *cas9/dcas9* genes, in spite of
255 high expression of the protein. But, even this strain, Atc induction of Cas9 expression resulted
256 in a reduction in colony size but not TE. This was not the case with *dcas9*, where the
257 transformation efficiency as well as colony size was unaffected on induction of the gene.
258 JM109 also seemed to tolerate Cas9/dCas9 expression relatively well when tested with

259 constitutively expressed genes. The Novablue strain of *E. coli* showed moderate toxicity with
260 the two genes, but on induction of Cas9, no transformants could be recovered. The results are
261 useful while choosing between *E. coli* strains for applications involving Cas9 and its variants.

262 In the last two years, investigations have indicated that Cas9/dCas9 toxicity is perhaps
263 due to non-specific binding to NGG sequences in the genome and the unwinding of the genome
264 for PAM searching [19]. The threshold concentration of dCas9 at which toxicity just appears
265 in *E. coli* could be increased by abolishing the PAM binding property in dCas9, lending
266 credibility to this theory. Further, earlier reports have shown high-affinity non-specific DNA
267 binding by the Cas9 in the absence of sgRNA [23].

268 This would also imply that a determinant of Cas9/dCas9 toxicity in an organism is
269 amenability of its chromosome to binding by such proteins. This would in turn almost certainly
270 depend on the GC content of the organism influencing PAM density on genome, the fraction
271 of the genome that is transcriptionally active and perhaps its epigenetic status. The microbes
272 that showed acute toxicity towards Cas9/dCas9 in this study are all GC rich, resulting in higher
273 occurrence of NGG in the genome leading to binding of the chromosome at higher density
274 resulting in disruption of normal DNA metabolism. This explains why an organism such as *D.*
275 *radiodurans* which is known for its ability to repair DNA damage, would also suffer from
276 Cas9/dCas9 related toxicity. Another observation from this study was that the toxicity in all
277 strains was marginally higher for *cas9* bearing plasmids than *dCas9* bearing plasmids. This is
278 again expected considering that Cas9 might also exert a non-specific, sgRNA independent
279 cleavage of the chromosome at a low frequency, that would be absent in *dcas9* expressing
280 strains.

281 In eucaryotes and other cell lines, the chromosome is more tightly packed and organized, that
282 may lead to low accessibility of the DNA for non-specific Cas9 interactions. Further even if

283 such interactions do occur at a low frequency, it may be buffered by the presence of a higher
284 percentage of ‘junk’ DNA than in procaryotes, therefore not interfering with DNA metabolism
285 sufficiently to cause toxicity. Nevertheless, reports on definitive Cas9/dCas9 mediated
286 toxicities in eucaryotes, especially single-celled organismssuch as *Toxoplasma gondii* [24],
287 yeast [25], *Trichomonas vaginalis* [26], have begun to appear in literature. Plasmids carrying
288 Cas9 have also shown toxicity in some cell lines where ribonucleoprotein delivery has
289 improved viability [27].

290 This study also attempted to evaluate effect of host genome methylation on the toxicity
291 of Cas9/dCas9. Since, dam^+/dcm^+ strains would carry methylated chromosomes, it is tempting
292 to assume that this modification would mask the DNA to discourage non-specific Cas9/dCas9
293 binding and lead to lower toxicity levels. In dam^-/dcm^- strains, a naked DNA would probably
294 make non-specific Cas9/dCas9 binding easier. It is another question, whether the frequency of
295 methylated sites on the *E. coli* chromosome in dam^+/dcm^+ strains would be enough to effect
296 non-specific binding resulting in toxicity at all or not. Our results indicate marginally lower
297 Cas9/dCas9 mediated toxicity in dam^-/dcm^- strains despite lower expression of proteins.
298 Therefore, the results are not sufficient to conclude no effect due to chromosomal methylation
299 as far as Cas9/dCas9 binding is concerned. Earlier, it was shown that cleavage by Cas9 was
300 unaffected by cpG methylation [28]. However, there are other studies which showed that there
301 was a negative co-relation between off-target binding and DNA methylation [29]. It remains
302 to be proven more rigorously, whether indeed bacterial methylation affects non-specific
303 binding if at all, hence influencing toxicity and possibly off-target effects or whether it also
304 affects targeting.

305

306

307 **Conclusion**

308 In view of results from this study, Cas9 expression in microbes may need careful modulation
309 to ensure effective applications in silencing and genome editing. Especially in complex systems
310 such as metabolic engineering, Cas9/dCas9 toxicity may need evaluation, since multiple gene
311 modifications may compound the toxicity problem. It may be useful to employ strategies such
312 as use of temperature sensitive plasmids, integrative plasmids or low copy number plasmids,
313 transient expression or direct use of the sgRNA-Cas9 nucleoprotein complex by electroporation
314 etc. to minimise the toxic effects of the protein. In addition, alternative CRISPR systems that
315 maybe associated with lower toxicity such as Type I (Cascade) [30] or Type V (Cpf1) [31] may
316 be explored where specifically essential genes need to be probed.

317 **Acknowledgements:**

318 We would like to acknowledge Addgene for providing the plasmids, pwtCas9 and
319 pdCas9, Dr. Magnus Lundgren, Uppsala University for *S. typhimurium* DA6192 strain and
320 Dr. Amit Singh, Indian Institute of Science, Bengaluru for *M. smegmatis*. We would like to
321 thank Dr. Shyam Sunder Rangu, Dr. Bhakti Basu and Dr. Yogendra S. Rajpurohit for their
322 inputs on the manuscript. We also acknowledge Dr. Hari S. Misra for constant help and support.

323 **Conflict of interest:** The authors have no conflict of interest to declare.

324

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- 420

421 Table 1. Bacterial strains used in the study.

Strains used	Genotype	Source
<i>E. coli</i> DH5alpha	F ⁻ , endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, purB20, φ80dlacZΔM15, Δ(lacZYA-argF)U169, hsdR17(rK-mK+), λ ⁻	Lab collection
<i>E. coli</i> JM110	rpsL, thr, leu, thi, lacY, galK, galT, ara, tonA, tsx, dam, dcm, glnV44, Δ(lac-proAB), e14-, [F' traD36 proAB+ lacIq lacZΔM15], hsdR17, (rK-mK+)	Lab collection
<i>E. coli</i> JM109	endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrB+, Δ(lac-proAB), e14-, [F' traD36 proAB+ lacIq lacZΔM15], hsdR17, (rK-mK+)	Lab collection
<i>E. coli</i> GM2163	F ⁻ , araC14, leuB6(Am), fhuA13, lacY1, tsx-78, glnX44(AS), galK2(Oc), galT22, λ ⁻ , mcrA0, dcm-6, hisG4(Oc), rfbC1, rpsL136(strR), dam-13::Tn9, xylA5, mtl-1, thiE1, mcrB9999, hsdR2	Lab collection
<i>E. coli</i> Novablue	Δ(srl-recA)30::Tn10(DE3), Tet ^r	Novagen
<i>Salmonella typhimurium</i> DA6192	Wild type	Gift from Dr. Magnus Lundgren
<i>Mycobacterium smegmatis</i> MC ² 155	Wild type	Snapper et al. [32]

<i>Xanthomonas campestris</i> pv. Campestris str 8004	Wild type	Lab collection
<i>Deinococcus radiodurans</i> R1	Wild type (ATCC BAA 816)	Lennon et al. [33]

422

423 Table 2: Plasmids used in this study

Plasmid	Description of construct	Source/Reference
pUC19	<i>E. coli</i> vector, Amp ^r , 2.68kb	Norrande <i>et al.</i> 1983
pwtCas9	pUC19 with the <i>cas9</i> gene under Atc inducible promoter, Amp ^r	Qi <i>et al.</i> 2013[1]
pACYCDuet-1	<i>E. coli</i> vector, Cm ^r	Novagen
pdCas9	pACYCDuet-1 with <i>dcas9</i> gene under Atc inducible promoter, Cm ^r	Qi <i>et al.</i> 2013[1]
pSTKT	<i>E. coli-M. smegmatis</i> shuttle vector, Kan ^r , 5 kb	Parikh <i>et al.</i> , 2013 [34]
pST-cas9	<i>E. coli-M. smegmatis</i> shuttle vector with the <i>cas9</i> gene (optimized for expression in <i>Mycobacterium</i>) under P _{myc1tetO} promoter and sgRNA Cas9 handle under P _{smyc} , Kan ^r , 9.5 kb	This study
pST-dcas9	<i>E. coli-M. smegmatis</i> shuttle vector with the <i>dcas9</i> gene (optimized for expression in <i>Mycobacterium</i>) under	This study

	$P_{myc1tetO}$ promoter and sgRNA Cas9 handle under P_{smyc} , Kan ^r , 9.5 kb	
pRAD1	<i>E. coli-D. radiodurans</i> shuttle vector, Ap ^r , Cm ^r , 6.28kb	Meima & Lidstrom, 2000 [17]
pRA-cas9	<i>E. coli-D. radiodurans</i> shuttle vector with the <i>cas9</i> gene (optimized for expression in <i>Mycobacterium</i>) under P_{groESL} , Ap ^r , Cm ^r , 10.6 kb	This study
pRA-dcas9	<i>E. coli-D. radiodurans</i> shuttle vector with the <i>dcas9</i> gene (optimized for expression in <i>Mycobacterium</i>) under P_{groESL} , Ap ^r , Cm ^r , 10.6kb	This study
pRA- cas9P-	<i>E. coli-D. radiodurans</i> shuttle vector with the <i>cas9</i> gene without any promoter (optimized for expression in <i>Mycobacterium</i>) Ap ^r , Cm ^r , 10.6 kb	This study
pRA- dcas9P-	<i>E. coli-D. radiodurans</i> shuttle vector with the <i>dcas9</i> gene without any promoter (optimized for expression in <i>Mycobacterium</i>), Ap ^r , Cm ^r , 10.6kb	This study
pBBR1MCS5	<i>E. coli-X. campestris</i> shuttle vector, Gen ^r , 4.7kb	Kovach et al., 1995 [35]
pBB-cas9	<i>E. coli-X. campestris</i> shuttle vector with the <i>cas9</i> -sgRNA cassette from pST-cas9, Gen ^r , 9.3 kb	This study
pBB-dcas9	<i>E. coli-X. campestris</i> shuttle vector with the <i>dcas9</i> -sgRNA cassette from pST-dcas9, Gen ^r , 9.3 kb	This study

424

425

426 Table 3. Electroporation conditions

Organism	Electroporation apparatus	Voltage applied	Recovery conditions
<i>S. typhimurium</i>	Eppendorf eporator	2.5kV	Luria Bertani broth at 37°C for 1h under shaking
<i>M. smegmatis</i>	Biorad Total Systems	2.5kV	Middlebrook 7H9 with glycerol and 0.1% Tween 80 at 37°C for 2h under shaking
<i>X. campestris</i>	Eppendorf eporator	1.4kV	Luria Bertani broth at 28°C for 4h under shaking

427

428

429

430 Legends:

431 Fig. 1. Transformation efficiency of plasmids bearing *cas9* (a) or *dcas9* (b) relative to the
432 absence of induction with anhydrotetracycline in *S. typhimurium*, *E. coli* Novablue and *E. coli*
433 DH5alpha. Transformation was done by electroporation for *S. typhimurium* and by CaCl₂
434 method for *E. coli* cells. Transformants were selected on antibiotic selection plates and colony
435 forming units were enumerated to determine transformation efficiency. (c) Reduction of colony
436 size on induction with Atc as observed for *S. typhimurium*. Results are from experiments that
437 were repeated three times each.

438 Fig. 2. Transformation of cas9/dcas9 bearing plasmids in *X. campestris* (a) and *M. smegmatis*
439 (b). Plasmids bearing cas9/dcas9 were electroporated along with empty vector controls into *X.*
440 *campestris* and *M. smegmatis*. The cells were spread on antibiotic selection plates and allowed
441 to grow.

442 Fig. 3. Transformation of plasmids pRAD1, and pRAD1 bearing cas9 and dcas9 with (pRA-
443 cas9 and pRA-dcas9) or without promoter (pRA-cas9P- and pRA-dcas9P-) in *D. radiodurans*.
444 Plasmids isolated from JM109(a) or JM110(b) strains of *E. coli* were used to transform *D.*
445 *radiodurans*. Cells were plated on chloramphenicol selection plates. (c) Transformation
446 efficiency of plasmids as determined from CFUs enumerated on antibiotic selection plates
447 where colonies could be recovered. Results are from experiments repeated three times.

448

449 Fig.4. Cas9/dCas9 associated toxicity in *E. coli* strains that are dam⁺/dcm⁺ or dam⁻/dcm⁻. Four
450 *E. coli* strains were transformed with pRAD1 or pRA-Cas9 or pRA-dCas9 and transformants
451 were elected on carbenicillin selection plates. The CFU were enumerated to determine TE (a).
452 Results are from experiments repeated four times. (b) Protein extracts from *E. coli* strains,
453 DH5alpha (Lanes A, B,C), JM110 (Lanes D, E, F), JM109 (Lanes G, H, I) and GM2163 (Lanes
454 J, K,L) carrying pRAD1 (Lanes A, D, G and J) or pRA-Cas9 (Lanes B, E, H and K) or pRA-
455 dCas9 (Lanes C, F, I and L) were separated by gel electrophoresis and stained with Coomassie
456 Brilliant Blue (b) or developed for Western blot using Anti-Cas9 antibody (c).

Fig. 1

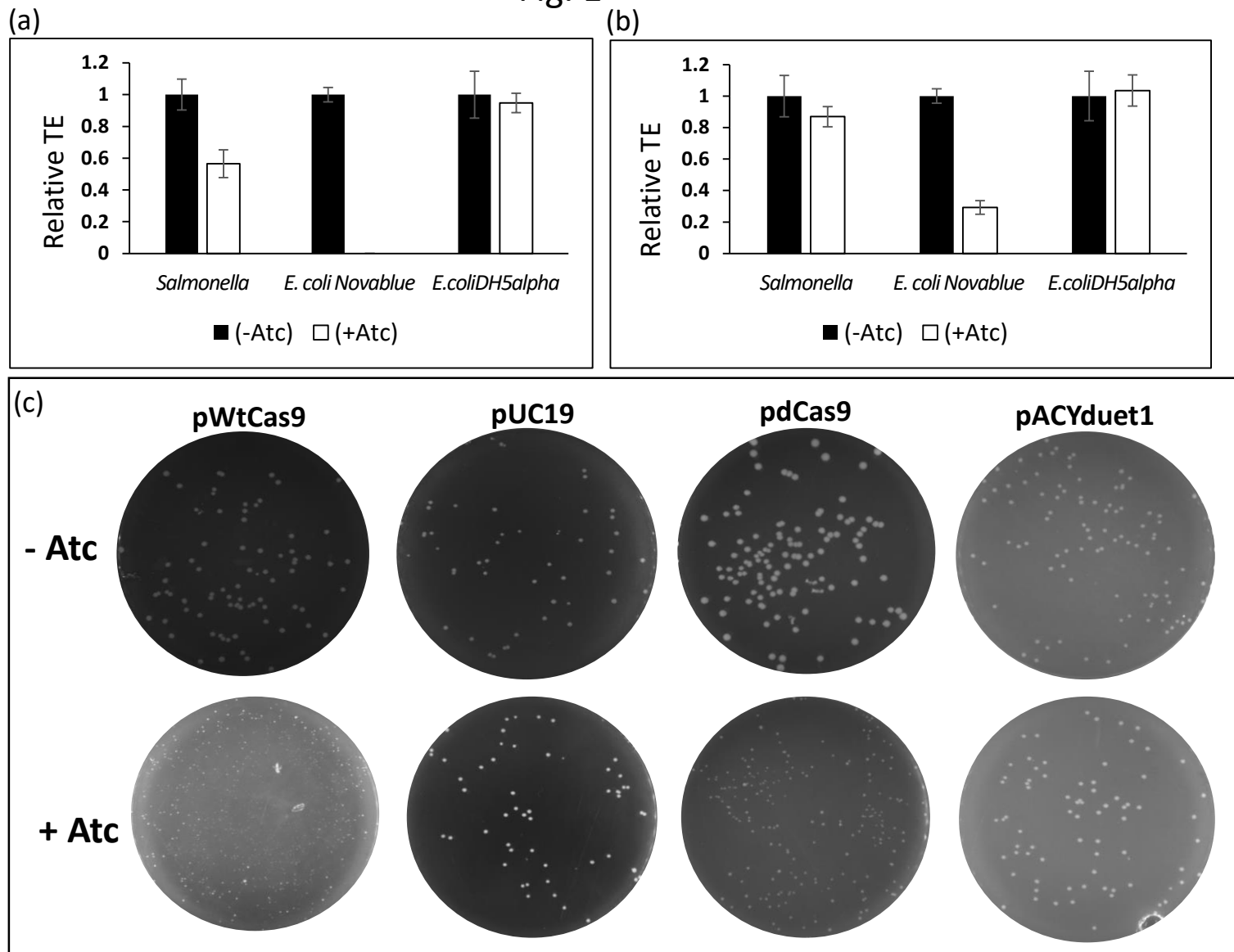
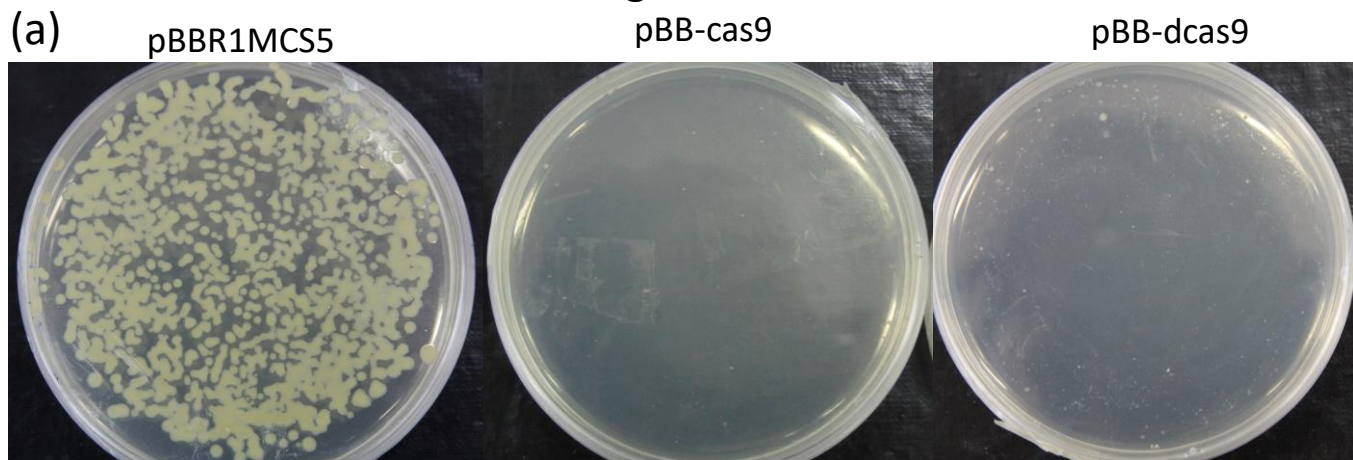


Fig. 2



(b)

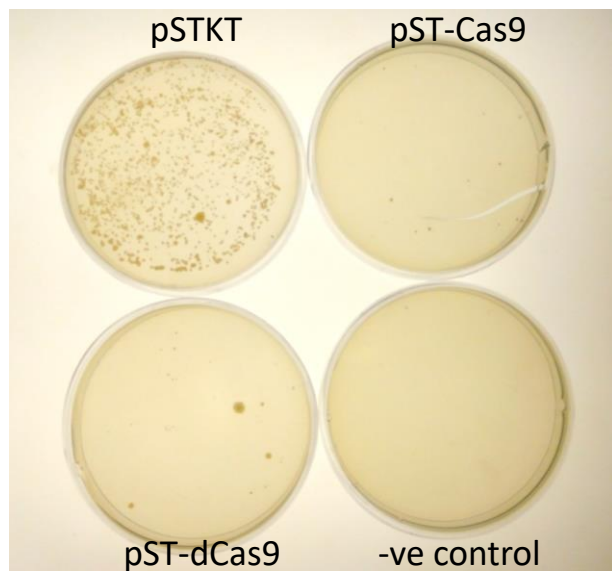
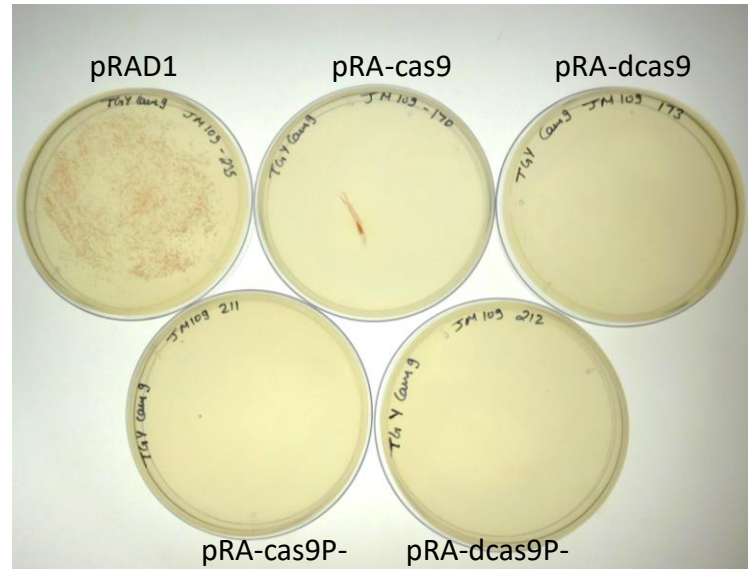
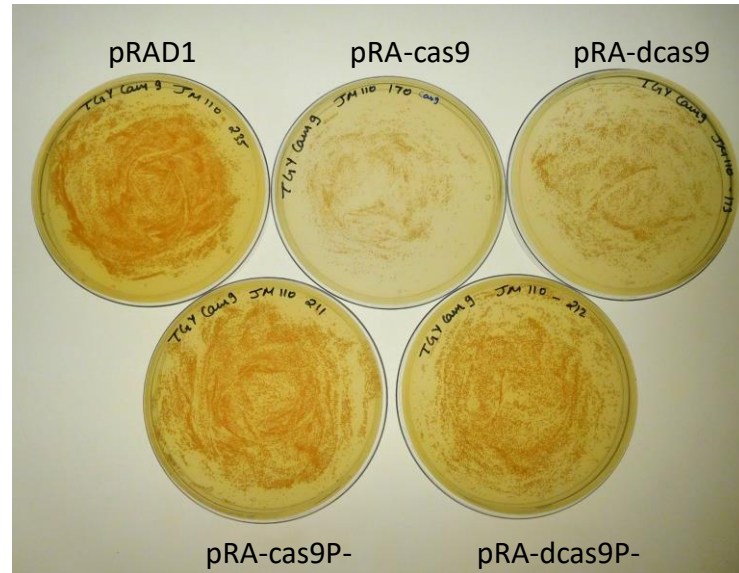


Fig. 3

(a)



(b)



(c)

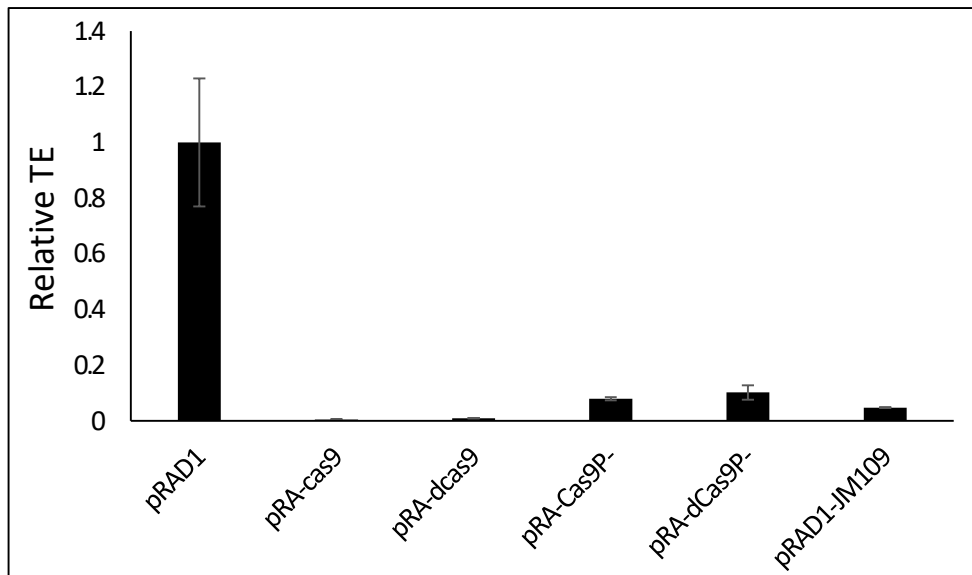
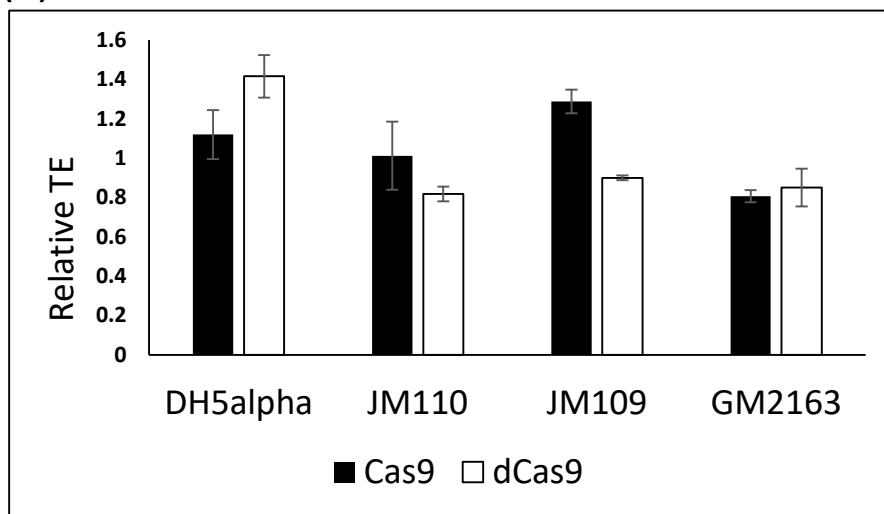
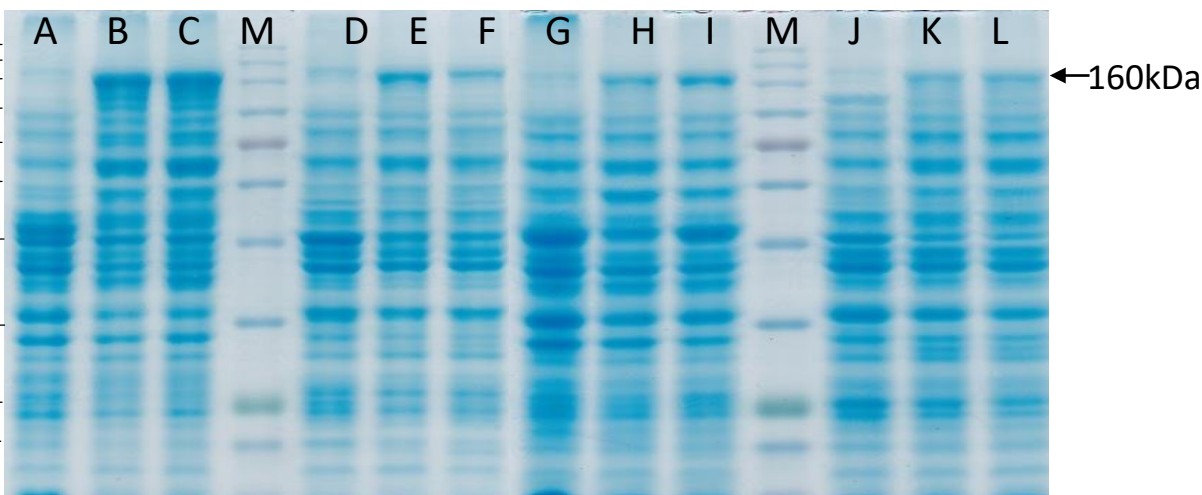


Fig. 4

(a)



(b)



(c)

