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1 **Influence of very short patch mismatch repair on SOS inducing lesions after**
2 **aminoglycoside treatment in *Escherichia coli*.**

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9

10 **Abstract (100 words max).**

11 Low concentrations of aminoglycosides induce the SOS response in *Vibrio cholerae*
12 but not in *Escherichia coli*. In order to determine whether a specific factor present in *E. coli*
13 prevents this induction, we developed a genetic screen where only SOS inducing mutants are
14 viable. We identified the *vsr* gene coding for the Vsr protein of the very short patch mismatch
15 repair (VSPR) pathway. The effect of mismatch repair (MMR) mutants was also studied. We
16 propose that lesions formed upon aminoglycoside treatment are preferentially repaired by
17 VSPR without SOS induction in *E. coli* and by MMR when VSPR is impaired.

18

19 **Keywords: SOS, antibiotics, sub-MIC, VSPR, DNA repair**

20

21 **Introduction**

22 Low concentrations of antibiotics are now known to lead to bacterial resistance
23 development [4]. Sub-minimal inhibitory concentrations (sub-MICs) of several antibiotics
24 induce the SOS response [8]. This temporary induction of SOS allows bacteria to mutate,
25 evolve and adapt to their environment. Thus understanding the mechanisms leading to SOS
26 induction is central to understand the connections between antibiotic treatments, bacterial
27 stress responses and resistance development. Interestingly, aminoglycosides induce SOS in
28 *Vibrio cholerae* and not in *Escherichia coli* [8]. The SOS regulon and DNA repair
29 mechanisms are very similar in both bacteria. One hypothesis that could explain the lack of
30 SOS induction in *E. coli* is that specific factor(s), absent from *V. cholerae*, prevents SOS
31 induction by sub-MIC aminoglycosides. In order to test this hypothesis, we submitted *E. coli*
32 to mariner transposition in mass and selected for mutants which became able to induce SOS in
33 response to tobramycin sub-MIC (TOB). We chose to use low doses of tobramycin (100 fold
34 below the MIC) because this antibiotic was used in our previous studies, the same effect was
35 also observed with other aminoglycosides [2, 6, 9]. Our screen led to the identification of the
36 very short patch mismatch repair pathway as an *E. coli* specific response to TOB treatment,
37 without impacting the induction of SOS by DNA damaging agents.

38

39 **Materials and methods**

40 **Plasmid construction.** [pACYC184-Pint-dapA]: *dapA* gene fused to the SOS regulated *V.*
41 *cholerae* *intIA* gene promoter was amplified using oligonucleotides 1514/1515 on *E. coli*
42 MG1655 genomic DNA; cloned into pTOPO^{Stratagene} plasmid (ampicilin 100 μ g/ml); extracted
43 by EcoRI and cloned into pACYC184 (tetracycline 15 μ g/ml). [pACYC184-PrecN-dapA]:
44 same as *Pint-dapA* with oligonucleotides 1513/1515. **pB453.** *vsr* gene of *E. coli* fused to the
45 constitutive *bla* promoter was amplified using oligonucleotides 2435/2436 on MG1655 and
46 cloned into pTOPO. **pB421, pB449** and **pB451** were constructed by site-directed mutagenesis
47 by whole plasmid amplification using respectively oligonucleotides 2429/2430, 2439/2440
48 and 2441/2442 on plasmid pB420 carrying wild type *mutS* gene under the control of its own
49 promoter (chloramphenicol 25 μ g/ml). The PCR product was digested with DpnI and
50 circularized in competent TOP10 cells. **Table S1** shows oligonucleotide sequences.

51 **Strain constructions.** MG1655 *vsr::km* (B599) and MG1655 *Δ dam::Tn9* (B371) were
52 constructed by P1 transduction of *vsr::km* and *Δ dam::Tn9* from strains Keio JW1943 and
53 M0099^{Fermentas}. MG1655 *mutS::Tn10 vsr::km* was constructed by P1 transduction of *vsr::km*
54 into MG1655 *mutS* strain (2580). Antibiotics were used at the following concentrations:
55 25 μ g/ml kanamycin (*km*) and chloramphenicol (Tn9); 15 μ g/ml tetracycline (Tn10).

56 **Construction of the *E. coli::mariner* insertion library** was performed as described [1] into
57 MG1655 *Δ dapA* *E. coli* strain. The library was spread on several Petri dishes containing 100
58 μ g/ml spectinomycin + 60 μ g/ml DAP in order to achieve a library size of 10.000 clones.

59 **Identification of mutants:** the mariner transposon insertion locus was determined by
60 sequencing as described in a previous study [1]. Antibiotics concentrations used for the
61 screen: TOB: tobramycin 0.01 μ g/ml; MMC: mitomycin C 0.02 μ g/ml, LEV: levofloxacin
62 0.01 μ g/ml.

63 **Flow cytometry** experiments were performed as described [8]. **Statistical significance.** Each
64 flow cytometry measurement was independently performed on at least four independent
65 samples. When necessary, in order to test if the means of two groups were different, we first
66 tested the equality of their variance with a Fisher test. When the variances of two groups were
67 found to be significantly different, an unequal variance t test was performed. Otherwise a
68 simple t test was performed. The chosen significance threshold was 0.05 for all tests. The
69 corresponding P values are given in Table S2.

70 Results

71 In order to search for genes that prevent SOS induction by AGs at low antibiotic
72 concentrations in *E. coli*, we used a random gene inactivation strategy using mariner
73 transposon insertions as described previously [13, 1]. The *E. coli* MG1655 Δ *dapA* (8120) was
74 used as the host for our genetic screen (**Fig.1A**). *dapA* encodes the dihydrodipicolinate
75 synthase, an enzyme essential for the synthesis of diaminopimelic acid (DAP), essential for
76 the peptidoglycan reticulation. Thus, MG1655 Δ *dapA* only grows in medium supplemented
77 with DAP [21], and dies without addition of DAP (**Fig.1B**). In parallel, we constructed a
78 plasmid where the *dapA* gene is under control of an SOS regulated promoter, the promoter of
79 the *V. cholerae* integrase gene *intIA*. In our setup, induction of SOS leads to transcription
80 from *intIA* promoter [3, 15] and biosynthesis of DAP. In order to verify that SOS induction
81 results in viability of the MG1655 Δ *dapA* carrying the plasmid *Pint_dapA* (**Fig.1B**), we grew
82 the strain in LB supplemented with DAP in order to allow growth in the absence of SOS
83 induction. We plated a 10^{-6} dilution of the overnight culture on several antibiotics at sub-MIC
84 (and without DAP). In *E. coli*, mitomycin C and levofloxacin are known SOS inducers
85 whereas tobramycin is not. We confirmed that MG1655 Δ *dapA* with plasmids carrying
86 *Pint_dapA* form colonies when SOS is induced (MMC and LEV) and that they are not viable
87 in the absence of SOS induction (TOB) (**Fig.1B**). The *recN* promoter was also tested but we
88 chose to pursue with the *intIA* promoter as it gave better results (**Fig.1B**), probably because
89 the basal expression of *recN* is higher than *intIA*.

90 We then transformed our MG1655 Δ *dapA* mariner insertion library with the plasmid
91 *Pint-dapA* and selected viable colonies on sub-MIC TOB in absence of DAP in the medium
92 (**Fig.1A**). Sub-MIC TOB does not induce SOS in wild type *E. coli* [7, 8], thus if clones are
93 able to grow on this medium, this means that DapA is expressed and that SOS is induced in
94 these conditions. With such a screen after transposition, we expected to get colonies where

95 inactivation of a gene would lead to SOS-dependent expression of *dapA* from the *intIA*
96 promoter (or that transposition created a new promoter upstream of the *dapA* gene). We
97 obtained two viable colonies on medium supplemented with sub-MIC TOB and devoid of
98 DAP. The sequencing of flanking regions in both colonies led to the identification of the *vsr*
99 gene (b1960) in these two clones. *Vsr* is involved in very short patch repair (VSPR) in *E. coli*
100 [19]. VSPR pathway comprises the *Vsr* endonuclease, DNA polI (*polA*), DNA ligase, MutL
101 and MutS [23]. The identification of *vsr* in our inactivation screen suggests that in the absence
102 of VSPR, SOS is induced by sub-MIC TOB in *E. coli*. This induction was confirmed by flow
103 cytometry using the *gfp* reporter under the control of the SOS dependent *recN* promoter
104 (Fig.2) that we developed and used in our previous studies [3, 5, 7, 8]. In order to confirm the
105 result obtained in our genetic screen, we constructed a MG1655 Δvsr strain and verified that
106 inactivation of *vsr* leads to SOS induction by TOB, and that complementation with a plasmid
107 expressing *vsr* restores the wild type phenotype (**Fig.2A**, Δvsr p_Vsr), showing that in the
108 absence of VSPR, SOS is induced by sub-MIC TOB in *E. coli*.

109 VSPR is involved in the repair of T-G mismatches methylated by Dcm and competes
110 with mismatch repair (MMR) [12]. Furthermore, VSPR in *E. coli* requires the presence of the
111 Dam protein, because *E. coli* Δdam strain has decreased levels of *Vsr* protein [11]. We tested
112 and observed that SOS is induced by TOB in the MG1655 Δdam strain (**Fig.2A**), which is
113 consistent with the fact that decreased VSPR leads to SOS induction. Note that the basal level
114 of SOS (in LB) is high in the Δdam mutant compared to wild type. This is because *E. coli*
115 Δdam undergoes massive DNA double strand break formation, which is a powerful SOS
116 inducer [22].

117 On the other hand, VSPR is also influenced by MutS levels. MutS is part of the Dam-
118 directed mismatch repair pathway in *E. coli*. VSPR is decreased in a *mutS*⁺ strain carrying
119 MutS⁺ in multicopy plasmids [17, 19], meaning that a high level of MutS inhibits VSPR,

120 possibly because mismatch repair competes with VSPR when MutS levels are high. However,
121 the presence of MutS is important for optimal VSPR [23]. MutS and MutL are proposed to
122 recruit Vsr on the mismatch [20] [14]. As a result, VSPR is not only inhibited by high MutS
123 levels, but also decreased in a *mutS* deficient background.

124 To address whether MutS can influence SOS induction by TOB through VSPR
125 modulation, we first measured the effect of TOB on SOS in MG1655 deficient for *mutS* and
126 we found that SOS is induced by TOB in the absence of MutS (**Fig.2A**). Moreover, upon
127 over-expression of Vsr in the *mutS* background, SOS goes back to wild type levels, meaning
128 that in the absence of *mutS*, SOS is induced by TOB because of decreased Vsr levels. We then
129 over-expressed MutS+ from a plasmid in MG1655 and observed that over-expression of
130 MutS+ which decreases VSPR, also induces SOS in the presence of TOB (**Fig.2B, MutS+**).
131 These observations are consistent with a possible role of MutS on VSPR in the presence of
132 TOB.

133 To further test this hypothesis, we decided to use previously characterized dominant
134 negative mutants of *mutS* which decrease MMR efficiency [24]. These mutants, expressed
135 from plasmids, were classified in two categories : class I mutants which decrease MMR
136 without affecting VSPR in a wild type (MutS proficient) (or $\Delta mutS$) background and class II
137 mutants which decrease both MMR and VSPR [18]. Some class I mutants even increased
138 VSPR. It was thus concluded that MutS protein lacking functions required for MMR can still
139 participate in VSPR. We decided to test S621N and T669I class I mutants and the G619D
140 class II mutant for their effect on SOS. Whether in a *mutS+* or a $\Delta mutS$ background, class I
141 mutants are expected to enhance VSPR and thus prevent SOS induction by TOB whereas the
142 class II mutant should decrease VSPR thus allow SOS induction by TOB. **Fig.2B** shows the
143 results obtained with these mutants in wild type and $\Delta mutS$ backgrounds. As expected, in
144 strains over-expressing VSPR proficient class I mutants S621N and T669I, SOS levels are

145 similar to the ones with the empty plasmid in the wild type *E. coli* strain, whereas over-
146 expression of MutS+ which decreases VSPR shows a high SOS induction. Conversely, in the
147 strain over-expressing the VSPR deficient class II mutant G619D, SOS is induced by TOB.
148 We obtained similar results in the $\Delta mutS$ background: VSPR proficient class I mutants S621N
149 and T669I have lower SOS levels than VSPR deficient class II mutant G619D and the MutS+
150 over-expressing strains. Altogether, our data suggest a key role for VSPR to repair the DNA
151 damage that would otherwise induce the SOS response in the presence of low concentrations
152 of TOB. Note that the double $\Delta vsr \Delta mutS$ mutant has a high basal level of SOS. Even though
153 we do not have an exact explanation for this observation, we can hypothesize that since the
154 double mutant has a slight growth defect compared to the wild type or single mutants in LB,
155 this could influence SOS induction, independently of tobramycin treatment, as observed.
156

157 Discussion

158 SOS induction can be seen as a bacterial adaptation mechanism, which has to be
159 tightly controlled by bacteria in order to prevent decrease of fitness in conditions where it is
160 not needed. The differences in the conditions inducing SOS between bacteria may account for
161 their different lifestyles and studying these conditions is important to understand different
162 defense strategies developed by bacteria.

163 In *E. coli*, inactivation of *vsr* leads to SOS induction by TOB. Interestingly, the VSPR
164 repair pathway is absent from *V. cholerae* [10] where TOB induces SOS (although a Dcm
165 homologue exists). On the other hand, MMR is present and functional in both bacteria. We
166 propose that in *E. coli*, unlike in *V. cholerae*, DNA lesions during TOB treatment can be
167 repaired by VSPR in a way which does not induce SOS. One can even think that when active,
168 VSPR may prevent the binding of RecA on DNA and subsequent SOS induction. However,
169 transduction and conjugation experiments involving recombination of incoming homologous
170 DNA in the chromosome did not yield any difference in recombination efficiency between
171 wild type and Δvsr strains (data not shown).

172 Interestingly, we have shown that inactivation of *mutS* in *V. cholerae* abolishes SOS
173 induction by TOB [1], which means that MutS is involved in TOB-mediated SOS induction in
174 *V. cholerae*. In *E. coli*, *mutS* phenotype is more complex to interpret as both its inactivation as
175 well as its over-expression induce SOS. This difference can likely be explained by the
176 presence of VSPR in *E. coli* (and its absence from *V. cholerae*). Importantly, our observation
177 that MutS presence or absence influences SOS induction in both bacteria shows that MMR is
178 involved in the repair of TOB-mediated lesions. We propose that in *E. coli*, TOB-mediated
179 DNA lesions are preferentially repaired by VSPR without inducing SOS. In the absence of
180 VSPR, the repair of these lesions engage SOS induction. This can possibly be due to single

181 stranded DNA gaps formed by MMR action. Conversely, in *V. cholerae* where VSPR is
182 absent, the repair always involves SOS induction.

183

184

185 **References**

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249

250

251 **Legend to figures**

252 **Figure 1: The genetic screen. A. Experimental setup. B. Validation of the host strain.**

253 MG1655 $\Delta dapA$ carrying the specified plasmid was grown in LB supplemented with DAP in
254 order to allow growth in the absence of SOS induction. A 10^{-6} dilution of the overnight culture
255 was plated on several antibiotics at sub-MIC (and without DAP). The growth of colonies was
256 monitored. "+" means growth of colonies. "-" means no colony development. TOB:
257 tobramycin $0.01 \mu\text{g/ml}$; MMC: mitomycin C $0.02 \mu\text{g/ml}$, LEV: levofloxacin $0.01 \mu\text{g/ml}$

258

259 **Figure 2: SOS induction levels after sub-MIC tobramycin treatment. A. Effect VSR. B.**

260 **Effect of plasmids over-expressing *mutS* mutants.** Histogram bars represent the GFP
261 fluorescence in LB or in the presence of tobramycin ($0.01 \mu\text{g/ml}$) and thus reflect the levels of
262 SOS. MMR and VSPR stand for: respectively mismatch repair and very short patch repair.
263 "+" means *proficient*; "-" means *deficient*. Error bars represent standard deviation. Each strain
264 was tested at least 4 times.

265

266

267 **Table 1: Strains and plasmids**

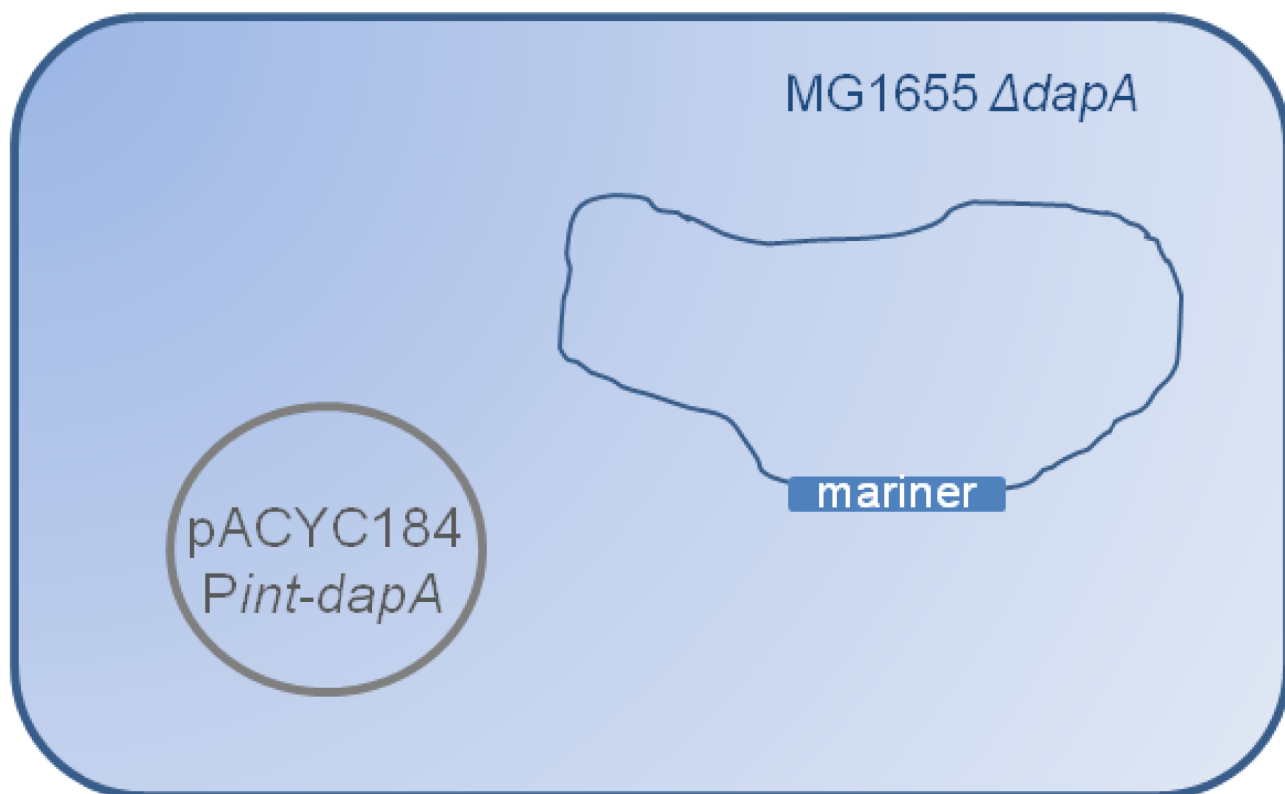
Strains	Genotype	Resistance	Construction
8120	MG1655 Δ dapA		Laboratory collection
9404	MG1655 Δ dapA pACYC184-Pint-dapA	Tetracyclin	This study
B599	MG1655 <i>vsr::km</i>	Kanamycin	This study
B371	MG1655 Δ dam:: <i>Tn9</i>	Chloramphenicol	This study
2580	MG1655 <i>mutS::<i>Tn10</i></i>	Tetracyclin	Laboratory collection
B600	MG1655 <i>mutS::<i>Tn10</i> vsr::km</i>	Tetracyclin, Kanamycin	This study
Plasmids			
pB453	pTOPO-Pbla- <i>vsr</i>	Ampicillin	This study
pB420	pACYC184- <i>mutS</i> ⁺	Chloramphenicol	[16]
pB421	pACYC184- <i>mutS</i> _{G619D}	Chloramphenicol	This study
pB449	pACYC184- <i>mutS</i> _{S621N}	Chloramphenicol	This study
pB451	pACYC184- <i>mutS</i> _{T669I}	Chloramphenicol	This study
p8356	pSW23T- <i>sfiA::PrecN-gfp</i>	Chloramphenicol	[7, 8]

268

269

270

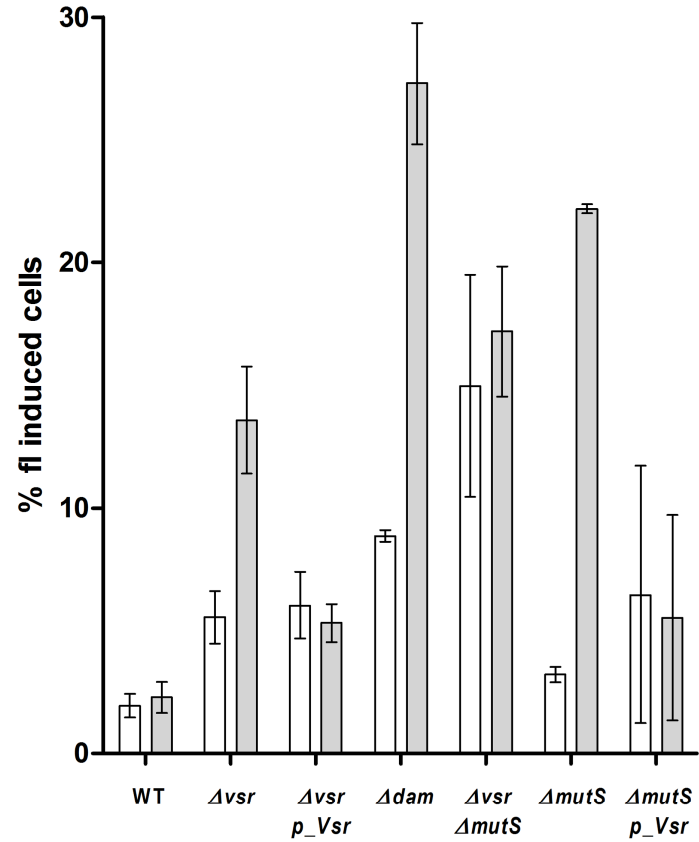
A



B

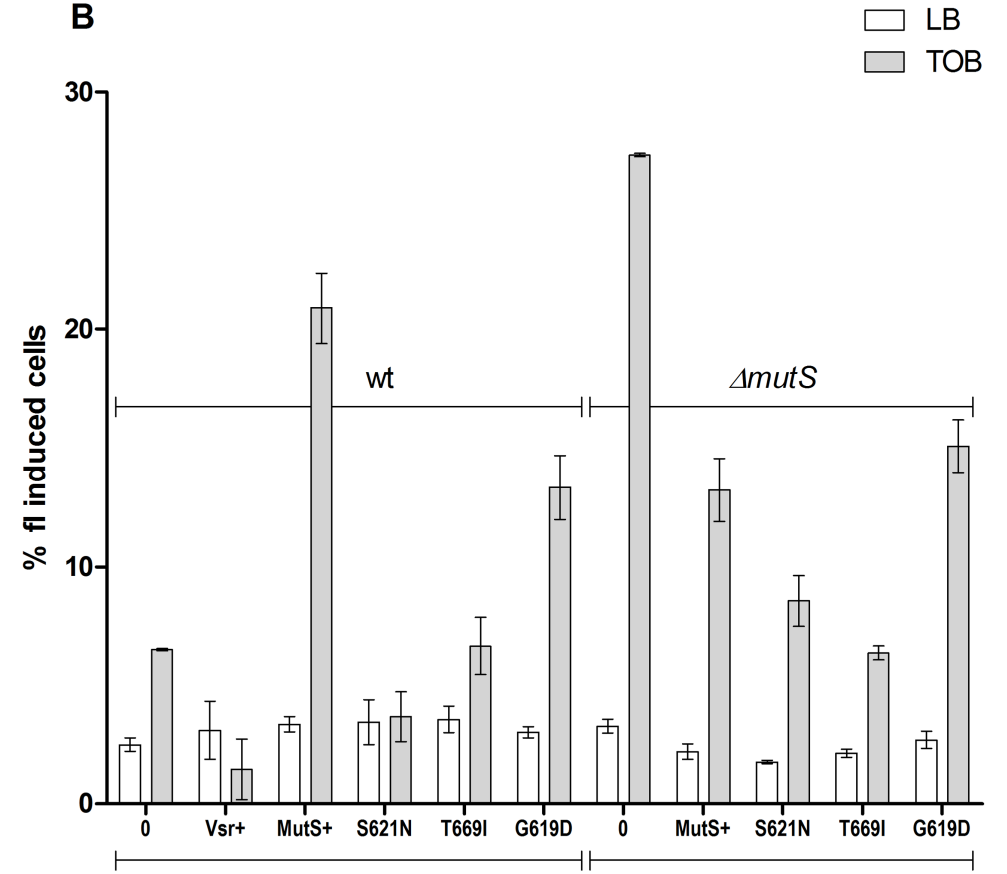
Strain MG1655 <i>dapA</i> with	Viability (colony growth)			
	DAP	TOB	MMC	LEV
No plasmid	+	-	-	-
Pint- <i>dapA</i>	+	-	+	+
PrecN- <i>dapA</i>	+	-	+	+

A



MMR	+	+	+	-	-	-	-
VSPR	+	-	+	-	-	-	+

B



MMR	+	+	+	-	-	-	-	+	-	-	-
VSPR	+	+	-	+	+	-	-	-	+	+	-