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

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Abstract (100 words max).

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

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

Introduction

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

Materials and methods

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

Strain constructions. MG1655 *vsr*::*km* (B599) and MG1655 *Δdam::*Tn*9* (B371) were constructed by P1 transduction of *vsr*::*km* and *Δdam::*Tn*9* from strains Keio JW1943 and M0099^{Fermentas}. MG1655 *mutS::*Tn*10 vsr*::*km* was constructed by P1 transduction of *vsr*::*km* into MG1655 *mutS* strain (2580). Antibiotics were used at the following concentrations: 25μg/ml kanamycin (*km*) and chloramphenicol (Tn*9*); 15 μg/ml tetracycline (Tn*10*).

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

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

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

Results

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

We then transformed our MG1655 *ΔdapA* mariner insertion library with the plasmid *Pint-dapA* and selected viable colonies on sub-MIC TOB in absence of DAP in the medium (**Fig.1A**). Sub-MIC TOB does not induce SOS in wild type *E. coli* [7, 8], thus if clones are able to grow on this medium, this means that DapA is expressed and that SOS is induced in 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.2**A), 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 *ΔmutS*) 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 *ΔmutS* 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 *ΔmutS* backgrounds. As expected, in
144 strains over-expressing VSPR proficient class I mutants S621N and T669I, SOS levels are

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

Discussion

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

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

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

186 [1] Baharoglu, Z., Babosan, A., Mazel, D., Identification of genes involved in low
187 aminoglycoside-induced SOS response in Vibrio cholerae: a role for transcription stalling and
188 Mfd helicase, Nucleic Acids Res (2013).
189 [2] Baharoglu, Z., Babosan, A., Mazel, D., Identification of genes involved in low
190 aminoglycoside-induced SOS response in Vibrio cholerae: a role for transcription stalling and
191 Mfd helicase, Nucleic Acids Res 42 (2014) 2366-2379.
192 [3] Baharoglu, Z., Bikard, D., Mazel, D., Conjugative DNA transfer induces the bacterial SOS
193 response and promotes antibiotic resistance development through integron activation, PLoS
194 Genet 6 (2010) e1001165.
195 [4] Baharoglu, Z., Garriss, G., Mazel, D., Multiple Pathways of Genome Plasticity Leading to
196 Development of Antibiotic Resistance, Antibiotics 2 (2013) 288-315.
197 [5] Baharoglu, Z., Krin, E., Mazel, D., Transformation-induced SOS regulation and carbon
198 catabolite control of the V. cholerae integron integrase: connecting environment and genome
199 plasticity, J Bacteriol (2012).
200 [6] Baharoglu, Z., Krin, E., Mazel, D., RpoS Plays a Central Role in the SOS Induction by
201 Sub-Lethal Aminoglycoside Concentrations in Vibrio cholerae, Plos Genetics 9 (2013).
202 [7] Baharoglu, Z., Krin, E., Mazel, D., RpoS Plays a Central Role in the SOS Induction by
203 Sub-Lethal Aminoglycoside Concentrations in Vibrio cholerae., PLoS Genetics 9(4):
204 e1003421. doi:10.1371/journal.pgen.1003421 (2013).
205 [8] Baharoglu, Z., Mazel, D., *Vibrio cholerae* triggers SOS and mutagenesis in response to a
206 wide range of antibiotics: a route towards multiresistance, Antimicrob Agents Chemother 55
207 (2011) 2438-2441.
208 [9] Baharoglu, Z., Mazel, D., Vibrio cholerae Triggers SOS and Mutagenesis in Response to a
209 Wide Range of Antibiotics: a Route towards Multiresistance, Antimicrobial Agents and
210 Chemotherapy 55 (2011) 2438-2441.
211 [10] Banerjee, S., Chowdhury, R., An orphan DNA (cytosine-5-)-methyltransferase in Vibrio
212 cholerae, Microbiology 152 (2006) 1055-1062.
213 [11] Bell, D.C., Cupples, C.G., Very-short-patch repair in Escherichia coli requires the dam
214 adenine methylase, J Bacteriol 183 (2001) 3631-3635.
215 [12] Bhagwat, A.S., Lieb, M., Cooperation and competition in mismatch repair: very short-
216 patch repair and methyl-directed mismatch repair in Escherichia coli, Mol Microbiol 44
217 (2002) 1421-1428.
218 [13] Chiang, S.L., Rubin, E.J., Construction of a mariner-based transposon for epitope-
219 tagging and genomic targeting, Gene 296 (2002) 179-185.
220 [14] Drotschmann, K., Aronshtam, A., Fritz, H.J., Marinus, M.G., The Escherichia coli MutL
221 protein stimulates binding of Vsr and MutS to heteroduplex DNA, Nucleic Acids Res 26
222 (1998) 948-953.
223 [15] Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S.,
224 Gonzalez-Zorn, B., Barbe, J., et al., The SOS response controls integron recombination,
225 Science 324 (2009) 1034.
226 [16] Gutierrez, A., Laureti, L., Crussard, S., Abida, H., Rodriguez-Rojas, A., Blazquez, J.,
227 Baharoglu, Z., Mazel, D., et al., beta-lactam antibiotics promote bacterial mutagenesis via an
228 RpoS-mediated reduction in replication fidelity, Nature communications 4 (2013) 1610.
229 [17] Lieb, M., Rehmat, S., Very short patch repair of T:G mismatches in vivo: importance of
230 context and accessory proteins, J Bacteriol 177 (1995) 660-666.

231 [18] Lieb, M., Rehmat, S., Bhagwat, A.S., Interaction of MutS and Vsr: some dominant-
232 negative mutS mutations that disable methyladenine-directed mismatch repair are active in
233 very-short-patch repair, J Bacteriol 183 (2001) 6487-6490.
234 [19] Macintyre, G., Doiron, K.M., Cupples, C.G., The Vsr endonuclease of Escherichia coli:
235 an efficient DNA repair enzyme and a potent mutagen, J Bacteriol 179 (1997) 6048-6052.
236 [20] Polosina, Y.Y., Mui, J., Pitsikas, P., Cupples, C.G., The Escherichia coli mismatch repair
237 protein MutL recruits the Vsr and MutH endonucleases in response to DNA damage, J
238 Bacteriol 191 (2009) 4041-4043.
239 [21] Richaud, C., Mengin-Lecreulx, D., Pochet, S., Johnson, E.J., Cohen, G.N., Marliere, P.,
240 Directed evolution of biosynthetic pathways. Recruitment of cysteine thioethers for
241 constructing the cell wall of Escherichia coli, J Biol Chem 268 (1993) 26827-26835.
242 [22] Robbins-Manke, J.L., Zdraveski, Z.Z., Marinus, M., Essigmann, J.M., Analysis of global
243 gene expression and double-strand-break formation in DNA adenine methyltransferase- and
244 mismatch repair-deficient Escherichia coli, J Bacteriol 187 (2005) 7027-7037.
245 [23] Robertson, A.B., Matson, S.W., Reconstitution of the very short patch repair pathway
246 from Escherichia coli, J Biol Chem 287 (2012) 32953-32966.
247 [24] Wu, T.H., Marinus, M.G., Dominant negative mutator mutations in the mutS gene of
248 Escherichia coli, J Bacteriol 176 (1994) 5393-5400.

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Legend to figures

Figure 1: The genetic screen. A. Experimental setup. B. Validation of the host strain. MG1655 *ΔdapA* carrying the specified plasmid was grown in LB supplemented with DAP in order to allow growth in the absence of SOS induction. A 10^{-6} dilution of the overnight culture was plated on several antibiotics at sub-MIC (and without DAP). The growth of colonies was monitored. "+" means growth of colonies. "-" means no colony development. TOB: tobramycin 0.01μg/ml; MMC: mitomycin C 0.02 μg/ml, LEV: levofloxacin 0.01 μg/ml

Figure 2: SOS induction levels after sub-MIC tobramycin treatment. A. Effect VSR. B. Effect of plasmids over-expressing *mutS* mutants. Histogram bars represent the GFP fluorescence in LB or in the presence of tobramycin (0.01 μg/ml) and thus reflect the levels of SOS. MMR and VSPR stand for: respectively mismatch repair and very short patch repair. "+" means *proficient*; "-" means *deficient*. Error bars represent standard deviation. Each strain was tested at least 4 times.

267 **Table 1: Strains and plasmids**

Strains	Genotype	Resistance	Construction
8120	MG1655 *ΔdapA*		Laboratory collection
9404	MG1655 *ΔdapA pACYC184-Pint-dapA*	Tetracyclin	This study
B599	MG1655 *vsr*::*km*	Kanamycin	This study
B371	MG1655 *Δdam::Tn9*	Chloramphenicol	This study
2580	MG1655 *mutS::Tn10*	Tetracyclin	Laboratory collection
B600	MG1655 *mutS::Tn10 vsr*::*km*	Tetracyclin, Kanamycin	This study
Plasmids			
pB453	pTOPO-P*bla-vsr*	Ampicillin	This study
pB420	pACYC184-*mutS*+	Chloramphenicol	[16]
pB421	pACYC184-$mutS_{G619D}$	Chloramphenicol	This study
pB449	pACYC184-$mutS_{S621N}$	Chloramphenicol	This study
pB451	pACYC184-$mutS_{T669I}$	Chloramphenicol	This study
p8356	pSW23T_*sfiA*::P*recN-gfp*	Chloramphenicol	[7, 8]

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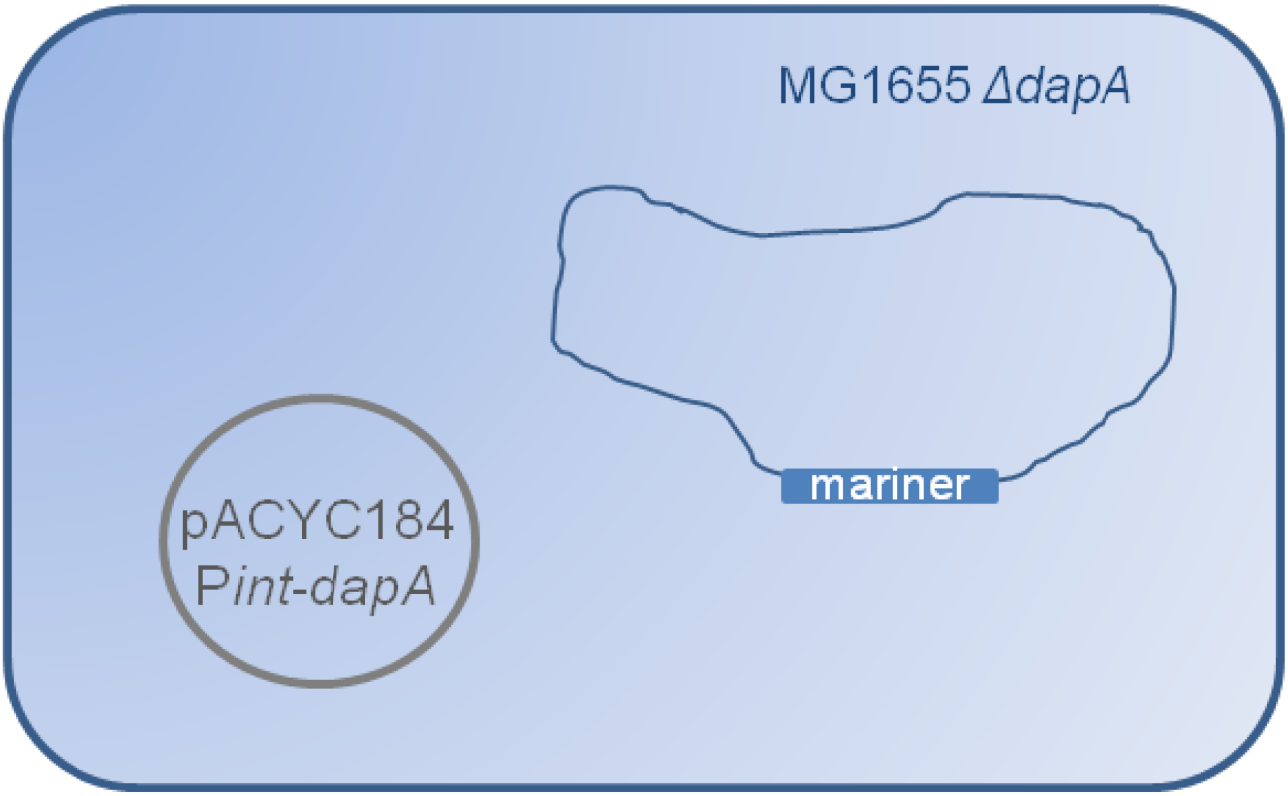
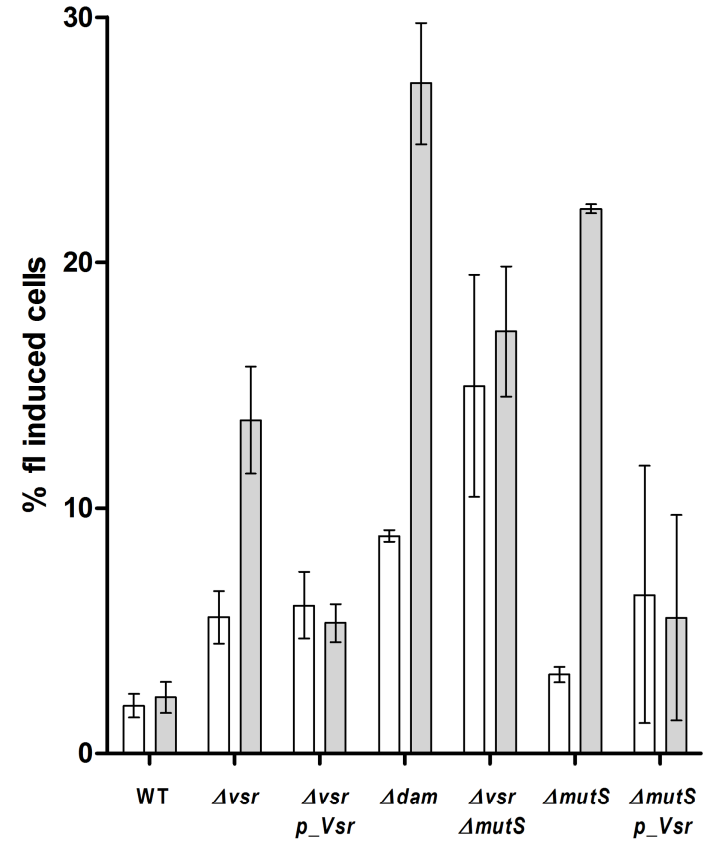
A



B

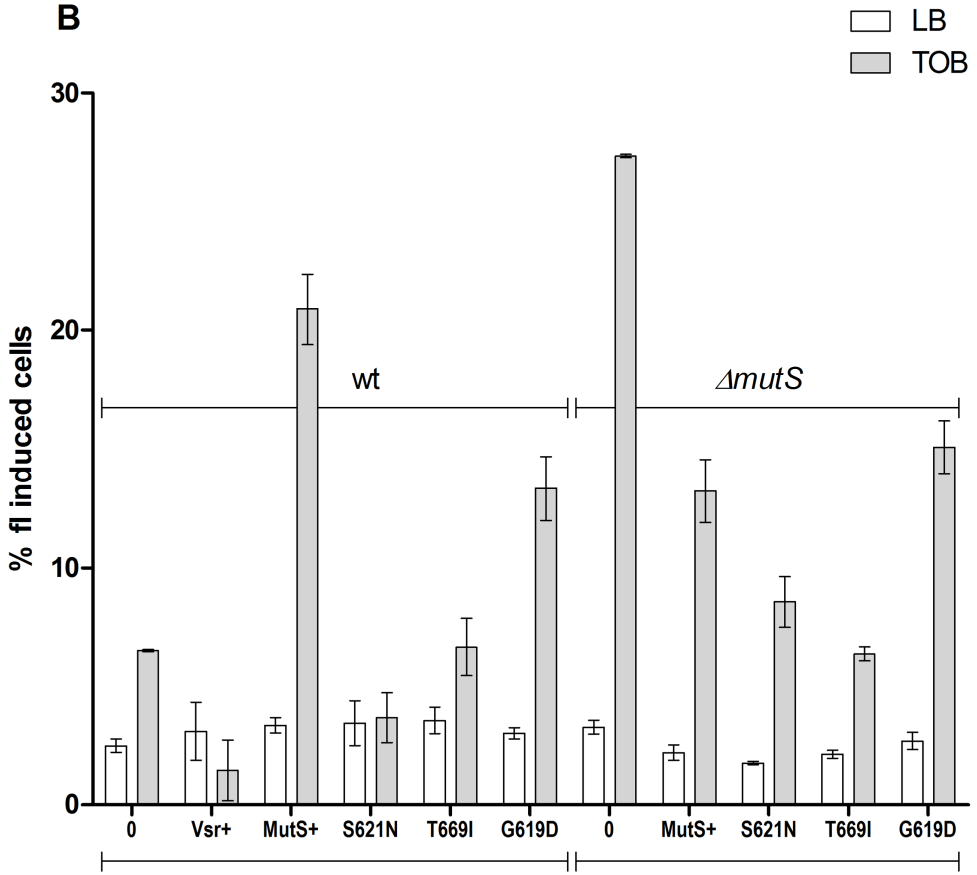
Strain MG1655 *dapA* with	Viability (colony growth)			
	DAP	TOB	MMC	LEV
No plasmid	+	-	-	-
Pint-dapA	+	-	+	+
PrecN-dapA	+	-	+	+

A



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

B



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