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

3 Zeynep Baharoglu^{1,2,*}, Didier Mazel^{1,2},

4 1. Institut Pasteur, Unité Plasticité du Génome Bactérien, Département Génomes et Génétique, 25 rue
5 du docteur Roux 75015 Paris, France

6 2. CNRS, UMR3525, Paris, France

7 * To whom correspondence should be addressed. Zeynep Baharoglu Tel: +33 144389483; Fax: +33 145688834;
8 E-mail: baharogl@pasteur.fr

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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.

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

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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-Pint-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-PrecN-dapA]: same as Pint-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 *Adam::Tn9* (B371) were constructed by P1 transduction of *vsr::km* and *Adam::Tn9* from strains Keio JW1943 and M0099^{Fermentas}. MG1655 *mutS::Tn10 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 (Tn9); 15 µg/ml tetracycline (Tn10).

Construction of the *E. coli::mariner* insertion library was performed as described [1] into MG1655 *AdapA 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.

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.

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 $\Delta dapA$ (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 $\Delta dapA$ 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 $\Delta dapA$ 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 $\Delta dapA$ with plasmids carrying *Pint_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 $\Delta dapA$ 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

inactivation of a gene would lead to SOS-dependent expression of *dapA* from the *int1A* promoter (or that transposition created a new promoter upstream of the *dapA* gene). We obtained two viable colonies on medium supplemented with sub-MIC TOB and devoid of DAP. The sequencing of flanking regions in both colonies led to the identification of the *vsr* gene (b1960) in these two clones. Vsr is involved in very short patch repair (VSPR) in *E. coli* [19]. VSPR pathway comprises the Vsr endonuclease, DNA polI (*polA*), DNA ligase, MutL and MutS [23]. The identification of *vsr* in our inactivation screen suggests that in the absence of VSPR, SOS is induced by sub-MIC TOB in *E. coli*. This induction was confirmed by flow cytometry using the *gfp* reporter under the control of the SOS dependent *recN* promoter (Fig.2) that we developed and used in our previous studies [3, 5, 7, 8]. In order to confirm the result obtained in our genetic screen, we constructed a MG1655 Δvsr strain and verified that inactivation of *vsr* leads to SOS induction by TOB, and that complementation with a plasmid expressing *vsr* restores the wild type phenotype (**Fig.2A**, Δvsr p_Vsr), showing that in the absence of VSPR, SOS is induced by sub-MIC TOB in *E. coli*.

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

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

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

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

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

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Legend to figures

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

MG1655 $\Delta dapA$ 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 $0.01 \mu\text{g/ml}$; MMC: mitomycin C $0.02 \mu\text{g/ml}$, LEV: levofloxacin $0.01 \mu\text{g/ml}$

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 ($0.01 \mu\text{g/ml}$) 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 $\Delta dapA$		Laboratory collection
9404	MG1655 $\Delta dapA$ pACYC184-Pint-dapA	Tetracyclin	This study
B599	MG1655 <i>vsr::km</i>	Kanamycin	This study
B371	MG1655 $\Delta dam::Tn9$	Chloramphenicol	This study
2580	MG1655 <i>mutS::Tn10</i>	Tetracyclin	Laboratory collection
B600	MG1655 <i>mutS::Tn10 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]

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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

B