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Zeynep Baharoglu, Didier Mazel. Vibrio cholerae triggers SOS and mutagenesis in response to a wide range of antibiotics: a route towards multiresistance.. Antimicrobial Agents and Chemotherapy, 2011, 55 (5), pp.2438-2441. 10.1128/AAC.01549-10 . pasteur-01423615

HAL Id: pasteur-01423615

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Submitted on 30 Dec 2016

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2011, p. 2438–2441 Vol. 55, No. 5
0066-4804/11/$12.00 doi:10.1128/AAC.01549-10
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Vibrio cholerae Triggers SOS and Mutagenesis in Response to a Wide Range of Antibiotics: a Route towards Multiresistance▽†

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Received 9 November 2010/Returned for modification 3 December 2010/Accepted 27 January 2011

Antibiotic resistance development has been linked to the bacterial SOS stress response. In *Escherichia coli*, fluoroquinolones are known to induce SOS, whereas other antibiotics, such as aminoglycosides, tetracycline, and chloramphenicol, do not. Here we address whether various antibiotics induce SOS in *Vibrio cholerae*. Reporter green fluorescent protein (GFP) fusions were used to measure the response of SOS-regulated promoters to subinhibitory concentrations of antibiotics. We show that unlike the situation with *E. coli*, all these antibiotics induce SOS in *V. cholerae*.

The emergence of multiply resistant bacteria has been correlated with widespread antibiotic usage. It has been noted that sub-MICs of antibiotics induce several changes in gene expression (8). Fluoroquinolones (FQs), beta-lactams, and trimethoprim (TMP) induce the SOS stress response in *Escherichia coli* (19, 23, 25), resulting in increased mutation frequency (17, 25), whereas other antibiotics, such as aminoglycosides (AGs), chloramphenicol (CAM), rifampin (RIF), or tetracycline (TCN), do not (23). However, TCN induces mutagenesis requiring SOS-regulated DNA polymerases (7), suggesting a link between tetracycline and SOS. Interestingly, AGs, as well as FQs and mitomycin C (MMC), induce the competence regulon (*com*) in *Streptococcus* (21). *Streptococcus* does not have any homologue for the SOS repressor LexA; however, most of the DNA repair genes, including *recA*, belong to the *com* regulon, considered a parallel of SOS in *Streptococcus*.

A recent study demonstrated how beta-lactams, FQs, and AGs stimulate production of reactive oxygen species (ROS) in bacteria (12). ROS can damage DNA and induce mutagenesis, leading to multiple resistances. Damaged DNA is a potent SOS inducer, suggesting that all antibiotics have the potential to induce the bacterial stress response, which is a fundamental mechanism of adaptation and resistance development.

Vibrio cholerae is a Gram-negative human pathogen which can be deadly if not treated appropriately with antibiotics (e.g., TCN, TMP, FQs, and sometimes CAM, according to WHO recommendations). AGs are also generally used against Gram-negative bacteria, although not specifically against cholera infections. All strains of *V. cholerae* carry a chromosomal superintegron (SI), composed of an array of promoterless adaptive gene cassettes, that can be recombined and summoned when necessary through the action of the integrase IntIA (3), which is regulated by SOS (5, 11).

The *V. cholerae* response to sub-MICs of antibiotics has not been thoroughly studied. In this study we addressed whether antibiotics that do not induce SOS in *E. coli* might act differently in *V. cholerae*.

Results and discussion. We constructed *V. cholerae* and *E. coli* reporter strains carrying the green fluorescent protein gene (*gfp*) fused to the *recN* promoter. RecN expression is upregulated during SOS induction in both bacteria (1). Figure 1 shows the percentage of GFP-induced cells after treatment with sub-MICs of specified antibiotics. Sub-MICs were determined as concentrations 100-fold lower than the MIC for each bacterium (Fig. 1). Bacteria were cultured overnight with specified antibiotics on Mueller-Hinton (MH) medium, and the percentage of fluorescence-induced cells was determined by flow cytometry as described previously (1).

Our data confirm that FQs and TMP are strong SOS inducers in *E. coli* (Fig. 1, black bars). The isogenic *lexAind* strain, which is not inducible for SOS (due to an uncleavable LexA repressor) corroborated that the increase in GFP expression was indeed due to SOS induction (Fig. 1, gray bars). Conversely, AGs, TCN, and CAM do not induce SOS in *E. coli*. Supporting this fact, abrogation of SOS (*lexAind* strain) does not impact fluorescence values obtained with TCN and AGs. In order to examine how these antibiotics act on *V. cholerae* SOS, we used the quorum-sensing-proficient N16961 *hapR*$^+$ strain (4, 16), because the overall SOS level (even without antibiotics) was three to five times higher (and thus easier to detect) in the *hapR*$^+$ context than in the *hapR*-deficient mutant (Fig. 1, red versus pink bars for MH medium; see also Fig. S1 in the supplemental material). Besides, many other natural strains of *V. cholerae*, such as the classical O1 strain responsible for the 6th pandemic (O395), the O1 strain El Tor MJ1236, and strains A1552, O37, HK1, CA401, and SG21, are *hapR*$^+$. As expected, ciprofloxacin (CIP) (an FQ) and TMP strongly induced SOS

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 7 February 2011.

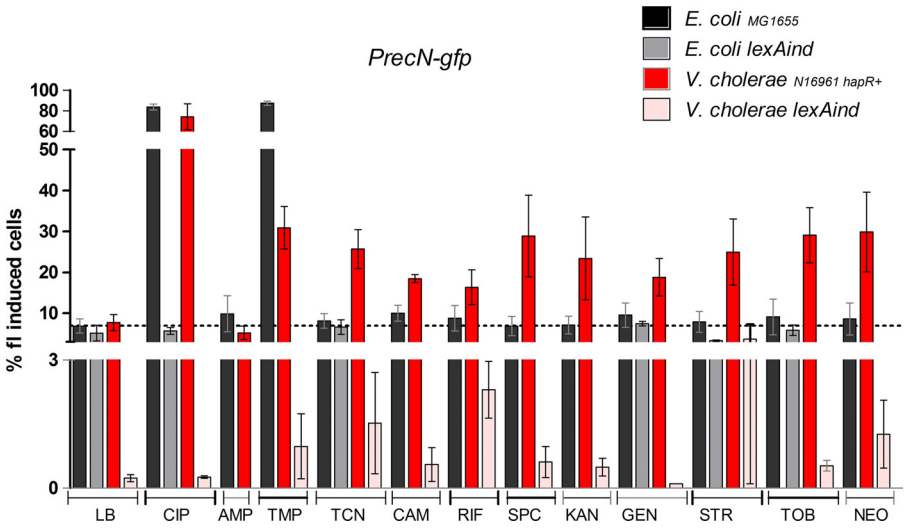


FIG. 1. SOS induction by subinhibitory concentrations of antibiotics in *E. coli* and *V. cholerae.* Bacteria were grown overnight in MH supplemented with sub-MICs of specified antibiotics as follows (final concentrations [μg ml^{-1}] of drug for organism [*E. coli*, *V. cholerae*]): ciprofloxacin (CIP), 0.05, 0.005; ampicillin (AMP) and rifampin (RIF), 0.05, 0.01; trimethoprim (TMP), 0.05, 0.005; tetracycline (TCN), 0.15, 0.015; chloramphenicol (CAM), 0.15, 0.005; spectinomycin (SPC), kanamycin (KAN), and streptomycin (STR), 0.2, 0.05; gentamicin (GEN), tobramycin (TOB), and neomycin (NEO), 0.1, 0.01. The samples were washed in PBS, and the fluorescence was measured using the FACSCalibur device (1). The percentage of fluorescent cells is represented on the *y* axis. Each measurement was reproduced at least four times. In order to test whether 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.

(Fig. 1, red bars). Strikingly, AGs, CAM, RIF, and TCN also induced SOS in *V. cholerae*, although to a lower degree than FQs and TMP. GFP expression was dramatically reduced in the *lexAind* strain regardless of the antibiotic, confirming that sub-MIC antibiotics induce SOS in *V. cholerae*. SOS induction by beta-lactams is variable, depending on experimental conditions (15, 18), and ampicillin did not induce SOS in our P_{recN}-*gfp* fusion strains.

SOS is also known to induce mutagenesis. A major pathway is through the activation of the translesion synthesis polymerases (such as DinB/VC2287) that lack proofreading activity and that belong to the SOS regulon in both bacteria. We determined the effect of incubation with sub-MIC antibiotics on mutation frequency, using RIF resistance appearance as a reporter. Indeed, spontaneous point mutations in the gene encoding the β-subunit of RNA polymerase (*rpoB*) are known to give rise to this phenotype and are commonly used for such testing (24).

As expected, mutagenesis was strongly induced by CIP (FQ) and TMP in the *E. coli* strain MG1655 (Fig. 2A). TCN, CAM, RIF, and spectinomycin (SPC) also slightly induced mutation frequency, suggesting a low effect of these antibiotics on SOS, which was undetectable by flow cytometry. It is important to note here that mutagenesis may not exclusively be due to SOS, as other pathways exist (2, 20).

Notably, in addition to the strong SOS inducers (CIP and TMP), ampicillin (AMP), TCN, and CAM strongly increased the mutation frequency in *V. cholerae* N16961 $hapR^+$. Some AGs (SPC, tobramycin [TOB], and neomycin) also significantly induced mutagenesis, whereas gentamicin and kanamycin did not consistently affect mutation frequency. No increase was observed in the isogenic *lexAind* strain, showing that increased frequencies in wild-type *V. cholerae* were indeed due to the induction of SOS (Fig. 2A). To ascertain that the effect of sub-MICs of antibiotics is a general property of *V. cholerae*, we also tested the mutagenic effect of selected antibiotics on O1 classical (0395/569B) and O139 (MJ1236) strains of *V. cholerae*, which differ from N16961 according to whole genome sequencing data comparison. MMC was used as a control SOS inducer. All *V. cholerae* strains show increased mutation frequency after CAM and TOB treatment, whereas no effect is observed in *E. coli* (Fig. 2B). Unlike *E. coli*, *V. cholerae* is thus prone to SOS response activation by a wide set of antibiotics at sub-MICs. We conclude that *E. coli* should not be taken as a paradigm regarding the effects of antibiotics on bacteria.

SOS induction by antibiotics has to be taken seriously, as it can result in undesired changes in the behavior of bacteria and their adaptation to hostile environments. Examples are numerous: SOS can induce the expression of the cholera toxin (22), can trigger SI cassette rearrangements conferring resistance to an originally sensitive *V. cholerae* (1), and may lead to persistence and eventually the development of multidrug resistance (9, 10, 13).

In conclusion, activation of SOS appears to be a major threat to the efficiency of antibiotic treatments. An engineered bacteriophage that suppresses the SOS network has been reported to enhance killing by antibiotics in *E. coli* and to increase survival of infected mice (14). Another example in treatment failure due to SOS is the resistance to FQs mediated by translesional DNA-polymerase-dependent mutations appearing upon SOS induction (6). Consequently, the inhibition of

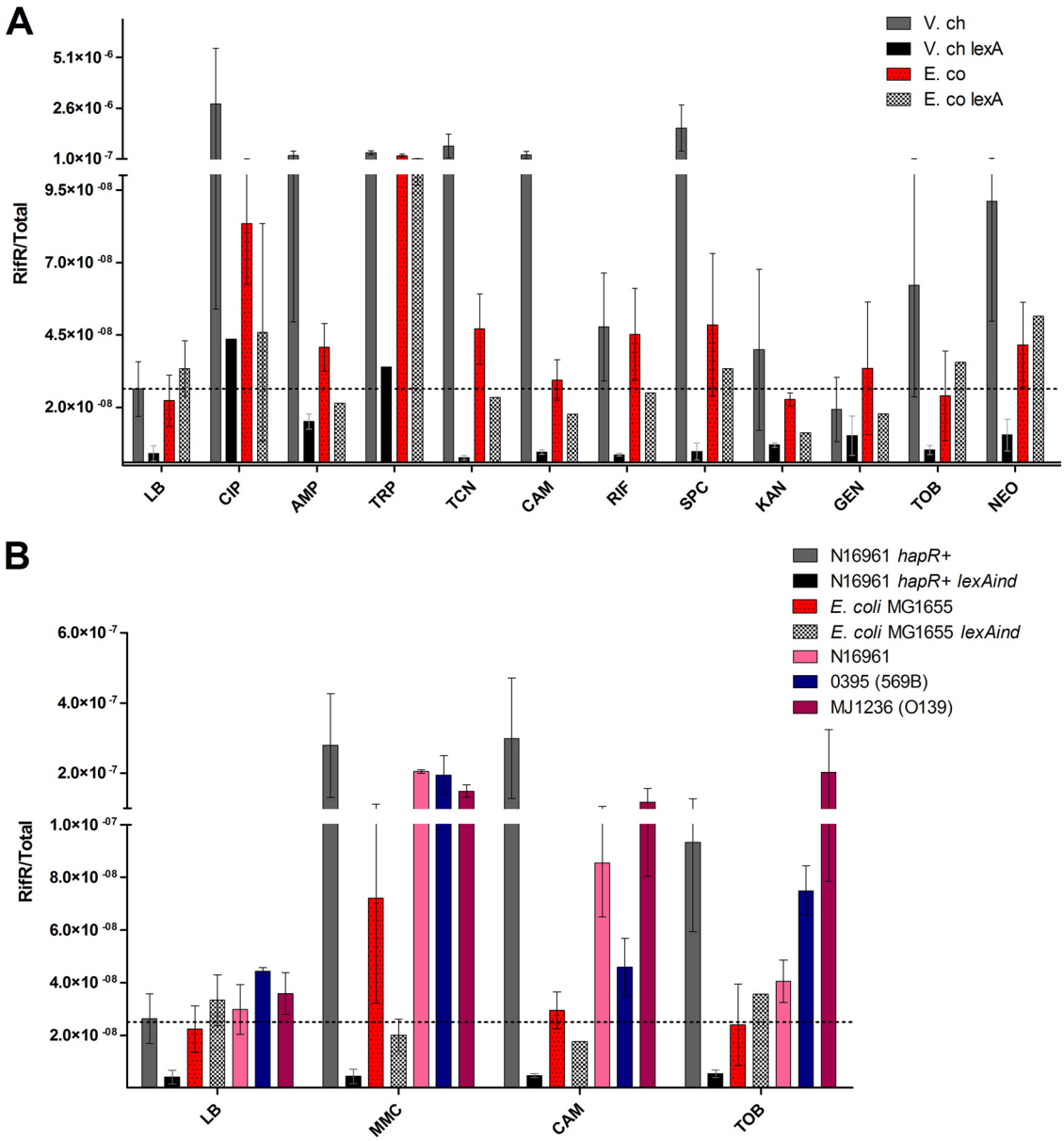


FIG. 2. Spontaneous mutation frequency after treatment with subinhibitory concentrations of antibiotics. Bacteria were grown as described in Fig. 1. Appropriate dilutions were plated on Luria broth, and 1 ml of culture was centrifuged and plated on 100-μg/ml rifampin plates. The mutation frequency, corresponding to the rifampin-resistant CFU count over the total number of CFU, was measured and represented on the *y* axis for each antibiotic. (A) *E. coli* MG1655 and *V. cholerae* N16961 *hapR*$^{+}$. (B) Comparison of various *V. cholerae* strains.

the SOS response in pathogens by the use of "anti-SOS" components could be promising for prevention of resistance development.

We thank David Bikard for his help with statistical analysis of the flow cytometry data.

This work was supported by the Institut Pasteur, the Centre National de la Recherche Scientifique (CNRS-URA 2171), the French National Research Agency (ANR-08-MIE-016), the EU (NoE EuroPathoGenomics, LSHB-CT-2005-512061). and the Fondation pour la Recherche Médicale (équipe FRM 2007). Z.B. is supported by a postdoctoral fellowship from the Roux Foundation and a DIM Malinf fellowship (Conseil régional d'Île-de-France).

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