RpoS Plays a Central Role in the SOS Induction by Sub-Lethal Aminoglycoside Concentrations in *Vibrio cholerae*

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Abstract

Bacteria encounter sub-inhibitory concentrations of antibiotics in various niches, where these low doses play a key role for antibiotic resistance selection. However, the physiological effects of these sub-lethal concentrations and their observed connection to the cellular mechanisms generating genetic diversification are still poorly understood. It is known that, unlike for the model bacterium *Escherichia coli*, sub-minimal inhibitory concentrations (sub-MIC) of aminoglycosides (AGs) induce the SOS response in *Vibrio cholerae*. SOS is induced upon DNA damage, and since AGs do not directly target DNA, we addressed two issues in this study: how sub-MIC AGs induce SOS in *V. cholerae* and why they do not do so in *E. coli*. We found that when bacteria are grown with tobramycin at a concentration 100-fold below the MIC, intracellular reactive oxygen species strongly increase in *V. cholerae* but not in *E. coli*. Using flow cytometry and *gfp* fusions with the SOS regulated promoter of *intIA*, we followed AG-dependent SOS induction. Testing the different mutation repair pathways, we found that over-expression of the base excision repair (BER) pathway protein MutY relieved this SOS induction in *V. cholerae*, suggesting a role for oxidized guanine in AG-mediated indirect DNA damage. As a corollary, we established that a BER pathway deficient *E. coli* strain induces SOS in response to sub-MIC AGs. We finally demonstrate that the RpoS general stress regulator prevents oxidative stress-mediated DNA damage formation in *E. coli*. We further show that AG-mediated SOS induction is conserved among the distantly related Gram negative pathogens *Klebsiella pneumoniae* and *Photorhabdus luminescens*, suggesting that *E. coli* is more of an exception than a paradigm for the physiological response to antibiotics sub-MIC.

Introduction

Vibrio cholerae is a severe human pathogen growing on crustacean shells or planktonically in the aquatic environment. *V. cholerae* couples environmental stress and adaptation through SOS response dependent gene expression and mutagenesis, induced, for instance, during ssDNA uptake [1–2] or after antibiotic treatment [3]. The SOS stress response occurs through the induction of an entire regulon controlled by the LexA repressor [4]. In the presence of abnormal levels of single stranded DNA (ssDNA) in the cell, RecA, the pivotal protein of homologous recombination, forms a nucleofilament on ssDNA which catalyses the self-cleavage of the LexA repressor. Inactivation of LexA releases the transcription of recombination and repair genes belonging to the SOS regulon. ssDNA levels increase in the cell during horizontal gene transfer but also when replication is blocked in the presence of any DNA damaging agent such as antibiotics that target DNA (like fluoroquinolones or mitomycin C) or UV irradiation.

We recently reported the effects on the SOS response induction of sub-Minimal Inhibitory Concentrations (sub-MIC) of antibiotics from different families in *V. cholerae* [3]. Sub-MIC of antibiotics

may be found in several environments [5], in particular in the mammalian hosts of pathogenic and commensal bacteria, where they can play a very important role for the selection of resistant bacteria [6]. Moreover, a large part of the ingested antibiotics is rejected unchanged in the environment [7–8]. Unlike above-MIC, the biological effects of sub-MIC of antibiotics have not been studied in detail. Transcriptome and proteome analyses have shown that many antibiotics exhibit contrasting properties when tested at low compared to high concentrations: it has been noted that sub-MIC of antibiotics induce several changes in expression profiles of a wide range of genes unrelated to the target function [9]. Sub-MIC of antibiotics induce phenotypic changes, including an increase in mutagenesis [3]. Strikingly, we observed that sub-MIC of aminoglycosides (referred to as AGs throughout this manuscript), chloramphenicol, rifampicin and tetracycline induce SOS in *V. cholerae* [3]. These antibiotics do not directly target DNA synthesis or the DNA molecule and they do not induce SOS in *E. coli* [3]. The fact that they induce SOS in *V. cholerae* suggests a role for intermediate factors that cause stress and lead to DNA damage in this bacterium. A recent study demonstrated how beta-lactams, fluoroquinolones (FQs) and AGs stimulate production of reactive oxygen species (ROS) in bacteria [10]. ROS can damage DNA

Author Summary

Bacteria can remodel their genome in order to adapt to new environments. This genomic plasticity can be mediated by horizontal gene transfer, genome rearrangements, and mutations. One important factor is the bacterial SOS response, induced upon DNA damaging stress conditions. SOS response induction leads to increased mutation frequencies and genome rearrangements. Antibiotics such as fluoroquinolones cause direct DNA damage and induce the SOS response. Other antibiotics such as aminoglycosides (AGs) do not target DNA and do not induce SOS in *E. coli*. However, it was recently shown that low concentrations of AGs, which do not inhibit bacterial growth, do induce SOS in *V. cholerae*. We show here that AG-dependent SOS induction is caused by oxidative stress in *V. cholerae* and that *E. coli* is well protected against this kind of oxidative insult thanks to efficient oxidative stress response through the RpoS general stress response sigma factor. We propose that the model bacterium *E. coli* is not a paradigm in this case, as other pathogens such as *Klebsiella pneumoniae* and *Photorhabdus luminescens* also induce SOS in response to sub-MIC AGs.

and proteins, and induce mutagenesis, increasing as such the odds of resistance conferring mutations, ultimately leading to multiple resistances.

DNA damage can indeed be caused by ROS [11]: DNA is the target of hydroxyl radicals (OH^-) and the Fenton reaction is the major source of OH^- formation [12] in the presence of iron ions [13–14]. Iron can localize along the phosphodiester backbone of nucleic acids and OH^- attacks DNA sugar and bases and ultimately causes double strand breaks, which are repaired by the RecBCD homologous recombination pathway [15].

Another type of DNA damage caused by oxidative attack is the incorporation of oxidized guanine residues (7,8-Dihydro-8-oxo-guanine or 8-oxo-G). *E. coli* and *V. cholerae* possess a defense system against 8-oxo-G which involves MutT, MutY and MutM [16]. MutT hydrolyses 8-oxo-G in the nucleotide pool, whereas MutY and MutM limit incorporation of 8-oxo-G and mismatch formation [17]. Here too, incomplete action of this base excision repair system may lead to double strand DNA breaks that are cytotoxic if unrepaired [18].

On the other hand, it is known that mistranslated and misfolded proteins are more susceptible to oxidation [19]. Moreover, mistranslational corruption of proteins may lead to replication fork collapse and induction of SOS [20]. It has been shown in *Deinococcus radiodurans* that cell death by radiation is not caused by direct DNA damage but primarily by oxidative damage on proteins, which eventually results in the loss of DNA repair [21]. Proteome protection against ROS thus seems to be at least as important as DNA protection for keeping cell integrity in times of oxidation. Knowing that AGs target protein translation, it was appealing to determine if sub-MIC of these antibiotics induce ROS formation in *V. cholerae* and to try to characterize mechanisms involved in the induction of SOS.

Finally, oxidative stress is known to induce the RpoS regulon [22–23]. RpoS is the stationary phase sigma factor, which is induced in exponential phase in response to stress [24–26]. Genes expressed following RpoS regulon induction, namely catalases (KatE, KatG) and iron chelators (Dps), protect cells from ROS related DNA damage [27], e.g. double strand DNA breaks caused by hydroxyl radicals generated through the Fenton reaction [12–13,28]. It has been shown in *Vibrio vulnificus* (a human pathogen) that RpoS is essential in exponential growth phase to overcome H_2O_2 related oxidative stress [29]. Moreover, it was observed that *V. vulnificus* is more sensitive to H_2O_2 than *E. coli* and that KatG catalase expression is more strongly reduced in a *ΔrpoS* mutant in *V. vulnificus* compared to *E. coli*. These observations pointed to an effect of RpoS in the differences on the response to H_2O_2 between these two species, and as *V. cholerae* is more phylogenetically related to *V. vulnificus* than to *E. coli*, one would expect more conservation for the RpoS response between the two *Vibrio* species.

RpoS levels are regulated at several stages in *E. coli*: (i) transcription [30] (ii) mRNA stability [31] and (iii) protein stability. Indeed, the RpoS protein is targeted to the ClpXP protease for degradation by the adaptor protein RssB [32–34]. *ΔclpP* mutants accumulate RpoS [34]. Anti-adaptors IraD, IraM, IraP are induced following stress (respectively: oxidative stress, magnesium and phosphate starvation) [32] and prevent this targeting by RssB, thus stabilizing the RpoS protein [33]. A *ΔrssB* mutant relieves H_2O_2 sensitivity of an *E. coli ΔiraD* mutant in an RpoS dependent fashion. Interestingly, the IraD anti-adaptor, which is induced during oxidative stress and DNA damage is conserved only in *E. coli* and *Salmonella* and is absent from *V. cholerae* [24–25], whereas RssB is present in *V. cholerae*, suggesting that *E. coli* RpoS is more effectively protected from degradation during stress than *V. cholerae* RpoS. Moreover, among Gram negative pathogens, *E. coli* and *Salmonella* seem to be the only species that carry these anti-adaptors, suggesting that other pathogens may behave like *V. cholerae* in terms of RpoS degradation.

Here, we test and show that sub-MIC of the AG tobramycin induces oxidative stress in *V. cholerae*. Moreover, we show that this SOS induction is mostly due to hydroxyl radical formation and 8-oxo-G incorporation in DNA using GFP as a reporter of SOS thanks to fusions of *gfp* with the SOS-dependent *intIA* promoter constructed for our previous studies [1,3]. Finally, we provide evidence for a role of RpoS in the protection of *E. coli* and *V. cholerae* cells against sub-MIC tobramycin induced stress and propose that most of the induction is linked to the rapid degradation of the RpoS protein in *V. cholerae*. We further show that the SOS induction by AGs is conserved among distantly related Gram negative pathogens.

Results

Oxidative stress and ROS formation is induced by tobramycin in *V. cholerae*

In order to understand how sub-MIC of antibiotics such as AGs that do not directly target DNA (AGs) impact the bacterial cell and induce a stress response in *V. cholerae*, we aimed at determining if SOS induction by sub-MIC takes place following oxidative stress.

First we addressed whether there is a difference in ROS formation between *E. coli* and *V. cholerae*. We chose to use low doses of tobramycin (100 fold below the MIC) in both bacteria because this antibiotic was used in previous studies [3]. These concentrations (0.1 µg/ml for *E. coli* and 0.01 µg/ml for *V. cholerae*) were previously shown to induce SOS in *V. cholerae* and not in *E. coli* [3]. We performed growth in LB medium with and without tobramycin in the presence of dihydrorhodamine 123 (DHR), a chemical agent that becomes fluorescent upon oxidation into rhodamine 123 in the presence of intracellular ROS [35]. DHR oxidation actually reports the presence of H_2O_2 and intracellular peroxidases [36], and peroxidases are induced in response to oxidative stress and catalyze the formation of ROS. Fluorescence is thus interpreted as peroxidase induction and generation of free oxygen radicals [36]. We measured the intracellular generation of free radicals/peroxidase induction through rhodamine

fluorescence at the middle and end of exponential phase (OD600 0.5 and 0.8). Our results showed that, when these bacteria were grown in the presence of tobramycin at 1/100 MIC, peroxidase induction (i.e. oxidative stress) took place in *V. cholerae* but not in *E. coli* (Figure 1). Ciprofloxacin (a fluoroquinolone) was used as a control known to induce ROS formation in *E. coli* [10]. Ciprofloxacin also induced SOS in both bacteria at sub-MIC (at a concentration of 1/100 of the MIC) [3]. As expected, sub-MIC of ciprofloxacin induced DHR oxidation in *E. coli* and in *V. cholerae*. These results point to a significant activation of the oxidative stress response and ROS formation in *V. cholerae* in response to sub-MIC tobramycin treatment.

Hydroxyl radicals and incorporation of 8-oxo-G during growth of *V. cholerae* on tobramycin leads to SOS induction

In order to study the nature of DNA damage caused by sub-MIC tobramycin, we alternatively inactivated the RecFOR gap repair and RecBCD double strand break repair pathways by deleting *recF* and *recB* respectively in the wild type *V. cholerae* strain (Figure 2). We used GFP fused to the *intIA* promoter as the SOS reporter (plasmid p4640 for *V. cholerae*) [1,3]. Mitomycin C (MMC) cross-links the two DNA strands, leading to double strand break formation, which induces SOS. MMC was thus used as an SOS inducer and tested as a positive control. The basal GFP fluorescence on LB was at the same level for all strains shown in Figure 2. The RecFOR pathway is involved in the induction of SOS by allowing RecA nucleo-filament formation on single strand DNA lesions, while the RecBCD pathway recruits RecA on double strand DNA breaks [15]. SOS induction following tobramycin treatment decreased from 3 fold for wild type to 1.2
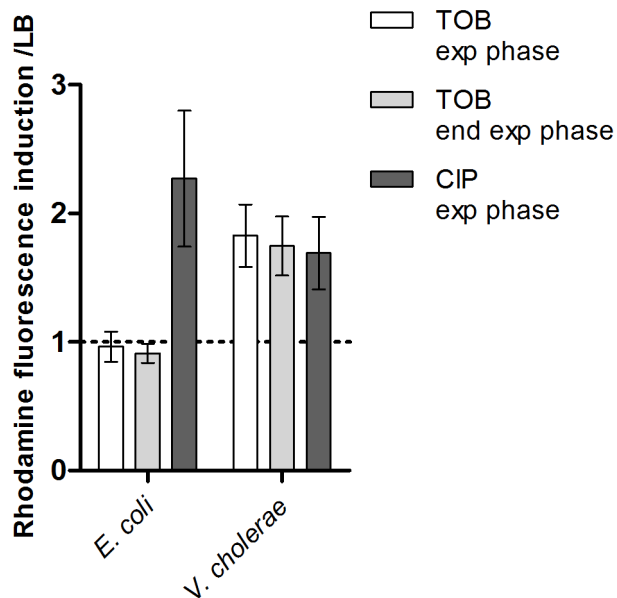

Figure 1. Sub-MIC tobramycin treatment induces ROS formation in *V. cholerae* and not in *E. coli*. Histogram bars show the ratio of rhodamine fluorescence in the presence of the specified antibiotic over fluorescence in LB and thus reflect the induction of ROS formation. Tobramycin (TOB) and ciprofloxacin (CIP) concentrations were respectively 0.01 µg/ml and 0.005 µg/ml for *V. cholerae*, and 0.1 µg/ml and 0.05 µg/ml for *E. coli*. Exp phase stands for exponential phase i.e. an OD600 nm of 0.5. End exp phase stands for end of exponential phase i.e. an OD600 nm of 0.8.
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fold for *recB* and 1.6 for *recF* mutants (Figure 2). This suggests that both double strand breaks and single strand lesions are formed on DNA, with a more dramatic effect on double strand DNA breaks. The *recB* mutant also grew more slowly in tobramycin than the *recF* mutant (data not shown).

The presence of double strand (ds) lesions is compatible with the fact that hydroxyl radicals (OH^-) target DNA and cause ds breaks. The Fenton reaction is the major source of OH^- radical formation in the presence of Fe^{2+} ions [12–13]. If OH^- radicals are responsible for SOS induction, then iron is also expected to be essential for inducing SOS. We used 2,2′-dipyridyl (DP), an iron chelator that prevents the Fenton reaction, to test whether iron depletion affects SOS induction by tobramycin. DP did not change the basal level of fluorescence in LB. In the presence of tobramycin, the SOS induction was decreased from 3 to 1.3 fold upon addition of DP (Figure 2).

Single strand and double strand lesions can occur during mismatch repair. Mismatches are formed when a DNA base is incorporated in a non Watson-Crick manner. This can happen during oxidative stress, which leads to the presence of higher levels of oxidized guanine residues (8-oxo-G). During replication, when the DNA polymerase encounters an 8-oxo-G residue, it pairs it to an adenine instead of a cytosine. The repair of such mismatches involves MutY [37]. MutT is also involved in the response to 8-oxo-G by decreasing its concentration in the nucleotide pool. We over-expressed MutY and MutT in *V. cholerae* from a plasmid. The empty plasmid showed higher SOS induction by tobramycin than the wild type strain without any plasmid. This is also reproducible with other plasmids (not shown), and can be explained by the fact that such high copy plasmids introduce more DNA to replicate and thus more potential for DNA damage and repair. When we over-expressed MutY or MutT, we found that they strongly decreased the SOS induction, from 6.2 fold for the wild type carrying the empty vector plasmid to 1.4 and 1.2 fold respectively, confirming that sub-MIC tobramycin treatment led to incorporation of 8-oxo-G residues (Figure 2).

Moreover, when the base excision repair pathway for 8-oxo-G was impaired in *E. coli*, (using the *E. coli* ΔmutT ΔmutM ΔmutY strain), sub-MIC tobramycin and kanamycin (another AG) treatments resulted in SOS response induction, whereas no SOS induction was observed in BER proficient *E. coli*, further implicating the presence of incorporated 8-oxo-G residues as responsible for SOS induction (Figure 3).

Altogether, this first set of results shows that *V. cholerae* is more prone to react to oxidative stress than *E. coli*, forming reactive oxygen species at low doses of antibiotics, which can explain SOS induction by sub-MICs in this species.

In order to understand and clarify the origin of these differences between *E. coli* and *V. cholerae*, we decided to address the role of the RpoS regulon on sub-MIC tobramycin-induced SOS.

RpoS is involved in the protection of *E. coli* and *V. cholerae* from ROS–induced SOS

It is well established that the RpoS-dependent general stress response is triggered in response to oxidative stress.

Several genes expressed after RpoS regulon induction, namely catalases (KatE, KatG) and iron chelators (Dps), protect cells from ROS related DNA damage [12–13,28]. It has been shown in *Vibrio vulnificus* that RpoS is essential in exponential growth phase to overcome H_2O_2 related oxidative stress [29]. We constructed a *V. cholerae* strain deleted for *rpoS*. We performed viability tests and found that the mutant strain did not grow in 2 mM H_2O_2 (data not shown), confirming that RpoS is essential for *V. cholerae* growth during oxidative stress.
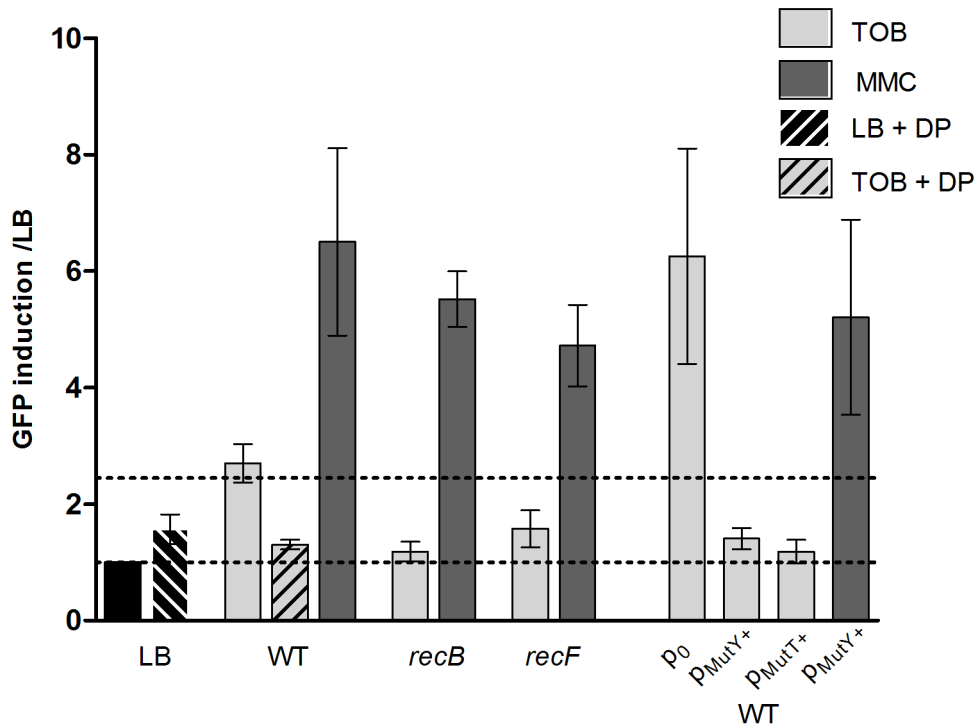
Figure 2. Factors modulating SOS induction by tobramycin in *V. cholerae*. Histogram bars show the ratio of GFP fluorescence in a given strain in the presence of antibiotic over fluorescence of the same strain grown in LB and thus reflect the induction of SOS by the antibiotic. p0 stands for empty pTOPO plasmid. pMutY+ stands for pTOPO-MutY+ (p9476). TOB: tobramycin 0.01 µg/ml. MMC: mitomycin C 0.1 µg/ml. DP: 2,2 Dipyridyl 0.5 mM.
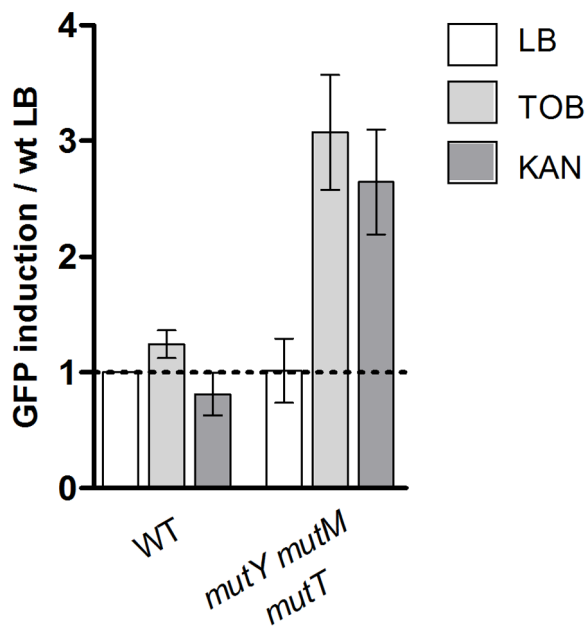doi:10.1371/journal.pgen.1003421.g002

Figure 3. Oxidized guanine incorporation after tobramycin treatment induces SOS in the *E. coli* base excision repair deficient mutant. Histogram bars show the ratio of GFP fluorescence in the presence of antibiotic over fluorescence of the wild type strain grown in LB and thus reflect the induction of SOS. TOB: tobramycin 0.1 µg/ml. KAN: kanamycin 0.2 µg/ml. WT is *E. coli* is BER proficient.
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RpoS is targeted to the ClpXP complex for degradation by the RssB protein. In *E. coli*, IraD binds to and titrates RssB during oxidative stress induction so that less free RssB is present in the cell to bind RpoS. IraD thus protects RpoS from degradation. As mentioned in the introduction, IraD is absent from the genome of a majority of bacterial species, whereas RssB is conserved. In order to address whether the absence of IraD plays a role in the induction of SOS by sub-MIC tobramycin in other species, we decided to test two other unrelated pathogens, *Klebsiella pneumoniae* and *Photorhabdus luminescens*. In order to follow SOS induction in these species, we transformed them, as we did for *E. coli*, with the plasmid p9092 carrying the *Pint-gfp* fusion. We found that sub-MIC tobramycin treatment induced SOS in both species, as it did for *V. cholerae* (Figure 4).

As the *iraD* gene is absent from the *V. cholerae* genome, we hypothesized that higher levels of RpoS are present in the cell during sub-MIC tobramycin treatment in *E. coli*, than in *V. cholerae*, allowing *E. coli* to cope more easily with oxidative stress and avoid eventual DNA damage. In order to test this hypothesis, we first deleted *iraD* in *E. coli*. [32]. We used the *Pint-gfp* fusion (GFP fused to the *intIA* promoter) as a reporter of SOS induction (p9092 for *E. coli*) [2]. *sfiA* is another gene regulated by the SOS response and commonly used for SOS measurement assays (as in [1]). GFP fused to the *sfiA* promoter was also tested and gave the same induction profiles as the *intIA* promoter (not shown). We chose to carry on with the *intIA* promoter in order to have the same reporter promoter in *E. coli* as in *V. cholerae*. IraD protects RpoS from degradation. Deleting *iraD* in *E. coli* MG1655 resulted in SOS induction following sub-MIC tobramycin treatment (Figure 5A). RssB targets RpoS to degradation. Conversely, we
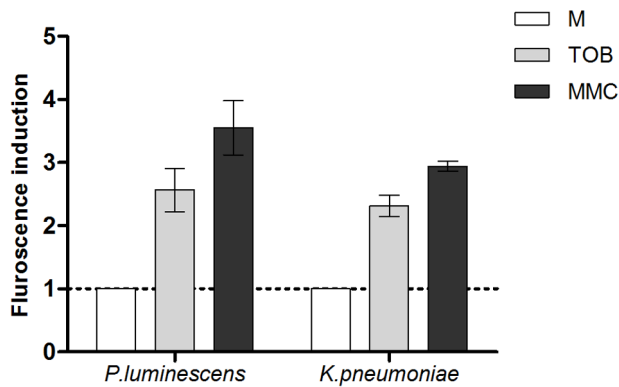
Figure 4. Sub-MIC aminoglycosides induce SOS in other pathogens. Histogram bars show the ratio of GFP fluorescence in the presence of antibiotic over fluorescence in LB and thus reflect the induction of SOS by the antibiotic. Tobramycin (TOB) concentrations were 0.01 µg/ml for *Klebsiella pneumoniae* and 0.0025 µg/ml for *Photorhabdus luminescens*. Mitomycin C (MMC) was used at 0.1 µg/ml.
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found that RssB over-expression strongly induced SOS in these conditions (Figure 5A).

We then expressed the *E. coli* anti-adaptor IraD in *V. cholerae* (Figure 5B) and found that in this context sub-MIC tobramycin dependent SOS induction was decreased in *V. cholerae*, whereas SOS induction by MMC was not affected (4 fold induction, not shown on the graph). Over-expression of RpoS also relieved SOS induction following sub-MIC tobramycin treatment. Conversely, when *rpoS* was deleted or when RssB was over-expressed in *V. cholerae*, SOS was significantly induced with or without tobramycin. The higher basal levels when RpoS is low (deletion or high level of RssB) could be a clue to impaired replication in these strains in LB. For instance, when sub-units of DNA polymerase are impaired (such as in *dnaEts* or *dnaNts* mutants at semi-permissive temperature), such SOS induction can also be detected [38]. Over-expression of IraD or RssB in a *V. cholerae ΔrpoS* strain had no effect on SOS, whereas RpoS over-expression complemented efficiently the V. cholerae *ΔrpoS* strain, confirming that the effect observed in the wild type strain is dependent on RpoS (Figure 5B). Western blotting and detection with an RpoS-specific antibody showed that in the presence of tobramycin, the amount of RpoS increased (or RpoS degradation decreased) in wild type *E. coli* and not in wild type *V. cholerae* during exponential phase (Figure 6). Moreover, these increased RpoS amounts were not observed in an *E. coli ΔiraD* strain. Altogether, these data show that protection of RpoS levels is sufficient to relieve sub-MIC AG induced SOS response in *V. cholerae*.

We then addressed whether cellular ROS levels were modified in *E. coli* and *V. cholerae* mutants. We performed the DHR assay on *rpoS* and *iraD* mutants. As the growth of these strains was impaired in tobramycin, instead of measuring fluorescence at OD600 nm 0.5 after TOB 0.01 µg/ml treatment as we did in Figure 1, we either measured fluorescence at the beginning of exponential phase (OD 0.2) for the same concentration of TOB (0.01 µg/ml, Figure 5C), or at the same OD (0.5) for a decreased TOB concentration (0.001 µg/ml, Figure 5D). In both cases, we observed an increase of ROS in *E. coli* and *V. cholerae* when *rpoS* or *iraD* (for *E. coli*) were deleted, showing that decreased RpoS levels lead to ROS formation after TOB treatment in both bacteria.

Finally, it has been observed that addition of polyamines (e.g. putrescine, spermidine) induces RpoS transcription [39] and reduces intracellular ROS production and DNA fragmentation [40–41]. Polyamines thus have antioxidant properties and a protective effect on DNA. We tested the effect of spermidine on tobramycin dependent SOS induction in *V. cholerae* wild type or *Δrpos*. We observed that spermidine decreases SOS induction after tobramycin treatment in an RpoS dependent way (Figure 5B).

These results support the idea that RpoS is involved in SOS induction by oxidative stress after AG treatment in *V. cholerae*, and suggest that the RpoS protein level is less stable in *V. cholerae*.

Discussion

We show here that *V. cholerae* is subject to oxidative stress in response to AG (here tobramycin) at concentrations 100 times below the MIC. These concentrations of AGs seem to lead to the formation of ROS and ultimately to DNA damage through double strand breaks and 8-oxo-G incorporation into DNA. *E. coli* on the other hand, has a stronger resistance to this kind of feeble stress triggered by AGs.

We found that a difference between *V. cholerae* and *E. coli* is that the RpoS sigma factor is protected from degradation by anti-adaptor proteins in *E. coli* upon oxidative stress induction, whereas it seems to be more easily degraded in *V. cholerae*. Indeed, we showed that the protection of *E. coli* RpoS from degradation by the anti-adaptor IraD is important for the protection of the cells against oxidative stress that is triggered in the presence of tobramycin. IraD is part of a genomic island [42]. We looked for other genomes that possess *iraD* orthologs in KEGG (http://www.genome.jp/kegg/) and MicroScope (http://www.cns.fr/agc/microscope/home/index.php) databases containing respectively 2020 and 872 bacterial genomes, including commensal bacteria (like *Bacillus subtilis*, *Streptococcus agalactiae*) and pathogens such as *Acinetobacter baumannii*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Vibrio cholerae*, *Yersinia pestis* and others. In KEGG, *iraD* orthologs were found in 69 genomes (49 *E. coli* and 5 *Shigella* with 70 to 100% protein identity; 15 *Salmonella* with 40 to 50% identity). In MicroScope, a total of 49 genomes appear to possess *iraD* orthologs where 44 are *E. coli*, 4 *Shigella* and 2 *Salmonella*. No sequenced bacterial organism other than *E. coli*, *Shigella* and *Salmonella* species possess *iraD*, which suggests that *E. coli* is more of an exception than a paradigm for the physiological response to antibiotics sub-MIC. We cannot rule out the possibility that other anti-adaptors exist in *V. cholerae* that are not homologous to IraD. Indeed, the three known *E. coli* anti-adaptors IraD, IraP (responding to phosphate starvation) and IraM (responding to magnesium starvation) do not resemble each other, although they all interact with RssB to protect RpoS from degradation. RpoS levels are also regulated at transcriptional level by *rprA* and *dsrA* small RNAs that bind the *rpoS* mRNA, and protect it from degradation in *E. coli*. Interestingly, like the IraD protein, *rprA* and *dsrA* small RNAs are also absent from *V. cholerae* [31].

Figure 7 represents the model we propose for the induction of SOS after AG treatment: the presence of sub-MIC AG leads to an increase of reactive oxygen species in the bacterial cell, causing oxidative stress. We propose that RpoS levels are insufficient to cope with oxidative stress caused by sub-MIC tobramycin in *V. cholerae*. A more steady presence of RpoS, as in *E. coli*, can lead to more efficient protection from oxidative stress and avoidance of DNA damage. The protective effect of RpoS can be through proteome protection, namely by decreasing the synthesis of iron rich proteins [43] and through genome protection by limiting available free iron and OH⁻ formation [43–44]. Mistranslated or misfolded proteins have indeed been shown to be more susceptible
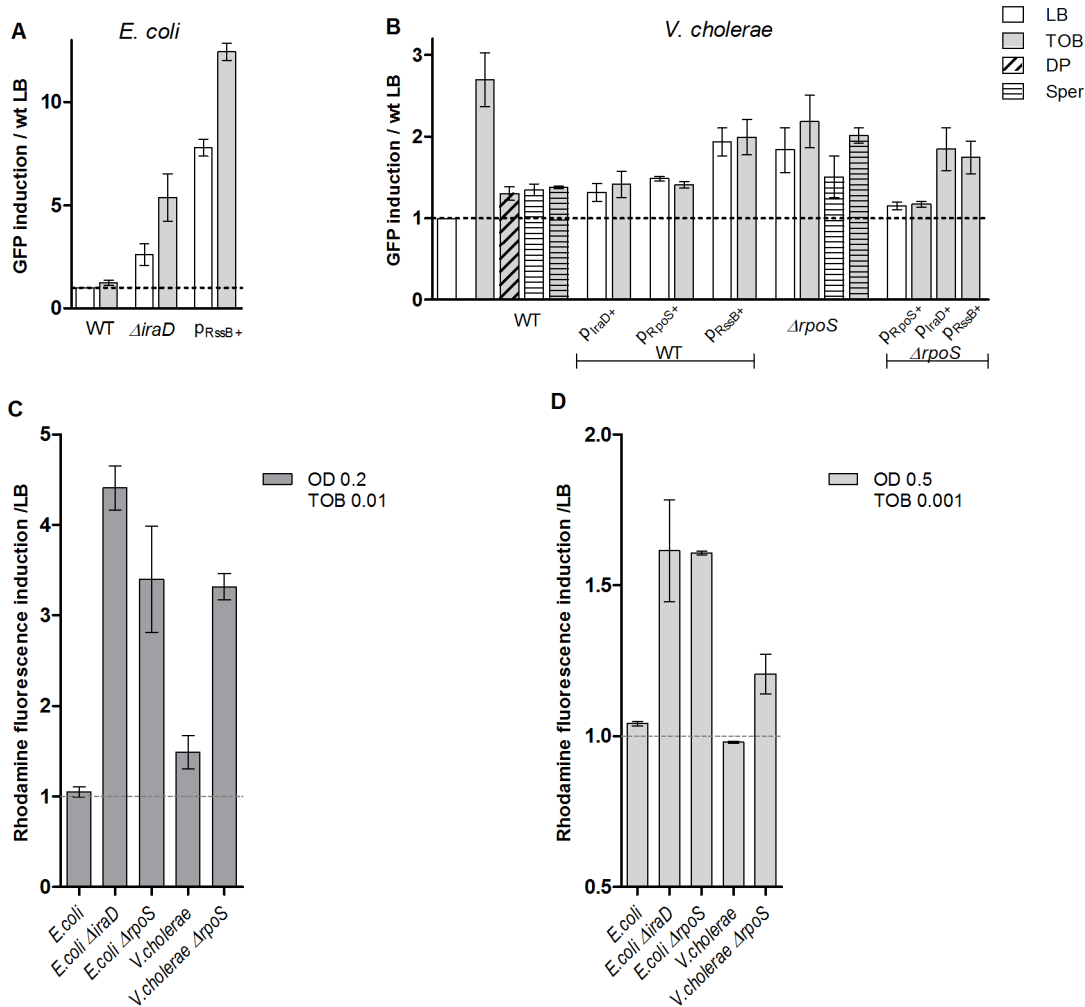
Figure 5. RpoS protects *E. coli* and *V. cholerae* from tobramycin-dependent SOS induction and ROS formation. A and B. Histogram bars show the ratio of GFP fluorescence in the presence of antibiotic over fluorescence of the wild type strain grown in LB and thus reflect the induction of SOS. pRssB+ is pTOPO-RssB+ (pA209). pIraD+ is pTOPO-IraD+ (pA208). pRpoS+ is pTOPO-RpoS+ (pA237). Tobramycin (TOB) concentrations were 0.01 µg/ml for *V. cholerae* and 0.1 µg/ml for *E. coli*. Spermidine (Sper) was used at a final concentration of 10 mM. 2,2 Dipyridil (DP) was used at a final concentration of 0.5 mM. A: *E. coli* p9092. B: *V. cholerae*::p4640. C and D. Histogram bars show the ratio of rhodamine fluorescence in the presence of Tobramycin (TOB). C. Cultures were treated with TOB 0.01 µg/ml and rhodamine fluorescence was measured at OD600 nm 0.2. D. Cultures were treated with TOB 0.001 µg/ml and rhodamine fluorescence was measured at OD600 nm 0.5.
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to oxidation [19]. Protein oxidation is thus not only a function of ROS availability but also of the levels of aberrant proteins. AGs target the ribosome and cause mistranslation, which could lead to oxidation of mistranslated proteins, and thus an increase in oxidized proteins and eventually DNA damage due to impaired replication and repair. Interestingly, stabilization of a single oxidative stress sensitive protein was shown to be sufficient to enhance oxidative stress resistance of the whole organism in *V. cholerae* [45], confirming the weight of protein oxidation on *V. cholerae*'s ability to cope with stress and RpoS could be a key factor in this process.

Moreover, it was shown in *V. cholerae* that cell envelope damage (caused by chemical treatment, genetic alterations, physical damage) leads to internal oxidative stress, formation of oxygen radicals, changes in iron physiology (increased iron storage) [46] and induces RpoS [46–48]. The presence of AGs could be responsible for such envelope stress in *V. cholerae*. RpoS regulates various factors that allow protection from oxidative stress, such as

antioxidant agents, but also iron availability sensors like Fur, which prevents iron acquisition when the free iron level is high [43–44]. Indeed, excess iron reacts with ROS formed as a natural consequence of aerobic metabolism, and generates hydroxyl radicals through the Fenton reaction. Iron availability is necessary for the toxicity of antibiotics [49]. Another protein induced by RpoS is Dps, a DNA binding iron chelator involved in the protection against killing by bactericidal antibiotics (through hydroxyl radicals) upon oxidative stress during exponential phase [49]. Dps binds and stores iron, which attenuates OH^- formation. It is tempting to state here that the effect of RpoS is synergistic with SOS for genome protection, in that RpoS prevents OH^- attack on DNA whereas SOS repairs it. Our results using DP are consistent with an effect of iron in the process of DNA damage by sub-MIC tobramycin. So why has *V. cholerae* selected for an RpoS system that is less efficient than that of *E. coli*?

This could be explained by the need for *V. cholerae* to quickly up- and down-regulate the RpoS regulon during infection. Indeed, in
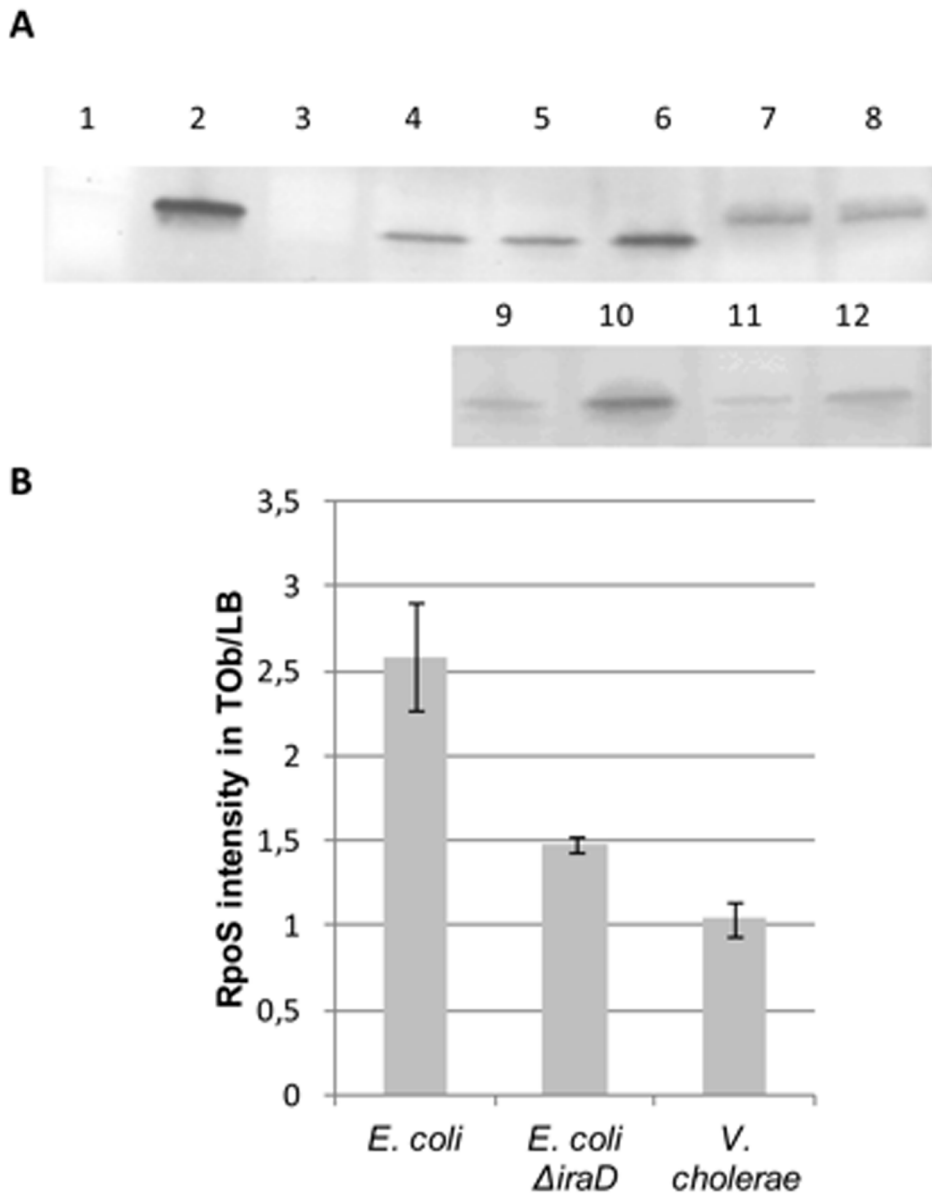
Figure 6. RpoS protein levels are higher in the presence of sub-MIC tobramycin in *E. coli* but unchanged in *V. cholerae*. Overnight cultures were diluted to OD 0.1. Tobramycin treatment at 0.5 µg/ml was initiated at OD 0.5 for one hour. The growth was then stopped and the western blotting was performed. Note that *V. cholerae* RpoS (335 aminoacids) is larger than *E. coli* RpoS (330 aminoacids). A: representative western blots using RpoS antibody. Wells: 1- *V. cholerae ΔrpoS*, 2- Purified *E. coli* RpoS (with His-tag, thus larger than native protein), 3- *E. coli ΔrpoS*, 4- and 9- *E. coli* grown in Tob 0.05 µg/ml, 5- *E. coli* grown in LB, 5- *E. coli* grown in Tob 0.05 µg/ml, 6- and 10- *E. coli* grown in Tob 0.5 µg/ml, 7- *V. cholerae* grown in LB, 8- *V. cholerae* grown in Tob 0.5 µg/ml, 11- *E. coli ΔiraD* grown in LB, 12- *E. coli ΔiraD* grown in Tob 0.5 µg/ml B: Bands corresponding to RpoS were quantified. The histogram represents the intensity of fluorescence of secondary antibody (Alexa) obtained in tobramycin over the intensity obtained in LB in at least two independent experiments.
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contrast to its role in stress response, the effects of RpoS on pathogenesis and virulence are highly variable and depend on bacterial species and their niches [50]. RpoS is conserved within alpha, beta and gamma proteobacteria but the RpoS regulon composition is subject to modification between species [51]. In *Salmonella*, RpoS is essential for virulence. In *E. coli*, RpoS induces virulence genes, but a *ΔrpoS* mutant can outcompete wild type cells in the mouse colon. A tradeoff has been shown between self preservation and nutritional competence in *E. coli* infected patients, determined by levels of RpoS that naturally occur in different *E. coli* cells from the same infecting strain [52–53]. In

Vibrio anguillarum, a fish pathogen, virulence is also up-regulated by RpoS [54]. Conversely, and illustrating the high variability of the RpoS regulon between even closely related species, in *V. cholerae* RpoS represses virulence genes (a *ΔrpoS* strains produces more cholera toxin), and is not required for intestinal survival, even though RpoS contributes to overcoming host-specific stresses in the gut [55]. Nevertheless, RpoS plays an important role in the pathogenicity of *V. cholerae* because it is required for detachment from the epithelial cells after infection and release of bacteria in the environment (the ''mucosa escape response'') [56]. This state is characterized by high RpoS levels, RpoS dependent up-regulation

Figure 7. Model of SOS induction by sub-MIC aminoglycosides. We propose that in the presence of sub-MIC AGs, ROS are formed, inducing DNA damage directly (8-oxo-G incorporation) or indirectly (impaired DNA replication and repair proteins). When present and stable, RpoS protects cells from this type of DNA damage by activating an efficient response to oxidative stress, for instance through the decrease of intracellular iron.
doi:10.1371/journal.pgen.1003421.g007

of chemotaxis, motility, flagella and reduced virulence gene expression. *V. cholerae* thus has to down-regulate RpoS in order to be virulent in the gut and up-regulate RpoS in order to spread in the environment and survive. This is not the case for *E. coli* where RpoS induces virulence and has no known role in its escape and spread to the environment. The absence of proteins protecting RpoS from degradation in *V. cholerae* could increase the efficiency of the switch between low RpoS (cholera) and high RpoS (spread). Considering this RpoS regulon variability, it was proposed that horizontally transferred genes, which may enhance host adaptation, integrate into the RpoS regulon [51,57].

In Gram-negative pathogens, acquired multiple antibiotic resistance is in many cases associated with mobile integrons. This natural genetic engineering system is composed of a gene coding an integrase belonging to the site-specific recombinase family and a primary recombination site where gene cassettes can be integrated. The existence of sedentary integron platforms gather-

ing up to 200 gene cassettes in the chromosomes of environmental proteobacteria has been demonstrated [58–61]. This is the case for *V. cholerae*, which carries a superintegron gathering more than 170 cassettes [52]. These cassettes can code for antibiotic resistance and other adaptive genes, and their integration by the integrase allows expression from a constitutive promoter upstream of the insertion site [62–63]. Moreover, integrase expression is controlled by the SOS response, suggesting that the evolutionary success of these elements largely accounts for the coupling of stress and cassette array remodeling [64–65]. We show here that sub-MIC AGs induce SOS and thus integron integrase expression in *V. cholerae*. This AG driven induction, which is apparently common in gamma proteobacteria that lack IraD, likely explains how the numerous cassettes coding for resistance to all AG (43 in 2009 [66]) characterized in mobile integrons were recruited. This implies that the use of these antibiotics may promote cassette rearrangements and expression of integron-borne resistance genes

to all families of antibiotics, including the ones that do not induce SOS in *E. coli*.

Bacteria face different growth conditions in different environments. *V. cholerae* has two different niches: the aquatic environment where it grows as a biofilm on crustacean shells where nutrients are scarce, and the host gut, a rich environment where virulence is induced. In both locales, *V. cholerae* may face varying antibiotic concentrations, due to rejections in the environment or antibiotic treatments of the host. Other pathogens are also known to encounter sub-MIC of antibiotics, such as *Pseudomonas aeruginosa*, which causes chronic lung infections where antibiotics subsist as gradients. Strikingly, it was shown that metronidazole treatment of a patient infected with *P. aeroginosa* induced β-lactamase and ceftazidime resistance through SOS-mediated integron rearrangements [67]. It is tempting to make a parallel here with our work with AGs, as neither AGs nor metronidazole cause direct DNA damage, though they are both able to induce the SOS response. It is thus important to better understand how different ecological niches and different life styles modulate evolution of stress responses, which have a major impact on the evolution of genome plasticity and antibiotic resistance, and understanding the molecular mechanisms that drive the emergence of drug resistance can facilitate the design of more effective treatments.

Materials and Methods

Strain and plasmids used in this study are shown in Table 1, oligonucleotides in Table 2.

Constructions

Strains. A267 was constructed by P1 preparation on strain JW5782-2 11574 (KEIO collection) and transduction in MG1655. A268: P1 preparation on strain JW5437-1 11387 (KEIO collection) and transduction in MG1655. A071: PCR amplification of *recF::cm* using oligonucleotides 1598/693 on plasmid pMEV227 (Marie-Eve Kennedy Val, unpublished) and chitin transformation [2] in strain 8637. A072: PCR amplification of *recB::cm* using oligonucleotides 1598/693 on plasmid pMEV228 (Marie-Eve Kennedy Val, unpublished) and chitin transformation [2] in strain 8637. A321: PCR amplification of regions flanking rpoS using oligonucleotides 2041/2042 and 2043/2044 on strain 8637. PCR amplification of *aadA1* conferring spectinomycin resistance on pAM34 using oligonucleotides 2045/2046 sur. PCR-assembly (as described [2]) of the *rpoS::aadA1* fragment using oligonucleotides 2041/2044 and chitin transformation.

Klebsiella pneumoniae was grown in LB medium at 37°C. Electroporation of *Klebsiella pneumoniae* was performed following the same protocol as for *E. coli* using ice-cold 10% glycerol solution. Tetracycline was used at a concentration of 15 μg/ml for the selection of clones carrying plasmid p9092. The MIC of tobramycin was determined using E-tests as 1 μg/ml as for *V. cholerae* so a sub-MIC concentration of 0.01 μg/ml was used for SOS induction assays (repeated 3 times).

Photorhabdus luminescens was grown on liquid Schneider Drosophila medium (Sigma) and solid Nutrient Agar (Sigma) at 30°C. Electroporation of *Photorhabdus luminescens* was performed as described [68] using ice-cold SH buffer (5% sucrose 1 mM Hepes). After 3 hours of incubation at 30°C following electroporation, 5 μg/ml tetracycline was added to the culture and incubation over-night at 30°C with shaking before plating on selective medium. Tetracycline was used at a concentration of 10 μg/ml for the selection of clones carrying plasmid p9092. The MIC of tobramycin was determined using E-tests to be 0.25 μg/ml so a sub-MIC concentration of 0.0025 μg/ml was used for SOS induction assays (repeated 3 times).

Table 1. Strains and plasmids.

Strain number	Genotype of interest	Ref. or construction
E. coli		
MG1655	WT	Laboratory collection
MK612	*mutT mutY zrd::tn10 mutM::tn10-km*	[71], Laboratory collection
A267	MG1655 *ΔiraD::kan*	This study
A268	MG1655 *ΔrpoS::kan*	This study
V. cholerae		
8637	N16961 *hapR+*	[3]
A071	N16961 *hapR+ recF::cm*	This study
A072	N16961 *hapR+ recB::cm*	This study
A321	N16961 *hapR+ rpoS::aadA1*	This study
Plasmids		
p9644	pTOPO	Stratagene (circularized)
p9476	pTOPO-MutY+	This study
pA208	pTOPO-Pbla-IraD+	This study
pA209	pTOPO-Pbla-RssB+	This study
pA237	pTOPO-Pbla-RpoS+	This study
p4640*	*gfp* induced by SOS, SOS reporter for flow cytometry in *V. cholerae*	[1]
p9092**	*gfp* induced by SOS, SOS reporter for flow cytometry in *E. coli*	[2]

*p4640 carrying *gfp* fused to the SOS inducible *intIA* promoter was integrated in the chromosome of the specified *V. cholerae* strains by conjugation as described [2].
**p9092, pACYC184 plasmid carrying *gfp* fused to the SOS inducible *intIA* promoter was introduced by heat shock transformation into specified CaCl2 competent *E. coli* strains.
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Table 2. Oligonucleotides.

Name	Sequence
693	TGTGTGGAATTGTGAGCGG
1598	ACGGCAGGTATATGTGATGG
1585	GTACAAAATACGGTGCGAGCCATTCACTCTTCC
1586	CTATTGCCTGCACATTACTAAATCAGAGC
2041	GTCAAAATTTGACTAAAAAAGATCCAGTTAAGACGG
2042	GCGAGCATCGTTTGTTCGCCCAGCTTCTGTATGGAACGGGAGCGGCCTCCCCCTGGCAACTTTGCGAGTCATTGCGATTTACAACC
2043	CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTTTTTCCAGACTCATCCAAAACTAAGGCACCCGG
2044	CCGAGTGGCTTGCCAAAGAGATTGGTGCC
2045	TAAATCGCAATGACTCGCAAAGTTGCCAGGGGGAGGCCGCCCCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGC
2046	GCACCCCACCGGGTGCCTTAGTTTTGGATGAGTCTGGAAAAAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACG
2206	TGTAAGTTTATACATAGGCGAGTACTCTGTTATGGATGATGCGACAATCACTTCAGG
2207	TTAGCTGACATTCTCCAGCGTCG
2208	TGTAAGTTTATACATAGGCGAGTACTCTGTTATGGATGACGCAGCCATTGGTCGG
2209	TCATTCTGCAGACAACATCAAGCGCAGTCG
2210	TGTAAGTTTATACATAGGCGAGTACTCTGTTATGGATGAGTGTCAGCAATACCGTAACC
2211	TTAGTTGTCGTATTCGACGTTAAACAGC

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Plasmids. p9476: PCR amplification of *mutY* with its own promoter using oligonucleotides 1585/1586 on strain 8637, cloned in pTOPO-TA. pA208: PCR amplification of *iraD* with the constitutive *bla* promoter using oligonucleotides 2206/2207 on strain MG1655, cloned in pTOPO-TA. pA209: PCR amplification of *rssB* with the constitutive *bla* promoter using oligonucleotides 2208/2209 on strain MG1655, cloned in pTOPO-TA. pA237: PCR amplification of *rpoS* with the constitutive *bla* promoter using oligonucleotides 2210/2211 on strain MG1655, cloned in pTOPO-TA.

Detection of intracellular ROS by DHR123. Overnight cultures of *E. coli* MG1655 and *V. cholerae* 8637 were diluted 100 fold in LB or LB+ sub-MIC tobramycin. DHR123 (Sigma) was added to the cultures at a final concentration of 0.9 µg/ml (i.e. $2.5.10^{-3}$ µM). Cultures were grown to the specified OD 600 nm (OD 0.5 for "exp phase" and OD 0.8 for "end exp phase"). DHR fluorescence was measured at 500 nm excitation wavelength and 550 nm emission wavelength on a TECAN infinite M200. Experiments were repeated at least twice.

Flow cytometry. Flow cytometry experiments were performed as described [1,3] and repeated at least 6 times: briefly, overnight cultures were diluted 100 fold in LB or LB+ sub-MIC antibiotic. Spermidine was used at a final concentration of 10 mM. 2,2 Dipyridil was used at a final concentration of 0.5 mM. After 6 to 8 hours of culture, the culture was washed in PBS and the GFP fluorescence was measured using the Miltenyi MACSQuant device.

RpoS immunoblot. Overnight cultures were diluted 100 fold in LB and grown at 37°C. At OD_{600} 0.3, 0.5 µg/ml tobramycin was added to cultures for one hour. Control cultures without tobramycin were also performed. Protein extracts were prepared as previously described [69]. SDS-PAGE was carried out in 4–15% polyacrylamide minigels (Mini Protean II; Bio-Rad). 20 µg of total proteins were loaded into each lane. The proteins were transferred to PVDF membrane with a Trans-Blot turbo (Bio-Rad). Western blots were performed in PBS-T with 1% BSA by incubating the membrane for one hour with a 1/20 000 dilution of polyclonal rabbit antibodies against the RpoS protein [70] and for one hour with a 1/5 000 dilution of Invitrogen Alexa fluor 750 goat anti rabbit antibody. The bands corresponding to RpoS were visualized and quantifyed using an Odyssey. Negative controls were performed with *E. coli* Δ*rpoS* and *V. cholerae* Δ*rpoS* strains. Experiments were repeated twice.

Acknowledgments

Author Contributions

Conceived and designed the experiments: ZB EK DM. Performed the experiments: ZB EK. Analyzed the data: ZB EK DM. Contributed reagents/materials/analysis tools: ZB EK. Wrote the paper: ZB DM.

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