Generation of reactive oxygen species by lethal attacks from competing microbes

Tao G. Dong[a,1], Shiqi Dong[a], Christy Catalano[a], Richard Moore[a], Xiaoye Liang[a], and John J. Mekalanos[b,1]

[a]Snyder Institute for Chronic Diseases and Department of Ecosystem and Public Health, University of Calgary, Calgary, AB, Canada T2N 4Z6; and [b]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115

Contributed by John J. Mekalanos, January 2, 2015 (sent for review December 22, 2014)

Whether antibiotics induce the production of reactive oxygen species (ROS) that contribute to cell death is an important yet controversial topic. Here, we report that lethal attacks from bacterial and viral species also result in ROS production in target cells. Using *soxS* as an ROS reporter, we found *soxS* was highly induced in *Escherichia coli* exposed to various forms of attacks mediated by the type VI secretion system (T6SS), P1*vir* phage, and polymyxin B. Using a fluorescence ROS probe, we found enhanced ROS levels correlate with induced *soxS* in *E. coli* expressing a toxic T6SS antibacterial effector and in *E. coli* treated with P1*vir* phage or polymyxin B. We conclude that both contact-dependent and contact-independent interactions with aggressive competing bacterial species and viruses can induce production of ROS in *E. coli* target cells.

T6SS | reactive oxygen species | interspecies competition | antibiotics | phage

Microbial species exist predominantly in complex communities in the natural environment and animal hosts. To survive in a multispecies environment, bacteria have developed various strategies to compete with other species. For example, some bacteria can exert long-range inhibitory effects by secreting diffusible molecules, such as antibiotics, bacteriocins, and H_2O_2 (1), whereas others require direct cell-to-cell contact to kill nearby organisms (2, 3). One such contact-dependent inhibitory system is the type VI secretion system (T6SS), a protein translocating nanomachine expressed by many Gram-negative bacterial pathogens that can kill both bacterial and eukaryotic cells (3–5). Structurally analogous to an inverted bacteriophage tail, the T6SS delivers effectors into target cells by using a contractile sheath to propel an inner tube out of the producer cell and into nearby target cells. The inner tube (composed of Hcp protein) is thought to carry toxic effector proteins within its lumen or on its tip, which is decorated with VgrG and PAAR proteins (4, 6, 7). Given that some cells can detect T6SS attack but not suffer any measurable loss in viability (8, 9), it would seem that cell killing is likely due to the toxicity of effectors rather than membrane disruptions caused by insertion of the spear-like VgrG/PAAR/Hcp tube complex. T6SS-dependent effectors can attack a number of essential cellular targets, including the cell wall (10, 11), membranes (11, 12), and nucleic acids (13), and thus can mimic the actions of antibiotics and bacteriocins. As a model prey or target organism, *Escherichia coli* can be killed by the T6SS activities of a number of bacteria including *Vibrio cholerae* (14), *Pseudomonas aeruginosa* (10, 15), and *Acinetobacter baylyi* ADP1 (7).

Collins and coworkers (16–18) have reported that antibiotic treatment of *E. coli* elicits the production of reactive oxygen species (ROS) resulting from a series of events involving perturbation of the central metabolic pathway, NADPH depletion, and the Fenton reaction. ROS can cause lethal damage to DNA, lipid, and proteins (19, 20) and thus can contribute to cell death in combination with the deleterious effects of antibiotics on their primary targets. The idea that antibiotics kill bacterial cells, in part, through the action of ROS has been supported by a number of follow-up studies (18, 21–23) but has also been challenged by others as a result of observations contradictory to a model where ROS is the sole mediator of antibiotic lethality (24–26). These observations include the fact that antibiotics kill under anaerobic conditions, oxidation of the hydroxyphenyl fluorescein fluorescence dye used to measure ROS levels is nonspecific, and the extracellular level of H_2O_2 is not elevated by antibiotic treatment (24, 26). To address these concerns, Dwyer et al. (27) used a panel of ROS-detection fluorescence dyes, a defined growth medium under stringent anaerobic conditions, and an in vivo H_2O_2 enzymatic assay to study the effects of antibiotics on cells. The results further support that antibiotics induce ROS generation, which contributes to the efficacy of antibiotics in addition to their primary lethal actions (18, 27, 28).

Results and Discussion

Because T6SS antibacterial effectors and antibiotics attack similar cellular targets, we reasoned that T6SS attacks might also induce ROS production and oxidative stress. To test this hypothesis, we examined the transcriptome response of *E. coli* O157:H7 EDL933 exposed to *V. cholerae* V52, a T6SS active strain known to kill *E. coli* (14), and compared it with the response to a T6SS null *vasK* mutant of V52 (3). Using RNA-sequencing (RNA-seq) analysis, we identified 15 *E. coli* genes that were highly induced by T6SS exposure (greater than fivefold change) (Fig. 1*A* and Dataset S1). These genes included many that are typically induced by cell envelope stress (e.g., *spy*, *cpxP*, *osmY*, *pspG*, *marA*). However, the gene showing the strongest up-regulation (nearly a 100-fold increase in expression) was *soxS*, which encodes a transcriptional activator of genes in a regulon that responds to reactive oxidative (redox) stress (29). It is well established that certain types of redox stress in *E. coli* are sensed by the SoxR regulatory protein, which goes on to activate transcription of the *soxS* gene (29). We further confirmed that T6SS attack caused the induction of *soxS* by quantitative RT-PCR (qPCR) (Fig. 1*B*). The

Significance

How microbes respond to lethal attacks from competing species is not fully understood. Here, we investigated the response of *Escherichia coli* to attacks from the type VI secretion system (T6SS), bacteriophage P1*vir*, and polymyxin B. We report that generation of reactive oxygen species (ROS) is a general outcome of potentially lethal activities mediated by contact-dependent or contact-independent interactions of aggressive competing bacterial species and phage. An ROS response gene, *soxS*, is highly induced in response to all sources of attacks tested. This discovery will likely prompt other investigations into why evolution has selected expression of this gene as a "first responder" to potentially lethal interspecies competition.

Author contributions: T.G.D. and J.J.M. designed research; T.G.D., S.D., C.C., R.M., and X.L. performed research; T.G.D. and J.J.M. analyzed data; and T.G.D. and J.J.M. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. Email: tdong@ucalgary.ca or john_mekalanos@hms.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1425007112/-/DCSupplemental.

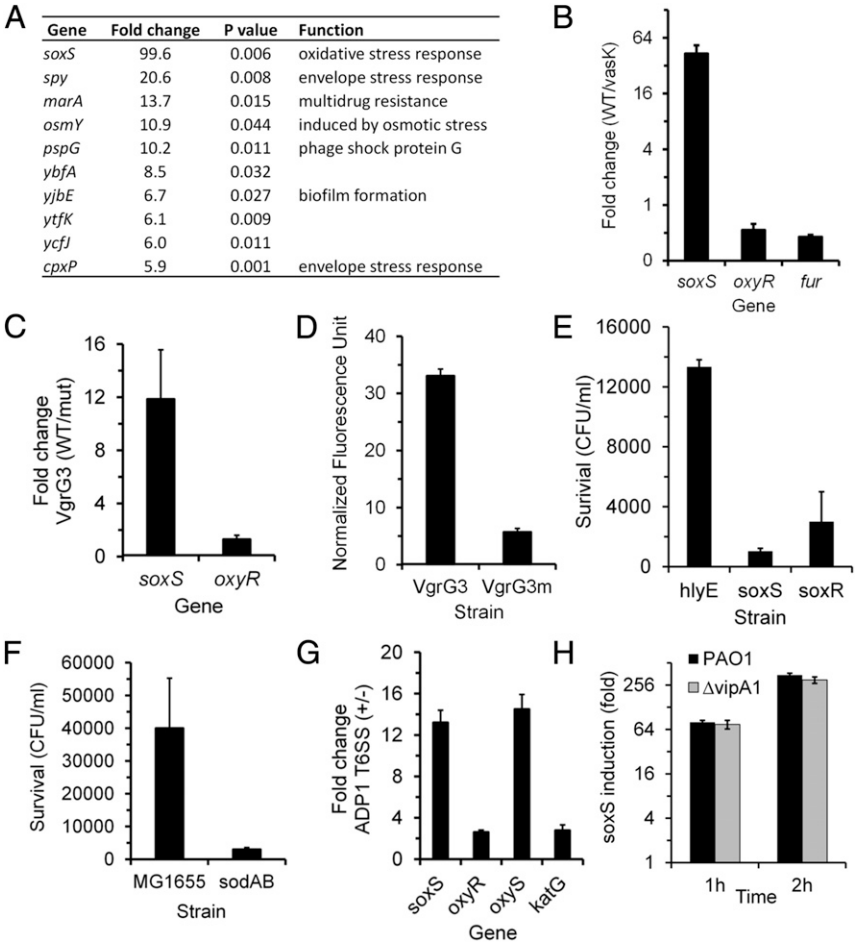

Fig. 1. T6SS of *V. cholerae* induces ROS. (*A*) Top 10 highly induced genes by T6SS in *E. coli*. WT V52 and its T6SS null *vasK* mutant were mixed with *E. coli* O157:H7 EDL933 at a 10:1 ratio and incubated on an LB plate for 30 min at 37 °C. RNA samples were extracted from the mixture and processed for RNA-seq analysis. (*B*) Confirmation of *soxS* induction by qPCR. The genes *oxyR* and *fur* were not induced by T6SS, consistent with the RNA-seq analysis. (*C*) Toxicity of VgrG3 induces *soxS* expression. RNA samples were collected from *E. coli* K-12 DH5alpha-expressing VgrG3 and its catalytic mutant D842A, respectively. Gene expression was quantified by qPCR. (*D*) Toxicity of VgrG3 induces ROS. ROS levels were measured using a fluorescence dye CM-H2DCFDA (1 μM) at excitation/emission wavelengths of 495/520 nm in a microplate reader. The mean and SEs of four replicates for each group are shown. VgrG3m, VgrG3^{D842A}. (*E*) Mutants of *soxS* and *soxR* were more susceptible to T6SS killing. The *hlyE* mutant, defective in hemolysin E synthesis, was used as a control for comparison. HlyE has little effect on oxidative response in *E. coli*. All mutants were obtained from the *E. coli* Keio collection. (*F*) Loss of *sodA* and *sodB,* encoding superoxide dismutases, renders *E. coli* more susceptible to T6SS killing. The double-deletion mutant of *sodA sodB* was compared with its parental strain *E. coli* K-12 MG1655. (*G*) Oxidative response genes induced by the T6SS of *Acinetobacter* ADP1. *E. coli* MG1655 was coincubated with ADP1 and its T6SS mutant. Gene expression was quantified by qPCR. (*H*) Induction of *soxS* by *P. aeruginosa* PAO1. Expression of *soxS* in *E. coli* MG1655 exposed to PAO1 and its *vipA1* mutant was compared with *soxS* expression in *E. coli* alone. Mean values and SEs of triplicate samples are indicated in *B*, *C*, and *E*–*H*.

expression of *oxyR* and *fur*, encoding regulators for H_2O_2 stress and iron acquisition, respectively, was not induced by T6SS, consistent with our RNA-seq results (Fig. 1*B*). We then determined whether T6SS-dependent effectors are important for inducing *soxS*. We have previously identified three T6SS antimicrobial effectors in V52 (11), one of which is VgrG3, a lysozyme-like enzyme that attacks the cell wall of target species. Expression of VgrG3 in *E. coli* is highly toxic, whereas a point mutation in its catalytic residue D842A abolishes such toxicity (11). We found that transcription levels of *soxS* were 12-fold higher in *E. coli* cells expressing VgrG3 than in cells expressing the catalytic mutant VgrG3^{D842A} (Fig. 1*C*). To test if the toxicity of VgrG3 results in increased ROS levels, we used a general ROS fluorescence sensor CM-H2DCFDA (30, 31) to compare ROS levels in VgrG3-expressing cells. We found ROS levels were about sevenfold higher in *E. coli* expressing WT VgrG3 than in cells expressing VgrG3^{D842A} (Fig. 1*D*), indicating the toxicity of this single T6SS effector is sufficient to induce ROS. Interestingly, certain mammalian innate immunity proteins that also target bacterial peptidoglycan have recently been shown to induce oxidative stress based on transcriptomic analysis (32).

Because SoxRS-induced gene products are important for mitigating damage caused by oxidative stress, and *soxS* is highly induced by T6SS, we wondered whether mutants of *soxS* and *soxR* would be more susceptible to T6SS-mediated killing. Using mutants from the Keio collection (33), we found that the survival of *soxS* and *soxR* mutants was significantly reduced in comparison to a control mutant lacking the *hlyE* gene encoding hemolysin E (Fig. 1*E*). We further tested the survival of the *sodA sodB* double mutant lacking both cytoplasmic superoxide dismutases SodA and SodB. The *sodA* gene is positively regulated by SoxS, and the *sodA sodB* double mutant is known to be sensitive to oxidative stress (34). As expected, the *sodA sodB* double mutant was more sensitive to T6SS killing than its parental strain MG1655 (Fig. 1*F*).

We next tested if other T6SS killer bacteria could also induce ROS in *E. coli*. *A. baylyi* ADP1 is known to kill *E. coli* via its T6SS (7). Expression of *soxS* was induced 13-fold by the T6SS of ADP1 compared with an isogenic T6SS null mutant of ADP1 (Fig. 1*G*). Interestingly, *oxyR* and its regulated genes *oxyS* and *katG* were also induced by ADP1, suggesting different T6SS system effectors might induce different types of oxidative damage and redox signals (Fig. 1*G*). The T6SS apparatus encoded by the H1 locus of *P. aeruginosa* secretes multiple antibacterial effectors that are toxic to *E. coli* (10), and the *vipA1* gene is crucial for the function of this antibacterial nanomachine (9). We found that both WT *P. aeruginosa* PAO1 and the *vipA1* mutant were able to induce *soxS* highly in *E. coli* (Fig. 1*H*). The induction of *soxS* by the *vipA*1 mutant might be due to toxic molecules secreted by the other T6SS H2 and H3 clusters in *P. aeruginosa* or might be simply independent of T6SS. Nevertheless, our data clearly show that interactions with multiple different bacterial species can trigger *soxS* induction in *E. coli*.

Because the T6SS apparatus is similar in structure to the contractile tails of certain bacteriophages (4), we predicted that phage attack might also induce ROS in *E. coli*. We tested this prediction using phage P1*vir* and its susceptible host *E. coli* K-12 MG1655. Indeed, *soxS* was induced 14-fold in MG1655 treated with P1*vir*, whereas *oxyR* and its regulated genes *oxyS* and *katG* were not induced (Fig. 2*A*). Using the fluorescence ROS sensor CM-H2DCFDA, we detected a high level of ROS in MG1655 treated with P1*vir* and little ROS signal in nontreated MG1655 cells (Fig. 2*B* and Movies S1 and S2). Because killing of *E. coli* by T6SS results from the toxicity of T6SS-delivered antibacterial effectors (4), we predicted that other antimicrobial molecules might also induce *soxS* expression. Polymyxin B is an antimicrobial peptide produced by *Paenibacillus polymyxa* that targets the cell envelope and is highly effective against *E. coli* (35). We treated *E. coli* with polymyxin B and found a 35-fold increase in *soxS* expression (Fig. 2*C*). Using the fluorescence sensor CM-H2DCFDA, we found ROS levels were much higher in polymyxin B-treated cells (Fig. 2*D*). Our result is consistent with previous reports that polymyxin B induces ROS production in *Acinetobacter baumannii* (36) and that treatment with pleurocidin, another antimicrobial peptide, results in hydroxyl radical formation (37). Interestingly, it has been shown recently that polymyxin B also induces activation of the T6SS of *P. aeruginosa,* a phenomenon that is thought to mimic the T6SS counterattack by this organism against other aggressive T6SS-positive species (15). Thus, ROS generation might contribute to the signal that activates *P. aeruginosa* T6SS counterattacks.

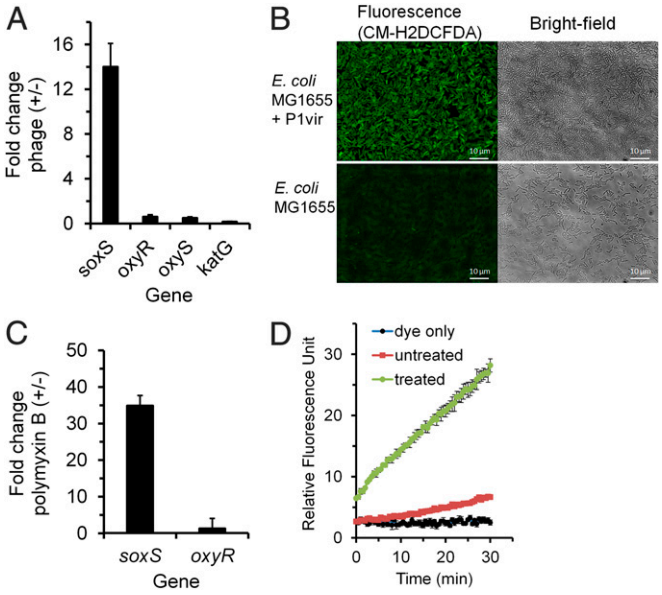

Fig. 2. Induction of *soxS* by P1*vir* phage, and polymyxin B. Gene expression was quantified by qPCR. (*A*) Phage P1*vir* induces *soxS*. *E. coli* MG1655 was infected with P1*vir* at a multiplicity of infection of 1 at 37 °C for 30 min. Mean values and SEs of triplicate samples are indicated. (*B*) Detection of ROS in *E. coli* MG1655 treated with P1*vir*. Examples of fluorescence and bright-field images of *E. coli* treated with and without P1*vir*, respectively, are shown. ROS were detected using the fluorescence dye CM-H2DCFDA (10 μM) [also Movies S1 (with phage) and S2 (no phage)]. (*C*) Polymyxin B induces *soxS*. *E. coli* MG1655 was treated with polymyxin B at 20 μg/mL for 15 min at room temperature. Mean values and SEs of triplicate samples are indicated. (*D*) Generation of ROS in *E. coli* MG1655 treated with polymyxin B. ROS levels were detected using CM-H2DCFDA (1 μM). Mean values and SEs of four replicates are shown.

Given that exogenous T6SS attack induces ROS in *E. coli*, we asked whether a ROS scavenger, thiourea, might rescue *E. coli* from T6SS-mediated killing. It has been shown that thiourea is protective against some bactericidal antibiotics (17, 27). We found that *E. coli* was fully protected from *V. cholerae* T6SS in the presence of thiourea (Fig. 3*A*). We then determined whether this protection is truly due to scavenging of ROS or to the inhibition of T6SS itself. We quantified the gene expression of *hcp* and *vipA* (two T6SS essential genes encoding the inner tube and outer sheath of T6SS apparatus) to assess the expression of other T6SS genes in the *hcp* and the core structural operons, respectively. We found the expression of *hcp* and *vipA* was substantially repressed by thiourea (Fig. 3*B*). We then used a known T6SS effector TseL (11) as an indicator of T6SS secretion and tested whether TseL is secreted in the presence of thiourea. By expressing TseL on an arabinose-inducible pBAD vector (11) rather than its native promoter, we minimized the effect of thiourea on the expression of TseL. Our results show that the secretion of TseL was inhibited by thiourea (Fig. 3*C*). In addition, thiourea did not protect *E. coli* from the toxicity of VgrG3 expression (Fig. S1). We conclude that thiourea protects *E. coli* from T6SS killing by inhibiting the expression of *V. cholerae* T6SS genes through an as yet unknown mechanism. We then tested whether thiourea inhibits other T6SS systems. Indeed, secretion of Hcp was impaired in ADP1 treated with thiourea, and killing of *E. coli* by ADP1 was abolished (Fig. 3*D*). A recent report suggests that other small molecules can interfere with T6SS function (38), but, to our knowledge, thiourea represents the first molecule identified that blocks transcription of T6SS genes. Thus, thiourea and its derivatives might be promising antibacterial drugs if they block expression of T6SS genes in organisms that rely on T6SS activity for their full virulence.

After the initial studies of Kohanski et al. (17), two prominent reports have focused on whether antibiotics can kill in the absence of ROS production and whether ROS production contributes significantly to cell death (24, 26). A subsequent paper by Dwyer et al. (27) provided further evidence that ROS production was a typical consequence of the cellular disruption caused by antibiotics, and thus could enhance the lethality of antibiotics beyond their primary modes of action. The results presented here resonate with all four of these studies in that the lethal insults we studied (T6SS effectors, P1*vir* phage, and polymyxin B) induced ROS production but cell killing could occur by nonoxidative (cell wall hydrolysis, phage replication, and envelope disruption, respectively) as well as oxidative mechanisms depending upon the insult. Thus, it seems prudent to consider the possibility that antibiotics and other noxious agents, such as T6SS effectors, could contribute to cell killing by multiple mechanisms that may vary in their relative contributions depending upon the physiological state of the target cell and its ability to respond to and repair primary and secondary damage.

In conclusion, we show that T6SS attack, a specific antibacterial T6SS effector, a virulent phage, and polymyxin B can all

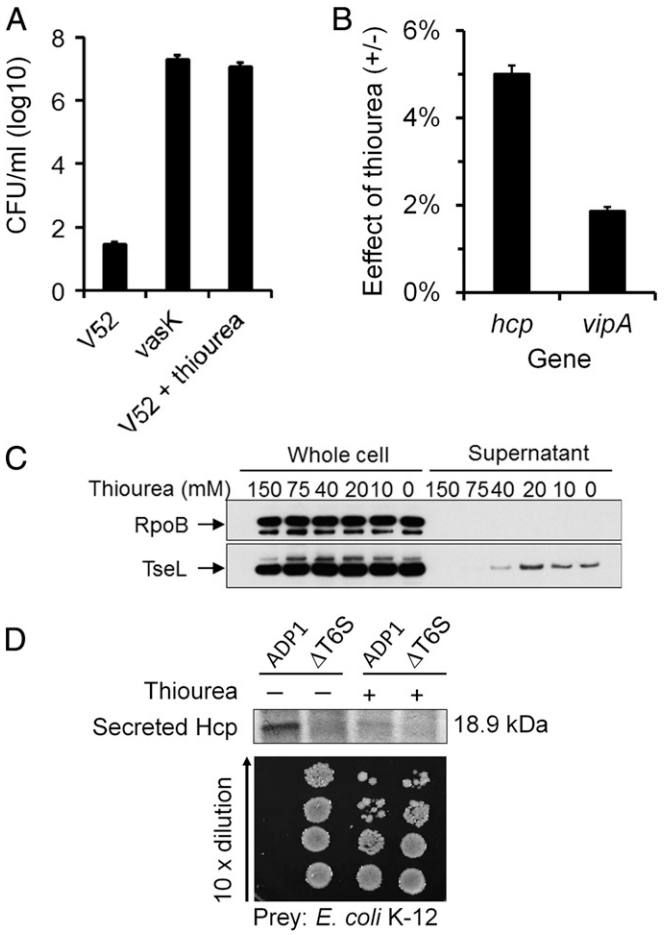

Fig. 3. Effect of thiourea on T6SS. (*A*) Killing of *E. coli* MG1655 by *V. cholerae* in the presence or absence of thiourea. Mean values and SEs of triplicate samples are indicated. Thiourea was used at 150 mM. (*B*) Effect of thiourea on T6SS gene expression by qPCR. Mean values and SEs of triplicate samples are indicated. Thiourea was used at 150 mM. RpoB, RNA polymerase subunit B. (*C*) Western blot analysis of TseL secretion at different concentrations of thiourea. (*D*) Thiourea inhibited Hcp secretion and T6SS killing by ADP1. Coomassie blue staining of secreted Hcp by ADP1 shows that Hcp secretion was inhibited by 150 mM thiourea. Thiourea also inhibited T6SS-mediated *E. coli* killing by ADP1. ΔT6S, the T6SS deletion mutant of ADP1.

stimulate *soxS* expression in *E. coli*. Previous reports suggested that the expression of *soxS* could be up-regulated by antibiotics (16, 27), volatile organic compounds (39), and hydrogen peroxide (40). Interestingly, all of these compounds can be produced by various bacterial species. Collectively, these results suggest that *soxS* can be considered as a "sentinel gene" whose expression may be up-regulated in response to competing bacterial species, their products, or virulent viruses (Fig. 4). In a complex multispecies community, elevation of *soxS* expression may be a strategy to prepare for potentially lethal interactions with aggressive species either by direct contact (e.g., T6SS organisms, phages) or through exposure to diffusible toxic molecules (e.g., antibiotics, metabolites). It follows that some of these lethal interactions probably produce toxic effects, at least in part, through the generation of ROS. Presumably, induction of SoxS-regulated genes within a narrow window of exposure to ROS or other toxic-inducing signals would provide an evolutionary fitness advantage for *E. coli* during such encounters. Although we observed that *soxS* was highly induced by lethal attacks, genes controlled by SoxS were not induced (with the one exception of *marA*). This finding is similar to previous observations that *E. coli* treated with norfloxacin also exhibited increased levels of *soxS* expression but not the genes controlled by SoxS (16). Induction of *soxS* by lethal signals may not result in activation of genes controlled by SoxS simply because these signals are generated by effectors that are also rapidly fatal to the cell under the experimental conditions used here. Although further work will be required to understand why *soxS* has been selected as a sentinel gene, our results nonetheless point to a key role for ROS in signaling the onset of antagonistic bacterial interactions that will likely have ecological and medical importance.

Materials and Methods

Bacterial Strains and Growth Conditions. *V. cholerae* V52, *A. baylyi* ADP1, *P. aeruginosa*, and their respective T6SS null mutants were described previously (3, 9). *E. coli* strains O157:H7 EDL933, K-12 DH5alpha, and MG1655, and phage P1*vir* were obtained from our culture collection. The *sodA sodB* double mutant is a gift from H. E. Schellhorn, McMaster University, Hamilton, ON, Canada. The *soxS::kan*, *soxR::kan*, and *hlyE::kan E. coli* mutants were obtained from the Keio collection. All strains were routinely grown aerobically at 37 °C in LB with 5 g/L sodium chloride. Antibiotics were used at the following concentrations: ampicillin (100 μg/mL), streptomycin (100 μg/mL), kanamycin (50 μg/mL), carbenicillin (75 μg/mL), and chloramphenicol (2.5 μg/mL for V52 and 25 μg/mL for *E. coli*).

T6SS Killing Assay. The T6SS killing assay was performed as described previously (11). Briefly, 1×10^9 cells of killer cells (V52, ADP1, or PAO1) and 1×10^8 *E. coli* cells were mixed together, spotted on LB, and incubated aerobically at 37 °C for 3 h. The mixture was then recovered in 1 mL of LB, and the survival of *E. coli* was quantified by serial plating on selective medium for *E. coli* growth. For RNA extraction, the killing mixture was filtered onto a nitrocellulose membrane and then placed on an LB plate. The mixture was incubated aerobically at 37 °C for 30 min before RNA extraction. All assays were performed in biological triplicates and at least twice.

RNA Extraction. To minimize RNA degradation during sample preparation, we used a fast RNA extraction approach by mixing membrane-bound cells with boiling lysis buffer (1% SDS and 8 mM EDTA) for 30 s and then with an equal volume of acidic phenol (pH 4.3) as previously described (41). After 5 min of incubation at 65 °C and 10 min on ice, samples were centrifuged at 20,000 × *g* for 10 min to collect the aqueous RNA phase. RNA was then purified using a Direct-zol RNA MiniPrep Kit (Zymo Research). Total RNA was treated using a MICROBExpress Bacterial mRNA Enrichment Kit (Life Technologies) to remove ribosomal RNA before the preparation of sequencing libraries.

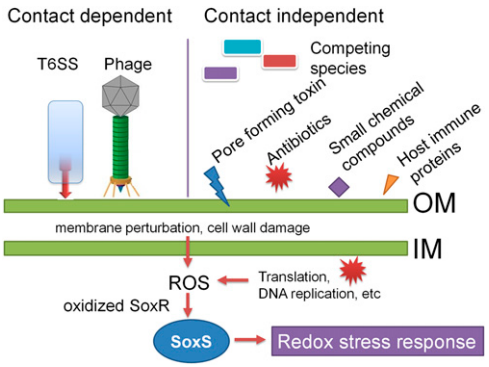

Fig. 4. *SoxS*, a sentinel gene in interspecies interaction. In a multispecies community, competing species can induce ROS production through contact-dependent (e.g., T6SS, phage) or contact-independent (e.g., antibiotics, polymyxin B, small chemical compounds) lethal attacks. ROS production may result from membrane perturbation, cell wall damage, or inhibition of essential cytoplasmic activities by diffusible molecules (e.g., bactericidal antibiotics). In addition to prokaryote-mediated insults, immune proteins from mammalian hosts (e.g., peptidoglycan recognition proteins) can induce ROS production. ROS oxidize SoxR. which, in turn, induces the transcription of *soxS*. As a transcriptional regulator, SoxS up-regulates a group of genes involved in the SoxRS redox stress response. IM, inner membrane; OM, outer membrane.

Sequencing Libraries Preparation and Data Processing. Sequencing libraries were prepared using a NEBNext mRNA Library Prep Kit (New England Biolabs) and a NEBNext Multiplex Oligos for Illumina Kit (New England Biolabs), as per the manufacturer's instructions. Briefly, RNA samples were fragmented, reversed-transcribed, and ligated to Illumina adaptors. Ligation products with 200 bp were selected by gel extraction and amplified using multiplex primers. Sequencing was performed using an Illumina HiSeq2000 platform in the Biopolymer core facility at Harvard Medical School. Sequencing reads (50 nt in length) were mapped to the *E. coli* EDL933 reference genome using the software CLC Genomics Workbench (QIAGEN) as previously described (41). Genes without mapped reads in any of the biological replicate samples were filtered and excluded from downstream analysis. Gene expression levels are represented as the reads per kilobase per million mapped reads values. The Student's *t* test was used for estimating statistical significance.

qPCR. qPCR was performed using the Fast SYBR green mix (Kapa Biosystems) and the RNA-to-C_T 1-Step Kit (Applied Biosystems) as previously described (41). The 16S RNA gene *rrsA* in *V. cholerae* and *E. coli* was used as an internal control to normalize samples with different initial quantities. All primers used in this study are listed in Dataset S2.

Thiourea Treatment. To test the effect of thiourea on T6SS gene expression, exponential phase growing cultures ($OD_{600} = 0.5$) were treated with thiourea (150 mM) for 20 min at 37 °C and then subjected to RNA extraction. Thiourea was also added to LB plates at 150 mM to test the effect on T6SS-mediated killing. Biological triplicates were tested in each experiment.

Killing Assay of *E. coli soxRS* and *sodAB* Mutants. Exponential phase ($OD_{600} = 0.5$) cultures of V52 killer and *E. coli* mutants were concentrated 10-fold and mixed at a 1:1 ratio. After coincubation on LB for 2 h at 37 °C, cells were collected in 1 mL of LB, serially diluted, and plated on selective medium for *E. coli* survival. For mutants from the Keio collection, LB supplemented with kanamycin was used. For comparing survival of the *sodAB* mutant and MG1655, cells were transformed with the pUC19 plasmid to confer carbenicillin resistance. The experiment was performed three times.

P1*vir* Phage Infection Assay. P1*vir* phage lysate titer was quantified by plating *E. coli* K-12 MG1655 cells infected with serial dilutions of phage lysates on LB plates containing 0.7% top agar, 5 mM $CaCl_2$, and 10 mM $MgSO_4$. For RNA extraction, exponential phase *E. coli* K-12 MG1655 culture was infected with P1*vir* phage at a multiplicity of infection of 1 and coincubated at 37 °C for 30 min. Biological triplicates were tested.

Fluorescence Dye-Based ROS Detection. CM-H2DCFDA (Life Technologies) was dissolved in 100% (vol/vol) ethanol to make 1 mM stock solution. To test the effect of VgrG3 expression, exponential phase cultures ($OD_{600} = 0.5$) of *E. coli* DH5alpha carrying a pBAD-VgrG3 or pBAD-VgrG3^{D842A} vector were induced with 0.1% arabinose for 1 h at 37 °C. One milliliter of culture was washed with PBS buffer by centrifugation and resuspended in 1 mL of PBS. One microliter of CM-H2DCFDA was added to the 1-mL cell suspension and incubated for 30 min at room temperature. To test the effect of polymyxin B treatment, exponential phase cultures ($OD_{600} = 0.5$) of *E. coli* were washed with PBS and treated with 20 μg/mL polymyxin B for 15 min at room temperature. One microliter of the stock CM-H2DCFDA solution was added to 1 mL of cell suspension. One hundred microliters of the resultant cell suspension samples was transferred to a 96-well black plate. Fluorescence was measured using a SpectraMax M2 Plate Reader (Molecular Devices) with excitation/emission wavelengths of 495/520 nm.

Detection of TseL and Hcp. Western blot analysis was used to monitor the expression of TseL in V52, as previously described (11). The previously constructed pBAD18-TseL-3V5 was induced with 0.1% L-arabinose for 30 min in the presence of different concentrations of thiourea. Secreted samples were collected by centrifugation and filtered using a 0.2-μm filter, followed by precipitation using 10% (wt/vol) trichloroacetic acid. Whole-cell and secreted samples were then mixed with SDS-loading dye and resolved on a 4–12% (wt/vol) SDS/PAGE gel. Proteins were then transferred to a PVDF membrane by electrophoresis. The membrane was blocked with 5% nonfat milk in TBST buffer [50 mM Tris, 150 mM NaCl, 0.05% Tween 20 (pH 7.6)] and incubated with a 1:10,000 dilution of the primary anti-V5 antibody (Sigma). The PVDF membrane was then washed with TBST buffer, incubated with a 1:10,000 dilution of HRP–anti-mouse second antibody (Pierce), and developed with the ECL substrate (Pierce). For Hcp secretion in ADP1, secreted samples were prepared similarly. Samples were run on a 4–12% (wt/vol) SDS/PAGE gel and stained with Coomassie Blue R250 (BIO-RAD). Hcp is a highly abundant secreted protein and can be readily detected by Coomassie staining (7).

Fluorescence Microscopy. Overnight cultures of *E. coli* MG1655 were diluted 1:100 into fresh LB supplemented with 5 mM $CaCl_2$ and 10 mM $MgSO_4$. Cells were grown aerobically to exponential phase ($OD_{600} = 0.5$). CM-H2DCFDA was added to the culture to a final concentration of 10 μM. One milliliter of the culture was treated with 100 μL of P1*vir* phage lysate (final titer = 5×10^8 pfu/mL) and incubated in a 37 °C water bath for 30 min. Both treated and untreated cultures were centrifuged and concentrated 10-fold. Samples were spotted on a 1% agarose pad containing 10 μM CM-H2DCFDA. Cells were imaged at room temperature after 30 min. Fluorescence signals of CM-H2DCFDA were detected using an FITC filter set. Multiple fields of cells (at least 30 fields per sample) were analyzed. A Nikon Ti-E inverted microscope with a perfect focus system and a CFI Plan Apo Lambda 100× Oil objective lens was used for imaging. Fiji was used for all image analysis and manipulations. Rolling-ball background correction (50 pixels) was used to correct for uneven background for individual fluorescence images. For bright-field images, auto-brightness/contrast was used. To create a montage, fluorescence and bright-field images were converted to an eight-bit image stack.

ACKNOWLEDGMENTS. We thank the Advancing Canadian Wastewater Assets program for providing infrastructure support. This work was supported by National Institute of Allergy and Infectious Diseases Grant AI-01845 (to J.J.M.) and by an internal research grant from the University of Calgary (to T.G.D.).

1. Wheater DM, Hirsch A, Mattick ATR (1952) Possible identity of lactobacillin with hydrogen peroxide produced by lactobacilli. *Nature* 170(4328):623–624.
2. Aoki SK, et al. (2005) Contact-dependent inhibition of growth in *Escherichia coli*. *Science* 309(5738):1245–1248.
3. Pukatzki S, et al. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci USA* 103(5):1528–1533.
4. Ho BT, Dong TG, Mekalanos JJ (2014) A view to a kill: The bacterial type VI secretion system. *Cell Host Microbe* 15(1):9–21.
5. Mougous JD, et al. (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312(5779):1526–1530.
6. Silverman JM, et al. (2013) Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. *Mol Cell* 51(5):584–593.
7. Shneider MM, et al. (2013) PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 500(7462):350–353.
8. Basler M, Mekalanos JJ (2012) Type 6 secretion dynamics within and between bacterial cells. *Science* 337(6096):815.
9. Basler M, Ho BT, Mekalanos JJ (2013) Tit-for-tat: Type VI secretion system counterattack during bacterial cell-cell interactions. *Cell* 152(4):884–894.
10. Hood RD, et al. (2010) A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* 7(1):25–37.
11. Dong TG, Ho BT, Yoder-Himes DR, Mekalanos JJ (2013) Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 110(7):2623–2628.
12. Miyata ST, Unterweger D, Rudko SP, Pukatzki S (2013) Dual expression profile of type VI secretion system immunity genes protects pandemic *Vibrio cholerae*. *PLoS Pathog* 9(12):e1003752–e1003752.
13. Koskiniemi S, et al. (2013) Rhs proteins from diverse bacteria mediate intercellular competition. *Proc Natl Acad Sci USA* 110(17):7032–7037.
14. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S (2010) The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc Natl Acad Sci USA* 107(45):19520–19524.
15. Ho BT, Basler M, Mekalanos JJ (2013) Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science* 342(6155):250–253.
16. Dwyer DJ, Kohanski MA, Hayete B, Collins JJ (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol Syst Biol* 3:91.
17. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130(5):797–810.
18. Dwyer DJ, Collins JJ, Walker GC (2015) Unraveling the Physiological Complexities of Antibiotic Lethality. *Annu Rev Pharmacol Toxicol* 55:313–332.
19. Cabiscol E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* 3(1):3–8.
20. Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC (2012) Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science* 336(6079):315–319.
21. Davies BW, et al. (2009) Hydroxyurea induces hydroxyl radical-mediated cell death in *Escherichia coli*. *Mol Cell* 36(5):845–860.
22. Wang X, Zhao X (2009) Contribution of oxidative damage to antimicrobial lethality. *Antimicrob Agents Chemother* 53(4):1395–1402.

23. Yeom J, Imlay JA, Park W (2010) Iron homeostasis affects antibiotic-mediated cell death in *Pseudomonas* species. *J Biol Chem* 285(29):22689–22695.
24. Liu Y, Imlay JA (2013) Cell death from antibiotics without the involvement of reactive oxygen species. *Science* 339(6124):1210–1213.
25. Hassett DJ, Imlay JA (2007) Bactericidal antibiotics and oxidative stress: A radical proposal. *ACS Chem Biol* 2(11):708–710.
26. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K (2013) Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* 339(6124):1213–1216.
27. Dwyer DJ, et al. (2014) Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc Natl Acad Sci USA* 111(20):E2100–E2109.
28. Zhao X, Drlica K (2014) Reactive oxygen species and the bacterial response to lethal stress. *Curr Opin Microbiol* 21C:1–6.
29. Nunoshiba T, Hidalgo E, Amábile Cuevas CF, Demple B (1992) Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. *J Bacteriol* 174(19):6054–6060.
30. Ro S-H, et al. (2014) Sestrin2 inhibits uncoupling protein 1 expression through suppressing reactive oxygen species. *Proc Natl Acad Sci USA* 111(21):7849–7854.
31. O'Donovan P, et al. (2005) Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* 309(5742):1871–1874.
32. Kashyap DR, et al. (2014) Peptidoglycan recognition proteins kill bacteria by inducing oxidative, thiol, and metal stress. *PLoS Pathog* 10(7):e1004280.
33. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* 2:0008.
34. Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: Is superoxide dismutase necessary for aerobic life? *EMBO J* 5(3):623–630.
35. Dixon RA, Chopra I (1986) Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in *Escherichia coli*. *J Antimicrob Chemother* 18(5):557–563.
36. Sampson TR, et al. (2012) Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob Agents Chemother* 56(11): 5642–5649.
37. Choi H, Lee DG (2012) Antimicrobial peptide pleurocidin synergizes with antibiotics through hydroxyl radical formation and membrane damage, and exerts antibiofilm activity. *Biochim Biophys Acta* 1820(12):1831–1838.
38. Sun K, et al. (2014) Screening for inhibition of *Vibrio cholerae* VipA-VipB interaction identifies small-molecule compounds active against type VI secretion. *Antimicrob Agents Chemother* 58(7):4123–4130.
39. Kim KS, Lee S, Ryu C-M (2013) Interspecific bacterial sensing through airborne signals modulates locomotion and drug resistance. *Nat Commun* 4:1809.
40. Zheng M, et al. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* 183(15):4562–4570.
41. Dong TG, Mekalanos JJ (2012) Characterization of the RpoN regulon reveals differential regulation of T6SS and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic Acids Res* 40(16):7766–7775.