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Massachusetts Institute of Technology

Lethality of MalE-LacZ hybrid protein shares mechanistic attributes with oxidative component of antibiotic lethality

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Contributed by Graham C. Walker, July 13, 2017 (sent for review May 8, 2017; reviewed by Carl F. Nathan and Evgeny Nudler)

Downstream metabolic events can contribute to the lethality of drugs or agents that interact with a primary cellular target. In bacteria, the production of reactive oxygen species (ROS) has been associated with the lethal effects of a variety of stresses including bactericidal antibiotics, but the relative contribution of this oxidative component to cell death depends on a variety of factors. Experimental evidence has suggested that unresolvable DNA problems caused by incorporation of oxidized nucleotides into nascent DNA followed by incomplete base excision repair contribute to the ROS-dependent component of antibiotic lethality. Expression of the chimeric periplasmic-cytoplasmic MalE-LacZ$_{72–47}$ protein is an historically important lethal stress originally identified during seminal genetic experiments that defined the SecY-dependent protein translocation system. Multiple, independent lines of evidence presented here indicate that the predominant mechanism for MalE-LacZ lethality shares attributes with the ROS-dependent component of antibiotic lethality. MalE-LacZ lethality requires molecular oxygen, and its expression induces ROS production. The increased susceptibility of mutants sensitive to oxidative stress to MalE-LacZ lethality indicates that ROS contribute causally to cell death rather than simply being produced by dying cells. Observations that support the proposed mechanism of cell death include MalE-LacZ expression being bacteriostatic rather than bactericidal in cells that overexpress MutT, a nucleotide sanitizer that hydrolyzes 8-oxo-dGTP to the monophosphate, or that lack MutM and MutY, DNA glycosylases that process base pairs involving 8-oxo-dGTP. Our studies suggest stress-induced physiological changes that favor this mode of ROS-dependent death.

cell death | ROS | antibiotics | MalE-LacZ | protein translocation

Agents or conditions that inhibit important biological processes can kill cells. However, physiological processes metabolically downstream of the initial inhibition can also contribute to cell death (1). For example, β-lactam antibiotics not only inhibit penicillin-binding proteins leading to lysis; they also induce a futile cycle of cell wall synthesis and degradation that contributes to their killing (2). In another example, lethal attacks on *Escherichia coli* mediated by the type VI secretion system P1*vir* phage and the antimicrobial peptide polymyxin B elicit the production of reactive oxygen species (ROS) that contribute to cell death (3). In bacteria, ROS production has been associated with the lethal effects of diverse stresses (4). In most cases, the detailed mechanisms responsible for ROS production are poorly understood; however, a variety of futile metabolic cycles can elicit H_2O_2 production, illustrating the breadth of possible metabolic perturbations that could potentially induce oxidative stress (5).

Despite widespread evidence that endogenous ROS produced as a consequence of metabolic stress can be lethal to bacteria and eukaryotes, the application of this concept to antibiotic lethality has been complicated. Evidence from multiple investigators using a variety of antibiotics to study different bacteria indicated that ROS generated metabolically downstream of the interaction of the antibiotics with their primary cellular targets contribute to drug lethality (1, 6, 7). This conclusion was challenged by critiques (8, 9) that focused particularly on an earlier paper that had carried out a systems-level analysis of the lethality of multiple classes of bactericidal antibiotics and proposed a model to account for the experimental observations (10). Substantial new evidence has subsequently been published that addresses concerns that were raised and strongly supports a contributing role for ROS in antibiotic lethality (11, 12). Recent reviews have discussed how the apparently contradictory results can be explained in part by the specifics of the experimental setup and the technical details of the assays used (1, 6, 7).

Notably, the challenges to the involvement of ROS in antibiotic lethality (8, 9, 13) did not consider the implications of prior

Significance

Understanding the molecular basis of the lethality of antibiotics and certain other stresses is complicated because cell death can result from direct inhibition of a critical biological process as well as from reactive oxygen species (ROS) generated by events metabolically downstream of the direct interaction of the agent with its target. Prior evidence has indicated that the ROS-dependent component of antibiotic lethality is due in part to lethal DNA problems resulting from the incorporation of oxidized nucleotides into DNA and incomplete DNA repair. Our observations unexpectedly indicate that the predominant mechanism of lethality from a hybrid protein that jams the machinery that translocates proteins across the cytoplasmic membrane shares attributes with the ROS-dependent component of antibiotic lethality.

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genetic and physiological evidence that oxidized nucleotides, especially 8-oxo-dGTP, contribute to cell death by bacterial antibiotics (14). Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) have only limited abilities to react with cellular constituents (15, 16). The oxidation of nucleotides and nucleic acids instead requires the highly reactive hydroxyl radicals and intermediates produced when H_2O_2 undergoes Fenton chemistry, collectively referred to as "Fenton oxidants" (17). It is the amount and target of Fenton oxidants that are relevant for antibiotic lethality, not the intracellular levels of endogenously produced O_2^- or H_2O_2. These and other results suggest that the incorporation of oxidized nucleotides into nascent DNA followed by incomplete base excision repair (BER) is an important molecular mechanism that contributes to the ROS-dependent component of antibiotic lethality (11, 12, 14, 18). To date, no alternative interpretation has been suggested for this body of experimental observations.

Since a mode of cell death involving nucleotide oxidation is common to three different classes of bactericidal antibiotics (β-lactams, quinolones, and aminoglycosides), it is plausible that other types of cellular stress induce death by a similar mechanism. We analyzed an historically important lethal stress originally identified by Jon Beckwith and his colleagues during their seminal genetic experiments that defined the highly conserved SecY-dependent protein translocation system (19). We show the lethality caused by the expression of a chimeric MalE-LacZ protein consisting of the NH_2-terminal sequences of MalE (periplasmic maltose-binding protein) joined to a modestly NH_2-terminally truncated LacZ (β-galactosidase) (20) shares attributes with the oxidative component of antibiotic lethality. Our analyses suggest additional physiological parameters that are important for this mode of cell death besides the levels of ROS. The induced changes in gene expression suggest that exposing bacteria to H_2O_2 does not fully recapitulate the intracellular environment caused by antibiotics and other stresses in which this mode of DNA-based oxidative cell death occurs, thereby helping explain some of the past confusion regarding the role of ROS-dependent death in antibiotic lethality.

Results

Induction of the MalE-LacZ Hybrid Protein Kills Cells but Does Not Elicit SecY Degradation. The MalE-Lac$Z_{72–47}$ fusion protein encoded by strain PB72-47/MM18 (21) (Table S1), hereafter referred to as "MalE-LacZ," was isolated using a bacteriophage Mu-based genetic strategy (21). The hybrid protein consists of the first N-terminal 212 amino acids of MalE (53.5% of MalE), followed by a short linker of 11 amino acids derived from Mu, followed by an N-terminally truncated LacZ missing its first 41 amino acids (Fig. 1*A*). As previously reported (21), induction of the MalE-LacZ fusion by maltose addition to minimal-glycerol medium results in cell killing that begins ≈2 h later (Fig. S1*A*). Continued incubation of the culture eventually results in resumed cell growth (21) due to the accumulation of variants that have lost the λ transducing phage or have accumulated suppressor mutations. Induction of a LamB-LacZ fusion protein elicits an FtsH-dependent degradation of SecY proposed to contribute to its lethality (22), but induction of MalE-LacZ does not (Fig. S1*B*), suggesting a different mechanism underlies its lethality.

MalE-LacZ Induction Does Not Cause Death Predominantly by Cell Lysis but Induces *soxS*. DAPI staining revealed that the elongated cells reported by Bassford et al. (21) upon MalE-LacZ induction are actually short chains of cells in which DNA partitioning and at least partial septum formation appear to have occurred but the cells have not separated (Fig. S1*C*). Using a live-dead stain to examine the timing of cell death after maltose induction, we observed that although the commitment to cell death and loss of colony-forming ability was evident at 3 h after induction, cells did not begin to exhibit substantial staining until 4 h. Importantly, most of the cell death occurred without cell lysis, suggesting that the lethality resulted from another cause (Fig. S1 *D* and *E*). Expression of the MalE-LacZ fusion protein

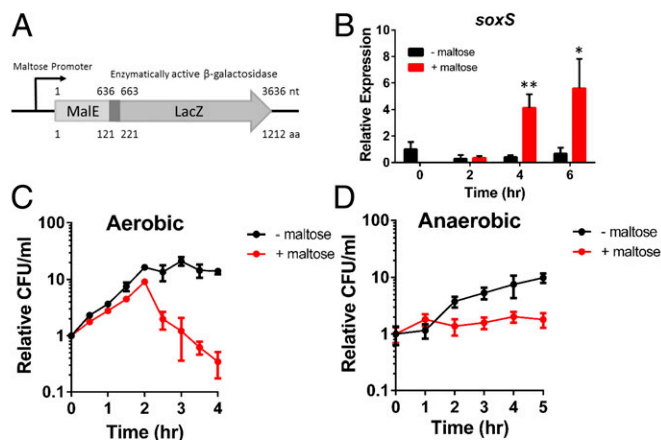

Fig. 1. Expression of MalE-LacZ increases expression of an oxidative response gene and requires oxygen for killing. (*A*) MalE-LacZ fusion protein. N terminus of periplasmic MalE is fused to a truncated but enzymatically active LacZ by a 19-amino acid linker derived from the bacteriophage Mu. (*B*) Expression of MalE-LacZ by the addition of 0.2% maltose increases the expression of the oxidative response gene *soxS* as determined by qPCR. (*C* and *D*) The induction of MalE-LacZ expression causes bacterial cell death after 2 h when grown aerobically in LB medium (*C*), whereas under strict anaerobic conditions MalE-LacZ expression is bacteriostatic (*D*). Data shown represent the mean ± SD with at least three biological replicates. Significant values are $*P \le 0.05$; $**P \le 0.01$.

jams translocation machinery, so the precursors of normally secreted proteins still containing their signal peptides accumulate after maltose addition, with some forming cytoplasmic aggregates (23). However, the normal localization of a fraction of translocated proteins (23) suggested that some other type of mechanism might be responsible for cell death. Similar to stress caused by the type VI secretion system, P1*vir* phage, and polymyxin B (3), we found that induction of the MalE-LacZ protein increases the expression of *soxS*, a marker of oxidative stress (Fig. 1*B*).

MalE-LacZ Induction Is Not Lethal in Anaerobic Conditions but Leads to ROS Production in Aerobic Conditions. To test whether oxidative stress might underlie MalE-LacZ–dependent cell death, we first compared the effect of maltose addition on cell survival under aerobic and strict anaerobic conditions. We used LB medium in these experiments since *E. coli* cannot grow anaerobically on the glycerol medium used previously (Fig. S1*A*) (21). Induction of MalE-LacZ under anaerobic conditions is bacteriostatic rather than bactericidal, indicating that molecular oxygen is required for cell death (Fig. 1 *C* and *D*).

Using four independent methods, we then tested whether expression of MalE-LacZ under aerobic conditions results in ROS production. First, to directly measure the intracellular concentration of H_2O_2 induced by MalE-LacZ, we used the recently described APX system, which uses a cytoplasmic-expressed variant of ascorbate peroxidase to convert Amplex Red into a readily detectable fluorescent product in an H_2O_2-dependent manner (12). We observed that the intracellular H_2O_2 levels increase over time following MalE-LacZ induction (Fig. 2*A*). Second, to directly measure the dynamic production of O_2^- after maltose addition, we used a miniaturized cytochrome *c*-based biosensor that has previously been used to detect the release of O_2^- from cells treated with bactericidal antibiotics (24). Using this technique, we found that expression of MalE-LacZ results in the release of O_2^- as early as 40 min after induction (Fig. 2*B*). Third, we used an ELISA to show that MalE-LacZ induction leads to increased amounts of 8-oxo-guanine (8-oxo-7,8-dihydroguanine) in DNA (Fig. 2*C*), a well-established biomarker for oxidative stress (11) that is relevant to experiments described below. Fourth, we used two dyes based on different chemistries that have been widely used for ROS detection in previous studies (3, 12, 25):

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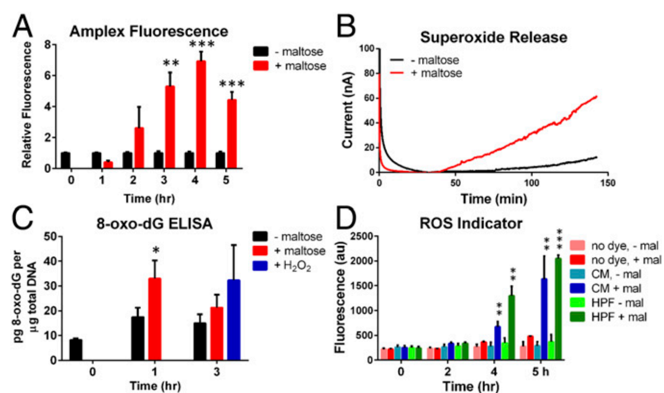

Fig. 2. MalE-LacZ expression causes the production of ROS. (*A*) Expression of MalE-LacZ induces the production of H_2O_2 detected by the intracellular enzymatic sensor APX, using Amplex Red fluorescence as an output. (*B*) Induction of MalE-LacZ leads to an increase in amperometric responses of the cytochrome *c* superoxide sensor, indicating an increase in the rate of superoxide release. (*C*) 8-Oxo-dG content of the total DNA determined by an ELISA-based assay. Expression of MalE-LacZ induces a significant increase in 8-oxo-dG content 1 h after induction, decreasing at later time points. Cells were treated with 10 mM H_2O_2 for 60 min as a positive control. (*D*) Expression of MalE-LacZ induces ROS detected by two different fluorescent dyes, CM and HPF. A one-way ANOVA was performed to determine statistical significance against the no-dye autofluorescence control. Data shown represent the mean ± SD with at least three biological replicates for *A* and *C*, representative data from three independent experiments for *B*, and three technical replicates for *D*. Significant values are $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

3′-(p-hydroxyphenyl) fluorescein (HPF) and 5/6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), whose fluorescence has been shown to correlate with O_2^- production determined by direct measurement (24). For both dyes, we observed statistically significant increases in fluorescence compared with controls for maltose-induced autofluorescence and morphology changes (26, 27) in the absence of a dye (Fig. 2*D*). Microscopic images demonstrated this increase in fluorescence was not a flow cytometry artifact (4) caused by autofluorescence or altered cell shape (Fig. S2).

Genetic Evidence That Oxidative Stress Plays a Causal Role in Cell Death Caused by MalE-LacZ Induction. If ROS contribute causally to cell death, as opposed to simply being generated by dying cells, then mutants sensitive to oxidative stress should be more sensitive to killing by MalE-LacZ induction. Consistent with H_2O_2 playing a causal role in lethality, we observed that null mutants lacking either of *E. coli*'s cytoplasmic catalases, KatE or KatG, were more sensitive to killing by maltose induction of the MalE-LacZ hybrid protein (Fig. 3 *A* and *B*), as were mutants lacking either subunit of *E. coli*'s *ahpCF*-encoded alkyl hydroperoxidase, which reduces H_2O_2 to water (Fig. 3 *C* and *D*). Moreover, the addition of exogenous catalase to the growth medium provided some protection (Fig. 3*E*). Since the O_2^- released into the medium cannot cross the membrane, and the exogenous catalase is external, some of the extracellular O_2^- produced upon MalE-LacZ induction may be converted to H_2O_2 that then enters the cells and contributes to lethality. Interestingly, a *ΔoxyR* mutant did not display increased sensitivity to killing (Fig. 3*F*). *katE*, whose deletion causes the strongest phenotype, is not regulated by OxyR, but *katG* and *ahpCF* are, indicating that basal levels of KatG and AhpCF play a physiologically significant role in protecting cells against endogenous H_2O_2 produced by MalE-LacZ induction. *soxR*- and *soxS*-null mutants exhibited an increased susceptibility to killing after maltose induction (Fig. 3 *G* and *H*), but a *sodA sodB* double mutant lacking both cytoplasmic superoxide dismutases was much more resistant to killing (Fig. 3*I*), which is similar the result reported for bactericidal antibiotics and has been attributed to the impaired ability of the strains to convert O_2^- to H_2O_2 (28).

Genetic Evidence That DNA Problems Related to the Processing of Incorporated Oxidized Nucleotides Contribute Causally to the Lethality of MalE-LacZ Induction. Our analyses of the lethality of DinB overexpression and bactericidal antibiotics led us to propose that oxidation of nucleotides, particularly those containing guanine, the most easily oxidized nucleic acid base (29), contributes causally to cell death (14). This model accounts for our observations that (*i*) an increased level of the nucleotide sanitizer MutT, which hydrolyzes nucleoside triphosphates containing 8-oxo-dG to their respective monophosphates, reduces killing (14); (*ii*) an increased level of the mismatch repair protein MutS that only modestly affects growth rate reduces killing (12); (*iii*) deletion of *mutM* and *mutY*, which encode BER enzymes involved in the processing of 8-oxo-dG lesions, reduces killing (14); and (*iv*) deletion of *recA* sensitizes cells to killing (14). The model suggests that incorporation of 8-oxo-dG into nascent DNA creates a situation in which incomplete MutM/MutY-dependent BER generates lethal DNA problems that can be partially ameliorated by RecA-dependent homologous recombination. We carried out parallel experiments to test whether this same mechanism might be responsible for MalE-LacZ–induced cell death.

Strikingly, increasing the expression of MutT resulted in MalE-LacZ induction being bacteriostatic rather than bactericidal (Fig. 4*A*), an observation suggesting that oxidation of dGTP to its corresponding 8-oxo-guanine derivative contributes to cell killing, as did increasing MutS expression (Fig. 4*B*). Together

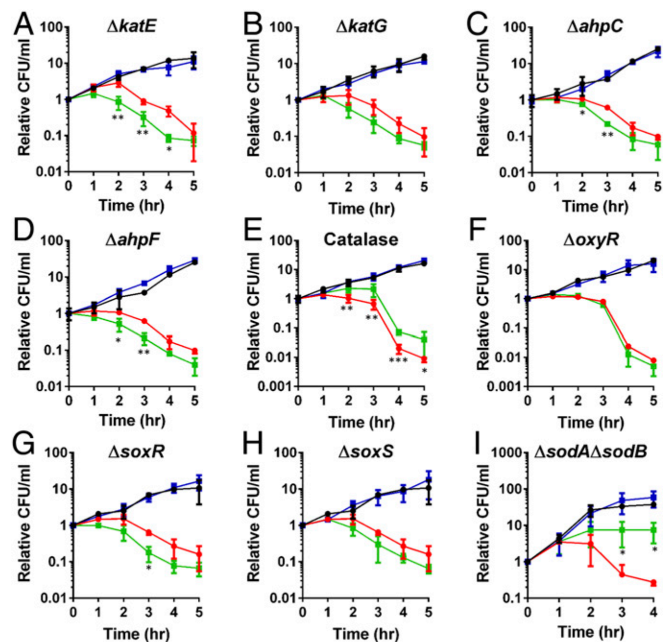

Fig. 3. ROS contributes causally to MalE-LacZ–induced cell death. (*A*–*D*) Knockouts of the catalases KatE (*A*) and KatG (*B*) and either subunit of the alkyl hydroperoxidase AhpC (*C*) or AhpF (*D*) are more sensitive to killing by MalE-LacZ. (*E*) Addition of exogenous catalase to the medium provides a protective effect. (*F*) Knockout of the major oxidative response sensor, OxyR, has no effect on killing. (*G* and *H*) Knockouts of either component of the other major oxidative sensor, SoxRS, are sensitive to MalE-LacZ killing. (*I*) A double knockout of both cytoplasmic superoxide dismutases *sodA* and *sodB* is insensitive to killing by MalE-LacZ. Black squares are wild type, red are wild type plus maltose, blue are mutant, and green are mutant plus maltose. Data shown represent the mean ± SD with at least three biological replicates. Significant values are $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

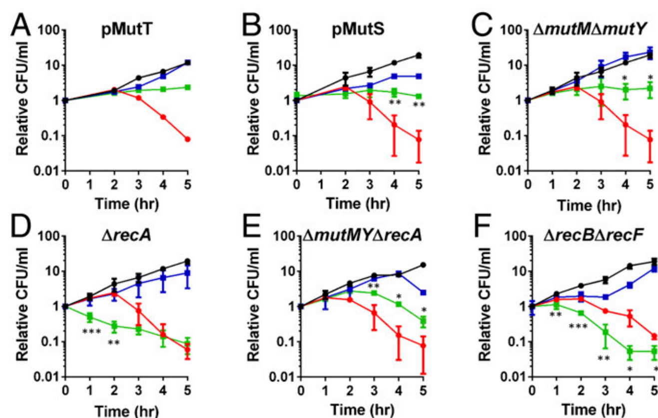

Fig. 4. Cell death due to MalE-LacZ results from attempted BER of oxidative DNA damage. (*A* and *B*) Overexpression of the 8-oxo-dGTP sanitizer MutT (*A*) or the mismatch-repair protein MutS (*B*) protects cells from MalE-LacZ expression. (*C*) Double knockout of MutM and MutY DNA glycosylases involved in BER of 8-oxo-dG lesions is resistant to killing by MalE-LacZ. (*D*) The Δ*recA* strain is more susceptible to killing by MalE-LacZ expression, with cell death beginning earlier. (*E*) Extra sensitivity of Δ*recA* to MalE-LacZ killing can be partially suppressed in a Δ*mutM* Δ*mutY* background. (*F*) The Δ*recB* Δ*recF* strain is more sensitive to killing by MalE-LacZ. Data shown represent the mean ± SD with at least three biological replicates. Black squares are wild type, red are wild type plus maltose, blue are mutant, and green are mutant plus maltose. Significant values are $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

these two observations suggest that the lethality stems in part from 8-oxo-dGTP or some other potentially miscoding oxidized nucleotide being incorporated into nascent DNA in a fashion that leaves it potentially repairable by mismatch repair, as previously suggested for mutagenesis induced by subinhibitory levels of antibiotics (30) and cell killing by higher levels of bactericidal antibiotics (12). While multicopy suppression is a widely used technique in microbial genetics, we note that, although cellular MutT and MutS levels were increased by introducing plasmids from the ASKA collection (31), we did not add the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), thereby avoiding the concern (4) that induction of extremely high levels of protein might possibly impair overall bacterial growth and metabolism. The maltose-induced levels of the MalE-LacZ protein in the strains carrying the *mutT* and *mutS* plasmids were indistinguishable from those in the parental strain (Fig. S3*A*).

Similarly, we found that deleting *mutM* and *mutY* also resulted in MalE-LacZ induction being bacteriostatic rather than bactericidal (Fig. 4*C*), an observation suggesting that the action of these BER DNA glycosylases involved in processing 8-oxo-dG and oxidized purine lesions contributes causally to cell death by generating BER intermediates (14). The level of the MalE-LacZ protein was not affected by loss of *mutM* or *mutY* (Fig. S3*B*).

Deleting the *recA* gene not only increased sensitivity to killing by MalE-LacZ induction; loss of colony-forming ability also could be observed as early as the 1-h time point (Fig. 4*D*). Loss of *recA* has no effect on the levels of the MalE-LacZ protein (Fig. S3*B*), but the increased sensitivity to killing could be largely suppressed by deleting *mutM* and *mutY* (Fig. 4*E*). These observations suggest that potentially lethal DNA problems involving strand breaks caused by MutM and MutY incisions at oxidized nucleotides begin to arise earlier after MalE-LacZ induction than we had suspected but are initially kept in check by a RecA-dependent process. A Δ*recB* Δ*recF* double mutant had a similar phenotype, suggesting that RecA's recombinational functions, rather than its SOS regulatory roles, are important (Fig. 4*F*).

Time-Resolved Microarray and Network Analysis of the Consequences of MalE-LacZ Induction. Additional mechanistic insights into the lethal physiological events triggered by MalE-LacZ induction were gained by a time-resolved microarray study (*SI Materials and Methods*) with qPCR verification of representative genes of interest (Fig. S4 and Table S2) and Network Component Analysis (NCA), a method that uses the connectivity from gene-regulatory networks to infer the activity of transcriptional regulators (*SI Materials and Methods* and Dataset S1) (32, 33). Many changes in gene expression were observed, including the rapid and robust induction of the *mal* operon as well as the previously reported (34) robust induction of heat-shock genes (Figs. S5 and S6). In addition to MalT and RpoH (Fig. 5 *A* and *B*), our NCA analysis detected significant changes in transcriptional activity for 14 transcription factors out of 131 analyzed (Dataset S2).

Several observations were particularly relevant. First, the canonical oxidative stress-response factor OxyR (35) did not exhibit statistically significant changes in activity following MalE-LacZ induction (Fig. 5*C*), a result consistent with the lack of an effect of a *ΔoxyR* mutation on MalE-LacZ–dependent killing. Second, of enzymes that scavenge ROS, we found transient but statistically significant induction of only *katE* and *sodB* (Fig. 5 *D* and *E*). Third, we observed significant induction at a minimum of three different time points of three genes, *mutM*, *mutY*, and *mutT*, whose products are associated with processing of 8-oxoguanine and also affect the sensitivity of cells to MalE-LacZ induction (Fig. 5 *F*–*H*). Fourth, NCA did not indicate significant LexA activation, but we observed significant induction of several genes (*dinG*, *mfd*, *nusA*, *recF*, *recJ*, and *recQ*) whose products participate in DNA-repair processes (Fig. S6). Interestingly, cells overexpressing DinB (14) exhibited changes in gene expression very similar to changes in cells expressing MalE-LacZ, including this noncanonical oxidative stress response (Fig. S7*A*) and induction of *mutM*, *mutY*, and other DNA-repair genes (Fig. S7*B*).

Discussion

The MalE-LacZ periplasmic-cytoplasmic fusion protein is historically important because of its use in the elucidation of SecY-dependent protein translocation (21). However, our observations suggest that cell death caused by MalE-LacZ induction does not result directly from jamming of protein translocation or SecY degradation. Rather our findings support a model in which physiological/metabolic stress from MalE-LacZ expression increases the production of low levels of O_2^- and H_2O_2. H_2O_2 does not accumulate to high intracellular levels because cellular conditions favor its participation in Fenton chemistry, resulting

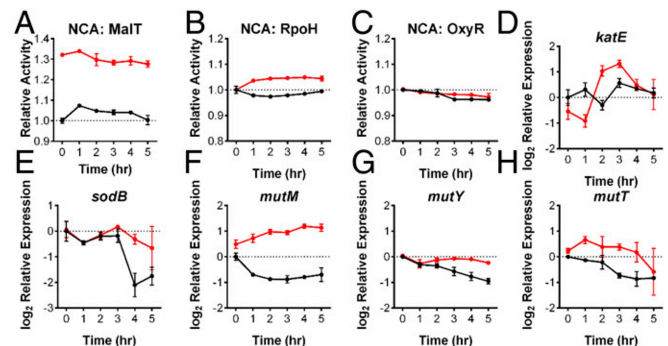

Fig. 5. Time-resolved microarrays reveal lack of induction of the OxyR oxidative stress-response regulon but increased expression of oxidized nucleotide-repair genes. (*A* and *B*) NCA shows strong induction of the MalT maltose regulon (*A*) and RpoH heat-shock response (*B*). (*C*) In contrast, the OxyR oxidative stress-response regulon is not activated. (*D*–*H*) Maltose addition induces expression of the *katE* (*D*) and *sodB* (*E*) oxidative stress-response genes and all three major components of oxidized nucleotide repair, *mutM* (*F*), *mutY* (*G*), and *mutT* (*H*). Black squares are control, red are maltose-induced. Data shown represent the mean ± SD with three biological replicates.

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in the oxidization of nucleotides, including 8-oxo-dGTP, which are subsequently incorporated into DNA. Cellular death does not result directly from the incorporation of oxidized nucleotides but rather from lethal DNA problems caused by intermediates of MutM/MutY-dependent BER. Initially these potentially lethal DNA problems can be ameliorated by RecA-dependent processes or by a combination of RecB- and RecF-dependent processes. Multiple, independent lines of evidence support the physiological relevance of each element of this model for cell death.

These physiological and mechanistic attributes of the lethality caused by MalE-LacZ expression are strikingly similar to those of the oxidative component of antibiotic lethality (1, 6, 7, 12, 14). In the case of MalE-LacZ, this mode of cell death involving oxidative DNA damage is the dominant cause of the lethality. The situation is more complicated for antibiotics because they can also cause cell death by their direct effect on their cellular targets and associated cellular processes. The relative contribution of this oxidative component to antibiotic-induced cell death can vary depending on factors such as the experimental conditions, the antibiotic, and the metabolic state of the bacterium (1, 6, 12).

Two observations related to the H_2O_2-inducible OxyR regulon might appear to be inconsistent with this model: (*i*) MalE-LacZ expression does not induce the OxyR regulon, and (*ii*) an *oxyR* mutant does not display increased sensitivity to MalE-LacZ lethality. However, these observations can be understood by (*i*) recognizing that the amount and targeting of Fenton oxidants are critical rather than the levels of endogenously produced O_2^- or H_2O_2, (*ii*) appreciating that only a small increase in oxidative damage in DNA can prove lethal, and (*iii*) considering the chemical and enzymatic requirements of the DNA-based cellular death pathway indicated by our experimental observations.

Our observations implicating 8-oxo-dG and potentially other oxidized nucleotides in cell death imply that Fenton chemistry is involved in the lethality since neither O_2^- nor H_2O_2 reacts significantly with nucleic acids or nucleotides (15, 16, 36). It has been argued that, since it is implausible for intracellular levels of endogenously produced H_2O_2 to rise to lethal levels, stresses would have to do more than accelerate H_2O_2 formation (4). One specific effect that stresses such as MalE-LacZ and antibiotics could induce that would additionally increase oxidative damage would be to accelerate the rate of the Fenton reaction, which would also prevent H_2O_2 from accumulating to sufficient levels to induce the OxyR regulon. Fenton oxidation can occur so rapidly relative to diffusion that H_2O_2 produced by histone demethylation introduces 8-oxo-dG lesions into the surrounding DNA that are then exploited to control local gene expression (37). Various physiological factors such as pH (38), cysteine levels (39), and anionic ligands (38) accelerate the Fenton reaction and could be affected by stresses such as MalE-LacZ and antibiotics. Interestingly, H_2S, the product of cysteine metabolism, protects bacteria from antibiotic killing (40). It is particularly relevant that nucleoside triphosphates and nucleic acids are anionic ligands that complex with Fe^{+2} and promote the Fenton reaction to an extent comparable to EDTA and nitrilotriacetate (38, 41). Furthermore, the proximity of the nucleic acid base of a dNTP or NTP to an Fe^{+2} complexed by its phosphates (42) favors its reaction with highly reactive Fenton oxidants (11, 16). Since the diffusion distance for a hydroxyl radical is only one carbon bond length (43), nucleotides and nucleic acids must be at higher risk of damage from Fenton oxidants than many other biomolecules because of their ability to complex Fe^{+2} and promote local production of Fenton oxidants.

Bacteria live on a knife's edge with respect to their ability to tolerate oxidative stress (4), so that only a small increase in oxidative damage in DNA can have mutagenic or lethal consequences. In the case of aerobically grown *E. coli*, the threat posed by endogenous ROS is narrowly balanced by the titers of scavenging enzymes (15). Minor oxidative reactions that impart a gain of function to the target can be particularly important even though they represent a negligible component of the overall reactions (16); 8-oxo-dG is such a gain-of-function oxidation product because it pairs with dA as well as dC. With respect to tolerating 8-oxo-dG, aerobically grown *E. coli* live so close to the edge that decreasing the level of MutT by only a factor of two increases the mutation rate (44). Moreover, for aerobically grown *E. coli*, simply increasing the frequency of initiation of DNA replication (45) or increasing DNA polymerase IV, which has a propensity to use 8-oxo-dGTP (14, 18), is enough to cause cell death because BER cannot be completed before encountering the next replication fork (45). An advantage that bacteria gain by living so close to this threshold is that they can mutate in response to stress by increasing the incorporation of oxidized deoxynucleotides into DNA while simultaneously suppressing mismatch repair (30). Thus, MalE-LacZ expression or bactericidal antibiotic stress seems to exaggerate a type of potential oxidative toxicity that lurks just below the threshold in unstressed wildtype cells.

A factor that likely contributes to cell death is that BER of oxidative damage can be slow to complete because the lyase activities associated with MutM and MutY catalyze β or β,δ eliminations, thereby generating ends that require further processing to expose the 3′-OH needed for DNA polymerases (46). This DNA-based death mechanism, which initially can be counteracted by RecA-dependent or RecB/RecF-dependent processes, is likely complex. Since it takes only a single unrepaired double-stand break (DSB) to kill a bacterial cell (47), one problem could be DSBs caused by MutM and MutY incisions at closely spaced lesions (14) or by replication forks encountering unrepaired BER intermediates (45). Other potentially lethal DNA problems include interstrand crosslinks mediated by abasic sites (48), LigA/MutY-dependent futile cycles of ligation/incision (49), and unresolvable collisions caused by stalled transcription or replicative complexes (50).

Importantly, key genes whose functions affect this mode of DNA-based oxidative cell death are not regulated by H_2O_2-inducible OxyR or by SoxRS but rather by other stress regulators: e.g., *mutM* (RpoH) (51), *mutY* (Fur/ArcA/Fnr, normally down-regulated upon oxidative stress but up-regulated upon MalE-LacZ induction) (52), and *mutT* (CpxA/CpxR in an operon with *secM* and *secA*) (53), and *dinB*/*recA* (SOS) (54). Thus, the intracellular environment of cells generating endogenous H_2O_2 as a consequence of a stress can be very different from the intracellular environment of cells treated with exogenous H_2O_2. For example, the increased levels of MutM glycosylase due to the powerful heat-shock response elicited by MalE-LacZ would result in more frequent initiations of BER and hence in a greater probability of a BER intermediate with a strand break being encountered by a replication fork before repair can be completed. Some of the past confusion in this research area may have arisen because the mode of oxidative death occurring in bacterial cells undergoing stress from agents such as bactericidal antibiotics or MalE-LacZ does not conform to expectations based on studies of cells treated with exogenous H_2O_2, as these other stresses induce additional key non–OxyR-regulated proteins that affect lethality.

Our evidence that a completely different stress besides bactericidal antibiotics causes ROS-based lethality resulting from nucleotide oxidation and incomplete BER shows that this type of cell death is not unique to antibiotics and suggests that it likely contributes to death from other stresses as well. As previously discussed (14), oxidation of ribonucleotides could also contribute to lethality by other mechanisms. Our results suggest that exploiting the oxidative component of antibiotic lethality is a plausible strategy to improve the efficacy of existing antibiotics or to identify new ones (55, 56).

Materials and Methods

Detailed materials and methods can be found in *SI Materials and Methods*.

MalE-LacZ Induction. MM18 and derivatives were streaked onto LB agar containing X-Gal and grown overnight (ON) at 37 °C. Blue, average-sized colonies were picked and grown ON in M63 medium or LB with any appropriate antibiotics. The cultures were then diluted 1:100 in fresh

medium, grown to OD_{600} 0.1, and then split into two populations with one receiving 0.2% maltose and grown at 37 °C on a rotating shaker. For the exogenous catalase experiment, 1,000 units of bovine catalase (Sigma) were added to each culture at time 0. Samples were taken every hour to determine cfus or to harvest cells for protein, DNA, or RNA extraction. The resulting colonies were counted on a ProtoCOL 3 colony counter. Student's *t* test was used to determine statistical differences between samples.

Microscopy. MM18 was grown as previously described. At the indicated time points, cultures were concentrated by centrifugation, washed in PBS, and then fixed in 4% paraformaldehyde. Cells were then washed, stained with DAPI, and washed again. Bacteria were plated on slide-mounted agarose pads and visualized with a Nikon Eclipse Ni microscope.

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