A DNA damage response in *Escherichia coli* involving the alternative sigma factor, RpoS

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We isolated an *Escherichia coli* mutant in the *iraD* gene, sensitive to various forms of DNA damage. Our data are consistent with the function of IraD to promote accumulation of the alternative transcription sigma factor, RpoS, by binding to the adaptor RssB protein that targets RpoS for degradation. Our results demonstrate the physiological importance of this mode of regulation for DNA damage tolerance. Although RpoS is best known for its regulation of genes induced in stationary phase, our work underscores the importance of the RpoS regulon in a DNA damage response in actively growing cells. We show that *iraD* transcription is induced by DNA damage by a mechanism independent of the SOS response. The IraD and SOS regulatory pathways appear to act synergistically to ensure survival of cells faced with oxidative or DNA damaging stress during cellular growth.

oxidative stress | posttranslational regulation | replication stress | SOS response | DNA repair

Throughout its life cycle, *Escherichia coli* is faced with different environmental challenges and regulates gene expression accordingly. One way is by changes in the promoter recognition of RNA polymerase via different situation-specific σ factors (1). In *E. coli*, the major alternative sigma factor is σS (RpoS), which is required for expression of specific genes on entry to stationary phase or as a response to stress (2–4). Although the RpoS dependence of many of these responses and the regulation of RpoS itself have been well studied, the relevance of this to DNA repair has not been a major focus.

To find genes important in DNA damage responses, we performed a random Tn*5* transposon insertion mutant screen, assaying sensitivity to, among other agents, phleomycin and azidothymidine (AZT). Phleomycin induces random single- or double-strand breaks in the backbone of DNA (5), whereas AZT blocks DNA synthesis, leading to single-strand gaps in the replication fork (6). One insertion mutant in *iraD* (previously an unknown gene, *yjiD*) was hypersensitive to phleomycin and AZT.

Recent work from Gottesman and coworkers (7) implicated IraD in posttranslational regulation of RpoS. The RssB adaptor protein targets RpoS to ClpXP for degradation during logarithmic growth, keeping RpoS protein levels low in the absence of stress (8–12). IraD was identified in a high-copy plasmid screen for genes promoting accumulation of an RpoS-LacZ fusion protein. The IraD gene product acts as an antiadaptor protein via direct binding and inhibition of the ability of RssB to target RpoS for proteolysis by ClpXP *in vitro* (7).

In the work presented here, we demonstrate that IraD is required for survival to DNA damage, providing evidence of the physiological importance of IraD in particular and the antiadaptor mechanism in general. The data presented suggest that IraD acts as an antagonist of RssB, regulating RpoS levels and stabilization, not only after DNA damage but constitutively. Our results establish the importance of RpoS stabilization in proliferating bacterial cells in which replication has been directly blocked, confirming a role for the RpoS regulon in DNA damage repair or tolerance. This model of regulation via protein stabilization may allow rapid and transient induction of the RpoS

regulon. We demonstrate induction of *iraD* by various forms of DNA damage by a mechanism independent of the SOS response. The loss of both IraD and SOS responses leads to a synergistic decrease in the ability to withstand DNA damage, indicating that both pathways function in a complementary fashion to ensure cell survival.

Results

Isolation and Characterization of an *iraD* Transposon Insertion Allele. To identify DNA damage response factors in *E. coli*, we mutagenized cells by random insertion of Tn*5*-EZ and screened for hypersensitivity to DNA damaging agents. One insertion mutant showing hypersensitivity to both AZT and phleomycin mapped to the ORF, *iraD*. Sequence analysis revealed an insertion 18 nt downstream from the ATG start site, followed by a 9-nt target site duplication. The orientation of the Tn*5* element was such that the *kan* promoter could potentially transcribe an IraD protein truncated by 3 amino acids at its N terminus. Because of the possibility that the Tn*5* allele does not completely inactivate IraD function and could disrupt its regulation, we also examined a complete deletion, *iraD*Δ, in the analysis that follows.

IraD Is Important for Survival of DNA Damage in Growing Cells. In a microarray study, *iraD* transcripts were induced almost 30-fold after hydrogen peroxide treatment, ranking as the seventh highest *E. coli* gene up-regulated after oxidative stress (13). We found that the original *iraD*::Tn*5* and the *iraD*Δ mutant were extremely sensitive to hydrogen peroxide, with a decrease in survival greater than 10-fold relative to WT strains after modest exposure (Table 1*A*) of early exponentially growing cultures. The *iraD*::Tn*5* mutant showed somewhat greater sensitivity to hydrogen peroxide than the null allele in this experiment; the basis for this finding is unknown, and this reduced sensitivity was not seen under all assay conditions.

A high-copy plasmid expressing the *iraD* gene from its natural promoter fully complemented peroxide sensitivity of the *iraD*Δ mutant, whereas expressing just the promoter region of *iraD* did not (Table 1*B*). (The Tn*5* allele could not be tested because it already carries the *kan* resistance gene for the plasmid.) At higher doses of peroxide, a lower copy ampicillin-resistance plasmid expressing *iraD* from its natural promoter only partially complemented the *iraD*Δ strain, although it fully complemented the *iraD*::Tn5 allele (Fig. 1). The difference between the two alleles confirms our suspicion that *iraD*::Tn*5* is not a null allele; incomplete complementation of the null allele may result from failure to express sufficient plasmid-encoded *iraD*+. In addition, we used P1 phage transduction to replace the mutant copy of *iraD* with a WT copy of the gene in both mutant strain

Table 1. H₂O₂ sensitivity of *iraD*Δ and *iraD*::Tn5

Strain genotype	Plating efficiency*	SD
MG1655	0.31	0.13
*iraD*Δ	0.032	0.014
iraD::Tn5	0.009	0.007
*rpoS*Δ	0.026	0.011
*rssB*Δ	0.23	0.11
*hsdR*Δ	0.42	0.14
*iraD*Δ *hsdR*Δ	0.028	0.010
*rpoS*Δ *hsdR*Δ	0.046	0.023
*rssB*Δ *hsdR*Δ	0.234	0.072
iraD::Tn5 *rpoS*Δ	0.006	0.003
iraD::Tn5 *rssB*Δ	0.190	0.12
*iraD*Δ *hsdR*Δ *rpoS*Δ	0.027	0.0077
*iraD*Δ *hsdR*Δ *rssB*Δ	0.32	0.19
MG1655/pDONR-P	0.18	0.02
MG1655/pDONR-P-*iraD*⁺	0.27	0.09
*iraD*Δ pDONR-P	0.017	0.007
*iraD*Δ/pDONR-P-*iraD*⁺	0.23	0.07

*After exposure to 5 mM H₂O₂ for 20 min.

backgrounds and found that the hypersensitivity was lost and the fractional survival recovered to the same levels as in the WT background (data not shown), demonstrating that no other mutation in these strains contributes to peroxide sensitivity.

We assayed colony formation in the presence of low levels of the replication inhibitor AZT, which causes DNA chain termination during DNA synthesis (6) and is only expected to damage replicating cells. Null mutants in *iraD* exhibited dramatic hypersensitivity to AZT (Fig. 2) at doses that only marginally affected WT strains. Similar results (data not shown) were obtained with chronic treatment with phleomycin. These data confirm that *iraD* is important for survival of proliferating cells to multiple types of DNA damage.

Mutants in RpoS Mimic Effects of IraD. IraD has been shown to function as an antiadaptor protein, controlling levels of RpoS via its ability to bind the RssB adaptor protein that targets RpoS for proteolysis by ClpXP (7). If this is the basis of its effects on DNA damage survival, we expect that *rpoS* mutants should have survival defects comparable to or greater than those in *iraD*. Mutants in *rpoS* have been shown to be sensitive to hydrogen peroxide (14), although most of these analyses examined stationary phase and not growing cultures. Exponentially growing
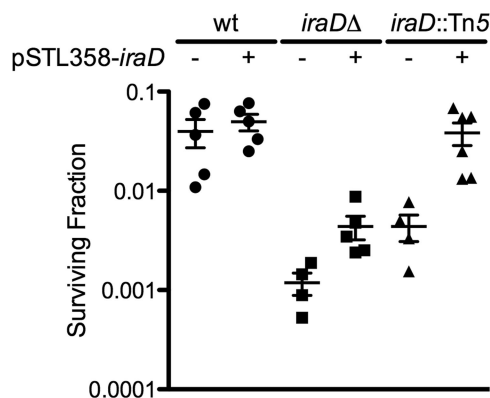

Fig. 1. Complementation of *iraD* with low-copy plasmid-encoded *iraD*. Plating efficiency of strains after 15-min exposure to 12.5 mM H₂O₂. Each point represents an independent determination. Strains denoted (+) carry the vector with *iraD*⁺ and its upstream noncoding region, whereas those denoted (−) carry only the upstream noncoding sequences. wt, wild type.
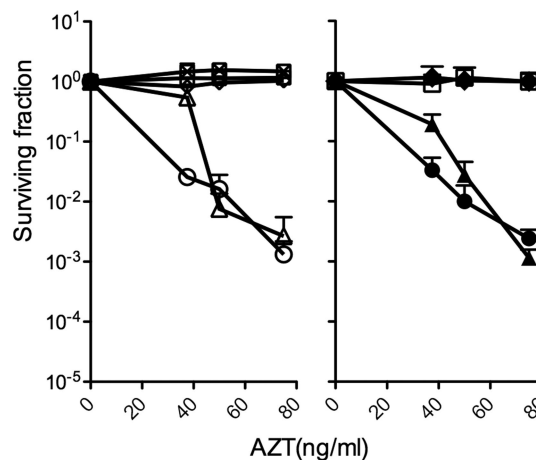

Fig. 2. Plating efficiency with chronic AZT exposure. Squares represent WT, triangles represent *iraD*Δ, circles represent *rpoS*Δ, diamonds represent *rssB*Δ, and squares with X through represent *rssB*Δ *iraD*Δ. Strains in the left panel carry an additional mutation in *hsdR*, used as a linked selective marker in certain *iraD* strain constructions, which does not affect survival in these assays. Strains in the right panel are *hsdR*⁺.

rpoS null mutants had similar sensitivity to hydrogen peroxide as the *iraD* null mutant assayed in parallel (Table 1) as well as to AZT (Fig. 2) and phleomycin (data not shown). Effects of RpoS and IraD appeared to be genetically epistatic (the effects of the double mutants were no greater than those of the single mutants), suggesting that they function in the same pathway.

IraD Suppression via Inactivation of RssB. During exponential growth, RpoS levels are kept low by ClpXP, with the RssB protein acting as a recognition factor for RpoS proteolysis (11, 12, 15, 16). If the role of IraD in promoting DNA damage survival is to antagonize the effects of RssB on RpoS stability, *iraD* phenotypes should be suppressed by *rssB* inactivation.

For both the truncation and null allele of *iraD*, we observed rescue of hypersensitivity to hydrogen peroxide when combined with an *rssB* deletion allele (Table 1). The sensitivity of the *iraD* null mutant to chronic doses of AZT is likewise dramatically suppressed by *rssB* inactivation (Fig. 2). Inactivation of *rssB* did not improve survival of *iraD*⁺ strains. This agrees with the expectation that IraD acts an inhibitor of RssB activity to promote survival to DNA damage by promoting accumulation of RpoS.

RpoS Levels and Stability Are Altered in IraD Mutants. To investigate whether IraD affects levels of RpoS protein, we examined exponentially growing WT, *iraD*Δ, and *iraD*::Tn5 strains by Western blot analysis (Fig. 3*A*). Steady-state levels of RpoS were lower both in the *iraD* null and the *iraD*::Tn5 mutants (2–3-fold), suggesting that IraD is a major determinant of RpoS levels during normal exponential growth in rich medium. The effect of the Tn5 was somewhat less than that of the null allele.

Western blot analysis of RpoS levels in cultures after treatment with chloramphenicol (Fig. 3*B*) to block new RpoS synthesis confirms that IraD affects RpoS levels by altering stability of the protein. In LB exponential cultures without peroxide addition (Fig. 3*B*), RpoS levels declined rapidly after inhibition of new synthesis, more so in the *iraD* null and *iraD*::Tn5 mutants than in WT strains. This constitutive control of RpoS stability *in vivo* is similar to that observed by Bougdour et al. (7). As a control, we examined similarly the stability of DnaJ, another putative ClpXP target (17), to determine the specificity of the *iraD* mutants. Steady-state DnaJ levels were not affected by IraD and did not decline significantly during this time course (data not shown).
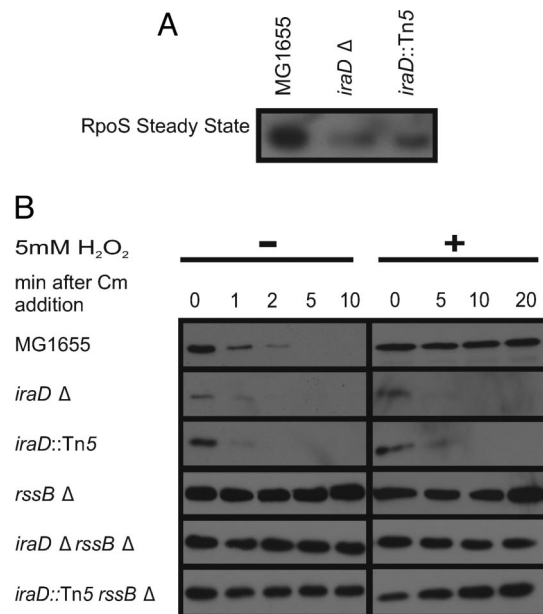
Merrikh *et al.*

Fig. 3. RpoS levels and stability in *iraD* mutants by RpoS Western blot analysis. (*A*) Western blot of steady-state RpoS levels during early log phase in the *iraD* mutants versus WT. (*B*) RpoS stability following chloramphenicol treatment to block new protein synthesis either with (+) or without (−) 5 mM hydrogen peroxide treatment for 10 min. Levels of RpoS are shown at the times indicated after chloramphenicol treatment.

To investigate RpoS stability after DNA damage (Fig. 3*B*), we treated cells with peroxide for 10 min, added chloramphenicol to block further RpoS synthesis, and measured RpoS levels by Western blotting. Under this regimen, RpoS was quite stable in WT strains (with a half-life >20 min) but disappeared rapidly in both the *iraD* null and the *iraD*::Tn*5* mutants. Mutation of RssB stabilized RpoS, even in the absence of IraD, both with and without peroxide treatment. Thus, IraD effects on RpoS stability correlate with effects on DNA damage survival.

DNA Damage Inducibility of IraD. Previous microarray analysis established that *iraD* transcript levels are dramatically increased after treatment with hydrogen peroxide in growing cultures, independent of *oxyR* (13). To establish whether IraD is induced generally by DNA damage, we used a luciferase reporter assay (18) in which the entire upstream region of *iraD* was inserted upstream of the *luxCDABE* operon and expression can be measured by light production. We found a dramatic increase in expression after treatment with AZT, phleomycin, or hydrogen peroxide, compared with controls (Fig. 4*A*), but not with mitomycin C, a classic SOS inducer. The increase in expression in response to hydrogen peroxide treatment is comparable to that seen in microarray studies (13).

In parallel, we also measured induction of *lux* operon fusions to known DNA damage-regulated SOS genes, *recA* and *dinB* (Fig. 4*A*). Although *recA* is more highly expressed than *dinB*, both promoters are induced by all 4 DNA damaging treatments, as we have shown previously for AZT (19). These results suggest that expression of IraD is induced transcriptionally not only after oxidative stress (13) but after other forms of DNA damage. IraD, however, differs from the SOS-regulated genes in that it is not significantly induced by the DNA cross-linker, mitomycin C. The transcriptional induction of IraD may be sufficient to explain the stabilization of RpoS after DNA damage: increased levels of IraD protein may sequester more RssB, thereby promoting the accumulation of RpoS.
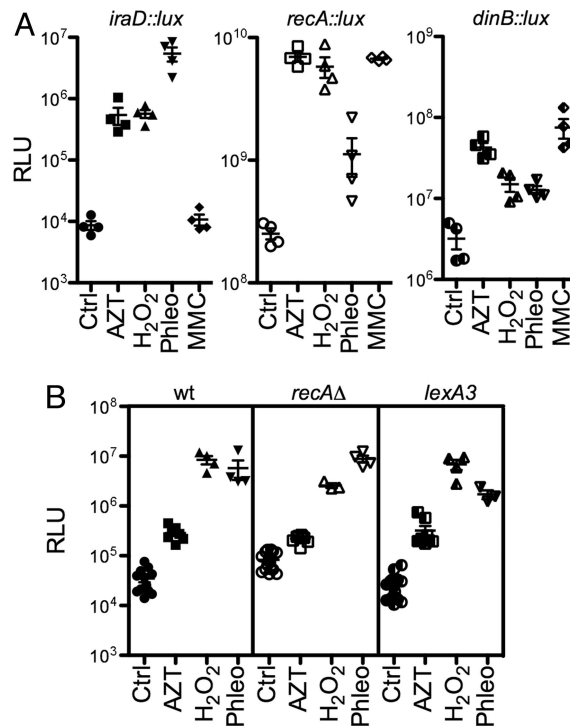

Fig. 4. Expression of *iraD*, *recA*, and *dinB* reporter constructs in response to DNA damage. (*A*) Expression of *iraD*, *recA*, and *dinB luxCDABE* reporters in response to H$_2$O (Ctrl), AZT, H$_2$O$_2$, phleomycin (Phleo), and mitomycin C (MMC) for at least 4 isolates. (*B*) Expression of *iraD*::*luxCDABE* in response to H$_2$O (Ctrl), AZT, H$_2$O$_2$, and Phleo for at least 3 isolates in WT, *recAΔ*, or *lexA3* background. RLU, relative luminescence unit (bioluminescence cpm normalized to OD$_{600}$).

DNA Damage Induction of IraD Is Independent of the SOS Response. The best-characterized DNA damage response is the "SOS response," regulated by the RecA and LexA proteins in *E. coli* (20). During DNA damage, the signal for induction of the SOS response is the formation of RecA filaments on single-stranded DNA, which activate the self-cleavage of LexA, the transcriptional repressor of many DNA damage-responsive genes. We introduced the promoter *lux* fusions into strains in which the SOS response was rendered noninducible by mutation in *recA* or *lexA*. Induction of the *iraD* promoter by AZT, peroxide, or phleomycin was independent of *recA* and *lexA* (Fig. 4*B*), suggesting that the DNA damage responsiveness of the *iraD* promoter is not mediated via the SOS response. In control experiments, induction of the *recA* promoter by these agents was largely abolished by *recA* or *lexA* mutations (data not shown) (19). The SOS independence of IraD induction was not unexpected, given its failure to respond to MitoC and the lack of apparent LexA binding consensus sites in the upstream region of *iraD*. It is interesting to note that the basal levels of *iraD*::*lux* expression were slightly higher in untreated cultures of the *recA* null mutants compared with WT strains, suggesting that spontaneous lesions left unrepaired by RecA can induce the IraD response.

IraD and RecA/LexA Contribute Independently and Additively to DNA Damage Survival. We investigated the relation of *iraD* to the SOS response by measuring colony formation during chronic AZT treatment. Mutants lacking *iraD* or *recA* or harboring a noncleavable (SOS noninducible) allele of *lexA* were comparably sensitive to low doses of AZT exposure (Fig. 5). The double mutants *iraD recA* and *iraD lexA3* were severely sensitive to AZT treatment (Fig. 5), much more so than the single mutants, suggesting a strong synergy between inactivation of *iraD* and the SOS
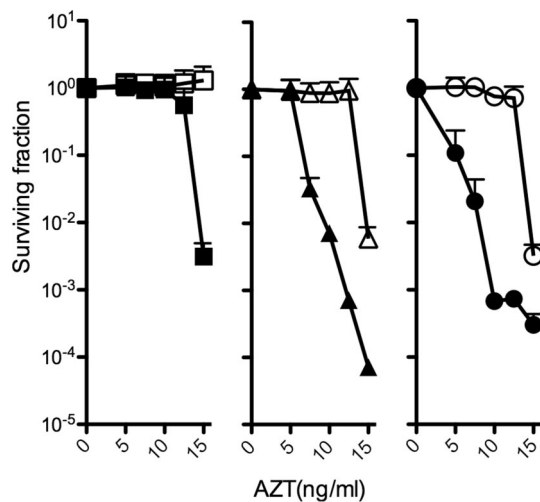
Fig. 5. Plating efficiency under chronic AZT exposure. WT (open squares), *iraD*Δ (filled squares), *lexA3* (open triangles), *iraD*Δ *lexA3* double mutant (filled triangles), *recA*Δ (open circles), and *iraD*Δ *recA*Δ double mutant (filled circles).

response. Therefore, the IraD regulatory system works in a manner independent of and complementary to the SOS response. Both are important for cell survival during sustained DNA damage.

Discussion

IraD, an Oxidative Stress and DNA Damage Response Protein. Using a forward genetic screen, we identified the unknown gene *yjiD*, referred hereto as *iraD*, as a factor aiding survival of proliferating *E. coli* cells to multiple forms of DNA damage, including oxidative stress. Previous studies have shown induction of the *iraD* gene after oxidative stress (13), implying its possible role in defense against such stress, but its role in a more general DNA damage response was unknown.

IraD Is a Regulator of RpoS Protein Levels via RssB. Our data are entirely consistent with the biochemical observation that IraD acts as an antiadaptor of RssB to promote stabilization of RpoS (7). In agreement with this mechanism, *iraD* mutant phenotypes with respect to survival of DNA damage can be rescued by loss of RssB and are mimicked by mutations in RpoS. Based on these biochemical properties and our genetic experiments, we and others (7) have renamed the previously unknown *yjiD* as *iraD*, for "*i*nhibition of *R*ssB *a*ctivity after *D*NA damage."

IraD joins IraP, induced after phosphate starvation (10), and IraM (YcgW/ElbA), induced by low magnesium, in a group of antiadaptors that regulate RpoS stability in response to specific physiological cues. We provide here biological evidence that antiadaptors have real and important physiological consequences: in the case of IraD, enhanced survival to DNA damage. Our results show that IraD is the primary determinant of steady-state levels of RpoS in exponentially growing cells in aerobic culture during normal growth and that IraD stabilizes RpoS against RssB-dependent degradation after DNA damage.

A Class of Regulatory Elements. Bacterial cells may employ antiadaptors as a general strategy to control RpoS levels, and potentially other proteins, in response to specific types of environmental stress. The known antiadaptors, IraP, IraM, and IraD are small unrelated proteins, 86, 107, and 133 aa in length, respectively, predicted to be mostly helical in secondary structure. IraD is a member of a large group of orthologous proteins ("COG3518"), sharing small size and a domain similarity to the T4 gp25 baseplate-wedge protein, and is the first member to have

defined biological function. This group may therefore be composed of specific protein sequestration factors, reflecting a general strategy for bacterial regulation.

RpoS: A DNA Damage Response. Our data implicate RpoS in a general DNA damage response, with IraD acting as a specific positive regulatory factor. The sensitivity of *rpoS* mutants to the thymidine analogue AZT, which specifically blocks DNA replication, suggests a role for the general stress response in the toleration or repair of DNA damage. The *iraD* gene is induced following DNA damage, as demonstrated by promoter fusion assays, in a manner independent of the RecA/LexA-controlled SOS response. The mechanism of this regulation is therefore quite interesting and will be pursued in further studies. Other investigations have identified a handful of genes induced by DNA damage, independent of LexA (20), but these have not been well characterized. Previous studies of transcripts induced by UV light or mitomycin C, traditional treatments to induce the SOS response, have not identified IraD/YjiD as DNA damage inducible (21, 22). The latter but not the former study did, however, identify a number of RpoS-regulated genes as DNA damage inducible, including *dps* and *uspA*. The physiological conditions necessary for *iraD* induction may be specific: *iraD* is strongly induced by peroxide in one microarray study but not in another (13, 23). The upstream region of *iraD* has been reported to possess an Fnr binding sequence, which controls genes expressed differentially in anaerobic growth conditions (24); however, the functional consequences of this site are unknown.

How Does RpoS Promote DNA Damage Survival? The oxidative stress response controlled by RpoS in stationary phase is well studied, and a number of proteins that metabolize reactive oxygen species, such as catalase and superoxide dismutase, are under regulation of σS (25, 26). Our demonstration of sensitivity of *rpoS* mutants to the replication inhibitor AZT during exponential growth suggests that σS may control other functions important for DNA damage repair or tolerance. We speculate that one downstream target is *xthA*, encoding exonuclease III (Exo III), which is known to be regulated by RpoS (27). Exo III, an abasic endonuclease and 3′ exonuclease (28), is important for repair of oxidative damage and removal of damaged nucleotides at 3′ ends of DNA chains (29). Damage to the sugar moiety, blocking replication elongation, is a common oxidative lesion and similar to the one presented by chain-terminating AZT. Mutants in *xthA* are profoundly sensitive to peroxide (30) and to AZT (D. Cooper and S.T.L., unpublished data). Other genes in the RpoS regulon, such as translesion DNA polymerase IV (*dinB*) (31), may also be critical for aspects of the DNA damage response. Although this polymerase does not contribute to survival to AZT (data not shown), its regulation by RpoS explains the observation that frameshift mutagenesis is elevated by *iraD* overexpression (32).

The Importance of RpoS Induction in Growing Cells. Our findings demonstrate a specialized regulatory mechanism controlling RpoS and that RpoS has a significant role in the response to DNA damage, not only in stationary phase but while cells are undergoing rapid growth and division. Cells incorporate AZT only when replicating; therefore, the requirement for IraD and RpoS to form colonies during chronic exposure to AZT confirms their role in growing cells. The complex regulation of σs, involving the induction of both positive and negative regulators after stress, allows for highly sensitive and quickly responding regulation, including rapid termination of the response, which is potentially critical for resumption of cell proliferation.

Materials and Methods

Strains and Strain Constructions. All strains are isogenic with MG1655 (F⁻ *rph*-1) (33). The *iraD*::Tn*5* Insertion mutant, STL9197, was isolated by electroporation of the EZ-Tn5 <R6K ori KAN-2> transpososome complex (Epicentre) into MG1655 (34); all other strains were constructed by P1 transduction (35). Cultures were grown at 37 °C in LB supplemented with appropriate antibiotics at 20 μg/mL kanamycin, 100 μg/mL ampicillin, and/or 10 μg/mL tetracycline. STL7180 carries *recA*::*cat*, and *lexA3* strain (STL12071) was constructed by transduction via its linkage with *malF*3180::Tn10*kan*. The *rssB*::*tetA* (10) and *iraD*::*tetA* alleles (S. Gottesman, National Institutes of Health) were introduced by P1 transduction into MG1655, generating STL11118 and STL11119, respectively. STL7291 (*rpoS*::Tn*10*) was derived by Winkler and coworkers (36), published previously as TX3740. The *hsdR*Δ::FRT *kan* allele from the Mori collection (37) was transduced into MG1655, generating STL11795, and was used as a linked marker with which to move *iraD*Δ::*tetA* in strains already resistant to tetracycline. Alleles were combined via transduction to generate STL11126 (*iraD*::Tn*5 rssB*Δ::*tetA*), STL11124 (*iraD*::Tn*5 rpoS*::Tn*10*), STL 11796 (*iraD*Δ::*tetA hsdR*Δ::FRT *kan*), STL11900 (*iraD*Δ::*tetA rssB*Δ::*tetA hsdR*Δ::FRT *kan*), STL11901(*iraD*Δ::*tetA rssB*Δ::*tetA hsdR*Δ::FRT *kan*), STL12798 (*recA*Δ::*cat iraD*Δ::*tetA*), and STL12803 (*malF*3180::Tn*10 kan lexA3 iraD*Δ::*tetA*).

Plasmids and Plasmid Constructions. All plasmids in this study were constructed using Gateway Cloning Technology (Invitrogen) from PCR products amplified using MG1655 chromosomal template DNA (Masterpure DNA purification kit; Epicentre) and *Pfu* Turbo (Stratagene) or Phusion polymerase (Finnzymes) and were recovered by transformation into *E. coli* K-12 strain DH5α. After purification (PCR purification kit; Qiagen), sequences were inserted into Gateway high-copy vector pDONR201, conferring kanamycin resistance, by the BP reaction (Invitrogen) and by LR reaction into destination vectors pSTL358 (38) and pDEW201::GW (34), respectively, to generate low-copy complementation plasmids and *lux* reporters. Plasmid pDONR-P-*iraD* carrying *iraD* ORF and its upstream region were constructed using the primers 5′ GGGGACAAGT TTG-TACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCGTAA ACAAATGACA TG-CATGTTTCT and 5′ GGGGACCACT TTGTACAAGA AAGCTGGGTC TTAGCT-GACA TTCTCCAGCG TCGCACTGCG. The control plasmid in the complementation analysis, pDONR-P, was constructed using the primers 5′-GGGGACAAGT TTGTACAAAA AAGCAGGCTTC GAAGGAGATA GAACCGTAAA CAAATGACAT GCATGTTTCT and 5′-GGGGACCACT TTGTACAAGA AAGCT-GGGTC TTTGCGCACT CCTGACGTTT AGCAA. Low-copy versions of these plasmids were constructed by LR reaction into the pSC101-derived low-copy vector, pSTL358, conferring ampicillin resistance. For luciferase assays, the 600-bp *iraD* upstream region was inserted upstream of the *Photorhabdus luminescence luxCDABE* operon in the pDEW201::GW derivative of *lux* reporter vector pDEW201 (18).

Survival Assays. Each experimental point was determined using at least 3 independent sets of data with a total of 8 or more isolates per strain. For hydrogen peroxide, cultures were grown with aeration to OD₆₀₀ 0.2–0.3 in LB medium and split. Hydrogen peroxide (Fisher Scientific) was added to 5 mM for 20 min or to 12.5 mM for 15 min; control cultures were incubated untreated. Treatment was terminated with catalase (Sigma–Aldrich, from *Aspergillus niger*, 180 ng/mL), and cell survival was determined by serial dilution and plating. Plating efficiency assays in the presence of AZT were done as described (34); phleomycin (Invitrogen) treatment was performed similarly with doses of 0.5–0.65 μg/mL.

Western Blot Analyses for Determination of RpoS Levels. Exponential-phase cultures in LB (OD of 0.2–0.3) were treated or not treated with 5 mM hydrogen peroxide as described above. Protein was precipitated in 20% wt/vol ice-cold trichloroacetic acid, and the pellet was resuspended in SDS buffer. Equal amounts of all samples (normalized to culture OD) were resolved on a 15% wt/vol polyacrylamide gel, transferred onto a nitrocellulose membrane, and developed by using antibodies to RpoS (mouse monoclonal; Neoclone), DnaJ (mouse monoclonal; Stressgen Biotechnologies), and anti-mouse IgG horseradish peroxidase–linked antibody (Amersham) and the ECL detection system (Pierce).

The stability of RpoS was determined after addition of chloramphenicol (200 μg/mL) to exponential-phase LB cultures harvested at the time points indicated and Western blot analyses as described previously.

Lux Reporter Assays. Luciferase reporter assays were performed as described (19). After transformation into MG1655, *lexA3*, or *recA*::*cat* strains, the promoter assays were performed with exponentially growing cultures (OD₆₀₀ 0.2–0.4) exposed to 20 μg/mL phleomycin, 25 mg/L hydrogen peroxide, 1 μg/mL AZT, or 1.5 μg/mL mitomycin C for 40 min. Arbitrary luciferase expression values are the amount of bioluminescence (cpm) divided by optical density of the culture at 600 nm.

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MICROBIOLOGY

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