

The Small RNA GcvB Promotes Mutagenic Break Repair by Opposing the Membrane Stress Response

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ABSTRACT

Microbes and human cells possess mechanisms of mutagenesis activated by stress responses. Stress-inducible mutagenesis mechanisms may provide important models for mutagenesis that drives host-pathogen interactions, antibiotic resistance, and possibly much of evolution generally. In *Escherichia coli*, repair of DNA double-strand breaks is switched to a mutagenic mode, using error-prone DNA polymerases, via the SOS DNA damage and general (σ^S) stress responses. We investigated small RNA (sRNA) clients of Hfq, an RNA chaperone that promotes mutagenic break repair (MBR), and found that GcvB promotes MBR by allowing a robust σ^S response, achieved via opposing the membrane stress (σ^E) response. Cells that lack *gcvB* were MBR deficient and displayed reduced σ^S -dependent transcription but not reduced σ^S protein levels. The defects in MBR and σ^S -dependent transcription in $\Delta gcvB$ cells were alleviated by artificially increasing σ^S levels, implying that GcvB promotes mutagenesis by allowing a normal σ^S response. $\Delta gcvB$ cells were highly induced for the σ^E response, and blocking σ^E response induction restored both mutagenesis and σ^S -promoted transcription. We suggest that GcvB may promote the σ^S response and mutagenesis indirectly, by promoting membrane integrity, which keeps σ^E levels lower. At high levels, σ^E might outcompete σ^S for binding RNA polymerase and so reduce the σ^S response and mutagenesis. The data show the delicate balance of stress response modulation of mutagenesis.

IMPORTANCE

Mutagenesis mechanisms upregulated by stress responses promote *de novo* antibiotic resistance and cross-resistance in bacteria, antifungal drug resistance in yeasts, and genome instability in cancer cells under hypoxic stress. This paper describes the role of a small RNA (sRNA) in promoting a stress-inducible-mutagenesis mechanism, mutagenic DNA break repair in *Escherichia coli*. The roles of many sRNAs in *E. coli* remain unknown. This study shows that $\Delta gcvB$ cells, which lack the GcvB sRNA, display a hyperactivated membrane stress response and reduced general stress response, possibly because of sigma factor competition for RNA polymerase. This results in a mutagenic break repair defect. The data illuminate a function of GcvB sRNA in opposing the membrane stress response, and thus indirectly upregulating mutagenesis.

Bacterial (1–7), yeast (8), and human cancer (9, 10) cells possess mechanisms of mutagenesis upregulated by stress responses. Stress-inducible mutation mechanisms may accelerate adaptation specifically when cells are poorly adapted to their environments, i.e., when stressed. Modeling studies indicate that stress-inducible mutagenesis can be selected on the basis of acceleration of adaptation even in asexual bacterial populations, in which deleterious mutations generated cannot be purged by recombination (11, 12). Stress-inducible mutation mechanisms drive evolution of antibiotic resistance (13–15) and cross-resistance (16), antifungal drug resistance (8, 17) and possibly much of evolution generally.

In *Escherichia coli*, repair of DNA double-strand breaks (DSBs) by homologous recombination is switched to a mutagenic mode using error-prone DNA polymerases under the control of the SOS DNA damage response and the general stress response (1, 2, 6, 18–20). Mutagenic break repair (MBR) requires proteins that perform DSB repair via homologous recombination (21–23), low-fidelity DNA polymerases (Pols) IV (18, 19, 24), V (19, 25), and II (26), and the activators of the general/starvation stress response (RpoS/ σ^S) (18, 19, 27, 28), the SOS DNA damage response (29, 30), and the RpoE/ σ^E membrane stress response (31). The σ^E response drives mutagenesis by promoting spontaneous DNA breakage (31) at some genomic locations, as do RNA/DNA hy-

brids (R-loops) caused by transcription (32). The SOS response promotes mutagenesis via its 10-fold transcriptional upregulation of Pol IV (33) and by allowing production of Pol V. The general stress response is activated by the σ^S transcriptional activator, a sigma factor of RNA polymerase, in response to starvation, antibiotics (16), and many other stresses (34, 35). The general stress response directly and indirectly up- and downregulates transcription of more than 500 *E. coli* genes (34, 35) and promotes mu-

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tagenesis by allowing the use of, or errors made by, the error-prone DNA Pols in DSB repair, by an as-yet-unknown mechanism (18, 19, 26). Thus, even in cells with a DSB, an activated SOS response and the resulting 10-fold-higher levels of Pol IV, DSB repair remains relatively nonmutagenic, using high-fidelity DNA Pol III (36), unless the general stress response is also activated either by stress or artificially (18, 19, 26). That is, σ^S -inducing stress is not itself needed for mutagenesis during DSB repair; artificial activation of the σ^S response is sufficient to make repair mutagenic even in growing cells (18, 19). The MBR mechanism, in which DNA Pol IV initiates mutagenic DNA synthesis from a D-loop (intermediate in recombinational repair), has been recapitulated in solution with purified proteins (37). Further, the mutation signatures of σ^S -promoted mutagenesis are overrepresented in extant bacterial genomes, suggesting that MBR is widespread in bacterial mutagenesis in the wild (38).

Mutagenic break repair in *E. coli* is promoted by a large network of more than 93 genes, mutations in any of which decrease mutagenesis (39). More than half of MBR network genes promote mutagenesis by sensing stress and transducing signals that lead to activation of the σ^S , SOS, and/or σ^E stress responses (39), indicating the importance of stress response control of mutagenesis to *E. coli*. Among the genes discovered in this screen for MBR-defective mutants is *hfq*, which encodes the Hfq RNA chaperone (39). Hfq is required for MBR in *E. coli* (39).

Hfq was discovered as a bacterial host factor required for synthesis of bacteriophage Q_B RNA (40) and is part of the conserved family of Sm-like RNA-modulating proteins found in eukaryotes, archaea, and eubacteria (41). Hfq is required for virulence of several bacterial species (42–49). Acting as an RNA chaperone, Hfq facilitates base pairing of a collection of small RNAs (sRNAs) to specific mRNA molecules, which allows the sRNAs to up- or downregulate translation from the mRNAs (50, 51). sRNAs are approximately 100 bp long and downregulate translation of some mRNAs by base pairing that blocks ribosome-binding sites (52). sRNAs also upregulate translation by melting mRNA secondary structures such as hairpins that would otherwise prevent ribosome recognition (53). Several sRNAs are upregulated during stress, including DsrA and RprA, both of which promote translation of the *rpoS* mRNA to σ^S protein (54). Of the approximately 100 sRNAs known in *E. coli* (55–58), 30 sRNAs require Hfq to function (59). Although the means by which Hfq promotes MBR is unknown, the fact that it does so suggests that one or more of the Hfq client sRNAs may promote mutagenesis. In this study, we examined nine sRNA clients of Hfq that are not encoded within protein-coding genes and that showed expression patterns potentially relevant to starvation stress (59). We report below that cells that lack the GcvB sRNA are MBR defective.

Found in diverse bacteria, GcvB is an Hfq-chaperoned sRNA that up- or downregulates translation of amino acid biosynthesis and transport proteins (60–63). In *Salmonella enterica*, GcvB is a master regulator of amino acid metabolism and directly up- or downregulates translation of ~1% of all mRNAs (64). GcvB regulates a network of mRNAs by inhibiting or enabling translation based on cellular environment. *E. coli* Δ *gcvB* mutant cells are acid sensitive, possibly caused partly by reduced σ^S levels, shown with a σ^S -LacZ fusion protein (65).

Many sRNAs in *E. coli* promote membrane integrity and do so by regulating outer membrane protein genes (66–68). The levels of various sRNAs are increased under different stresses (56, 69),

and many are upregulated by the σ^E membrane stress response (70–72). sRNAs, such as MicA, RybB, and MicL, are induced by cell envelope stress, and then they downregulate translation of outer membrane porins and lipoproteins, aiding membrane integrity (73–77). Transcription of *rpoE* and σ^E -dependent promoter use is increased in an *hfq* mutant, supporting the roles of sRNAs in averting the σ^E response and promoting membrane integrity (78). Here, we show that the GcvB sRNA is required for MBR. We find that GcvB promotes MBR by allowing a robust σ^S response. We report that Δ *gcvB* mutant cells display reduced σ^S -regulated promoter activity and MBR but not reduced σ^S protein levels. Artificial upregulation of σ^S restored σ^S -regulated promoter activity and MBR, implying that normal quantities of σ^S are insufficient to activate the general stress response in Δ *gcvB* cells. We provide evidence that the MBR and σ^S response deficiency in Δ *gcvB* cells result from hyperactivation of the σ^E membrane stress response. We suggest that GcvB may promote the σ^S response, and so also MBR, indirectly by keeping membrane stress low enough for σ^S to compete successfully with σ^E for RNA polymerase. The data illuminate a function of GcvB in opposing the membrane stress response, and thus indirectly upregulating mutagenesis.

MATERIALS AND METHODS

Strains and materials. *E. coli* K-12 strains and plasmids used are shown in Table 1. Strains were constructed using phage lambda Red-mediated recombineering as described previously (79) and phage P1-mediated transduction as described previously (80). M9 minimal medium (80) had carbon sources added at 0.1% and vitamin B₁ (B1) at 10 μ g/ml. LBH medium was as described previously (81). Antibiotics and other additives were used at the following final concentrations: ampicillin, 100 μ g/ml; chloramphenicol, 25 μ g/ml; kanamycin, 50 μ g/ml; tetracycline (Tet), 10 μ g/ml; rifampin, 100 μ g/ml; and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), 40 μ g/ml.

Each of nine nonpolar deletions of sRNA genes was constructed by recombineering using pKD3 as the PCR template (79). The nucleotides deleted for each new deletion allele are shown in Table S1 in the supplemental material.

Quantitative Lac mutagenesis assays with spontaneous DSBs. The Lac assay (29) for stress-inducible MBR measures reversion of an F'-borne *lacI-Z* gene with a +1-bp frameshift allele during starvation stress, and the assay was performed as described previously (23). Viable Lac⁻ starving cells on the lactose-containing plates were measured daily throughout the experiments as described previously (29) and varied less than 2-fold during the days of the experiments reported. Lac⁺ revertant CFU (indel mutants) are counted to day 5. The mutation rates (Lac⁺ CFU per 10⁸ CFU per day) shown are means \pm standard errors of the means (SEM) from four separate experiments with four independent cultures for each strain and were calculated as described previously (28) by subtracting the number of colonies counted on day 3 from the number of colonies counted on day 5 and dividing by 2.

Chromosomal Tet reversion assay with I-SceI-induced DSBs in plasmid-free cells. The Tet reversion assay of Shee et al. (19, 20, 39) was performed as described previously. A chromosomally encoded arabinose-inducible, glucose-repressible I-SceI endonuclease, produced weakly by leaky expression, cleaves a chromosomal I-SceI cut site (I-site) near a *tet* mutation reporter gene in liquid-starved plasmid-free cells (19). The chromosomal *tet* gene with a +1-bp frameshift allele resides 8.5 kb from the I-site (I-site A, *tet2*) (19). Cells are grown for 12 h to saturation in liquid, starved for 72 further hours, then rescued from starvation, plated on LBH solid medium containing glucose and tetracycline (LBH glucose tetracycline solid medium), and incubated at 37°C to select *tet*⁺ revertant tetracycline-resistant (Tet^r) colonies, which are counted the following

TABLE 1 *Escherichia coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference(s), source, or construction
Strains		
CAG45114	MG1655 (λ <i>rpoHP3-lacZ</i>)	106
ENZ280	Δ (<i>srlR-recA</i>)306::Tn10 [mini-F <i>recA</i> ⁺]	107, 108
FC29	Δ (<i>lac-proB</i>) _{XIII} <i>thi ara</i> [F'128 <i>proAB</i> ⁺ <i>lacI</i> ^q]	29
FC40	Δ (<i>lac-proB</i>) _{XIII} <i>thi ara</i> Rif ^r [F'128 <i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacI33-lacZ</i>]	29
JW3677	BW25113 Δ <i>recF</i> ::FRT-Kan ^r -FRT	109
JW5437	BW25113 Δ <i>rpoS</i> ::FRT-Kan ^r -FRT	109
SP874	MC4100 Δ <i>rpoE</i> :: <i>cat</i>	T. Silhavy (Princeton)
SMR820	FC40 <i>lexA3</i> (Ind ⁻)	24
SMR3856	SMR4562 Lac ⁺ day 5	86
SMR4562	Independent construction of FC40	30
SMR5236	SMR4562 <i>rpoE2072</i> ::Tn10dCam	31
SMR5535	SMR4562 Δ <i>recA</i>	SMR4562 \times P1 (ENZ280)
SMR5833	SMR4562(pKD46)	SMR4562 \times pKD46
SMR8842	CAG45114 <i>rpoE2072</i> ::Tn10dCam	CAG45114 \times P1 (SMR5236)
SMR10336	SMR4562 Δ <i>rpoS</i> ::FRT-Kan ^r -FRT	SMR4562 \times P1 (JW5437)
SMR10582	SMR4562 <i>viaG-yfp</i> FRT- <i>cat</i> -FRT	39
SMR10777	SMR4562 Δ <i>zie3920.5</i> ::3Chi-Kan-I-SceI cut site	19
SMR10808	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT	19
SMR10823	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT <i>rpoE2072</i> ::Tn10dCam	SMR10808 \times P1 (SMR5236)
SMR10832	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT Δ <i>rpoS</i> ::FRT	19
SMR10854	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT Δ <i>zie3920.5</i> ::3Chi-Kan-I-SceI cut site <i>rpoE2072</i> ::Tn10dCam	SMR10823 \times P1 (SMR10777)
SMR10862	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT Δ <i>zie3920.5</i> ::3Chi-Kan-I-SceI cut site Δ <i>rpoS</i> ::FRT	19
SMR10866	FC36 Δ <i>araBAD567</i> Δ <i>attλ</i> ::P _{BAD} I-SceI <i>tet2</i> FRT Δ <i>zie3920.5</i> ::3Chi-Kan-I-SceI cut site	19
SMR12566	SMR4562 Δ <i>rssB</i> ::Tet ^r	39
SMR12661	SMR4562 Δ <i>rpoS746</i> ::FRT-Kan ^r -FRT <i>viaG-yfp</i> FRT- <i>cat</i> -FRT	39
SMR12692	SMR4562 Δ <i>rssB</i> ::Tet ^r <i>viaG-yfp</i> FRT- <i>cat</i> -FRT	39
SMR12848	SMR4562 <i>viaG-yfp</i> FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR10582)
SMR13014	SMR4562 Δ <i>rpoS</i> ::FRT	SMR10336 \times pCP20
SMR13096	SMR4562 <i>viaG-yfp</i> FRT	39
SMR17962	MG1655 Δ <i>attλ</i> ::P _{<i>sulA</i>} <i>mCherry</i> FRT- <i>cat</i> -FRT	83
SMR17966	MG1655 Δ <i>attλ</i> ::P _{<i>sulA</i>} <i>mCherry</i> FRT <i>lexA3</i> (Ind ⁻) <i>malB</i> ::Tn9	83
SMR20177	SMR5833 Δ <i>oxyS</i> ::FRT- <i>cat</i> -FRT	This work
SMR20181	SMR5833 Δ <i>rprA</i> ::FRT- <i>cat</i> -FRT	This work
SMR20183	SMR5833 Δ <i>dsrA</i> ::FRT- <i>cat</i> -FRT	This work
SMR20185	SMR5833 Δ <i>rybB</i> ::FRT- <i>cat</i> -FRT	This work
SMR20201	SMR5833 Δ <i>micF</i> ::FRT- <i>cat</i> -FRT	This work
SMR20203	SMR5833 Δ <i>spf</i> ::FRT- <i>cat</i> -FRT	This work
SMR20205	SMR5833 Δ <i>ryhB</i> ::FRT- <i>cat</i> -FRT	This work
SMR20207	SMR5833 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT	This work
SMR20219	SMR4562 Δ <i>oxyS</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20177)
SMR20220	SMR4562 Δ <i>dsrA</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20181)
SMR20221	SMR4562 Δ <i>rybB</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20185)
SMR20230	SMR4562 Δ <i>rprA</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20181)
SMR20232	SMR4562 Δ <i>micF</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20201)
SMR20234	SMR4562 Δ <i>spf</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20203)
SMR20236	SMR4562 Δ <i>ryhB</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20205)
SMR20238	SMR4562 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20207)
SMR20290	SMR4562 Δ <i>cyaR</i> ::FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR20179)
SMR21332	SMR3856 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT	SMR3856 \times P1 (SMR20207)
SMR21361	SMR4562 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT Δ <i>rssB</i> ::Tet ^r	SMR12566 \times P1 (SMR20207)
SMR21448	SMR4562 Δ <i>gcvB</i> ::FRT	SMR20238 \times pCP20
SMR21450	SMR4562 Δ <i>micF</i> ::FRT	SMR20232 \times pCP20
SMR21467	SMR4562 Δ <i>rybB</i> ::FRT	SMR20221 \times pCP20
SMR21471	SMR4562 Δ <i>gcvB</i> ::FRT <i>viaG-yfp</i> FRT- <i>cat</i> -FRT	SMR21448 \times P1 (SMR12848)
SMR21553	SMR21641 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT	SMR21641 \times P1 (SMR20207)
SMR21633	SMR10866 Δ <i>gcvB</i> ::FRT- <i>cat</i> -FRT	SMR10866 \times P1 (SMR20207)
SMR21641	SMR4562 Δ <i>attλ</i> ::P _{<i>sulA</i>} <i>mCherry</i> FRT- <i>cat</i> -FRT	SMR4562 \times P1 (SMR17962)
SMR21725	SMR4562 Δ <i>rpoS</i> ::FRT Δ <i>attλ</i> ::P _{<i>sulA</i>} <i>mCherry</i> FRT- <i>cat</i> -FRT	SMR13014 \times P1 (SMR17962)

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Relevant genotype or description	Reference(s), source, or construction
SMR21728	SMR4562 $\Delta att\lambda::P_{sulA}mCherry$ FRT- <i>cat</i> -FRT $\Delta recF::FRT$ - <i>Kan</i> ^r -FRT	SMR21641 \times P1 (JW3677)
SMR21909	SMR4562 $\Delta rrsB::FRT$ $\Delta gcvB::FRT$	SMR21361 \times pCP20
SMR21933	SMR21553 $\Delta rrsB::FRT$	SMR21909 \times P1 (SMR17962)
SMR21934	SMR4562 $\Delta gcvB::FRT$ $\Delta rrsB::Tet^r$ <i>yiaG-yfp</i> FRT- <i>cat</i> -FRT	SMR21909 \times P1 (SMR12848)
SMR21996	SMR4562 $\Delta gcvB::FRT$ <i>rpoE2072::Tn10dCam</i>	SMR21448 \times P1 (SMR5296)
SMR21998	SMR4562 $\Delta gcvB::FRT$ <i>yiaG-yfp</i> FRT	SMR21471 \times pCP20
SMR22047	SMR4562 $\Delta gcvB::FRT$ <i>rpoE2072::Tn10dCam</i> <i>yiaG-yfp</i> FRT	SMR21998 \times P1 (SMR5236)
SMR22064	SMR4562 <i>yiaG-yfp</i> FRT <i>rpoE2072::Tn10dCam</i>	SMR13096 \times P1 (SMR5236)
SMR22066	SMR10866 $\Delta gcvB::FRT$	SMR21633 \times pCP20
SMR22074	SMR21633 <i>rpoE2072::Tn10dCam</i>	SMR22066 \times P1 (SMR5236)
SMR22216	CAG45114 $\Delta gcvB::FRT$ - <i>cat</i> -FRT	CAG45114 \times P1 (SMR20207)
SMR22296	CAG45114 $\Delta gcvB::FRT$	SMR22216 \times pCP20
SMR22310	SMR22296 <i>rpoE2072::Tn10dCam</i>	SMR22296 \times P1 (SMR5296)
SMR22549	SMR4562 $\Delta dsrA::FRT$	SMR20220 \times pCP20
SMR22551	SMR4562 $\Delta rprA::FRT$	SMR20230 \times pCP20
SMR22554	SMR4562 $\Delta dsrA::FRT$ - <i>cat</i> -FRT $\Delta rprA::FRT$	SMR22551 \times P1 (SMR20220)
SMR22556	SMR4562 $\Delta cyaR::FRT$	SMR20290 \times pCP20
SMR22558	SMR4562 $\Delta oxyS::FRT$	SMR20219 \times pCP20
SMR22560	SMR4562 $\Delta rylB::FRT$	SMR20236 \times pCP20
SMR22562	SMR4562 $\Delta spf::FRT$	SMR20234 \times pCP20
SMR22936	SMR3856 $\Delta rprA::FRT$ - <i>cat</i> -FRT	SMR3856 \times P1 (SMR20181)
SMR22940	SMR10866 $\Delta rprA::FRT$ - <i>cat</i> -FRT	SMR10808 \times P1 (SMR20181)
SMR22950	SMR3856 $\Delta rprA::FRT$	SMR22936 \times pCP20
SMR22954	SMR10866 $\Delta rprA::FRT$	SMR22940 \times pCP20
SMR22960	SMR3856 $\Delta rprA::FRT$ $\Delta dsrA::FRT$ - <i>cat</i> -FRT	SMR22950 \times P1 (SMR20183)
SMR22964	SMR10866 $\Delta rprA::FRT$ $\Delta dsrA::FRT$ - <i>cat</i> -FRT	SMR22954 \times P1 (SMR20183)
Plasmids		
pCP20	Temperature-inducible yeast F1p recombinase gene controlled by λ Cts857 in a temperature-sensitive replicon	110
pKD3	Source of FRT- <i>cat</i> -FRT	79
pKD46	<i>ori101 repA101ts P_{BAD}gam-bet-exo Amp^r</i>	79

day, as are the total viable CFU assayed on medium without tetracycline. Mutant frequencies are the titers of Tet^r mutant CFU per milliliter on LBH glucose tetracycline medium divided by those of total CFU/ml from LBH glucose plates. Data presented are the values (means \pm SEM) from 10 independent experiments with three cultures for each strain.

Flow cytometric assays for σ^S and SOS response-regulated promoter activity. σ^S and SOS response activation for σ^S activity were quantified by flow cytometry as described previously (39) for σ^S . The method of Pennington and Rosenberg (82) as modified by Nehring et al. (83) was used for SOS. The chromosomal *yiaG-yfp* σ^S response reporter gene (39) and $\Delta att\lambda::P_{sulA}mCherry$ SOS reporter gene (83) were used in separate cells. Strains were grown at 37°C for 48 h with aeration in liquid M9 medium containing vitamin B₁ and glycerol (liquid M9 B1 glycerol). σ^S and SOS response-dependent promoter activity was quantified in two ways. First, for the SOS response, in which only a small subpopulation of growing cells is induced spontaneously relative to negative-control, SOS-off mutant cells (82), we set gates by the method of Pennington and Rosenberg (82). Gates were calibrated using negative-control SOS-off *lexA*(Ind⁻) cells, and SOS response “on” was scored as the fluorescence intensity shown by the most fluorescent 1% of events observed in wild-type cultures. Cells that fell below this gate (less fluorescence) were scored as negative. The values (percent positive cells; means \pm SEM) from five independent SOS activity experiments with three independent cultures for each strain are given. Second, for both SOS and σ^S responses, we report the mean fluorescence intensity per cell, a measure more useful for responses and mutants in which cells display a unimodal distribution of fluorescence intensities, and a majority or all of the cells in the population of mutants examined are shifted relative to the wild-type strain or the negative-control strains.

Western blot analyses of σ^S and σ^E protein levels. Western blot analyses for quantification of σ^S and σ^E protein levels in stationary cultures were performed by the methods of Galhardo et al. (33) and Gibson et al. (31), respectively. The optical density at 600 nm (OD₆₀₀) was taken of 5-ml samples from 48-h cultures grown in M9 B1 glycerol, and the concentrations were adjusted to standardize the different strains. The cells were pelleted and resuspended in 1 ml of lysis buffer/loading sample (62.5 mM Tris [pH 6.8], 25% glycerol, 2% SDS, 0.01% bromophenol blue, 0.5% β -mercaptoethanol) and boiled. Fifteen-microliter portions of each sample were electrophoresed on 13% SDS-polyacrylamide gels, and the proteins were transferred to Hybond-LFP polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked with 2% blocking buffer and probed with 1:700 dilution of polyclonal mouse anti- σ^S antibody (Neoclone) (84) or 1:5,000 dilution of polyclonal rabbit anti- σ^E antibody (85) (gift of Carol Gross, University of California at San Francisco [UCSF]). Goat anti-mouse and anti-rabbit secondary antibodies conjugated to Cy5 fluorescent dye (Amersham Biosciences) were used at a 1:5,000 dilution to detect σ^S and σ^E proteins, respectively. Fluorescence was assessed on a Typhoon scanner with a photomultiplier voltage (PMT) of 500 and a 670-nm bandpass (670BP) 30Cy5 emission filter, and the bands were quantified using ImageJ software (NIH). Quantifications from four separate Western blots for σ^S and σ^E are reported, each with band intensities normalized to isogenic wild-type control strain SMR4562 and means \pm 1 SEM are shown.

Reconstruction experiments. Reconstruction experiments were used to demonstrate that Lac⁺ $\Delta gcvB$ and Lac⁺ $\Delta dsrA \Delta rprA$ cells form colonies normally under selective assay conditions in the presence of neighbor cells, using assays described previously (86) as reviewed in reference 87, such that their defect in producing Lac⁺ mutant colonies reflects reduced

mutagenesis, not impaired colony formation. We quantified the timing of colony appearance and fraction of known numbers of $\Delta gcvB$ and $\Delta dsrA \Delta rprA$ CFU that formed colonies under precise reconstructions of experimental conditions and compared these with those of isogenic nonmutant strains.

UV light sensitivity assays. Saturated liquid M9 B1 glycerol cultures were starved as in the Lac mutagenesis assays described above, diluted, plated at various concentrations onto LBH solid medium, and exposed to various doses of UVC light in a Stratalinker (Artisan Technology Group). CFU titers were quantified and graphed. Data are normalized to viable-cell titers with no UVC irradiation. *lexA*(Ind⁻) mutant cells, which produce an uncleavable LexA transcriptional repressor of the SOS genes, and so are SOS response defective, were used as a positive control for SOS response deficiency. *ΔrecA* cells have stronger UV sensitivity and were also used as a positive control.

Semiquantitative SDS-EDTA sensitivity assay for σ^E response deficiency. σ^E response-defective cells are sensitive to SDS-EDTA, which disrupts the membrane (88). Strains grown to saturation and starved, per Lac mutagenesis experiments, were diluted, and 10- μ l spots containing ~30 and ~300 CFU deposited onto solid M9 B1 glycerol medium with and without 0.01% SDS and 0.25 mM EDTA, incubated for 48 h at 30°C (the permissive temperature for a $\Delta rpoE$ control strain [89]) and scored. We used control isogenic cells carrying the *rpoE2072::Tn10dCam* separation-of-function allele, which confers σ^E response deficiency but maintains the essential function of σ^E (31), and are SDS-EDTA sensitive (31). Although *rpoE* is an essential gene (89), the $\Delta rpoE$ mutant is viable because of acquisition of compensatory extragenic “suppressor” mutations that permit viability (90).

Catalase colony assays for σ^S response activity. Catalase colony assays for σ^S response activity were performed as described previously (91). Wild-type control, isogenic $\Delta gcvB$, and $\Delta rpoS$ strains were grown into colonies on M9 B1 glycerol medium for 48 h at 37°C. Three microliters of 30% hydrogen peroxide was dropped onto each colony, and the time elapsed before bubbles appeared was measured. Six colonies were tested in four independent experiments, and the times to bubbling (means \pm SEM) (in seconds) were reported. *P* values compared with the values for wild-type colonies were determined using two-tailed Student's *t* test.

β -Galactosidase assay for σ^E activity. Because the *rpoHP3* promoter is σ^E dependent, the *rpoHP3-lacZ* fusion gene is a reporter for σ^E -dependent transcription (92), which is measured as β -galactosidase activity in liquid cultures. β -Galactosidase assays of saturated M9 B1 glycerol cultures were performed as described previously (31). The values (means \pm SEM) from three experiments and four independent cultures for each strain are reported.

Acid sensitivity assays. Acid sensitivity assays were performed as described previously (65). Saturated overnight cultures of wild-type control and isogenic $\Delta gcvB$, *rpoE2072::Tn10dCam* (*rpoE::Tn*), and $\Delta gcvB rpoE::Tn$ cells were diluted 1:50 in LB medium and grown at 37°C with aeration for 5 h. Acid challenge was performed by adding 3 volumes of acidified LB medium (pH 1.9) to cultures, resulting in a final pH of 2.0. Cells were grown in acidified liquid culture for 30 min. The challenge was interrupted with the addition of 3 volumes of alkalized LB medium (pH 9.3), resulting in a final pH of 7.0. The optical density at 600 nm was taken after 3 h of recovery. The values (means \pm SEM) for three experiments containing three independent cultures per strain are reported.

RESULTS

MBR deficiency caused by deletion of Hfq client genes *gcvB* and *dsrA* or *rprA*. We deleted nine genes that encode sRNA clients of Hfq (see Table S1 in the supplemental material), genes that are not embedded in another gene and that showed expression patterns potentially relevant to starvation stress (59). We assayed the deletion mutants for MBR proficiency/deficiency using the Lac MBR assay (29). The Lac assay quantifies reversion via MBR of a conjugative-plasmid-borne *lac* gene with a +1-bp frameshift allele dur-

ing prolonged starvation for days on minimal lactose solid medium (1–3, 6, 7). Colonies visible on day 2 are roughly 50% preexisting generation-dependent Lac⁺ reversion mutants, and colonies from day 3 onward are DSB-, DinB/Pol IV-, SOS-, σ^S -dependent, MBR-generated revertants. Δhfq cells show a strong 16-fold \pm 2-fold deficiency in MBR in the Lac assay (39). We found that $\Delta gcvB$ cells showed a significant 12-fold \pm 2-fold defect in Lac⁺ MBR revertant accumulation (Fig. 1A and B, mean \pm SEM mutation rate compared with the WT), a defect smaller than that of the Δhfq mutant. The double mutant $\Delta gcvB \Delta hfq$ was inviable and could not be tested. In addition, the $\Delta dsrA \Delta rprA$ double mutant showed a smaller but significant 2-fold \pm 0.4-fold reduction in the accumulation of Lac⁺ revertants (Fig. 1A and B). Because neither the $\Delta dsrA$ nor $\Delta rprA$ single mutant showed reduction, the data imply that either DsrA or RprA can function in MBR (they are redundant functions), such that loss of neither sRNA singly reduces accumulation of revertants. Both sRNAs promote translation of σ^S (54). Here, we follow up the role of sRNA GcvB.

GcvB and RprA or DsrA are required for mutagenesis, not mutant colony formation. We show that the failure of $\Delta gcvB$ cells to produce Lac⁺ revertant colonies (Fig. 1A and B) is not merely the inability of Lac⁺ revertants carrying a $\Delta gcvB$ mutation to form colonies under experimental conditions. We performed reconstruction experiments in which a functional Lac⁺ allele is moved into $\Delta gcvB$ cells, and their efficiency and speed of colony formation under precise reconstructions of experimental conditions are measured (~100 Lac⁺ $\Delta gcvB$ cells mixed with ~10⁹ Δlac nonrevertible neighbor cells on selective plates). The data show that $\Delta gcvB$ cells form colonies normally and do not have decreased viability or growth rate under experimental conditions (Fig. 1C and D). Similar reconstruction experiments showed that $\Delta dsrA \Delta rprA$ mutant cells also do not have decreased ability to form colonies under experimental conditions (see Fig. S1 in the supplemental material). We conclude that GcvB is required for mutagenesis, not outgrowth of mutant cells into colonies, as are DsrA or RprA.

GcvB and either DsrA or RprA are required for MBR in the chromosomal Tet assay. We confirmed the MBR deficiency of $\Delta gcvB$ and $\Delta dsrA \Delta rprA$ cells using the chromosomal Tet MBR assay (19) in which a chromosomal *tet* gene in plasmid-free cells reverts by indel mutation during prolonged starvation in liquid and then the cells are rescued from starvation and selected for tetracycline-resistant (Tet^r) mutant CFU. A chromosomally encoded I-SceI endonuclease is weakly induced and cleaves an I-SceI cut site near the *tet* gene, provoking repair, presumably with a sister chromosome (present in ~40% of stationary-phase *E. coli* [93]). The *tet* reversions that result are dependent on DSBs, DSB repair protein, SOS, DinB, and σ^S (19), all as observed in the Lac assay (18). We found that GcvB and either DsrA or RprA promoted a significant 69% \pm 11% and 43% \pm 9% of MBR, respectively, in the chromosomal Tet assay (Fig. 1E). We conclude that GcvB promotes much of stress-inducible MBR in *E. coli*, generally.

GcvB promotes MBR other than or in addition to by promoting SOS, DSB repair, the σ^E response, or spontaneous DNA breakage. We tested $\Delta gcvB$ cells for possible defects in several known components of MBR reactions. Cells defective for homologous recombinational (HR) DSB repair or the SOS response both show sensitivity to UV light (94, 95) [Fig. 2A, *lexA*(Ind⁻) and

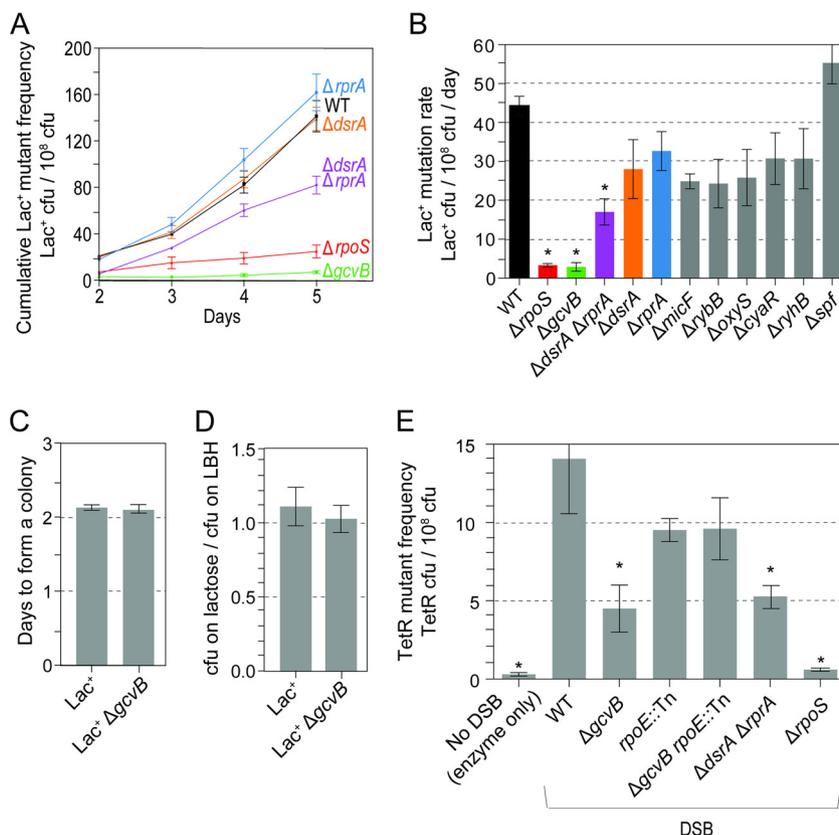


FIG 1 sRNA GcvB promotes mutagenic break repair in chromosomal and F'-based MBR assays. (A) Lac MBR assay. Lac⁺ CFU are revertants of a conjugative-plasmid-borne *lac* frameshift allele during starvation on solid medium. The results of a representative experiment are shown. (B) Quantification of Lac⁺ MBR mutation rates as described in Materials and Methods. The values are means \pm standard errors of the means (SEM) (error bars) from three experiments for each strain. Asterisks indicate values that are significantly different from the value for the isogenic wild-type (WT) control strain ($P = 0.0002$ for the $\Delta rpoS$ strain, $P = 0.0004$ for the $\Delta gcvB$ strain, and $P = 0.01$ for the $\Delta dsrA \Delta rprA$ strain; two-tailed Student's *t* test used in all comparisons here). From left to right, isogenic strains are SMR4562, SMR10336, SMR21448, SMR22554, SMR22549, SMR22551, SMR21450, SMR21467, SMR22558, SMR22556, SMR22560, and SMR22562. (C and D) Reconstruction experiments show that, when constructed, $\Delta gcvB$ mutant Lac⁺ cells are proficient at colony formation (Materials and Methods). (C) Normal speed of colony formation by $\Delta gcvB$ cells under MBR assay conditions. (D) Similar efficiencies of colony formation under selective conditions, compared with CFU on rich (LBH) medium without neighbor cells. (E) Chromosomal Tet MBR assay in plasmid-free cells. $\Delta gcvB$ cells display a MBR defect that is relieved by the *rpoE::Tn* separation-of-function mutation, which blocks the membrane stress response (31). Asterisks indicate values that are significantly different from the value for the isogenic wild-type (WT) control strain ($P = 0.04$ for the $\Delta gcvB$ strain, $P = 0.04$ for the $\Delta dsrA \Delta rprA$ strain, $P = 0.001$ for the $\Delta rpoS$ strain, and $P = 0.001$ for "No DSB" [I-SceI enzyme present with no cut site]). Values are means \pm SEM from seven experiments with positive controls. From left to right, the strains are SMR10808, SMR10866, SMR21633, SMR10854, SMR22074, SMR22964, and SMR10862. TetR, tetracycline resistant.

ΔrecA positive-control strains]. We found that $\Delta gcvB$ cells were as UV resistant as isogenic *gcvB*⁺ cells ("wild-type" [WT] cells in Fig. 2A), indicating that they have neither SOS nor HR defects. Two lines of evidence show that a defective σ^E membrane stress response does not underlie the MBR deficiency of $\Delta gcvB$ cells. First, cells with defects in the σ^E membrane stress response show sensitivity to SDS-EDTA, which disrupts the cell membrane (88). We found that $\Delta gcvB$ cells were as resistant to SDS-EDTA as the wild-type isogenic control (Fig. 2B). Second, σ^E promotes MBR in the Lac assay by promoting spontaneous DNA breakage (31); thus, σ^E is not required in the Tet assay in which DSBs are provided by I-SceI endonuclease (19, 39). Our finding that GcvB also promotes MBR in the Tet assay (Fig. 1E) indicates that GcvB promotes MBR other than or in addition to by allowing a σ^E response and other than or in addition to by promoting spontaneous DNA breakage. Because $\Delta gcvB$ cells showed a greater reduction of MBR in the Lac assay than in the Tet assay, it remains possible that GcvB plays two roles: one that affects spontaneous DNA breakage and another DSB-/ σ^E -independent role.

Decreased σ^S response but normal σ^S protein levels in cells that lack GcvB. We found that $\Delta gcvB$ cells display reduced activity of σ^S -upregulated promoters in two assays (Fig. 3). First, cells defective for the σ^S response have decreased *katE* transcription and thus decreased catalase activity and a defect in metabolizing hydrogen peroxide (H₂O₂) (96). When a drop of hydrogen peroxide is placed on an *E. coli* colony, H₂O₂ is metabolized to H₂O and O₂, and bubbles appear on the colony. The rapidity of onset of bubbling indicates σ^S -upregulated promoter activity (96). For wild-type and $\Delta rpoS$ strains, bubbles appeared after 2 ± 0.3 and 22 ± 1 s, respectively (Fig. 3C, plotted as 1/time to bubbling). $\Delta gcvB$ cells take 11 ± 1 s (mean \pm SEM) to produce bubbles, which is significantly different from the wild-type cells and not significantly different from the σ^S -null $\Delta rpoS$ strain data (Fig. 3C).

Second, we measured activity of the σ^S -upregulated *yiaG* promoter using flow cytometry of cells carrying a chromosomal *yiaG-yfp* reporter gene (39), which exploits the σ^S specificity of the *yiaG* promoter (Fig. 3A). We found that $\Delta gcvB$ cells showed a significant 1.6-fold \pm 0.08-fold decrease in mean yellow fluorescence

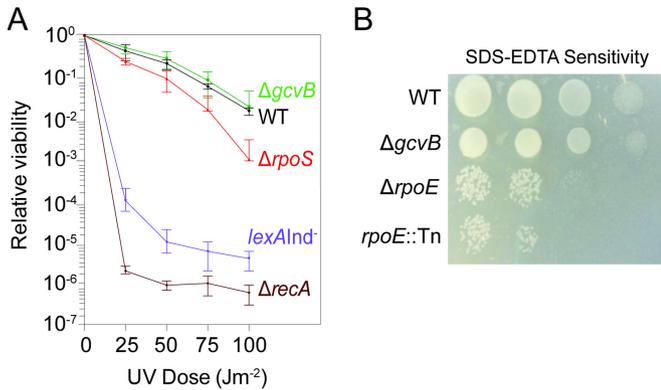


FIG 2 $\Delta gcvB$ cells show normal recombinational DNA repair and activation of the SOS and σ^E stress responses. (A) UV sensitivity assay. Values are means \pm SEM from three experiments with two cultures for each experiment. The UV resistance of $\Delta gcvB$ cells indicates homologous recombinational (HR) repair and SOS response proficiency. $\Delta recA$ HR- and SOS-defective cells and SOS-uninducible $lexA(Ind^-)$ cells are UV sensitive ($P < 0.02$ compared with the value for the WT; Student's two-tailed paired t test for each UV dose). The mild UV sensitivity of $rpoS$ -null cells was observed previously (105). The lack of UV sensitivity of $\Delta gcvB$ cells indicates that their σ^S response impairment (Fig. 3) is not as severe as in σ^S -null cells (Fig. 3B). From top to bottom, the strains are SMR20238, SMR4562, SMR10336, SMR820, and SMR5535. (B) $\Delta gcvB$ cells are SDS-EDTA resistant, indicating a functional σ^E response. Shown is a representative image of cultures spotted onto solid medium containing membrane-disrupting detergent (SDS) and EDTA at ~ 300 CFU per spot (left two spots) and ~ 30 CFU per spot (right two spots). SDS and EDTA retard growth of σ^E -response-defective $\Delta rpoE$ (88) and $rpoE::Tn10dCam$ (31) mutant cells. Although $rpoE$ is an essential gene (89), the $\Delta rpoE$ mutant is viable because of acquisition of compensatory extragenic "suppressor" mutations that permit viability (90). From top to bottom, the strains are SMR4562, SMR20238, MC4100, and SMR5236.

intensity (per cell) compared with the WT control (Fig. 3B). The decreased production of yellow fluorescent protein (YFP) from P_{yiaG} is not as great as in σ^S -null $\Delta rpoS$ cells (Fig. 3A and B), implying that σ^S -regulated promoter activity is reduced but not abolished in $\Delta gcvB$ cells. The reductions in expression of σ^S -upregulated genes in both assays were reversed by artificial upregulation of σ^S via deletion of $rssB$ (Fig. 3B and C). $RssB$ is a protein chaperone that brings σ^S to the ClpXP protease for degradation (97), such that its removal causes artificially high σ^S levels (97).

Although σ^S -upregulated promoter activity is reduced in $\Delta gcvB$ cells, we found that σ^S protein levels are not detectably reduced relative to isogenic $gcvB^+$ ("wild-type") cells. A representative Western blot is shown in Fig. 3D. Quantification from multiple Western blots shows that the relative levels of σ^S protein in $\Delta gcvB$ cells compared to wild-type cells are 1.1 ± 0.1 and 1.0 ± 0.1 , respectively (Fig. 3D, wild-type normalized to 1.0, mean \pm SEM from four experiments, the SEM on the WT is normalized proportionally to 1 to show the day-to-day variability in measurement). These data imply that GcvB does not regulate σ^S production or stability in stationary cells. We conclude that σ^S is present in $\Delta gcvB$ cells but that σ^S -upregulated promoter activity is decreased.

Mutagenesis and high spontaneous SOS induction in $\Delta gcvB$ cells are relieved by artificial upregulation of σ^S . We observed previously that $\Delta rpoS$ cells, which lack a functional σ^S response, display increased spontaneous induction of the SOS response (98) (Fig. 4A) for unknown reasons. Perhaps damaged cellular components and/or high levels of reactive oxygen

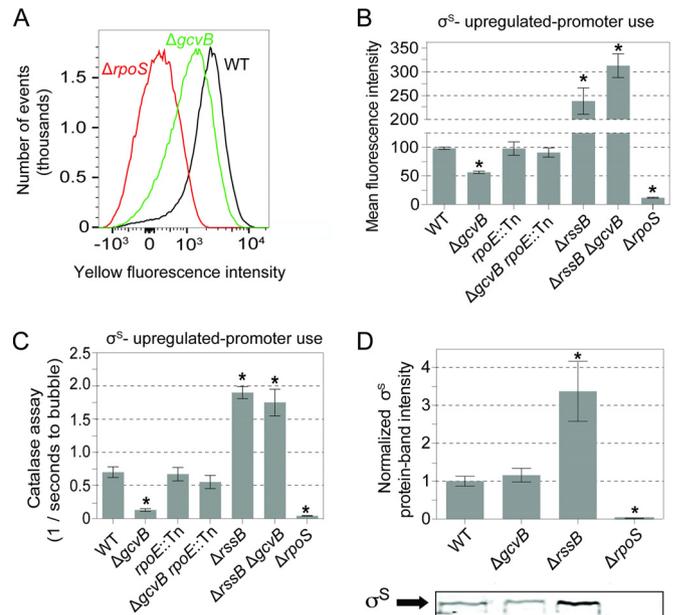


FIG 3 Reduced σ^S -upregulated transcription, but not σ^S protein levels, in stationary-phase $\Delta gcvB$ cells and its dependence on the σ^E response. In all experiments, measurements and assays are from stationary-phase cells, grown under experimental conditions as for mutagenesis experiments. (A) Reduced σ^S -regulated promoter activity in $\Delta gcvB$ cells. A flow cytometric fluorescence assay of stationary-phase starved cultures shows σ^S -dependent yellow fluorescence (in arbitrary fluorescence units) from the $yiaG$ - yfp σ^S response reporter gene (39). The results of a representative experiment are shown. (B) Quantification of mean fluorescence intensities per cell from five independent experiments. Fluorescence intensity is shown in arbitrary fluorescence units. Values are means \pm SEM. The $rpoE::Tn$ σ^E -response-defective mutation (31) restored σ^S response activity to $\Delta gcvB$ cells, indicating that the σ^S response reduction in $\Delta gcvB$ cells is σ^E response dependent. Asterisks indicate values that are significantly different from the value for the WT strain ($P = 1 \times 10^{-6}$ for the $\Delta gcvB$ strain, $P = 4 \times 10^{-8}$ for the $\Delta rssB$ strain, $P = 9 \times 10^{-8}$ for the $\Delta rssB \Delta gcvB$ strain, and $P = 2 \times 10^{-10}$ for the $\Delta rpoS$ strain) by Student's two-tailed t test. The value for the $\Delta gcvB rpoE::Tn$ double mutant is significantly different from the value for the $\Delta gcvB$ single mutant ($P = 4 \times 10^{-3}$). There is no significant difference between the values for the $\Delta rssB$ mutant and $\Delta rssB \Delta gcvB$ mutant ($P = 0.56$ by Student's two-tailed t test). From left to right, the strains are SMR10582, SMR21471, SMR22064, SMR22047, SMR12692, SMR21934, and SMR12661. (C) σ^E -response-dependent reduction of σ^S response activity in $\Delta gcvB$ cells by the catalase colony assay (Materials and Methods). Asterisks indicate values that are significantly different from the value for the WT strain ($P = 8 \times 10^{-5}$ for the $\Delta gcvB$ strain, $P = 4 \times 10^{-13}$ for the $\Delta rpoS$ strain, $P = 2 \times 10^{-4}$ for the $\Delta rssB$ strain, and $P = 0.01$ for the $\Delta rssB \Delta gcvB$ strain). Values are means \pm SEM from four experiments with five colonies per experiment. The strains are the same strains used in panel B. (D) Western blot analyses show σ^S protein levels in stationary phase unaffected by the $\Delta gcvB$ mutation. (Top) Results from three quantified immunoblots normalized to the WT value. Values are means \pm SEM. Asterisks indicate values that are significantly different from the value for the WT strain ($P = 1 \times 10^{-3}$ for the $\Delta rpoS$ strain and $P = 7 \times 10^{-4}$ for the $\Delta rssB$ strain). (Bottom) Representative immunoblot. From left to right, the strains are SMR4562, SMR20238, SMR12566, and SMR10336.

species, normally reduced by the σ^S response, cause DNA damage. We also found increased spontaneous SOS induction in $\Delta gcvB$ cells (Fig. 4A and B). We found that both the mutagenesis defect and the high spontaneous SOS response in $\Delta gcvB$ cells were reversed by reducing ClpXP-mediated proteolytic degradation of σ^S (97). Deletion of $rssB$ increases the general stress response (97), and it also increases MBR (39). $rssB$ deletion fully restored mu-

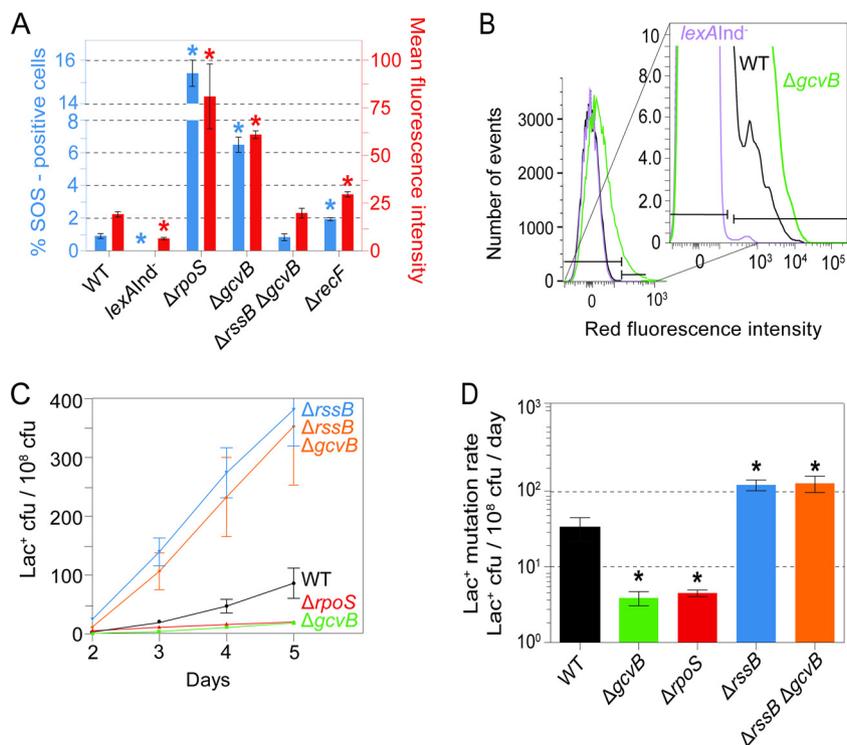


FIG 4 Artificial upregulation of σ^S substitutes for GcvB in mutagenesis and suppression of spontaneous SOS induction. (A) Increased spontaneous SOS response in $\Delta rpoS$ or $\Delta gcvB$ cells and its reversibility in $\Delta gcvB$ cells by the $\Delta rssB$ mutation, which promotes σ^S stability. SOS activity was measured by the method of Pennington and Rosenberg (82) as modified by Nehring et al. (83) by flow cytometry of strains with a chromosomal SOS-upregulated fluorescence reporter transgene, $\Delta att\lambda::P_{sulA}mCherry$. This assay quantifies single cells with spontaneous DNA damage that triggers the SOS response (not spurious promoter firing, shown in reference 82). On the left-hand y axis, the percentage of SOS-positive cells quantifies the fraction of cells in a subpopulation with higher fluorescence than the main population. Gates (horizontal brackets shown in panel B) are set per Materials and Methods for the bimodal distribution in wild-type cells, also shown in panel B. The right-hand y axis shows the mean fluorescence intensity (in arbitrary fluorescence units per cell) of all single cells assayed. The mean fluorescence intensity reports on shifts that affect most of the cells in the population, as seen in $\Delta gcvB$ cells (shown also in panel B). The data imply that the σ^S response prevents some spontaneous DNA damage. Asterisks indicate values that are significantly different from the value for the WT strain [$P = 2 \times 10^{-6}$ for the $lexA(Ind^-)$ strain, $P = 4 \times 10^{-10}$ for the $\Delta gcvB$ strain, $P = 5 \times 10^{-4}$ for the $\Delta rpoS$ strain, and $P = 5 \times 10^{-6}$ for the $\Delta recF$ strain]. Values are means \pm SEM from four experiments. From left to right, the strains are SMR21641, SMR17966, SMR21725, SMR21553, SMR21933, and SMR21728. (B) Representative flow cytometry analysis of spontaneous SOS induction showing the small cell subpopulation with spontaneous SOS-activating DNA damage, as described previously (82), in arbitrary fluorescence units. (C) The $\Delta gcvB$ MBR-deficient phenotype is suppressed by $\Delta rssB$, a mutation that increases σ^S protein levels by reducing σ^S proteolytic degradation (97), and increases MBR as shown here and shown previously (39, 98). The results of a representative experiment are shown. (D) Quantification of mutation rates from three experiments. Values are means \pm SEM. Asterisks indicate values that are significantly different from the value for the WT strain ($P = 0.05$ for the $\Delta gcvB$ strain, $P = 0.05$ for the $\Delta rpoS$ strain, $P = 0.006$ for the $\Delta rssB$ strain, and $P = 0.04$ for the $\Delta gcvB \Delta rssB$ strain) by Student's two-tailed t test. From left to right, the strains are SMR4562, SMR20238, SMR10336, SMR12566, and SMR21361.

tagenesis to $\Delta gcvB$ cells, elevated mutagenesis to the greater-than-wild-type levels seen in $\Delta rssB$ cells (Fig. 4C and D; see also Table S2 in the supplemental material), and ameliorated the $\Delta gcvB$ high-SOS phenotype (Fig. 4A). The data indicate that GcvB promotes MBR by allowing a robust σ^S response, such that in $\Delta gcvB$ cells, MBR is reduced via σ^S response deficiency.

Blocking the σ^E membrane stress response restores mutagenesis and σ^S response activity to $\Delta gcvB$ cells. We tested the hypothesis that reduced transcription of σ^S -upregulated genes and reduced MBR in $\Delta gcvB$ cells (Fig. 1, 3, and 4) might result from hyperinduction of the σ^E membrane stress response. The σ^E response promotes MBR by promoting spontaneous DNA DSBs by as yet unknown means (31). Although σ^E is an essential protein, encoded by *rpoE*, we previously isolated a separation-of-function *rpoE* mutation, *rpoE2072::Tn10dCam* (*rpoE::Tn*), that retains the σ^E essential function but is incapable of mounting a σ^E stress response (31). Cells carrying this special *rpoE::Tn* mutation show a ≥ 10 -fold reduction in spontaneous MBR but no reduction

if DSBs are supplied by I-SceI double-strand endonuclease (31). Because the Tet MBR assay measures mutagenesis activated by I-SceI cleavage at a nearby *tet* gene (19), MBR in the Tet assay is σ^E independent. We used the Tet MBR assay to test whether a σ^E response interferes with σ^S -dependent promoter activity and thus MBR. We found that the *rpoE::Tn* mutation restored normal levels of MBR to $\Delta gcvB$ cells (Fig. 1E, $\Delta gcvB$ *rpoE::Tn* compared with WT) and normal σ^S -dependent-promoter activity measured with flow cytometric and colony/catalase assays (Fig. 3B and C, $\Delta gcvB$ *rpoE::Tn* compared with WT). We conclude that the σ^E role in the membrane stress response underlies the defects in σ^S -dependent promoter activity and MBR in $\Delta gcvB$ cells. This might result from hyperinduction of the σ^E response in $\Delta gcvB$ cells, in which excessive σ^E molecules titrate RNA polymerase (RNAP), decreasing normal levels of σ^S -RNAP enzyme in favor of the σ^E -RNAP enzyme, thus reducing the σ^S response. Whereas *rpoE::Tn* increased σ^S -dependent promoter activity and MBR in $\Delta gcvB$ cells (Fig. 1E and 3B and C), *rpoE::Tn* did not increase σ^S protein levels in $\Delta gcvB$

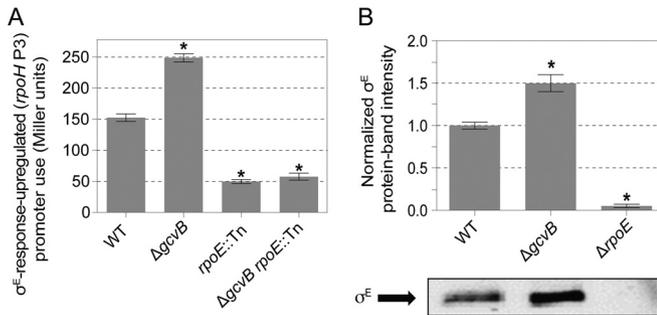


FIG 5 Hyperactivation of the σ^E membrane stress response in cells that lack sRNA GcvB. (A) Increased activation of the σ^E -dependent *rpoHP3* promoter in stationary-phase $\Delta gcvB$ cells measured as β -galactosidase activity from the *rpoHP3-lacZ* fusion gene. Values are means \pm SEM from three experiments. Asterisks indicate values that are significantly different from the value for the WT strain ($P = 4 \times 10^{-3}$ for the $\Delta gcvB$ strain, $P = 2 \times 10^{-3}$ for the $\rho E::Tn$ strain, and $P = 3 \times 10^{-3}$ for the $\Delta gcvB \rho E::Tn$ strain). From left to right, the strains are SMR8841, SMR22216, SMR8842, and SMR22310. (B) σ^E protein levels are increased in cells that lack GcvB. (Top) Quantified Western immunoblots normalized to WT bands. Values are means \pm SEM from three experiments. Asterisks indicate values that are significantly different from the value for the WT strain ($P = 7 \times 10^{-4}$ for the $\Delta gcvB$ strain and $P = 5 \times 10^{-6}$ for the $\Delta \rho E$ strain). (Bottom) Representative immunoblot. From left to right, the strains are SMR4562, SMR20238, and MC4100.

cells (see Fig. S2 in the supplemental material), supporting the σ^E/σ^S competition hypothesis.

σ^E activity and protein levels are increased in $\Delta gcvB$ cells. We measured σ^E activity using the *rpoHP3-lacZ* promoter fusion reporter gene (92), which reports on σ^E stress response-dependent transcription as β -galactosidase activity (92). We found a significant 1.6-fold \pm 0.04-fold increase in σ^E -dependent β -galactosidase activity in $\Delta gcvB$ cells relative to their isogenic *gcvB*⁺ parent cells (Fig. 5A). We also found that σ^E protein levels were increased 1.5-fold \pm 0.1-fold in $\Delta gcvB$ cells (mean \pm SEM for four Western blots [a representative blot shown in Fig. 5B]). The data imply that normally, GcvB plays a role that suppresses the σ^E stress response. GcvB might simply function in some way that promotes membrane integrity by, for example, maintaining proper levels of membrane proteins such that upon its removal, the σ^E response is induced.

The σ^E response is required for acid resistance. We attempted to test whether the acid sensitivity of the *E. coli* $\Delta gcvB$ mutant (65) might, like MBR, result from hyperinduction of the σ^E response. Surprisingly, we found that cells that carry the *rpoE2072::Tn* allele, which blocks the σ^E stress response without impairing the σ^E essential function (31), were also acid sensitive, and more acid sensitive than $\Delta gcvB$ cells were (Fig. 6). We conclude that a functional σ^E response is required for acid resistance. This is not incompatible with the possibility that the acid sensitivity of the *E. coli* $\Delta gcvB$ cells (65) results from a hyperinduced σ^E response, implying that both too much of a σ^E response and too little result in acid sensitivity. Further experiments would be needed to establish that specific mechanism.

DISCUSSION

We found that GcvB, an sRNA client of Hfq, promotes mutagenic break repair (MBR) during starvation stress in *E. coli* in two different MBR assays (Fig. 1) and presented evidence that it does so by allowing a robust σ^S general/starvation stress response, appar-

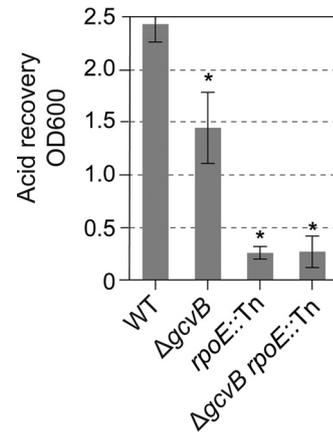


FIG 6 The σ^E response is required for acid resistance. The *rpoE2072::Tn* mutation, which ablates the σ^E response without affecting the σ^E essential function (31), caused strong acid sensitivity, indicating that the σ^E response is required for acid resistance. Because $\Delta gcvB$ cells display a hyper- σ^E response (Fig. 5) and *rpoE::Tn* cells have no σ^E response (31), the data suggest that both too much and too little σ^E response activity may result in acid sensitivity and that the σ^E response must occur at just the right level for resistance. The OD₆₀₀ of cultures after 3-h recovery from a 30-min acid challenge was measured. Values are means \pm SEM from three experiments with three independent cultures for each strain. Asterisks indicate values that are significantly different from the value for the wild-type (WT) control strain ($P = 0.01$ for the $\Delta gcvB$ strain, $P = 5 \times 10^{-8}$ for the *rpoE::Tn* strain, and $P = 3 \times 10^{-9}$ for the $\Delta gcvB \rho E::Tn$ strain) by Student's two-tailed paired *t* test. From left to right, isogenic strains are SMR4562, SMR20238, SMR5236, and SMR21996.

ently by suppressing the σ^E membrane stress response. First, MBR proficiency was restored to $\Delta gcvB$ cells by artificial upregulation of σ^S using an *rssB* mutation, which blocks σ^S protein degradation (Fig. 4C and D), implying that the $\Delta gcvB$ MBR defect is caused by failure to mount a robust σ^S response. Moreover, we found that (i) cells that lack GcvB showed decreased σ^S -regulated gene expression in two assays (Fig. 3A to C) and that (ii) the σ^S -dependent reduction in gene expression was also reversible by σ^S upregulation (Fig. 3B and C) but that (iii) σ^S protein levels were not reduced in $\Delta gcvB$ cells (Fig. 3D).

Second, blocking the σ^E membrane stress response, but not the σ^E essential function, with an *rpoE::Tn* separation-of-function mutation (31) restored σ^S -regulated promoter activity (Fig. 3B and C) and MBR (Fig. 1E) to $\Delta gcvB$ cells without increasing σ^S protein levels (see Fig. S2 in the supplemental material). The data imply that a too-active σ^E response inhibits the σ^S response and MBR, possibly via sigma factor competition for RNA polymerase (RNAP) (model below). Supporting this possibility, σ^E protein levels and activity were abnormally high in $\Delta gcvB$ cells (Fig. 5). We suggest that GcvB may promote membrane integrity and thus avert membrane stress response hyperinduction.

Our data demonstrate that GcvB promotes stress-inducible MBR and suggest a possible function for GcvB in *E. coli* in membrane maintenance. These data reinforce the importance and delicacy of stress response regulation of mutagenesis (39; for recent reviews, see references 2, 6, and 10).

Model in which sigma factor competition reduces MBR in $\Delta gcvB$ mutant cells. In Fig. 7 we outline a possible model in which sigma factor competition for RNAP could promote the delicate regulatory balance between the σ^S and σ^E responses and thus modulate MBR in response to the presence or absence of GcvB

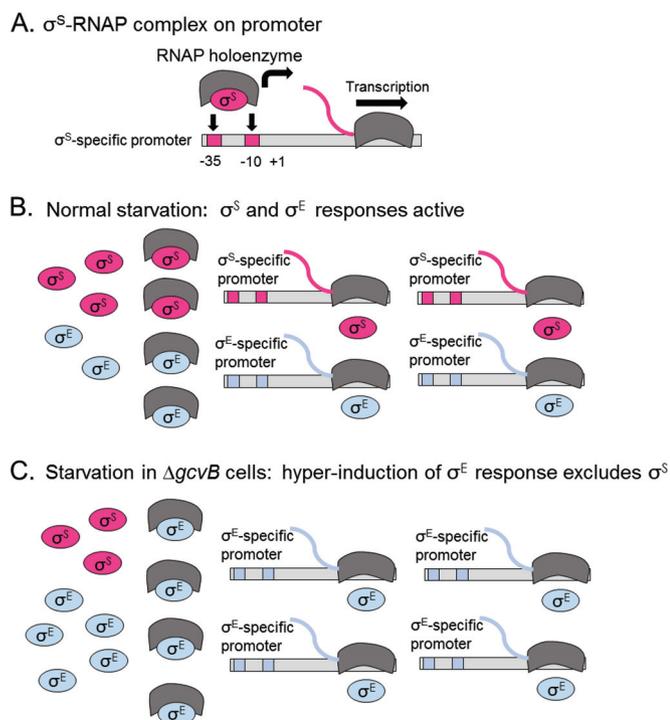


FIG 7 Model in which σ^E competition for RNA polymerase reduces the σ^S response and mutagenic break repair in $\Delta gcvB$ mutant cells. The observed σ^E -dependent reductions of both MBR and σ^S -dependent gene expression in $\Delta gcvB$ cells could result from competition of σ^E with σ^S for RNA polymerase (RNAP) (gray arc shapes) in cells that lack GcvB. (B) We suggest that sRNA GcvB is required for normal membrane integrity and thus keeps the σ^E response low in σ^S -response-induced stationary cells. (C) In the absence of GcvB, excessive membrane stress is proposed to hyperactivate the σ^E response, producing increased σ^E -RNAP complexes, at the expense of σ^S -RNAP complexes. Reduced σ^S -RNAP complexes could cause a reduced general stress response, which causes MBR deficiency (18, 19, 27, 28). The pink and blue lines represent σ^S - and σ^E -dependent transcripts, respectively.

sRNA. Sigma factor competition for RNAP has been implicated in shifting transcriptional patterns under various circumstances, and it is affected by both the number of sigma factors present in the cell and their affinity for RNAP (99, 100). σ^E has higher affinity for RNAP than σ^S has (101). Several bacterial sRNAs promote membrane integrity (67) (see the introduction), making it a reasonable hypothesis that GcvB suppresses the σ^E response (Fig. 5) because it, too, is needed for integrity of the cell membrane. GcvB would thus indirectly suppress hyper- σ^E response induction. We suggest that normally GcvB promotes membrane integrity and that when σ^S is induced during starvation, σ^S -RNAP complexes can form, allowing activation of the σ^S response (Fig. 7B). We found previously that there is some σ^E response induction in *E. coli* under MBR starvation conditions (31), so we infer that normally during starvation both σ^S and σ^E responses are activated (Fig. 7B). We suggest that in $\Delta gcvB$ cells, membrane stress-promoted hyperinduction of σ^E blocks σ^S access to RNAP via competition (Fig. 7C). This model is supported by our findings that loss of σ^E response induction capability restores MBR and σ^S -regulated promoter activity to $\Delta gcvB$ cells (Fig. 1E and 3B and C) without increasing σ^S protein levels (see Fig. S2 in the supplemental material). This model might also explain some of the acid sensitivity reported for $\Delta gcvB$ cells (65). Perhaps a hyperinduced σ^E response contributes

to σ^S response depression and acid sensitivity of $\Delta gcvB$ cells in addition to the reduced production of σ^S (a σ^S -LacZ fusion protein) observed in that study (65). We found that GcvB and a functional σ^E response both promote acid resistance (Fig. 6). The data suggest that both too much and too little σ^E response activity may result in sensitivity, and the data are not incompatible with the possibility that some of the acid sensitivity of *E. coli* $\Delta gcvB$ cells (65) results from a hyper- σ^E response. Other models are possible.

Which GcvB target gene(s) may affect membrane integrity under MBR starvation conditions is not known. A list of known and predicted targets of the GcvB sRNA is given in Table S3 in the supplemental material. An outer membrane protein gene, *ompF*, is an experimentally implicated GcvB target (102), and it might contribute to destabilization of the membrane when upregulated due to loss of GcvB. GcvB regulation of other genes on, and possibly not on, the lists in Table S3 might contribute to membrane integrity additionally or alternatively.

sRNAs play many and various roles in bacterial biology and across the tree of life, yet the functions of many sRNAs remain obscure, even in *E. coli* (68, 103, 104). GcvB is now implicated in membrane integrity and demonstrated to regulate mutagenesis. Other possible roles and the specific mechanism(s) of action of GcvB await future exploration.

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