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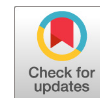

# Appropriate Regulation of the $\sigma^E$-Dependent Envelope Stress Response Is Necessary To Maintain Cell Envelope Integrity and Stationary-Phase Survival in *Escherichia coli*

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**ABSTRACT** The alternative sigma factor $\sigma^E$ is a key component of the *Escherichia coli* response to cell envelope stress and is required for viability even in the absence of stress. The activity of $\sigma^E$ increases during entry into stationary phase, suggesting an important role for $\sigma^E$ when nutrients are limiting. Elevated $\sigma^E$ activity has been proposed to activate a pathway leading to the lysis of nonculturable cells that accumulate during early stationary phase. To better understand $\sigma^E$-directed cell lysis and the role of $\sigma^E$ in stationary phase, we investigated the effects of elevated $\sigma^E$ activity in cultures grown for 10 days. We demonstrate that high $\sigma^E$ activity is lethal for all cells in stationary phase, not only those that are nonculturable. Spontaneous mutants with reduced $\sigma^E$ activity, due primarily to point mutations in the region of $\sigma^E$ that binds the −35 promoter motif, arise and take over cultures within 5 to 6 days after entry into stationary phase. High $\sigma^E$ activity leads to large reductions in the levels of outer membrane porins and increased membrane permeability, indicating membrane defects. These defects can be counteracted and stationary-phase lethality delayed significantly by stabilizing membranes with $Mg^{2+}$ and buffering the growth medium or by deleting the $\sigma^E$-dependent small RNAs (sRNAs) MicA, RybB, and MicL, which inhibit the expression of porins and Lpp. Expression of these sRNAs also reverses the loss of viability following depletion of $\sigma^E$ activity. Our results demonstrate that appropriate regulation of $\sigma^E$ activity, ensuring that it is neither too high nor too low, is critical for envelope integrity and cell viability.

**IMPORTANCE** The Gram-negative cell envelope and cytoplasm differ significantly, and separate responses have evolved to combat stress in each compartment. An array of cell envelope stress responses exist, each of which is focused on different parts of the envelope. The $\sigma^E$ response is conserved in many enterobacteria and is tuned to monitor pathways for the maturation and delivery of outer membrane porins, lipoproteins, and lipopolysaccharide to the outer membrane. The activity of $\sigma^E$ is tightly regulated to match the production of $\sigma^E$ regulon members to the needs of the cell. In *E. coli*, loss of $\sigma^E$ results in lethality. Here we demonstrate that excessive $\sigma^E$ activity is also lethal and results in decreased membrane integrity, the very phenotype the system is designed to prevent.

**KEYWORDS** cell envelope, stress response, transcriptional regulation

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Stress responses allow cells to rapidly adapt their gene expression to cope with changing conditions. Signal transduction pathways sense an inducing stress and transduce that information to a transcription factor, which, in turn, regulates the expression of specialized sets of genes required to combat the stress. Once the stress

is removed, the response is downregulated and gene expression returns to the basal state. In bacteria, major cellular stress responses are mediated by alternative sigma factors, which rapidly reprogram gene expression by replacing the housekeeping sigma factor and directing RNA polymerase to the genes in their regulons (1–3). Gram-negative bacteria have compartmentalized responses to address stress in their two cellular compartments, the cytoplasm and the cell envelope. The alternative sigma factors $\sigma^E$ and $\sigma^{32}$ mediate the envelope and cytoplasmic stress responses, respectively (4–6). Although mutants lacking $\sigma^{32}$ are very temperature sensitive, *Escherichia coli* can grow without $\sigma^{32}$ at temperatures below 20°C (7). In contrast, *rpoE*, the gene encoding $\sigma^E$, is essential for viability (8). When $\sigma^E$ activity is depleted, cells develop envelope defects and lyse, indicating that $\sigma^E$ is required to maintain cell envelope integrity and that combating envelope stress via $\sigma^E$ is critical for survival (5).

$\sigma^E$ directs the transcription of a regulon that has a significant impact on the cell envelope (9). There are two arms of the response, one mediated by proteins and the other by small RNAs (sRNAs) (9, 10). $\sigma^E$ transcribes genes encoding proteases, which degrade misfolded envelope proteins, and chaperones and assembly factors, which escort outer membrane proteins and lipopolysaccharide (LPS) as they transit from the inner membrane across the periplasm to the outer membrane (9, 11, 12). $\sigma^E$ also transcribes genes encoding several sRNAs that block the expression of a series of mRNAs, including those encoding every major outer membrane porin and several lipoproteins (10, 13). As a result, when conditions in the cell envelope are unfavorable, $\sigma^E$ serves to increase the capacity of the pathways that deliver LPS and porins to the outer membrane, while decreasing the load on the system by reducing *de novo* synthesis of outer membrane constituents. The sRNA arm of the response is of particular importance because expression of the RybB, MicA, or MicL $\sigma^E$-dependent sRNAs can restore viability when $\sigma^E$ activity is severely reduced (10, 13).

The activity of $\sigma^E$ is tuned to the state of outer membrane protein folding through a regulatory pathway that is activated by unfolded outer membrane proteins (14). The activity of $\sigma^E$ is directly regulated by the anti-sigma factor RseA. RseA is an inner membrane protein whose cytoplasmic domain binds to $\sigma^E$ and prevents $\sigma^E$ from associating with RNA polymerase (15–17). Unfolded outer membrane proteins trigger a proteolytic cascade that results in the complete degradation of RseA, freeing $\sigma^E$ to direct transcription (18, 19). In addition to the pathway that controls the proteolysis of RseA, transcription by the $\sigma^E$-RNA polymerase holoenzyme is activated by the global stress alarmone guanosine-3′,5′-bisdiphosphate (ppGpp) (20, 21). ppGpp is a potent signal of starvation, and its levels increase during entry into stationary phase in rich medium or when growth slows due to depletion of specific nutrients (22, 23). The activity of $\sigma^E$ increases at such times in a ppGpp-dependent manner, independent of RseA and the envelope stress signaling pathway, suggesting that $\sigma^E$ might facilitate the survival of *E. coli* after the exponential phase of growth (20, 24).

The role of $\sigma^E$ in stationary-phase survival remains relatively unexplored. $\sigma^E$ has been proposed to direct a pathway that leads to the lysis of viable but nonculturable cells (VBNC) in early stationary phase. This proposal is based on observations that cultures with elevated $\sigma^E$ activity due to $\sigma^E$ overexpression or deletion of the *rseA* gene have lower optical densities (ODs) in early stationary phase than do isogenic wild-type cells, although the CFU per milliliter for the two strains are comparable. Cultures with elevated $\sigma^E$ activity also have higher levels of proteins released into the growth medium than wild-type cultures, suggesting that cells that are not able to form colonies have lysed (25–27).

To better understand $\sigma^E$-directed cell lysis and its contribution to the role of $\sigma^E$ in stationary-phase survival, we investigated the effects of elevated $\sigma^E$ activity in cultures grown for 10 days, into stationary phase, in rich medium. Importantly we found that $\sigma^E$-directed cell lysis is likely to be due to membrane defects in cells with elevated $\sigma^E$ activity, rather than a purposeful regulated process. In fact, elevated $\sigma^E$ activity is toxic in stationary phase for all cells, and mutants with reduced $\sigma^E$ activity take over cultures

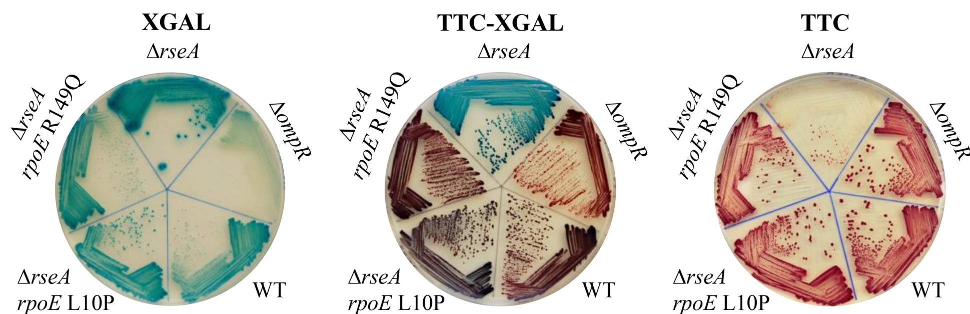


**FIG 1** Cells with a wide range of $\sigma^E$ activities can be distinguished on lactose–TTC–X-Gal agar. The activity of $\sigma^E$ from the $\sigma^E$-dependent *rpoH*p3-*lacZ* reporter is shown on LB–X-Gal (left), lactose–TTC–X-Gal (center), and lactose-TTC (right) plates for the Δ*ompR* strain with very low levels of $\sigma^E$ activity, the wild-type (WT) strain with moderate levels of $\sigma^E$ activity, and the Δ*rseA* strain with high levels of $\sigma^E$ activity. Also shown are two mutants, isolated from stationary-phase Δ*rseA* cultures, that have mutations in *rpoE* that reduce its activity to different extents (8-fold decrease for the L10P mutation and 33-fold decrease for the R149Q mutation) (Table 1).

within 4 to 5 days. The toxicity is due almost entirely to the RybB, MicA, and MicL sRNAs, indicating that proper regulation of their expression is a critical role of the $\sigma^E$ response.

## RESULTS

**High $\sigma^E$ activity causes a lethal phenotype in stationary phase.** In previous studies of $\sigma^E$-directed cell lysis with a Δ*rseA* mutant in early stationary phase, the optical densities, CFU per milliliter, and release of protein into culture supernatants were examined, but $\sigma^E$ activity was not investigated (25–27). Because we had observed that mutants with reduced $\sigma^E$ activity occasionally appeared when overnight cultures of a Δ*rseA* strain were plated, we decided to monitor $\sigma^E$ activity using the $\sigma^E$-dependent *rpoH*p3-*lacZ* fusion, in addition to measuring the optical density and CFU per milliliter. To better distinguish colonies with a wide range of $\sigma^E$ activities, we developed a method that combined two indicators of β-galactosidase activity, with different dynamic ranges, i.e., 2,3,5-triphenyltetrazolium chloride (TTC) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). Cells with high levels of *lacZ* expression, such as the Δ*rseA* strain, form dark blue colonies on X-Gal agar, white to light pink colonies on lactose-TTC agar, and blue colonies on lactose–TTC–X-Gal agar (Fig. 1). Cells with intermediate levels of $\sigma^E$ activity, such as the wild-type strain, form medium blue colonies with X-Gal, red colonies with TTC, and purple colonies with TTC and X-Gal (Fig. 1). Cells with low levels of $\sigma^E$ activity, such as a Δ*ompR* strain (6), form light blue colonies with X-Gal, deep red colonies with TTC, and red colonies with the combination (Fig. 1).

To gain a more complete view of the role of $\sigma^E$-directed cell lysis during stationary phase, we grew cultures of wild-type and Δ*rseA* strains in Luria-Bertani (LB) broth for 10 days, into the start of long-term stationary phase. Previous studies focused only on early-stationary-phase cultures grown for 72 h or less (25–27). The optical density at 600 nm ($OD_{600}$) of the Δ*rseA* cultures decreased approximately 4-fold during the first 5 days in stationary phase and then remained at the same level for the following 5 days (Fig. 2A, blue squares). In contrast, the $OD_{600}$ of the wild-type cultures did not change significantly for the entire 10 days (Fig. 2A, green squares). Despite the observation that the $OD_{600}$ of the Δ*rseA* cultures was less than that of the wild-type cultures, the CFU per milliliter of the two strains were similar (Fig. 2B). The CFU per milliliter began to decrease after 2 to 3 days and dropped by 90 to 99% before leveling off by day 7 (Fig. 2B), as expected for cultures grown in rich medium that are transitioning from early stationary phase through death phase and into long-term stationary phase (28). When examined by phase-contrast microscopy, the Δ*rseA* cultures had numerous small translucent cells indicative of lysis, which were likely responsible for the lower optical density. The remaining phase-dense cells looked similar to those in the wild-type cultures and had no obvious morphological defects. Our results from the first 72 h in

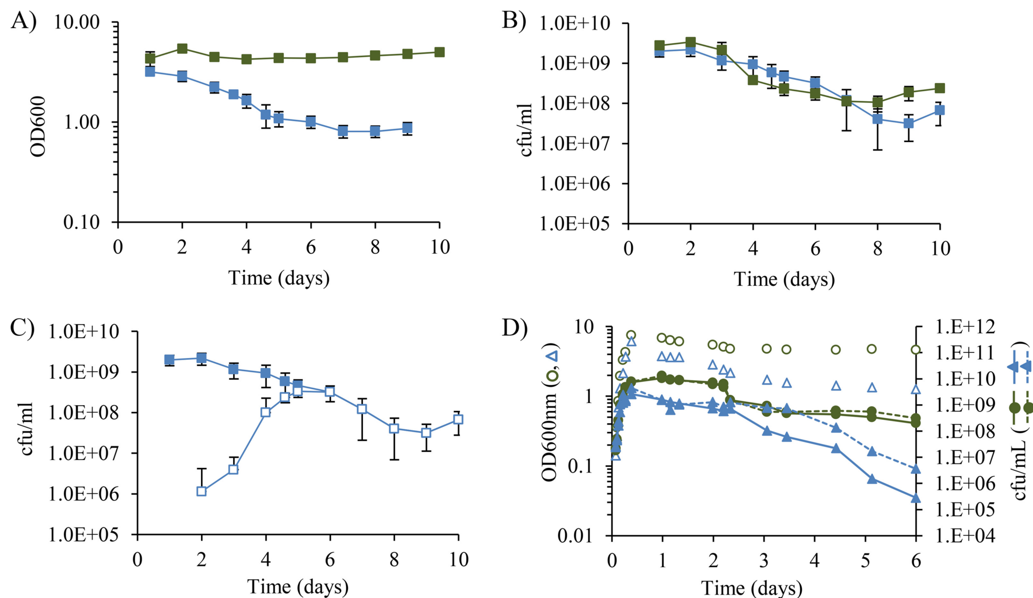


**FIG 2** Mutants with reduced $\sigma^E$ activity outcompete the parental Δ*rseA* strain in stationary-phase cultures. (A and B) $OD_{600}$ (A) and CFU per milliliter (B) of Δ*rseA* (blue squares) and wild-type (green squares) cultures grown for 10 days are shown. (C) Total CFU per milliliter of Δ*rseA* cultures (closed squares) and CFU per milliliter of mutants with reduced $\sigma^E$ activity that arose in Δ*rseA* cultures (open squares) are shown. Data presented are averages with standard deviations from 4 independent cultures. (D) $\sigma^E$ overexpression from the plasmid pLC245 also reduces survival in stationary phase. The $OD_{600}$ values for a wild-type strain carrying pLC245 (blue triangles) or the control plasmid pTrc99a (green circles) grown under inducing conditions in the absence of selective pressure for the plasmids are shown as open symbols. The total CFU per milliliter (closed symbols, dashed lines) and the CFU per milliliter of cells still carrying the plasmid, as measured by ampicillin resistance (closed symbols, solid lines), are shown for each strain.

stationary phase for wild-type and Δ*rseA* cultures were similar to those reported previously (25–27).

A critical difference between the wild-type and Δ*rseA* strains was observed when $\sigma^E$ activity was monitored. The colonies from wild-type cultures were a purple color throughout the experiment on TTC–X-Gal agar, indicating that the levels of $\sigma^E$ activity remained relatively constant. Within 2 to 3 days, however, the population of cells from the Δ*rseA* cultures with high $\sigma^E$ activity that formed blue colonies in the presence of lactose, TTC, and X-Gal started to decrease and purple colonies with lower levels of $\sigma^E$ activity began to appear (Fig. 2C, open squares). By day 5 or 6 of stationary phase, all colonies were purple (Fig. 2C), indicating that bacteria with high levels of $\sigma^E$ activity did not survive more than few days in stationary phase and were replaced by a population of cells with lower levels of $\sigma^E$ activity. Colonies of two mutants with reduced $\sigma^E$ activity, the Δ*rseA rpoE* L10P and Δ*rseA rpoE* R149Q strains, are shown in Fig. 1. The $OD_{600}$ of the Δ*rseA* cultures also stopped decreasing around day 6 (Fig. 2A), consistent with replacement of the dying Δ*rseA* strain by cells with lower levels of $\sigma^E$ activity. Similar results were obtained with two Δ*rseA* strains, each carrying a different tightly linked marker, *nadB*::Tn*10* or *yfiC*::*kan*.

**The stationary-phase defects are due to elevated $\sigma^E$ activity and not loss of RseA.** The decrease in the number of cells with high levels of $\sigma^E$ activity could be caused by toxic effects associated with high $\sigma^E$ activity or by a $\sigma^E$-independent defect caused by deletion of the *rseA* gene. To distinguish between these possibilities, we compared the growth of a wild-type strain containing a plasmid with the *rpoE* gene under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter with the growth of a strain with the empty vector plasmid. The strains were grown into early exponential phase in the presence of ampicillin to ensure that all cells had the plasmid. Cells were then transferred to LB medium without ampicillin to allow plasmid loss, if it proved to be toxic, and with IPTG to induce overexpression of *rpoE*. The $OD_{600}$ of the strain overexpressing *rpoE* was lower in stationary phase than was that of the

**TABLE 1** Effects of *rpoE* mutations on $\sigma^E$ amounts and activity in the Δ*rseA* strain background

| ***rpoE*** **variant** | **Relative amount of** $\sigma^{Ea}$ | **Activity of** $\sigma^E$ **(arbitrary units)** | **Specific activity of** $\sigma^{Eb}$ |
|---|---|---|---|
| Wild type | 1.00 | 36.37 ± 1.04 | 36.37 ± 1.04 |
| R173C | 0.50 ± 0.12 | 0.25 ± 0.00 | 0.50 ± 0.00 |
| S172A | 0.60 ± 0.20 | 0.47 ± 0.03 | 0.79 ± 0.05 |
| L127Q | 0.58 ± 0.14 | 0.65 ± 0.02 | 1.11 ± 0.04 |
| R176Q | 0.41 ± 0.15 | 0.75 ± 0.38 | 1.82 ± 0.92 |
| F175S | 0.52 ± 0.17 | 2.08 ± 0.08 | 4.01 ± 0.16 |
| R171C | 0.20 ± 0.11 | 0.69 ± 0.01 | 3.53 ± 0.07 |
| E126K | 0.24 ± 0.12 | 1.48 ± 0.47 | 6.22 ± 2.00 |
| R149Q | 0.16 ± 0.05 | 1.08 ± 0.01 | 6.87 ± 0.09 |
| A177V | 0.19 ± 0.02 | 1.51 ± 0.03 | 7.81 ± 0.15 |
| V170M | 0.11 ± 0.05 | 1.62 ± 0.05 | 15.01 ± 0.46 |
| A60P | 0.06 ± 0.03 | 2.11 ± 0.04 | 33.09 ± 0.67 |
| S155I | 0.11 ± 0.05 | 3.76 ± 0.86 | 35.41 ± 8.07 |
| L10P | 0.05 ± 0.00 | 4.47 ± 1.23 | 84.57 ± 23.28 |

[a]Amount of the $\sigma^E$ variant relative to the amount of wild-type $\sigma^E$ in the parental Δ*rseA* strain.
[b]Activity of $\sigma^E$ normalized to the relative amount of $\sigma^E$.

control strain (Fig. 2D), similar to findings observed with the Δ*rseA* strain. Overexpression of *rpoE* also reduced survival (measured as the total CFU per milliliter) in stationary phase, compared to the control strain without the plasmid or the overexpression strain without induction of *rpoE* (Fig. 2D). Ninety percent of the cells overexpressing *rpoE* were sensitive to ampicillin after 3 days in stationary phase, indicating that the *rpoE* overexpression plasmid had been lost and/or cells with the plasmid were not able to survive (Fig. 2D). In contrast, the pTrc99a control plasmid was stably maintained even in the absence of selective pressure (Fig. 2D). These data indicate that failure to survive in stationary phase is a property of elevated $\sigma^E$ activity and is not due to a function of RseA that is independent of $\sigma^E$.

**Spontaneous mutations with lower $\sigma^E$ activity map primarily to the *rpoE* gene.** Several of the strains isolated from the stationary-phase cultures were analyzed to identify the mutations causing lower $\sigma^E$ activity. In cultures of the Δ*rseA nadB*::Tn*10* strain, many mutants also lost tetracycline resistance. PCR amplification using primers for the promoter region failed, suggesting that the *rpoE* promoter was disrupted by recombination events due to excision of Tn*10* from *nadB*, which is directly upstream of *rpoE*. In mutants isolated from cultures of the Δ*rseA* Δ*yfiC*::*kan* strain, the kanamycin resistance marker was stable and *rpoE* could be successfully amplified by PCR. Fifteen mutants isolated from stationary-phase cultures of the Δ*rseA* Δ*yfiC*::*kan* strain and the Δ*rseA nadB*::Tn*10* strain that retained tetracycline resistance were analyzed. Thirteen mutants had point mutations in *rpoE* and two had mutations mapping outside *rpoE*, one to the *rpoBC* region encoding the β and β′ subunits of RNA polymerase and one to *nsrR*, a nitrite-sensitive transcriptional repressor. All mutations were moved by P1 transduction into a clean Δ*rseA* background, to verify that the mutations were responsible for the phenotypes. $\sigma^E$ activity and protein levels were measured for the isolates with mutations in *rpoE*. We found that all of the mutations decreased $\sigma^E$ activity to levels similar to or below those found in the wild-type strain with a functional *rseA* gene.

The mutations could be roughly divided into three classes: those that significantly decreased the specific activity and had only minor effects on the amount of $\sigma^E$ in the cell, those that had moderate effects on both the specific activity and the amount of $\sigma^E$, and those that had stronger effects on the amount than on the specific activity of $\sigma^E$ (Table 1 and Fig. 3). The L127Q, S172A, R173C, F175S, and R176Q mutations belong to the first class (Fig. 3). The mutations decreased the specific activity of $\sigma^E$ 9- to 72-fold, with only small effects on the amount of $\sigma^E$. These residues have been shown to form direct contacts with the −35 region of the promoter DNA, with the exception of L127, which is buried in the hydrophobic core of $\sigma^E$ domain 4 (15, 29). The *rpoE* gene is

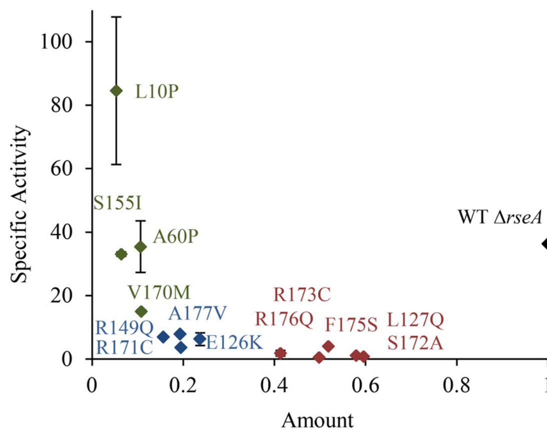


**FIG 3** Mutants with reduced $\sigma^E$ activity that have been recovered from stationary-phase cultures of the Δ*rseA* strain have mutations in the *rpoE* gene that reduce the amount and/or specific activity of $\sigma^E$. The amount of $\sigma^E$ in each strain was measured by Western blotting and normalized to the level of wild-type (WT) $\sigma^E$ in the parental Δ*rseA* strain. The specific activity was determined by measuring $\sigma^E$ activity from the *rpoH*p3-*lacZ* reporter gene in liquid cultures and normalizing the activity to the amount of $\sigma^E$ in that particular strain. Mutations that primarily reduce the amount of $\sigma^E$ but not the specific activity are shown in green, those that reduce the amount and specific activity of $\sigma^E$ are shown in blue, and those that reduce the specific activity but not the amount of $\sigma^E$ are shown in red.

transcribed from both $\sigma^{70}$- and $\sigma^E$-dependent promoters, and the slightly reduced amounts of $\sigma^E$ in these mutants could be due to reduced transcription from the $\sigma^E$-dependent promoter or reduced stability of the protein.

The E126K, R149Q, R171C, and A177V mutations affected both the amount of $\sigma^E$ (4- to 6-fold reductions) and the specific activity (5- to 10-fold reductions) (Fig. 3). E126 is exposed on the surface and potentially at the interface between core RNA polymerase and $\sigma^E$, R149 and R171 make DNA contacts in the −35 promoter region, and A177 forms part of the hydrophobic core (15, 29).

The remaining mutations, L10P, A60P, S155I, and V170M, reduced the amount of $\sigma^E$ in the cell by ≥10-fold, with little to no reduction in specific activity (Fig. 3). These mutations are likely to destabilize the folded structure of the protein, leading to increased degradation by proteases. The L10P and A60P mutations introduce prolines in the first position of a turn and in the middle of an $\alpha$ helix, respectively (30, 31). An isoleucine substitution at position 155 likely exposes a hydrophobic residue on the surface of the protein, and V170 is buried in the hydrophobic core (15, 29).

**Reduced $\sigma^E$ activity is sufficient to eliminate the stationary-phase defects.** In addition to having elevated $\sigma^E$ activity, cells lacking RseA cannot modulate $\sigma^E$ in response to envelope stress, either of which could lead to the stationary-phase defects. To distinguish between these possibilities, we asked whether Δ*rseA* mutants with reduced $\sigma^E$ activity exhibited the same phenotype as the parent Δ*rseA* strain in stationary phase. Two mutants belonging to the first two classes described above, the Δ*rseA rpoE* S172A and Δ*rseA rpoE* R171C strains, were selected for further examination. When cultures of these strains were grown for 10 days, the $OD_{600}$ did not decrease (Fig. 4A), the CFU per milliliter were comparable to those of the wild-type strain (Fig. 4B), and the colonies remained the same color (purple) on lactose–TTC–X-Gal plates, indicating that decreasing $\sigma^E$ activity reversed the survival defect of Δ*rseA* strains.

**High $\sigma^E$ activity reduces outer membrane integrity.** Given the impact of $\sigma^E$ regulon members on the outer membrane (9, 10, 13, 32, 33), we reasoned that high $\sigma^E$ activity might lead to an imbalance in the synthesis of outer membrane components, altering the permeability of the cell envelope and potentially making the Δ*rseA* cells less fit in stationary phase. To test this idea, we examined the sensitivity of the Δ*rseA* strain to the detergent SDS and rifampin, a large hydrophobic antibiotic that normally is not able to cross the outer membrane of *E. coli* but can enter cells with increased permeability. Exponential-phase Δ*rseA* cultures rapidly lost viability (a decrease in CFU

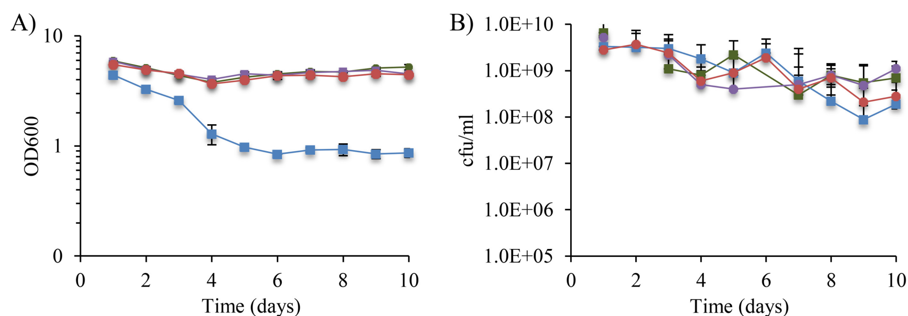


**FIG 4** Mutations that decrease $\sigma^E$ activity reverse the stationary-phase defects of the Δ*rseA* strain. The $OD_{600}$ (A) and total CFU per milliliter (B) of Δ*rseA* (blue squares), wild-type (green squares), Δ*rseA rpoE* S172A (red circles), and Δ*rseA rpoE* R171C (purple circles) cultures are shown. Mutants with lower levels of $\sigma^E$ activity did not appear for the wild-type, Δ*rseA rpoE* S172A, and Δ*rseA rpoE* R171C strains. Data shown are averages with standard deviations from 2 independent cultures.

per milliliter of >5 orders of magnitude) in the presence of 5% SDS and stopped growing within 90 min after the addition of rifampin to a final concentration of 8 $\mu$g/ml (Fig. 5A). In contrast, growth of the wild-type strain was not affected at these concentrations of SDS and rifampin over the same time frame (Fig. 5A). Approximately one-half of the Δ*rseA* cells that survived SDS treatment had reduced $\sigma^E$ activity, as indicated by colony color on lactose–TTC–X-Gal agar, whereas no colonies with reduced $\sigma^E$ activity were seen for untreated Δ*rseA* cultures. These data indicated that a small population of cells with low $\sigma^E$ activity were already present in exponential-phase cultures and were enriched by SDS treatment.

To determine whether the membrane defects persisted into stationary phase, 28- to 30-h cultures (before mutants took over the population) were treated with 5% SDS. The CFU per milliliter of cultures of the Δ*rseA* strain decreased by approximately 4 orders of magnitude within 30 min after SDS treatment, while the wild-type strain was not affected (Fig. 5B). Of the cells that survived SDS treatment, 99% exhibited reduced $\sigma^E$ activity on lactose–TTC–X-Gal agar, suggesting that Δ*rseA* mutants with low $\sigma^E$ activity were less sensitive to SDS and were present in the cultures after 28 h of growth at a frequency of about $0.5 \times 10^{-4}$ to $1 \times 10^{-4}$.

To determine whether elevated $\sigma^E$ activity was responsible for the SDS sensitivity of the Δ*rseA* strain, stationary-phase cultures of the Δ*rseA rpoE* S172A and Δ*rseA rpoE* R171C mutants were treated with SDS. Both mutations reversed the SDS sensitivity of the Δ*rseA* strain (Fig. 5C), indicating that high $\sigma^E$ activity is responsible for reduced outer membrane integrity as well as the stationary-phase lethality phenotypes.

**Stabilization of the outer membrane reduces stationary-phase lethality and reverses membrane permeability defects.** $Mg^{2+}$ is known to stabilize the outer membrane, most likely by stabilizing interactions between adjacent LPS molecules (34). If the increased membrane permeability of the Δ*rseA* strain is related to its instability in stationary phase and is due to a problem with the LPS component of the outer membrane, then supplementing the growth medium with $Mg^{2+}$ should decrease both defects associated with the Δ*rseA* strain. Indeed, we found that stationary-phase Δ*rseA* cultures grown in LB medium with 20 mM $MgCl_2$ (LB-Mg) were no longer sensitive to 5% SDS (Fig. 5B). In addition, the decrease in optical density during the first 5 days of stationary phase was significantly reduced when the Δ*rseA* strain was grown in LB-Mg, compared to growth without $Mg^{2+}$ supplementation (Fig. 6A), the appearance of mutants with low $\sigma^E$ activity was delayed an average of 48 h in the LB-Mg cultures (Fig. 6B), and mutants with low $\sigma^E$ activity did not take over even after 10 days in stationary phase (Fig. 6B). The number of mutants stopped increasing by day 8, and the mutants constituted approximately 30% of the total CFU per milliliter for the last 3 days of culture growth (Fig. 6B).

**Growth in buffered medium partially reverses SDS sensitivity.** The pH of the growth medium increased in the stationary-phase cultures over time, as is known to

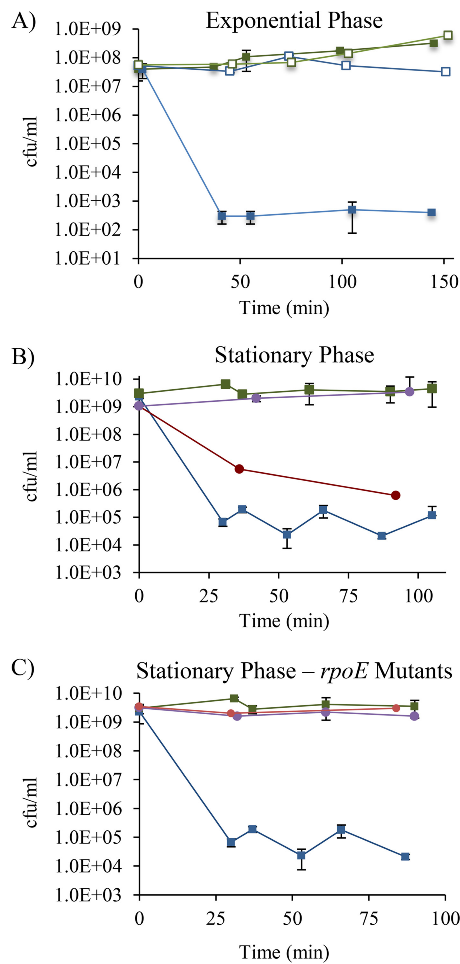


**FIG 5** The Δ*rseA* strain exhibits membrane permeability defects that are stabilized by $Mg^{2+}$ and by mutations that reduce $\sigma^E$ activity. (A) CFU per milliliter of the Δ*rseA* (blue squares) and wild-type (green squares) strains from exponentially growing cultures in LB medium treated with 5% SDS (closed symbols) or 8 $\mu$g/ml rifampin (open symbols) is shown. (B) CFU per milliliter of 1-day-old stationary-phase cultures treated with 5% SDS is shown for Δ*rseA* cultures in LB medium (blue squares), LB-MOPS (red circles), and LB-Mg (purple circles) and wild-type cultures in LB medium (green squares). (C) CFU per milliliter of Δ*rseA* (blue squares), Δ*rseA rpoE* S172A (red circles), Δ*rseA rpoE* R171C (purple circles), and wild-type (green squares) stationary-phase cultures in LB medium treated with 5% SDS is shown. Data presented are averages with standard deviations from at least 2 independent cultures. In all panels, time indicates minutes after treatment.

occur for *E. coli* grown in LB medium, which could contribute to the reduced survival of Δ*rseA* cells. To relieve the alkali stress, cultures were grown in LB medium buffered to pH 7.3 with 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (LB-MOPS). The pH remained constant for the 10 days of culture growth. Mutants with lower $\sigma^E$ activity appeared at approximately the same time as they did in cultures grown in unbuffered LB medium (Fig. 6C), and the decrease in $OD_{600}$ was not reversed (Fig. 6A). However, survival after the addition of SDS increased by 1 order of magnitude for stationary-phase cultures of the Δ*rseA* strain grown in LB-MOPS compared to the same strain grown in unbuffered LB medium (Fig. 5B), although the sensitivity was not fully reversed. A strong effect of growth medium buffering on stationary-phase survival was not observed; however, buffering potentiated the stabilization seen with $Mg^{2+}$. Mutants with reduced $\sigma^E$ activity appeared in MOPS-buffered LB medium supplemented with 20 mM $MgCl_2$ 24 h later than in cultures grown in LB-Mg and 72 h later than in cultures grown in LB medium (Fig. 6B and D). The percentage of mutants with reduced $\sigma^E$ activity stopped increasing by day 8, and mutants constituted approximately 14% of the total CFU per milliliter for the last 3 days of the experiment (Fig. 6D).

 

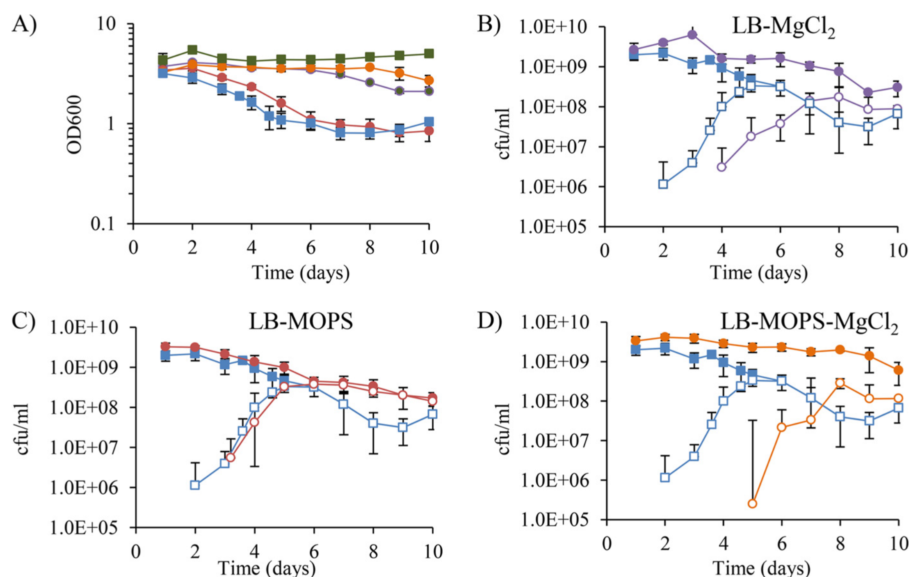


**FIG 6** Supplementation of LB medium with $Mg^{2+}$ and buffering with MOPS stabilize the Δ*rseA* strain in stationary phase. The Δ*rseA* strain was grown into stationary phase for 10 days in LB medium (blue squares), LB-Mg (purple circles), LB-MOPS (red circles), or LB-MOPS-Mg (orange circles). The wild-type strain grown in LB medium (green squares) is also shown. (A) $OD_{600}$ of the cultures is shown. (B to D) CFU per milliliter is shown as total CFU per milliliter (closed symbols) and CFU per milliliter of mutants with reduced $\sigma^E$ activity (open symbols) for cultures grown in LB-Mg (B), LB-MOPS (C), or LB-MOPS-Mg (D). Data shown are averages with standard deviations from at least 4 independent cultures.

Buffering of the growth medium did not have a large effect on survival of the Δ*rseA* strain. However, mutants that arose in Δ*rseA* cultures grown in LB-MOPS exhibited a wider range of $\sigma^E$ activity than did those obtained from cultures grown in unbuffered LB medium or in LB-Mg. Pale purple colonies with about 2-fold lower $\sigma^E$ activity than the parent Δ*rseA* strain were seen frequently on lactose–TTC–X-Gal agar, while only deep red colonies with 6- to 70-fold lower $\sigma^E$ activity were isolated from cultures in unbuffered LB medium or LB-Mg. A similar high level of diversity of mutants with various amounts of $\sigma^E$ activity was found when the Δ*rseA* strain was grown in the MOPS-based EZ rich defined medium. The phenotype of a pale purple mutant isolated from a culture grown in LB-MOPS was stable when the mutant was grown in MOPS-buffered LB medium for 10 days. When the same mutant was grown in unbuffered LB medium for 10 days, deep red mutants with further decreased $\sigma^E$ activity accumulated, although their accumulation was delayed by 6 days, compared to a Δ*rseA* strain. Thus, 2-fold decreased $\sigma^E$ activity was sufficient to allow survival for 10 days in MOPS-buffered medium, but lower $\sigma^E$ activity was required for survival beyond day 6 in unbuffered LB medium. This result indicates that buffering of the medium partially relieves the lethal phenotype associated with elevated $\sigma^E$ activity.

**Overexpression of $\sigma^E$-dependent sRNAs in the Δ*rseA* mutant makes a significant contribution to SDS sensitivity and stationary-phase lethality.** During envelope stress, $\sigma^E$ reduces the load on the outer membrane biogenesis systems by transcribing the RybB and MicA sRNAs, which inhibit the expression of every major porin as well as several other genes that affect the envelope (10). These sRNAs are highly expressed in the Δ*rseA* mutant, resulting in clear reductions in the levels of OmpC, OmpF, and OmpA in the outer membrane (Fig. 7A). To examine whether sRNA overexpression contributed to the phenotype of the Δ*rseA* strain, double and triple mutants lacking one or both of the sRNAs were made in the Δ*rseA* background. OmpC amounts were restored in the Δ*rseA* Δ*rybB* mutant (Fig. 7A), but SDS sensitivity was only partially reversed (Fig. 7B). OmpA amounts in the Δ*rseA* Δ*micA* mutant were about one-half of those found in the wild-type strain (Fig. 7A), and a moderate reduction in SDS sensitivity was observed (Fig. 7B). Deletion of both the *rybB* and *micA* genes in the

 

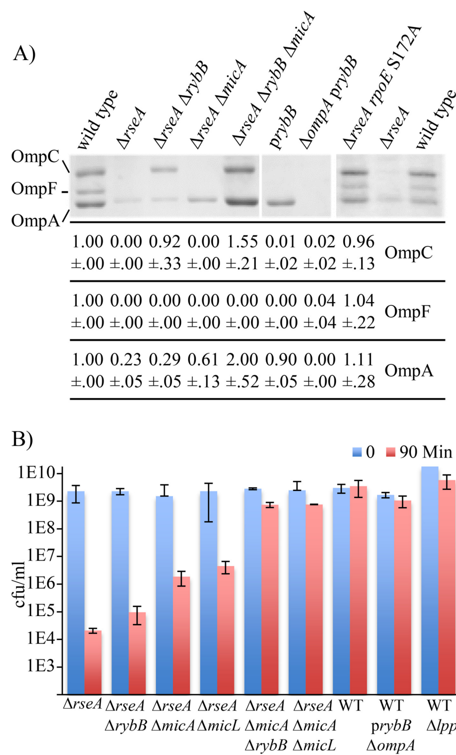


**FIG 7** Levels of the major outer membrane porins are severely reduced in the Δ*rseA* strain and are completely or partially restored by deletion of sRNAs or mutations that reduce $\sigma^E$ activity. (A) A representative gel with outer membrane preparations separated by 6 M urea-SDS-PAGE is shown. Quantification of the levels of OmpC, OmpF, and OmpA relative to those in the wild-type (WT) strain (averages and standard deviations from multiple biological repeats) are shown in the table below. (B) CFU per milliliter of the indicated strains before (0) and 90 min after treatment of stationary-phase cultures with 5% SDS is shown. Data presented are averages with standard deviations from at least 2 independent cultures.

Δ*rseA* strain restored OmpC and OmpA but not OmpF to levels found in wild-type cells (Fig. 7A) and reversed the SDS sensitivity of the strain (Fig. 7B). We do not understand why OmpF levels were not restored in the Δ*rseA* Δ*rybB* mutant, despite the fact that RybB has been demonstrated to negatively regulate *ompF* expression (10). Regulation of *ompF* expression is known to be complex and is inhibited by other regulators such as CpxR and OmpR, either of which could keep expression levels low in the Δ*rseA* Δ*rybB* background (35, 36). Levels of all of the porins, as well as SDS tolerance, were restored in the Δ*rseA rpoE* S172A mutant (Fig. 5C and 7A). Interestingly, low porin levels alone are not sufficient to explain the SDS sensitivity of the Δ*rseA* mutant. When the *ompA* gene was deleted and the *rybB* gene was overexpressed from a plasmid in a wild-type strain, OmpC, OmpF, and OmpA were nearly undetectable but the SDS sensitivity was similar to that of wild-type cells (Fig. 7).

To determine whether high levels of RybB and MicA play a role in stationary-phase lethality in addition to SDS sensitivity, the Δ*rseA* Δ*rybB* Δ*micA* strain was grown into stationary phase. The $OD_{600}$ did not decrease to the same extent as that of the Δ*rseA* strain, although it did decrease more than that of the wild-type culture (Fig. 8A). The appearance of mutants with low $\sigma^E$ activity was delayed by 5 days for the triple mutant, compared to the Δ*rseA* strain, and approximately 5 to 10% of the culture still had high levels of $\sigma^E$ activity by day 10, suggesting that aberrant expression of MicA and RybB made a significant contribution to the reduced viability of the Δ*rseA* mutant in stationary phase (Fig. 8B). These effects were not due to a reduction in $\sigma^E$ activity in the triple mutant, because the $\sigma^E$ activity of the Δ*rseA* Δ*rybB* Δ*micA* strain was similar to that of the Δ*rseA* single mutant.

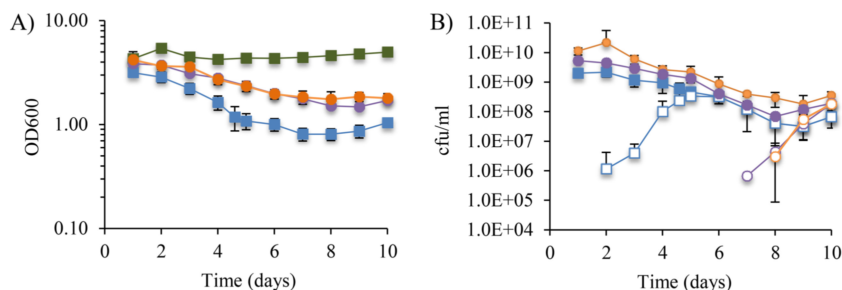


**FIG 8** MicA, RybB, and MicL sRNAs are responsible for the instability of the Δ*rseA* strain during the first part of stationary phase. The $OD_{600}$ (A) and CFU per milliliter, as total CFU per milliliter (closed symbols) and CFU per milliliter of mutants with reduced $\sigma^E$ activity (open symbols) (B), of Δ*rseA* (blue squares), Δ*rseA* Δ*micA* Δ*rybB* (purple circles), Δ*rseA* Δ*micA* Δ*rybB* Δ*micL* (orange circles), and wild-type (green squares) cultures grown in LB medium, measured at the indicated times, are shown. Data are averages with standard deviations from at least 2 independent cultures.

In addition to the sRNAs that regulate porin expression, $\sigma^E$ transcribes the MicL sRNA, which blocks translation of the mRNA encoding Lpp (13). Lpp is the most abundant protein in *E. coli* and connects the outer membrane to the peptidoglycan layer, enhancing the structural integrity of the cell envelope (37). Deleting MicL in the Δ*rseA* background increased the survival of stationary-phase cells after SDS treatment to a similar extent as deleting MicA alone (Fig. 7B). Deleting both the *micL* and *micA* genes in the Δ*rseA* strain prevented lysis (Fig. 7B), indicating that the Δ*rseA* strain can be stabilized by alleviating the repression of target gene expression by either MicA and RybB or MicA and MicL. As we observed with porin levels, reduced amounts of Lpp alone were not responsible for the SDS sensitivity, because stationary-phase cultures of a Δ*lpp* mutant in an otherwise wild-type strain were not sensitive to SDS (Fig. 7B).

To determine whether high levels of MicL were responsible for the remaining stationary-phase instability of the Δ*rseA* Δ*micA* Δ*rybB* strain, we constructed the Δ*rseA* Δ*micA* Δ*rybB* Δ*micL* quadruple mutant. Stationary-phase survival was marginally improved (Fig. 8). Colonies with reduced $\sigma^E$ activity did not appear until after 8 days of culture growth, and approximately 50% of the colonies still had elevated $\sigma^E$ activity by day 10, as opposed to 5 to 10% for the Δ*rseA* Δ*micA* Δ*rybB* strain.

## DISCUSSION

**Tuning $\sigma^E$ activity to the proper level is necessary for outer membrane homeostasis.** The cell envelope of Gram-negative bacteria is critical for maintaining cell integrity. Its composition can be altered to meet challenges imposed by the environment, and $\sigma^E$ plays an essential role in maintaining envelope homeostasis (5). Here we demonstrate not only that loss of $\sigma^E$ activity is toxic to *E. coli* but also that elevated levels of $\sigma^E$ activity are deleterious. Both the Δ*rseA* strain with excess $\sigma^E$ activity and Δ*rpoE* strains with no $\sigma^E$ activity (containing suppressor mutations to support viability) exhibit increased membrane permeability (38). It is only when $\sigma^E$ activity is within the appropriate range, neither too low nor too high, that proper envelope homeostasis is maintained. Interestingly, expression of the RybB, MicA, and MicL sRNAs plays a central role in cellular defects arising from both limited and excessive $\sigma^E$ activity. Cell viability following depletion of $\sigma^E$ activity could be restored by expressing the *micL*, *rybB*, or *micA* gene from the $\sigma^{70}$-dependent promoter (10, 13). Conversely, deleting the *micL*, *rybB*, and *micA* genes from the Δ*rseA* mutant restored membrane integrity and significantly delayed the onset of stationary-phase defects. Taken together, these data demonstrate that the sRNAs are a critical part of the $\sigma^E$ response and their expression must be appropriately fine-tuned to match the needs of the cells.

Recent work with the Cpx envelope stress response parallels our finding that $\sigma^E$ activity must be properly controlled (39). The Cpx response is activated by disruption of peptidoglycan homeostasis and helps *E. coli* survive treatment with $\beta$-lactam antibiotics that block peptidoglycan synthesis. Similar to $\sigma^E$, the level of Cpx activity must

be appropriately modulated; both too little activity and too much are deleterious. Mutants lacking the Cpx response are more sensitive to $\beta$-lactam antibiotics, while constitutive activation causes peptidoglycan-associated problems, including morphological changes, cell division defects, and increased sensitivity to $\beta$-lactams (39).

**What is the cause of stationary-phase lethality due to elevated $\sigma^E$ activity?** A large part of the stationary-phase lethality of the Δ*rseA* strain can be attributed to increased membrane permeability. Stabilizing the membranes of Δ*rseA* cells by supplementing the growth medium with $Mg^{2+}$, which bridges negative charges on the LPS and strengthens the permeability barrier, eliminated the SDS sensitivity, nearly reversed the decrease in optical density following entry into stationary phase, and delayed the appearance of mutants with reduced $\sigma^E$ activity. Increased pH of the growth medium in stationary phase appeared to exacerbate the membrane defects of the Δ*rseA* strain because buffering to neutral pH decreased the SDS sensitivity, although not to the same extent as supplementation with $Mg^{2+}$. Growth in medium that was both buffered and supplemented with $Mg^{2+}$ had the largest effect on survival of the Δ*rseA* strain in stationary phase. Mutants with low levels of $\sigma^E$ activity arose but did not take over the cultures during the time frame of the experiment. The appearance of these mutants with low levels of $\sigma^E$ activity suggests that Δ*rseA* cells are sensitive to stresses present later in stationary phase that are different, in nature and/or severity, from those encountered during early stationary phase and the deleterious effects of these stresses cannot be fully mitigated by the extent of membrane stabilization provided by $Mg^{2+}$ supplementation and pH neutralization.

The membrane permeability defects and stationary-phase lethality are caused by elevated $\sigma^E$ activity and are not due to an unrelated effect of the *rseA* deletion. Both defects were reversed by mutations in the Δ*rseA* strains that decreased $\sigma^E$ activity. Since the only known role of $\sigma^E$ in cells is to direct transcription, aberrant expression of $\sigma^E$-dependent genes is responsible for the phenotypes. Our data show that high levels of the RybB and MicA sRNAs are major contributing factors. Deleting RybB and MicA from a Δ*rseA* strain reversed the permeability defects and significantly delayed the appearance of mutants with low $\sigma^E$ activity. MicA and RybB regulate the expression of all major porin genes, such that Δ*rseA* cells exhibit dramatic reductions in outer membrane porins. Low porin levels are known to result in the appearance of phospholipids in the outer leaflet of the outer membrane, which destabilizes the structure of the outer membrane and increases the sensitivity of the bacteria to SDS and hydrophobic antibiotics (40, 41). However, the membrane permeability defects of the Δ*rseA* strain are not due solely to low levels of porins. When porin levels were reduced by deleting the *ompA* gene and overexpressing *rybB* in a wild-type background, the cells were still resistant to 5% SDS. MicA has additional targets beyond porin mRNAs, and reductions in their expression in addition to *ompA* may contribute to the phenotype (10). For example, the MicA regulon includes *pal*, *lpxT*, and *ycfS*, all of which encode proteins that have the potential to affect outer membrane integrity (10). LpxT is involved in lipid A biosynthesis, Pal plays a role in maintaining outer membrane integrity in complex with the Tol protein, and YcfS is a L,D-transpeptidase involved in peptidoglycan maturation (42–44).

In addition to MicA and RybB, the MicL sRNA contributes to phenotypes associated with the Δ*rseA* strain. Lpp, the only known target of MicL, stabilizes the outer membrane, and complete deletion of *lpp* results in leakage of periplasmic contents and increased outer membrane vesiculation (13, 45). Deletion of MicL from the Δ*rseA* strain increased survival following SDS treatment, completely reversed SDS sensitivity when combined with the Δ*micA* allele, and increased stationary-phase survival when combined with deletions of the *rybB* and *micA* genes. The observations that the stationary-phase instability of the Δ*rseA* strain was not completely eliminated in the quadruple mutant and was achieved only by decreasing $\sigma^E$ activity indicate either that aberrant overexpression of members of the protein arm of the $\sigma^E$ regulon is deleterious when

the cultures persist longer into stationary phase or that expression of $\sigma^E$-dependent sRNAs is required for survival later in stationary phase.

The membrane permeability defects of the Δ*rseA* strain are also apparent in exponential phase, indicating that these defects are inherent to elevated $\sigma^E$ activity and are not due to stationary-phase-specific changes in physiology. We do not currently know whether the Δ*rseA* cells would fail to survive if the cultures were maintained in exponential phase for sufficient time to allow selection of mutants with reduced $\sigma^E$ activity or whether stationary phase imposes additional stresses that make membrane integrity important for survival.

**Mutations in the *rpoE* gene reduce $\sigma^E$ activity and appear at many residues shown to contact the −35 region of promoter DNA.** In the course of these experiments, we isolated a series of mutations in *rpoE* that reduced its activity. Sigma factors bind to promoter DNA with two conserved domains (3). Domain 4 binds to the −35 promoter motif and helps position RNA polymerase properly on the DNA. Domain 2 binds to the −10 promoter motif and helps to separate the DNA strands so that the polymerase can access the template strand and initiate RNA synthesis. Most of the mutations we recovered in *rpoE* map to domain 4, and many were shown to form contacts with the −35 motif in the cocrystal structure of isolated domain 4 bound to DNA (29). Interestingly, only one mutation in domain 2 was recovered, and the mutation reduced the stability of $\sigma^E$ in the cells, while having little effect on specific activity. The failure to recover mutations in domain 2 residues that contact the DNA suggests that domain 2 of $\sigma^E$ is less tolerant to mutation than domain 4. The importance of domain 2-promoter interactions has been clearly demonstrated for the housekeeping sigma factor $\sigma^{70}$, which can direct transcription from promoters with an extended −10 region and no −35 region (2). However, alternative sigma factors have more stringent promoter recognition properties, leading to the idea that interactions with both regions of the promoter are critical (2). The appearance of mutations in many of the residues that contact the DNA in the −35 region demonstrates that the interaction between domain 4 of $\sigma^E$ and the −35 promoter motif can be weakened while still supporting transcription initiation.

**Does $\sigma^E$ direct lysis of viable but nonculturable cells during early stationary phase?** *E. coli* cultures are known to accumulate a population of nonculturable cells following entry into stationary phase (28, 46). The discrepancies between the optical densities and CFU per milliliter for wild-type and Δ*rseA* strains have led to the hypothesis that elevated $\sigma^E$ activity activates a $\sigma^E$-dependent cell death pathway that results in lysis of this subpopulation of nonculturable cells and concomitant reduction in the optical density (25–27). The work presented here suggests that lysis of this nonculturable cell population is due to the physical consequences of the cellular defects associated with high $\sigma^E$ activity. Early-stationary-phase *E. coli* cultures have been shown to contain relatively large subpopulations of cells that do not form colonies and exhibit extensive protein oxidation (46). Both the Cpx and $\sigma^E$ envelope stress responses are activated to a greater extent in these cells than in the culturable cells, indicating that cell envelope homeostasis is disrupted (46). Therefore, it is likely that the membrane perturbations and other defects associated with the Δ*rseA* mutation further destabilize the nonculturable population, resulting in lysis, whereas these cells remain intact but do not form colonies in a wild-type background. Furthermore, our findings show that the deleterious effects of high $\sigma^E$ activity are not limited to the nonculturable population present during the first few days in stationary phase but ultimately lead to lethality for all cells with elevated $\sigma^E$ activity.

**What is the role of $\sigma^E$ during entry into stationary phase?** The activity of $\sigma^E$ does increase during entry into stationary phase, although not to the same high levels seen in strains lacking *rseA* or overexpressing the *rpoE* gene (20, 21). Entry into stationary phase is accompanied by a large change in cell physiology that involves remodeling of the outer membrane, including decreases in outer membrane protein contents and increases in LPS contents (47). Rather than directing cell lysis, a more likely role for $\sigma^E$

**TABLE 2** Strains and plasmids used

| Strain or plasmid | Genotype or characteristics[a] | Source or reference, P1$_{vir}$ donor strain, and/or description |
|---|---|---|
| Strains | | |
| CAG45113 | MG1655 Δ*lacX74* | |
| SEA001 | MG1655 ϕλ[*rpoH*p3::*lacZ*] Δ*lacX74* | 20 |
| SEA2000 | SEA001 Δ*rseA nadB*::Tn*10* | 20 |
| SEA6462 | SEA001 Δ*rseA* Δ*yfiC*::*kan* | This work; P1 donor JW2559 (51), Δ*yfiC*::*kan* transduced into SEA2000 |
| SEA6647 | CAG45113(pJV300) | This work; CAG45113 transformed with plasmid pJV300 |
| SEA6649 | CAG45113(pKP109-6) | This work; CAG45113 transformed with plasmid pKP109-6 |
| SEA6713 | SEA001 Δ*rseA* Δ*yfiC* | This work; *kan* marker excised from SEA6462 by Flp recombinase |
| SEA6718 | SEA001 Δ*rseA* Δ*yfiC* Δ*rybB*::*kan* | This work; P1 donor KMT197 (32), Δ*rybB*::*kan* transduced into SEA6713 |
| SEA6759 | SEA001 Δ*rseA* Δ*yfiC* Δ*rybB*::*kan* Δ*micA*::*cam* | This work; P1 donor G897 (52), Δ*micA*::*cam* transduced into SEA6718 |
| SEA6767 | SEA001 Δ*rseA* Δ*yfiC* Δ*micA*::*cam* | This work; P1 donor G897 (52), Δ*micA*::*cam* transduced into SEA6713 |
| SEA6771 | CAG45113 Δ*ompA*::*kan*(pKP109-6) | This work; P1 donor JW0940 (51), Δ*ompA*::*kan* transduced into SEA6649 |
| SEA278 | SEA001 Δ*rseA* Δ*yfiC* Δ*micL*::*kan* | This work; P1 donor JW1863 (51), Δ*micL*::*kan* transduced into SEA6713 |
| SEA276 | SEA001 Δ*rseA* Δ*yfiC* Δ*micA*::*cam* Δ*micL*::*kan* | This work; P1 donor G897 (52), Δ*micA*::*cam* transduced into SEA278 |
| SEA281 | SEA001 Δ*rseA* Δ*yfiC* Δ*micL* Δ*micA*::*cam* Δ*rybB*::*kan* | This work; P1 donor KMT197 (32), Δ*rybB*::*kan* transduced into SEA276 after excision of *kan* marker from *micL* by Flp recombinase |
| SEA272 | SEA001 Δ*lpp*::*kan* | This work; P1 donor JW1667 (51), Δ*lpp*::*kan* transduced into SEA001 |
| Spontaneous mutants | | |
| SEA6525 | SEA001 Δ*rseA yfiC*::*kan rpoE* R171C | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6526 | SEA001 Δ*rseA yfiC*::*kan rpoE* L10P | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6527 | SEA001 Δ*rseA yfiC*::*kan rpoE* S172A | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6542 | SEA001 Δ*rseA yfiC*::*kan rpoE* L127Q | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6543 | SEA001 Δ*rseA yfiC*::*kan rpoE* V170 M | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6544 | SEA001 Δ*rseA yfiC*::*kan rpoE* R149Q | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6663 | SEA001 Δ*rseA yfiC*::*kan rpoE* R173C | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA6666 | SEA001 Δ*rseA yfiC*::*kan rpoE* A177V | This work; spontaneous mutant of SEA6462 isolated after growth in LB broth |
| SEA7008 | SEA001 Δ*rseA nadB*::Tn*10 rpoE* E126K | This work; spontaneous mutant of SEA2000 isolated after growth in LB broth |
| SEA7012 | SEA001 Δ*rseA nadB*::Tn*10 rpoE* R176Q | This work; spontaneous mutant of SEA2000 isolated after growth in LB broth |
| SEA7035 | SEA001 Δ*rseA nadB*::Tn*10 rpoE* A60P | This work; spontaneous mutant of SEA6462 isolated after growth in EZ rich medium |
| SEA7036 | SEA001 Δ*rseA nadB*::Tn*10 rpoE* S155I | This work; spontaneous mutant of SEA6462 isolated after growth in EZ rich medium |
| SEA7037 | SEA001 Δ*rseA nadB*::Tn*10 rpoE* F175S | This work; spontaneous mutant of SEA6462 isolated after growth in EZ rich medium |
| Plasmids | | |
| pJV300 | Vector, ColE1 ori rep, IPTG inducible, Ap$^r$ | 53 |
| pKP109-6 | *rybB* in pJV300, Ap$^r$ | Gift from J. Vogel; *rybB* under control of Plac promoter |
| pLC245 | *rpoE* in pTrc99a, Ap$^r$ | 9 |
| pTrc99a | Vector, pBR322 ori, IPTG inducible, Ap$^r$ | |

[a]Ap$^r$, ampicillin resistant.

in stationary phase is to help orchestrate these changes through transcription of its regulon to the appropriate extent at the appropriate time.

**Concluding thoughts.** Stress responses provide extra protection for cells in the face of harm. Therefore, it might be predicted that having a response on at an elevated level all the time would render the cells more stress resistant. For example, the Rcs phosphorelay is activated by damage to the peptidoglycan layer, and constitutively activating it increases survival in the presence of β-lactam antibiotics (48). However, other stress responses are tuned to combat particular sets of importune conditions, and expression of the genes required to cope with such conditions can actually be deleterious in the absence of stress. As shown for the $\sigma^E$ and Cpx responses, it is the precise regulation of the stress responses, both activation to the appropriate extent in response to specific signals and downregulation as stress is alleviated, that ensures cell survival.

## MATERIALS AND METHODS

**Strains, plasmids, media, and growth conditions.** Strains and plasmids used in this work are described in Table 2. Cultures were grown in LB medium adjusted to pH 7.3 and supplemented when indicated with 20 mM $MgCl_2$ or 40 mM MOPS adjusted to pH 7.3. The defined rich MOPS medium (EZ rich medium) was purchased from Teknova. Lactose–TTC–X-Gal agar contained 1% lactose, 50 μg/ml

TTC, 50 $\mu$g/ml X-Gal, 2 g/liter yeast extract, 10 g/liter tryptone, 5 g/liter NaCl, and 1.5% agar. Selection for the plasmids was performed using 100 $\mu$g/ml ampicillin. Because studies of $\sigma^E$ can involve mutants with thermosensitivity at elevated temperatures, all cultures were grown at 30°C, with aeration. Mutant alleles were moved into the indicated strains using P1 transduction.

**Stationary-phase lethality studies.** For the stationary-phase growth studies, strains were first streaked onto lactose–TTC–X-Gal agar to verify the expected levels of $\sigma^E$ activity. One isolated colony for each strain was grown at 30°C in 1 ml LB broth until an $OD_{600}$ of ~1.0 was reached. Cultures were diluted by a factor of $10^{-5}$ in fresh LB medium, and 5 $\mu$l of the diluted cells was used to inoculate 4 ml LB broth, supplemented with MOPS and/or $MgCl_2$ as indicated. The low cell count in the inoculates (<50 bacteria) reduced the probability that a mutant with low $\sigma^E$ activity would be present at the beginning of the growth of Δ*rseA* strains. Cultures were incubated for 10 days at 30°C, with aeration. At the indicated times, the absorbance of the cultures was measured, and the CFU were determined on LB agar or lactose–TTC–X-Gal agar. The pH of MOPS-buffered cultures was monitored and was shown to remain the same, whereas the pH of unbuffered cultures increased over the 10-day period.

**Plasmid-induced lethality and plasmid stability studies.** Overnight cultures in LB medium with ampicillin of the wild-type strain SEA6003 carrying the plasmid pLC245 (for overexpression of $\sigma^E$) or the empty vector control plasmid pTrc99a were diluted into fresh LB medium with ampicillin to an $OD_{600}$ of 0.025 and were incubated at 30°C, with aeration. When the cultures reached an $OD_{600}$ of ~0.15, the cells were pelleted by centrifugation and the pellets were resuspended in fresh LB medium with 25 $\mu$M IPTG and without ampicillin. The cultures were then incubated for 6 days at 30°C, with aeration. At the indicated times, the $OD_{600}$ was measured and the CFU per milliliter were determined on LB plates (total CFU per milliliter) and LB plates supplemented with ampicillin (CFU per milliliter of bacteria still carrying the plasmid).

**Membrane sensitivity tests.** To assess membrane sensitivity in stationary phase, strains were first streaked onto lactose–TTC–X-Gal agar to verify the expected levels of $\sigma^E$ activity. Cultures were then inoculated with isolated colonies and grown for ~28 h at 30°C, with aeration. Aliquots of the cultures were mixed with SDS to a final concentration of 5% or with an equal volume of water as a control and were incubated at 30°C, with aeration. At the indicated times, the $OD_{600}$ of the cultures was measured and the CFU per milliliter were determined. For membrane sensitivity in exponential phase, overnight cultures were diluted to an $OD_{600}$ of 0.025 and incubated at 30°C, with aeration. When the cultures reached an $OD_{600}$ of 0.1 to 0.2, SDS was added to a final concentration of 5%, rifampin was added to a final concentration of 8 $\mu$g/ml, or an equal volume of water was added. The $OD_{600}$ was measured and the CFU per milliliter were determined at the indicated times after the addition of SDS or rifampin.

**$\beta$-Galactosidase assays.** $\beta$-Galactosidase assays were performed as described previously (19).

**Western blot hybridization.** Cells were grown in LB medium to an $OD_{600}$ of 0.8, and whole-cell extracts were prepared as described previously (49). Proteins extracted from 0.16 OD units of cells were separated by SDS-PAGE and transferred to Amersham Hybond-P polyvinylidene difluoride (PVDF) transfer membranes. Blots were probed with anti-$\sigma^E$ and anti-RpoB polyclonal antibodies, and bound complexes were detected with horseradish peroxidase-coupled donkey anti-rabbit IgG. The secondary antibody was visualized with the Amersham ECL reagent according to the manufacturer's recommendations, using HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ). Films were scanned using an Epson Perfection 3170 photo scanner, and the protein bands were quantified using ImageQuant 5.2 software. The intensity of each $\sigma^E$ band was normalized to that of RpoB to control for loading differences.

**Outer membrane preparations and electrophoresis.** Outer membranes were separated from inner membranes on the basis of their insolubility in 0.5% sarcosyl, as described previously (6). Proteins in the resulting membrane extracts were separated by 6 M urea-SDS-PAGE (10% polyacrylamide) and visualized using Coomassie blue. Gels were scanned, and the amounts of proteins were quantified using ImageQuant 5.2 software.

**Mapping of spontaneous mutations.** Because the mutants had reduced $\sigma^E$ activity, we first sequenced the *rpoE* gene to determine whether the mutations were located in that region. The *rpoE* gene and its promoter were amplified with the primers rpoEnadBmid (5′-CCGCTACCGATAATCAACACG-3′) and rseB1R (5′-GCTGATGAATGACAGCTCG-3′), using *Taq* polymerase. Amplified fragments were purified using QIAquick spin columns and were sequenced using the primers rpoE1 (5′-ACTGGTAGTGC GCTATCAGC-3′) and rpoE1R (5′-TTTACAGCAATCCGATACAGCC-3′). All *rpoE* mutations were moved by P1 transduction to a clean Δ*rseA* strain, to ensure that the phenotypes were linked to the mutations.

To map mutations not located in *rpoE*, a library of mini-Tn*10*::*kan* insertions was made in the wild-type *E. coli* strain. The resulting transposon library was moved into the mutant strains by P1 transduction, and colonies reversing the phenotype of interest were isolated. The mini-Tn*10*::*kan* insertion site was determined using a PCR-based protocol, with primers hybridizing to the transposon and a random primer (32, 50). The resulting PCR fragments were sequenced to identify the region of the chromosome linked to the mutation, although the precise mutation was not identified.

**Structural analysis.** *In silico* analysis of the $\sigma^E$ point mutations was performed using the $\sigma^E$ structures with Protein Data Bank (PDB) accession numbers 1OR7 (15), 4LUP (30), and 2H27 (29).

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