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Activation of Toxin-Antitoxin System Toxins Suppresses Lethality Caused by the Loss of σE in Escherichia coli

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ABSTRACT

σE, an alternative σ factor that governs a major signaling pathway in envelope stress responses in Gram-negative bacteria, is essential for growth of Escherichia coli not only under stressful conditions, such as elevated temperature, but also under normal laboratory conditions. A mutational inactivation of the hicB gene has been reported to suppress the lethality caused by the loss of σE, hicB encodes the antitoxin of the HicA-HicB toxin-antitoxin (TA) system; overexpression of the HicA toxin, which exhibits mRNA interferase activity, causes cleavage of mRNAs and an arrest of cell growth, while simultaneous expression of HicB neutralizes the toxic effects of overproduced HicA. To date, however, how the loss of HicB rescues the cell lethality in the absence of σE and, more specifically, whether HicA is involved in this process remain unknown. Here we showed that simultaneous disruption of hicA abolished suppression of the σE essentiality in the absence of hicB, while ectopic expression of wild-type HicA, but not that of its mutant forms without mRNA interferase activity, restored the suppression. Furthermore, HicA and two other mRNA interferase toxins, HigB and YafQ, suppressed the σE essentiality even in the presence of chromosomally encoded cognate antitoxins when these toxins were overexpressed individually. Interestingly, when the growth media were supplemented with low levels of antibiotics that are known to activate toxins, Escherichia coli cells with no suppressor mutations grew independently of σE. Taken together, our results indicate that the activation of TA system toxins can suppress the σE essentiality and affect the extracytoplasmic stress responses.

IMPORTANCE

σE is an alternative σ factor involved in extracytoplasmic stress responses. Unlike other alternative σ factors, σE is indispensable for the survival of E. coli even under unstressed conditions, although the exact reason for its essentiality remains unknown. Toxin-antitoxin (TA) systems are widely distributed in prokaryotes and are composed of two adjacent genes, encoding a toxin that exerts harmful effects on the toxin-producing bacterium itself and an antitoxin that neutralizes the cognate toxin. Curiously, it is known that inactivation of an antitoxin rescues the σE essentiality, suggesting a connection between TA systems and σE function. We demonstrate here that toxin activation is necessary for this rescue and suggest the possible involvement of TA systems in extracytoplasmic stress responses.
known function. Either deletion of hicB or overexpression of ptsN downregulates multiple E. coli, Cpx, and Bae systems, presumably because they suppress the σ^E essentiality by somehow alleviating envelope stresses (12, 13).

Subsequently, hicB was revealed to encode an antitoxin of the HicAB toxin-antitoxin (TA) system (14). A TA system is a set of two adjacent genes, encoding a toxin and an antitoxin, that form an operon. Toxins are proteins that exert a variety of harmful effects on the toxin-producing bacterium itself, but their toxicity is normally suppressed by their cognate, unstable antitoxins, which are either proteins or RNAs (15). TA systems are classified into five classes according to the antitoxin type (16). In the type I TA systems, antitoxins are antisense RNAs that repress toxin synthesis, while the antitoxins of the type II TA systems are proteins that form stable complexes with toxins to inhibit their functions (15). The antitoxin of the type III TA systems is also an RNA, but in this case, the antitoxin RNA directly binds to and suppresses the cognate toxin (17). Antitoxins of the type IV (18) and V (19) TA systems are sequence-speciﬁc endoribonucleases termed mRNA interferases (20). Stress conditions that inhibit transcription or translation, such as nutritional starvation or exposure to antibiotics, promote the degradation of antitoxins by cellular proteases, such as Lon and ClpAP/ClpXP, leading to the liberation of active toxins to cleave mRNAs (21). Although TA systems were first identified as Lon and ClpAP/ClpXP, leading to the liberation of active toxins and thereby compensates for the lack of σ^E in a HicA-independent manner. In this study, we demonstrated that the mRNA interferase activity of HicA is necessary for suppression of σ^E essentiality by the hicB null mutation. We also found that two other toxins, when activated, have a potential ability to support the cell growth of E. coli in the absence of σ^E and that low concentrations of antibiotics that activate toxins suppress the σ^E essentiality. Our results thus highlight the relationship between ESR systems and toxin functions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, plasmids, and primers used in this study are listed in Tables S1 to S3, respectively, in the supplemental material. Their construction is described in Text S1. E. coli K-12 strain W3110 was used as the wild-type strain. The Keio collection (34), the ASKA library (35), and pCP20 (36) were provided by the National BioResource Project-E. coli (National Institute of Genetics, Mishima, Japan). BD2314 was provided by the Coli Genetic Stock Center (Yale University, New Haven, CT). Unless indicated otherwise, cells were grown in liquid or on solid L medium (containing 10 g/liter Bacto trypticase, 5 g/liter yeast extract, and 5 g/liter NaCl; the pH was adjusted to 7.2 by using NaOH).

Cotransduction frequency analysis. Strains SN332 and YD710, carrying the linked ΔrpoE::kan ung::Tn10 and ΔrpoE::kan zfh::Tn10 markers, respectively, were used as donor strains to examine the essentiality of rpoE by P1-mediated transduction (37). Tetracycline (TC)-resistant transductants that had received ung::Tn10 or zfh::Tn10 were selected on an L agar plate supplemented with 12.5 μg/ml TC by incubation for 48 h at 30°C. After purification on an L agar plate without antibiotics, single colonies were replicated on an L agar plate supplemented with 12.5 μg/ml kanamycin (KM) or 12.5 μg/ml TC to determine whether the ΔrpoE::kan allele had been cotransduced with ung::Tn10 or zfh::Tn10. Where specified, isopropyl-β-D-thiogalactopyranoside (IPTG) or antibiotics were added to the media at the indicated concentrations throughout the procedures. The cotransduction frequency (CF) was calculated according to the following equation: CF (%) = number of colonies resistant to both KM and TC/total number of TC-resistant colonies screened. To confirm that Km-resistant transductants carried the FRT-kan-FRT gene, TC-resistant transductants were randomly picked up and subjected to colony PCR with primers that were designed to amplify the rpoE gene region.

Disk diffusion assay. A W3110-derived, suppressor-free ΔrpoE::kan ung::Tn10 strain was constructed as described above, except that 10 μg/ml erythromycin (EM) was included in the medium throughout the procedure. Cells of this strain were suspended in 300 μl of L medium (the optical density at 660 nm was approximately 0.05), washed with L medium, and resuspended in 1 ml of L medium. Five microliters of the cell suspension was mixed with 2.5 ml of melted soft L agar (0.55% agar) and quickly poured onto a solid L agar plate. A sterile filter paper disk containing 5 μl of 50-mg/ml ampicillin, 20-mg/ml chloramphenicol (CP), 50-mg/ml EM, 50-mg/ml spectinomycin, 5-mg/ml rifampin, 10-mg/ml nalidixic acid, ethanol, or sterile water was placed onto the solidified soft agar (for preparation of the antibiotic solutions, CP, EM, and rifampin were dissolved in ethanol, whereas the others were dissolved in water). Plates were incubated for 24 h at 30°C. For evaluation of the effect of serine hydroxamate, a lawn of the suppressor-free ΔrpoE::kan ung::Tn10 strain was prepared by spreading 5 μl of the cell suspension on an L agar plate, and a sterile filter paper disk containing 20 μl of 50-mg/ml serine hydroxamate (in water) was placed on it. Plates were incubated for 36 h at 30°C.

In vivo RNA cleavage analysis by primer extension. To evaluate the mRNA interference activity of HicA derivatives, YD105 cells carrying pTH19cr, pTH-HicA, pTH-HicA(H23A), or pTH-HicA(H38A) were grown in L medium at 30°C. At the mid-exponential growth phase, IPTG was added to a final concentration of 100 μM, and the expression of HicA or its derivatives was induced for 30 min. For YafQ derivatives, W3110 cells carrying pCA24ΔNot, ASKA-YaqQ, or ASKA-YaqQ(H76A) were
used, and protein expression was induced with 500 μM IPTG for 10 min. Total RNA was extracted from cells by using the hot phenol method essentially as described previously (38). Cleavage of the ompA mRNA was analyzed by the fluorescence-labeled primer extension procedure (39), with the following modifications. One nanomole of the 6-carboxyfluorescein (FAM)-labeled primer 6-FAM-ompA-1 (see Table S3 in the supplemental material), which was complementary to the coding region of the ompA gene (nucleotides +606 to +625 with respect to the transcription start site) (14), was mixed with 300 μg of total RNA in a final volume of 300 μl. The primers and RNAs were denatured by incubation at 90°C for 2 min and annealed by cooling to 30°C at a rate of 2°C/min, using a thermal cycler. cDNA synthesis was then carried out with Moloney murine leukemia virus reverse transcriptase (Superscript II; Invitrogen) at 42°C for 1 h in a final volume of 60 μl, according to the manufacturer’s recommendations. Next, the RNA was degraded by adding 200 μg of 1 M NaOH and incubating the sample at 70°C for 10 min, followed by neutralization with 200 μl of 1 M HCl. The synthesized cDNA was purified with an affinity column (Nucleosip; Macherey-Nagel) and precipitated with ethanol. The dried pellets were dissolved in a solution of 6 μl of Hi-Di formamide loading buffer (Applied BioSystems), heated at 95°C for 5 min, and placed on ice for 5 min. The DNA sequencing ladder used to assess cleavage sites was prepared by dideoxy DNA sequencing with a Thermo Sequenase dye primer manual cycle sequencing kit (USB), using the same primer as that used for the primer extension reactions and an ompA DNA fragment amplified by PCR as a template. The sequencing ladder and the primer extension products were separated in a 6% polyacrylamide-7 M urea sequencing gel and visualized by using a fluorescence scanner (Typhoon FLA9000; GE Healthcare).

Immunoblotting. SDS-PAGE and Western blotting were carried out under standard conditions (40, 41). To raise anti-HicA antibodies, a pentadcapeptide comprising the amino-terminal 14 amino acid residues of HicA followed by Cys (MKQSEFRRLVESQGC) was synthesized and used to immunize a rabbit. To reduce nonspecific binding of the anti-HicA antisera during immunoblotting, antibodies that react with proteins other than HicA were adsorbed by using total proteins of the ΔhicA strain as follows. Total proteins of YD626 were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. After incubation in the blocking reagent for a Can Get Signal kit (Toyobo, Osaka, Japan) for 30 min, the membranes were incubated overnight with anti-HicA antisera diluted 5,000-fold with Can Get Signal immunoreaction enhancer solution (Toyobo). The non-immune-adsorbed fraction was used to detect HicA. For immunological detection of hexahistidine-tagged YafQ, a Penta-His HRP conjugate kit (Qiagen) was used. Proteins were visualized by using an enhanced chemiluminescence substrate (ECL Prime LAS 4000 luminescence image analyzer (GE Healthcare)).

β-Galactosidase assay. Cells were cultivated overnight, diluted 5,000-fold into L medium supplemented with IPTG, and grown at 30°C for 10 h. β-Galactosidase (LacZ) activity was determined essentially as described previously (42).

RESULTS

hicA is required for the σE dispensability caused by a hicB null mutation. Previous work suggested that disruption of the hicB gene suppresses σE essentiality, based on the observation that the ΔrpoE::cat mutation can be introduced into a ΔhicB strain, but not into a wild-type (hicB+) strain or a ΔhicB strain with a complementing hicB+ plasmid, by directly selecting CP-resistant ΔrpoE transductants after P1-mediated transduction (12). However, because revertants carrying suppressor mutations in the ΔrpoE background arise easily (3), it is not completely clear whether the disruption of hicB alone is sufficient for the suppression or whether the suppression also involves some other mutation(s). To more rigorously evaluate the contribution of the hicB disruption to the suppression of the σE essentiality, we employed a cotransduction assay in which the ΔrpoE::kan allele was cotransduced as a nonselective marker with a nearby ung::Tn10 marker conferring TC resistance (Fig. 1A). When the wild-type strain W3110 carrying pTH-RpoE, a plasmid with the rpoE gene under the control of the lac promoter/operator, was used as a recipient, the cotransduction frequency of the ΔrpoE::kan allele with the ung::Tn10 allele was 87.5% (Table 1), a value comparable to that expected from the distance between the rpoE (58.28 min) and ung (58.44 min) genes (43). In contrast, the cotransduction frequencies were very low when W3110 without pTH-RpoE or with the empty vector (pTH188cr) was used as the recipient, which is in accordance with the σE essentiality. Induction of rpoE expression from pTH-RpoE by use of IPTG was not necessary for cotransduction of the ΔrpoE::kan allele, presumably due to the leaky expression of rpoE from the plasmid. Next, we examined the effects of the ΔhicB mutation on the ΔrpoE::kan cotransduction frequency. We hypothesized that if the ΔhicB mutation was sufficient to suppress the σE essentiality, then cotransduction of the ΔrpoE::kan allele would occur at similar frequencies in the presence and absence of pTH-RpoE. Indeed, this proved to be the case (Table 1). We also introduced pTrcHis2B, which overproduces the LacI repressor, enabling tight
Suppression of σ^E Essentiality by TA System Toxins

regulation of the lac promoter, into the hicB^+ ΔpoE/pTH-RpoE and ΔhicB ΔpoE/pTH-RpoE strains, and we found that the growth of the hicB^+ ΔpoE/pTH-RpoE/pTrcHis2B strain was IPTG dependent, while its ΔhicB counterpart grew irrespective of the addition of IPTG (Fig. 1B). Collectively, these results confirmed that single disruption of the hicB gene is sufficient to suppress the lethality caused by the lack of σ^E.

HicB acts as the antitoxin against the HicA toxin. To check whether HicA is involved in the suppression of σ^E essentiality, cotransduction experiments were performed using YD105, a ΔhicAB ΔpoE/pTH-RpoE strain, and we found that the growth of the hicB^+ ΔpoE/pTH-RpoE/pTrcHis2B strain was IPTG dependent, while its ΔhicB counterpart grew irrespective of the addition of IPTG (Fig. 1B). Collectively, these results confirmed that single disruption of the hicB gene is sufficient to suppress the lethality caused by the lack of σ^E.

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TABLE 2 CF of the ΔrpoE::kan marker with the ung::Tn10 marker in wild-type cells carrying ASKA clones

<table>
<thead>
<tr>
<th>ASKA clone</th>
<th>TA system type</th>
<th>IPTG concn (μM)</th>
<th>CF (%)</th>
<th>n³</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCA24NΔNot</td>
<td>II</td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>chpB</td>
<td>II</td>
<td>1,000</td>
<td>1.6</td>
<td>63</td>
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<tr>
<td>gnsA</td>
<td>II</td>
<td>1,000</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>hicA</td>
<td>II</td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>hicA</td>
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<td>19</td>
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<td>44</td>
</tr>
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<td>48</td>
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<tr>
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³ Number of transductants screened to calculate CF.

from the ASKA clone with higher concentrations of IPTG inhibited cell growth (data not shown), a sub-MIC of IPTG should be required to screen the ASKA library for TA system toxins that suppress the σ⁸ essentiality of W3110. Thus, we first determined the MICs of IPTG against strains carrying each of the ASKA clones for TA system toxins, and we then investigated the suppression of the σ⁸ essentiality at sub-MIC levels. Interestingly, cells carrying the ASKA clones for HigB and YafQ, the toxins of the type II HigB/HigA and YafQ/DinJ systems, respectively, allowed efficient cotransduction of the ΔrpoE::kan allele with the ung::Tn10 allele, suggesting that these toxins can also suppress the σ⁸ essentiality (Table 2). It should be noted that the suppression of the σ⁸ essentiality by either HigB or YafQ did not depend on HicA, because the ΔrpoE::kan allele was introductory to YD626 (ΔhicA) carrying the HigB- or YafQ-overexpressing plasmid, with cotransduction frequencies of 83.3% (n = 48) and 64.5% (n = 48), respectively. As in the case of HicA, the mRNA interferase activity of YafQ was necessary for the suppression, because the H87A mutant of YafQ, which is known to lack mRNA interferase activity (45, 46), failed to suppress the σ⁸ essentiality (Table 2). Unlike hicB, disruption of neither higA nor dinJ, the genes encoding the cognate antitoxins for HigB and YafQ, respectively, suppressed the σ⁸ essentiality (data not shown), presumably because the expression levels of HigB and YafQ from the chromosome are insufficient for suppression even in the absence of the antitoxins, at least under the growth conditions tested.

Previously identified suppressors of σ⁸ essentiality have been reported to downregulate multiple ESR systems (12, 13). We thus examined the effects on ESR systems of the toxin overexpression that suppressed the σ⁸ essentiality by using a σ⁸ reporter, rpoHP3-lacZ, and a Cpx reporter, cpxP-lacZ (see Fig. S1 in the supplemental material), failed to suppress the σ⁸ essentiality (data not shown). The effects of toxin overexpression on ESR systems were dependent on their mRNA interferase activities, as HicA (H23A) and YafQ (H87A) did not exhibit such effects.

**Suppression of σ⁸ essentiality by low levels of antibiotics.** Stress-inducing treatments, such as exposure to heat, nutrient starvation, DNA-damaging reagents, and antibiotics, destabilize antitoxins that normally neutralize their cognate toxins, resulting in release and activation of the free toxins (15, 21). Antibiotics that activate TA systems include translational inhibitors, such as CP (23, 47), spectinomycin (23), and EM (48), as well as rifampin (23) and nalidixic acid (24), which inhibit transcription and DNA replication, respectively. We supposed that stress conditions that destabilize antitoxins would also suppress the σ⁸ essentiality. To examine this possibility, we performed a cotransduction assay by using W3110 as the recipient, in the presence of low concentrations of CP or EM. We indeed found that the presence of these antibiotics at sub-MICs allowed cotransduction of the ΔrpoE::kan allele into wild-type cells at significantly high efficiencies (Table 2). It suggested that the expression levels of HigB and YafQ from the chromosome are insufficient for suppression even in the absence of the antitoxins, at least under the growth conditions tested.

Completely abolished by either the H23A or H38A mutation (Fig. 2C). These results indicate that His-23 and His-38 are functionally important residues for the mRNA interferase activity of HicA and support the previous result that the mRNA interferase activity is responsible for the detrimental effect of HicA on cell growth (14). Our results showing that the function of YD105 carrying the pTH-HicA derivative encoding the H23A or H38A mutation (Table 1) and the mRNA interferase activity of YafQ are necessary for suppression of the σ⁸ essentiality.

**Increased expression of the YafQ or HigB toxin also suppresses the σ⁸ essentiality.** The E. coli genome contains at least 36 TA systems, 17 and 19 of which belong to the type I and type II classes, respectively (15). Our results showing that the function of HicA as a toxin is responsible for the suppression of the σ⁸ essentiality prompted us to examine whether TA system toxins other than HicA have a similar activity. Eight clones for type I toxins and 14 clones for type II toxins are available from the ASKA library (without a green fluorescent protein [GFP] fusion), a complete archival set of E. coli K-12 open reading frames (ORFs) (35). In contrast to pTH-HicA, which neither suppressed the σ⁸ essentiality nor inhibited growth of W3110, the ASKA plasmid clone carrying hicA supported the growth of W3110 in the absence of σ⁸ when the expression of HicA was induced with 100 μM IPTG (Table 2). It is very likely that, in this case, a much higher level of expression of HicA from the ASKA plasmid than from pTH-HicA (data not shown) overcame the neutralization effect of the chromosomally encoded HicB protein, although it cannot be ruled out that the difference came from some effects of the N-terminally attached His tag and/or the C-terminally attached artificial peptide on the activity of ASKA-encoded HicA. As induction of HicA
of ClpP from the ASKA plasmid suppressed the $\sigma^B$ essentiality of W3110 even in the absence of low levels of antibiotics (Table 3). The protease activity of ClpP was required for the suppression, because ClpP(S111A), a proteolytically inactive form of ClpP (50), was inactive in the suppression (Table 3). We found that either loss or overexpression of Lon markedly decreased the number of TC-resistant transductants in the transduction of the ung::Tn10 allele, making it difficult to accurately estimate the cotransduction frequency (data not shown). A loss of Lon stabilizes its cellular target SulA (51), which acts as a cell division inhibitor. Disruption of the ung gene, encoding uracil-DNA glycosylase, is known to increase the mutation rate (52). It is thus conceivable that a higher mutation rate caused by the ung::Tn10 allele might induce the SOS response, which upregulates sulA expression, leading to a cell growth defect in the absence of Lon. In addition, the ung disruption might increase the frequency of spontaneous suppressor mutations for the rpoE null mutation. To address these possibilities, the rpoE::kan cotransduction assay was conducted using another nearby Tn10 marker, the zif::Tn10 allele (Table 3). W3110 containing the zif::Tn10 allele was 18.8% when W3110 carrying pTH-RpoE was used as the recipient (Table S4). Therefore, Lon is also required for the suppression of $\sigma^B$ essentiality by low levels of antibiotics.

While hicA, higB, and yafQ were identified as multicopy suppressors of $\sigma^B$ essentiality, the antibiotic-induced suppression of $\sigma^B$ essentiality was still observed with a $\Delta hicA$ $\Delta higB$ $\Delta yafQ$ triple mutant (Table 3). Also, overexpression of ClpP suppressed the $\sigma^B$ essentiality of the triple mutant, although ClpP overexpression in this strain had a detrimental effect on growth, which made it difficult to accurately determine the cotransduction frequency (Table 3). Thus, it is possible that some toxin or combination of toxins other than HicA, HigB, and YafQ can act in the antibiotic-induced suppression of $\sigma^B$ essentiality in the absence of these three toxins.

**DISCUSSION**

In this study, we demonstrated that the suppression of $\sigma^B$ essentiality by the hicB null mutation depends on the hicA gene, encoding the cognate toxin, and that the hicA(H23A) and hicA(H38A) mutants cannot act in place of wild-type hicA in this suppression. The ompA mRNA cleavage assay showed that HicA(H23A) and HicA(H38A) are defective in mRNA interferase activity. In addi-

#### Table 3 CF of ung::Tn10 and ΔrpoE::kan markers in the presence of antibiotics

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Antibiotic</th>
<th>Antibiotic concn (μg/ml)</th>
<th>CF* (%)</th>
<th>n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>CP</td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>0.5</td>
<td>2.1</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>1</td>
<td>19.0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>2</td>
<td>73.0</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>2.5</td>
<td>68.7</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>5</td>
<td>87.5</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>10</td>
<td>78.5</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>20</td>
<td>89.6</td>
<td>48</td>
</tr>
<tr>
<td>+ pCA24NΔNotI</td>
<td></td>
<td>0</td>
<td>2.1</td>
<td>47</td>
</tr>
<tr>
<td>+ ASKA-ClpP</td>
<td></td>
<td>0</td>
<td>56.3</td>
<td>48</td>
</tr>
<tr>
<td>+ ASKA-ClpP(S111A)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
</tbody>
</table>

^a CF was measured in the presence of 1 mM IPTG when the recipient cells carried ASKA plasmids.

^b Number of transductants screened to calculate CF.

ΔrpoE::kan allele in the presence of 10 μg/ml EM. Suppression at appropriate concentrations of antibiotics would be manifested as the formation of a ring of colonies around the disks. Indeed, as expected, paper disks containing EM and CP gave rise to the formation of rings around the disks (Fig. 3B), supporting the idea that these antibiotics suppressed the $\sigma^B$ essentiality when present at appropriate concentrations. Spectinomycin, rifampin, and nalidixic acid gave similar results, demonstrating the ΔrpoE strain-suppressing ability of these antibiotics. In contrast, no detectable ring was observed with ampicillin and streptomycin (Fig. 3B), although these antibiotics inhibited the growth of the ΔrpoE cells to the same degree when the $\sigma^B$ essentiality had been suppressed by the addition of 10 μg/ml EM (see Fig. S3 in the supplemental material). These results indicate that the deficiencies in the ΔrpoE strain-suppressing ability of ampicillin and streptomycin are not due to resistance or to a high susceptibility to these antibiotics. We also tested serine hydroxamate, a structural analogue of 1-serine that inhibits charging of seryl-tRNA and thereby induces amino acid starvation and liberation of toxins (47, 49). As shown in Fig. 3C, this drug caused ring formation around the disk.

The following results supported the idea that the above-described effects of antibiotics resulted from the activation of toxins. Antitoxins are preferentially degraded by Lon and ClpAP/ClpXP, which are major cytoplasmic proteases, under stressed conditions (47, 49). Therefore, a lack of these proteases should lead to the inactivation of toxins by stabilized antitoxins, whereas their overexpression should promote the activation of toxins. We found that disruption of clpP, which encodes the proteolytic subunit of ClpAP/ClpXP, abolished the suppression of $\sigma^B$ essentiality by CP or EM (Table 3). The MICs of these antibiotics for the clpP mutant were essentially the same as those for the wild-type strain (data not shown), indicating that the clpP mutation did not interfere with entrance of the antibiotics into a cell. Conversely, overexpression of rpoE::kan markers in the presence of antibiotics.
tion, we identified two other mRNA interferase toxins, HigB and YafQ, as multicopy suppressors of the $\sigma^E$ null mutation. As observed for HicA, the suppression of $\sigma^E$ essentiality by YafQ required its mRNA interferase activity. These results strongly suggest that the observed suppression of $\sigma^E$ essentiality results from activation of the mRNA interferase activities of these toxins. It has been reported that some antibiotics activate toxins (23, 24, 47, 48). Here we found that low levels of antibiotics suppressed the $\sigma^E$ essentiality. This suppression required the antitoxin-degrading proteases, supporting the idea that toxin activation can induce $\sigma^E$ dispensability. The levels of toxins required for suppression of the $\sigma^E$ essentiality appeared to be considerably low, because leaky expression of plasmid-borne hicA from the lac promoter was sufficient to support the growth of the $\Delta rpoE$ strain. Under these conditions, no detectable effect of toxins on cell growth or the cleavage of ompA mRNA was observed (data not shown). Also, the hicB disruption, which should activate intrinsic HicA, had little effect on cell growth and ompA mRNA cleavage (data not shown), which agrees with the previous observation that the overall protein profiles of cellular envelope fractions are unchanged by the hicB null mutation (12, 14).

Overexpression of mRNA interferase toxins, such as HicA, causes global translational inhibition and growth arrest (14). Therefore, global translational inhibition by toxins or inhibitors, which could reduce the accumulation of stress factors such as misfolded cell surface proteins, might be responsible for suppression of the lethality conferred by the lack of $\sigma^E$. If this were so, then translation inhibitors would generally induce $\sigma^E$ dispensability. However, this is not the case, as streptomycin did not suppress the $\sigma^E$ essentiality. It is thus possible that the suppression effect of toxins is exerted through cleavage of a specific target mRNA(s). In this case, HicA, HigB, and YafQ may have shared targets whose
cleavage results in the suppression effect. The suppression induced by antibiotics and ClpP overexpression occurred even in the absence of HicA, HigB, and YaQ. Although it cannot be ruled out that suppression occurs through toxin-independent mechanisms, these results suggest the possibility that activation of some other toxins also induces σE dispensability. However, we did not observe the suppression of σE essentiality with other toxins encoded by ASKA library plasmids, at least when they were individually overexpressed. Although it is possible that the N-terminally attached His tag and/or the C-terminally attached artificial peptide compromised their functions, the finding that toxins other than HicA, HigB, and YaQ induced no suppression may have been at least partly attributable to differences in the cellular targets. Also, some of these toxins might need to cooperate to suppress the σE essentiality.

Unlike HicA, HigB and YaQ are ribosome-dependent mRNA interferases (45, 46, 53, 54), indicating that an mRNA cleavage mechanism does not define the ability of toxins to suppress the σE essentiality. Previous primer analyses of HicA and YaQ did not reveal common target motifs (14, 45, 46); HicA apparently has no obvious consensus recognition motif, whereas YaQ preferentially cleaves mRNA at the 5′ side of adenine residues (46). However, it is possible that more accurate identification of the cleavage specificities of these toxins by using alternative techniques, such as transcriptome sequencing (RNA-seq) (28), to identify their common targets could help us to understand the mechanisms by which these TA systems function in response to envelope stress. It should be noted that the possible targets of these toxins for the suppression of σE essentiality might include non-coding RNAs that are directly or indirectly involved in cellular resistance to extracytoplasmic stresses.

Emerging evidence supports the involvement of TA systems in stress responses (21). The present study suggests that they may also be involved in ESR systems. Although the mechanism by which toxin-mediated RNA cleavage induces σE dispensability remains to be clarified, these toxins might reduce intrinsic cell surface stresses through unknown mechanisms, which could lead to less dependency on σE and other ESR systems under normal growth conditions and to increased survival upon exposure to environmental stresses. Consistent with this idea, the disruption of hicB has been reported to downregulate ESR systems (12, 13), and overexpression of HicA, HigB, or YaQ exerted similar effects (see Fig. S2 in the supplemental material). Further exploration of their cellular targets will be required to elucidate the mechanisms of suppression of the σE essentiality and the physiological roles of the TA systems in ESR systems.

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