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Activation of Toxin-Antitoxin System Toxins Suppresses Lethality Caused by the Loss of σ^E in *Escherichia coli*

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ABSTRACT

σ^E, 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 σ^E. *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 σ^E and, more specifically, whether HicA is involved in this process remain unknown. Here we showed that simultaneous disruption of *hicA* abolished suppression of the σ^E 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 σ^E 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, *E. coli* cells with no suppressor mutations grew independently of σ^E. Taken together, our results indicate that the activation of TA system toxins can suppress the σ^E essentiality and affect the extracytoplasmic stress responses.

IMPORTANCE

σ^E is an alternative σ factor involved in extracytoplasmic stress responses. Unlike other alternative σ factors, σ^E 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 σ^E essentiality, suggesting a connection between TA systems and σ^E function. We demonstrate here that toxin activation is necessary for this rescue and suggest the possible involvement of TA systems in extracytoplasmic stress responses.

Bacteria are equipped with extracytoplasmic stress response (ESR) systems that sense cell envelope stresses and cope with them by altering gene expression. *Escherichia coli* has five ESR systems (σ^E, Cpx, Bae, Rcs, and phage shock protein responses), whose functions have been well characterized (1, 2). Among them, the σ^E-dependent ESR system is considered to be the most important, because it is the only ESR system that is essential for the viability of this bacterium (3). σ^E is an alternative σ factor which is normally sequestered by the inner membrane anti-σ^E protein RseA (4, 5). Stress conditions that endanger the integrity of the cell envelope trigger a signal transduction cascade that results in sequential proteolysis of RseA by the membrane proteases DegS and RseP, leading to release from the membrane of σ^E complexed with the RseA cytoplasmic domain. The RseA cytoplasmic domain is then further degraded by cytoplasmic proteases, such as ClpXP (6–9). σ^E thus liberated interacts with the RNA polymerase core enzyme to promote transcription of σ^E regulon genes that encode stress-combating and membrane biogenesis factors, including periplasmic chaperones, proteases, proteins that assist in the assembly of outer membrane proteins into the outer membrane, and small RNAs that downregulate outer membrane proteins (2, 10, 11).

σ^E is indispensable even under conditions of considerably low envelope stress (3). It remains unclear why σ^E is essential for the viability of *E. coli*. Several suppressors that enable *E. coli* cells to survive without σ^E have been isolated and characterized. Button et al. isolated suppressors of the null mutation of the *rpoE* gene, encoding σ^E, and revealed that a loss-of-function mutation of *hicB* (formerly known as *ydcQ*) suppresses the lethality conferred by the lack of σ^E (12). Hayden and Ades (13) isolated two genes, *ptsN* and *yhbW*, as multicopy suppressors of the *rpoE* null mutation; the former encodes a protein homologous to enzyme IIA of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system, whereas the latter encodes a protein with an un-

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known function. Either deletion of *hicB* or overexpression of *ptsN* downregulates multiple ESR systems, including the σ^E, 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 proteins that antagonize the toxin activity and degrade the toxin RNA, respectively. Toxins of many type II 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 plasmid-encoded postsegregation killing systems that kill plasmid-free segregant cells (22), they have been identified in most prokaryotic genomes. TA systems have been shown to play a role in various physiological processes, such as programmed cell death (23, 24), formation of persister cells upon antibiotic treatment (25, 26), and generation of specialized ribosomes (27, 28). The *E. coli* chromosome contains at least 36 TA loci, 17 and 19 of which belong to the type I and type II TA systems, respectively (15).

The HicAB toxin-antitoxin pair belongs to the type II TA systems. Ectopic expression of HicA results in cleavage of the mRNAs of *ompA*, *dksA*, and *lpp*, as well as transfer-messenger RNA (tmRNA) at specific sites, and causes growth arrest, while coexpression of HicB restores cell growth (14). HicA adopts an $\alpha1\beta1\beta2\beta3\alpha2$ fold characteristic of a double-stranded RNA-binding domain (29, 30), indicating that HicA may cleave mRNAs and tmRNA around their double-stranded regions. Recent biochemical and structural analyses of the HicA3-HicB3 complex, a HicAB family TA system of *Yersinia pestis*, revealed that the tetrameric HicB3 antitoxin binds two HicA3 toxin monomers, occluding the catalytic His28 residue of HicA3 (31). Both the HicB3 tetramer and the HicA3-HicB3 complex act as transcriptional autorepressors of the operon (31). The physiological function of the HicAB TA system is not known; its involvement in cell death or dormancy appears unlikely, as cells lacking HicB show an apparently normal growth phenotype (14).

Why the disruption of *hicB* suppresses the σ^E essentiality remains an enigma. Interestingly, it has been shown that some antitoxins are involved in repression of not only their own promoter but also the promoters of other genes. For example, the MqsA antitoxin of the MqsR/MqsA toxin-antitoxin system represses transcription of *rpoS* (32), and DinJ of the YafQ/DinJ TA system represses *cspE* (33). Therefore, it cannot be ruled out that the absence of HicB causes the expression of some unidentified HicB-controlled genes 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 tryptone, 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 Δ*rpoE*::*kan ung*::Tn*10* and Δ*rpoE*::*kan zfh*::Tn*10* 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*::Tn*10* or *zfh*::Tn*10* 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 Δ*rpoE*::*kan* allele had been cotransduced with *ung*::Tn*10* or *zfh*::Tn*10*. 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 Δ*rpoE*::*kan ung*::Tn*10* 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 500 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, 50-mg/ml streptomycin, 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 Δ*rpoE*::*kan ung*::Tn*10* 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 interferase 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 pCA24NΔNot, ASKA-YafQ, or ASKA-YafQ(H87A) 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 600 μl, according to the manufacturer's recommendations. Next, the RNA was degraded by adding 200 μl 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 (Nucleospin; 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 pentadecapeptide comprising the amino-terminal 14 amino acid residues of HicA followed by Cys (MKQSEFRRWLESQGC) was synthesized and used to immunize a rabbit. To reduce nonspecific binding of the anti-HicA antiserum during immunoblotting, antibodies that react with proteins other than HicA were adsorbed by using total proteins of the Δ*hicA* 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 antiserum diluted 5,000-fold with Can Get Signal immunoreaction enhancer solution (Toyobo). The non-membrane-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 Western blotting detection reagent; GE Healthcare) and an ImageQuant 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 σ^E dispensability caused by a *hicB* null mutation.** Previous work suggested that disruption of the *hicB* gene suppresses σ^E essentiality, based on the observation that the Δ*rpoE*::*cat* mutation can be introduced into a Δ*hicB* strain, but not into a wild-type (*hicB*$^+$) strain or a Δ*hicB* strain with a complementing *hicB*$^+$ plasmid, by directly selecting CP-resistant Δ*rpoE* transductants after P1-mediated transduction (12). However, because revertants carrying suppressor mutations in the Δ*rpoE* 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 σ^E essentiality, we employed a cotransduction assay in which the Δ*rpoE*::*kan* allele was cotransduced as a nonselective marker with a nearby *ung*::Tn*10* 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 Δ*rpoE*::*kan* allele with the *ung*::Tn*10* 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 (pTH18cr) was used as the recipient, which is in accordance with the σ^E essentiality. Induction of *rpoE* expression from pTH-RpoE by use of IPTG was not necessary for cotransduction of the Δ*rpoE*::*kan* allele, presumably due to the leaky expression of *rpoE* from the plasmid. Next, we examined the effects of the Δ*hicB* mutation on the *rpoE*::*kan* cotransduction frequency. We hypothesized that if the Δ*hicB* mutation was sufficient to suppress the σ^E essentiality, then cotransduction of the Δ*rpoE*::*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

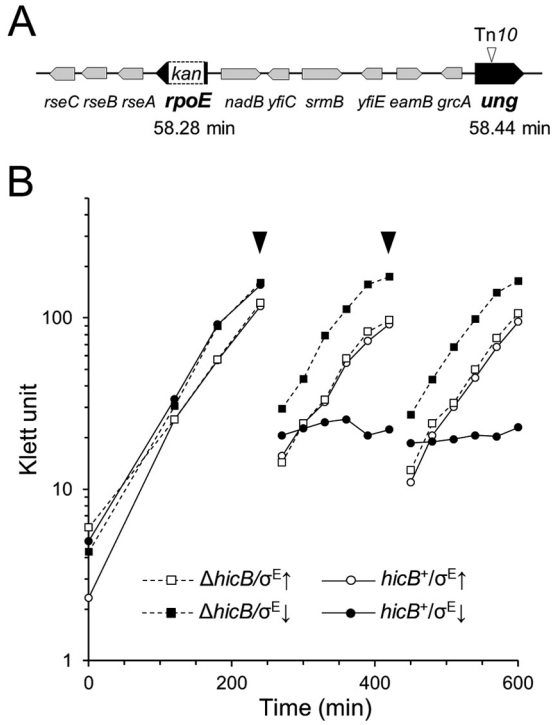

FIG 1 Disruption of the *hicB* gene is sufficient for suppression of the lethality caused by the lack of σ^E. (A) Genomic organization of the *rpoE* locus. The Δ*rpoE*::*kan ung*::Tn*10* strain (SN322) was used as a donor for P1-mediated cotransduction frequency assays. (B) Effects of σ^E depletion on cell growth in the presence or absence of *hicB*. Cells of YD221 (*hicB*$^+$ Δ*rpoE*)/pTH-RpoE/pTrcHis2B (circles) and YD224 (Δ*hicB* Δ*rpoE*)/pTH-RpoE/pTrcHis2B (squares) were grown overnight at 30°C. Cells were diluted 100-fold in L media supplemented with 20 μg/ml CP, 50 μg/ml ampicillin, and either 1 mM IPTG (open symbols) or 0.4% glucose (solid symbols) and grown at 30°C. Cells were diluted 10-fold in prewarmed fresh media in the late exponential phases (indicated by arrowheads). The turbidity of the cells was measured using a Klett-Summerson colorimeter (filter no. 54). Growth curves representative of three independent cultivations are shown.

TABLE 1 Cotransduction frequencies (CF) of the Δ*rpoE*::*kan* marker with the *ung*::Tn*10* marker

Recipient strain	IPTG concn (μM)	CF (%)	*n*[a]
W3110			
No plasmid	0	2.6	112
+ pTH18cr	1,000	0	48
+ pTH-RpoE	0	77.0	48
+ pTH-RpoE	1,000	87.5	48
+ pTH-HicA	1,000	0	81
YD44 (Δ*hicB*)			
No plasmid	0	63.1	149
+ pTH18cr	1,000	66.6	48
+ pTH-RpoE	1,000	78.2	46
YD105 (Δ*hicAB*)			
No plasmid	0	0	144
+ pTH19cr	0	0	43
+ pTH-HicA	0	68.3	79
+ pTH-HicA(H23A)	0	0	77
+ pTH-HicA(H38A)	0	0	31

[a] Number of transductants screened to calculate CF.

regulation of the *lac* promoter, into the *hicB*$^+$ Δ*rpoE*/pTH-RpoE and Δ*hicB* Δ*rpoE*/pTH-RpoE strains, and we found that the growth of the *hicB*$^+$ Δ*rpoE*/pTH-RpoE/pTrcHis2B strain was IPTG dependent, while its Δ*hicB* 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 Δ*hicAB* strain, as a recipient. The results showed that no KM-resistant clones were found among the 144 TC-resistant transductants (Table 1). The cotransduction frequency was recovered when the transduction was conducted using YD105 transformed with pTH-HicA, carrying *hicA* under the control of the *lac* promoter/operator (Table 1). Addition of IPTG was not necessary for suppression of the σ^E essentiality of YD105 by pTH-HicA, and induction with 100 μM IPTG actually inhibited cell growth (data not shown). It should be noted that induction of HicA from pTH-HicA with up to 1 mM IPTG neither affected the growth of the wild-type cells (data not shown) nor suppressed the σ^E essentiality (Table 1), presumably because HicA is neutralized by intrinsic HicB in this case. These results suggest that an appropriate level of free HicA is required for the efficient suppression of the σ^E essentiality.

The mRNA interferase activity of HicA is necessary for the suppression of σ^E essentiality. Ectopic production of HicA causes cleavage of mRNAs and tmRNA, resulting in global translation inhibition and growth arrest (14). To examine whether the mRNA interferase activity is required for suppression of the σ^E essentiality by HicA, we constructed HicA mutants lacking mRNA interferase activity by mutating one of the two evolutionally conserved His residues (His-23 or His-38) to Ala. The importance of His-23 for mRNA interferase activity was recently suggested based on structural and functional analyses of the H24A and H28A mutant proteins of the HicA homologues from *Burkholderia pseudomallei* and *Y. pestis*, respectively (30, 31). His-38 is located in the β3-α2 loop (30), in which basic residues are implicated in substrate binding of the double-stranded RNA-binding domain proteins (44). pTH-HicA derivatives encoding an H23A or H38A mutant form of HicA were introduced into the Δ*hicAB* strain. Immunoblotting analysis showed that significant amounts of the wild-type and mutant forms of HicA accumulated when their expression was induced with 100 μM IPTG (Fig. 2A). The levels of the H23A and H38A mutant proteins were higher than that of the wild-type protein, suggesting that these mutations somehow affect the expression or stability of the HicA protein. After induction of HicA and its H23A and H38A derivatives in YD105 by use of 100 μM IPTG for 30 min, total RNAs were prepared and subjected to primer extension analysis with *ompA* primers to evaluate the mRNA interferase activity of these proteins (Fig. 2B). Whereas expression of wild-type HicA induced cleavage of the *ompA* mRNA, just as reported previously (14), no signal for the *ompA* mRNA cleavage was detectable upon expression of HicA(H23A) and HicA(H38A), irrespective of the increased accumulation of these proteins. Accordingly, the toxic effect of HicA was almost

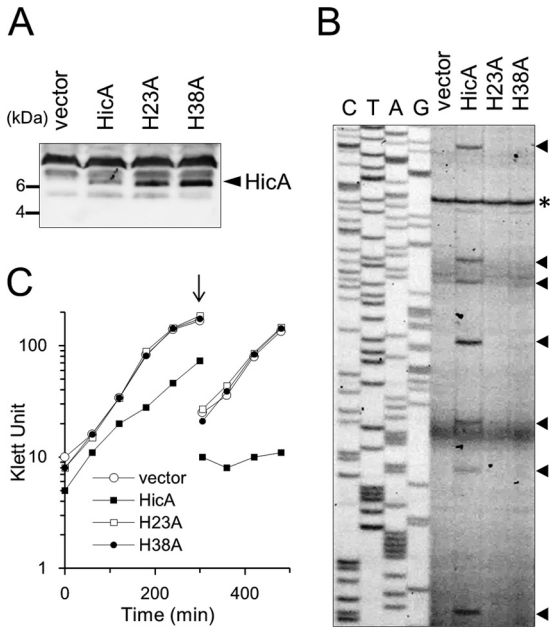

FIG 2 The H23A and H38A mutations abolish the mRNA interferase activity of HicA. YD105 (Δ*hicAB*) cells carrying pTH19cr (vector), pTH-HicA, pTH-HicA(H23A), or pTH-HicA(H38A) were grown in L medium supplemented with 20 μg/ml CP at 30°C. (A) Total cellular proteins were prepared from cells grown in the presence of 100 μM IPTG and were subjected to SDS-PAGE followed by immunoblotting with anti-HicA antiserum as described in Materials and Methods. Migration positions of molecular mass markers are shown on the left. (B) Cleavage patterns of *ompA* mRNA after induction of HicA or its active site mutant derivatives. Cells were grown in L medium and induced with 100 μM IPTG in the mid-exponential growth phase. Cells were harvested at 30 min postinduction, and total RNA was extracted. Primer extension analysis was performed as described in Materials and Methods. DNA sequencing ladders obtained using the same primer were loaded in the adjacent lanes. Arrowheads indicate the positions of cleavage, whereas the asterisk indicates a nonspecific band. (C) Effects of induction of HicA derivatives on cell growth. Cells were grown in the presence of 100 μM IPTG and diluted 10-fold in the late exponential phase (indicated by an arrow). Cell density was monitored using a Klett-Summerson colorimeter (filter no. 54). Growth curves representative of two independent cultivations are shown.

TABLE 2 CF of the Δ*rpoE*::*kan* marker with the *ung*::Tn*10* marker in wild-type cells carrying ASKA clones

ASKA clone	TA system type	IPTG concn (μM)	CF (%)	*n*[a]
pCA24NΔNot		0	0	48
chpB	II	1,000	1.6	63
gnsA	II	1,000	0	55
hicA	II	0	0	48
hicA	II	100	55.0	100
higB	II	1,000	69.9	103
hipA	II	100	0	20
mazF	II	30	0	60
mqsR	II	8	5.0	20
rlnA	II	100	0	19
yafO	II	30	0	68
yafQ	II	20	54.5	44
yafQ(H87A)	II	20	2.1	48
yjhX	II	1,000	0	64
ykfI	II	30	0	16
ypjF	II	30	0	15
yoeB	II	8	0	20
ldrC	I	30	0	20
ldrD	I	30	0	15
hokA	I	15	0	20
hokB	I	30	0	15
hokC	I	1,000	0	20
hokD	I	8	0	20
hokE	I	15	0	10
symE	I	30	0	20

[a] Number of transductants screened to calculate CF.

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). No cotransduction of the Δ*rpoE*::*kan* marker was observed with YD105 carrying the pTH-HicA derivative encoding the H23A or H38A mutant (Table 1), indicating that the mRNA interferase activity of HicA is necessary for suppression of the σ^E essentiality.

Increased expression of the YafQ or HigB toxin also suppresses the σ^E 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 σ^E 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 σ^E essentiality nor inhibited growth of W3110, the ASKA plasmid clone carrying *hicA* supported the growth of W3110 in the absence of σ^E 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 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 σ^E 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 σ^E 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 Δ*rpoE*::*kan* allele with the *ung*::Tn*10* allele, suggesting that these toxins can also suppress the σ^E essentiality (Table 2). It should be noted that the suppression of the σ^E essentiality by either HigB or YafQ did not depend on HicA, because the Δ*rpoE*::*kan* allele was introducible to YD626 (Δ*hicA*) 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) (see Fig. S1 in the supplemental material), failed to suppress the σ^E essentiality (Table 2). Unlike *hicB*, disruption of neither *higA* nor *dinJ*, the genes encoding the cognate antitoxins for HigB and YafQ, respectively, suppressed the σ^E 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 σ^E 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 σ^E essentiality by using a σ^E reporter, *rpoHP3-lacZ*, and a Cpx reporter, *cpxP-lacZ* (see Fig. S2 in the supplemental material). We found that overexpression of HicA, HigB, and YafQ significantly downregulated the expression of the σ^E reporter. The expression of the Cpx reporter was also downregulated by overexpression of either HigB or YafQ, while a less prominent effect was observed with HicA overexpression. 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 σ^E 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 involve 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 σ^E 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 Δ*rpoE*::*kan* allele into wild-type cells at significantly high efficiencies (Table 3). The Δ*rpoE* strain thus constructed in the presence of 10 μg/ml EM ceased to grow upon removal of EM (Fig. 3A). Together, these results suggest that cells can grow without σ^E in the presence of these antibiotics. We next performed a disk diffusion assay in which a paper disk containing an antibiotic was placed onto a lawn of Δ*rpoE* cells that had been prepared by transduction of the

TABLE 3 CF of *ung*::Tn*10* and Δ*rpoE*::*kan* markers in the presence of antibiotics

Recipient strain	Antibiotic	Antibiotic concn (μg/ml)	CF[a] (%)	*n*[b]
W3110		0	0	48
	CP	0.5	2.1	48
	CP	1	19.0	21
	CP	2	73.0	89
	EM	2.5	68.7	48
	EM	5	87.5	48
	EM	10	78.5	144
	EM	20	89.6	48
+ pCA24NΔNotI		0	2.1	47
+ ASKA-ClpP		0	56.3	48
+ ASKA-ClpP(S111A)		0	0	48
YD619 (Δ*clpP*)		0	0	48
	CP	2	0	66
	EM	10	0	96
YD649 (Δ*hicA* Δ*higB* Δ*yafQ*)		0	0	48
	CP	2	60.4	48
	EM	10	75.0	48
+ ASKA-ClpP		0	20.5	44

[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.

Δ*rpoE*::*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 σ^E essentiality when present at appropriate concentrations. Spectinomycin, rifampin, and nalidixic acid gave similar results, demonstrating the Δ*rpoE* 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 Δ*rpoE* cells to the same degree when the σ^E 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 Δ*rpoE* 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 L-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 σ^E 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 ClpP from the ASKA plasmid suppressed the σ^E 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*::Tn*10* 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*::Tn*10* 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 Tn*10* marker, the *zfh*::Tn*10* allele (Tn*10* is inserted into the nonessential *hcaT* gene, encoding a putative 3-phenylpropionic transporter). The cotransduction frequency of the Δ*rpoE*::*kan* allele with the *zfh*::Tn*10* allele was 18.8% when W3110 carrying pTH-RpoE was used as the recipient (see Table S4 in the supplemental material). This low cotransduction frequency was expected because of the longer distance between the two genes (0.92 min). Comparable frequencies of Δ*rpoE*::*kan* cotransduction were observed with a strain carrying the Δ*hicB* mutation or with the wild-type strain in the presence of a low level of EM (see Table S4), just as observed in the cotransduction experiments using the *ung*::Tn*10* allele. These results suggest that the increased mutation rate caused by the *ung*::Tn*10* allele did not significantly, if at all, affect the estimation of the Δ*rpoE*::*kan* cotransduction frequencies. Next, we tried cotransduction of the Δ*rpoE*::*kan* allele with the *zfh*::Tn*10* allele in the presence of a low level of EM, using the Δ*lon* strain as the recipient. The number of TC-resistant transductants was still smaller when the recipient carried the Δ*lon* mutation, but it was much larger than that obtained by *ung*::Tn*10* transduction into the Δ*lon* strain. Among the TC-resistant transductants, none received the Δ*rpoE*::*kan* marker (see Table S4). Therefore, Lon is also required for the suppression of σ^E essentiality by low levels of antibiotics.

While *hicA*, *higB*, and *yafQ* were identified as multicopy suppressors of σ^E essentiality, the antibiotic-induced suppression of σ^E essentiality was still observed with a Δ*hicA* Δ*higB* Δ*yafQ* triple mutant (Table 3). Also, overexpression of ClpP suppressed the σ^E 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 σ^E essentiality in the absence of these three toxins.

DISCUSSION

In this study, we demonstrated that the suppression of σ^E 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-

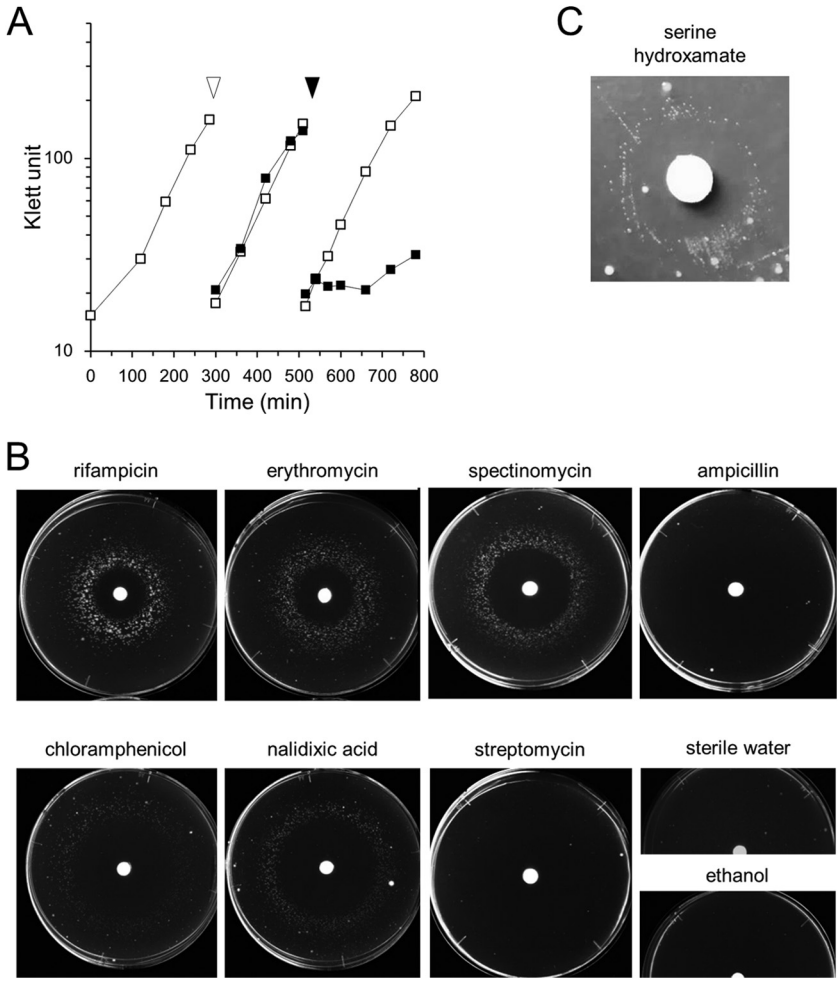

FIG 3 Low levels of antibiotics suppress the σ^E essentiality. (A) The Δ*rpoE*::*kan* marker was cotransduced with the *ung*::Tn*10* marker into W3110 in the presence of 10 μg/ml EM as described in Materials and Methods. The resulting, suppressor-free Δ*rpoE*::*kan ung*::Tn*10* strain was grown at 30°C in L medium supplemented with 10 μg/ml EM at the point indicated by the open arrowhead. Cells were then harvested, washed three times with L medium, diluted 10-fold with prewarmed L medium supplemented with (open squares) or without (solid squares) EM, and cultured further at 30°C. Cells were diluted 10-fold at the point indicated by the solid arrowhead. The turbidity of the cells was measured using a Klett-Summerson colorimeter (filter no. 54). Growth curves representative of four independent cultivations are shown. (B and C) Drug-containing disk diffusion assays. Paper disks containing the indicated antibiotics or their solvents (sterile water and ethanol; one half of each plate is shown) (B) or serine hydroxamate (C) were placed onto a lawn of the suppressor-free Δ*rpoE*::*kan ung*::Tn*10* strain constructed in the presence of 10 μg/ml EM, and the plates were incubated for 24 h (B) or 36 h (C) at 30°C. Large colonies that formed outside the rings included revertants carrying a suppressor mutation(s) in the Δ*rpoE* background.

tion, we identified two other mRNA interferase toxins, HigB and YafQ, as multicopy suppressors of the σ^E null mutation. As observed for HicA, the suppression of σ^E essentiality by YafQ required its mRNA interferase activity. These results strongly suggest that the observed suppression of σ^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 σ^E essentiality. This suppression required the antitoxin-degrading proteases, supporting the idea that toxin activation can induce σ^E dispensability. The levels of toxins required for suppression of the σ^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 Δ*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 σ^E. If this were so, then translation inhibitors would generally induce σ^E dispensability. However, this is not the case, as streptomycin did not suppress the σ^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 YafQ. 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 σ^E dispensability. However, we did not observe the suppression of σ^E 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 YafQ 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 σ^E essentiality.

Unlike HicA, HigB and YafQ 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 σ^E essentiality. Previous primer extension analyses of HicA and YafQ did not reveal common target motifs (14, 45, 46); HicA apparently has no obvious consensus recognition motif, whereas YafQ 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 σ^E essentiality might include noncoding 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 σ^E dispensability remains to be clarified, these toxins might reduce intrinsic cell surface stresses through unknown mechanisms, which could lead to less dependency on σ^E 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 YafQ 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 σ^E essentiality and the physiological roles of the TA systems in ESR systems.

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