HicA of *Escherichia coli* Defines a Novel Family of Translation-Independent mRNA Interferases in Bacteria and Archaea[†]

Mikkel G. Jørgensen,^{1,3} Deo P. Pandey,² Milena Jaskolska,³ and Kenn Gerdes^{1*}

Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Newcastle NE2 4HH, United Kingdom¹; Department of Cell Biology, Sciences III, University of Genève 30, quai Ernest-Ansermet, 1211 Geneva 4, Switzerland²; and Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark³

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Toxin-antitoxin (TA) loci are common in free-living bacteria and archaea. TA loci encode a stable toxin that is neutralized by a metabolically unstable antitoxin. The antitoxin can be either a protein or an antisense RNA. So far, six different TA gene families, in which the antitoxins are proteins, have been identified. Recently, Makarova et al. (K. S. Makarova, N. V. Grishin, and E. V. Koonin, *Bioinformatics* 22:2581–2584, 2006) suggested that the *hicAB* loci constitute a novel TA gene family. Using the *hicAB* locus of *Escherichia coli* K-12 as a model system, we present evidence that supports this inference: expression of the small HicA protein (58 amino acids [aa]) induced cleavage in three model mRNAs and tmRNA. Concomitantly, the global rate of translation was severely reduced. Using tmRNA as a substrate, we show that HicA-induced cleavage does not require the target RNA to be translated. Expression of HicB (145 aa) prevented HicA-mediated inhibition of cell growth. These results suggest that HicB neutralizes HicA and therefore functions as an antitoxin. As with other antitoxins (RelB and MazF), HicB could resuscitate cells inhibited by HicA, indicating that ectopic production of HicA induces a bacteriostatic rather than a bactericidal condition. Nutrient starvation induced strong *hicAB* transcription that depended on Lon protease. Mining of 218 prokaryotic genomes revealed that *hicAB* loci are abundant in bacteria and archaea.

Toxin-antitoxin (TA) loci that encode protein antitoxins share certain characteristics. They are two-component systems that encode two small proteins, a toxin and an antitoxin; the antitoxin neutralizes the toxin by direct protein-protein contact; the antitoxin has a DNA binding domain and autoregulates TA operon transcription; many such toxins cleave mRNA; the toxin families exhibit complex phylogenetic patterns due to extensive horizontal gene transfer; and members of the same family are often detected on phages and plasmids, as well as on chromosomes (3, 14, 33). A previous comprehensive analysis of ≈120 prokaryotic genomes led to the identification of ≈600 loci belonging to the six known TA gene families (*relBE*, *mazEF*, *vapBC*, *phd-doc*, *ccdAB*, and *parDE*). The genes are distributed in a striking pattern: slowly growing free-living organisms particularly have many TA loci, while obligatory intracellular organisms have few or none (27, 33). For example, *Mycobacterium tuberculosis* has more than 60, and *Sulfolobus* spp. has ≈30 TA loci, while *Mycobacterium leprae* and rickettsiae have none.

The RelE and MazF families of toxins inhibit translation by mRNA cleavage (34, 45) and were coined mRNA interferases (45). Doc of P1 also inhibits translation (26). Two other TA families, typified by *ccd* of plasmid F and *parDE* of plasmid RK2, encode inhibitors of DNA gyrase (1, 4, 20, 30). The biological functions of chromosome-encoded TA loci have been the subjects of considerable debate. In almost all cases

investigated, chromosome-encoded TA loci are induced by nutritional stress, raising the possibility that these TA loci function to help the cells cope with environmental stress (5, 14). In further support of the stress response hypothesis, transcription of TA loci was induced by heat shock in *Sulfolobus solfataricus* and by exposure to chloroform in *Nitrosomonas europaea* (18, 40). There is also evidence that a MazF toxin homologue elicits programmed cell death during *Myxococcus* species development (32). It has been suggested that *mazEF* of *Escherichia coli* also mediates programmed cell death (2, 24, 25), although we and others have not been able to reproduce these results (10, 42).

A bicistronic locus closely linked to the pilus gene cluster of *Haemophilus influenzae* (*hif*) was called *hic* (for *hif* contiguous) (29). In a recent bioinformatic study, it was suggested that the *hicAB* loci constitute a novel TA gene family with many members in bacteria and archaea (27). Predicted HicA proteins (COG1724) are small and have a double-stranded RNA-binding fold, while predicted HicB proteins (COG1598/4226) have a DNA binding domain fused to a degraded RNase H fold (27). The bioinformatic analyses did not predict with certainty which of the proteins would be the toxin or the antitoxin, although HicB has a DNA binding domain similar to that of known antitoxins.

Here, we present evidence showing that the *hicAB* genes of *E. coli* K-12 have properties very similar to those of previously characterized TA loci. Thus, induction of *hicA* inhibited translation and induced multiple cleavages in three model mRNAs and tmRNA. Induction of *hicB* neutralized the detrimental effects of HicA, indicating that HicB can function as an antitoxin. Similar to bona fide TA loci, transcription of *hicAB* was induced by amino acid and carbon starvation by a Lon-dependent mechanism. Finally, we present a comprehensive update of TA gene phylogeny.

* Corresponding author. Mailing address: Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Newcastle NE2 4HH, United Kingdom. Phone: 44 0191 222 5318. Fax: 44 0191 222 2467. E-mail: kenn.gerdes@ncl.ac.uk.

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TABLE 1. Strains and plasmids used and constructed

Strain or plasmid	Genotype or plasmid property ^a	Reference or source
MG16655	Wild-type <i>E. coli</i> K-12	Laboratory collection
MGJ36	Same as MG1655, except Δ <i>hicAB</i>	This work
BW25113	<i>lacI^q rrmB ΔlacZ hsdR ΔaraBAD ΔrhaBAD</i>	11
MG1655 Δ <i>ssrA</i>	Same as MG1655, except Δ <i>ssrA</i>	7
SG22093	<i>clpP1::Cml^r rcsA166::Kan^r</i>	S. Gottesman, NIH
SG22095	<i>lon146::Tet^r rcsA166::Kan^r</i>	S. Gottesman, NIH
pNDM220	mini R1; <i>bla lacI^q</i> ; P _{A1/O4/O3} MCS	15
pBAD33	p15A; <i>cat</i> P _{BAD} ::MCS	17
pMJ221	P _{A1/O4/O3} :: <i>hicA</i>	This work
pMJ331	P _{BAD} :: <i>hicB</i>	This work
pSC320	pBR322; <i>tet ssrA smgB</i>	7
pSC321	pBR322; <i>tet ssrA' smgB</i>	7
pKD3	<i>cat</i> template plasmid	11
pKD46	<i>bla</i> , λ Red recombinase expression	11
pCP20	pSC101; <i>rep</i> (Ts) <i>bla</i>	11

^a *cat*, chloramphenicol transacetylase gene; *bla*, β -lactamase gene; *tet*, tetracycline resistance gene; MCS, multiple cloning sites.

TABLE 2. DNA primers

Primer	Sequence
<i>hicA</i> -f.....	5'-CCCTCTAGAGGATCCGTTTACTTTGTTGATATACTCAG
<i>hicA</i> -r.....	5'-CCCAAGCTTGAATTCTTAACTCAAACCGAGTTGTTTC
<i>hicB</i> -f.....	5'-CCCTCTAGAGGATCCCGCCAATTAATAAAGGTTAATGAC
<i>hicB</i> -r.....	5'-CCCAAGCTTGAATTCTTAAACCATCACCAGCGATAAC
qPCR- <i>hic</i> -f.....	5'-ATCTCAGGGCGTCGATGTAG
qPCR- <i>hic</i> -r.....	5'-CCGAGTTGTTTCAGGATTGC
qPCR- <i>tatA</i> -f.....	5'-TGAGCGATGATGAACCAAAG
qPCR- <i>tatA</i> -r.....	5'-TCGCGTCTTCTGTTTTAGCC
delta <i>hicAB</i> -f.....	5'-TAATGTTTACTTTGTTGATATACTCAGCGGCAGGGAGATGTGTAGGCTGGAGCTGCTTC
delta <i>hicAB</i> -r.....	5'-ATTGAGAACTGTTAAAAATGACAACCTTTTCGTAACTGTAACATATGAATATCCTCTTA
<i>hicA</i> -20.....	5'-CTGAGATTCGAGCCAAC
ompA-ctr-ccw.....	5'-GGTATCCAGACGGGTAGCG
dkSA PE2.....	5'-ATCTGTTTTTCGCGAATTTTCAG
rpoD PE1.....	5'-GATTCCACGCTGGAAAGCAC
10SA-2.....	5'-GCCCTCGGCATGCACC

Our analysis revealed 1,340 TA loci in 218 prokaryotic genomes, 119 of which belong to the new *hicAB* TA gene family.

MATERIALS AND METHODS

Growth conditions and media. Cells were grown either in standard Luria-Bertani (LB) broth or in M9 minimal medium at 37°C, supplemented with 0.2% glucose, 1 μ g/ μ l thiamine, and amino acids in defined concentrations. When appropriate, the medium was supplemented with ampicillin (30 or 100 μ g/ml) or chloramphenicol (50 μ g/ml). When amino acid starvation was induced in M9 minimal medium by the addition of 0.4 mg/ml serine hydroxymate (SHX; Sigma-Aldrich), serine was excluded from the medium. Glucose starvation was induced in M9 minimal medium containing 0.05% glucose by the addition of methyl α -D-glucopyranoside (Sigma-Aldrich). Expression of the P_{A1/O4/O3} promoter was induced by the addition of 2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and expression of the P_{BAD} promoter was induced by the addition of 0.2% arabinose.

Bacterial strains and plasmids. Bacterial strains and plasmids used and constructed are listed in Table 1, and DNA primers are listed in Table 2.

(i) **Strain MGJ36.** Strain MG1655 Δ *hicAB* was constructed by deletion of *hicAB* from the chromosome of MG1655, by the procedure described in reference 11. A PCR product was synthesized with the primers delta *hicAB*-f and delta *hicAB*-r, with pKD3 as template. The PCR product was electroporated into strain BW25113/pKD46, and the cells were spread on Luria agar plates containing 25 μ g/ml chloramphenicol and incubated at 37°C. The Δ *hicAB::cat* allele from BW25113 Δ *hicAB* was then transduced into MG1655. The *cat* gene was removed with the plasmid pCP20, resulting in MG1655 Δ *hicAB*.

(ii) **Plasmid pMJ221.** The *hicA* gene was amplified from MG1655 chromosomal DNA, using the primers *hicA*-f and *hicA*-r. The PCR product was digested with BamHI and EcoRI and inserted into pNDM220. This plasmid expresses *hicA* upon addition of IPTG.

(iii) **Plasmid pMJ331.** The *hicB* gene was amplified from MG1655 chromosomal DNA, using *hicB*-f and *hicB*-r as primers. The PCR product was digested with HindIII and XbaI and inserted into pBAD33. Cells carrying this plasmid express *hicB* upon addition of arabinose. PCR fragments were confirmed by sequencing.

Rates of protein, RNA, and DNA synthesis. Cells were grown at 37°C in M9 minimal medium with 0.2% glucose and amino acids in defined concentrations to an optical density at 450 nm (OD₄₅₀) of $\approx$ 0.5. The cultures were diluted 10 times, and 2 mM IPTG was added at an OD₄₅₀ of 0.3. Samples of 0.5 ml were added to 5 μ Ci of [³⁵S]methionine (protein synthesis), 2 μ Ci of [methyl-³H]thymidine (DNA synthesis), or 0.1 μ Ci of [2-¹⁴C]uracil (RNA synthesis) plus a 100-fold excess of unlabeled carrier. After 1 min of incorporation, samples were chased for 10 min with 0.5 ml/mg of cold methionine, 0.5 mg/ml thymidine, or 0.5 mg/ml uracil, respectively.

The samples were harvested and resuspended in 200 μ l cold 20% trichloroacetic acid and centrifuged at 20,000 $\times$ g for 30 min at 4°C. The samples were washed twice with 200 μ l cold 96% ethanol. Precipitates were transferred to vials, and the amounts of incorporated radioactivity were counted in a liquid scintillation counter.

Primer extension analysis. Cells were grown in LB medium at 37°C. At an OD₄₅₀ of 0.5, the cultures were diluted 10 times and grown to an OD₄₅₀ of 0.5, and transcription of the toxin was induced by the addition of 2 mM IPTG. To inhibit translation, chloramphenicol (50 μ g/ml) was added. Primer extension analysis was used to map the *hicAB* promoter and the cleavage patterns of the *dkSA*, *ompA*, *rpoD*, and *ssrA* mRNAs, using ³²P-labeled primers, following extension by reverse transcriptase. The primer (3 pmol) was labeled with 2 μ l of [γ -³²P]ATP at a concentration of 6,000 Ci/mmol by addition of 0.4 μ l polynucleotide kinase (New England Biolabs Inc.) in polynucleotide kinase buffer and incubated for 1 h at 37°C. Labeled primer was hybridized to 10 to 20 μ g total RNA and extended with reverse transcriptase (Finnzymes). The labeled cDNA was fractionated by using a 6% polyacrylamide gel electrophoresis, which was dried and placed on a phosphorimager screen.

Reverse transcription qPCR. RNA was extracted from all cell samples using an RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR (qPCR) reactions were run in duplicate simultaneously. Briefly, 10 to 20 ng cDNA was mixed with 0.3 μ M primers and 10 μ l of 2 $\times$ PCR Master Mix for Sybr green kit from Eurogentec (Seraing, Belgium), and the qPCR was run on a LightCycler 480 real-time PCR system (Roche). Relative expression was analyzed using qBase software (19).

RESULTS

Overproduction of HicA is bacteriostatic. The *hicAB* locus of *E. coli* K-12 is shown schematically in Fig. 1A. The *hicA* reading frame (formerly *yncN*) consists of 58 codons and has a GTG start codon and a predicted Shine-Dalgarno sequence (AGGGAGG). The *hicB* gene (formerly *ycdQ*) has 145 codons and starts 24 bp downstream of *hicA*. We used primer extension analysis to map the transcriptional start site upstream of *hicA*. Interestingly, the *hicA* mRNA 5' end was not detectable with total RNA prepared from exponentially growing cells (Fig. 1B, -2' sample). However, the addition of chloramphenicol induced the appearance of a distinct cDNA band, consistent with a transcriptional start site located 66 bp upstream of the *hicA* start codon (Fig. 1A and B).

Thus, blockage of translation induced *hicAB* transcription. No additional promoter was detected in the region between *hicA* and *hicB* (data not shown).

The *hicA* gene was PCR amplified and inserted downstream of the IPTG-inducible $P_{A1/03/04}$ promoter of the low-copy-number vector pNDM220, resulting in pMJ221. Similarly, *hicB* was inserted downstream of the arabinose-inducible P_{BAD} promoter of pBAD33, resulting in pMJ331. Strains MG1655/pMJ221 ($P_{A1/03/04}::hicA$) and MG1655/pMJ331 ($P_{BAD}::hicB$) were grown exponentially in rich medium. Induction of *hicA* resulted in a rapid cessation of cell growth (Fig. 2A) and simultaneously inhibited cell proliferation (Fig. 2B). These results show that ectopic production of HicA is bacteriostatic. By contrast, induction of *hicB* had no effect on cell growth.

HicB neutralizes HicA-mediated inhibition of growth. Next, we deleted the chromosome-encoded copy of the *hicAB* locus and repeated the *hicA* induction experiment described above. In this case, HicA also inhibited cell growth (data not shown) and, moreover, reduced the number of colony-forming cells (Fig. 2B). This result indicated that HicA was more active in the $\Delta hicAB$ strain than in the wild-type strain. Therefore, we tested whether HicB could neutralize HicA. Cells containing plasmids carrying the inducible *hicA* (IPTG) and *hicB* (arabinose) genes were grown exponentially. As described before, induction of *hicA* inhibited cell growth (Fig. 2C). Strikingly, however, later induction of *hicB* resulted in an immediate resumption of cell growth, showing that HicB can indeed counteract HicA. Moreover, the rapid resumption of cell growth indicated that HicA was bacteriostatic rather than bactericidal, at least for the 90 min of *hicA* induction used here.

HicA inhibits the global rate of translation. Since the molecular target of HicA is unknown, we measured the rates of protein, DNA, and RNA syntheses after induction of *hicA*. As shown in Fig. 3, the rate of translation was strongly inhibited (20-fold after 1 h with *hicA* induction), while replication and transcription continued unaffected during this period.

Induction of *hicA* induces mRNA cleavage. Ectopic production of mRNA interferases, such as RelE and MazF, results in a dramatic inhibition of translation (7, 10). Therefore, we investigated whether ectopic production of HicA would induce mRNA cleavage in three different test mRNAs. The primer extension analysis illustrated in Fig. 4 shows that HicA induced multiple cleavage sites in the *ompA*, *dksA*, and *rpoD* mRNAs. By comparison of the band patterns obtained with RNA prepared from cells treated with chloramphenicol, HicA-mediated cleavages were distinguished from premature terminations of reverse transcriptase (Fig. 4, Cml). The HicA-mediated cleavage patterns in the three test mRNAs did not yield any obvious consensus recognition motifs.

FIG. 1. Genetic organization of the *hicAB* locus. (A) Genetic map of *hicAB*. The enlargement shows the *hicAB* promoter region. (B) The 5' ends of the *hicAB* mRNA were mapped by primer extension using a ^{32}P -labeled *hicA*-20 primer (see Materials and Methods). Cells of *E. coli* MG1655 were grown exponentially in LB medium at 37°C. At an OD_{450} of ≈ 0.5 , chloramphenicol (Cml) (50 $\mu\text{g}/\text{ml}$) was added, and samples were taken at the time points indicate promoter sequences. Numbers at left SD, Shine-Dalgarno sequence.

FIG. 2. *hicAB* of *E. coli* is a TA locus. (A) Growth of MG1655 wild-type (wt) cells carrying pMJ221 ($P_{A1/O3/O4}::hicA$) or pMJ331 ($P_{BAD}::hicB$) were grown exponentially in LB medium at 37°C. At an OD₄₅₀ of ≈ 0.5 , transcription of *hicA* and *hicB* was induced by the addition of 2 mM IPTG and 0.2% arabinose, respectively. (B) CFU counts after induction of *hicA* and *hicB* in wild-type and in $\Delta hicAB$ strains (*hicA* induction only) are shown. Cell samples were diluted and plated on rich solid medium containing ampicillin (30 μ g/ml) for the *hicA*-carrying plasmid pMJ221 and chloramphenicol (50 μ g/ml) plates for the *hicB*-carrying plasmid pMJ331. (C) Growth of MG1655/pMJ221 ($P_{A1/O3/O4}::hicA$)/pBAD33 (vector control lacking HicB [-HicB]) and MG1655/pMJ221 ($P_{A1/O3/O4}::hicA$)/pMJ331 ($P_{BAD}::hicB$) (+HicB). Cultures were grown in LB medium at 37°C to exponential phase and then diluted to an OD₄₅₀ of ≈ 0.1 , and growth was continued for 1 h. At this point, *hicA* was induced by addition of IPTG (2 mM). Ninety minutes later, *hicB* was induced by addition of arabinose (0.2%).

HicA-induced RNA cleavage does not depend on translation. Next, we investigated whether HicA also induced cleavages in tmRNA. Figure 4D shows a primer extension analysis of the coding region of tmRNA before and after induction of

FIG. 3. Rates of protein, DNA, and RNA synthesis after induction of *hicA*. Cells of *E. coli* MG1655 carrying pMJ221 ($P_{A1/O3/O4}::hicA$) were grown exponentially in M9 minimal medium, and *hicA* was induced by addition of IPTG (2 mM) at time zero. Samples were taken at the time points indicated, and synthesis rates were measured by pulse-labeling for 1 min and a 10-min chase as described in Materials and Methods. The preinduction rates were set to 100%.

hicA. As shown, HicA induced cleavage of wild-type tmRNA at two sites (A $\downarrow$ AAC). Addition of chloramphenicol did not lead to any appreciable cleavage of tmRNA, thus showing that the cleavages were induced by HicA. Primer extension analysis of the nontranslated regions of tmRNA did not reveal additional cleavage sites (data not shown).

Both of the HicA-induced cleavage sites were located within the translated part of tmRNA. Therefore, we tested HicA cleavage of a nontranslated variant of tmRNA that had its GCA resume codon changed to a UAA ochre codon (44). The mutant tmRNA exhibited a cleavage pattern indistinguishable from that of the wild-type tmRNA (Fig. 4D, third and fourth panels). Thus, the HicA-mediated cleavages in tmRNA were independent of tmRNA translation.

Amino acid and carbon starvation induce *hicAB* transcription. We investigated whether *hicAB* would be induced during nutrient starvation. Amino acid starvation was induced by the addition of SHX to exponentially growing cells, and the relative level of *hicAB* mRNA was measured by qPCR. As shown in Fig. 5A, SHX induced a rapid and dramatic increase in the level of *hicAB* transcripts. Sixty minutes after the onset of amino acid starvation, the level of *hicAB* mRNA was ≈ 15 -fold increased. Assuming that the metabolic stability of *hicAB* mRNA was not changed by amino acid starvation, as was the case with *relBE* mRNA (9), we conclude that amino acid starvation activates transcription of *hicAB*.

Next, we induced glucose starvation by the addition of α -methyl glucoside to cells growing in glucose minimal medium (Fig. 5B). In this case, *hicAB* transcription was also stimulated, although to a lesser degree ($\approx$ sixfold). Finally, we measured the effect of chloramphenicol on *hicAB* transcription (Fig. 5C). As shown, *hicAB* transcription was stimulated strongly by chloramphenicol (≈ 12 -fold), consistent with the result shown in Fig. 1B.

Activation of *hicAB* depends on Lon. The above-described results are consistent with the notion that HicB is an unstable antitoxin that autoregulates *hicAB* transcription. If so, induction of *hicAB* transcription might depend on a cellular protease. To test this possibility, we investigated the transcriptional response to amino acid starvation in *lon* or *clpP* protease mutant strains. Deletion of *clpP* did not reduce induction of *hicAB* during amino acid starvation (Fig. 5D). However, dele-

FIG. 4. HicA induces cleavage of the *ompA*, *dksA*, and *rpoD* mRNAs and tmRNA. Primer extension analysis of the *ompA* (A), *dksA* (B), and *rpoD* (C) mRNAs and tmRNA (D) using the 32 P-labeled primers *ompA*-ctr-ccw, *dksA* PE1, *rpoD* PE1, and 10SA-2, respectively, are shown. (A to C) Total RNA was prepared from cells growing exponentially in LB medium at 37°C. At time zero, 50 μ g/ml chloramphenicol (first panels, labeled Cml) or 2 mM IPTG (second panels, labeled *hicA*) was added. The strains used were MG1655 (first panels) and MG1655/pJM221 ($P_{A1/O4/O3}::hicA$) (second panels). (D) The first panel shows results with strain MG1655; second panel with strain MG1655/pJM221 ($P_{A1/O4/O3}::hicA$); third panel with strain MG1655 Δ *ssrA*/pMJ221 ($P_{A1/O3/O4}::hicA$)/pSC320 (encoding wild-type tmRNA and *smpB*); and fourth panel with strain MG1655 Δ *ssrA*/pMJ221 ($P_{A1/O3/O4}::hicA$)/pSC321 (encoding tmRNA carrying a resume codon mutation and *smpB*). Square dots indicate HicA-induced cleavages in the RNA sequences. The numbers above the lanes are time points of cell sampling relative to *hicA* induction or the addition of chloramphenicol (left panels). GCA* and UAA* are wild-type and mutated tmRNA resume codons, respectively.

FIG. 5. Nutrient starvation induces *hicAB* transcription by a Lon-dependent mechanism. (A) Cells were grown exponentially in LB medium, and samples were withdrawn before and after the addition of SHX (1 mg/ml). (B) Cells were grown exponentially in M9 minimal medium containing 0.05% glucose, and samples were withdrawn before and after the addition of methyl- α -D-glucopyranoside (2%). (C) Samples were withdrawn before and after the addition of 50 μ g/ml chloramphenicol as indicated. Strains SG22093 ($\Delta clpP$) (D) and SG22095 (Δlon) (E) were grown exponentially in LB medium at 37°C, and starvation was induced by the addition of SHX (1 mg/ml).

tion of *lon* severely reduced the activation of *hicAB* transcription during amino acid starvation (Fig. 5E). This observation suggests that HicB is an autorepressor of *hicAB* transcription and that Lon degrades HicB during starvation.

Distribution of *hicAB* and other TA loci in prokaryotic genomes. Previously, we generated a comprehensive phylogenetic analysis of the six known TA locus families in 126 completely sequenced prokaryotic genomes (RelE, MazF, VapC, Doc, CcdB, and ParE) (33). Here, we extended the previous survey to encompass 218 (22 archaeal and 196 bacterial) genomes and included the *hicAB* gene family in the analysis. In total, we identified 1,340 TA loci divided among 1,069 loci of bacteria (≈ 5.4 loci per genome) and 272 loci of archaea (≈ 12.4 loci per genome), of which 119 were *hicAB* loci (Table 3). Figure 6 shows the two bacterial and two archaeal genomes with the highest number of *hicAB* loci together with members of the six other TA families. *Treponema denticola*, a gram-negative spirochete, has at least 32 TA loci, 9 of which belong to the *hicAB* family. *Photorhabdus luminescens*, an enteric insect pathogen, has at least 59 TA loci, 8 of which belong to the *hicAB* family. *Methanosarcina activorans* and *M. mazei* have 21 and 18 TA loci, respectively. In both cases, 8 of these are *hicAB* loci. Thus, genomes carrying multiple TA loci are common.

Table S1 in the supplemental material gives an overview of the TA loci that we identified in 218 prokaryotic genomes. Detailed information for all these genes is given in Table S2 in the supplemental material. Our extensive survey supports conclusions drawn from a previous study that encompassed 118 genomes (33). All archaea, even *Nanoarchaeum* spp., with its tiny ≈ 490 -kb genome, have at least two TA loci (see Table S1 in the supplemental material), whereas almost all obligate intracellular organisms are devoid of TA loci (49 bacterial species) (see Table S3 in the supplemental material). Thus, the average number of TA loci in free-living bacteria is estimated here to be ≈ 7.2 per genome. Even with this correction, it appears that archaea have, on average, significantly more TA loci than bacteria. Some organisms, both archaeal and bacterial particularly, have many TA loci. Organisms with more than 10 TA loci are listed in Table S4 in the supplemental material. Many of these organisms are characterized by slow growth (e.g., *Mycobacterium tuberculosis* and species of *Geobacter*, *Synechocystis*, *Nostoc*, *Nitrosomonas*, and *Caulobacter* and many archaea).

TABLE 3. Phyletic distribution of seven TA families in 218 prokaryotic genomes^a

Organism	No. of indicated loci							Total
	<i>relBE</i> ^b	<i>parDE</i>	<i>vapBC</i>	<i>hicAB</i>	<i>mazEF</i>	<i>phd-doc</i>	<i>ccdAB</i>	
Bacteria	369	91	352	91	117	39	10	1,069
Archaea	31	0	208	28	2	3	0	272
Total	400	91	560	119	119	42	10	1,341
Stand alone	20	2	21	23	7	4	0	80

^a Gene loci were divided into gene families based on sequence similarities between the toxins. Note that *hicAB* loci have a reversed gene order (toxin gene upstream of antitoxin gene).

^b The RelE toxin family includes the HigB, YhaV, YoeB, and YafQ subfamilies that all share weak but significant sequence similarity and may have similar tertiary folds (21, 41).

FIG. 6. Bacterial and archaeal genomes with many *hicAB* loci. The bacterial (two upper diagrams) and archaeal (two lower diagrams) genomes with the highest number of *hicAB* loci are shown. Other TA loci are included for comparison.

DISCUSSION

Here, we show that *hicAB* of *E. coli* K-12 has many characteristics in common with a number of well-characterized TA loci such as *relBE* and *mazEF*. (i) The *hicA* gene encodes an inhibitor of translation whose ectopic production leads to mRNA cleavage. (ii) *hicB* encodes a protein that neutralizes HicA. (iii) Nutritional stress induces *hicAB* transcription by a Lon-dependent mechanism. (iv) Chloramphenicol also induced transcription of the *hicAB* locus. Observations (iii) and (iv) are consistent with the proposal that HicB is an unstable transcriptional repressor of *hicAB* transcription and that Lon degrades HicB. This proposal is consistent with the presence of an HTH DNA binding motif in the C terminus of HicB (6). (v) The phylogenetic pattern of the *hicAB* locus is similar to that of the *relBE* and *vapBC* loci (Table 3 and see Table S1 in the supplemental material).

Ectopic production of HicA-induced cleavages in three model mRNAs and in tmRNA. We do not know if these cleavages are direct or indirect, that is, if HicA overproduction activates an endogenous RNase or if HicA itself is an RNase (Fig. 4). Although many TA loci encode mRNA-cleaving enzymes, there are examples of such translational inhibitors that mediate indirect mRNA cleavage (13), and the final test to determine whether HicA is an RNase awaits biochemical experiments.

The *hicA* (formerly *yncN*) gene of *E. coli* K-12 has not been described before (23), whereas *hicB* (formerly *ycdQ*) has been implicated in genetic interaction with σ^E , the sigma factor of *E. coli* that responds to stress in the cell membrane and periplasmic space (12). Thus, in wild-type cells, σ^E is essential. However, suppressor mutations that render σ^E nonessential are easily obtained, and one class of such suppressors had IS1

elements inserted into *hicB* (6). Deletion of *hicB* from wild-type cells resulted in a significant downregulation of extracytoplasmic stress responses (both the σ^E and the Cpx-dependent responses) and a severe reduction in the formation of outer membrane vesicles (6). The reason(s) for these complex phenotypes is not known. However, we suggest that deletion of *hicB* (*ycdQ*) leads to hyperactivation of HicA that, in turn, leads to degradation of mRNAs encoding proteins involved in extracytoplasmic stress. We are now testing this proposal.

Including that of *hicAB*, seven TA families are now known (Table 3). Some free-living organisms have a plethora of these gene systems. For example, *M. tuberculosis* has more than 60 TA loci and *N. europaea* has at least 49 (see Table S1 and S3 in the supplemental material). The phylogenetic pattern is consistent with the proposal that TA loci are particularly beneficial to slowly growing organisms. The reason for this apparent correlation is not known. *E. coli* has at least seven TA loci that encode mRNA interferases: four *relBE* loci (*relBE* [15], *yefM yoeB* [16], *dinJ yafQ* [31], and *prfF yhaV* [35]), two *mazEF* loci (*mazEF* and *chpB* [28]), and *hicAB* (this work). In all cases, transcription of these loci is induced by amino acid starvation. Thus, even some rapidly growing organisms have multiple TA loci. The reason for this apparent redundancy is not known.

The literature contains a number of hypotheses that explain the physiological function(s) of TA loci. One group suggested that *mazEF* mediates programmed cell death during amino acid starvation (2), although we and others (10, 42) have not been able to confirm this. By contrast, we have obtained solid evidence that *relBE* and *mazEF* of *E. coli* are induced during nutrient starvation and reduce the global level of translation without cell killing (8–10). There are several reasons why a reduced global level of translation may be beneficial rather than detrimental to starving cells. Most importantly, it may reduce the level of translational errors by reducing the level of uncharged tRNAs (37, 38). Second, a reduced cellular level of translation may help the cells adapt more rapidly to nutrient starvation. The phylogenetic distribution of TA loci (i.e., their absence from obligate intracellular organisms) is consistent with the proposal that they function as stress response elements that are triggered by nutrient starvation and other forms of environmental stresses. This hypothesis is supported by the observations that TA loci were induced by heat shock in *S. solfataricus* and by exposure to chloroform in *Nitrosomonas europaea* (18, 40). However, we are aware that this hypothesis does not explain the apparent massive redundancy of TA loci.

It has also been proposed that TA loci may function to help the cells enter a dormant state called persister cells (22, 36, 43). Persister cell formation can be viewed as an extreme case of translational inhibition, and the phenomenon is compatible with the proposal that TA loci reduce the cellular level of translation during nutritional stress.

Due to the differential metabolic stabilities of the toxins and antitoxins, TA loci may also lead to genetic stabilization of the chromosomal regions to which they are closely linked (39). To test these hypotheses more stringently, we are now analyzing model organisms devoid of all known TA loci.

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