Transcriptional Repressor HipB Regulates the Multiple Promoters in *Escherichia coli*

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Key Words

HipB · HipA · RelA · Persistence · Toxin-antitoxin system · *Escherichia coli* · ppGpp synthesis · Dormancy

Abstract

HipB is a DNA-binding protein in *Escherichia coli* and negatively regulates its own promoter by binding to the palindromic sequences [TATCCN_xGGATA (N represents any nucleotides)] on the *hipBA* promoter. For such sequences, bioinformatic analysis revealed that there are a total of 39 palindromic sequences (TATCCN_(x)GGATA: N is any nucleotides and x is the number of nucleotides from 1 to 30) in the promoter regions of 33 genes on the *E. coli* genome. Notably, *eutH* and *fadH* have two and three TATCCN_(x)GGATA palindromic sequences located in their promoters, respectively. Another significant finding was that a palindromic sequence was also identified in the promoter region of *hipAB* locus, known to be involved in the RelA-dependent persisters cell formation in bacteria. Here, we demonstrated that HipB binds to the palindromic structures in the *eutH*, *fadH*, as well as the *relA* promoter regions and represses their expressions. We further demonstrated that HipA enhances the repression of the *relA* promoter activity by HipB. This effect was not ob-

served with D291A HipA mutant which was previously shown to lack an ability to interact with HipB, indicating that HipA enhances the HipB's repressor activity through direct interaction with HipB.

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Free-living bacteria contain developed toxin-antitoxin (TA) systems to control the rate of intercellular metabolisms to regulate cell growth and death under stress conditions [Gerdes et al., 2005; Yamaguchi and Inouye, 2011]. Each TA system is transcribed from a single operon so that a stable TA complex is formed; hence, the toxin activity is inhibited by its cognate antitoxin under normal growth conditions. In the TA systems, toxins are stable, whereas the antitoxins are unstable. Under stress conditions, such as DNA damage and antibiotic treatment, antitoxins are preferentially degraded by stress-inducible proteases, releasing toxins from the TA complexes. The toxins thus released exert toxic effects on individual cellular targets, causing growth arrest and

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eventually cell death. This process is reminiscent of eukaryotic programmed cell death [Engelberg-Kulka and Glaser, 1999].

In *Escherichia coli*, at least 36 TA systems have been identified [Yamaguchi et al., 2011]. The HipBA system is unique as, in contrast to other toxins, HipA toxin is an unusually large protein consisting of 440 residues and causes a persistence phenotype [Black et al., 1991]. When HipA is induced, cell growth is arrested resulting in the formation of persister cells, which become tolerant to various antibiotics such as penicillin, streptomycin, chloramphenicol, tetracycline and rifampicin. Persister cells, in contrast to antibiotic-resistant cells, do not have any mutations on the genome; nevertheless, they are still resistant to antibiotics as they are in the metabolic dormant state. This poses a serious threat to human health as pathogenic bacteria are able to survive through drug treatment [Glickman and Sawyers, 2012], as persister cells always exist a fraction of the population which survive the environmental stress or antibiotic treatment [Black et al., 1994, 1991; Moyed and Bertrand, 1983; Moyed and Broderick, 1986; Scherrer and Moyed, 1988]. Formation of persister cells has been shown to be controlled by modulating the level of (p)ppGpp [Korch and Hill, 2006]. In *E. coli*, RelA and SpoT control the synthesis of (p)ppGpp. RelA and SpoT synthesize (p)ppGpp from either GTP, ATP, or GDP, and SpoT can also hydrolyze (p)ppGpp yielding GDP and pyrophosphate. Stresses, such as accumulation of uncharged tRNA at ribosomes during amino acid starvation, trigger the expression of RelA [Haseltine and Block, 1973]. Induced expression of RelA increases the amount of (p)ppGpp which in turn interacts with RNA polymerase to repress the rRNA operon expression [Gentry and Cashel, 1996; Wendrich et al., 2002].

High persister (*hip*) mutants have been identified following the exposure of growing *E. coli* to ampicillin [Korch et al., 2003]. A particular mutant allele, *hipA7*, produces a high number of persister cells. The *hipA* gene is a part of the *hipBA* operon which was characterized to be one of the TA systems in *E. coli* [Black et al., 1994, 1991]. Antitoxin HipB forms a 1:1 complex with HipA, thus blocking the HipA toxicity [Black et al., 1994]. HipB is also a negative regulator for the *hipBA* transcription [Black et al., 1991]. The HipB binding sequence in the *hipBA* promoter has been shown to be four palindromic sequences TATCCN₈GGATA (N: any nucleotides) upstream of the *hipBA* promoter [Black et al., 1994]. In the present paper, we demonstrate that similar palindromic sequences exist in close proximity to the *eutH*, *fadH* and

relA promoters, as well as in the promoter regions of a total of 30 other genes on the *E. coli* genome. Using in vitro and in vivo analyses, we show that the expressions of these genes are repressed by HipB. Our results unveil a complexity in the regulatory mechanism of cellular dormancy and a novel function of HipBA. We also demonstrate that HipB modulates the expression of RelA, and its physiological significance will be discussed.

***E. coli* Has 39 Putative HipB-Binding Palindromic Sequences**

By bioinformatic analysis of the *E. coli* genome, we identified a total of 43 genes having the HipB-binding palindromic sequences (TATCCN_(x)GGATA: N is any nucleotides and x is the number of nucleotides from 1 to 30) in both promoters (39 sequences in 33 genes; table 1) or in coding regions (10 sequences; not shown). We demonstrate in the present paper that HipB binds 4 palindromic sequences in the *hipBA* promoter simultaneously and cooperatively. Notably, genes such as *eutH* and *fadH* also contain 2 and 3 TATCCN_(x)GGATA palindromic sequences in their promoter regions. The functions of these 33 genes are highly diverse, as their putative functions are associated with persistence (*relA*) [Metzger et al., 1989], metabolism (*ubiD*, *fadH*, *frsA*, *ydeT*, *fkpB*, *can*, and *ygeW*) [Harvie et al., 2006; He et al., 1997; Hottenrott et al., 1997; Koo et al., 2004; Leppik et al., 1976; Smith and Ferry, 2000; Xi et al., 2000], transcriptional regulation (*insH*, *rfaH*, *xapR*, *yijO*, *nhaR*, *hipB*, and *yedW*) [Beutin et al., 1981; Engler and van Bree, 1981; Hirakawa et al., 2003; Moyed and Bertrand, 1983; Perez-Rueda et al., 2004; Rahav-Manor et al., 1992; Seeger et al., 1995], and mismatch repair (*mutH*) [Welsh et al., 1987]. Notably, among the 33 genes, there are a number of membrane proteins and transporter proteins such as, *yiaB*, *ynjI*, *ygiH*, and *eutH* encoding inner membrane proteins [Daley et al., 2005; Drew et al., 2002; Stojiljkovic et al., 1995], and the genes for transporter proteins, *alsA*, *modF*, and *yjhF* [Kim et al., 1997; Peekhaus et al., 1997; Saurin et al., 1999].

EutH, an ethanolamine transporter, transports uncharged ethanolamine [Penrod et al., 2004]. Bacteria use ethanolamine as sole carbon and nitrogen source under aerobic conditions [Roof and Roth, 1988; Roth et al., 1996]. FadH is 2, 4-dienoyl-CoA reductase and degrades unsaturated fatty acids in *E. coli* [Liang et al., 2000; You et al., 1989]. The multiple HipB-binding sites located on the promoters indicate a possibility that HipB plays a significant role in regulation of *eutH* and *fadH* expressions.

Table 1. HipB-binding palindromic sequences on the *E. coli* genome

Nucleotides between TATCC and GGATA (GAP) ¹	Gene ²	Distance from +1 ³	GAP ⁴
G	<i>yqhG</i>	35	1
CGCT	<i>alsA</i>	174	4
CCTTAAGC	<i>hipB</i>	22	8
TCACATAA	<i>hipB</i>	50	8
CGTAGAGC	<i>hipB</i>	89	8
GCGATCGC	<i>hipB</i>	117	8
AGGTCGCA	<i>ydeT</i>	53	8
AACAGGAT	<i>yhiI</i>	264	8
CAGCTTTCTGT	<i>mutH</i>	210	11
CCTCCTGGCGGC	<i>ybdM</i>	111	12
GACCTACAGGGA	<i>yehS</i>	98	13
TAAGTAAACAGAA	<i>yjtD</i>	198	13
AGTTTGGATCGTCG	<i>insH</i>	103	14
GCTCCATCGCGGGC	<i>ppdD</i>	166	14
TTGCGCCCCGATTAAAC	<i>rfaH</i>	36	17
GTTTAATCGGGGCGCAA	<i>ubiD</i>	131	17
TTTTCCATATAAATATT	<i>ygaQ</i>	123	17
ATTGTGTTTATGTATGTC	<i>xapR</i>	69	18
CCTGCCTGACCGGGTGGG	<i>yiaB</i>	164	18
AGGCATAACCTTTACTACAG	<i>yehE</i>	8	20
AGCGGATCAAGAAAATTCGTT	<i>ynjI</i>	57	21
TTGTCATGTGAATGAGTTTTGA	<i>yedW</i>	96	22
TCAAAACTCATTCACATGACAA	<i>yedX</i>	37	22
CACGAAGCGCCTGAACGCCGCCT	<i>can</i>	148	23
TGTAACCCTGCAACGCTGGCTCG	<i>relA</i>	158	23
CTGTCAGTAATCGCTGCACAAAGTG	<i>modF</i>	5	25
GGCCTACAGATCGCGGCGGTAGGCT	<i>yijO</i>	169	25
AGCCTACGGGATTGCACATGTAGGGC	<i>eutH</i>	54	26
AGCCTACGGGATTGCACATGTAGGGC	<i>eutH</i>	154	26
GCCCTACATGTGCAATCCGTAACCT	<i>fadH</i>	191	26
GCCCTACATGTGTGTTCCCGTAGGTC	<i>fadH</i>	291	26
GCCCTACATGTGCAATCCTCCTGGCC	<i>fadH</i>	391	26
GTCAAATTAAGTCTTTTGACGCCGCGC	<i>ygeW</i>	146	26
GGCCTACAAATGCGCTCCCCGTAGGTC	<i>phnL</i>	46	27
CGCAAGATACCTGGATTGAACAGCCGTG	<i>frsA</i>	167	28
TGGTCGGTTCTATCTCTTCGGCGGTAATT	<i>nhaR</i>	135	29
ATCACGTCCTGCCTGCTTTTGGCTAACC	<i>ygiH</i>	112	29
GGCCTACAGATTGCTGCGAAATCGTAGGCC	<i>fkpB</i>	92	30
GACTCTGCATCATTCGTTATTTGCTCAGTC	<i>yjhF</i>	35	30

¹ The sequences between TATCC and GGATA.² The name of downstream gene.³ Distance of palindromic sequence from the transcription initiation site.⁴ The length of sequences between TATCC and GGATA.

Another interesting observation was that one of the putative HipB-binding sequences resides in close proximity to the *relA* promoter (158 nucleotides upstream of the translation initiation site). Due to its relevance to persistence and (p)ppGpp synthesis, we analyzed whether HipBA indeed regulates the expression of RelA in addition to *eutH* and *fadH*.

HipB Binds to the *eutH*, *fadH*, and *relA* Promoters

First, the interaction of HipB with the *eutH*, *fadH*, and *relA* promoters was examined using double-stranded DNA composed of the putative HipB-binding sites in the *relA*, *eutH*, and *fadH1* promoters (fig. 1 and online suppl. fig. 1; for all online suppl. material, see www.

Fig. 1. HipB-binding palindromic sequences in the promoter region of the *hipBA* operon and the *relA* gene. Four palindromic sequences (*hipB1*, *hipB2*, *hipB3* and *hipB4*), found upstream of the *hipBA* promoters (each having 8-base spacer sequences, as shown). The HipBA-binding palindromic sequence in the promoter region of the *relA* gene has 23-base spacer sequences.

karger.com/doi/10.1159/000354311). The formation of HipB-DNA complex was analyzed by electrophoretic mobility shift assay. As shown in figure 2 and online supplemental figure 2, the HipB protein formed a complex with the *hipB1*, *hipB2*, *relA*, *eutH*, and *fadH1* promoter sequences. In all cases, the amount of free DNA was significantly reduced by the addition of the HipB protein. The band patterns of the HipB-DNA complex, however, appeared different. While the HipB-*hipB* promoter complexes exhibited a distinct band, the addition of HipB to the *relA*, *eutH*, and *fadH1* promoter sequences yielded smear patterns. We speculated that the absence of a distinct band, when the *relA*, *eutH*, and *fadH1* sequences were used, is most likely due to weaker (possibly unstable) interaction between HipB and the *relA*, *eutH*, and *fadH1* sequences. A crude estimate of the binding constant for HipB-*relA* and HipB-*hipB1* complexes was calculated based on figure 2a with the dissociation constant (K_d) of HipB-*hipB1* and HipB-*relA* of approximately 0.9×10^{-7} and 2.9×10^{-6} M, respectively. No significant interaction was observed between HipB and DNA fragment without a palindromic sequence (fig. 2c). To confirm the specificity of HipB binding to the *relA* promoter, we performed the same experiment with use of HipA, which was added to the reaction mixture containing HipB prior to the addition of DNA. When both HipB and HipA were added to the reaction mixture, the smear band was shifted higher

Fig. 2. Protein-DNA interactions. **a** *hipB* and *relA* promoter fragments with HipB. **b** HipB and HipBA with *relA* promoter fragment. **c** HipB and HipBA with negative control DNA fragment. All samples contained 8×10^{-7} M DNA and 0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, or 5.6×10^{-6} M HipB and HipBA, respectively. The binding buffer consisted of 10 mM HEPES-KOH (pH 7.9 at 20°C), 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, and 5% glycerol. Samples are resolved in 8% polyacrylamide gel. Running buffer was composed of 25 mM Tris-base, 190 mM glycine, and 1 mM EDTA. F = Free double-strand annealed DNA. Arrow indicates the position of the HipBA-DNA complex. Arrowhead and asterisk indicate the positions of the HipB-DNA complex.

Fig. 3. Effects of HipB on the *relA*, *euthH*, and *fadH* promoter activity. **a** The *relA*, *euthH*, and *fadH* promoters were transcriptionally fused to *lacZ*. **b** BW25133 cells carrying designated plasmids were grown in M9 medium overnight. Cells were diluted 100 times by fresh medium containing 25 ng/ml chloramphenicol and 100 ng/ml ampicillin. When the cell density reached OD₆₀₀ 0.2, arabinose (0.2%) was added. After 1 h and 3 h of induction, 0.1 ml of the cell culture was collected, and 0.9 ml of Z buffer (0.06 M Na₂HPO₄·7H₂O,

0.04 M NaH₂PO₄·H₂O, 0.01 M KCl, 0.001 M MgSO₄·7H₂O, and 0.05 M β-mercaptoethanol; pH 7.0), 2 drops of chloroform, and 1 drop of 0.1% SDS were added. Samples were incubated at 28°C for 5 min and 0.2 ml *o*-nitrophenol-β-galactopyranoside was added. Reactions were stopped by adding 0.5 ml of 1 M Na₂CO₃ and absorbance at 420 nm was measured by a spectrophotometer. β-Galactosidase activity was normalized by the β-galactosidase activity at the time of induction (cells in 0.2 in OD₆₀₀).

Table 2. A list of primers used to clone the *euthH*, *fadH*, and *relA* promoters

Name	Sequence
euthP-F	5'-GAATTCCCGGGGATCCTGCCGCATCTTATCCAGC-3'
euthP-R	5'-TAAAACGACGGGATCCCCAATTCCTATCGCCTTTAC-3'
fadP-F	5'-GAATTCCCGGGGATCCTAAACGCCTTATCCGCCC-3'
fadP-R	5'-TAAAACGACGGGATCCCCGTAGCTCATAAAATTGTC-3'
relAp-F	5'-GAATTCCCGGGGATCCAACTGGAACGCTTTTCGC-3'
relAp-R	5'-TAAAACGACGGGATCCCCCGCAACCATCGTCTCTC-3'

than that of HipB alone (fig. 2b). Significantly, this HipBA-DNA complex appeared as a sharp and distinctive band, in contrast to smear HipB-DNA complex band. This confirms that the DNA shift observed is not due to a trace amount of contaminated non-specific DNA-binding proteins but due to specific interaction between HipB and the *relA* promoter, and that the addition of HipA somehow stabilizes these HipB-DNA interactions.

We then assessed whether HipB binds to other putative HipB-binding sequences (table 1). HipB indeed bound to all 8 palindromic sequences tested (data not shown), suggesting that HipB is a global regulator for *E. coli* gene expression regulating not only *hipB*, *relA*, *euthH*, and *fadH* genes but also many other genes.

HipB Negatively Regulates RelA, EuthH and FadH Expression

Next, the interaction between HipB and the *relA*, *euthH*, and *fadH* promoters in vivo was examined using a β-galactosidase reporter assay system as depicted in figure 3a. A reporter gene for β-galactosidase was cloned in pRS414 plasmid immediately downstream of the *relA*, *euthH*, and *fadH* promoters (table 2), while *hip* genes were cloned into pBAD33 under the control of the arabinose-inducible promoter. Two plasmids were co-transformed into *E. coli* BW25113 (Δ*hipBA*; fig. 3a). The levels of the *relA*, *euthH*, and *fadH* promoter activities at 1 and 3 h after HipB induction were determined [Zhang and Bremer, 1995] (fig. 3b). Compared to the empty pBAD33 plasmid,

Fig. 4. Effects of HipB, HipBA and HipBA-D291A on the *relA* promoter activity. The *relA* promoter was transcriptionally fused to *lacZ*. BW25133 cells carrying designated plasmids were grown in M9 medium overnight. β -Galactosidase assay was carried out as described in figure 3.

cell expressing HipB showed decreased *relA*, *eutH*, and *fadH* promoter activities by 27.4, 43.7, and 42.0% in 1 h and 59.5, 50.2, and 57.2% in 3 h, respectively. This is consistent with the proposed role of HipB as a repressor of the *hipBA* promoter [Black et al., 1994]. As predicted, HipB repressed an expression of genes with multiple palindromic sequences (*eutH* and *fadH*) at a higher degree than that of a single palindromic sequence (*relA*). There are Crp, FadR, and ArcA-binding sites between the HipB-binding sites and the translational start site of the *fadH* promoter [Feng and Cronan, 2010]. CRP-cAMP complex activates the *fadH* promoter; however, the absence of CRP-cAMP reduces the repression of *fadH* expression by FadR and ArcA-ArcB. This suggests a possibility that HipB may regulate *fadH* expression in cooperation with other DNA-binding proteins.

HipA Plays an Additional Role in Repression of RelA Expression

Since HipA has been reported to play a role in persister cell formation in a RelA-dependent manner [Korch et al., 2003], we examined whether HipA plays an additional role in repression of the *relA* promoter activity. To achieve equal molar expression of HipB and HipA, both proteins were coexpressed from pBAD33. The HipBA complex was found to repress the *relA* promoter by 36.8%, significantly higher than by HipB alone

(fig. 4). This result is also consistent with the previous notion that HipA protects HipB from degradation and that apparent increase in repression is likely due to increase in available HipB for DNA binding [Hansen et al., 2012]. We also performed the same experiment with a mutant allele of HipA [D291A]. HipA with this mutation was proposed to have a weaker interaction with HipB [Schumacher et al., 2009], and therefore was not expected to affect the repressor activity of HipB. Consistently, we observed that HipBA (D291A) exhibited no difference from HipB alone in repression of RelA expression (fig. 4).

In this study, we discovered that HipB can regulate the expression of *EutH*, *FadH*, and *RelA* in *E. coli*. Our results also suggest that HipA plays an additional role in the regulation of persistence as it enhances the repressor activity of HipB. Previously, the study of HipA was limited to a search for a target of its toxicity and how that influences the degree of persistence. Our study unveiled another possible mechanism for regulating persister formation through the *hipBA* loci.

HipA was first identified to make a dramatic increase in persistence in *E. coli* [Korch et al., 2003]. This increase in persistence depends on the presence of (p)ppGpp synthetase (RelA). However, the direct evidence of how HipA alters persistence has not been clearly demonstrated. Recent studies showed that HipA has a serine/threonine kinase domain and can phosphorylate EF-Tu at Thr382 [Schumacher et al., 2009]. The phosphorylated EF-Tu results in a loss of its ability to bind aa-tRNA [Alexander et al., 1995; Lippmann et al., 1993]. These observations suggested that HipA is toxic since it inhibits protein synthesis by modulating activity of EF-Tu [Schumacher et al., 2012, 2009]. Its antitoxin, HipB, is a DNA-binding protein that regulates the transcription of the *hipBA* module [Black et al., 1994, 1991]. It was suggested that disordered C-terminus of HipB releases a degradation signal for Lon protease [Hansen et al., 2012]. The binding of HipA to HipB blocks the access of Lon to HipB. Our observation that HipA co-expression with HipB enhances the repressor activity of HipB, supports this hypothesis. The HipB-*relA* promoter complex showed a smear band in gel electrophoresis, possibly indicating unstable binding in the in vitro buffer condition. Weak interaction between HipB and the *relA* promoter was stabilized by the addition of HipA, as a sharp distinct band appeared, indicating that the HipBA complex may be more stable than HipB alone for binding to the *relA* promoter. The mechanism of how HipA stabilizes HipB-DNA binding requires further investigation.

HipB-binding palindromic sequences are widely distributed on the *E. coli* chromosome (table 1). The functions of these genes also seem highly diverse. Some genes encode enzymes that regulate the important physiological functions in metabolism and transportation. These enzymes may be involved in regulating the internal cellular environment during the transition from the active growth state to the state of persistence. Others may alter transcription pattern as IS5 transposase (*insH*), or as DNA-binding transcriptional regulators (*rfaH*, *hipB*, and

yedW). It remains to be determined what types of the genes are regulated by these regulators and how that regulation affects *E. coli* physiology.

Acknowledgements

The authors thank Dr. Elliot Campbell for critical reading of the manuscript. This work was supported by a US National Institute of Health grant (1R01GM081567).

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