Metal ion-dependent anti-termination of transcriptional regulation of ribonucleoprotein complexes

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Abstract Anti-terminator proteins are frequently used by bacteria to sense a specific metabolite signal and direct RNA polymerase to either terminate or continue transcription of the genes downstream of an operon. One such protein is HutP, which binds to upstream *cis*-regulatory sequences to regulate expression of the histidine utilization (*hut*) operon in *Bacillus subtilis*. HutP must be activated by L-histidine and divalent metal ions before binding to *hut* mRNA; binding of activated HutP prevents termination of transcription. Thus, HutP appears to regulate the *hut* operon in a unique fashion in this class of regulatory proteins. To understand gene (*hut* operon) regulation by HutP, we performed several biochemical and structural studies. These studies reveal events in the regulatory mechanism, starting with the activation of HutP and ending with the unwinding of *hut* terminator RNA. In this review, we describe the unique regulatory mechanisms commonly used by many *Bacillus* species.

Keywords Anti-termination · *hut* operon · *Bacillus subtilis* · Ribonucleoprotein complex · Crystal structure

Introduction

Bacteria utilize a variety of mechanisms for regulating gene expression at the posttranscriptional level in response to changes in the environment. Among these, a common pathway is the stabilization of alternative mRNA secondary structures by RNA-binding proteins. These proteins direct transcription either to pause or to proceed to the synthesis of the

full-length transcript. RNA-binding proteins modulate the nascent mRNA in two ways, referred to as termination and anti-termination. Termination proteins bind to the nascent mRNA and prevent the formation of an alternative structure. Anti-termination proteins promote destabilization of the terminator structure. In the latter case, the nascent mRNA forms the terminator structure by default, while in the former case, the mRNA is set for transcription elongation. Several bacterial termination and anti-termination proteins that regulate nascent mRNA have been described in *Escherichia coli* and *Bacillus subtilis*, including TRAP, PyrR, LacT, BglG, SacT/SacY, GlpP, and HutP; these mRNAs regulate the *trp*, *pyr*, *lac*, *bgl*, *sac*, *glpP* and *hut* operons, respectively (Houman et al. 1990; Aymerich and Steinmetz 1992; Oda et al. 1992; Babitzke and Yanofsky 1993; Arnaud et al. 1996; Glatz et al. 1996; Lu et al. 1996; Alpert and Siebers 1997). To facilitate or destabilize terminator structures, regulatory proteins require specific ligands for activation before binding to their cognate RNAs (Table 1). For example, the *trp* RNA-binding attenuation protein (TRAP) is a well-characterized protein that inhibits the formation of the anti-terminator structure by modulating the leading region of mRNA (Babitzke and Yanofsky 1993). In contrast to the TRAP regulation mechanism, HutP binds directly to the terminator and stabilizes the anti-terminator structure. HutP regulates *hut* (histidine utilization) operon transcription by an anti-termination mechanism in *Bacillus subtilis*. HutP is unique in its regulation of mRNA in that it requires not only a specific ligand L-His (L-histidine) but also a divalent metal ion for its activation. In this review, we focus on this unique regulatory mechanism that is unraveled by structural and functional analyses

This article does not present data from any studies with human or animal subjects performed by the authors.

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The *hut* operon and HutP protein

The *hut* operon is responsible for the degradation of histidine to ammonia, glutamate and a one-carbon compound.

Table 1 Proteins that regulate termination and anti-termination of transcription and their cognate ligands required for activation

Operons	Proteins	Ligands required for activation
<i>trp</i>	TRAP	Tryptophan
<i>pyr</i>	PyrR	Uridine monophosphate
<i>lac</i>	LacT	Lactose
<i>bgl</i>	BglG	β -Glucosides
<i>sac</i>	SacT/SacY	Phosphoenol pyruvate
<i>glpP</i>	GlpP	Glycerol-3-phosphate
<i>hut</i>	HutP	L-Histidine

Although it is not yet clear just how widely distributed the *hut* pathway is among bacteria, about 1,164 bacterial species are currently known to possess the histidine utilization (Hut) system (Bender 2012). The regulation of the *hut* pathway has been extensively studied in enteric bacteria (such as *Klebsiella*), *Pseudomonas* species, and *Bacillus subtilis*. However, the mechanisms of regulation in these bacteria differ significantly, as discussed in a recent review (Bender 2012). In our review, we concentrated primarily on the histidine utilization system that is operating in *Bacillus subtilis*. The *hut* genes in *B. subtilis* are located in a single *hut* operon which consists of five structural genes, namely, *hutH*, *hutU*, *hutI*, *hutG*, and *hutM*, which code for the histidase, urocanase, imidazolone propionate amino hydrolase, formimino L-glutamate formimino hydrolase, and histidine permease enzymes, respectively. In addition, there is a positive regulatory gene, *hutP* (Fig. 1a) (Chasin and Magasanik 1968; Kimmhi and Magasanik 1970; Oda et al. 1988; Yoshida et al. 1995). These five enzymes are responsible for the degradation of L-histidine and its use as a carbon and nitrogen source. The structural genes are located far downstream of the promoter, whereas the regulatory gene *hutP* is located much closer to the promoter. The nucleotide sequence located between the structural genes and *hutP* forms a stem-loop terminator structure (nucleotides +515 to +553) which is known to be modulated by HutP after HutP activation by L-histidine and divalent metal ions (Fig. 1b). Once the terminator structure is attenuated, the RNA polymerase can synthesize a full-length *hut* mRNA transcript, which is then translated into five structural proteins. HutP is a 16.2-kDa protein consisting of approximately 148 amino acid residues, depending upon the bacterial species. HutP is present in various *Bacillus* species, including highly pathogenic bacteria such as *Bacillus anthracis*. The amino acids at the C-terminus of HutP are highly conserved across *Bacillus* species compared to the N-terminus residues (Kumarevel et al. 2004a), suggesting that the C-terminus residues are important for HutP regulatory functions. HutP has no sequence similarity with other known transcription-regulating proteins, indicating that HutP controls transcription in a unique fashion. The HutP protein is essential for high levels of expression of the *hut* operon (Oda et al. 1988).

Essential ligands for activating HutP and binding to *hut* mRNA

Earlier studies showed that HutP required L-histidine for binding to the *hut* terminator (Oda et al. 2000; Kumarevel et al. 2004a), suggesting that L-histidine controls the binding of HutP to the terminator by modifying the conformational state of HutP. Kumarevel et al. (2004a) observed that 10 mM L-histidine was required for the complete activation of HutP and for efficient binding to *hut* mRNA. To address which chemical groups of L-histidine were important for the activation of HutP, these authors screened analogs of L-histidine for their ability to activate HutP and induce binding to RNA. Among the analogs tested, L-histidine β -naphthylamide (HBN) and L-histidine benzylester showed tenfold higher affinity than unmodified L-histidine. Other analogs, such as L-histidine methyl ester and L- β -imidazole lactic acid, showed similar activation as L-histidine. In contrast, urocanic acid, histamine, and L-histidinamide showed only weak activation of HutP. The presence of imidazole alone or of several analogs that were modified at the imidazole ring failed to activate HutP for binding to the terminator RNA. These analyses suggested that the imidazole ring and the backbone of L-histidine are essential for activating HutP (Kumarevel et al. 2004a).

To evaluate the divalent metal ion requirement for the HutP–RNA interaction, an important step was to remove the endogenous divalent metal ions by denaturing the protein in 7 M urea followed by refolding HutP in binding buffer without any divalent metal ions. Kumarevel et al. (2005a) used this treated HutP protein to analyze HutP binding to the terminator RNA in the presence and absence of divalent metal ions (Fig. 1c). The results of these binding analyses suggested that HutP requires not only L-histidine but also divalent ions such as MgCl_2 . To address which other divalent metal ions support the activation of HutP, these authors then screened 15 divalent metal ions, differing in ionic radii and preferred coordination, at a concentration of 10 mM and observed that Mn^{2+} , Zn^{2+} , and Cd^{2+} were the most efficient ions, followed by Mg^{2+} , Co^{2+} , and Ni^{2+} . The other divalent metal ions, which only weakly supported HutP activation, were Ba^{2+} , Sr^{2+} , Pt^{2+} , Ca^{2+} , Pb^{2+} , and Ag^{2+} . The divalent metal ions Cu^{2+} , Yb^{2+} and Hg^{2+} and monovalent metal ions such as NaCl (100 mM) and KCl (100 mM) failed to activate HutP for RNA binding (Kumarevel et al. 2005a).

HutP protein interaction with *hut* mRNA

The *hut* mRNA between nucleotides +459 to +572 was found to fold into alternative conformations (Oda et al. 2000). These authors also observed that the *hut* mRNA appeared to form a stable terminator structure (nucleotides +498 to +572) in the absence of activated HutP, and that the region between

Fig. 1 A histidine utilization (*hut*) operon of *Bacillus subtilis* and its regulation are mediated by the interaction of HutP (bacterial anti-termination protein which regulates *hut*) with *hut* terminator RNA in the presence of L-histidine and metal ions. **a** A schematic representation of the *hut* operon of *B. subtilis*. **b** The proposed *hut* mRNA terminator structure is known to be destabilized by activated HutP. Dashed lines Terminator region, red dashed lines within the terminator HutP binding regions. **c** Binding analyses to evaluate the role of L-histidine and divalent metal ions in the activation of HutP and binding to the *hut* terminator RNA. The binding assay was carried out with 200 nM of HutP and 1 nM unlabeled RNA (5′-CAUAGAUCUUAGACGAUAGGG-3′, 21-mer)

nucleotides +459 to +537 seemed to form a destabilized RNA structure (anti-terminator structure) upon binding of activated HutP. To map the HutP binding site between nucleotides +459 to +537, these authors analyzed shorter RNAs of varying lengths. They initially found that nucleotides +459 to +572 were sufficient for recognizing activated HutP, but further deletion analyses revealed the a 20-mer RNA (between nucleotides +496 to +515, 5′-CAUAGAUCUUAGACGAUAGGG-3′) was the shortest that was sufficient for efficient recognition of activated HutP (Oda et al. 2000) (Fig. 1d). Interestingly, in this region, three UAG repeats and four non-conserved spacer residues were found (Kumarevel et al. 2004a). Upon

containing the same RNA with 10,000 cpm of 5′ end-labeled product. To this reaction mixture 10 mM of L-histidine and 10 mM of MgCl₂ were added in the case of test samples; for controls, either MgCl₂ or L-histidine was omitted. Upon completion of a 1-h incubation at 25 °C, the reaction mixture was filtered, washed, and quantitated similar to that reported previously (Kumarevel et al. 2004a). **d** Identification of activated HutP binding sites within the *hut* terminator RNA region. Letters in red Important residues (UAG) within the terminator region. Shorter (20 mer) RNA fragments were analyzed for HutP binding to define the binding region and to identify the important nucleotides for HutP recognition

further trimming to UAG, AUAG, UAGA, or UUUAGUU or when UAG was reversed in the 20-mer RNA (5′-CAGAUUAGUACGAGAUAGG-3′), the HutP failed to bind. These studies show that the UAG sequence is important and that efficient recognition requires multiple UAG repeats to facilitate cooperative binding (Kumarevel et al. 2004a). To understand the role of the four-spacer nucleotides, Kumarevel et al. (2004b) carried out base substitutions to replace the spacers with four U nucleotides. These authors found that the spacers had only a limited role in the recognition of activated HutP. Upon substitution of other bases for the A and G within the UAG motifs, HutP failed to recognize the mutated RNAs.

Taken together, these studies reveal that the activated HutP core recognition motif is XAG, where X indicates any base. To clarify which functional groups of the A and G residues were important, site-specific base modifications were introduced into the 20-mer RNA and analyzed for binding, revealing that the 6-amino moiety of the A base and the 2-amino moiety of the G base at the second and third positions, respectively, were the key functional groups for HutP recognition. Further analyses of the chemical groups within the UAG motif unveiled only one 2' OH of the A base that was important for HutP recognition (Kumarevel et al. 2004b).

Crystal structures of HutP

To understand regulation of the *hut* operon by HutP, a number of studies were carried out to resolve a series of crystal structures of HutP and its complexes (Table 2). These complexes included apo-HutP, HutP-L-His, HutP-L-His-Mg²⁺ (HutP-L-His-Mn²⁺, HutP-L-His-Ba²⁺, HutP-L-His-Zn²⁺), HutP-L-His-Mg²⁺-RNA (21 mer), and HutP-L-His-Mg²⁺-RNA (55 mer, a model RNA), as summarized in Table 2. The HutP-Mg²⁺ complex was also resolved, but no specific binding site for Mg²⁺ was found, suggesting that the structure of the HutP-Mg²⁺ complex is the same as that of apo-HutP. Among these crystal structures, the highest resolution structure was 1.48 Å, obtained for the quaternary complex (HutP-L-His-Mg²⁺-RNA). For clarity, crystal structure details of free HutP and the HutP complexes are described separately in the following sections.

Apo-HutP

Analysis of the crystal structure of apo-HutP (in the absence of any ligand) revealed that two HutP monomers form a dimer

with a non-crystallographic twofold axis, and three dimers are symmetrically arranged along a crystallographic threefold axis to form a hexamer (Fig. 2a). The hexamer structure resembles a flattened cylinder approximately 83 Å wide and 48 Å thick along the threefold axis. A close-up view in Fig. 2b shows the HutP dimer. The monomer belongs to the α/β family of proteins whose structure consists of four α helices and four β strands arranged in the order $\alpha\alpha\beta\alpha\alpha\beta\beta$ in the primary structure. Four antiparallel strands form a β sheet in the order $\beta1\beta2\beta3\beta4$ with two α helices each at the front and back of the β sheet. A hydrophobic pocket is visible in the vicinity of the twofold axis (Fig. 2c), and this pocket is employed for binding of L-His as shown in the following section.

HutP-L-His and -L-histidine analogs

Of all the HutP series structures, HutP-L-His was the first structure to be solved by crystallography. Phasing information was obtained from multi-wavelength anomalous dispersion (MAD) experiments (Kumarevel et al. 2004a). All other crystal structures of the HutP series were solved by molecular replacement methods. HBN was employed as an L-histidine analog for the X-ray study of this binary complex and showed the highest activation of HutP and subsequent RNA binding among all of the L-histidine analogs tested (Kumarevel et al. 2004a). In the crystal structure of HutP-L-histidine analog (HutP-HBN), HBN is bound in the hydrophobic pocket (Fig. 2c), resulting in three L-histidine analogs in the HutP hexamer (Fig. 3a). As described in Table 2, crystals of the HutP-L-histidine analog are isomorphic with apo-HutP. Indeed, HutP in the binary complex is very similar to the apo form of HutP, indicating that HBN fits in the hydrophobic pocket without inducing significant structural changes (Fig. 3b). It is clear that the existence of the hydrophobic

Table 2 Summary of the crystallographic data collected for HutP in the presence and absence of different ligands

HutP complex	Space group	Unit cell (Å, °)	Number of molecules In ASU	Resolution (Å)	PDB code	Reference
Apo-HutP	P2 ₁ 3	a=b=c=96.82	2	2.78	1WPS	Kumarevel et al. 2005b
HutP- Mg ²⁺	P2 ₁ 3	a=b=c=95.41	2	2.6	1WPT	Kumarevel et al. 2005a
HutP-HBN	P2 ₁ 3	a=b=c=95.41	2	2.8	1VEA	Kumarevel et al. 2004a
HutP-L-His-Mg ²⁺	P2 ₁ 2 ₁ 2	a=77.79, b=80.88, c=75.17	3	1.70	1WPV	Kumarevel et al. 2005b
HutP-L-His-Mn ²⁺	P2 ₁ 2 ₁ 2	a=77.76, b=81.40, c=76.04	3	2.2	1WRN	Kumarevel et al. 2005a
HutP-L-His-Ba ²⁺	P2 ₁ 2 ₁ 2	a=78.22, b=81.14, c=75.97	3	2.35	1WRO	Kumarevel et al. 2005a
HutP-L-His-Zn ²⁺	P3	a=b=129.33, c=76.67, γ =120.0	12	2.5	4H4L	Balasundaresan et al. 2013
HutP-L-His-Mg ²⁺ -RNA (21 mer)	H3	a=b=75.80, c=133.35, γ =120.0	2	2.20	1WRQ	Unpublished
HutP-L-His-Mg ²⁺ -RNA (21 mer)	R3	a=b=76.21, c=133.56, γ =120.0	2	1.60	1WMQ	Kumarevel et al. 2005b
HutP-L-His-Mg ²⁺ -RNA (21 mer)	H3	a=b=76.06, c=133.99, γ =120.0	2	1.48	1WPU	Unpublished
HutP-L-His-Mg ²⁺ -RNA (55 mer)	C2	a=99.64, b=76.47, c=62.79, β =109.06	3	1.70	3BOY	Gopinath et al. 2008

ASU Asymmetric Unit; PDB Protein Data Bank; HBN L-histidine β -naphthylamide

Fig. 2 Structure of apo-HutP (PDB code: 1WPS). Different HutP monomers are shown in *different colors*. **a** C α backbone chain presentation of the HutP hexamer, viewed along the threefold symmetry axis, **b** ribbon presentation of the HutP dimer, viewed along the pseudo twofold axis, **c** alternative view of the HutP dimer surface reveals the hydrophobic pocket (*boxed*) in the center of dimer interface

pocket is a prerequisite for the recognition of the L-His imidazole group and its discrimination from other amino acid residues. This is the necessary step to verify the incoming residue in the pocket because L-His in the following step does not employ the imidazole group but rather the backbone amino and carboxyl groups of the L-His to coordinate with Mg^{2+} , as described later in this review. The details of the L-His binding site are shown in Fig. 3c. Arg88 and Glu81 of another monomer form a salt bridge at the gateway to the hydrophobic pocket, which may provide stabilizing interactions. Mutational analyses of Arg88 and Glu81 suggest that these residues are important for L-His binding. The imidazole side-chain of HBN is buried in the hydrophobic pocket and hydrogen bonds with Glu139, Phe141, and Glu81. The backbone nitrogen of HBN bonds with Phe141 and the backbone oxygen of HBN bonds with Gly85 of another monomer. The naphthyl ring of HBN is at the gateway to the hydrophobic pocket, placing it in the vicinity of the methylene chains of Arg88 and Glu81 that are involved in the salt bridge mentioned above (Kumarevel et al. 2004a). When the structure of HutP, co-crystallized with L-histidine benzylester and L-histidine, was also solved, the mode of binding was found to be the

same as in the case of HBN, and the HutP structure was also similar to that in the HBN complex (Kumarevel et al. 2004a).

In biochemical experiments, a filter-binding assay showed that HutP failed to bind to RNA in the absence of Mg^{2+} ions, despite the presence of L-His (Fig. 1c; Kumarevel et al. 2005a, b). When Mg^{2+} ions were added to the binding buffer, formation of the RNA-bound quaternary complex was observed (Fig. 1c). These studies suggest that the L-His-bound HutP is not activated to bind RNA and that L-His and Mg^{2+} ions are required to activate HutP. The next step to understanding the role of divalent metal ions in the activation of HutP was to be resolved a ternary complex structure of HutP (HutP-L-His- Mg^{2+}).

HutP-L-His- Mg^{2+}

Crystals of the HutP-L-His- Mg^{2+} complex provide better atomic resolution than those of apo-HutP and HutP-L-His and improve the accuracy of the structure. Indeed, in HutP-L-His- Mg^{2+} , the entire HutP peptide chain (Leu2-Ile148) is visible (the N-terminal Met1 must be fMet and may be removed during translation) except for some side-chain atoms that are likely

Fig. 3 Structure of HutP-L-His (PDB code: 1VEA). The L-histidine analog (L-histidine β -naphthylamide; HBN) is shown as a *stick model*. Different HutP monomers are shown in *different colors*. A *yellow-colored stick model* represents HBN. **a** C α backbone chain presentation of the HutP hexamer and HBN, viewed along the threefold symmetry axis, **b** ribbon presentation of the HutP dimer and HBN, **c** L-His binding site in the HutP-L-His complex. *Dotted lines* Hydrogen bonds

exposed to solvent regions. In contrast, N-terminus residues 1–5 of one chain and 1–4 of another chain were not visible in the HutP-L-His structure. Analysis of the crystal structure of HutP-L-His-Mg²⁺ showed that the Mg²⁺ ion and L-His interact. This interaction site is at the interface of three HutP monomers (Fig. 4a, b; Kumarevel et al. 2005b); thus, the HutP hexamer has six L-His-Mg²⁺ binding sites, as shown in Fig. 4a. A close-up view of the L-His-Mg²⁺ binding site is shown in Fig. 4c. The bound Mg²⁺ ion is located at the interface between HutP monomers, forming a six-way coordination with the L-His ligand and the histidine cluster of the HutP protein. Of the six coordination interactions, two are with the amino and carboxyl groups of the L-His ligand, and three others are with the imidazole nitrogens of His73, His77, and His138. The sixth coordination site of the Mg²⁺ ion is with a water molecule that is anchored by a hydrogen bond to the side-chain of Glu81. When the structures of the HutP-L-His-Mg²⁺ complex and the HutP-HBN complex are superimposed, the L-His-Mg²⁺ binding site is approximately 12 Å from the L-His binding pocket in the HutP-HBN complex. This result suggests that when the Mg²⁺ ion is added to the HutP-L-His complex, the ion binds to

the amino and carboxyl groups of the L-His ligand and induces the conformational change of the L-His imidazole which in turn loosens the binding of L-His to HutP, resulting in L-His being pulled out of the hydrophobic pocket. In this way, Mg²⁺-bound L-His can move along the dimer interface of HutP to a new binding site (Kumarevel et al. 2005b). This movement is linked to the movement of Arg88 in the opposite direction, leading to the disruption of the hydrophobic pocket; the salt bridge between Arg88 and Glu81 in the HutP-HBN complex is disrupted, and a different type of salt bridge is newly formed between Arg88 and Glu81, as shown in Fig. 4c. The carboxyl group of the L-His ligand also forms salt bridges with Arg88 and Arg98. The L-His-Mg²⁺ binding site is thus created by the drastic rearrangement of these amino acid residues as they become involved in hydrogen bonds, salt bridges, and Mg²⁺ ion coordination, as mentioned above. The rearrangement of Arg98 (C α position shifted by 5.4 Å) is accompanied by a large change (5.0 Å) in the C α position of the next residue, Thr99. Consequently, the Thr99 side-chain forms two hydrogen bonds with the N3 and 2' OH of the A4 base of the RNA, as described below (Fig. 5).

Fig. 4 Structure of HutP-L-His-Mg²⁺ (PDB code: 1WPV). L-His and Mg²⁺ are shown as a yellow-colored stick model and a blue sphere model, respectively. Different HutP monomers are shown in different colors. **a** C α backbone chain presentation of the HutP hexamer, L-His, and Mg²⁺, viewed along the threefold symmetry axis, **b** ribbon presentation of the HutP dimer, **c** L-His-Mg²⁺ binding site in the HutP-L-His-Mg²⁺ complex. Dotted lines Coordination interactions and hydrogen bonds, red sphere water molecule. **d** Alternative-binding modes of L-His with respect to the metal ions

HutP-L-His complexes with different divalent metal ions

In a series of biochemical analyses Kumarevel et al. (2004a) demonstrated that several divalent metal ions were able to influence the activation of HutP. Among these, Mn²⁺, Zn²⁺,

and Cd²⁺ were the most efficient, followed by Mg²⁺, Co²⁺, and Ni²⁺ ions. Other divalent metal ions that weakly supported activation were Ba²⁺, Sr²⁺, Pt²⁺, Ca²⁺, Pb²⁺, and Ag²⁺. To clarify the importance of these divalent metal ions in the activation of HutP, three of these divalent cations (Mn²⁺,

Fig. 5 Crystal structure of the HutP-L-His-Mg²⁺-RNA (21 mer) complex (PDB code: 1WPU). The HutP hexamer complex is presented as a surface model (gray), as is the RNA (color). Close-up views of specific HutP-RNA nucleotide (A and G) interactions are shown in the box

Ba^{2+} and Zn^{2+}) were also used for crystallographic studies (Kumarevel et al. 2004a; Balasundaresan et al. 2013). The crystallization conditions for the HutP-L-His- Mn^{2+} and HutP-L-His- Ba^{2+} complexes were essentially the same as those for HutP-L-His- Mg^{2+} . The obtained crystals were isomorphous with HutP-L-His- Mg^{2+} , as shown in Table 2, and their structures were very similar to that of HutP-L-His- Mg^{2+} . These similarities occurred despite slight changes in ion coordination; the coordination distances between the Ba^{2+} ions and the N atoms of the histidine residues were approximately 0.3 Å larger than the Mg^{2+} and Mn^{2+} distances, thereby widening the dimer interface (Kumarevel et al. 2004a).

However, in the case of the HutP-L-His- Zn^{2+} complex, crystals were obtained under conditions different from those used for the Mg^{2+} , Mn^{2+} , and Ba^{2+} complexes. The structure was solved at a resolution of 2.5 Å (Balasundaresan et al. 2013), and the structure of HutP was very similar to that of the HutP-L-His- Mg^{2+} complex. Differences were observed in the active site where Zn^{2+} formed a distorted four-coordinate complex, and the orientation of the L-His ligand coordinated to Zn^{2+} was reversed relative to its orientation with Mg^{2+} , Mn^{2+} , and Ba^{2+} . This resulted in the coordination of Zn^{2+} with the N ϵ atom of the imidazole ring of L-His. The coordination structure observed in the complexes with the Mg^{2+} , Mn^{2+} , and Ba^{2+} ions is Form I, and the structure in the complex with the Zn^{2+} ion is Form II (Fig. 4d). Interestingly, the crystal structure of the HutP-L-His- Zn^{2+} complex has 12 HutP monomers in an asymmetric unit (Table 2). Therefore, it may be possible for the orientation of L-His in some monomers to be Form I, however, the coexistence of Form I and Form II is possible in the HutP active site; the imidazole side chain and the nitrogen-carboxyl oxygen backbone chain of the L-His are similar in size and metal coordination ability and are thus exchangeable. In this context, it is possible to speculate that Zn^{2+} is the best metal ion to mediate the activation of RNA binding (Kumarevel et al. 2004a). As described later Thr99 is an important residue that specifically interacts with the adenosine of the UAG motif. The next residue Arg98 binds tightly to L-His through the salt bridge in Form I as seen in Fig. 7c, but weakly to L-His in Form II. The positional flexibility of Arg98 is transmitted to Thr99 and the side chain of Thr99 is adjusted to a preferable position for better binding to RNA (Balasundaresan et al. 2013).

HutP-L-His- Mg^{2+} -RNA

A minimal 20-base region for HutP binding to *hut* mRNA was identified as the region between nucleotides +496 to +515 (Fig. 1d). In this region, three UAG repeats and four non-conserved spacer residues were found to be important for RNA binding (Kumarevel et al. 2004b). In the quaternary complex structural analysis, a 21-mer RNA (5'-UUUAGU UUUUAGUUUUUAGUU-3') was employed. The resolved

structure (Kumarevel et al. 2005b) shows that the orientation of HutP, L-His, and the Mg^{2+} ions in the quaternary complex with RNA is very similar to that of the HutP-L-His- Mg^{2+} complex, indicating that the L-His- Mg^{2+} -bound form of HutP is able to bind RNA. Therefore, it is natural to reason that the L-His- Mg^{2+} -bound form is the active form of HutP. In contrast, apo-HutP and HutP-L-His are inactive forms of HutP that correspond to pre-structural rearrangements. The 21-mer RNA is bound on each side of the HutP hexamer with a triangle shape (Fig. 5). Each of the bound 21-mer RNAs has three repeated seven-nucleotide sequences (UUUAGUU) arranged with a crystallographic threefold symmetry. The central A4-G5 of the UAG motif forms an extensive hydrogen-bonding network with HutP (Fig. 5), and bases A4 and G5 are stacked on top of each other. Figure 5 shows that A4 hydrogen bonds to side-chains of Thr99 and Thr128 and that G5 bonds with a side-chain of Glu137. These amino acid residues are critical at the RNA-binding site, as shown by the failure of the mutant proteins Thr99Ala, Thr128Ala, and Glu137Ala to bind the RNA (Kumarevel et al. 2004b). Thus, the specific interactions between the bases A4 and G5 and HutP demonstrate the importance of these nucleotides within the UAG motif. On the other hand, the bases U1, U2, U3, and U6 do not interact with HutP, and U7 interacts with the neighboring dimer within the hexamer. These U nucleotides most likely serve as spacers; their basic function is to appropriately place the next UAG binding site on the HutP hexamer. The backbone conformation at the U2 nucleotide is unusual, with the RNA bent to form a triangular shape on the surface of the HutP hexamer. In Fig. 5, the negatively charged phosphate backbone of the RNA is directed outwards toward the solvent region, and the RNA bases bury into HutP for hydrogen-bonding interactions. This orientation readily explains the RNA sequence-dependent nature of the recognition by HutP.

There is one RNA-binding site on each surface of the HutP hexamer. In addition, there are two UAG-rich regions (highlighted in red letters in Fig. 6a) within the *hut* terminator structure. These are considered to be potential HutP binding sites and are flanked by a 20-nucleotide spacer region (site I, +498 to +514; site II, +535 to +549). Each binding site consists of three XAG motifs (X denotes any base). Of these two potential binding sites, the first recognizes one surface (site I) of the HutP hexamer, and the second recognizes the other surface (site II). Between these two binding sites, there is a 17- to 20-nucleotide spacer region (depending on the first XAG motif count at site II; cyan-highlighted region in Fig. 6a), which is most likely sufficient to reach the RNA on the other surface of the hexamer. To evaluate whether the two XAG motifs separated by the spacer region are important for HutP binding, a model RNA was designed, synthesized, and tested for its binding to the activated HutP (Fig. 6b; Gopinath et al. 2008). These binding studies revealed that the model 55-mer RNA binds in a cooperative manner to HutP. To

Fig. 6 Presence of two potential RNA-binding regions within the *hut* terminator RNA, and the structure of the model 55-mer bound to HutP (PDB code: 3BOY). **a** *hut* terminator regions showing two potential binding sites for the HutP protein separated by the spacer region, highlighted in *cyan*. Important residues for *hut* binding are shown in *red letters*. **b** A model 55-mer RNA sequence mimicking the *hut* terminator RNA for HutP binding. The spacer region flanking sites I and II is shown in *red letters*. **c** Molecular surface representation of the quaternary complex structure showing the model 55-mer RNA binding the two surfaces (site I and site II) of HutP

understand this mode of HutP-RNA recognition, the quaternary complex (HutP-L-His-Mg²⁺-55 mer RNA) was resolved at 1.70 Å. As predicted, the structure revealed that the XAG motifs in site I (nucleotides 1–20) bind in a triangular fashion to one surface of HutP. The following linker nucleotides carry the remaining XAG motif to site II (nucleotides 37–55) on the other surface of HutP, where binding occurs in the same fashion as in site I (Fig. 6c). The structural analysis was further confirmed by in-line mapping, mutational, and in vivo *hut* anti-termination studies which demonstrated that the model RNA bound to the both surfaces of HutP, as shown in Fig. 6c.

Structural rearrangement of HutP

A large structural change in the HutP protein is observed between the structures of apo-HutP and the HutP-L-His-Mg²⁺ complex [root-mean-square deviation (RMSD) 3.1 Å], as mentioned earlier. However, the structure of the HutP-L-His-Mg²⁺ complex closely resembles that of the HutP-L-His-Mg²⁺-RNA complex (RMSD 0.69 Å), suggesting that divalent metal ions and L-His, but not RNA, cause structural rearrangement of the HutP. Structural differences between the HutP-L-His-Mg²⁺ complex and the apo-HutP complex are observed mainly in the region of the L-His-Mg²⁺-binding site of HutP. Figure 7 shows a structural comparison between the two complexes. It appears that His73, His77, and His138 are rearranged to coordinate with the Mg²⁺ ion. The rearrangement of His138 is linked to the movement of the following residue, Glu137, whose side-chain binds specifically with G5 of the UAG motif (Fig. 5). Arg98 rearranges to form a salt

bridge with L-His, and this rearrangement is linked to the movement of the next residue, Thr99, whose side chain binds specifically with A4 of the UAG motif (Fig. 5). Arg88 is also rearranged to form a salt bridge with L-His, and this arrangement is linked to the movement of Glu81, whose side chain

Fig. 7 Superposition between HutP-L-His (PDB code: 1VEA) and HutP-L-His-Mg²⁺-RNA (PDB code: 3BOY) showing structural rearrangement of HutP. C backbone chain models of the HutP of 1VEA and 3BOY are shown in *orange* and *green*, respectively. HBN and L-His are represented by *stick models with labels*. Important amino acid residues involved in structural rearrangement are shown by *thick stick models with bold labels* in 3BOY, and by *thin stick models with small labels* in 1VEA.

Fig. 8 Conformational changes in the *hut* terminator RNA upon binding to activated HutP

oxygen forms both a salt bridge with Arg88 and water-mediated coordination with a Mg^{2+} ion (Fig. 4c). After rearrangement, the above residues are located at the interface between three HutP monomers, and they appear to contribute directly or indirectly to the formation of the L-His- Mg^{2+} -binding site, which is necessary for RNA binding.

Destabilization of *hut* terminator structure

The above section describes how the HutP protein is activated through structural rearrangement caused by divalent metal ions and the L-His ligand. The activated HutP is able to bind to RNA, and the bound RNA is arranged in a novel triangular

Fig. 9 Anti-termination of the *hut* operon mediated by activated HutP

fashion (Fig. 5). Before HutP binding, the *hut* mRNA forms a stem-loop terminator structure in between the HutP and HutH coding regions. After HutP binding, the terminator structure is destabilized, allowing the RNA polymerase to synthesize a full-length *hut* mRNA transcript. A simple destabilization pathway is shown in Fig. 2. Taking into account the structural, biochemical, and in vivo studies described in preceding section, we propose a detailed mechanism of *hut* terminator structure destabilization, as shown in Fig. 8. There are two potential HutP binding sites (site I, +498 to +514; site II, +535 to +549). Each binding site consists of three XAG motifs. The first binding site recognizes one surface of the HutP hexamer, and the second site recognizes the other surface. Between these two binding sites, there is a 20-nucleotide spacer region (Fig. 8), which is sufficient to reach the RNA on the other surface. The quaternary complex was tested using model RNA, which consisted of a 17-nucleotide spacer (U_{17}) region flanked by two UAG-rich regions (Fig. 8). In this structure, the first binding site, consisting of 21-mer RNA ($U1-U21$) forms a triangle shape on one surface of the HutP hexamer. The second binding site ($U35-U55$) forms another triangle shape on the other surface via the spacer region ($U22-U34$). In vivo analyses using the native *hut* terminator (nucleotides +489 to +599 in Fig. 8; Gopinath et al. 2008) suggest that HutP recognizes the first two XAG motifs in site I and then rolls over the RNA towards the third XAG motif. The adjacent G–C-rich base-pair stem will start to melt. Consequently, the XAG motif in site II is exposed and binds to the other surface of HutP, resulting in the disruption of the stable stem region. Taken together, in vitro, in vivo, and structural analyses reveal that HutP destabilizes the *hut* terminator RNA structure by accessing two XAG-rich sites.

Summary

The histidine utilizing (Hut) pathway is highly conserved among bacteria, but the mechanisms of regulation in these bacteria differ. In *Bacillus subtilis*, the Hut pathway is known to be regulated by a positive regulatory protein, HutP. Several studies have shown that HutP binds to the *hut* mRNA terminator and destabilizes the terminator structure to allow polymerase to synthesize the full-length *hut* mRNA. A series of structural analyses were carried out to unravel the overall events starting from activation of HutP to binding to the terminator RNA. These studies revealed snapshots of the conformational changes at each stage, and these snapshots have enabled clarification of the entire activation process of HutP in the presence of two ligands (L-histidine and a divalent metal ion), as summarized in Fig. 9. These conformational changes appear to be important for binding to the terminator RNA in order to facilitate the unwinding of the *hut* terminator RNA. This kind of regulation appears to be unique among the

known termination and anti-termination proteins, where a divalent metal ion renders help in this process. We suggest that such a metal-ion dependent anti-termination regulatory mechanism is also being utilized by other species of bacteria.

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Conflict of Interest The authors certify that there is no actual or potential conflict of interest in relation to this article.

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