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COMMENTARY

HU Knew? *Bacillus subtilis* HBsu Is Required for DNA Replication Initiation

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ABSTRACT The prokaryotic nucleoid-associated protein (NAP) HU is both highly conserved and ubiquitous. Deletion of HU causes pleiotropic phenotypes, making it difficult to uncover the critical functions of HU within a bacterial cell. In their recent work, Karaboja and Wang (J Bacteriol 204:e00119-22, 2022, https://doi.org/10.1128/JB.00119-22) show that one essential function of *Bacillus subtilis* HU (HBsu) is to drive the DnaA-dependent initiation of DNA replication at the chromosome origin. We discuss the possible roles of HBsu in replication initiation and other essential cellular functions.

KEYWORDS chromosome structure, nucleoid-associated protein, NAP, HU, HBsu, *hbs*

HU IS REQUIRED FOR CELL VIABILITY ACROSS THE BACTERIAL DOMAIN

Prokaryotic nucleoid-associated proteins (NAPs) are a diverse class of DNA-binding proteins spatially organizing DNA through wrapping, bending, or bridging. NAPs are globally involved in compacting and structuring the bacterial chromosome and regulating DNA topology (1). While the DNA-structuring activities of NAPs have been a major focus of attention, it is less clear how these activities translate into the many functions that NAPs fulfill within a bacterial cell (see below).

The protein HU is an abundant NAP that is highly conserved throughout the bacterial domain (2, 3). In some species such as the proteobacteria *Escherichia coli* and *Caulobacter crescentus*, HU is encoded by two closely related genes and can thus assemble into homo- and heterodimers, while in others like the actinomycete *Mycobacterium smegmatis* and the firmicute *Bacillus subtilis*, HU is encoded by a single gene and occurs exclusively as a homodimer. Removing or impairing HU activity *in vivo* is generally detrimental, causes strongly reduced viability in *E. coli*, and is lethal in *B. subtilis*. *E. coli* cells lacking HU show defects affecting growth, metabolic gene expression, lysogeny, transposition, cell division, resistance against DNA damage, and DNA supercoiling (4–8). In the absence of HU, *E. coli* frequently produces anucleate cells, indicating defects in chromosome duplication or segregation (6). Additionally, a recent study in *Mycobacterium smegmatis* showed that the loss of HU causes delayed DNA replication initiation, suggesting a role of HU homologs in DNA replication initiation (9). Critically, however, the pleiotropic phenotypes associated with HU mutants have obfuscated attempts to ascertain its essential function(s) within the bacterial cell.

The *B. subtilis* HU homolog, termed HBsu and encoded by the single gene *hbs*, is the only known major NAP in this organism (10). HBsu appears to have a DNA architectural function similar to that of *E. coli* HU (11–13), and cells harboring low levels or defective variants of HBsu display an array of phenotypes, including a classical sporulation defect (14–16). While these previous studies have pointed toward important functions of HBsu *in vivo*, the essentiality of HBsu made it challenging to pinpoint its most critical activities.

HBsu IS DIRECTLY INVOLVED IN DNA REPLICATION INITIATION IN *BACILLUS SUBTILIS*

Using two independent methods for rapidly depleting HBsu from the cell, Karaboja and Wang (17) have recently shown that HBsu is directly involved in DNA replication initiation at the chromosomal origin of *B. subtilis*. By tracking fluorescently labeled chromosomal origins

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and performing genome-wide marker frequency analyses, Karaboja and Wang authors show that chromosomal replication initiation is quickly halted upon the depletion of HBsu, phenocopying the depletion of the essential replication initiation protein DnaB. This is an exciting finding since a role of HU homologs in DNA replication initiation has been proposed for several bacteria based on knockout phenotypes and *in vitro* studies (6, 9, 18) but until now was unknown for firmicutes like *B. subtilis*. Importantly, Karaboja and Wang show that HBsu activity is specifically required for replication initiation at the native chromosomal origin, which depends on the master bacterial DNA replication initiator DnaA (17). Cells initiating replication from an artificially introduced plasmid origin, *oriN*, which requires the heterologous initiator RepN and is independent of DnaA (19, 20), are not blocked for DNA replication. This finding opens important questions regarding the function of HBsu in DNA replication initiation and why HBsu essentiality is specific to the DnaA-dependent chromosome origin (*oriC*).

HBsu AS PART OF THE INITIATION COMPLEX AND A POSSIBLE ROLE IN DNA LOOPING AT THE ORIGIN?

The role of HU in DNA replication initiation has been most studied in *E. coli*, where it promotes the unwinding of the *E. coli* origin *in vitro* (18). Further *in vitro* studies showed that HU directly interacts with DnaA to stabilize DnaA binding at the origin (21), and it was suggested that HU might be specifically incorporated into a DnaA-origin complex (22). It is important to note that HU is not the only NAP stimulating DNA replication initiation in *E. coli*. The *E. coli* origin harbors several binding sites for the protein factor for inversion stimulation (Fis), a NAP initially thought to inhibit DNA replication initiation based on *in vitro* results (23, 24) but later shown to stimulate replication initiation *in vivo* under rapid-growth-promoting conditions (25). Additionally, the integration host factor (IHF) binds to a single specific site in the *E. coli* origin. IHF is a distant homolog of HU that introduces a sharp bend of >120° into double-stranded DNA (1, 26) and promotes replication initiation *in vitro* and *in vivo* (18, 27, 28). IHF is not essential for replication initiation in *E. coli*, but control over replication timing is lost upon its deletion (22). Importantly, the combined deletion of HU and IHF produces a strong growth defect in *E. coli*, suggesting that these NAPs have at least partially redundant functions (27). The essential role of HBsu in DnaA-dependent *oriC* activity in *B. subtilis* is consistent with NAPs being generally involved in DNA replication initiation. Critically, however, binding of HBsu to the *B. subtilis* chromosomal origin has not yet been shown, nor is HBsu a known interaction partner of DnaA (29). Future investigations might specifically test for HBsu recruitment to the origin and its relationship to DnaA activity.

While HU has a mode of action on DNA different from that of IHF (1), its activity in modulating DNA flexibility could play an important role in *B. subtilis* origin topology. *In vitro*, HU proteins can alter the persistence length of the DNA double helix (30, 31), and in *E. coli*, HU facilitates the formation of repression loops between two *lacO* sites via the repressor LacI (32). It has been suggested that DnaA molecules recruited between 50 and 100 bp upstream of the unwinding site of the *B. subtilis* origin are delivered to the latter via a DNA loop (33). Furthermore, *B. subtilis* and other firmicutes as well as some epsilonproteobacteria have been suggested to possess bipartite origins of replication in which the origin consists of two clusters of DnaA-binding sites that are separated by the ~1,300-bp-long *dnaA* gene. *In vitro*, upon the binding of DnaA to the separate clusters, these can join while looping out the *dnaA* coding region (34–36). It is currently unknown whether looping between DnaA-binding-site clusters in bipartite origins takes place or is essential for chromosomal replication initiation *in vivo*. It is possible that in *B. subtilis*, HBsu plays a role in stabilizing DNA loops formed within the chromosomal origin. Such structures could facilitate the delivery of DnaA and other initiation proteins between regions of *oriC*, or they could promote an architecture of the DNA polymer more labile to unwinding (37).

DOES HBsu AFFECT DnaA-CHROMOSOMAL ORIGIN-DEPENDENT DNA REPLICATION INITIATION VIA ACTING ON SUPERCOILED DNA?

In addition to stabilizing DNA loops, HU homologs influence DNA superhelicity (38). *In vitro*, HU forms multimers around which DNA can be wrapped, leading to restrained negative DNA supercoiling (39). Negative supercoiling is a requirement for bacterial origin

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unwinding *in vitro* (40) and *in vivo* (41, 42). In *E. coli*, the introduction of negative supercoiling into the chromosomal origin via the transcription of flanking genes promotes replication initiation (41). Similarly, negative supercoiling is also important for initiation at the chromosomal origin of *B. subtilis in vivo*. A global reduction in negative supercoiling through inhibition of gyrase restricts the recruitment of DnaA to the chromosomal origin and inhibits DNA replication initiation (42). Importantly, in *E. coli*, mutations that suppress the loss of HU are frequently found in a gyrase-encoding gene, suggesting a functional link between HU activity and negative supercoiling *in vivo* (43). It is intriguing that, similar to the HBsu depletion phenotype recently reported by Karaboja and Wang (17), gyrase inactivation blocked DnaA-dependent DNA replication initiation at *oriC*, while the plasmid-derived origin *oriN* was much less affected (42). Because RepN-*oriN* requires the same downstream replication initiation proteins, DnaD and DnaB, as does DnaA-*oriC* (44), the sensitivity of *oriC* to both HBsu depletion and changes in supercoiling might reflect critical differences in the architecture and/or initiation mechanism between the two replication initiation systems.

WHAT ARE OTHER ESSENTIAL FUNCTIONS OF HBsu *IN VIVO*?

While Karaboja and Wang have shown that HBsu plays an essential role in DnaA-dependent DNA replication initiation, they also found that restoring initiation via *oriN* is not sufficient for *B. subtilis* viability in the absence of HBsu (17). Therefore, HBsu must perform at least one other essential function in the cell.

One property of HU homologs to consider is their ability to bind a wide range of nucleic acid polymers (45–49). *E. coli* HU binds specifically and with high affinity to nucleic acid structures associated with DNA replication, recombination, and repair (e.g., nicked or gapped DNA and DNA-RNA hybrids, DNA invasions, and DNA forks) (45–49). Using nonlethal alleles, previous work showed that HBsu plays important roles in DNA repair, homologous recombination, and β-protein-mediated site-specific recombination (16). As a consequence, HBsu-defective *B. subtilis* cells are extremely sensitive to DNA-damaging agents, a phenotype akin to that observed for *E. coli* strains lacking HU (4, 8). It is not yet clear whether HBsu's involvement in DNA repair and recombination is essential for viability under standard laboratory conditions. The observed cell elongation phenotype of HBsu-deficient *B. subtilis* may be due to the activation of the DNA damage response, which is known to inhibit cytokinesis. This model predicts that (i) conditions dampening replication and repair stress might compensate for the loss of HBsu and (ii) one of the DNA damage responses in *B. subtilis* (LexA or DnaA dependent) is being activated following the depletion of HBsu.

It is also plausible that HBsu fulfills one or several essential functions in gene expression. Many NAPs are wide-ranging transcriptional regulators that positively or negatively affect gene expression (1, 3). In *E. coli*, HU regulates 8% of the genome, including genes responding to SOS induction, high osmolarity, acid stress, and anaerobic growth (7). The regulation of these genes is thought to occur through two mechanisms: (i) constraining of superhelical DNA, such as for operons responding to hyperosmolarity, and (ii) DNA loop stabilization, which promotes or inhibits the binding of the transcription factors LexA, GadX, and FNR. In addition to affecting gene expression at the level of transcription, HU is also involved in regulating translation. HU interacts with RNA *in vitro* (49), and in *E. coli*, HU binds to the mRNA encoding the alternative sigma factor σ^S (encoded by *rpoS*), enhancing translation *in vitro* and the expression of σ^S *in vivo*. Moreover, HU was also shown to bind to the small RNA (sRNA) DsrA *in vitro* (49), a positive regulator of σ^S expression (50). In *B. subtilis*, HBsu interacts with small cytoplasmic RNA (scRNA), a component of the signal recognition particle involved in protein secretion (51). It remains to be investigated whether HBsu plays a general role in translation through interaction with mRNAs and/or regulatory sRNAs. Although the regulon of HBsu in *B. subtilis* has not yet been determined, a plausible model is that misregulation of transcription and/or translation of one or several genes/operons in the absence of HBsu contributes to lethality. Future work investigating HU enrichment at chromosomal sites via chromatin immunoprecipitation sequencing (ChIP-Seq), as well as transcriptome sequencing (RNA-Seq) and proteomics in the presence and absence of HBsu, is needed to identify transcriptional and translational regulatory functions of HBsu.

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Through their roles in regulating DNA topology and gene expression, NAPs have been suggested to contribute to overall chromosome architecture and compaction albeit to various degrees depending on the NAP and the bacterial species (1, 52). Highly transcribed genes set supercoil diffusion barriers, leading to the organization of bacterial chromosomes into dozens of isolated chromosomal interaction domains of 30 to 420 kbp (53–56), which themselves are further demarcated into subregions that display a high level of self-interaction (52, 53, 56). The loss of HU in *C. crescentus* does not strongly affect the chromosomal domain boundaries, but short-range interactions between chromosomal sites up to 100 kbp apart are significantly reduced, suggesting a role of HU in packing and stabilizing DNA plectonemes (53). Conversely, in *E. coli*, the loss of HU activity disrupts DNA interactions occurring between chromosomal sites up to a megabase apart, implying a role of HU in the macrodomain organization of the *E. coli* chromosome (56). Although intrachromosomal interactions have been studied in *B. subtilis* (55, 57, 58), the impact of HBsu deletion on chromosome structure has not yet been investigated. The improper spatial organization of the *B. subtilis* chromosome upon the depletion of HBsu could hamper structural separation between sister chromosomes and negatively affect chromosome segregation. Analysis of chromosome conformation (e.g. Hi-C) during different stages of HBsu depletion using the strains constructed by Karaboja and Wang will be critical for understanding the potential role of HBsu in *B. subtilis* chromosome organization.

CONCLUDING REMARKS

The work by Karaboja and Wang on HBsu (17) has added an exciting new component to the process of DNA replication initiation in *B. subtilis*. Investigating the potential roles of HBsu in origin loop formation, superhelicity, and binding *oriC* initiation complexes will be key to developing a comprehensive understanding of bacterial DNA replication initiation. Furthermore, investigations of HBsu's other essential function(s) will likely unveil new perspectives on the importance of NAPs in bacterial cell biology.

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