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RESEARCH ARTICLE

HBsu Is Required for the Initiation of DNA Replication in *Bacillus subtilis*

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ABSTRACT Nucleoid-associated proteins (NAPs) help structure bacterial genomes and function in an array of DNA transactions, including transcription, recombination, and repair. In most bacteria, NAPs are nonessential in part due to functional redundancy. In contrast, in *Bacillus subtilis* the HU homolog HBsu is essential for cell viability. HBsu helps compact the *B. subtilis* chromosome and participates in homologous recombination and DNA repair. However, none of these activities explain HBsu's essentiality. Here, using two complementary conditional HBsu alleles, we investigated the terminal phenotype of the mutants. Our analysis revealed that cells without functional HBsu fail to initiate DNA replication. Importantly, when the chromosomal replication origin (*oriC*) was replaced with a plasmid origin (*oriN*) whose replication does not require the initiator DnaA, cells without HBsu initiated DNA replication normally. However, HBsu was still essential in this *oriN*-containing strain. We conclude that HBsu plays an essential role in the initiation of DNA replication, likely acting to promote origin melting by DnaA, but also has a second essential function that remains to be discovered.

IMPORTANCE While it is common for a bacterial species to express multiple nucleoid-associated proteins (NAPs), NAPs are seldomly essential for cell survival. In *B. subtilis*, HBsu is a NAP essential for cell viability. Here, using conditional alleles to rapidly remove or inactivate HBsu, we show that the absence of HBsu abolishes the initiation of DNA replication *in vivo*. Understanding HBsu's function can provide new insights into the regulation of DNA replication initiation in bacteria.

KEYWORDS replication initiation, HU, HBsu, nucleoid-associated proteins, NAPs, *Bacillus subtilis*

All living organisms are challenged with organizing their DNA within the confines of their cells. In eukaryotes, histones are a major player for packaging the DNA in the nucleus. Bacteria do not have histones, but they contain histone-like proteins called nucleoid-associated proteins, or NAPs (1). The vast majority of bacterial species contain many well-known NAPs, including HU, H-NS, IHF, and Fis (2). Generally, these proteins bind to DNA and introduce constraints like bending, wrapping, and bridging, which compact the DNA and structure the nucleoid. In addition to these structuring roles, many NAPs play other functions, such as global regulation of gene expression, controlling DNA replication initiation, and facilitating DNA recombination (3 to 9).

One of the most well-studied NAPs is HU. In *Escherichia coli*, the heterodimer of HU is among the most abundant proteins during exponential growth (10). *In vitro*, HU binds to DNA nonspecifically and induces or stabilizes DNA bending (10, 11), consistent with the role of facilitating nucleoid compaction and long-range DNA contacts in live cells (12). Using a plasmid that contains the chromosomal replication origin, *oriC*, *in vitro* work showed that HU interacts with the replication initiator protein, DnaA, and stimulates the unwinding of *oriC* at replication initiation (13–15). *In vivo* studies have also shown that HU influences DNA replication initiation. In *E. coli*, HU mutants exhibit asynchronous replication (16), cell division defects, and anucleate cell formation (17,

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18). In *Mycobacterium smegmatis*, mutations in the HU homolog caused a delay in DNA replication initiation as well as lowered the frequency for reinitiation of replication (19). Despite these important roles, HU is not essential in *E. coli* or *M. smegmatis* (17, 19).

The HU homolog in the Gram-positive bacterium *Bacillus subtilis* is called HBsu, which is encoded by the *hbs* gene. HBsu is the only prominent NAP in *B. subtilis* and has been shown to be essential for viability (20–22). Like HU, HBsu binds nonspecifically to DNA *in vitro* and compacts DNA (23–25). *In vivo*, HBsu localizes to the entire nucleoid and is implicated in constraining DNA supercoiling (24, 26) and facilitating DNA repair and recombination (27). However, none of these functions explain why HBsu is essential for cell growth and survival.

Here, we investigate the function of HBsu in *B. subtilis*. We constructed conditional mutants either to remove the protein by combined depletion and degradation or to inactivate the protein with a temperature-sensitive allele. Using fluorescence microscopy and whole-genome sequencing, we found that when HBsu was absent or inactivated, DNA replication initiation was blocked. Interestingly, this effect is specific to the chromosomal replication origin, *oriC*, and not when replication is initiated from a plasmid replication origin, *oriN*. Thus, HBsu is required for replication initiation in *B. subtilis in vivo* and is likely acting through *oriC* or its initiator, DnaA.

RESULTS

Construction of conditional mutants of HBsu. To investigate how the HBsu protein impacts chromosome organization and cell physiology, we sought to remove the protein by depletion or degradation. For depletion, at the *hbs* endogenous gene locus, we replaced the *hbs* promoter with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (*Phy*) and grew the cells in the absence of inducer. For degradation, we replaced the *hbs* gene with *hbs-ssrA* fusion (28) and, at a separate genetic locus (*lacA*), expressed a xylose-inducible (*E. coli*) *sspB* gene, which encodes an adaptor protein for the degradation of HBsu-SsrA through the ClpXP proteosome (28). We found that either depleting or degrading HBsu was not efficient at removing the protein. Therefore, we combined these two approaches and constructed a system that could simultaneously deplete and degrade HBsu (Fig. 1A). We first grew the cells in the presence of IPTG to produce HBsu-SsrA. We then washed off the IPTG and resuspended the cells in fresh medium containing 0.5% xylose, so that transcription of *hbs-ssrA* was halted because of the absence of IPTG and the existing HBsu-SsrA proteins were degraded because of the xylose-induced SspB protein. In the presence of IPTG, cells grew similarly to the wild type (WT). In the absence of IPTG and presence of xylose, cell growth was severely retarded (Fig. 1A). Based on the immunoblot analysis and quantification, the HBsu-SsrA protein levels dropped to 21%, 15%, and 13% after depletion-degradation for 1 h, 2 h, and 3 h, respectively (Fig. 1B).

As an alternative approach, we isolated temperature-sensitive (*ts*) alleles of *hbs* using a method described before (29). Briefly, the *hbs* gene was amplified by error-prone PCR and assembled with a spectinomycin resistance gene as well as a 2.5-kb region upstream and a 2.2-kb region downstream of the *hbs* gene using isothermal assembly (30). The product was transformed directly into *B. subtilis*, replacing the wild-type gene and selecting for spectinomycin resistance at 30°C. Transformants were arrayed and then screened for colony formation at permissive (30°C) and restrictive (42°C) temperatures. Approximately 6,900 transformants were screened. Mutants with growth rates and nucleoid morphologies most similar to those of the wild type at 30°C were characterized further. We selected the mutant that had the strongest growth defect at 42°C for our analysis and named it the *hbs*(*ts*) mutant (Fig. 1C), which contains two amino acid substitutions, D40G and P77S. Immunoblot analysis revealed an additional HBsu species that was smaller than the intact protein, indicating that HBsu(ts) is not as stable as the WT (Fig. 1D). After temperature shift, there was still a substantial fraction of the protein remaining, indicating that HBsu(ts) was inactivated at 42°C largely due to changes in protein conformation.

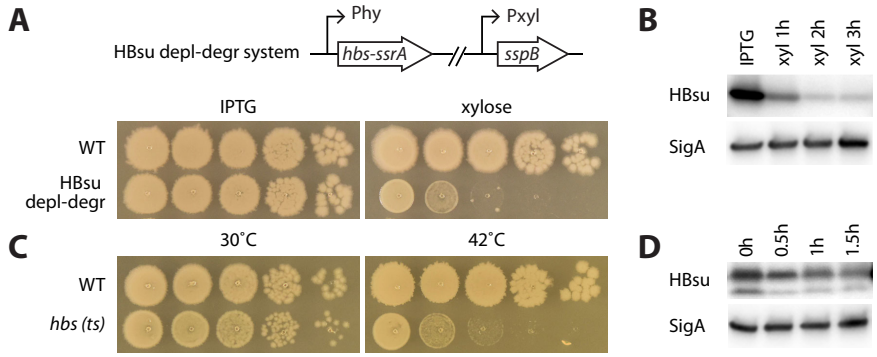


FIG 1 HBsu is required for the growth of *Bacillus subtilis*. (A) An HBsu depletion-degradation (depl-degr) system was constructed. The gene fusion *hbs-ssrA* was expressed from an IPTG-inducible promoter (*Phy*) at the endogenous locus of *hbs*. *E. coli sspB* adaptor protein was expressed from a xylose-inducible promoter (*Pxyl*). The HBsu depletion-degradation strain (BWX1336) was grown in liquid medium containing 0.5 mM IPTG. Cultures were washed three times using medium without the inducer and then normalized to an OD_{600} of 2. The cultures were serially diluted and spotted on agar plates containing 0.5 mM IPTG (HBsu+) or 0.5% xylose (depletion-degradation). Wild-type *B. subtilis* (PY79) was used as a control. The plates were incubated at 37°C for 12 h. (B) Immunoblot analysis of the HBsu depletion-degradation system. The HBsu depletion-degradation strain (BWX1336) was grown in CH medium in the presence of 0.5 mM IPTG. Cells were washed three times using CH medium and then resuspended into CH medium containing 0.5% xylose (xyl). Samples were taken 1 h, 2 h, and 3 h after resuspension. When OD_{600} reached 0.5, the culture was diluted 1:5 in warm CH containing 0.5% xylose to maintain exponential growth. The same growth medium and condition was used for microscopy and whole-genome sequencing experiments throughout the study. Samples were blotted using HBsu polyclonal antibodies. SigA controls for loading. (C) Serial dilutions of WT and *hbs*(*ts*) (BWX4331) strains grown at 30°C and 42°C. The two strains were grown at 30°C, normalized to an OD of 2, serially diluted, and spotted on LB plates, which were incubated at indicated temperatures for 14 h. (D) Immunoblot analysis of *hbs*(*ts*) (BWX4331) strain. Cells were grown in CH medium at 30°C and shifted to 42°C for indicated times. When OD_{600} reached 0.5, the culture was diluted 1:5 in warm CH to maintain exponential growth. The same growth condition was used for microscopy and whole-genome sequencing experiments in this study. The membranes were blotted using HBsu polyclonal antibodies. SigA controls for loading.

Removing or inactivating HBsu generated anucleate cells and larger gaps between nucleoids. To investigate the cytological phenotype upon depletion-degradation of HBsu-SsrA, we visualized the nucleoid morphology using fluorescence microscopy. Cells were grown in the presence of 0.5 mM IPTG to mid-exponential phase. The cultures were washed three times and resuspended using prewarmed medium containing 0.5% xylose. Cultures were diluted as necessary so that the optical density at 600 nm (OD_{600}) was below 0.6 at all time points. In the presence of IPTG, cells grew similarly to the wild type. Upon HBsu-SsrA depletion-degradation, we observed bigger gaps between nucleoids (Fig. 2A) and a dramatic increase of anucleate cells or minicells. Two hours after depletion-degradation, 10% of cells (n = 1,656) were anucleate; after another hour, the proportion of anucleate cells became 40% (n = 2,069) (Fig. 3D). A similar phenotype was observed for *hbs*(*ts*) mutant when HBsu was inactivated by shifting the temperature to 42°C (Fig. 2B). This phenotype was reminiscent of cells arrested for replication initiation, such as in *dnaB*(*ts*) (31) (Fig. 2C).

Removing or inactivating HBsu decreased the number of replication origins per nucleoid. To test the effect of HBsu on DNA replication initiation, we analyzed the number of replication origins upon HBsu depletion-degradation. Replication origins were visualized using an array of 48 *tetO* genes bound by TetR-CFP (32). In the presence of IPTG, there were 2 to 4 origins per cell (Fig. 3A), with an average of 2.8 origins per cell (n = 1,546) (Fig. 3D). After depletion-degradation of HBsu-SsrA, the number of origins decreased to an average of 1.5 origins per cell (n = 2,069) (Fig. 3A and D). A similar effect was observed for *hbs*(*ts*) mutant when HBsu was inactivated at 42°C (Fig. 3B and D). The removal or inactivation of HBsu phenocopied the *dnaB*(*ts*) mutant, which is known to arrest DNA replication initiation (31) (Fig. 3C and D). These results indicate that HBsu is required for replication initiation in *B. subtilis*.

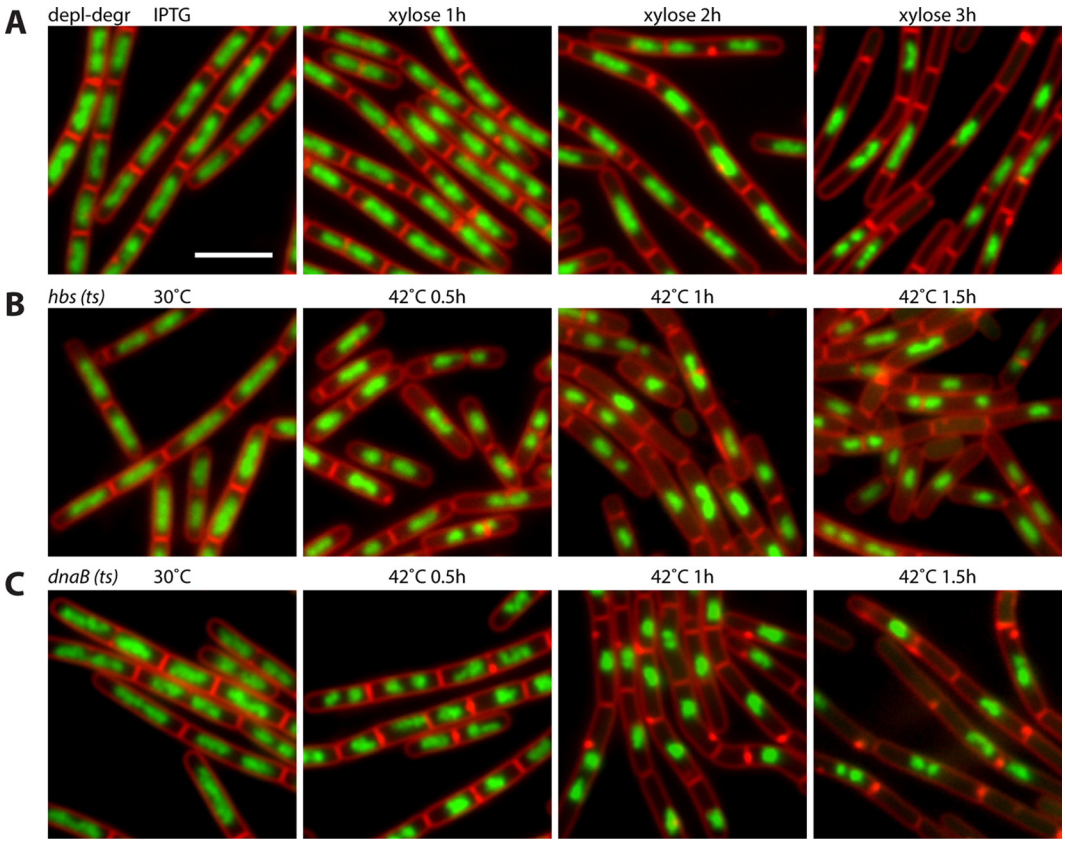
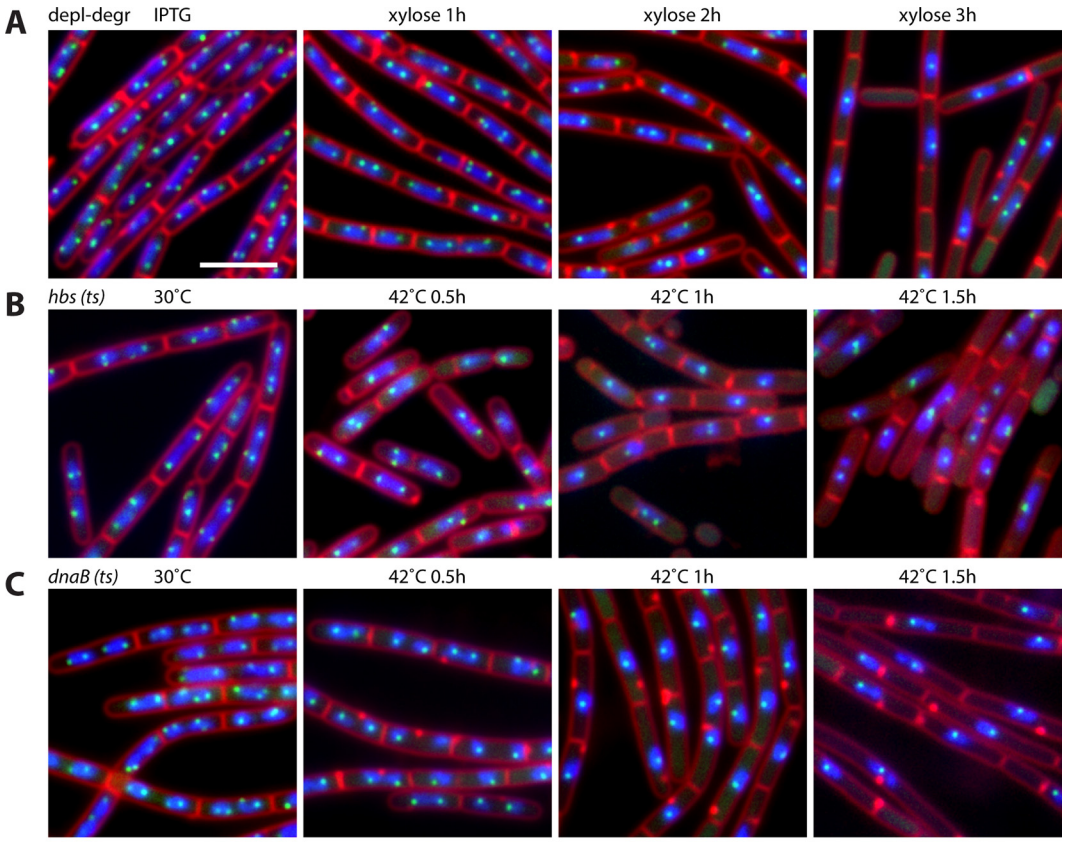


FIG 2 Gaps between nucleoids increased upon HBsu removal or inactivation. (A) HBsu depletion-degradation strain (BWX1340) was grown in the presence of 0.5 mM IPTG and then washed three times and resuspended in warmed medium containing 0.5% xylose for indicated times. FM4-64-stained membrane (red) and DAPI-stained nucleoids (green) are shown. Bar, 4 μm. (B) *hbs*(*ts*) (BWX4414) strain was grown at 30°C, shifted to 42°C, and imaged at the indicated time points. (C) *dnaB*(*ts*) (BWX2533) strain was grown at 30°C, shifted to 42°C, and imaged at the indicated time points.

Removing or inactivating HBsu decreased *ori*-to-*ter* ratio. To quantify the relative copy number of chromosome loci across the entire genome, we performed whole-genome sequencing (WGS). Cells were grown as described in previous sections, and samples were taken before and after HBsu depletion-degradation. The genomic DNA was extracted, processed, and sequenced using Illumina NextSeq. The samples were normalized for the total number of reads and mapped to the wild-type genome (PY79). Before HBsu-SsrA depletion-degradation, the WGS result showed the typical profile for exponential growth, with an *ori*-to-*ter* ratio of 2.8 (Fig. 4A). Upon depletion-degradation, the WGS profile became flat, and the *ori*-to-*ter* ratio dropped to 1.3 and 1.0 at 2 h and 3 h (Fig. 4A). A similar phenomenon was observed when HBsu was inactivated in *hbs*(*ts*) mutant (Fig. 4B). Again, removing or inactivating HBsu phenocopied the *dnaB*(*ts*) mutant, in which the *ori*-to-*ter* ratio dropped from 2.6 to 1.2 after temperature shift from 30°C to 42°C (Fig. 4C). These data further corroborate the conclusion that HBsu is required for replication initiation. We note that WGS measures the *ori*-to-*ter* ratio, which is lower than the origin/cell ratio measured by microscopy (Fig. 3D).

HBsu was not required for replication initiation when *oriC* was replaced by a plasmid replication origin, *oriN*. To test whether the requirement of HBsu for replication initiation is specific to *B. subtilis oriC*, we introduced the HBsu depletion-degradation system to a strain containing the plasmid replication origin, *oriN*, as the only replication origin on the chromosome (33, 34). This strain does not require *oriC* or DnaA for replication. Upon HBsu depletion-degradation, the *oriN* strain had HBsu protein levels drop to levels similar to that of the *oriC* (WT) strain (compare Fig. 5C with 1B). However, in contrast to the *oriC* strain in which we saw a decrease in origin number per nucleoid (Fig. 3B), the average



D

depl-degr	IPTG	xylose 1h	xylose 2h	xylose 3h
No. of cells	1546	1859	1656	2069
*origin/cell	2.8	2.6	2.0	1.5
anucleate cells	0%	0%	10%	40%
hbs (ts)	**30˚C**	**42˚C 0.5h**	**42˚C 1h**	**42˚C 1.5h**
No. of cells	1650	1897	2009	3126
*origin/cell	2.2	1.8	1.5	1.5
anucleate cells	5%	5%	30%	51%
dnaB (ts)	**30˚C**	**42˚C 0.5h**	**42˚C 1h**	**42˚C 1.5h**
No. of cells	1247	1151	790	745
*origin/cell	3.1	2.0	1.1	1.1
anucleate cells	0%	0%	16%	45%

* average number of origins in cells containing a nucleoid

FIG 3 Number of replication origins was reduced upon HBsu removal or inactivation. (A to C) Representative images of DAPI-stained nucleoids (blue), FM4-64-stained membrane (red), and replication origin (*tetO*/TetR-CFP, green) of cells growing under indicated conditions in CH medium. (A) HBsu depletion-degradation strain (BWX1340) was grown in the presence of 0.5 mM IPTG, washed three times, and resuspended in warmed medium containing 0.5% xylose. Bar, 4 μm. (B) *hbs*(*ts*) strain (BWX4414) was grown at 30°C, shifted to 42°C, and imaged at the indicated time points. (C) *dnaB*(*ts*) strain (BWX2533) was grown at 30°C, shifted to 42°C, and imaged at the indicated time points. (D) Analysis of number of origins per cell that contained nucleoids and the percentage of anucleate cells.

number of origin foci per cell in *oriN* strain did not decrease (Fig. 5A and B). In parallel, we performed whole-genome sequencing on samples collected before and after HBsu depletion-degradation in the *oriN* strain. We found that the replication profiles of the *oriN* strain remained largely unchanged upon HBsu depletion-degradation (Fig. 5D), consistent with the microscopy data (Fig. 5A and B). From these results, we concluded that HBsu is not required for replication initiation at *oriN* but is only required for replication initiation at *oriC*. Thus, the requirement of HBsu for replication initiation is likely acting through DnaA.

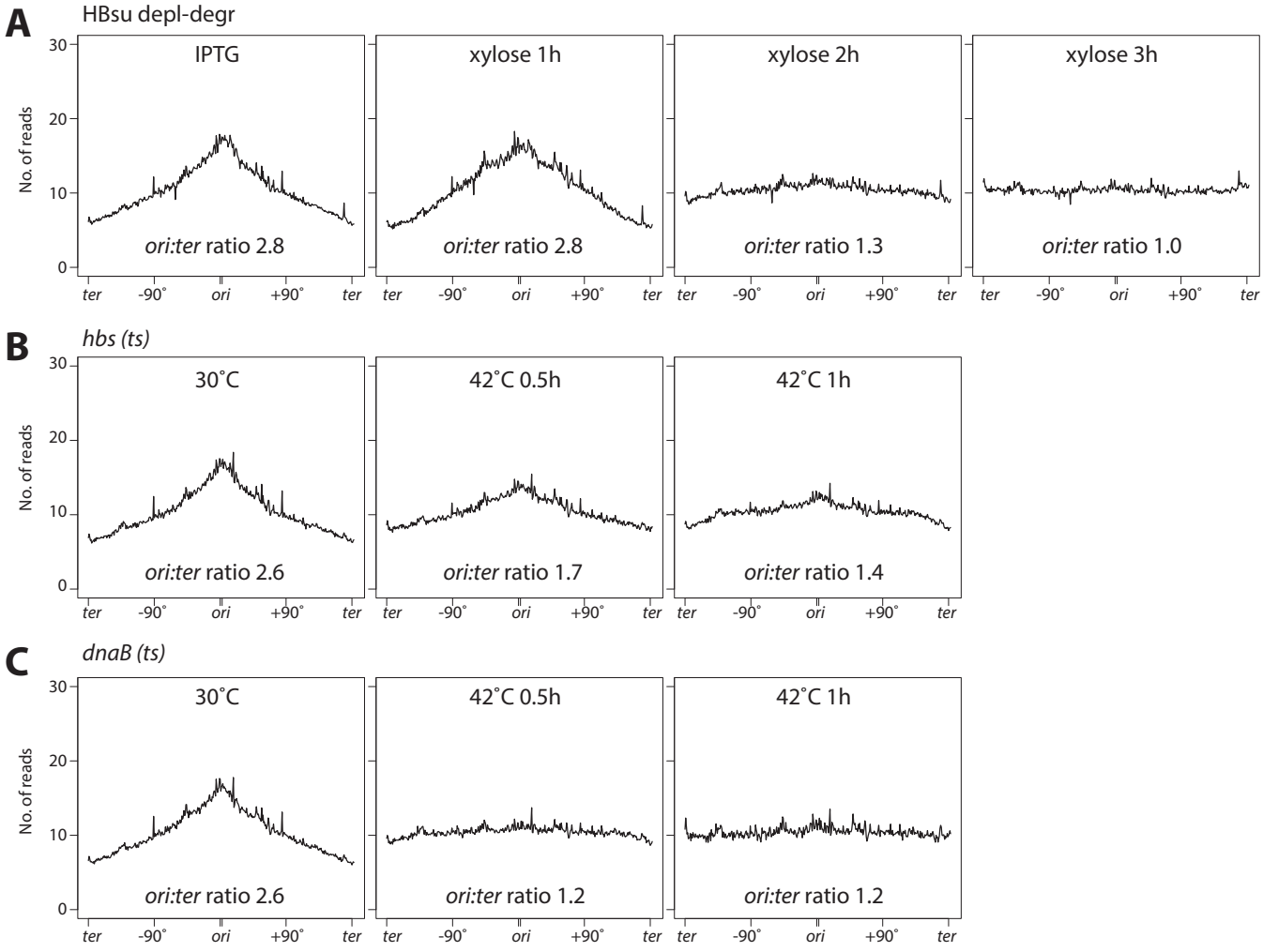


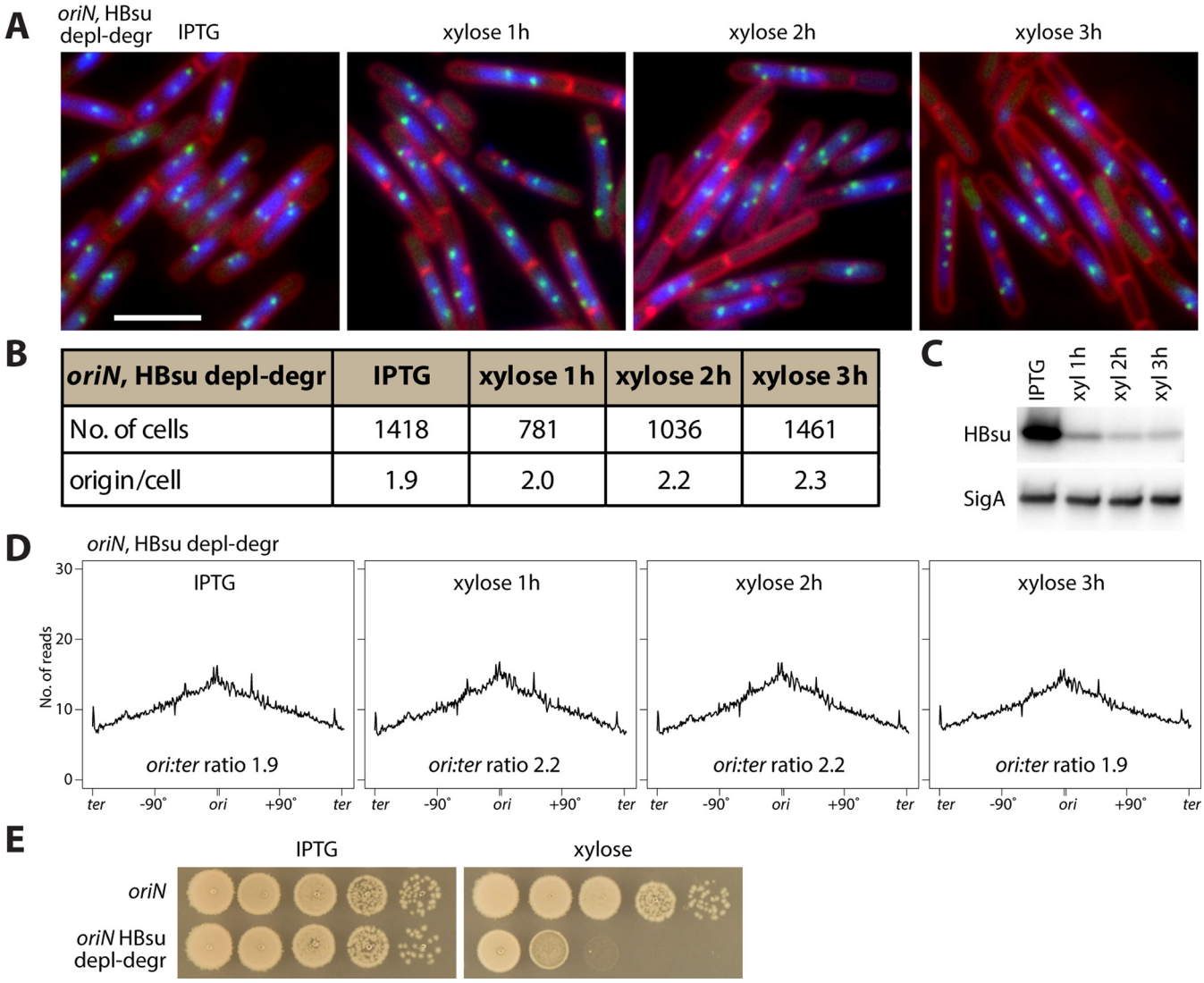
FIG 4 Ratio of *ori* to *ter* was reduced upon HBsu removal or inactivation. Whole-genome sequencing analysis of cells growing under indicated conditions (see Materials and Methods). The *x* axis represents genome location. The *y* axis represents the normalized number of reads. Data are plotted in 10-kb bins. (A) HBsu depletion-degradation strain (BWX1336) was grown in the presence of 0.5 mM IPTG, washed three times, and resuspended in warmed medium containing 0.5% xylose. (B) The *hbs*(*ts*) strain (BWX4331) was grown at 30°C, shifted to 42°C, and collected at indicated time points. (C) The *dnaB*(*ts*) strain (BWX2299) was grown at 30°C, shifted to 42°C, and collected at indicated time points.

Interestingly, in the absence of HBsu, although the *oriN* strain could replicate normally, it could not survive (Fig. 5E). Thus, HBsu has one or more other essential functions that are independent of its role in replication initiation.

DISCUSSION

Although HU homologs have been shown to be required for *in vitro* replication experiments (13, 15), *in vivo*, HU is generally not essential for cell survival. The absence of HU only causes mild defects in DNA replication, such as asynchrony or delay in initiation (16, 19). In contrast, *B. subtilis* HBsu is essential for cell survival. Using conditional alleles to remove or inactivate HBsu, here we show that HBsu is essential for DNA replication initiation *in vivo*. We postulate that this essentiality is likely due to the lack of redundancy: in *E. coli*, two other NAPs, IHF and Fis, have been implicated in replication initiation (5), while *B. subtilis* lacks IHF and Fis. Consistent with this idea, although *E. coli* could survive the absence of one or two of HU, IHF, and HN-S, it could not survive the absence of all three (35).

We found that HBsu's requirement for replication initiation is specific for the chromosomal origin, *oriC*; when *oriC* was changed to a plasmid origin, *oriN*, which uses a different initiator protein (33, 34), initiation was unchanged without functional HBsu. In



oriN, HBsu depl-degr	IPTG	xylose 1h	xylose 2h	xylose 3h
No. of cells	1418	781	1036	1461
origin/cell	1.9	2.0	2.2	2.3

FIG 5 HBsu is not required for replication initiation at *oriN*. (A) Visualizing replication origin in *oriN* strain containing HBsu depletion-degradation system. BWX4530 was grown in the presence of 0.5 mM IPTG, washed three times, and resuspended in warmed medium containing 0.5% xylose. DAPI-stained nucleoids (blue), FM4-64-stained membrane (red), and replication origin (*tetO*/TetR-CFP, green) are shown. Bar, 4 μm. (B) Analysis of number of origins per cell that contained nucleoids and the percentage of anucleate cells. (C) Immunoblot analysis of the *oriN* strain containing the HBsu depletion-degradation system (BWX4397). Cells were grown in CH medium in the presence of 0.5 mM IPTG. Cells were washed three times using CH medium and then resuspended into CH medium containing 0.5% xylose. Samples were taken 1 h, 2 h, and 3 h after resuspension. When OD_{600} reaches 0.5, the cultures were diluted 1:5 in warm CH containing 0.5% xylose to maintain exponential growth. SigA controls for loading. The same growth condition was used for panels A, B, and D. (D) Whole-genome sequencing analysis of cells growing under indicated conditions. The *x* axis represents genome location. The *y* axis represents the normalized number of reads. Data are plotted in 10-kb bins. (E) Serial dilutions of cells containing *oriN* as the only replication origin with (bottom, BWX4397) or without (top, BWX4349) the HBsu depletion-degradation system. The agar plates contain 0.5 mM IPTG (HBsu+, left) or 0.5% xylose (depletion-degradation, right). The plates were incubated at 37°C for 18 h.

E. coli, HU was shown to interact with DnaA, stabilize DnaA oligomers that are assembled on *oriC*, and facilitate strand opening (13). We speculate that HBsu plays a similar role in *B. subtilis*. Alternatively, HBsu may act on *oriC* DNA first, like bending the DNA, to facilitate DnaA acting on *oriC*. Biochemical and structural work has provided much understanding of DnaA interactions with *oriC* DNA sequence (36) and with initiation regulators Soj and SirA (37–39). Similar approaches aimed at identifying how HBsu interacts with DnaA or *oriC* will lead to better understanding of DNA replication initiation.

Here, we show that one reason HBsu is essential is its being required for replication initiation, but simply restoring replication was not sufficient to rescue cell growth. Specifically, although *oriN* permitted DNA replication in the absence of HBsu, it did not rescue cell viability (Fig. 5E). We infer that HBsu has other roles that are likely unrelated

TABLE 1 Strains used in this study

Strain	Genotype	Reference or source	Figure
PY79	Wild type	40	1A–D
BWX1336	*Phy hbs-ssrA* (*spec*), *lacA*::*PxylA* (*Ec*) *sspB* (*loxP-erm-loxP*)	This study	1A and B, 2A, 4A
BWX4331	*hbs*(*ts*) (*loxP-spec-loxP*)	This study	1C and D, 2B, 4B
BWX2299	*dnaB134*(*ts*), *zhb83*::*Tn917* (*erm*)	This study	2C, 4C
BWX1340	*yycR* (−7[3])::*tetO48* (*cat*), *ycgO*::*PftsW tetR-cfp* (*phleo*), *lacA*::*PxylA* (*Ec*) *sspB loxP* (no antibiotic marker) *Phy hbs-ssrA* (*spec*)	This study	3A and D
BWX4414	*yycR* (−7[3])::*tetO48* (*cat*), *ycgO*::*PftsW tetR-cfp* (*phleo*), *hbs*(*ts*) (*loxP-spec-loxP*)	This study	3B and D
BWX2533	*yycR* (−7[3])::*tetO48* (*cat*), *ycgO*::*PftsW tetR-cfp (phleo*), *dnaX-yfp* (*spec*), *dnaB134*(*ts*), *zhb83*::*Tn917* (*erm*)	43	3C and D
BWX4530	*trpC2*, *pheA1*, *359*[3]::*oriN* (*kan*), Δ*oriC-S*, *oriN*-based replication, *Phy hbs-ssrA* (*spec*), *lacA*::*PxylA* (*Ec*) *sspB* (*loxP-erm-loxP*), *yycR* (−7[3])::*tetO48* (*cat*), *ycgO*::*PftsW tetR-cfp* (*phleo*)	This study	5A and B
BWX4397	*trpC2*, *pheA1*, *359*[3]::*oriN* (*kan*), Δ*oriC-S*, *oriN*-based replication, *Phy hbs-ssrA* (*spec*), *lacA*::*PxylA* (*Ec*) *sspB* (*loxP-erm-loxP*)	This study	5C, D, and E
BWX4349 (MMB208)	*trpC2*, *pheA1*, *359*[3]::*oriN* (*kan*), Δ*oriC-S*, *oriN*-based replication	34	5E
KPL69	*trpC2*, *pheA1*, *dnaB134* (*ts*) - *zhb83*::*Tn917* (*erm*)	31	
MMB208	*trpC2*, *pheA1*, *359*[3]::*oriN* (*kan*), Δ*oriC-S*, *oriN*-based replication	34	
BWX1214	*hbs-ssrA* (*kan*)	This study	
BWX1332	*Phy hbs-ssrA* (*spec*)	This study	

to DNA replication. Since HBsu is shown to be a nucleoid-associated protein and binds to the entire nucleoid (24), it is possible that structuring the nucleoid is important for various aspects of cell physiology, including but not limited to cell division and/or chromosome segregation. Moreover, pleiotropy may explain why the HU homolog is essential in *B. subtilis* but not in other organisms. In the future, isolation of separation-of-function mutants could identify HBsu's essential functions and reveal the contribution of HBsu to different aspects of cell biology.

MATERIALS AND METHODS

General methods. *Bacillus subtilis* strains were derived from the prototrophic strain PY79 (40). *oriN* strains were derived from JH642 (34). Strains used in this study are listed in Table 1; oligonucleotides and next-generation sequencing samples used in this study can be found in Tables S1 and S2 in the supplemental material. Cells were grown in defined rich casein hydrolysate (CH) medium (41). When *oriN* strains were grown, CH medium was supplemented with 20 μg/mL tryptophan and 50 μg/mL phenylalanine. For depletion and degradation experiments, cells were grown in the presence of 0.5 mM IPTG, washed three times with warm medium, and resuspended in medium containing 0.5% xylose. Cells were grown at 37°C, 30°C, or 42°C as indicated. Cultures were diluted in warm medium so that the cells were in mid-exponential-phase growth with an OD_{600} below 0.6 at all time points.

Fluorescence microscopy. Fluorescence microscopy was performed using a Nikon Eclipse Ti2 microscope equipped with a Plan Apo 100×/1.45-numeric-aperture phase contrast oil objective and a scientific complementary metal-oxide semiconductor (sCMOS) camera. Membranes were stained with *N*-(3-triethylammoniumpropyl)-4-(6-(4-[diethylamino] phenyl) hexatrienyl) pyridinium dibromide (FM4-64; Molecular Probes) at 3 μg/mL. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) at 2 μg/mL. Images were cropped and adjusted using MetaMorph software. Final figure preparation was performed in Adobe Illustrator.

Construction of *Phy hbsu-ssrA* (*spec*) strain. *Phy hbsu-ssrA* (*spec*) strain (BWX1332) was built in two steps (see below). First, a degradation-only strain, *hbs-ssrA* (*kan*) (BWX1214), was built. Secondly, *hbs-ssrA* (*kan*) strain was used to build *Phy hbsu-ssrA* (*spec*) strain.

hbs-ssrA (*kan*) strain (BWX1214) was built by direct transformation of an isothermal assembly product that contained three PCR fragments: (i) a 0.6-kb fragment containing the upstream region and open reading frame of the *hbs* gene (amplified from wild-type genomic DNA using oligonucleotides oWX442 and oWX443); (ii) the *ssrA kan* fragment from pWX499 (1.7 kb) (amplified using oligonucleotides oWX444 and oWX445); and (iii) a 0.6-kb fragment downstream of the *hbs* gene (amplified from wild-type genomic DNA using oligonucleotides oWX446 and oWX447). pWX499 is a cloning vector that contains the *ssrA* tag and the kanamycin resistance gene. After transformation into wild-type *B. subtilis*, the *hbs* gene region of the resulting strain was amplified and sequenced using oWX448 and 449.

Phy hbsu-ssrA (*spec*) strain (BWX1332) was built by direct transformation of an isothermal assembly product that contained five PCR fragments: (i) a 1.3-kb fragment containing the upstream region of the *hbs* gene (amplified from wild-type genomic DNA using oligonucleotides oWX475 and oWX476); (ii) a spectinomycin resistance gene and *Phy* promoter from pDR111 (1.6 kb) (amplified using oligonucleotides oWX477 and oWX478); (iii) the *hbs-ssrA* region (amplified from BWX1214 genomic DNA using

oligonucleotides oWX479 and oWX480); (iv) a *lacI* fragment from pDR111 (amplified using oligonucleotides oWX481 and oWX482); and (v) a 1.2-kb fragment downstream of the *hbs* gene (amplified from wild-type genomic DNA using oligonucleotides oWX483 and oWX484). pDR111 is a cloning vector that contains the *Phy* promoter, a spectinomycin resistance gene, and the *lacI* gene. After transforming the isothermal assembly product to wild-type *B. subtilis*, the entire *Phy hbsu-ssrA* (*spec*) region was amplified using oWX475 and oWX484 and sequenced using oWX485-oWX489.

Generation of *hbs* mutant libraries. We constructed an *hbs* mutant library by direct transformation of an isothermal assembly product that contained four PCR fragments: (i) a 2.5-kb fragment containing the upstream region of the *hbs* gene (amplified from wild-type genomic DNA using Phusion polymerase and oligonucleotides oWX853 and oWX854); (ii) the *hbs* gene (352 bp) generated by error-prone PCR (GeneMorph II random mutagenesis kit; Stratagene) and oligonucleotides oWX855 and oWX856, under conditions in which each amplicon had, on average, 1 mutation per 300 bp; (iii) a spectinomycin resistance cassette (amplified from pWX466 using oligonucleotides oWX438 and oWX439); and (iv) a 2.2-kb fragment downstream of the *hbs* gene (amplified from wild-type genomic DNA using Phusion polymerase and oligonucleotides oWX857 and oWX858). The transformation was plated on LB agar plates supplemented with spectinomycin (100 μg/mL) at 30°C. More than 40,000 colonies were pooled, aliquoted, and frozen in LB medium containing 14% glycerol.

Screening for *hbs*(*ts*) mutants. Frozen aliquots of the mutant library were thawed, diluted, and plated onto rectangular plates (OmniTray; Thermo Scientific) containing LB agar supplemented with spectinomycin at a density of ~200 colonies per plate. The plates were incubated 16 to 18 h at 30°C. A colony-picking robot (BioMatrix; S&P Robotics) picked and arrayed independent transformants onto rectangular LB agar plates, which were incubated overnight at 30°C. The rearrayed colonies were replica plated onto two LB agar plates. One was placed at 30°C and the other at 42°C. Mutants that grew well at 30°C and grew poorly at 42°C were streaked for single colonies and retested to make sure the phenotype is reproducible. Genomic DNA from these mutants were used to transform the wild type to confirm linkage. The mutagenized region was then sequenced. Approximately 6,900 transformants were screened. Fifteen candidate clones were retested for streaking. Four mutants had growth close to that of the wild type at 30°C. These 4 mutants were characterized further for nucleoid morphologies. The mutant that was the most similar to the wild type for growth rate and nucleoid morphology and had the strongest defect at 42°C was named the *hbs*(*ts*) mutant and used for our analysis. The *hbs* region was amplified using oWX447 and oWX448 and sequenced using oWX442. Two amino acid substitutions were present in *hbs*(*ts*) (D40G and P77S). Note that the *hbs*(*ts*) mutant has a lower growth rate than the wild type at 30°C (Fig. 1C).

Whole-genome sequencing for DNA replication profile. Cells were grown in CH medium containing desired supplements (such as 0.5 mM IPTG or 0.5% xylose) at 37°C, 30°C, or 42°C as indicated. Samples were collected at the desired time points. Genomic DNA was extracted using the Qiagen DNeasy blood and tissue kit (69504; Qiagen). DNA was sonicated using a Qsonica Q800L sonicator for 12 min at 20% amplitude to achieve an average fragment size of 250 bp. DNA library was prepared using NEBNext Ultra II kit (E7645; NEB) and sequenced using Illumina NextSeq. Sequencing reads were mapped to the *B. subtilis* PY79 genome (NCBI reference sequence NC_022898.1) using CLC Genomics Workbench (Qiagen). The mapped reads were normalized to the total number of reads for that sample and plotted in R.

Immunoblot analysis. Cells were collected at appropriate time points and resuspended in lysis buffer (20 mM Tris, pH 7.0, 1 mM EDTA, 10 mM $MgCl_2$, 1 mg/mL lysozyme, 10 μg/mL DNase I, 100 μg/mL RNase A, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% proteinase inhibitor cocktail (P-8340; Sigma) to a final OD_{600} of 10 for equivalent loading. The cell resuspensions were incubated at 37°C for 10 min for lysozyme treatment, followed by the addition of an equal volume of 2× Laemmli sample buffer (1610737; Bio-Rad) containing 10% β-mercaptoethanol. Samples were heated for 5 min at 80°C prior to loading. Proteins were separated by precast 4 to 20% polyacrylamide gradient gels (Bio-Rad 4561096) and electroblotted onto mini-polyvinylidene difluoride membranes using Bio-Rad Transblot Turbo system and reagents (1704156; Bio-Rad). The membranes were blocked in 5% nonfat milk in phosphate-buffered saline (PBS) with 0.5% Tween 20 and then probed with anti-HBsu (1:5,000) (gift from David Rudner) or anti-SigA (1:10,000) (42) diluted into 3% bovine serum albumin in 1× PBS–0.05% Tween 20. Primary antibodies were detected using Immun-Star horseradish peroxidase-conjugated goat anti-rabbit antibodies (1705046; Bio-Rad) and Western Lightning Plus ECL chemiluminescence reagents as described by the manufacturer (NEL1034001; Perkin Elmer). The signal was captured using ProteinSimple FluorChem system. The intensity of the bands was quantified using ProteinSimple AlphaView software.

Data availability. Whole-genome sequencing data were deposited in the NCBI Sequence Read Archive (SRA) (accession no. PRJNA817244).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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