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Degradation of gene silencer is essential for expression of foreign genes and bacterial colonization of the mammalian gut

Jeongjoon Choi[a,1], Matias Schmukler[a], and Eduardo A. Groisman[a,b,2]

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Horizontal gene transfer drives bacterial evolution. To confer new properties, horizontally acquired genes must overcome gene silencing by nucleoid-associated proteins, such as the heat-stable nucleoid structuring (H-NS) protein. Enteric bacteria possess proteins that displace H-NS from foreign genes, form nonfunctional oligomers with H-NS, and degrade H-NS, raising the question of whether any of these mechanisms play a role in overcoming foreign gene silencing in vivo. To answer this question, we mutagenized the *hns* gene and identified a variant specifying an H-NS protein that binds foreign DNA and silences expression of the corresponding genes, like wild-type H-NS, but resists degradation by the Lon protease. Critically, *Escherichia coli* expressing this variant alone fails to produce curli, which are encoded by foreign genes and required for biofilm formation, and fails to colonize the murine gut. Our findings establish that H-NS proteolysis is a general mechanism of derepressing foreign genes and essential for colonization of mammalian hosts.

antisilencing | *Escherichia coli* | H-NS | Lon | protease

Significance

To express foreign genes, enteric bacteria must overcome the silencing effects caused by the heat-stable nucleoid structuring (H-NS) protein. Overcoming gene silencing by H-NS has been largely ascribed to proteins that compete with H-NS for binding to foreign DNA. However, we now report that H-NS proteolysis is a general mechanism of overcoming foreign gene silencing and essential for colonization of the murine gut by the enteric bacterium *Escherichia coli*. Both *E. coli* and *Salmonella enterica* serovar Typhimurium express foreign genes by degrading H-NS but do so under disparate conditions, likely reflecting their distinct lifestyles.

Horizontal gene transfer is critical for bacterial evolution (1, 2). Horizontally transferred genes (HTGs) (also referred to as foreign genes) can confer new properties upon an organism, including utilization of specific carbon sources, resistance to antimicrobial agents, and survival within mammalian tissues (1–3). These properties emerge only upon expression of HTGs in a recipient organism. Expression of HTGs often requires overcoming the silencing effects that xenogeneic silencing proteins exert on AT-rich foreign DNA (4–6).

The best-characterized xenogeneic silencer is the heat-stable nucleoid structuring (H-NS) protein from enteric bacteria (6–9). H-NS binds to AT-rich DNA, thereby silencing expression of the corresponding genes by preventing RNA polymerase from accessing or escaping promoters (4, 6). Because H-NS amounts were believed to stay constant (8, 10–12), overcoming foreign gene silencing was largely ascribed to antisilencing proteins that hinder H-NS binding to foreign DNA or that activate expression from different promoters (5, 6, 13).

By contrast, our laboratory reported that the enteric pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) degrades H-NS when inside macrophages, which reduces H-NS amounts by 95% and relieves silencing of foreign genes (14), including several required for virulence (15–17). *S.* Typhimurium also degrades H-NS when grown in laboratory media of mildly acidic pH (14), which *S.* Typhimurium experiences inside a macrophage phagosome (18), but not in media of neutral pH (14). A decrease in H-NS amounts may also impact translation because H-NS binding to mRNA impacts mRNA stability (19).

In vitro, the protease Lon degrades H-NS except when H-NS is bound to foreign DNA (14). By displacing H-NS from foreign DNA, the master virulence regulator PhoP renders H-NS a Lon substrate (14). In agreement with these biochemical data, *lon*- and *phoP*-null mutants fail to decrease H-NS amounts and to derepress HTGs (14). Although these data suggest that H-NS degradation is necessary for expression of HTGs, the Lon and PhoP proteins may theoretically derepress foreign genes independently of their role in decreasing H-NS abundance because Lon degrades dozens of substrates (20) and PhoP regulates expression of hundreds of genes (21, 22), including some required for expression of HTGs (22–24). Therefore, to determine whether H-NS proteolysis is essential to derepress HTGs, an H-NS variant competent for gene silencing but resistant to proteolysis must be identified and an organism expressing such variant investigated for the ability to express HTGs. Such an investigation avoids the confounding effects resulting from mutations in the *lon* and *phoP* genes, which are pleiotropic.

We now report that *Escherichia coli* K-12 degrades H-NS in a *lon*- and *phoP*-dependent manner, like *S.* typhimurium. However, unlike *S.* Typhimurium, *E. coli* degrades H-NS

Author affiliations: [a]Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT 06536; and [b]Microbial Sciences Institute, Yale University, West Haven, CT 06516

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[1]Present address: Department of Genetics, Yale School of Medicine, New Haven, CT 06520.

[2]To whom correspondence may be addressed. Email: eduardo.groisman@yale.edu.

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when grown in laboratory media of neutral pH (14). We identify an H-NS variant that resists degradation by Lon but retains the ability to bind and silence HTGs. An engineered *E. coli* strain expressing this variant in lieu of wild-type H-NS fails to express HTGs silenced by H-NS and is defective for colonization of mammalian tissues. Our findings establish that H-NS degradation is essential for expression of HTGs and that closely related species differ in the conditions under which H-NS degradation takes place, likely reflecting the distinct environments they inhabit.

Results

Lon Targets a Region in the H-NS Oligomerization Domain. H-NS consists of an N-terminal oligomerization domain (amino acids 1–83) and a carboxyl-terminal DNA-binding domain (amino acids 91–137) connected by a short linker (amino acids 84–90) (Fig. 1*A*) (9). A chimera consisting of the 50 N-terminal amino acids of H-NS fused to the full-length GFP protein (H-NS^{50aa}-GFP) accumulates to >10 times higher amounts in the *lon* mutant than in wild-type *S.* Typhimurium, recapitulating the behavior of the full-length H-NS protein (14). By contrast, the *phoP* mutant harbors wild-type amounts of the H-NS^{50aa}-GFP chimera, unlike the full-length H-NS, indicating that *phoP* is dispensable for H-NS degradation if H-NS lacks its DNA-binding domain. These results indicate that Lon targets a region(s) within the 50 N-terminal amino acids of H-NS.

To identify the H-NS region targeted by Lon, we investigated the abundance of H-NS-GFP chimeric proteins consisting of different portions of the H-NS N terminus fused to the full-length GFP (Fig. 1*A*) in isogenic wild-type and *lon* *S.* Typhimurium strains. The abundance of the H-NS-GFP chimeras harboring 26, 35, or 50 N-terminal amino acids of H-NS was four- to eightfold higher in the *lon* mutant than in the wild-type strain (Fig. 1*B*), indicating that they include a region(s) responsible for Lon-dependent degradation. By contrast, wild-type and the *lon* mutant displayed similar amounts of a chimera with 20 N-terminal amino acids of H-NS fused to GFP (Fig. 1*B*). Thus, Lon targeting involves a region corresponding to amino acids 21–26 (Fig. 1*C*), which are located between the second and third alpha-helices of H-NS (Fig. 1*D*).

Further support for the notion that H-NS residues at positions 21–26 are critical for Lon-dependent degradation was obtained with a derivative of the H-NS^{50aa}-GFP chimera in which wild-type H-NS residues CTLETL at these positions were replaced by residues NNQENQ. Amounts of the H-NS^{50aa}-GFP derivative were approximately sevenfold higher than those of the original H-NS^{50aa}-GFP chimera in wild-type *S.* Typhimurium (Fig. 1*E*). By contrast, similar amounts of both proteins were present in the *lon* mutant (Fig. 1*E*). These data argue that H-NS amino acids 21–26 are critical for Lon-dependent degradation. These residues are almost perfectly conserved in multiple enteric species (*SI Appendix*, Fig. S1) (14) that harbor highly conserved PhoP and Lon proteins, indicating that Lon targets a conserved H-NS region.

***E. coli* Degrades H-NS at Neutral pH in a Lon- and PhoP-Dependent Manner.** To determine whether H-NS degradation is essential for expression of HTGs in living bacteria, we set out to engineer a strain expressing a variant of the full-length H-NS

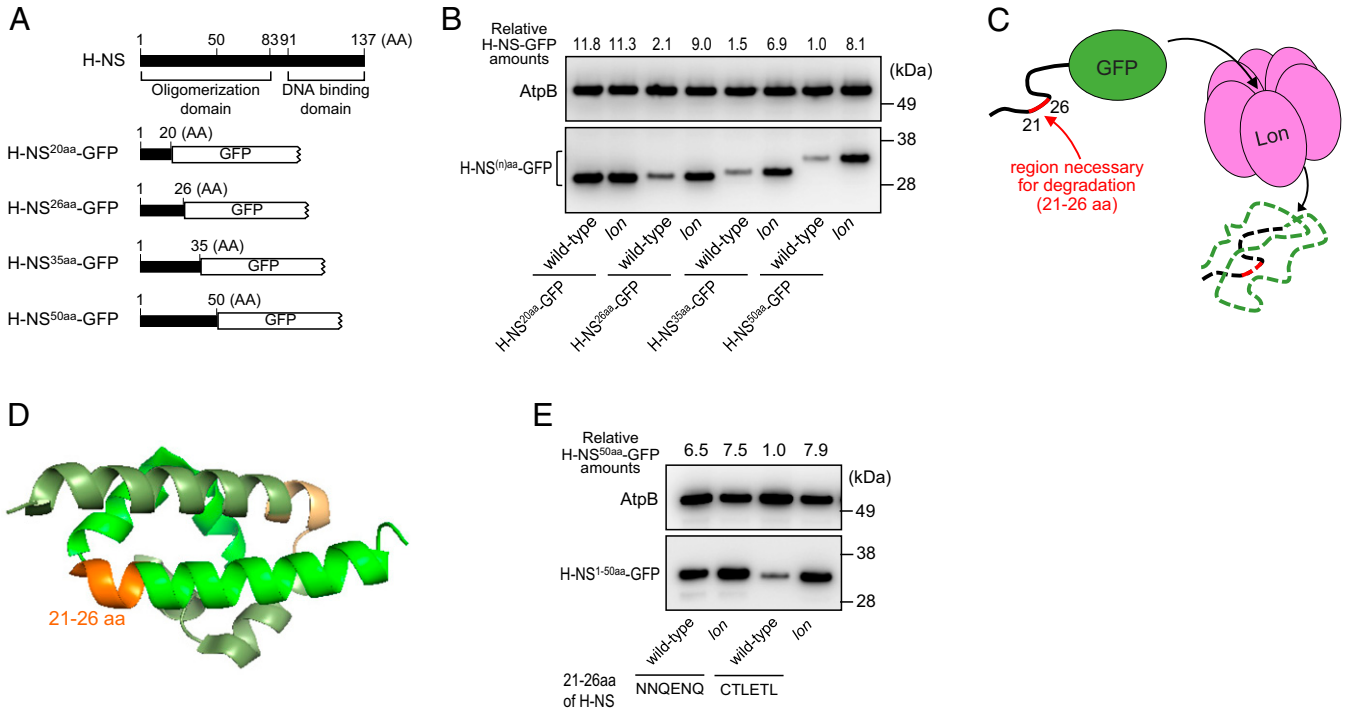

Fig. 1. H-NS amino acids at positions 21–26 are necessary for degradation by Lon. (*A*) Schematics of the full-length H-NS and chimeric proteins consisting of the indicated portions of H-NS and full-length GFP. AA, amino acid. (*B*, *E*) Western blot analysis of crude extracts prepared from (*B*) wild-type (JC805) and *lon* (JC864) *S.* Typhimurium harboring plasmids expressing the indicated H-NS-GFP fusion proteins, and (*E*) wild-type (JC805) and *lon* (JC864) *S.* Typhimurium–harboring plasmids expressing H-NS$^{1\text{-}50aa}$-GFP fusion protein and its variant containing substitutions at positions 21–26 amino acid residues of H-NS using antibodies recognizing GFP or the loading control AtpB. Bacteria were grown to midlog phase in *N*-minimal media with 1 mM of Mg^{2+} at pH 4.9 (acidic pH) with 20 μM of isopropyl β-D-1-thiogalactopyranoside. Nos. above the blots correspond to the relative amounts of H-NS-GFP chimeric proteins normalized to the H-NS$^{1\text{-}50aa}$-GFP fusion protein in the wild-type strain and AtpB abundance. A representative of at least three independent experiments is shown. (*C*) A schematic of H-NS region necessary for proteolysis by Lon protease; region corresponding to residues 21–26 is highlighted in red. (*D*) Structure of the N terminus of H-NS (4icg), where residues 21–26 are shown in orange, indicating the region necessary for H-NS degradation is located between the second and third alpha helixes of H-NS.

protein resistant to Lon and retaining a wild-type ability to bind and silence AT-rich HTGs. Our attempts to engineer a derivative of wild-type *S.* Typhimurium expressing a full-length H-NS protein with the CTLETL-to-NNQENQ substitutions from the normal *hns* chromosomal location and promoter were unsuccessful. That is to say, the resulting *S.* Typhimurium strains had additional nucleotide substitutions in the *hns* gene. The resulting H-NS protein variants were present in higher amounts than the wild-type H-NS protein but appeared to be nonfunctional because they were defective in silencing HTGs in *S.* Typhimurium (*SI Appendix*, Fig. S2). These results prompted us to explore whether H-NS is also degraded in *E. coli*, a species closely related to *S.* Typhimurium (25) that tolerates *hns* inactivation (26) and, thus, may also tolerate accumulation of a protease-resistant H-NS variant.

Both *lon* and *phoP* mutants of *E. coli* harbored higher amounts of H-NS than the isogenic wild-type strain (*SI Appendix*, Fig. S3*A*), mimicking the behavior displayed by *lon*, *phoP*, and wild-type *S.* Typhimurium (14). Curiously, *E. coli* decreased H-NS amounts when grown at neutral pH, but not in mildly acidic pH (*SI Appendix*, Fig. S3*A*), which is surprising given that *S.* Typhimurium decreases H-NS amounts when grown in mildly acidic pH but not at neutral pH (14). Furthermore, when grown in complex media, such as Luria broth (LB), *E. coli* reduced H-NS abundance (*SI Appendix*, Fig. S3*B*), but *S.* Typhimurium did not (*SI Appendix*, Fig. S3*C*), and the reduction exhibited by *E. coli* was *phoP* and *lon* dependent (*SI Appendix*, Fig. S3*B*). These results indicate that *E. coli* and *S.* Typhimurium employ the same genes to control H-NS abundance, but they do so under different conditions, possibly reflecting the distinct lifestyles of these closely related species (27).

HTGs Remain Silent in an *E. coli* Strain Expressing a Functional H-NS Variant Resistant to Lon. To test the hypothesis that H-NS degradation is necessary for expression of HTGs, we developed a genetic strategy to identify *E. coli* mutants expressing variants of the full-length H-NS protein resistant to Lon but retaining the ability to silence HTGs. We synthesized a library of oligonucleotides corresponding to the DNA region specifying amino acids 2–44 of H-NS, in which the DNA sequence specifying amino acids 21–26 was expected to harbor one or more mutations (Fig. 2*A*). The library of annealed oligonucleotides was introduced into an *E. coli* strain harboring a

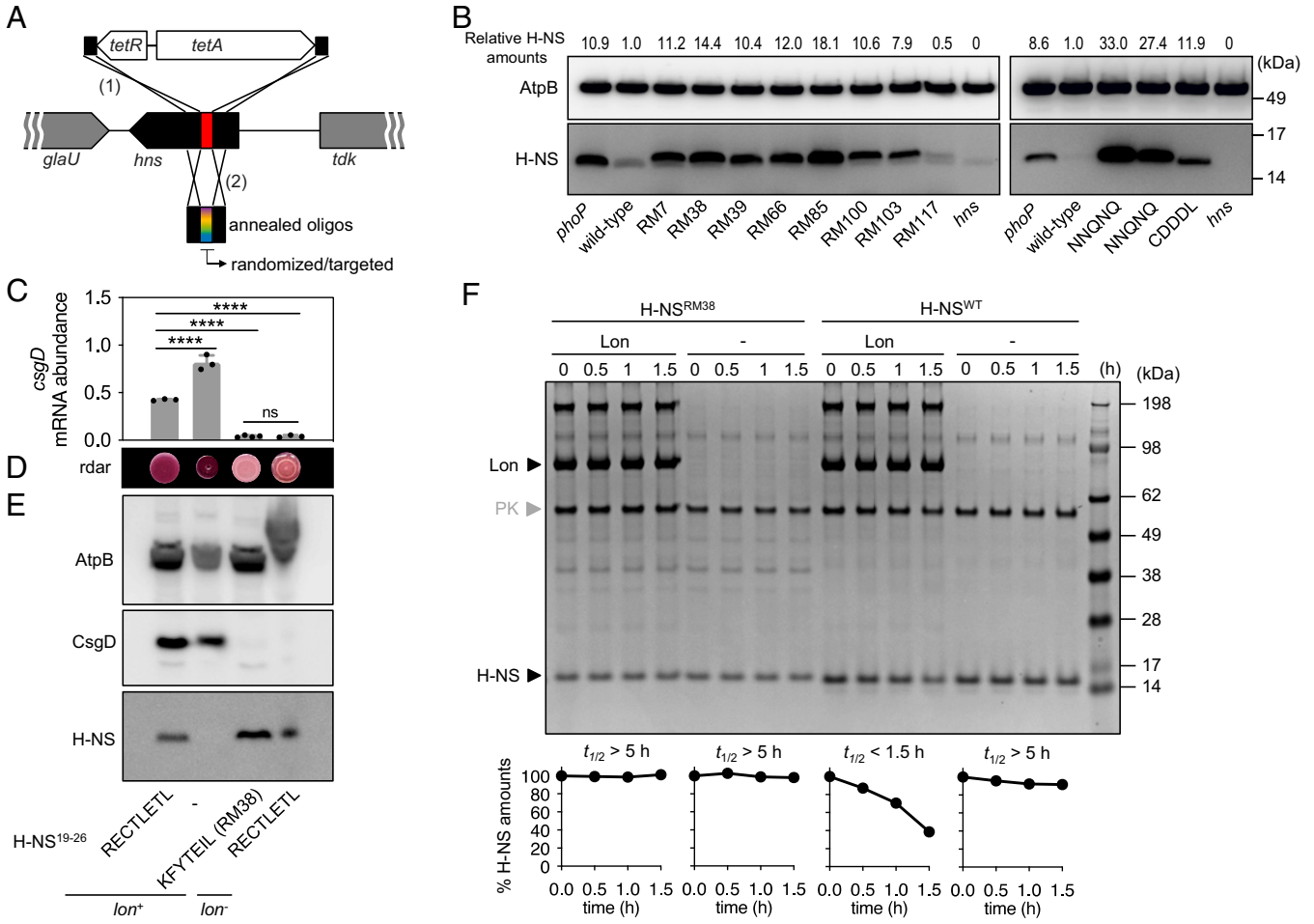

Fig. 2. A genetic screen identifies H-NS variants resistant to degradation by Lon. (*A*) Schematic of the procedure used to engineer chromosomal *hns* gene variants. (1) A *tetAR* cassette was inserted between nucleotides encoding residues 20–27 amino acids of *hns*, replacing nucleotides encoding residues 21–26. Then, (2) the cassette was replaced by annealed randomized or targeted oligonucleotides, generating mutations in the corresponding region. (*B*, *E*) Western blot analysis of crude extracts prepared from (*B*) wild-type (MG1655), *phoP* (EG12976), *hns* (JC1620), as well as mutant *E. coli* strains specifying variant H-NS proteins grown to midlog phase in LB broth at 37 °C and (*E*) wild-type (MG1655), *lon* (JC1292), RM38, and *hns* (JC1620) *E. coli* strains grown on plates containing Congo red at 23 °C for 50 h, using antibodies recognizing H-NS, CsgD, or the loading control AtpB. Nos. above the blots correspond to the normalized relative amounts of H-NS proteins. (*C*) mRNA abundance of the *csgD* gene produced by *E. coli* strains grown as described in *E*. The mean and SD from three independent experiments are shown. ****$P < 0.0001$, one-way ANOVA (wild-type vs. others, the RM38 mutant vs. the *lon* mutant); ns, not significant. (*D*) rdar phenotype of *E. coli* strains grown as in *E*. A representative of at least three independent experiments is shown. (*F*) In vitro degradation of purified H-NS proteins (wild type and RM38) by purified Lon protease. Pyruvate kinase (PK) is part of the adenosine triphosphate generation mix. $t_{1/2}$, the half-life of H-NS. Graphs under the $t_{1/2}$ correspond to the % amounts of remaining H-NS proteins at the indicated times.

tetRA cassette in the *hns* gene, and transformants were selected on fusaric acid–containing LB agar plates, as described (28). Because *tetRA*-expressing strains are sensitive to fusaric acid (28), clones resistant to fusaric acid should have lost or mutated the *tetRA* genes. We looked for colonies exhibiting wild-type growth because the *hns*::*tetRA* mutant forms smaller colonies than the wild-type strain (29). We screened ∼200 clones and identified several expressing H-NS variants that accumulated to greater than eightfold higher abundance than the wild-type strain (Fig. 2*B*), resembling the behavior of the *phoP*-null mutant, which accumulates wild-type H-NS to ∼11-fold higher amounts than wild-type *E. coli* (Fig. 2*B*) and served as control.

To determine the silencing ability of the identified H-NS variants, we examined the mRNA abundance of the H-NS-silenced *csgD* gene. This gene was chosen because it displayed the highest mRNA reduction in the *phoP* mutant (i.e., when H-NS accumulated) among the tested genes (*SI Appendix*, Fig. S4*A*). Mutant RM38, which accumulated the variant H-NS to >14-fold higher amounts than wild-type *E. coli* accumulated wild-type H-NS (Fig. 2*B*), displayed the most-pronounced reduction in *csgD* mRNA amounts, resembling the *lon* mutant (Fig. 2*C* and *SI Appendix*, Fig. S4*B*). The low *csgD* mRNA abundance exhibited by the RM38 mutant resulted in as low CsgD protein abundance as the *lon*-null mutant (Fig. 2*E* and *SI Appendix*, Fig. S4*D*). DNA sequence analysis of the hsn^{RM38} gene revealed replacement of RECTLETL, the deduced amino acid sequence of residues 19–26 of wild-type H-NS, by KFY-TEIL (Fig. 2*E*).

The *csgD* gene specifies curli, an amyloid protein that is part of biofilms. Binding of the dye Congo red to curli confers a characteristic red colony morphology (Fig. 2*D*) (30). The RM38 mutant exhibited low Congo red binding (Fig. 2*D*), mimicking the behavior of the *lon* mutant (Fig. 2*D* and *SI Appendix*, Fig. S4*C*) and in agreement with decreased abundance of the CsgD protein (Fig. 2*E*).

The purified H-NS^{RM38} protein was not degraded by purified Lon from *E. coli*, whereas the purified wild-type H-NS protein from *E. coli*—used as positive control—was degraded (Fig. 2*F*). (We used purified Lon from *S.* Typhimurium to proteolyze wild-type H-NS from *S.* Typhimurium in our previous publication (14).) Moreover, the hns^{RM38} single mutant and the hns^{RM38} *lon* double mutant accumulated H-NS to similar amounts (Fig. 3*A*). Cumulatively, these results indicate that the

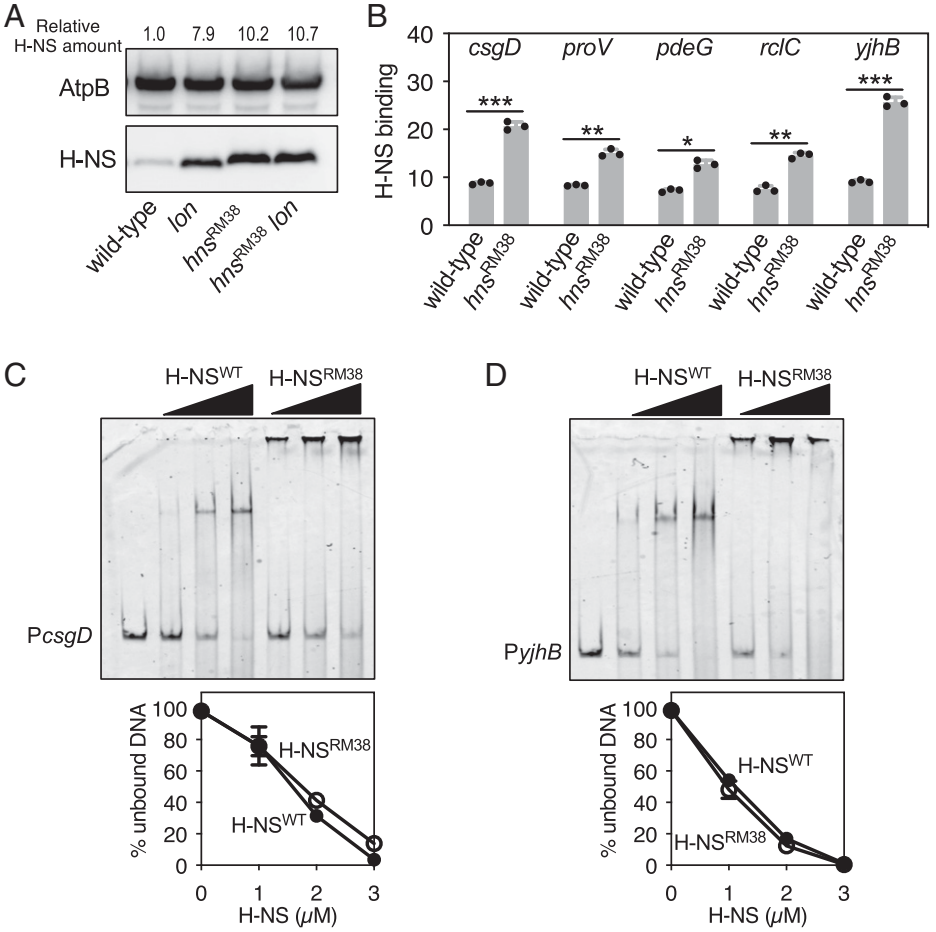

Fig. 3. The H-NS^{RM38} variant accumulates in *lon*$^+$ *E. coli*, increasing H-NS binding to foreign gene promoters. (*A*) Western blot analysis of crude extracts prepared from wild-type (MG1655), *lon* (JC1292), hns^{RM38} (JC1791), and hns^{RM38} *lon* (JC1805) *E. coli* strains grown to midlog phase in LB broth using antibodies recognizing H-NS or the loading control AtpB. Nos. above the blot correspond to the normalized relative amounts of H-NS proteins. (*B*) In vivo binding of H-NS to the promoter regions of the *csgD*, *proV*, *pdeG*, *rclC*, and *yjhB* genes was determined in wild-type (MG1655) and hns^{RM38} (JC1791) *E. coli* grown to midlog phase in LB broth by chromatin immunoprecipitation (ChIP). Following crosslinking, DNA fragments bound by wild-type and RM38 mutant H-NS proteins were precipitated by anti-H-NS antibodies. Following reversal of crosslinking, precipitated DNA fragments were analyzed by qRT-PCR as described in the *Materials and Methods*. The mean and SD from three independent experiments are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed *t* test with wild-type versus hns^{RM38}. (*C* and *D*) In vitro binding of purified wild-type H-NS and H-NS^{RM38} to *csgD* and *yjhB* promoter DNAs. Eighty femtomole of promoter DNAs were incubated with purified wild-type H-NS or H-NSP^{RM38} (0, 1, 2, and 3 μM) proteins. Representatives of at least two independent experiments are shown. Unbound DNA amounts are shown in graph underneath.

different amounts of H-NS and H-NS^{RM38} detected in *E. coli in vivo* (Fig. 2*B*) reflect direct effects of Lon on these proteins and argue against the possibility of the H-NS^{RM38} protein exhibiting altered susceptibility to a protease other than Lon.

In sum, we identified an H-NS variant resistant to Lon and with a wild-type gene-silencing ability. This variant provides a means to critically test the requirement for H-NS degradation in expression of HTGs.

H-NS Degradation Is Necessary to Derepress HTGs. We determined the whole genome sequence of *E. coli* mutant RM38 to rule out the possibility of its phenotypes resulting from mutations in genes other than or in addition to *hns*. We established that RM38 also harbors a mutation in the *rhsC* gene that is predicted to change the amino acid sequence of the RhsC protein at position 186 from threonine to alanine (*SI Appendix*, Fig. S5*A*). Thus, we engineered mutant RM38 by mutating the *rhsC* gene back to wild type so it would specify a wild-type RhsC protein with threonine instead of alanine at position 186 (*SI Appendix*, Fig. S5*A*). The resulting strain exhibited the same curli-defective phenotype as its parent RM38, behaving like the *csgD*-null mutant (*SI Appendix*, Fig. S5*B*), reflecting the highly reduced *csgD* mRNA abundance of the *hns*^{RM38} strain compared with wild-type *E. coli* (*SI Appendix*, Fig. S5*C*). (Please note that the mRNA abundance of disparate genes was altered differentially by the *hns*^{RM38} allele (*SI Appendix*, Fig. S5*C*).) These results indicate that the behavior of strain RM38 is due to expression of the H-NS^{RM38} variant and that the identified amino acid substitution in RhsC does not alter curli production.

The increased H-NS abundance exhibited by the RM38 mutant resulted in increased H-NS occupying foreign genes in vivo (Fig. 3*B*), as determined by chromatin immunoprecipitation (ChIP). The increased occupancy does not appear to be due to increased DNA-binding activity of the H-NS^{RM38} variant because purified wild-type and H-NS^{RM38} proteins displayed similar binding to foreign genes in vitro (Fig. 3 *C* and *D*). Curiously, the H-NS^{RM38} protein produced a supershift in the two examined DNA targets that was not displayed by the wild-type H-NS protein (Fig. 3 *C* and *D*). The supershift may be due to higher oligomeric forms in the H-NS^{RM38} protein, which differs from the wild-type H-NS protein in residues located within the oligomerization domain (9). Consistent with this notion, in vitro oligomerization analysis revealed that the purified RM38 mutant H-NS protein forms higher oligomeric states than wild-type H-NS protein (*SI Appendix*, Fig. S6). The results in this section indicate that the higher H-NS binding to and silencing of HTGs exhibited by mutant RM38 is due to increased abundance of the H-NS^{RM38} protein resulting from being resistant to cleavage by Lon.

H-NS Degradation Is Necessary for Colonization of the Murine Gut by *E. coli*. *E. coli* requires mucus-derived β-5 acetyl neuraminic acid (β-Neu5Ac) to colonize the murine gut (31). The protein NanT takes up β-Neu5Ac (*SI Appendix*, Fig. S7) and isomerizes β-Neu5Ac into α-Neu5Ac, which proceeds into glycolysis via the aldolase NanA (32, 33) (*SI Appendix*, Fig. S7). An alternative form of mucus-derived sialic acid, 2,7-anhydro-Neu5Ac, supports *E. coli* growth in vitro independently of NanT (34). The latter pathway requires YjhB to import 2,7-anhydro-Neu5Ac and YjhC to convert 2,7-anhydro-Neu5Ac into α-Neu5Ac (33, 34) (*SI Appendix*, Fig. S7). Because YjhB and YjhC are encoded by HTGs, we reasoned that an *E. coli* strain expressing the Lon-resistant H-NS^{RM38} protein would display reduced expression of these genes, thereby compromising gut colonization.

First, we determined that purified wild-type and H-NS^{RM38} proteins bind similarly to *yjhB* DNA in vitro (Fig. 3*D*). These results agree with those obtained with *csgD* DNA (Fig. 3*B*) and support the notion that the increased binding to *yjhB* DNA exhibited by the H-NS^{RM38} protein in vivo (Fig. 3*B*) is due to H-NS^{RM38} accumulation (Fig. 3*A*) resulting from H-NS^{RM38} resistance to cleavage by Lon (Fig. 2*F*).

And second, we determined that the *E. coli* strain expressing the Lon-resistant H-NS^{RM38} protein in lieu of wild-type H-NS is defective in gut colonization. In this experiment, Balb/C mice that had been treated with antibiotics for 3 d to enable colonization were inoculated with a mixture of four *E. coli* strains: wild-type, *hns*^{RM38} mutant, *yjhB*-null mutant, and *nanT*-null mutant. To help distinguish and enumerate the strains, we introduced plasmids expressing the fluorescent proteins GFP or mCherry constitutively (Fig. 4*A* and *SI Appendix*, Fig. S8). The four strains displayed similar fecal carriage up to 3 d postinoculation (Fig. 4*B*), mimicking the behavior exhibited by the strains grown in laboratory media containing glucose as the sole carbon source (Fig. 4*C*). The wild-type strain maintained its abundance 7 d postgavage (Fig. 4*B*).

By contrast, the *hns*^{RM38} mutant was recovered in much-lower amounts than the wild-type strain at 5 and 7 d postgavage (Fig. 4*B*). The behavior of the *hns*^{RM38} mutant resembled that of the *yjhB*-null mutant (Fig. 4*B*), which is defective in gut colonization because it cannot utilize 2,7-anhydro-Neu5Ac (33, 34) (*SI Appendix*, Fig. S7). The *hns*^{RM38} and *yjhB* mutants were as defective as the *nanT* mutant (Fig. 4*B*), which was used as control because it displayed a defect like that previously reported by others (31). These results support the notion that the ability to reduce H-NS abundance is critical for gut colonization by *E. coli*.

Discussion

We previously reported that *S.* Typhimurium proteolyzes H-NS when inside macrophages, thereby decreasing H-NS amounts by 95% and derepressing foreign genes (14). We have now determined that *E. coli* degrades the H-NS protein in a *lon*- and *phoP*-dependent manner (Figs. 2*B* and 3*A* and *SI Appendix*, Fig. S3 *A* and *B*) and that H-NS proteolysis is essential for derepression of HTGs (Figs. 2*C* and 3*B* and *SI Appendix*, Fig. S4*B*) and colonization of the mammalian gut by *E. coli* (Fig. 4*B*). Our findings demonstrate the essential role that regulated proteolysis plays in host colonization by enteric bacteria (35). H-NS degradation likely impacts expression of HTGs silenced by other nucleoid-associated proteins because H-NS protects the nucleoid-associated protein StpA from degradation by forming hetero-oligomers with it (36).

Apart from enabling expression of HTGs, a reduction in H-NS abundance may independently enhance bacterial survival during infection. This is because both lack of H-NS and overexpression of H-NS impair bacterial growth (37), even in species that tolerate deletion of the *hns* gene (26), and also because a reduction in bacterial growth rate renders bacteria phenotypically resistant to many antibacterial agents (38). For instance, *S.* Typhimurium degrades >95% of H-NS when inside macrophages (14), where it replicates at a much-slower rate compared with in vitro culture conditions (39). This behavior appears to be conserved in evolution because excessive histone amounts are toxic to eukaryotes, and histone degradation is critical for cellular function (40, 41). Even a small (approximately two-fold) decrease in H-NS abundance allows expression of acid-resistance genes in *E. coli* K-12 (42) and virulence genes in enterohemorrhagic *E. coli* (43).

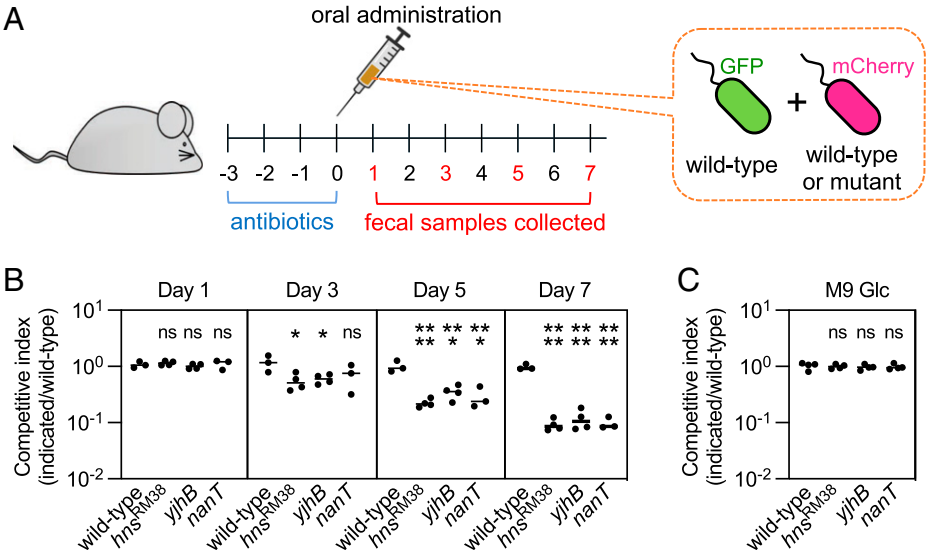

Fig. 4. H-NS degradation is necessary for gut colonization by *E. coli*. (*A*) Schematic of experiment. Mice were pretreated daily with 250 mg kg^{-1} ampicillin for 3 d, followed by inoculation of a mixture composed of wild type expressing GFP and an indicated mutant or wild type expressing mCherry. Fecal samples were collected at indicated times after inoculation. (*B*) Fecal contents were determined at indicated times and shown as competitive index (i.e., the ratio between wild-type *E. coli* expressing GFP and mutants expressing mCherry). (*C*) Bacterial Nos. were determined as described in *B* but grown in M9 media with glucose as a carbon source. Dots indicate values from individual mice (n = 3–4), and bars indicate the mean of each group (data are representatives of two independent experiments, which gave similar results). *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVA (wild-type vs. others); ns, not significant.

H-NS Degradation Is Necessary for Expression of Foreign Genes. The mechanism of H-NS proteolysis and ts requirement for expression of HTGs (Fig. 5) (14) are conserved between *E. coli* and *S. enterica*, which diverged from a common ancestor >100 million years ago (44). The identified mechanism likely operates in other enteric bacteria because (i) Lon is highly conserved across organisms, including bacteria, archaea, and eukaryotes (20); (ii) antisilencing proteins with the ability to dissociate H-NS from DNA and render it susceptible to proteolysis exist in numerous bacterial species (5, 6); and (iii) residues 21–26 of H-NS, which are necessary for H-NS degradation by Lon, are almost perfectly conserved in members of the family *Enterobacteriaceae* (*SI Appendix*, Fig. S1) (14, 45).

The identified mechanism explains how enteric bacteria respond to environmental signals experienced during infection by derepressing HTGs. For example, increased osmolarity promotes H-NS dissociation from DNA (46), which renders H-NS a Lon substrate (14). Thus, the high osmolarity that *E. coli* encounters in the intestine may result in H-NS degradation, thereby favoring expression of HTGs necessary for gut colonization. In support of this notion, an *E. coli* strain expressing a Lon-resistant H-NS variant failed to depress gut-colonization

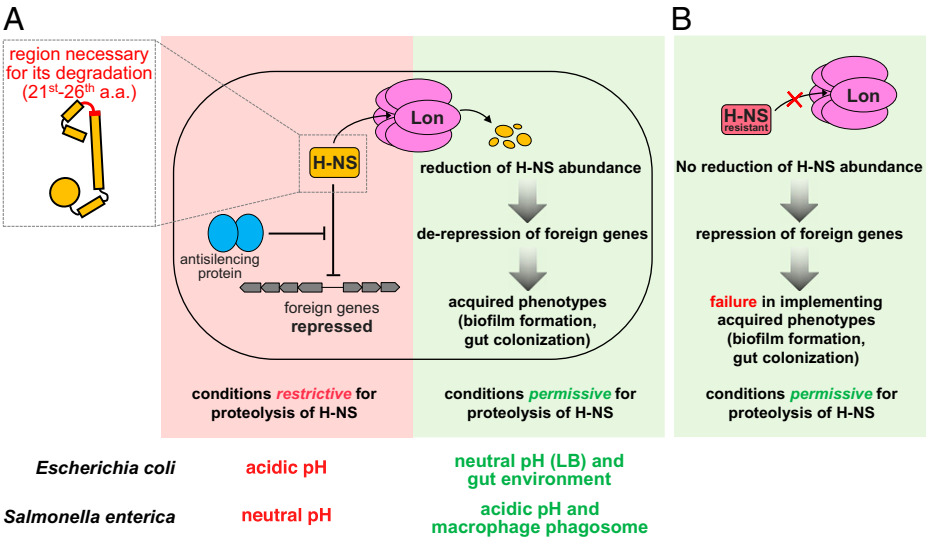

Fig. 5. Condition-dependent H-NS proteolysis governs derepression of foreign genes and bacterial behavior. (*A*) (*Left*) Under conditions restrictive for H-NS degradation, foreign genes are silenced by abundant H-NS. Antisilencing proteins specifically derepress their target genes by antagonizing H-NS binding to the corresponding DNAs. (*Right*) Under conditions permissive for H-NS proteolysis, protease Lon degrades H-NS, decreasing cellular H-NS amounts, reducing H-NS binding to foreign DNA, and allowing expression of the corresponding genes and the display of the specified phenotypes. The closely related bacterial species *Escherichia coli* and *Salmonella enterica* utilized this mechanism to derepress foreign gene expression but implement it under different conditions, which may reflect their distinct lifestyles. (*B*) An H-NS variant resistant to Lon-mediated degradation fails to express foreign genes under the permissive conditions for the H-NS proteolysis, because its abundance does not decrease. Thus, such a variant renders the organism's behavior like being under the restrictive conditions, even when experiencing the permissive conditions and the presence of proteins that displace H-NS from foreign DNA.

genes (*SI Appendix*, Fig. S3) and was defective in gut colonization (Fig. 4*B*). The decrease in H-NS amounts here reported results from H-NS proteolysis and contrasts with that mediated by the *E. coli* SspA protein, which appears to result from decreased translation of *hns* mRNA, as wild-type and *sspA E. coli* exhibit similar H-NS stability (42).

H-NS affinity for DNA decreases as temperature increases (47, 48). Therefore, as bacteria in the environment transition to the higher temperature of warm-blooded animals, decreased H-NS binding to DNA would make it more likely for H-NS to be degraded (14). Our findings help explain how a temperature shift induces ∼25% of bacterial genes (49, 50) when H-NS is proficient in binding both thermo-regulated and non-thermo-regulated genes (49, 50).

The distant species *Shewanella spp.* utilizes a different mechanism to promote expression of foreign genes when experiencing changes in temperature. *Shewanella spp.* phosphorylates H-NS at high temperatures, which favors binding to and silencing of foreign genes at high temperature but not at low temperature (51). This mechanism may have emerged due to the lack of conservation between the *Shewanella* and enteric H-NS proteins at residues 21–26 (*SI Appendix*, Fig. S1; only 38% shared amino acid identity in full-length H-NS protein), which may render the *Shewanella* H-NS refractory to proteolysis by Lon.

***Salmonella* and *E. coli* Degrade H-NS under Different Conditions.** Both *S.* Typhimurium and *E. coli* utilize Lon to proteolyze H-NS (14) (Figs. 2*F* and 3*A* and *SI Appendix*, Fig. S3 *A* and *B*), and in both species, inactivation of the *phoP* gene hinders H-NS degradation (Fig. 2*B* and *SI Appendix*, Fig. S3) (14), most likely due to PhoP's role in displacing H-NS from DNA, thereby rendering it a Lon substrate. However, *S.* Typhimurium degrades H-NS when inside a macrophage phagosome or experiencing laboratory media of mildly acidic pH (but not neutral pH) (14), whereas *E. coli* degrades H-NS when grown in laboratory media of neutral pH (Figs. 2*B* and 3*A* and *SI Appendix*, Fig. S3 *A* and *B*) but not mildly acidic pH (*SI Appendix*, Fig. S3*A*). The different conditions under which H-NS is proteolyzed may reflect the disparate lifestyles of these closely related species and the demands on specific HTGs to occupy different niches. Thus, H-NS proteolysis appears to be a general mechanism utilized by bacterial species having distinct habitats.

H-NS Proteolysis Suggests Alternative Roles for Antisilencing Proteins. Overcoming xenogeneic silencing has been ascribed to H-NS displacement from foreign DNA by antisilencing proteins (5, 6), formation of hetero-oligomers with proteins that prevent H-NS binding to foreign DNA (5, 52), and/or use of alternative promoters to transcribe HTGs (13). However, that Lon degrades H-NS in *S.* Typhimurium, *E. coli*, and likely other enteric bacteria suggests that antisilencing proteins that displace H-NS from foreign DNA derepress HTGs by enhancing H-NS degradability. For example, PhoP displaces H-NS from DNA, rendering it a Lon substrate (Fig. 2*B* and *SI Appendix*, Fig. S3) (14). The likelihood of an antisilencing protein promoting H-NS degradation may depend on its specific mechanism of action because an antisilencing protein that binds the oligomerization domain of H-NS (52), where amino acids 21–26 are located, may potentially compromise H-NS degradation despite hindering H-NS binding to DNA (52).

The decrease in cellular H-NS abundance resulting from H-NS proteolysis may impact, in turn, other processes responsible for overcoming foreign gene silencing. For example, by reducing the requirement for antisilencing proteins, H-NS degradation may enable bacteria to efficiently achieve foreign gene expression with a limited No. of antisilencing proteins. In addition, H-NS proteolysis may favor expression of HTGs by promoting dissociation of other nucleoid-associated proteins (NAPs), such as StpA, Hha, and YdgT (53, 54). This is because H-NS forms hetero-oligomeric filaments with these NAPs (53, 54) and because the function of these NAPs is dependent on H-NS (37, 55). Thus, changes in H-NS abundance broadly impact gene expression controlled by multiple NAPs, derepressing foreign genes silenced by H-NS, altering translation of H-NS-bound transcripts, and enabling bacteria to have a robust response to changes in their surroundings.

Materials and Methods

Bacterial Strains, Plasmids, Oligodeoxynucleotides, and Growth Conditions. Bacterial strains and plasmids used in this study are listed in *SI Appendix*, Table S1. All *S. enterica* serovar Typhimurium strains were derived from wild-type strain 14028s (56) and constructed by phage P22-mediated transductions as described (57). All *E. coli* strains were derived from wild-type strain MG1655.

Bacteria were grown at 37 °C in LB; *N*-minimal media (58) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated pH (pH 7.6 or pH 4.9) and 1 mM of $MgCl_2$ unless specified; or M9 media supplemented with 20 mM glucose. *E. coli* DH5α was used as the host for preparation of plasmid DNA (59). To induce expression of plasmid-linked genes under the control of LacI-repressed and AraC-regulated promoters, isopropyl β-D-1-thiogalactopyranoside and L-arabinose, respectively, were added at the indicated concentrations (∼0–1 mM). When necessary to select for plasmid maintenance, appropriate antibiotics were added at the following final concentrations: ampicillin at 50 μg mL^{-1}, chloramphenicol at 20 μg mL^{-1}, kanamycin at 50 μg mL^{-1}, and tetracycline at 10 μg mL^{-1}.

To determine curli fiber production ("rdar" phenotype), bacteria were grown overnight in LB broth and 3 μL of normalized bacteria by optical density at 600 nm were spotted on LB agar supplemented with Congo red (40 μg mL^{-1}) and Coomassie brilliant blue (20 μg mL^{-1}). Agar plates were then incubated at 25 °C for the indicated times.

DNA oligonucleotides used in this study are listed in *SI Appendix*, Table S2.

Genetic Screen to Identify *E. coli* Chromosomal *hns* Mutants. To engineer chromosomal *hns* gene variants, the *tetRA* cassette was amplified by PCR using primers 17238/17239 from transposon Tn*10* of strain MS7953s, then introduced into wild-type *E. coli* (MG1655) harboring plasmid pKD46 (60) using the one-step disruption method (60). The *tetRA* cassette insertion removed *hns* nucleotides encoding amino acids 21–26 from the H-NS protein. Then, the *tetRA* cassette was replaced with annealed oligonucleotides 17616/17617, which generated randomized mutations in the region of *hns* specifying residues 21–26 of H-NS. The resulting strains were obtained following selection against tetracycline resistance on media containing fusaric acid (28) and purified on the same plate. Mutations in selected candidates were then identified by DNA sequencing.

Whole-genome sequencing of the RM38 mutant revealed that this mutant harbored one additional mutation on its chromosome: the mutant *rhsC* gene specified a variant with the T186A substitution in the RhsC sequence (*SI Appendix*, Fig. S5).

To restore the wild-type sequence to *rhsC*, a *tetRA* cassette was amplified by PCR using primers 17885/17886 from transposon Tn*10* of strain MS7953s, then introduced into the RM38 mutant *E. coli* harboring plasmid pKD46 (60) using the one-step disruption method (60). The *tetRA* cassette insertion was then replaced with annealed oligonucleotides 17887/17888. The resulting strains were obtained following selection against tetracycline resistance on media containing fusaric acid (28), and mutations were verified by DNA sequencing.

qRT-PCR. Total RNA was isolated using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. The purified RNA was quantified using a Nanodrop machine (NanoDrop Technologies). Complementary DNA (cDNA) was synthesized using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). The mRNA amount of the *STM14_1977*, *ycjE*, *csgD*, *proV*, *pdeG*, *rclC*, and *yjhB* genes was determined by quantification of cDNA using Fast SYBR Green PCR Master

Mix (Applied Biosystems) and appropriate primers (*ycjE*: 16675/16676; *STM14_1977*: 15973/15974; *csgD*: 17620/17621; *proV*: 17618/17619; *pdeG*: 17622/17623; *rclC*: 17624/17625; *yjhB*: 17626/17627; *ompA*: W3961/W3962; *rrs*: 3203/3204) and monitored using a QuantStudio 6 machine (Applied Biosystems). Data were normalized to the levels of 16S ribosomal RNA (rrs) or *ompA*.

ChIP. Bacterial cells were crosslinked with 1% formaldehyde at room temperature for 15 min, quenched with 200 mM glycine at room temperature for 10 min, and washed three times with cold phosphate-buffered saline (PBS). Then, cells were lysed in cell lysis solution A (10 mM Tris [pH 8.0], 50 mM NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 20% sucrose, and 10 mg mL^{-1} lysozyme) and 10× radioimmunoprecipitation assay (RIPA) solution (Millipore). DNA was fragmented to an average size of 500 bp by sonication (VirTis), and a 50 μL aliquot was taken as "input DNA". Immunoprecipitation of H-NS–DNA complex was performed using antibodies recognizing H-NS and using Magna-Chip protein A/G magnetic beads (Millipore). Samples were then washed twice with 1× RIPA solution, twice with LiCl immune complex wash buffer (Millipore), twice with TE buffer (20 mM Tris [pH 8.0] and 1 mM EDTA), and eluted in elution buffer (50 mM Tris [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulfate [SDS]) with incubation at 65 °C for 15 min. Both immunoprecipitated (IP) and input DNA samples were incubated at 65 °C for 9 h to reverse crosslinks, purified using Qiagen PCR purification column, and quantified using qRT-PCR using primers (*csgD*: 17620/17621; *proV*: 17618/17619; *pdeG*: 17622/17623; *rclC*: 17624/17625; *yjhB*: 17626/17627; *ompA*: W3961/W3962). Binding of H-NS to DNA was calculated as the ratio of "IP DNA/input DNA" and normalized to binding to the *ompA* gene.

Western Blot Analysis. Crude extracts were prepared in B-PER bacterial protein extraction reagent (Pierce) with 100 μg mL^{-1} lysozyme and EDTA-free protease inhibitor (Roche). Samples were separated in 4–12% NuPAGE gels (Life Technologies) and analyzed by Western blotting using antibodies recognizing H-NS (1:2,000), FLAG (Sigma; 1:2,000), GFP (Invitrogen, 1:3,000), or AtpB (Abcam; 1:5,000). Secondary horseradish peroxidase–conjugated antisera recognizing rabbit or mouse antibodies (GE Healthcare) were used at 1:5,000 dilution. The blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce) and visualized using LAS-4000 (Fuji Film). The density of protein bands was determined by quantification using ImageJ software version 1.52 (NIH).

In Vitro Protein Degradation Assay. Protein degradation assays were performed in a buffer containing 25 mM Tris (pH 8.0), 100 mM KCl, 12 mM $MgCl_2$, 2 mM adenosine triphosphate, 4 mM phosphoenol pyruvate (Roche), and 20 μg mL^{-1} pyruvate kinase (Sigma) at 30 °C. Purified H-NS (wild type or RM38 variant) and Lon (Sino Biological, Inc.) proteins were used at 5 μM and 1 μM, respectively. Samples were taken at desired time points, quenched with SDS loading dye, and frozen in dry ice. Samples were separated in 4–12% NuPAGE gels (Life Technologies), and proteins were detected by Coomassie blue G-250 staining.

Mouse Infection and Determination of Bacterial Nos. in Mouse Organs. Six-week-old female Balb/C mice were purchased from Charles River Laboratories. Three to four mice in each group were treated daily with 250 mg kg^{-1} ampicillin for 3 d prior to bacteria inoculation. After 3 d of antibiotics treatment, mice were inoculated via oral gavage with 0.1 mL of PBS containing 1:1 mixture of $\sim 1 \times 10^9$ of indicated *E. coli* strains that had been grown overnight in LB broth containing 50 μg mL^{-1} ampicillin and resuspended and diluted in PBS. Animals were housed in temperature- and humidity-controlled rooms and maintained on a 12 h light/12 h dark cycle. Fecal samples were collected at indicated times to determine bacterial No. in the fecal content. Fecal colonization was determined by homogenizing fecal content in 0.5 mL of ice-cold PBS, followed by serial dilution in PBS and then plating the diluents on LB agar media. Each bacterial strain was distinguished by visualizing fluorescence on a portable transilluminator (Clare Chemical Research, DR46). All procedures complied with regulations of the Institutional Animal Care and Use Committee of the Yale School of Medicine.

Statistical Analyses. Sample sizes (biological replicates) for each experimental group or condition are described in each figure legend. For comparisons of two groups, *t* tests were applied. For comparisons of more than three groups, one-way ordinary ANOVA test was applied. These analyses provide *P* values for each comparison.

Other experimental procedures. Detailed descriptions of constructing strains and plasmids, purification of H-NS proteins, electrophoretic mobility shift assay, in vitro protein oligomerization analysis, and amino acid sequence comparisons are in *SI Appendix, SI Materials and Methods*.

Data, Materials, and Software Availability. All data are included in the manuscript and/or *SI Appendix*.

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