Xenogeneic Silencing and Its Impact on Bacterial Genomes

Kamna Singh,[1] Joshua N. Milstein,[2,3] and William Wiley Navarre[1]

[1]Department of Molecular Genetics, University of Toronto, Ontario M5S 1A8, Canada; email: william.navarre@utoronto.ca

[2]Department of Chemical and Physical Sciences, University of Toronto Mississauga, Ontario L5L 1C6, Canada

[3]Department of Physics, University of Toronto, Ontario M5S 1A7, Canada

Annu. Rev. Microbiol. 2016. 70:199–213

First published online as a Review in Advance on June 17, 2016

The *Annual Review of Microbiology* is online at micro.annualreviews.org

This article's doi: 10.1146/annurev-micro-102215-095301

Keywords

horizontal gene transfer, xenogeneic silencing, H-NS, nucleoid-associated proteins, genome evolution

Abstract

The H-NS (heat-stable nucleoid structuring) protein affects both nucleoid compaction and global gene regulation. H-NS appears to act primarily as a silencer of AT-rich genetic material acquired by horizontal gene transfer. As such, it is key in the regulation of most genes involved in virulence and in adaptation to new environmental niches. Here we review recent progress in understanding the biochemistry of H-NS and how xenogeneic silencing affects bacterial evolution. We highlight the strengths and weaknesses of some of the models proposed in H-NS-mediated nucleoprotein complex formation. Based on recent single-molecule studies, we also propose a novel mode of DNA compaction by H-NS termed intrabridging to explain over two decades of observations of the H-NS molecule.

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INTRODUCTION

Horizontal gene transfer (HGT; or lateral gene transfer) allows bacteria to acquire foreign genes to enrich their genetic diversity and plays a key role in bacterial speciation (14, 21, 63). HGT enables bacterial cells to rapidly acquire phenotypes necessary to explore and acclimatize to previously inaccessible environments (33). Conversely, horizontally acquired genes may have direct, negative effects on fundamental cellular and metabolic processes. Furthermore, the costs associated with expressing foreign genes when not required can put cells at a competitive disadvantage in their corresponding environments (50, 70). Indeed, despite the fact that HGT is a central driver of prokaryotic speciation, it is clear that bacteria employ extensive systems to protect their genomes from invading DNA, such as restriction enzymes and CRISPR systems (12, 57, 69, 79). Thus bacterial cells must constantly balance two important evolutionary pressures—the need to rapidly adapt and the need to protect their genomic and regulatory integrity.

In 2006, four groups independently reported that the heat-stable nucleoid structuring (H-NS) protein downregulates gene expression from foreign-derived (xenogeneic) DNA segments in the chromosomes of *Escherichia coli* (42, 61) and *Salmonella enterica* (54, 59). All four groups utilized chromatin immunoprecipitation assays to determine the specific localization of H-NS (or epitope-tagged versions of H-NS) and analyzed the global gene expression of cells lacking H-NS using microarray technology. These experiments demonstrated that the H-NS protein specifically binds to and represses transcription from regions that contain significantly more adenine and thymine bases (i.e., are more AT rich) than either the *E. coli* or the *S. enterica* chromosomal average (average percentage AT $\approx$ 48). With rare exception, AT-rich regions are indicative of the DNA having been acquired by HGT.

The transcriptional repression of foreign DNA on the basis of its atypical base composition has been termed xenogeneic silencing. Xenogeneic silencers have since been identified in a diverse array of bacterial species where they regulate the majority of xenogeneic sequences (i.e., sequences derived from a foreign source) (3, 58). As a result, xenogeneic silencing proteins play a central role in the regulation of most virulence-associated genes in several important pathogens. Several recent and excellent reviews have been written about xenogeneic silencing (3, 31, 47, 58, 73). We limit this review to three areas where recent progress has been particularly rapid or insightful. Specifically, we discuss (*a*) new findings that point to the mechanism of DNA recognition by these

proteins, (*b*) a new model for the structure of a silenced complex based on recent single-molecule studies, and (*c*) recent studies that have experimentally assessed the impact of silencing on bacterial genome structure and content.

XENOGENEIC SILENCING PROTEINS

Xenogeneic silencing proteins have been classified into three distinct families based on differences in their evolutionary origins and the structure of their DNA-binding domains: the H-NS family, the MvaT family, and the Lsr2 family (3). Members of the H-NS and MvaT families are widespread in the gram-negative alpha-, beta-, and gammaproteobacteria, whereas the Lsr2 silencers are found almost exclusively in the high-GC, gram-positive *Actinobacteria* (68). Notably, no silencing proteins have yet been found in clades where AT-rich bacterial species are the norm, like the phylum *Firmicutes*.

H-NS Family

The H-NS family of DNA-binding proteins are grouped on the basis of a highly conserved motif in their C-terminal domains (TWTG$\underline{X}_1$G$\underline{X}_2$X$_3$P) that is critical for DNA binding (30, 40). The nearly identical H-NS proteins of *E. coli* and *Salmonella* comprise two functionally and structurally autonomous domains: an N-terminal oligomerization domain (residues 1–83) and the C-terminal DNA-binding domain (residues 91–137) (77). The N-terminal domain is composed of (*a*) two short α helices followed by an elongated third helix, which together form the dimerization domain; and (*b*) a short fourth helix that forms a secondary dimerization site by associating to the distal end of the third helix via a helix-turn-helix structure (5). The N-terminal domains of other H-NS family members are diverse in sequence but are also predicted to be largely α helical. In the enteric bacteria the N-terminal domain of H-NS also interacts with an additional accessory factor, Hha, discussed further below (**Figure 1**).

Among xenogeneic silencers, the H-NS-like proteins are the most diverse in both their primary sequence and their phylogenetic distribution (68). Most isolates of *Yersinia* only encode a single H-NS-like molecule. *E. coli* and *Salmonella* encode a minimum of two H-NS-like proteins (H-NS and StpA), but paralogs can be found on mobile genetic elements like plasmids or genomic islands (68), which means some strains of *E. coli* can harbor several additional H-NS-like proteins. The H-NS-like proteins seem to have proliferated greatly in the betaproteobacterial order *Burkholderiales*. *Burkholderia vietnamiensis* strain G4, for example, encodes no fewer than 17 H-NS-like proteins distributed on its three chromosomes and on three of its five plasmids. Not all members of the H-NS family act as silencers. Ler, for example, is an H-NS paralog encoded on the *LEE* pathogenicity island of pathogenic strains of *E. coli* that acts as a countersilencer to activate gene expression (37, 51, 67, 85). Therefore, it should not be assumed that any uncharacterized H-NS family member functions as a xenogeneic silencer.

MvaT Family

The MvaT family is a distinct group of DNA-binding proteins that have clear evolutionary relationships to the H-NS family but that lack the canonical H-NS motif (29). They are restricted largely to the family *Pseudomonadaceae*. Like H-NS, MvaT has been shown to specifically target AT-rich DNA segments in *Pseudomonas aeruginosa* by chromatin immunoprecipitation assays, and MvaT and its paralog MvaU silence gene expression in *P. aeruginosa* in a fashion similar to H-NS (18, 29). MvaT can complement *E. coli* strains lacking H-NS for several phenotypes, indicating that it is similar to H-NS in its mode of binding (75).

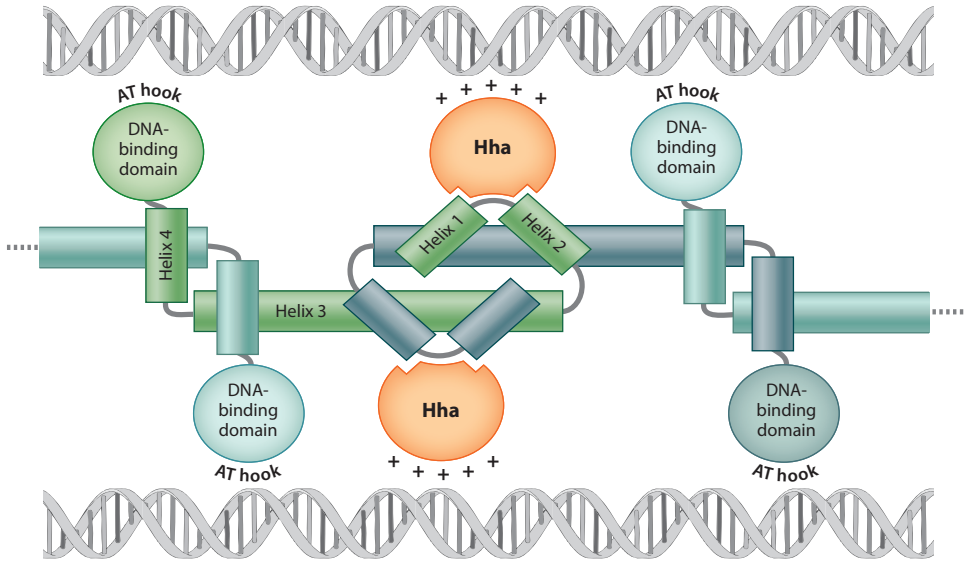

Figure 1

Diagram of an H-NS polymer in complex with Hha (*orange*) and DNA (*gray*). Each H-NS monomer within the oligomer is shown in a different color (*green*, *aqua*, and *teal*). Hha monomers flank either side of the N-terminal dimerization domain of H-NS (composed of helices 1 and 2 and part of helix 3). The central dimerization domain of H-NS is formed at the interface of the C termini of helix 3 and helix 4. This arrangement likely projects the DNA-binding surfaces of Hha and H-NS on opposite sides of the oligomer. This model is based on structural observations of the H-NS oligomerization domain (PDB 3NR7), and of Hha in complex with the H-NS N-terminal domain (PDB 4ICG).

Lsr2 Family

The Lsr2 family of silencing proteins is completely unrelated to H-NS and MvaT proteins in sequence, structure, and phylogenetic distribution. Whereas the oligomerization domain of H-NS is elongated and entirely α helical, the equivalent domain from Lsr2 is globular and largely composed of β sheets (74). Likewise, the C-terminal DNA-binding domain of H-NS shares no overall structural homology with Lsr2, save a short motif that is involved in targeting AT-rich DNA (40). This suggests that the origins of Lsr2 and H-NS are distinct and their functional similarities are the result of convergent evolution.

Lsr2 has been shown to be a xenogeneic silencer in *Mycobacterium tuberculosis* through studies similar to those used for H-NS and MvaT. Chromatin immunoprecipitation and microarray expression assays showed that Lsr2 binds to and downregulates expression from the AT-rich regions of the mycobacterial genome (40, 41). Remarkably, despite their near-complete lack of sequence or structural similarity, H-NS and Lsr2 are functionally interchangeable when expressed in silencing-deficient strains of either *E. coli* or *M. tuberculosis* or *M. smegmatis* (39).

Hha Family of Accessory Silencing Factors

The H-NS proteins of the enteric bacteria, including *Yersinia* spp., *E. coli*, and *Salmonella* spp., associate directly with a class of secondary accessory proteins, of which the prototypical member is Hha (called YmoA in *Yersinia* spp.) (36, 38, 56, 62). *E. coli* and *Salmonella* spp. contain a second paralog of Hha, YdgT. These small (<10 kDa) proteins are almost entirely restricted to the

Enterobacteriaceae and are not found in other species, including those of the genus *Vibrio*. Why these accessory proteins exist is something of an enigma given that the H-NS molecules encoded in the nonenteric species apparently do not need a cofactor to act as effective silencers.

Hha (and YdgT) directly interacts with the N-terminal dimerization domain of H-NS (and StpA) and appears to stabilize the formation of higher-order H-NS nucleoprotein filaments along long stretches of AT-rich DNA (2, 23). Hha is not needed by H-NS when it is bound to short stretches of DNA that contain specific high-affinity binding sites, which corresponds with observations that Hha/YdgT generally affect xenogeneic DNA and not housekeeping genes that tend to have short, well-defined H-NS binding sites (8). Notably, loss of Hha and YdgT does not have a dramatic effect on the overall binding of H-NS to the *E. coli* or *Salmonella* chromosome, as assessed by electrophoretic mobility shift or chromatin immunoprecipitation assays, but has a profound effect on the ability of H-NS to silence foreign DNA (2, 76).

The X-ray crystal structure of Hha in complex with the N-terminal dimerization domain of H-NS revealed that two monomers of Hha bind to symmetrical sites on either side of the H-NS dimer (2). This arrangement projects a cluster of conserved basic amino acids found in each Hha monomer on opposite faces of the H-NS dimerization domain (**Figure 1**). Hha mutants that lack those basic residues are capable of binding to H-NS but incapable of regulating the expression of *bilA*, an H-NS/Hha-regulated promoter, in vivo (2). This suggests that the positively charged residues may directly interact with DNA to further stabilize the H-NS:DNA complex (2, 23).

DNA-BINDING PROPERTIES OF XENOGENEIC SILENCERS

The interaction of H-NS with DNA occurs in two separate but connected steps (78). The first step is target selection, whereby H-NS monomers preferentially bind AT-rich DNA. The second step involves oligomerization to make a nucleoprotein structure that is conducive to silencing. This second step is absolutely critical for H-NS function given that mutations in the oligomerization domain can abolish the ability of H-NS to downregulate transcription while having little, if any, effect on DNA binding (1, 16, 71, 77).

Targeting AT-Rich DNA

An intuitive (and well-supported) model for nucleoprotein formation is that H-NS first nucleates at high affinity prior to its oligomerization along AT-rich tracts of lesser affinity to assemble a final higher-order nucleoprotein complex. This model has been supported by a time-resolved analysis of H-NS assembly at the *virF* promoter (78). Several recent studies have examined how the C-terminal DNA-binding domain of H-NS and related proteins (e.g., Ler, MvaT, and Lsr2) are able to selectively target AT-rich DNA and revealed what constitutes a high-affinity binding site. These studies have employed nuclear magnetic resonance (NMR), protein-binding microarrays, and more standard footprinting and gel-shift assays to paint a fairly complete picture of the reasons AT-rich DNA is preferred only slightly over GC-rich DNA.

Early studies that addressed the preferred DNA targets of H-NS found that H-NS selectively binds curved DNA and that this binding can be inhibited by drugs like distamycin that intercalate into the minor groove and straighten DNA. Confounding factors in these studies include the fact that the model curved sequence was considerably more AT rich than the control fragment; we now know that distamycin competes directly with H-NS and other silencers for binding to the minor groove (40). Furthermore, detailed analyses of H-NS binding sites, both chromosome-wide and at specific promoters, have largely failed to find any strong correlation between the affinity of

H-NS binding and DNA curvature (15, 54, 59). We therefore believe the oft-repeated assertion that H-NS has an intrinsic preference for curved DNA is incorrect.

Recent studies suggest that it is the shape and structural parameters of the target DNA that are recognized by all three families of silencers rather than a specific sequence. AT-rich sequences possess structural properties distinct from those of GC-rich DNA (88). A-tract sequences, where there is a run of several adjacent dAdA, dTdT, or dAdT dinucleotide steps (e.g., AAAATTTT), have a minor groove that is considerably more narrow than either mixed-base or GC-rich DNA owing to tight base stacking (43). At the opposite end of the spectrum is the structure formed by the dTdA dinucleotide (also known as the TpA or TA step). This dinucleotide step, owing to steric clashes that occur between the adjacent adenine bases in the major groove, disrupts close base stacking interactions to distort the minor groove and greatly increase flexibility of the DNA (49). Finally, G:C base pairs have an exocyclic amino group that projects into the base of the minor groove, whereas A:T base pairs do not. This means the minor groove of GC-rich DNA is more shallow than the minor groove of AT-rich DNA.

A specific DNA motif (5′-TCGATATATT-3′) targeted by H-NS was first identified during a detailed analysis of the *proU* promoter in *E. coli* (15). This sequence was found to bind an H-NS monomer with a K_d of ∼50 nM. More recently the selective preferences of the H-NS, MvaT, and Lsr2 DNA-binding domains have been systematically analyzed using protein-binding microarrays (11). Also, structural studies of Ler (24), H-NS (40), Lsr2 (41), and MvaT (29) binding domains in complex with DNA have been completed. The results indicate that the H-NS-like and Lsr2 proteins each intercalate a specific protein loop into the minor groove (**Figure 2**). For H-NS this loop is contained within its canonical motif (TWTG**X**$_1$**G**X$_2$X$_3$P), where the X$_1$GX$_2$ (usually QGR) sequence forms a prokaryotic AT hook that is reminiscent of the AT-hook motif critical for DNA binding by the HMG-IY proteins of eukaryotes (25). Remarkably, despite the fact that

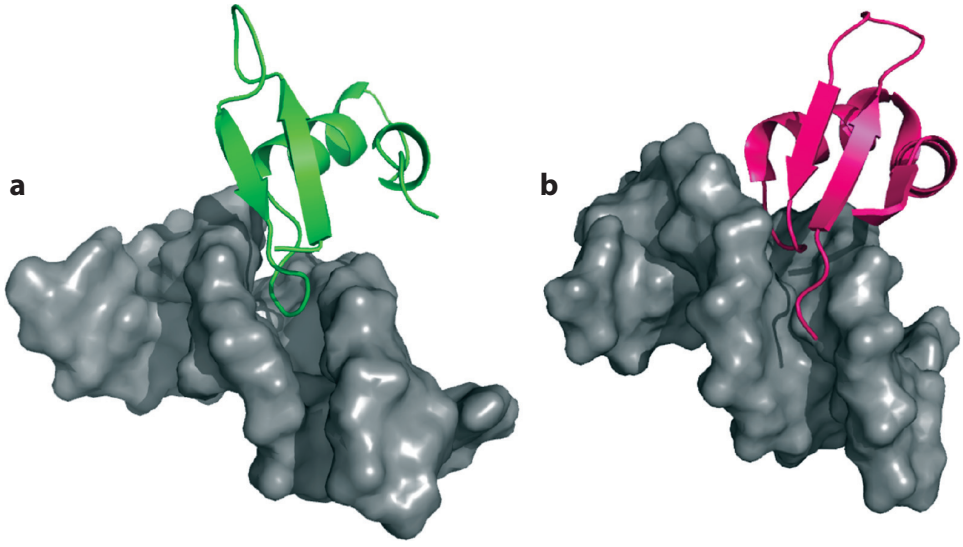

Figure 2

Structures of the C-terminal DNA-binding domains of (*a*) H-NS (*green*) and (*b*) MvaT (*magenta*) in complex with DNA (*dark gray*). These structures (PDB 2L93 and 2MXF) show how the AT-hook motif of H-NS and AT pincer of MvaT insert into the minor groove of AT-rich sequences. Specific features of the minor groove enable xenogeneic silencing proteins to discriminate between GC-rich and AT-rich DNA.

it is unrelated to H-NS, Lsr2 also intercalates an AT hook (sequence RGR) into the minor groove of AT-rich DNA (40). Substitution of either the X_1 or X_2 residues with alanine or drugs that bind in the minor groove (distamycin, netropsin) disrupt DNA binding by both proteins (40).

The highest-affinity DNA sequences for H-NS and Lsr2 contain one or two adjacent TpA steps flanked by A-tract sequences, which is consistent with both the H-NS high-affinity motif first identified at the *proV* promoter and a strong binding DNA target identified for Ler (24). The AT-hook structure of these proteins nestles into the minor groove of AT-rich DNA, not unlike a sausage in a bun. The central glycine and the side arms of the X_1 and X_2 residues of the hook lie along the floor of the groove, the width of which can easily be accommodated by the flexible TpA steps. A-tracts bring the phosphate backbones together, which dramatically increases the electronegative potential of the minor groove. The electronegative groove interacts with charged moieties of the H-NS or Lsr2 X_1 and X_2 residues (usually lysine and arginine). GC-rich DNA has reduced affinity for the proteins because the minor groove is wider (less electronegative) and the exocyclic amino group of guanine interferes with the ability of the hook to nestle into the groove.

A similar analysis of MvaT, which lacks the canonical H-NS motif and has no AT-hook structure, revealed that this class of proteins also targets the minor groove (29). However instead of intercalating into the minor groove with a single AT hook, MvaT intercalates into the groove with two separate structures, one of which resembles an AT hook, forming a novel AT-pincer structure (**Figure 2**). Furthermore, the protein makes extensive contacts with the phosphate backbones of the DNA using a network of lysine, arginine, and glutamate residues. The extensive contact MvaT makes with its target DNA may allow these proteins, which come from species with a higher GC content, to better tolerate GC base pair interruptions in their binding targets.

Higher-Order Nucleoprotein Complex Formation

Oligomerization is essential for gene silencing by H-NS, MvaT, and Lsr2. However, different findings from several single-molecule studies have led to disagreement as to what the relevant structure of a nucleoprotein oligomer is. Oligomerization of the *E. coli/Salmonella* H-NS protein occurs via two independent dimerization domains contained within the N-terminal 80 residues of the protein. In solution, at low concentrations, the H-NS protein exists primarily as a dimer via homotypic interactions within the first 46 residues (13). At higher concentrations in solution, and likely when in complex with DNA, the protein further oligomerizes by interactions with the central dimerization domain contained within residues 60–80. This head-to-head/tail-to-tail organization allows H-NS to form extended polymeric chains (**Figure 1**). Extended chains of the first 80 residues of H-NS have been visualized using X-ray crystallography, showing that the structure forms a helical scaffold from which the DNA-binding domains are predicted to protrude in opposing directions along the filament (5). This general arrangement is supported by subsequent solid-state NMR studies of the full-length H-NS protein (65).

Models of the H-NS Nucleoprotein Structure

Several laboratories have attempted to gain insight into the H-NS:DNA complex using a combination of electron microscopy, atomic force microscopy, and other single-molecule experimental approaches. These studies have found that the H-NS nucleoprotein complex can adopt at least two different modes: stiffened, where the protein forms stiff oligomeric filaments along the DNA, and bridging, where distant regions of the nucleoprotein complex bridge to form loops in the DNA.

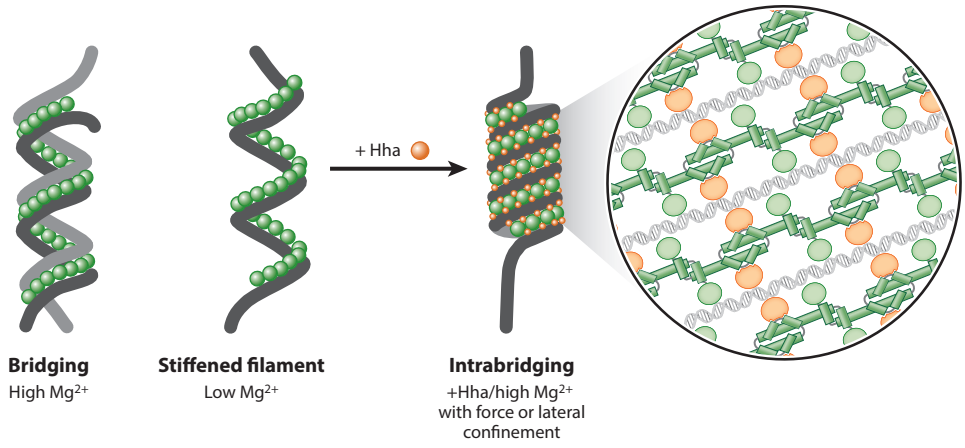

Figure 3

Three proposed models of the H-NS nucleoprotein complex. This diagram depicts how a stiffened H-NS nucleoprotein filament could be compacted by intrabridging in the presence of Hha or high concentrations of Mg^{2+}.

(4, 27) (**Figure 3**). These two conformations have also been observed to occur for Lsr2 and MvaT (20, 26, 64, 84). Whether the protein:DNA complex adopts a bridged or stiffened conformation depends largely on the experimental conditions employed, leaving it open to interpretation as to which conformation is biologically relevant (53).

A key parameter that determines which of the two modes predominates in vitro was determined by Liu et al. (53), who reported that the switch between the two modes of DNA binding is mediated by the presence of magnesium and calcium ions. Specifically, at higher concentrations of divalent cations the H-NS:DNA complex was observed to condense, as would be expected if the complex adopted bridging behavior, whereas at lower concentrations the structure would adopt a stiffened state. Ions like Mg^{2+} could theoretically affect the nucleoprotein complex via effects on H-NS, the DNA, or both. DNA flexibility increases in response to Mg^{2+} and other ions (10). Also, in vivo studies indicate that DNA inside of a cell is much more flexible than DNA in a low-ionic-strength buffer, likely due to a combination of cytoplasmic ions (K^+, Mg^{2+}, spermidine), and DNA-binding proteins (83). Therefore, the in vitro conditions under which the stiffened H-NS nucleoprotein filaments are observed may not represent the physical state of the DNA polymer within the cell.

Whether the stiffened or bridged complexes of DNA are the biologically relevant modes for silencing has been addressed in a few recent studies, once again with conflicting results. One study found that H-NS-mediated silencing was only counteracted by a known H-NS antagonist (SsrB) when bound in the stiffening mode, suggesting it is the relevant form of the complex in vivo (80). A separate study came to the opposite conclusion when the effect of bridging or stiffening on the ability of H-NS to block progression of RNA polymerase (RNAP) was examined with respect to pause sites that trigger Rho-dependent termination (52). This study found that bridged H-NS filaments strongly favored a longer duration of pauses by RNAP at a subset of pause sites. Moreover, it was bridged complexes, and not stiffened ones, that promoted Rho-dependent termination. Previously, it had been shown that RNAP can be stalled by an increase in DNA torque (55). The authors proposed that the formation of bridged H-NS nucleoprotein filaments constrains DNA in plectonemes, preventing twist diffusion and causing accumulation of DNA

torsional stress in front of the polymerase, which in turn leads to a stall that inhibits transcription (52).

Two recent single-molecule studies point to yet another possible nucleoprotein structure that combines aspects of bridging and stiffening (**Figure 3**). In the first study, the mechanics of the H-NS/Hha nucleoprotein complex were probed using optical tweezers and tethered particle motion (TPM) analysis (81). These approaches were combined to observe short stretches of DNA anchored at one end to a surface and on the other end to a polystyrene bead. In TPM experiments, the motion of the bead can be observed directly to gain some information about the properties of the DNA to which it is attached. Mg^{2+} caused naked DNA to soften, and upon addition of H-NS the DNA compacted, as would be expected if bridges formed. However, when mechanical tension was briefly applied to the bead (via an optical trap), the nucleoprotein structure collapsed into a highly compacted state. This force-induced compaction occurred more rapidly when Hha was added even in the absence of Mg^{2+}. These findings suggest that Hha may play a role in helping H-NS compact DNA into an ordered nucleoprotein complex. It is likely that DNA, in the absence of ions like Mg^{2+}, is simply too stiff to be compacted by H-NS owing to electrostatic repulsion between the negatively charged phosphates in the DNA backbone and several glutamate residues in helix 3 of H-NS. However, the presence of positively charged surfaces of Hha allows the complex to overcome this electrostatic repulsion, thereby leading to DNA condensation.

The second study examined H-NS-induced DNA elongation and compaction by using nanofluidic chips where the DNA was trapped in a long, but thin, nanochannel (87). It was shown that besides the presence of magnesium and salt, the confinement of the nucleoprotein complex in a nanochannel also facilitates DNA compaction. The authors suggested that the interplay between the space confinement, H-NS-mediated attraction, and filamentation dictates H-NS-induced DNA conformation and compaction.

Combining structural information about the H-NS/Hha oligomer with these single-molecule studies, which reveals a highly compact nucleoprotein structure, points to a novel H-NS/Hha/DNA arrangement we term intrabridging. Unlike bridging, where H-NS cross-links two adjacent DNA duplexes or distant segments of the same duplex, intrabridging occurs when a single DNA molecule is toroidally intercalated within the corkscrew-shaped H-NS oligomer, bound on its top and bottom sides by surfaces of a continuous H-NS filament (**Figure 3**). This structure is consistent with recent observations that H-NS, in the presence of Mg^{2+}, is able to trap supercoils in the form of plectonemes (86).

The intrabridging model avoids several limitations of both the stiffening and bridging models. It does not involve long-distance interactions between duplexes that would tangle and disorganize the nucleoid, and intrabridging would compact DNA instead of extending it as stiffening would do. Intrabridging may not have been observed in earlier studies because such studies did not include Hha and because interduplex bridges spontaneously arose when the DNA was allowed to fold back on itself. The two recent single-molecule studies prevented this folding back by applying force or through confinement in a nanochannel.

IMPACT OF SILENCING ON EVOLUTION OF BACTERIAL GENOMES

S. enterica and *E. coli* are closely related bacterial species known to have last shared a common ancestor approximately 100 Mya (28). Their genomes are largely syntenic, and they share a nearly identical set of ancestral core genes that perform basic cellular functions. Since diverging, however, each has acquired vast amounts of DNA via HGT. An early and key evolutionary event in *Salmonella* evolution was the acquisition of the SPI-1 pathogenicity island, which enabled these bacteria to

invade intestinal epithelial cells and provoke an intense inflammatory response—whereas most *E. coli* lineages evolved as commensal and opportunistic pathogens (66). A second major HGT event, the acquisition of SPI-2, enabled *Salmonella* spp. to persist inside of host cells and, in some cases, spread systemically. Other key HGT, gene loss, and mutational events have further diversified the genus, leading to a multitude of subspecies that have varying host specificities (28, 48, 60).

Of the approximately 4,500 genes found in any typical isolate of *S. enterica*, only about 2,700 (~60%) will be found in any single isolate of *E. coli*. However, a comparison of a large number of *Salmonella* strains with a multitude of *Escherichia* strains reveals that fewer than 1,000 genes are universally conserved in all isolates. The %GC distribution of these highly conserved core genes is relatively narrow, centered at an average of 54%. In contrast, genes that are present in only one or a few *Salmonella* isolates (and therefore likely to be recent acquisitions) are overwhelmingly AT rich. Remarkably, even the genes found across all *Salmonella* species (but not in *E. coli*), like those in SPI-1, typically have a lower overall %GC than the core set of highly conserved genes. This suggests that, even after 100 MYr, these critical *Salmonella*-specific genes have not ameliorated GC-content of the host genome, perhaps because doing so would remove the ability of H-NS to regulate them (28).

Given that xenogeneic silencing proteins silence AT-rich sequences it is possible they play a role in promoting their acquisition. One way to determine specifically how xenogeneic silencing affects the bacterial cell (its fitness, its regulatory networks, and its genome) is to search for secondary mutations that exacerbate or alleviate the fitness consequences of the loss of H-NS. Although loss-of-function mutations in *hns* are generally well tolerated in laboratory strains of *E. coli*, they are poorly tolerated in *S. enterica* (54, 59) and lethal in *Yersinia* spp. (7, 19, 45). Several studies have found that, in both *E. coli* and *S. enterica*, additional mutations in the RpoS (σ^{38}) sigma factor improve growth of strains lacking H-NS in laboratory culture (44, 59, 89). This phenomenon is likely due to the fact that levels of RpoS are greatly elevated in strains lacking H-NS owing to the influence of H-NS on the anti-adaptor IraD and IraM proteins (9). Increased expression of RpoS is deleterious for bacterial growth in lab culture, and in fact loss of RpoS occurs rapidly during regular lab passaging of both *E. coli* and *Salmonella* (34, 35). Also found to improve the growth of *S. enterica* strain 14028s variants that lack H-NS are disruptions in the genes encoding the PhoP/PhoQ two-component regulatory system. The reasons for this are unknown but are independent of *rpoS* (1, 59).

To explore how H-NS affects bacterial fitness, our group performed experimental evolution of an *hns* mutant of *S. enterica* strain 14028s (1). Here, six independently derived *Salmonella rpoS hns* double mutant cultures were passaged for 300 generations (30 days) in laboratory media to select for variants that would display improvements in growth. Whole-genome sequencing revealed that five of the six *hns rpoS* mutant lineages independently acquired large deletions in the SPI-1 pathogenicity island, suggesting SPI-1 misregulation is a major cause of the growth defect of *Salmonella* strains in lab culture. This was verified in freshly reconstructed Δ*SPI-1 hns* mutant strains where it was found that removal of SPI-1 doubled the growth rate of the *hns* mutant. This study provides experimental evidence that silencing acts as a buffer against the negative fitness consequences of HGT and suggests that *Salmonella* would not have arisen if H-NS did not buffer the negative fitness impact of SPI-1, the defining gene locus of the species (1). Notably, a separate study had observed a fitness improvement in *S. enterica* strain LT2 *hns*-null mutants upon deletion of the SPI-2 gene cluster, encoding a second type 3 secretion system.

Another finding was that all six evolved *hns* mutant lineages acquired mutations in the gene encoding the H-NS paralog StpA (1). In all cases these were gain-of-function mutations in the

N-terminal 80 amino acids of StpA encoding the oligomerization domains. A similar finding had serendipitously been observed in an *hns* mutant of *E. coli* (46). Experimental evolution using 24 additional *hns* mutant lineages found, yet again, that each rapidly acquired compensatory point mutations in the oligomerization domain of StpA (1). Remarkably, when expressed at high levels from a plasmid, StpA was largely ineffective as a substitute for H-NS. In contrast, many (but not all) of the isolated *stpA* mutants were able to complement an *hns* mutant (1). Equally surprising was the fact that the change of a single residue in StpA could confer H-NS-like behavior on the StpA molecule both in vivo and in vitro. The location of these mutations within StpA suggest that functional differences between the two paralogs result from differences in their oligomerization rather than in their DNA targets.

A worrying result of this study is that for the last two decades, laboratories have studied *hns* mutants without appreciating how rapidly compensatory mutations can arise in loci including *rpoS* or *stpA*. Special measures should be undertaken while maintaining H-NS mutant strains to minimize the time spent growing in laboratory culture, and all strains should have their *stpA* and *rpoS* loci sequenced for mutations (if not occasional sequencing of the whole genome). Finally, phenotypes should be verified using several independently derived mutants, and complementation analysis should always be performed.

Experimental evolution has also been performed on *hns stpA* mutants of *E. coli* where independent lineages were passaged and subjected to deep sequencing (72). Mutations in *rpoS* were again identified in a large percentage of the isolates. Furthermore, the authors noted that *hns stpA* mutant strains frequently lost sequences within the *E. coli rac* prophage and, more strikingly, that an extremely large 2-Mb section of the chromosome flanking the origin of replication appears to be duplicated in many isolates. The duplicated region is flanked by IS2 elements that are found frequently in the genomes of *E. coli* and related bacteria. This suggests that loss of H-NS and StpA leads to chromosomal instability in *E. coli*, perhaps because of spurious activation of some component within the IS2 elements or by allowing inappropriate recombination to occur between insertion sequences.

Another study assessed the impact of MvaT and its paralog MvaU on *P. aeruginosa* strain PAO1 (17). Specifically, the study addressed the underlying reason *Pseudomonas* could not tolerate the loss of both proteins simultaneously. Here MvaT was depleted using a ClpX protein degradation system in an *mvaU* mutant. A *Pseudomonas* transposon library was used to identify genes that, when deleted, would enable *P. aeruginosa* to tolerate the loss of both silencers. Remarkably, the authors of this study found that MvaT and MvaU were dispensable in strains carrying mutations in a prophage (Pf4) and/or genes encoding a type IV pilus. The authors subsequently found that the type IV pilus is a receptor for the Pf4 phage. This indicates that MvaT and MvaU are critical for repressing expression of both virulence-associated and prophage genes in strain PAO1, although this study has not been extended to determine whether MvaT and MvaU play this role in other strains of *Pseudomonas*.

These experimental evolution studies, and other more directed studies (6, 32), indicate that silencing plays a critical role in fitness and genome stability by repressing the inappropriate expression of horizontally acquired sequences—thereby allowing cells to bank potentially useful AT-rich sequences in a cryptic state with a minimal impact on fitness. A corollary to this hypothesis is that AT-rich sequences would, over evolutionary time, accumulate in genomes at a higher rate than GC-rich sequences. These sequences would eventually be lost or, in rare jackpot events, integrate successfully into the cell's physiological and regulatory networks. Several studies have examined how xenogeneic sequences can evolve to integrate into the regulatory network of a bacterial cell, and we point the reader to some excellent recent papers on the subject (22, 82, 90).

CONCLUDING REMARKS

Over the last 10 years we have greatly improved our understanding of how xenogeneic silencers target foreign DNA and their impact on bacterial fitness and evolution. That said, many aspects of H-NS and related molecules remain controversial, and in this review we bring together evidence from a variety of sources to put forth testable models about how these proteins can affect nucleoid structure and bacterial evolution.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We sincerely apologize to authors whose work in this large field we did not have space to cover.

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