REVIEW

Silencing of xenogeneic DNA by H-NS—facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA

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Lateral gene transfer has played a prominent role in bacterial evolution, but the mechanisms allowing bacteria to tolerate the acquisition of foreign DNA have been incompletely defined. Recent studies show that H-NS, an abundant nucleoid-associated protein in enteric bacteria and related species, can recognize and selectively silence the expression of foreign DNA with higher adenine and thymine content relative to the resident genome, a property that has made this molecule an almost universal regulator of virulence determinants in enteric bacteria. These and other recent findings challenge the ideas that curvature is the primary determinant recognized by H-NS and that activation of H-NS-silenced genes in response to environmental conditions occurs through a change in the structure of H-NS itself. Derepression of H-NS-silenced genes can occur at specific promoters by several mechanisms including competition with sequence-specific DNA-binding proteins, thereby enabling the regulated expression of foreign genes. The possibility that microorganisms maintain and exploit their characteristic genomic GC ratios for the purpose of self/non-self-discrimination is discussed.

The recent availability of hundreds of complete bacterial genome sequences has shed light on the important role that lateral (horizontal) interspecific gene transfer (LGT) plays in microbial evolution (Hacker and Kaper 2000; Ochman et al. 2000; Lerat and Ochman 2004; Binnewies et al. 2006). Analysis of these genome sequences indicates that LGT is the primary source of genetic diversity in microorganisms and reveals that microbial genomes are highly dynamic over evolutionary time (Jain et al. 2002; Lerat et al. 2005). The role of LGT in microbial

evolution has been most extensively studied in the context of pathogens, and it is now well appreciated that many virulence factors of bacterial pathogens were acquired from exogenous sources on phages, plasmids, transposons, and integrating conjugative elements (Hacker and Kaper 2000; Ochman et al. 2000). LGT is not limited to pathogens, however, and evidence for LGT can be found in the genomes of virtually all free-living microbes sequenced thus far (Ochman and Davalos 2006). Genes acquired via LGT share certain features that can aid in their identification, such as association with phage or transposon genes and/or integration adjacent to tRNA loci (Hacker and Kaper 2000; Ingersoll et al. 2002). Foreign sequences frequently exhibit variant base composition and codon usage from that of their host genome; in a majority of cases, genes acquired by LGT are relatively rich in adenine and thymine (AT-rich) (Lawrence and Ochman 1997; Daubin et al. 2003).

Comparative genomic analysis reveals that *Salmonella enterica* serovar Typhimurium strain LT2 has acquired and retained >200 discrete regions of >100 bases in length acquired via LGT since it diverged from its last common ancestor with *Escherichia coli*, representing ~1400 ORFs or slightly more than one-quarter of its total genetic material (Porwollik and McClelland 2003), with comparable gene acquisition observed in *E. coli* over the same time period (Lawrence and Ochman 1998; Ochman and Jones 2000). Several *S. enterica* Typhimurium sequences obtained via LGT are known to be involved in virulence, but the functions of many are still obscure. Although 200 horizontally acquired sequences may seem like an impressive number, the ~100 million years since *Salmonella* and *E. coli* shared a common ancestor (Ochman and Wilson 1987; Doolittle et al. 1996) indicate that a new sequence is acquired and stably maintained by enteric bacteria on an average of only once every several hundred thousand years. The rate at which genetic information is gained and lost is not uniform between bacterial clades and depends on the lifestyle of the

organism (Ochman 2005). Once the host range of a bacterium narrows, as occurred in *Salmonella* serovars Typhi and Paratyphi A, many LGT-derived genes rapidly become nonfunctional and undergo the process of removal (Parkhill et al. 2001; McClelland et al. 2004).

Although LGT has played a major role in microbial evolution, foreign sequences are more likely to decrease rather than increase the fitness of the recipient organism. Phages can compromise bacterial survival (Buckling and Rainey 2002), but conjugative plasmids and transposons may also decrease fitness (Lee and Edlin 1985) by integrating into functionally useful regions of the genome or by carrying genes detrimental to the recipient cell. Even in the cases in which a newly acquired sequence might provide a useful function, the fitness of the recipient may be adversely impacted if new sequences are not integrated into pre-existing regulatory networks. Expression of novel genes can disrupt cellular function in unanticipated ways. For example, expression of the bacteriophage λ cII regulatory protein is highly toxic for *E. coli* due to effects on DNA replication (Kedzierska et al. 2003), and expression of the plasmid-encoded Ysc type III secretory system of *Yersinia* elicits an extracytoplasmic stress response in host cells (Darwin and Miller 2001).

This raises the question of how genomes can expand their functional repertoire through LGT while maintaining their physiological, structural, and regulatory integrity. Recent findings have revealed a crucial role for the H-NS protein in protecting the cell from detrimental effects of LGT by recognizing and silencing sequences exhibiting significantly lower GC content than the host genome (Lucchini et al. 2006; Navarre et al. 2006; Oshima et al. 2006). This phenomenon, which we term "xenogeneic silencing" ("xenogeneic" means obtained from an organism of a different species), is the first defense against foreign genetic material to be described since the discovery of restriction enzymes >40 years ago. Ironically, a system to protect cells from foreign DNA can also facilitate its acquisition. Xenogeneic silencing provides important new insights into bacterial evolution by showing how cells can acquire new DNA encoding virulence or antibiotic resistance determinants without compromising fitness.

H-NS

Although now best known for its inhibitory effects on gene expression, H-NS was originally identified as an *E. coli* DNA-binding protein that stimulated transcription from bacteriophage templates (Cukier-Kahn et al. 1972). H-NS, along with Dps, Fis, HU, IHF, YdgT, Hha, Hfq, Lrp, StpA, CbpB, and IciA, is often described as one of the "nucleoid-associated proteins," a group of DNA-binding proteins implicated in the organization and higher-order structure of the bacterial chromosome (Ali Azam and Ishihama 1999; Dorman 2004). H-NS is one of the most abundant DNA-binding proteins in *E. coli* at 20,000 copies per cell. H-NS can constrain supercoils in vitro (Tupper et al. 1994), and a recent study determined that H-NS

can function as a "domainin," a protein that prevents global unwinding of the chromosome following double-stranded breaks by acting as a barrier that constrains supercoiling to local domains (Hardy and Cozzarelli 2005).

Due to technical constraints, it has been difficult to determine which, if any, of the nucleoid-binding proteins are responsible for compacting the nucleoid or controlling higher-order chromosomal structure in vivo. Overexpression of H-NS results in chromosomal compaction and global transcriptional silencing (Spurio et al. 1992; McGovern et al. 1994). However, two recent studies of the higher-order structure of the *E. coli* chromosome have failed to detect a role for H-NS, StpA, Fis, IHF, HU, or Dps in maintaining the global looped structure of the bacterial nucleoid (Brunetti et al. 2001; Zimmerman 2006). Many roles that have been ascribed to H-NS, including maintaining the higher-order structure of the nucleoid and compacting and partitioning the bacterial chromosome between daughter cells (Kaidow et al. 1995), may actually be carried out directly by the multiprotein "condensing" complex (MukBEF in γ-proteobacteria) (Fennell-Fezzie et al. 2005). At the present time, "nucleoid-associated" may be best regarded as a descriptive term referring to localization and DNA binding without specific implications as to function.

DNA binding and control of gene expression by H-NS

Multiple studies have demonstrated that H-NS regulates the expression of a large number of genes in both *E. coli* and *Salmonella*, and that its effects on gene expression are largely inhibitory. Sequences bound by H-NS are AT-rich but highly degenerate, and the affinity of H-NS for bona fide sites differs from that of nonspecific sites by less than an order of magnitude (Lucht et al. 1994; Tupper et al. 1994). Many reports have shown that H-NS has higher affinity for intrinsically curved DNA in vitro (Owen-Hughes et al. 1992; Lucht et al. 1994; Tupper et al. 1994). At low concentrations in solution, H-NS exists primarily as a dimer, but at higher concentrations or at promoters, H-NS can multimerize into tetramers or higher-order complexes (Falconi et al. 1988; Ceschini et al. 2000; Smyth et al. 2000). DNase I protection assays have revealed that H-NS multimerizes cooperatively along AT-rich tracts within promoters leading to extended footprints, and this multimerization appears to be requisite for its silencing function (Spurio et al. 1997; Ueguchi et al. 1997; Rimsky 2004; Dame 2005).

A complete, high-resolution structure of the H-NS protein has proven elusive, but several studies have supported a model of an N-terminal domain and flexible linker that are involved in multimerization of the H-NS protein and a C-terminal DNA-binding domain (Ueguchi et al. 1996; Smyth et al. 2000). Structures of the N-terminal (Renzoni et al. 2001; Bloch et al. 2003) and C-terminal (Shindo et al. 1995, 1999) domains have been resolved by NMR analysis, but no comparable structure exists of H-NS in complex with target DNA. Despite this limitation, significant insight has been gained into the

DNA-binding properties of H-NS and related molecules through the use of atomic (scanning)-force microscopy, which has revealed that H-NS and related proteins are capable of "bridging" adjacent helices of DNA (Dame et al. 2000, 2001, 2002, 2005). This mode of binding is unusual for a DNA-binding protein and suggests that two DNA-binding sites are found on opposing faces of the tetramer. Due to discrepancies in the NMR structures of the H-NS multimerization domains, the exact orientation of each dimer within the tetramer remains enigmatic (Dorman 2004). However, the model shown in Figure 1 explains how the ability of of H-NS to bridge adjacent strands could simultaneously explain its preference for curved DNA (or its higher affinity for regions with AT bases on the same face of the helix) (Bracco et al. 1989; Yamada et al. 1990; Owen-Hughes et al. 1992), its ability to constrain supercoiling (Hulton et al. 1990; Hardy and Cozzarelli 2005), its requirement of tetramerization for DNA bending (Spurio et al. 1997), and an ability to silence gene expression by binding regions downstream from the transcription start site (Owen-Hughes et al. 1992; Atlung and Ingmer 1997; Jordi and Higgins 2000; Dole et al. 2004b; Chen et al. 2005; Yang et al. 2005).

Functions of H-NS

For most of the past two decades, H-NS has been thought of as a global regulator that can alter the expression of a large number of genes in response to certain environ-
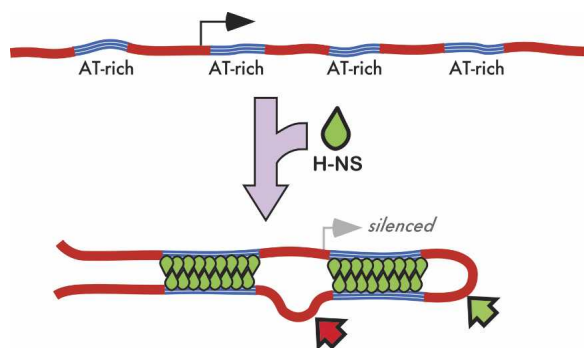

Figure 1. Bridging model for H-NS binding. The affinity of H-NS for AT-rich DNA (blue) and its ability to polymerize along and bridge adjacent regions of DNA provides an explanation for several previous observations. H-NS (teardrop shape) is capable of multimerizing into tetramers or higher-order oligomers with diametrically opposed binding surfaces. Intrinsic curvature or a protein-induced bend (green arrow) can serve to arrange nearby AT-rich sites to facilitate H-NS binding. This model can explain why many H-NS-binding sites lie downstream from the promoter (black arrow) and provides an explanation as to why H-NS displays higher affinity for repeating A-tracts on the same face of the double helix in vitro (an arrangement that also induces a bend). Furthermore, this arrangement can account for the ability of H-NS to constrain supercoiling to local domains: A nick or break in one domain (green arrow) would not alter supercoiling in an adjacent topologically isolated domain (red arrow).

mental conditions such as pH, temperature, or osmolarity (Atlung and Ingmer 1997; Amit et al. 2003; Dorman 2007). This model is attractive but not completely explanatory, as the subsets of genes regulated by pH, temperature, and osmolarity are distinct; for example, H-NS-silenced genes expressed during conditions of increased osmolarity do not generally overlap with those regulated by temperature (Atlung and Ingmer 1997; Hommais et al. 2001). Expression of H-NS relative to DNA content appears to be relatively constant under a range of environmental conditions (Free and Dorman 1995; Atlung and Ingmer 1997; Dorman 2004). Since H-NS concentrations do not vary significantly in response to pH, temperature, or osmolarity, the H-NS protein has instead been postulated to undergo structural and functional alteration under these environmental conditions. While some biochemical evidence for such structural changes has been obtained, the correlation with H-NS-dependent gene expression has been less than perfect.

Much effort has focused on the possibility that H-NS might act as a thermosensor that globally regulates a large subset of genes in response to temperature. A recent report noted that more than three-quarters of the 531 *Salmonella* genes exhibiting altered expression when cultures were shifted from 25°C to 37°C are dependent on H-NS (Ono et al. 2005). Notable among these genes were SPI-1 and the cobalamin biosynthetic operon (*cob*), as well as genes involved in flagellar biosynthesis and chemotaxis. Both SPI-1 and *cob* were constitutively expressed at high levels in the absence of H-NS regardless of temperature, whereas motility genes were constitutively repressed. Temperature-induced regulation of SPI-1 was rapid, occurring within minutes. Further work found that the purified H-NS N-terminal domain had altered oligomerization properties, and full-length H-NS had decreased affinity for the *hilC* promoter at 37°C, but no temperature dependence in the affinity of the C-terminal domain for DNA was observed. A model was proposed in which the oligomerization properties of H-NS change at 37°C to favor a dimeric conformation, thereby reducing DNA binding and permitting a rapid transcriptional response to change in temperature. This model is supported by additional observations suggesting that H-NS structure changes in response to temperature or osmolarity (Amit et al. 2003).

However, although most temperature-dependent *Salmonella* genes were found to be regulated by H-NS, the converse was not the case. More than 200 genes were silenced equally well by H-NS at 25°C and 37°C, including *virK*, *pipB2*, *mig-14*, *pagC*, *yciEFG*, and the pathogenicity islands SPI-2, SPI-3, and SPI-5. The *proU* (*proVWX*) operon, whose promoter contains a well-characterized H-NS-binding site (Lucht et al. 1994; Tupper et al. 1994), was paradoxically more effectively silenced at 37°C than at 25°C. Other studies also question whether H-NS loses its ability to repress transcription at higher temperatures. The H-NS-silenced *invA* promoter of *Yersinia* is active at 30°C and inactive at 37°C (Heroven et al. 2004; Ellison and Miller 2006). Several loci in the locus of enterocyte effacement (LEE) carried by patho-

genic *E. coli* are repressed directly by H-NS at all temperatures (Umanski et al. 2002). H-NS silences *eltAB*, encoding the heat-labile enterotoxin of *E. coli*, both at 22°C and at 37°C (Yang et al. 2005). Thermal regulation of the *Shigella virF* promoter has been attributed to temperature-induced alterations in promoter confirmation rather than in the H-NS protein itself (Falconi et al. 1998; Prosseda et al. 2004). Changes in promoter conformation might also account for the persistent silencing of the *virB* promoter at 37°C when the *Shigella* virulence plasmid is integrated into the chromosome (Colonna et al. 1995).

Interestingly, another recent study analyzing H-NS structure and function in response to temperature concluded that H-NS tetramerization and activity is actually higher at elevated temperatures (Stella et al. 2006). Chimeric molecules were constructed whereby the N-terminal oligomerization and linker domains of H-NS were fused to the DNA-binding domains of phage repressors. H-NS dimerization and oligomerization were determined through the use of reporter constructs situated downstream from different combinations of phage operator sequences. This experimental approach allowed the assay of β-galactosidase activity as a measure of DNA binding by the H-NS chimera as a dimer or higher-order oligomer (Stella et al. 2005). This approach, along with gel-filtration studies of purified H-NS protein, demonstrated that H-NS tetramerization is inhibited at temperatures <25°C, providing a possible explanation for the derepression of H-NS-regulated genes at lower temperatures. However, some *hns* mutant strains have a survival deficit at low temperatures (Dersch et al. 1994), and it is difficult to explain how H-NS could be inactive yet essential for survival under cold shock. This model would predict the widespread activation of hundreds of H-NS-silenced genes during cold shock, which is in contrast to the ~25 genes actually induced under these conditions (La Teana et al. 1991).

H-NS has also been posited to act as a global regulator in response to osmolarity, and *hns* mutants display altered osmosensitivity (Barth et al. 1995; Levinthal and Pownder 1996; Hommais et al. 2001). H-NS was identified as a silencer of the osmo-regulated *proU* operon and subsequently as a silencer of other osmo-regulated genes including *osmC*, *osmY*, *otsAB*, and the SPI-1 regulatory locus *hilA* (Atlung and Ingmer 1997; Schechter et al. 2003; Olekhnovich and Kadner 2006). Force-extension measurements of individual λ DNA molecules complexed with H-NS indicate that H-NS increases the rigidity of DNA at temperatures <32°C or in the presence of 200 mM KCl, which Amit et al. (2003) interpreted to mean that H-NS alone might act as a temperature or osmolarity sensor. However, these observations must be interpreted with caution, as the concentrations of H-NS used in the study were in vast excess to the DNA (Dame and Wuite 2003; Amit et al. 2004). Moreover, the use of KCl to alter osmolarity may have more pronounced effects on protein–DNA interactions than the physiologic salt potassium glutamate, whose cytoplasmic concentrations rise during osmotic stress (Gralla and Vargas 2006).

The notion of H-NS as a simple osmosensor appears to be undermined by the same reasoning that questions its role as a thermosensor, namely, that the expression of many H-NS-silenced genes is unaffected by changes in osmolarity (Hulton et al. 1990; Atlung and Ingmer 1997). The *proU* promoter maintains much of its osmo-regulation in the absence of H-NS (Fletcher and Csonka 1995), presumably due to changes in DNA supercoiling, and many other osmo-regulated H-NS-silenced genes are activated by factors like OmpR or the alternative σ-factor σ^S (RpoS, σ^{38}). It is interesting to note that H-NS-silenced *Shigella* virulence genes are inhibited by low osmolarity, even at temperatures normally associated with induction (Porter and Dorman 1994). Collectively, these observations illustrate the inadequacy of a simple model in which H-NS is unable to bind DNA under certain environmental conditions, but rather point the way to an alternative model in which H-NS and environmental parameters such as temperature and osmolarity have competing or interactive effects on DNA superhelicity, thereby modulating the expression of certain genes in concert with additional factors like sequence-specific DNA-binding proteins. Below we summarize the various factors that influence silencing and countersilencing of H-NS-regulated genes.

Silencing of foreign sequences by H-NS

Four independent laboratories including our own have exploited recent advances in microarray technology to examine the entire complement of genes regulated by H-NS in *Salmonella* (Lucchini et al. 2006; Navarre et al. 2006) or *E. coli* (Grainger et al. 2006; Oshima et al. 2006). Each of these studies used chromatin immunoprecipitation and hybridization to a microarray (ChIP-on-chip) to identify sites of H-NS binding throughout the bacterial chromosome. Despite the differences in strains and conditions, the studies come to very similar conclusions about the functional properties of H-NS.

The two studies in *Salmonella* identified genes that display altered expression in *hns* mutant *Salmonella* by cDNA microarray analysis (Lucchini et al. 2006; Navarre et al. 2006). In accordance with previous estimates, increased expression of >400 genes was documented in an *hns* mutant. Approximately 90% of H-NS-repressed genes showed evidence of being acquired by LGT, and 65% of H-NS-repressed genes were unique to *Salmonella* spp. Most H-NS-repressed genes were AT-rich; the average GC content of an H-NS-repressed ORF was 46.8%, in comparison to 52.2% for the overall genome. To verify that H-NS could silence the expression of an AT-rich foreign gene, we introduced an AT-rich gene from *Helicobacter pylori* along with its promoter into a large nonessential GC-neutral region within the Gifsy-2 prophage. As predicted, H-NS both bound and silenced the foreign gene but not the adjacent downstream GC-neutral prophage gene. This finding demonstrated that H-NS is capable of silencing a newly introduced foreign gene based on increased adenine and thymine content per se and irrespective of its position in the chromosome. The re-

sults of the ChIP-on-chip analyses revealed a striking correlation between percentage AT content and H-NS binding. More than 400 AT-rich regions of the *Salmonella* chromosome, including the plasmid virulence region, all five pathogenicity islands, and nearly every AT-rich islet, were bound by H-NS. The magnitude of interaction with H-NS correlated strongly with AT content. A high-resolution oligonucleotide array demonstrated that binding is not necessarily restricted to promoter regions, as several AT-rich coding regions were also bound by H-NS, consistent with the idea that H-NS can silence gene expression by polymerizing along DNA and bridging adjacent helices (Dame et al. 2002). H-NS binding within coding regions has been previously noted in the *bgl*, *proU*, and *eltAB* operons (Lucht et al. 1994; Dole et al. 2004a; Madhusudan et al. 2005; Yang et al. 2005). By using identical growth conditions for both expression analysis and ChIP, Lucchini et al. (2006) were able to verify that H-NS acts almost exclusively as a silencer of gene expression.

The two recent studies on H-NS in *E. coli* that also used ChIP-on-chip technology came to conclusions similar to those of the studies in *Salmonella.* Namely, H-NS primarily targets AT-rich sequences and, although biased toward intergenic regions, frequently binds within coding sequences. Like Lucchini et al. (2006), Oshima et al. (2006) found that H-NS acts primarily as a repressor of transcription as most H-NS-bound loci were transcriptionally repressed. Not all aspects of these studies were in perfect agreement, however. In their study of H-NS-binding sites in *Salmonella*, Lucchini et al. (2006) observed that H-NS did not coprecipitate with RNA polymerase at the vast majority of promoters, suggesting that a previously proposed "trapping" model of silencing (Schroder and Wagner 2000; Dame et al. 2002; Shin et al. 2005), in which $E\sigma^{70}$ is trapped by H-NS in the initiation complex, may not be a universal mechanism by which H-NS silences transcription. Their results, however, appear to be contradicted by the observations of Oshima et al. (2006) and Grainger et al. (2006), each of whom observed that H-NS and RNA polymerase coprecipitate at more than half of all H-NS-binding sites. The reasons for the discrepancy between these studies are unclear.

Notably, none of these high-resolution mapping studies of H-NS binding revealed a significant correlation between calculated intrinsic curvature and interaction with H-NS. Lucchini et al. (2006) determined from their data set that AT-rich genes are 20 times more likely to be repressed by H-NS than GC-rich ones, whereas genes with strong intrinsic curvature are only twofold more likely to be regulated by H-NS than those with little or no curvature. In retrospect, it appears that support for the curvature-binding hypothesis may have been based on a misinterpretation. Yamada et al. (1990) identified H-NS as a protein that would preferentially bind multimers of a curved sequence (CGGGCAAAAA) in the presence of excess noncurved sequence (CGGGCCCGGCAAAAA), but the curved sequence used in these experiments was also significantly more AT-rich. Subsequently these investigators demonstrated that H-NS

binding to the curved sequence could be inhibited by distamycin, an antibiotic that binds the minor grove in AT base pairs and disrupts intrinsic curvature (Yamada et al. 1991), but this intervention might also have occluded H-NS-binding sites if they were merely A+T-rich. A highly curved synthetic DNA sequence (Ulanovsky et al. 1986) used in several other H-NS-binding studies is also incidentally extremely AT-rich (TCTCTAAAAA ATATATAAAAA; percentage GC = 9.5) (Zuber et al. 1994). Another study, which found that H-NS preferred both GC-rich and AT-rich curved sequences more than their noncurved counterpart sequences, did not directly compare the affinity of the GC-rich sequences to the AT-rich sequences (Jordi et al. 1997).

Analysis of H-NS-binding sites by DNase footprinting at several promoters including *proU*, *bgl*, *virF*, *hns*, and *invA*, combined with computer analysis of intrinsic curvature, suggests that in many cases the H-NS protein does not bind curved DNA per se, but rather binds to AT-rich sequences on the arms of DNA immediately flanking a region of predicted curvature (Jordi and Higgins 2000; Heroven et al. 2004; Prosseda et al. 2004; Ellison and Miller 2006). Moreover, preferential binding of curved DNA sequences is not an exclusive characteristic of H-NS, but is also displayed by CbpA, Hfq, and IciA (Ali Azam and Ishihama 1999). It is possible that the architecture of the H-NS tetramer with its diametrically opposing binding surfaces is another contributor to a higher affinity for curved DNA in vitro (Fig. 1). Curvature might play a particular role at a subset of promoters in which the AT-rich domains are small and need to be properly aligned. "A-tracts," AT-rich sequences that introduce maximal DNA curvature, are, in fact, overrepresented in the *E. coli* genome and suggested to facilitate DNA looping (Tolstorukov et al. 2005). However, a genomewide analysis of H-NS-binding sites suggests that curvature does not play a major role for large AT-rich sequences where sufficient flexibility might exist for the sequence to fold back on itself (Lucchini et al. 2006).

Both our study and that of Lucchini et al. (2006) noticed that mutations in *hns* have a more severe effect on *Salmonella* growth than has been previously reported in *E. coli* (Barth et al. 1995; Hommais et al. 2001). We observed that disruptions in the *hns* gene were impossible to introduce in wild-type *Salmonella* strain 14028s, but could be made in strains with defects in the alternative σ-factor σ^S. The study by Lucchini et al. (2006) used a laboratory strain of *Salmonella* (LT2) that contains a mutation in the start codon of the *rpoS* gene (Lee et al. 1995). A similar growth advantage for *hns* mutants carrying a second-site *rpoS* mutation has also been observed in *E. coli* (Barth et al. 1995), possibly because the marked nonphysiological accumulation of σ^S during logarithmic growth of an *hns* mutant strain is deleterious to the cell (Yamashino et al. 1995, 1998; Zhou and Gottesman 2006). Additional mutations have been identified that improve growth rates of *Salmonella hns* mutants, including mutations in genes encoding the transcription factor PhoP or *Salmonella* pathogenicity island SPI2, which encodes a type III secretion system (TTSS). To-

gether these observations suggest that the growth defect caused by an *hns* mutation results from dysregulated expression of incompatible genes. Two independent laboratories have failed in their attempts to construct an *hns* mutation in *Yersinia* spp., suggesting that this phenomenon is not unique to *Salmonella* (Heroven et al. 2004; Ellison and Miller 2006).

An earlier attempt to characterize the H-NS regulon in *E. coli* K-12 was performed using proteomic and cDNA microarray analysis (Hommais et al. 2001). Approximately 5% of the transcriptome was altered in the *hns* mutant, and many of the genes identified were involved in stress adaptation or cell envelope biogenesis. Although a correlation with foreign DNA was not noted, several genes acquired by LGT were found to be H-NS regulated, including several adhesion loci. A correlation between H-NS binding and foreign genes may have been overlooked because the K-12 laboratory strain lacks obvious large LGT-derived genomic regions such as pathogenicity islands, and the experiments were performed before many related genome sequences were available. More recently, genes under the control of H-NS were determined in a uropathogenic strain of *E. coli* (UPEC) (Muller et al. 2006), using an expanded "pathoarray" designed specifically to analyze genes involved in virulence. In that study, every UPEC virulence locus was found to be repressed by H-NS, strongly corroborating the findings in *Salmonella* and indicating that H-NS function is conserved between the two species. The specificity of H-NS binding for AT-rich sequences explains the predilection of H-NS for virulence genes, since most virulence genes are A+T-rich.

The xenogeneic silencing (XS) model

Data obtained from recent studies suggest that the primary role of H-NS is to specifically silence AT-rich DNA, presumably as a mechanism of defense against foreign sequences. The ability of bacteria to exploit their characteristic genome GC/AT ratios to recognize foreign DNA is an extension of the traditional concept of "self" versus "non-self" discrimination for the purpose of innate immunity. As outlined in Figure 2, the binding properties of H-NS suggest a model for how such silencing is likely to occur. The XS hypothesis provides a coherent and unifying model for a large amount of data regarding the regulation of horizontally acquired genes in several bacterial species.

XS represents a bacterial analog to the epigenetic silencing of repetitive and potentially harmful mobile genetic elements by histones and RNA interference systems in eukaryotes (Bender 2004; Zilberman and Henikoff 2004; Cam et al. 2005). Interestingly, histones first evolved in the archaea, which have very small genomes, long before the evolution of the massive multichromosome genomes found in eukaryotes (Cubonova et al. 2005). With this in mind, it is possible that the early evolution of nucleoid protein diversity, including the development of what later became the eukaryotic histone, was driven more by the need to silence parasitic DNA
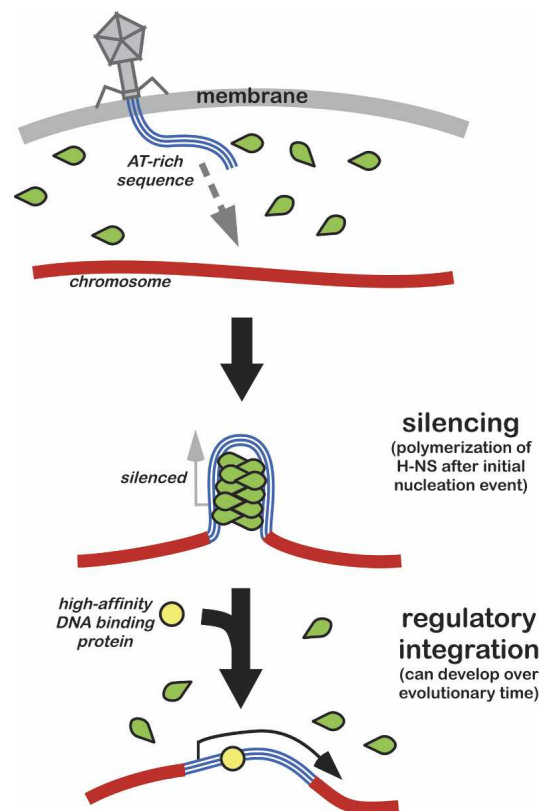

Figure 2. Facilitation of foreign DNA acquisition by XS. H-NS binding to AT-rich foreign DNA silences expression. A sequence-specific DNA-binding protein with greater affinity can compete with H-NS to relieve silencing and allow gene expression under suitable conditions. In this way, XS allows the bacterial host cell to tolerate the presence of a foreign sequence and ultimately to recruit and harness the sequence into a pre-existing regulatory network.

elements than by chromosome structural requirements per se.

An important consequence of XS is the role it is likely to have played in shaping microbial evolution. As mentioned previously, sequences acquired via LGT are usually more AT-rich than the resident genome (Daubin et al. 2003), even in cases in which the host genome is itself AT-rich. For example, the pathogenicity island of *Francisella tularensis*, which has an overall genome GC content of 32.7%, harbors a pathogenicity island with 27.5% GC (Larsson et al. 2005). XS provides an underlying molecular mechanism that can explain why AT-rich sequences have preferentially accumulated in genomes over evolutionary time (Fig. 2). Unlike restriction enzymes, which protect cells by destroying foreign DNA, it appears that H-NS functions to ameliorate the potentially negative consequences of xenogeneic sequences by silencing their expression. The protection that H-NS affords the bacterial cell might better be termed "tolerance" as opposed to "immunity," because H-NS can actually facilitate the acquisition of AT-rich sequences over evolutionary time. From a different perspective, it is also conceivable that "selfish genes" benefit from being

AT-rich because they are better tolerated than GC-rich ones.

If bacteria with GC-rich genomes have systems to silence AT-rich sequences, it might be assumed that AT-rich bacteria possess analogous mechanisms to silence GC-rich DNA. However, a few lines of evidence suggest this may not be the case. First, with the notable exception of *Haemophilus influenzae*, species containing H-NS-like molecules have genomic percentage GC > 45% (Tendeng and Bertin 2003). Second, silencing proteins designed to target GC-rich sequences would largely fail to bind promoter regions, which are almost always more AT-rich than their associated ORFs (Pedersen et al. 2000; Ussery et al. 2004). Third, a striking correlation has been observed between GC content and genome size. The largest genomes, presumably containing the most sequence of LGT origin, are GC-rich, while smaller genomes tend to be highly AT-rich (Moran 2002; Bentley and Parkhill 2004; Wang et al. 2006). Among the species with small AT-rich genomes are obligate endosymbionts that reside in isolated or highly specialized niches and would not be expected to frequently encounter phages or other bacteria, and therefore would not benefit from XS. Bacteria with large, GC-rich genomes tend to be generalists that can live under a wide variety of environmental conditions and exist in the close proximity of several other bacterial species; such species might derive considerable benefit from XS. Thus, whether xenogeneic silencers targeting GC-rich sequences exist remains an open question.

Countersilencing and the integration of foreign genes into global regulatory circuits

Although the XS hypothesis can explain how foreign sequences might be selectively targeted for repression by H-NS, the question remains how H-NS-silenced genes of potential benefit to the host can be integrated into functional regulatory networks and expressed under appropriate conditions (Fig. 3). It is clear from a survey of the literature that many genes regulated by H-NS are also controlled by other global regulators including Lrp, Fis, IHF, CRP-cAMP, ppGpp, and σ^S. Perhaps this is because regulatory proteins with relatively degenerate target specificities (e.g., H-NS, HU, IHF, Hfq) are more likely than highly sequence-specific transcriptional regulators to recognize newly introduced genetic elements and recruit them into global regulatory networks.

Many loci appear to be directly coregulated by σ^S and H-NS including *spv*, *LEE1* (Ler) (Laaberki et al. 2006), *asr* (Seputiene et al. 2004), *gadBC* (De Biase et al. 1999; Waterman and Small 2003), *proU* (Rajkumari and Gowrishankar 2001), *hdeAB* (Shin et al. 2005), *csgBA* (Olsen et al. 1993; Arnqvist et al. 1994), *spvR* (Robbe-Saule et al. 1997), *csiD* (Marschall et al. 1998), and *yciEFG* (Navarre et al. 2006). Several disparate lines of evidence support an intriguing hypothesis whereby RNA polymerase (E) in association with σ^S (Eσ^S) can selectively activate xenogeneic loci under conditions of stress, where they may prove useful. A recent biochemical analysis of the *hdeAB* promoter revealed that Eσ^S is able to transcribe this H-NS-silenced locus much more efficiently than polymerase associated with the housekeeping σ-subunit σ^D (RpoD, σ^{70}) (Shin et al. 2005). Furthermore, although the basal promoter elements recognized by Eσ^S and Eσ^D are highly similar, recent analyses of several σ^S-dependent promoters have revealed that they are less conserved and more AT-rich than Eσ^D-dependent promoters (Becker and Hengge-Aronis 2001; Typas and Hengge 2006; Typas et al. 2007), features that might explain a greater tendency for Eσ^S to initiate the transcription of newly acquired AT-rich genes. Associations between H-NS and Eσ^S are complicated by the fact that H-NS negatively regulates σ^S (Yamashino et al. 1995; Zhou and Gottesman 2006) and by the fact that H-NS and σ^S are inversely regulated by the small RNA DsrA, which en-
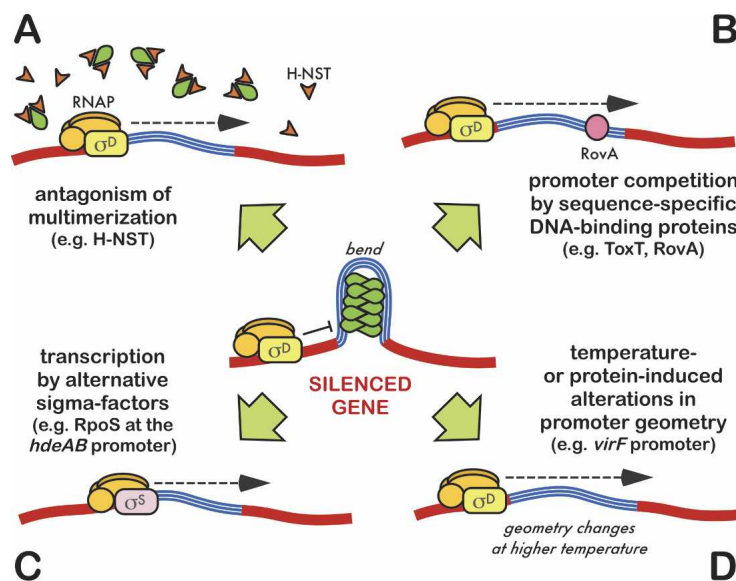

Figure 3. Mechanisms of countersilencing. At least four different mechanisms of countersilencing have been proposed for a variety of different promoters. These include disruption of H-NS complexes by multimerization antagonists such as H-NST and the 5.5 protein from phage T7 (*A*), competition for DNA binding by a high-affinity sequence-specific DNA-binding protein such as ToxT or RovA (*B*), activation of certain promoters such as the *Salmonella spv* promoter or the *E. coli hdeAB* promoter by the alternative σ-factor RpoS (*C*), and changes in promoter geometry due to protein binding or environmental changes that disrupt H-NS complex formation such as may occur at the *virF* promoter in *Shigella* (*D*). None of these four mechanisms is necessarily mutually exclusive, and it is possible that more than one mechanism may be operating at any given promoter.

hances RpoS translation and stability while enhancing degradation of *hns* mRNA (Lease et al. 1998; Majdalani et al. 1998). It should be noted that the effects of the DsrA RNA on H-NS levels were observed with DsrA in multicopy, so the physiological relevance of this interaction remains unclear. Nevertheless, the hypothesis that a stress-activated σ-factor can selectively drive the expression of certain H-NS-silenced genes (or, conversely, that H-NS can selectively repress Eσ^D) may provide one pathway by which foreign genes can be rapidly assimilated into global regulatory networks and might also account for the relationship between H-NS and certain stresses (e.g., osmolarity) without invoking a structural change in the H-NS protein itself.

Another mechanism by which H-NS-silenced genes can be activated is direct competition with sequence-specific DNA-binding proteins (Fig. 3). Several instances of direct competition between virulence regulators and H-NS have been described. SlyA/RovA is a MarR family transcription factor that antagonizes H-NS binding at specific promoters in *Yersinia* and *E. coli* (Westermark et al. 2000; Heroven et al. 2004; Wyborn et al. 2004; Ellison and Miller 2006). In *Salmonella*, it has been shown that all of the genes positively regulated by SlyA were acquired by LGT, and all but one of these are H-NS repressed (Navarre et al. 2005, 2006). H-NS has also been shown to repress virulence genes in *Vibrio cholerae*, and the ToxT transcription factor antagonizes H-NS-mediated silencing at the *ctx* and *tcpA* promoters (Nye et al. 2000; Yu and DiRita 2002). In addition to their ability to antagonize H-NS-mediated silencing, both ToxT and SlyA/RovA have the ability to activate transcription by directly interacting with RNA polymerase (Nye et al. 2000; Yu and DiRita 2002; Tran et al. 2005). A third example of countersilencing by direct competition has been shown for the divergent *pap* promoters of *E. coli*, where two factors, PapB and CRP, appear to work in tandem to antagonize H-NS-mediated silencing (Forsman et al. 1992). It is notable that the consensus recognition sites for ToxT (Hulbert and Taylor 2002) and SlyA/RovA (Stapleton et al. 2002) are very AT-rich, and the critical recognition motif for PapB is a run of three adjacent T/A bases (Xia et al. 1998), which may explain why these proteins have evolved to act as countersilencers. Synthetic promoters allow countersilencing even by nonnative DNA-binding proteins such as TyrR or the Lac and λ repressors (Caramel and Schnetz 1998; Gowrishankar and Pittard 1998), supporting the argument that any DNA-binding protein with sufficient affinity might be able to function as a countersilencer.

The Ler (LEE-encoded regulator) protein provides a distinctive example of H-NS antagonism, as Ler itself is an H-NS homolog acquired by LGT (Mellies et al. 1999; Park et al. 2005). Ler is encoded within the LEE pathogenicity island of enteric bacterial strains capable of causing attaching and effacing (A/E) intestinal lesions (Barba et al. 2005). Ler regulates the expression of multiple promoters by antagonizing H-NS-mediated silencing at several promoters both within and outside of the LEE island (Elliott et al. 2000; Bustamante et al. 2001;

Umanski et al. 2002). Whether Ler antagonizes H-NS by directly competing for binding sites, interfering with multimerization, or some other mechanism remains uncertain. Like ToxT and SlyA, Ler has additional regulatory activity at certain promoters in the absence of H-NS, but the nature of this activity has not been determined (Sperandio et al. 2000; Umanski et al. 2002; Haack et al. 2003). A few other examples of H-NS homologs acquired by LGT have recently been described, but their role in gene expression is thus far unknown (Beloin et al. 2003; Forns et al. 2005a; Park et al. 2005).

Truncated H-NS molecules (H-NST) associated with small genomic islands in UPEC, EPEC, and EAEC *E. coli* strains (Williamson and Free 2005) provide another mechanism of H-NS antagonism. As opposed to the sequence-specific transcription factors that compete with H-NS for binding to specific sites on DNA, the H-NST interfere with H-NS multimerization and hence are predicted to broadly activate the H-NS regulon. Indeed, overexpression of H-NST stimulates the transcription of the unlinked *proU* and *bgl* operons (Williamson and Free 2005), suggesting that H-NST act as dominant-negative versions of H-NS. Such truncated H-NS molecules might be tolerated poorly by species in which *hns* mutations are toxic, and hence it is not surprising that H-NST homologs have not thus far been identified in *Yersinia* or *Salmonella* spp.

Foreign sequences fight back—antagonism of XS by mobile genetic elements

Horizontally acquired H-NS antagonists such as Ler and H-NST are evocative of an evolutionary arms race in which "selfish sequences" have devised strategies to avoid XS. An obvious role of XS is protection against phages, and phages, in turn, might be anticipated to use strategies to evade or resist XS. Of note, the major structural and regulatory genes of all four *Senterica* Typhimurium strain LT2 prophages have a GC content similar to or higher than the genome average. In fact, the largest nonessential GC-neutral regions of the *Salmonella* chromosome exist within these prophages. Not coincidentally, H-NS was not observed to interact with these prophages in recent analyses of the *Salmonella* H-NS regulon (Lucchini et al. 2006; Navarre et al. 2006). Adoption of the GC signatures of their hosts represents a straightforward mechanism by which temperate bacteriophages can evade XS.

Evidence suggests that phages may have other ways of controlling XS. More than a decade ago, the 5.5 protein of coliphage T7 was found to act as an H-NS antagonist, but the relevance of this observation was unclear at the time (Liu and Richardson 1993). The average genome GC content of T7 is ~48%, and many T7 promoters are highly AT-rich, which may explain the necessity for the 5.5 protein. The 5.5 protein is one of the most abundant phage proteins produced during infection and permits T7 growth on λ lysogens (Pao and Speyer 1975). This protein binds H-NS directly and can activate expression of the *proU* locus (Liu and Richardson 1993). Although nearly

all T7-like phages encode the 5.5 protein, no homologs have appeared in any other sequenced phage outside of the T7 family, leaving open the question of how other AT-rich phages avoid XS.

Another function of H-NS might be protection against transposons and insertion sequences. However, although H-NS inhibits transposition of the Mu transposon (Falconi et al. 1991), transposition of IS1, IS903, Tn10, and Tn5 appears to be enhanced by H-NS (Rouquette et al. 2004; Swingle et al. 2004). H-NS binds the Tn10 transposome complex and facilitates productive intermolecular strand transfer by maintaining the transposome in an open configuration (Wardle et al. 2005). Such observations suggest that many transposons not only avoid XS but may even have mechanisms to exploit H-NS for their own ends.

How widespread is xenogeneic silencing?

Considerable evidence gathered from the literature, including a recent analysis of genes regulated by H-NS in pathogenic *E. coli* (Muller et al. 2006), indicates that H-NS functions as a xenogeneic silencer in all enteric bacteria and perhaps in a large number of other proteobacteria. Bertin and colleagues have explored the presence of H-NS homologs in several bacterial genomes through both computational (Tendeng and Bertin 2003) and empirical approaches (Goyard and Bertin 1997; Tendeng et al. 2000, 2003a,b; Bertin et al. 2001; Cerdan et al. 2003). H-NS is highly conserved in primary sequence only within the Enterobacteriaceae. More distant homologs have been found throughout the α-, β-, and γ-proteobacteria, with several species encoding multiple H-NS paralogs (Tendeng and Bertin 2003). Interestingly, kin17/btcd, a zinc-finger DNA-binding protein in mammals, has no significant primary amino acid homology with H-NS yet can complement an *hns* mutation in *E. coli* (Timchenko et al. 1996; Tissier et al. 1996). The kin17 protein is stress-activated and appears to be involved in DNA replication in mammalian cells (Masson et al. 2003). Whether kin17 plays any role in silencing foreign DNA in higher organisms has not been addressed.

Analysis of H-NS-like proteins has revealed that their sequences are considerably more divergent at the N-terminal domain involved in multimerization than at the C-terminal DNA-binding domain (Tendeng and Bertin 2003). This suggests that there may be considerable evolutionary pressure to modify the multimerization domain, but the DNA-binding domain is more constrained in order to maintain function. Functional H-NS chimeric molecules have been generated by swapping the N-terminal domain with the multimerization domains of other H-NS-like proteins, supporting the idea that the multimerization domains retain their function despite sequence diversity (Deighan et al. 2003; Tendeng et al. 2003a; Rodriguez et al. 2005). Extensive variability observed between the N-terminal domains of H-NS-like molecules may prevent interactions with antagonists

that abrogate silencing by interfering with H-NS multimerization (e.g., H-NST and phage T7 5.5 protein).

GC content in bacterial self-discrimination and speciation

Species-specific GC ratios were first described by Erwin Chargaff in the early 1950s (Zamenhof et al. 1952), and GC contents of microbial genomes can vary between 25% and 75% (Sueoka 1962). Since that time several attempts have been made to explain why GC content varies so widely between bacterial species (Sueoka 1992; Lobry and Sueoka 2002; Naya et al. 2002; Rocha and Danchin 2002; Daubin and Perriere 2003; Musto et al. 2004, 2005, 2006; Foerstner et al. 2005; Wang et al. 2006). Growth temperature was suggested as a possible explanation, since AT-rich DNA is more readily denatured, but was found to correlate poorly with genomic percentage GC (Kawashima et al. 2000; Musto et al. 2006; Wang et al. 2006). Although the genomes of psychrophilic organisms sequenced thus far are fairly AT-rich, the GC content of hyperthermophilic species can vary between 35% and 70%. AT-rich thermophiles may maintain their DNA in double-stranded form through supercoiling (Gagua et al. 1981; Kawashima et al. 2000). A weak but consistent correlation between strict anaerobic growth and decreased GC content has also been observed (Naya et al. 2002; Musto et al. 2006), which is counterintuitive given that guanine oxidation (leading to GC-to-AT transitions and GC-to-TA transversions) is the most common type of oxidative DNA damage. The side-by-side coexistence of bacterial species with widely differing GC contents suggests that there may be no overriding influence of environment on GC content.

Several factors might be predicted to favor the accumulation of AT in genomes: Guanine and cytosine are more energetically costly than adenine and uracil (Rocha and Danchin 2002), ATP is abundant within cells, oxidative cytosine deamination and guanine oxidation generate AT base pairs (Wang et al. 1998; Bentley and Parkhill 2004), and protein-coding sequences are more constrained in organisms with extreme GC (or AT) bias (Sueoka 1961; Lobry 1997). Nevertheless, several bacterial species maintain GC-rich genomes in the absence of an obvious benefit. The XS hypothesis suggests that one possible benefit of maintaining a characteristic GC content is to distinguish "self" from foreign DNA in order to maintain genomic integrity and facilitate the acquisition of beneficial sequences. H-NS and related proteins that bind DNA on the basis of regional AT content may provide a mechanism by which selection pressure to maintain GC content can be applied.

Future directions

XS provides a novel paradigm to understand how bacteria interact with foreign genetic material, which generates new avenues for future research. There is considerable evidence that H-NS functions in concert with other

proteins to silence gene expression, suggesting that XS may be carried out by a multiprotein complex. For example, the Hha (YmoA) protein has been demonstrated both to bind H-NS and to play a role in H-NS-mediated silencing at several loci in enteric bacteria (Cornelis 1993; Nieto et al. 2000; Madrid et al. 2002; Forns et al. 2005a,b; Sharma et al. 2005; Ellison and Miller 2006). Hha does not appear to be necessary for H-NS-mediated silencing at all loci, and it is an open question how Hha might interact with H-NS at some promoters but not others. It has also been demonstrated that H-NS can form heteromeric complexes with paralogous proteins like StpA and Sfh in vitro, as well as with the RNA-binding protein Hfq (Kajitani and Ishihama 1991; Williams et al. 1996; Beloin et al. 2003; Paytubi et al. 2004). The components of the XS complex and the possibility that variant complexes act at different promoters should be amenable to investigation by the same high-throughput methods that have been used to study H-NS.

Although some doubt has been cast on the primary role of H-NS as an environmental sensor, it is not surprising that a highly abundant DNA-binding protein might have pleiotropic effects on gene expression (Dorman 2004) and DNA metabolism (Falconi et al. 1991; Katayama et al. 1996; Atlung and Hansen 2002; Wardle et al. 2005). Given the wealth of observations linking H-NS with environmental parameters such as temperature and osmolarity, it will be of interest to perform global analyses of H-NS binding under a variety of experimental conditions. Such studies may help to elucidate how a protein recognizing the simple characteristic of adenine and thymine content could play an important role in modulating gene expression in response to stress.

The explanation for the presence of more than one H-NS-like protein in some bacteria is presently enigmatic. A mutation in *stpA*, encoding the H-NS paralog StpA, has no discernable phenotype on its own in *E. coli* but derepresses the expression of a subset of genes in the absence of H-NS (Muller et al. 2006). StpA levels are low in cells that express H-NS, accounting for the absence of *stpA* phenotypes unless *hns* is also mutated (Francetic et al. 2000; Free et al. 2001; Sonnenfield et al. 2001; Muller et al. 2006). Similarly, H-NS and Hha paralogs encoded on plasmid R27 can also functionally complement mutations in *hns* or *hha* (Forns et al. 2005a). Such backup systems might be useful for maintaining functional gene regulatory circuits in the presence of H-NS antagonists, such as those encoded by certain phages, and may also prevent detrimental H-NS titration by multicopy genetic elements (Doyle et al. 2007).

Also unresolved is the precise mechanism by which H-NS recognizes AT-rich DNA. Attempts to define a sequence-specific binding site for H-NS have been unsuccessful (Ali Azam and Ishihama 1999). AT-rich DNA has a narrow minor groove that might provide some degree of specificity as has previously been observed for the binding of certain drugs that interact with the minor groove (Aymami et al. 1999). Alternatively, H-NS may be able to recognize fluctuations in hydrogen bonding of the DNA double helix that are dependent on local AT

content (Sullivan and Lilley 1986). High-resolution analysis of H-NS binding throughout the chromosome revealed that H-NS binding does not correlate perfectly with AT content; that is, there are a few examples in which regions of moderately high AT content interact with H-NS more strongly than other regions with extremely high AT content (Lucchini et al. 2006; Navarre et al. 2006). Ultimately the analysis of such exceptions may reveal the basis of binding specificity. Difficulties in obtaining a complete high-resolution structure of H-NS have thus far thwarted attempts to determine the specific mechanism of DNA binding (Dorman 2004). As Hha stabilizes the H-NS protein in vitro, perhaps someday it will be possible to obtain a complete structure of an Hha/H-NS/promoter complex (Pons et al. 2004).

The most far-reaching questions to be answered are whether XS is widespread throughout the bacterial kingdom and has played an important role in driving bacterial evolution. Many bacterial species that do not possess obvious H-NS homologs interact frequently with foreign DNA and have accumulated significant amounts of genetic material from foreign sources (Tendeng and Bertin 2003). What mechanisms might these species have to recognize and interact with incoming sequences? This question is particularly relevant for bacterial species with plastic genomes that exchange DNA at very high rates. Studies of H-NS represent only the beginning of what should be a fruitful area of exploration for many years to come.

Acknowledgments

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Silencing of xenogeneic DNA by H-NS—facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA

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