

Review

Correspondence
Charles J. Dorman
cjorman@tcd.ie

Anti-silencing: overcoming H-NS-mediated repression of transcription in Gram-negative enteric bacteria

Daniel M. Stoebel,¹ Andrew Free² and Charles J. Dorman¹

¹Department of Microbiology, School of Genetics and Microbiology, Moyné Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland

²Institute of Evolutionary Biology, University of Edinburgh, Room 714a, Darwin Building, The King's Buildings, Mayfield Road, Edinburgh EH9 3JR, UK

The H-NS nucleoid-associated DNA-binding protein is an important global repressor of transcription in Gram-negative bacteria. Recently, H-NS has been implicated in the process of xenogeneic silencing, where it represses the transcription of foreign genes acquired by horizontal transfer. This raises interesting questions about the integration of the horizontally acquired genes into the existing gene regulatory networks of the microbe. In particular, how do bacteria derepress silenced genes in order to benefit from their expression without compromising competitive fitness through doing so inappropriately? This article reviews current knowledge about the derepression of genes that are transcriptionally silenced by H-NS. It describes a variety of anti-silencing mechanisms involving (i) protein-independent processes that operate at the level of local DNA structure, (ii) DNA-binding proteins such as Ler, LeuO, RovA, SlyA, VirB, and proteins related to AraC, and (iii) modulatory mechanisms in which H-NS forms heteromeric protein–protein complexes with full-length or partial paralogues such as StpA, Sfh, Hha, YdgT, YmoA or H-NST. The picture that emerges is one of apparently ad hoc solutions to the problem of H-NS-mediated silencing, suggesting that microbes are capable of evolving anti-silencing methods based on the redeployment of existing regulatory proteins rather than employing dedicated, bespoke antagonists. There is also evidence that in a number of cases more sophisticated regulatory processes have been superimposed on these rather simple anti-silencing mechanisms, broadening the range of environmental signals to which H-NS-repressed genes respond.

Introduction

It has been appreciated for some time that the H-NS nucleoid-associated protein affects the transcription of a very wide variety of genes in Gram-negative bacteria. In the past two years, the application of DNA microarray whole-genome transcriptomic methods and chromatin immunoprecipitation on chip, or ChIP-on-chip, has illustrated the extent of the influence exerted by H-NS, at least in the bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (Grainger *et al.*, 2006; Lucchini *et al.*, 2006; Navarre *et al.*, 2006, 2007; Oshima *et al.*, 2006; Wade *et al.*, 2007). The family of H-NS-like proteins is spread very widely among Gram-negative bacteria, but the most detailed information on its role in transcription repression comes from just a few species (Dorman, 2004; Rimsky, 2004; Tendeng & Bertin, 2003).

Transcription silencing is generally regarded as a feature of eukaryotes (Grewal & Elgin, 2007; Morse, 2007), but there are examples of the phenomenon in the *Enterobacteriaceae*.

As in eukaryotes, silencing in bacteria usually involves the formation of a nucleoprotein complex that renders the affected DNA inaccessible to sequence-specific DNA-binding proteins such as RNA polymerase that are required for transcription to take place. These nucleoprotein structures are usually more extensive than those associated with more conventional modes of transcription repression (Rine, 1999).

The H-NS DNA-binding protein has been described as a transcription silencer, although it is clear that it can also possess the characteristics of a repressor (Dorman, 2007b). Its negative effects on transcription are pervasive and extend throughout the bacterial genome. H-NS is not the only bacterial protein to have been classified as a silencer: bacterial plasmid partitioning proteins can silence the promoters of genes in the vicinity of their *cis*-acting binding *parS*-like sites over distances of several kilobase pairs (Kim & Wang, 1999; Rine, 1999; Rodionov *et al.*, 1999; Rodionov & Yarmolinsky, 2004; Yarmolinsky, 2000).

In keeping with the distinction between silencing and repression, the silencing complexes of H-NS have been found to be relatively extensive in a number of examples. Here we review briefly the properties of H-NS and its potential to act as a silencer of transcription. The main focus of this article will be on the mechanisms used by bacteria to relieve the silence imposed on the genome by this protein.

H-NS and transcription repression

H-NS is a small, abundant protein with DNA- and RNA-binding activity (Brescia *et al.*, 2004). It consists of an amino-terminal oligomerization domain that is attached to a carboxyl-terminal nucleic acid-binding domain by a flexible linker peptide (Badaut *et al.*, 2002; Dorman *et al.*, 1999). H-NS forms at least dimers in solution and these have the ability to create DNA–protein–DNA bridges both between separate DNA molecules and between different portions of the same DNA molecule (Fig. 1a) (Dame *et al.*, 2005, 2006; Dorman, 2007a; Noom *et al.*, 2007). H-NS has also been implicated in closing the looped domains that form an important part of the higher organization of the bacterial chromosome (Noom *et al.*, 2007).

H-NS–DNA nucleoprotein structures can impede the movement of RNA polymerase and so H-NS can act to repress transcription through its DNA-binding and bridging activities (Dame *et al.*, 2002; Schröder & Wagner, 2000). The result has been described as H-NS-mediated transcriptional silencing (Bouffartigues *et al.*, 2007; Fang & Rimsky, 2008; Göransson *et al.*, 1990; Lang *et al.*, 2007; Lucchini *et al.*, 2006; Madhusudan *et al.*, 2005; McGovern *et al.*, 1994; Murphree *et al.*, 1997; Navarre *et al.*, 2006; Nye *et al.*, 2000; Petersen *et al.*, 2002; Westermarck *et al.*, 2000; Will *et al.*, 2004). It has been estimated from single-molecule studies using optical tweezers that the force required to disrupt an H-NS–DNA bridge is 7 pN at an unzipping rate of 70 bp s⁻¹, which is the speed of RNA polymerase; RNA polymerase can exert a force of up to 25 pN (Dame *et al.*, 2006). Perhaps the H-NS–DNA bridge is strong enough to contain a stationary RNA polymerase (Fig. 1a) but not to block the movement of one already under way (Fig. 1e).

Much effort has been invested in understanding the DNA-binding preferences of H-NS. The sequences to which it binds are usually A + T-rich and are often associated with regions of intrinsic curvature (Dame *et al.*, 2001; Prosseda *et al.*, 2004; Tolstorukov *et al.*, 2005; Yamada *et al.*, 1990). In addition, a recent study has identified a discrete DNA sequence, 5'-TCGATATATT-3', to which H-NS binds with higher affinity than other A + T-rich elements (Lang *et al.*, 2007). It seems likely that this sequence, or related sequences, can form nucleation sites from which the H-NS protein can spread laterally along DNA, forming H-NS filaments and possibly DNA–H-NS–DNA bridges (Lang *et al.*, 2007; Rimsky *et al.*, 2001). Currently our impressions of H-NS behaviour following binding at nucleation sites

remain somewhat speculative as they are derived from data obtained in highly artificial *in vitro* single-molecule studies carried out in flow chambers using optical tweezers (Dame *et al.*, 2006).

ChIP-on-chip studies have shown that H-NS binds to the A + T-rich portions of the genomes of *Salmonella* Typhimurium (Lucchini *et al.*, 2006; Navarre *et al.*, 2006) and *E. coli* (Grainger *et al.*, 2006; Oshima *et al.*, 2006). In the case of *S. Typhimurium*, this includes the part of the genome that contains the major virulence genes, many of which are located in pathogenicity islands that are believed to have been acquired by horizontal gene transfer (Abrahams & Hensel, 2006; Ellermeier & Slauch, 2007; Rhen & Dorman, 2005) and a virulence plasmid (O'Byrne & Dorman, 1994). H-NS and its homologues bind to A + T-rich sequences in other strains and species too, many of which harbour virulence genes thought to have been acquired by lateral gene transfer. Examples include H-NS-repressed virulence genes in pathogenic *E. coli* (Bustamante *et al.*, 2001; Corbett *et al.*, 2007; Haack *et al.*, 2003; Laaberki *et al.*, 2006; Müller *et al.*, 2006; Torres *et al.*, 2007), *Erwinia* spp. (Nasser & Reverchon, 2002), *Proteus mirabilis* (Coker *et al.*, 2000), *Shigella flexneri* (Beloin & Dorman, 2003; Prosseda *et al.*, 2004), *Vibrio cholerae* (Ghosh *et al.*, 2006; Nye *et al.*, 2000) and *Yersinia* spp. (Cathelyn *et al.*, 2007; Ellison & Miller, 2006b; Heroven *et al.*, 2007).

An evolutionary dilemma

At the heart of the xenogeneic silencing hypothesis is the assumption that the cell benefits from the downregulation of the transcription of horizontally acquired genes by the H-NS protein because this prevents their inappropriate expression and, presumably, an associated reduction in the competitive fitness of the bacterium (Dorman, 2007b; Lucchini *et al.*, 2006; Navarre *et al.*, 2007), although the cause of the fitness reduction is obscure in all but a few cases (Stoebel *et al.*, 2008). Clearly, the horizontally acquired genes in modern bacteria are expressed in specific circumstances. This suggests that the bacteria possess tools to counteract the transcription silencing activity of H-NS in ways that allow the genes to be expressed for the benefit of the microbe. What kinds of regulatory systems were recruited for this purpose? Was there one dramatically successful solution that has been widely replicated or have many distinct answers to the problem of H-NS repression emerged? Even the most cursory survey of the extant examples strongly indicates that the latter is the case. What does this reveal about the nature of the H-NS–DNA relationship and about the flexibility and evolvability of gene regulatory circuits in bacteria? Here, we review a number of examples of anti-H-NS activity and consider what this information tells us about the evolution of bacterial gene regulatory circuits and their capacity for further development in the future.

A protein-independent mechanism

The bridging activity of H-NS makes the protein sensitive to the structure of the DNA to which it binds. Curvature can facilitate bridge-formation, and curvature is also sensitive to environmental stress such as temperature and osmolarity. Increases in temperature can reduce the degree of curvature and displace the apex of the curve in ways that undermine the ability of H-NS to maintain a bridged structure (Fig. 1b), as has been demonstrated in the case of the *virF* virulence gene promoter in *Shigella flexneri* (Prosseda *et al.*, 2004). The promoter of the *proU* operon encoding a transport system for the uptake of the osmoprotectant glycine betaine is also negatively regulated by H-NS binding to *cis*-acting sequences located upstream and downstream of the transcription start site. With the exception of the HU protein at the P2 promoter (Manna & Gowrishankar, 1994) no *trans*-acting factors are known to be involved in derepression of the *proU* promoter at high osmolarity, although changes in DNA superhelicity, which occur following osmotic shock, affect H-NS binding to its regulatory regions (Bouffartigues *et al.*, 2007). Like increases in temperature, increasing salt concentration can diminish DNA curvature (Sinden *et al.*, 1998). While H-NS binding to the *proU* regulatory region is temperature-sensitive *in vitro* (Badaut *et al.*, 2002), temperature does not appear to affect repression *in vivo* (Bouffartigues *et al.*, 2007).

The VirB protein, an ad hoc solution?

Shigella flexneri maintains its principal virulence genes in a 31 kbp segment of A+T-rich DNA on a 230 kbp plasmid (Dorman *et al.*, 2001). The H-NS protein represses the promoters that are responsible for the transcription of the *S. flexneri* virulence genes in bacteria growing below the permissive temperature for their expression, 37 °C (Beloin & Dorman, 2003). Activation of transcription involves a regulatory cascade in which the product of the *virF* gene activates the downstream *virB* promoter (Dorman *et al.*, 2001). The VirB regulatory protein in turn alleviates H-NS-mediated repression of the operons coding for the structural virulence genes (Adler *et al.*, 1989; Le Gall *et al.*, 2005; McKenna *et al.*, 2003). VirB shows strong amino acid sequence homology to plasmid partitioning proteins such as ParB from plasmid/phage P1 and SopB from the F plasmid (Beloin *et al.*, 2002). The DNA sequence to which it binds resembles the *parS* sequences that are bound by the plasmid partitioning proteins (Taniya *et al.*, 2003; Turner & Dorman, 2007).

In vitro transcription assays show that VirB does not act as a conventional transcription factor, recruiting RNA polymerase to the promoter and/or enhancing the rate of formation of open transcription complexes. Instead, VirB acts to antagonize the repressive activity of the H-NS protein. The mechanism by which this is achieved involves VirB remodelling the DNA within the H-NS–DNA nucleoprotein complex (Fig. 1d). This can be seen by the

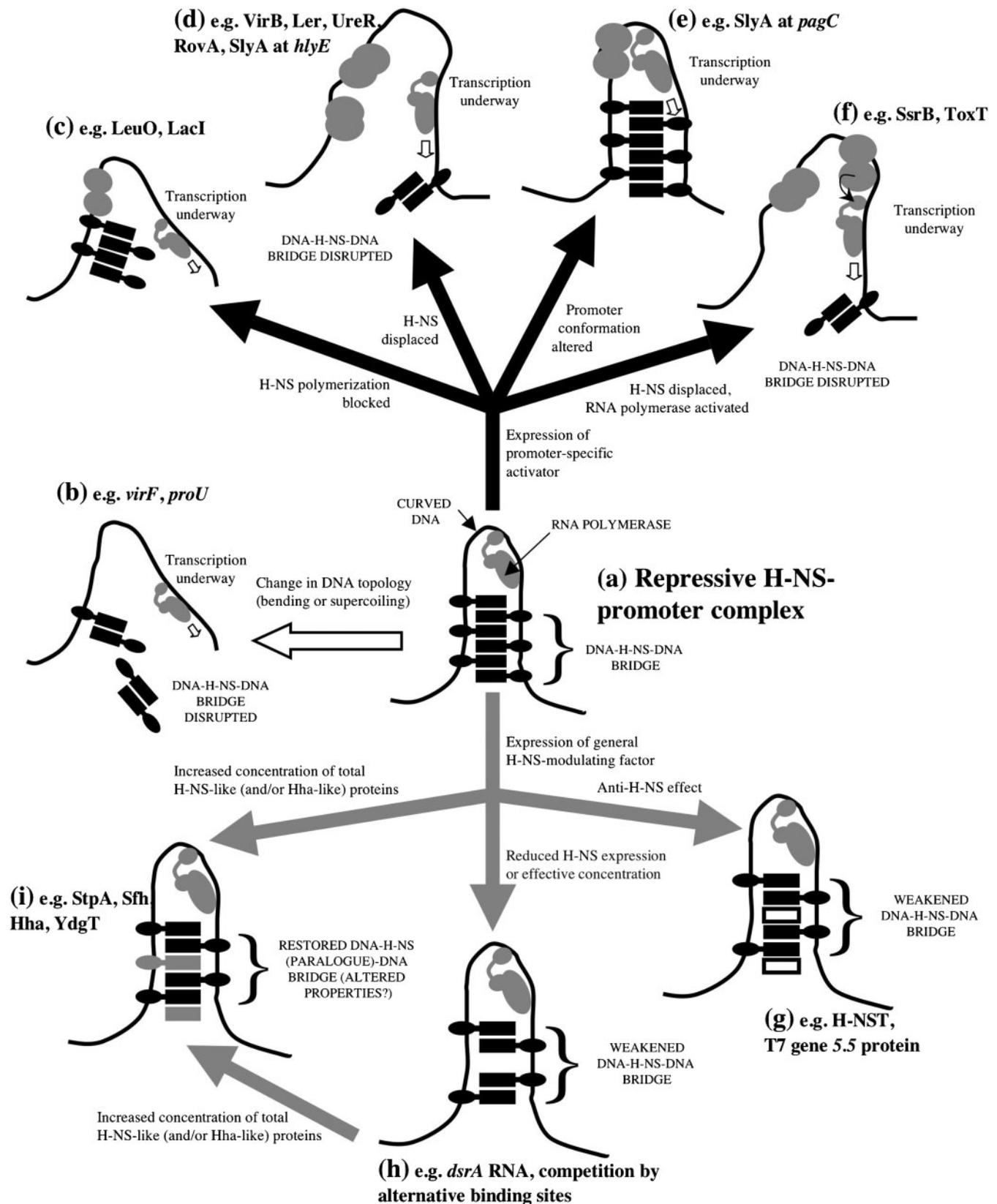
appearance of VirB-dependent hypersensitive sites in DNase I footprinting assays in regions known to be protected by H-NS. Such hypersensitive sites arise when DNA winds around a protein, making particular bases even more susceptible to cleavage by DNase I (Nickerson & Achberger, 1995; Wagner, 2000). In the *icsB* promoter of *S. flexneri*, the hypersensitivity originates at the *parS*-like sequence and extends through the region that is bound by H-NS. The current model of VirB action involves binding of the protein to the *parS*-like sequence followed by propagation of a VirB multimeric complex along the DNA with associated wrapping of the DNA duplex by the protein. This action is detrimental to the maintenance of the H-NS–DNA repression complex and facilitates the initiation of transcription by RNA polymerase (Fig. 1d). The derepression mechanism only requires RNA polymerase, VirB, H-NS and the target promoter DNA; it does not rely on any chemical or physical signal. This was shown by activating an H-NS-repressed promoter by overexpression of the *virB* gene under conditions that were otherwise non-permissive for transcription (Beloin & Dorman, 2003).

The involvement of the ParB/SopB-like protein VirB in antagonizing a gene-silencing activity is particularly interesting in the light of evidence that ParB and SopB can themselves silence transcription, possibly by spreading from their native initial binding sites *parS* (ParB) or *sopC* (SopB) to generate a nucleoprotein filament (Yarmolinsky, 2000). Clearly, it is important to consider each case in the context of the molecular details and not to fall into the trap of assigning unique biological properties to these DNA-binding proteins.

H-NS antagonism by SlyA-like proteins

The SlyA DNA-binding protein has been studied in some detail as a regulatory agent that counteracts the transcriptional silencing activity of H-NS. SlyA is related to winged-helix proteins such as MarR (*E. coli*), RovA (*Yersinia*) and PecS (*Erwinia*) and it has been studied in *Salmonella* and *E. coli*, where it controls the expression of a large group of genes (Cathelyn *et al.*, 2006, 2007; Ellison & Miller, 2006a; Heroven *et al.*, 2004; Revell & Miller, 2000; Reverchon *et al.*, 1994; Wilkinson & Grove, 2006). This contrasts with other anti-silencers, such as VirB, that act at far fewer promoters. The members of the SlyA regulon encode secreted, membrane-associated and periplasmic proteins, leading to the suggestion that a major role of SlyA involves controlling the composition of the bacterial cell envelope (Navarre *et al.*, 2005; Spory *et al.*, 2002; Stapleton *et al.*, 2002). SlyA also contributes to *Salmonella* virulence and is important in resisting the oxidative stress and antimicrobial peptides that are encountered in macrophages (Libby *et al.*, 1994; Stapleton *et al.*, 2002).

The *hlyE* haemolysin gene in *E. coli*, also known as *clyA* and *sheA*, is repressed by H-NS and activated by SlyA (Westermarck *et al.*, 2000). Its promoter lies in a region of A+T-rich DNA that is bound by both SlyA and H-NS



(Lithgow *et al.*, 2007; Westermark *et al.*, 2000) (Fig. 1d). H-NS prevents binding of the promoter by RNA polymerase. In contrast, SlyA permits RNA polymerase to bind the

promoter but inhibits binding by the H-NS protein. Lithgow *et al.* (2007) discovered that SlyA and H-NS engage in mutual antagonism at the regulatory region of

Fig. 1. Illustration of the ways in which a repressive H-NS–promoter complex (a) can be modified to permit transcription. At certain promoters (b), specific derepression occurs via a protein-independent mechanism involving environmentally induced changes in DNA bending or supercoiling (open arrow). Many promoters are derepressed via promoter-specific DNA-binding proteins (black arrows), which can work by preventing the polymerization of H-NS along the DNA (c), displacing H-NS from the promoter (d), modifying the conformation of the promoter while H-NS remains *in situ* (e), or displacing H-NS and directly activating transcription by RNA polymerase (f). Other derepression mechanisms are general for many H-NS-repressed promoters (grey arrows): these include weakening of the H-NS–promoter complex by an anti-H-NS factor (g), reduced effective H-NS concentration caused by expression changes or increased competition between binding sites (h), and expression of a paralogue of H-NS or the H-NS-interacting protein Hha (i). The latter may also enhance repression or alter its sensitivity to other derepression mechanisms dependent upon the exact properties of the heteromeric protein–DNA complex. Specific examples of each derepression mechanism that are discussed in detail in the text are shown in bold.

the *hlyE* promoter. SlyA can displace H-NS, but H-NS can also displace SlyA when the relative abundances of the two proteins favour H-NS over SlyA. This is a very interesting insight because it suggests a mechanism for the re-establishment of H-NS-mediated transcription repression at target promoters such as that of the *E. coli hlyE* gene. It has similarities to an earlier description of antagonism between H-NS and the AraC-like urease gene activator UreR in *Proteus mirabilis*, where not only can UreR displace H-NS, but H-NS can also displace UreR from an intrinsically curved A+T-rich DNA sequence located between the divergently transcribed *ureR* and *ureD* genes (Poore & Mobley, 2003).

Antagonism of H-NS by SlyA at other promoters is more complex. The SlyA protein governs the expression of a subset of the genes in the PhoP/PhoQ regulon, and the *slyA* gene is itself under the control of PhoP/PhoQ. This two-component regulatory system governs the expression of many genes involved in *Salmonella* virulence in response to magnesium ions. The PhoP/PhoQ system regulates *slyA* positively while the SlyA protein is a repressor of its own gene (Norte *et al.*, 2003; Shi *et al.*, 2004); H-NS is a repressor of the *phoP* promoter (Kong *et al.*, 2008).

The *ugtL* and *pagC* genes in *S. Typhimurium* are known to be repressed by H-NS and positively regulated by the PhoP/PhoQ two-component regulator and SlyA (Table 1). The purified SlyA protein can counteract H-NS repression *in vitro* but it has no ability to activate transcription. This shows that SlyA cannot act as a conventional transcription activator and it is not required for transcription activation when the H-NS repressor is absent. The PhoP protein is a transcription factor and it displays a need for SlyA, but only when H-NS is present. In the absence of the H-NS repressor, PhoP can activate its target promoters without the assistance of SlyA (Perez *et al.*, 2008). This is an example of layered control in which a more targeted and

specific regulatory switch is superimposed on the anti-H-NS anti-silencing mechanism, in this instance one imposed by SlyA. Similarly, at the *E. coli hlyE* promoter, Crp and Fnr also contribute to transcriptional control in addition to the mutual antagonism of SlyA and H-NS (Westermarck *et al.*, 2000). However, it is not known if Crp and/or Fnr affect the expression of SlyA, creating the type of feed-forward loop that arises at *pagC* and *ugtL* due to the influence of PhoP/PhoQ on SlyA levels. Not all response regulators need a separate protein to act as an H-NS antagonist; the SsrB response regulator encoded by the SPI2 pathogenicity island of *Salmonella enterica* combines the roles of H-NS antagonist and transcription activator (Walthers *et al.*, 2007) (Fig. 1f).

Examination of the *pagC* and *ugtL* promoters *in vitro* using DNase I footprinting techniques shows that SlyA does not displace H-NS from the DNA. Instead the presence of SlyA results in hypersensitivity of certain bases in the DNA to DNase I cleavage (Fig. 1e). The SlyA/H-NS relationship with the *pagC* and *ugtL* promoters is reminiscent of that involving VirB at the *icsB* promoter in *Shigella flexneri*, described above (Fig. 1d) (Turner & Dorman, 2007). Remodelling of the H-NS–DNA nucleoprotein complex may allow RNA polymerase to obtain access to and to activate the hitherto H-NS-repressed promoter. These results show that a single H-NS antagonist can act via two mechanisms: by displacing H-NS at the *hlyE* promoter and by remodelling the H-NS–DNA complex to permit transcription at *pagC* and *ugtL* (Table 1).

RovA, antagonizing H-NS in *Yersinia* species

The RovA protein is a homologue of SlyA that was identified originally as a positive regulator of *inv*, the gene coding for invasins, in response to temperature and growth phase in *Yersinia* (Cathelyn *et al.*, 2007). RovA is now

Table 1. SlyA antagonism of H-NS-mediated repression

Promoter	Mode of H-NS antagonism	Additional activators
<i>hlyE</i>	SlyA displacement of bound H-NS (and vice versa)	Crp, Fnr
<i>pagC/ugtL</i>	SlyA remodelling of H-NS-bound promoter	PhoP/PhoQ

known to control the transcription of a regulon of genes that, like *inv*, are subject to repression by the H-NS protein. It has been proposed that the principal function of RovA in *Yersinia enterocolitica* is to act as an antagonist of H-NS-mediated transcriptional silencing (Cathelyn *et al.*, 2007).

Despite the fact that the RovA protein is highly conserved in *Y. enterocolitica* and *Y. pestis*, microarray analysis reveals little overlap in the sets of genes that are subject to RovA control in these two species. This is explained, at least in part, by the fact that each species lacks orthologues of some of the RovA-dependent genes that are found in the other. RovA-dependent genes that are present in both species frequently have promoters that have diverged strongly, suggesting the evolution of novel regulatory interactions. The joint action of horizontal acquisition of different groups of H-NS-repressible genes and the rapid evolution of promoter sequences has led to the evolution of distinct RovA regulons in *Y. enterocolitica* and *Y. pestis* (Cathelyn *et al.*, 2007). This may point to the fact that the mechanism by which RovA relieves H-NS-imposed silencing relies on molecular interactions that are easy to arrange and therefore highly likely to evolve.

The molecular detail of transcriptional upregulation by the RovA protein has also been examined in the enteropathogenic bacterium *Y. pseudotuberculosis*. Here, the invasin gene *inv* is repressed by a silencing complex consisting of H-NS and an extended region of A + T-rich DNA at the *inv* promoter. When the bacterium is grown under *inv*-inducing conditions, RovA displaces H-NS from the silencing element, leading to transcription of the *inv* gene (Heroven *et al.*, 2007).

Interestingly, the transcription of *rovA*, the gene that encodes the RovA protein, is itself subject to repression by H-NS. Much like the VirB regulon in *Shigella flexneri*, anti-silencing of the RovA regulon is also integrated into the H-NS regulon. In *Y. pseudotuberculosis*, the repression of *rovA* by H-NS is reinforced by a co-repressor, RovM, a protein related to LysR-like transcription factors, bringing an

opportunity for fine-tuning the operation of the anti-silencing mechanism (Heroven *et al.*, 2007).

Opposing H-NS in *Vibrio cholerae*

The major virulence factors of *V. cholerae*, the aetiological agent of Asiatic cholera, are encoded by genes within A + T-rich horizontally transmissible genetic elements (Davis & Waldor, 2003; McLeod *et al.*, 2005; Murphy & Boyd, 2008). These genes are regulated by several environmental signals to ensure that their products are expressed when the bacterium arrives at appropriate sites in the host and that they are repressed elsewhere (Lee *et al.*, 1999; Schild *et al.*, 2007). Among the major virulence factors expressed by *V. cholerae* are cholera toxin, CTX, and the toxin co-regulated pilus, Tcp (Skorupski & Taylor, 1997). H-NS silences the transcription of the genes encoding these major virulence factors by targeting their A + T-rich promoters (Nye *et al.*, 2000). This silencing is opposed by the ToxT regulatory protein, an AraC-like DNA-binding protein that derepresses transcription of a number of virulence gene promoters in *V. cholerae* (Yu & DiRita, 2002). The mechanism is thought to involve not only the displacement of H-NS but also the activation of transcription by ToxT, possibly due to direct interaction between ToxT and RNA polymerase (Hulbert & Taylor, 2002; Yu & DiRita, 2002). This is more than anti-silencing and hints at a form of regulation that is more intricate than simply displacing H-NS from a promoter (Fig. 1f).

AraC-like proteins and H-NS

The antagonistic relationship between the AraC-like ToxT transcription activator and the H-NS repressor in *V. cholerae* is one of many examples where AraC-like DNA-binding proteins positively regulate genes that are subject to repression by H-NS (Table 2). In those cases where the matter has been investigated, the AraC-like protein has been found both to antagonize H-NS repression and to exert a positive influence (albeit a modest one) on

Table 2. AraC-like protein antagonists of H-NS

AraC-like protein	Regulatory target	Reference
AppY	Genes contributing to anaerobic and growth phase adaptation in <i>E. coli</i>	Atlung <i>et al.</i> (1996)
CfaD	Virulence genes in enterotoxigenic <i>E. coli</i>	Jordi <i>et al.</i> (1992)
GadW	Glutamate decarboxylase (<i>gadA</i>) in <i>E. coli</i>	Tramonti <i>et al.</i> (2006)
GadX	Glutamate decarboxylase (<i>gadA</i>) in <i>E. coli</i>	Tramonti <i>et al.</i> (2006)
HilC	Virulence genes in SPI1 pathogenicity island of <i>Salmonella enterica</i>	Olekhovich & Kadner (2007)
HilD	Virulence genes in SPI1 pathogenicity island of <i>Salmonella enterica</i>	Olekhovich & Kadner (2007)
PerA	Virulence genes in the LEE pathogenicity island of enteropathogenic <i>E. coli</i>	Porter <i>et al.</i> (2004)
RegA	Putative virulence genes in <i>Citrobacter rodentium</i>	Yang <i>et al.</i> (2008)
Rns	Pilus genes in enterotoxigenic <i>E. coli</i>	Murphree <i>et al.</i> (1997)
UreR	Urease genes in <i>Proteus mirabilis</i>	Poore & Mobley, (2003)
VirF	The <i>virB</i> regulatory virulence gene in <i>Shigella flexneri</i> and enteroinvasive <i>E. coli</i>	Falconi <i>et al.</i> (1998); Porter & Dorman (2002)

promoter function in the absence of the repressor (Jordi *et al.*, 1992; Murphree *et al.*, 1997). Those AraC-like proteins that derepress and activate thermally responsive virulence genes do not appear to bind chemical ligands; they respond instead to a physical signal (temperature). Recently, an AraC-like protein that opposes H-NS has been described that binds a chemical co-factor: this is the sodium carbonate-responsive RegA protein of *Citrobacter rodentium* (Yang *et al.*, 2008). A role in H-NS displacement has not so far been described for other ligand-binding AraC-like proteins, including the prototypic example, AraC itself. Overall, the majority of the transcription factors in this family that have been shown to displace H-NS are non-ligand binders that upregulate virulence genes.

Temperature sensitivity is a common theme among those H-NS-antagonizing AraC-like proteins that are involved in virulence gene activation, yet the molecular basis of temperature sensitivity remains obscure in most cases (Porter & Dorman, 2002). Perhaps the effect of temperature on the topology of the DNA to which the AraC-like protein binds influences its interaction with RNA polymerase, allowing it to act as a transcription activator as well as an anti-repressor that disrupts H-NS–DNA complexes. It has been shown in the case of VirF activation of the *virB* virulence regulatory gene in *Shigella flexneri* that over-expression of the VirF protein is essential but not sufficient for upregulation of the *virB* promoter at the normally non-permissive temperature of 30 °C (Tobe *et al.*, 1995). However, if the topology of the *virB* promoter is adjusted by the creation of a local domain of negatively supercoiled DNA, then VirF can activate *virB* transcription at a temperature where this would otherwise not be possible (Tobe *et al.*, 1995). This sensitivity to changes in local DNA structure recalls the activation of the *virF* and *proU* promoters, where derepression is achieved through the removal of H-NS as a result of DNA structural adjustments alone.

The LeuO protein: setting boundaries

The ability to form nucleoprotein filaments with DNA plays an important role in H-NS-mediated transcriptional silencing. LeuO has been identified as a protein that can set limits to the polymerization of H-NS along the genetic material. It is a LysR-like DNA-binding protein that was identified as a transcription activator in the promoter relay that governs the expression of the *leuABCD* operon in *Salmonella* Typhimurium (Chen & Wu, 2005; Chen *et al.*, 2005; Fang & Wu, 1998). One of the functions of LeuO is to counteract the repressive activity of the H-NS protein at the *leuO* promoter. It does this by binding between the promoter and an upstream binding-and-nucleation site for H-NS (Fig. 1c). By imposing itself at this position, LeuO blocks the propagation of the H-NS–DNA filament, preserving the *leuO* promoter in a transcriptionally active state (Chen & Wu, 2005; Chen *et al.*, 2005). The ability of LeuO to act as a boundary element in this way is enhanced

if it can interact simultaneously with additional binding sites, in a manner analogous to the interaction of the LacI repressor protein with two operator sequences when repressing the *lac* operon in *E. coli*. In fact, Wu and colleagues have succeeded in substituting the LacI protein for LeuO at the *leuO* promoter and demonstrating that the *lac* repressor can also act as a boundary element in the face of encroachment by a self-propagating H-NS–DNA filament (Chen & Wu, 2005; Chen *et al.*, 2005) (Fig. 1c). This suggests that there may be a great deal of flexibility in the kinds of proteins that can be co-opted as boundary elements.

The regulatory effects of LeuO are not confined to the *leuABCD-leuO* region of the *S. Typhimurium* chromosome. Instead, this protein is now recognized as one with widespread effects on gene expression in *S. Typhi*, *S. Typhimurium* and *E. coli* (De la Cruz *et al.*, 2007; Hernández-Lucas *et al.*, 2008; Stratmann *et al.*, 2008). The LeuO protein displaces H-NS at the *ompS1* promoter in *S. Typhi* (De la Cruz *et al.*, 2007), showing that its mechanism of action is not confined to impeding H-NS polymerization along DNA. It can relieve H-NS-imposed silencing of *bgl*, a normally cryptic operon that confers on *E. coli* the ability to utilize β -glucosides (Ueguchi *et al.*, 1998). Importantly, LeuO also controls the translation of the mRNA specifying the RpoS sigma factor by regulating the expression of the DsrA small regulatory RNA (sRNA; Klauck *et al.*, 1997) that promotes efficient translation of *rpoS* mRNA (Majdalani *et al.*, 1998). This places LeuO at the heart of the regulatory nexus responsible for adaptation to stationary phase and stress.

In addition to its positive influence on expression of RpoS, the DsrA sRNA acts to impede the translation of *hns* mRNA, reducing the cellular pool of the H-NS protein (Fig. 1h). It does this by direct RNA–RNA interaction with the *hns* mRNA (Lease *et al.*, 1998). This explains an earlier observation that DsrA acts as an anti-silencer in the case of H-NS-repressed genes in *E. coli* (Sledjeski & Gottesman, 1995). It also closes the regulatory loop that includes the antagonists H-NS and LeuO.

The AraC-like proteins Rns and CfaD upregulate the H-NS-repressed genes that code for CS1 and CFA/1 fimbriae, respectively. In each case the positive regulator has been shown to require two binding sites, one located upstream and one downstream of the promoter (Jordi *et al.*, 1992; Murphree *et al.*, 1997). The requirement for the downstream site has never been explained adequately (Egan, 2002) and it is tempting to speculate that it is involved in the establishment of an Rns- or CfaD-mediated bridge that protects the promoter from incursion by H-NS.

HU, Fis, RpoS and H-NS

Other nucleoid-associated proteins can antagonize H-NS binding to DNA. Experiments with magnetic tweezers and atomic force microscopy have suggested that the abundant

HU protein can compete with H-NS for the same binding sites in DNA, opening up H-NS-condensed promoter regions (van Noort *et al.*, 2004). The Fis protein has also been reported to antagonize H-NS repression, for example at rRNA gene promoters where its binding sites are distributed among those of H-NS (Schneider *et al.*, 2003). At later stages of growth when Fis levels are low, H-NS represses the rRNA gene promoters (Afflerbach *et al.*, 1998). The nucleoid-associated protein HU and the RpoS stress and stationary-phase sigma factor of RNA polymerase have been described as having positive regulatory roles at the H-NS-repressed *proU* promoter in *E. coli* (Manna & Gowrishankar, 1994), and a wider overlap between the H-NS and RpoS regulons has been described (Barth *et al.*, 1995). This may indicate a role for RpoS in overcoming H-NS-mediated repression in bacteria undergoing stress.

Full-length, truncated and partial paralogues and orthologues of H-NS

An intriguing group of proteins is made up of small polypeptides with homology to the oligomerization domain of H-NS. Those with the closest amino acid sequence similarity to this domain are members of the H-NST family, so-called because they resemble H-NS truncates that lack the nucleic acid binding and linker domains (Williamson & Free, 2005). The genes coding for these truncates have been detected in pathogenicity islands of various pathogenic enterobacteria including enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli*. The protein from EPEC, H-NST(EPEC), co-purifies with H-NS. This protein can interfere with the ability of H-NS to repress the *proU* operon in *E. coli*, but it has a reduced ability to do so with an H-NST derivative that harbours a leucine-to-proline mutation at codon 30 that is known to impair dimer formation. Perhaps H-NST weakens the bridging activity of H-NS oligomers by replacing some full-length H-NS proteins, reducing the number of DNA-binding domains in the complex (Fig. 1g). This has been exploited to create a pseudo-H-NS⁻ phenotype in *Y. enterocolitica*, a bacterium in which *hns* mutations are very difficult, if not impossible, to construct (Baños *et al.*, 2008). The existence of genes encoding these H-NS inhibitors within A+T-rich pathogenicity islands which are targets for H-NS repression points to a mechanism by which horizontally acquired genetic elements may not simply displace H-NS from DNA but directly modulate the repressive activity of the host-encoded H-NS protein. However, one should consider that a strategy with the potential to alter the influence of H-NS throughout the cell might be selected against. In this context it should be noted that the biological effects of H-NST described so far were obtained by expressing the protein to higher-than-natural levels (Baños *et al.*, 2008; Williamson & Free, 2005). The T7-phage-encoded protein 5.5, which interacts with H-NS in a similar manner to H-NST in order to derepress T7 RNA polymerase-mediated transcription (Liu &

Richardson, 1993) (Fig. 1g), does not suffer from the evolutionary constraint noted above for H-NST.

Genes coding for small proteins that interact directly with H-NS are found in the ancestral chromosome and on horizontally acquired islands. The YmoA protein of *Yersinia* was recognized originally as a regulator of virulence gene expression in *Y. enterocolitica* (Cornelis *et al.*, 1991). It is related to the Hha protein, discovered initially as a modulator of haemolysin gene expression in *E. coli*, and the two proteins can substitute for one another functionally (Balsalobre *et al.*, 1996; Mikulskis & Cornelis, 1994). The Hha protein must interact with H-NS in order to exert its effect on haemolysin gene expression; YmoA also interacts with H-NS and this relationship was exploited in the isolation of the H-NS protein from *Yersinia* (Nieto *et al.*, 2000, 2002). The solution structure of YmoA has been solved using nuclear magnetic resonance spectroscopy (McFeeters *et al.*, 2007). The results lend weight to the view that YmoA (and Hha) should be regarded as independent oligomerization domains of H-NS. Potentially, the proteins may oligomerize to produce YmoA–H-NS and Hha–H-NS heteromers. The absence of a nucleic acid-binding domain on the YmoA and Hha partners may result in a failure of the heteromers to participate in DNA–protein–DNA bridging, compromising (or at least modifying) the structure of repression complexes, although all the available evidence indicates that this does not happen. It has also been pointed out that the insertion of YmoA or Hha dimers into H-NS–DNA complexes may alter the supra-structuring of the nucleoprotein complexes in ways that alter biological function (McFeeters *et al.*, 2007). In cases where the contribution of these proteins has been examined, such as *inv* gene repression in *Y. enterocolitica*, the effect of YmoA has been to enhance the repressive effect of H-NS (Ellison & Miller, 2006b) (Fig. 1i). It seems paradoxical that the introduction of a protein with the potential to reduce the amount of DNA–H-NS–DNA bridging can result in better repression. This provides a stark illustration of the limitations of our current models of how these proteins work.

The discovery of paralogues of Hha-like proteins has added a further layer of complexity. The *ydgT* gene codes for an Hha-like protein in *E. coli* and *Salmonella*, and it can interact with H-NS and the H-NS paralogue, the StpA protein (Paytubi *et al.*, 2004). The YdgT protein is important in *Salmonella enterica* for the proper contextual regulation of the virulence genes in the SPI2 pathogenicity island: in the absence of YdgT, the bacterium upregulates its SPI2 genes too early during infection, leading ultimately to a loss of virulence (Coombes *et al.*, 2005). Other work has established a role for Hha as a negative regulator of SPI2 genes in *Salmonella* (Silphaduang *et al.*, 2007), while investigations that used microarrays have shown that inactivation of the *hha* and *ydgT* genes in *Salmonella* leads to upregulation of A+T-rich genes that have been acquired horizontally. This suggests that these proteins target at least some of the same genes as H-NS, probably by

direct protein–protein interaction with H-NS (Vivero *et al.*, 2008).

The StpA protein is a full-length paralogue of H-NS and these two can form heterodimers (Deighan *et al.*, 2000; Dorman, 2004; Dorman *et al.*, 1999; Johansson & Uhlin, 1999; Williams *et al.*, 1996). There is no evidence that StpA interferes with or enhances the ability of H-NS to repress transcription and so the biological role of StpA–H-NS heterodimers remains obscure. So too does the significance of the Sfh–H-NS heteromer, in which H-NS interacts with the Sfh protein, a plasmid-encoded full-length H-NS orthologue (Beloin *et al.*, 2003; Deighan *et al.*, 2003; Doyle & Dorman, 2006; Doyle *et al.*, 2007). A study carried out in uropathogenic *E. coli* using microarray analysis suggests that H-NS–H-NS homodimers and H-NS–StpA heterodimers may control distinct regulons (Müller *et al.*, 2006). However, these data could equally be explained by the ability of StpA to compensate for H-NS at just a subset of H-NS-regulated promoters in the *hns* mutant; the results do not necessarily support a distinct role for H-NS–StpA heteromers. H-NS-mediated silencing of the *bgl* operon in *E. coli* requires the full-length H-NS protein with both its oligomerization and DNA-binding domains. However, the H-NS oligomerization domain alone can silence *bgl* if StpA is available to act as a molecular adaptor (Free & Dorman, 1997; Free *et al.*, 2001), although this is not the case with every H-NS-repressed promoter (Wolf *et al.*, 2006). It is unclear if the interaction with H-NS influences the RNA chaperone activities of StpA (Mayer *et al.*, 2007), although the fact that oligomerization with H-NS protects StpA from Lon-mediated proteolysis might suggest that it does to the extent that H-NS promotes the stability of this RNA chaperone (Johansson & Uhlin, 1999).

Not all H-NS analogues are thought to act by direct protein–protein interaction with H-NS. The Ler DNA-binding protein is encoded by the LEE (locus of enterocyte effacement) pathogenicity island of enterohaemorrhagic *E. coli* (EHEC) and EPEC. It activates the transcription of the major virulence operons in the island at 37 °C by opposing the silencing activity of H-NS (Barba *et al.*, 2005; Bustamante *et al.*, 2001; Haack *et al.*, 2003; Umanski *et al.*, 2002). Ler and H-NS are partial analogues whose oligomerization domains are highly divergent coiled-coils; there is no evidence that Ler and H-NS form heterodimers. Instead, Ler is thought to displace H-NS (Haack *et al.*, 2003). It also acts on gene expression outside the LEE (Elliott *et al.*, 2000). For example, Ler counteracts the silencing activity of H-NS at the *lpf* operon in EHEC, which encodes long polar fimbriae (Torres *et al.*, 2007). Thus, despite its homology to H-NS, Ler acts more like VirB or SlyA.

Perspective

The finding that partial and full-length orthologues of H-NS are encoded by mobile genetic elements or genetic elements that probably were mobile in the past has important

implications for our view of the evolvability and flexibility of control circuits that include the H-NS protein. We are still at an early stage in the investigation of H-NS functional modulation, if any, by interaction with analogues encoded by the bacterial chromosome. Now we must take into account the contributions of H-NS-interacting proteins that are encoded by imported genes. The emerging picture is indeed very dynamic and complicated.

The rather ad hoc nature of some of the mechanisms used to counteract H-NS-mediated transcriptional silencing suggests that these may emerge relatively easily to meet the regulatory needs of the bacterium. Evidence from the *Shigella flexneri virF* gene suggests that the very DNA sequences that attract H-NS may themselves be remodelled in response to temperature in ways that dislodge the protein. The action of the VirB regulatory protein in the same *S. flexneri* virulence gene cascade shows that a presumptive former plasmid partitioning protein can find a new role as an H-NS antagonist. The fact that H-NS–DNA nucleoprotein structures can be remodelled by a range of unrelated DNA-binding proteins reinforces the impression that transcriptional silencing by H-NS can be overcome relatively easily. It is also consistent with the observation that H-NS has relatively weak DNA-binding activity (Shin *et al.*, 2005). This suggests that new regulatory circuits can emerge that place H-NS-repressed genes under the control of new regulatory proteins and the regulatory signals that affect the biological activities of those proteins. This provides a basis for evolution of new gene control switches in which rather simple mechanisms aimed at H-NS displacement may be refined to respond to one or more environmental signals. It is clear that studies of H-NS biology have much to teach us not only about how regulatory switches have evolved in the past but also about how they may evolve in the future.

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