INTERACTION OF DNA-BINDING PROTEIN H-NS WITH THE FLAGELLAR MOTOR OF *E.COLI*

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

The histone-like nucleoid-structuring protein (H-NS) is well known as a global regulator of transcription. A number of studies have suggested that H-NS also positively influences the function of the flagellar motor, but the details of its motility-regulating action remain unclear. In an effort to characterize the actions of H-NS in the flagellar motor, we sought to test the effects of specific mutations in H-NS that are predicted to alter its state of multimerization. As a foundation for this work, we examined the effects of H-NS expression in strains that expressed the flagellar regulatory proteins FlhDC at various levels, from various plasmids. The results gave indications that certain plasmids previously used to provide FlhDC constitutively did not, in fact, express the proteins at levels sufficient to stimulate flagellar assembly. This complicates the interpretation of previous work, because the cells retained the chromosomal copies of the *flhDC* genes whose expression is known to be influenced by H-NS. Thus, effects in the previous experiments may have been the result of up-regulation of chromosomal *flhDC* rather than direct actions at the flagellar motor. To overcome this problem, I constructed new strains in which the chromosomal copies of *flhDC* were deleted, and revisited the question of H-NS action in the motor. For these experiments, the *flhDC* genes were expressed from a regulatable plasmid that had been verified by complementation of the *flhDC* deletion strain, and H-NS was expressed from a second regulatable plasmid. The results indicate that H-NS contributes to flagellar motility in ways other than its stimulatory effect *flhDC*

expression, as was suggested on the basis of the previous work. Details of its action are different from those reported previously. An analysis of mutants altered at interfaces needed for H-NS multimerization gives evidence that H-NS must act as a dimer or larger multimer, in both its gene-regulatory and motility regulating.

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ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Many species of bacteria respond to changes in the environment by moving towards favorable conditions, using a style of motility based on rotating flagella. The flagellum is a thin helical filamentous organelle that is inserted into the membrane and extends a considerable distance (several microns) from the cell. The rotation of the flagellum is driven by a large, multiprotein molecular machine in the inner membrane (Blair, 1995; Glagolev & Skulachev, 1978; Kojima & Blair, 2004a; Macnab, 2003; Namba & Vonderviszt, 1997)

The flagellar motor is the most complex and largest molecular engine known in bacteria. It is driven by the flow of ions across the cell membrane, utilizing energy stored in the transmembrane ion gradient. The rotating flagella produce thrust that enables cells to move towards favorable environments and avoid unfavorable ones.(Glagolev & Skulachev, 1978; Larsen, Adler, Gargus, & Hogg, 1974; Manson, Tedesco, Berg, Harold, & Van der Drift, 1977). In most bacterial species, including the well-studied model organisms *E.coli* and *Salmonella enterica serovar Typhimurium,* the motors are driven by a proton motive force, but some others, including marine *Vibrio* species and certain alkalophiles, are driven by sodium ions (Berg, 2003; Hirota & Imae, 1983; Kawagishi, Maekawa, Atsumi, Homma, & Imae, 1995). The bacterial flagellum has been studied for more than 45 years and a great deal is known about flagellar structure, components, assembly, genetics, and function in chemotaxis. However, the mechanism of rotation is still not fully understood (Berg, 2003; Sowa & Berry, 2008).

The flagellar motor can spin either clockwise (CW) or counter- clockwise (CCW), altering the CW/CCW rotational bias in response to environmental cues, such as changes in concentration of chemical effectors. Thus, motor reversals are the basis of

chemotaxis. When the motors turn CCW, the cell forms a bundle of filaments, which push the cell forward in a smooth motion termed a "run." When flagella switch to CW rotation, the bundle of filaments falls apart, producing a rapid re-orienting movement termed a tumble (Figure 1.1; Berg, 2003; Bourret & Stock, 2002; Kojima & Blair, 2004a). The switch from CCW to CW rotation is initiated by interaction with the signaling molecule cheY-phosphate, which favors CW rotation (Sarkar, Paul, & Blair, 2010; Welch, Oosawa, Aizawa, & Eisenbach, 1993). In the presence of increasing concentration of an attractant such as glucose, intervals of CW rotation are suppressed, so that the cells move toward regions of higher concentration. In the presence of increasing concentrations of repellent, such as phenol, the cells swim, on average, down the gradient (Berg, 2003; Bourret & Stock, 2002; Stock & Koshland, 1978).

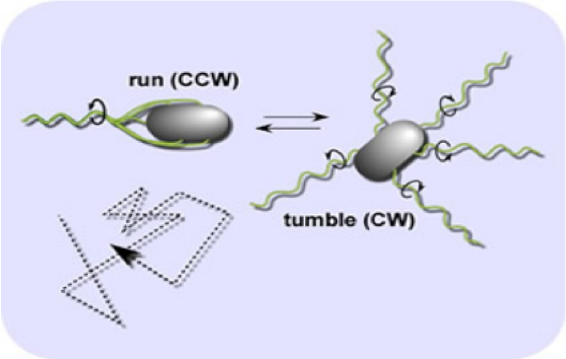


Figure 1.1. Flagellar rotation and its relationship to cellular motion. When flagellar rotates, CCW, cells move along relatively smooth paths. When one or more motors turn CW, the cells tumble (Figure courtesy of J.S. Parkinson).

Classification and regulation of flagellar genes

More than 50 genes are involved in assembly and operation of the bacterial flagellar motor (Berg, 2003; R. M. Macnab, 2003) In *E. coli*, these genes are organized in about a dozen operons, grouped into 3 classes, called early, middle and late (or class 1, 2 and 3), according to the relative timing of their expression. The early operon consists of the genes *flhD* and *flhC*, which are required for the expression of all the middle (class 2) genes. The class 2 genes encode proteins that form the basal body and hook. Class 2 genes are in turn needed for expression of the late (class 3) genes, which encode proteins that form the filament and also the proteins that function in rotation and chemotaxis (Figure 1.2). Defects in early or middle gene expression, or in assembly of a functional basal body, down-regulate late gene expression (Chilcott & Hughes, 2000; Kutsukake, Ohya, & Iino, 1990). This involves the action of two competing regulatory proteins, FlgM and FliA (s28), which are encoded by class 2 operons. FliA is required for class 3 transcription, but is antagonized by the action of FlgM, until FlgM is exported from the cell through the assembled hook-basal body. Expression of the master regulator genes *flhDC* is responsive to a variety of environmental clues, such as temperature, osmolarity, and salt concentration (Chilcott & Hughes, 2000), thus determining the conditions under which cells assemble flagella.

Flagellar structure

On average, an *E. coli* cell contains four to six flagella. These arise at random points on the cell surface and extend several body lengths out into the external medium. The long, filamentous part of the flagellum is made from many thousands of subunits of

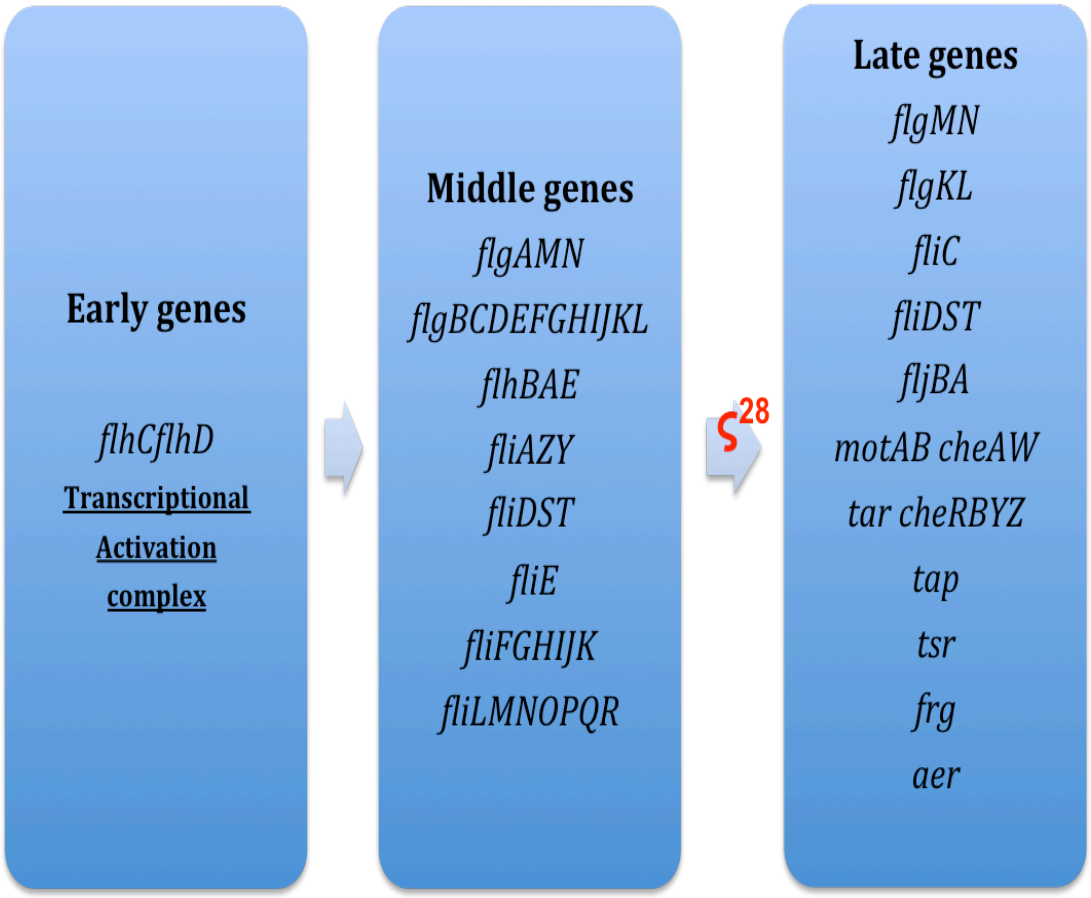


Figure 1.2. Three transcriptional classes of flagellar genes. The early genes *flhDC* encode a transcription factor that functions as the master regulator for directing transcription of genes in the second regulatory class. The middle class genes encode components of the flagellar basal structure and hook, and the late class genes encode late assembled proteins including those of the filament, the stator (MotA/B), and the chemosensory pathway (e.g., CheA and CheW). A few genes are transcribed from both middle- and late-class promoters. (Adapted from Chilcott & Hughes, 2000; Kutsukake, Ohya, & Lino, 1990)

the flagellin protein, FliC. The filament is joined to a flexible structure termed the hook, which is in turn joined to the basal body (Berg, 2003; DePamphilis & Adler, 1971).

The hook is formed from the protein FlgE. At the junction between hook and filament, and also at the tip of the filament, are the hook-associated proteins FlgK, FlgL and FliD. The basal body contains a set of rings, termed MS, L, and P, mounted on a rod (Berg, 2003, 2008; Blair, 1995; Kojima & Blair, 2004a). The LP ring is located at the level of the outer membrane (Lipopolysaccharide) and peptidoglycan, while the MS ring is within and above the cytoplasmic membrane. The rod is formed from the proteins FlgB, FlgC, FlgF and FlgG, and functions to connect the MS ring to the hook (Homma, Ohnishi, Iino, & Macnab, 1987). The MS ring is formed from the protein FliF. On its cytoplasmic face, the MS-ring attaches to a large drum-shaped structure called the C ring. The MS ring is the first structure built during assembly of the motor, and serves a central structural role (Homma et al., 1987).

The motor is subdivided into the rotor, or rotating part, and the stator. The stator consists of two proteins, MotA and MotB, which are embedded in the cell membrane around the MS ring (Kojima & Blair, 2004a; Ueno, Oosawa, & Aizawa, 1992). The function of the stator is to conduct ions across the membrane and somehow couple this proton flow to rotation (Kojima & Blair, 2004a). The C ring, also termed " the switch complex," is formed from the proteins FliG, FliM, and FliN. This complex regulates the direction of motor rotation. It also functions in assembly; cells lacking FliG, FliM, or FliN are nonflagellate (Fgure 1.3; Francis, Sosinsky, Thomas, & DeRosier, 1994; Ueno et al., 1992; Yamaguchi et al., 1986).

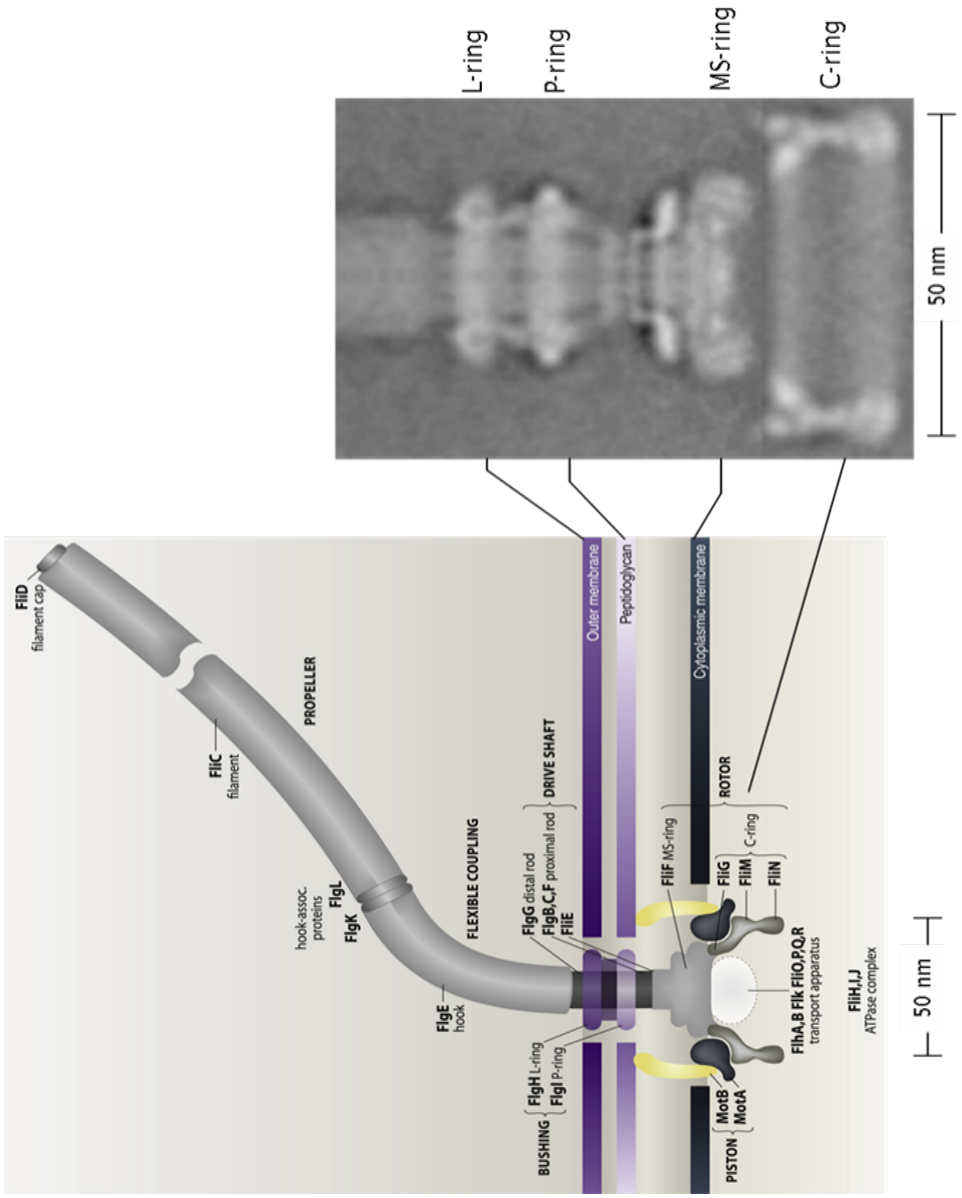


Figure 1.3. A schematic diagram of the flagellar motor, drawn to scale, compared to a rotationally averaged reconstruction of images of hook basal bodies seen in the electron microscope. (See text and Table 1.1 for details; adapted from Berg, 2008)

Table 1.1. Proteins of *E. coli* involved in flagellar motor assembly and function.

Protein Symbol[a]	Common name and Function	Cellular location	Approximate Stoichiometry[b]
MS ring			
FliF	MS-ring protein. Platform upon which flagellar structures are build.	Cytoplasmic	26
C-ring			
FliG	Rotor/switch protein, torque generation and interacts with the stator protein, MotA/MotB	periphral	26
FliM	Rotor/switch protein, a target for CW switching protein Che Y-Phospho.	periphral	32-34
FliN	Rotor/switch protein, flagellar assembly	periphral	110
Stator			
MotA	Stator protein, involve in torque generation by interacting with rotor protein (FliG).	Inner membrane	4
MotB	Stator protein, convert proton energy into torque.	Inner membrane	2
FT3SA			
FlhA	Export component, target for soluble export complex.	Inner membrane	~2
FlhB	Export component, Involve in determining substrate specificity	Inner membrane	~2
FliO	Export component	Inner membrane	~1
FliQ	Export component, essential for flagellar export.	Inner membrane	~4
FliR	Export component, essential for flagellar export.	Inner membrane	~1

Table 1.1 continued

Protein Symbol[a]	Common name and Function	Cellular location	Approximate Stoichiometry[b]
FliH	Negative regulator of FliI, important for flagellar export.	cytoplasm	~1
FliI	ATPase, important for flagellar export.	cytoplasm	
FliJ	General chaperone	cytoplasm	
Rod Protein			
FlgB	Rod protein	Periplasmic space	7
FlgC	Rod protein	Periplasmic space	6
FlgF	Rod protein	Periplasmic space	6
FlgG	Distal rod protein	Periplasmic space	26
FlgJ	Rod capping protein, muraminidase	Periplasmic space	
FlgE	MS-rod junction protein,export gate	Periplasmic space	~9
P-ring			
FlgI	P-ring protein	Periplasmic space	24
FlgA	Chaperone for P-ring protein.	Periplasmic space	
L-ring			
FlgH	L-ring protein. Lipoprotein.	Outer membrane	28
Hook protein			
FlgE	Hook protein.	Cell exterior	132
FlgD	Hook-capping protein	Cell exterior	
Filament associated protein			
FliC	Filament protein, flagellin.	Cell exterior	20,000
FlgK	HAP1, first hook filament junction.	Cell exterior	11
FlgL	HAP3, second hook filament junction.	Cell exterior	11
FliD	HAP2, filament-capping protein, flagellin folding chaperone.	Cell exterior	10

Table 1.1 continued

[a] The original flagellar gene and gene products nomenclature for *Salmonella* and *E. coli* were replaced by a unified and simplified nomenclature in 1988 (Iino et al., 1988).

[b] Per flagellum, for reference see text. (Adapted from Macnab RM., 2003)

Structure of the rotor

Crystal structures have been solved for major portions of the switch-complex proteins FliG, FliM and FliN (Brown, Hill, & Blair, 2002; Brown, Mathews, Joss, Hill, & Blair, 2005). Some general features of subunit organization in the complex have been examined using biochemical and mutational approaches in conjunction with electron microscopic images of the basal body. The crystal structure of a FliG fragment that includes the C-terminal two-thirds of the protein shows two compact globular domains, $FliG_M$ (middle) and $FliG_C$ (C-terminal), joined by a helix and a short extended linker (Thomas, Morgan, & DeRosier, 1999). FliG is the rotor protein most closely involved in rotation and contains a set of conserved charged residues that interact directly with the stator. These residues lie together at the top of $FliG_C$ (Zhou, Lloyd, & Blair, 1998). $FliG_c$ also has a conserved surface hydrophobic patch, at the end opposite the charged ridge, which has been shown to interact with FliM. An additional conserved surface feature, termed the EHPQR motif after its constituent residues is found on the $FliG_M$ domain, and has been shown to also interact with FliM (Blair, 1995; Sowa & Berry, 2008). FliM is closely involved in direction switching and contains a segment near its N terminus that binds to the clockwise-signaling molecule CheY-phosphate (Bren & Eisenbach, 1998; Sockett, Yamaguchi, Kihara, Irikura, & Macnab, 1992; Welch et al., 1993). FliM is located under FliG in the C-ring. FliN is at the bottom of the C-ring, where it functions to facilitate the export of flagellar subunits by providing binding sites for the flagellar export protein FliH (Irikura, Kihara, Yamaguchi, Sockett, & Macnab, 1993; Paul, Harmon, & Blair, 2006). Mutational studies and binding studies indicate that FliN also provides a second interaction site for CheY-phosphate (in addition to that on FliM; Paul

et al., 2006). Basic features of subunit organization in the C-ring, and its relationship to the stator complexes, are outlined in Figure 1.4.

MotA and MotB are both integral to the cytoplasmic membrane, but have different topologies. MotA has four transmembrane (TM) segments, relatively large domains in the cytoplasm, and only relatively short segments in the periplasm (Lloyd, Whitby, Blair, & Hill, 1999; Zhou, Fazzio, & Blair, 1995). MotB has a short segment in the cytoplasm, a single TM segment, and a large periplasmic domain (Chun & Parkinson, 1988; Stader, Matsumura, Vacante, Dean, & Macnab, 1986). Each motor contains several, probably about a dozen, $MotA_4$ $MotB_2$ complexes (Kojima & Blair, 2004b; Reid et al., 2006; Sato & Homma, 2000). These complexes function independently to conduct protons across the membrane and couple this proton flow to rotation.

While structural studies of the rotor proteins have made fair progress, the structure and mechanism of the stator are less understood (Brown et al., 2002; Brown et al., 2005; Lloyd et al., 1999; Sato & Homma, 2000). The mechanism of rotation is not known in molecular detail, but a number of observations suggest that it might involve conformational changes in the stator driven by ion association at a critical Asp residue of MotB (Braun, al-Mawsawi, Kojima, & Blair, 2004; E. A. Kim, Price-Carter, Carlquist, & Blair, 2008; Kojima & Blair, 2001). A rough model for the arrangement of membrane segments in the $MotA_4$ $MotB_2$ complexes has been proposed, on the basis of data from disulfide-cross-linking studies using Cys residues introduced by mutagenesis (Braun et al., 2004; Braun & Blair, 2001; E. A. Kim et al., 2008). Each stator complex appears to contain two copies of MotB, and thus two of the critical Asp 32 proton-

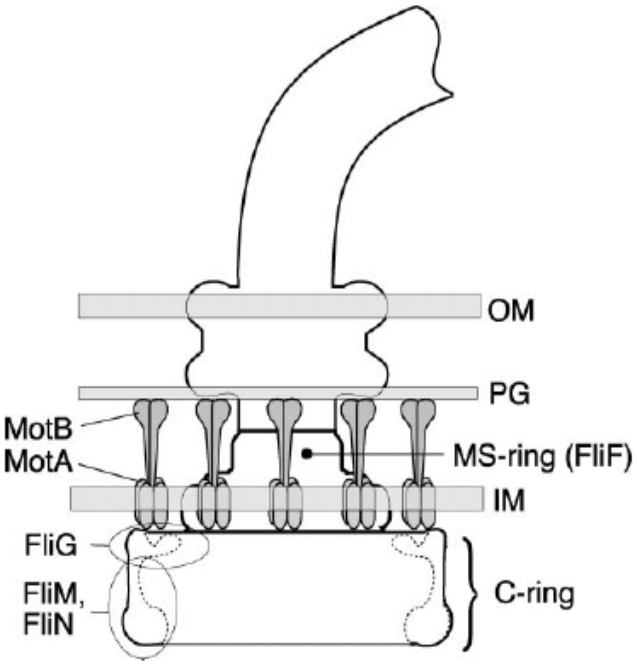


Figure 1.4. Image of the basal body indicating the approximate locations of the proteins that form the stator (MotA and MotB) and the switch complex (FliG, FliM and FliN). OM, outer membrane; PG, peptidoglycan,; IM, inner membrane. (Adapted from Brown et al., 2007)

binding sites. These sites are accessed through channels formed primarily by MotA segments 3 and 4, together with the MotB segment (E. A. Kim et al., 2008; Kojima & Blair, 2001).

Sequence of flagellar assembly

Bacterial flagella are assembled in a precise sequence, generally proceeding from inner (membrane-proximal) structures to outer (distal) ones (Figure 1.5). The first step is assembly of the MS ring in the membrane, from about 25 copies of the protein FliF. In a

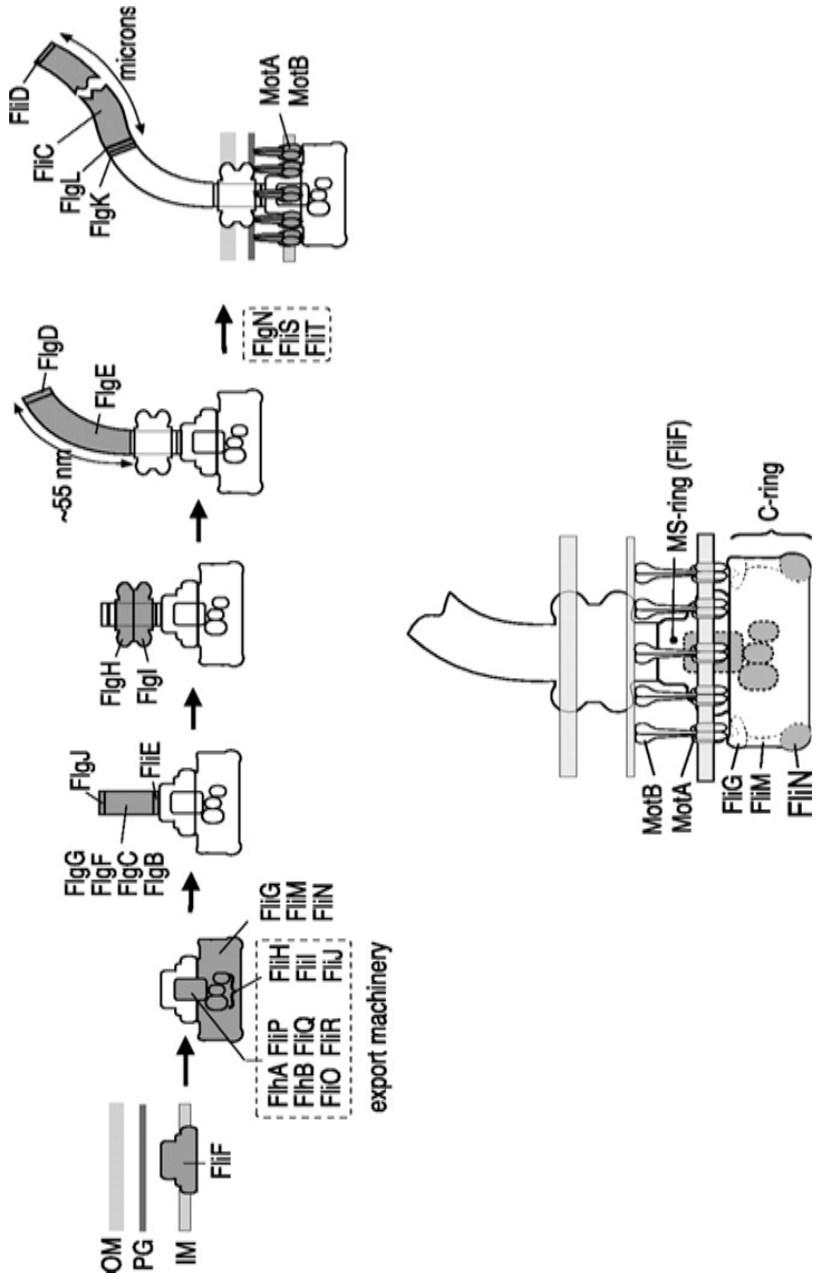


Figure1.5. Assembly pathway of the bacterial flagellum. Protein components of the export apparatus are indicated. (See text and Table 1.1. for details). At each step, the most recently added components are shaded. (Adapted from Paul et.al, 2006)

subsequent step, the C ring is assembled onto the MS ring, from about 25 copies of FliG, 34 copies of FliM, and more than 100 copies of FliN (Kubori, Shimamoto, Yamaguchi, Namba, & Aizawa, 1992; Ueno et al., 1992). In the absence of the C ring, further steps in assembly are prevented, or at least greatly slowed (Yamaguchi et al., 1986). Assembly of most of the external structures involves the action of a specialized secretion apparatus in the basal body that exports the protein subunits that form the structures outside the membrane, including the rod, hook, and filament. Once this secretion apparatus begins operating, it enables formation of the rod first, formed from the proteins FlgB, FlgC, FlgF, and FlgG, attached to the MS ring by the protein FliE and capped by the protein FlgJ (Homma, Kutsukake, Hasebe, Iino, & Macnab, 1990). FlgJ has a muramidase activity that enables the growing rod to penetrate the peptidoglycan (Nambu, Minamino, Macnab, & Kutsukake, 1999). In the next step, The LP ring is assembled from FlgI and FlgH, which, unlike the other exterior components, are exported by the conventional Sec pathway. Once the rod is complete and has penetrated the outer membrane, the hook is assembled from many copies of the protein FlgE. Hook assembly also occurs with the aid of a capping protein, called FlgD. The hook grows to a fairly well determined length (55+/-6 nm), and then FlgD is replaced by the hook-associated proteins FlgK, FlgL and FliD. FlgK and FlgL form short zones that function as the junction between hook and filament (Homma, Komeda, Iino, & Macnab, 1987; Homma, Ohnishi, et al., 1987). FliD acts as a cap to initiate polymerization of the filament; flagellin (FliC) monomers are then inserted between the FlgL and FliD zones. Mature filaments are typically several microns in length and contain many thousands of subunits (Bennett, Thomas, Fraser, & Hughes, 2001; Yokoseki, Kutsukake, Ohnishi, & Iino, 1995).

Chemotaxis

Bacteria are capable of responding and adapting to environmental change, often by moving to more favorable environments. As mentioned, motor reversals are regulated by the signaling protein CheY-phosphate. The level of phosphorylation of CheY is in turn determined by the chemotaxis pathway, which consists of several kinds of membrane-bound chemoreceptors, a regulated protein kinase called CheA, and a coupling protein, CheW, that links the receptors to CheA (Parkinson, 2003; Stock & Koshland, 1978). Other proteins of the chemotaxis system include CheZ, a phosphatase that terminates the CW signal by catalyzing CheY dephosphorylation, and CheR and CheB, which set the level of methylation of the chemoreceptors (described further below) (Parkinson, 1993, 2003; Parkinson & Kofoid, 1992).

Chemoreceptors contain sizable periplasmic domains that include the sites for binding chemoeffectors. Attractant (e.g., serine) binding to the periplasmic domain causes a conformational change that is transmitted across the membrane to the cytoplasmic domain, which in turn alters the structure or organization of an array of CheW and CheA subunits near the inner surface of the membrane. The cytoplasmic domains of the chemoreceptors also include sites for methylation and demethylation, at conserved glutamyl residues. Receptor methylation is involved in sensory adaptation, and enables cells to make temporal comparisons between ligand concentrations experienced in the immediate past (within about the last second) with those encountered a few seconds before (Parkinson, 2003).

Changes in the receptor's ligand occupancy and level of methylation regulate the rate of autophosphorylation of CheA (Sowa & Berry, 2008), which transfers its

phosphoryl groups to aspartate residues on either of the two proteins, CheY or CheB (Bennett et al., 2001; Paul et al., 2006; Welch et al., 1993). CheY-phosphate (CheY-P) binds to the switch complex to promote CW rotation, as already noted (Cluzel, Surette, & Leibler, 2000; Hess, Oosawa, Kaplan, & Simon, 1988). CheY-P can dephosphorylate spontaneously over the course of a few seconds, but in the cell its dephosphorylation is accelerated by CheZ. CheB is a methylesterase, which becomes more active upon phosphorylation. The regulation of CheB resets the level of receptor methylation so that the CheA kinase activity is restored to approximately its prestimulus value (Figure 1.6; Hess et al., 1988; Parkinson, 2003).

Flagellar motility contributes to pathogenesis: *Helicobactor pylori*

Flagellar motility is known to be a determinant of virulence for some pathogens. *Helicobacter pylori* provides a well documented example of the involvement of motility in pathogenesis. *Helicobacter pylori* is a Gram-negative microaerophilic bacterium that is linked to gastritis and peptic ulcers and that in rare cases can contribute to development of gastric cancer (Peterson, 1991). It has been estimated that more than half of the world population has been colonized by *Helicobactor pylori* (Peterson, 1991). *H. pylori* infections are persistent; however, over 80% of people infected with *H. pylori* are asymptomatic, and only a small percentage of people infected are at increased risk for developing gastric cancer (Marshall, 1993; T. Watanabe, Tada, Nagai, Sasaki, & Nakao, 1998). Flagella and chemotaxis have been shown to be critical for *H. pylori* to successfully colonize the human stomach and establish an infection that penetrates into

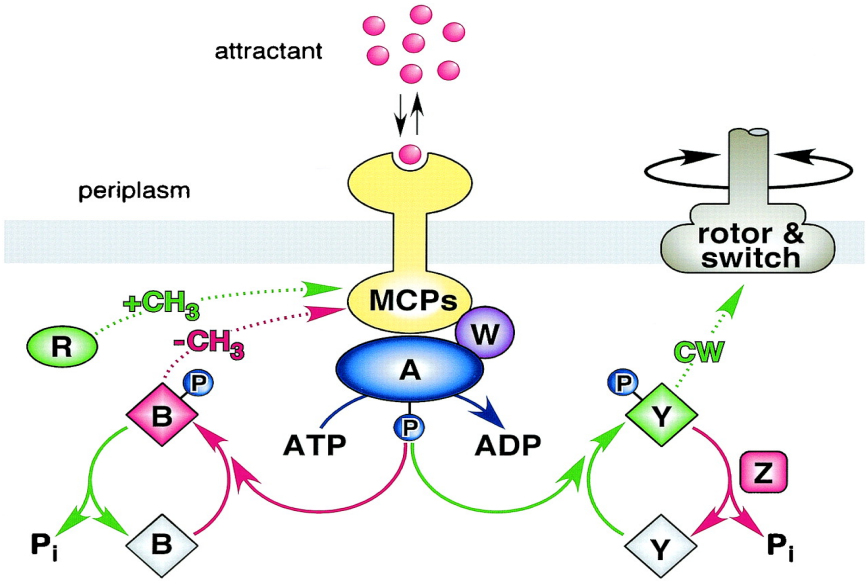


Figure 1.6. A circuit diagram of bacterial chemotaxis showing the key proteins and protein complex including the flagellar motor. (Adapted from Parkinson et al., 2003)

the gastric mucus (Aspholm-Hurtig et al., 2004; Eaton, Suerbaum, Josenhans, & Krakowka, 1996; J. S. Kim, Chang, Chung, & Yum, 1999; Terry, Williams, Connolly, & Ottemann, 2005). *H. pylori* swims by means of a unipolar bundle of two to six sheathed flagella, each about 3-4 µm long. The bundle typically exhibits a bulb-like structure at its distal end (Geis, Suerbaum, Forsthoff, Leying, & Opferkuch, 1993). Like that of enteric bacteria, the flagellum of *H. pylori* comprises a basal body, hook, and filament (Chevance & Hughes, 2008; Geis et al., 1993), and is driven by the proton motive force (Alm et al., 1999; Geis et al., 1993).

H. pylori must cope with highly acidic conditions in order to colonize its host. Within the gastric mucus layer, there is a pH gradient in which the regions closer to the

epithelial cell surface have more neutral pH. *H. pylori* senses this pH gradient and swims away from the more highly acidic environment of the lumen so that it can stay within the more neutral pH environment of the epithelial cell surface (Figure 1.7; Ho et al., 1995). As a further measure to protect against acid, *H. pylori* produces urease to help neutralize the stomach acid (Figure 1.7; Schreiber et al., 2004). Several studies suggest that motility is a critical factor in the infectivity of *H. pylori* (McGee et al., 2005; Osaki et al., 2006; S. Watanabe et al., 1997) Motility-defective *H. pylori* strains gave a reduced density of infection, and decreased inflammation, in a gerbil model of gastric disease (Osaki et al., 2006). Kao et al. (2012) found that patients infected with higher-motility strains have a higher density of *H. pylori* and inflammation in the stomach. It appears that higher-motility strains can reach upper parts of the stomach more easily than lower-motility strains, increasing the severity of infection in stomach (Kao et al., 2012). Details of this process, and the specific mechanisms by which motility enhances infection, are unknown.

H. pylori flagella exhibit the phenomenon of phase variation, a regulatory mechanism that changes the expression of flagellar genes to evade the host immune response (Gewirtz et al., 2004; Lee et al., 2003). Because flagellin from *Salmonella typhimurium* can activate the Toll-like receptor, TLR5, to elicit the host immune response, it has been used in vaccinology as an antigen. Unlike flagellin from *Salmonella,* however, flagellin from *H. pylori* does not induce strong antibody production (Huleatt et al., 2007; Sanders, Yu, Moore, Williams, & Gewirtz, 2006). Many studies have been done to guide the development of a vaccine against *H.pylori*, but no effective vaccine is currently available.

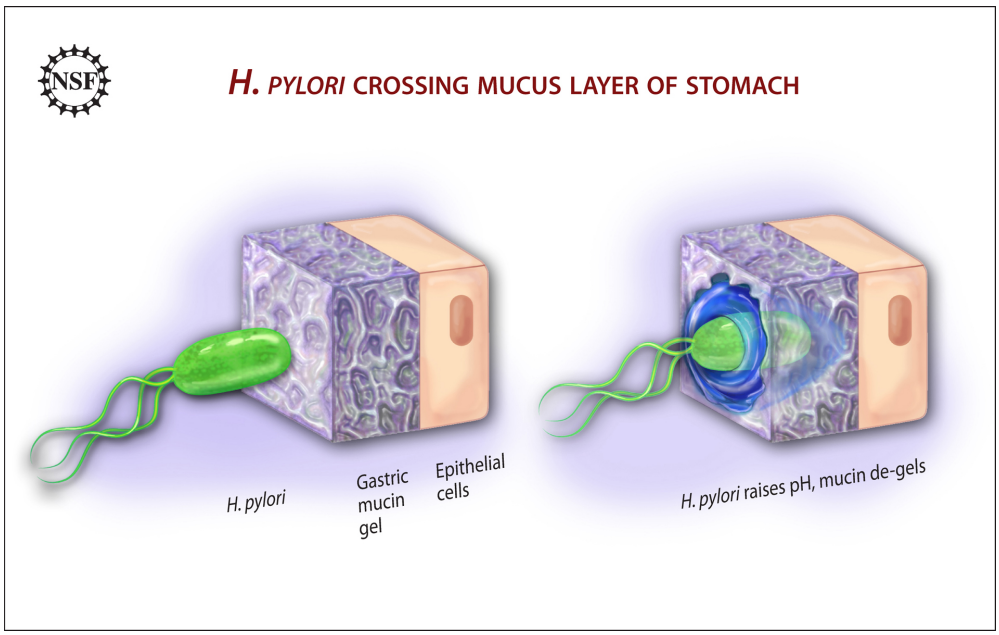


Figure 1.7. *Helicobacter pylori* penetrates the mucin layer to move closer to epithelial cells. (Adapted from Deretsky, 2009)

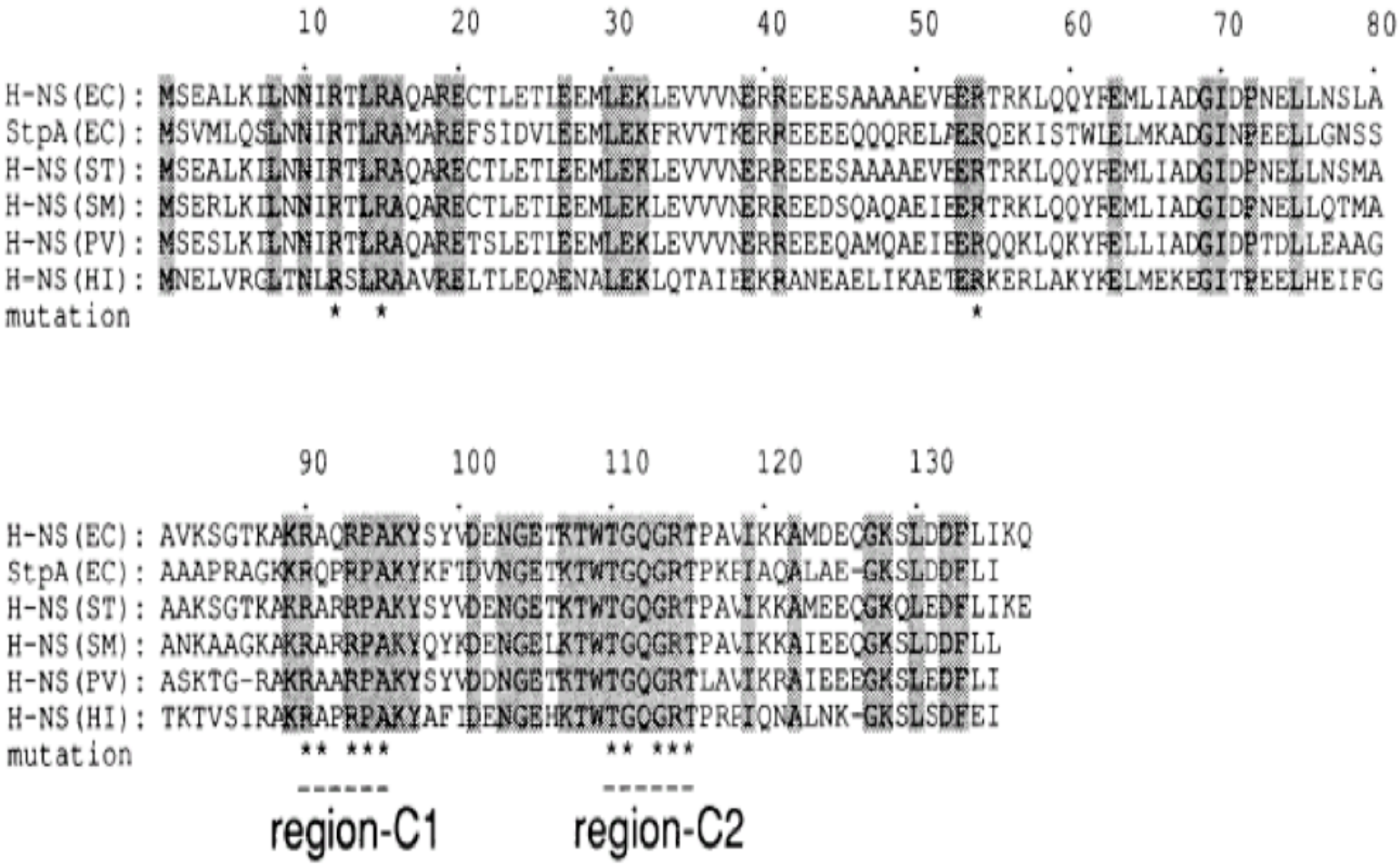
The DNA–binding protein HNS

The histone-like nucleoid-structuring protein (H-NS) is a small (15.6 kDa; 136 residues) DNA-binding protein that functions as a global regulator of transcription. H-NS has also been implicated in packaging of the bacterial chromosomal DNA (Dorman, 2004; Drlica & Rouviere-Yaniv, 1987; Gualerzi et al., 1986; Spassky, Rimsky, Garreau, & Buc, 1984; Ueguchi & Mizuno, 1993; Ueguchi, Suzuki, Yoshida, Tanaka, & Mizuno, 1996). It is one of the most abundant nucleoid-associated proteins found in Gram-negative bacteria (Berg, 2003). In *E. coli*, H-NS has been shown to control gene expression in response to certain environmental factors, in part by altering DNA supercoiling (Blot, Mavathur, Geertz, Travers, & Muskhelishvili, 2006; Hommais et al., 2001).

Homologs of H-NS have been identified in several Gram-negative bacteria (Fleischmann et al., 1995; Hulton et al., 1990; La Teana, Falconi, Scarlato, Lammi, & Pon, 1989; Zhang & Belfort, 1992). The first homolog identified, StpA, was found in *E.coli* and exhibits roughly 60% identity with H-NS at the amino acid level (Zhang & Belfort, 1992). Sequence alignment (Figure 1.8) shows strong conservation of certain amino acids in all H-NS homologs, but it is not known whether these homologs function similarly to H-NS as regulators of transcription, nor is it known whether H-NS homologs of other species carry out the same functions as H-NS in *E.coli* (Ueguchi et al., 1996).

H-NS is believed to regulate transcription by binding DNA differentially in response to changes in osmolarity, pH and temperature. It binds DNA with some preference for curved and AT-rich regions, and while displaying some sequence preferences, it does not conform to any strict consensus sequence (Bracco, Kotlarz, Kolb, Diekmann, & Buc, 1989; Durrenberger et al., 1991; Spassky et al., 1984). While details of its action are not yet clear, it has been proposed that H-NS could prevent the binding of RNA polymerase to promoter regions by altering DNA topology, a mechanism that would distinguish it from more typical sequence–specific regulators. H- NS monomers undergo self-association to form dimers and larger multimers, to an extent that depends on experimental conditions and also the method of analysis (Blot et al., 2006; Falconi, Gualtieri, La Teana, Losso, & Pon, 1988; Goransson et al., 1990; Hommais et al., 2001; Spurio, Falconi, Brandi, Pon, & Gualerzi, 1997; Tupper et al., 1994).

Figure 1.8. Alignment of amino acid sequence of H-NS and StpA from *E.coli*, and H-NS from *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, and *Haemophilus influenza*. Shading indicates conserved positions. The region C2 is crucial for DNA–binding, and the region C1, involved in the interaction with DNA, is a carboxyl-terminal domain of H-NS from *E.coli* for which the structure has been solved by NMR. (Shindo et al., 1995; Ueguchi et al., 1996). (Adapted from Ueguchi, et al., 1996)


10 20 30 40 50 60 70 80
H-NS(EC): MSEALKILNNIRTLRAQARECTLETIEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYFEMLIADGIDPNELLNSLA
StpA(EC): MSVMLQSLNNIRTLRAMAREFSIDVIEEMLEKFRVVTKERREEEEQQQRELAERQEKISTWLELMKADGINPEELLGNSS
H-NS(ST): MSEALKILNNIRTLRAQARECTLETIEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYFEMLIADGIDPNELLNSMA
H-NS(SM): MSERLKILNNIRTLRAQARECTLETIEEMLEKLEVVVNERREEDSQAQAEIEERTRKLQQYFEMLIADGIDPNELLQTMA
H-NS(PV): MSESLKILNNIRTLRAQARETSLETIEEMLEKLEVVVNERREEEQAMQAEIEERQQKLQKYFELLIADGIDPTDLLEAAG
H-NS(HI): MNELVRGLTNLRSLRAAVRELTLEQAENALEKLQTAIEEKRANEAELIKAETERKERLAKYKELMEKEGITPEELHEIFG
mutation * *
90 100 110 120 130
H-NS(EC): AVKSGTKAKRAQRPAKYSYVDENGETKTWTGQGRTPAVIKKAMDEQGKSLDDFLIKQ
StpA(EC): AAAPRAGKKRQPRPAKYKFTDVNGETKTWTGQGRTPKPIAQALAE-GKSLDDFLI
H-NS(ST): AAKSGTKAKRARRPAKYSYVDENGETKTWTGQGRTPAVIKKAMEEQGKQLEDFLIKE
H-NS(SM): ANKAAGKAKRARRPAKYQYKDENGELKTWTGQGRTPAVIKKAIEEQGKSLDDFLL
H-NS(PV): ASKTG-RAKRAARPAKYSYVDDNGETKTWTGQGRTLAVIKRAIEEEGKSLEDFLI
H-NS(HI): TKTVSIRAKRAPRPAKYAFIDENGEHKTWTGQGRTPREIQNALNK-GKSLSDFEI
mutation ** *** ** ***
region-C1
region-C2

Biochemical and structural studies of H-NS demonstrated that it has at least two functionally distinct domains, joined through a flexible linker. The C-terminal domain is involved in DNA binding, whereas the N-terminal domain enables self-association (Arold, Leonard, Parkinson, & Ladbury, 2010; Ceschini et al., 2000; Spassky & Buc, 1977). A relatively recent crystallographic study revealed the structure of a large N-terminal part of H-NS protein (residues 1-83) in an oligomerized state (Arold et al., 2010). The structural model (Figure 1.9) shows that H-NS dimerizes through two short helices in the N-terminal domain, H1 and H2, with a second region of self-association (termed site 2) formed by helices H3 and H4 in the central part of the protein, which adopt a helix-turn-helix structure. Site 2 is involved in oligmerization to form higher-order (tetramer and larger) complexes (Arold et al., 2010; Shindo et al., 1995).

As discussed above, the flagellar operons are regulated in a hierarchical fashion with the master regulators *flhDC* being required for the expression of all other flagellar genes (Berg, 2003; Chilcott & Hughes, 2000; Macnab, 2003). H-NS functions as a repressor, down-regulating expression of a wide variety of genes, but in the case of flagellar genes it is in effect a positive regulator, because one of the genes it represses is *hdfR*, which encodes a negative regulator of the *flhDC* operon. Thus, *hns*-deletion mutants do not express f*lhDC* (as a result of hyperexpression of HdrR) and are nonflagellate. Ko and Park (Ko & Park, 2000b) found that when *hns* was expressed from a plasmid in an *hns*-deletion strain, flagella were assembled but motility remained poor. They suggested that H-NS must somehow directly influence the function of the flagellar motor (Donato & Kawula, 1998; Ko & Park, 2000a, 2000b), but the mechanism of this effect was unknown. Ko and Park found that motility was rescued weakly by

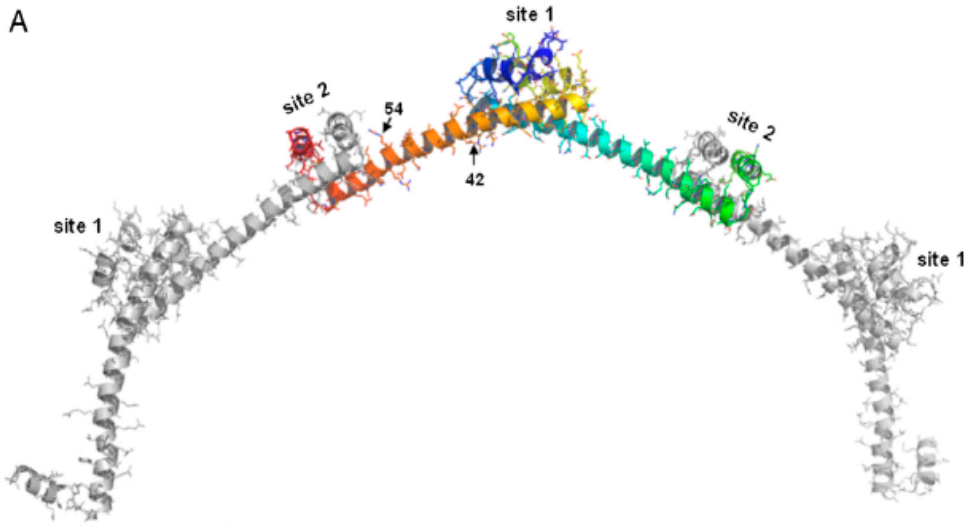


Figure 1.9. Structural basis for H-NS oligomerization. Shown is an oligomer of three symmetry–related dimers (each formed by association through site1), connected through their secondary dimer interface (site 2). (Adapted from Arold et al., 2010)

overexpression of MotA, which might suggest that in the absence of HNS, MotA or MotA/B complexes fail to associate normally with the rotor. In an earlier study, Marykwas et al., (1996) had obtained evidence that H-NS interacts with the rotor protein FliG, and in a subsequent study Donato et al. (1998) found that an H-NS mutation that increased its affinity for FliG also improved the performance of the motor. These findings suggest that the action of H-NS in the motor likely involves an interaction with FliG (Donato & Kawula, 1998; Ko & Park, 2000b).

Following up on these studies, Paul et al. (2011) attempted a closer examination of the H-NS/FliG interaction, mapping the site of interaction to the EHPQR motif in the

middle domain of FliG, a region that has also been shown to interact with FliM (Brown, Terrazas, Paul, & Blair, 2007; Paul, Carlquist, & Blair, 2011c). They measured motility under various conditions and obtained evidence suggesting that H-NS might contribute to motor function under challenging conditions such as increased load or increased ionic strength of the medium. A model was proposed in which H-NS functions, as a dimer or larger multimer, to organize the FliG subunits in the rotor and stabilize the rotor–stator interface (Paul et al., 2011c).

Present goal/aims

In this work, we sought to extend the work of Paul et al. to define more precisely the effects of H-NS on the motor. An initial goal was to determine whether H-NS must bind to the motor as a dimer, or as a larger multimer, in order to exert its effects. As discussed above, structural information on the H-NS multimer is available to guide the introduction of mutations that should destabilize H-NS self-association. We sought to test the effects of such mutations on the action of H-NS at the motor. In the course of the experiments, however, we found that the strains used in the previous study of Paul et al., though supposed to express FlhDC constitutively, did not actually express the proteins at levels sufficient for normal flagellar gene expression. Thus, a partial defect in flagellar gene expression might underlie part or all of the previously reported motility defects. Conversely, when the strain used by Ko and Park was re-constructed, motility was found to be unexpectedly good. These results prompted a fresh start, using strains constructed to more fully separate the hypothesized action(s) of H-NS at the motor from its known role in flagellar gene expression. The results indicate that H-NS does appear to make some

direct (non-*flhDC*-related) contribution to motor function, as previously reported, but this contribution appears different from what was suggested in the earlier work.

CHAPTER 2

CHARACTERIZATION OF H-NS MUTANTS AND THE ROLE OF H-NS IN THE FLAGELLAR MOTOR

Introduction

The H-NS (histone–like nucleoid-structuring) protein of *E. coli* functions to organize the chromosome and down-regulate the expression of diverse genes. Among the genes repressed by H-NS is *hdfR*, which in turn represses transcription of the flagellar master regulators *flhDC*; thus, H-NS is required for normal expression of the flagellar regulon. Ko and Park, (2000a, 2000b) found that in strains engineered to express *flhDC* constitutively, deletion of H-NS still disrupted motility, indicating a role for H-NS in the flagellar motors. The mechanism of action of H-NS in the motor remains unknown. It seems likely to involve the rotor protein FliG, to which H-NS has been shown to bind (Donato & Kawula, 1998; Ko & Park, 2000a, 2000b; Paul et al., 2011c). Together with FliM and FliN, FliG forms the flagellar "switch complex" that is essential for flagellar motor rotation and the regulation of direction (CW vs. CCW) switching. FliG interacts with the stator protein MotA, and Paul et al. (2011c) proposed that H-NS might function to organize the rotor and stabilize the rotor-stator interface. The DNA-organizing action of H-NS requires its association into dimers, tetramers, and larger multimers (Arold et al., 2010). Results of Paul et al. were taken to indicate that H-NS must associate into at least dimers in order to exert its action at the motor, but the possible role of larger multimers was not addressed (Arold et al., 2010; Ceschini et al., 2000; Spassky & Buc, 1977).

The crystallographic structure of a large N-terminal part of H-NS (residues 1-83) is shown in Figure 2.1. In the crystal, this H-NS construct is in a polymerized state composed of dimers stabilized through interactions involving helices H1 and H2 in the N-terminal domain (the interface called site 1), linked end-to-end with other dimers

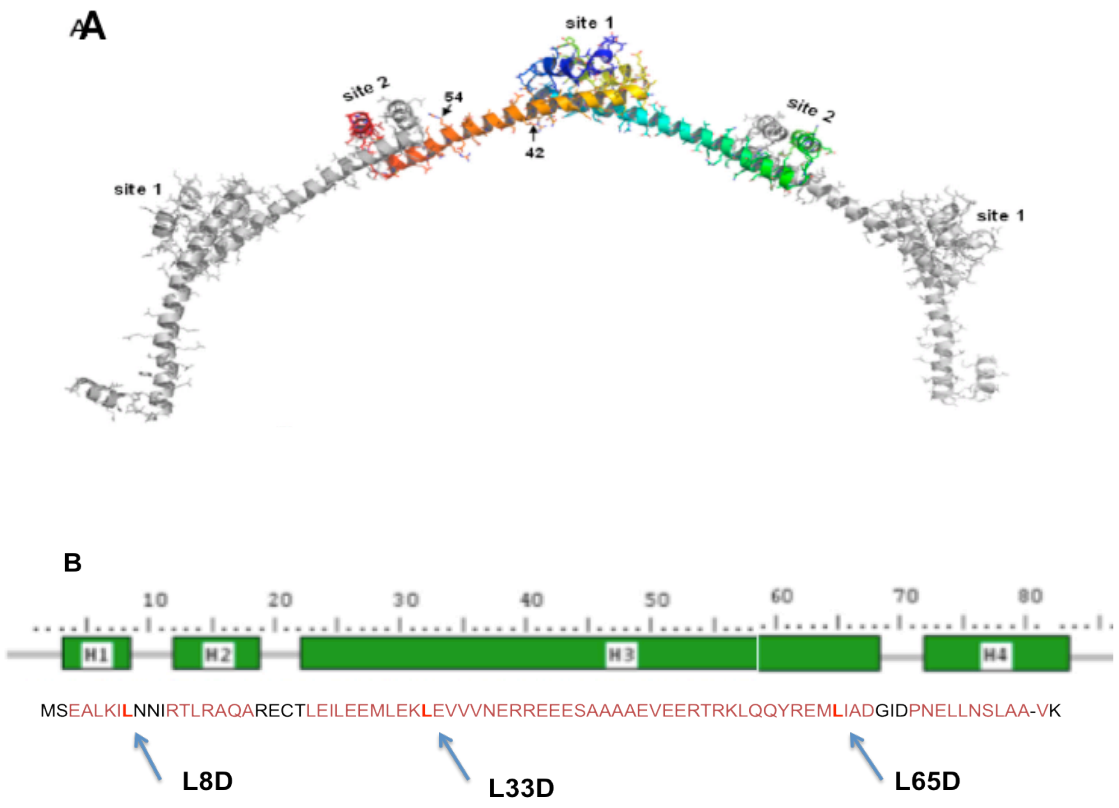


Figure 2.1. Structural basis for H-NS oligomerization. (A) Oligomer of three symmetry-related H-NS dimers (middle dimer shown in color). Dimers form through interactions in the N-terminal domain, at the interface termed site1. These oligomerize through another interface, called site 2, involving the central region of the protein. The C-terminal domain of H-NS (residues 84-137) is not present in this structure. B) Amino acid sequence of H-NS from *E. coli*, indicating positions of mutations made in the present study. (Adapted from Arold et al., 2010)

through interactions that involve helices H3 and H4 in the central region of the protein (the interface called site 2; Arold et al., 2010; Shindo et al., 1995). To examine the role of multimeric state in the function of H-NS at the motor, we replaced hydrophobic residues at each of these interfaces with aspartate and measured the effects on function. Residues targeted were Leu8 and Leu33 in dimerization site 1 and Leu65 within site 2 (Figure 2.1B; Arold et al., 2010; Shindo et al., 1995). Mutant *hns* genes were expressed from a salicylate-inducible plasmid, in cells with the master regulators *flhDC* expressed from another, compatible plasmid. Initial experiments gave reason to believe that the *flhDC*-expressing plasmid used previously by Paul et al. (2011c) was faulty. This in fact proved to be the case, as will be described below. Experiments were also attempted with the plasmid used previously by Ko and Park (2000b), but in this case motility was unexpectedly strong, even in the absence of any H-NS. This suggested that the previous results, of both Paul et al. and Ko and Park, may have been confounded by the known influence of H-NS on the chromosomal *flhDC* genes still present in the strains. To allow cleaner separation of the effects of H-NS in the motor from its effects on *flhDC* expression, I constructed new strains having the chromosomal *flhDC* genes deleted and containing a different, better-tested plasmid construct to express *flhDC*. The results indicate that HNS is needed for optimal motility for reasons other than its regulation of *flhDC*, as concluded previously. Its contribution to motility is different from what was reported before, and does not appear specifically related to challenging conditions such as high concentration of agar or ionic strength.

Results

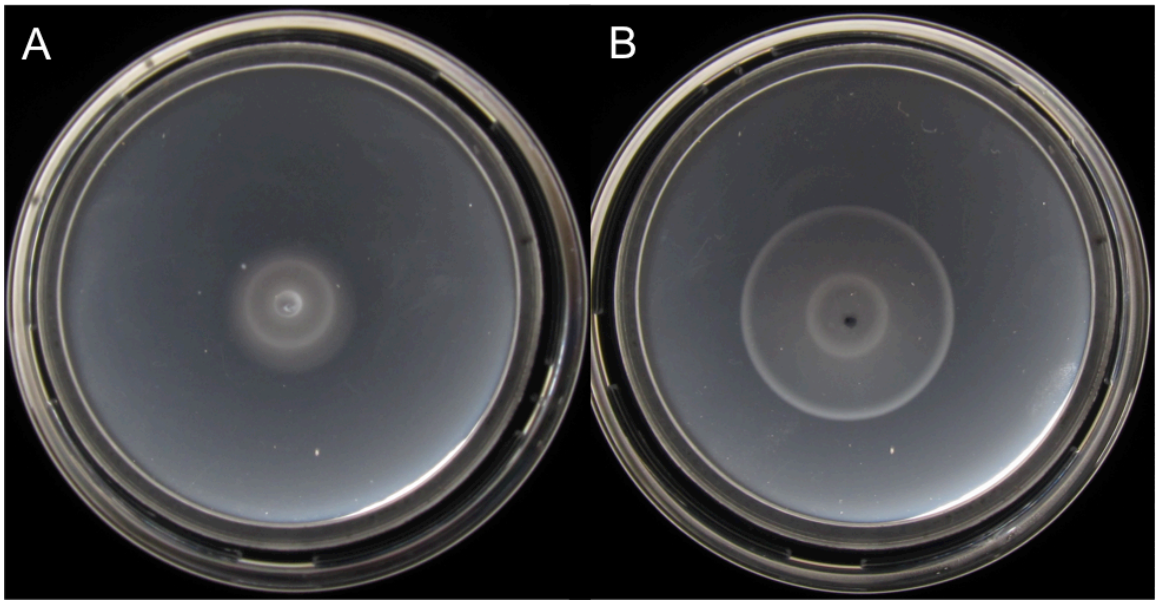
Unexpected behavior of Δ*hns* strains expressing *flhDC* from plasmids. As a foundation for the study of H-NS mutants, we re-tested the motility of Δ*hns* strains containing plasmids encoding *flhDC* under control of regulatable (IPTG or salicylate-inducible) promoters. Previous studies (Ko & Park, 2000b; Paul et al., 2011c) had indicated that in the absence of H-NS, motility remained poor even when *flhDC* was expressed from plasmids at levels supposedly sufficient for normal flagellar gene expression. Unexpectedly, Δ*hns* cells expressing *flhDC* from the salicylate-regulated plasmid pKP147 exhibited fairly strong motility even with the plasmid uninduced or induced at a low level (Table 2.1). Plasmid pKP141, used previously by Ko and Park, gave motility that was poorer than that seen with pKP147, but still better than expected on the basis of their report (where it displayed a severe impairment on motility plates). Similar results were observed whether the experiment used their original Δ*hns* strain or a Δ*hns* strain constructed in this laboratory in the RP437 background. Finally, whereas motility was unexpectedly strong with pKP147 or pKP141, the *flhDC* plasmid used by Paul et al., pKP85, did not restore any measurable motility to a Δ*hns* strain (Figure 2.2).

Sequencing showed that pKP141 expresses FlhD with an additional 15 residues at the N-terminus. A reconstruction of events (as described in the methods of Ko and Park, 2000b) indicates that the construct is as intended, and resulted from the use of available restriction sites in various vectors rather than PCR inserting the gene. Plasmid pKP85 was found to encode an out-of-frame fusion to several additional N-terminal residues, and would not be expected to express *flhDC*. The cause of this defect is uncertain, but in any event the failure of pKP85 to express *flhDC* means that in the physiological

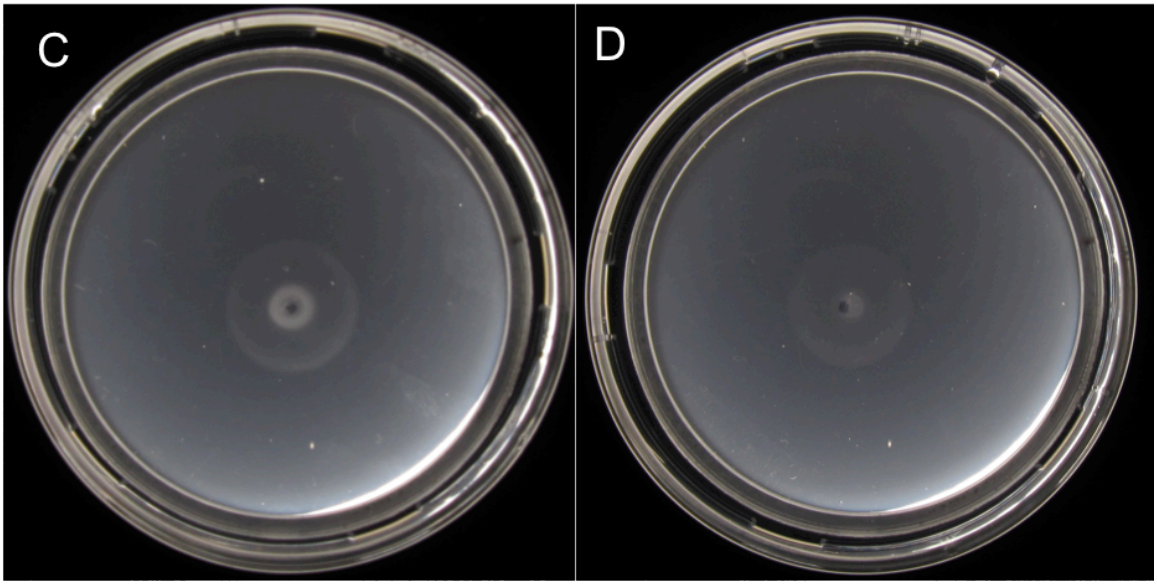
Table 2.1. Strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or property	Source or reference
Strains		
DB9 (RP437)	Wild-type strain	J.S. Parkinson
MS365	Wild type *E. coli* strain from Park; a.k.a. MS296	(Ko & Park, 2000a)
MS1162	Δ*hns* (hns:: FRT-FRT) in MS296	(Paul et al., 2011c)
EK1	Δ*flhDC* in MS365	This study
EK2	Δ*flhDC* in MS1162	This study
Plasmids		
pRR48	P*tac* expression vector; Amp^R	J.S. Parkinson
pKG116	P*salicylate* expression vector; Cm^R	J.S. Parkinson
pTM30	P*tac* expression vector; Amp^R	(Tang, Billings, Wang, Sharp, & Blair, 1995)
pKP58	*hns* in pKG116	(Paul et al., 2011c)
pKP85	*flhDC* in pTM30; Amp^R, out of frame	(Paul et al., 2011c)
pKP141	*flhDC* in pTRC99A; Amp^R	(Ko & Park, 2000b)
pKP147	*flhDC* in pKG116; Cm^R	
pKP148	*flhDC* in pRR48; Amp^R	
pKD46	Recombineering, carrying lamda-red recombinase gene (arabinose inducible) Amp^R	(Datsenko & Wanner, 2000)

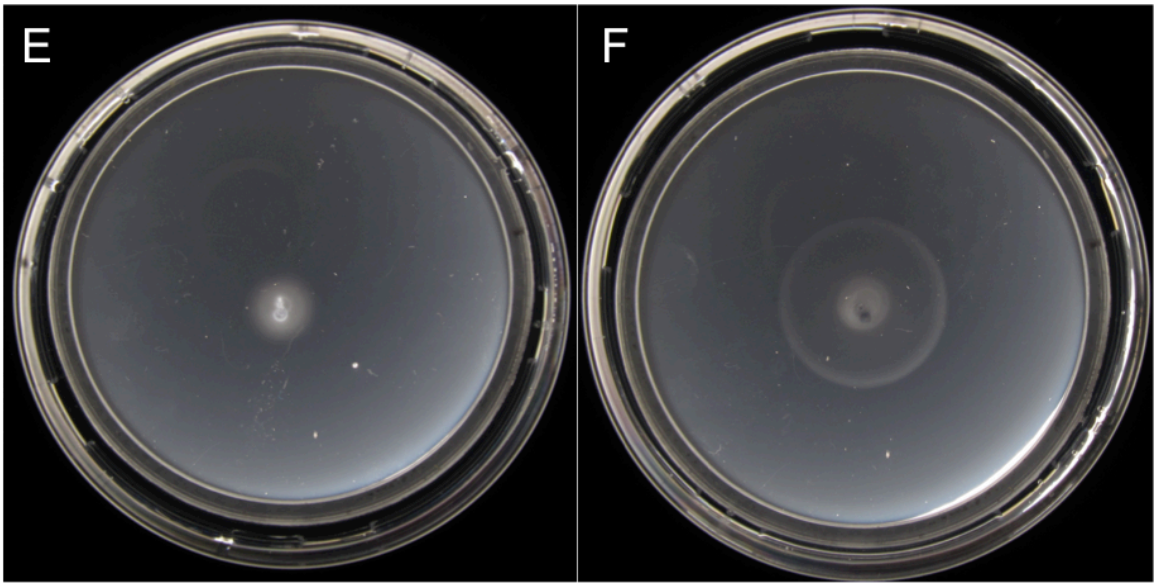
Figure 2.2 Comparison of *flhDC* plasmids. Plasmids were introduced into the chromosomal *flhDC* deletion strain, which was constructed in the wild-type background of Ko and Park(2000b). Cells were cultured overnight in TB containing the appropriate antibiotic, and 5-uL aliquots were spotted onto tryptone motility plates containing inducer at the indicated concentrations. Agar concentration was 0.26%. The plates were incubated at 32°C for 5 hrs. A) IPTG inducible *flhDC* expressing plasmid pKP148 without induction; B) IPTG inducible *flhDC* plasmid pKP148 induced with 1mM IPTG; C) IPTG inducible *flhDC* expressing plasmid pKP141without induction; D) IPTG inducible *flhDC* expressing plasmid pKP141 induced with 1mM IPTG; E) Na-salicylate inducible *flhDC* expressing plasmid pKP147 without induction; F) Na-salicylate inducible *flhDC* expressing plasmid pKP147, induced with 2.5μM Na-salicyte; G) IPTG inducible *flhDC* expressing plasmid pKP85 without induction; H) IPTG inducible *flhDC* expressing plasmid pKP85 induced with 1mM IPTG.



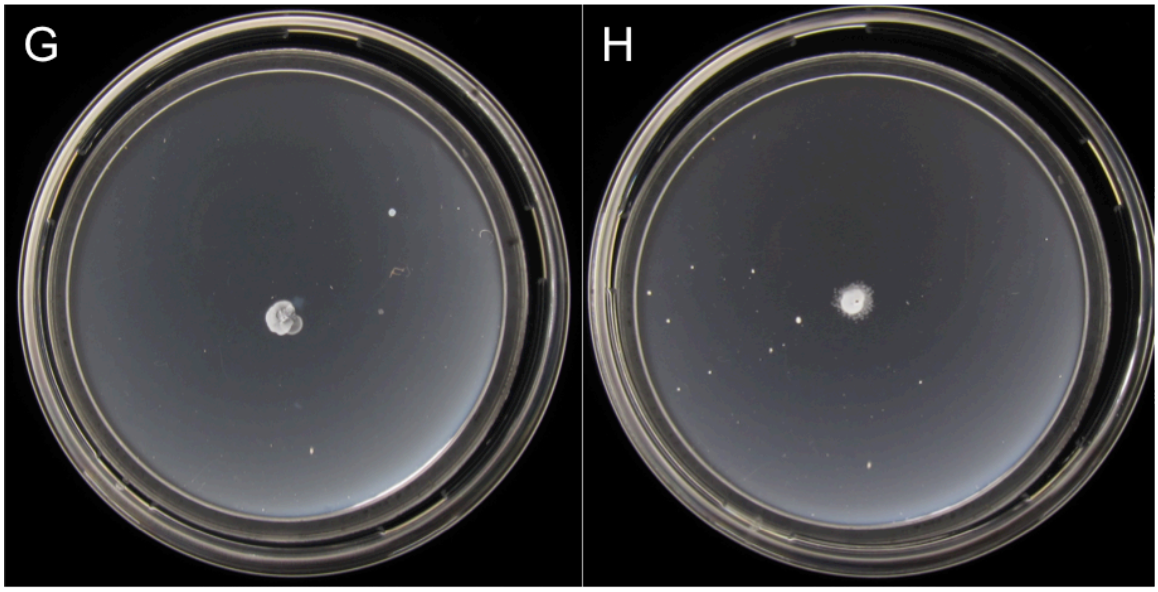
pKP148 (uninduced) **pKP148 (1mM IPTG)**



pKP141 (uninduced) **pKP141 (1mM IPTG)**



pKP147 (uninduced) **pKP147 (2.5 µM Na-salicylate)**



pKP85 (uninduced) **pKP85 (1 mM IPTG)**

Figure 2.2 continued

experiments of Paul et al. H-NS would have acted mainly to stimulate needed expression of chromosomal *flhDC*, preventing any interpretation of the results in terms of a direct action of H-NS at the motor. To address this shortcoming, new strains, with *flhDC* deleted from the chromosome, were made.

***flhDC* and Δ*flhDC* Δ*hns* strains.** The lambda-Red method was used to make deletions removing all but the first 4 codons of *flhD* and the last 26 codons of *flhC* (a fuller 3' deletion was tried initially but was found to remove sequences of *flhC* needed for expression of the downstream *mocha* operon). The *flhDC* plasmids were introduced into this strain, and motility was tested on soft-agar plates (Figure 2.2).

Plasmid pKP141 of Ko and Park (2000b) gave fair complementation of the Δ*flhDC* strain. pKP147 showed stronger complementation, and was inducible with salicylate, as expected. Plasmid pKP85 failed to complement the *flhDC* deletion, whether uninduced or induced with 1 mM IPTG (Table 2.2). Complementation was most effective with plasmid pKP148, which restored motility comparable to or better than the wild-type when induced with 0.5 mM IPTG or above (Figures 2.2 and 2.3).

Revisiting the question of H-NS action in the motor. To re-examine the role of H-NS in the flagellar motor, we made the same *flhDC* deletion in the Δ*hns* background. The *flhDC* genes were expressed from plasmid pKP148, with IPTG induction at various levels, and *hns* was expressed from the compatible plasmid pKP58, inducible with salicylate.

The strain was first used in an experiment to look at agar-concentration effects. Previous work had suggested that H-NS greatly improves motility of cells moving through agar of relatively high concentration. An example experiment is shown

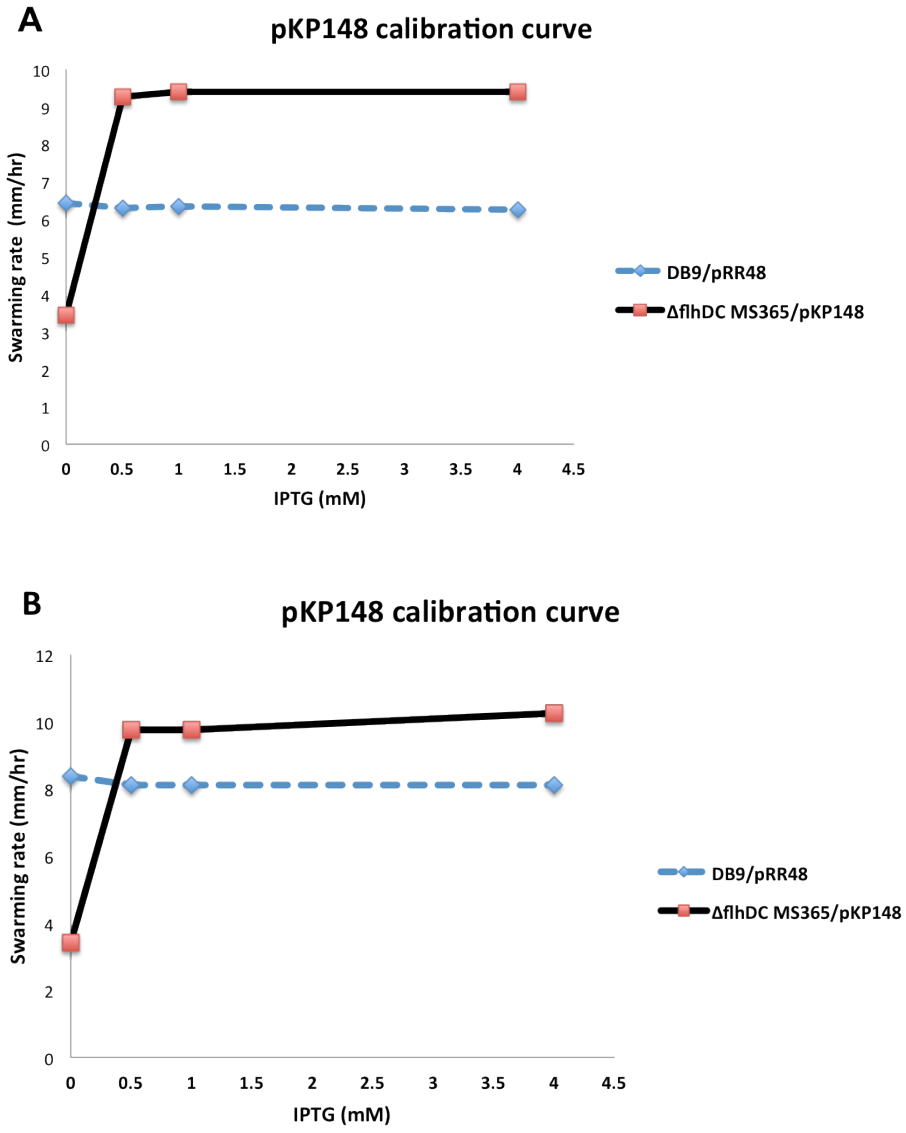


Figure 2.3. Complementation of the Δ*flhDC* strain by plasmid pKP148 induced with IPTG at various levels. Comparison is to a wild-type strain (DB9, identical to RP437) containing the control (parent) vector pRR48. Results of two experiments are shown. A) The first time pKP148 calibration, B) The second time pKP148 calibration.

in Figure 2.4, and results are summarized in Figure 2.5. Induction of the plasmid-encoded H-NS enhanced motility in the soft-agar plates, ($p=0.0061$ for samples induced with 4 mM IPTG, 2.5 μM Na-sal. and agar at 0.26%; $p=0.019$ for those induced with 4 mM IPTG, 2.5 μM Na-sal. and agar at 0.30%) but the effect was most pronounced in low agar concentrations, becoming increasingly less important as agar concentration increased from 0.26% to 0.38%. In the highest-concentration plates, cells failed to move whether or not H-NS was present. Thus, the previous finding of increased H-NS importance in high-concentration agar is not confirmed, and it appears instead that H-NS

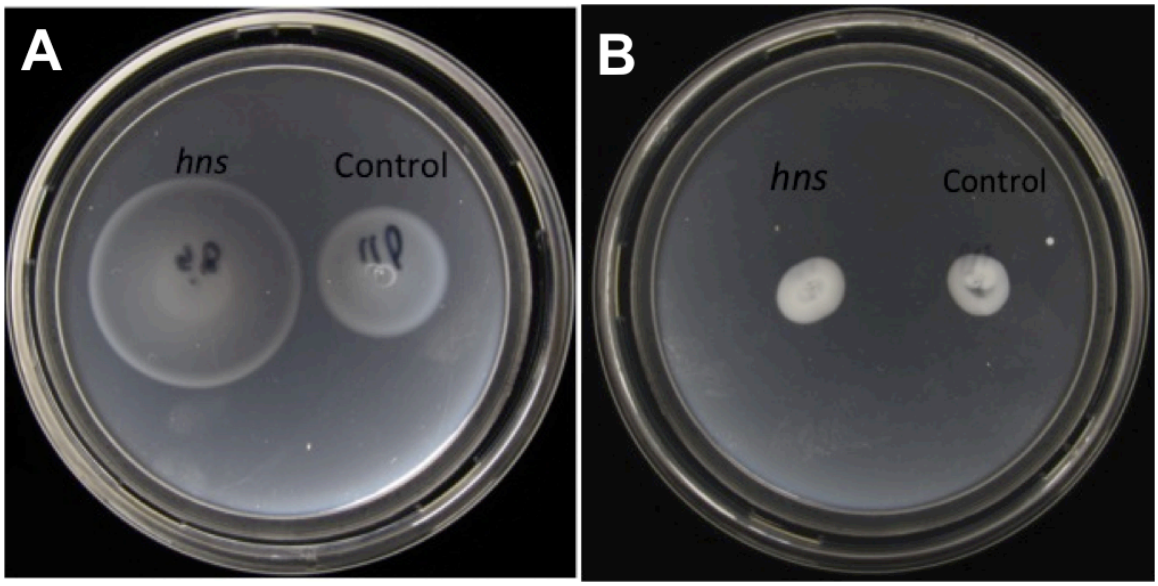
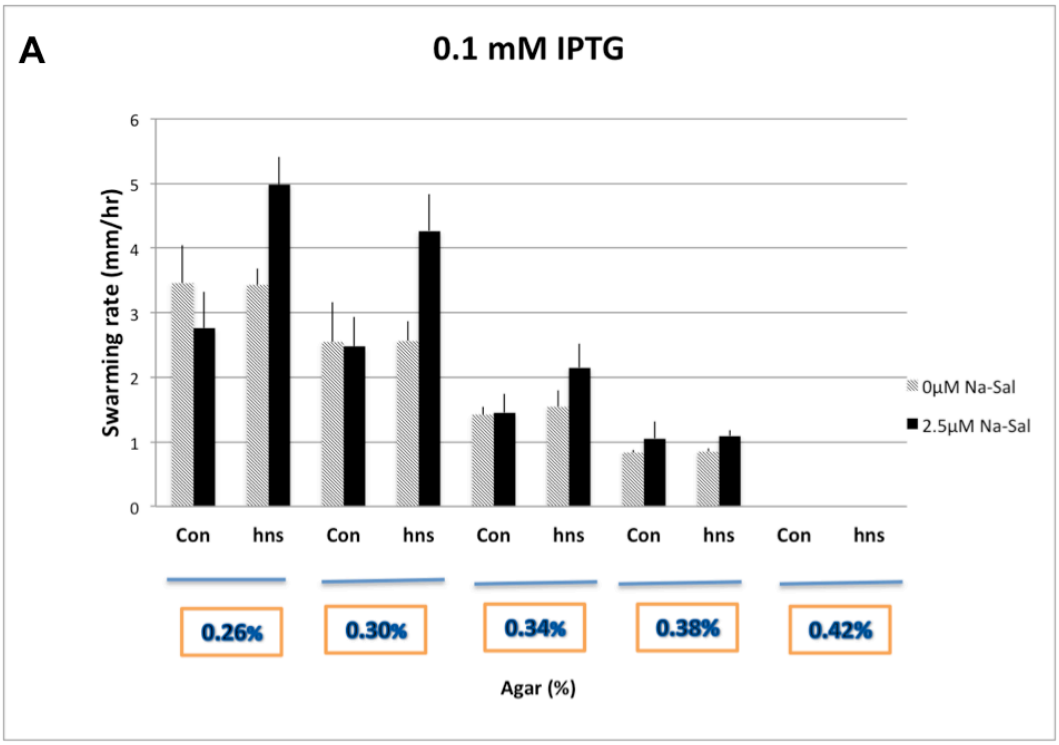
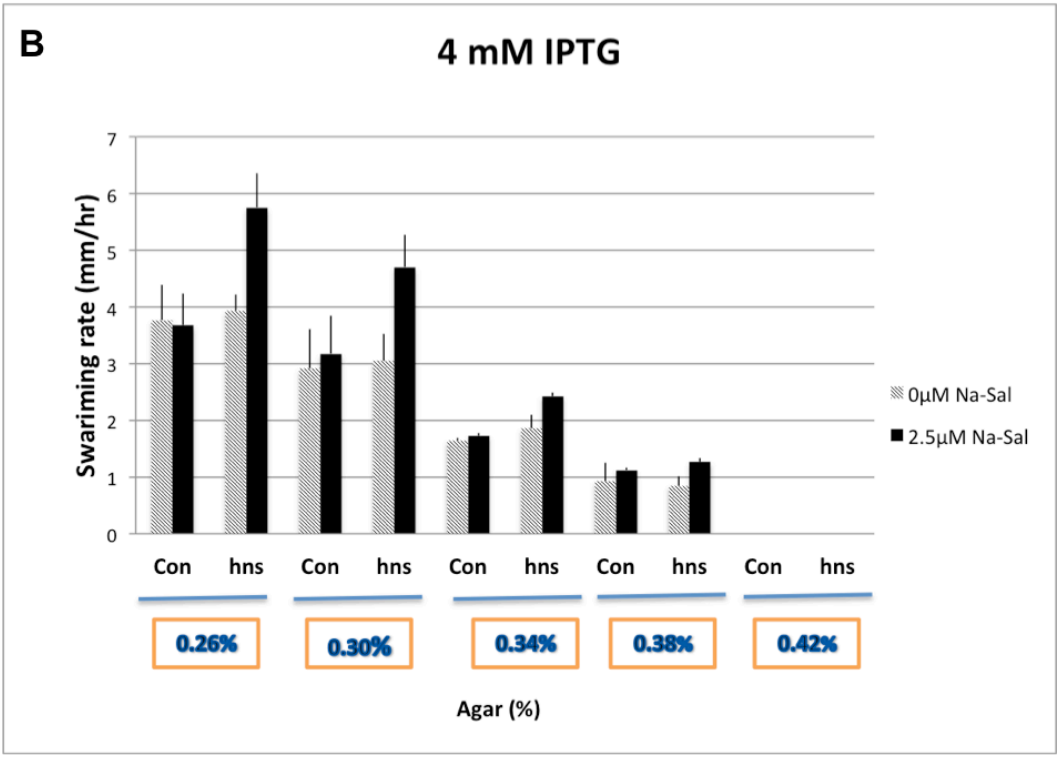


Figure 2.4) Example of the effect of H-NS on motility in soft-agar plates. (A) 0.26% agar. Plate contained 4 mM IPTG 2.5 uM Na-salicylate. The plate was incubated at 32°C for 9 hrs. (B) 0.42% agar, with IPTG at 4 mM and Na-salicylate at 20 μM. Results were similar (also negative) with induction of H-NS at a lower level. The plate was incubated at 32°C for 15 hrs.

Figure 2.5 Effect of H-NS on motility in plates containing agar at various concentrations. (A) FlhDC induced with 0.1 mM IPTG. (B) FlhDC induced with 4mM IPTG. Con, strain containing the control parent vector pKG116; *hns*, strain containing the *hns*-expressing plasmid pKP58.


A
0.1 mM IPTG
Swarming rate (mm/hr)
0
1
2
3
4
5
6
Con
hns
Con
hns
Con
hns
Con
hns
Con
hns
0.26%
0.30%
0.34%
0.38%
0.42%
Agar (%)
0μM Na-Sal
2.5μM Na-Sal


B
4 mM IPTG
Swariming rate (mm/hr)
0
1
2
3
4
5
6
7
Con
hns
Con
hns
Con
hns
Con
hns
Con
hns
0.26%
0.30%
0.34%
0.38%
0.42%
Agar (%)
0μM Na-Sal
2.5μM Na-Sal

contributes most to motility in plates with relatively low agar concentration.

Effects of ionic strength were examined next. In the previous report (87), H-NS was found to enhance motility, especially in plates containing more than the usual amount of salt. Here, effects of H-NS were compared in standard tryptone plates ([NaCl]~ 85 m*M*) and in plates with 0.3 *M* NaCl. Results are shown in Figures 2.6 and 2.7. Experiments used the Δ*flhDC* Δ*hns* strain, with *flhDC* expressed from plasmid pKP148 as before.

In standard motility plates, with agar at 0.26%, induction of H-NS again caused a roughly 40%-50% increase in the rate of spreading relative to the strain without H-NS ($p=0.0015$ for samples induced with 0.1 mM IPTG and 2.5 μM Na-sal.; $p=0.0022$ for those induced with 4 mM IPTG and 2.5 μM Na-sal). Motility was decreased somewhat in plates containing 0.3 M NaCl. The motility reduction was similar in cells with or without H-NS. This motility enhancement by H-NS does not appear to be specifically related to motor operation at high ionic strength, in contrast to previous suggestions.

Measurements of motility under various conditions of agar and salt concentration thus reinforce the proposal that H-NS contributes something to motility in addition to its stimulation of *flhDC* expression, but do not support the hypothesis that it serves to improve motor function under the challenging conditions of high agar- or salt-concentration. Previous results were very likely due to the involvement of H-NS in enabling *flhDC* expression, and the more-justified conclusion would be that depressed levels of FlhDC (resulting from the lack of any *flhDC* expression from the plasmid) are especially detrimental to motor function under those challenging circumstances.

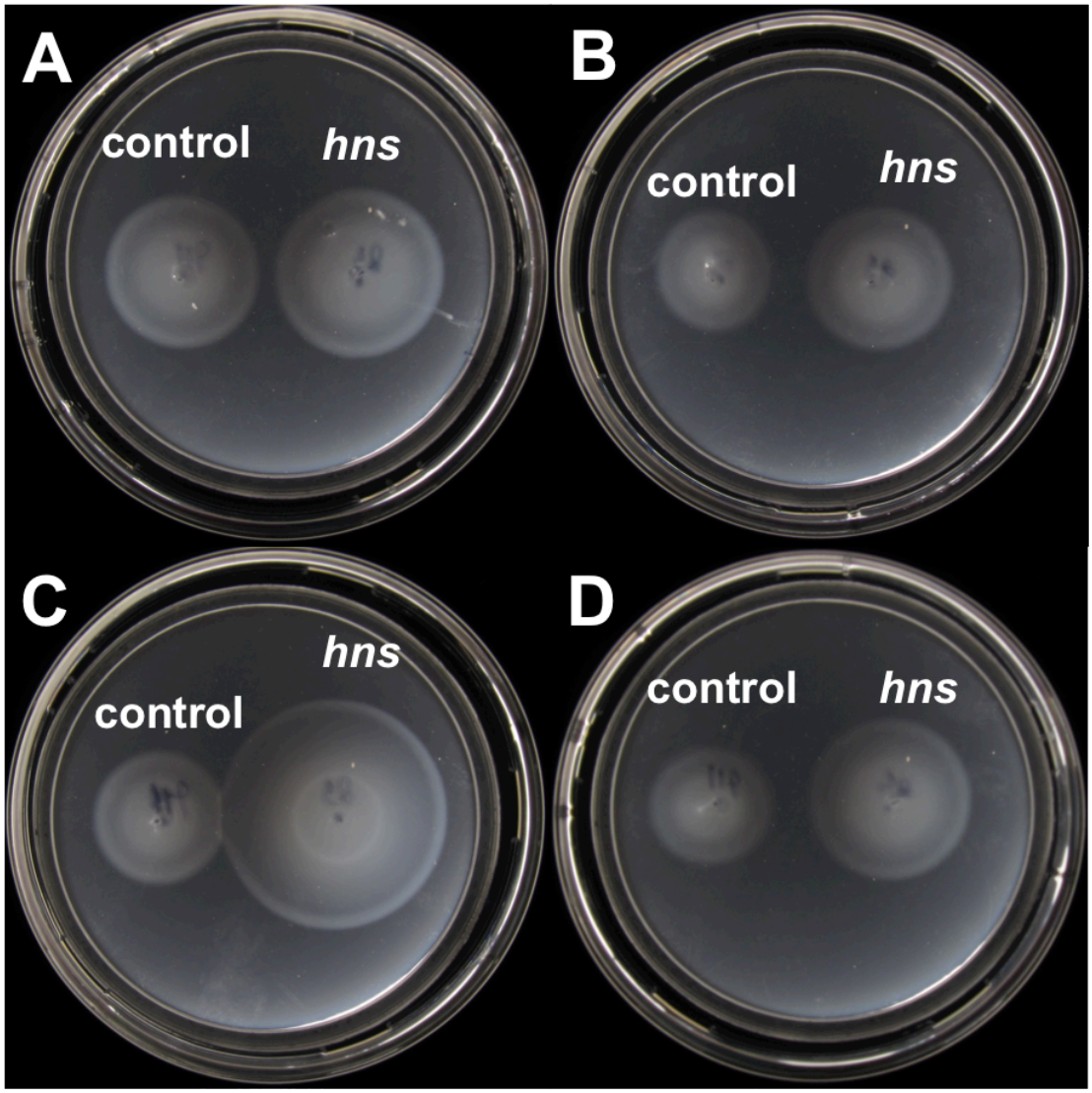


Figure 2.6. Effect of HNS on motility in soft-agar plates containing NaCl at 85 mM (panels A and C) or 0.30 M (B and D). H-NS was not induced in plates A and B, and was induced with 2.5μM Na-sal in plates C and D. FlhDC was induced with 4 mM IPTG in all plates. Agar concentration was 0.26%. Plates were incubated at 32°C for 7hrs. Con, strain with parent vector pKG116; *hns*, strain with pKP58.

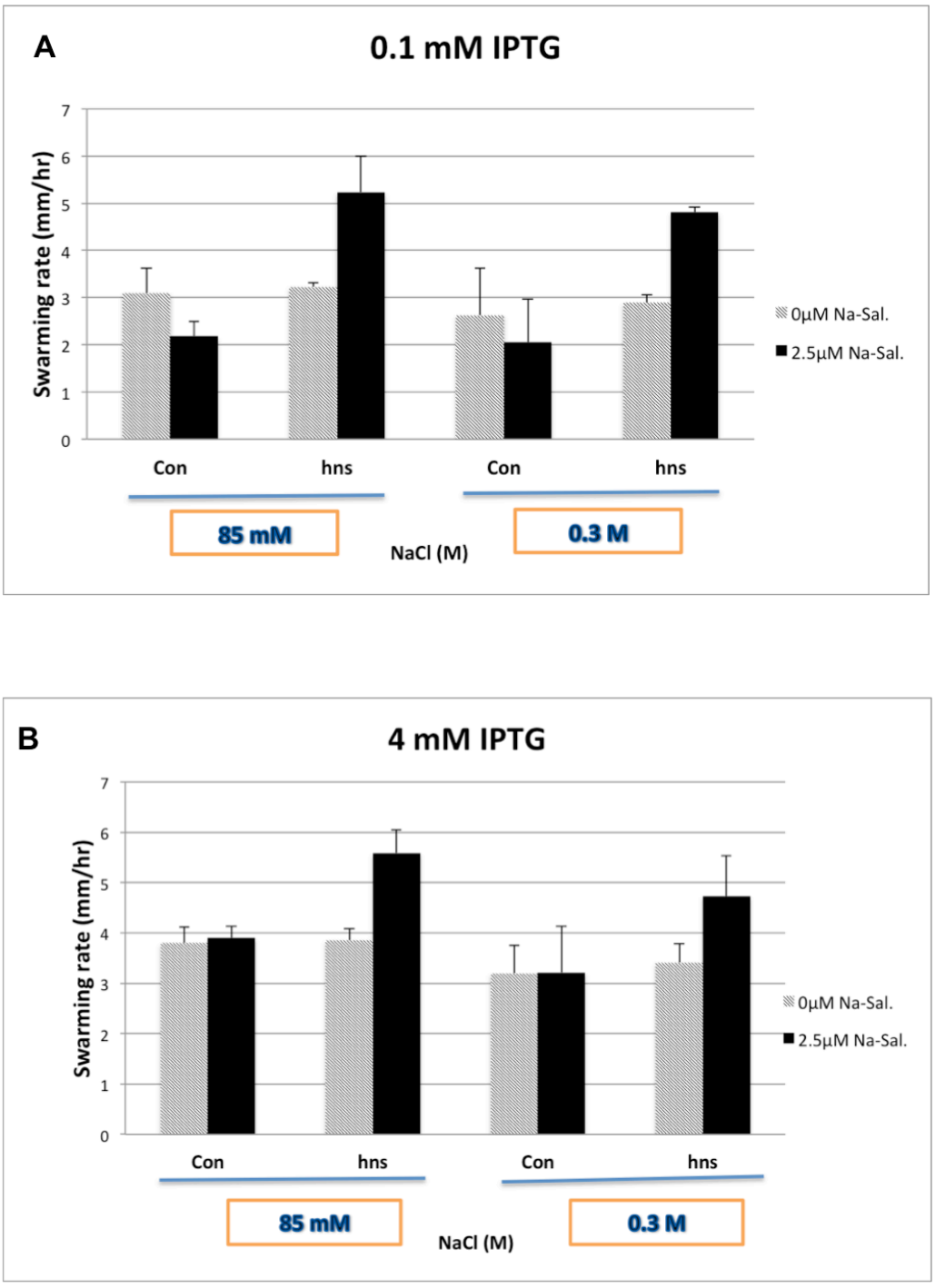
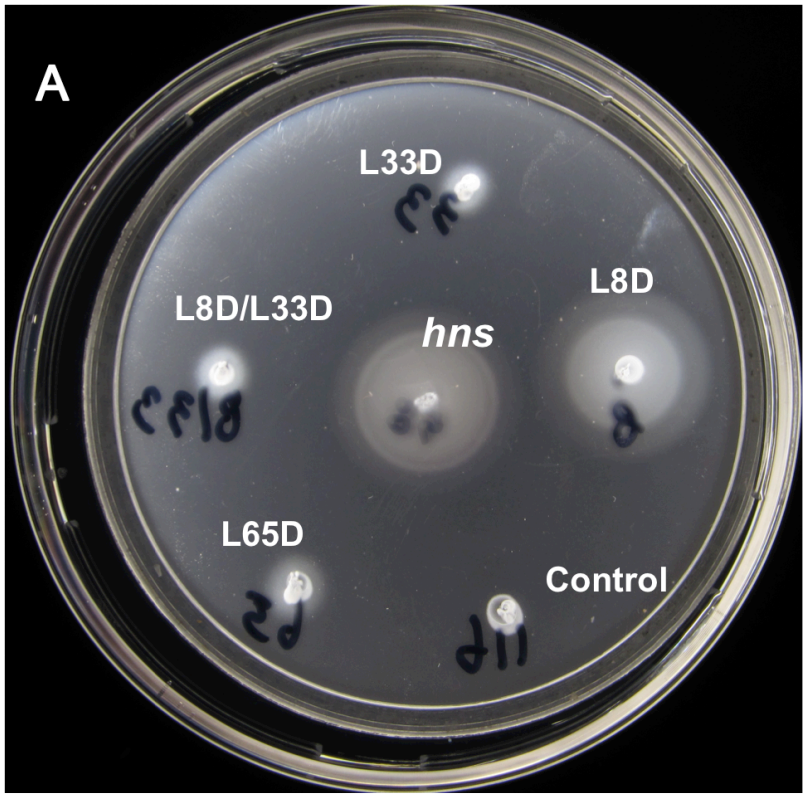


Figure 2.7. Summary of H-NS effects in low and high salt, with FlhDC expressed at a low level (0.5 mM IPTG, panel A) or high level (4 mM IPTG, panel B).

Functional importance of H-NS self-association. As discussed above, the initial goal of the experiments was to assess the role of H-NS self-association in its hypothesized function in the motor. To address this, H-NS structural data are available to guide a rational mutagenesis-based approach to destabilizing H-NS dimers and multimers by altering hydrophobic residues at the relevant interface. We made H-NS mutations that replace hydrophobic residues at the interfaces with aspartic acid (L8D, L33D, and L8D/L33D at site 1; L65D at site 2) and measured the effects on H-NS function, once again using soft-agar motility plates. Function was tested both in the Δ*hns* background and in the Δ*flhDC* Δ*hns* strain with *flhDC* expressed at various levels from plasmid pKP148. Results are presented in Figures 2.8 and 2.9.

In the Δ*hns* background, where the plasmid-expressed H-NS is needed for induction of chromosomally encoded *flhDC*, all of the mutations except L8D caused strong motility defects, even with the *hns* plasmid induced strongly using 20 μM Na-salicylate. Both interfaces thus appear important for the function of H-NS in this background, where it acts primarily to stimulate expression of *flhDC*. This accords with current thinking on the molecular basis of transcriptional repression by H-NS, which is hypothesized to involve large multimers of H-NS that associate cooperatively to 'cover' large regions of DNA (Arold et al., 2010; Shindo et al., 1995; Spurio et al., 1997; Tupper et al., 1994). Although Leu8 is partially buried in site 1, it is significantly more exposed than Leu33, and also makes fairly extensive contacts with itself (i.e., the same residue in the other subunit). Replacement of Leu8 by aspartate might therefore be tolerated, particularly if one or both of the Asp residues is protonated (Asp and Leu have similar volumes, and since the introduced Asp residues would be in proximity to each other, an

Figure 2.8 Effects of H-NS mutations on motility. (A) Motility in the Δ*hns* background. Plates contained 0.26% agar and 20 μM Na-salicylate to induce the chromosomally plasmid encoded *hns* gene. (Plate incubated at 32 °C for 9 h.) (B) Motility in the Δ*hns* Δ*flhDC* background, with *flhDC* expressed from plasmid pKP148 induced with 4 mM IPTG. The plate contained 0.26% agar and 2.5 μM Na-salicylate. Results were similar with higher-level (20 μM) induction of *hns* (data not shown). (Plate incubated at 32 °C for 6 h.)


A
L33D
L8D
L8D/L33D
hns
L65D
Control

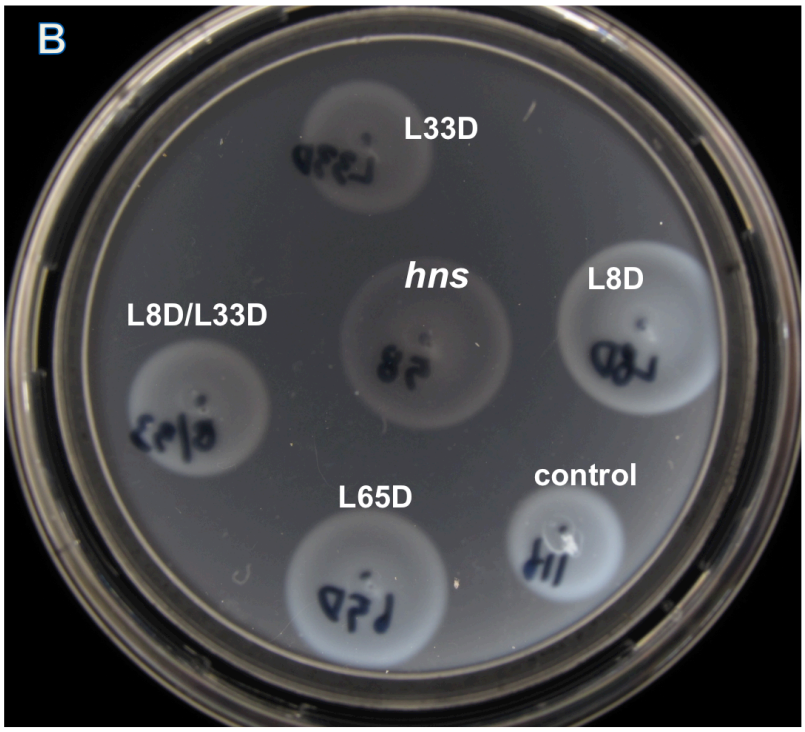
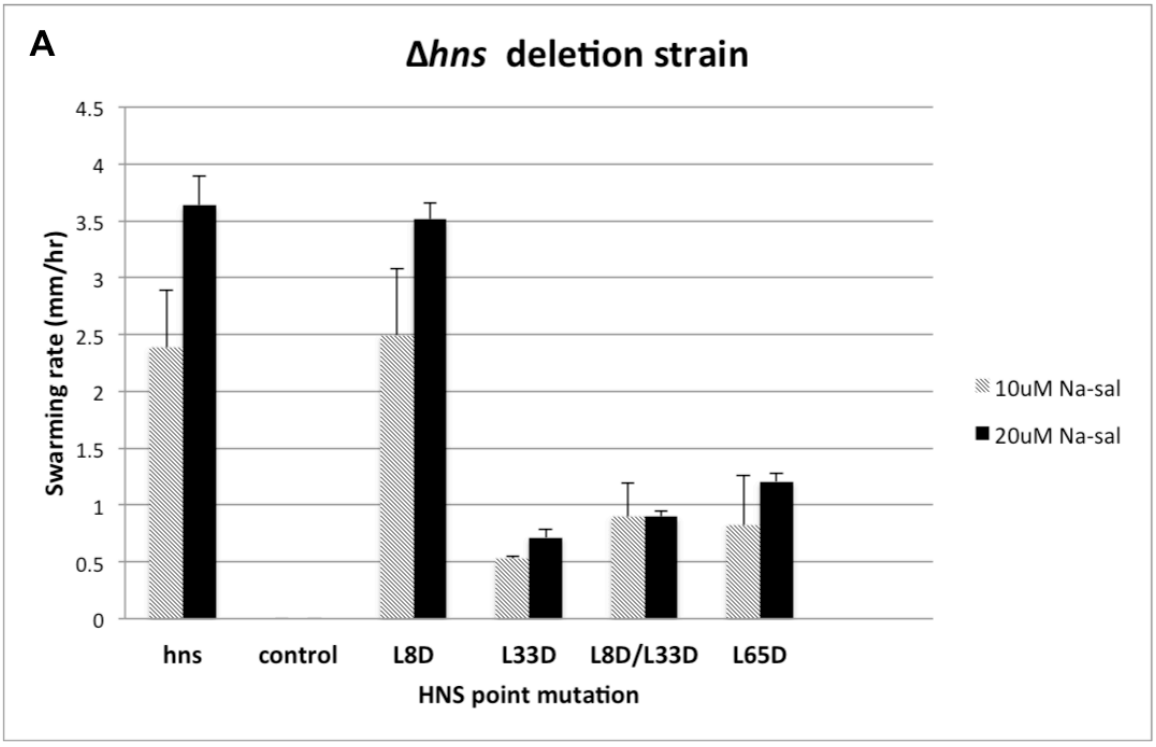
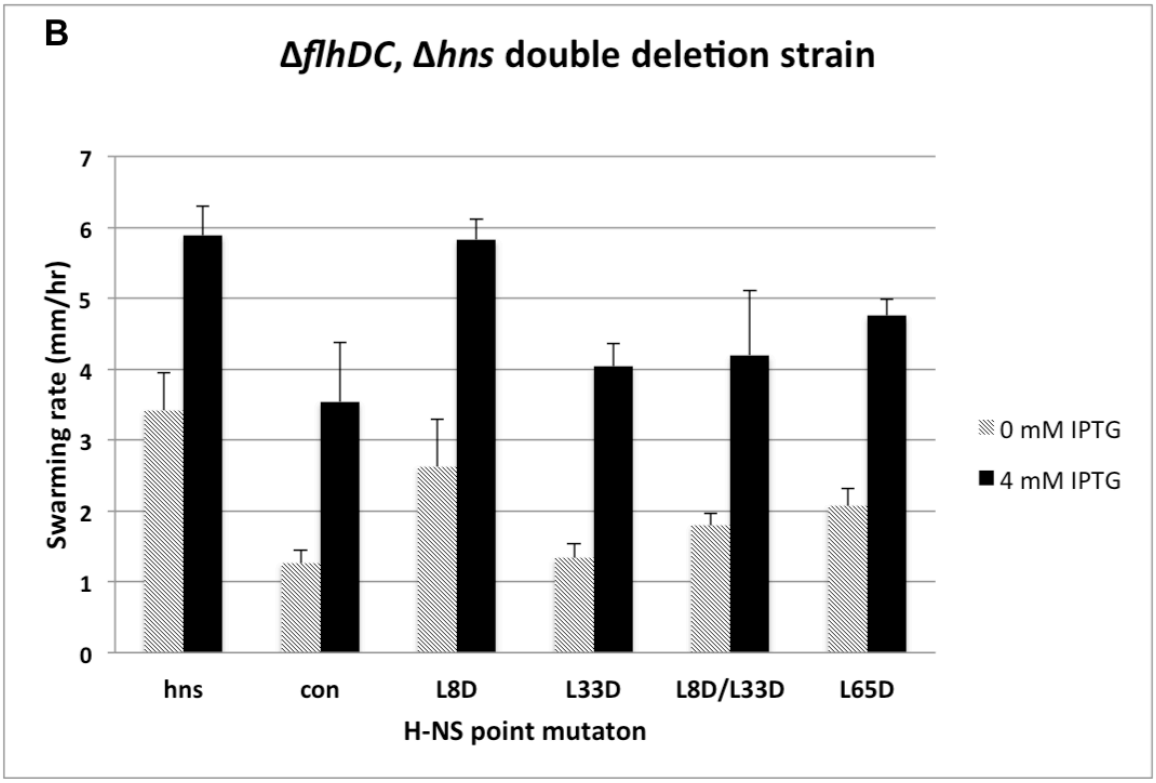

B
L33D
hns
L8D
L8D/L33D
control
L65D

Figure 2.9. Effects of mutations at the H-NS self-association interfaces. (A) Motility in the Δ*hns* background, at two levels of induction of H-NS by Na-salicylate. (B) Motility in the Δ*hns* Δ*flhDC* background, with *flhDC* expressed from pKP148 at two different levels. Experiments used 2.5 μM Na-salicylate to induce expression of the H-NS proteins.


A
Δhns deletion strain
Swarming rate (mm/hr)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
hns
control
L8D
L33D
L8D/L33D
L65D
HNS point mutation
10uM Na-sal
20uM Na-sal


B
ΔflhDC, Δhns double deletion strain
Swarming rate (mm/hr)
7
6
5
4
3
2
1
0
hns
con
L8D
L33D
L8D/L33D
L65D
H-NS point mutaton
0 mM IPTG
4 mM IPTG

OH---O hydrogen bond might take the place of what was formerly a hydrophobic contact). The L33D mutation at the same interface caused a large reduction of motility, as did the L65D mutation at site 2 (still in the Δ*hns* background).

Experiments in the double-deletion background with *flhDC* expressed from pKP148 allowed us to study the effects of the interfacial replacements on the other (putatively motor-related) function of H-NS (Figure 2.8). With FlhDC expressed at either a moderate (0.5 mM IPTG) or high level (4 mM), and agar and salt at normal levels, a substantial motility improvement was once more seen upon induction of plasmid-borne wild-type *hns* (almost two-fold in this set of experiments). The growth rate of all mutants, however, showed no significant difference from the wild-type (*hns*; strains, growth rates, and p-values: wild-type *hns*, 1.090 gen./hr , $p>0.05$; no *hns*, 1.068 gen/hr, $p>0.05$; L8D, 1.074 gen/hr, $p>0.05$; L33D, 1.045 gen/hr, $p>0.05$, L8D/L33D, 1.073 gen/hr, $p>0.05$; L65D, 0.953 gen/hr, $p>0.05$ induced with 4 mM IPTG and 2.5 μM Na-sal.) The mutations in *hns* caused various levels of impairment, all mild compared to those in the Δ*hns* background. The L8D mutation was, again, without any effect. The L65D replacement caused a mild (approximately 19%) motility reduction, and the L33D and L33D/L8D double mutants had approximately 31% and 29% motility reduction. ($p=0.0017$ for L33D ; $p=0.0069$ for L65D ; $p=0.213$ for L8D/L33D induced with 4mM IPTG and 2.5 μM Na-sal.) The pattern was the same whether *flhDC* were moderately (0.5 mM IPTG) or strongly induced (4 mM IPTG). The milder effects of the site 2 replacement might indicate that this interface, while essential for the DNA-related functions of H-NS, may be less important for its motility function(s).

Discussion

An unexpected finding from this study was that plasmids used previously to express *flhDC* do not in fact express functional proteins at acceptable levels. When a suitable Δ*flhDC* strain is used to assess pKP85 function, the plasmid gives only a very slight indication of motility, only upon strong induction. The maker of the plasmid asserts that its ability to complement a Δ*flhDC* strain was tested (though using a different strain than here). The plasmid was reprepped from storage strains and was found to be the same, in both its sequence and the failure to complement. The origin of the problem and the failure to detect it earlier are thus mysterious. In contrast with the problems encountered with pKP85, plasmid pKP141 (a renamed but unaltered version of the plasmid used by Ko and Park (2000b)) was found to give unexpectedly strong motility when tested in the Δ*hns* background. In the work of Ko and Park, this same strain produced flagella at close to the wild-type level yet remained only very poorly motile. They also found that the motility varied in severity according to the genetic background, with motility substantially improved in a Δ*hns* strain derived from a different wild-type strain called OW1 (Ko & Park, 2000b) We examined a strain reconstructed from the same components as Ko and Park's (generously provided by Dr. Park), yet found motility stronger than expected. We cannot presently account for this stronger motility, but suggest that it might be taken as an indication that the motility defect arises from a particular combination of circumstances, probably including relatively weak expression of *flhDC*. In the *flhDC*-deletion background, the plasmid did not show strong complementation, in spite of being based on a pTrc99A vector that should enable very strong expression. We suggest that this failure to complement well

might be due to the nonnative character of the FlhD protein encoded by the plasmid, which is predicted to contain 16 additional residues at the N-terminus as a result of the construction method.

Present results obtained using the Δ*flhDC* Δ*hns* strain should be more reliable than those obtained before, and though the results are different in many respects, the conclusion appears to stand that H-NS participates in some motility-related function(s) other than its regulation of *flhDC* expression. Cells containing H-NS had stronger motility than cells without the protein, even in cells with *flhDC* expressed at what should be ample levels from a plasmid. The contribution of H-NS was found to be greatest in plates that allowed rapid movement, however, in contrast to the previous results suggesting greater importance in high-agar plates. Whereas the previous suggestion was that H-NS adjusts the motor for operation under high load or weakened rotor-stator interactions (i.e., high ionic strength), it seems more likely that H-NS either facilitates motor operation at high speed, or facilitates some step(s) in flagellar assembly so that cells in a motility plate are, on average, more highly motile. Further characterization of the motility characteristics, including some study of the kinetics of motility onset, may shed light on this question.

Alternatively, it is possible that H-NS plays some other, as yet uncharacterized roles in flagellar gene expression. While high-level expression of *flhDC* was not sufficient to restore optimal motility here, constitutive expression of other (class 2 or 3) operons might, if H-NS has a role in regulating one or more of the downstream operons in addition to its well-documented involvement in *flhDC* regulation.

Although experiments of Paul et al. were taken to indicate that H-NS must act as a

dimer or larger multimer in the motor, that conclusion must also be discounted in light of the difficulties with *flhDC* expression. The present results with interfacial-residue mutants appear to indicate that self-association is important for the function of H-NS: A mutation at dimerization site 1 (L33D) impaired the function of H-NS not only in the Δ*hns* strain where it functions mainly to stimulate *flhDC* expression, but also in the Δ*hns* Δ*flhDC* strain with *flhDC* expressed constitutively. The mutation at site 2 (L65D) showed somewhat different behavior, causing a severe motility defect in the first strain but a milder reduction of motility (compared to wild-type H-NS) in the latter strain. This could indicate either that a dimer of H-NS (associated through site 1) is sufficient for the hypothesized function of H-NS in the motor, or that the L65D mutation only partially disrupts association through site 2 (enough to prevent multimer formation on DNA, but still allowing sufficient oligomer formation for the motility function). Further work, including the study of additional mutants at site 2, will be needed to address this question. Also, it will be important to ascertain the stability of the various mutant proteins, by use of suitably tagged constructs. Experiments along these lines, and other proposals for continued study, are outlined in the concluding chapter.

Materials and methods

Strains and media. The *E. coli* strains and plasmids used are listed in Table 2.1. Procedures for transformation and plasmid isolation were described previously (Tang et al., 1995; Tang & Blair, 1995). TB contained 10 g tryptone and 5g NaCl per liter, and LB contained these plus 5 g yeast extract. Ampicillin was used at 125 μg/ml in liquid medium and 50 μg/ml in motility plates. Chloramphenicol was used at 50 μg/ml in liquid

medium and 12.5μg /ml in motility plates. IPTG was prepared as a 0.1 M stock in water and used at the concentrations indicated. Sodium salicylate was prepared as 10 mM aqueous stocks and used at the concentrations indicated. DNA sequencing and oligonucleotide synthesis were carried out by core facilities at the University of Utah.

Site-directed mutagenesis. Site-directed mutagenesis was carried out using the Quick change protocol (Stratagene, LA Jolla, CA) on the *hns* gene encoded in plasmid pKP58, and mutations were confirmed by sequencing.

Motility assay. Swimming motility in soft-agar plates was measured as described (Tang et al., 1995), using various deletion strains (Table 2.1) and corresponding plasmids as described in the figures. Motility plates contained tryptone, agar at 0.26% unless indicated otherwise, antibiotics, and inducers (IPTG or Na-salicylate) at the concentrations indicated in the figures or figure legends.

To examine the effects of the *hns* mutations in the "FlhDC-constitutive" background, cells of the Δ*flhDC* Δ*hns* containing the *flhDC*-expression plasmid pKP148 were transformed with the pKP58 mutant variants. Controls were transformed with pKG116, the parent of pKP58. Other experiments used just the Δ*hns* strain, transformed with the pKP58 variants (or the pKG116 control).

The same procedures were used in the experiments examining agar and salt effects, except the composition of the plates was changed as indicated in the text and figures. Plates made according to the standard recipe contain 0.5%, or nominally about 85 mM, NaCl.

Construction of *flhDC* deletions. Chromosomal deletions were introduced using the lambda-red method (Dean, Macnab, Stader, Matsumura, & Burke, 1984). This

involved amplifying the *tetRA* genes using primers that included ~40 bp of homology to the targeted upstream and downstream regions. Amplified *tetRA* DNA was introduced by electroporation into target strains that were previously transformed with plasmid pKD46, encoding the lambda recombinase under control of the *ara* promoter. Integrates were selected on tetracycline (5 mg/ml) plates, and recultured on LB plates at 42° C to drive loss of the pKD46 plasmid, which has a temperature sensitive origin of replication.

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTION

H-NS makes a contribution to motor functions

A number of studies suggested that H-NS, in addition to its DNA-binding function and the associated effects on gene expression (Blot et al., 2006; Dorman, 2004; Drlica & Rouviere-Yaniv, 1987; Gualerzi et al., 1986; Spassky et al., 1984; Ueguchi & Mizuno, 1993; Ueguchi et al., 1996; Yoshida, Ueguchi, Yamada, & Mizuno, 1993), might act directly at the flagellar motor. Paul et al. suggested, specifically, that H-NS might stabilize the flagellar rotor so that productive rotor-stator interactions are maintained under functionally challenging conditions such as increased load or ionic strength. The present experiments, using strains constructed to more cleanly separate the gene-regulatory from other functions of H-NS, show that H-NS does contribute to motor function, but this contribution does not appear to be specifically related to conditions of high ionic strength or agar concentration. The precise action of H-NS remains to be determined. It might facilitate steps in assembly, for example, by organizing FliG subunits to enable more-rapid installation of other switch-complex proteins. Alternatively, H-NS might organize rotor subunits so as to facilitate installation of the stators, which interact with FliG or regulate other flagellar genes. With the strains now available, it should be possible to study the contributions of H-NS further to unravel the molecular nature of its contributions to motility.

Previous experiments (Paul et al., 2011) were taken to indicate that H-NS must act as a dimer or larger multimer in the flagellar motor. (Paul et al., 2011c). In light of the findings here, we suggest that those experiments mainly reported on the level of *flhDC* expression, rather than any function in the motor, and the results reflected the well-accepted requirement for H-NS self-association in its DNA-binding roles. In the present

experiments, it was found that the specific motility-related function(s) of H-NS do appear to require at least dimer formation, and possibly the formation of larger multimers. Whereas the L65D mutant of H-NS failed to support function in the Δ*hns* background, its effect was milder in the Δ*hns* Δ*flhDC* background with plasmid-borne *flhDC* expressed from a non-native promoter. The strong defect in the Δ*hns* background indicates that the gene-regulatory properties of the protein are disrupted in this mutant, presumably because dimerization through the interface is disrupted or at least weakened significantly. The retention of much of the motility-specific function (as measured in the Δ*hns* Δ*flhDC* background) would then indicate that this function is less dependent on the site-2 interaction. In this interpretation, an H-NS dimer, formed by association at the site-1 interface, would suffice for the motility function. The study of additional site-2 mutations, including multiple mutations more certain to disrupt the interaction, could provide a further test of this proposal.

Future directions

In its role as a DNA-binding protein, H-NS acts as a sensor of fundamental environmental variables, binding DNA differentially in response to changes in osmolarity, pH and temperature (Dorman, 2004; Drlica & Rouviere-Yaniv, 1987; Gualerzi et al., 1986; Hommais et al., 2001; Spassky et al., 1984; Ueguchi & Mizuno, 1993; Ueguchi et al., 1996; Yoshida et al., 1993). This raises the possibility that the motility-specific function(s) of H-NS might also be responsive to one or more environmental parameters. Thus, further study of H-NS and its motility-related functions should include some study of the effects of temperature, osmolarity, and pH. H-NS is

especially important in cellular response to low temperature. Thus, it might be worthwhile examining the contributions of H-NS to flagellar function at low temperature, and in the recovery of motility following incubation at low temperature.

As mentioned above, H-NS might enhance motility in soft-agar plates by speeding steps in flagellar assembly. It might be possible to test this proposal in experiments using an inducible *flhDC* construct to induce flagellar synthesis in cells with or without H-NS, monitoring various motility-related functions such as the appearance of FliC (flagellin) in the medium, the growth of flagella (by EM), and the onset of motility. A key question, related to whether H-NS facilitates assembly or enhances function of the fully assembled flagellum, is whether or not the protein actually localizes to the mature basal body. To address this, fluorescent H-NS constructs might be used, in conjunction with advanced microscopic methods for protein localization.

Finally, as mentioned in Chapter 2, in addition to its known role in regulating *flhDC*, H-NS might have a role in regulating one or more of class 2 or class 3 flagellar operons. This possibility might be addressed using strains with reporter constructs in various class 2 and 3 operons, and experiments to measure the abundance of class 2 and class 3 mRNAs in *flhDC*-constitutive backgrounds having H-NS expressed at various levels. Also, H-NS might effect gene expression of other motility genes. To address this, real time PCR might be used for gene expression.

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