**Enhanced Binding of Altered H-NS Protein to Flagellar Rotor Protein FliG Causes Increased Flagellar Rotational Speed and Hypermotility in *Escherichia coli***

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H-NS is an *Escherichia coli* nucleoid protein known only to function as a modulator of gene expression. In this study, we found that specific single amino acid substitutions in H-NS caused an approximately 50% increase in flagellum rotational speed. In fluorescence anisotropy and chemical cross-linking assays, H-NS interacted with the flagellar torque-generating rotor protein FliG to form a complex with a *Kd* of 2.15 µM. Furthermore, one of the altered H-NS proteins that exhibited high speed flagellum rotation bound FliG 50% tighter than wild-type H-NS. These results demonstrate the first non-regulatory role for H-NS and provide a direct correlation between H-NS-FliG binding affinities, flagellar rotation, and motor torque generation.

Motility in many bacterial species is achieved by rotating surface-exposed organelles called flagella. In *Escherichia coli* and *Salmonella typhimurium* about 50 genes are required for the biosynthesis and operation of these peritrichous, multicomponent structures. Gene expression is hierarchical, whereby one class of genes must be turned on before the next class can be expressed. At the top of this cascade lies the *flhCD* master operon, whose expression is required for the expression of all other flagellar genes (reviewed in Refs. 1 and 2).

Flagellar filament rotation is controlled by a motor embedded in the inner membrane (reviewed in Refs. 1 and 3). Energy to drive this motor is derived from the transmembrane gradient of protons, or proton-motive force (4, 5). Bacteria swim by a random walk consisting of a series of runs and tumbles (6). When the motor rotates counterclockwise (CCW), flagella filaments form a tight bundle that propels the bacterium into a smooth swimming pattern. Conversely, during clockwise (CW) rotation, flagellar filaments separate, causing tumbling and directional reorientation (7).

The *E. coli* flagellar motor consists of a MotA-MotB stator complex which forms a transmembrane proton channel (8–10), and a rotor of three interacting proteins, FliG, FliM, and FliN (11, 12). All three rotor proteins are involved in the processes of flagellar assembly, switching, and rotation (13, 14). However, FliG is predominately involved in torque generation (12, 14, 15), whereas FliM mainly functions in switching rotor direction (16). The precise role of FliN is the least well defined, but it may participate in flagella protein-specific export and assembly (17, 18).

It has been shown that *hns* insertion mutations render bacteria non-motile, suggesting that H-NS is a positive regulator of flagellar gene expression (19, 20). H-NS is a 15.4-kDa nucleoid-associated DNA-binding protein (21–23) that modulates the expression of many unrelated genes in *E. coli* (24–26). In most instances, such as *fimB* (27) and *proU* (28) expression, H-NS acts as a direct transcriptional repressor.

Here we characterized two independent *hns* point mutations, *hns*T108I and *hns*A18E, in relation to *E. coli* motility. Rather than displaying a non-motile behavior, strains carrying these mutations exhibited an unprecedented hypermotility. This novel hypermotile phenotype was a result of enhanced binding of mutant protein H-NST108I to the flagellar rotor, causing increased flagellar rotational speed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Phage, Media, and Genetic Techniques**—Table I lists all bacterial strains, plasmids, and phage used. Media consisted of Luria-Bertani (LB) broth, LB agar, T broth (1% tryptone, 0.5% NaCl), and soft-agar (T broth, 0.3% agar) (Difco). Antibiotics were added to a final concentration of 100 µg (ampicillin), 20 µg (tetracycline), 20 µg (chloramphenicol), or 50 µg (kanamycin) per ml of medium. Isopropylthiogalactoside was used at a final concentration of 1 mM. P1 vir generalized transductions were carried out as described previously (29). Cultures were grown at 37 °C for protein purification and 30 °C for motility and flagella assays.

**Swarming and Motility**—AL127 was transformed with various plasmids. Fresh colonies were picked, inoculated onto low agar (0.3%) tryptone plates, and incubated at 30 °C. Swarming diameters were measured every 2–3 h, beginning 9 h post-inoculation. Differences in motility rates were calculated by comparing the swarm diameter of AL127 expressing an *hns* mutant plasmid relative to the same strain expressing a wild-type *hns* gene, on the same plate for three individual experiments.

**Flagellation**—Strains were grown at 30 °C overnight in T broth with the appropriate antibiotics and then diluted 25-fold into fresh media and incubated until the *A600* reached 0.3–0.5. Cells from these cultures were incubated on polylysine-coated coverslips for 15 min at room temperature, rinsed twice with phosphate-buffered saline, and fixed in 3% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.4). Bacteria were observed by dark field microscopy, recorded to videotape, played back at 1/5 the speed, and scored using The Observer 3.1 videotape analysis system software (Noldus Information Technology). Typically, 30 individual fields were quantitated for 30 s each for CWA or 1 min each for
flagellum rotational speed.

**Protein Purifications—*E. coli*** wild-type H-NS and H-NST108I were each purified as described before (27) except the mutant protein lysate was washed from the double stranded DNA-cellulose column at a lower salt concentration (125 versus 250 mM NaCl) than the wild-type cell lysate. His-tagged FlIG was nickel-affinity purified basically by the method of Toker and Macnab (31) from BL21(DE3) cultures harboring pET28NdefliG. Protein solutions were concentrated with Centricon-10 columns as instructed by the manufacturer (Amicon). Protein concentrations were measured with the Bio-Rad DC protein assay kit (Bio-Rad).

**Fluorescence Anisotropy—***H-NS* was labeled with the thiol-reactive probe, fluorescein 5-maleimide, after reduction with tris-(2-carboxyethyl)sulfosuccinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (Pierce) by incubating for 1 h at room temperature. Reactions were quenched by the addition of 1 M Tris (pH 7.5), H-NS (wild-type or T108I) was added, and the entire reaction was incubated another hour. The fluorescence reactions were terminated by the addition of 5 × Laemmli denaturing sample buffer. Reactions containing equal amounts of each protein (5 μM) were electrophoresed on a 4–20% denaturing gradient gel (Jule Biotechnologies, Inc.) and visualized by Coomassie or Sypro-Orange (Molecular Probes) staining. Complexes were quantitated free, uncomplexed H-NS protein.

**Chemical Cross-linking—***FlIG* was first attached to the heterobifunctional cross-linker, sulfosuccinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (Pierce) by incubating for 1 h at room temperature. Reactions were quenched by the addition of 1 M Tris (pH 7.5), H-NS (wild-type or T108I) was added, and the entire reaction was incubated another hour. The cross-linking reactions were terminated by the addition of 5 × Laemmli denaturing sample buffer. Reactions containing equal amounts of each protein (5 μM) were electrophoresed on a 4–20% denaturing gradient gel (Jule Biotechnologies, Inc.) and visualized by Coomassie or Sypro-Orange (Molecular Probes) staining. Complexes were quantitated free, uncomplexed H-NS protein.

**Antibody Production and Western Blotting—***A wild-type* H-NS and H-NST108I were non-motile. Motility was restored in these strains upon the addition of a wild-type *hns* clone in trans. However, the *hnsT108I* and *hnsA18E* mutations each conferred a greater than 2-fold increase (2.1 ± 21 and 2.5 ± 17, respectively) in swim rate as compared with the wild-type strain. These data represent the first instance in which any *E. coli* mutation, *hns* or otherwise, resulted in a hypermotile phenotype.

**Flagellation—***H-NS* is a positive transcriptional regulator of the *flhCD* operon, the master operon which controls expression of all other flagellar components (1). In the absence of *H-NS*, flagellar genes are not expressed resulting in the loss of intact flagella and motility (20). One explanation for the hypermotile phenotype that we observed with specific *hns* point mutations was that these alleles affected flagella biosynthesis and/or assembly. To test this hypothesis, we examined strains expressing different *hns* alleles by scanning electron microscopy (SEM). Visually, there was no discernible difference in cell size or shape or flagellum length or distribution between strains carrying a wild-type *hns* allele (Fig. 2A) or either *hns* mutation (Fig. 2B and C). There was also no statistical difference in the average number of flagella/cell between the control and *hnsA18E* strains and only a very slight increase expressed from the *hnsT108I* allele (Table II). We concluded that the increased motility rates exhibited by the *hnsT108I* and *hnsA18E* mutations each conferred a greater than 2-fold increase (2.1 ± 21 and 2.5 ± 17, respectively) in swim rate as compared with the wild-type strain. These data represent the first instance in which any *E. coli* mutation, *hns* or otherwise, resulted in a hypermotile phenotype.

**Flagellar Rotational Behavior—***E. coli* swim by rotating their flagella filaments (35, 36) either CCW or CW (7). Motion is an alternating series of smooth runs (CCW rotation) and abrupt directional changes called tumbles (CW rotation) (6). It is possible that the *hnsT108I* and *hnsA18E* mutations caused a shift in flagellar rotational bias resulting in hypermotility.

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Table I
Bacteria, plasmids, and bacteriophage used

| Bacteria | Plasmids/Phage | Source |
|----------|---------------|--------|
| AL127    | pMSS46-1      | This study |
| AL90     | pTHK116       | This study |
| RP437    | pE2T8NdefliG  | This study |
| RBB1041  | pET28NdefliG  | This study |
| RP1616   | pTHK113       | This study |
| BL21(DE3)| pQE-60        | This study |
| M15(pREP4)| pDMG1        | This study |

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2. G. M. Donate and T. H. Kawula, manuscript in preparation.
Strains bearing \( hns \) mutant alleles may “run” (versus tumble) a higher percentage of the time than their wild-type counterparts. To address this issue, we performed tethering experiments (30) to compare the rotational bias of strains containing wild-type or mutant \( hns \) genes. In this assay, individual cells harboring plasmid-based \( hns \) alleles were tethered to a microscope slide via an anti-flagella antibody. The spinning direction of cell bodies was observed and bias was calculated to be the proportion of the time cells spent rotating CCW (Table III).

Wild-type cells generally spin CCW with occasional pauses and direction reversals (36). Both of the wild-type controls (Table III, lines 1 and 4) adopted this spinning mode, rotating CCW 85% of the time. Additional controls included a “gutted” strain (RBB1041) which is deleted for the chemotaxis genes and spins only in the default CCW direction (37) (line 2), and a CW-biased \( cheZ \) mutant (RP1616) (38) which only tumbles (line 3). Strains with \( hns \) mutant alleles \( T108I \) or \( A18E \) (lines 5 and 6) did not favor a higher running (CCW) bias over wild-type strains. In fact, these strains along with two other \( hns \) mutations which did not exhibit a hypermotile phenotype (data not shown), tumbled or paused slightly more frequently than wild-type cells (lines 7 and 8). Thus, the \( hns \) mutations did not affect the function of the chemotaxis apparatus or the mechanism of flagellar switching.

**Flagellar Speed**—Flagella propel bacteria by rotating motor-driven helical filaments (35, 36) whereby swimming speed is directly related to flagellar rotational speed (39). Free-swimming bacteria can transverse 20–60 \( \mu \)m/s in liquid media (40), whereas tethered bacteria can rotate their cell bodies 2–9 revolutions/s (36). We postulated that the \( hnsT108I \) and \( hnsA18E \) mutations caused hypermotile bacterial swimming behavior by directly increasing the speed at which individual flagella rotated. Once again, we employed bacterial tethering to measure rotational speed using a host strain (AL128) containing a \( cheA-\)\( cheZ \) deletion as well as an \( hns2-tetR \) mutation. This strain enabled us to survey cells that were all rotating in one direction, CCW, without the complications of switching. Rotational speed data (Table IV) were accumulated from cells of the same size and tethered at the same point to maintain similar drag coefficients, overall cell geometry, and load. The controls (Table IV, lines 1 and 2) displayed similar rotational speeds regardless of the location of \( hns \) (chromosome- or plasmid-based). In contrast, the addition of either \( hns \) point mutation in \emph{trans} (lines 3 and 4) resulted in significant \(( p < .005 \text{ and } p < .025, \text{ respectively}) \) increases in the speed of the tethered cell body. We concluded that \( hnsA18E \) and \( hnsT108I \) accelerated flagellar speeds 44–62% (Table IV) over wild-type levels without affecting flagellar assembly (Fig. 2 and Table II) or switching (Table III). This direct effect on flagellum rotational speed likely caused the original swarm plate hypermotile phenotype displayed by these \( hns \) mutant alleles.

**H-NS-FliG Binding via Fluorescence Anisotropy**—Flagellar rotation is driven by a reversible rotary motor anchored in the inner membrane at the base of the flagellum (reviewed in Refs. 1 and 3). The motor consists of a stator (MotA and MotB) and a rotor (FliG, FliM, and FliN). The three rotor proteins interact...
with each other (11, 12) to form a switch complex peripherally attached to the inner membrane, facing the cytoplasm (41–43). All three proteins are involved in flagellar assembly, switching, and rotation (13, 14). Since Flg is the motor protein most involved in speed and torque generation (12, 14, 15), we examined the binding between H-NS and Flg in fluorescence anisotropy assays. This technique quantitates protein-protein interactions by measuring the change in anisotropy of a fluorescently labeled protein upon the addition of a second unlabeled protein (44).

Increasing amounts of purified N terminus His-tagged Flg was added to fluorescein-labeled H-NS and emission anisotropy of the fluorophore was assessed (Fig. 3). As the concentration of Flg increased the anisotropy values also increased, reflecting the formation of a slower rotating complex and protein binding. The leveling off of the curve at higher Flg concentrations demonstrated protein binding saturation (45). We calculated the dissociation constant ($K_d$) for the H-NS-Flg complex to be $2.15 \pm 0.25 \mu M$, well within the range of biologically relevant protein associations. These data represent the first direct biochemical evidence that H-NS binds to Flg and forms a complex with the flagellar motor machinery.

**H-NS-Flg Binding via Chemical Cross-linking**—Since wild-type H-NS bound Flg in the anisotropy studies (Fig. 3), we wanted to determine if the mutant H-NST108I also bound Flg. A difference in binding affinities between Flg and wild-type or mutant H-NS protein might account for the hypermotile swarm phenotype seen with the mutant. We compared binding capabilities of each of these proteins to Flg by chemical cross-linking.

Equal amounts of either wild-type or T108I H-NS were cross-linked to Flg. H-NS-Flg complexes were trapped, run on SDS-polyacrylamide gels and stained (Fig. 4A). Tandem Western blots were also performed on identical reactions with anti-H-NS (Fig. 4B) and anti-Flg antisera (Fig. 4C) to confirm that the complexes contained both proteins. Several conclusions can be drawn from these results. First, in agreement with previous work (11, 12), Flg self-associated (Fig. 4C) in the absence (reaction 6) and presence (reaction 7) of cross-linker. Second, both wild-type and T108I H-NS dimerized (Fig. 4C) at the same protein molar ratios. Although H-NS and Flg obviously bound to each other in vitro, this complex did not seem to be the major Flg interaction since most of the protein remained in the monomer form (Fig. 4, reactions 4 and 5). Also, wild-type H-NS (Fig. 4, A, B, and C, reaction 7) and H-NST108I (Fig. 4, A, B, and C, reaction 8) each bound to Flg forming a complex of approximately 55 kDa. Furthermore, H-NST108I bound Flg 50% tighter than wild-type H-NS (compare Fig. 4, A-C, reactions 7 and 8) at the same protein molar ratios. Although H-NS and Flg obviously bound to each other in vitro, this complex did not seem to be the major Flg interaction since most of the protein remained in the monomer form (Fig. 4, A, B, and C, reactions 7 and 8). These results demonstrated that the hnsT108I mutation conferred an enhanced binding affinity of H-NS for Flg. This increased attachment of H-NST108I to the torque-generating flagellar rotor protein Flg probably accounted for the increased motility rates and flagellum rotational speeds in *E. coli* strains carrying the *hns* mutation.

**DISCUSSION**

**Hypermotility**—We studied the effect of two *hns* point mutations, *hnsT108I* and *hnsA18E*, on *E. coli* motility. In swarm plate assays, we observed that each of these *hns* mutations bestowed a hypermotile phenotype (Fig. 1). Swarm rates for strains carrying the *hns* mutant alleles were approximately twice as fast as strains with the wild-type *hns* allele in trans. Previously characterized mutations in genes involved in flagella functions have led to three basic mutant phenotype classifications: non-flagellated (Fla−) (15), non-rotating flagella (“paralyzed”, Mot−) (14), and skewed chemotaxis, either CCW-biased (14) or CW-biased (46). In terms of swimming, mutations typically cause a decrease in motility (47). To the best of our
knowledge, hnsT108I and hnsA18E are the first documented mutations that cause a 2-fold increase in swarm rates.

The mutations studied here represent single H-NS amino acid substitutions of a C-terminal threonine to isoleucine (T108I) and an N-terminal alanine to glutamic acid (A18E). As a modulator of gene expression, H-NS usually functions by binding DNA and exerting an effect on transcription (reviewed in Refs. 32 and 33). It has been suggested by suppressor mutation isolation (52) and co-purification (53) that H-NS may interact with other E. coli proteins other than itself or homologs. As a modulator of gene expression, H-NS usually functions by binding DNA and exerting an effect on transcription (reviewed in Refs. 32 and 33). It has been suggested by suppressor mutation isolation (52) and co-purification (53) that H-NS may interact with other E. coli proteins other than itself or homologs.

In our anisotropy studies, it was evident that wild-type H-NS bound FlIG, yielding a typical binding curve (Fig. 3). We estimated the $K_d$ to be in the micromolar range, confirming that the H-NS-FlIG interaction was significant and biologically relevant. Our data represent the first biochemical evidence to support Marykwas’ (11) genetic-based conclusion that H-NS binds to FlIG. It also represents the first time H-NS has directly been shown to bind an E. coli protein other than itself or homologs. As a modulator of gene expression, H-NS usually functions by binding DNA and exerting an effect on transcription (reviewed in Refs. 32 and 33). It has been suggested by suppressor mutation isolation (52) and co-purification (53) that H-NS may interact with other E. coli proteins other than itself or homologs. However, the only other protein proven to bind H-NS is the bacteriophage T7 gene 5.5 protein product (54).

We carried out cross-linking reactions (Fig. 4) in order to compare the relative binding affinities of FlIG for either wild-type H-NS or H-NST108I. We were careful to equalize and monitor H-NS quantities such that any differences in complex intensity were due to binding tightness rather than unequivocal protein concentrations. Each protein dimerized and FlIG bound each H-NS species forming 55-kDa heterodimeric complexes. The caveat of such a cross-linking experiment is that the hypermotility exhibited by the hns mutation is a direct consequence of enhanced binding of H-NST108I to the flagellar rotor protein, FlIG.
Models—No existing model sufficiently accounts for the unexpected effects of H-NS on flagella rotation. Thus, we present a speculative hypothesis of H-NS action (Fig. 5) based on the data presented here as well as previous investigators results (10–12, 31, 41). We postulate that H-NS is involved in torque generation through its interactions with FliG. Given the fact that (i) hns mutations resulting in the hypermotile phenotype specifically affected flagellar rotational speed, (ii) speed, and presumably proton-motive force are all directly related (39, 55), and (iii) the C-terminal domain of FliG functions specifically in torque generation (15), we position H-NS at the interface between the rotor and stator, directly linked to the C terminus of FliG (Fig. 5A). Tighter binding of mutant H-NST108I to FliG (Fig. 5B) may cause increases in flagellar speed by altering the conformation of FliG relative to the other rotor proteins and/or the MotA-B complex, thus, compacting the motor complex and allowing faster rotation by creating less friction within the surrounding stationary MotA-B ring complex (56). Alternatively, the movement or rate of proton flux through this channel could also be affected, or the H-NS-FliG complex may play a regulatory role by altering expression of genes downstream of fliG in the flagellar assembly cascade.

In totality, this study provides several unique observations about H-NS and E. coli motility. We have defined a new swelling mutant class illustrated by a hypermotile phenotype. We have shown that H-NS plays a rare, non-regulatory role in motility. We provided the first biochemical evidence that H-NS binds to the flagellar rotor protein FliG and demonstrated that the tightness of this interaction determines flagellar rotational speed. Future directions will include testing our model in order to provide a suitable mechanistic view of H-NS activity on bacterial motility.

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Fig. 5. Model of H-NS activity at the flagellar rotor. A, wild-type H-NS interactions with FliG; B, proposed conformational changes induced by the binding of H-NST108I to FliG. PD, peptidoglycan; IM, inner membrane. See “Discussion” for details.
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