ARTÍCULOS
Evidence for Direct Protein-Protein Interaction between Members of the Enterobacterial Hha/YmoA and H-NS Families of Proteins

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Escherichia coli nucleoid-associated H-NS protein interacts with the Hha protein, a member of a new family of global modulators that also includes the YmoA protein from Yersinia enterocolitica. This interaction has been found to be involved in the regulation of the expression of the toxin α-hemolysin. In this study, we further characterize the interaction between H-NS and Hha. We show that the presence of DNA in preparations of copurified His-Hha and H-NS is not directly implicated in the interaction between the proteins. The precise molecular mass of the H-NS protein retained by Hha, obtained by mass spectrometry analysis, does not show any posttranslational modification other than removal of the N-terminal Met residue. We constructed an H-NS–His recombinant protein and found that, as expected, it interacts with Hha. We used a Ni²⁺-nitrilotriacetic acid agarose method for affinity chromatography copurification of proteins to identify the H-NS protein of Y. enterocolitica. We constructed a six-His–YmoA recombinant protein derived from YmoA, the homologue of Hha in Y. enterocolitica, and found that it interacts with Y. enterocolitica H-NS. We also cloned and sequenced the hns gene of this microorganism. In the course of these experiments we found that His-YmoA can also retain H-NS from E. coli. We also found that the hns gene of Y. enterocolitica can complement an hns mutation of E. coli. Finally, we describe for the first time systematic characterization of missense mutant alleles of hha and truncated Hha' proteins, and we report a striking and previously unnoticed similarity of the Hha family of proteins to the oligomerization domain of the H-NS proteins.

The Hha protein from Escherichia coli (32) and the YmoA protein from Yersinia enterocolitica (9) belong to a new family of modulators of gene expression. Both are small (about 8.5 kDa), show extensive homology in their amino acid sequences (9, 22). Hha has been identified in Escherichia coli (33). H-NS is considered a general negative regulator of transcription. H-NS affects the expression of a large number of genes (1, 24), and it is among the best-characterized examples of central modulators of gene expression (34). H-NS is also altered in H-NS mutants, depending on the medium osmolarity (2). Homologues of Hha have also been found in conjugative plasmids, where they could be involved in the regulation of sexual transfer (34).

Protein-protein interaction studies performed by immobilizing His-tagged Hha on nickel-nitrilotriacetic acid (Ni²⁺-NTA) agarose led us to demonstrate that Hha interacts with the nucleoid-associated protein H-NS and that the Hha–H-NS complex is responsible for the thermo-osmotic modulation of the expression of the hemolysin operon in E. coli (33). H-NS (15.4 kDa) and homologues are widespread in enterobacteria and other genera of gram-negative bacteria (4). E. coli and Salmonella enterica serovar Typhimurium H-NS proteins are among the best-characterized examples of central modulators implicated in the response to changes in osmolarity and temperature (for reviews see references 1, 49, and 51). H-NS affects the expression of a large number of genes (1, 24), and it is considered a general negative regulator of transcription. This protein binds preferentially to curved DNA (52), and it is also able to generate bends in noncurved DNA (44). Three isoforms of the H-NS protein have been identified, although the biological significance of each isoform is unclear (43, 49).

We extend here our observations concerning the interaction between Hha and H-NS and present further evidence demonstrating a direct protein-protein interaction, even in the absence of DNA. We also show that this interaction is common to other members of both families of proteins: the use of His-tagged YmoA allowed us to identify the H-NS protein from Y. enterocolitica, thus extending this newly described mechanism of regulation of gene expression to other genera of gram-negative bacteria. Finally, the H-NS-binding properties of altered Hha proteins together with the acidic amino sequence comparison presented here lead us to propose that Hha can be seen as an independent oligomerization domain of H-NS.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used are listed in Table 1. Liquid Luria-Bertani (LB) medium (10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter) was used. Solid medium was LB medium plus 15 g of agar per liter. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 50 μg/ml; tetracycline, 12.5 μg/ml; β-Glucoside indicator plates with salicin were prepared as described previously (40).

**Genetic and molecular procedures.** Isolation of plasmids, restriction digestion of DNA, ligation of DNA, and transformation were carried out by standard methods. Chromosomal DNA from Y. enterocolitica was isolated as described elsewhere (42). PCR amplification and sequencing of DNA were done according to standard methodology.

Plasmids for overexpression of proteins based on the T7 RNA polymerase system (45) were constructed as follows. For pETH-NSHIS, oligonucleotides used to amplify the hns gene of E. coli were HNSNPRO (5’-GAGATTACTCTATATGGAGC-3’) and HNSHR (5’-CCGGATCTTATATATGGTGTGCAC-3’) which adds after the last hns codon six codons for His residues plus two stop codons and a BamHI site. The PCR fragment was cut with NdeI and cloned into pET3b. For pETHYMOA,
Plasmid phage P1 Cultures of the mutant strains previously obtained were infected with bacteriophage P1. A linker was inserted into it, as described previously (33).

\[ /\text{H}1 1032 \text{GGATCCTTAAAATACCGCC-3}\]

\[ /\text{H}1 1032 \text{HHAT2 (5'phenotype (large hemolytic haloes) were purified as potential}\]

\[ /\text{H}29 5\text{K}\]

Chemical mutagenesis of E. coli E. coli H29 was used to distinguish hemolytic phenotypes. Whole cells from overnight cultures of strains BSN27(pHNS-1), BSN27(pUC19), and BSN26 were resuspended in standard denaturing sample buffer and analyzed by SDS-PAGE. Western blot analysis of proteins transferred to nitrocellulose membranes was performed with polyclonal antibodies raised against E. coli Hha and HNS. Hha-specific antibodies were prepared as described previously (33).

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<th>Strain or plasmid</th>
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<td>E. coli BL21 hsl8 gel (AcW857 ind-1)</td>
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| Plasmids tagged Hha by Ni\(_{\text{2+}}\)-NTA technology (16, 17, 38) as described previously (33). 

**Electrophoresis and Western analysis of proteins.** Protein samples were analysed in a Tricine–gel/dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE) system (16, 17, and 45%) and stained with Coomassie blue. Bio-Rad protein standards and prestained protein standards were used for molecular weight estimation. Whole cells from overnight cultures of strains BSN27(pHNS-1), BSN27(pUC19), and BSN26 were resuspended in standard denaturing sample buffer and analyzed by SDS-PAGE. Western blot analysis of proteins transferred to nitrocellulose membranes was performed with polyclonal antibodies raised against E. coli Hha and HNS. Hha-specific antibodies were prepared as described previously (33).

**Overexpression of proteins by the T7 RNA polymerase system and purification of His-tagged proteins.** E. coli strain BL21(DE3)(pLysE) was used as a host for induction of expression of proteins. Plasmids containing the desired cloned genes (pET plasmids) were introduced into BL21(DE3)(pLysE) by transformation. One-liter cultures were grown at 30°C to an optical density at 600 nm of 1.0. At this point, IPTG (isopropyl-\(\beta\)-d-thiogalactopyranoside) was added to 0.5 mM, and incubation was carried out for 15 min. Cells were then pelleted by centrifugation and resuspended in 20 ml of buffer A (20 mM HEPES [pH 7.9], 10% glycerol, 100 mM KC\(_{\text{2}}\), 5 mM MgCl\(_{\text{2}}\), 50 mM imidazole). Clear cellular extracts were obtained as described previously (33). His-tagged proteins were purified by immobilized-metal affinity chromatography by using Ni\(_{\text{2+}}\)-NTA technology (16, 17, 38) as described previously (33).

**Microsequencing of proteins.** N-terminal analysis of amino acid composition of proteins was performed by automatic Edman degradation. Prior to analysis, proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad) by semidy electrophoblotting. The membrane was then stained with Coomassie blue. The target protein was cut from the membrane and subjected to sequence analysis as previously described (36).

**Results**

DNA is not required for the interaction between His-Hha and H-NS. We previously showed in vitro interaction of Hha and H-NS by demonstrating that upon purification of His-tagged Hha by Ni\(_{\text{2+}}\)-NTA technology, H-NS coeluted with His-Hha when the agarose matrix was washed with a buffer containing 200 mM imidazole (33). Prior to His-Hha elution with imidazole, H-NS can be separated from the Ni\(_{\text{2+}}\)-NTA agarose matrix when the KCl concentration in the buffer in-
creases (0.5 to 1 M) (33). This has been reported for other proteins retained by His-tagged partners bound to Ni\(^{2+}\)-NTA agarose beads (37). When we further analyzed the fractions released upon KCl washing, we found that DNA was also present. This could be interpreted as His-Hha retaining DNA and as H-NS binding to DNA and not directly to His-Hha. To rule out this possibility, we tested the ability of His-Hha to bind H-NS in the absence of DNA. To do this, the fractions eluted at 0.5 and 1 M KCl (containing H-NS and DNA) were dialyzed and then treated with DNase. Samples that had been treated identically except for the DNase added were used as controls. Purified His-Hha bound to the agarose matrix was then mixed (2 h, 4°C) with H-NS in the presence and absence of DNA. Upon several washings of the agarose matrix with buffer A containing increasing KCl concentrations, H-NS could be visualized by immunodetection mainly in the fractions eluted at high salt concentration, thus demonstrating that the interaction between Hha and H-NS occurs in the absence of DNA (Fig. 1A and B).

**Mass spectrometry analysis of the H-NS preparation copurifying with Hha.** Two or three H-NS isoforms, whose properties have not been described, can be observed by two-dimensional PAGE in cellular extracts (49). To analyze which of the H-NS isoforms copurifies with His-Hha, we determined its molecular mass by mass spectrometry analysis. We obtained masses of 15,407.8 and 15,410.1 Da in two separate experiments; these values closely resemble the mass calculated from the amino acid sequence (15,408.5 Da), without the first Met residue.

**H-NS–His binds Hha.** Considering that the amino-terminal end of H-NS is responsible for protein–protein interaction and the carboxy-terminal end is responsible for protein–DNA interaction (13, 41, 48), we decided to add the six-His tag to the carboxy-terminal end of H-NS to test if, in vitro, immobilized H-NS is able to interact with Hha. The hns gene of *E. coli* was amplified with the oligonucleotides HNSNPRO and HNSHIR, which adds a six-His tag to the H-NS protein at the carboxy-terminal end, and then cloned in pET3b, yielding plasmid pETHISYMOA. His-YmoA was overexpressed in *E. coli* strain Y754 and purified by using Ni\(^{2+}\)-NTA agarose. No other proteins copurified with H-NS–His (Fig. 1C). It is relevant that whereas H-NS is an abundant protein in the cell, this is not the case for Hha. When not overexpressed, this protein is barely detectable in SDS-PAGE gels (28). To overcome this problem, a cellular extract containing overexpressed Hha was prepared and mixed with Ni\(^{2+}\)-NTA agarose already containing H-NS–His. A protein exhibiting a molecular mass similar to that of Hha copurified with H-NS (Fig. 1D). Western blot analysis demonstrated that this protein is Hha (Fig. 1E). It is thus apparent that, in vitro, H-NS–His is able to bind Hha too, as expected.

**His-YmoA binds both *Y. enterocolitica* and *E. coli* H-NS.** We next decided to test if the interaction between Hha and H-NS could be extended to other members of both families of proteins, particularly to YmoA and a hitherto-undescribed member of the H-NS family from *Y. enterocolitica*. The ymoA gene from *Y. enterocolitica* strain Y754 was first cloned in plasmid pET3b. Next, a histidine tag was added, yielding plasmid pHISYMOA. His-YmoA was overexpressed in *E. coli* strain BL21(DE3)(pLysE) and purified by using Ni\(^{2+}\)-NTA agarose. A second protein copurified with His-YmoA. As expected, this protein turned out to be *E. coli* H-NS (Fig. 2A and B). The next step was to release *E. coli* H-NS from the His–YmoA–H-NS complex by repeated washing of the Ni\(^{2+}\)-NTA agarose matrix with buffer A containing 1 M KCl. The Ni\(^{2+}\)-NTA agarose matrix containing His-YmoA was then split in two aliquots. One was used for a control experiment. The other was
mixed with a *Y. enterocolitica* Y754 crude cell extract, and again, several washings with buffer A containing increasing concentrations of imidazole were performed. Two accompanying proteins coeluted with His-YmoA (Fig. 2C). One of them, with a larger molecular mass, was shown not to specifically interact with His-YmoA; it was also detected upon imidazole washing of a Ni$^{2+}$-NTA agarose matrix with which a crude extract of the *Y. enterocolitica* culture was mixed (lacking bound His-YmoA) (Fig. 2C). The second protein, with a molecular mass of about 15 kDa, copurified specifically with His-YmoA and reacted with *E. coli* H-NS-specific antibodies (Fig. 2D). We suspected that this protein was *Y. enterocolitica* H-NS. This was confirmed by N-terminal microsequencing. The sequence obtained (SEALKILNIRRTLRAQAREXTLTE) matched that of the *E. coli* H-NS, except for the Cys residue at position 20 (Cys residues are commonly not found in N-terminal Edman sequencing).

*Y. enterocolitica* hns gene complements some hns-induced phenotypes in *E. coli*. In order to confirm the identification in *Y. enterocolitica* crude cell extracts of a protein belonging to the H-NS family, we decided to clone the *Y. enterocolitica* hns gene and determine its nucleotide sequence. Chromosomal DNA was isolated from *Y. enterocolitica* Y754, subjected to partial Sau3A digestion, and then ligated to pUC19 plasmid previously digested with BamHI. This ligation was transformed into *E. coli* strain BSN27 (*hns*). About 4,000 colonies were lifted with toothpicks and streaked onto Bgl indicator plates, where *hns* + colonies appear greenish and *hns* colonies appear yellow due to derepression of the bgl operon (15). One of the clones able to complement the Bgl phenotype was selected, and plasmid DNA was isolated from it. The resulting plasmid (pYN-1) contained about 3 kbp of *Y. enterocolitica* DNA. Immunodetection of an ~20kDa protein in crude cell extracts from strain BSN27(pYN-1) suggested that plasmid pYN-1 carries the *hns* gene from *Y. enterocolitica* (Fig. 3A). This was confirmed by DNA sequencing (GenBank accession number AJ302639). Analysis of the DNA sequence showed an open reading frame whose translation closely matches the amino acid sequence of the H-NS proteins. Additionally, it perfectly matches the sequence of residues obtained in the N-terminal Edman degradation of the protein bound to His-YmoA. Other authors have identified the same *Y. enterocolitica* hns gene (5). We next tested whether the *Y. enterocolitica* hns gene is also able to complement other hns-related phenotypes of strain BSN27. Specifically, we tested the deregulation of hemolysin expression caused by the *hns* allele (33). *E. coli* BSN27(pHly152, pYN-1) cells showed a significant decrease in hemolysin production compared to BSN27(pHly152, pUC19) cells (Fig. 3B), thus showing that the *hns* gene of *Y. enterocolitica* is also able to complement the deregulation of hemolysin expression caused by the *hns* allele of *E. coli* strain BSN27.

**Isolation of single amino acid substitutions in Hha protein and truncated Hha proteins which affect binding to H-NS.** To gain information about the functional domain organization of the Hha protein we chose a genetic approach. We used mutagenesis to obtain single amino acid substitutions that showed the characteristic Hha$^{-}$ phenotype, i.e., derepression of the hly operon. *E. coli* SK(pANN202-312) was used for chemical mu-
tagenesis. Approximately $4 \times 10^4$ colonies on blood agar plates were screened, and a total of 42 clones were isolated which showed an increase in the hemolytic haloes (phenotype of hha mutants). In order to identify the mutations in the hha gene, these mutants were infected with bacteriophage P1 obtained on E. coli strain KL743 (which carries a Tcr marker close to the hha gene). Association of acquisition of Tcr and reversion to low hemolysin production (about 50% frequency) was observed in mutants H11, H18, H21, H29, and H39. Mutants showing no reversion at all, unstable phenotypes, or unclear results were not studied further.

The hha genes of mutants H11, H18, H21, H29, and H39 were sequenced. All of them had single point missense mutations in the hha ORF which result in amino acid substitutions: hha11 and hha21, R50 to H (CGC to CAC); hha18, R16 to C (CGT to TGT); hha29, P64 to S (CCT to TCT); and hha39, P64 to L (CCT to CTT). In order to evaluate if binding to H-NS protein was affected by these mutations, we cloned the ORFs of hha11, hha18, hha29, and hha39 in pET3b and then added a six-histidine tag at the amino end. The recombinant mutant proteins were expressed in BL21(DE3)(pLysE) host cells and purified from the cleared cellular extracts by mixing with Ni$^{2+}$-NTA agarose followed by elution with increasing concentrations of imidazole, Tricine-SDS-PAGE, and Western blotting with specific anti-H-NS antibodies. We found that the interaction with H-NS was impaired in all four His-Hha proteins (Fig. 4). The truncated His-HhaT1, His-HhaT2 and HisT3 proteins were analyzed in the same way. We found that none of the three truncated proteins retained H-NS in our assay. The addition of the six-histidine tag at the carboxy-terminal end of the Hha protein was evaluated and found to impair interaction with H-NS, too (Fig. 4). These results indicated that most of the Hha protein is involved in binding to H-NS and prompted us to take a closer look at the amino acid sequences of the Hha/YmoA family of proteins. We compared them to the oligomerization domain of the members of H-NS family (first 70 amino acids) and found a certain degree of similarity (Fig. 5) in the amino acid sequences. The similarity in length is also remarkable.

**DISCUSSION**

Interaction of H-NS with other proteins apart from itself or homologues (50) has been described in very few instances. H-NS has been proven to bind the bacteriophage T7 gene 5.5 protein product. The significance of this interaction seems clear, since it would favor bacteriophage T7 multiplication by inhibiting H-NS repressive function (25). H-NS also binds to the flagellar rotor protein FliG, but the significance of this interaction is unclear (11, 26). The significance of the interaction of H-NS with HF-I (21), an RNA-binding protein that participates in translation of rpoS RNA (31), is also unclear.
Our previous observations about the interaction between Hha and H-NS (33) not only suggested the existence of an H-NS-Hha complex but also assigned a modulatory role to it: thermos- and osmmodulation of the expression of the toxin α-hemoly-sin. The data in this paper confirm a direct protein-protein interaction between Hha and H-NS and extend it to other members of both families of proteins. The protein complex can be evidenced in vitro in the absence of DNA, and it is also detected when HNS-His is the protein bound to the Ni²⁺-NTA agarose matrix. In this case, overexpression of Hha is required because of the relatively low abundance of Hha in the cell that could already be bound to native H-NS, being thus inaccessible for interaction with H-NS–His. This suggests that other low-abundance proteins interacting with tagged H-NS might not be apparent in similar copurification experiments.

The existence of H-NS isoforms has been previously documented (23, 43, 49). It has been suggested that the differences in isoelectric points detected between H-NS isoforms of similar abundance proteins interacting with tagged H-NS might not be for interaction with H-NS–His. This suggests that other low-abundance proteins interacting with tagged H-NS might not be apparent in similar copurification experiments.

The existence of H-NS isoforms has been previously documented (23, 43, 49). It has been suggested that the differences in isoelectric points detected between H-NS isoforms of similar molecular weights found in two-dimensional PAGE must correspond to posttranslational modifications located in the amino half of the protein (12), but the nature of such modifications remains unknown. The accuracy of MALDI-TOF permits the identification of changes in the molecular mass of the protein due to posttranslational modifications. Thus, the mass spectrometry data presented here allow us to assume that the H-NS isoform that interacts with Hha does not have any modification other than the removal of the initial Met residue.

E. coli H-NS and Y. enterocolitica H-NS have identical amino-terminal domains. This probably explains the ability of His-YmoA to bind E. coli H-NS and suggests that the H-NS proteins from both microorganisms have similar capacities for interacting with other protein partners. The strategy we used to clone the hns gene of Y. enterocolitica showed that it is able to complement in E. coli some of the defects caused by the hns mutation, such as the Bgl phenotype and the deregulation of hemolysin expression. The high degree of similarity of the H-NS proteins suggests that, as we show here for Y. enterocolitica H-NS, they are functionally interchangeable. The fact that H-NS of Y. enterocolitica interacts with YmoA, a known modulator of the expression of virulence genes (9), suggests that HNS of Y. enterocolitica may play a significant role modulating expression of virulence in Y. enterocolitica.

Whereas the role of the N-terminal domain of H-NS in generating protein oligomers is well characterized (reviewed in reference 13), H-NS binding to DNA is the main factor considered in modeling H-NS-mediated regulation of gene expression (18, 20, 46, 47). Nevertheless, focusing on the H-NS modulation of the bgl operon, the need for H-NS to interact with other proteins (6) and the relevance of the N-terminal domain, perhaps to provide an anchoring point for other regulatory factors (48), have been pointed out. The results we present here further support the relevance of protein-protein interaction for H-NS-mediated modulation of gene expression.

The data obtained from the Hha proteins containing amino acid substitutions and truncations suggest that the entire protein is involved in the interaction with H-NS. Nevertheless, the carboxy-terminal end must be more closely implicated, since the addition of the six-histidine tag at the carboxy-terminal end severely impairs interaction with H-NS, which is not the case when the six-histidine tag is placed at the amino-terminal end. Additionally, the fact that YmoA, despite lacking the first five amino acid residues of Hha, still interacts with H-NS suggests that the very N-terminal end is not essential for Hha to interact with H-NS. These genetic and biochemical data are consistent with the results obtained from the alignment of the amino acid sequence of the different members of the Hha family and the oligomerization domain of the members of the H-NS family (Fig. 5). Apart from the similarity in length, it is apparent that there are identity boxes scattered along the sequence alignment.

Taking the results together, we conclude that Hha can be considered a specialized homologue of the amino-terminal oligomerization domain of H-NS and that this observation is very likely valid for other members of the Hha/YmoA family of proteins. Furthermore, the interaction of Hha-type and H-NS-type proteins is probably present in many enteric bacteria as a mechanism of regulation of gene expression.

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Functional Replacement of the Oligomerization Domain of H-NS by the Hha Protein of Escherichia coli

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Members of the H-NS family of proteins play a relevant role as modulators of gene expression in gram-negative bacteria. Interaction of these proteins with members of the Hha/YmoA family of proteins has been previously reported. It has been hypothesized that the latter proteins are functionally equivalent to the N-terminal domain of H-NS-like proteins. In this report we test this assumption by replacing the N-terminal domain of Escherichia coli H-NS by Hha. It has been possible to obtain a functional protein that can compensate for some of the hns-induced phenotypes. These results highlight the relevance of H-NS-Hha interactions to generate heterooligomeric complexes that modulate gene expression in gram-negative bacteria.

The H-NS protein of Escherichia coli was described almost 30 years ago as a chromosome-associated protein. Since then it has been thoroughly studied as an architectural protein playing an important role in the global regulation of gene expression. H-NS protein and homologues (the H-NS family of proteins) are widespread in enterobacteria and other genera of gram-negative bacteria (38). Best characterized in different enteric bacteria such as E. coli or Salmonella enterica serovar Typhimurium, the H-NS protein is a relevant example of a global modulator exerting its effects in response to different environmental signals (for a recent review, see reference 8). The expression of approximately 5% of the genes of E. coli has been found to be directly or indirectly altered in hns mutant strains (13).

One of the outstanding features of H-NS is the ability to generate higher-order homomeric and heteromeric complexes. H-NS oligomerization depends upon the N-terminal domain of the protein, extending up to residue 65 (1, 9). Generation of dimers, trimers, and tetramers has been reported (5, 36). H-NS oligomerization appears as a process necessary for transcriptional repression (30). H-NS is able not only to generate homodimers and homooligomers but also to interact with other proteins. Generation of heterodimers and heterooligomers with the H-NS parologue StpA is a well-documented process (15, 16, 39). Interaction of H-NS with StpA protects the latter protein from Lon-mediated proteolysis (16). It has also been reported that StpA can act as a molecular adapter for some species of truncated H-NS proteins to repress the bgl operon (11).

Members of the H-NS family also interact with members of the Hha/YmoA family (26, 27, 29). These small proteins (M_r, about 8 kDa) were initially described in E. coli (Hha) and Yersinia enterocolitica (YmoA) as thermomodulators of the expression of virulence factors (6, 23, 25). Both were independently considered new nucleoid-associated proteins that modulate gene expression (4, 6, 21). Since then, many other members of the Hha/YmoA family have been identified, both in the chromosomes of gram-negative bacteria and in conjugative plasmids (19). Interaction of Hha and H-NS was first evidenced when the biological role of Hha as a modulator of the expression of the operon encoding the E. coli toxin α-hemolysin (Hly) was assessed. Rather than showing affinity and specificity for DNA sequences, Hha showed high H-NS binding affinity and specificity (27). Further work demonstrated that, in fact, an Hha–H-NS complex modulates the expression of the hly operon (20). Recent studies have extended the Hha-H-NS interaction to other members of both families: YmoA interacts with Y. enterocolitica H-NS (26), and Hha and its E. coli parologue YdgT interact with StpA. Interaction of Hha/YdgT with StpA prevents proteolytic degradation of this latter protein (29).

A mutational analysis of Hha focused to identify domains of the protein showed that almost all the protein sequence corresponds to a unique protein-binding domain. A comparison of the amino acid sequences corresponding to the Hha family and the N-terminal end of the H-NS family showed the existence of conserved regions (26). These results suggested that Hha-like proteins might have evolved to be functionally equivalent to the amino-terminal oligomerization domain of H-NS and, hence, interact with full-length H-NS proteins to generate heterodimers and heterooligomers that modulate gene expression in gram-negative bacteria (26). We show in this report that the replacement of the N-terminal domain of H-NS by Hha sequences generates a functional chimeric protein that, when expressed in hns mutants, restores different hns mutant phenotypes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used are listed in Table 1. The different strains were grown either in Luria-Bertani medium or in minimal medium M63 supplemented with serine (40 mg/liter) and prepared as described previously (22). Antibiotics, when required, were used at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 12.5 μg/ml; kanamycin, 25 μg/ml; and chloramphenicol, 50 μg/ml.

Genetic and molecular procedures. Isolation of plasmids, restriction digestion, ligation of DNA, and transformation were carried out by standard methods. PCR amplification and sequencing of DNA were done according to standard methodology. All of the oligonucleotides used are listed in Table 2.
To construct plasmid pUCHhahaHnsHyb, the hha gene was first PCR amplified using the oligonucleotides HhaNde and HhaBam, which adds a BamHI site, and Hha-Bam2, which add BamHI sites, and cloned into pHNM. The hns gene was then PCR amplified using the oligonucleotides HNSBProx and HNSBDist, which add BamHI sites. The BamHI PCR fragment (508 bp) was cloned into the BamHI site of pUCHhahaHnsHyb, obtaining plasmids pUCHhahaHnsHyb2, pUCHhahaHnsHyb30, and pUCHhahaHnsHyb45.

To construct plasmid pHLaHhaHnsHyb2, the sequence cloned into pUCHhahaHnsHyb2 was PCR amplified using the oligonucleotides HylProx and HNSBDist, which add BamHI sites, and cloned into pHNM. Plasmid pHLaHhaHnsHyb2 was obtained by cloning into pET15b a PCR fragment amplified from pUCHhahaHnsHyb2 using the oligonucleotides Haa-Hla, which adds six codons of His residues and an NdeI site, and HNSBDist, which adds a BamHI site.

**Measurement of hemolysin production.** Hemolysin production was assayed by measuring hemolytic activity as previously described (24).

**Measurement of β-galactosidase activity.** β-Galactosidase activity was evaluated as previously described (24).

**Overexpression of proteins by the T7 RNA polymerase system and purification of His-tagged proteins.** E. coli strain BL21(DE3) Δhns was used as a host for induction of the expression of protein Hha-Hns-Hyb2. Plasmid pHLaHhaHnsHyb2 was transformed into strain BL21(DE3) Δhns. Clear cellular extracts were obtained as described previously (27). His-tagged recombinant protein was purified by immobilized metal affinity chromatography by using Ni²⁺-nitrilotriacetic acid (NTA) technology (12) as described previously (27). His-Hla and H-NS-His were purified as previously described (26, 27).
Electrophoretic analysis of proteins. Protein samples were analyzed in a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (16.5%, 10%, and 4%) (31) and stained with Coomassie blue.

Gel retardation assays. Gel retardation assays were performed as described previously (20). The fragments used were obtained by amplification of plasmid pHly152, using HlyR0 and HlyBam for the R0 fragment (2.7 kb) and HlyS and HlyP for the S-P fragment (1.1 kb).

RESULTS

Construction of a chimeric Hha-H-NS protein. To obtain a hybrid Hha-Hns protein, we decided to generate random fusions between fragments corresponding to the N-terminal part of Hha and the C-terminal part of H-NS. As a preliminary step we generated a recombinant DNA fragment containing (i) the hns promoter, (ii) the hha gene (full-length), and (iii) the hns gene (full-length) (Fig. 1A) (see Materials and Methods for details). This DNA fragment, flanked by recognition sites for the enzymes NdeI and BamHI, was cloned into pUC19 (plasmid pUCHhaHnsHyb). The construction contained unique MfeI (inside hha) and BamHI (located at the 3′ end of hns) restriction sites. To obtain random fusions between hha and hns, both genes were PCR amplified and Bal31 digested. Upon Klenow filling and ligation, the ligation products were amplified with the oligonucleotides HhaBamMet and HNSBDist. Amplification products of the proper size (about 500 bp) were then MfeI-BamHI digested and cloned into plasmid pUCHhaHnsHyb. Upon transformation into strain E. coli 5K, inserts from 60 different clones were sequenced. Three of them contained an in-frame open reading frame (ORF) corresponding to an hha-hns construct. The corresponding plasmids were termed pUCHhaHnsHyb2, pUCHhaHnsHyb30, and pUCHhaHnsHyb45. To avoid undesirable recombinational events, they were introduced into strain E. coli HB101 by transformation. Upon transformation, the inserts were sequenced again. Such clones were then used as a source of recombinant plasmids for subsequent work.

We next tested the ability of these plasmids to complement hns phenotypes. We decided to test expression of both hly and bgl operons (increased in hns mutants) in strains BSN26, BSN27(pUC19), BSN27(pUCHhaHnsHyb2), BSN27(pUCHhaHnsHyb30), and BSN27(pUCHhaHnsHyb45) (Fig. 2). To test hemolysin expression, plasmid pHly152 was introduced by conjugation. To test bgl expression, plasmid pDFY167(bgl::lacZ) was introduced by transformation. Plasmid pUCHhaHnsHyb45 did not complement, plasmid pUCHhaHnsHyb30 yielded intermediate levels of complementation, and plasmid pUCHhaHnsHyb2 significantly com-
implemented both \textit{hns} mutant phenotypes. Figure 3 shows the amino acid sequence of protein Hha-Hns-Hyb2, as well as details of the structural domains of both Hha and H-NS proteins that are present in the chimeric protein.

**Complementation of \textit{hns} mutant phenotypes by protein Hha-Hns-Hyb2 depends upon gene dosage.** To further test the ability of protein Hha-Hns-Hyb2 to complement \textit{hns} phenotypes, we decided to supply the protein in a low-copy-number system to mimic physiological expression levels. To do this, DNA from plasmid pUCHhaHnsHyb2 was amplified with the oligonucleotides HibBProx and HNSBam2 (thus yielding two BamHI restriction sites flanking the recombinant gene). The amplification product was then ligated into BamHI-digested pLG388-30. The recombinant plasmid was termed pLGHhaHnsHyb2. Plasmid pLGHhaHnsHyb2 was also tested for complementation of the \textit{hns} mutation (Fig. 4). With respect to the deregulation of hemolysin expression that is apparent in \textit{hns} mutants, a moderate effect was evident when plasmid pLGHhaHnsHyb2 was supplied in transcript. In contrast, cells harboring this plasmid did not modify expression of the \textit{bgl} operon when compared to plasmid-free \textit{hns} cells.

We further investigated whether protein Hha-Hns-Hyb2 was

![Graph](image)

FIG. 2. Effect of the different Hha-Hns-Hyb constructions on the expression of the \textit{bgl} (A) or \textit{hly} (B) operons in an \textit{hns} genetic background. All strains carried either plasmid pDFY436 (\textit{bglG: lacZ}) (A) or pHly152 (B). Activity of strain BSN27(pUC19), considered as 100%, was 1,500 units (A) or 1,200 units (B). Samples were collected at the exponential phase of growth (optical density at 600 nm, 0.4). The data shown are means ± standard deviations (error bars) of at least three independent experiments.

![Graph](image)

FIG. 3. Amino acid sequence and structural domains of the proteins Hha, H-NS (N-terminal domain), and Hha-Hns-Hyb2 (N-terminal domain). Boxes indicate the α-helix domains of each protein. Boldface residues correspond to those preserved among members of both families of proteins. The flexible linkers that connect the N- and C-terminal domains in both H-NS and Hha-Hns-Hyb2 are shown.
able to complement a global physiological effect of the \textit{hns} mutation, i.e., the serine sensitivity of \textit{hns} mutants (18). In vivo complementation of the serine susceptibility of \textit{hns} mutants has been used to identify H-NS-like proteins in different gram-negative microorganisms (38). Strains BSN26, BSN27, BSN27 (pUCHhaHnsHYb2), and BSN27 (pLGHhaHnsHyb2) were grown in minimal medium supplemented with serine, and growth was monitored (Fig. 5). It could be shown that, when expressed in the high-copy-number vector pUC19, the chi-meric protein partially alleviates the severe growth defects of strain BSN27 in this medium.

**DISCUSSION**

In this report we bring biochemical evidence supporting the hypothesis that, in fact, in spite of low similarity at the level of the amino acid sequence, Hha-like proteins can be functionally equivalent to the N-terminal domain of H-NS-like proteins and, hence, participate in the generation of heterooligomeric protein partially alleviates the severe growth defects of strain BSN27 in this medium.

**Binding of Hha-Hns-Hyb2 to DNA sequences of the regulatory region of the \textit{hly} operon.** We also tested Hha-Hns-Hyb2 for its ability to specifically bind to sequences that have been shown to be preferential binding sites for H-NS in the regulatory region of the \textit{hly} operon (19). Previously, we obtained a purified preparation of Hha-Hns-Hyb2 by His-tagging this protein, overexpressing it in strain BL21(DE3) \textdagger{hns}, and purifying it by using nickel-NTA agarose technology (Fig. 6A). We first tested the ability of Hha-Hns-Hyb2 to bind to the R0 fragment that corresponds to the \textit{hly} regulatory region and includes specific binding sites for H-NS (20). The results obtained (Fig. 6B) showed that, when compared to H-NS, the hybrid protein must be supplied in higher concentrations to obtain similar low-migrating protein-DNA complexes. In spite of this, binding of Hha-Hns-Hyb2 protein to the R0 sequence showed specificity. When an additional DNA fragment that contains no specific sequences for H-NS is added to the reaction mixture (fragment S-P) (20), the R0 fragment is specifically retarded both by the H-NS and Hha-Hns-Hyb2 proteins (Fig. 6C). These results are consistent with the fact that the C-terminal domain of H-NS, responsible for protein-DNA interaction, is intact in the hybrid protein.

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**FIG. 4.** Ability of the Hha-Hns-Hyb2 protein, supplied in a low-copy-number system, to complement \textit{hns} phenotypes. (A) Expression of the \textit{bgl} operon. (B) Expression of the \textit{hly} operon. All strains carried either plasmid pDFY436 (A) or pHly152 (B). Activity of strain BSN27 (pLG88-30), considered as 100%, was 1,000 units (A) or 3,000 units (B). Samples were collected at the exponential phase of growth (optical density at 600 nm, 0.4). The data shown are means ± standard deviations (error bars) of at least three independent experiments.

**FIG. 5.** Serine sensitivity of strains BSN26 (squares), BSN27 (circles), BSN27 (pLGHaHnsHyb2) (triangles), and BSN27 (pUCHaHnsHyb2) (diamonds). Growth curves of control strains BSN27 (pUC19) and BSN27 (pLG88-30) (not shown) were similar to those of strains BSN27 and BSN27 (pLGHaHnsHyb2), respectively. OD\textsubscript{600}, optical density at 600 nm.
FIG. 6. Interaction of Hha-Hns-Hyb2 with DNA. (A) Purification of His-Hha-Hns-Hyb2 by Ni$^{2+}$-NTA agarose technology. Lane 1, crude extract; lanes 2 to 4, fractions obtained upon washing with buffer A; lanes 5 to 9, fractions eluted upon washing with increasing concentrations of imidazole (50 to 250 mM). Bands other than the 14.4-kDa band (arrow) correspond to truncated form (low band) or to multimeric forms (upper bands) of the protein. (B) Band shift assays with the R0 fragment (10 ng of DNA) and various concentrations of H-NS, Hha, or Hha-H-NS-Hyb2 proteins. (C) Competition gel retardation assay with fragments R0 and S-P (5 ng of each DNA fragment). Variable concentrations of H-NS or Hha-Hns-Hyb2 were added.
complexes that modulate gene expression in gram-negative bacteria. Substitution of the N-terminal domain of H-NS (amino acid residues 1 to 65) by the first 61 amino acid residues of Hha yields a chimeric protein that partially compensates for an H-NS defect in E. coli cells. Considering that the structure of both the N-terminal domain of H-NS (33) and full-length Hha are known (41), it is possible to predict the structure of the hybrid protein, which includes the first three α-helices of Hha connected by the flexible linker to the C-terminal domain of H-NS (Fig. 3). It is apparent that not every substitution of the N-terminal end of H-NS by Hha yields proteins that can, at least partially, replace H-NS. From the 60 random fusions that were sequenced, only three corresponded to in-frame ORFs, and only the Hha-Hns-Hyb2 protein, when expressed at high level, can significantly alleviate hns mutant phenotypes. We assessed the ability of Hha-Hns-Hyb2 to compensate for H-NS loss by measuring the chimeric protein in both high-copy- and low-copy-number vectors. These results showed that a high level of expression is needed for the hybrid protein to efficiently compensate some of the phenotypes that exhibit hns mutants. This is not surprising and may correspond to the reduced efficiency of the chimeric protein to generate higher-order oligomeric complexes with itself and/or Hha. On the other hand, the presence of the intact H-NS C-terminal domain accounts for the fact that Hha-Hns-Hyb2 shows affinity and specificity for DNA sequences that are targets for H-NS. Recent reports about the role of Hha in modulating the hilA promoter in S. enterica serovar Typhimurium and the esp operon of enterohemorrhagic E. coli O157:H7 have suggested that Hha specifically binds to its target sequences in hilA (10) and in esp (34). In both examples, authors purified the Hha protein used to test specific binding to DNA by using protein fusion strategies. In neither of the examples was it demonstrated that the purified Hha preparations were H-NS free. Thus, it cannot be ruled out that contaminating H-NS accounts for specific binding to the hilA and esp sequences. Because the molecular mass of Hha is about the half that of H-NS and because Hha tends to dimerize and oligomerize in solution, contamination by H-NS can only be detected by analyzing Hha preparations by Western blotting with H-NS-specific antibodies. Interestingly, for the hilA promoter different groups have demonstrated that Hha specifically binds to its target sequences in hilA (10) and in esp (34).

References


