The structural and functional organization of H-NS-like proteins is evolutionarily conserved in Gram-negative bacteria

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Summary
The structural gene of the H-NS protein, a global regulator of bacterial metabolism, has been identified in the group of enterobacteria as well as in closely related bacteria, such as Erwinia chrysanthemi and Haemophilus influenzae. Isolated outside these groups, the BpH3 protein of Bordetella pertussis exhibits a low amino acid conservation with H-NS, particularly in the N-terminal domain. To obtain information on the structure, function and/or evolution of H-NS, we searched for other H-NS-related proteins in the latest databases. We found that HvrA, a trans-activator protein in Rhodobacter capsulatus, has a low but significant similarity with H-NS and H-NS-like proteins. This Gram-negative bacterium is phylogenetically distant from Escherichia coli. Using theoretical analysis (e.g. secondary structure prediction and DNA binding domain modelling) of the amino acid sequence of H-NS, StpA (an H-NS-like protein in E. coli), BpH3 and HvrA and by in vivo and in vitro experiments (e.g. complementation of various H-NS-related phenotypes and competitive gel shift assay), we present evidence that these proteins belong to the same class of DNA binding proteins. In silico analysis suggests that this family also includes SPB in R. sphaeroides, XrvA in Xanthomonas oryzae and VicH in Vibrio cholerae. These results demonstrate that proteins structurally and functionally related to H-NS are widespread in Gram-negative bacteria.

Introduction
Prokaryotic cells contain many proteins involved in the organization and/or function of their chromosomal DNA. These proteins are generally small, abundant and basic (Hayat and Mancarella, 1995). Among them, HU, IHF, FIS and H-NS have been studied extensively during the past decade. HU is present in E. coli as a heterodimer capable of wrapping DNA into nucleosome-like particles. HU homologous proteins have been found in numerous prokaryotes as well as in organelles of eukaryotes (Oberto et al., 1994). IHF shows considerable amino acid sequence similarity with HU but, unlike it, IHF binds to a specific DNA sequence (Goosen and van de Putte, 1995). FIS is involved in recombination, replication and transcription processes (Finkel and Johnson, 1992).

H-NS is one of the most abundant DNA binding proteins in enterobacteria. Numerous phenotypes have been associated with hns mutations, especially a modification in the expression of several plasmid and chromosomal genes. Most of these target genes are also regulated by environmental parameters, such as pH, osmolarity and temperature (Atlung and Ingmer, 1997). Moreover, a possible role of H-NS in many other biological processes such as replication, recombination and transposition has also been suggested (Ussery et al., 1994). H-NS is a neutral protein isolated more than 20 years ago as a transcription factor (Jacquet et al., 1971). It was later shown to be involved in the organization of the bacterial chromosome by affecting the level of DNA condensation (Spassky et al., 1984). H-NS exists essentially as a homodimer (Falconi et al., 1988) and binds preferentially to curved DNA in vitro (Bracco et al., 1989; Yamada et al., 1991; Owen-Hughes et al., 1992; Jordi et al., 1997).

The three-dimensional structures of HU, IHF and FIS have been determined and their DNA binding domain identified (Tanaka et al., 1984; Yuan et al., 1991; Rice et al., 1996). In contrast, no information is available concerning H-NS structure, except for the organization of its C-terminal domain that has been resolved by nuclear magnetic resonance (NMR; Shindo et al., 1995). However, by characterization of dominant negative mutants and

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mutational analysis, it has been proposed that H-NS contains at least two distinct domains. The N-terminal region is implicated in protein–protein interactions and the C-terminal region in DNA binding (Ueguchi et al., 1996; Williams et al., 1996).

H-NS exhibits strong amino acid conservation in enterobacteria (Atlung and Ingmer, 1997) as well as in closely related bacteria such as Erwinia chrysanthemi (GenBank accession number X89444) and Haemophilus influenzae (Fleischmann et al., 1995). Moreover, a protein (StpA) showing 57% identity with H-NS has been characterized in E. coli (Zhang et al., 1996). Outside these groups, only one H-NS-like protein has been characterized so far: BpH3 from Bordetella pertussis, a phylogenetically more distant bacterium (Goyard and Bertin, 1997). Using theoretical and experimental studies, we provide evidence that H-NS-like proteins are ever more widespread and that they represent a large family of functionally and structurally related DNA binding proteins.

Results

To search for H-NS-related DNA binding proteins, we screened the latest SWISSPROT database (release 35.0) using the BLITZ program (Smith and Waterman, 1981). We found a protein, HvrA, that shows a low (30%) but significant similarity to the whole sequence of E. coli H-NS (Fig. 1). HvrA is a 103-amino-acid protein (137 in H-NS) known as a trans-acting positive regulator involved in the control of photosystem gene expression in R. capsulatus (Buggy et al., 1994).

**Fig. 1.** Alignment of R. capsulatus HvrA sequence with the H-NS sequence of E. coli (Pon et al., 1988). Residues conserved in both sequences are in grey boxes. The MULTALIGN method (Corpet, 1988) was used for initial alignment and refined manually (see below).

**Fig. 2.** Motility assay on semi-solid medium. A. FB8 wild-type strain. B. BE1411 hns::SmR mutant. C. BE1411/pLGH-NS. D. BE1411/pDStpA. E. BE1411/pSUH3.19. F. BE1411/pDIAS23. Plasmids pLGH-NS, pDStpA, pSUH3.19 and pDIAS23 produce H-NS, StpA, BpH3 and HvrA respectively. The results are representative of three independent experiments. White bars represent 10 mm.

**Effect of H-NS and H-NS-related proteins on various hns-related phenotypes**

Because of the low similarity between H-NS and HvrA, it was of interest to determine whether HvrA was able to reverse the effects of H-NS deficiency in a mutant strain. Mutations in the hns gene abolish motility in Salmonella typhimurium (Hinton et al., 1992) and in E. coli (Bertin et al., 1994). Therefore, we tested the effect of H-NS, StpA, BpH3 and HvrA synthesis on the swarming behaviour of an hns mutant. As expected, the hns::SmR mutant was non-motile on semi-solid medium (Fig. 2), while hns strains producing H-NS or BpH3 showed a motility close to that of the wild type (Bertin et al., 1994; Goyard and Bertin, 1997). Introduction of a plasmid encoding StpA or HvrA into an hns mutant also restored swarming but to a somewhat lesser extent (Fig. 2). Motility examination by light microscopy of the hns gene expressing hns and hns-related genes gave similar results (data not shown). Moreover, synthesis of H-NS, StpA, BpH3 and HvrA in a hns strain carrying a transcriptional fusion between the flagellin structural gene and lacZ resulted in increased β-galactosidase activity compared with the hns mutant (Table 1).

Besides the loss of motility, numerous phenotypes have been associated with hns mutations, e.g. β-glucoside utilization (Defez and De Felice, 1981), serine susceptibility (Lejeune et al., 1989), mucoidy (Sledjeski and Gottesman, 1995) and reduced growth yield (Barth et al., 1995). Like H-NS, synthesis of StpA, BpH3 and HvrA in the hns mutant strain reversed, at least in part, the hns phenotype with regard to β-glucoside utilization, mucoidy, growth yield and/or serine susceptibility (Table 2).
and secondary structures were predicted (Fig. 4). These on various H-NS-related phenotypes. E. coli strain
BE1414 hns-1001
BE1414 hns-1001 pDIA547
BE1414 hns-1001 pYCSstpA
BE1414 hns-1001 pSUH3.19
BE1414 hns-1001 pDIA523

a. Plasmids pDIA547, pYCSstpA, pSUH3.19 and pDIA523 produce
b. Data are the mean values ± standard deviations of three independent assays. β-Galactosidase activities are expressed as described previously (Bertin et al., 1994).

Proteome analysis of hns mutant producing H-NS and H-NS-related proteins

The protein pattern of an hns strain has been drawn up recently using two-dimensional electrophoresis (Laurent-Winter et al., 1997). To study the ability of the different H-NS-like proteins to substitute for H-NS in E. coli further, we evaluated their effects on the hns mutant proteome. In agreement with our previous results, the hns mutation decreased the accumulation of GlyA and OmpF proteins compared with the wild-type strain (Fig. 3) and increased the accumulation of DnaK, ProX, Gnd and GroEL. Synthesis of H-NS, StpA, BpH3 and HvrA in the hns strain restored, at least in part, the wild-type protein profile on a two-dimensional gel.

Structural and functional organization of the N-terminal domain in H-NS and H-NS-like proteins

The N-terminal domain seems to play a crucial role in the oligomerization process of H-NS and StpA. This has been demonstrated in vitro as well as in vivo (Williams et al., 1996; Ueguchi et al., 1997). These observations prompted us to see whether, despite a low amino acid conservation in their N-terminal domain, H-NS-like proteins such as BpH3 and HvrA were able to form dimers. To achieve high-level conditional expression of StpA and HvrA in E. coli, Ndel–Xhol DNA fragments including the stpA and hvrA coding regions were cloned into the expression vector pET-22b. The resulting plasmids pDIA536 and pDIA540 carry a recombinant gene that encodes a hybrid protein with six histidines at the C-terminus of StpA or HvrA respectively. Recombinant proteins were purified from E. coli extracts as described in Experimental procedures. The ability of these proteins to interact in vitro was analysed in EDC-NHS-catalysed cross-linking experiments (Fig. 5). Like H-NS (kindly provided by S. Rimsky) and StpA, BpH3 (Goyard and Bertin, 1997) and HvrA could be cross-linked into species with a twofold increase in their apparent molecular weight. This indicates that each

Table 1. Expression of fiIC–lacZ transcriptional fusion.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmida</th>
<th>β-Galactosidase specific activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>YK3421</td>
<td>Wild type</td>
<td>−</td>
<td>5950 ± 255</td>
</tr>
<tr>
<td>BE1414</td>
<td>hns-1001</td>
<td>−</td>
<td>173 ± 3</td>
</tr>
<tr>
<td>BE1414</td>
<td>hns-1001</td>
<td>pDIA547</td>
<td>4140 ± 143</td>
</tr>
<tr>
<td>BE1414</td>
<td>hns-1001</td>
<td>pYCSstpA</td>
<td>2658 ± 106</td>
</tr>
<tr>
<td>BE1414</td>
<td>hns-1001</td>
<td>pSUH3.19</td>
<td>3293 ± 71</td>
</tr>
<tr>
<td>BE1414</td>
<td>hns-1001</td>
<td>pDIA523</td>
<td>1163 ± 44</td>
</tr>
</tbody>
</table>


Table 2. Effect of H-NS and H-NS-like proteins on various H-NS-related phenotypes.

<table>
<thead>
<tr>
<th>E. coli strain (relevant genotype)a</th>
<th>β-Glucoside utilizationb</th>
<th>Serine susceptibilityc</th>
<th>Mucoidyd</th>
<th>Growth yeild*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB8 (wild-type)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BE1411 (hns::SmR)</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BE1411/pLGH-NS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BE1411/pDStpA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BE1411/pSUH3.19</td>
<td>±</td>
<td>−</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>BE1411/pDIA523</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

a. Plasmids pLGH-NS, pDStpA, pSUH3.19 and pDIA523 were introduced into BE1411 hns strain to synthesis H-NS, StpA, BpH3 and HvrA respectively.
b. +, β-Glucoside metabolism was revealed by the appearance of red colonies on MacConkey–salicin agar plates; −, no metabolism by white colonies; ±, intermediate metabolism by pink colonies.
c. +, No growth on 0.4% glucose M63 medium containing 40 μg ml⁻¹ L-serine; −, growth on the same medium; ±, intermediate growth level.
d. +, Mucoid phenotype was observed on 0.4% glucose M63 medium; −, no mucoid phenotype.
e. +, Growth yield was evaluated by measurement of the OD₆₀₀ in stationary phase; −, 20 ± 2% reduction in the growth yield in comparison with the wild type; ±, intermediate growth yield.

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Fig. 3. Effect of H-NS and H-NS-like proteins on the accumulation of various H-NS-dependent proteins. Quantitative data were obtained from Coomassie blue-stained two-dimensional gels. The level of protein accumulation is plotted relative to the level of synthesis in the wild-type strain, which was assigned a value of 100%. Plasmids pLGH-NS, pDStpA, pSUH3.19 and pDIA523 produce H-NS, StpA, BpH3 and HvrA respectively.

Fig. 4. Structurally based alignment and prediction of secondary structures of H-NS, StpA, BpH3 and HvrA. The alignment was achieved using both mult align and HCA plots. Identical residues in comparison with H-NS are in grey boxes. The secondary structures were predicted using the Chou–Fasman algorithm (Chou and Fasman, 1977) modified by Ralph et al. (1987). Bold is plotted the STRIDE assignment for H-NS C-terminal domain from NMR structure and for StpA, BpH3 and HvrA C-domains from MODELLER H-NS = α-helix; G = 3_10-α-helix; E = β-strand; B = β-bridge; and T = turn.
protein is able to interact with itself and to form dimers (at least) in vitro.

**Structural and functional organization of the C-terminal domain in H-NS and H-NS-like proteins**

The C-terminal domain is the most conserved region in H-NS-like proteins. The amino acid composition of the C-domain of StpA (residues 91–134) is 70% identical to that of H-NS (residues 91–137), while the C-domains of BpH3 (residues 82–123) and HvrA (residues 61–102) are both 40% identical to that of H-NS. Sequence conservation and distribution of hydrophobic clusters suggest that the secondary and tridimensional structures of all these C-domains are similar. On the basis of the alignment of H-NS, StpA, BpH3 and HvrA (Fig. 4), we calculated the three-dimensional structure of their C-domains by homology with the NMR structure of H-NS using MODELLER (Sali and Blundell, 1993). Three-dimensional structures were obtained by satisfying spatial restraints derived from the structure of the C-terminal domain of H-NS obtained by NMR experiments (Shindo et al., 1995). The all-atom models calculated for the C-domain of H-NS and H-NS-like proteins are shown in Fig. 6. The secondary structures of the C-domains were deduced using STRIDE, which assigns structure from the atomic co-ordinates of a protein (Fig. 4). After energy minimization, all secondary structures were restricted to a common core of $\beta$-turns and helices ($\alpha$ or $3\alpha_0$). Structural topology of the C-terminal domain is conserved: the hydrophobic core of H-NS that holds this structure packed is built around the interactions of W109 and Y97, Y99, F134 and I135. Distances between Y97, Y99 and W109 are less than 5 Å. Y97 in H-NS is conserved in all proteins and corresponds to Y97, Y87 and Y67 in StpA, BpH3 and HvrA respectively. W109 is also strictly conserved and corresponds to W109, W96 and W67 in StpA, BpH3 and HvrA respectively. W109 is also strictly conserved and corresponds to W109, W96 and W67 in StpA, BpH3 and HvrA respectively.

**Fig. 5.** Analysis of the in vitro protein–protein interactions in H-NS and H-NS-like proteins by chemical cross-linking experiments. After 1 h incubation at room temperature with (+) or without (−) EDC-NHS cross-linking reagents, proteins were loaded onto a SDS–14% acrylamide gel and silver stained.

**Fig. 6.** Three-dimensional structures of the C-domains of H-NS, StpA, BpH3 and HvrA. H-NS structure was determined by NMR (PDB code 1HNR), and the three other structures were calculated with MODELLER (Sali and Blundell, 1993) using the alignment presented in Fig. 4.
W77 in StpA, BpH3 and HvrA respectively. W109 is surrounded by two polar residues, threonine or serine, in all proteins.

The strong amino acid conservation in the C-terminal domain of the various H-NS-like proteins prompted us to determine whether the DNA binding properties of H-NS-related proteins were similar to those of H-NS, i.e. a preferential binding to the bla promoter region (Lucht et al., 1994; Zuber et al., 1994). The binding properties of H-NS, StpA, BpH3 and HvrA were evaluated in gel shift experiments. DNA of plasmid pBR322 was restricted by Taq I and Ssp I restriction enzymes to generate a 217 bp fragment encompassing the bla promoter region and additional small DNA fragments used as competitors for binding. As shown in Fig. 7, a low concentration of all proteins (from 0.25 to 2 \( \mu M \), depending on the protein used) resulted in significant retardation of the 217 bp fragment carrying the bla promoter. Such an alteration in electrophoretic mobility was not observed with other DNA fragments. A twofold increase in concentration resulted in a full retardation of the bla promoter. This result is in agreement with previous data on H-NS, suggesting a co-operative binding of this protein to DNA (Rimsky and Spassky, 1990; Lucht et al., 1994). No such retardation was observed with other DNA fragments of plasmid pBR322. These data suggest that StpA, BpH3 and HvrA, like H-NS, have functional similarities with respect to DNA binding.

H-NS and H-NS-like proteins constitute a large class of DNA binding proteins

As the C-terminal domain is highly conserved in the four proteins studied, we looked for similar domains in proteins of the databases. A 9-amino-acid sequence corresponding to the pattern centred around Gln at position 112 in H-NS (Fig. 4) was used to screen the latest databases using the BLITZ program (Smith and Waterman, 1981). Two additional H-NS-like proteins were identified in Gram-negative bacteria. SPB is a 104-amino-acid transcription factor isolated from *R. sphaeroides* (Shimada et al., 1996). XrvA is a 132-amino-acid protein that regulates virulence to rice in *Xanthomonas oryzae* (GenBank accession number X97866). Moreover, we recently identified VicH (*Vibrio cholerae* H-NS-like protein), a 135-amino-acid protein (GenBank accession number AJ010791) showing a strong amino acid conservation between its C-terminal domain and that of H-NS and H-NS-like proteins (unpublished results). As in H-NS, StpA, BpH3 and HvrA, theoretical analysis suggests that the N-terminal domains of SPB, XrvA and VicH are mainly \( \alpha \)-helical and able to adopt a coiled-coil conformation, frequently identified as the dimerization element in several transcription factors (Lupas, 1996). Moreover, they appear to be organized in two sub-domains separated by a loop (data not shown). Finally, analysis of conserved residues in the C-terminal part supports the existence of the following consensus motif in the DNA binding domain of all these proteins: [AP]–K–Y–x(5,6)–[GS]–[ED]–x(0,2)–T–W–[TS]–[G][QR][G][RK][TAK][PL].

A structurally based alignment of all H-NS and H-NS-like proteins was achieved using both MULTALIGN and HCA methods (data not shown). This multiple alignment was used to generate a phylogenetic tree using protein parsimony analysis (Fig. 8). H-NS-like proteins form three obvious clusters in a parsimony tree, which correspond to H-NS of enterobacteria, to H-NS of *H. influenzae*, StpA of *E. coli* and *S. typhimurium* and VicH of *V. cholerae*, and to H-NS-like proteins isolated from *Rhodobacter* and *Bordetella* genera.

Discussion

During the past decade, genetic and biochemical analysis
of the DNA binding protein H-NS has advanced our understanding of its structure and function in *E. coli* as well as in other enterobacteria (Ussery *et al.*, 1994; Atlung and Ingmer, 1997). Recently, *hns*-like genes have been identified in *Erwinia chrysanthemi* (GenBank accession number X89444), in *H. influenzae* (Fleischmann *et al.*, 1995) and in *B. pertussis* (Goyard and Bertin, 1997), but no information is available concerning the role of these proteins. Moreover, in a very recent report, it has been suggested on the basis of a multiple alignment that H-NS-like protein sequences (Buggy *et al.*, 1994; Shimada *et al.*, 1996; Atlung and Ingmer, 1997; Goyard and Bertin, 1997) (GenBank accession numbers U82566, X89444, X97866, AJ006983 and AJ010791). Species abbreviations are as follows: *ecoli*, *Escherichia coli*; *shifl*, *Shigella flexneri*; *salty*, *Salmonella typhimurium*; *serma*, *Serratia marcescens*; *erwch*, *Erwinia chrysanthemi*; *provu*, *Proteus vulgaris*; *haein*, *Haemophilus influenzae*; *xanor*, *Xanthomonas oryzae*; *borpe*, *Bordetella bronchiseptica*; *rhoca*, *Rhodobacter chrysanthemi*; *provu*, *Proteus vulgaris*; *haein*, *Haemophilus influenzae*; *serma*, *Serratia marcescens*; *erwch*, *Erwinia chrysanthemi*; *provu*, *Proteus vulgaris*; *haein*, *Haemophilus influenzae*. Studies of mutant H-NS proteins suggest that the N-terminal domain plays a crucial role in transcriptional repression of the *proU* operon and in the oligomerization process (Ueguchi *et al.*, 1996; Williams *et al.*, 1996). Only a few amino acids are conserved in both regions in H-NS, StpA, BpH3 and HvrA. For example, the Leu residue in position 26 in H-NS and in StpA corresponds to Leu at positions 20 and 14 in BpH3 and HvrA respectively (Fig. 4). However, despite a low amino acid conservation in their N-terminal domain, we observed that H-NS, StpA, BpH3 and HvrA decreased, at least in part, the accumulation of ProX in an *hns* mutant (Fig. 3). This provides evidence that all proteins are able to repress *proU* expression at low osmolality. Moreover, by chemical cross-linking experiments, we showed that BpH3 and HvrA are able to form homodimers in vitro like H-NS and StpA (Ueguchi *et al.*, 1996; Williams and Rimsky, 1997) (Fig. 5). Taken together, these data reinforce the idea that similarities exist in the functional and structural organization of the N-terminal region in H-NS and H-NS-like proteins. Theoretical analysis of amino acid sequences further supports such a structural conservation. Indeed, the N-terminal domain was predicted mainly as α-helical in all four proteins (Fig. 4). In addition, the N-domain of H-NS and H-NS-like proteins is organized in two parts separated by a loop and connected to the C-domain by a second loop, which corresponds to a protease-sensitive linker recently identified in H-NS and StpA (Cusick and Belfort, 1998). Both loops are rich in positively charged amino acids. Their length seems to be the major structural difference between the four proteins (Fig. 4).

A similar organization of the C-terminal part in H-NS, StpA, BpH3 and HvrA is suggested by secondary structure prediction (Fig. 4) and C-domain modelling (Fig. 6). These observations are corroborated by gel shift experiments. Indeed, like H-NS (Lucht *et al.*, 1994; Zuber *et al.*, 1994), StpA, BpH3 and HvrA were shown to bind preferentially to the *bla* promoter region (Fig. 7). Unlike the N-terminal domain, a strong amino acid conservation (i.e. more than 40%) was observed in the DNA binding domain of the four proteins (Fig. 4), as in that of other H-NS-related proteins such as *vicH* of *V. cholerae* (unpublished results). This observation supports a key role for these highly conserved residues in the function of all H-NS-related proteins. Mutagenesis of most of them in H-NS and StpA has been shown to result in an altered pattern of gene expression (Ueguchi *et al.*, 1996; Williams *et al.*, 1996). In particular,

**Fig. 8.** Phylogenetic tree constructed by a parsimony method (Felsenstein, 1989) from a structurally based alignment of H-NS-like protein sequences (Buggy *et al.*, 1994; Shimada *et al.*, 1996; Atlung and Ingmer, 1997; Goyard and Bertin, 1997) (GenBank accession numbers U82566, X89444, X97866, AJ006983 and AJ010791). Species abbreviations are as follows: *ecoli*, *Escherichia coli*; *shifl*, *Shigella flexneri*; *salty*, *Salmonella typhimurium*; *serma*, *Serratia marcescens*; *erwch*, *Erwinia chrysanthemi*; *provu*, *Proteus vulgaris*; *haein*, *Haemophilus influenzae*; *xanor*, *Xanthomonas oryzae*; *borpe*, *Bordetella pertussis*; *borbr*, *Bordetella bronchiseptica*; *rhoca*, *Rhodobacter chrysanthemi*; *provu*, *Proteus vulgaris*; *haein*, *Haemophilus influenzae*. Synthesis of H-NS, StpA, BpH3 and HvrA in *E. coli* clearly showed that all these proteins were able to complement, at least in part, various phenotypes of an *hns* mutant such as loss of motility (Fig. 2 and Table 1), β-glucoside utilization, serine susceptibility, mucoidy and reduction in growth yield (Table 2). Moreover, expression of their structural gene in an *hns* mutant resulted in a protein pattern on two-dimensional electrophoresis close to that of the wild-type strain (Fig. 3). The difference between H-NS, StpA, BpH3 and HvrA in their ability to restore the wild-type pattern could be accounted for by the proteins themselves and/or the level of expression of their structural gene in the *hns* strain.

In the present paper, using an approach involving theoretical analysis of functional and/or structural protein domains and/or the level of expression of their structural gene in the *hns* strain.
mutations affecting Y97 abolish H-NS and StpA function. W109 also seems to be important in this respect. In fact, substitution of this residue impairs H-NS binding to curved DNA but has no effect on binding to non-curved DNA (Spurio et al., 1997).

The results presented in the present paper provide evidence that proteins of the H-NS family are widespread at least in Gram-negative bacteria. However, the physiological role of all these proteins as well as the genetic organization of their structural gene and the regulation of their expression present several differences between microorganisms. H-NS protein is involved in a number of cellular functions such as the modulated expression of numerous environmentally regulated genes. The hns gene is not part of an operon in E. coli (Ussery et al., 1994; Atlung and Ingmer, 1997). In the presence of H-NS, the homologous protein StpA of E. coli is expressed at a low level. Inactivation (Sondén and Uhlin, 1996; Zhang et al., 1996) or overexpression of stpa (Fig. 3) results in altered transcription of some H-NS-regulated genes. However, unlike H-NS, StpA acts as an RNA chaperone in vitro (Zhang et al., 1996). In B. pertussis, bph3 is present as a single gene. Even if the expression of bph3 in E. coli hns mutants reversed numerous H-NS-dependent phenotypes to the wild type (Fig. 2 and Table 2), the role of BpH3 in the metabolism of the Bordetella genus is not yet known (Goyard and Bertin, 1997). Similarly, the precise function of XrvA in the physiology of X. oryzae has not been reported, except its involvement in virulence to rice. In contrast, the role of SPB and HvrA in the regulation of the puf operon in R. sphaeroides and in R. capsulatus, respectively, has been demonstrated (Buggy et al., 1994; Shimada et al., 1996). Both proteins play a role in the light-mediated expression of these genes by a specific binding to their promoter region. However, spb is transcribed monocistronically, and SPB is a repressor of the puf operon. In contrast, hvrA is transcribed polycistronically with the regulatory protein RegA structural gene, and HvrA is a trans-activator of puf expression. It is, therefore, tempting to speculate that proteins of the H-NS class have evolved from an ancestral protein (Fig. 8) of broad specificity (like H-NS in enterobacteria) to more specialized proteins, either by duplication (like the paralogous proteins StpA in E. coli and in S. typhimurium) or by divergence (like the orthologous proteins BpH3, BbH3, XrvA, SPB, HvrA and VicH in B. pertussis, B. bronchiseptica, X. oryzae, R. sphaeroides, R. capsulatus and V. cholerae respectively).

Experimental procedures

Bacterial strains, plasmids and growth conditions

The hns::Sm<sup>R</sup> mutation of strain BE1411 results from the insertion into the FB8 wild-type strain (Bruni et al., 1977) of a Sm<sup>R</sup>/Sp<sup>R</sup> cassette at the HpaI site located at the 38th codon of the hns gene. This strain was constructed by allele replacement using the M13mp8 phage (Blum et al., 1989). The hns-1001 mutation was introduced into YK3421 containing a fillC–lacZ transcriptional fusion (Komedu, 1982) by P1 transduction as described previously (Bertin et al., 1994), giving rise to BE1414. Rhodobacter capsulatus strain CIP 104408 was used for polymerase chain reaction (PCR) amplification of the hvrA gene.

Plasmids pLGH-NS and pDStpa (Williams et al., 1996), pSUH3.19 (Goyard and Bertin, 1997) and pDIAS23 were introduced into the hns strain by transformation to produce H-NS, StpA, BpH3 and HvrA, respectively, without the addition of IPTG. For compatibilities reasons with resident Kan<sup>R</sup> and Ap<sup>R</sup> markers, plasmids pYCStpA (Williams et al., 1996) and pDIA547 were introduced into YK3421 to evaluate the effect of H-NS and H-NS-like proteins on flagella synthesis. To overproduce StpA-His6 and HvrA-His6 recombinant proteins, plasmids pDIA536 and pDIA540, respectively, were introduced into the BL21(DE3) strain (Novagen) in the presence of plasmid pDIA17 synthesising the lacI repressor (Munier et al., 1991).

Bacterial growth was carried out at 37°C in Luria–Bertani medium or L-M63 medium supplemented when required with 40 µg ml<sup>−1</sup> serine (Miller, 1972). Swarm plates containing 25 ml of tryptone medium were used to assess the motility of bacteria as described previously (Bertin et al., 1994). Metabolism of β-glucosides was tested on MacConkey indicator agar plates with 1% salicin as a carbon source. β-Galactosidase activity was measured as described previously (Bertin et al., 1994) from cells grown in M63 medium supplemented with 0.01% uracil, 0.1% casamino acids and 0.4% succinate as a carbon source. When required, antibiotics were added at the following concentrations: kanamycin or ampicillin, 25 µg ml<sup>−1</sup>; streptomycin, 10 µg ml<sup>−1</sup>; chloramphenicol, 20 µg ml<sup>−1</sup>.

DNA manipulations

The hvrA gene of R. capsulatus with a putative ribosome binding site was PCR amplified as follows: one clone of R. capsulatus was resuspended in 50 µl of 10 mM Tris–1 mM EDTA buffer containing 100 µg ml<sup>−1</sup> Proteinase K. The suspension was incubated for 1 h at 65°C. Proteinase K was inactivated by heating for 10 min at 95°C, and the suspension was then centrifuged at 10 000 x g for 5 min. DNA amplification was carried out with 20 µl of supernatant using Pfu polymerase (Stratagene) and PCR core reagents from Perkin-Elmer as well as synthetic oligonucleotides 5'-AACAGGTCCATGGAAACCCTACTGAG-3' containing a PsI site and 5'-CGGATCCCGGGTGCGCTGAACCGCGGCC-3' containing a BamHI site. The final concentration of MgCl<sub>2</sub> was adjusted to 2 mM. A first step, at 98°C for 45 s, was followed by 30 cycles consisting of 45 s at 95°C, 45 s at 56°C and 45 s at 72°C. The resulting DNA fragment of about 0.4 kb was purified on a Millipore Ultrafree-MC column. The hvrA gene was cloned into PsI and BamHI restriction sites of pSU19 plasmid (Bartolome et al., 1991) under the control of the lac promoter, giving rise to plasmid pDIA523.

Plasmid pDIA547 was constructed by inserting the hns structural gene and its promoter region into the pSU19 plasmid in the opposite orientation with respect to the resident Plac promoter.

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To overproduce HvrA-His6 and StpA-His6 proteins, their structural gene was PCR amplified from genomic DNA using primers introducing a Ndel cloning site at the 5’ end and a Xhol cloning site at the 3’ end of each fragment. PCR products were inserted into the Ndel and Xhol sites of the pET-22b vector (Novagen), giving rise to plasmids pDIA536 and pDIA540 respectively.

**Preparation of total protein extracts and two-dimensional gel electrophoresis**

Protein samples were prepared by ultrasonic treatment, and detection of proteins was performed by Coomassie blue staining of two-dimensional gels as described previously (Laurent-Winter et al., 1997). Gels were digitalized using a JX-330 scanner (Sharp). Quantitative data were obtained using the PD Quest software package (PDI).

**Protein purification**

Recombinant proteins HvrA-His6 and StpA-His6 were purified from *E. coli* using NiSO₄ chelation columns (Qiagen). BL21(DE3) strain, carrying either plasmid pDIA536 or plasmid pDIA540, was grown to mid-log phase in LB medium. Cultures of cells synthesising StpA and HvrA were induced by 0.5 mM IPT6 for 1 h and 3 mM IPTG for 2 h respectively. Bacterial pellets of 1 l culture of cells containing overexpressed proteins were disrupted by sonication in buffer A (50 mM NaH₂PO₄, pH 8, and 300 mM NaCl) containing 20 mM and 70 mM imidazole for HvrA and StpA respectively. Cell debris was removed by centrifugation, and crude extracts were loaded onto a 3 ml NiSO₄ chelation column. After adsorption on the column and washing with buffer A containing 100 mM imidazole, proteins were eluted with a 200–500 mM imidazole gradient in buffer A. Buffer was changed to 500 mM NaCl and 40 mM NaH₂PO₄, pH 8, using a PD 10 desalting column (Pharmacia). Protein concentration was measured colorimetrically with the Bio-Rad reagent using the Bradford procedure. The purity of both HvrA and StpA preparations was estimated to be more than 95% on SDS–PAGE stained with Coomassie brilliant blue.

**Protein–protein cross-linking**

Chemical cross-linking experiments with wild-type H-NS and recombinant StpA-His6, His6-BpH3 and HvrA-His6 proteins were performed using the chemical cross-linker 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) and the catalyst N-hydroxysuccinimide (NHS) as described previously (Williams et al., 1996). Reaction mixtures containing each protein at a final concentration of 25 μM were loaded onto an SDS–14% acrylamide gel (Prosiebe) and silver stained.

**Gel retardation assay**

DNA fragments were obtained by digestion of plasmid pBR322 with TaqI and SspI restriction enzymes and incubated for 15 min at room temperature with H-NS, StpA, BpH3 or HvrA in 10 μl of reaction mixture containing 40 mM HEPES, pH 8, 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.022% NP40 and 0.1 mg ml⁻¹ BSA. Protein–DNA complexes were resolved as described previously (Goyard and Bertin, 1997).

**Sequence analysis**

Analysis of sequence homology was carried out using the BLAST algorithm (Smith and Waterman, 1981). The MULTALIGN method (Corpet, 1988) was used for sequence alignments, refined by the hydrophobic cluster analysis (HCA) method (Lemesle-Varloot et al., 1990). Identity and similarity scores were calculated by reference to H-NS. Secondary structure prediction was performed using the Chou and Fasman algorithm (Chou and Fasman, 1978) and the STRIDE algorithm was used for protein secondary structure assignment from atomic co-ordinates (Frishman and Argos, 1995). Tridimensional structures of the C-terminal domain of BpH3, HvrA and StpA were calculated by homology with the NMR structure of the C-terminal domain (47 amino acids) of H-NS (Shindo et al., 1995) using MODELLER (Sali and Blundell, 1993). Phylogenetic relationships were investigated using the protein parsimony method (PROTPARS) from the PHYLIP package (Felsenstein, 1989).

**Acknowledgements**

We are grateful to P. Glaser, I. Martin and T. Pugsley for critical reading of the manuscript. We thank S. Rimsky and C. Badaut for providing us with H-NS protein and various plasmids and for helpful advice. We also thank S. Ngo for help in some experiments. Financial support came from the Institut Pasteur and the Centre National de la Recherche Scientifique (URA D1129). N.B. was supported by a grant from FNRS-Televie.

**References**


