Differential effects and interactions of endogenous and horizontally acquired H-NS-like proteins in pathogenic Escherichia coli

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Summary

The nucleoid-associated protein H-NS is important for gene regulation in Escherichia coli. We have studied H-NS interaction with StpA and an uncharacterized H-NS-like protein, Hfp, in the uropathogenic E. coli isolate 536 that expresses all three nucleoid-associated proteins. We found distinct interactions of the three proteins at the protein level, resulting in the formation of heteromers, as well as differences in their gene expression at the transcriptional level. Mutants lacking either StpA or Hfp alone did not exhibit a phenotype at 37°C, which is consistent with a low level of expression at that temperature. Expression of the hfp and stpA genes was found to be induced by apparently diametrical conditions, and StpA and Hfp levels could be correlated to modulatory effects on the expression of different H-NS targets, the bgl operon and operons for virulence factors such as fimbriae and capsular polysaccharide. The hns/hfp and hns/stpA double mutants displayed severe growth defects at low and high temperatures respectively. Our findings demonstrated different requirements for the alternative H-NS/Hfp/StpA combinations under these growth conditions. We propose that Hfp and StpA have distinct functions and roles in a dynamic pool of nucleoid-associated proteins that is adapting to requirements in a particular environment.

Introduction

The bacterial nucleoid is organized in a heterogeneous manner containing areas with highly condensed, quiescent DNA, and areas that are accessible for proteins involved in DNA processes such as replication, repair and transcription. The distribution of transcriptional activity along the chromosome is dynamic and changes rapidly according to the current requirements of the cell (Travers and Muskhelishvili, 2005). In Escherichia coli, several small proteins with DNA-binding activity play an important role in shaping the structure of the nucleoid and, simultaneously, in ensuring its flexibility. These so-called nucleoid-associated proteins affect the architecture of the nucleoid by either bending or bridging DNA (Dame, 2005; Lujisterburg et al., 2006).

One of the most abundant nucleoid-associated proteins is the histone-like nucleoid structuring protein H-NS. The protein consists of a C-terminal DNA-binding domain and an N-terminal oligomerization domain (Ueguchi et al., 1997; Renzoni et al., 2001; Bloch et al., 2003). The precise mechanism of DNA binding and nucleoprotein complex formation by H-NS is still uncertain, despite many efforts in the last years. For instance, higher-order complexes of H-NS molecules bound along the DNA, or bridges between two adjacent DNA stretches mediated by H-NS have been shown (Rimsky et al., 2001; Dame et al., 2005), both with potential huge impact on DNA processes. The effects of H-NS have been studied in detail in non-pathogenic E. coli, where it first was suggested to cause transcriptional silencing and now is considered as a global regulator of gene expression (Göransson et al., 1990; Atlung and Ingmer, 1997; Hommais et al., 2001). In uropathogenic E. coli strain
536, we could also demonstrate a major role of H-NS on the expression of more than 500 genes, including many virulence factors such as fimbriae, cytotoxins and siderophores (Müller et al., 2006).

A second member of the H-NS-like protein family that is found in both pathogenic and non-pathogenic E. coli variants is the StpA protein. StpA shares 58% sequence identity to H-NS and has a similar domain structure (Cusick and Belfort, 1998). It was shown to bind both DNA and RNA, the latter conferring RNA chaperone activity (Zhang et al., 1996; Sonnenfield et al., 2001; Mayer et al., 2007). Mutations in the stpA gene do not result in a notable phenotype under standard growth conditions, but StpA can compensate for the lack of hns in repression of some genes, thereby being considered as a molecular back-up for H-NS (Sondén and Uhlin, 1996; Zhang et al., 1996). Moreover, StpA is directly interacting with the H-NS protein by forming heteromers (Johansson et al., 2001).

The progress in genome sequencing led to the discovery of several genes encoding homologues or interaction partners for H-NS (Bertin et al., 2001; Tendeng and Bertin, 2003). Interestingly, many of those are encoded on transmissive genomic elements and many are also involved in virulence. One example is the plasmid-encoded H-NS-like protein of *Shigella flexneri*, Sfh (Beloin et al., 2003; Doyle and Dorman, 2006). Sfh shares about 60% amino acid homology with H-NS and StpA and it is able to interact with both proteins (Deighan et al., 2003). Furthermore, it is able to complement an hns mutation and affects virulence gene expression when overexpressed, but also plays an additional role in transmission and maintenance of its encoding plasmid (Beloin et al., 2003; Doyle et al., 2007). Acquisition of horizontally acquired proteins that are able to interact with H-NS might be an important step in fine tuning the pre-existing regulatory network of the host cell, which might explain the occurrence of several H-NS-like proteins within some bacterial species.

In this study, we provide evidence that the uropathogenic *E. coli* strain 536, but not non-pathogenic K-12 laboratory strains, harbours a third member of the H-NS-like protein family, the Hfp protein, which shares homology with both H-NS and StpA. The gene coding for Hfp is located on a genomic island inserted at the *serU* locus, and its expression is repressed by H-NS. Like StpA, the Hfp protein has biological activity and can complement several hns phenotypes. To study the functions and interplay of the horizontally acquired Hfp protein and the endogenous regulators H-NS and StpA, a series of mutant derivatives were constructed and searched for differences in their phenotype. Possible interactions between the three regulators were studied at the protein and the transcriptional level. The results obtained verified that a horizontally acquired regulator such as Hfp can be successfully integrated into the regulatory circuits of the cell. Moreover, our results from growth experiments and transcription analysis of the three corresponding genes led to the hypothesis of a flexible composition of the nucleoid-associated protein pool with changing temperature or with growth phase, resulting in distinct heteromeric protein complexes.

**Results**

Extraintestinal pathogenic *E. coli* variants commonly express three members of H-NS-like proteins

The genes coding for H-NS and StpA can be found in the chromosome of all characterized *E. coli* variants. In this study, the genome of uropathogenic *E. coli* strain 536 (Accession No. CP000247), as a newly sequenced representative of extraintestinal pathogenic *E. coli* (ExPEC) isolates, was searched for the presence of H-NS-like proteins. Like *E. coli* K-12 strains, UPEC (uropathogenic *E. coli*) strain 536 possesses both the *hns* and the *stpA* equivalents. However, a third hit was obtained represented by gene ECP_1927, which is annotated as a putative DNA-binding protein (Accession No. ABG69928). The sequence encodes a protein of 134 amino acids (a.a.), which was now termed Hfp for H-NS-family protein. Hfp exhibits high homology to both H-NS and StpA (57.7% and 52.2% a.a. identity respectively) over the entire length of the sequence (Fig. 1A). When comparing the chromosomal region in the vicinity of the *hfp* gene with the corresponding region in the genome of the K-12 strain MG1655, a 23 kb large DNA stretch including the *hfp* gene was only present in the genome of UPEC strain 536 (Fig. 1B). The insertion was flanked by direct repeats associated with the *serU* tRNA locus and the overall GC content (38%) differed from the rest of the genome (50%), all of which are qualifiers for horizontally acquired DNA regions, so-called genomic islands.

In order to further investigate the occurrence of Hfp in other *E. coli* sero- and pathotypes, a collection of 133 isolates comprising both extraintestinal and intestinal pathogenic *E. coli* isolates (ExPEC and IPEC) as well as faecal isolates from healthy volunteers was screened for the presence of the *hfp* gene by PCR. A 355 bp PCR product could be amplified in 55 out of the 133 strains (Table 1). Most of the *hfp*-positive strains belonged to the ExPEC group (58% positive isolates), with UPECs as prevalent subgroup (79% positive isolates). The *hfp* gene was also present in a high number of faecal strains (39% positive isolates).

Taken together, these results indicate that UPEC strain 536, and probably a majority of other uropathogenic *E. coli* isolates, carries genes for three highly homologous nucleoid-associated proteins: the core chromosome-encoded proteins H-NS and StpA, as well as the so far uncharacterized Hfp protein.
Hfp is directly interacting with the H-NS protein

To test whether the \textit{hfp} gene encodes a functional gene product, plasmid pCM9 carrying the \textit{hfp} gene and an approximately 200 nt upstream region was introduced into \textit{hns} mutants of the K-12 strains MG1655 and MC4100, and its ability to complement for the lack of H-NS was assessed in the cases of some well-described \textit{hns} mutant phenotypes (Fig. S1). In brief, we provide evidence that the \textit{hfp} gene produces a functional protein which can complement for the lack of H-NS in terms of several H-NS-dependent operons.

Heteromeric interactions between H-NS and its homologue StpA have been described (Johansson et al., 2001). Evidence for a direct protein–protein interaction between H-NS and Hfp were obtained when a His-tagged version of the Hfp protein was overexpressed and purified using Ni-affinity chromatography. When the 16 kDa His-Hfp protein was eluted from the chromatography column, a second protein of approximately 15.5 kDa was co-purified in equimolar ratio (Fig. 2A). N-terminal sequencing of the smaller protein resulted in the sequence SEALKILM, which matches the H-NS sequence. Immunoblotting with antiserum recognizing H-NS confirmed its presence in all purification fractions (data not shown). When performing the same purification procedure in an\textit{hns} mutant of the expression strain BL21, only the 16 kDa His-Hfp protein was eluted from the chromatography column, a second protein of approximately 15.5 kDa was co-purified in equimolar ratio (Fig. 2A). N-terminal sequencing of the smaller protein resulted in the sequence SEALKILM.

\begin{table}[h]
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\begin{tabular}{|l|c|c|c|}
\hline
Variant & \% \textit{hfp}+ & \% \textit{hfp}– & \% total \\
\hline
\textit{ExPEC} (\textit{n} = 67) & 39 & 28 & 58.2 \\
\text{whereof UPEC (\textit{n} = 42)} & 33 & 9 & 78.6 \\
\text{Faecal isolates (\textit{n} = 39)} & 15 & 24 & 38.5 \\
\text{whereof known ECOR B2 (\textit{n} = 11)} & 5 & 6 & 45.5 \\
\textit{IPEC} (\textit{n} = 27) & 1 & 26 & 3.7 \\
\text{Total (\textit{n} = 133)} & 55 & 78 & 41.4 \\
\hline
\end{tabular}
\caption{Occurrence of the \textit{hfp} gene in different \textit{E. coli} variants.\textit{}}
\end{table}

\textbf{Fig. 1.} Comparison of amino acid sequences and chromosomal location of the \textit{hfp} gene.

\begin{itemize}
\item A. Alignment of the three nucleoid-associated proteins of UPEC strain 536 (H-NS = ECP_1284; StpA = ECP_2632; Hfp = ECP_1927). Identical residues are depicted with light shading; dark shading indicates residues with > 50% similarity. The domain structure is indicated above the sequence. Residues that are essential for the function of H-NS are marked with filled arrowheads; residues that confer a dominant-negative phenotype when substituted are marked with open arrowheads (as summarized by Williams and Rimsky (1997)).
\item B. Genome comparison of the \textit{hfp} region of UPEC strain 536 and non-pathogenic \textit{E. coli} K-12 strain MG1655. Dark shading indicates regions of high homology, while horizontally acquired sequences can be identified as white regions with no homology. The localization of the \textit{hfp} gene in the genome of the pathogen, as well as of tRNA loci is marked within the sequences. The corresponding \textit{serU}-associated genomic island is located in close vicinity to the ‘high pathogenicity island’ (HPI) (Carniel, 2001). The GC content of the UPEC strain 536 sequence is depicted at a window size of 500 nucleotides.
\end{itemize}

\textbf{Table 1.} Occurrence of the \textit{hfp} gene in different \textit{E. coli} variants.\textit{a}

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\textit{a.} The presence of the \textit{hfp} gene was monitored in clinical isolates by PCR screening using primers Hfp-RT1 and Hfp-RT2. Extraintestinal pathogenic \textit{E. coli} (\textit{ExPEC}) included uropathogenic \textit{E. coli} isolates (\textit{n} = 42), strains causing sepsis (\textit{n} = 15) and newborn meningitis (\textit{n} = 10); intestinal pathogenic \textit{E. coli} (\textit{IPEC}) included enterotoxigenic (\textit{n} = 4), enterohaemorrhagic (\textit{n} = 12), enteropathogenic (\textit{n} = 4), enteroinvasive (\textit{n} = 2) and enteroaggregative \textit{E. coli} (\textit{n} = 5).
linked fraction (Fig. 2B). To investigate if this heteromerization with H-NS has a stabilizing function for Hfp, as it has been shown for StpA (Johansson and Uhlin, 1999), protein stability was analysed by expressing the native Hfp protein from plasmid pCM9 in E. coli derivatives lacking both StpA and H-NS, and/or the Lon protease. No degradation of Hfp in an hns- lon+ strain could be observed 3 h after stopping protein synthesis by addition of spectinomycin (Fig. S2), which suggests that the Hfp protein, in contrast to StpA, is stable even in absence of its interaction partner H-NS.

**Table 2.** Phenotypic characterization of mutants.

| Phenotype               | 536 wt | stpA | hns | hfp | hns/stpA | hfp/stpA | hns/hfp | hfp/hns/stpA |
|-------------------------|--------|------|-----|-----|----------|----------|---------|-------------|
| bgI phenotype           | +      | -    | +   | -   | +        | -        | +       | +           |
| Motility                | ++     | -    | +   | -   | +        | -        | -       | -           |
| Haemolytic activity (30°C) | 14    | 15   | 85  | 14  | 76       | 18       | 127     | 70          |
| Curli fimbriae: 37°C    | +      | -    | +   | -   | +        | -        | +       | +           |
| Curli fimbriae: 25°C    | +      | +    | +   | -   | +        | +        | -       | -           |
| Autoaggregation         | -      | -    | -   | -   | +        | ++       | -       | +++         |
| siderophore kinetics    | Slow   | Slow | Fast | Slow | Fast     | Slow     | Fast    | Fast        |
| Generation time (min): 25°C | 51    | 50   | 55  | 50  | 62       | 49       | 111     | 83          |
| 37°C                   | 24     | 24   | 29  | 25  | 47       | 24       | 27      | 53          |
| 45°C                   | 30     | 28   | 27  | 29  | 103      | 29       | 29      | 145         |

**Assay systems:** Generation times were assessed from cultures grown in LB medium at indicated temperatures with aeration; the bgI phenotype was assessed on agar plates containing the substrate X-Glu; motility was assessed on 0.3% agar plates; haemolytic activity was measured from culture supernatants and is defined as the dilution at which 50% haemolysis occurred; expression of curli fimbriae was assessed on agar plates containing congored dye; assays for autoaggregation and siderophore production were performed as described in Experimental procedures.
the hns/stpA and the hns/hfp double mutants at the lowest and highest temperatures: in liquid medium at a temperature range from 25°C to 45°C. Samples for β-galactosidase measurements were taken after 16 h of incubation. Mean values and standard deviations of three independent experiments are shown.

The results derived from the growth experiments suggested distinct requirements for Hfp and StpA at low or high temperatures respectively. Therefore, it was investigated whether these differences in the requirement are reflected in the expression levels of the corresponding genes at those temperatures. When studying hfp expression using a chromosomal hfp–lacZ fusion derivative of UPEC strain 536 at a temperature range from 25°C to 45°C, a significant reduction in hfp expression could be observed with increasing temperature (Fig. 3A). While the hfp gene was clearly expressed at both 25°C and 30°C, β-galactosidase activity dropped markedly when cultures were grown at 37°C and was undetectable in cultures grown at 45°C.

The transcript levels of hns, stpA and hfp were also determined by reverse transcription-PCR (RT-PCR) using...
RNA samples from wild-type (wt) strain 536 grown at a temperature range of 25–45°C. Relatively constant levels of hns transcript were observed at every temperature, while stpA transcript levels increased with increasing temperature (data not shown), thereby indicating that the temperature-regulated expression of stpA described in E. coli K-12 (Free and Dorman, 1997) also occurs in pathogenic isolates. On the other hand, the level of hfp transcript decreased with increasing temperature in agreement with the hfp–lacZ fusion data. Moreover, we observed a clear difference in the abundance of the transcripts in the wt at 37°C when compared with each other, with hns being much more abundant than stpA or hfp (see also Fig. 3C, first lane). This is consistent with previous observations that stpA levels are usually low at 37°C (Sondén and Uhlin, 1996; Zhang et al., 1996) and indicates that the same holds true for hfp.

The expression of stpA is growth phase dependent and occurs transiently during exponential growth (Free and Dorman, 1997). To analyse expression of the hfp gene through the batch growth phases, β-galactosidase activity from the chromosomally encoded hfp–lacZ fusion was measured every second hour during growth at 30°C. Only basal hfp expression levels could be detected throughout logarithmic phase (Fig. 3B). However, β-galactosidase activity markedly increased upon entry into stationary phase and stayed constant thereafter. The observed stationary-phase induction of hfp does not seem to be strictly dependent on RpoS, an alternative sigma-factor regulating many genes expressed in stationary phase. Expression of hfp in an rpoS mutant derivative of the hfp–lacZ fusion strain was significantly reduced (60% reduction compared with wt) but followed the same pattern of induction upon entry into stationary phase as observed in the wt (Fig. 3B). Temperature and growth phase are not the only factors that divergently regulate the expression of the H-NS homologues. Table S1 in Supporting information lists the differences between the expression of the stpA gene and the hfp gene as described in the literature and according to our present studies. In summary, we provide evidence that the expression levels of the H-NS-related genes hfp and stpA, which are relatively low during standard growth at 37°C, can be induced at certain, seemingly diametrical conditions, e.g. by temperature variations. Moreover, the results suggest that Hfp and StpA probably operate at temperatures other than 37°C.

Expression of the hfp, stpA and hns genes is subject to negative auto- and cross-regulation

Next, the interplay between the nucleoid-associated proteins at the transcriptional level was investigated. H-NS and StpA have been shown to negatively regulate transcription of their own genes (autoregulation) as well as of their homologue (cross-regulation). To include Hfp, transcript amounts of all three nucleoid-associated protein-encoding genes were detected by semi-quantitative RT-PCR in UPEC strain 536 and the hfp, hns and stpA mutant derivatives (Fig. 3C). De-repression of both hfp and stpA transcription occurred in the absence of H-NS, thereby indicating a role for H-NS as a repressor of both its homologues, in agreement with published data (Sondén and Uhlin, 1996). The negative effect of H-NS on hfp expression was also observed using an hns mutant derivative of the hfp–lacZ fusion strain (Fig. 3B and D). A slightly higher amount of hns transcript could be detected in the stpA mutant, as it was described before in E. coli K-12 (Sondén and Uhlin, 1996), but also in mutants lacking Hfp, suggesting that both StpA and Hfp negatively affect hns expression. A lack of StpA did not affect hfp transcription; vice versa, expression of stpA was not altered in an hfp mutant. To further quantify the effect of StpA on hfp expression, a mutation in the stpA gene was introduced into the hfp–lacZ fusion strain and β-galactosidase activity was measured from cultures grown at 30°C for 16 h (Fig. 3D). In accordance with the RT-PCR results (Fig. 3C), no alteration in hfp expression was observed in the stpA mutant background, which corroborates disparate effects of H-NS and StpA on hfp expression.

The hfp promoter region is target for DNA binding by nucleoid-associated proteins

The repressive effect of H-NS-like proteins on target gene expression involves DNA binding to promoter regions, which also might be the mechanism for the transcriptional cross-regulation described above. To first characterize the hfp promoter region, various parts of the upstream region of the hfp gene were fused to a plasmid-encoded lacZ gene and transcriptional activity at different growth stages was assessed in a lacZ mutant derivative of strain 536 (Fig. S4A). In brief, the results indicated that transcriptional activity required upstream sequences of more than 70 nucleotides in length, with maximal activity measured with a construct encompassing 412 nucleotides upstream of the translational start codon, and confirmed both the growth phase-dependent and H-NS-regulated expression of hfp.

To further characterize the hfp promoter region, the start point of hfp transcription was determined by primer extension analysis. The most prominent transcriptional start point was identified at a T residue 339 bp upstream of the hfp translation initiation codon (see Fig. S5). Transcription from this promoter presumably is repressed by H-NS, since the amount of extension product was significantly increased in samples derived from an hns mutant. Analysis of the preceding sequence revealed a region of high
similarity to the proposed H-NS binding motif (Lang et al., 2007) between the −10 and −35 elements, which would explain the observed H-NS-mediated repression of hfp expression. A second transcriptional start site with an RpoS-dependent −10 box was predicted 87 bp upstream of the translation initiation codon, but the transcript was barely detectable by primer extension analyses. This proximal promoter also contained a predicted Fis site (Fig. S5), which might explain the modest degree of activation of hfp expression observed using hfp–lacZ reporter fusion derivatives (Table S1 and data not shown). Taken together, these results suggest that hfp is transcribed monocistronically from promoters located within a region of −70 to −412 nucleotides counting backwards from ATG and that several global regulators might be involved in regulating hfp expression.

H-NS was shown to preferentially bind to AT-rich, intrinsically curved DNA sequences (Yamada et al., 1990; Owen-Hughes et al., 1992). The AT content of a 400-bp-long upstream region of hfp was calculated to be 63.8%, which is similar to the AT content of the corresponding hns and stpA upstream regions (60.6% and 63.5% AT respectively). A computer prediction software suggested strong intrinsic curvature of the 400 bp fragment upstream of hfp (data not shown). This apparent feature, and the presence of the predicted H-NS binding site, made us hypothesize that the hfp promoter region is a potential target for binding of nucleoid-associated proteins. To elucidate the DNA-binding activities of all three nucleoid-associated proteins to the promoter regions of hfp, hns and stpA, electrophoretic mobility shift assays (EMSA) were performed. Purified Hfp protein caused a minor shift of DNA fragments containing the hfp promoter or the hns promoter region, respectively, at a protein concentration of 5–11 μM, while no such shift was observed with the stpA promoter fragment (Fig. 4A). When studying the potential for H-NS- or StpA-mediated cross-regulation at the hfp promoter, H-NS was found to bind to the hfp fragment in a cooperative manner, resulting in extensive, stepwise shifts (Fig. 4B). No specific binding could be observed with purified StpA protein at the same conditions, thereby supporting the result of unaltered hfp expression in the stpA mutant (Fig. 3D). The DNA-binding activity of the StpA protein preparation was verified in a control experiment where binding of StpA was monitored (data not shown) to a DNA fragment representing the promoter of the E. coli K-12 stpA locus (Sondén and Uhlin, 1996). In summary, our results with DNA representing the hfp and hns promoter regions suggested that Hfp has a recognizable albeit weak DNA-binding activity. Furthermore, the difference in the shifts caused by H-NS and Hfp at a given protein concentration indicated that there would be differences in the arrangement or the stability of the DNA–protein complexes formed by the two proteins.

**Differential modulation of gene expression by Hfp and StpA**

The data obtained from transcriptional analysis suggested that the effects of Hfp and StpA are the strongest at temperatures other than 37°C. This was also confirmed by the observation that both the hfp and the stpA single mutants exhibited no obvious phenotype when tested for the expression of the known major virulence traits of strain 536 at 37°C (Table 2). Lack of H-NS on the other hand affected the expression of all factors tested, resulting in a hyper-fimbriated, hyper-haemolytic, but non-motile phenotype at 37°C, as described before (Müller et al., 2006).
The phenotypes at 37°C of the double and triple mutants were solely dependent upon H-NS but not of its homologues StpA or Hfp at that temperature.

Fimbrial adhesins are crucial factors for virulence of uropathogenic *E. coli*. When comparing expression of two of the fimbrial operons of UPEC strain 536 in the *hns*/*stpA* and the *hns*/*hfp* mutant, a clear difference was observed: as determined from the immunoblot analyses of samples from 37°C, lack of StpA resulted in a less pronounced de-repression of Prf-pili in the absence of H-NS, whereas expression of Sfa-pili was even higher compared with the *hns* single and *hns*/*hfp* double mutants (Fig. 5A; middle panel), which indicates differences in the ability of StpA and Hfp to modulate fimbriation at 37°C. The expression of fimbriae was also tested at 25°C and 45°C (Fig. 5A; left and right panel respectively). A de-repression of the Sfa-pili in strains lacking H-NS could be observed at 25°C, as described for 37°C (Fig. 5A; middle panel), while at 45°C Sfa-pili were only produced by the *hns*/*hfp* double mutant, which indicates that StpA alone was not able to repress sfa expression at this temperature. Prf-pili could not be detected in any of the strains at 25°C and 45°C. This seems consistent with a tight regulation of the expression of those virulence traits that would be optimized to the conditions in a mammalian host, and also suggests that StpA and Hfp probably do not play any major role in regulation of virulence factor expression in that specific environmental setting.

The expression of the capsule determinant was investigated as a second potential target operon, since H-NS was shown to affect the expression of the region 1 genes of the K5 capsule in a temperature-dependent manner by repressing transcription at 25°C, but being required for maximum expression at 37°C (Corbett *et al.*, 2007). The role of the nucleoid-associated proteins in the production of capsular polysaccharide in UPEC strain 536 was assessed by ELISA. In 536 wt bacteria, the highest expression level of capsular polysaccharide was detected at 37°C, in agreement with published data (Corbett *et al.*, 2007), whereas the expression level dropped to 60% at 44°C, and only background levels were reached at 25°C (Fig. 5B). When the mutant series was analysed, only the *hns* and *hns*/*hfp* double mutants (but not the *hns*/*stpA* mutant) showed alterations in capsule expression. At 25°C, reactivity with the K15 antiserum was 3.1-fold and 4.5-fold increased in the *hns* and *hns*/*hfp* mutants, respectively, as compared with wt, which also coincided with a mucoid phenotype of these mutants at this temperature on plates. At 37°C, all mutants behaved like the wt, except the *hns*/*hfp* mutant that showed a slightly but significantly higher capsule production. At 44°C, only the *hns* mutant yielded significantly higher values as compared with wt. These results confirm a role of nucleoid-associated proteins in the production of the capsular polysaccharide of serotype K15 in a temperature-dependent manner.

To further characterize the roles of Hfp and StpA in transcription, a reporter system for *bgl* expression was used as a well-described target for regulation by H-NS-like proteins. A plasmid containing a transcriptional *bglG::lacZ* fusion (pKENV61) was introduced into strain 536 and the set of mutants and *β*-galactosidase activity was measured from cultures grown at 25°C, 37°C or 42°C (Fig. 5C and D). Since the strains used are all lac<sup>+</sup> on the chromosome, a control experiment was performed using plasmid pKENV61<sub>D</sub>, where a part of the *bglG::lacZ* fusion was deleted. In all strains and conditions tested, the background *β*-galactosidase activity measured was less than 20 Miller units (data not shown). When measuring *β*-galactosidase activity in strains carrying pKENV61, all strains lacking H-NS exhibited de-repressed *bgl* transcription at any of the temperatures tested, with the strongest effect observed at 25°C (Fig. 5C and D). Interestingly, *bgl* expression was four to five times higher in the *hns*/*stpA* double mutant compared with the *hns* single mutant at 37°C and 42°C, but not at 25°C (Fig. 5D). The same effect was observed in an *hns·<sup>+</sup>* background, where de-repression in both *stpA* and *hfp*/*stpA* mutants compared with the wt increased with increasing temperatures (Fig. 5C). These results suggest that StpA has an additive effect in the repression of the *bgl* operon and that the strength of repression correlates with the expression levels of the *stpA* gene, i.e. high StpA levels at 42°C coincide with a strong repressive effect on *bgl* transcription. Lack of Hfp alone did not result in de-repression of the *bgl* operon (Fig. 5C). However, lack of both H-NS and Hfp resulted in higher *β*-galactosidase values than those obtained by the lack of H-NS alone, and this additional increase in *bgl* expression was the highest at 25°C, where the difference between the *hns*/*hfp* and the *hns* single mutant was more than 1000 Miller units (Fig. 5D). From this, we can conclude that Hfp, in contrast to StpA, does not contribute to repression of the *bgl* operon when H-NS is present. In the absence of H-NS, however, Hfp can exert a repressive effect that is the strongest at low temperatures, two conditions where Hfp levels are induced.

Taken together, as exemplified by the *bgl* operon and the capsule determinant, a clear correlation could be made between *hfp* and *stpA* operon levels and the strength of modulatory effects of the encoded proteins on the expression of H-NS-regulated genes. This suggests that the H-NS homologues Hfp and StpA can act as modulators of gene expression at divergent environmental conditions.

**Discussion**

In this study, we provide evidence that many ExPEC, as exemplified by UPEC strain 536, encode three members of
the H-NS-like protein family, the global regulator H-NS itself, as well as two intraspecies homologues StpA and Hfp, similar to the situation described for the StpA and Sfh proteins of *Shigella* (Beloin et al., 2003). We suggest that the so far uncharacterized *hfp* gene and its gene product are not to be considered simply the redundant result of gene duplication, but that Hfp represents a distinct functional member in a homeostatically regulated pool of H-NS-

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**Fig. 5.** Effect of Hfp and StpA on gene expression.

A. Effect of the lack of nucleoid-associated proteins on fimbriation. The protein levels of the major subunits of S and P fimbriae (SfaA and PrfA respectively) were determined by Western blotting from cultures of UPEC strain 536 and mutants lacking one or two of the nucleoid-associated proteins H-NS, Hfp and StpA. All strains were grown to mid-log phase at 25°C, 37°C and 45°C respectively. The alpha-subunit of the RNA polymerase was used as a control for equal loading of whole cell lysates. Images depict representative blots of at least two independent experiments.

B. Effect of the H-NS homologues on the production of capsular polysaccharide on the cell surface. Strain 536 and the series of isogenic *hns*, *stpA* and *hfp* mutant derivatives were grown overnight at 25°C, 37°C and 44°C, and cultures were adjusted to the same optical density. The K15 capsule was detected by ELISA using pre-absorbed K15 capsule-specific antiserum and HRP-conjugated secondary antibodies and quantified by measuring the optical density at 490 nm. Mean values and standard deviations from three independent experiments are shown.

C and D. Effect of the H-NS homologues on expression from the H-NS-regulated *bgl* promoter. Transcriptional activity was measured in strain 536 and isogenic mutants lacking *stpA* and/or *hfp* (C) and the series of *hns* mutant derivatives (D). In both (C) and (D), all strains carried a transcriptional *bgl–lacZ* fusion on a plasmid (pKEN61). The strains were grown to mid-log phase at 25°C, 37°C and 42°C, as indicated in the figure. Mean values and standard deviations from three independent experiments are shown.

In (B–D): Values marked with one asterisk are statistically significant with a *P*-value of < 0.05, two asterisks indicate a *P*-value < 0.01, and three asterisks indicate a *P*-value < 0.001 as calculated using the Student’s *t*-test.
like proteins. In this respect, our findings support existing hypotheses (Dorman, 2004). This protein pool includes full-length H-NS homologues such as StpA or Hfp, and, as shown by Juarez and co-workers (Madrid et al., 2007), also more distant homologues like members of the Hna/ YmoA family. In particular, our study demonstrates that the composition of the pool of nucleoid-associated proteins in *E. coli* S36 is distinctly regulated and changes according to environmental conditions, resulting in variable levels of Hfp or StpA as interaction partners for the abundant H-NS protein. The latter suggestion is derived from our transcription studies of the *hfp* and *stpA* genes. The expression levels of both genes are relatively low under standard laboratory growth conditions as described here and elsewhere (Sondén and Uhlin, 1996), which might account for the apparent lack of phenotype of *hfp, stpA* or *hfp/stpA* double mutants at 37°C. Presumably, it indicates that the major effects of Hfp or StpA are not exerted at this temperature. On the other hand, as summarized in Table S1, expression of the two H-NS homologues can be markedly increased under certain conditions, e.g. at lower temperature and upon entry into the stationary growth phase for *hfp* expression, or at higher temperatures (>40°C) during active growth for *stpA* expression. The inducing conditions for elevated Hfp and StpA levels thereby seem to be quite diametrical, suggesting that Hfp and StpA rarely encounter each other in the cell. Therefore, we propose a scenario in which Hfp and StpA have evolved to being divergently regulated through selective forces exerted by different environmental niches (see Fig. 6).

Despite the differences in expression levels, the biochemical parameters of the histone-like proteins are very similar. Like StpA, the Hfp protein exhibits high homology to H-NS, thereby also suggesting a similar two-domain structure. Homology between Hfp and H-NS is the highest in the region corresponding to the N-terminal oligomerization domain, which might account for the observed heteromerization between Hfp and H-NS. In contrast to StpA, which has been reported to be readily degraded by the Lon protease if not protected by complex formation with H-NS (Johansson and Uhlin, 1999), heteromerization with H-NS did not seem to be required for stability of Hfp, which demonstrates distinct properties of the Hfp and StpA proteins. Heteromerization with H-NS might be an important aspect in the role of Hfp and StpA in the cell. Due to the observed differences between H-NS and Hfp in their interaction with DNA (Fig. 4), we need to consider the possibility that a heteromeric complex might exhibit yet another distinct DNA binding property. Two examples of promoters that are regulated by alternating nucleoprotein complexes is the region 1 promoter of the K5 capsule determinant and the promoter of the H-NS-silenced gene for the cryptic haemolysin HlyE, also known as ClyA (Westermark et al., 2000; Corbett et al., 2007; Lithgow et al., 2007). In these cases, both H-NS and the SlyA protein bind to overlapping sites within the promoter region, resulting in higher-order protein : DNA complexes, depending on the relative concentrations of the DNA binding proteins. Our results using strain S36 suggest that H-NS represses the production of the K15 capsule at low temperatures, as it was described for other group 2 capsule determinants (Corbett et al., 2007). In contrast to the K5 capsule, H-NS seems not to be required for maximal expression of the K15 capsule at 37°C, since no reduction was observed in the *hns* mutant compared with the wt. At lower temperatures, on the other hand, Hfp has a clear repressive effect on the K15 determinant, since the level of de-repression in the *hns/hfp* mutant differed significantly from that of the *hns* mutant at 25°C (Fig. 5B). As determined by EMSA, both purified H-NS and Hfp proteins were able to bind to a DNA fragment spanning the region 1 promoter (our unpublished data). Therefore, a participation of Hfp in the nucleoprotein complex formed at the capsule determinant might occur.

Differential effects of Hfp and StpA were observed for other operons as well, such as the fimbrial and *bgl* determinants. Nevertheless, most of the effects of Hfp and StpA on gene expression could be revealed when H-NS was absent, i.e. in the *hns/hfp* and *hns/stpA* double
mutants. This might partly result from the low expression levels of stpA and hfp in the wt due to repression by H-NS, thus rendering H-NS much more abundant, which might mask the effects of the H-NS homologues. In the absence of H-NS, on the other hand, Hfp and StpA levels increase and they can assume some of the H-NS functions. However, the results obtained from our growth studies clearly indicate that Hfp and StpA are not simple ‘molecular backups’ for H-NS.

Characterization of the H-NS-like genes and proteins also involves questions about what selective forces may promote establishment of genes moved by lateral gene transfer. In this study, we also investigated the presence of the hfp gene and the serU-associated genomic island by PCR screening of a large set of different E. coli pathotypes and variants. The hfp gene was mainly detected in extraintestinal pathogenic isolates, but is also present in some faecal isolates. Since the faecal flora is considered to be the reservoir for uropathogenic E. coli, and many of the faecal isolates that carried the hfp gene belonged to ECOR group B2, the phylogenetic lineage that comprises mostly extraintestinal pathogenic isolates, the distribution pattern of hfp among faecal ECOR B2 strains observed can be explained. It is also very striking that only one out of 27 IPEC strains included in our PCR screen, EAEC isolate O42, was positive for the hfp gene. However, both uropathogenic and faecal isolates were over-represented in the strain collection compared with IPEC strains, which could result in a certain bias. Therefore, it remains to be elucidated whether there is a specific selection for the hfp gene in non-IPEC variants. Moreover, amplification of a PCR product of the right size does not imply an unaltered nucleotide sequence or a functional protein. Hence, not all hfp-positive strains can be necessarily considered to express the hfp gene.

In conclusion, many pathogenic E. coli isolates encode two distinct H-NS homologues, Hfp and StpA, which differ in their function and in the expression levels of the respective genes. All three proteins exhibit distinct interactions at the transcriptional and at the protein level. These interactions result in fine-tuned expression of a broad subset of target genes, including virulence-associated genes such as fimbriae genes and the capsular polysaccharide genes in UPEC strain 536. Moreover, Hfp exemplifies a regulatory protein encoded on a genetic element acquired by lateral gene exchange which made an impact on the regulatory circuits of the cell as an integrated partner in the pool of nucleoid-associated proteins.

**Experimental procedures**

*Bacterial strains and culture conditions*

All strains and plasmids used in this study are listed in Table S2. The strain collection used for the PCR screening has been described elsewhere (Dobrindt et al., 2001). Unless stated otherwise, bacteria were grown with aeration in Luria broth (LB) at 37°C. The temperature 44°C or 45°C was used as high temperature condition, except when using plasmid containing strains, since the hns/hfp and hns/stpA double mutants were unable to grow at 45°C in the presence of antibiotics in the growth medium. For the detection of siderophores, cultures were grown overnight in MM9 medium (Schwyn and Neilands, 1987) and iron-limited conditions were induced during 3 h by adding 0.1 mM of the chelator 2,2’-dipyridyl. When required, antibiotics carbenicillin and kanamycin were used at concentrations of 100 μg ml⁻¹ and 30 μg ml⁻¹ respectively.

**Mutagenesis**

The construction of deletion mutants was performed as described earlier (Müller et al., 2006) using the λ-Red mutagenesis method (Datenscho and Wanner, 2000). The chromosomal hfp–lacZ translational fusion strain was constructed in a similar approach: a 6.5 kb linear DNA fragment containing a promoterless lacZ gene followed by lacY, lacA and the neo gene, promoting kanamycin resistance, was PCR amplified with primers Hfp–lacZ1 and Hfp–lacZ2, using plasmid pVIK112 as template. The primers contained 54 nt and 48 nt homology extensions that allowed λ-Red recombinase-mediated recombination into the intergenic region between the hfp gene and the downstream ORF ECP_1928, thus leaving an intact copy of the hfp gene on the chromosome. Bacterial clones with successful integration were selected on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and kanamycin. All mutations were confirmed by both PCR and Southern hybridization.

**Plasmid construction**

For overexpression of His-tagged Hfp protein, the hfp sequence was amplified from the chromosome of UPEC strain 536 by using primers Xhol-His-Hfp and Hfp-HindIII, which contained a hexa-His sequence, a factor XA cleavage site, as well as Xhol or HindIII restriction sites. After enzymatic digest, the fragment was ligated into the Xhol/HindIII sites of the expression plasmid pASK75, resulting in plasmid pCM8. For complementation experiments, the native hfp gene with its own promoter was amplified by PCR using primers Hfp-fw-HindIII and Hfp-rv-XbaI, and the 750 bp fragment was cloned into the HindIII/XbaI sites of the low-copy-number plasmid pWKS30, resulting in plasmid pCM9. The promoter fusion constructs were created by PCR amplification of different parts of the upstream region of the hfp gene using the Hfp-EcoRI series of forward primers in combination with the reverse primer Hfp-BamHI binding immediately after the translational start codon of the hfp sequence. The PCR products were cloned into the EcoRI/BamHI sites of plasmid pRZS202, resulting in plasmids pCM15-pCM19. All inserts were sequenced for verification.

**RNA isolation and RT-PCR analysis**

Total RNA extraction and semi-quantitative RT-PCR were performed as described previously (Müller et al., 2006). The
sequences of the oligonucleotides used for RT-PCR are listed in Table S3 in Supporting information.

Phenotypic tests

The utilization of beta-glucosides (bgl phenotype) was detected on agar plates containing indicator dye and 40 µg ml⁻¹ 5-Bromo-4-chloro-3-indolyly-D-glucopyranoside (X-Glu; Sigma) as substrate, where white colonies were scored as bgl-negative, and blue colonies as bgl-positive (Johansson et al., 1998). Motility, haemolytic activity and the production of siderophores were assessed as described before (Müller et al., 2006). Production of curli adhesin was detected after 3 days of incubation on no-salt LB plates supplemented with 40 mg l⁻¹ congored dye and 20 mg l⁻¹ Coomassie Brilliant Blue R-250. Results were scored as + = red colonies or − = white colonies. Auto-aggregation was assessed at room temperature in M63BI medium after adjusting optical densities of the cultures as described elsewhere (Roux et al., 2005) and reported here as absent (−), slow (+) and fast (++)

β-Galactosidase assay

The β-galactosidase activity was determined as described by Miller (1972). Experiments were performed in triplicate from independent cultures, with three separate measurements for each sample.

Purification of recombinant His-Hfp protein

*Escherichia coli* strain BL21 (DE3) Δhns was used for overexpression of recombinant Hfp protein from plasmid pCM8. Approximately 800 ml of LB cultures were grown at 30°C to OD₆₀₀ = 0.2, before adding the inducer anhydro-tetracycline at a final concentration of 0.2 µg ml⁻¹. Protein synthesis was carried out for 6 h. Cells were pelleted by centrifugation and resuspended in 10 ml of LEW buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8) containing protease inhibitor cocktail (Complete Mini, Roche). Cells were lysed by sonication for 10 x 20 s pulses with 20 s cooling periods in between. After two rounds of centrifugation at 18,000 g for 20 min at 4°C, the cleared lysate was loaded onto Ni²⁺- columns (Protino Ni1000, Macherey-Nagel). Affinity chromatography was performed following the manufacturer’s recommendations. Eluted protein was dialysed into storage buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5 mM DTT, 20% glycerol] and concentrated using Vivaspin 10 000 columns (Vivasience). The His-tag was removed by factor XA digest (New England Biolabs) overnight at room temperature. For final purification by gel filtration, 1 ml of protein samples after factor XA treatment were loaded onto a HiLoad Superdex 75 prep grade column (Amersham Biosciences) and eluted with a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) in 2 ml fractions.

In vivo protein stability assay

Stability of native Hfp protein expressed from pCM9 was tested as described by Johansson and Uhlin, (1999). Briefly, 20 ml of cultures were grown to OD₆₀₀ = 0.4, before inhibiting protein synthesis by the addition of 100 µg ml⁻¹ spectinomycin. Samples for Western blotting were taken before and 180 min after the addition of spectinomycin.

Electrophoretic mobility shift assay (EMSA)

DNA–protein interactions were studied by a gel retardation assay using pure H-NS and StpA proteins or recombinant Hfp protein purified from *E. coli* BL21 Δhns as described above. As substrate for binding, 400 bp PCR products containing the promoter regions of hfp, hns and stpA were used. The composition of the reaction buffer was: 25 mM HEPES (pH 7.3), 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 50 mM KCl and 0.1 µg of poly(dIdC) non-specific competitor DNA. Purified protein (0–27 µM) was incubated with 100 ng of PCR products for 20 min at room temperature. Samples were loaded onto 6% Tris-glycine gels and DNA was visualized by ethidium bromide staining.

Chemical cross-linking

Interactions between Hfp and H-NS were studied by chemical cross-linking for 20 min at room temperature using 2.5 mM glutaraldehyde as described previously (Lindberg et al., 2008). Cross-linked products were analysed by immunoblotting.

Immunoblotting

The detection of fimbral proteins using specific antisera was performed as described previously (Müller et al., 2006). Primary antisera against H-NS or StpA were derived from immunogenized rabbits and were used in 10 000-fold dilutions. The serum originally directed against StpA strongly reacted with the Hfp protein and therefore could be used for the detection of Hfp. Antiserum recognizing the alpha-subunit of the DNA polymerase was used as a loading control on stripped membranes as performed previously (Müller et al., 2006).

Capsule ELISA

Quantitative measurements of the production levels of the K15 capsule were performed as described (Schneider et al., 2004).

In silico analyses

Promoter regions were analysed using the bend.it and model.it servers, which are accessible at http://hydra.icgeb.trieste.it/~krystian/dna (Munteanu et al., 1998). Promoter prediction was performed using the BPREM software available at the SoftBerry Homepage (http://www.softberry.com).

Statistical analyses

Differences between average values were tested for significance by performing an unpaired, two-sided Student’s t-test.
The levels of significance of the resulting P-values are reported by the following symbols: * = \( P < 0.05 \), ** = \( P < 0.01 \), *** = \( P < 0.001 \) and n.s. = non-significant.

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