A global modulatory role for the *Yersinia enterocolitica* H-NS protein

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The H-NS protein plays a significant role in the modulation of gene expression in Gram-negative bacteria. Whereas isolation and characterization of *hns* mutants in *Escherichia coli*, *Salmonella* and *Shigella* represented critical steps to gain insight into the modulatory role of H-NS, it has hitherto not been possible to isolate *hns* mutants in *Yersinia*. The *hns* mutation is considered to be deleterious in this genus. To study the modulatory role of H-NS in *Yersinia* we circumvented *hns* lethality by expressing in *Y. enterocolitica* a truncated H-NS protein known to exhibit anti-H-NS activity in *E. coli* (H-NST EPEC). *Y. enterocolitica* cells expressing H-NST EPEC showed an altered growth rate and several differences in the protein expression pattern, including the ProV protein, which is modulated by H-NS in other enteric bacteria. To further confirm that H-NST EPEC expression in *Yersinia* can be used to demonstrate H-NS-dependent regulation in this genus, we used this approach to show that H-NS modulates expression of the YmoA protein.

INTRODUCTION

In enteric bacteria, the nucleoid-associated H-NS protein plays a role both as an architectural protein and as a global modulator of gene expression (Dorman, 2004). In many instances, gene regulation processes that require H-NS respond to environmental changes such as osmolarity, pH or temperature variations (Tupper *et al.*, 1994). It is therefore not surprising that the H-NS protein has been found to play an important role in the regulation of the expression of virulence determinants. H-NS-modulated operons usually contain two target sequences, which have often been characterized as being AT-rich curved DNA stretches (Rimsky, 2004). A current hypothesis that is supported by experimental evidence considers that H-NS binding to its target sequences is followed by an oligomerization process. A DNA loop should then be generated, bringing the two target sites into closer contact. The resulting nucleoprotein complex influences expression of the genes affected (Dorman & Deighan, 2003; Falconi *et al.*, 1998; Rimsky, 2004). When considering such a model, changes in physico-chemical properties of the DNA region to which H-NS binds may be essential to facilitate DNA bridging. Thus, temperature-mediated changes in DNA supercoiling and hence in DNA flexibility may affect the ability of H-NS to modulate expression of certain genes or operons (Falconi *et al.*, 1998; Madrid *et al.*, 2002). In addition to indirect effects on DNA, temperature and/or other factors may also influence the ability of H-NS itself to dimerize/oligomerize and to facilitate the interaction with DNA (Ono *et al.*, 2005; Stella *et al.*, 2006).

The modulatory properties of H-NS can also be influenced by heteromeric interactions (Dorman, 2004), such as those with members of the Hha-YmoA family of proteins (Madrid *et al.*, 2007a, b; Nieto *et al.*, 2000, 2002). These proteins show structural mimicry of the H-NS oligomerization domain and form complexes with H-NS that modulate gene expression (Ellison & Miller, 2006; Madrid *et al.*, 2002; Nieto *et al.*, 2002). The chromosomes of many members of the *Enterobacteriaceae* encode paralogues of both H-NS [the StpA protein (Sondén & Uhlin, 1996)] and Hha [the YdgT protein (Paytubi *et al.*, 2004)]. Remarkably, members of the genus *Yersinia* contain single copies of the *hns* and *hha* genes.

Virulence gene expression in *Yersinia enterocolitica* has been extensively studied by different laboratories. Thermoregulation of virulence gene expression in this micro-organism is a well-documented process (Rohde *et al.*, 1994; Straley & Perry, 1995), but the mechanism by which temperature regulates virulence expression is not completely understood. YmoA is a regulatory protein found to participate in this process (Cornelis *et al.*, 1991). *ymoA* mutants of *Y. enterocolitica* show a pleiotropic phenotype very reminiscent of that of classical *hns* mutants of *Escherichia coli* and *Shigella*, e.g. alterations in the supercoiling state of the DNA and in the expression of temperature-regulated genes (Mikulskis & Cornelis, 1994). YmoA shows extensive similarity to the *E. coli* Hha protein and other proteins of the same family (Madrid *et al.*, 2007a, b). We described that Hha and H-NS interact to modulate gene expression (Madrid *et al.*, 2002; Nieto *et al.*, 2000).
This interaction is also apparent for other members of both families of proteins, i.e. YmoA and H-NS or YdgT and H-NS (Ellison & Miller, 2006; Nieto et al., 2000; Paytubi et al., 2004). Whereas hns mutants have been isolated in different members of the Enterobacteriaceae such as E. coli, Salmonella and Shigella, it has not been hitherto possible to isolate hns mutants in the genus Yersinia. The few examples reported of Yersinia genes that are modulated by H-NS used the surrogate hns background (Ellison & Miller, 2006; Heroven et al., 2004; Pérez-Gutierrez et al., 2007). In this study we altered H-NS function in Y. enterocolitica by expressing the H-NST EPEC protein, and hns mutants were isolated and transformed into the protein expression pattern. We further used this strategy to show that H-NS modulates expression of the ymoA gene.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. The strains were grown in Luria–Bertani (LB) medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per litre). Antibiotics, when required, were used at the following concentrations: ampicillin (Ap), 50 μg ml⁻¹; kanamycin (Km), 50 μg ml⁻¹; chloramphenicol (Cm), 12.5 μg ml⁻¹.

To construct plasmid pJOBI01-Km a mini-Tn5-based mutagenesis was used (De Lorenzo et al., 1990). First, pUTmini-Tn5 Km1 was mobilized from donor strain E. coli S17-1 pir to recipient strain E. coli 5K(pJOBI01) by a filter mating technique (Herrero et al., 1990). Plasmids from kanamycin-resistant (Km r) transconjugants were isolated and transformed into E. coli 5K. Km r transformants were selected and replica printed on Ap plates to test for the loss of the Ap' marker. Therefore it could be verified that no essential function of plasmid pJOBI01 was affected by mini-Tn5 insertion. To mobilize plasmid pJOBI01-Km from E. coli to Yersinia, E. coli S17.1 was used as donor strain.

To construct plasmid pETHNSYhis, the hns gene of Y. enterocolitica strain W22703 was PCR amplified using oligonucleotides HNSYHISBAM (5'-GGGAATTCATATGAGCGAAGCGTTAAAGATTC-3'), which adds an NdeI site to the sequence encoding the N-terminus of H-NS protein, and HNSYHISBAM (5'-CGGATCTATTAATGCTGATGTTG-3'), which adds a His6-tag-encoding sequence plus a BamHI site to the sequence encoding the C-terminus of H-NS protein. The NdeI–BamHI PCR fragment was cloned into a modified plasmid pET15b, which contains the Pet–XbaI fragment with the cloning/expression region of plasmid pET3b.

To construct plasmid pETHNSTEPEC, the hns gene of Y. enterocolitica strain W22703 was PCR amplified using oligonucleotides TENDE (5'-GAGTGATGGTGCAGCAGGAAATCATCCAGTG-3'), which adds an NdeI site to the sequence encoding the N-terminus of H-NSTEPEC protein, and TEBAM (5'-GGGAATTCATATGAGCGAAGCGTTAAAGATTC-3'), which adds a His6-tag-encoding sequence plus a BamHI site to the sequence encoding the C-terminus of H-NSTEPEC protein. The NdeI–BamHI PCR fragment was cloned into the modified plasmid pET15b as described above.

Genetic and molecular procedures. Isolation of plasmids and transformation were carried out by standard methods. Electroporation of Y. enterocolitica cells was performed as previously described (Conchas & Carniel, 1990).

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 induction of expression of proteins. Plasmids containing the desired cloned genes (pET plasmids) were introduced by transformation into the strain used. One-litre cultures were grown to an OD₆₀₀ of 0.3, and at this point IPTG was added to 0.5 mM. Incubation was continued for 2 h. Cells were pelleted by centrifugation and resuspended in 20 ml buffer A (20 mM HEPES pH 7.9, 200 mM NaCl, 10 mM MgCl₂, 20% (vol/vol) glycerol, 0.1% (vol/vol) β-mercaptoethanol).

| Table 1. Strains and plasmids used in this study |
|-----------------|-----------------|-----------------|
| Strains or plasmid | Description | Source or reference |
| Y. enterocolitica W22703 | Na', serotype O:9, R⁻ M⁺ | Cornelia & Colson (1975) |
| Y. enterocolitica W22711 | W22703 ymoA::Tn5-TcI | Cornelia et al. (1991) |
| E. coli 5K | F⁻ hsdR hsdM thi-1 rpsL leuZ | Juarez et al. (1984) |
| E. coli S17-1 pir | Tp' Sm', recA thi pro hsd R⁻ M⁻ RP4:2-Tc : Mu : Km | Miller & Mekalanos (1988) |
| E. coli S17-1 | recA thi pro hsdR Rpb4-2 (Tn1::ISR1 tet::Mu Km::Tn7) | Simon et al. (1983) |
| E. coli BL21(DE3) Δhns | Δhns::Km | Zhang et al. (1996) |
| Plasmids | | |
| pJOBI01 | oriBamHI hns+ Ap' | Johansson et al. (2001) |
| pUTmini-Tn5Krn1 | oriBamHI mini-Tn5Krn1 | De Lorenzo et al. (1990) |
| pJOBI01-Km | pJOBI01, Ap' mini-Tn5Krn1 | This work |
| pHSGHNSTE | hnsT_EPEC in pHSG576 | Williamson & Free (2005) |
| pHSG576 | oriBamHI lacZa Cm' | NRBP (NIG, Japan): E. coli |
| pHyl152 | hlyR hlyC hlyA hlyB hlyD | Noege et al. (1981) |
| pET3b | Ap', T7 promoter | Studier et al. (1990) |
| pET15b | oriBamHI Ap', T7 promoter | Novagen (Madison) |
| pETHNSHis | pET3b hns E. coli His-tag Ap' | Nieto et al. (2002) |
| pETHNSYHis | pET + hns Y. enterocolitica His-tag Ap' | This work |
| pETHNSTEPEC | pET + hnsT_EPEC Ap' | This work |
H-NS modulates *Y. enterocolitica* gene expression

**Fig. 1.** Interaction between H-NSHis and H-NST\textsubscript{EPEC}-Coomassie-blue-stained SDS-PAGE gel loaded with fractions eluted from Ni\textsuperscript{2+}-NTA agarose matrix after binding to two different cellular extracts from IPTG-induced cultures of *E. coli* BL21(DE3) Δhns transformed with plasmids pETHNSYHis and pETHNSTEPEC. Lanes 1, 2 and 3 correspond to buffer A washes of the agarose matrix. Lanes 4, 5 and 6 correspond to elution of the bound proteins with buffer A plus 200 mM imidazole. Lane 7: overproduced H-NST\textsubscript{EPEC} cell extract.

100 mM KCl, 5 mM MgCl\textsubscript{2}, 20 mM imidazole). The cells were lysed by three passages through a French press at 1000 p.s.i. The lysed extract was centrifuged at 12000 g for 30 min at 4 °C. His-tagged *Y. enterocolitica* H-NS protein was purified by immobilized metal-affinity chromatography by using Ni\textsuperscript{2+}-NTA technology (Hoffmann & Roeder, 1991), as described previously (Nieto et al., 2000).

**Preparation of cell extracts and 2D electrophoretic analysis of proteins.** For 2D gel electrophoresis, cells were grown in LB medium at 30 °C. Samples (250 ml) were collected at the exponential growth phase (OD\textsubscript{600} 0.6) and cells harvested by centrifugation (10 min, 5000 g, 4 °C). The pellets were washed four times by centrifugation for 10 min at 2000 g, 4 °C in 10 ml low-salt washing sample buffer (3 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 68 mM NaCl, 9 mM NaH\textsubscript{2}PO\textsubscript{4}). Cells were then resuspended in 300 μl of a buffer containing 10 mM Tris/ HCl pH 8.0, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1 % Triton X-100, and stored at −20 °C. Crude extracts were prepared by mixing 15 μl of the corresponding cell suspension with 300 μl of a solution containing urea (7 M), thiourea (2 M), CHAPS (4 % w/v), DTT (65 mM) and a trace of bromophenol blue. After centrifugation at 10 000 g for 20 min, 4 °C, the supernatant was collected and samples were stored at −80 °C.

Immobiline DryStrips (24 cm, pH 3–11 NL, Amersham Biosciences) were rehydrated at 50 V for 10 h with 150 μl protein from crude extract supplemented with 0.5 % (v/v) of the appropriate IPG buffer (Amersham Biosciences). Isoelectric focusing was carried out according to the manufacturer's protocol (IPGphor, Amersham Biosciences). Prior to second-dimension electrophoresis, strips were equilibrated for 15 min in equilibration buffer [30 % (v/v) glycerol, 2 % (w/v) SDS, 6 M urea, 50 mM Tris/HCl, trace of bromophenol blue, pH 8.8] containing 65 mM DTT. This step was repeated using equilibration buffer supplemented with 100 mM iodoacetamide. The strips were then embedded in 0.5 % agarose and the proteins resolved by electrophoresis through 12.5 % SDS-PAGE (Ettan DALT six, Amersham Biosciences) at 2.5 W per gel for 30 min, followed by 100 W for 3–4 h. For protein identification, gels were silver-stained and digitized by transmission scanning (ImageScanner, Amersham Biosciences). Spots excised from the gel were stored at 4 °C until identification by MALDI-TOF MS or ESI-MS-MS.

**In-gel digestion and acquisition of mass spectra.** Proteins were in-gel digested with trypsin (Sequencing grade modified, Promega) in a Genomic Solutions automatic Investigator ProGest robot. Spots excised from the 2D gels were analysed by either MALDI-TOF/TOF MS (4700 Proteomics Analyser, Applied Biosystems) or ESI-MS-MS (Q-TOF Global, Micromass-Waters). Data were submitted for database searching in the MASCOT server.

**Western blot analysis.** Cells were grown in LB medium at 30 °C. Aliquots were collected at the exponential growth phase (OD\textsubscript{600} 0.6). After centrifugation, cells were resuspended in 10 mM potassium phosphate buffer pH 7.0, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 0.5 M NaCl (Straley & Perry, 1995). Cell lysates were obtained by sonication and the protein concentration was evaluated (Bradford Bio-Rad). Proteins were electrophoretically separated and transferred to nitrocellulose membranes. To obtain H-NS-specific antibodies, H-NSHis\textsubscript{b} protein was overproduced from plasmid pETHNSHis as described previously (Nieto et al., 2002) and eluted from Tricine-SDS-PAGE gels in 0.2 M Tris/HCl pH 8.9 prior to injection into rabbits by standard procedures. Testing the extracted serum allowed us to confirm that it was able to recognize H-NS protein and not give any cross-reaction to H-NST\textsubscript{EPEC} (data not shown). To immunodetect YmoA, we used polyclonal antibodies raised against *E. coli* Hha protein, which also recognize YmoA (Balsalobre et al., 1996).

**Total RNA isolation.** To be used in RT-PCR assays, total RNA from different strains was isolated by using the SV Total RNA Isolation System (Promega). Total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies). When necessary, DNA was eliminated with Turbo DNase (Ambion).

![Graph](http://mic.sgmjournals.org) **Fig. 2.** Growth of strains W22703 (○), W22703(pHSG576) (△) and W22703(pHSGHNSTE) (□) in LB medium aerobically at 37 °C and 30 °C. The mean ± σ of three independent cultures for each strain is presented (error bars not shown where smaller than symbols).
RT-PCR. To determine the specific mRNA levels of different genes, we used Ready-to-Go RT-PCR beads (Amersham Biosciences). The primer pairs used were HNSTUP/HNSTDOWN (5'-CGCAACCATGACCTCAA-3')/5'-AGATCTTCTGGCGAAACC-3'), (GLNH5/GLNH3 (5'-ATCACCTACCCGAGCAAG-3')/5'-AAGTGCCGTCTTCTTGGAGG-3'), PROU5/PROU3 (5'-CGATACGGAACCACAAATC-3')/5'-GGTTTCAGTACGTTCTGGA-3'), YEN16S (5'-TCAGTGTTATTGTGGGAAACT-3')/5'-CGCAACCA-3'), (PLNH3/PLNH5 (5'-GTCTTCTTTCAGG-3')/5'-TTAAACGCATCAGGTAGTCA-3'), YmoA-RT/YmoA-PCR (5'-ACATGTTGGCATAAGTGG-3')/5'-AACTGACTACCTGATCGT-3'). The RNA was reverse transcribed for 1 h at 42 °C. To inactivate the reverse transcriptase, samples were incubated at 95 °C for 5 min. The amplification was accomplished by 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at the appropriate temperature for each primer pair, and extension for 30 s at 72 °C. The RT-PCR was terminated by a final extension of 10 min at 72 °C. The PCR products were analysed by agarose gel electrophoresis. 16S rRNA was reverse transcribed for 1 h at 42 °C. Each primer pair, and extension for 30 s at 72 °C. A fragment corresponding to the internal control (using primers YEN16S-5'/YEN16S-3'). First, saturation curves with increasing amounts of total RNA were performed to determine the interval of linear increase in the relative amount of RT-PCR product and total RNA (data not shown).

**Band-shift assays.** Electrophoretic band-shift assays were performed as described previously (Madrid et al., 2002). A fragment corresponding to the promoter region of the ymoA gene was amplified using primers pYmoA-F/pYmoA-R (5'-CTCTGTTTAGTAGTTACGGGA-3'/5'-TTAAACGCATCAGGTAGTCA-3'). A PCR fragment corresponding to the upstream region of the hly operon of plasmid pHy152 amplified with primers CONT-1/CONT-2 (5'-TTTACGCCCGTAAGGTGATG-3') was used as non-specific DNA.

**RESULTS**

Expression of H-NST<sub>EPEC</sub> in *Y. enterocolitica* affects the growth rate and alters the protein expression pattern

As a strategy to study the modulatory role of H-NS in *Yersinia* we decided, instead of isolating hns<sub>T</sub> mutants, to interfere with H-NS's repressive ability. It has been previously shown in *E. coli* that some truncated forms of H-NS exhibit a dominant-negative phenotype (Williams et al., 1996). It has also been shown that some naturally occurring truncated H-NS proteins, such as H-NST<sub>EPEC</sub>, exhibit anti-HNS activity. The gene encoding H-NST<sub>EPEC</sub> was first identified in a genomic island of an enteropathogenic *E. coli* strain (Williamson & Free, 2005).

Considering that the amino acid sequences from the *E. coli* and *Y. enterocolitica* H-NS proteins are very similar and, in addition, that *Y. enterocolitica* H-NS protein can substitute for *E. coli* H-NS function (Nieto et al., 2002), it should then be reasonable to expect that H-NST<sub>EPEC</sub> protein would also interfere with *Y. enterocolitica* H-NS function. We first tested that H-NST<sub>EPEC</sub> does actually interact with *Y. enterocolitica* H-NS. Overexpressed *Y. enterocolitica* H-NSHis was bound to a Ni<sup>2+</sup>-NTA agarose matrix and mixed with a cell extract containing overproduced H-NST<sub>EPEC</sub>. Imidazole-mediated elution of H-NSHis protein resulted in co-elution of H-NST<sub>EPEC</sub> (Fig. 1). Next, *Y. enterocolitica* strain W22703 was transformed with plasmid pHSGHNSTE, which carries the hns<sub>T</sub>EPEC gene cloned in plasmid pHSG576. Plasmid pHSG576 was used as a control. Transformants were selected and their growth rate was compared to that of the parental strain. Transformants harbouring pHSGHNSTE showed a significantly reduced growth rate (0.77 ± 0.018 h<sup>-1</sup> at 37 °C; 0.59 ± 0.041 h<sup>-1</sup> at 30 °C) compared to those carrying plasmid pHSG576 (0.94 ± 0.007 h<sup>-1</sup> at 37 °C; 0.89 ± 0.015 h<sup>-1</sup> at 30 °C) (Fig. 2). Expression in *Y. enterocolitica* of the hns<sub>T</sub>EPEC gene carried by plasmid pHSGHNSTE was confirmed by RT-PCR analysis using specific oligonucleotides complementary to hns<sub>T</sub>EPEC mRNA (data not shown).
We next analysed the protein expression pattern in strains W22703 and W22703(pHSGHNSTE). Total cell extracts were obtained and analysed by 2D-PAGE as described above. The results (Fig. 3) showed that, when compared to plasmid-free cells, cells harbouring plasmid pHSGHNSTE exhibit a detectable alteration of the protein expression pattern. Sixteen proteins corresponding to spots showing altered expression were excised and identified by MALDI-TOF MS/ESI-MS-MS (Table 2). The identified proteins participate in several physiological processes. One of them (ProV) corresponds to one of the best-studied examples of an H-NS-modulated protein in *E. coli* and other enteric bacteria (Jordi & Higgins, 2000). W22703 cells harbouring plasmid pHSGHNSTE showed increased ProV expression, as has been shown in *E. coli* (Williamson & Free, 2005). Two other proteins (UreG and GalU) have been reported to be modulated by H-NS in *Proteus mirabilis* and *Salmonella enterica* serovar Typhimurium respectively (Ono et al., 2005; Poore & Mobley, 2003). It is remarkable that several of the identified proteins (ProV, UreG, FabA, FabZ and glnH gene product) show temperature-dependent expression in *Yersinia pestis* (Han et al., 2004; Motin et al., 2004). To correlate alterations in protein expression with transcription levels, we used RT-PCR to measure transcription of some of the genes encoding proteins showing expression differences between strains W22703 and W22703(pHSGHNSTE). As expected,

**Table 2.** Proteins showing altered expression levels in 2D-PAGE gels from strains W22703 and W22703(pHSGHNSTE) that could be identified by MALDI-TOF MS/ESI-MS-MS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Identification no. in Fig. 3</th>
<th>Gene</th>
<th>Effect of H-NS protein</th>
<th>Previous reported effects on expression</th>
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</thead>
<tbody>
<tr>
<td>Methylaspartate ammonia-lyase</td>
<td>1</td>
<td>YE4043</td>
<td>Induction</td>
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<tr>
<td>WbcV, O-antigen biosynthesis</td>
<td>2</td>
<td>wbcV</td>
<td>Induction</td>
<td></td>
</tr>
<tr>
<td>Glycine betaine-binding periplasmic protein</td>
<td>3</td>
<td>proU</td>
<td>Induction</td>
<td>Repression at 37 °C in <em>Y. pestis</em> (Motin et al., 2004), induction in <em>E. coli</em> and <em>Salmonella hns</em> mutants (Hommais et al., 2001; Jordi &amp; Higgins, 2000)</td>
</tr>
<tr>
<td>Urease accessory protein UreG</td>
<td>4</td>
<td>ureG</td>
<td>Induction</td>
<td>Repression at 37 °C in <em>Y. pestis</em> (Han et al., 2004), induction in <em>E. coli hns</em> mutant and at 37 °C in <em>Proteus mirabilis</em> (Poore &amp; Mobley, 2003)</td>
</tr>
<tr>
<td>Hypothetical protein YE2691</td>
<td>5</td>
<td>YE2691</td>
<td>Induction</td>
<td></td>
</tr>
<tr>
<td>Hypothetical protein YE4025</td>
<td>6</td>
<td>YE4025</td>
<td>Induction</td>
<td></td>
</tr>
<tr>
<td>GalU, UTP-glucose-1-phosphate uridylyltransferase</td>
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<td>galU</td>
<td>Induction</td>
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<tr>
<td>Putative LuxR-family transcriptional regulatory protein</td>
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<td>YE1956</td>
<td>Induction</td>
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<tr>
<td>Hypothetical protein YE3387</td>
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<td>Repression</td>
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<td>Repression</td>
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</tr>
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<td>rpe</td>
<td>Repression</td>
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<tr>
<td>FabA, 3-hydroxydecanoyl-(acyl carrier protein) dehydratase</td>
<td>12</td>
<td>fabA</td>
<td>Repression</td>
<td>Repression at 37 °C in <em>Y. pestis</em> (Motin et al., 2004)</td>
</tr>
<tr>
<td>TrpS, tryptophanyl-tRNA synthetase</td>
<td>13</td>
<td>trpS</td>
<td>Repression</td>
<td></td>
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<tr>
<td>FabZ, 3R-hydroxymyristoyl-(acyl carrier protein) dehydratase</td>
<td>14</td>
<td>fabZ</td>
<td>Repression</td>
<td>Repression at 37 °C in <em>Y. pestis</em> (Motin et al., 2004)</td>
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<tr>
<td>Putative amino-acid-binding protein precursor</td>
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<td>glnH</td>
<td>Repression</td>
<td>Repression at 37 °C in <em>Y. pestis</em> (Motin et al., 2004)</td>
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<tr>
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<td>16</td>
<td>yggE</td>
<td>Repression</td>
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</table>

We next analysed the protein expression pattern in strains W22703 and W22703(pHSGHNSTE). Total cell extracts were obtained and analysed by 2D-PAGE as described above. The results (Fig. 3) showed that, when compared to plasmid-free cells, cells harbouring plasmid pHSGHNSTE exhibit a detectable alteration of the protein expression pattern. Sixteen proteins corresponding to spots showing altered expression were excised and identified by MALDI-TOF/TOF MS or ESI-MS-MS (Table 2). The identified proteins participate in several physiological processes. One of them (ProV) corresponds to one of the best-studied examples of an H-NS-modulated protein in *E. coli* and other enteric bacteria (Jordi & Higgins, 2000). W22703 cells harbouring plasmid pHSGHNSTE showed increased ProV expression, as has been shown in *E. coli* (Williamson & Free, 2005). Two other proteins (UreG and GalU) have been reported to be modulated by H-NS in *Proteus mirabilis* and *Salmonella enterica* serovar Typhimurium respectively (Ono et al., 2005; Poore & Mobley, 2003). It is remarkable that several of the identified proteins (ProV, UreG, FabA, FabZ and glnH gene product) show temperature-dependent expression in *Yersinia pestis* (Han et al., 2004; Motin et al., 2004). To correlate alterations in protein expression with transcription levels, we used RT-PCR to measure transcription of some of the genes encoding proteins showing expression differences between strains W22703 and W22703(pHSGHNSTE). As expected,

**Fig. 4.** RT-PCR analysis of transcription of the *glnH*, *proV* and *ymoA* genes from strains W22703 and W22703(pHSGHNSTE). 16S rRNA was used as a control to confirm equivalent quantity of template loading.
the transcriptional data corroborated the protein expression data (Fig. 4).

**H-NS represses transcription of the *Y. enterocolitica ymoA* gene**

The above results show that proteins exhibiting altered expression levels in cells expressing H-NSETSPEC are likely candidates to be regulated by H-NS in *Y. enterocolitica*. To further show that expression of H-NSETSPEC can be a useful approach to test H-NS-dependent gene expression in *Yersinia*, we decided to investigate whether H-NS modulates expression of the ymoA gene. Whereas cross-regulation between H-NS-StopA and Hha-like proteins has been previously reported in *E. coli* (Hommais et al., 2001; Paytubi et al., 2004; Zhang et al., 1996), information about H-NS modulating ymoA expression in *Yersinia* was not available. We decided to measure ymoA transcription both in cells expressing abnormally high H-NS levels and in cells expressing H-NSETSPEC. RT-PCR experiments showed that, when compared to plasmid-free W22703 cells, cells harbouring plasmid pHSGHNSTE increased ymoA expression (Fig. 4). These results suggest that H-NS represses YmoA expression. This was confirmed by determining the effect of increased H-NS levels on ymoA transcription. Plasmid pJOB101 contains the *hns* gene cloned under the control of the *tac* promoter. As strain W22703 is Ap’, we inserted a mini-Tn5 transposon in the Ap’ determinant, generating plasmid pJOB101-Km. W22703(pJOB101-Km) cells growing in the presence of IPTG overexpressed H-NS and showed a reduced growth rate (Fig. 5A, B). ymoA transcription was measured in strains W22703, W22703(pJOB101-Km) and W22703(pJOB101-Km) induced with 10 μM and 50 μM IPTG at 30 °C. 16S rRNA was used as a control to confirm equivalent quantity of template loading. (D) Immunodetection of YmoA protein in extracts from strains W22703, W22711 and W22703(pJOB101-Km) induced with 50 μM IPTG and grown at 30 °C.

![Fig. 5. Overexpression of the *hns* gene reduces the growth rate of strain W22703 and represses ymoA transcription.](image-url)
The results presented here strongly suggest that interference with H-NS function accounts for the altered expression levels of many proteins of strain W22703(pHSGHNSTE). Some of the identified proteins have already been reported to be modulated by H-NS in other enteric bacteria (ProV, UreG, GalU). A significant number have also been identified in Y. pestis as temperature-modulated (Han et al., 2004; Motin et al., 2004). Considering that H-NS is a well-characterized example of a temperature-dependent modulator (Ono et al., 2005) the above-referred results suggest that these proteins belong to the H-NS regulon.

Experiments showing that H-NS influences ymoA transcription further confirm that expression of H-NSTEPEC protein represents a valuable strategy to test H-NS-dependent modulation of Yersinia genes. H-NS overexpression resulted in ymoA downregulation, and H-NSTEPEC interference with H-NS activity resulted in ymoA upregulation. The observation that H-NS levels modulate ymoA expression in Yersinia matches results reported in E. coli for the hns and hha genes: hha expression increases in hns mutants (Hommais et al., 2001).

A still unanswered question is the reason why the hns gene is essential in Yersinia. The fact that (i) E. coli hns mutants are viable and (ii) the E. coli paralogue StpA is overexpressed in hns mutants (Sondén & Uhlin, 1996) would suggest that the lack of an H-NS paralogue accounts for the lethality of the hns allele in Yersinia. However, the fact that E. coli double hns stpA mutants are viable argues against this. When considering the recent view that H-NS silences large AT-rich stretches of laterally acquired DNA (Dorman, 2007; Lucchini et al., 2006; Navarre et al., 2006; Pfum, 2006), it could be suggested that deregulated expression of genes located within these genomic islands can be lethal in Yersinia. Nevertheless, hns mutants cannot be isolated in strains lacking some of these sequences (e.g. the pYV plasmid). As an alternative hypothesis, we propose the following. Temperature transition in Yersinia results in drastic alterations in the protein expression pattern and cell physiology (Han et al., 2004; Motin et al., 2004). Both the fact that H-NS interacts with YmoA and results presented in this work suggest a role for H-NS in modulating proteins from the temperature regulon. H-NS loss would then result in major global physiological alterations rendering cells unable to grow in conventional culture media.

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H-NS binds to the ymoA promoter region

To confirm that H-NS is a repressor of the ymoA gene, we tested H-NS binding to the ymoA promoter region. A competitive band-shift assay (Fig. 6) showed that H-NS exhibits preferential binding to the ymoA promoter region, hence supporting the hypothesis that H-NS modulates ymoA expression.

DISCUSSION

Several reports have shown that H-NS modulates expression of different genes from E. coli, Salmonella and Shigella (Dorman, 2004). In contrast, only few reports have shown a modulatory role for H-NS in Yersinia (Ellison & Miller, 2006; Heroven et al., 2004; Perez-Gutierrez et al., 2007). The most likely reason is that the hns gene is essential in this genus. Hence, deregulated mutants obtained by random mutagenesis procedures are not going to map in the Yersinia hns gene. The previous finding that the YmoA protein is a relevant modulator in Yersinia (Cornelis et al., 1991; Ellison et al., 2003) and that this protein interacts with H-NS (Nieto et al., 2002) strongly suggested that H-NS must also play an important modulatory role in this genus. All experimental approaches used by us (unpublished results) and others (Ellison & Miller, 2006; Heroven et al., 2004) failed to isolate hns mutants in Yersinia. Therefore, alternative strategies that would allow study of the regulatory role of the H-NS protein in Yersinia should be developed. Rather than searching for the loss of H-NS function, we decided to partially interfere with H-NS modulatory activity.
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secretion system is modulated by lipopolysaccharide O-antigen status. 


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