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 *E. coli hns* background (Ellison & Miller, 2006; Heroven *et al.*, 2004; Pérez-Gutiérrez *et al.*, 2007). In this study we altered H-NS function in *Y. enterocolitica* by expressing the H-NST_{EPEC} protein, reported to antagonize H-NS in *E. coli* (Williamson & Free, 2005). *Y. enterocolitica* cells expressing H-NST_{EPEC} showed an altered growth rate and significant differences in 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 pJOB101-Km a mini-Tn5-based mutagenesis was used (De Lorenzo *et al.*, 1990). First, pUTmini-Tn5Km1 was mobilized from donor strain *E. coli* S17-1 λ pir to recipient strain *E. coli* 5K(pJOB101) 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^r

marker. Therefore it could be verified that no essential function of plasmid pJOB101 was affected by mini-Tn5 insertion. To mobilize plasmid pJOB101-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 HNSYNDE (5'-GGGAATTCATATGAGCGAAGCGTTAAAGATTC-3'), which adds an *NdeI* site to the sequence encoding the N-terminus of H-NS protein, and HNSYHISBAM (5'-CGGGATCCTATTAATGGTG-ATGGTGATGGTGCAGCAGGAAATCATCCAGTG-3'), which adds a His₆-tag-encoding sequence plus a *Bam*HI site to the sequence encoding the C-terminus of H-NS protein. The *NdeI*–*Bam*HI PCR fragment was cloned into a modified plasmid pET15b, which contains the *PstI*–*XbaI* fragment with the cloning/expression region of plasmid pET3b.

To construct plasmid pETHNSTEPEC, the *hnsT_{EPEC}* gene from plasmid pHSGHNSTE was amplified by PCR using oligonucleotides TENDE (5'-AGTCTATCCAAGGAGCAAACATATGATTGATG-3'), which adds an *NdeI* site to the sequence encoding the N-terminus of H-NST_{EPEC} protein, and TEBAM (5'-CACGGATCCGTCAATG-AGATCTTCTGGCG-3'), which adds a *Bam*HI site after the *hnsT_{EPEC}* gene. The *NdeI*–*Bam*HI 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) Δ *hns* 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,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>Y. enterocolitica</i> W22703	Nal ^r , serotype O:9, R ⁻ M ⁺	Cornelis & Colson (1975)
<i>Y. enterocolitica</i> W22711	W22703 <i>ymoA</i> ::Tn5-Tc1	Cornelis <i>et al.</i> (1991)
<i>E. coli</i> 5K	F ⁻ <i>hsdR hsdM thr thi rpsL leu lacZ</i>	Juárez <i>et al.</i> (1984)
<i>E. coli</i> S17-1 λ pir	Tp ^r Sm ^r , <i>recA thi pro hsd R</i> ⁻ M ⁺ RP4:2-Tc : Mu:Km Tn7 λ pir	Miller & Mekalanos (1988)
<i>E. coli</i> S17-1	<i>recA thi pro hsdR</i> Rp4-2 (Tn1::ISR1 <i>tet</i> ::Mu Km::Tn7)	Simon <i>et al.</i> (1983)
<i>E. coli</i> BL21(DE3) Δ <i>hns</i>	Δ <i>hns</i> ::Km	Zhang <i>et al.</i> (1996)
Plasmids		
pJOB101	<i>ori_{RSF1010} hns</i> ⁺ Ap ^r	Johansson <i>et al.</i> (2001)
pUTmini-Tn5Km1	<i>ori_{R6K} mini-Tn5Km1</i>	De Lorenzo <i>et al.</i> (1990)
pJOB101-Km	pJOB101, Ap::mini-Tn5Km1	This work
pHSGHNSTE	<i>hnsT_{EPEC}</i> in pHSG576	Williamson & Free (2005)
pHSG576	<i>ori_{pSC101} lacZα</i> Cm ^r	NRBP (NIG, Japan): <i>E. coli</i>
pHly152	<i>hlyR hlyC hlyA hlyB hlyD</i>	Noegel <i>et al.</i> (1981)
pET3b	Ap ^r , T7 promoter	Studier <i>et al.</i> (1990)
pET15b	<i>ori_{PMB1} Ap</i> ^r , T7 promoter	Novagen (Madison)
pETHNSHis	pET3b <i>hns E. coli</i> His-tag Ap ^r	Nieto <i>et al.</i> (2002)
pETHNSYHis	pET + <i>hns Y. enterocolitica</i> His-tag Ap ^r	This work
pETHNSTEPEC	pET + <i>hnsT_{EPEC}</i> Ap ^r	This work

Fig. 1. Interaction between H-NSHis and H-NST_{EPEC}. Coomassie-blue-stained SDS-PAGE gel loaded with fractions eluted from Ni²⁺-NTA agarose matrix after binding to two different cellular extracts from IPTG-induced cultures of *E. coli* BL21(DE3) Δ *hns* 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_{EPEC} cell extract.

100 mM KCl, 5 mM MgCl₂, 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 Ni²⁺-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 (OD₆₀₀ 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 KH₂PO₄, 68 mM NaCl, 9 mM NaH₂PO₄). Cells were then resuspended in 300 μ l of a buffer containing 10 mM Tris/HCl pH 8.0, 1.5 mM MgCl₂, 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 μ l of the corresponding cell suspension with 300 μ l 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 μ g 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 (Etan 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 (OD₆₀₀ 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-NS-His₆ 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_{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).

Fig. 2. Growth of strains W22703 (◆), W22703(pHSG576) (◇) and W22703(pHSGHNSTE) (■) in LB medium aerobically at 37 °C and 30 °C. The mean $\pm$ SD of three independent cultures for each strain is presented (error bars not shown where smaller than symbols).

Fig. 3. 2D-PAGE analysis of cell extracts from strains W22703 (A) and W22703(pHSGHNSTE) (B). Spots 1 to 8 (in squares) correspond to proteins that, when compared to the plasmid-free strain, show induced expression in strain W22703(pHSGHNSTE). Spots 9 to 16 (circled) correspond to proteins showing increased expression in strain W22703. See Table 2 for identities of spots 1–16.

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'-CGCAACCACTGACCTCAAA-3')/(5'-AGATCTTCTGGCGAAACCC-3'), (GLNH5/GLNH3 (5'-ATCACTTACACCGACGAACG-3'/5'-AAGTGCCGTCTTCTTCAGG-3'), PROU5/PROU3 (5'-CGATACGGTAACCCACAATC-3'/5'-GGTTCGAGTAGTCAGTTGGA-3'), YEN16S-5'/YEN16S-3' (5'-TGAGTAATGTCTGGGAAACT-3'/5'-TTCTTCTGCAGTAACGTC-3') and YmoA-RT/YmoA-PCR (5'-ACATGTTGCCATACAGTAGG-3'/5'-AAACTGACTACCTGATGCGT-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 used as 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'-CTCTGTTTAGTAGTTACGGA-3'/5'-TTAAACGCATCAGGTAGTCA-3'). A PCR fragment corresponding to the upstream region of the *hly* operon of plasmid pHly152 amplified with primers CONT-1/CONT-2 (5'-TTTACGCCCGTAAGGTGATG-3'/5'-TGAGTCACCTCTGACTGAGA-3') was used as non-specific DNA.

RESULTS

Expression of H-NST_{EPEC} 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* 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_{EPEC}, exhibit anti-HNS activity. The gene encoding H-NST_{EPEC} 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_{EPEC} protein would also interfere with *Y. enterocolitica* H-NS function. We first tested that H-NST_{EPEC} does actually interact with *Y. enterocolitica* H-NS. Overexpressed *Y. enterocolitica* H-NSHis was bound to a Ni²⁺-NTA agarose matrix and mixed with a cell extract containing overproduced H-NST_{EPEC}. Imidazole-mediated elution of H-NSHis₆ protein resulted in co-elution of H-NST_{EPEC} (Fig. 1). Next, *Y. enterocolitica* strain W22703 was transformed with plasmid pHSGHNSTE, which carries the *hnsT*_{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⁻¹ at 37 °C; 0.59 ± 0.041 h⁻¹ at 30 °C) compared to those carrying plasmid pHSG576 (0.94 ± 0.007 h⁻¹ at 37 °C; 0.89 ± 0.015 h⁻¹ at 30 °C) (Fig. 2). Expression in *Y. enterocolitica* of the *hnsT*_{EPEC} gene carried by plasmid pHSGHNSTE was confirmed by RT-PCR analysis using specific oligonucleotides complementary to *hnsT*_{EPEC} mRNA (data not shown).

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

Protein	Identification no. in Fig. 3	Gene	Effect of H-NS _{EPEC} protein	Previous reported effects on expression
Methylaspartate ammonia-lyase	1	YE4043	Induction	
WbcV, O-antigen biosynthesis	2	<i>wbcV</i>	Induction	
Glycine betaine-binding periplasmic protein	3	<i>proU</i>	Induction	Repression at 37 °C in <i>Y. pestis</i> (Motin <i>et al.</i> , 2004), induction in <i>E. coli</i> and <i>Salmonella hns</i> mutants (Hommais <i>et al.</i> , 2001; Jordi & Higgins, 2000)
Urease accessory protein UreG	4	<i>ureG</i>	Induction	Repression at 37 °C in <i>Y. pestis</i> (Han <i>et al.</i> , 2004), induction in <i>E. coli hns</i> mutant and at 37 °C in <i>Proteus mirabilis</i> (Poore & Mobley, 2003)
Hypothetical protein YE2691	5	YE2691	Induction	
Hypothetical protein YE4025	6	YE4025	Induction	
GalU, UTP-glucose-1-phosphate uridylyltransferase	7	<i>galU</i>	Induction	Induction in a <i>Salmonella hns</i> mutant (Ono <i>et al.</i> , 2005)
Putative LuxR-family transcriptional regulatory protein	8	YE1956	Induction	
Hypothetical protein YE3387	9	YE3387	Repression	
Hypothetical protein YE3397	10	YE3397	Repression	
Rpe, ribulose-phosphate 3-epimerase	11	<i>rpe</i>	Repression	
FabA, 3-hydroxydecanoyl-(acyl carrier protein) dehydratase	12	<i>fabA</i>	Repression	Repression at 37 °C in <i>Y. pestis</i> (Motin <i>et al.</i> , 2004)
TrpS, tryptophanyl-tRNA synthetase	13	<i>trpS</i>	Repression	
FabZ, 3R-hydroxymyristoyl-(acyl carrier protein) dehydratase	14	<i>fabZ</i>	Repression	Repression at 37 °C in <i>Y. pestis</i> (Motin <i>et al.</i> , 2004)
Putative amino-acid-binding protein precursor	15	<i>glnH</i>	Repression	Repression at 37 °C in <i>Y. pestis</i> (Motin <i>et al.</i> , 2004)
Hypothetical protein YE3403	16	<i>yggE</i>	Repression	

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*, *proU* and *ymoA* genes from strains W22703 and W22703(pHSGHNSTE). 16S rRNA was used as a control to confirm equivalent quantity of template loading.

Fig. 5. Overexpression of the *hns* gene reduces the growth rate of strain W22703 and represses *ymoA* transcription. (A) Growth of strains W22703 (◆), W22703(pJOB101-Km) (◇), W22703(pJOB101-Km) induced with 10 µM IPTG (▲) and W22703(pJOB101-Km) induced with 50 µM IPTG (△), in LB medium aerobically at 37 °C and 30 °C. The mean ± SD of three independent cultures for each strain is presented (error bars not shown where smaller than symbols). (B) Immunodetection of H-NS protein in cell extracts. (C) RT-PCR analysis of *ymoA* transcription 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.

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-NST_{EPEC} are likely candidates to be regulated by H-NS in *Y. enterocolitica*. To further show that expression of H-NST_{EPEC} 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-StpA 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-NST_{EPEC}. 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^r, we inserted a mini-Tn5 transposon in the Ap^r 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 and W22703(pJOB101-Km) in the presence of different amounts of IPTG. H-NS overexpression resulted in *ymoA* downregulation (Fig. 5C). Immunodetection of YmoA confirmed that cells overexpressing H-NS contain reduced levels of the YmoA protein (Fig. 5D).

Fig. 6. Interaction of the *ymoA* promoter region with H-NS. The DNA fragments used correspond to the *ymoA* promoter (pYmoA) and a fragment from the *hly* operon (control) to which H-NS does not show specific binding. No protein (lane A), 30 pmol (lane B) and 60 pmol (lane C) of purified H-NSHis protein was added to the reactions.

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; Pérez-Gutiérrez *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.

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-NST_{EPEC} protein represents a valuable strategy to test H-NS-dependent modulation of *Yersinia* genes. H-NS overexpression resulted in *ymoA* downregulation, and H-NST_{EPEC} 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; Pflum, 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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