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A new target for an old regulator: H-NS represses transcription of *bolA* morphogene by direct binding to both promoters

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ABSTRACT

The *Escherichia coli bolA* morphogene is very important in adaptation to stationary phase and stress response mechanisms. Genes of this family are widespread in gram negative bacteria and in eukaryotes. The expression of this gene is tightly regulated at transcriptional and post-transcriptional levels and its overexpression is known to induce round cellular morphology. The results presented in this report demonstrate that the H-NS protein, a pleiotropic regulator of gene expression, is a new transcriptional modulator of the *bolA* gene. In this work we show that *in vivo* the levels of *bolA* are down-regulated by H-NS and *in vitro* this global regulator interacts directly with the *bolA* promoter region. Moreover, DNaseI footprinting experiments mapped the interaction regions of H-NS and *bolA* and revealed that this global regulator binds not only one but both *bolA* promoters. We provide a new insight into the *bolA* regulation network demonstrating that H-NS represses the transcription of this important gene.

1. Introduction

The *Escherichia coli bolA* gene is induced at the onset of stationary phase and in response to many forms of stress [1]. The overexpression of *bolA* leads to substantial changes in the cell and the bacterial bacilli transform into spheres [1–3]. The fact that BolA affects the expression of numerous genes highlights its importance, and previous reports show that *bolA* can act as a transcription factor. For instance, BolA has been demonstrated to specifically interact with the *mreB* promoter, repressing its transcription [4]. This leads to a reduction in MreB protein levels and consequently to an abnormal MreB polymerization. BolA was also shown to directly regulate the transcript levels of the important D,D-carboxypeptidases PBP5 and PBP6, and to modulate the expression levels of the β-lactamase *ampC* [2,5]. Furthermore, *bolA* is involved in biofilm development and promotes changes in the outer membrane that affect permeability and resistance to antibiotics such as vancomycin [6,7]. It is not surprising that the expression of a gene involved in the control of several cellular processes is tightly regulated at transcriptional and post-transcriptional levels. In optimal growth conditions, *bolA* is under the control of a weak σ^{70}-dependent constitutive promoter, *bolAp2*. During stress and stationary phase it is mostly transcribed from the strong gearbox promoter, *bolAp1*, induced by the sigma factor σ^s [3,8]. As a σ^s-regulated gene, *bolA* expression is sensitive to ppGpp [9] and cAMP [10] intracellular levels. *bolA* was also shown to be repressed by the direct binding of OmpR in its phosphorylated form [11]. Ribonuclease III (RNase III) and polyA polymerase (PAPI) are involved in post-transcriptional control of *bolA* expression [12–14].

The histone-like (or heat-stable) protein H-NS was shown to affect some σ^s-dependent genes [15]. This 15 kDa nucleoid-associated protein is abundant in bacterial cells and is often compared to eukaryotic histones because of its high affinity for DNA. It binds preferentially to curved AT-rich regions that are found in certain promoter regions [16].

In this work we show that H-NS down-regulates *bolA* levels. We demonstrate that this regulation is mediated by a specific binding of H-NS to the *bolA* promoter region, involving both promoters. The interaction region of H-NS with *bolA* promoters was mapped and the implications of *bolA* regulation by H-NS are discussed.

2. Materials and methods

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 polynucleotide kinase were purchased from Fermentas. DNaseI was purchased from Sigma. All the enzymes were used according to the supplier's instructions. Oligonucleotide primers used in this work are listed in Table 1 and were synthesized by STAB Vida, Portugal.

2.1. Bacterial strains and plasmids

The *E. coli* strains used were: DH5α (F′ *fhuA2* Δ(*argF-lacZ*)*U169* phoA glnV44 Φ80 Δ(*lacZ*)*M15* gyrA96 recA1 relA1 endA1 thi-1 hsdR17a) for cloning experiments; BL21(DE3) ($F^- r_B^- m_B^-$ *gal ompT* (*int::*P_{lacUV5} T7 gen1 imm21 nin5) for overexpression and

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Table 1
Oligonucleotides used in this work.

Oligonucleotides	Sequence
hnsNdeI	5′-GGAATTCCATATGAGCGAAGCACTTAAAATTCTG-3′
hnsBamHI	5′-CGGGATCCCGTTATTGCTTGATCAGGAAATCGTCGAGGG-3′
X2	5′-GTCACAATGTCCCAGCCG-3′
X7	5′-CGATGCTTCCTGCTCCAC-3′
16sF	5′-AGAGTTTGATCCTGGCTCAG-3′
16sR	5′-ACGGCTACCTTGTTACGACTT-3′
bolAFw	5′-GGGGTACCTGTTTGGTAAAAATTCCCG-3′
RNM012	5′-TCTATCCGCTCACGTATCAT-3′
RblrealT	5′-AGTTCCTCCGCTAAAGTACTG-3′
P2	5′-CTTGACGGAAAAACCAGGACG-3′
FblrealT	5′-AACCCGTATTCCTCGAAGTAG-3′

purification of the H-NS protein; JW1225 (Δ*hns::kan*^{r}) [17]; MG1655; MG1693 (a spontaneous Thy$^-$ derivative of strain MG1655); and CMA92 (MG1655 Δ*hns::kan*^{r}), this work. These strains were grown in Luria Broth medium (LB) at 37 °C, supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin, when required.

The *hns* coding sequence was amplified by PCR using *E. coli* MG1693 chromosomal DNA and the primers hnsNdeI and hnsBamHI. The amplified fragment was cut with NdeI and BamHI restriction enzymes and cloned into the pET-15b vector (Novagen) previously cleaved with the same enzymes. The resulting plasmid (pCDA1) encoding H-NS fused to an N-terminal His6-tag was used to transform *E. coli* BL21(DE3) resulting in CMA93 strain (BL21(DE3) + pCDA1).

The *hns* deletion mutant was obtained from the Keio collection [17]. P1-mediated transduction to transfer the mutation to the MG1655 background (CMA92) was performed as previously described [18]. All constructions were confirmed by DNA sequencing at STAB Vida, Portugal.

2.2. Expression and purification of H-NS

BL21(DE3) containing pCDA1 was grown overnight at 37 °C, 120 rpm in LB media supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin. Fresh 250 ml of LB was inoculated with the overnight culture to a final OD_{600} of 0.1 and the culture was incubated at 37 °C, 180 rpm. At OD_{600} – 0.5, the expression of *hns* was induced with 1 mM IPTG for 2 h in the same growing conditions. Cells were harvested by centrifugation and the pellets stored at −80 °C. The cellular pellets were resuspended in 6 ml of buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then disrupted using a French press at 9000 psi and the crude extracted was treated with Benzonase (Sigma) to degrade the nucleic acids. After 30 min incubation on ice, the suspension was centrifuged for 30 min, at 48,000*g*, 4 °C. The supernatant was collected and loaded into a HisTrap Chelating Sepharose 1 ml column (GE Healthcare) equilibrated in buffer A using an AKTA HLPC system (GE Healthcare). Elution was performed using a gradient of buffer B (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) from 0% to 100% in 20 min. Collected fractions containing the pure protein were pooled together and buffer exchanged to buffer C (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) using a desalting 5 ml column (GE Healthcare). Eluted proteins were then concentrated by centrifugation at 4 °C with an Amicon Ultra Centrifugal Filters Devices (Millipore) with a mass cutoff of 10 kDa. Protein concentration was determined by the Bradford quantification method and 50% (v/v) glycerol was added to the final fractions prior to storage at −20 °C. More than 90% homogeneity was revealed by analyzing the purified protein in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue.

2.3. RNA extraction and RT-PCR

Total RNA was extracted by the Trizol (Ambion) according to the supplier instructions with some modifications. Briefly, an overnight CMA92 culture was diluted to a final OD_{600} of 0.1 and incubated at 37 °C, 180 rpm. Samples were collected at different points corresponding to the different phases of the bacterial growth curve (exponential – 0.4; late exponential – 1.2; and stationary phase – 2.5). Each aliquot containing 20 ml of bacterial cell culture was mixed with an equal volume of TM buffer (10 mM Tris, 25 mM NaN_3, 500 μg/ml chloramphenicol, 5 mM $MgCl_2$, pH 7.2) and harvested by centrifugation. The cell pellet was resuspended in 600 μl of lysis buffer (10 mM Tris, 5 mM $MgCl_2$, 300 μg/ml lysozyme, pH 7.2) followed by five cycles of freeze and thaw. The suspension was supplemented with 1% SDS and 0.33 mM AcOH. One milliliter of Trizol reagent (Ambion) was added and the suspension was vortexed 5 min at room temperature, followed by a 10 min centrifugation at 16000*g*, 4 °C. The aqueous phase was collected and mixed with 200 μl of chloroform. The mixture was vortexed again for 15 min at room temperature and centrifuged for 15 min at 4 °C. The aqueous phase was collected and total RNA was precipitated with isopropanol. After drying, the pellet was resuspended in H_2O and the RNA concentration was measured with a spectrophotometer (NanoDrop 1000).

Reverse transcription-PCR (RT-PCR) was carried out with 50 ng of total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions, using oligonucleotides X2 and X7. As an independent control, the 16S rRNA-specific primers 16sF and 16sR were used. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

2.4. Electrophoretic mobility shift assays

All the fragments used in the electrophoretic mobility shift assays (EMSA) experiments were generated by PCR and were radioactively labeled at their 5′-end. For this purpose the reverse primer in each PCR reaction was previously end-labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase. PCR reactions were carried out using genomic DNA from *E. coli* MG1693 as template. Four different substrates were obtained with different primer pairs: bolAFw and RNM012; bolAFw and RblrealT; P2 and RblrealT; FblrealT and RbrealT. The resulting PCR fragments were run in a 5% non-denaturing polyacrylamide (PAA) gel and purified by the crush and soak method previously described [18]. The concentration of the purified fragments was measured in a Biophotometer Plus (Eppendorf).

Binding reactions were performed in a total volume of 10 μl containing EMSA buffer (10 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0.5 mM DTT, 5% glycerol), 1 nM of labeled substrate and increasing concentrations of purified H-NS. H-NS was diluted to the desired concentrations prior to the assay in 2 mM Tris–HCl pH 8, 0.2 mM DTT, 10 mM KCl and 10 mM NaCl. In all the assays a control reaction without protein was performed. The binding reactions were incubated at room temperature for 20 min and the samples were then analyzed in a 5% non-denaturing PAA gel. DNA–protein complexes were detected using the PhosphorImager system from Molecular Dynamics.

2.5. DNaseI footprinting

DNaseI footprinting assays were performed as described by Leblanc and Moss [19] with some modifications. Briefly, the DNA–protein complexes obtained as described above (but in a total volume of 50 μl), were supplemented with a cofactor solution (5 mM $CaCl_2$, 10 mM $MgCl_2$) and 5×10^{-3} Kunitz units/μL of DNaseI, and incubated 2 min at room temperature. The digestion reaction

was stopped with addition of stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0) followed by phenol-chloroform-isoamylalcohol (Sigma) extraction of the digested DNA. The extracted DNA was resuspended in formamide dye mix [95% deionized formamide, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 5 mM EDTA pH 8.0, 0.025% (w/v) SDS], resolved in a 8% denaturing 8.3 M urea PAA sequencing gel at 1500 V in 1X TBE. Digested fragments were detected using a PhosphorImager system from Molecular Dynamics. M13 sequencing reaction was performed with Sequenase Version 2.0 sequencing kit according to the instructions manual and resolved in the same gel.

2.6. DNA curvature analysis

The online available DNA curvature analysis software (<http://www.lfd.uci.edu/~gohlke/dnacurve/>) was used with AA Wedge algorithm. This bioinformatics tool enables the compilation of the curvature values and the calculation of the global 3D structure of a DNA molecule from its nucleotide sequence. This program was used to obtain the 3D model of the *bolA* promoter region.

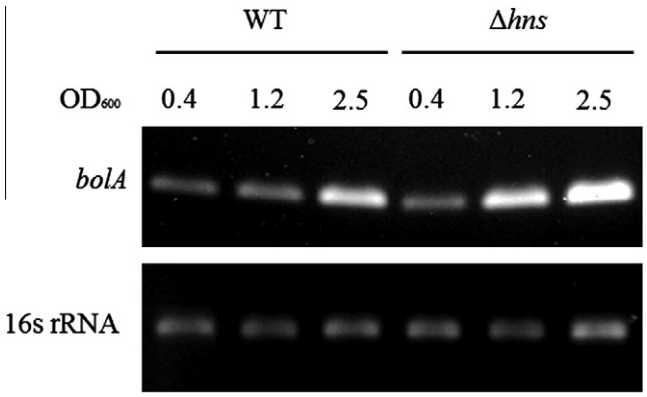

Fig. 1. Down-regulation of the *bolA* transcript level by H-NS. RT-PCR amplification of *bolA* transcript from total RNA extracted in different growth phases: *E. coli* exponential (OD_{600} 0.4), late exponential (OD_{600} 1.2) and stationary phase (OD_{600} 2.5) (upper image). Control experiments performed with 16s rRNA specific primers (image on the bottom) indicated that there were no significant differences in the amounts of RNA in each sample.

3. Results and discussion

3.1. Effect of H-NS in bolA expression

In optimal growth conditions during exponential phase *bolA* is regulated by σ^{70} and only relatively low mRNA levels are detected in the cell. However in stationary phase, *bolA* expression is under the control of σ^{s} and a 5-fold increase of the transcript level is observed [1]. Under stress conditions the *bolA* levels can increase further [1]. Since H-NS is a global regulator shown to affect the expression of several genes that respond to stress and are regulated by σ^{s}, we wanted to test whether H-NS could also be involved in the control of *bolA* expression. Taking into account that *bolA* is growth-phase regulated, the influence of H-NS on its expression was assayed during different phases of bacterial growth. Three points were analyzed (OD_{600} of 0.4, 1.2 and 2.5) corresponding to exponential, late exponential and stationary phase. For each optical density, samples were taken and total RNA was extracted from a WT cell culture and the isogenic Δ*hns* strain. *bolA* mRNA levels were then estimated by RT-PCR using a pair of specific primers (Fig. 1). In agreement with the previous results for the wild type strain, *bolA* levels are low during exponential phase and reach a maximum during stationary phase. In the absence of H-NS, the *bolA* levels in late exponential phase are significantly higher than in the wt and increase ~2.4-fold. In the *hns* strain there is also an increase of *bolA* in stationary

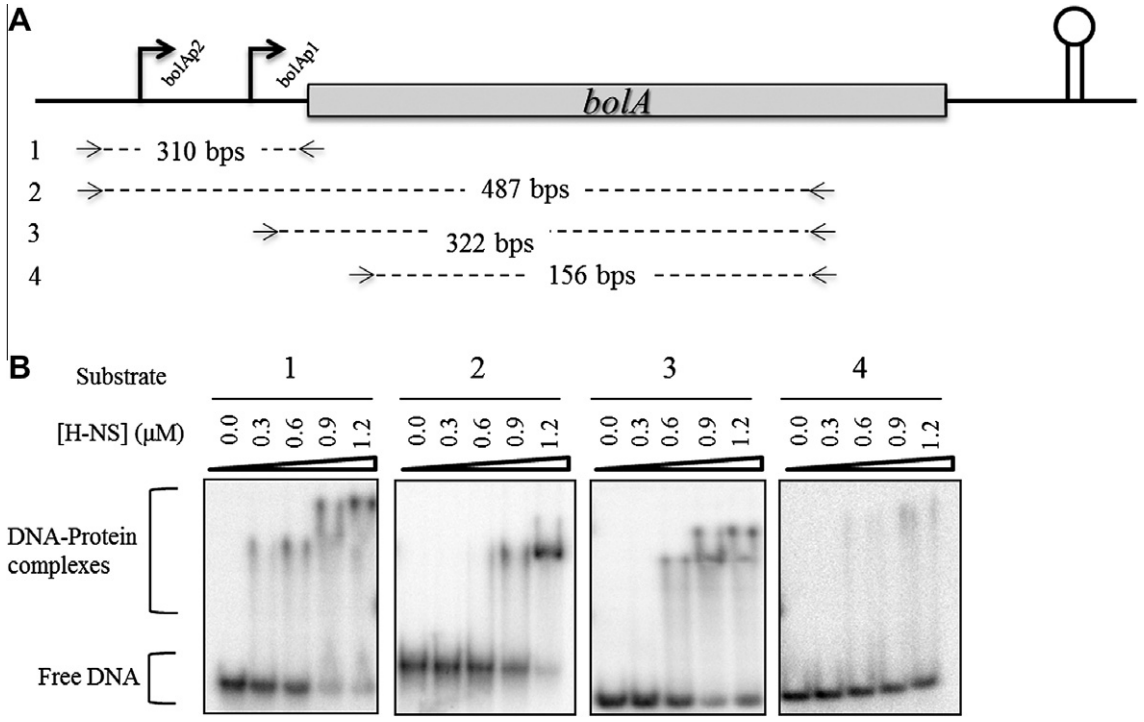

Fig. 2. DNA–protein interactions of *bolA* promoters and H-NS. (A) Schematic representation of *bolA* genomic region. The different substrates used in the electro-mobility shift assays (EMSA) are represented. (B) Representative EMSA of H-NS with 1 nM of the indicated substrates above the respective image. A control reaction without protein ([H-NS] = 0.0 μM) was performed in all experiments. Binding reactions using an increasing concentration of H-NS (indicated at the top of each lane) were resolved in a 5% non-denaturing polyacrylamide gel. Free DNA and DNA–protein complexes are indicated.

phase but the difference is quite lower. H-NS is probably repressing the expression of *bolA* during late exponential growth similarly to what happens when it regulates the *hchA* gene [20]. On the other hand, in stationary phase competes with the higher σ^s levels in the cell, and that is probably why it can not exert the same level of repression.

Taken together, these results indicate that H-NS downregulates *bolA* expression, supporting our hypothesis that H-NS could be a transcriptional repressor of *bolA* expression. Moreover, these results provide another evidence for the key function of H-NS as a selective silencer of genes that rapidly respond to environmental changes [15,21,22].

3.2. H-NS binds specifically to bolA promoter(s)

The RT-PCR results indicate that H-NS is involved in the modulation of *bolA* mRNA levels. However the nature of this regulation remains unknown. H-NS is known to be able to modulate gene expression in at least two different ways: by directly binding to specific targets or indirectly through the down-regulation of the σ^s transcript [15]. Thus we tested if H-NS could be acting directly over *bolA* as a transcriptional regulator. For this purpose, the *E. coli* H-NS protein was purified near homogeneity (Figure S1) and the pure protein was used in gel mobility shift assays with the *bolA* genomic region. Four different substrates were used in order to discriminate the ability of H-NS to bind the *bolA* upstream region (Fig. 2A). As a protein that binds DNA with high affinity, H-NS was able to retard the mobility of all the DNA fragments tested, generating retardation bands that correspond to DNA–protein complexes (Fig. 2B). However, some relevant differences were observed among the substrates tested. The substrate 1, comprising only *bolAp2* and *bolAp1* is clearly the preferred H-NS substrate. For this substrate DNA–protein complexes could be observed with only 0.3 μM of H-NS, while at least a 2-fold excess was needed for the formation of DNA–protein complexes with any of the other substrates. In addition, when using substrate 1 almost all DNA was bound with only 0.9 μM of H-NS, whereas the amount of protein needed to completely bind the substrate 2 (*bolAp2* + *bolAp1* + ORF) raised to 1.2 μM. This amount of H-NS was not even sufficient to completely bind substrate 3 (missing *bolAp2*), and at this protein concentration free DNA was still detected. At higher H-NS concentrations, a retarded band of higher molecular mass could be detected. The appearance of this band was concomitant with the disappearance of the complex of lower mass. The higher band probably corresponds to the binding of more than one molecule per substrate. H-NS is indeed known to form higher order structure complexes with its targets [23]. With substrate 1, at 1.2 μM almost all DNA molecules seem to be bound by more than one protein molecule. When using substrate 2 with the same H-NS concentration, this higher order complex is almost absent, indicating that the majority of DNA is still bound by only one H-NS molecule. The substrate missing *bolAp2* presents an intermediate situation since both protein complexes are equally detected. These experiments show that *in vitro* the presence of the whole *bolA* coding region (substrate 2) or the deletion of *bolAp2* (substrate 3) seems to affect the efficiency of the H-NS binding to *bolA*. Finally, H-NS was also able to bind to the substrate which comprises only the *bolA* coding region (substrate 4), although with a significantly lower affinity. Together, these results suggest that H-NS binds preferentially the *bolA* promoter region (with both promoters). H-NS is not only sequence but also structure sensitive [21]. Despite *bolAp1* and *bolAp2* being present in substrate 2, the additional presence of the *bolA* ORF may change the conformation of the promoter region (see below), thus affecting H-NS binding. This likely explains the partial loss of H-NS binding affinity for the longer substrate.

These experiments clearly show that the efficiency of H-NS binding is affected by both *bolA* promoters. Together with the *in vivo* data these results provide strong evidence that H-NS represses *bolA* transcription through a direct interaction with the entire *bolA* promoter region. Our results indicate that the reported co-immunoprecipitation of *bolA* with H-NS [16] was probably due to a direct interaction with this *bolA* region.

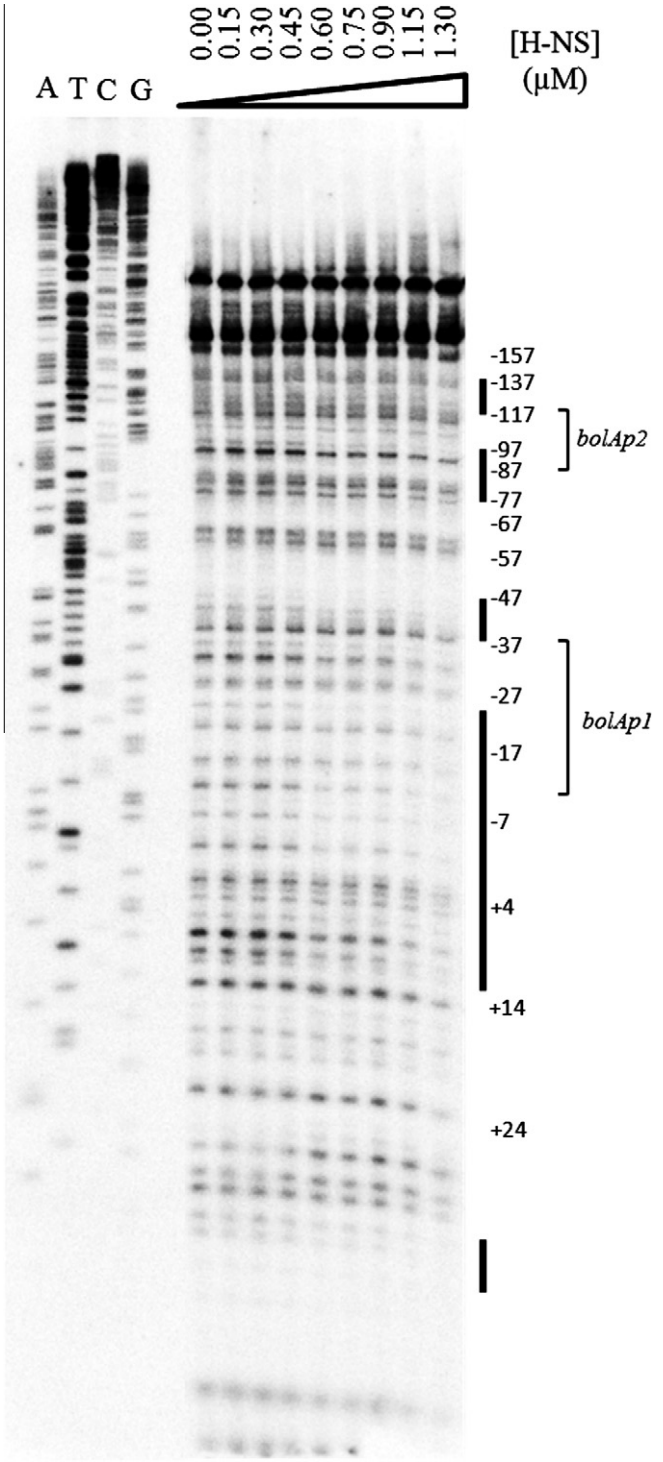

Fig. 3. Mapping of H-NS binding sites on *bolA* promoters by DNaseI foot-printing. Electrophoretic separation of a fragment with *bolAp2* and *bolAp1* after H-NS binding followed by DNaseI digestion. The different lanes correspond to different H-NS concentrations, as indicated on top. The lanes labeled with A, T, C and G represent the M13 sequencing reaction. The sites that are protected by H-NS are indicated with black lines. The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site.

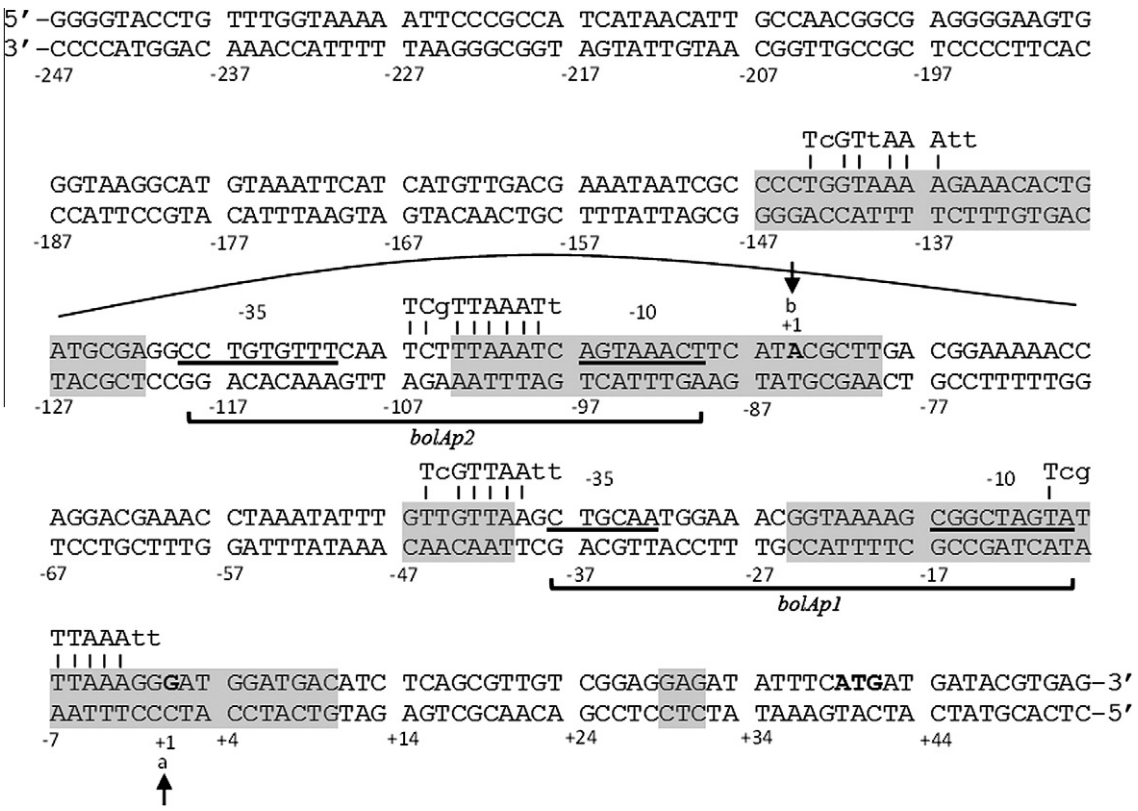

Fig. 4. Schematic representation of *bolA* promoter region. The numbers indicate nucleotide positions with respect to the *bolAp1* transcription start site. The sites revealed by H-NS-mediated DNaseI protection are highlighted in gray and the -35 and -10 regions of the promoters are underlined. The transcription start sites (a and b), and the initiation codon are in bold. The H-NS-binding consensus sequence is indicated above the DNA duplex, and the vertical bars indicate the base match between consensus and promoter sequence. The region of DNA predicted to have significant curvature is indicated by a curved line immediately above the sequence.

3.3. DNA curvature of bolA promoter region

DNA curvature analysis bioinformatics tool was used to calculate the bending region(s) to evaluate if 3D structure of the *bolA* promoters could influence H-NS binding. The double helix of a DNA fragment containing both *bolA* promoters displayed an accentuated curvature, possibly favoring the interaction with H-NS (Figure S2). However, when a DNA fragment lacking *bolAp2* was analyzed (such as substrate 3), the curvature is close to null. Hence, the DNA curvature seems to be directly dependent on the *bolAp2* region. Since the DNA structure is an important factor for the proper H-NS binding specificity, this could be one reason for the variations observed above (in the gel retardation assays).

3.4. Mapping of H-NS interaction sites

To pursue our studies and clearly identify the region(s) of interaction between H-NS and the *bolA* promoters we have performed DNaseI foot-printing assays to map the H-NS binding sites to the *bolA* promoters. We used a DNA fragment containing both *bolAp2* and *bolAp1* (substrate 1 – Fig. 2) and protection zones were identified (Fig. 3). The interaction regions were evenly distributed through the entire region analyzed, which demonstrates that H-NS can bind to several sites covering both *bolA* promoters. Sequence analysis demonstrated that the largest protection site was detected in the gearbox promoter *bolAp1* (Fig. 4). A narrower protection zone was found upstream of the -35 box of this promoter. Two other main interaction regions were mapped around *bolAp2*. The last protection zone corresponds only to a 3 bps sequence and it may not be significant. Even though H-NS was considered a non-sequence specific binding protein, recent studies defined that this global regulatory protein interacts with AT-rich regions commonly found in bacterial gene promoters [21]. A consensus region, and a consensus structure (DNA curvature) for protein–DNA interaction has also been identified [21,24,25]. In these experiments, the regions of interaction were confirmed to be AT-rich, matching the characteristics of the high affinity H-NS interaction zones and, all the main interaction zones identified share a partial similarity with the 10 bp described consensus (TCGTTAAATT) [21] (see Fig. 4). Altogether, our results support H-NS ability to bind simultaneously to several sites within the entire regulatory region of *bolA*, and form higher order structures originating a repressive nucleoprotein complex that modulates the activity of *bolAp1* and *bolAp2*.

In this report we showed that the pleiotropic histone-like protein H-NS is a new transcription regulator of *bolA* and we have characterized its mode of action. We demonstrated that H-NS is directly repressing *bolA* expression by binding to different locations along its entire promoter regions. Four major interaction zones were identified encompassing both *bolAp2* and *bolAp1* promoters. Moreover, the binding sites are confined to a curved DNA region, acknowledged to be the H-NS preferred consensus structure.

BolA has been shown to be a pleiotropic protein that affects several cellular functions. It has been described as a transcription factor, as well as a morphogene [2,4,26]. It was also shown to be important for cell survival [7]. In this context, a fine tuned regulation of this gene may be essential for the cell. This work adds a new regulator, H-NS, to the already complex network of BolA modulators. H-NS is known to be involved in flagella biosynthesis [27]. Additionally, in *E. coli*, bacterial motility influence biofilms architecture [28]. We have previously shown that *bolA* can induce biofilm formation [6], therefore H-NS and BolA may be involved in

the molecular mechanisms that control the link between motility and biofilm development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.084.

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