A putative curved DNA region upstream of rcsA in Escherichia coli plays a key role in transcriptional regulation by H-NS

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It is well established that in Escherichia coli, the histone-like nucleoid structuring (H-NS) protein also functions as negative regulator of rcsA transcription. However, the exact mode of regulation of rcsA transcription by H-NS has not been studied extensively. Here, we report the multicopy effect of dominant-negative hns alleles on the transcription of rcsA based on expression of cps-lac transcriptional fusion in Δlon, Δlon rpoB12, Δlon rpoB77 and lon+ strains. Our results indicate that H-NS defective in recognizing curved DNA fails to repress rcsA transcription significantly, while nonoligomeric H-NS molecules still retain the repressor activity to an appreciable extent. Together with bioinformatics analysis, our study envisages a critical role for the putative curved DNA region present upstream of rcsA promoter in the transcriptional regulation of rcsA by H-NS.

In Escherichia coli, the genetic material is organized in the form of nucleoid and the DNA-binding proteins such as histone-like proteins serve as a dynamic scaffold for nucleoid organization [1–4]. The histone-like nucleoid structuring (H-NS, previously denoted as H1) protein of E. coli is one of the major components of the nucleoid. hns gene was identified by Pon et al. [5], and it maps at 27 min of E. coli chromosome. H-NS protein comprises 137 amino acids with 15.5 kDa molecular weight. Although initial studies suggested that H-NS is involved only in the organization of chromosome, the identification that H-NS has higher propensity to bind to DNA, especially the AT-rich sequences, clearly indicated the regulatory function associated with H-NS [6–8]. H-NS was found to affect gene expression in a number of different ways, and it has been reported that expression of over 5% of the E. coli genes is affected in hns mutant [9–11].

H-NS binding does not seem to occur with any obvious sequence specificity [12,13]. Different mechanisms for transcriptional regulation by H-NS have been proposed; the most accepted models are as follows: H-NS might indirectly regulate initiation by binding to region distal from the promoter which causes change in supercoiling that in turn affects the supercoiling-sensitive promoters; and H-NS can also directly inhibit transcription by preferential binding to the promoter region. Many of the preferred H-NS binding sites contain an A/T-rich region, suggesting that a sequence-induced curvature is causing the preferential binding [14–18]. Studies on structural aspects of H-NS revealed that H-NS is comprised of a C-terminal DNA-binding domain and a coiled-coil N-terminal domain that mediates oligomerization, forming higher-order homomeric or heteromeric complexes. At least two dimerization sites have been identified that

Abbreviations
Ces, capsule expression suppression; Cps, capsular polysaccharide; H-NS, histone-like nucleoid structuring; LB medium, Luria–Bertani medium; OD, optical density; RcsA, regulator of capsule synthesis A; rpoB, RNA polymerase beta subunit.
allow H-NS to form higher-order oligomers [19–22]. The oligomerization and DNA-binding domains are joined via a flexible linker.

H-NS itself acts as a repressor for its own promoter, and apart from H-NS, StpA, Fis and CspA also play a role in the regulation of H-NS expression [23,24]. There is also a post-transcriptional negative regulatory mechanism which involves a small RNA called DsrA and an RNA-binding chaperon protein called Hfq [25,26]. The expression of H-NS is also increased by an unknown mechanism during growth at elevated hydrostatic pressure. H-NS and other nucleoid-associated proteins can recognize horizontally acquired DNA and transcriptionally silence it through xeno-genic silencing under environmental conditions that do not require expression of horizontally acquired genes [27,28]. In addition to its role in nucleoid architecture, H-NS plays a pleiotropic role in bacterial response to environmental stimuli such as starvation and changes in pH, temperature and osmolarity [29–31].

Very recently, we have reported the suppression of overexpression of genes implicated in colanic acid capsular polysaccharide (Cps) synthesis in Δlon mutant of E. coli by two novel rpoB mutations, namely rpoB12 and rpoB77. Genetic and molecular analyses clearly showed that downregulation of rcsA transcription is the primary reason for the elicitation of this capsule expression suppression (Ces) phenotype by these two rif alleles. Furthermore, our study clearly indicated that the presence of functional H-NS is mandatory for both the rpoB mutations to function as capsule expression suppressors in the Δlon strain of E. coli [32]. Sledjesky and Gottesman [25] have shown that H-NS functions as a repressor for rcsA transcription, and their study also revealed the involvement of a small RNA, namely DsrA located downstream of rcsA, in the regulation of rcsA by H-NS. DsrA binds to H-NS and thereby inhibits the action of H-NS on rcsA transcription. However, the mode of binding and the exact binding region for H-NS in the promoter region of rcsA have not been reported so far. They have suggested that the upstream region of rcsA promoter might possess bending/curved DNA region [25]. In our earlier study, we have provided evidence for the occurrence of bendable DNA region upstream of rcsA promoter through bioinformatics analyses [32]. In this study, perhaps for the first time we have given the genetic evidence that supports the presence of putative curved DNA region upstream of rcsA promoter. Furthermore, we have shown that the H-NS molecule which is defective in the formation of higher-order oligomers can still function as a repressor at the rcsA promoter. The bioinformatics analyses show that the region around 400 bp upstream of rcsA promoter might serve as H-NS binding site.

Materials and methods

Media composition, chemicals, fine chemicals and genetic and molecular techniques used in this study

The media (conventional LB and minimal media) composition used in this entire study is essentially as described in Ref. [33]. Materials used for media, buffer, solutions, most of the antibiotics and other fine chemicals were purchased from HiMedia, India. Streptomycin was purchased from Sarabhai Chemicals, India, and the final concentration of each of them is quoted wherever appropriate. All the genetic techniques were according to Ref. [33] (with minor modifications), and molecular techniques employed in this study were as per Ref. [34].

Bacterial strains and phages used in this study

Table 1 gives the list of bacterial strains, phages and plasmids used in this study. All the bacterial strains are the derivatives of E. coli K-12, and the genetic nomenclature is according to Refs [35] and [36].

Plasmid isolation, transformation and construction of strains bearing clones of dominant-negative alleles of hns

The strains bearing the plasmids harbouring the dominant-negative alleles of hns, namely pLGhns-D64, pLGhns-P116S, pLGhns-T55P, pLGhns-L26P and pLGhns+*, were procured from J Gowrishankar, CDFD, Hyderabad, India. The plasmids were isolated from relevant strains using the alkaline lysis method [37], and the presence of insert was confirmed through restriction digestion analyses. The strains, namely SG20780 (Δlon cps-lac), SG201781 (lon+ cps-lac), MMRT6 (Δlon cps-lac rpoB12) and MMRT23 (Δlon cps-lac rpoB77), were transformed with the relevant clones. The CaCl2-mediated transformation technique was followed. As the vector backbone (pLG339) bears kanamycin as selection marker, the transformants were selected on LB plates containing kanamycin. Representative transformants from each case were selected and purified for further use.

Beta-galactosidase assay

0.1 mL of overnight cultures of each strain (carrying cps-lac fusion) was subcultured into 5 mL of M9
minimal medium containing glucose as carbon source and grown at 30 °C. The cultures were allowed to attain mid-log phase, and then, the optical density of the cultures was recorded at 600 nm wavelength. The beta-galactosidase expressed from \( \text{cps-lac} \) fusion was assayed as described in Ref. [33] with minor modifications.

Bioinformatics analyses

The DNA sequence of the coding region of \( \text{rcsA} \) including the upstream region of \( \text{rcsA} \) promoter (till -600) was retrieved from Ecocyc.org, and the structure of the DNA sequence was elucidated using the software \\textit{MODEL.IT} (http://hydra.icgeb.trieste.it/dna/index.php). Further analyses/manipulations of the structure were carried out using \\textit{PYMOL} (http://pymol.org/edu/?q=educational).

Results

\textbf{H-NS which is defective in recognizing curved DNA fails to repress \( \text{rcsA} \) transcription}

In our earlier study pertaining to the isolation and characterization of novel \( \text{rpoB} \) mutations capable of suppressing the overproduction of colanic acid Cps in \( \text{lon} \) mutant of \( \text{E. coli} \), we have substantiated the role of functional H-NS in the elicitation of Ces phenotype by the two \( \text{rpoB} \) mutations, namely \( \text{rpoB12} \) (C1576 to T1576; His526 to Tyr526) and \( \text{rpoB77} \) (C1535 to T1535; Ser512 to Tyr512) [32]. As a continuation to this aspect, the effect of dominant-negative alleles of \( \text{hns} \) in Ces strains (\( \Delta\text{lon} \text{rpoB12} \) and \( \Delta\text{lon} \text{rpoB77} \) and in parental strains (\( \Delta\text{lon} \) and \( \text{lon}^+ \)) was studied (strains bearing the dominant-negative \( \text{hns} \) alleles were procured...
from Gowrishankar, CDFD, India). For information of different dominant-negative alleles of hns used in this study (Table 2). All the hns alleles, namely hnsP116S, hnsΔ64, hnsT55P and hnsL26P, have been cloned into a vector with its native promoter. The clone bearing hns<sup>+</sup> (pLG-hns<sup>+</sup>) was also used, as in this case the result could be presumed and it can be used for better comparison. All the clones were

| Strain/plasmid harbouring hns variant alleles and the levels of expression of β-galactosidase from cps-lac fusion (in Miller units) in the indicated strains. Values are average of seven different experiments | Inference on rcsA transcription based on cps-lac expression |
|---|---|
| SG20780/pLGhns-P116S | 431 Introduction of pLGhns-P116S significantly increased the cps-lac expression in all the four strains. It is very clear that in the strains bearing pLGhns-P116S, the expression level of cps-lac is increased to an appreciable degree when compared to that of the strains bearing pLG-hns<sup>+</sup> and relevant strains without any plasmid. These results clearly indicate that the mutant H-NSP116S molecules could no longer serve as repressors for rcsA transcription. As H-NSP116S molecules are reported to be defective in recognizing curved DNA region (although it retains nonspecific DNA-binding activity), this observation leads to the inference that the upstream region of rcsA promoter should bear curved DNA region |
| SG20780/pLGhnsΔ64 | 335 In the presence of pLGhnsΔ64, there is little increase in β-galactosidase activity from cps-lac fusion when compared to the cps-lac expression in the respective strains bearing no plasmid. Although C-terminally deleted H-NS molecules are shown to have deficiency in DNA binding, they are reported to have more binding affinity towards chromosomally encoded wild-type H-NS molecules. As pLGhnsΔ64 did not result in significant level of increase in cps-lac expression, it suggests that binding of H-NSΔ64 with chromosomally encoded wild-type H-NS might probably help in retaining the repressor activity to some extent. However, when compared with the strains bearing pLGhns<sup>+</sup>, there was a considerable elevation in the level of cps-lac expression in all strains. These results signify the fact that although HNSΔ64-H-NS<sup>+</sup> hetero-oligomers retain repressor activity, it perhaps cannot be equated to the activity of H-NS<sup>+</sup>-H-NS<sup>+</sup> homo-oligomers |
| SG20781/pLGhns-P116S | 200 |
| MMRT6/pLGhns-P116S | 382 |
| MMRT23/pLGhns-P116S | 272 |
| SG20780/pLGhns-T55P | 92 Introduction of pLGhns-T55P unexpectedly decreased the β-galactosidase activity from cps-lac fusion in all the strains to an appreciable degree. Comparison of the levels of expression of cps-lac in the strains bearing pLGhns-T55P with those of the relevant strains without plasmid clearly indicates the drastic reduction in the expression level of cps-lac due to multicopy pLGhns-T55P. It is possible when the oligomerization-defective H-NST55P molecules can still repress rcsA transcription, and it is perhaps due to nonspecific binding of the H-NST55P molecules along the rcsA promoter region |
| SG20780/pLGhns-L26P | 139 In a similar fashion to pLGhns-T55P, introduction of pLGhns-L26P also decreased the β-galactosidase activity from cps-lac fusion in the relevant strains. The inference and explanations could be the same as above (as in the case of pLGhns-T55P) |
| SG20781/pLGhns* | 88 Overexpression of the wild-type functional H-NS molecules represses the rcsA transcription much better as was expected |
| MMRT6/pLGhns* | 20 |
| MMRT23/pLGhns* | 14 |
| SG20780 | 323 |
| SG20781 | 9 |
| MMRT6 | 149 |
| MMRT23 | 84 |

As was expected in the absence of any clone, in the Δlon strain a higher level of expression of cps-lac was seen. However, in Δlon rpoB12 and Δlon rpoB77 mutants due to elicitation of Ces phenotype, the cps-lac expression was less as was expected. In the lon<sup>+</sup> strain, in accordance with expectation, a very low level of expression of cps-lac was seen due to RcsA degradation
transformed into the relevant strains, namely SG20780 (Δlon cps-lac), SG20781 (lon+ cps-lac), MMRT6 (Δlon cps-lac rpoB12) and MMRT23 (Δlon cps-lac rpoB77). In each case, a transformant was purified, cells were grown overnight in minimal glucose medium containing kanamycin till mid-log phase and β-galactosidase assay was carried out as described in Materials and methods. As was expected, introduction of the hns+ clone reduced the expression of cps-lac fusion to an appreciable degree in all the strains (Fig. 1). These results once again signify the role of H-NS as a repressor of rcsA transcription. As shown in Fig. 1, introduction of clones bearing variant alleles of hns, namely pLGhns-P116S and pLGhnsΔ64, into the relevant strains revealed the following: with reference to the mutant H-NS (with P116S amino acid substitution) which is defective in binding to curved DNA, the expression level of cps-lac has gone up to appreciable levels in all the above-mentioned strains. Comparative analyses of the multicopy effect of hns+ and hnsP116S alleles on the expression of cps-lac fusion in the relevant strains clearly show the inability of the mutant form of H-NSP116S molecules to exert complete repressor activity like the wild-type although it is reported to retain nonspecific DNA-binding activity (for the comparative values, see Table 2). These results strongly support the view that the upstream region of rcsA promoter is likely to contain a bendable/curved region and the inability of mutant form of H-NS (H-NSP116S) to bind to such putative bendable/curved region of rcsA promoter could be the cause for higher level of cps-lac expression in the relevant strains. Introduction of pLG-hnsΔ64 increased the cps-lac expression to some extent that clearly implies that the H-NS molecules bearing only the N-terminal region are not completely defective in repression at rcsA promoter.

**Oligomerization-defective H-NS can still function as a repressor at rcsA promoter**

Structural analyses have revealed that the N-terminal (amino acids 1–46) region of H-NS is involved in the oligomerization [8]. The clones, namely pLGhnsL26P and pLGhnsT55P, when introduced into the strains, namely SG20780 (Δlon cps-lac), SG20781 (lon+ cps-lac), MMRT6 (Δlon cps-lac rpoB12) and MMRT23 (Δlon cps-lac rpoB77), surprisingly decreased the cps-lac expression remarkably. The hns alleles cloned into these vectors result in the amino acid substitution at N-terminal region (at amino acid positions T55P and L26P), which is expected to affect the oligomerization property of H-NS molecules. This indirectly but strongly supports the view that even the oligomerization-defective H-NS could interfere with cps-lac expression perhaps by repressing rcsA transcription (Fig. 1).

**Bioinformatics analyses reveal that H-NS binding region could be present ~ 400 bp upstream of rcsA promoter**

H-NS has been shown to bind to the curved DNA region preferentially [14–16]. In our earlier report, we have given evidence for the involvement of functional H-NS in the elicitation of Ces phenotype by rpoB12 and rpoB77 mutations, and we have predicted the presence of bendable DNA sequence upstream of the rcsA promoter [32]. Here, we show that the DNA sequence around 400 bp upstream of the rcsA promoter is probably bendable in nature. Using the software modellit, we have modelled the DNA region present upstream of rcsA, and the image was further manipulated by pymol software. Figure 2A,B clearly shows that the region -130 to -400 does exist as a putative curved DNA. Therefore, the sequence underlined in Fig. 2C could be the most probable region where the H-NS might bind to and repress rcsA transcription.

**Discussion**

Overproduction of colanic acid Cps and extreme sensitivity to DNA-damaging agents are considered as the iconic phenotypes of a lon mutant of E. coli. Detailed study on these aspects revealed that stabilization of two Lon substrates, namely RcsA (the positive regulator of cps transcription) and SulA (cell division inhibitor that gets induced upon DNA damage), is the main reason for the elicitation of the above-mentioned phenotypes, respectively [38–40]. Previous studies pertaining to isolation of suppressor(s) for these two hallmark phenotypes implicated a vital role of mutation in ssrA and a novel allele of dnaJ (faa) [41–44]. Earlier using an unorthodox, wee bit strategy, we sought for rif (rpoB) mutations capable of suppressing either one or both of the phenotypes of lon mutant. In such an attempt, we were indeed successful in isolating two such novel rif alleles (rpoB12 and rpoB77) that could suppress only the overproduction of capsule synthesis. Detailed analyses showed that the elicitation of this Ces phenotype by these rif mutations primarily stems from the downregulation of rcsA transcription. Our study also revealed the requirement of functional H-NS in the elicitation of Ces phenotype [32]. Although the role of H-NS in the transcriptional
regulation of \( rcsA \) has been reported, the exact mode of regulation of \( rcsA \) transcription by H-NS has not been reported to date.

Much of the information about the properties of different domains of H-NS came from the analyses of effect of different mutations on the functionality of H-NS. Systematic mutational analyses with H-NS revealed that C-terminal region is crucial for DNA binding and the central and N-terminal regions are involved in the formation of oligomer/higher-order
During the course of such analyses, dominant-negative variants of hns have been isolated [19,20]. In this study, the effect of different clones bearing dominant-negative alleles of hns such as hnsP116S, hnsT55P, hnsL26P, hnsΔ64 and hns+, in phenotypically Ces strains such as MMRT6 (Δlon rpoB12) and MMRT23 (Δlon rpoB77) and also in Δlon and lon+ strains has clearly revealed that the hnsP116S allele that codes for H-NS but is defective in recognizing curved DNA almost completely abolished the elicitation of Ces phenotype in both Δlon rpoB12 and Δlon rpoB77 strains. This effect was seen even in Δlon and lon+ strains. This indirectly implies that the region upstream of rcsA promoter might possess putative curvature which might play an important role in the regulation of rcsA transcription by H-NS.

Further, the clones bearing hns alleles coding for the amino acid substitutions, namely T55P and L26P (which are defective in the formation of higher-order oligomers), significantly reduced the cps-lac expression not only in the Δlon rpoB12 and Δlon rpoB77 strains but also in the Δlon and lon+ strains; that is, the effect is albeit closer to that of wild-type H-NS. This was totally unexpected as we imagined that the mutant forms of H-NS cannot form higher-order oligomers and therefore will not be able to repress rcsA transcription. But the fact that we have made such an observation compelled us to make a model that in a nonoligomeric state and even without forming higher-order oligomers, these mutant forms of H-NS perhaps might be able to bind to DNA and function as repressors for rcsA transcription. Williams et al. [19] have reported that the introduction of clone(s) bearing oligomerization-defective hns alleles, namely L26P and T55P, drastically decreased the expression of semisynthetic 5A6Agal promoter. Similar analyses with one
other H-NS-regulated gene, namely proU, indicate that the expression of its promoter was not found to be identical to that of 5A6Agal promoter. These observations signify the fact that the regulatory function of H-NS depends on the sequence features of the promoters also. In similar analyses, it was also found that introduction of clone bearing hns allele (P116S) elevated the expression of 5A6Agal promoter to an appreciable degree, while the same was once again not found to be true with proU promoter. It has been reported that the upstream region of 5A6Agal promoter bears a curvature [45]. However, in the case of proU, the presence of any curved DNA region has not been reported and notably the repression by H-NS essentially needs extensive nucleoprotein formation at the proU promoter region. The expression pattern of 5A6Agal promoter and rcsA promoter is found to be similar in the presence of different dominant-negative hns alleles. These observations clearly favour the notion that the upstream region of rcsA might possess curved DNA which would serve as binding region for H-NS, thus aiding H-NS to transcriptionally regulate rcsA expression.

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Author contributions

SM designed the study, performed experiments, analysed the results and wrote the manuscript. MK performed the experiments. MHM analysed the results, wrote the manuscript and provided resources for the study.

Conflict of interest

The authors declare no conflict of interest.

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