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A role for histone-like protein H1 (H-NS) in the regulation of hemolysin expression by *Serratia marcescens*

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Abstract

The histone-like protein H1 (H-NS) is an abundant structural component of the bacterial nucleoid and influences many cellular processes including recombination, transcription and transposition. Mutations in the *hns* gene encoding H-NS are highly pleiotropic, affecting the expression of many unrelated genes. We have studied the role of H-NS on the regulation of hemolysin gene expression in *Serratia marcescens*. The *Escherichia coli hns* mutant carrying *S. marcescens* hemolysin genes on a plasmid constructed by ligation of the 3.2-kb *Hin*dIII-*Sac*I fragment of pR02 into pBluescriptIIKS, showed a high level of expression of this hemolytic factor. To determine the osmoregulation of wild-type and *hns* defective mutants the cells were grown to mid-logarithmic phase in LB medium with 0.06 or 0.3 M NaCl containing ampicillin and kanamycin, whereas to analyze the effect of pH on hemolysin expression, the cells were grown to late-logarithmic phase in LB medium buffered with 0.1 M Tris-HCl, pH 4.5 to 8.0. To assay growth phase-related hemolysin production, bacterial cells were grown in LB medium supplemented with ampicillin and kanamycin. The expression of *S. marcescens* hemolysin genes in wild-type *E. coli* and in an *hns*-defective derivative at different pH and during different growth phases indicated that, in the absence of H-NS, the expression of hemolysin did not vary with pH changes or growth phases. Furthermore, the data suggest that H-NS may play an important role in the regulation of hemolysin expression in *S. marcescens* and its effect may be due to changes in DNA topology influencing transcription and thus the amount of hemolysin expression. Implications for the mechanism by which H-NS influences gene expression are discussed.

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Key words
- Hemolysin expression
- H-NS
- Global regulation
- *Serratia marcescens*

Introduction

Serratia marcescens is an important opportunistic pathogen in nosocomial infections such as septicemia, pneumonia, keratitis, and wound infections (1,2). Hemolysin production is a common attribute of *S. marcescens* strains and has been shown to be involved in the virulence of this pathogen (3-5). *S. marcescens* hemolysin is determined

by two chromosomal genes termed *shlA* and *shlB*. The ShlA (162 kDa) polypeptide is the hemolysin itself, whereas ShlB (61 kDa) is required for activation and secretion of ShlA (6). The hemolytic activity is growth dependent and declines noticeably once cells leave the phase of exponential growth (7). The expression of the *shlB* gene is regulated by iron levels in the medium, a function that may be related to the *fur* gene of *Escherichia coli* (8).

It has been recently shown that H-NS protein is able to mediate the response of many operons to environmental changes (9,10). H-NS is one of the two most abundant proteins in the bacterial nucleoid of enteric bacteria, including *S. marcescens* (9-11). This neutral and low-molecular mass protein (5.5 kDa) binds DNA as a dimer in a relatively non-specific fashion or exhibits specificity for AT-rich sites and in almost all known cases interacts with heterologous curved sequences (12). The complexes formed between H-NS and non-specific binding sites appear to be different from the complexes formed with specific binding sites (10-12). Little is known about H-NS regulation of hemolysin genes in bacteria. The data presented here support the hypothesis that H-NS acts at specific sites to influence DNA topology and hence the transcription and expression of two chromosomal genes, *shlA* and *shlB*, responsible for hemolysin production by *S. marcescens*. To our knowledge, this is the first report to analyze the role of H-NS in the expression of a virulence factor of *S. marcescens*.

Material and Methods

The bacterial strains and plasmids used in this study are listed in Table 1. Cells were grown in LB medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 50 mM $MgCl_2$, pH 7.0.

Plasmid construction

Plasmid p3H was constructed by ligation of the 3.2-kb *Hin*dIII-*Sac*I fragment of pR02 (a gift from V. Braun, University of Tübingen, Tübingen, Germany) (5) into pBluescriptIIKS (Stratagene, La Jolla, CA, USA) digested with *Hin*dIII and *Sac*I. The truncated *shlA* gene in p3H was restored to the whole *shlA* gene for *shl*A PCR amplification of *S. marcescens* SN8 (a gift from Dr. V. Braun). The 3.9-kb *Sac*I-*Eco*RI fragment of the entire PCR-amplified *shlA* was ligated to p3H digested with *Sac*I-*Eco*RI. The hemolysin expression in *E. coli* DH10B was demonstrated by a liquid hemolysis assay, with minor modi-

Table 1. Bacterial strains and plasmids used in the present study.

Strain or plasmid	Relevant genotype	Source or reference
Strain		
Escherichia coli		
MC4100	Wild-type	9
MC41	MC4100 *hns*::kan^R	10
Salmonella typhimurium		
CH946	ProU1702::Mud 1-8	17
CH1839	CH946, *hns*-1::kan^R	17
Plasmid		
pR02	*shlB shlA* (Δ256-1578) in pT7-6	3
pBluescriptIIKS (pKS)	High-copy number cloning vector, amp^R	Stratagene
p3H	pR02-derivative carrying the entire *shlA* in pKS	Present study

amp^R = ampicillin resistant; kan^R = kanamicin resistant.

fications, as described below (7).

PCR amplification

The synthetic oligonucleotide primers ShlAI - 5'TGGATGAAAAATAATAACTT CAGACTTTCG3' and ShlAII - 5'ATGAA TTCCGCGTTATTTGCCGCTGAAC3' were designed based on the sequence reported for the *S. marcescens* hemolysin genes (13) and used to amplify a 4.8-kb sequence corresponding to the entire *shlA* gene. PCR was performed with a PTC-200 thermal cycler (MJ Research, Watertown, MS, USA) using a 50-µl reaction mixture containing 1X PCR buffer plus 2.0 mM $MgCl_2$, 2.5 U of long template DNA polymerase (Gibco BRL, Rockville, MD, USA), 200 µM (each) dNTPs, and 50 pmol of each primer. The cycling conditions were as follows: 2 min at 94ºC followed by 35 cycles of 1 min at 94ºC, 45 s at 56ºC, and 4 min at 72ºC, and ending with 5 min at 72ºC. Amplified products from PCR were electrophoresed on 0.8% agarose gels in the presence of ethidium bromide and recorded with a UV-gel Doc System (BioRad Laboratories Inc., Hercules, CA, USA).

Liquid hemolysis assay

Blood samples were obtained from healthy volunteers and stored at 4ºC for no more than 4 days. Immediately before the hemolysis assay, the erythrocytes were collected by centrifugation and washed with 0.9% NaCl until the supernatant was practically free of hemoglobin. The hemolysis assay medium contained 0.5 ml of washed erythrocytes (8%, v/v), 0.1 ml of *Serratia* cells (3 x 10^8, A_{540} = 0.9) harvested by centrifugation at 2260 *g* for 20 min at 4ºC and resuspended in 0.9% NaCl, and 0.1 mg/ml kanamycin sulfate. This mixture was incubated at 30ºC for 90 min and then centrifuged for 3 min at 2260 *g*. The supernatant was diluted 10-fold in 0.9% NaCl and absorbance was measured at 405 nm. The absorbance at 405 nm for total lysis was obtained by resuspending the washed erythrocytes in distilled water.

Environmental regulation

To determine the osmoregulation of wild-type and *hns* mutants the cells were grown to mid-logarithmic phase (A_{490} of 0.7) in LB medium with 0.06 or 0.3 M NaCl containing ampicillin and kanamycin. The hemolytic activity was measured as described above. To analyze the effect of pH on hemolysin expression, strains were grown to logarithmic phase in LB buffered with 0.1 M Tris-HCl, pH 4.5 to 8.0, supplemented with appropriate antibiotics from an overnight culture grown under the same conditions. To assay growth phase-related hemolysin expression, bacterial strains were grown in LB medium containing ampicillin and kanamycin. Two 1.0-ml samples of cells were removed every 20 min and A_{490} was measured. Hemolysis was measured as described above.

Results

Effect of *hns* mutations on hemolysin expression

As mentioned above, previous studies have shown that H-NS can modulate expression of a number of genes in response to environmental signals. To determine whether hemolysin expression was affected by H-NS, the p3H plasmid (carrying *shlA*, *shlB*) was transformed into *E. coli* and *Salmonella typhimurium* cells (both the wild-type and *hns* mutant). As shown in Figure 1, the level of hemolytic activity was 2-fold lower in two wild-type strains (MC4100 and CH946) compared with two strains harboring *hns* mutations (MC41 and CH1839), while it was essentially the same in the two wild-type or *hns* mutant strains. Densitometric analysis showed that the MC41 strain expressed ShlA and ShlB at levels 2-fold higher than the

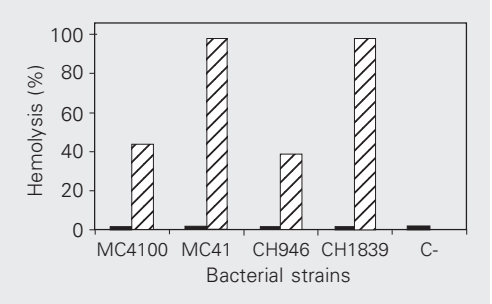

Figure 1. Effect of *hns* mutations on *Serratia marcescens* hemolysin expression. Hemolysis was measured by absorbance at 405 nm. C– = negative control, no bacteria added to cells (filled columns to the left of each column). MC4100 and MC41 = *E. coli* strains *hns*+ and *hns*, respectively. CH946 and CH1839 = *S. typhimurium hns*+ and *hns*, respectively. Each bar indicates the mean ± SEM of six independent experiments. The level of hemolytic activity was 2-fold lower in the two wild-type strains compared with the two strains harboring *hns* mutations (MC41 and CH1839). P < 0.001 (chi-square significance test).

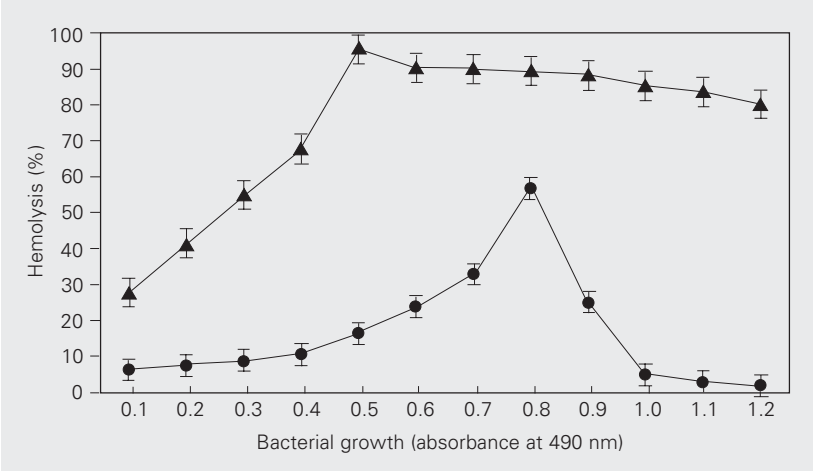

Figure 2. Effect of bacterial growth and H-NS on hemolysin synthesis. Erythrocyte lysis by *Escherichia coli* MC4100 (circles) and MC41 (triangles) was measured by absorbance at 405 nm. Each point indicates the mean ± SEM for four independent experiments. Hemolysin expression in *E. coli* MC41 was not growth phase dependent, compared to the wild-type strain MC4100. P < 0.001 (chi-square significance test).

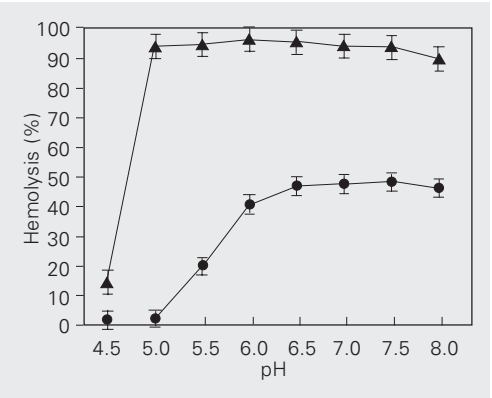

Figure 3. Effect of pH on hemolysis by an *hns* mutant. Hemolysis by *Escherichia coli* MC4100 (circles) and MC41 (triangles) was measured by absorbance at 405 nm. Each point indicates the mean ± SEM of four independent experiments. The hemolytic activity is highly reduced at pH 5.0 in wild-type strains compared to *hns* mutant strains. P < 0.001 (chi-square significance test).

MC4100 strain (data not shown). The results suggest that hemolysin production is sensitive to the *hns* mutation.

Effect of growth phase

The hemolysin activity of *S. marcescens* is regulated according to the growth state of the culture. ShlA and ShlB expression is maximal in the late logarithmic growth phase (3-5). To test whether hemolysin expression in the *hns* mutant is growth phase dependent, the hemolytic activity was analyzed during different growth phases. Strains were grown under optimal conditions using LB medium and samples were removed periodically and assayed for hemolytic activity. The data presented in Figure 2 show that hemolysin expression in the *hns* mutant was not growth phase dependent. In contrast to the wild-type strain (MC4100), the hemolytic activity of the *hns* mutant (MC41) was significantly stronger during the different growth phases analyzed. In addition, the maximal hemolytic activity of the wild-type strain was at $OD_{490} = 0.8$, whereas in the *hns* mutant the maximal activity was obtained at $OD_{490} = 0.5$. Similar results were obtained with the CH946 and CH1839 strains (data not shown). The results suggest that H-NS may control hemolysin expression in a growth phase-dependent manner.

Effect of pH

Acid adaptation is likely to be an important variable in bacterial pathogenicity since pH has been identified as a regulator of the expression of genes involved in virulence. These genes include the *invF* and *pagC* genes of *S.* typhimurium (14) and *virF* of *S. flexineri* (15). To determine whether *S. marcescens* hemolysin genes are regulated by pH, the hemolytic activity was analyzed at pH 4.5 to 8.0. Bacterial strains from overnight cultures were grown to the mid-logarithmic phase in LB medium buffered with 0.1 M Tris-HCl at

different pH. As shown in Figure 3, the hemolytic activity was found to be highly reduced at pH 5.0 in wild-type strains, while it increased in *hns* mutants. The results indicate that hemolysin is repressed at low pH and that H-NS may influence hemolysin expression.

Effect of osmolarity

Previous studies have shown that high osmolarity may induce a number of genes involved in bacterial virulence (16). To determine the effect of osmolarity on hemolysin expression, bacterial strains were grown under optimal conditions using LB medium supplemented with 0.06 or 0.3 M NaCl and were assayed for hemolytic activity (Table 2). The hemolytic activity of the wild-type or *hns* mutant strains was higher under low osmolarity conditions. However, no difference in hemolytic activity was observed between wild-type and *hns* mutants. These results suggest that osmolarity may be an environmental signal controlling hemolysin expression independent of H-NS activity.

Discussion

The ability of bacteria to persist in the human host depends upon prompt adaptation to changing environmental conditions such as temperature, pH, osmolarity, oxygen tension, and nutrients (16,17). Previous studies have suggested that certain environmental factors and the action of H-NS on the nucleoid modulate the expression of several virulence genes (6,7). Many H-NS-dependent genes are environmentally regulated and are sensitive to changes in DNA supercoiling. H-NS can alter DNA topology by constraining negative supercoiling (18). H-NS is also known to affect the expression of genes primarily at the transcriptional level (9,18). The molecular basis of H-NS influence on transcription is probably due to its preferential interaction with intrinsically curved DNA (10). Interestingly, it has been shown that H-NS affects gene expression by binding to a downstream regulatory element in the structural genes, one of the few binding sites that does not contain a detectable curvature (9,10). This, together with the observation that *hns* mutants express altered plasmid linking numbers, suggest that H-NS modifies gene expression through changes in DNA topology (18). However, recent data indicate that H-NS may also play a direct role in gene expression (10,18).

Several DNA-binding proteins, including H-NS, have been identified in Gram-negative bacteria using genetic and biochemical strategies (19,20). However, relatively little is known about the effects of H-NS and environmental signals on the expression of *S. marcescens* virulence genes. Thus, the present study was carried out in order to determine whether H-NS and environmental factors modulate the expression of hemolysin, an important virulence factor of *S. marcescens*.

In this report we have shown that *hns* mutations had double the hemolytic activity of wild-type *E. coli* strains harboring the *S. marcescens shlA* gene, suggesting that hemolysin expression may be regulated by this DNA-binding protein. Similarly, the *hns* mutant increases α-hemolysin expression in *E. coli*, although not so strongly (8). Previ-

Table 2. Effect of osmolarity on the hemolytic activity of *Escherichia coli* strains transformed with plasmids carrying the cloned *Serratia marcescens shlA* gene.

Strain[a] (plasmid)	Genotype	Mean hemolytic activity (%)[b]		Ratio[c]
		LB - 0.06 M NaCl	LB - 0.3 M NaCl	
MC4100 (p3H)	*hns*$^+$	66 ± 7	34 ± 4	1.9
MC41 (p3H)	*hns*	94 ± 8	44 ± 5	2.1
CH946 (p3H)	*hns*$^+$	68 ± 7	24 ± 2	2.8
CH1839 (p3H)	*hns*	95 ± 8	33 ± 4	2.9

[a]Strains were grown to mid-logarithmic phase (OD_{490} = 0.7) in LB supplemented with 0.06 or 0.3 M NaCl and with kanamycin and ampicillin. [b]Hemolytic activity was measured as described in Material and Methods. The data are the means ± SEM of four independent experiments. [c]Ratio of hemolytic activity LB - 0.06 M NaCl/LB - 0.3 M NaCl.

ous studies have shown that *hns/hha* mutants strongly enhance α-hemolysin expression. Hha is a temperature- and osmolarity-dependent modulator of the expression of the *E. coli* hemolysin operon (8). However, to our knowledge, no Hha-related protein has been identified thus far in *S. marcescens*. On the other hand, *hns* mutations are also involved in the expression of high levels of cholera toxin from *Vibrio cholerae* (20). Thus, as observed for a number of virulence genes that are H-NS regulated, our results provide some evidence that *S. marcescens* hemolysin is also H-NS regulated. H-NS may bind to a bent sequence or to a downstream regulatory element located in the promoter region of the *shl* structural genes influencing hemolysin expression. Our data also suggest that *E. coli* H-NS may also be involved in the regulation of the *S. marcescens* hemolysin operon. This finding is not surprising since the sequence of *E. coli* H-NS and *S. marcescens* H-NS appears to be highly conserved (17).

Despite the functional similarity between *E. coli* and *S. marcescens* H-NS, the mechanism by which H-NS modulates *S. marcescens* hemolysin is not clear. Previous studies have shown that H-NS can alter DNA supercoiling and that variation of DNA supercoiling may modulate expression of virulence genes in response to the same environmental factors (17). We have shown that environmental factors such as pH, osmolarity and growth phase affect hemolysin expression in *hns* mutants and in wild-type strains. However, in contrast to pH and growth phase, the modulation of hemolysin expression by osmolarity appears to be independent of the binding of H-NS to the nucleoid. Thus, our data indicate that changes in DNA topology may be a regulatory mechanism for the control of hemolysin gene expression. This is in contrast to previous studies showing that enhancement of *E. coli* α-hemolysin expression in the *hha/hns* mutant is not correlated with a global alteration of DNA topology, since a reporter plasmid isolated from this mutant displayed a topoisomer distribution similar to that of the parental strain (8). On the other hand, the increased expression of *S. marcescens* hemolysin in an *E. coli hns* mutant may be attributed to higher sigma S factor concentration in this bacterial species. The expression of the *rpoS* gene encoding the RNA polymerase sigma S factor is affected by H-NS. In *hns* mutants the concentration of sigma S is increased by a factor of 10 relative to the wild-type strain grown exponentially at low osmolarity (8). Moreover, to our knowledge, it is not known whether *S. marcescens* hemolysin genes are transcribed in a sigma S-dependent manner. Finally, H-NS may influence hemolysin expression by more than one mechanism.

The data presented here suggest that H-NS influences hemolysin expression and that specific environmental factors act as signals to induce *S. marcescens* hemolysin expression in *E. coli*, a close relative of *S. marcescens*.

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