MOLECULAR INTERACTION OF THE *SHIGELLA FLEXNERI* PROTEIN H-NS AND ITS SIGNIFICANCE IN THE TEMPERATURE REGULATION OF VIRULENCE

1999

ROGERS

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION OFFICE
(301) 295-3913
FAX: (301) 295-6772

January 22, 1999

APPROVAL SHEET

Title of Thesis: "Molecular Interaction of the *Shigella flexneri* protein H-NS and its Significance in the Temperature Regulation of Virulence"

Name of Candidate: Francine C. Rogers
Master of Science
Department of Microbiology & Immunology

Thesis and Abstract Approved:

Anthony Maurelli, Ph.D. — 1/22/99
Chairman — Date

Eleanor Metcalf, Ph.D. — 1/22/99
Member — Date

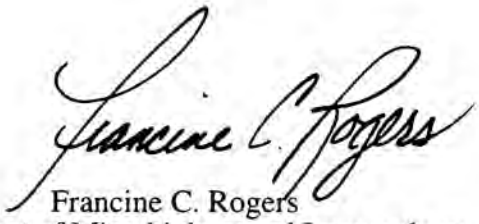
Paul Rick, Ph.D. — 1/22/99
Member — Date

Franziska Grieder, Ph.D. — 1.22.99
Member — Date

The author hereby certifies that the use of copyrighted materials in the thesis manuscript entitled:

"Molecular Interaction of the *Shigella flexneri* protein H-NS and its Significance in the Temperature Regulation of Virulence"

beyond brief excerpts is with permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such a copyright violation.



Francine C. Rogers
Department of Microbiology and Immunology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Thesis:
Molecular Interaction of the *Shigella flexneri* protein H-NS and its Significance in the Temperature Regulation of Virulence

Francine C. Rogers; Candidate, Masters of Science, 1999

Thesis directed by: Anthony T. Maurelli, Ph.D., Associate Professor, Department of Microbiology and Immunology

Shigella flexneri is an enteric pathogen and the causative agent of bacillary dysentery. Virulence is regulated by many environmental signals, although temperature is thought to be the primary signal. Virulence gene expression is repressed at 30°C and induced at 37°C. The regulatory cascade begins with the antagonistic relationship between H-NS, a negative regulator of *virB*, and VirF, a positive regulator of *virB*. The C-terminal portion of the H-NS protein binds to the curved DNA sequence upstream of the *virB* promoter inhibiting access to VirF and the RNA polymerase at 30°C. At 37°C, VirF binds upstream of *virB* and positively regulates *virB* transcription. Subsequently, VirB activates transcription of the virulence genes for attachment and invasion, encoded on the 220 kb plasmid. The significance of H-NS is its inhibition of transcription of the virulence genes at temperatures ‹37°C allowing the bacterium to conserve energy when not inside a mammalian host. In this thesis, the role of H-NS as a global regulator in the bacterial cell is explored in relation to *Shigella* virulence gene expression and other stress-induced genes.

Molecular Interaction of the *Shigella flexneri* protein H-NS and its Significance in the Temperature Regulation of Virulence

by
Francine C. Rogers

Thesis submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of
Master of Science 1999

DEDICATION

To the Frog and the Smurf
In Remembrance of Samuel

ACKNOWLEDGEMENTS

Anthony T. Maurelli – Thank you for being my advisor. For mentoring me in your lab and constantly asking me, "Why?"

To the Maurelli Lab, past and present – Thank you for your advice, answers to my questions and most importantly your friendship, especially Robin Sandlin, Raymond Schuch and Rey Fernández.

Thesis committee, Dr. Grieder, Dr. Maurelli, Dr. Metcalf, and Dr. Rick – Thank you for your time and energy reviewing my thesis and answering my questions. To all the faculty – Thank you for guiding and shaping my educational future. You have been an inspiration.

Mom and Dad - Thank you for all your love and your never ending support of my education and dreams.

Paul D. Rogers – Thank you for your love, support and assistance. You will always be, "my hand to hold, shoulder to lean on in the dark, someone to hear me...someone to love." (P.D.R., 1995)

Walter Sprague – Thank you for always being there for those late night phone conversations, for being my devil's advocate I never wanted to listen to but always needed to hear.

Agnes Jones-Trower – Thank you for being a fellow graduate student, but more importantly, a friend. Thanks for always answering my questions, taking classes and for going on those stress relieving walks and candy runs.

Katherine Marie Irons – Thank you for your wide-eyed amazement of the "World of Science."

Friends, past and present, old and new – Thanks for your friendship, especially Maria Scott, Tammy Crowder, Karen K., C.A.T., D.M., D.A.C., G.G., M.O'C., G.B.R., R.W.C., R.G.B., and B.G.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Epidemiology and pathogenesis of shigellosis

Bacteria of the genus *Shigella* are the causative agents of bacillary dysentery or shigellosis. *Shigella* spp. are gram-negative nonmotile rods, 2-4 μm in length and 0.4-0.6 μm in width. The genus is divided into four species (spp.), *S. flexneri*, *S. dysenteriae*, *S. sonnei* and *S. boydii*. The pathogenicity of *Shigella* is based on its ability to invade human colonic epithelial cells, replicate, and spread (LaBrec *et al.*, 1964).

Shigella infection is an important public health problem. This organism is responsible for a significant occurrence of the acute diarrheal diseases worldwide (Kopecko *et al.*, 1985) with an estimated incidence of over 200 million cases annually. Over half a million people die from shigellosis each year. The populations most at risk are children under six years of age (Keusch and Formal, 1984), and persons who are subject to conditions of crowding and poor sanitation.

Transmission of *Shigella* is by the fecal/oral route, with higher primates being the only significant reservoir (Kopecko *et al.*, 1985). Therefore, *Shigella* can be easily spread through contaminated food

and water (Maurelli and Lampel, 1997). It can also be spread by insects, such as houseflies (Levine and Levine, 1991) in open sewage and garbage areas. The best protection against infection is proper sanitation, both personal and municipal, and insect control.

Shigella spp. are highly infectious. The oral infectious dose 50% (ID50) is less than two hundred organisms. Full clinical disease can be demonstrated with the ingestion of as few as ten organisms (Levine *et al.*, 1973). *Shigella* are considered acid resistant (Formal and Levine, 1983), since they are able to traverse the stomach without significant mortality. Once through the stomach, they continue through the small intestine and colonize in the colon. In 1965, Takeuchi *et al.*, performed electron micrographic studies that elucidated bacterial invasion into intestinal epithelial cells. After attaching to the brush border, the bacteria presumably are internalized through the M cells (specialized antigen presenting cells) by induced phagocytosis (Parsot and Sansonetti, 1996). Once in the M cells, the bacteria can escape from the vacuole within fifteen minutes and multiply in the cytoplasm. An actin tail formation is induced at one end of the bacterium. The actin tail provides the mechanism for intra- and intercellular motility (Bernardini *et al.*, 1989). Two model pathways, as shown in Figure 1, have been proposed for cell to cell spread: 1) the bacterium is released from

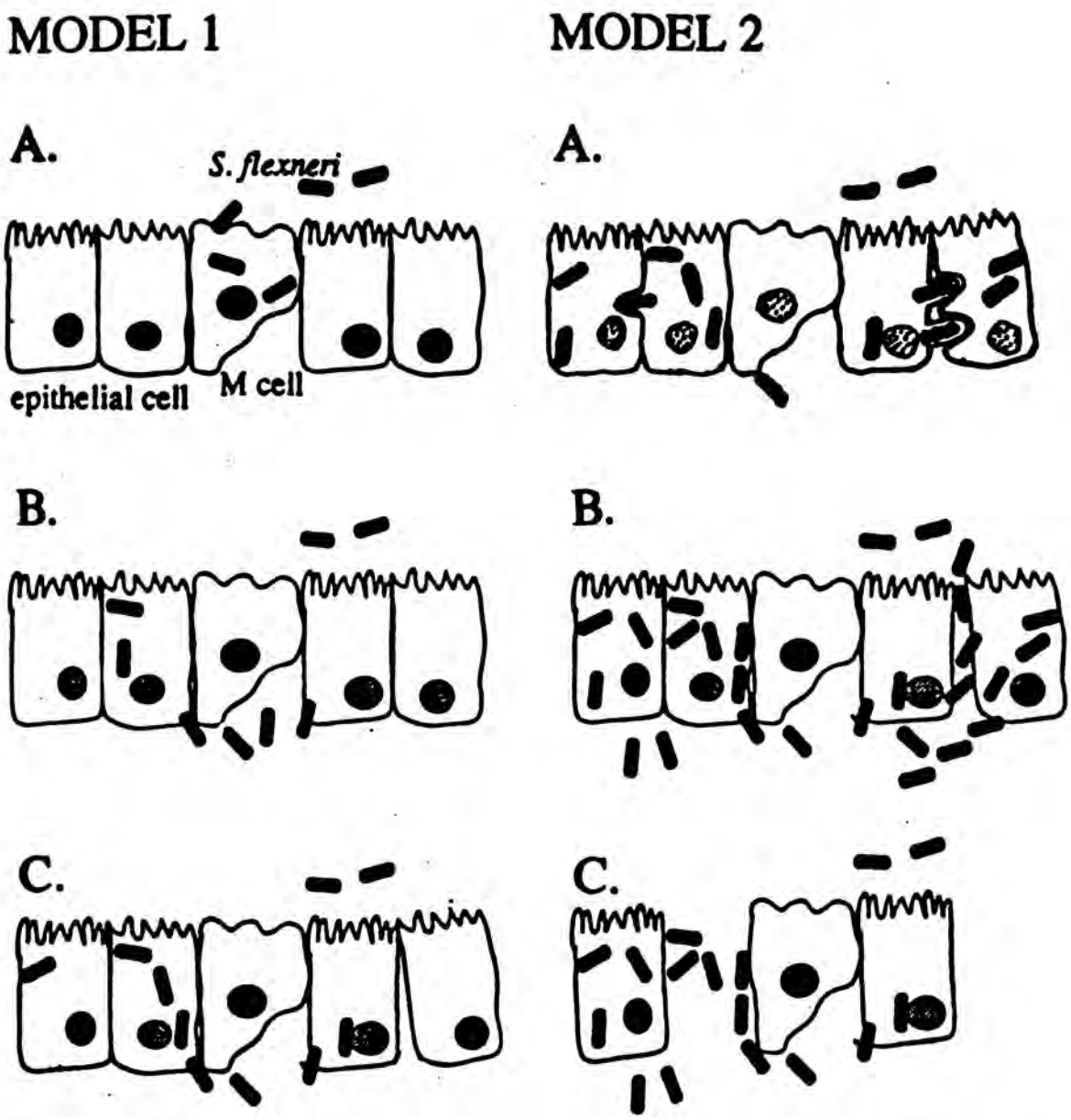


Figure 1. Two model pathways proposed for *S. flexneri* cell to cell spread. Model 1: A. Transcytosis of bacterium through the M cell. B. Bacterium enters lamina propia and reinvades the epithelial cells through the basolateral surfaces. C. Continued dissemination of *S. flexneri*. Model 2: A. Direct passage between cells through bacteria-generated protrusions. B. Bacterial entry through the rupturing epithelial barrier and continued cell to cell spread. C. Cell death leading to destruction of the mucosa. (Adapted from Zychlinsky *et al.*, 1994, with permission from Dr. P. J. Sansonetti)

the killed cells into the lamina propria and reinvades the epithelial cells through the basolateral surface, and 2) direct passage between cells through bacteria-generated protrusions. The cell to cell spread results in cell death and destruction of mucosa. Ulceration is seen as the cells slough off. The infection is primarily localized in the mucosal surface and dissemination to the bloodstream is rare since bacteria that reach the lamina propria evoke an immune response (Takeuchi *et al.*, 1965). The infection, usually self-limiting (Formal and Levine, 1983), causes an acute inflammation of the colon with disease symptoms ranging from mild diarrhea to acute abdominal pain, fever, watery diarrhea, and/or bloody mucoid stools.

Virulence genes and the regulation cascade in *Shigella flexneri*

Virulent *Shigella* carries a large 220-kb virulence plasmid. The plasmid encodes the temperature regulated genes essential for attachment, invasion, and intra- and intercellular spread. A partial list of the virulence factors associated with the expression of plasmid-linked virulence genes is shown in Table 1 and a map of the virulence plasmid in Figure 2. The transcriptional regulation of the plasmid-encoded genes is controlled by a multicomponent process that will be discussed in a subsequent section of this thesis.

Table 1. Virulence Factors of *Shigella flexneri* associated with expression of plasmid-linked virulence genes

Virulence Factor	Role	Reference
H-NS	Repressor of *virB* transcription	Tobe *et al.*, 1993
VirF	Regulates *virB* and *icsA* expression	Adler *et al.*, 1989
VirB	Activates plasmid virulence genes	Adler *et al.*, 1989
Mxi & Spa proteins	Excretion of Ipa proteins	Parsot *et al.*, 1995
Ipa B, C	Invasion and escape from the phagocytic vesicle	Parsot *et al.*, 1995
IpaD	Adherence	Parsot *et al.*, 1995
IpgC	Molecular chaperone of IpaB and IpaC	Menard *et al.*, 1994
IscA	Intercellular spread	Bernardini *et al.*, 1989
IcsB	Intercellular spread, lysis of protrusions	Allaoui *et al.*, 1992
VirK	Post-transcriptional control of *icsA* gene	Nakata *et al.*, 1992
VirA	Invasion and spreading	Uchiya *et al.*, 1995
VacB	Post-transcriptional control of *ipa* and *icsA* expression	Tobe *et al.*, 1992

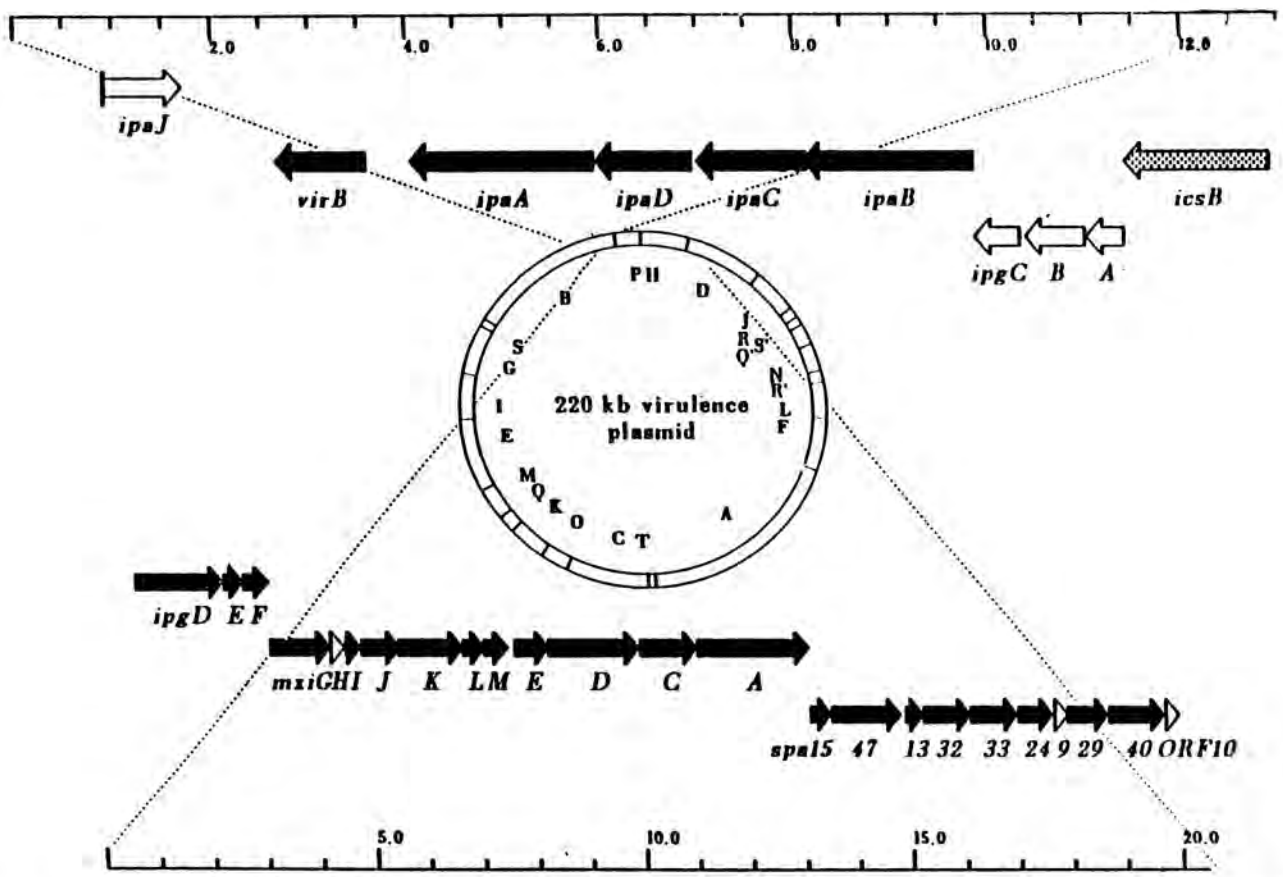


Figure 2. Map of virulence plasmid from *S. flexneri* 2a. A *Sal*I restriction map of the 220-kb plasmid is shown in the center. Sections of *Sal*I fragments B and P (upper map) and fragments P, H, and D (lower map) are expanded to illustrate the virulence loci encoded in these regions. Arrows mark the direction of transcription of each open reading frame. The extended regions are contiguous and include 32 kb. The open reading frame from *isc*B is separated from that of *ipg*D by 314 bp. (From Maurelli and Lampel, 1997)

The first major set of genes encoded on the virulence plasmid are the *mxi* and *spa* operon. Mxi and Spa proteins comprise the Type III secretion system responsible for the release of the Ipa invasion proteins from the cytoplasm of the bacterium (Parsot *et al.*, 1995). The *ipa* operon encodes IpaB, C, D, and A. IpaB and IpaC are required for host cell entry and escape from the phagocytic vesicle while IpaD is presumably required for adherence (Parsot *et al.*, 1995). IpaB and IpaC are secreted proteins and IpgC acts as a molecular chaperone to prevent aggregation of homo-oligomers of the Ipa proteins in the cytoplasm (Menard *et al.*, 1994). An actin tail is induced by the product of the *icsA* gene. The bacterium uses the actin tail formation to spread into adjacent epithelial cells (Bernardini *et al.*, 1989). Protrusions inside the adjacent cell are lysed by IscB (Allaoui *et al.*, 1992). Another protein, VirA is required for invasion and intercellular spread (Uchiya *et al.*, 1995).

Many of these invasion proteins are also subjected to post-transcriptional regulation. VacB controls *ipa* and *icsA* expression (Tobe *et al.*, 1992). VirK also controls expression of the *icsA* gene (Nakata *et al.*, 1992).

Temperature and virulence regulation of *Shigella flexneri*

It is advantageous for pathogens to exploit their environmental conditions and reduce synthesis of genes that are nonessential in the niche they currently occupy. For *Shigella* spp., expression of virulence genes is repressed at 30°C but not at 37°C (Maurelli *et al.*, 1984). This adaptation allows the bacterium to conserve energy.

Temperature is an efficient cue for virulence regulation. Outside the host, temperatures are usually <37°C. When the bacterium is ingested, the increase in temperature results in production of the virulence proteins necessary for survival. *Shigella* also use a change in osmolarity of their environment to signal residence within the colon (Porter and Dorman, 1994). Once the pathogen has reached its desired niche within the gastrointestinal tract, *Shigella* begins transcription of the plasmid-encoded genes for invasion, replication and cell-to-cell spread.

The protein responsible for the regulation of virulence gene expression is H-NS, which is encoded by the *virR* gene (Maurelli and Sansonetti, 1988). As illustrated in Figure 3, H-NS mediates the transcription of the virulence regulon by repressing transcription of *virB*, the positive transcriptional activator of the plasmid virulence regulon at 30°C (Hromockyj *et al.*, 1992). VirF acts antagonistically to

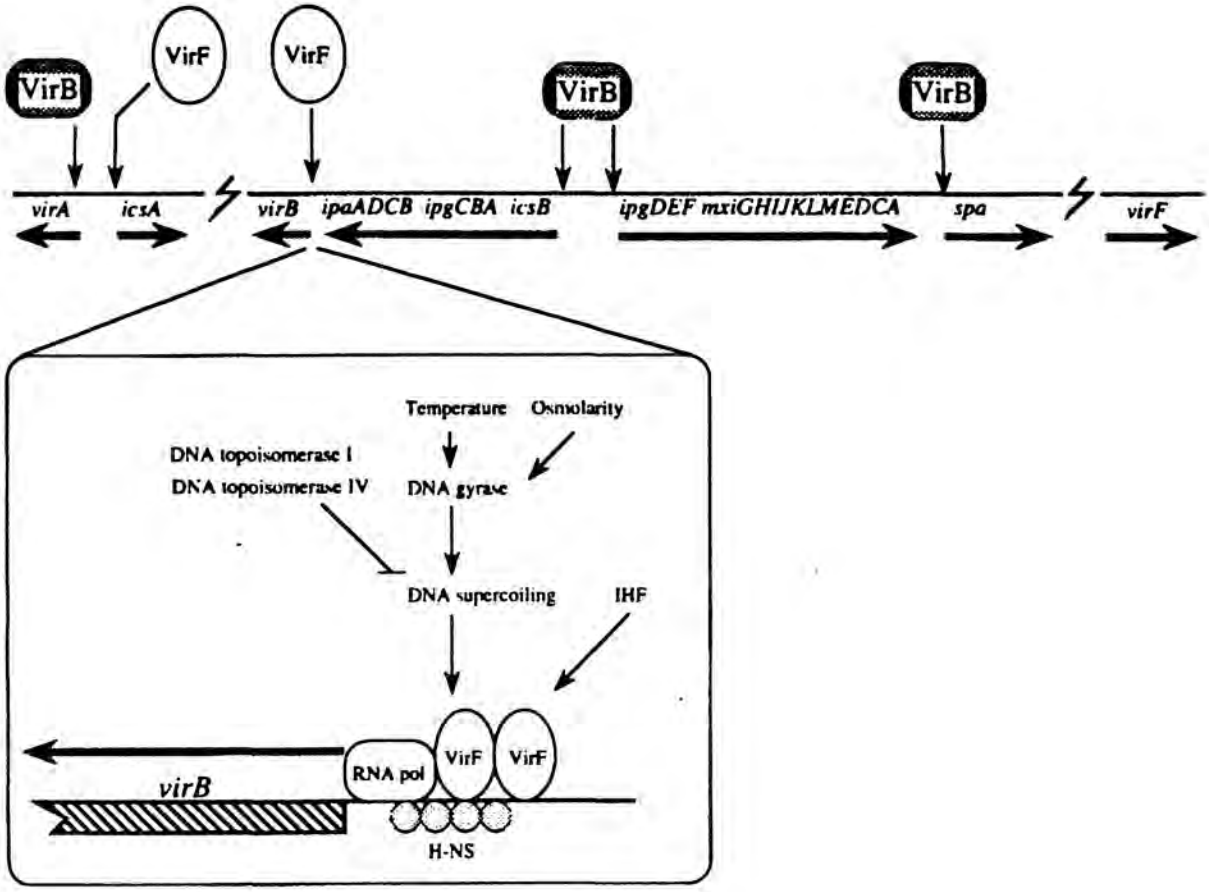


Figure 3. Summary of the key features of the *S. flexneri* virulence gene regulatory cascade. The upper portion of the figure summarizes the chief components of the virulence gene regulon located within a 31 kb region on the 230 kb plasmid. The sites at which positive transcriptional regulatory inputs are made by the VirF and VirB proteins are shown by vertical arrows. The regulatory region of *virB* is shown in enlargement at the lower left. At the *virB* promoter, the overlapping binding sites of the positively acting VirF protein and the negatively acting H-NS protein are shown. The positive inputs of IHF and DNA supercoiling are indicated by arrows, with supercoiling being promoted by DNA gyrase (probably under environmental control) and antagonized by the DNA-relaxing enzymes topoisomerases I and IV. Diagrams not drawn to scale. (Adapted from Dorman and Porter, 1998)

H-NS (Dorman *et al.*, 1990; Hale, 1991; Higgins *et al.*, 1990b). VirF is responsible for activating transcription of *virB* at 37°C (Porter and Dorman, 1994). The interactions between H-NS, VirF, and the *virB* promoter will be discussed in more detail below. H-NS is also a global regulatory protein (Falconi *et al.*, 1993; Ueguchi and Mizuno, 1993; Yoshida *et al.*, 1993; Laurent-Winter *et al.*, 1997) and a cold-shock protein (Dersch *et al.*, 1994). The implications for H-NS's diverse role in regulation of bacterial function will also be discussed.

CHARACTERIZATION OF *virR*(*hns*) and H-NS

Before the discussion of the regulatory effects of H-NS, an introduction is necessary to define the properties of *hns* and its conservation within the *Enterobacteriaceae* family. Therefore, the following sections have been divided into: Characterization of *Shigella flexneri virR*(*hns*), Comparison of *virR*/H-NS within *Enterobacteriaceae*, and Characterization of H-NS of *Escherichia coli*. It is the intent of the author to sequentially present the information known about the gene and then its protein product. Since H-NS has been predominantly studied in *E. coli*, the majority of the data presented is from *E. coli*.

a. Characterization of *Shigella flexneri virR(hns)*

The product of the *virR* gene, required for temperature regulation in *S. flexneri* (Maurelli and Sansonetti, 1988), encodes the 137 amino acid nucleoid protein, H-NS (Falconi *et al.*, 1988). The gene is located at 27 minutes on the chromosome between the *galU* and *trp* genes (Maurelli and Sansonetti, 1988; Hromockyj *et al.*, 1992) and transcribed in the opposite orientation of the aforementioned genes (Pon *et al.*, 1988). It is composed of a 411 base pair open reading frame (ORF; Hromockyj *et al.*, 1992). The promoter is preceded by a sequence of curved DNA that is believed to play a role in autoregulation (Falconi *et al.*, 1993; Dersch *et al.*, 1993). Autoregulation and the significance of curved DNA will be discussed in more detail in a subsequent section. There is a weak Shine-Dalgarno sequence preceding the coding region of the gene with the initiation codon being AUG and the termination codon being UAA (Pon *et al.*, 1988). A Rho-independent transcription termination site is located 29 bases away from the translational termination site which is composed of a stem-loop structure, followed by a row of 6 Ts (Pon *et al.*, 1988). *virR* is, therefore, transcribed as a monocistronic unit. Because *virR* is allelic to the regulatory locus, *hns* in *E. coli*, this gene has been named *hns* (Hromockyj *et al.*, 1992).

b. Comparison of *hns* and H-NS homologues within *Enterobacteriaceae*

The clinically significant members of the *Enterobacteriaceae* include two major groups: 1) the indigenous members of the GI tract, *Escherichia*, *Klebsiella*, *Proteus*, and *Enterobacter*; and 2) the pathogens of the gastrointestinal tract (GI) tract, *Salmonella*, *Shigella*, and *Yersinia*. *Serratia* is another member of the *Enterobacteriaceae* family and is known to cause opportunistic infections. Mutations in other regulatory loci of *Escherichia coli* K-12 (*drdX*, *bglY*, *osmZ* and *pilG*) and *Salmonella typhimurium* (*osmZ*) have been identified as alleles of *hns* and also map to 27 minutes on the *E. coli* chromosome and 34 minutes on the *S. typhimurium* chromosome (Hulton *et al.*, 1990; Goransson *et al.*, 1990; May *et al.*, 1990; Kawula and Orndorff, 1991; Pon *et al.*, 1988). This discovery led to comparisons of *hns* alleles within *Enterobacteriaceae*, and the results showed that H-NS structure is conserved in many bacteria. In *E. coli*, *Proteus vulgaris*, *S. typhimurium*, *Serratia marcescens* and *S. flexneri*, there is a high degree of homology both at the nucleotide and amino acid level (Goransson *et al.*, 1990; Hromockyj *et al.*, 1992; Hulton *et al.*, 1990; La Teana *et al.*, 1989; Marsh and Hillyard, 1990). The DNA homology is restricted to the coding regions (the structural gene and its

transcriptional and translational signals), but little homology occurs in the flanking regions. Figure 4 shows the amino acid alignment of several of these homologues. The most conserved residues are the basic amino acids and all the aromatic residues (La Teana *et al.*, 1989). Therefore, *hns* must be an important gene- though not necessarily essential gene- since it is highly conserved within this family. For the remainder of this thesis, the gene will be referred to as *hns* and the translated product, H-NS.

c. Characterization of H-NS of *Escherichia coli*

H-NS was first identified in the early 1970's (Jaquet *et al.*, 1971) from *E. coli*. It is a neutral protein, with an isoelectric point (pI) of ~7.5 (Spassky *et al.*, 1984; Gualerzi *et al.*, 1986) and a molecular mass of 15.6 kDa (Varshavsky *et al.*, 1977; Spassky *et al.*, 1984). Three isoforms of H-NS have been identified that differ by their pI, the pH at which the protein is immobile in an electric field (Spassky *et al.*, 1984). The isoforms are found in about equal concentration within the bacterium. H-NS is a major component of the bacterial chromatin and it appears to be involved in its organization (Varshavsky *et al.*, 1977). There are approximately 20,000 copies present per cell (Spassky *et al.*, 1984; Lammi *et al.*, 1984), which exist as homodimers

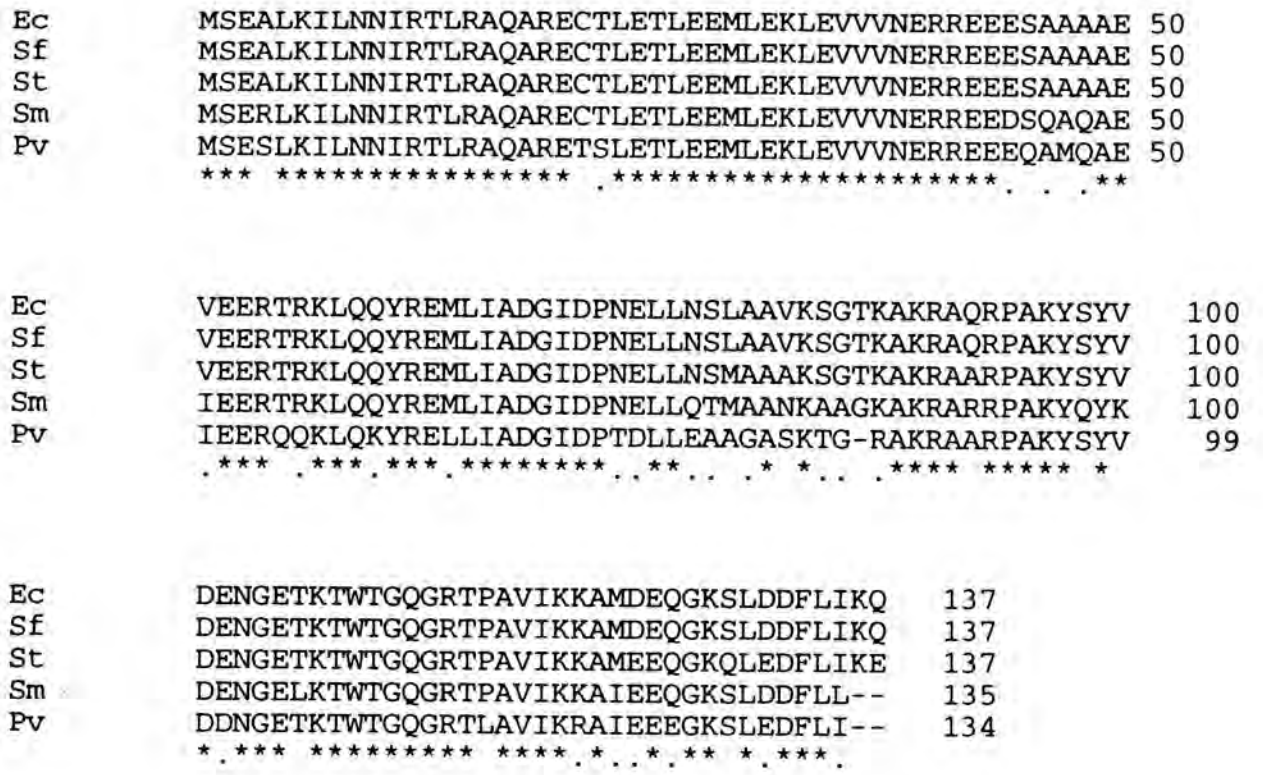
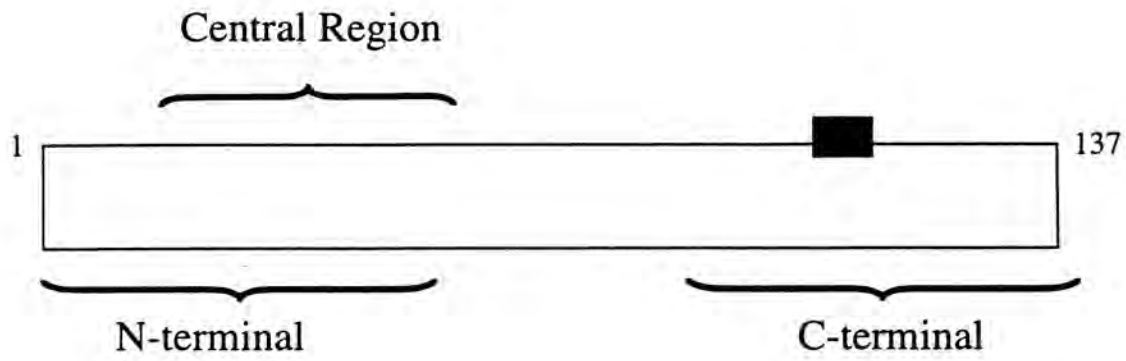


Figure 4. Comparison of the amino acid sequences of H-NS from members of the *Enterobacteriaceae* family. Ec-*Escherichia coli*, Sf-*Shigella flexneri*, St-*Salmonella typhimurium*, Sm-*Serratia marcescens*, and Pv-*Proteus vulgaris*. Comparison completed by Clustal program in PCgene. Perfectly conserved amino acids are designated by a "*". Amino acids that are considered "similar" are designated by a ".".

or higher oligomers (Falconi *et al.*, 1988; Ueguchi *et al.*, 1996), and are located in the nucleoid (Durrenberger *et al.*, 1991).

H-NS functions as a global regulator of gene expression (reviewed in Williams and Rimsky, 1997). It negatively regulates the transcriptional expression of a diverse group of unlinked genes (Bertin *et al.*, 1990; Yoshida *et al.*, 1993). Numerous studies have assessed and elucidated the mechanism by which H-NS inhibits transcription at 30°C. The protein's ability to interact with DNA and protein which results in the inhibition of transcription, was analyzed predominately in mutant studies (Ueguchi and Mizuno, 1993; Ueguchi *et al.*, 1996; Ueguchi *et al.*, 1997). The results from these experiments concluded that the 137 amino acid sequence of H-NS can be divided into three functional domains as illustrated in Figure 5. The N-terminal domain is responsible for transcriptional repression (Ueguchi *et al.*, 1996; Ueguchi *et al.*, 1997). Results from mutational studies showed regulation of transcription by H-NS is allele specific, having varying degrees of influence on different genes (Ueguchi *et al.*, 1996). The central region, residues 21 to 63, is responsible for oligomer formation (Ueguchi *et al.*, 1997). The formation of dimers and oligomers is essential for transcriptional repression (Ueguchi *et al.*, 1997). The C-terminal domain, residues 91 to 137, is responsible for DNA binding (Tippner and Wagner, 1995; Shindo *et al.*, 1995;



The N-terminal domain from amino acid 1 thru ~60, is responsible for transcriptional repression.

The central region from amino acid 21 thru 63, is responsible for oligomer formation.

The C-terminal domain from amino acid 91 to 137, is responsible for binding to the DNA. The black box superimposed in the C-terminal domain corresponds to the stretch of residues from Trp-108 thru Thr115.

Figure 5. Schematic representation of the H-NS protein divided into hypothesized domains as proposed by previous studies (Ueguchi *et al.*, 1996; Ueguchi *et al.*, 1997)

Ueguchi *et al.*, 1996; Ueguchi *et al.*, 1997). Trp-108 of H-NS is believed to be in close contact with the DNA through hydrophobic contact with the staggered base pairs of the DNA major groove (Tippner and Wagner, 1995). The amino acid stretch from Thr110 to Thr115 is determined to be crucial for DNA binding through mutational analysis (Ueguchi *et al.*, 1996). H-NS/DNA binding will be discussed in relation to the protein's DNA binding affinity in subsequent sections.

THE H-NS PROTEIN OF *Shigella flexneri*: CURRENT STATUS

Current Questions

The number of questions regarding H-NS and its regulatory role seems to increase as we learn more about the biochemistry and molecular biology of this protein. Thus, we know that *hns* is a highly conserved regulatory gene responsible for the temperature regulation of virulence gene expression in *S. flexneri*. H-NS is known to bind curved regions of double stranded DNA and influence transcription of a variety of unlinked genes at the transcriptional level. But explanations as to how this protein performs its regulatory role are only speculative. The next section discusses current

research that has been undertaken to elucidate the role of *hns*. It is the goal of this thesis to shed light on H-NS and its regulatory role. More specifically, this thesis plans to explicate how all the present data, along with the proposed experiments discussed in the research protocol, can be applied to answer the major questions of the role of H-NS in the temperature regulation of virulence in *S. flexneri*: Does H-NS bind to DNA specifically at 30°C and not 37°C? What are the specific amino acids in the protein that are responsible for the binding to DNA? Is it the primary, secondary or tertiary structure of the protein that is responsible for the temperature regulation? Does supercoiling of DNA play a major factor in preventing H-NS from binding at 37°C thereby allowing VirF to bind? Will a tertiary structural change altering the loop formation within H-NS make it more capable of binding supercoiled DNA than VirF?

A considerable amount of research has been done to characterize H-NS. The information below has been divided into sections pertaining to H-NS's ability to bind DNA, its effects on DNA topology, transcriptional regulation of selected genes, and DNA repair.

a. H-NS/DNA binding affinity

The ability of H-NS to bind DNA is the fundamental starting point for analyzing its function within the bacterial cell. As was discussed previously, the C-terminal domain of H-NS is responsible for this protein's ability to bind DNA (Tippner and Wagner, 1995; Shindo *et al.*, 1995; Ueguchi *et al.*, 1996; Ueguchi *et al.*, 1997).

H-NS has also been found to bind preferentially to nonspecific sequences of curved DNA (Bracco *et al.*, 1989; Yamada *et al.*, 1990, 1991; Owen-Hughes *et al.*, 1992; Falconi *et al.*, 1993, Zuber *et al.*, 1994). Curved DNA is characterized by stretches of short homopolymeric dA:dT tracts (Koo *et al.*, 1986). Each tract is approximately half the face of a helical turn and repeated at 10-11 bp intervals (Crothers *et al.*, 1990) Synthetic promoters with nucleotide sequences containing tracts of 5 adenines (A) or 6As were designed to be curved based on the above information. The promoters were fused with a reporter gene, *lacZ* (Zuber *et al.*, 1994). ß-galactosidase expression was repressed *in vivo* because the promoters were inhibited, as H-NS was able to recognize and bind to the curved DNA structure. The three-dimensional structure of the C-terminal domain of H-NS is shown in Figure 6. Mutagenesis studies presented evidence that mutant H-NS proteins, with an internal deletion of 4 amino acids from G112 through P115, corresponding to

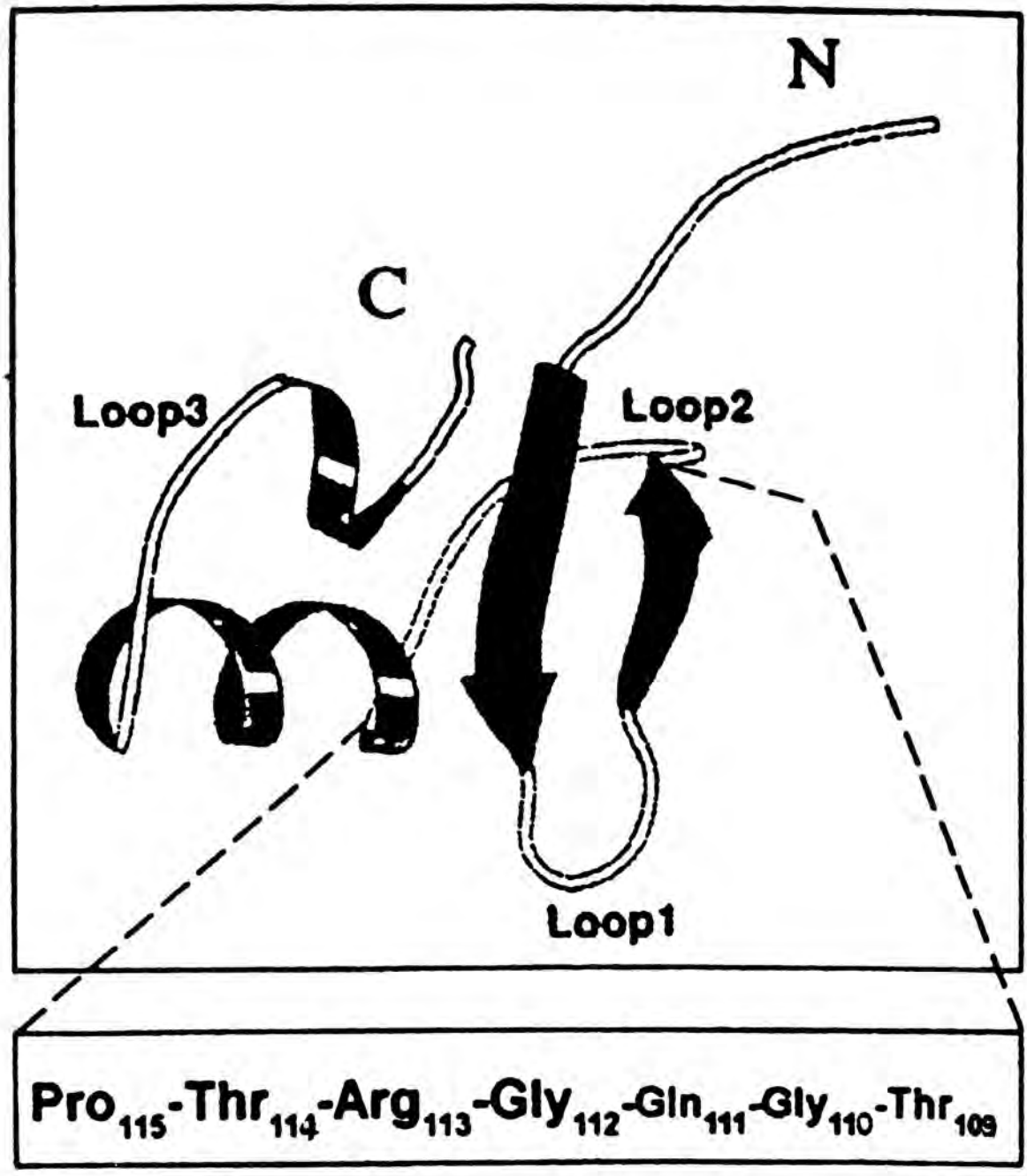


Figure 6. Three dimensional structure of the C-terminal domain (47 residues) of H-NS as elucidated by Shindo *et al.*, (1995) by two-dimensional NMR spectroscopy showing loop 2 which contains the Gly112-Arg113-Thr114-Pro115 tetrapeptide deleted in the H-NSΔ12 mutant (Spurio *et al.*, 1992) and mutagenized as described in Spurio *et al.*, (1997). (Adapted from Spurio *et al.*, 1997)

the distal portion of loop 2, lost the ability to preferentially recognize curved DNA (Spurio *et al.*, 1997). The Trp-108 amino acid within the C-terminal has been postulated to bind in the major grooves of the DNA helix through hydrophobic interactions (Tippner and Wagner, 1995). It is believed that H-NS recognizes the DNA secondary structure, not the primary sequence. Therefore, the exact sequence of the nucleic acids in the DNA molecule is not necessarily important, but the secondary structure is. Many of the promoters regulated by H-NS are believed to have curved DNA associated with them. Accordingly, the next series of experiments should investigate how the deletion mutant affects the expression of these genes.

b. Effect of H-NS on DNA supercoiling

The bacterial chromosome is compacted in a negatively supercoiled nucleoid using the opposing activities of DNA gyrase and DNA topoisomerase I (Drlica *et al.*, 1990). This compaction allows the chromosome to fit within the bacterial membrane in a more energetically favored state. H-NS and other DNA-binding proteins are responsible for constraining about half of the supercoils while the unconstrained supercoils are available for transcription and replication (Bliska and Cozzarelli, 1987). DNA supercoiling changes in

response to environmental signals such as osmolarity, temperature, and oxygen tension (Goldstein and Drlica, 1984, Higgins *et al.*, 1988, 1990; Dorman *et al.*, 1988; Drlica *et al.*, 1990). Supercoiling also varies with growth phase (Dorman *et al.*, 1988). To evaluate changes in the level of supercoiling, changes in the linking number, the number of times one strand of the double helix crosses over the other strand if the DNA molecule is conceptually constrained to lie on a plane (Drlica, 1984), of a reporter plasmid are measured. The linking number fluctuation is indicative of changes in the *in vivo* levels of DNA supercoiling in bacterial cells (Dorman *et al.*, 1988; Higgins *et al.*, 1988; Ni Bhriain *et al.*, 1989; Drlica, 1984).

H-NS binding to DNA results in a change in supercoiling (Dorman *et al.*, 1990; Higgins *et al.*, 1988). H-NS is able to constrain negative supercoils *in vitro* and has a structural role in the organization of the bacterial chromatin. Approximately 6 dimers of H-NS are required to constrain one negative supercoil (Tupper *et al.*, 1994). It is speculated that H-NS can wrap around the DNA at the preferred sites of curved DNA to form a nucleosome-like structure. The resulting structure would be able to constrain the supercoils. This major alteration in the DNA topology could effectively silence a nearby promoter (Tupper *et al.*, 1994).

Osmolarity is an environmental signal used by the bacterium to sense its niche. Variations in osmolarity can induce changes in DNA supercoiling that occur very rapidly and do not require protein synthesis (Park *et al.*, 1989; McCellan *et al.*, 1990). Since no new proteins are made, the amount of H-NS in the cell does not change. Potassium glutamate is accumulated within an eukaryotic cell in response to an increase in extracellular osmolarity (Epstein and Schultz, 1965; Sutherland *et al.*, 1986) It has been found that changes in the concentration of potassium glutamate influence the ability of H-NS to constrain DNA supercoils *in vitro* (Tupper *et al.*, 1994). Therefore, the effect of osmolarity on H-NS binding and DNA topology may be due to an alteration in the activity of H-NS, not its concentration (Hulton *et al.*, 1990). Also, increases in supercoiling in *E. coli* are independent of transcription or changes in the activity of topoisomerase I (Dorman *et al.*, 1989; Lejuene and Danchin, 1990). Recently, using *hns*::Tn*10* insertion mutants, an increase in plasmid and chromosomal DNA supercoiling in *E. coli* was demonstrated *in vivo* using the DNA cross-linking reagent trimethylpsoralen (Monjica and Higgins, 1997). The trimethylpsoralen penetrates into the cell and intercalates the DNA in a supercoiling-dependent fashion. When irradiated with long-wavelength UV light, covalent cross-links form.

The *hns* mutant strain had a greater net level of unconstrained negative supercoiling than the wild-type *E. coli*.

There are 20,000 copies of H-NS in the nucleoid and 10,000 negative superhelices on the chromosome (Worcel and Burgi, 1972). Six dimers are required to constrain one supercoil and constrained supercoils result in a change in DNA topology. H-NS's ability to constrain supercoils appears to supports the hypothesis that H-NS influences the transcription of various genes via changes in DNA topology (Tupper *et al.*, 1994; Dorman *et al.*, 1990; Higgins *et al.*, 1988).

c. H-NS effects on transcriptional regulation and autoregulation

It has been demonstrated that H-NS can bind to DNA, usually at nonspecific curved sequences, and this binding affects supercoiling (Dorman *et al.*, 1990; Higgins *et al.*, 1988). The combination of these effects has been studied with regard to regulation of transcription. H-NS has been shown to negatively regulate the transcription of a diverse group of unlinked genes, including regulation of itself (Bertin *et al.*, 1990; Yamada *et al.*, 1991; Yoshida *et al.*, 1993; Falconi *et al.*, 1993; Dersch *et al.*, 1993; Ueguchi *et al.*, 1993; Free and Dorman, 1995). Transcriptional regulation has been studied using operon

fusions to many genes. However, the most detailed studies are fusions to *hns*. Accordingly, the studies below describe transcriptional regulation of *hns* by H-NS.

Transcriptional regulation has been studied by fusing a reporter gene with a promoter suspected to be regulated by H-NS. Comparisons between wild-type *hns* and mutant *hns* have resulted in data, discussed below, which support the hypothesis that H-NS may bind either upstream or downstream of a promoter and inhibit transcription. In one study, a promoterless *cat* gene was fused to a 400 bp DNA fragment containing the upstream region and part of the coding region of the *E. coli* gene, *hns*. The *cat* gene was significantly repressed in *E. coli* and *S. typhimurium* strains containing the wild-type *hns* allele, but not mutant *hns* alleles (Falconi *et al.*, 1993).

The structure of the *hns* promoter region appears to display features of curved DNA as indicated by its migration on 2-dimensional polyacrylamide gels run at different temperatures (Dersch *et al.*, 1993). Since *hns* transcription was repressed in the wild-type strain, but not the mutant *hns* strain (Falconi *et al.*, 1993), and H-NS can bind to curved DNA (Dersch *et al.*, 1993), this result implied that the protein was binding directly to the *hns* promoter. Gel shift experiments indicated that wild-type H-NS was able to bind

to the DNA fragment containing the *hns* promoter region while the mutant H-NS was unable to bind (Falconi *et al.*, 1993).

DN*ase*1 footprinting studies have suggested that H-NS binds cooperatively to DNA (all or none fashion; Falconi *et al.*, 1993; Rimsky and Spassky, 1990). A critical concentration of H-NS must be reached before it can protect an area of DNA from digestion. From these results, it was concluded that H-NS protects at least 3 DNA segments upstream of the *hns* promoter (Falconi *et al.*, 1993). The initial site bound is located -150 bp upstream of the transcriptional start site, followed by yet another site further upstream. Once these sites are occupied, an additional site between -20 bp and -65 bp upstream of the transcriptional start site is bound. Binding to this last site is believed to be the ultimate cause of transcriptional repression (Falconi *et al.*, 1993), because this site overlaps the promoter. Binding of H-NS to this site could directly preclude access of the RNA polymerase to the transcriptional start site.

Additional promoter fusions have been constructed to confirm the transcriptional regulation of H-NS. A set of experiments conducted with *hns-lacZ* protein and operon fusions integrated at the *attB* locus of *E. coli* K-12 demonstrated negative autoregulation of *hns* at the level of transcription (Dersch *et al.*, 1993). Another group of experiments using transcriptional fusions integrated at the *attλ*

locus of *E. coli* also demonstrated negative autoregulation, at least during the logarithmic growth phase (Ueguchi *et al.*, 1993). The repression was confirmed in experiments that compared the growth of functional *hns* strains to *hns* deletion strains and *hns* plasmid-complemented strains (Ueguchi *et al.*, 1993). These experiments involving the promoter region of *hns* demonstrated that the promoter structure is required for transcriptional autoregulation by H-NS.

d. Growth phase autoregulation of H-NS

Because H-NS is able to autoregulate, it was believed that the concentration of H-NS must change in order to regulate changes in transcription (Falconi *et al.*, 1993; Dersch *et al.*, 1993; Ueguchi *et al.*, 1993; Free and Dorman, 1995). Debate has occurred over whether this regulation is growth phase-dependent (Table 2). The first report of growth phase variation was documented by Spassky and collegues in 1984. They measured the concentration of cellular extracts and found that one isoform of H-NS (H1a) increased dramatically from exponential phase to late stationary phase. They believed, therefore, that the amount of H-NS present in the cell was dependent on the growth phase.

Table 2. Growth Phase Autoregulation of H-NS

Induction in stationary phase	Methods	References
yes, of H1a isoform	two-dimensional gel analysis of cellular extracts	Spassky *et al.*, 1984
no	Immunoblot	Tanaka *et al.*, 1991
no	*hns-luxAB* plasmid transcriptional fusion Immunoblot	Hinton *et al.*, 1992
no	Immunoblot	Yawuzawa *et al.*, 1992
yes	*hns-lacZ* fusion at *attB* site ß-galactosidase assays Immunoblot	Dersch *et al.*, 1993
yes	*hns-lacZ* transcriptional fusion at *attλ* site ß-galactosidase assays Immunoblot	Ueguchi *et al.*, 1993
no	DNA synthesis blocking Immunoblots repeat of Dersch *et al.*, 1993 fusion assay experiments coupled with DNA synthesis blocking experiments	Free & Dorman, 1995

Transcription of *hns* is positively regulated by the Fis protein. The Fis protein counteracts H-NS inhibition in early-log phase growth and has an overlapping DNA target site for binding (Falconi *et al.*, 1993). Since the concentration of Fis declines after logarithmic phase growth, it could be postulated that *hns* transcription would be repressed since the positive regulator is gone. However, experiments have yielded contradictory results. Experiments using *hns-lacZ* fusions integrated into *attB* (Dersch *et al.*, 1993; Ueguchi *et al.*, 1993) demonstrated an increase in H-NS in the stationary phase. Dersch *et al.*, (1993), found a ten-fold increase in this protein while Ueguchi *et al.*, (1993), detected a three-fold increase. But, the fusion construct was inserted in a site not normally regulated by H-NS and could have been affected by the local topology. Also, the resultant product was not H-NS but ß-galactosidase, so no regulatory product was made. Therefore, the gene fusion could continue to be transcribed and translated, resulting in the detected increase in ß-galactosidase. In contrast, other researchers found no induction of *hns* transcription in stationary phase (Hinton *et al.*, 1992; Yasuzawa *et al.*, 1992, Tanaka *et al.*, 1991; Free and Dorman, 1995). Hinton *et al.*, (1992) found no increase in H-NS in *S. typhimurium* with a *hns-luxAB* transcriptional fusion on a plasmid. Also, immunoblot analysis of H-NS in *E. coli*

yielded results demonstrating no stationary phase induction (Yasuzawa *et al.*, 1992; Free and Dorman, 1995).

The most convincing evidence for relatively constant transcription of *hns* comes from experiments designed to study *hns* transcription during periods of blocked DNA synthesis. When DNA synthesis was blocked, transcription of *hns* was also down-regulated (Free and Dorman, 1995). This result is not surprising because if *hns* were continuously transcribed and translated, the protein would be able to bind to all of the available sites on the DNA thus resulting in excessive compaction and cell death. Excessive H-NS is deleterious to the cell (Spurio *et al.*, 1992). Repression of *hns* transcription was relieved when DNA synthesis resumed (Free and Dorman, 1995). When the synthesis of DNA proceeded, there was an increase in unbound DNA. Therefore, autoregulation serves to keep the relative amounts of DNA and H-NS constant and to match *de novo* H-NS synthesis to the demands of DNA synthesis and maintain a relatively constant H-NS:DNA ratio (Free and Dorman, 1995).

e. Effects of H-NS on *Shigella* temperature regulated virulence genes

Mutations in *hns* have pleiotropic effects on gene expression (Reviewed in Williams and Rimsky, 1997). Indeed, H-NS is

autoregulated and it is a regulator of approximately 30 unlinked genes (Falconi *et al.*, 1993; Dersch *et al.*, 1993; Ueguchi *et al.*, 1993; Free and Dorman, 1995). Many of these genes appear to be transcribed in response to some type of stress. Two hypothetical models have been postulated to explain the global effect of H-NS on transcription: transcriptional silencing (Goransson *et al.*, 1990), and repression via changes in DNA topology (Higgins *et al.*, 1988). In both models, H-NS binds in the vicinity of the target promoter and forms a nucleoprotein complex. However, in the first model, silencing is believed to occur by directly occluding the RNA polymerase. In the second model, H-NS significantly alters the topology of the DNA and the RNA polymerase is incapable of functionally interacting with the promoter. The two models are not mutually exclusive and do not apply only in *Shigella*.

Regulation of virulence in *S. flexneri* is a complex, multicomponent process. Environmental signals such as temperature and osmolarity are cues that modify the expression of various genes, and an array of cellular responses occur as a result of this expression.

S. flexneri are viable and free-living in the environment. When the organisms are ingested by a primate host, the bacteria can sense the increase in temperature. The bacteria begin to transcribe virulence genes necessary for colonization of the desired site in the

GI tract. The regulation of virulence gene expression is tiered. At 30°C, expression of the virulence regulon is repressed as a result of H-NS (Hromockyj *et al.*, 1992; Maurelli and Sansonetti, 1988) binding to the *virB* promoter and inhibiting transcription (Tobe *et al.*, 1993). At 37°C, repression by H-NS is lifted and *virB* is positively regulated by binding of VirF. In the absence of VirF at 37°C, no activation of transcription from the *virB* promoter occurs (Porter and Dorman, 1997; Tobe *et al.*, 1993). VirF also regulates the transcription of *icsA* (Tobe *et al.*, 1993). The interaction between H-NS and VirF appears to be antagonistic at the *virB* promoter with nearly overlapping binding sites. The transcriptional start point of *virB* is 54 bp upstream of the translational initiation codon. VirF binds from -17 bp to -117 bp upstream and H-NS binds upstream from -20 bp to +20 bp (Tobe *et al.*, 1993). The proposed model of H-NS, VirF and *virB* promoter interaction is depicted in Figure 7.

Mutations in *hns* that inactivate the protein abolish the thermoregulation and allow *virB* to be transcribed at 30°C (Tobe *et al.*, 1993; Hromockyj *et al.*, 1992; Maurelli and Sansonetti, 1988). In contrast, an increase in the concentration of H-NS at 37°C mimics the effects of reduced temperature and downregulates transcription of *virB* in *E. coli* (Dagberg and Uhlin, 1992). H-NS has been shown to

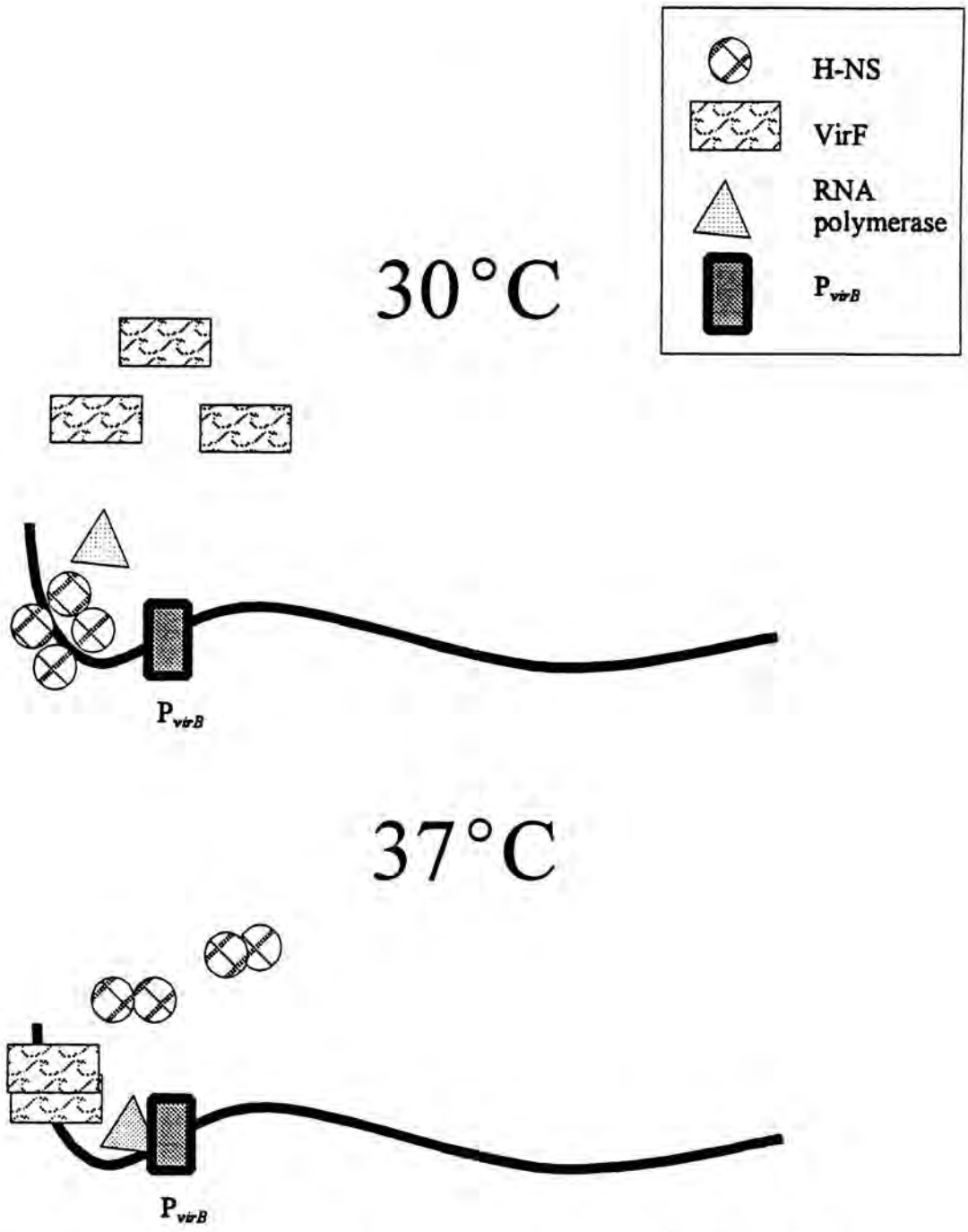


Figure 7. Proposed mechanism of molecular interaction of H-NS, VirF and RNA polymerase at the *virB* promoter. At 30°C, H-NS is predicted to bind the curved region of DNA upstream of the *virB* promoter, blocking access of VirF and the RNA polymerase. Due to increased DNA supercoiling at 37°C, VirF binds upstream of the promoter, excluding H-NS from its binding site and allowing RNA polymerase to bind and initiate transcription.

affect the topology of DNA by constraining supercoils (Monjica and Higgins, 1997; Tupper *et al.*, 1994; Dorman *et al.*, 1990; Lejuene and Danchin 1990; Higgins *et al.*, 1988). It is hypothesized that VirF is able to bind upstream of the *virB* promoter at 37°C, because the promoter takes on a different conformation than it had at 30°C. The topology change may not inhibit H-NS binding but it does promote VirF binding. *In vitro* transcription assay results, using VirF and a supercoiled plasmid containing *virB*, showed activation of *virB* transcription (Tobe *et al.*, 1993). However, when the plasmid superhelicity was altered either by topoisomerase I or linearized by a restriction enzyme digest, the activation of *virB* transcription was reduced (Tobe *et al.*, 1993). Also, *in vivo* studies showed that *S. flexneri* grown at 37°C in the presence of novobiocin, a gyrase inhibitor, had *virB* transcription levels similar to the wild-type grown at 30°C (Tobe *et al.*, 1993). *virB* activation was seen only when negatively supercoiled DNA was used as a template (Tobe *et al.*, 1993). Therefore, if there is an increase in the concentration of a DNA-binding protein, like H-NS, that binds to the DNA and confers changes on the structure of the promoters, the topology may be altered enough that another protein cannot bind. Alternatively, the change in structure of the promoter due to the temperature change to 37°C may facilitate VirF binding. VirF binding would allow

initiation of transcription by RNA polymerase which can now access the *virB* promoter, rather than allowing H-NS to bind and block the RNA polymerase and, therefore, transcription (Tobe *et al.*, 1993). In this situation, H-NS would no longer be bound to the DNA.

It is known that H-NS is not a factor at the virulence gene promoters since excess VirB can still turn on the virulence genes (Dagberg and Uhlin, 1992). Also, changes in H-NS concentration do not affect transcription from the invasion gene promoters themselves (Dagberg and Uhlin, 1992). H-NS must exert its regulatory effect locally on the *virB* promoter while downstream regulation of the virulence genes is regulated by VirB.

Shigella spp. sense changes in osmolarity and respond by altering DNA supercoiling (Higgins *et al.*, 1988, 1990a; Dorman *et al.*, 1988; Drlica *et al.*, 1990). Regulation of virulence gene expression by detection of two environmental signals, like temperature and osmolarity, is a more efficient method of regulation than reaction to one signal. *S. flexneri* virulence gene transcription is regulated by both temperature and osmolarity (Porter and Dorman, 1994), and both signals are needed for full activation of its virulence genes reducing the likelihood of an inappropriate response (Porter and Dorman, 1994). In the case of *S. flexneri*, H-NS actively inhibits transcription of *virB* at 30°C and at low osmolarity even though VirF

is present in the cell. At 37°C and increased osmolarity, VirF positively regulates transcription of *virB* even though the same concentration of H-NS is present in the cell as was present at 30°C and low osmolarity. Transcriptional regulation is, therefore, a combination of the two models, transcriptional silencing and changes in DNA topology. Changes in DNA topology result from environmental signals as well as from the ability of various proteins to bind. When the DNA binding proteins are bound, they can alter the topology of the DNA further. As the structure of the DNA changes, proteins which normally are bound may not be able to recognize their binding site, and as a consequence these proteins will not be able to regulate. Transcriptional silencing occurs because RNA polymerase is not able to initiate.

f. Effect of H-NS on other bacterial proteins

Initial research on H-NS as a global regulator of gene transcription found that a diverse group of unlinked genes was affected; examples are given in Table 3. Research on global regulation has involved comparing subsets of proteins produced in either a *hns* mutant strain or a wild-type strain (Laurent-Winter *et al.*, 1997; Yoshida *et al.*, 1993). The proteins were electrophoresed

Table 3. Examples of H-NS regulated genes in *Enterobacteriaceae*

Gene	Regulation	Function	Reference
hns	autoregulated cold-shock	global regulator chromatin structure	Dersch *et al.*, 1993; Falconi *et al.*, 1993; La Teana *et al.*, 1991
virB	temperature	activator of virulence genes	Tobe *et al.*, 1993; Dorman *et al.*, 1990; Maurelli & Sansonetti, 1988
proU	osmolarity	glycine betaine transport	May *et al.*, 1986; Higgins *et al.*, 1988; Barr *et al.*, 1992; Ueguchi & Mizuno, 1993; Lucht *et al.*, 1994; Jordi *et al.*, 1997
fimA	temperature	type 1 fimbriae	Higgins *et al.*, 1988; Kawula & Orndorff, 1991; Dorman & Ni Bhriain, 1992b
fimB *fimE*	temperature	type 1 fimbrial recombinases	Higgins *et al.*, 1988; Kawula & Orndorff, 1991; Olsen *et al.*, 1998
pap	temperature	pili	Goransson *et al.*, 1990; Forsman *et al.*, 1992; White-Ziegler *et al.*, 1998
ompC *ompF*	osmolarity/ temperature	outer membrane porins	Graeme-Cook *et al.*, 1989; Dorman & Ni Bhriain, 1992a
lux	unknown	bioluminescence	Ulitzur *et al.*, 1997
cfa	temperature	fimbriae	Jordi *et al.*, 1992; Smyth & Smith, 1992

csgA	temperature/ osmolarity	curli subunit	Olsen *et al.*, 1989, 1993; Arnqvist *et al.*, 1994
bgl	unknown	ß-glucosidase	Higgins *et al.*, 1988; Kawula & Lelivelt, 1994
osmC	osmolarity	periplasmic protein	Bouvier *et al.*, 1998
osmY	osmolarity	periplasmic protein	Barth *et al.*, 1995
bolA	stationary phase	morphogene	Barth *et al.*, 1995
otsA	osmolarity/ stationary phase	trehalose synthesis	Barth *et al.*, 1995
rrnB	growth rate	ribosomal RNA	Tippner *et al.*, 1994
stpA	unknown	RNA chaperone	Zhang *et al.*, 1996; Sonden & Uhlin, 1996
cadA	pH	lysine decarboxylase	Shi *et al.*, 1993
adi	pH	arginine decarboxylae	Shi *et al.*, 1993; Shi & Bennett, 1994
neo	unknown	kanamycin resistance	Bertin *et al.*, 1992
10K-L	unknown	unknown	Yoshida *et al.*, 1993
10K-S	unknown	unknown	Yoshida *et al.*, 1993
orf II/III	stationary phase	unknown	Yoshida *et al.*, 1993
hde	stationary phase	acid resistance	Yoshida *et al.*, 1993

and stained. From the synthesis of the additional proteins found in the *hns* mutant strain, it was concluded that expression of these proteins was directly or indirectly regulated by H-NS (Laurent-Winter *et al.*, 1997). Additional characterization has been done on many of the proteins believed to be directly regulated by H-NS, as well as, experiments analyzing how H-NS regulates these proteins. This section is an elaboration of some of the examples in Table 3.

The *proU* operon in *E. coli* is stimulated in response to hyperosmotic stress (May *et al.*, 1986). The operon encodes a high affinity glycine-betaine transport system activated by high osmolarity. The activation of the *proU* operon correlates with increases in the DNA supercoiling produced in response to osmotic stress (Higgins *et al.*, 1988). The linking number of the operon, when carried on a plasmid, is altered (Dattananda *et al.*, 1991). *In vitro* studies showed that H-NS selectively inhibits transcription of the *proU* operon by binding downstream of the *proU* promoter region (Lucht *et al.*, 1994). Other studies showed that inhibition of the early step(s) of transcription initiation, namely open and/or closed complex formation were affected (Ueguchi and Mizuno, 1993). The *proU* operon is derepresssed in an *hns* mutant strain, but the osmotic control is not abolished (Lucht *et al.*, 1994).

Another protein synthesized in *E. coli* in response to an increase in temperature from 30°C to 37°C, is the type 1 fimbrial protein, FimA. Type 1 fimbriae undergo phase variation by site-specific recombination of a 314 bp invertible segment with 9 bp inverted repeat ends. The recombinases required for recombination are *fimB* (on/off orientation) and *fimE* (off orientation; Higgins *et al.*, 1988; Kawula and Orndorff, 1991).

H-NS is involved in *fimA* site-specific recombination (Higgins, *et al.*, 1988; Kawula and Orndorff, 1991) and reduces the inversion rate (Kawula and Orndorff, 1991). H-NS also regulates transcription negatively from the *fimA* promoter (Dorman and Ni Bhriain, 1992b) and mutations in *hns* result in a 100-fold accelerated rate of switching (Kawula and Orndorff, 1991; Spears *et al.*, 1986). Mutations also result in a five-fold increase in *fimB* expression since H-NS normally binds cooperatively to the *fimB* promoter (Donato *et al.*, 1997). Thus, when H-NS is bound to the *fimB* promoter at 30°C, the protein reduces the transcription of *fimB*. Decreased amounts of FimB recombinase favors the off orientation of *fimA* since FimE is the predominant recombinase.

The major regulatory role for H-NS in the type 1 fimbrial system is its ability to modulate *fimB* and *fimE* expression in response to temperature (Olsen *et al.*, 1998). Although fimbriae are

made at 30°C, the regulation favors the fimbriated state in the mammalian host (Olsen *et al.*, 1998), since fimbriae are responsible for adherence of the bacteria to various D-mannose-containing biomolecules on host cell surfaces. Fimbriae are of principal importance in establishing a successful colonization of mammalian host tissues. In terms of virulence, the pathogen is better equipped to establish itself and cause an infection in the fimbriated state.

Pap pili in *E. coli* are encoded by the *pap* operon and are used to facilitate attachment to uroepithelial cells (Roberts *et al.*, 1989). The operon encodes approximately 11 genes and is subject to phase variation at 37°C. At temperatures below 26°C, Pap pili expression is repressed (Goransson and Uhlin, 1984), and Pap transcription is negatively regulated by H-NS (reviewed by Smyth and Smith, 1992). H-NS binds to the monocistronic *papI* operon (transcriptional regulator) and the polycistronic *papB* operon (White-Ziegler *et al.*, 1998). H-NS regulates expression of these operons in a temperature-dependent fashion. *In vivo* experiments showed that H-NS blocks DNA methylation of the *pap* regulatory region at 23°C, but not at the mammalian body temperature of 37°C apparently by binding to the DNA and forming a nucleoprotein complex (White-Ziegler *et al.*, 1998). This complex represses *pap* gene expression. At

37°C, H-NS does not seem to be bound, allowing these sites to be methylated. Methylation appears to be essential for the activation of the *papBAp* transcription by the global regulator Lrp. This temperature regulation is significant because ingested bacteria are able to sense the increase in temperature, relieving the repression of the *pap* operon by H-NS. This allows the bacteria to make pili, attach to epithelial cells, and establish an infection.

Porins OmpC and OmpF are in the membranes of *E. coli* and *Salmonella typhimurium* (Pratt *et al.*, 1996). The ratio of the two porins changes with response to the osmotic environment. When the osmolarity of the environment is low, OmpF dominates because its pore formation has a larger diameter and allows the bacterium to scavenge nutrients (Pratt *et al.*, 1996). When the bacterium is inside the GI tract and exposed to high osmolarity, OmpC is the dominant porin. The smaller diameter pore excludes compounds greater than 200 Da and, therefore, can keep out detergent molecules like bile salts (Pratt *et al.*, 1996). Both porins undergo transcriptional and post-transcriptional regulation (reviewed in Dorman and Ni Bhriain, 1992a) and are sensitive to changes in DNA supercoiling (Graeme-Cook *et al.*, 1989; Huang *et al.*, 1990; Tsui *et al.*, 1988). As previously mentioned, H-NS activity is modulated in response to changes in osmolarity. *hns* mutants increase expression of OmpC and decrease

expression of OmpF (Graeme-Cook *et al.*, 1989). This result is consistent with what occurs when the osmolarity around the bacteria is increased, as in the human intestinal tract. The bacteria will synthesize smaller porins of OmpC to protect their internal environment. Therefore, an *hns* mutant mimics the response to an increase in osmolarity by altering the ratio of OmpC and OmpF.

H-NS is able to repress genes transcribed in *E. coli* from other genera, such as *Vibrio*, as can be demonstrated when the *lux* operon was transformed into *E. coli*. *hns* mutant strains resulted in emitted light, while transformed wild-type strains did not (Ulitzur *et al.*, 1997). Further characterization of the *lux* operon showed DNA regions with homopolymeric stretches of poly d(A) and poly d(T) (Ulitzur *et al.*, 1997). Highly rich A-T sequences are features of curved DNA. H-NS preferentially binds to curved DNA as was demonstrated using synthetic promoters (Zuber *et al.*, 1994). The probable model of transcriptional regulation is a change in DNA topology due to the binding of the H-NS to the curved sequences. The binding of H-NS to the promoter region could possibly inhibit RNA polymerase from interacting with DNA and result in the "transcriptional silencing".

g. Effect of H-NS on DNA repair

Most recently, H-NS has been implicated in the down-regulation of DNA repair in *Shigella* (Palchaudhuri *et al.*, 1998). The results showed that H-NS suppresses DNA repair caused by UV irradiation at 30°C. In addition, suppression of repair was not found in cultures incubated at 37°C after irradiation. The investigators believed that H-NS was responsible for the regulation of repair because *hns* mutant strains grown at 30°C and 40°C consistently showed higher survival rates than wild-type strains grown at 30°C, 37°C and 40°C. Results from similar experiments did not demonstrate regulation of repair by H-NS in *E. coli* lab strains because such lab strains may have been subjected to undocumented irradiation previously. However, H-NS and temperature have opposite effects on the *E. coli* chromosome and the *Shigella* and *Salmonella* chromosomes. While the *hns* mutants cause relaxation of plasmid DNA in *Shigella* and *Salmonella* (Dorman *et al.*, 1990), *hns* mutants in *E. coli* showed increased negative supercoiling of DNA (Higgins *et al.*, 1988). The difference in supercoiling, and the access to the chromosome by repair proteins, could also result in the observed differences.

DISCUSSION

Current status implications

It has long been known that H-NS regulates virulence gene expression in *Shigella*. It is believed that regulation occurs by the direct binding of the H-NS protein to the *virB* promoter and possible alterations in the local topology of the DNA. However, new studies are elucidating H-NS's role within the bacterial cell. The protein has been shown to block methylation of the *pap* operon of *E. coli* at 23°C, but not at 37°C. Also, it has been shown to suppress DNA repair at 30°C, but not 37°C. Both these systems show thermoregulation similar to the control of virulence gene expression. H-NS binds at temperatures lower than 37°C and inhibits transcription.

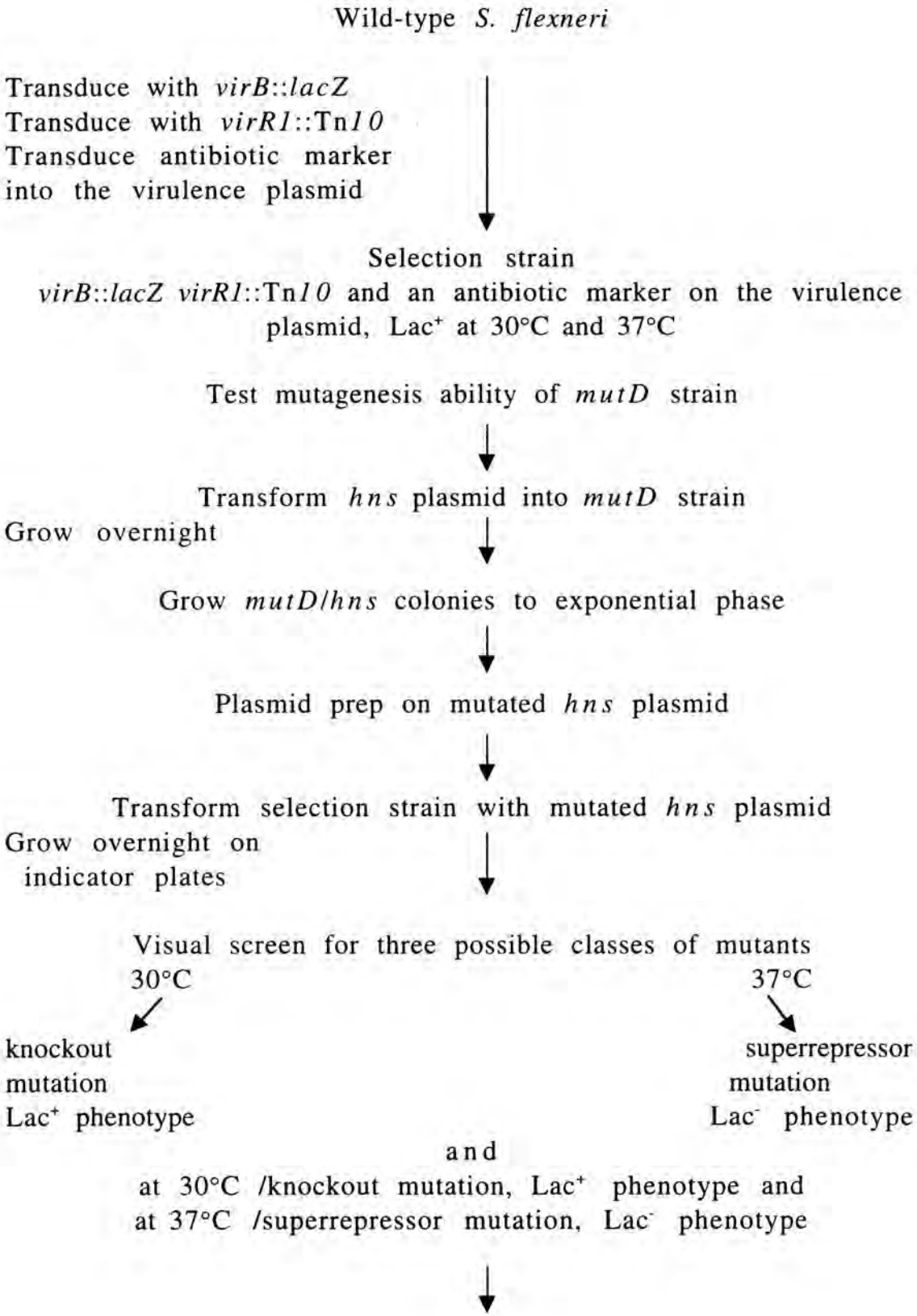
The H-NS protein can be divided into 3 domains responsible for DNA binding, oligomerization and transcriptional repression. The available data do not allow a conclusion as to which domain of the protein is responsible for thermoregulation. Likewise, it is not known whether the protein's ability to function as a thermoregulator is due to its structure or the ability of the protein to bind to DNA at elevated temperature. The following section outlines a research protocol designed to answer the question: which aspects of the

Shigella hns gene are essential for the temperature regulation phenotype?

Research proposal to study H-NS mutants in *Shigella flexneri*

The purpose of the proposed experiments is to identify temperature deregulated mutants of *hns* in an attempt to elucidate regions and/or amino acids of H-NS that are necessary for binding to *virB* and for thermoregulation of virulence gene transcription. Figure 8 is a flowchart diagramming the experiments below.

The first step will be to assemble the necessary components for the experiments. A low copy number plasmid encoding *hns* will be used since overexpression of H-NS can be deleterious to the cell (McGovern *et al.*, 1994; Spurio *et al.*, 1992). Also, the plasmid containing *hns* will encode for antibiotic resistance. The selection strain requires that *virB* be fused to the indicator since we are studying the direct interaction of H-NS and *virB*, and that *hns* be functionally deleted. Therefore, a *virB*::*lacZ* operon fusion must be made in a wild-type strain of *S. flexneri*. This fusion can be transduced into the chromosome. Due to the instability of the large virulence plasmid (Schuch and Maurelli, 1997), an antibiotic resistance cassette will be transduced into a downstream virulence


Wild-type S. flexneri
Transduce with virB::lacZ
Transduce with virR1::Tn10
Transduce antibiotic marker into the virulence plasmid
Selection strain
virB::lacZ virR1::Tn10 and an antibiotic marker on the virulence plasmid, Lac+ at 30°C and 37°C
Test mutagenesis ability of mutD strain
Transform hns plasmid into mutD strain
Grow overnight
Grow mutD/hns colonies to exponential phase
Plasmid prep on mutated hns plasmid
Transform selection strain with mutated hns plasmid
Grow overnight on indicator plates
Visual screen for three possible classes of mutants
30°C
37°C
knockout mutation
Lac+ phenotype
superrepressor mutation
Lac- phenotype
and
at 30°C /knockout mutation, Lac+ phenotype and
at 37°C /superrepressor mutation, Lac- phenotype

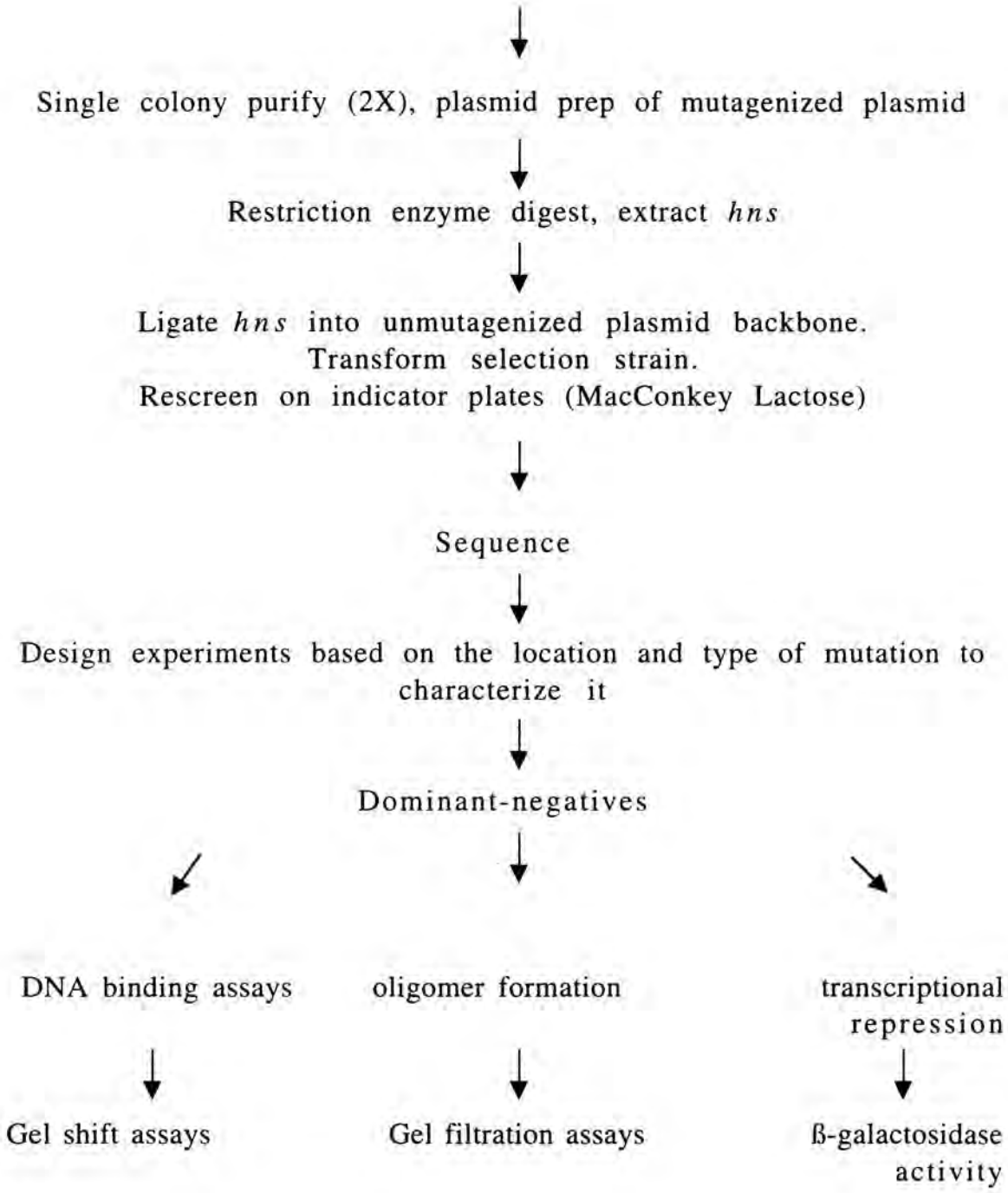


Figure 8. Diagram of proposed research protocol to examine *hns* mutants in *S. flexneri.*

gene like *mxiC*. The antibiotic resistance marker in *mxiC* will be used to select for maintenance of the virulence plasmid. The *virB* on the virulence plasmid is left intact. The transduction of *virR1*::Tn10 (Hromockyj et al, 1992) represses the temperature regulation. The selection strain will have the genotype *virR1*::Tn*10*, *virB*::*lacZ* and an additional antibiotic marker, such as ampicillin, on the virulence plasmid. The phenotype will be non-invasive at both 30°C and 37°C, since an invasion gene, such as *mxiC*, is interrupted by the insertion of the antibiotic marker on the virulence plasmid. It will be Lac^+ at both temperatures because *hns* is insertionally inactivated and, therefore, does not repress the *virB*::*LacZ* fusion.

Random mutagenesis will be performed on the *hns* containing plasmid in *mutD* strains (Ueguchi et al, 1997; Wu *et al.*, 1990). These strains have a mutation in the *mutD* allele resulting in a mutator phenotype responsible for an increase in the spontaneous mutation rate. Specifically, the products of the *mutD* strains are involved in proofreading and repair by DNA polymerase III of potential transversion mutations (Wu *et al.*, 1990). Initially, isolates of *mutD* will be tested for mutational ability by growth on Luria agar plates with 50 µg/ml nalidixic acid and scoring for spontaneously resistant colonies. The frequency of spontaneous mutations is about 1000-fold

above the wild-type level (Wu *et al.*, 1990). The *hns*-containing plasmid will be transformed into the *mutD* strain and grown overnight at 37°C. Then, colonies will be grown to exponential phase in 2X yeast extract Luria broth (LB) since the rich medium increases the possibility of point mutations occurring in the plasmid. The mutated plasmid from the bacterial culture will then be harvested. The mutagenized plasmid pool will be transformed into the selection strain and grown for one hour in broth to express the antibiotic markers on the *hns* and virulence plasmids. The culture will then be plated on MacConkey lactose indicator plates containing appropriate antibiotics for selection of transformants and virulence plasmid maintenance. The plates will be divided in half and incubated overnight at 30°C and 37°C, respectively. The plates are subsequently screened for aberrant morphologies. The wild-type phenotype for *hns* in the *virB*::*lacZ* fusion strain is Lac^- at 30°C and Lac^+ at 37°C. There are three classes of possible mutants that will be screened (see figure 8). The mutant phenotypes that will be screened will be Lac^- colonies at 37°C and/or Lac^+ colonies at 30°C. All mutant colonies will be single colony purified twice and then a plasmid prep will be performed on the bacterial culture generated from each colony to harvest the mutagenized plasmid. The plasmid is subsequently

digested with restriction enzymes to extract the coding region for *hns*. The *hns* fragment will then be ligated into an unmutagenized plasmid backbone and re-screened for the mutant phenotype. Any mutant identified will then be sequenced, since the H-NS gene is only 411 bp.

The most interesting mutants will be those that enables H-NS to bind to the *virB* promoter and inhibit transcription at 37°C, a super-repressor. Theoretically, H-NS is out-competed at the *virB* promoter at 37°C by VirF due to supercoiling changes (Higgins *et al.*, 1990a). H-NS that is able to bind to more highly supercoiled DNA would also have a more global effect. The global effect, unfortunately, could cause too much compaction of the DNA and inhibit transcription of vital genes resulting in cell death. The reason these mutants might be so rare is the fine balance between enough and too much H-NS (McGovern *et al.*, 1994; Spurio *et al.*, 1992). The other class of mutants screened will be those that are unable to repress virulence gene transcription at 30°C. Deletions and alterations in the amino acids of these mutants will show which parts of the protein are specifically needed to interact with and regulate *virB*.

Ideally, the mutation frequency attained from the *mutD* strains on the *hns* plasmid will be 1.0 - 2.0%. The background mutation rate

is approximately 0.1%. At least, 1000 colonies must be screened, and the expected results would be 10 colonies with aberrant morphologies. Super-repressor mutations might be more difficult to quantitate due to supercoiling sensitivity as was discussed earlier.

Additional experiments will be performed to characterize the mutation. The experimental design will be determined by where the mutations occur in the gene. Experiments with dominant-negatives (Ueguchi *et al.*, 1997; Williams *et al.*, 1996) will aid in the clarification of transcriptional repression, DNA binding, and oligomer formation.

Transcriptional repression will be studied with ß-galactosidase assays. Genes believed to be directly regulated by H-NS will be fused to *lacZ*. The cells will be grown under varying conditions of temperature and osmolarity. Expression of the gene will be measured via the ß-galactosidase assay in both mutant *hns* and wild-type *hns* backgrounds.

DNA binding will be analyzed in gel shift assays. DNA fragments will be incubated with varying amounts of the mutant H-NS protein. After incubating, the samples will be electrophoresed on a 1% (w/v) agarose gel and stained with ethidium bromide to visual protein-DNA complexes.

Gel-filtration assays will be used to study oligomer formation. The protein is applied to a fast protein liquid chromatography column system. Fractions are collected and an aliquot of each fraction is electrophoresed in a non-denaturing polyacrylamide gel. Duplicates will be run. One gel will be stained with Coomassie brilliant blue to visualize all protein. H-NS will be detected on the other gel by immunoblotting.

Conclusion

H-NS research is an exciting field. In the last decade alone, its role has gone from simply being a nucleoid structural protein, to a major regulatory factor in *Shigella* virulence. However, there is much more to be learned about the mechanisms of H-NS. The more data collected, the better understanding we will have of this protein, its role in regulation of bacterial function including virulence and how we may be able to use this protein to limit and, hopefully, eliminate infection.

REFERENCES

1. **Adler, B., C. Sasakaawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa.** 1989. A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol. Microbiol. **3**:627-635.
2. **Allaoui, A., J. Mounier, M. C. Prevost, P. J. Sansonetti, and C. Parsot.** 1992. *icsB*: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intercellular spread. Mol. Microbiol. **6**:1605-1616.
3. **Arnqvist, A., A. Olsen, and S. Normark.** 1994. σ^S - dependent growth phase induction of the *csgBA* promoter in *Escherichia coli* can be achieved in vivo by σ^{70} in the absence of the nucleoid-associated protein H-NS. Mol. Microbiol. **13**:1021-1032.
4. **Barr, G. C., N. Ni Bhriain, and C. J. Dorman.** 1992. Identification of two genetically active regions associated with the *osmZ* locus of *Escherichia coli*: role in regulation of *proU* expression and mutagenic effect at *cya*, the structural gene for adenylate cyclase. J. Bacteriol. **174**: 998-1006.
5. **Barth, M., C. Marschall, A. Muffler, D. Fischer, and R. Hengge-Aronis.** 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of the σ^S and the many σ^S-dependent genes of *Escherichia coli*. J Bacteriol. **177**: 3455-3464.

6. **Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86**: 3867-3871.

7. **Bertin, P., P. Lejeune, C. Colson, A. Danchin.** 1992. Mutations in *bglY*, the structural gene for the DNA binding protein H1 of the *Escherichia coli*, increases the expression of the kanamycin resistance gene carried by plasmid pGR71. Mol. Gen. Genet. **233**: 184-192.

8. **Bertin, P., P. Lejeune, W. C. Laurent, and A. Danchin.** 1990. Mutations in *bglY*, the structural gene for the DNA-binding protein H1, affect expression of several *Escherichia coli* genes. Biochimie **72**: 889-891.

9. **Bliska, J. B., and N. R. Cozzarelli.** 1987. Use of site-specific recombination as a probe of DNA structure and metabolism in vivo. J. Mol. Biol. **194**: 205-218.

10. **Bouvier, J., S. Gordia, G. Kampmann, R. Lange, R. Hengge-Aronis, and C. Gutierrez.** 1998. Interplay between global regulators of *Escherichia coli*: effects of RpoS, Lrp and H-NS on transcription of the gene *osmC*. Mol. Microbiol. **28**: 971-980.

11. **Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic curved DNA sequences can act as transcriptional activators in *E. coli*. EMBO J. **8**: 4289-4296.

12. **Crothers, D. M., T. E. Haran, and J. G. Nadeau.** 1990. Intrinsically bent DNA. J. Biol. Chem. **265**: 7093-7096.

13. **Dagberg, B., and B. E. Uhlin.** 1992. Regulation of virulence-associated genes in enteroinvasive *Escherichia coli*. J. Bacteriol. **174**: 7606-7612.

14. **Dattanada, C. S., K. Rajkumari, and J. Gowrishankar.** 1991. Multiple mechanisms contribute to osmotic inductibility of *proU* operon expression in *E. coli*: demonstration of two osmoresponsible promoters and a negative regulatory element with the first structural gene. J. Bacteriol. **173**: 7481-7490.

15. **Dersch, P., K. Schmidt, and E. Bremer**. 1993. Synthesis of the *Escherichia coli* K-12 nucleiod-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. Mol. Microbiol. **8**: 875-889.

16. **Dersch, P., S. Kneip, and E. Bremer**. 1994. The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. Mol Gen. Genet. **245**: 255-259.

17. **Donato, G. M., M. J. Lelivelt, and T. H. Kawula**. 1997. Promoter-specific repression of *fimB* expression by the *Escherichia coli* nucleiod-associated protein H-NS. J. Bacteriol. **179**: 6618-6625.

18. **Dorman, C. J., A. S. Lynch, N. Ni Bhriain, and C. F. Higgins**. 1989. DNA supercoiling in *Escherichia coli*: *topA* mutations can be suppressed by DNA amplification involving the *tolC* locus. Mol. Microbiol. **3**: 531-540.

19. **Dorman, C. J., and N. Ni Bhriain**. 1992a. Global regulation of gene expression during environmental adaptation: implications for bacterial pathogens. In C. E. Hormaeche, C. W.

Penn, and C. J. Smyth, (eds.), Molecular Biology of Bacterial Infection: Current Status and Future Prospects, pp. 193-230. Cambridge University Press.

20. **Dorman, C. J., and N. Ni Bhriain.** 1992b. Thermal regulation of *fim*A, the *Escherichia coli* gene coding for the type 1 fimbrial subunit. FEMS Microbiol Lett. **99**: 125-130.

21. **Dorman, C. J., G. C. Barr, N. Ni Bhriain, and C. F. Higgins.** 1988. DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. J. Bacteriol. **170**: 2816-2826.

22. **Dorman, C. J., N. Ni Bhriain, and C. F. Higgins.** 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri.* Nature **344**: 789-792.

23. **Dorman, C. J., and M. E. Porter.** 1998. The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. Mol. Micro. **29**: 677-684.

24. **Drlica, K.** 1984. Biology of bacterial deoxyribonucleic acid topoisomerases. Microbiol. Rev. **48**: 273-289.

25. **Drlica, K., G. J. Pruss, R. M. Burger, R. J. Franco, L. S. Hsiesh, and B. A. Berger.** 1990. Roles of DNA topoisomerases in bacterial chromosome structure and function. In K. Drlica and M Riley, (eds.), The Bacterial Chromosome, pp. 195-204. American Society for Microbiology Press. Washington, D.C.

26. **Durrenberger, M., A. La Teana, G. Citro, F. Venanzi, C. O. Gualerzi, and C. L. Pon.** 1991. *E. coli* DNA-binding

protein H-NS is localized in the nucleoid. Res. Microbiol. **142**: 373-380.

27. **Epstein, W., and S. G. Schultz.** 1965. Cation transport in *Escherichia coli* V. Regulation of cation content. J. Gen Physiology 49: 221-234.

28. **Falconi, M., C. F. Higgins, R. Spurio, C. L. Pon, and C O. Gualerzi**. 1993. Expression of the gene encoding the major bacterial nucleoid protein H-NS is subject to transcriptional autorepression. Mol. Microbiol. **10**: 273-282.

29. **Falconi, M., M. T. Gualtieri, A. La Teana, M. A. Losso, and C. L. Pon**. 1988. Proteins from the prokaryotic nucleoid: primary and quaternary structure of the 15kD *Escherichia coli* DNA-binding protein H-NS. Mol. Microbiol. **2**: 323-329.

30. **Formal, S. B., and M. M. Levine.** 1983. Shigellosis, pp. 167-186. In R. Germanier (ed.), Bacterial Vaccines. Academic Press, New York.

31. **Forsman, K., B. Sonden, M. Goransson, and B. E. Uhlin.** 1992. Antirepression function in *E. coli* for the cAMP-cAMP receptor protein transcriptional activator. Proc. Natl. Acad. Sci. USA **89**: 9880-9884.

32. **Free, A., and C. J. Dorman**. 1995. Coupling of *Escherichia coli* mRNA levels to DNA synthesis by autoregulation: implications for growth phase control. Mol. Microbiol. **18**: 101-113.

33. **Goldstein E., and K. Drlica.** 1984. Regulation of bacterial DNA supercoiling: plasmid linking numbers vary with growth temperature. Proc. Natl. Acad. Sci. USA. **81**: 4046-4050.

34. **Goransson, M., and B. E. Uhlin**. 1984. Environmental temperature regulates transcription of a virulence pili operon in *E. coli.* EMBO **3**: 2885-2888.

35. **Goransson, M., B. Sonden, P. Nilsson, B. Dagberg, K. Forsman, K. Emanuelsson, and B. E. Uhlin**. 1990. Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli.* Nature **344**: 682-685.

36. **Graeme-Cook, K. A., G. May, E. Bremer, and C. F. Higgins**. 1989. Osmotic regulation of porin expression: role of DNA supercoiling. Mol Microbiol. **3**: 1287-1294.

37. **Hale, T. L.** 1991. Genetic basis of virulence in *Shigella* species. Microbiol. Rev. **55**: 206-224.

38. **Higgins, C. F., C. J. Dorman, and N. Ni Bhriain.** 1990a. Environmental influences on DNA supercoiling: a novel mechanism for the regulation of gene expression. In Drlica, K. & Riley, M. (eds) The Bacterial Chromosome, pp. 421-432. American Society for Microbiology Press, Washington, D. C.

39. **Higgins, C. F., J. C. D. Hinton, C. S. J. Hulton, T. A. Owen-Hughes, G. D. Pavitt, and A. Seirafi.** 1990b Protein H1: a role for chromatin in the regulation of bacterial gene expression and virulence? Mol Microbiol. **4**: 2007-2012.

40. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer**. 1988. A physiological role of DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli.* Cell **52**: 569-584.

41. **Hinton, J. C. D., D. S. Santos, A. Seirafi, C. S. J. Hulton, G. D. Pavitt, and C. F. Higgins.** 1992. Expression and

mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium*. Mol. Microbiol. **6**: 2327-2337.

42. **Hromockyj, A. E., S. C. Tucker, and A. T. Maurelli.** 1992. Temperature regulation of *Shigella* virulence: identification of the repressor gene *virR*, an analogue of *hns*, and partial complementation by tyrosyl transfer RNA ($tRNA_1^{Tyr}$). Mol. Microbiol. **6**: 2113-2124.

43. **Huang, L., P. Tsui, and M. Freundlich.** 1990. Integration host factor is a negative effector of *in vivo* and *in vitro* expression of *ompC* in *Escherichia coli*. J. Bacteriol. **172**: 5293-5298.

44. **Hulton, C. S. J., A. Seirafi, J. C. D. Hinton, J. M. Sidebotham, L. Waddell, G. D. Pavitt, T. Owen-Hughes, A. Spassky, H. Buc, and C. F. Higgins.** 1990. Histonelike protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. Cell **63**: 631-642.

45. **Jacquet, M., R. Cukier-Kahn, J. Pla, and F. Gros.** 1971. A thermostable protein factor acting in *in vitro* DNA transcription. Biochem. Biophys. Res. Commun. **45**: 1597-1607.

46. **Jordi, B. J. A. M., A. E. Fielder, C. M. Burns, J. C. D. Hinton, N. Dover, D. W. Ussery, and C. F. Higgins.** 1997. DNA binding is not sufficient for H-NS mediated repression of *proU* expression. J. Biol. Chem. **272**: 12083-12090.

47. **Jordi, B. J. A. M., B. Dagberg, L. A. M. de Haan, A. M. Hamers, B. A. M. van der Zeijst, W. Gaastra, and B. E. Uhlin.** 1992. The positive regulator CfaD overcomes the repression mediated by histonelike protein H-NS (H1) in the

CFA/I fimbrial operon of *Escherichia coli*. EMBO J. **11**: 2627-2632.

48. **Kawula, T. H., and M. J. Lelivelt.** 1994. Mutations in a gene encoding a new HSP-70 suppress rapid DNA inversion and *bgl* activation, but not *proU* derepression in *hns*-1 mutant *Escherichia coli*. J. Bacteriol. **176**: 610-619.

49. **Kawula, T. H., and P. E. Orndorff.** 1991. Rapid site specific DNA inversion in *Escherichia coli* mutants lacking the histonelike protein H-NS. J. Bacteriol. **173**: 4116-4123.

50. **Keusch T. G., and S. B. Formal.** 1984. Shigellosis, pp. 723-733. In K. S. Warren, and A. F. Matimoud (eds.), Tropical and Geographical Medicine. McGraw Hill Book Company, New York.

51. **Koo, H-S., H-W. Wu, and D. M. Crothers.** 1986. DNA bending at adenine-thymine tracts. Nature **320**:501-506.

52. **Kopecko, D. J., L. S. Baron, and J. Buysse.** 1985. Genetic determinants of virulence in *Shigella* and dysenteric strains of *Escherichia coli*: their involvement in the pathogenesis of dysentery. Curr. Top. Microbiol. Immunol. **118**: 71-95.

53. **La Teana, A., A. Brandi, M. Falconi, R. Spurio, C. L. Pon, and C. O. Gualerzi.** 1991. Identification of the cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. Proc. Natl. Acad. Sci. USA **88**: 10907-10911.

54. **La Teana, A., M. Falconi, V. Scarlato, and C. L. Pon.** 1989. Characterization of the structural genes for the DNA-binding protein H-NS in *Enterobacteriaceae*. FEBS Lett. **244**: 34-38.

55. **LaBrec, E. H., H. Schneider, T.K. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**: 1503-1518.

56. **Lammi, M., M. Paci, C. L. Pon, K. A. Losso, A. Miano, R. T. Pawlik, G. L. Gianfranceschi, and C. O. Gualerzi.** 1984. Proteins from the prokaryotic nucleoid. Biochemical and ^{1}H-NMR studies of three bacterial histone-like proteins. In J. Huscher, and S. Spadari, (eds.), Proteins Involved in DNA Replication. pp. 467-477. Plenum, New York.

57. **Laurent-Winter, C., S. Ngo, A. Danchin, and P. Bertin.** 1997. Role of *Escherichia coli* histone-like nucleoid-structuring protein in bacterial metabolism and stress response-identification of targets by two-dimensional electrophoresis. Eur. J. Biochem. **244**: 767-773.

58. **Lejeune, P., and A. Danchin.** 1990. Mutations in the *bglY* gene increase the frequency of spontaneous deletions in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **87**: 360-363.

59. **Levine, M. M., H. L. DuPont, S. B. Formal, R. B. Hornick, A. Takeuchi, E. J. Gangarosa, M. J. Snyder, and J. P. Libonati.** 1973. Pathogenesis of *Shigella dysenteriae* 1 (Shiga) dysentery. J. Infect. Dis. **127**: 261-270.

60. **Levine, O. S., and M. M. Levine.** 1991. Houseflies (*Musca domestica*) as mechanical vectors of shigellosis. Rev. Infect. Dis. **13**:688-696.

61. **Lucht, J. M., P. Dersch, B. Kempf, and E. Bremer.** 1994. Interactions of the nucleoid-associated DNA-binding protein H-

NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. **269**: 6578-6586.

62. **Marsh, M., and D. R. Hillyard.** 1990. Nucleotide sequence of *hns* encoding the DNA-binding protein H-NS of *Salmonella typhimurium*. Nucleic Acids Res. **18**: 3397.

63. **Maurelli, A. T., and K. A. Lampel.** 1997. *Shigella* Species. In M. P. Doyle, L. R. Beauchat, and T. J. Montville, (eds.), Food Microbiology Fundamentals and Frontiers, pp. 216-227. American Society for Microbiology Press. Washington D. C.

64. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Identification of the chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85**: 2820-2824.

65. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**: 195-201.

66. **May, G., E. Faatz, M. Villarejo, and E. Bremer.** 1986. Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. Mol. Gen. Genet. **205**: 225-233.

67. **May, G., P. Dersch, M. Haardt, A. Middendorf, and E. Bremer.** 1990. The *osmZ* (*bglY*) gene encodes the DNA-binding protein H-NS (H1a) a component of the *Escherichia coli* K-12 nucleoid. Mol. Gen. Genet. **224**: 81-90.

68. **McClellan, J. A., P. Boublikova, E. Palecek, and D. M. J. Lilley.** 1990. Superhelical torsion in cellular DNA responds

directly to environmental and genetic factors. Proc. Natl. Acad. Sci. USA **87**: 8373-8377.

69. **McGovern, V., N. P. Higgins, R. S. Chiz, and A. Jaworski.** 1994. H-NS overexpression induces an artificial stationary phase by silencing global transcription. Biochimie **76**: 1019-1029.

70. **Menard, R., P. J. Sansonetti, C. Parsot, and T. Vasselon.** 1994. Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. Cell **79**:515-525.

71. **Monjica, F. J. M., and C. F. Higgins.** 1997. In vivo supercoiling of plasmid and chromosomal DNA in an *Escherichia coli hns* mutant. J. Bacteriol. **179**: 3528-3533.

72. **Nakata, N., C. Sasakawa, N. Okada, T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu and M. Yoshikawa.** 1992. Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. Mol. Microbiol. **6**:2387-2395.

73. **Ni Bhriain, N., C. J. Dorman, and C. F. Higgins.** 1989. An overlap between osmotic and anaerobic stress responses: a potential role of DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3**: 933-942.

74. **Olsen, A., Jonsson, A. & Normark, S.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature **338**: 652-655.

75. **Olsen, A., A. Arnqvist, S. Sukupolvi, and S. Normark.** 1993. The RpoS sigma factor relieves H-NS-mediated

repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. Mol Microbiol. **7**: 523-536.

76. **Olsen, P. B., M. A. Schembri, D. L. Gally, and P. Klemm.** 1998. Differential temperature modulation by H-NS of the *fimB* and *fimE* recombinase genes which control the orientation of the type 1 fimbrial phase switch. FEMS Microbiol. Lett. **162**: 17-23.

77. **Owen-Hughes, T. A., G. D. Pavitt, D. S. Santos, J. M. Sidebotham, C. S. J. Hulton, J. C. D. Hinton, and C. F. Higgins.** 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell **71**: 1-20.

78. **Palchaudhuri, S., B. Tominna, and A. Leon.** 1998. H-NS regulates DNA repair in *Shigella*. J. Bacteriol. **180**: 5260-5262.

79. **Park, S. F., D. A. Stirling, C. S. J. Hulton, I. R. Booth, C. F. Higgins, and G. S. A. B. Stewart.** 1989. A novel, non-invasive promoter probe vector: cloning of the osmoregulated *proU* promoter of *Escherichia coli* K12. Mol. Microbiol. **3**: 1011-1023.

80. **Parsot, C., R. Menard, P. Gounon, and P. J. Sansonetti**. 1995. Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocation leads to assembly of extracellular proteins into macromolecular structures. Mol. Microbiol. **16**: 291-300.

81. **Parsot C., and P. J. Sansonetti.** 1996. Invasion and the pathogenesis of *Shigella* infections. Curr. Top. Microbiol. Immunol. **209**: 25-42.

82. **Pon, C. L., R. Calogero, and C. Gualerzi.** 1988. Identification, cloning, nucleotide sequence and chromosomal map location of *hns*, the structural gene for the *Escherichia coli* DNA-binding protein H-NS. Mol. Gen. Genet. **212**: 199-202.

83. **Porter, M. E., and C. J. Dorman.** 1994. A role for H-NS in the thermo-osmotic regulation of virulence genes expression in *Shigella flexneri*. J. Bacteriol. **176**: 4187-4191.

84. **Porter, M. E., and C. J. Dorman.** 1997. Differential regulation of the plasmid-encoded genes in the *Shigella flexneri* virulence regulon. Mol. Gen. Genet. **256**: 93-103.

85. **Rimsky, S., and A. Spassky.** 1990. Sequence determinants for H1 binding on *E. coli lac* and *gal* promoters. Biochemistry **29**: 3765-3771.

86. **Roberts, J. A., M. B. Kaack, and E. N. Fussell.** 1989. Bacterial adherence in urinary tract infection: preliminary studies in a primate model. Infection **17**: 401-404.

87. **Schuch, R., and A.T. Maurelli.** 1997 Virulence plasmid instability in *Shigella flexneri* 2a is induced by virulence gene expression. Infect. Immun. **65**: 3686-3692.

88. **Shi, X., and G. N. Bennett.** 1994. Plasmids bearing *hfq* and the *hns*-like gene *stpA* complement *hns* mutants in modulating arginine decarboxylase gene expression in *Escherichia coli* . J. Bacteriol. **176**: 6769-6775.

89. **Shi, X., B. C. Waasdorp, and G. N. Bennett.** 1993. Modulation of acid-induced amino acid decarboxylase gene expression by *hns* in *Escherichia coli*. J. Bacteriol. **175**:1182-1186.

90. **Shindo, H., T. Iwaki, R. Ieda, H. Kurumizaka, C. Ueguchi, T. Mizuno, S. Morikawa, H. Nakamura, and H. Kuboniwa.** 1995. Solution structure of the DNA binding domain of the nucleoid-associated protein, H-NS from *Escherichia coli*. FEBS Lett. **360**: 125-131.

91. **Smyth, C. J., and S. G. J. Smith.** 1992. Bacterial fimbriae: variation and regulatory mechanisms. In C. E. Hormaeche, C. W. Penn, and C. J. Smyth, (eds.), Molecular Biology of Bacterial Infection: Current Status and Future Prospects, pp. 267-297. Cambridge University Press.

92. **Sonden, B. and B. E. Uhlin.** 1996. Coordinated and differential expression of histone-like proteins in *Escherichia coli*: regulation and function of the H-NS analog StpA. EMBO J. **15**: 4970-4980.

93. **Spassky, A., S. Rimsky, H. Garreau, and H. Buc.** 1984. H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. Nucleic Acids Res. **12**: 5321-5340.

94. **Spears, P. A., D. Schauer, and P. E. Orndorff.** 1986. Metastable regulation and type 1 piliation in *Escherichia coli* and isolation of a phenotypically stable mutant. J. Bacteriol. **168**: 179-185.

95. **Spurio, R., M. Durrenberber, M. Falconi, A. La Teana, C. L. Pon, and C. O. Gualerzi.** 1992. Lethal overproduction of the *Escherichia coli* nucleoid protein H-NS: ultramicroscopic and molecular autopsy. Mol. Gen. Genet. **231**: 201-211.

96. **Spurio, R., M. Falconi, A. Brandi, C. L. Pon, and C. O. Gualerzi.** 1997. The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending. EMBO J. **16**: 1795-1805.

97. **Sutherland, L., J. Cairney, M. J. Elmore, I. R. Booth, and C. F. Higgins.** 1986. Osmotic regulation of transcription: Induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. J. Bacteriol. **168**: 805-814.

98. **Takeuchi, A., H. Spring, E. H. Labrec, and S. B. Formal.** 1965. Experimental bacillary dysentery: an electron microscopic study of the response of intestinal mucosa to bacterial invasion. Am. J. Pathol. **4**: 1011-1044.

99. **Tanaka, K., H. Yamada, T. Yoshida, and T. Mizuno.** 1991. Overproduction and rapid purification of the *Escherichia coli* histone-like protein, H-NS. Agric. Biol. Chem. **55**: 3139-3141.

100. **Tippner, D., Afflerbach, H., Bradaczek, C. & Wagner, R.** 1994. Evidence for the regulatory function of the histone-like *Escherichia coli* protein H-NS in ribosomal synthesis. Mol. Microbiol. **11**: 589-604.

101. **Tippner, D., and R. Wagner.** 1995. Fluorescence analysis of the *Escherichia coli* transcription regulator H-NS reveals two distinguishable complexes dependent on binding to specific or nonspecific DNA sites. J. Biol. Chem. **270**: 22243-22247.

102. **Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa**. 1992. *vacB*, a novel chromosomal gene required

for the expression of virulence genes on the large plasmid of *Shigella flexneri*. J. Bacteriol. **174**:6359-6367.

103. **Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa.** 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: Activation of VirF and repression by H-NS. J. Bacteriol. **175**: 6142-6149.
104. **Tsui, P., V. Helu, and M. Freudlich.** 1988. Altered osmoregulation of *ompF* in integration host factor mutants of *Escherichia coli*. J. Bacteriol. **170**: 4950-4953.
105. **Tupper, A. E., T. Owen-Hughes, D. W. Ussery, D. S. Santos, D. J. Ferguson, J. M. Sidebotham, J. C. Hinton, and C. F. Higgins.** 1994. The chromatin associated protein H-NS alters DNA topology in vitro. EMBO J. **13**: 258-268.
106. **Uchiya, K., T. Tobe, K. Komatsu, T. Suzuki, M. Watarai, I. Fukuda, M. Yoshikawa, and C. Sasakawa.** 1995. Identification of a novel virulence gene, *virA*, on the large plasmid of *Shigella*, involved in invasion and intercellular spreading. Mol. Microbiol. **17**:241-250.
107. **Ueguchi, C., and T. Mizuno.** 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12**: 1039-1046.
108. **Ueguchi, C., C. Seto, T. Suzuki, and T. Mizuno.** 1997. Clarification of the dimerization domain and its functional significance for the *Escherichia coli* nucleoid protein H-NS. J. Mol. Biol. **274**: 145-151.
109. **Ueguchi, C., T. Suzuki, T. Yoshida, K. Tanaka, and T. Mizuno.** 1996. Systematic mutational analysis revealing the

functional domain organization of *Escherichia coli* nucleoid protein H-NS. J. Mol. Biol. **263**: 149-162.

110. **Ulitzur, S., A. Matin, C. Fraley, and E. Meighen.** 1997. H-NS protein represses transcription of the *lux* systems of *Vibrio fisheri* and other luminous bacteria cloned into *Escherichia coli.* Curr. Microbiol. **35**: 336-342.

111. **Varshavsky, A. J., A. Nedospasov, V. V. Bakayeva, T. G. Bakayeva, and G. Georgiev.** 1977. Histonelike proteins in the purified *Escherichia coli* deoxyribonucleoprotein. Nucleic Acids Res. **4**: 2725-2745.

112. **Williams, R.M., Rimsky, S. & Buc, H.** 1996. Probing the structure, function, and interactions of the *Escherichia coli* H-NS and StpA proteins by using dominant negative derivatives. J. Bacteriol. **178**: 4335-4343.

113. **Williams, R. M., and S. Rimsky.** 1997. Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. FEMS Microbiol. Lett. **156**:175-185.

114. **White-Ziegler, C. A., M. L. A. Hill, B. A. Braaten, M. W. van der Woude, and D. A. Low.** 1998. Thermoregulation of *Escherichia coli pap* transcription: H-NS is a temperature-dependent DNA methylation blocking factor. Mol. Microbiol. **28**: 1121-1137.

115. **Worcel, A. and E. Burgi.** 1972. On the structure of the folded chromosome of *Escherichia coli.* J. Mol. Biol. 71:127-147.

116. **Wu, T. -H., C. H. Clarke, and M. G. Marinus.** 1990. Specificity of *Escherichia coli mutD* and *mutL* mutator strains. Gene. **87**:1-5.

117. **Yamada, H., S. Muramatsu, and T. Mizuno.** 1990. An *Escherichia coli* protein that preferentially binds to sharply curved DNA. J. Biochem. **108**: 420-425.

118. **Yamada, H., T. Yoshida, K. -I. Tanaka, C. Sasakawa, and T. Mizuno.** 1991. Molecular analysis of the *Escherichia coli hns* gene encoding the DNA-binding protein, which preferentially recognizes curved DNA sequences. Mol. Gen. Genet. **230**: 332-336.

119. **Yasuzawa, K., N. Hayashi, N. Goshima, K. Kohno, F. Imamoto, and Y. Kano.** 1992. Histone-like proteins are required for cell growth and restraint of supercoils in DNA. Gene **122**: 9-15.

120. **Yoshida, T., C. Ueguchi, H. Yamada, and T. Mizuno.** 1993. Function of the *Escherichia coli* nucleoid protein, H-NS: molecular analysis of a subset of proteins whose expression is enhanced in a *hns* deletion mutant. Mol. Gen. Genet. **237**: 113-122.

121. **Zhang, A., S. Rimsky, M. E. Reaban, H. Buc, and M. Belfort.** 1996. *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. EMBO J. **15**: 1340-1349.

122. **Zuber, F., D. Kotlarz, S. Rimsky, and H. Buc.** 1994. Modulated expression of promoters containing upstream

curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. Mol. Microbiol. **12**: 231-240.

123. **Zychlinsky, A., J. J. Perdomo, and P. J. Sansonetti.** 1994. Molecular and cellular mechanisms of tissue invasion by *Shigella flexneri*. Annals N. Y. Acad. Sci. **730**: 197-208.