

**Characterization of Regulation and Expression Patterns of
Escherichia coli Hsp31 Protein**

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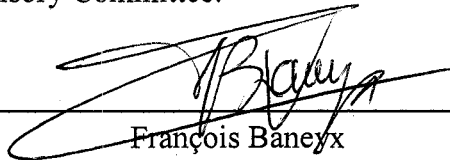
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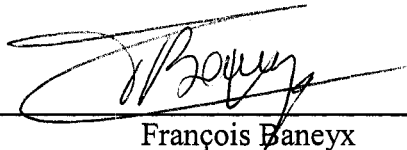
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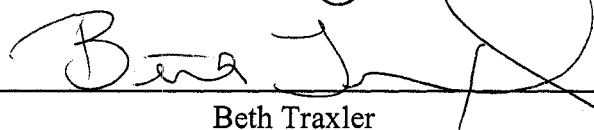
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Abstract

Characterization of Regulation and Expression Patterns of *Escherichia coli*
Hsp31 Proteins

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Escherichia coli is exposed to a variety of environmental stresses in its natural habitats. On a cellular level, these stresses wreak havoc by disrupting the structure and function of proteins, which ultimately leads to a breakdown of finely tuned and complex cellular machineries. To cope with constant environmental onslaughts, *Escherichia coli* employs an elaborate system of protein networks whose primary role is to prevent and alleviate stress-inflicted damaged. Although many components of its various stress response systems are known, the genome of *Escherichia coli* is still strewn with open reading frames whose cellular roles remain elusive. One such open reading frame, named *hchA*, codes for Hsp31, a homodimeric protein with structural similarities to archaeobacterial PfpI-type peptidases and human DJ-1 protein, which has been implicated in early onset Parkinson's disease. *hchA* was initially identified in an analysis of *Escherichia coli*'s transcriptome as one of 23 genes of unknown function whose expression is thermally induced. *In vitro* studies have shown that under heat shock conditions Hsp31 functions as a molecular chaperone - a type of a stress protein whose role is to help proteins reach their proper folded form - by capturing partially folded proteins and releasing them in an active form once the thermal stress has abated. To investigate the role of Hsp31 under physiological conditions, we studied its regulation and expression patterns. We have found that Hsp31 is a bona fide heat shock protein whose heat-induced expression is regulated by the housekeeping sigma factor σ^D and the global gene silencer H-NS. Its absence under severe heat shock conditions greatly impairs cell survival and leads to a decrease in protein integrity of a subset of cellular proteins. Moreover, its expression is

induced under a variety of stress conditions, while its absence under such conditions leads to an increase in protein aggregation. Hsp31 also accumulates in the stationary phase of growth through the action of the stationary phase sigma factor σ^S .

Consequently, stationary phase $\Delta hchA$ cells exhibit a decrease in fitness when exposed to a number of stress stimuli, with a greatly diminished survival in the presence of severe acidic stress.

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DEDICATION

Za moju porodicu.

CHAPTER 1: Overview of Major Stress Response Networks in *E. coli*

For enteropathogenic bacteria such as *Escherichia coli* coping with stress is the norm. In order to colonize the intestinal tract of their host where they thrive, these organisms need to pass through and survive stomach's acidic surroundings. They also have to be capable of withstanding oxidative attacks mounted by host's defense system in the form of superoxide anions, hydroxyl radicals, nitric oxide and other oxidizing agents produced by phagocytes. Likewise, life outside of the host is far from being stress-free. Once expelled from the host, and thus from a nutrient-rich environment, cells have to adjust to a life in a nutrient-limited milieu, abundant in potentially toxic metals and organic solvents, while coping with thermal stresses caused by temperature fluctuations. With so many factors against them, one might wonder how these organisms manage to survive. The answer lies in complex and elaborately controlled transcriptional and translation networks, some of which overlap to provide simultaneous protection against several different stress stimuli, which give these organisms the resilience to endure even under the most challenging of conditions.

1.1. Heat Shock Response

The fact that exposure to high temperatures leads to the accumulation of a specific subset of cellular proteins was serendipitously discovered in 1962 by Ferrucci Ritossa of the International Laboratory of Genetics and Biophysics in Naples, Italy. He observed a pattern of chromosomal puffing in fly's salivary glands following exposure to temperatures higher than the temperature at which normal growth and development occur. The puffing occurred a minute or two after the administration of heat shock and continued to increase in size for as long as 30 to 40 minutes (Ritossa, 1962). Two years later, it was shown that these chromosomal puffs represent actively transcribed genes (Pelling, 1964). Thus, the stage was set for the discovery of heat shock genes and of one of the most important classes of proteins – molecular chaperones.

Higher than physiological temperatures cause proteins to denature, in other words, to lose their proper structure and activity. A properly folded protein buries its hydrophobic patches inside of its structure, away from the solvent. Once the protein is denatured, its hydrophobic patches are exposed, which can lead to their self-association or association with hydrophobic regions of other denatured proteins. Interactions between hydrophobic patches prevent a protein from reaching its native conformation and may lead to formation of large, non-functional protein aggregates. Molecular chaperones are a class of proteins which help unfolded proteins achieve their proper conformation by binding to their solvent-exposed hydrophobic segments thus preventing their self-association while allowing for productive inter- and intra-molecular interactions. Once released from the chaperone, unfolded protein has an opportunity to reach its native conformation (Hartl and Hayer-Hartl, 2002). Molecular chaperones were first discovered as being heat-inducible and are therefore classified as heat shock proteins. However, they are not only important in the cellular response to heat shock, rather their induction is a common characteristic of a variety of stress response mechanisms since many stresses adversely affect folding status of proteins.

1.1.1. Regulation of cytoplasmic heat shock response

An organism's enzymes, required for cellular proliferation and maintenance, have evolved to function optimally at the organism's physiological growth temperature. For *Escherichia coli*, the optimal growth temperature is 37°C - temperature of its host cells. At a given temperature optimum, many proteins are marginally more stable in their folded form than in their unfolded form (Dill, 1990). Therefore, even slight temperature deviations above the optimum can lead to denaturation of enzymes and to a subsequent decline in cellular functions, which ultimately results in death if an organism does not respond in time. Thus, it is crucial for a cell to be able to sense temperature changes quickly in order to mount an appropriate response. Temperature-induced changes in the

degree of DNA supercoiling, RNA conformation and protein stability serve as cellular thermal sensors (Hurme and Rhen, 1998).

Induction of about 30 proteins in *Escherichia coli* occurs following a shift from 37°C to 42°C (Lemaux *et al.*, 1978; Yamamori *et al.*, 1978). Their heat-stimulated expression is regulated by a specific sigma factor σ^H (aka σ^{32}), which is temperature-regulated on transcriptional, translational and post-translational levels. Sigma factors are bacterial proteins that promote transcription of certain genes by recruiting RNA polymerase (RNAP) core enzyme to specific DNA regions (i.e. promoters) located upstream of genes' transcriptional start sites. Genes belonging to σ^H regulon contain the consensus promoter sequence consisting of a -35 CTTGAA box separated by 13-17 bp from -10 CCCCAT box (Cowing *et al.*, 1985). *E. coli* strains lacking the σ^H gene, *rpoH*, are extremely temperature sensitive with a maximal growth temperature of 20°C. Additionally, *rpoH* mutants lack the ability to induce production of heat shock proteins following temperature upshift (Zhou *et al.*, 1988). Transcription of *rpoH* gene is regulated by at least four different promoters. Three of these promoters are recognizable by the housekeeping RNAP- σ^D complex, while the fourth promoter is transcribed only under severe temperature conditions (45 to 50°C) by RNAP- σ^E , where σ^E is a sigma factor involved in envelope stress response (Erickson *et al.*, 1989; Erickson and Gross, 1989; Yura *et al.*, 2000).

Despite the fact that the levels of *rpoH* mRNA in *Escherichia coli* are high even in the absence of thermal stress, RpoH protein levels are kept low through a combined action of translational repression and protein instability. Under stress-free conditions, region A of *rpoH* mRNA, spanning the area from nucleotide 6 to 20 and containing a translational enhancer sequence, base-pairs with region B located 100 nucleotides downstream. This base-pairing results in a formation of a secondary stem-loop mRNA structure, which obscures ribosomal binding site and the start codon and thus inhibits translation (Kamath-Loeb and Gross, 1991; Nagai *et al.*, 1991; Lund, 2001). Exposure of cells to elevated temperatures is thought to lead to dissolution of the secondary

mRNA structure, which in turn results in efficient translation. Temperature melting profiles of *rpoH* transcript combined with primer extension inhibition (aka toe-print) analysis support this view (Morita *et al.*, 1999).

On a post-translational level, the stability of σ^H is greatly enhanced immediately upon the temperature upshift and remains so for 4 to 5 minutes (Straus *et al.*, 1987). This heat-induced instantaneous stabilization accounts for the observed immediate induction of heat shock proteins following exposure to elevated temperatures. Molecular chaperone system DnaK-DnaJ-GrpE has been implicated in regulation of σ^H stability (Straus *et al.*, 1987; Craig and Gross, 1991; Bukau, 1993). Under stress-free conditions, the level of misfolded cellular proteins is low, and thus there is an excess of available molecular chaperones. As a result, DnaK, facilitated by DnaJ and ATP hydrolysis, binds to σ^H and sequesters it from RNAP. Association of σ^H with DnaK seems to lead to its degradation by the membrane-associated protease FtsH, as well as by the cytoplasmic proteases HslVU, ClpAP and Lon (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995; Kanemori *et al.*, 1997). Accumulation of misfolded proteins following temperature upshift leads to titration of DnaK away from σ^H , which allows σ^H to bind to RNAP and promote transcription of heat shock genes, including DnaK. As DnaK levels increase due to σ^H -mediated transcriptional induction, DnaK is once again able to bind to σ^H and target it for degradation. A recent paper by Guisbert and colleagues implicates GroEL-GroES molecular chaperone pair as also being capable of binding to σ^H *in vivo* and decreasing its activity (Guisbert *et al.*, 2004). Thus, σ^{32} appears to be regulated by a network of molecular chaperones.

1.1.2. Cytoplasmic heat shock proteins

Owing to transient stabilization of σ^H , *E. coli* cells shifted from 30°C to 42°C increase their production of heat shock proteins (HSPs) by 15-20 fold within 5 minutes (Herendeen *et al.*, 1979; Lemaux *et al.*, 1978). As a result, immediately after heat shock, HSPs account for approximately 20% of total cellular proteins (Yura *et al.*, 2000). Heat-

inducible proteins can be grouped into two major categories – molecular chaperones and proteases. As already mentioned, molecular chaperones serve as folding modulators by helping unfolded proteins reach their native conformations. They also play an important role in maintaining proteome integrity under stress-free conditions by promoting proper folding of newly synthesized polypeptides. Proteins denatured beyond help are degraded by cytoplasmic proteases. However, as the genome-wide search of *E. coli*'s proteome has revealed, there is a subset of heat-inducible genes whose function is unknown (Blattner *et al.*, 1997). One can speculate that these genes code for novel molecular chaperones or proteases, or that their products might be involved in as-of-yet uncharacterized heat-inducible pathways.

Table 1.1 lists major families of known *E. coli* cytoplasmic molecular chaperones. They can be further divided into chaperones which promote net folding/unfolding of bound substrates (aka foldases), those that maintain partially folded client proteins on their surface to await the availability of folding chaperones following stress abatement (aka holdases), and those that disentangle thermal aggregates (aka disaggregases) (Mujacic and Baneyx, 2004).

DnaK-DnaJ-GrpE and GroEL-GroES foldases are the best characterized molecular chaperoning systems and are required for cell survival at elevated or all temperatures (Fink, 1999; Gross, 1996). DnaK is a 69-kDa monomer with substrate specificity for heptameric stretches of amino acids composed of a 4-5 residues-long hydrophobic core enriched in leucine and flanked by basic residues. This motif is a common occurrence, appearing every 35 residues on the average protein (Rüdiger *et al.*, 1997). In an unfolded protein, DnaK-binding motifs are solvent-exposed. Hydrolysis of DnaK-bound ATP, triggered by DnaK's co-chaperone DnaJ, accounts for tight binding of DnaK to the exposed hydrophobic domains of client proteins (Karzai and McMacken, 1996). A switch from the ADP- to ATP-bound state, stimulated by GrpE, facilitates substrate release. Ejected proteins either fold into their native conformation, cycle once again through DnaK-DnaJ-GrpE machinery, or are passed on to the GroEL-GroES for subsequent folding (Baneyx and Mujacic, 2004). Nonnative protein intermediates with

sizes up to 60 kDa are given a chance to fold in an infinite dilution environment inside GroEL's hollow cavity composed of two homoheptameric rings stacked back-to-back. The process of substrate capture and release is mediated by the capping of heptameric rings with GroES and by ATP binding and hydrolysis (Hartl and Mayer-Hartl, 2002). Substrates are expelled from GroEL-GroES either in their native form or in a partially folded form which may require further chaperone-assisted cycling. In the case of certain proteins, conversion of X-Pro bonds from a *trans* to a *cis* conformation is the rate-limiting step in folding. Such conversions are catalyzed by peptidyl *cis/trans* isomerases (PPIases) such as trigger factor. Trigger factor, whose substrate pool overlaps that of DnaK (Deurling *et al.*, 2003), also plays an important role in *de novo* protein folding by associating with polypeptides emerging from ribosomes and passing them on to DnaK for further folding assistance (Deurling *et al.*, 1999; Teter *et al.*, 1999; Hoffmann *et al.*, 2006).

Under heat shock conditions, large quantities of unfolded proteins accumulate in the cells, which may lead to titration of DnaK-DnaJ-GrpE and GroEL-GroES folding machineries. Holding chaperones, such as small heat shock proteins IbpA and IbpB and, as has been recently proposed, a novel molecular chaperone Hsp31, maintain partially folded substrates on their surfaces until stress has been abated or until folding chaperones become available (Veinger *et al.*, 1998; Sastry *et al.*, 2002). However, under severe stress conditions, folding and holding chaperones tend to get overloaded which leads to formation of large, insoluble protein aggregates. ClpB, a member of Hsp100 family of heat shock proteins, untangles these aggregates most likely through its crowbar-like action and threads them through its central pore in an ATP-dependent manner (Lee *et al.*, 2003a; Weibezahn *et al.*, 2004; Schlieker *et al.*, 2004). DnaK-DnaJ-GrpE system assists ClpB in this endeavor by fragmenting large aggregates into smaller ones (Zietkiewicz *et al.*, 2006), and also by associating with ClpB-threaded proteins to facilitate their refolding attempts (Goloubinoff *et al.*, 1999; Zolkiewski, 1999; Weibezahn *et al.*, 2005).

If numerous attempts at folding fail, aggregated proteins are cleared from the stressed cells through the action of the following ATP-dependent cytoplasmic proteases: Lon, ClpYQ (HslUV), ClpAP, ClpXP, and FtsH (HflB) (Miller, 1996; Mujacic and Baneyx, 2004). Lon and ClpYQ appear to play a central role in the degradation of thermosensitive proteins that accumulate following heat shock (Tomoyasu *et al.*, 2001; Rosen *et al.*, 2002; Mujacic and Baneyx, 2004). ClpA, which belongs to the Hsp100 family of heat shock proteins and assembles as a hexamer in the presence of Mg-ATP, associates with one of the two heptameric rings of the proteolytic component ClpP. ClpAP and ClpXP, which exhibit distinct substrate specificities, are involved in degradation of proteins that have been modified at their C-terminals by the addition of a non-polar tail through the action of the SsrA system (Gottesman *et al.*, 1998). In addition to their role during heat shock, Clp proteases also appear to be important in stationary phase adaptation of *E. coli* (Weichart *et al.*, 2003). Unlike the rest of cytoplasmic proteases, FtsH is associated with the cytoplasmic membrane via its N-terminal domain, while its active site is located in the cytoplasm (Tomoyasu *et al.*, 1995). Completion of the protein degradation process is believed to involve peptidases which attack 2-5 residue long sequences (Miller, 1996).

1.1.3. Heat shock response in cellular envelope

In addition to the inner, cytoplasmic lipid bilayer, *E. coli*, like its gram-negative relatives, possesses an outer membrane composed of phospholipids, murein lipoproteins, lipopolysaccharides and membrane-embedded protein channels and pores. Viscous, peptidoglycan and the protein-rich periplasmic region fills the 13-25 nm-thick space between the inner and the outer membranes. The diffusion rate in this dense cellular region is predicted to be 100-fold lower than that in the cytoplasm (Brass *et al.*, 1986). Proteins destined for periplasm and outer membrane are produced with specific polypeptide signal sequences which target them to one of the three main (Sec-, Tat- and SRP-dependent) export pathways (Mujacic and Baneyx, 2004). Like their cytoplasmic

counterparts, periplasmic and membrane proteins are prone - maybe even more so than cytoplasmic proteins due to such high protein crowding in the periplasm - to thermal stress-induced misfolding and aggregation. Periplasmic heat shock response is distinct from the cytoplasmic response, and it involves the induction of periplasm-specific molecular chaperones, proteases and PPIases.

Periplasmic heat stress response is controlled by the alternative sigma factor σ^E (or σ^{24}) and by the two component regulatory pair made up of sensor histidine kinase CpxA and the response regulator CpxR (Raivio and Silhavy, 2000). Sigma 24 responds to the presence of misfolded outer membrane or periplasmic proteins but not to the misfolding in the cytoplasm (Meccas *et al.*, 1993). As was the case with σ^H , σ^E levels are finely tuned through the action of several periplasmic and inner membrane proteins. In the absence of stress, σ^E is sequestered by the inner membrane anti-sigma factor protein RseA, whose N-terminal and C-terminal domains are located in the cytoplasm and periplasm, respectively. It has been postulated that additional binding of periplasmic protein RseB ensures an even tighter interaction between RseA and σ^{24} (De Las Peñas *et al.*, 1997; Missiakas *et al.*, 1996). Temperature-induced misfolding of outer membrane proteins leads to the removal of RseB and to DegS-mediated degradation of RseA (Walsh *et al.*, 2003), which results in the release of σ^E and its subsequent association with RNAP. RNAP- σ^E is thought to regulate expression of at least 11 genes, among them *rpoH*, *rseA*, *rseB* and those coding for periplasmic chaperones and proteases (Raina *et al.*, 1995; Rouviere and Gross, 1996).

CpxA/R pathway responds to disturbances in envelope protein biogenesis and possibly to accumulation of misfolded proteins in the inner membrane (Raivio and Silhavy, 2000). Under physiological conditions, histidine kinase CpxA, which is anchored in the inner membrane via two transmembrane α helices (Weber and Silverman, 1988), is in a phosphatase state with its periplasmic domain likely engaged in an interaction with CpxP. Stress-caused disruption in the assembly of outer membrane pili results in the accumulation of misfolded protein subunits on the periplasmic side of

the inner membrane. As a result, CpxA switches to its kinase mode and phosphorylates response regulator CpxR. In turn, CpxR stimulates expression of periplasmic genes involved in the folding and the assembly of pili and periplasmic PPIases. Transcription of *cpxA*, *cpxR* and *cpxP* genes is also stimulated (Raivio and Silhavy, 1997; Raivio and Silhavy, 2000).

1.1.4. Cell envelope heat shock proteins

As is the case with cytoplasmic heat shock proteins, thermal stress in the periplasm induces an array of folding modulators and proteases. One important characteristic distinguishes periplasmic heat shock proteins from their cytoplasmic counterparts – their ATP-independence caused by the absence of a periplasmic ATP pool. The majority of periplasmic chaperones and PPIases act in a specialized manner to promote proper folding and assembly of the specific outer membrane proteins (Table 1.2). For example, SurA specifically recognizes Ar-X-Ar (where Ar stands for aromatic ring and X for any amino acid) motifs characteristic of outer membrane proteins and facilitates their maturation (Bitto and McKay, 2003). Generic chaperones Skp and FkpA have a broader substrate range with FkpA acting as a dual chaperone/isomerase folding modulator (Walton and Sousa, 2004; Missiakas *et al.*, 1996; Arie *et al.*, 2001).

Along with the lack of an ATP pool, another hallmark of the periplasmic space is its oxidizing, disulfide bond friendly environment. Thus, the periplasm contains a set of proteins involved in disulfide-bond catalysis (Table 1.2). Twenty one kDa monomeric DsbA, which is Cpx-regulated and is present in an oxidized form *in vivo*, facilitates disulfide bond formation in the periplasm and thus becomes reduced. The inner membrane protein DsbB, with two cysteine-containing periplasmic loops, maintains DsbA in an oxidized form. Isomerization of incorrect disulfide bonds in proteins with more than two cysteines is handled by the periplasmic disulfide bond isomerase DsbC and its inner membrane-anchored partner DsbD which keeps it in its reduced state (Ritz and Beckwith, 2001). DsbA, DsbC and DsbG also exhibit chaperoning activity,

presumably because a partially folded structure is needed to allow for efficient disulfide formation and isomerization in substrate proteins.

Heat-inducible periplasmic proteases handle degradation of irretrievably misfolded periplasmic proteins. DegP, a member of σ^E regulon, is the major periplasmic protease and is required for cellular survival under heat shock conditions (Oliver, 1996). DegP and its family members - periplasmic proteases DegQ and DegS - have similar structural features consisting of a N-terminal segment believed to have a regulatory function, a conserved trypsin-like protease domain, and one (DegS) or two (DegP and DegQ) PDZ domains (Clausen *et al.*, 2002). The proteolytic site of DegP is located inside its hexameric cavity and is shielded by mobile side walls formed by the PDZ domains which appear to regulate access to the protease chamber (Krojer *et al.*, 2002; Jones *et al.*, 2002). An interesting feature of DegP is its ability to switch function at low temperature (28°C) from a protease to a chaperone (Spiess *et al.*, 1999). Additional envelope proteases with specific recognition motifs include DegS, DegQ, protease III and OmpT (Mujacic and Baneyx, 2004). In addition to its role in heat shock regulation of σ^E , DegS recognizes misfolded proteins by binding to Y-Q-F and Y-X-F motifs commonly found at the C-terminus of immature outer membrane porins (Walsh *et al.*, 2003). DegQ cleaves substrates at V/I-X locations (Waller and Sauer, 1996), while protease III prefers short peptide chains, but is also capable of degrading proteins with abnormal structures (Baneyx and Georgiou, 1991). Finally, OmpT exhibits a preference for paired basic residues (Miller, 1996).

1.2. Acid Stress

E. coli, a neutrophile, is capable of surviving in an extremely acidic (pH 1.5 to 2.5) gastric environment of its mammalian host. Compared to the acid-sensitive prokaryote *Vibrio cholera* which has an infectious dose of 10^6 - 10^{11} organisms, *E. coli*'s infectious dose (defined as the amount of the organism that needs to be consumed in order to give rise to disease symptoms) can be as low as 10 organisms (Boyd, 1995). What sets *E.*

coli apart from acid-sensitive prokaryotes is the presence of at least three cellular pathways specialized in battling against acidic shock. *E. coli*'s three acid resistant systems are stationary phase-dependent and are expressed in both laboratory, non pathogenic strains, as well as in the infamous *E. coli* pathogenic strain O157:H7 (McClure and Hall, 2000).

1.2.1. pH homeostasis vs. acid stress survival

Enteric organisms strive to maintain their internal pH in a 7.6-7.8 range even if the pH of the external environment fails to remain constant (Foster, 2000). Small fluctuations (1 pH unit or less) in the internal pH of bacterial cells are handled by pH homeostasis mechanisms such as antiporters. Potassium/proton antiporters handle pH homeostasis as the surrounding environment becomes more acidic by extruding H^+ ions from the cells while taking in K^+ ions. On the other hand, shifts to alkaline environments stimulate Na^+/H^+ antiporters (Zilberstein *et al.*, 1984). pH adjustments through homeostatic mechanisms are thought to occur through the activation of existing cellular components rather than through the induction of their synthesis (Foster, 2000; Zilberstein *et al.*, 1982). However, dealing with large changes in pH is beyond housekeeping mechanisms' capabilities and necessitates induction of cellular systems specialized in handling pH emergencies. Several such systems have been described in *E. coli* and its relative *Shigella flexneri*. Acid habituation measures the ability of cells pre-grown until the exponential (or log) phase of growth in a mildly acidic medium to survive a short, 7-minute exposure to pH 3 complex medium. Acid tolerance response or ATR (aka log phase acid tolerance) refers to the capacity of pre-adapted log phase cells to survive in pH 3 medium for several hours. Finally, the ability of stationary phase-grown cells to survive acidic challenges has been termed acid resistance. Whereas acid habituation and ATR do not protect cells at pH values lower than 3, stationary phase-induced acid resistance systems offer cells the most dramatic acid stress protection by allowing them to survive at pH 2 for several hours (Foster, 2000; Richard and Foster, 2003).

1.2.2. Acid resistance systems of *E. coli*

So far, three distinct stationary phase-induced acid resistance systems have been described in *E. coli* (Foster, 2004). Acid resistance 1 (AR1) system, also called oxidative system, is glucose-repressed and dependent on an alternate sigma factor σ^S (aka σ^{38}) and cAMP receptor protein CRP. This system is activated in the stationary phase of cells grown in a mildly acidic complex medium such as LB in the absence of glucose. Cells grown in this manner are capable of surviving in minimal glucose medium with pH of 2.5. However, mild acidic induction during stationary growth does not seem to be required for stimulation of this system since it has been shown that AR1 is induced upon the entry into the stationary phase of growth irrespective of growth medium's pH (Castanie-Cornet *et al.*, 1999). Of the three AR systems, AR1 is the least understood. It appears to be the key element for the survival during acid stress above pH 3, however its components have yet to be identified (Audia *et al.*, 2001). There is a link between F_0/F_1 proton translocating ATPase and AR1, but the mode of action of this complex in AR1 has not yet been elucidated (Richard and Foster, 2003). It has been postulated that it prevents intracellular accumulation of H^+ ions by pumping them out of the cell while consuming ATP, as this is the way in which *Streptococcus spp.* battle acid stress (Foster, 2000). Two other components implicated in AR1 are the products of *cfa* and *hdeA* genes. The *cfa* gene codes for cyclopropane fatty acid (CFA) synthase which dictates membrane fatty acid content and whose expression is induced upon the entry into the stationary phase of growth (Wang and Cronan, 1994). It has been postulated that Cfa-driven increase in membrane CFAs might decrease permeability of the outer membrane to protons. On the other hand, HdeA appears to be acid-inducible periplasmic molecular chaperone. During stationary phase of growth in rich medium, HdeA is one of the most abundant periplasmic proteins (Link *et al.*, 1997)). Acidic pH-stimulated dimer to monomer transition activates HdeA and allows it to suppress aggregation of acid-denatured proteins (Gajiwala and Burley, 2000). Deletion of *hdeA* gene in *S. flexneri* results in an acid-sensitive phenotype (Waterman and Small, 1996).

The second and the most protective acid resistance system is glutamate-dependent. AR2 is also the best characterized of the three systems. It is induced in cells during stationary phase of growth in complex medium supplemented with glucose (which represses AR1). Such cells endure pH 2 challenge in minimal glucose medium when exogenous glutamate is present. The components of AR2 are glutamate decarboxylases GadA and GadB and the antiporter GadC (Hersh *et al.*, 1996). Decarboxylases convert glutamate to γ -amino butyric acid (GABA) which consumes one intracellular H^+ ion and releases carbon dioxide (Figure 1.1). Since γ -carboxyl group of glutamate should be fully protonated in pH 2-2.5 extracellular environment and would deprotonate upon the entry into the less acidic cytoplasm and thus create a futile proton cycle, it has been postulated that the extra H^+ ion is removed in the periplasm during glutamate's transport into the cytoplasm (Richard and Foster, 2003). GadC antiporter transports GABA out of the cell while simultaneously replenishing cytoplasmic glutamate concentration. Exported GABA might offer acid stress relief by acting as a proton acceptor in the periplasm and the immediate extracellular space (Foster, 1993). At pH 2.5, the two decarboxylases are interchangeable, i.e. the presence of either GadA or GadB with GadC is enough to confer acid resistance (Castanie-Cornet *et al.*, 1999). However, at pH 2, both GadA and GadB are required for survival. Both GadA and GadB have pH optima of 3.8 which makes them highly functional under acid stress when cytoplasmic pH is known to drop down to 4.2 ± 0.1 (Smith *et al.*, 1992; Richard and Foster, 2004). Regulation of AR2 is complex and involves 11 known regulators with GadE acting as the central activator under all conditions (Foster, 2004). In cells grown in LB, AR2 is induced only during the stationary phase of growth and this induction is regulated by CRP, σ^S , GadX and GadW. In minimal medium, AR2 can be induced during the exponential phase of growth through the action of AraC-like regulator YedO and two-component signal transduction system EvgAS (Masuda and Church, 2003). A third AR2 regulatory circuit has been recently discovered. It is regulated by GTPase protein TrmE and is only observed under the growth in LB medium with glucose (Cabedo *et al.*, 1999; Gong *et al.*, 2004).

The third acid resistance system is similar in its mode of action to AR2. It is induced in low pH complex medium under anaerobic conditions or following growth in brain heart infusion broth supplied with 0.4% glucose (Castanie-Cornet *et al.*, 1999). Its structural components are arginine decarboxylase AdiA and arginine:agmatine antiporter AdiC (Foster, 2004). As was the case with AR2, decarboxylation of arginine to agmatine consumes one H^+ ion and releases carbon dioxide (Figure 1.1). Agmatine, unlike GABA, cannot provide any extracellular buffering since it is fully protonated at pH 4.5, which is the cytoplasmic pH under acid stress conditions in cells with an engaged AR3 (Dawson *et al.*, 1979; Richard and Foster, 2003). Regulation of AR3 has not been as thoroughly researched as that of AR2 but, nonetheless, appears to be as complex. CysB is the main activator with additional regulation provided by σ^S , AraC-like regulator AdiY, histone-like protein HNS, integration host factor, RpoA and CRP (Shi and Bennett, 1994).

1.2.3. Proposed mechanism of cellular defense against acid stress via AR1 or AR2

Measurements of *E. coli*'s membrane potential under acidic conditions in the presence or absence of glutamate or arginine led to several interesting observations. Membrane potential of *E. coli*, which is kept at -90 mV in cells growing at pH 7, is around 0 mV in pH 2.5 environment in the absence of glutamate or arginine and +30 mV in the presence of one of the amino acids (Richard and Foster, 2004). Membrane potential of 0 mV is thought to be due to the loss of membrane integrity. But why such hyperpolarization in cells with engaged AR2 or AR3? The observed membrane potential flip is a strategy employed by acidophiles as a survival method in low pH environments; positive membrane potential repulses H^+ ions and therefore slows their entry into the cells. A plausible explanation for the similar phenomenon observed in *E. coli* was proposed by Foster. He postulates that the influx of H^+ ions and accumulation of positively charged decarboxylation products of AR2 or AR3 account for this switch in the membrane potential. ClC H^+/Cl^- antiporters, which were shown to be important in AR2 and AR3

(Iyer *et al.*, 2003), might act as modulators of the positive membrane potential by allowing Cl^- ions in while letting protons out. As acid stress abates, decarboxylation will slow down and eventually stop, positively charged GABA or agmatine will be exported out of the cell while $\text{Cl}^- \text{H}^+/\text{Cl}^-$ antiporters will import more Cl^- ions. As a result, membrane potential should depolarize back to its pre-stress value of -90 mV (Foster, 2004).

1.2.4. In search of new acid resistance components

Salmonella enterica serovar Typhimurium contains all of the components of AR3 system but is incapable of surviving in a pH 2.5 environment. This implies that cellular factors other than the essential components of AR2 and AR3 are needed for cells to mount an effective resistance to acid stress. Therefore, several groups have used gene expression and proteomic profiling to identify these additional factors (Blankenhorn *et al.*, 1999; Rallu *et al.*, 2000; Tucker *et al.*, 2002; Yohannes *et al.*, 2005). The majority of acid-inducible genes have no assigned function. Many of them form chromosomal clusters, with one of the biggest clusters located at 78 minutes on *E. coli*'s chromosome (Foster, 2004). Some of the genes induced during acid stress are also known to be associated with other types of cellular stress responses such as oxidative stress or heat shock (Blankenhorn *et al.*, 1999). This is not a surprising finding since one of the regulators of acid stress is σ^S , a sigma factor implicated in general stress response (Hengge-Aronis, 2000). Another category of acid-inducible genes is composed of genes involved in handling of protein and DNA damage, which presumably allows stressed cells to cope with acid-inflicted damage while also providing them with a degree of cross-protection against other types of environmental stresses.

1.3. Oxidative Stress

Oxidative stress is one of the inherent dangers of aerobic life. Aerobic organisms use oxygen as their terminal electron acceptor in the electron transport chain. Occasionally,

molecular oxygen accidentally oxidizes electron carriers, which leads to formation of two highly reactive oxygen intermediates - superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Imlay, 2003). In *E. coli*, the respiratory chain can account for up to 87% of total intracellular H_2O_2 production (González-Flecha and Demple, 1995). Furthermore, *E. coli* walks a tight rope between oxidative stress and homeostasis. It produces just enough of protective enzymes to maintain intracellular O_2^- and H_2O_2 concentrations at 10^{-10} M and 10^{-7} to 10^{-6} M, respectively, which is just beneath the toxicity levels of these reactive oxygen species (González-Flecha and Demple, 1995; Gort and Imlay, 1998). In addition to the endogenous pool of superoxide anions and hydrogen peroxide, bacteria have to battle various forms of oxidative damage inflicted on them by plants, their mammalian hosts, and microbial competitors. For example, mammalian phagocytes attack bacteria by showering them with O_2^- , nitric oxide (NO) and hyperchlorous acid (HOCl), and with their chemical by-products H_2O_2 , hydroxyl radicals (HO), peroxynitrite (HOONO) and nitrosothiols (RSNO) (Storz and Imlay, 1999). Therefore, bacterial cells have evolved to contain elaborate and finely-tuned systems for management of oxidative stress.

1.3.1. Types of damage inflicted by oxidative stress

Cellular toxicity of superoxide anions is in large part due to their ability to disrupt the activity of proteins with iron-sulfur clusters [4Fe-4S]. O_2^- oxidizes catalytic iron atom in such clusters which results in cluster instability and degradation and, subsequently, in the loss of enzymatic activity (Imlay, 2003). Proteins with dehydratase activity employ such clusters. Therefore, it is not surprising that cells exposed to oxidative stress exhibit damage in pathways involving dehydratases, such as control of spontaneous mutagenesis, growth in the absence of amino acid supplements, and catabolism of non-fermentable carbon sources (Carlioz and Touati, 1986). Disruption of iron-sulfur clusters has an additional negative side-effect of releasing Fe^{2+} atoms into the cytosol. Fenton reaction of H_2O_2 and Fe^{2+} produces hydroxyl radicals which are potent DNA

mutagens. DNA mutagenesis is what ultimately leads to cell death after prolonged exposure to H_2O_2 . Ten minute exposure of cells to millimolar levels of H_2O_2 is enough to result in mutagenesis-induced death (Storz and Imlay, 1999; Imlay, 2003). In addition to its link with DNA damage, H_2O_2 is also capable of disrupting [4Fe-4S] clusters, but with slower reaction rates (Imlay, 2003).

Concurrent with the disruption of certain protein groups (i.e. dehydratases), oxidative stress leads to general protein damage due to the ability of oxidizing species to modify amino acids in various ways. For example, H_2O_2 and NO are very efficient in oxidizing protein thiols, which leads to disulfide bond formation, or to creation of sulfinic acid moieties (Storz and Imlay, 1999; Radi *et al.*, 1991). Under stress-free conditions, *E. coli*'s cytoplasm is a highly reducing environment, thus majority of cytoplasmic proteins maintain their cysteine residues in their native (i.e. reduced) form. Therefore, a switch from a reducing cytoplasmic environment to an oxidizing one has profound effects on folding and activity of proteins with cysteinyl residues (Åslund *et al.*, 2001). Another common modification of amino acids under oxidative stress is their OH-mediated oxidation to reactive carbonyl derivatives (Stadtman, 1993). The ability of carbonyl moieties to react with dinitrophenylhydrazine has been exploited to directly measure oxidative stress in cells, as well as to identify individual proteins susceptible to oxidative modifications (Shacter *et al.*, 1994; Tamarit *et al.*, 1998). In addition to carbonylation and disulfide bond formation, the laundry list of protein modifications stimulated by oxidative stress includes increase in acidity, decrease in thermal stability, fragmentation, formation of protein-protein cross-links, changes in fluorescence, and increase in proteolytic susceptibility (Cabedo *et al.*, 1999).

1.3.2. Oxidative stress regulons

E. coli has two main regulons which respond to oxidative stress: *oxyR* and *soxRS* (Table 1.3). Transcriptional activator OxyR is a tetrameric protein with two cysteine residues per monomer (Christman *et al.*, 1989). In the absence of oxidative stress, glutaredoxin 1

catalyzes reduction of OxyR by glutathione. However, elevated levels of H_2O_2 (or RSNO) lead to oxidation of cysteine residues and to disulfide bond formation between Cys199 and Cys208 residues of each monomer (Zheng *et al.*, 1998). Oxidized OxyR is active and able to induce transcription of the members of its regulon. Protein products of OxyR-inducible genes either act to directly eliminate oxidizing agents or are involved in protecting the integrity of proteins and DNA (Table 1.3). Hydroperoxidase (KatG) reduces H_2O_2 to water and oxygen, while alkyl hydroperoxide reductase (AhpCF) functions as the primary scavenger of H_2O_2 (Loewen *et al.*, 1985; Seaver and Imlay, 2001). Induction of ferric reductase (Fur) results in repression of ferric ion uptake, which indirectly lowers OH levels by making less iron available for Fenton reaction (Zheng *et al.*, 1999). On the other hand, glutathione reductase (GorA), glutaredoxin 1 (GrxA) and thioredoxin 2 (TrxC) work to rectify oxidation-caused protein damage by removing improper disulfide bonds (Ritz and Beckwith, 2001). Finally, Dps binds to DNA and protects it from hydroxyl radical-induced damage (Wolf *et al.*, 1999). Considering that Dps is a ferritin homolog, it may also provide indirect DNA protection by sequestering iron (Grant *et al.*, 1998).

The second major oxidative stress responsive regulon is stimulated by O_2^- and is controlled by SoxR and SoxS transcriptional factors. SoxR is a homodimer with two $[\text{2Fe-S}]$ clusters (Hidalgo and Demple, 1994; Hidalgo *et al.*, 1995). In the absence of oxidative stress, each cluster has a +1 charge. Oxidation of two clusters leads to activation of SoxR (Storz and Zheng, 2000). It is not yet clear whether O_2^- directly oxidizes the clusters or whether alterations in NADPH, reduced flavodoxin or ferredoxin levels lead to oxidation (Storz and Imlay, 1999). Activated SoxR induces expression of SoxS, which induces expression of the regulon. *soxRS* regulon contains more genes (so far) than *oxyR* regulon (Table 1.3). Some of the *soxRS*-regulated genes are manganese superoxide dismutase (SodA) which converts O_2^- to H_2O_2 , DNA repair enzyme endonuclease IV (Nfo), aconitase (AcnA), and O_2^- -resistant isoform of fumarase (FumC). Induction of a couple of membrane-associated proteins (TolC, ArcAB) and an outer membrane protein regulator (MicF) reflects cells' efforts to stave off oxidative

damage by excluding compounds capable of generating O_2^- species (Storz and Zheng, 2000).

As is evident from Table 1.3, many genes that are induced under oxidative stress are not under OxyR or SoxRS control. Among these genes are a couple of superoxide dismutases, DNA repair enzymes and molecular chaperone Hsp33 (*hslO* gene), to name a few. Hsp33 is unique among molecular chaperones based on its mode of activation. In the absence of stress, the protein is present in a reduced, monomeric form with a zinc ion coordinated by four of its cysteine residues. However, in the presence of heat and oxidative stress, zinc is ejected and two intramolecular bonds are formed, which leads to dimerization of Hsp31 and its subsequent activation as a molecular chaperone (Jakob *et al.*, 1999).

An interesting characteristic of many antioxidant genes is the control of their expression by more than one regulator. For example, the majority of genes belonging to *soxRS* regulon are also regulated by MarA and Rob transcriptional factors (Storz and Zheng, 2000). MarA is involved in regulation of genes involved in antibiotic resistance (Miller and Sulavik, 1996). Another regulator common to many antioxidant genes is the stationary phase and general stress sigma factor σ^S , which has been shown to regulate expression of at least eight antioxidant genes (Table 1.3). Involvement of σ^S in regulation of antioxidant genes is a variation on a theme since this sigma factor has been implicated in regulation of genes involved in the defense against osmotic and acidic stresses, among others. It is therefore not surprising that starved, stationary phase cells are intrinsically resistant to high levels of H_2O_2 (Loewen *et al.*, 1998).

1.4. σ^S and the General Stress Response

E. coli cells exhibit four distinct stages of growth that can be easily observed during propagation in a nutrient-rich broth. Dilution of cells into a fresh medium triggers the onset of the lag phase of growth during which cells do not divide but attempt to acclimate to the new environment. After the initial adjustment period, cells enter the

exponential or log phase of growth in which they grow and rapidly divide with a doubling time of approximately 20 minutes. Media exhaustion (i.e. depletion of carbon sources) and the build-up of toxins excreted during the log phase prompt the entry into the stationary phase of growth during which cells become dormant and exhibit no net growth. Prolonged exposure to nutrient-limited and toxin-rich environments leads to cell death.

E. coli stationary phase cells exhibit several interesting characteristics. They forgo their rod shape and assume smaller, ovoid appearance (Loewen and Hengge-Aronis, 1994), while the volume of periplasm increases (Ishihama, 1997). The major characteristic that sets apart stationary phase cells from their rapidly dividing exponential phase counterparts is their ability to survive under the variety of adverse environmental conditions, from high osmolarity and acidic pH to oxidative stress and nonoptimal growth temperatures. Unlike specific stress responses, such as heat shock or oxidative stress, which induce genes whose products help cells repair stress-caused damage, general stress response acts in a preventive manner by allowing cells to avoid the damage rather than to deal with its consequences. The general stress resistance characteristic of stationary phase cells has been attributed to the alternative sigma factor σ^S (or RpoS). It is the second most important bacterial sigma factor, after vegetative sigma factor σ^D , and is the master regulator of the general stress response (Hengge-Aronis, 2000).

1.4.1. σ^S and its hierarchy of regulation

E. coli's σ^S , the product of the *rpoS* gene, is a 38 kDa protein (thus also known as σ^{38}) that exhibits a significant level of similarity to the housekeeping sigma factor σ^D . Its homologs have been identified in a variety of gram-negative bacteria, many of which are pathogenic (Hengge-Aronis, 2000). σ^S is virtually undetectable in rapidly dividing, exponential phase cultures of *E. coli*. However, a switch from the log to the stationary phase of growth or the exposure to various environmental stresses elevates cellular σ^S

levels. Because of its importance under a variety of growth conditions, regulation of σ^S is complex and occurs on all possible levels – transcriptional, translational and post-translational – with different environmental stimuli affecting different parts of the regulatory circuit (Figure 1.2). The involvement of many cellular factors in regulation of σ^S makes a tangled web of its regulatory circuit (Table 1.4).

The *rpoS* mRNA is transcribed from several different promoters. *nlpDp1* and *nlpDp2* promoters of *nlpD* gene, which is located upstream of *rpoS* gene, produce a polycistronic *nlpD-rpoS* transcript and account for a small fraction of *rpoS* transcription (Lange *et al.*, 1995; Lange and Hengge-Aronis, 1994b). On the other hand, *rpoSp* promoter, located within *nlpD* gene, is the main source of *rpoS* mRNA. Even though all three promoters synthesize mRNA during the exponential phase of growth, *rpoSp* is the only promoter stimulated by the entry into the stationary phase of growth (Lange *et al.*, 1995; Takayanagi *et al.*, 1994). Among the *trans*-acting factors that affect *rpoS* transcription is cAMP-CRP, which has been shown to act as a negative regulator during the exponential phase of growth and as a positive regulator upon the entry into the stationary phase of growth (Lange and Hengge-Aronis, 1994a; McCann *et al.*, 1991; Hengge-Aronis, 2002a), guanosine-3',5'-bipyrophosphate (ppGpp), which positively regulates transcription probably through its effect on transcriptional elongation (Gentry *et al.*, 1993; Lange *et al.*, 1995), and polyphosphate, which has been shown to promote stationary phase transcription of *rpoS* *in vivo* but not *in vitro*, thus implying indirect influence (Shiba *et al.*, 1997).

One of the first indications of possible post-transcriptional regulation of *rpoS* came from the observation that while osmotic shock increased transcription of σ^S -dependent genes, there was no significant induction in *rpoS* transcription (Hengge-Aronis *et al.*, 1993). Subsequent studies showed that elevated levels of σ^S observed under such conditions as acidic pH, low growth temperature, hyperosmolarity and reaching of a certain minimal optical density during the late phase of exponential growth were caused by stimulation of *rpoS* translation (Hengge-Aronis, 2002a; Sledjeski and Gottesman, 1995; Lange and Hengge-Aronis, 1994a). Translational silencing of *rpoS* mRNA under

the stress-free conditions during the exponential phase of growth is thought to be due in part to the formation of a secondary mRNA structure involving the translational initiation region (TIR). Computer-predicted models suggest that *rpoS* mRNA forms an extensive secondary structure in which TIR is inaccessible to the ribosomes (Lange and Hengge-Aronis, 1994a). Based on this, it has been postulated that various stress conditions dissolve the secondary structure, which leads to active translation (Hengge-Aronis, 2000). In addition to the *cis*-acting element of translational regulation, several cellular factors have also been implicated in exerting control over *rpoS* translation (Hengge-Aronis, 2002a). Among the positive regulators of translation are RNA-binding protein Hfq and a small regulatory RNA DsrA. Hfq is required for efficient *rpoS* translation (Brown and Elliott, 1996; Muffler *et al.*, 1996b), while DsrA is required for low temperature induction of σ^S (Sledjeski and Gottesman, 1995; Sledjeski *et al.*, 1996). Modes of action of these two proteins are not completely clear. Hfq seems to bind to *rpoS* mRNA and promote its translation (Hengge-Aronis, 2002a), while it is likely that DsrA, which is partially complementary to the part of *rpoS* RNA that base-pairs with TIR, stimulates *rpoS* translation via an anti-antisense mechanism. On the other hand, OxyS regulatory RNA, which is a member of the *oxyR* antioxidant regulon, acts as a negative regulator, most likely by creating a translationally incompetent complex with Hfq and *rpoS* (Zhang *et al.*, 1998). Among other negative translational regulators are histone-like protein H-NS, a negative regulator of DsrA expression LeuO, and UDP-glucose (Hengge-Aronis, 2002a). The precise modes of action of the majority of these translational regulators and their connections with environmental signals known to induce *rpoS* translation remain yet to be elucidated.

Upon the onset of starvation, translation of *rpoS* mRNA, like those of the majority of other cellular mRNAs, is reduced (Lange and Hengge-Aronis, 1994a; Hengge-Aronis, 2000). However, σ^S levels continue to increase. This apparent discrepancy was explained by the observation that σ^S stability rapidly increases in carbon-starved, heat-, acid- and hyperosmotically-shocked cells (Takayanagi *et al.*, 1994; Muffler *et al.*, 1997; Bearson *et al.*, 1996; Lange and Hengge-Aronis, 1994a). During the exponential

growth, σ^S is synthesized but its half-life is only 1-4 minutes because it is actively degraded by ClpXP protease (Hengge-Aronis, 2000). Response regulator RssB acts as an accessory protein in degradation of σ^S , and its presence is essential for efficient proteolysis by ClpXP (Bearson *et al.*, 1996; Muffler *et al.*, 1996a; Pratt and Silhavy, 1996; Becker *et al.*, 1999). Phosphorylated RssB recognizes and binds to K173, E174 and V177 residues of σ^S , termed the turnover element, and thus presents the protein to ClpXP for degradation. RssB may also act to inhibit the ability of σ^S to initiate transcription of the members of its regulon since K173 residue seems to be important in transcription initiation by σ^S (Becker and Hengge-Aronis, 2001). Environmental stimuli are thought to dephosphorylate RssB, which greatly reduces its affinity for σ^S , thus increasing stability of σ^S (Hengge-Aronis, 2000). Molecules involved in phosphorylation and dephosphorylation of RssB have not yet been identified. Finally, several other factors are known to affect σ^S stability. Contrary to its role in σ^H proteolysis, molecular chaperone DnaK increases stability of σ^S during the stationary phase of growth and possibly during heat shock (Muffler *et al.*, 1997; Rockabrand *et al.*, 1998). RssA protein also exhibits a protective role (Muffler *et al.*, 1996a), while H-NS appears to have a dual role in σ^S regulation by promoting σ^S proteolysis in addition to inhibiting translation of *rpoS* mRNA (Barth *et al.*, 1995; Yamashino *et al.*, 1995).

1.4.2. Promoter recognition and transcription initiation by σ^S

Compared to the other sigma factors, σ^S has the lowest affinity for the RNA polymerase core enzyme *in vitro*. Moreover, during the stationary phase of growth, when σ^S is responsible for transcription of a significant fraction of genes, it reaches only about 30% of the cellular level of σ^D (Jishage and Ishihama, 1995; Jishage *et al.*, 1996). One is left wondering how σ^S manages to outcompete other sigma factors for the core enzyme and transcribe any of the members of its regulon. It appears that a combination of specific

promoter elements, involvement of certain *trans*-acting cellular factors and particular changes in DNA topology creates an environment conducive for σ^S -driven transcription.

Unlike other sigma factors which recognize promoter sequences highly divergent from σ^D recognition sequence, -10 consensus sequence recognized by σ^S (TATACT) is almost identical to that σ^D (TATAAT) (Hengge-Aronis, 2002b). However, in terms of promoter specificity, σ^S exhibits a higher level of flexibility than σ^D . Whereas σ^D recognizes promoters with well conserved -10 and -35 regions, σ^S is able to drive transcription from promoters with degenerate -35 boxes situated at non-optimal spacing of 15-19 upstream from the -10 box (Becker and Hengge-Aronis, 2001; Espinosa-Urgel *et al.*, 1996; Typas and Hengge, 2006). Moreover, *in vitro*, σ^S exhibits much higher contact with a promoter's -10 box than with its -35 box (Colland *et al.*, 1999). Therefore, promoters with highly divergent -35 boxes are preferentially recognized by σ^S . Another promoter feature that increases the affinity of σ^S for the promoter is the presence of cytosine at the -13 position of the promoter, which occurs in 80% of σ^S -dependent promoters (Becker and Hengge-Aronis, 2001; Espinosa-Urgel *et al.*, 1996; Weber *et al.*, 2005). This cytosine appears to interact directly with K173 residue located in region 2.5 of σ^S , which has been postulated to make a direct contact with the extended -10 promoter region (Becker and Hengge-Aronis, 2001; Murakami *et al.*, 2002; Murakami *et al.*, 2002b; Vassilyev *et al.*, 2002). Finally, σ^S selectivity can also be increased by the presence of an AT-rich sequence downstream and upstream of the -10 promoter box and, in some cases, by an upstream UP element-like motif, where the UP element is a specific sequence found in strong ribosomal promoters that is known to be contacted by the carboxyl-terminal domain of the α subunit of RNAP (Typas and Hengge, 2006; Hengge-Aronis, 2002b; Estrem *et al.*, 1999; Gourse *et al.*, 2000).

In addition to promoter specificity, several regulatory factors appear to be involved in generation of promoter selectivity for σ^S . For example, H-NS, integration host factor (IHF), and leucine-responsive regulatory protein (Lrp) are known to bind to the promoter regions of genes and regulate their transcription, usually in a negative manner

(Hengge-Aronis, 2002a). In the case of certain promoters, only σ^S is capable of overcoming this regulatory interference and activating transcription of these genes. For example, some σ^S -dependent genes cannot be transcribed in a *rpoS*-null background. However, in the absence of H-NS and σ^S , transcription of σ^S -dependent genes occurs, indicating that the absence of H-NS allows another sigma factor, most likely σ^D , to activate transcription of these genes (Arnquist *et al.*, 1994; Barth *et al.*, 1995; Yamashino *et al.*, 1995). Other factors known to promote $E\sigma^S$ -driven transcription are the alarmone ppGpp and DNA topology. The absence of ppGpp results in poor induction of regulons controlled by the alternative sigma factors such as σ^S and σ^H . ppGpp seems to help σ^S and σ^H compete with σ^D for binding to the RNA polymerase core enzyme (Jishage *et al.*, 2002). Similarly, decrease in DNA superhelicity, a feature of stationary phase cells, and elevated potassium glutamate levels, a characteristic of osmotically shocked cells, enhance selectivity for $E\sigma^S$ (Kusano *et al.*, 1996).

1.4.3. σ^S regulon

So far, through various genetic and molecular biology studies, at least 60 genes of known function (Loewen *et al.*, 1998) and around 20 genes with no known function (Lacour and Landini, 2004) have been identified as belonging to the σ^S regulon. However, a recent genome-wide analysis of bacterial stress response in *E. coli* suggests that up to 10% of all *E. coli* genes are subject to σ^S regulation (Weber *et al.*, 2005). Cellular roles of a large fraction of these σ^S regulon members are still unknown, while many of the genes of known function are involved either in adaptation to various stresses or in regulation of stress response systems (Ishihama, 1997; Loewen *et al.*, 1998; Weber *et al.*, 2005). The importance σ^S in regulation of stress genes is obvious when one considers that *E. coli rpoS* mutants are rapidly killed when exposed to hydrogen peroxide, high temperature (above 50°C), high osmolarity, acidic pH and ethanol (Lange and Hengge-Aronis, 1994a; McCann *et al.*, 1991; Small *et al.*, 1994; Lee *et al.*, 1995; Farewell *et al.*, 1998). The involvement of σ^S in regulation of acid and oxidative stress-

inducible genes has already been mentioned (Sections 1.2 and 1.3). In addition to its role in regulation of specific stress genes, σ^S also oversees the expression of cell envelope genes and genes involved in regulation of overall cell morphology, programmed cell death, redirection of metabolism under the stationary phase of growth, virulence, and nitrogen starvation response (Hengge-Aronis, 2000; Kabir *et al.*, 2004). Finally, σ^S regulates the expression of secondary regulatory genes such as *bolA* and *robA* which are master regulators of genes involved in control of cell shape and oxidative and multiple antibiotic resistances, respectively (Hengge-Aronis, 2000).

1.5. Hsp31

1.5.1. In vitro characterization of Hsp31

The *hchA* gene (aka *yedU*), which codes for Hsp31, was identified in the transcriptome analysis of *Escherichia coli* as one of the 23 heat-inducible loci of unknown function whose transcription increases 31-fold upon heat shock (Richmond *et al.*, 1999). In the initial biochemical characterization, the protein product of *hchA* gene was found to accumulate at the molecular weight of 31 kDa; thus, the protein was named Hsp31, where Hsp stands for heat shock protein. Purified protein was found to assume a homodimeric quaternary structure and was tested for the ability to function as a molecular chaperone. *In vitro* aggregation suppression experiment with model substrates citrate synthase (CS) and alcohol dehydrogenase (ADH) showed that addition of Hsp31 suppressed aggregation of these proteins under heat shock conditions (Sastry *et al.*, 2002). Similarly, Hsp31 was found to promote reactivation of thermally and chemically denatured model substrates following stress abatement and to form complexes with permanently unfolded reduced carboxymethyl α -lactalbumin (Sastry *et al.*, 2002; Malki *et al.*, 2003). ATP, which drives the chaperoning activities of the majority of the cytoplasmic molecular chaperones, is not required for chaperoning activity of Hsp31; in fact, it inhibits its activity under heat shock conditions (Sastry *et al.*, 2002). Studies with bis-ANS, a molecule that exhibits little intrinsic fluorescence in

its free form but becomes highly fluorescent upon binding to the solvent-exposed structured hydrophobic patches, revealed that elevated temperatures lead to the exposure of Hsp31's structured hydrophobic domains (Sastry *et al.*, 2002). In summary, *in vitro* biochemical characterization of Hsp31 suggests that under heat shock conditions, Hsp31 exposes its hydrophobic domains, which react with early unfolding intermediates of client proteins and rapidly release them in an active form after transfer to low temperatures (Sastry *et al.*, 2002).

1.5.1. Crystal structure of Hsp31

In the span of one year, crystal structures of Hsp31 and its human (DJ-1) and yeast (YDR533Cp) orthologs were solved by several different groups (Quigley *et al.*, 2003; Zhao *et al.*, 2003; Lee *et al.*, 2003b; Quigley *et al.*, 2004; Wilson *et al.*, 2004). Based on its structure, Hsp31 was found to belong to the DJ-1/ThiJ/PfpI superfamily of proteins. Its monomer is composed of two α - β domains (A and P) with a total of 13 β -sheets and 12 α -helices (Figure 1.3). Sixty-five-residue P domain is composed of four antiparallel β -sheets and two α -helices and is connected to the A domain via the linker region composed of residues 32-45. The interaction between P domain of one monomer with the P and A domains of the second monomer results in a dimer formation, which buries surface area of $\sim 1900 \text{ \AA}^2$ (Figure 1.4A). The homodimer contains a system of canyons, grooves and hydrophobic patches that may function as docking sites for partially unfolded proteins (Figure 1.4B), and it also appears to coordinate a zinc(II) ion (Zhao *et al.*, 2003). The major, 20 $\text{\AA}$ in diameter, hydrophobic patch of the protein involves seven residues from each subunit and is located in a shallow bowl that represents dimerization interface of P domains (Figure 1.4B). Hsp31 structure obtained from an alternate crystal showed a degree of flexibility (i.e. disorder) in certain parts of each monomer (D1-D5 in Figure 1.5A), with the linker region containing the majority of flexible residues (D2 in Figure 1.5A; Quigley *et al.*, 2004). In particular, the mobility of D2 and D3 domains was predicted to lead to the exposure of a sizable hydrophobic patch

made up of surface areas of both subunits near the dimer interface (Figure 1.5B). The prediction was confirmed in a series of *in vitro* experiments with various mutants of Hsp31 (Sastry *et al.*, 2004). Mutations in amino acid residues located in the bowl region or in the region that gets exposed when linker becomes mobile resulted in a decrease in chaperoning activity of Hsp31, with residues in the latter region proving to be crucial for substrate-binding at high temperature. Immobilization of the flexible linker regions resulted in a complete abolishment of chaperoning activity at elevated temperatures. Based on these findings, it was proposed that the mobile linker regions act as thermally-activated gates, the opening of which leads to the exposure of additional hydrophobic residues crucial for binding of proteins denature by heat stress (Sastry *et al.*, 2004).

Crystallographic studies of Hsp31 revealed another interesting feature of the protein—the presence of a catalytic triad often found in proteases, hydrolases, transglutaminases and amidotransferases (Figure 1.6A-B; Quigley *et al.*, 2003). Each monomer contains a triad composed of His186, Cys185 and Asp214 residues. In a dimer, two triads are connected by the negatively charged canyon. This catalytic triad appears to be a conserved feature of the DJ-1/ThiJ/PfpI superfamily members, as it is present in some capacity in yeast and human orthologs of Hsp31 (Lee *et al.*, 2003b; Wilson *et al.*, 2004). Furthermore, superposition of Hsp31's catalytic triad with the triad from protease I of *Pyrococcus horikoshii* (PhpI), an ortholog of Hsp31, revealed similar overall architecture of the triad between the two orthologs with the exception of the third triad member, glutamine in PhpI and aspartic acid in Hsp31 (Figure 1.7). However, unlike PhpI catalytic triad, which is active and solvent-exposed, the accessibility of Hsp31's triad is fairly restricted, as it lies at the bottom of a deep two-cavity, S-shaped pocket, which is ~17 Å long, 8 Å wide, and between 8 and 11 Å deep (Figure 1.6A; Quigley *et al.*, 2003; Zhao *et al.*, 2003). Indeed, initial attempts to detect any proteolytic activity by Hsp31 failed (Malki *et al.*, 2003; Sastry and Baneyx unpublished data). However, recent reports indicate that Hsp31 is capable of hydrolyzing single, small amino acids conjugated to fluorogenic groups (Lee *et al.*, 2003b; Malki *et al.*, 2004), and that it exhibits an exceedingly weak proteolytic activity towards bovine serum albumin (Lee *et*

al., 2003b), which suggests that it might function as a peptidase rather than a protease. Based on its ability to act as a molecular chaperone and due to the presence of a catalytic triad, it is possible that Hsp31 is a multi-functional protein, switching its role from a chaperone to a protease/peptidase based on the environmental conditions.

1.6. Significance of the Performed Research

Assignment of function to the protein products of unknown heat-inducible loci is of interest since the majority of heat stress proteins are either molecular chaperons or proteases. Besides the obvious importance that characterization of new genes has on the general understanding of cellular events and protein mechanisms, the added advantage of discovering a novel molecular chaperone lies in the possibility that the protein can be exploited as a folding helper for recombinant protein production. Over the years, molecular chaperones have been successfully used to increase the production yields of recombinant proteins in *E. coli* (Thomas *et al.*, 1997; Baneyx and Mujacic, 2004). Therefore, from the biotechnological perspective, characterization of a new molecular chaperone broadens the pallet of the available folding modulators commonly employed in the production of pharmaceutically important proteins. The elucidation of Hsp31's function is of further interest since the absence of DJ-1, human ortholog of Hsp31, is one of the causes of neurodegenerative Parkinson's disease. Thus, it is highly possible that characterization of Hsp31 can provide additional insights into DJ-1's mode of action.

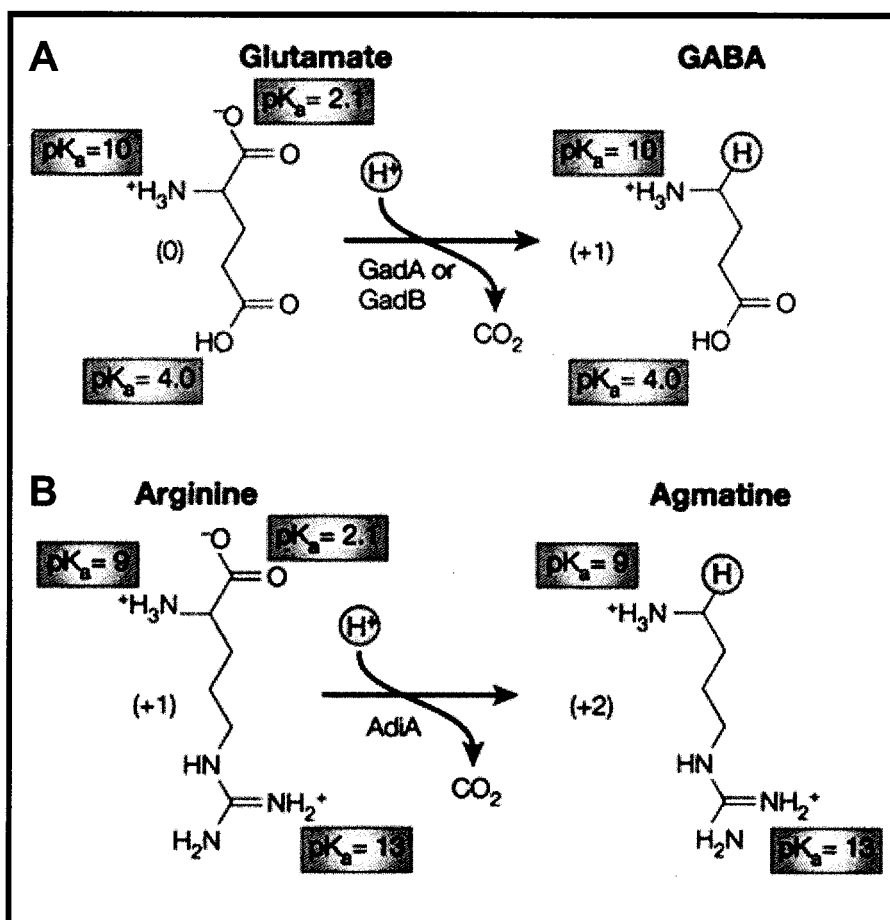


Figure 1.1. Consumption of protons during decarboxylation of glutamate (**A**) and arginine (**B**). Numbers in green indicate pK_a values of ionizable groups. Numbers in parentheses represent the charge of the compounds during the process. Orange circles mark locations of proton addition. GABA stands for γ -amino butyric acid (taken from Foster (2004)).

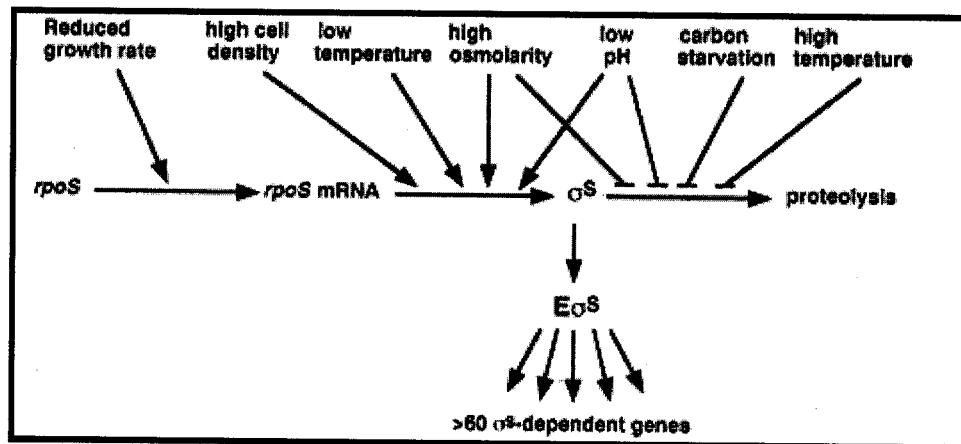


Figure 1.2. The effect of stress conditions on various levels of σ^S regulation (taken from Hengge-Aronis (2002a)).

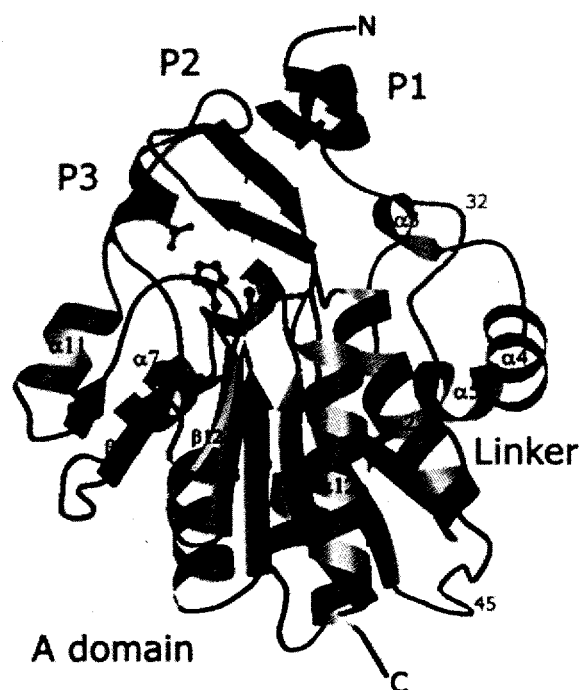


Figure 1.3. Ribbon representation of the Hsp31 monomer. A domain is in blue, P domain is in green, and the linker region is in purple. The catalytic triad is shown as a red ball-and-stick model (taken from Quigley *et al.* (2003)).

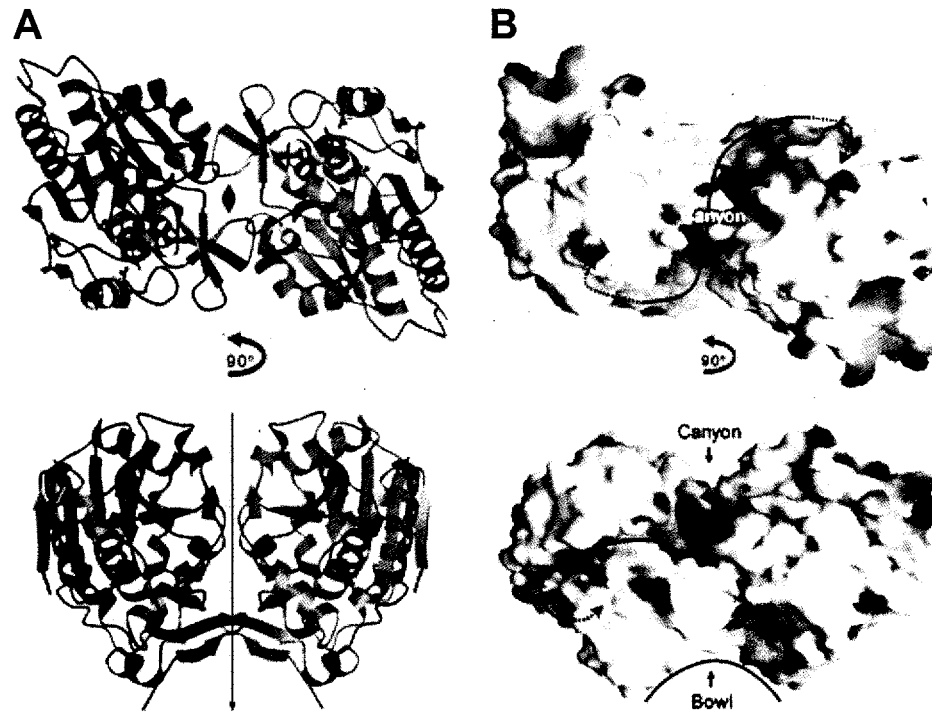


Figure 1.4. Two views of the ribbon (A) and electrostatic potential (B) representations of Hsp31 dimer. In (A), P domains are green, A domains are blue, the linker region is purple, while the catalytic triad is shown in red as a ball-and-stick model. In (B), electrostatic surfaces are colored between -10 kT (red) and +10 kT (blue), the negative groove connecting the putative triads is shown by a black line with arrows pointing to the triads (taken from Quigley *et al.* (2003)).

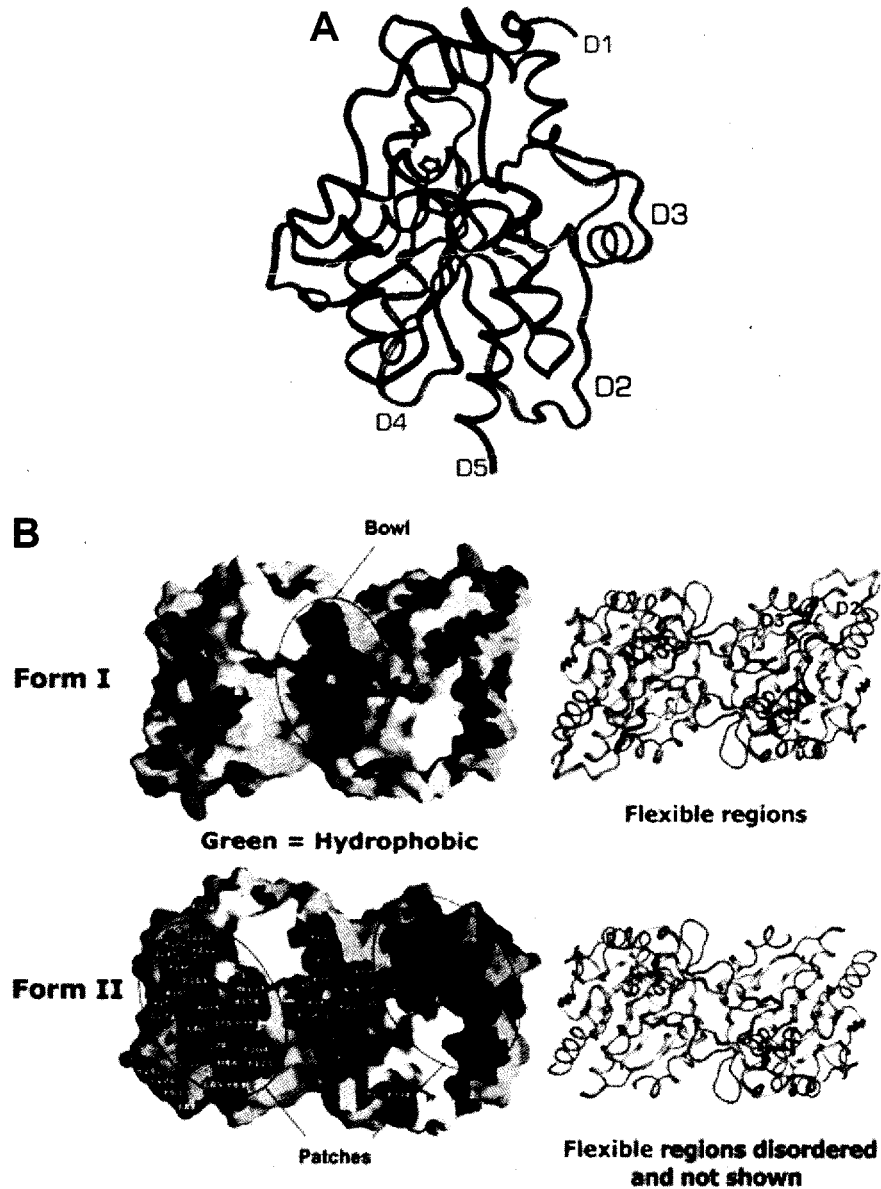


Figure 1.5. Disorder in Hsp31 structure. (A) Regions of disorder in Hsp31 monomer labeled D1-D5. The P domain is green, the A domain is blue. (B) Molecular surface of dimers shown with hydrophobic patches in green, and ribbon representation of dimers in the same orientation as shown on the right. Form I represents Hsp31 when flexible regions (in red) are not disordered. Form II shows how distortion of flexible regions exposes additional hydrophobic residues (taken from Quigley *et al.* (2004)).

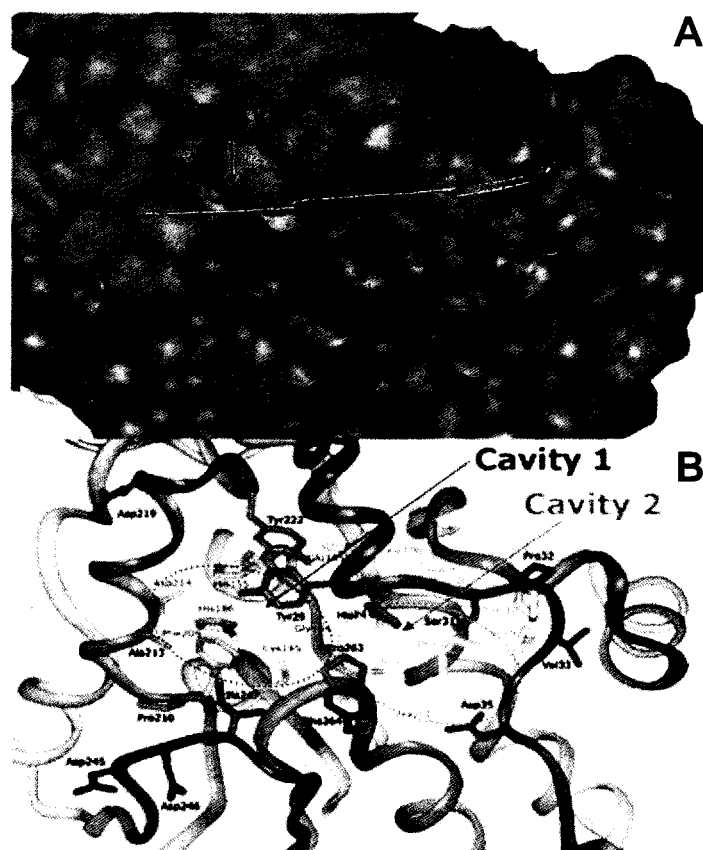


Figure 1.6. Putative active site pocket of Hsp31. (A) Surface representation of the active site pocket. Surfaces from the A and P domains are blue and green, respectively. Two residues of the triad, Cys-185 in yellow and His-186 in red, are visible. (B) Residues contributing to the active site pocket. Residues surrounding the pocket are blue (A domain) and green (P domain). The catalytic triad is colored by atom type: carbons are green, oxygens are red, nitrogens are blue, and sulfurs are yellow. Cavity 1 residues are red and cavity 2 residues are orange (taken from Quigley *et al.* (2003)).

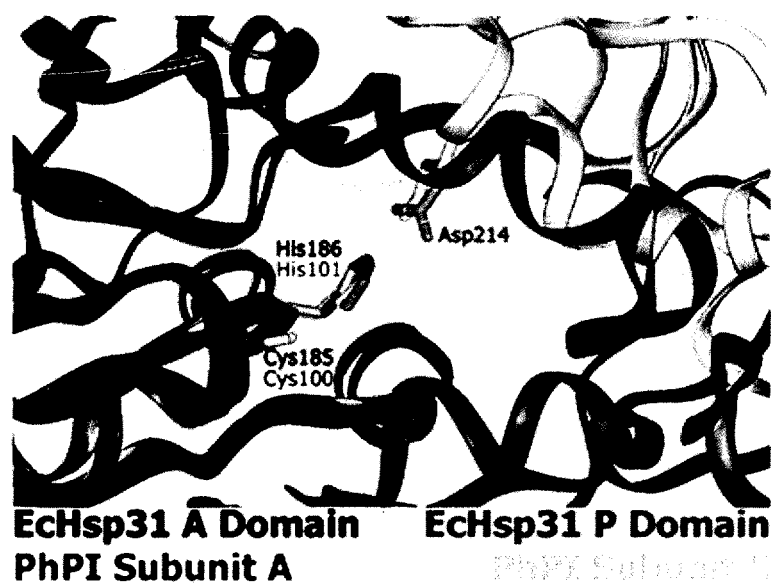


Figure 1.7. View of the catalytic triads of Hsp31 and PhPI. Subunits A and C of PhPI are orange and yellow, respectively. The PhPI triad residues for Cys-100 and His-185 are orange, and Glu-74' is yellow (taken from Quigley *et al.* (2003)).

Table 1.1. Cytoplasmic chaperones involved in heat shock response[†]

Family	Name	Co-factors	Function	Substrate specificity	ATP requirement
Hsp100(AAA+) ^a	ClpB		Disaggregase	Segments enriched in aromatic and basic residues	+
Hsp90	HtpG		Possible folding/secretory chaperone	Unknown	+
Hsp70	DnaK	DnaJ, GrpE	Folding chaperone	Segments of 4-5 hydrophobic amino acids, enriched in leucine and flanked by basic residues	+
Hsp60	GroEL	GroES	Folding chaperone	α/β folds enriched in hydrophobic and basic residues	+
Hsp33	Hsp33		Holding chaperone	Unknown	-
DJ-1 superfamily	Hsp31		Holding chaperone	Unknown	^b
Small Hsps	IbpA, IbpB		Holding chaperone	Unknown	^c
PP1ase	TF		Holding chaperone, PP1ase	Eight amino acid motif enriched in aromatic and basic residues	-

[†] Taken from Baneyx and Mijacic (2004). ^a AAA, ATPases associated with a variety of cellular activities. ^b ATP binding negatively regulates the chaperone activity of Hsp31 at high temperatures (Sastry *et al.*, 2002). ^c Binding of ATP to certain small Hsps triggers conformational changes and substrate release (Narberhaus, 2002).

Table 1.2. Periplasmic folding modulators[†]

Classification	Protein	Substrates
Generic chaperones	Skp (OmpH) FkpA	Outer membrane proteins and misfolded periplasmic proteins Broad substrate range
Specialized chaperones	SurA LolA PapD (and its family) FimC	Outer membrane proteins Outer membrane lipoproteins Proteins involved in P pili biosynthesis Proteins involved in type 1 pili biosynthesis
PPases	SurA PpiD FkpA PpiA (RotA)	Outer membrane β -barrel proteins Outer membrane β -barrel proteins Broad substrate range Unknown
Proteins involved in disulfide bond formation	DsbA DsbB DsbC DsbG DsbD DsbE (CcmG) CcmH	Reduced cell envelope proteins Reduced DsbA Proteins with nonnative disulfides Proteins with nonnative disulfides Oxidized DsbC, DsbG and CcmG Cytochrome <i>c</i> biogenesis Cytochrome <i>c</i> biogenesis

[†]Taken from Baneyx and Mujacic (2004).

Table 1.3. Antioxidant activities in *E. coli*[†]

Gene	Activity	Additional regulator(s)
<i>oxyR</i> regulon		
<i>katG</i>	Hydroperoxidase I	σ^S
<i>ahpCF</i>	Alkyl hydroperoxide reductase	
<i>gorA</i>	Glutathione reductase	σ^S
<i>grxA</i>	Glutaredoxin 1	
<i>trxC</i>	Thioredoxin 2	
<i>fur</i>	Ferric uptake repressor	SoxRS
<i>dps</i>	Nonspecific DNA binding protein	σ^S
<i>oxyS</i>	Regulatory RNA	
<i>agn43</i>	Outer membrane protein	
<i>fhuF</i>	Ferric reductase	
<i>soxRS</i> regulon ^a		
<i>sodA</i>	Manganese superoxide dismutase	ArcAB, FNR
<i>nfo</i>	Endonuclease IV	
<i>zwf</i>	Glucose-6-phosphate dehydrogenase	
<i>fumC</i>	Fumerase C	ArcAB, σ^S
<i>acnA</i>	Aconitase A	ArcAB, FNR, σ^S
<i>tolC</i>	Outer membrane protein	
<i>fur</i>	Ferric uptake repressor	OxyR
<i>micF</i>	RNA regulator of <i>ompF</i>	
<i>acrAB</i>	Multidrug efflux pump	
<i>nfsA</i>	Nitroreductase A	
<i>fpr</i>	Ferredoxin/flavodoxin reductase	
<i>fldA</i>	Flavodoxin	
<i>fldB</i>	Flavodoxin	
<i>ribA</i>	GTP cyclohydrolase	
Other defense activities		
<i>katE</i>	Hydroperoxidase II	σ^S
<i>xthA</i>	Exonuclease III	σ^S
<i>polA</i>	DNA polymerase I	
<i>recA</i>	RecA	
<i>mutM (fpg)</i>	Formamidopyrimidine glycosylase	FNR
<i>nth</i>	Endonuclease III	
<i>mutY</i>	Adenine glycosylase	
<i>msrA</i>	Methionine sulfoxide reductase	
<i>hslO</i>	Molecular chaperone	
<i>sodB</i>	Superoxide dismutase	
<i>sodC</i>	Copper-zinc superoxide dismutase	σ^S , FNR
<i>iscS</i>	NifS homolog	
<i>iscU</i>	NifU homolog	

[†]Adapted from Storz and Zheng (2000). ^aAlso regulated by MarA and Rob.

Table 1.4. *cis*-acting elements and *trans*-acting factors in the control of RpoS[†]

Element or factor	<i>rpoS</i> transcription	<i>rpoS</i> translation	σ^S proteolysis
<i>cis</i> -acting elements	<i>nlpD</i> _{p1+p2} , <i>rpoS</i> _p	Translational initiation (TIR) region in a specific secondary structure	Turnover element (crucial amino acids: K173, E174, V177)
<i>trans</i> -acting factors			
Macromolecular regulators	CRP-cAMP (-)	Hfq(+) DsrA RNA (+) OxyS RNA (-) H-NS (-) LeuO (-) DnaK (+)	
Small molecules	ppGpp (+) homoserine lactone (+)	UDP-glucose (-)	Acetyl phosphate (+)

[†]Taken from Hengge-Aronis (2000). Plus and minus signs indicate whether a factor stimulates or inhibits the corresponding step in σ^S regulation.

CHAPTER 2: The Effect of *hchA* Deletion on *E. coli* Survival and Protein Integrity under Heat Shock Conditions¹

2.1. Objectives

In vitro data with several, often-used, protein substrates have shown that Hsp31 exhibits chaperoning activity by acting as a molecular holdase (Section 1.5). However, while *in vitro* biochemical studies are very useful tools for assigning function and a mode of action to a protein, they do not provide much insight into the importance of a particular protein in the *in vivo* cellular processes. Therefore, to probe the cellular function of Hsp31, we constructed an *hchA* null mutant and characterized its phenotype alone or in combination with lesions in other cytoplasmic molecular chaperones.

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmids and routine growth conditions

Strains and plasmids used in this study are described in Table 2.1. Transformations were performed using either electrocompetent or CaCl₂ competent cells. Routine growth was at 30°C, unless otherwise stated, in Luria-Bertani (LB) medium supplemented with appropriate antibiotics.

2.2.2. Strain constructions

The λRed-mediated method (Datsenko and Wanner, 2000) was used to delete *hchA* from the chromosome of MC4100. A linear DNA fragment encoding the *cat* gene flanked by FRT recognition sites and *hchA* homology regions was obtained by PCR amplification of pKD3 using 70-nt long primers. The forward primer (5'-AGTCGCAAATATAGTGAC TACCCTAACTAAGCAACAATAAGGAATACACTCATATGAATATCCTCCTTAG -3') consisted of a 20-nt priming sequence for pKD3 and a tail homologous to the 50-nt region located immediately before the of *hchA* initiator. The reverse primer (5'-ACTAACGCGGCCGCGATTGATTATGCGCTTACATTCAAACGTAACAGGGAG

¹This work has been published (see Mujacic *et al.* (2004)). M. Bader provided assistance with 2D PAGE.

TGTAGGCTGGAGCTGCTTC-3') consisted of a 20-nt priming sequence for pKD3 and a 50-nt tail homologous to the intergenic region located 50-nt downstream of the *hchA* stop codon. Approximately 100 ng of the amplified fragment (984-bp) were mixed with 50 μ l of electrocompetent MC4100 cells harboring pKD46. Production of the λ Red proteins was induced by addition of 0.1% arabinose during the preparation of electrocompetent cells. Cells were grown at 30°C for 4-5 h, centrifuged at 2040 g for 5 min, resuspended in 140 μ l of LB medium and plated on LB-agar plates supplemented with 34 μ g/ml chloramphenicol. Following overnight incubation at 37°C, carbenicillin-sensitive candidate colonies were tested for a successful deletion event by PCR using primer pairs P1 (5'-CCCCTCCTGGCACAATAACATCA - 3') and P2 (5'-TATCCAGTGATTTTTTCTCCAT-3') and P3 (5'-GAATACAACAGTACTGCGATGA-3') and P4 (5'-GGTTACGCTCAGTCAAACCAGAAG-3'). One such colony, named MIR400, was selected for further studies. To obtain an unmarked *hchA* deletion, MIR400 was transformed with pCP20, grown at 40°C for 5 h and plated onto LB-agar medium supplemented with 50 μ g/ml streptomycin. Candidate colonies were tested for sensitivity to chloramphenicol and carbenicillin and excision of the *cat* gene was confirmed by PCR analysis. In the final strain (MIR401), the *hchA* gene has been replaced by one FRT site flanked by two 20-bp long stretches that were used as priming regions during amplification from pKD3. The presence of the correct mutation was confirmed by sequencing. P1 transduction and FLP-mediated excision were used to accumulate mutations. Screening phenotypes are listed in Table 2.1.

2.2.3. Growth curves

Overnight cultures grown at 30°C in 25 ml of LB supplemented with 50 μ g/ml streptomycin were used to inoculate triplicate 125 ml flasks containing 25 ml of medium so that the starting A_{600} would be ≈ 0.05 . All flasks were pre-warmed for 20 min at the appropriate temperature before inoculation. Culture turbidity was monitored at 600 nm at 30 min intervals for five hours.

2.2.4. Cell survival and recovery following challenge at 50°C

Overnight cultures grown at 30°C in 5 ml of LB supplemented with 50 µg/ml streptomycin were used to inoculate 125 ml flasks containing 25 ml of LB/streptomycin at a 1:50 dilution. Cells were grown at 30°C to mid-exponential phase ($A_{600} \approx 0.4$) and transferred to a water bath held at 50°C for 15, 60 or 120 min. Diluted aliquots were plated in duplicate on LB-agar plates supplemented with 50 µg/ml streptomycin and colonies were counted after overnight incubation at 30°C. The fraction surviving is defined as the number of colonies at a given time point after temperature upshift divided by the number of colonies immediately before transfer to 50°C. The experiment of Figure 2.2B was repeated on several occasions with similar results. For the experiment of Figure 2.2C, triplicate cultures inoculated as above were grown at 30°C for 120 min, transferred to 50°C for 30 min and returned to 30°C for an additional 150 min. Culture turbidity was monitored at 600 nm at 30 min intervals.

2.2.5. Disaggregation studies and quantification of insoluble proteins

Mid-exponential phase cultures grown at 30°C as described for the survival experiments were transferred to a water bath held at 48°C for 30 min and cells were allowed to recover at 30°C for 90 min. Samples (2 ml) were collected immediately before temperature upshift and at 30 min intervals thereafter and the A_{600} was recorded. Cells were sedimented by centrifugation at 10,000g for 10 min, resuspended in 2 ml of 50 mM potassium phosphate monobasic, pH 6.5, and disrupted with a French press at 10,000 psi. Insoluble fractions were recovered by centrifugation at 20,000g for 14 min. Pellets corresponding to aggregated and membrane fractions were resuspended in 1X SDS-DTT loading buffer. Buffer volumes were adjusted based on A_{600} values so that each sample would correspond to an identical amount of cells. Aliquots were fractionated on 12.5% SDS-PAGE mini-gels and proteins were visualized by Coomassie blue staining. The gels were digitized and protein content in each lane was determined by densitometric

analysis using the NIH IMAGE software 1.61 for PowerPC. Results shown are normalized to the intensity of the OmpF band (identified by a star on the figures). Quantification of insoluble proteins following heat shock was performed essentially as described above except that samples were only collected before shift and after 1h incubation at high temperature (48 or 50°C).

2.2.6. 2D-gel electrophoresis and MALDI analysis

Wild-type and $\Delta hchA$ cultures (250 ml) were grown to mid-exponential phase at 30°C in LB medium supplemented with 50 µg/ml streptomycin, transferred to 50°C for 1h and samples (150 ml) were centrifuged at 10,000g for 15 min. Cells were resuspended in 3 ml of 50 mM potassium phosphate monobasic, pH 6.5, disrupted with a French press at 10,000 psi and incubated on ice for 1 h in the presence of DNase (50 U) and RNase (100 µg). Insoluble fractions were isolated by centrifugation at 20,000g for 15 min. Pellets were washed twice with ice-cold water before solubilization in a buffer consisting of 9M urea, 2% Triton X-100, 2% Pharmalytes pH 3-10 (Pharmacia), 2% β-mercaptoethanol, and 2 µg/ml of the protease inhibitors pepstatin, aprotinin, and leupeptin. Buffer volumes were adjusted based on A_{600} values so that each sample would correspond to an identical amount of cells. Proteins were first separated by isoelectric focusing using a Pharmacia Multiphor II electrophoresis unit with an immobilized pH 4-7 gradient and next on 12% SDS polyacrylamide gels. Proteins were visualized by staining the gels with Coomassie brilliant blue. Gels were photographed with a Nikon Coolpix 4300 digital camera. Spots that were present or had greater intensity in $\Delta hchA$ insoluble fractions and were absent or less intense in wild-type insoluble fractions were excised from the gel loaded with $\Delta hchA$ samples. Gel fragments were dehydrated in acetonitrile for 10 min. Excess liquid was removed under vacuum and gel fragments were rehydrated for 30 min on ice in 50 µl of 20 µg/ml sequencing-grade modified trypsin (Promega). Excess trypsin was removed, 20 µl of 50 mM ammonium bicarbonate were added, and the mixture was incubated at 37°C for 5 h.

Peptides were eluted from the gel by several changes of extraction buffer (200 μ l of 50% acetonitrile, 5% formic acid), dried by evaporation, solubilized in 5% acetonitrile, 0.5% acetic acid, 0.1% trifluoroacetic acid and analyzed by MALDI-TOF. Protein identification was carried out with the help of MS-Fit (Baker, P. R. and Clauser, K. R.; <http://prospector.ucsf.edu>).

2.3. Results

2.3.1. *Hsp31 is important for E. coli growth and survival at elevated temperatures*

In an effort to gain information on the *in vivo* function of Hsp31, we used λ Red-mediated gene deletion (Datsenko and Wanner, 2000) to construct an unmarked *hchA* null mutant (Figure 2.1). Briefly, 70-nt long oligonucleotides containing 50-nt tails hybridizing immediately before the *hchA* gene start codon and 50-nt downstream of its stop codon were used to amplify a chloramphenicol resistance cassette flanked by FRT recognition sequences (Datsenko and Wanner, 2000). Following λ Red-assisted recombination into the MC4100 chromosome, the chloramphenicol resistance gene was excised using plasmid pCP20 which encodes the FLP recombinase gene on a temperature sensitive replicon (Table 2.1). The final strain, cured of the FLP expression plasmid (MIR401 in Table 2.1), retains an 84 bp scar in place of *hchA* between the divergently transcribed *yedS* and *yedV* open reading frames (Figure 2.1)

Because strains containing mutations or deletions in Hsps typically exhibit a temperature sensitive for growth phenotype, we compared the growth of *hchA* null cells to that of the isogenic wild-type at a variety of temperatures in rich medium. While there was essentially no difference between the two genetic backgrounds up to 46°C, the mutant displayed more severe growth defects than the wild-type after 150 min of incubation at 48°C (Figure 2.2A). Interestingly, $\Delta hchA$ cells reproducibly grew slightly better than MC4100 in the first 90 min of their transfer to 48°C (Figure 2.2A). The reasons for this phenotype remain unclear.

We next examined the impact of the *hchA* deletion on the ability of *E. coli* to recover from severe heat shock. For these experiments, mid-exponential phase cultures of wild-type or $\Delta hchA$ cells were incubated for increasing periods of time at 50°C and the number of survivors determined by plating at 30°C. Figure 2.2B shows that the loss of Hsp31 led to a pronounced increase in mortality with an about three orders of magnitude reduction in the number of viable cells after a 1h challenge at 50°C. Defects in septation were not responsible for this effect since $\Delta hchA$ cells were found to be non-filamentous under all growth conditions examined (data not shown). Because a similar phenotype was previously reported in *clpB* null mutants (Squires *et al.*, 1991; Thomas and Baneyx, 1998) we repeated the above experiment using isogenic $\Delta clpB$ and $\Delta clpB \Delta hchA$ cells (Table 2.1). While the *clpB* deletion had a more deleterious effect on viability than the *hchA* deletion (about one order of magnitude difference in CFU between the two strains following 1h of incubation at 50°C), combining the two alleles did not further reduce cell survival relative to the $\Delta clpB$ single mutant (Figure 2.2B).

Finally, we examined how exposure at 50°C for 30 min would affect the ability of various mutants to recover at 30°C. Figure 2.2C shows that the lack of either Hsp31 or ClpB had a similar deleterious impact on recovery from transient heat shock. However, while $\Delta hchA$ cells restarted healthy growth after 1.5 h of incubation at the lower temperature, the growth rate of $\Delta clpB$ cells remained significantly lower. In agreement with the data of Figure 2.2B, there was little difference in the behavior of $\Delta clpB$ and $\Delta hchA \Delta clpB$ cells. Thus, while both ClpB and Hsp31 play a role in the ability of *E. coli* to survive and recover from exposure to severe stress, the effects of the *clpB* mutation dominate and are not aggravated by the absence of Hsp31.

2.3.2. *Hsp3* does not function as a disaggregase

ClpB cooperates with the DnaK-DnaJ-GrpE system to mediate the disaggregation and reactivation of thermally and chemically denatured model proteins *in vitro* (Goloubinoff *et al.*, 1999; Zolkiewski, 1999) and that of thermolabile polypeptides *in vivo* (Mogk *et*

al., 1999). To determine if Hsp31 exhibits a similar function, mid-exponential phase cultures of wild-type, $\Delta clpB$, $\Delta hchA$ or $\Delta hchA \Delta clpB$ cells were transferred to 48°C for 30 min to induce host protein aggregation and cultures were allowed to recover for 1.5 h at 30°C. Insoluble fractions collected at 30 min intervals from the onset of the experiment were resolved by SDS-PAGE and the protein content was quantified by videodensitometry and normalized to the intensity of the OmpF band (Figure 2.3A). Whereas the levels of insoluble proteins remained elevated during the recovery period in *clpB* and *clpB hchA* null mutants, they decreased to pre-stress values within one hour of transfer to 30°C in both wild-type and $\Delta hchA$ cells (Figure 2.3B; data not shown). The rate of aggregate clearance estimated from the slope of the linear portion of the curves was about 1.2-fold lower in $\Delta hchA$ cells compared to the wild-type. By contrast, it was 3.3 fold less in $\Delta clpB$ cells relative to MC4100 and there was no significant variation in clearance rates between the $\Delta clpB$ and $\Delta hchA \Delta clpB$ strains (Figure 2.3B; data not shown). Taken together, these data suggest that the small difference in disaggregation efficiency between wild-type and $\Delta hchA$ cells reflects a lack of experimental sensitivity and that Hsp31 does not function as a disaggregase or plays a central role in ClpB-DnaK-DnaJ-GrpE-mediated protein renaturation at 30°C.

2.3.3. *Hsp31 modulates host protein aggregation under severe thermal stress conditions*

To gain further information on the role of Hsp31 in cellular protein folding, we compared the levels of insoluble proteins in wild-type and $\Delta hchA$ cells after subjecting mid-exponential phase cultures to a 1h heat shock at various temperatures. Using SDS-PAGE analysis, we were unable to detect any significant difference between insoluble fractions recovered from the two strains at temperatures as high as 48°C, although we reproducibly observed slightly higher levels of insoluble proteins in the *hchA* null strain relative to the wild-type prior to temperature upshift (data not shown). On the other hand, when cultures were exposed to 50°C for 1h, the aggregation of several proteins increased significantly in the $\Delta hchA$ background (arrows in Figure 2.4).

Although these results are consistent with a chaperone function at very high temperatures, the effects of misfolding and proteolysis can be difficult to delineate with such experiments. For instance, protein aggregation increases in *lon* mutants due to an impaired ability to turn over abnormal polypeptides (Tomoyasu *et al.*, 2001; Rosen *et al.*, 2002). Since each Hsp31 subunit contains a putative catalytic triad that is conserved among family members (Quigley *et al.*, 2003), we addressed the possibility that a lack of protease activity rather than a lack of chaperone activity was responsible for increased host protein aggregation in the $\Delta hchA$ mutant. For these experiments, BL21(DE3) cells harboring plasmid pKV111 (which encodes native Hsp31 under T7 transcriptional control; Table 2.1) or the control vector pET22b(+) were grown to mid-exponential phase at 30°C, induced with 1 mM IPTG and transferred for 1 or 2h at 50°C. There was no detectable difference in soluble or insoluble protein patterns between the two strains, as would have been expected if a broad substrate specificity protease was overproduced under conditions that promote host protein misfolding (data not shown). Similar results were obtained when Hsp31 was allowed to accumulate at 30°C before transfer to 50°C and are consistent with the fact that this protein exhibits very weak proteolytic activity (Lee *et al.*, 2003b). We therefore ascribe the increased aggregation of host proteins in the *hchA* mutant to the absence of Hsp31 chaperone activity.

2.3.4. Identification of Hsp31 substrates

The combination of two-dimensional gel electrophoresis and mass spectrometry has proven a powerful tool to catalog thermolabile host proteins that require the assistance of DnaK and/or trigger factor (TF) for proper folding (Mogk *et al.*, 1999; Deurling *et al.*, 2003). We used a similar strategy to identify *in vivo* substrates of Hsp31. For these experiments, insoluble fractions from MC4100 or isogenic $\Delta hchA$ cells were harvested after the cultures had been subjected to a 1h challenge at 50°C and samples were resolved by 2D-PAGE (Figure 2.5). While the differences between the two gels were much less dramatic than those observed in DnaK-depleted strains, the identity of six

spots that were only present in the insoluble fraction of *hchA* null cells were unambiguously determined by MALDI-TOF. Table 2.2 shows that three Hsp31 substrates (DeoA, DeoB and DeoC) are involved in nucleoside and deoxynucleoside catabolism and recycling. Two of these proteins (DeoA and DeoB) have previously been reported to interact with DnaK/TF (Mogk *et al.*, 1999; Deurling *et al.*, 2003). In addition, enolase, an enzyme of the glycolysis pathway, co-immunoprecipitates with GroEL (Houry *et al.*, 1999). AhpC does not belong to the list of known DnaK/TF or GroEL substrates.

2.3.5. Cross-talk with other cytoplasmic molecular chaperones

To determine if Hsp31 cooperates with folding chaperones of *E. coli*, the $\Delta hchA$ allele was moved into the *dnaK756*, *grpE280* and *groES30* genetic backgrounds which exhibit a temperature sensitive for growth phenotype at 42.5°C (Table 2.1, Figure 2.6A; Tilly *et al.*, 1981; Thomas and Baneyx, 1998). Growth curves recorded at this temperature revealed that the *hchA* deletion aggravated the defects of both *dnaK756* and *grpE280* cells relative to single mutants (compare open and closed symbols in Figure 2.6A). The pattern was reminiscent of that observed in $\Delta hchA$ cells cultivated at 48°C (Figure 2.1A) with the effect of Hsp31 inactivation becoming manifest after 150 min of incubation at high temperatures. By contrast, the absence of *hchA* had no influence on the growth of *groES30* cells at 42.5°C (Figure 2.6A, inset).

To further characterize the link between Hsp31 and DnaK-DnaJ-GrpE, disaggregation experiments similar to those described in Figure 2.3 were conducted using isogenic *dnaK756* and *dnaK756* $\Delta hchA$ cells. Figure 2.6B shows that the presence of the double mutation led to an increase in the amount of aggregated proteins present in the cell upon completion of heat shock at 48°C and to a slower clearance rate of insoluble materials following temperature downshift when

compared to the *dnaK756* single mutant. While the latter result may reflect participation of Hsp31 in DnaK-DnaJ-GrpE-ClpB-mediated disaggregation, it should be interpreted with care in light of the results of Figure 2.3 and based on the fact that the growth rate of *dnaK756* $\Delta hchA$ cells was about 4.5-fold lower than that of *dnaK756* or wild-type cultures during the low temperature recovery period. On the other hand, the above data lend support to the idea of a functional cooperation between the DnaK-DnaJ-GrpE system and Hsp31 in the management of thermal stress.

Because ClpB and IbpB are also known to cooperate with DnaK-DnaJ-GrpE, we finally investigated the effect of combining the *hchA* deletion with either *clpB* or *ibpAB* null mutations. The growth of the isogenic strain set (Table 2.1) was compared at 46°C, a temperature at which MC4100 derivatives containing $\Delta clpB$ or Δibp mutations start exhibiting growth defects (Thomas and Baneyx, 1998). Under these conditions, the absence of Hsp31 had essentially no impact on the growth of either $\Delta clpB$ or Δibp mutants (Figure 2.7).

2.4. Discussion

Escherichia coli Hsp31 is a representative member of a previously unrecognized family of Hsps (Sastry *et al.*, 2002) that has been subdivided into three classes based on variations in the structural P domain, oligomeric status and catalytic triad architecture (Quigley *et al.*, 2003). Purified Hsp31 exhibits the hallmark characteristics of a molecular chaperone. It promotes the reactivation of chemically unfolded polypeptides, forms stable binary complexes with reduced carboxymethylated α -lactalbumin, suppresses the thermal aggregation of several model proteins and releases a fraction of bound species in an active form following transfer to low temperatures (Sastry *et al.*, 2002; Malki *et al.*, 2003).

To explore the possible involvement of Hsp31 in the conformational quality control of the proteome and to determine whether it collaborates with other

cytoplasmic chaperones in the management of thermal stress, we constructed and characterized an isogenic strain set containing an *hchA* null allele (Figure 2.1, Table 2.1). Hsp mutants typically exhibit growth defects when cultivated under high temperature conditions and $\Delta hchA$ cells are no exception. However, whereas mutations in the DnaK-DnaJ-GrpE and GroEL-GroES folding systems lead to significant growth defects at 42.5°C (Figure 2.6A; Tilly *et al.*, 1981) and strains carrying deletions in *clpB*, *ibpAB* or *hspG* grow more poorly than isogenic wild-type cells at 46°C (Thomas and Baneyx, 1998), the deleterious effect of the *hchA* deletion only became apparent at 48°C (Figure 2.2A). Thus, Hsp31 seems to play a less important “housekeeping” function than ClpB, IbpA/B or HtpG in supporting cell growth at elevated temperatures. Furthermore, it does not appear to operate in series with these molecular chaperones since deletion of *hchA* does not aggravate the growth defects of $\Delta clpB$ or $\Delta ibpAB$ cells at 46°C (Figure 2.7). On the other hand, the absence of Hsp31 significantly impairs the ability of *E. coli* to recover from extreme heat shock at 50°C (Figure 2.2B-C), a phenotype exhibited by *dnaK756* and $\Delta clpB$ mutants but not by *groES30*, $\Delta htpG$ or $\Delta ibpAB$ cells (Squires *et al.*, 1991; Thomas and Baneyx, 1998).

ClpB is believed to play a central role in the ability of *E. coli* to survive a severe heat challenge by shearing apart large aggregates of misfolded thermosensitive proteins in an ATP-driven process and handing out partially folded species to the DnaK-DnaJ-GrpE system for reactivation (Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999; Zolkiewski, 1999; Weibezahn *et al.*, 2005). Because the clearance rates of thermally aggregated proteins at 30°C are within 20% of each other in wild-type and $\Delta hchA$ cells while disaggregation is highly impaired in $\Delta clpB$ cells (Figure 2.3), Hsp31 does not appear to function as a disaggregase. This result is in agreement with the observation that Hsp31 lacks an ATPase activity (Sastry *et al.*, 2002; Malki *et al.*, 2003), which would presumably be necessary to fuel the active remodeling of protein aggregates, and the fact that efficient substrate binding requires high temperature-induced conformational changes (Sastry *et al.*, 2002; unpublished data).

Although Figure 2.6B suggests that Hsp31 may assist DnaK-DnaJ-GrpE-ClpB in the clearance of thermal aggregates (possibly in a manner similar to IbpB; Mogk *et al.*, 2003), more extensive biochemical studies will be needed to determine if this is the case.

On the basis of biochemical data, we have previously proposed that Hsp31 captures and stabilizes early unfolding intermediates to alleviate overloading of the DnaK-DnaJ-GrpE machinery under conditions of severe thermal stress (Sastry *et al.*, 2002). Several lines of evidence support this hypothesis. First, the absence of Hsp31 leads to increased aggregation of a subset of host proteins when cells are incubated at 50°C (Figures 2.4-2.5), some of which are known substrates of DnaK/TF (Table 2.2). Second, the *hchA* deletion aggravates the growth defects of *dnaK756* and *grpE280* mutants at 42.5°C (Figure 2.6A). Third, host protein aggregation is enhanced in severely stressed *dnaK756 ΔhchA* cells relative to *dnaK756* cultures (Figure 2.6B).

Taken together with the recent findings of Bukau and coworkers (Mogk *et al.*, 2003), our results suggest that efficient handling of temperature-induced cellular protein misfolding involves multiple complementary mechanisms that revolve around the ability of DnaK-DnaJ-GrpE to actively refold proteins and the level of saturation of this system (Figure 2.8). Under severe stress conditions, thermolabile proteins would follow one of four possible fates: capture by DnaK-DnaJ and active refolding (path 1), capture and transient stabilization by Hsp31 (path 2), capture and permanent stabilization by small Hsps (path 3) or aggregation (path 4). The particular path followed would depend on the substrate unfolding pathway, its degree of unfolding and the availability of free DnaK. Direct capture by DnaK-DnaJ would be the most efficient way to deal with a heat shock of short duration. However, if stress persists, any protein released in a native conformation would start unfolding again, tying up the cellular supply of DnaK (path 1''). Under these conditions, Hsp31 and IbpB-dependent mechanisms would become important for efficient handling of protein misfolding. Hsp31 may stabilize early unfolding compact

intermediates or dissociated protomers from oligomeric proteins, and release them after stress abatement in an environment that is permissive for direct refolding or reassociation (path 2'). We note that such a role for Hsp31 would explain why the opening of its substrate binding site is negatively regulated at high temperatures by ATP but not by ADP (Sastry *et al.*, 2002) since efficient capture of unfolding intermediates would become important when the local ATP pool has become depleted by abortive cycles of DnaK-dependent refolding. IbpB would tightly bind more unstructured unfolding intermediates and retain them on its surface for subsequent DnaK-DnaJ-GrpE-ClpB-mediated refolding once the temperature has returned to physiological value (path 3'). Finally, the thermal aggregates that have escaped all protective systems would be renatured under physiological temperature conditions by DnaK-DnaJ-GrpE-ClpB (path 4').

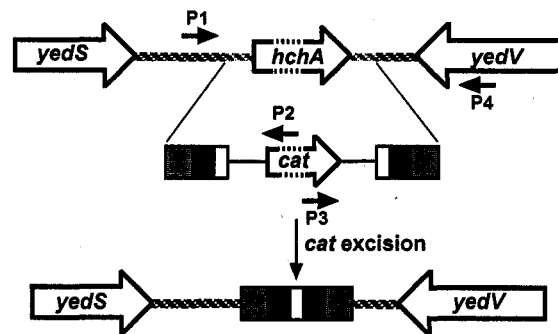


Figure 2.1. Deletion of the *hchA* gene. The strategy used to construct an unmarked *hchA* null strain is schematically highlighted. Grey boxes represent the 50-nt homology regions upstream and downstream of the 851 bp long *hchA* gene, black boxes the 20-nt pKD3 priming sequences and white boxes the FRT sites. Primer pairs P1 and P2, P3 and P4, and P1 and P4 were used for deletion and excision verifications. Figure is not drawn to scale.

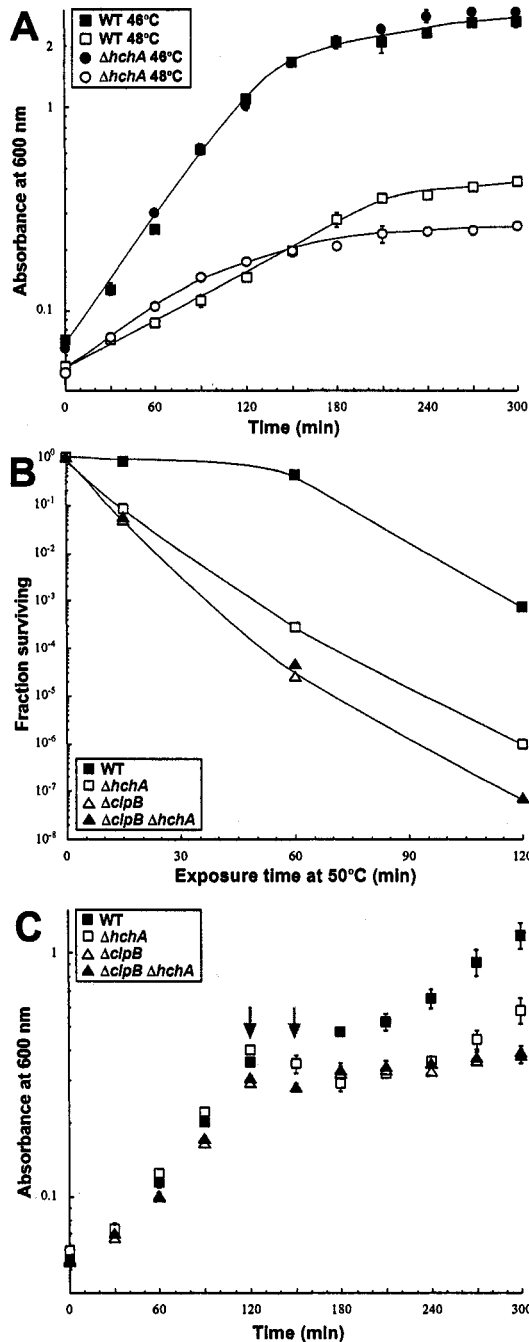


Figure 2.2. Effects of the *hchA* deletion on cell growth and survival at elevated temperatures.

A. Wild type and $\Delta hchA$ cells were grown at 46°C (closed symbols) or 48°C (open symbols) in LB medium. Culture absorbance was monitored at 600 nm for 5 h. Error bars correspond to triplicate experiments and are often smaller than symbols.

B. Wild type, $\Delta hchA$, $\Delta clpB$ and $\Delta clpB \Delta hchA$ cells were grown to mid-exponential phase at 30°C in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin and transferred to 50°C. Dilutions of samples collected immediately before temperature upshift, and 15, 60 and 120 min thereafter were plated in duplicate and the number of colony forming units determined after overnight incubation at 30°C. Data points shown are the averages of at least two separate experiments.

C. Wild type, $\Delta hchA$, $\Delta clpB$ and $\Delta clpB \Delta hchA$ cells were grown at 30°C in LB medium for 120 min, transferred to 50°C for 30 min (black arrow), shifted back to 30°C (grey arrow) and allowed to recover at this temperature. Culture absorbance was monitored at 600 nm. Error bars correspond to triplicate experiments.

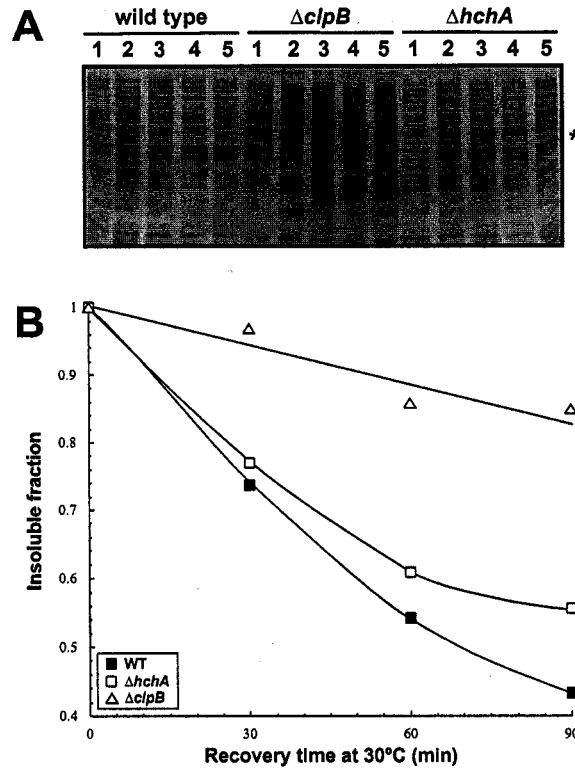


Figure 2.3. Role of Hsp31 in protein disaggregation.

A. Wild type, $\Delta hchA$ and $\Delta clpB$ cells were grown to mid-exponential phase at 30°C in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin, transferred to 48°C for 30 min, and allowed to recover at 30°C for 1.5 h. Insoluble protein fractions collected before temperature upshift (lanes 1), after 30 min of heat shock at 48°C (lanes 2), and after 30 (lanes 3), 60 (lanes 4) and 90 min (lanes 5) of transfer to 30°C were fractionated by SDS-PAGE. The star indicates the migration position of OmpF.

B. Amounts of insoluble proteins during recovery at 30°C were quantified by densitometric analysis of the gels, normalized to the intensity of the OmpF band and to the levels of insoluble proteins after 30 min of heat shock at 48°C (arbitrarily set at 100). Data points shown are the averages of two separate experiments.

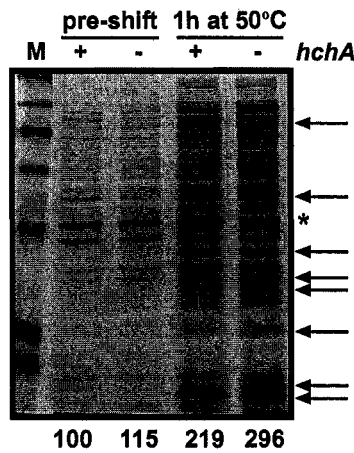


Figure 2.4. Effects of Hsp31 on *in vivo* protein aggregation. Wild type (+) and $\Delta hchA$ (-) cells were grown to mid-exponential phase at 30°C in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin and transferred to 50°C for 1 h. Insoluble protein fractions collected before temperature upshift and after 1 h of incubation at 50°C were fractionated by SDS-PAGE. Numbers at the bottom of the gel correspond to the amount of insoluble proteins normalized to the intensity of the OmpF band (star) and to the levels of insoluble proteins in wild type cells prior to the shift (arbitrarily set at 100). Arrows show the migration position of proteins that are present at higher levels in the insoluble fraction of the heat-shocked *hchA* null strain. Markers (lane M) are 148, 98, 64, 50, 36, 22 and 16-kDa from top to bottom.

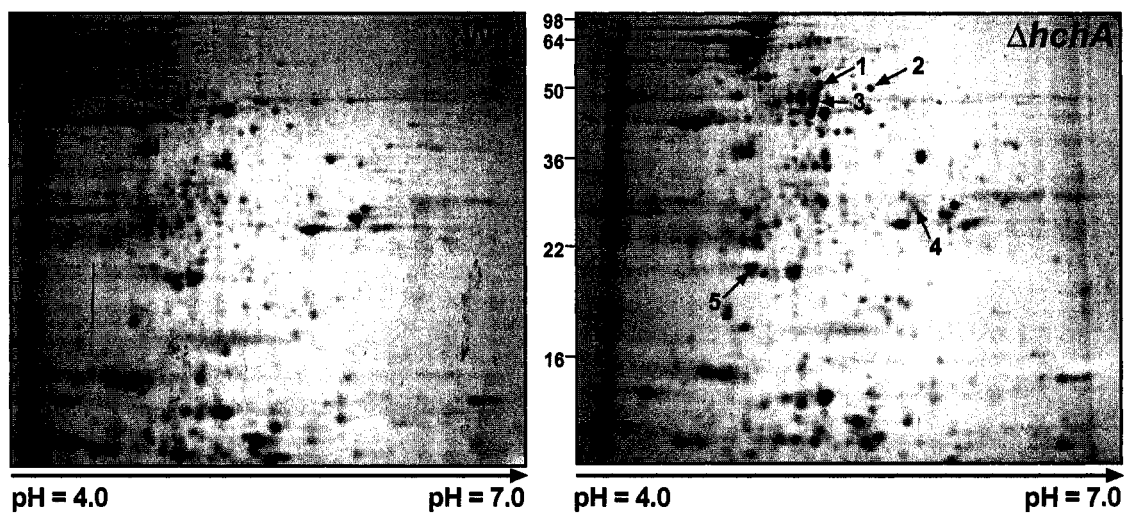


Figure 2.5. Two-dimensional gel electrophoresis of insoluble fractions from *hchA*⁺ (left) and $\Delta hchA$ (right) cells isolated after a 1-h heat shock at 50°C. Arrows indicate the position of proteins aggregating in $\Delta hchA$ but not in wild type cells and correspond to DeoA (1), Eno (2), DeoB (3), DeoC (4), and AhpC (5). Although two isoforms and a truncated version of DeoB were identified only one of the full-length isoforms is labeled with an arrow. Insoluble fractions contain both membrane proteins and aggregated proteins.

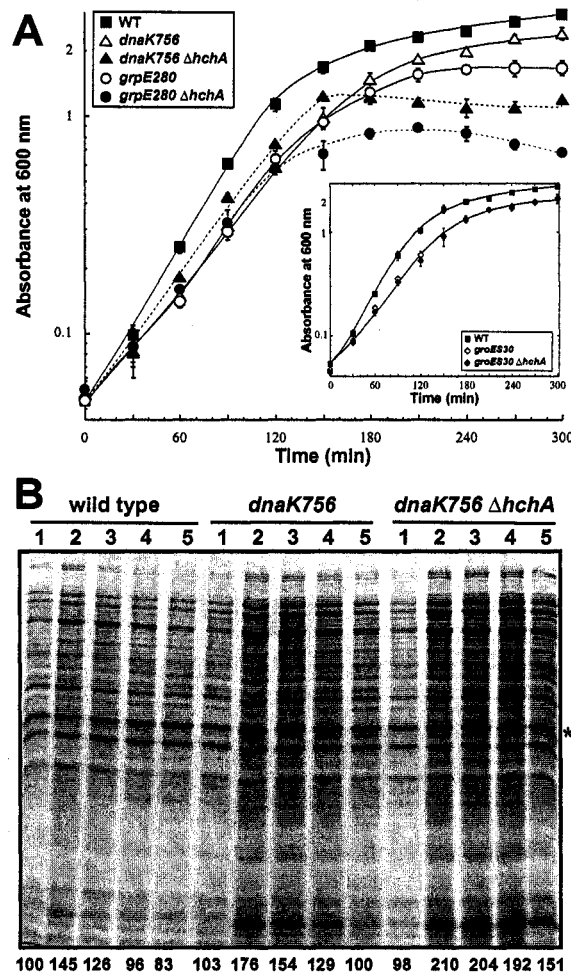


Figure 2.6. Interaction of Hsp31 with cytoplasmic chaperone systems.

A. Wild type, *dnaK756*, *grpE280*, *dnaK756 ΔhchA* and *grpE280 ΔhchA* cells were grown at 42.5°C in LB medium. Culture absorbance was monitored at 600 nm for 5 h. The inset shows the growth of wild type, *groES30* and *groES30 ΔhchA* cells under the same conditions. Similar experiments were conducted in *dnaJ259* and *dnaJ259 ΔhchA* cells but the effects of the *dnaJ259* allele on cell growth were severe and masked those of the *hchA* deletion (data not shown). Error bars correspond to triplicate experiments and are often smaller than symbols.

B. Wild type, *dnaK756* and *dnaK756 ΔhchA* cultures were heat-shocked at 48°C for 30 min and allowed to recover at 30°C as described in the legend of Figure 2.3. Insoluble protein fractions collected before temperature upshift (lanes 1), after 30 min of heat shock at 48°C (lanes 2), and after 30 (lanes 3), 60 (lanes 4) and 90 min (lanes 5) of transfer to 30°C were fractionated by SDS-PAGE. Numbers below the gel correspond to the videodensitometric quantification of proteins in each lane. Values were normalized to the intensity of the OmpF band (star) and to the levels of insoluble proteins present in the wild type strain prior to temperature upshift (arbitrarily set at 100).

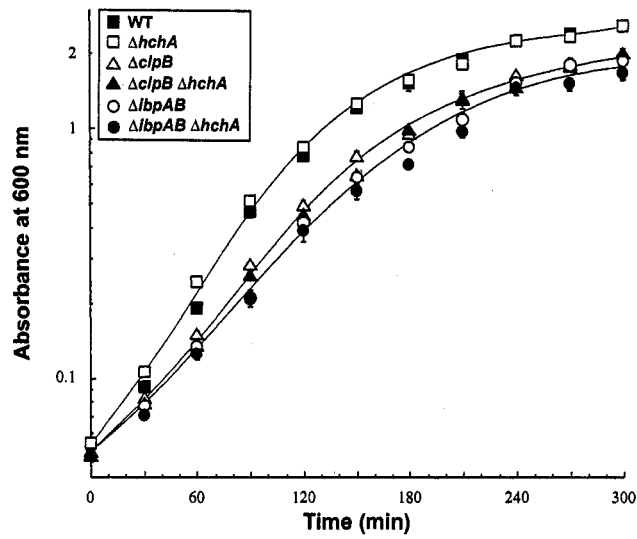


Figure 2.7. Wild type, $\Delta hchA$, $\Delta clpB$, $\Delta clpB \Delta hchA$, $\Delta ibpAB$ and $\Delta ibpAB \Delta hchA$ cells were grown at 46°C and culture turbidity was monitored as described in the legend of Figure 2.6.

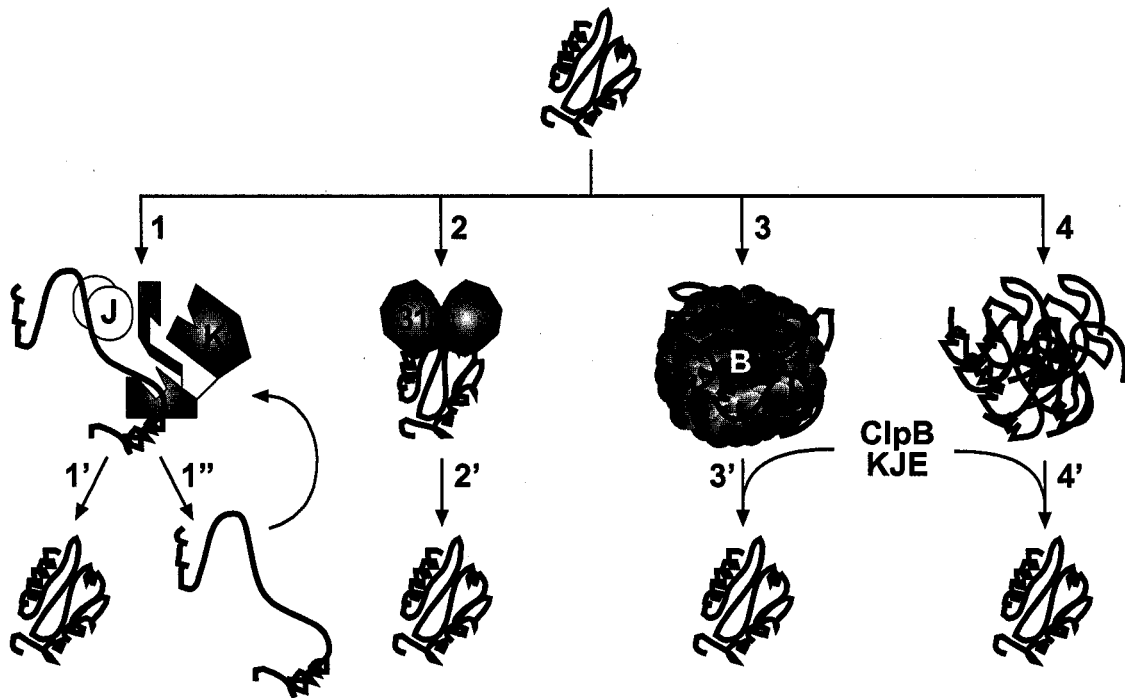


Figure 2.8. Possible model for handling of thermally induced protein misfolding in *E. coli*. Upon temperature increase, thermolabile proteins (top) lose their native conformation and start unfolding. Capture of solvent-exposed hydrophobic segments by DnaK (K) and DnaJ (J) and subsequent folding in a GrpE (E)-dependent manner is the most efficient way to restore the native conformation if the temperature rapidly returns to normal values (paths 1 and 1'). If the temperature remains high, proteins ejected from DnaK are unable to reach a proper conformation and cycle back to the chaperone (path 1''). Under these conditions, little free DnaK is available to interact with client proteins and paths 2-4 become prevalent. Early unfolding intermediates exposing a fluctuating hydrophobic core to the solvent or dissociated monomers from oligomeric proteins are captured and stabilized by Hsp31 which prevents further loss of structure (path 2). It is also possible that partially folded proteins ejected from DnaK in a structured but non-native form are captured by Hsp31 (not drawn). As physiological conditions return, conformational changes in Hsp31 modify the architecture of the substrate binding site (Sastry *et al.*, 2002; unpublished data) leading to substrate release and direct folding or oligomerization under permissive conditions (path 2'). More unstructured intermediates stably interact with surface-exposed hydrophobic patches exposed to the solvent by the small Hsp IbpB (B; path 3; Shearstone and Baneyx, 1999) or escape all defence mechanisms and form large aggregates (path 4). Following stress abatement, IbpB-bound proteins are engaged and refolded by the DnaK-DnaJ-GrpE system (Veinger *et al.*, 1998) with or without the assistance of ClpB (Mogk *et al.*, 2003) (path 3'). Renaturation of large thermal aggregates requires concerted action of the ClpB disaggregase and the DnaK-DnaJ-GrpE system (path 4'; Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999; Zolkiewski, 1999) and may be assisted by Hsp31 (not drawn).

Table 2.1. *Escherichia coli* strains and plasmids used in Chapter 2 studies

Construct	Genotype or description	Source, reference or construction details ^a
Strain		
BL21(DE3)	F ⁺ <i>ompT hsdS_B (h_B m_B) gal dcm</i> (DE3)	Novagen
MC4100	<i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Laboratory stock
JGT3	MC4100 <i>ΔclpB::kan</i>	Thomas and Baneyx (1998)
JGT5	MC4100 <i>grpE280 pheA::Tn10</i>	Thomas and Baneyx (1998)
JGT6	MC4100 <i>zjd::Tn10 groES30</i>	Thomas and Baneyx (1998)
JGT10	MC4100 <i>Δibp1::kan</i>	Thomas and Baneyx (1998)
JGT20	MC4100 <i>dna756 thr::Tn10</i>	Thomas and Baneyx (1998)
JGT25	MC4100 <i>dnaJ259 thr::Tn10</i>	Thomas and Baneyx (2000)
MIR400	MC4100 <i>ΔhchA::cat</i>	This study
MIR401	MC4100 <i>ΔhchA</i>	MIR400/FLP → Chl ^s , Str ^r
MIR412	MC4100 <i>thr::Tn10 dnaK756 ΔhchA::cat</i>	P1(MIR400)XJGT20 → Chl ^r , Tet ^r , Str ^r , Ts45; fil
MIR413	MC4100 <i>thr::Tn10 dnaK756 ΔhchA</i>	MIR412/FLP → Chl ^s , Tet ^r , Str ^r , Ts45; fil
MIR421	MC4100 <i>zjd::Tn10 groES30 ΔhchA::cat</i>	P1(MIR400)XJGT6 → Chl ^r , Tet ^r , Str ^r , Ts45
MIR422	MC4100 <i>zjd::Tn10 groES30 ΔhchA</i>	MIR421/FLP → Chl ^s , Tet ^r , Str ^r , Ts45
MIR430	MC4100 <i>dnaJ259 thr::Tn10 ΔhchA::cat</i>	P1(MIR400)XJGT25 → Chl ^r , Tet ^r , Str ^r , Ts45; fil
MIR431	MC4100 <i>dnaJ259 thr::Tn10 ΔhchA</i>	MIR430/FLP → Chl ^s , Tet ^r , Str ^r , fil; Ts45
MIR440	MC4100 <i>grpE280 pheA::Tn10 ΔhchA</i>	P1(JGT5)XMIR401 → Tet ^r , Str ^r , Ts45; fil
MIR460	MC4100 <i>Δibp1::kan ΔhchA::cat</i>	P1(MIR400)XJGT10 → Chl ^r , Kan ^r , Str ^r
MIR461	MC4100 <i>Δibp1::kan ΔhchA</i>	MIR460/FLP → Chl ^s , Kan ^r , Str ^r
MIR470	MC4100 <i>ΔclpB::kan ΔhchA::cat</i>	P1(MIR400)XJGT3 → Chl ^r , Kan ^r , Str ^r
MIR471	MC4100 <i>ΔclpB::kan ΔhchA</i>	MIR470/FLP → Chl ^s , Kan ^r , Str ^r
Plasmids		
pET22b(+)	T7 promoter expression vector	Novagen
pKV111	pET22b(+) derivative encoding <i>hchA</i> under T7 transcriptional control	Sastry <i>et al.</i> (2002)
pKD3	Contains <i>oriTγ</i> origin of replication and encodes <i>cat</i> gene flanked by FRT sites	Datsenko and Wanner (2000)
pKD46	Low copy number plasmid with temperature sensitive <i>oriT101</i> replicon encoding λRed genes under <i>P_{araBAD}</i> promoter control	Datsenko and Wanner (2000)
pCP20	Contains temperature sensitive replicon and encodes a thermally inducible FLP recombinase	Datsenko and Wanner (2000)

^aP1 transductions are described as P1(donor) X recipient → phenotypes used for selection and screening. FLP recombinase mediated excisions of the chloramphenicol resistance cartridge are described as strain/FLP → phenotypes used for selection and screening. Abbreviations are: Ts45, inability to grow at 45°C; fil, filamentous at 42°C; Amp, ampicillin; Chl, chloramphenicol; Tet, tetracycline; Str, streptomycin; r superscript, resistant; s, superscript, sensitive.

Table 2.2. Hsp31 substrates

Name	Function	M_r (kDa)	Structure	DnaK/TF Substrate ^a
DeoA	Thymidine phosphorylase	47.2	Homodimer	Yes
Eno	Enolase	45.5	Homodimer	No
DeoB	Phosphopentomutase	44.4	Monomer	Yes
DeoC	Deoxyribose-phosphate-aldolase	27.7	Homodimer	No
AhpC	Alkyl hydroperoxide reductase C22 protein	20.6	Homodimer	No

^aBased on the DnaK and TF substrates identified in Mogk *et al.* (1999) and Deuerling *et al.* (2003).

CHAPTER 3: Regulation of *hchA* expression²

3.1. Objectives

After establishing the importance of Hsp31 in survival and protein quality control under severe heat shock conditions, we set out to confirm and further justify classification of Hsp31 as a heat shock protein by investigating the expression profile of the *hchA* gene under heat shock conditions. To carry out *hchA* expression studies, we constructed an *hchA-lacZ* translational fusion and used it in conjunction with Western blotting technique. Finally, we identified cellular regulators of *hchA* expression during heat shock, as well as during various stages of growth, using primer extension analysis to determine the promoter region and transcriptional start site of the *hchA* gene.

3.2. Materials and Methods

3.2.1. Plasmid constructions

Strains and plasmids used in this study are listed in Table 3.1. The promoter region of *hchA* (defined as the 591 bp intergenic region between the *yedS* stop codon and the *hchA* initiator codon), along with the first 10 codons of the *hchA* gene were polymerase chain reaction (PCR)-amplified using primers 5'-AGTCTACCAGGCATGCTCAGTCAACTC-3' and 5'-AATGCATTATCTTCAGCAATAGTCGACTGCGGATT-3', which introduced 5'-SphI and 3'-SalI sites. Amplified DNA was digested with SalI and SphI and ligated into the same sites of pTBGM, which encodes a promoter-less *lacZ* gene, to yield pMM110. The integrity of the construct was confirmed by DNA sequencing. The translation product is a hybrid protein consisting of first 10 amino acids of Hsp31 followed by a Ser-Thr-Asp-Pro linker and amino acids 10–1015 of *E. coli* β -galactosidase.

To generate pMM120, the *hchA* promoter region, along with the complete *hchA* gene, was amplified with primers 5'-AGTCTACCAGGCATGCTCAGTCAACTC-3' and

²This work has been published (see Mujacic and Baneyx (2006)).

5'-TTCAAACGTAAGCTTGATTAACCCGCG-3'. The PCR product was ligated into pCR2.1TOPO.

To construct the RpoS (σ^S) expression vector pMM121 the *rpoS* gene was amplified from MC4100 chromosomal DNA using primers 5'-AGCCACCATATGAGTCAGAAT-3' and 5'-CTGGCCTTTCCTCGAGATGCTTAC-3', which introduced 5'-NdeI and 3' XhoI sites. The amplified DNA was digested with NdeI and XhoI and ligated into the same sites of pSR22 to place *rpoS* under the control of isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *trc* promoter. The integrity of the construct was confirmed by DNA sequencing.

To generate an N-terminally His-tagged version of σ^S (His₆- σ^S), the *rpoS* gene was isolated from pMM121 via NdeI and XhoI digestion and ligated into the same sites of pET28a (Novagen, Madison, Wisconsin), yielding plasmid pMM122.

3.2.2. Strain constructions

The *hchA::lacZ* translational fusion encoded by pMM110 was moved to the *att λ* site of MC4100 and RH90 through homologous recombination of the *bla* and *lacZ* genes, using λ RS45 (Simons *et al.*, 1987). The resulting strains were named MIR510 and MIR512 respectively. High titer lysate preparations and spot titting were performed as described (Silhavy *et al.*, 1984). The presence of a single chromosomal copy was verified by PCR amplification of chromosomal DNA with primers 5'-CCCCTCCTGGCACAATAACATCA-3' (which hybridizes to the 5' end of the *hchA* promoter region) and 5'-GCCAGCTTTCGGCACCGCT-3' (which hybridizes within *lacZ*).

The *hns::cat* null mutation was moved from BSN1 to MC4100, MIR510 and MIR512 using P1 transduction. The resulting chloramphenicol-resistant strains were named MIR517, MIR514 and MIR516 respectively.

3.2.3. Culture conditions

For the experiments of Figure 3.1, overnight cultures grown at 30°C in 25 ml of LB supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin were used to inoculate 25 ml of the same medium to a starting OD₆₀₀ of ≈ 0.05 . Cells were grown at 37°C and the OD₆₀₀ was recorded at 30 min intervals for 10 h. Soluble protein fractions for β -galactosidase assays and whole cells samples for Western blotting were collected every hour and 24 h after initiation of the experiment. For the experiments of Figure 3.3, overnight cultures of MIR510 harboring either pSR22 or pMM121 and grown at 37°C in 5 ml of LB/streptomycin were used to inoculate 500 ml shake flasks containing 100 ml of the same medium. Cells were grown at 37°C to OD₆₀₀ ≈ 0.4 , at which point 25 ml from each culture was transferred to a 125 ml flask and supplemented with 1 mM IPTG. Whole cells samples for SDS-PAGE and Western analyses were collected immediately before and 1 h after IPTG addition.

For the experiments of Figure 3.7, 25 ml of LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin were inoculated with frozen cells and cultures were grown at 20°C to OD₆₀₀ ≈ 0.4 . For wt* samples, an overnight starter culture grown at 30°C in 5 ml of LB/streptomycin was used to inoculate 25 ml of LB and cells were grown at 37°C to OD₆₀₀ ≈ 0.4 . Whole cells samples were collected prior to and 30 min after heat shock to 42°C.

For the experiment of Figure 3.9, overnight cultures grown at 37°C in 5 ml of LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin were used to inoculate 250 ml shake flasks containing 60 ml of the same broth. Cells were grown at 37°C to mid-exponential phase (OD₆₀₀ ≈ 0.4) and 25 ml of culture were transferred into duplicate 125 ml flasks. One flask was held at 37°C while the other was transferred to 42°C. Soluble cell fractions for β -galactosidase assays and whole cells samples for Western blotting experiments were collected immediately before and 15 and 60 min after heat shock as described below.

3.2.4. Protein purification

To express His₆-H-NS and RpoS, plasmids pTAC4632 and pMM122 were introduced into T7 expression strain BL21(DE3). Cells harboring pTAC4632 or pMM122 were grown in 500 ml of LB supplemented with 50 $\mu\text{g ml}^{-1}$ of appropriate antibiotic. Synthesis of His₆-H-NS or His₆- σ^S was induced in mid-exponential phase by addition of 1 mM IPTG. Cells were harvested 3 h post induction and proteins were purified using Ni-NTA His-Bind resin (Novagen, Madison, Wisconsin) according to manufacturer's instructions. His₆-H-NS was purified under native conditions while His₆- σ^S , the majority of which is produced in inclusion bodies, was purified under denaturing conditions in the presence of 6 M guanidine hydrochloride. Buffer exchange was performed with 10 kDa Centricon microconcentrators (Millipore, Billerica, Massachusetts). His₆-H-NS was stored in 10 mM Tris-HCl pH = 7.5, 1 mM EDTA, 5 mM DTT, 150 mM NaCl, 5% glycerol (Dersch *et al.*, 1993), while His₆- σ^S was stored in 10 mM Tris-HCl pH = 7.6, 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 5% glycerol. Proteins were more than 95% pure as judged by SDS-PAGE analysis. The presence of an N-terminal hexahistidine tag does not affect functionality of either protein (Gowrishankar *et al.*, 2003; T. Atlung, personal communication).

3.2.5. Transcriptional start site mapping

Overnight cultures grown at 30°C in LB/streptomycin were used to inoculate 125 ml flasks containing 25 ml of antibiotic-supplemented LB medium. Cells were grown at 37°C and samples for RNA extraction were collected at exponential and stationary phases of growth (Figure 3.4A). Alternatively, for the experiment of Figure 3.8 (lanes 2 and 3), frozen stocks of cells were inoculated into 125 ml flasks containing 25 ml of LB/streptomycin medium and were incubated at 20°C to mid-exponential phase, at which point they were transferred to 42°C. In this case, samples for RNA extraction were collected 30 min after heat shock. RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations except that

lysozyme-treated cells were sonicated for 5–10 min on 20% duty cycle in a Branson Sonifier to increase RNA yields. Transcription start sites were mapped using a reverse primer (5'-TAGTCCACGCCATCAAGATCAG-3') that hybridizes 100 nt downstream of the *hchA* start codon. [γ^{32}]ATP (6000 Ci mmol⁻¹, Perkin Elmer, Wellesley, MA) end-labelled primer was mixed with 4–12 μ g of RNA to synthesize cDNA using the ThermoScript reverse transcription kit (Invitrogen, Carlsbad, California). The same primer and the pMM120 plasmid template were used for sequencing reactions performed with the T7 Sequenase kit (Amersham Pharmacia Biotech, Piscataway, NJ).

3.2.6. Run-off transcription assays

Single-round transcription reactions by reconstituted RNA polymerase holoenzymes were carried out using the 878 bp PstI pMM120 fragment as a template. To prevent formation of extraneous transcripts by RNA polymerase (Schenborn and Mierendorf, 1985), 3' overhangs were blunted using T4 DNA polymerase according to the manufacturer's instructions (Fermentas International, Burlington, Canada). Approximately 0.6 pmol of double-stranded DNA was combined with either transcription buffer alone (40 mM Tris-HCl pH = 7.8, 10 mM MgCl₂, 1 mM DTT, 25 μ g ml⁻¹ BSA, 100 mM KCl) or with transcription buffer supplemented with 528 ng of purified His₆-H-NS. Mixtures were incubated at 37°C for 20 min and 1.8 pmol of either E σ^D (Epicentre Biotechnologies, Madison, Wisconsin) or E σ^S were added in a final reaction volume of 15 μ l. E σ^S was reconstituted by mixing *E. coli* RNA polymerase core enzyme (Epicentre Biotechnologies, Madison, Wisconsin) with His₆- σ^S at a 1:4 molar ratio followed by 30 min incubation at 37°C. After 20 min incubation at 37°C to allow for open complex formation, run-off transcription was initiated by addition of 2 μ Ci of [α -³²P]UTP (Perkin Elmer, Wellesley, MA) and 5 μ l of a pre-warmed mixture consisting of 600 μ M ATP, CTP, GTP and CTP, 30 μ M UTP, and 600 μ g ml⁻¹ heparin. Reactions were stopped after 10 min at 37°C by addition of 20 mM EDTA. Excess nucleotides were removed by chromatography on Sephadex G-

25 columns (Sigma-Aldrich, St. Louis, Montana). Samples were mixed with 10 μl of RNA loading buffer (Sambrook *et al.*, 1989), heated for 2 min at 95°C, chilled on ice, loaded onto a 6% acrylamide-urea gel and the dried gel was exposed to CL-XPosureTM film (Pierce, Rockford, Illinois).

3.2.7. Long-term survival under limited nutrient conditions

Frozen cells were inoculated into 125 ml flasks containing 25 ml of W salts (10.5 g K_2HPO_4 , 4.5 g KH_2PO_4 , 0.241 ml of 1 M MgSO_4) supplemented with 50 $\mu\text{g ml}^{-1}$ streptomycin, 0.2% ammonium sulphate and 0.2% glucose, and cultures were grown at 37°C. The number of colony-forming units (cfu) obtained 24 h after inoculation was obtained by plating onto LB-agar-streptomycin (0 h time point in Figure 3.6).

Thereafter, samples were collected every 24 h for 4 days, and the fraction of surviving cells was obtained by dividing the number of cfu at each time point by the number of cfu in the 0 h samples (arbitrarily set at 1). Each sample was plated in triplicate. Errors bars were obtained by propagation of errors analysis.

3.2.8. General techniques

Gel retardation experiments were conducted as described (Dersch *et al.*, 1993). Briefly, approximately 1 μg of pMM120 DNA was digested with PvuII and BlnI and combined with 0–1 μg of purified His₆-H-NS or bovine serum albumin (BSA) in 40 μl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 80 mM NaCl, 5 mM DTT and 5% glycerol. After 30 min incubation at 23°C, reaction mixtures were loaded onto a 4% polyacrylamide gel. The gel was run in 1× TBE buffer (Sambrook *et al.*, 1989) and stained with ethidium bromide.

To analyse the *hchA* promoter for intrinsic curvature, pMM120 was digested with ApaI and PstI and the resulting DNA fragments were subjected to the two-dimensional electrophoresis analysis (Mizuno, 1987). DNA fragments were first separated on a 4%

polyacrylamide gel at 55°C, followed by second dimension separation at 4°C. TBE (1×) was used as the running buffer and the DNA was stained with ethidium bromide.

For enzymatic assays, 2 ml culture samples were sedimented by centrifugation at 6500 g for 10 min, resuspended in an equal volume of 50 mM potassium phosphate monobasic (pH 6.5) and lysed with a French press operated at 10,000 psi. Soluble fractions recovered after centrifugation at 20,000 g for 20 min were assayed in triplicate for β -galactosidase activity using the chromogenic substrate ortho-nitrophenyl- β -galactoside (ONPG) and the method of Miller (Miller, 1972).

Whole cells samples for Western analysis were obtained by centrifuging 1 ml of cells at 10 000 g for 10 min and resuspending the pellet in 1× SDS-DTT loading buffer. Buffer volumes were adjusted so that each sample would correspond to an identical amount of cells (based on OD₆₀₀). Proteins were separated on 12.5% mini-gels, transferred to nitrocellulose, and the blots were incubated with rabbit polyclonal anti-Hsp31 antiserum (a gift from Peer Norbert Jørgenson, Novo Nordisk, Denmark) at a 1:600 dilution, or with rabbit polyclonal anti-DnaK antiserum (a gift from Anthony Gatenby, DuPont, DE) at a 1:2000 dilution.

3.3. Results

3.3.1. σ^S and H-NS are implicated in *hchA* regulation

In an early inventory of genes regulated by the nucleoid-binding protein H-NS, Yoshida and coworkers identified four unknown proteins whose production was upregulated in a Δhns mutant (Yoshida *et al.*, 1993). The N-terminal sequence of one of these is a perfect match to that of Hsp31 suggesting that H-NS is implicated in *hchA* regulation. However, *hchA* was not identified in a genome-wide analysis of H-NS-dependent genes (Hommais *et al.*, 2001). The combined observations that Hsp31 upregulation in an *hns* null strain occurs during the early stationary phase of growth (Yoshida *et al.*, 1993), that many $E\sigma^S$ -transcribed genes are silenced by H-NS in exponential phase cultures (Barth *et al.*, 1995), and that the growth defects of $\Delta hchA$ cells at 48°C manifest themselves at

the transition between exponential and stationary phases (Figure 2.2A; Mujacic *et al.*, 2004) led us to consider the possibility that *hchA* is a member of the σ^S regulon.

To gain a better understanding of *hchA* regulation, we constructed an MC4100 derivative bearing a single copy *hchA::lacZ* translational fusion at the *att λ* site of the chromosome. The complete 591-nt-long *yedS-hchA* intergenic region was included to avoid the exclusion of any promoter or 5' regulatory elements. The *hchA::lacZ* fusion was next moved to isogenic *rpoS359*, Δhns and Δhns *rpoS359* genetic backgrounds (Table 3.1).

To examine Hsp31 expression patterns during various phases of growth, we monitored *lacZ* transcription from the *hchA* promoter by β -galactosidase assays and Hsp31 synthesis levels by immunoblotting following inoculation of the various strains in Luria–Bertani (LB) medium at 37°C. In wild-type cells, the β -galactosidase activity increased approximately sixfold and the Hsp31 level approximately threefold between the third and fourth hour of cultivation (Figure 3.1A and B). These time points bracket the transition between the exponential and the stationary phases of growth (Figure 3.1A, inset). A progressive rise in enzyme and protein levels was observed thereafter, culminating, after 24 h of incubation, in a ninefold increase in β -galactosidase activity and an 11-fold increase in Hsp31 concentration relative to 1 h samples (Figure 3.1A and B).

To determine whether σ^S was involved in the stationary phase induction of *hchA* transcription, the above experiments were repeated in the *rpoS* null lysogen MIR512. In this genetic background, transcription from the *hchA* promoter (Figure 3.1A) and Hsp31 accumulation levels (Figure 3.1B and C) were approximately half those observed in *rpoS*⁺ cells. Nevertheless, *rpoS* inactivation did not completely eliminate stationary phase induction of Hsp31 synthesis, suggesting that although it is a significant contributor to the process, σ^S is not the only sigma factor responsible for *hchA* transcription in stationary phase cells. In addition, because both β -galactosidase activities and Hsp31 protein levels were lower in the *rpoS* strain 1–3 h post inoculation

compared with the wild-type (Figure 3.1), $E\sigma^S$ is likely involved in *hchA* transcription in exponentially growing cultures.

In agreement with the results of Yoshida and coworkers (Yoshida *et al.*, 1993), β -galactosidase activities were approximately 2.5-fold higher in *hns* null cells relative to control cultures for the first 3 h of exponential growth (Figure 3.1A and inset). Nevertheless, intracellular β -galactosidase concentrations became comparable in the two genetic backgrounds at later time points, suggesting that H-NS has little influence on transcription from the *hchA* promoter once cultures have entered the stationary phase. Immunoblotting experiments revealed a similar trend at the protein level: Δhns cells contained elevated amounts of Hsp31 (approximately 2.5-fold more than wild-type cells) in the first 3 h of growth, but the steady-state accumulation levels became similar as cells progressed deeper into stationary phase (Figure 3.1B and C). Finally, in Δhns *rpoS359* cells, both β -galactosidase activities and protein levels were intermediates between those found in Δhns and *rpoS359* single mutants (Figure 3.1). In summary, the above results indicate that σ^S is one of the sigma factors involved in *hchA* transcription, while H-NS mainly acts as a silencer of *hchA* gene expression during the exponential phase of growth.

3.3.2. H-NS binds to the *hchA* promoter region which exhibits features of curved DNA

To determine whether H-NS directly or indirectly regulates Hsp31 expression, we first tested its ability to bind to the *hchA* promoter using a gel mobility shift assay. For this experiment, plasmid pMM120 was digested with PvuII and BlnI to generate a series of fragments including an 858 bp fragment encompassing the 591-bp-long *hchA* promoter region and the first 83 nucleotides of the *hchA* gene. The DNA was incubated with increasing amounts of hexahistidine-tagged H-NS or BSA as a control, and mixtures were fractionated on a polyacrylamide gel. Figure 3.2A shows that H-NS supplementation decreased the electrophoretic mobility of the *hchA* promoter fragment in a concentration-dependent manner while BSA addition had no effect. Consistent with

the fact that H-NS interacts with the promoter of the β -lactamase (*bla*) gene (Lucht *et al.*, 1994; Atlung *et al.*, 1996), the mobility of the *bla*-containing fragment was also affected upon H-NS addition (Figure 3.2A).

The two-dimensional polyacrylamide gel electrophoresis method of Mizuno (1987) allows easy identification of curved DNA in a mixture of fragments by relying on the fact that curved DNA is less mobile than non-curved DNA when electrophoresed at 4°C compared with 55°C. On such gels, non-curved DNA migrates on a straight diagonal line while curved DNA deviates from the diagonal. Because H-NS preferentially binds to curved DNA (Yamada *et al.*, 1990), we tested the *hchA* 5' region for intrinsic curvature as follows. Plasmid pMM120 was digested with ApaLI and PstI to generate an 880 bp fragment encompassing the entire *hchA* promoter region as well as four additional fragments of various lengths. The mixture was first electrophoresed at 55°C, followed by a second dimension electrophoresis at 4°C. While the non-curved digestion products migrated in a straight diagonal line, both the *hchA* promoter fragment and a fragment containing the curved *bla* region (Lucht *et al.*, 1994; Atlung *et al.*, 1996) migrated anomalously (Figure 3.2B). Taken together, the above results suggest that H-NS modulates *hchA* transcription by directly interacting with its curved promoter region.

3.3.3. *hchA* is transcribed from $E\sigma^S$ - and $E\sigma^D$ -dependent promoters

To obtain additional evidence for the involvement of $E\sigma^S$ in *hchA* transcription, we constructed plasmid pMM121, a ColE1 derivative encoding authentic *rpoS* under transcriptional control of the IPTG-inducible *trc* promoter (Table 3.1). Wild-type cells harboring either pMM121 or the cloning vector pSR22 were grown to mid-exponential phase at 37°C, treated with 1 mM IPTG, and samples collected immediately before and 1 h after induction were analysed by SDS-PAGE and subjected to immunoblotting with anti-Hsp31 antiserum. Figure 3.3 shows that even before IPTG addition – and likely due to the leakiness of the *trc* promoter – pMM121 transformants contained approximately threefold more Hsp31 than control cultures. One hour post induction, large amounts of

σ^S were present in MIR510 (pMM121) cells and the Hsp31 intracellular concentration had risen by an additional 40%. Under these conditions, about 3.5-fold more Hsp31 was present in σ^S -overproducing cells compared with the control. Thus, an increase in the levels of σ^S in exponential phase cultures is accompanied by an increase in Hsp31 synthesis, bringing further evidence for a role of σ^S in *hchA* transcription.

In order to map the promoter(s) of the *hchA* gene, we conducted primer extension analysis using mRNA samples collected from the mid-exponential and stationary phases of wild-type and *rpoS359* cultures. Exponential phase cells contained one major transcript (Figure 3.4A, lanes 1–2, lower arrow) whose start site is adenine – 55. Inspection of the upstream sequence revealed the presence of σ^D - (σ^{70})-like –10 and –35 promoter sequences proximal to the transcription start site (Figure 3.4B, solid boxes). Four out of six nucleotides of the putative –10 hexamer match the TATAAT consensus of the vegetative sigma factor σ^D , while the putative –35 box exhibits a single nucleotide mismatch relative to the TTGACA –35 consensus at the expected spacing of 17 nt. In addition, a TG doublet, known to enhance transcription and found in the extended –10 region of about a quarter of all $E\sigma^D$ -dependent promoters (Burr *et al.*, 2000), is located immediately upstream of the –10 box (grey box in Figure 3.4B).

A faint band corresponding to a 155 nt longer transcript starting at adenine –211 was also detected in exponentially growing *rpoS*⁺ cells but was absent in the *rpoS* null strain (Figure 3.4A, lanes 1–2, top arrow). The same transcript was however, dominant in wild-type cultures harvested in stationary phase and was completely missing in stationary phase *rpoS359* cells, suggesting that it is produced by $E\sigma^S$ (Figure 3.4A, lanes 3–4). The DNA region upstream of this second transcription start site contains several features characteristic of σ^S -dependent promoters. First, the nucleotide sequence between the transcription start point and the putative –10 box is A/T-rich, a common occurrence in $E\sigma^S$ -recognized promoters (Hengge-Aronis, 2002b). Second, we were able to identify a possible –10 hexamer (Figure 3.4B, dashed box) preceded by a TG doublet and containing hallmark C and T residues at positions –13 and –14 respectively (Becker and Hengge-Aronis, 2001). Third, and consistent with the observation that σ^S is

less sensitive to degeneration in the -35 region because $E\sigma^S$ interacts mainly with the -10 extended region (Hengge-Aronis, 2002b; Lacour and Landini, 2004), there was no obvious -35 hexamer. On the other hand, the -35 region contains CG and CCG motifs that are often found upstream of the -10 hexamer in σ^S -dependent promoters (Wise *et al.*, 1996; Lacour and Landini, 2004).

To confirm that $E\sigma^S$ transcribes *hchA*, we performed run-off *in vitro* transcription assay on an 878 bp pMM120 PstI fragment containing the complete 591 bp *yedS-hchA* intergenic region and the first 270 nt of the *hchA* gene. Based on the results of Figure 3.4A, and because $E\sigma^S$ recognizes σ^D -dependent promoter structures *in vitro* (Gaal *et al.*, 2001), we expected at least two different products: a 320 nt transcript corresponding to initiation at adenine -55 and a 470 nt transcript corresponding to initiation at adenine -211 . Two transcripts of correct size were indeed synthesized by $E\sigma^S$ (Figure 3.5). Similar experiments conducted with $E\sigma^D$ yielded the expected 320 nt transcript, an approximately 200 nt product that is the likely result of abortive synthesis and several bands in the 400–800 nt region (Figure 3.5). While the identity of these longer RNAs cannot be unambiguously ascertained, they may result from spurious transcription from upstream $E\sigma^D$ -like promoter regions (underlined in Figure 3.4B). Pre-incubation of the DNA template with H-NS completely abolished both $E\sigma^S$ and $E\sigma^D$ -dependent transcription (Figure 3.5) lending further support to the idea that H-NS acts as a direct negative regulator of *hchA* expression and suggesting that it silences both promoters.

In summary, *hchA* is transcribed from dual σ^D - and σ^S -dependent promoters with $E\sigma^S$ -driven transcription playing a minor role in exponential phase but a major role upon entry in stationary phase.

3.3.4. *Hsp31* contributes to the viability of stationary phase cells

To explore the physiological relevance of *Hsp31* upregulation in the stationary phase of growth, wild-type, $\Delta hchA$ and *rpoS359* cells were cultivated for 24 h and the number of

viable cells was assigned an arbitrary value of 1 (Figure 3.6, 0 h time point). Cultures were incubated for an additional 4 days and the number of survivors determined by plating at 24 h intervals. Figure 3.6 shows that whereas 2 days were necessary for the *rpoS* null mutation to exert a noticeable impact on cell survival, an approximately 25% reduction in the viability of $\Delta hchA$ cells was detected as early as 1 day after initiation of the experiment. Thus, Hsp31 appears to be important in the early stages of nutritional starvation management.

3.3.5. *Hsp31 remains heat-inducible in $\Delta rpoH$ cells*

Hsp31 has been classified as an Hsp based on the observation that the concentration of the *hchA* transcript increases 30-fold when mid-exponential phase cells growing at 37°C are subjected to a 7 min heat shock at 50°C (Richmond *et al.*, 1999). Because many heat-shock genes encoding cytoplasmic chaperones and proteases are transcribed by the $E\sigma^H$ ($E\sigma^{32}$) holoenzyme, we investigated the role of σ^H in *hchA* transcription. For these experiments, wild-type or isogenic $\Delta rpoH$ cells were grown to mid-exponential phase at 20°C (strains lacking σ^H are not viable above this temperature; Zhou *et al.*, 1988), subjected to a 30 min heat shock at 42°C, and culture aliquots were analysed by immunoblotting using antiserum raised against Hsp31 or DnaK. As expected, DnaK, whose structural gene is transcribed from two $E\sigma^H$ -dependent promoters (Cowing *et al.*, 1985; Zhou *et al.*, 1988), experienced an about twofold induction in *rpoH*⁺ cells but was virtually undetectable at both 20 and 42°C in the *rpoH* null mutant (Figure 3.7A, lanes 1–2 and 5–6). By contrast, thermal stress led to an increase in Hsp31 synthesis in both genetic backgrounds, although the concentration of Hsp31 in heat-shocked $\Delta rpoH$ cells was approximately half that present in the wild-type (Figure 3.7B, compare lanes 2 and 6).

Interestingly, whereas the DnaK levels were comparable in wild-type cells cultivated at either 20 or 37°C, about fivefold less Hsp31 was synthesized at 20°C relative to 37°C

(Figure 3.7B, compare lanes 1 and 3). However, the concentration of Hsp31 in Δhns cells grown at 20°C was similar to that present in wild-type cells grown at 37°C (Figure 3.7B, compare lanes 3 and 7). Results were confirmed using wild-type and Δhns cells carrying the *hchA::lacZ* fusion (we were unable to obtain an *hchA::lacZ* derivative of the $\Delta rpoH$ strain). At 20°C the levels of enzymatic activity was 3.5-fold higher in Δhns cells relative to the wild-type and whereas 30 min incubation at 42°C resulted in a 10-fold increase in activity in wild-type cells, there only was a twofold increase in β -galactosidase activity in the *hns* null background (data not shown). We conclude that σ^H is not absolutely required for the heat-induction of Hsp31 and that Hsp31 synthesis is downregulated at low temperatures in a process that involves H-NS.

3.3.6. Heat-shock transcription of hchA is primarily driven by its $E\sigma^D$ -dependent promoter and involves relief of H-NS silencing

To determine whether an alternative (and possibly σ^H -dependent) transcript was synthesized upon thermal stress, we conducted primer extension experiments with RNA samples extracted from wild-type and $\Delta rpoH$ cells that had been heat-shocked for 30 min at 42°C. Under heat shock conditions, the major *hchA* transcript originated from the σ^D -dependent promoter although there was a small contribution from the $E\sigma^S$ -dependent promoter (Figure 3.8, lane 2). The same pattern was observed in $\Delta rpoH$ cells, suggesting that $E\sigma^H$ is not directly involved in transcription of *hchA* under heat shock conditions. This result is consistent with the fact that the *hchA* 5' region does not contain nucleotide sequences matching the consensus of σ^H -dependent promoters (Cowing *et al.*, 1985).

In an effort to gain information on the dynamics of *hchA* heat-activation and to delineate the roles of σ^S and H-NS in the induction process, we measured β -galactosidase activities and Hsp31 protein levels in *hchA::lacZ* lysogens following heat shock. After 15 min incubation at 42°C, β -galactosidase levels in wild-type cells were 3.5-fold higher than in control cultures and six times more elevated after a 60 min heat shock. There was also good agreement between activity and protein levels (Figure 3.9A

and B). Consistent with a minor role of $E\sigma^S$ in *hchA* transcription in exponential phase cultures (Figures 3.1 and 3.4), β -galactosidase activities were approximately 20% lower in the *rpoS* null strain relative to the wild-type. However, the pattern of heat induction was similar to that observed in the wild-type (Figure 3.9A and B). In the *hns* null mutant, basal enzymatic activity and Hsp31 levels were threefold higher than in wild-type cells and these values only increased 1.5- to twofold upon heat shock. Because the Δhns *rpoS359* double mutant behaved comparably to the Δhns strain, it is unlikely that this H-NS-independent induction is the result of increased transcription from the σ^S -dependent *hchA* promoter. Finally, unlike the archetypal Hsp DnaK whose levels increased following a 15 min heat shock but returned to basal levels after 60 min of high temperature incubation (Figure 3.9C), *hchA* transcription and Hsp31 protein levels remained elevated even after 60 min incubation at 42°C (Figure 3.9). Overall, the above results suggest that *hchA* heat-induction relies primarily on the relief of H-NS silencing upon thermal upshift, possibly resulting from a conformational change in the DNA (Falconi *et al.*, 1998), and on a secondary mechanism that may involve direct or indirect activation of its $E\sigma^D$ -dependent promoter.

3.4. Discussion

Escherichia coli hchA was originally identified on the basis of its induction by heat shock (Richmond *et al.*, 1999) and Hsp31, the *hchA* gene product, was later shown to possess molecular chaperone (Sastry *et al.*, 2002; Malki *et al.*, 2003) and aminopeptidase functions (Lee *et al.*, 2003b; Malki *et al.*, 2005). However, while the genes encoding most cytoplasmic chaperones and stress-inducible proteases are transcribed from at least one $E\sigma^H$ -dependent promoter (Yura *et al.*, 1993; Gross, 1996), our results indicate that *hchA* is not a member of the σ^H regulon. Rather, the core polymerase vegetative sigma factor holoenzyme ($E\sigma^D$) is chiefly responsible for *hchA* transcription under heat shock conditions with a minor contribution from the upstream $E\sigma^S$ -dependent promoter (Figure 3.8). The mechanism by which Hsp31 becomes upregulated upon thermal stress is activation of *hchA* transcription, primarily through relief of H-NS-mediated silencing

(Figure 3.9). An H-NS independent mechanism that may be related to the effect of temperature on the supercoiling and topology of the promoter or involve unidentified cellular factors also plays a minor role in *hchA* thermal induction (Figures 3.7 and 3.9). This alternative to the use of a dedicated heat-shock sigma factor also means that high-level synthesis of Hsp31 is sustained throughout the length of the high temperature incubation phase instead of being transient as would be the case for an $E\sigma^H$ -transcribed gene (Figure 3.9). A need for high concentrations of Hsp31 is consistent with its proposed role as a holdase that stabilizes unfolding intermediates until stress has abated, thereby preventing overloading of the DnaK-DnaJ-GrpE system (Sastry *et al.*, 2002; Chapter 2).

Although $E\sigma^H$ does not directly transcribe *hchA*, we found that the concentration of Hsp31 in heat-shocked $\Delta rpoH$ cells was about half that present in the wild-type (Figure 3.7). There are several possible explanations for this observation. First, σ^H contributes to *rpoD* transcription under heat shock conditions from an upstream promoter located within the *dnaG* gene (Taylor *et al.*, 1984; Grossman *et al.*, 1985). Lower level of Hsp31 in heat-shocked $\Delta rpoH$ cultures could therefore be an indirect result of a decrease in the intracellular concentration of $E\sigma^D$. Second, the pool of free $E\sigma^D$ may be reduced in *rpoH* null strains as cells attempt to compensate for the lack of σ^H by driving the transcription of heat shock genes from their σ^D -dependent promoters. Finally, the DnaK-mediated stabilization of σ^S under heat shock conditions and concomitant increase in σ^S intracellular concentration (Muffler *et al.*, 1997) should be impaired in *rpoH* cells owing to the fact that they contain low levels of DnaK. This may in turn reduce *hchA* transcription from its $E\sigma^S$ -dependent promoter. Most likely, all three mechanisms contribute to some extent to the decrease in Hsp31 accumulation in heat-shocked $\Delta rpoH$ cells.

Interestingly, we also found that low temperature growth leads to a reduction in Hsp31 intracellular concentration (Figure 3.7B). Because this effect is abolished in *hns* null cells and because the levels of H-NS increase under cold shock conditions (La Teana *et al.*, 1991), this phenomenon is adequately explained by more effective H-NS-

mediated negative regulation of *hchA* transcription. In summary, Hsp31 expression is highly sensitive to the growth temperature and H-NS plays an important role in modulating Hsp31 concentration in exponential phase cells, silencing *hchA* transcription more efficiently as the temperature decreases while allowing RNA polymerase access to its $E\sigma^D$ - and $E\sigma^S$ -dependent promoters in times of thermal stress.

Our experimental results firmly establish that *hchA* is an H-NS-regulated protein belonging to the σ^S regulon. Consistent with this assignment, a number of genes transcribed by $E\sigma^S$ in stationary phase are silenced by H-NS in the exponential phase (Barth *et al.*, 1995) and *hchA* was recently identified as a σ^S -dependent gene in two independent microarray studies (Patten *et al.*, 2004; Weber *et al.*, 2005). One of the hallmarks of members of the general stress regulon is that their transcription increases during the transition from exponential to stationary phases of growth due to higher levels of σ^S (Weichart *et al.*, 1993; Schellhorn *et al.*, 1998). Hsp31 is no exception: in wild-type *E. coli*, protein levels triple upon entry into stationary phase, remain elevated for extended periods of time (Figure 3.1), and *hchA* becomes primarily transcribed from its $E\sigma^S$ -dependent promoter (Figure 3.4). The fact that $E\sigma^D$ -driven transcription partially compensates for the absence of σ^S in *rpoS* null cells (Figure 3.1) further suggests that high concentrations of Hsp31 are required for *E. coli* to handle nutrient-limited growth. This hypothesis is supported by our observation that *hchA* inactivation exerts a deleterious effect on long-term cell survival (Figure 3.6). Interestingly, stationary phase induction seems to be a conserved feature of Hsp31 family members as carbon starvation-stimulated entry into stationary phase is one of the conditions that lead to the upregulation of YDR533C, the yeast homologue of Hsp31 (Wilson *et al.*, 2004).

What might be the role of Hsp31 in stationary phase cultures? One possibility is that it helps support the folding and/or maintain the integrity of proteins that become important for survival under nutrient-limited conditions. As we have reported in the previous chapter (Table 2.2), enolase, a component of the RNA degradosome (Miczak *et al.*, 1996), and DeoA, DeoB and DeoC, three proteins implicated in deoxynucleoside and nucleoside catabolism and recycling (Hammer-Jespersen, 1983), are Hsp31

substrates. Starved *E. coli* cells catabolize rRNA in a process that involves the degradasome (Ben-Hamida and Schlessinger, 1966; Kaplan and Apirion, 1975; Davis *et al.*, 1986; Bessarab *et al.*, 1998). The resulting nucleosides must be further processed to be useful as a nutrient source. DeoABC catalyse downstream reactions that yield nitrogenous bases and acetaldehyde (which can be used as nitrogen sources under starvation conditions) as well glyceraldehyde 3-phosphate (which can be fed into the glycolytic cycle) (Hammer-Jespersen, 1983). A role for Hsp31 in the conformational quality control of DeoABC and enolase would be consistent with recent evidence for a σ^S -dependency of *deoA*, *deoB* and *deoC* (Kabir *et al.*, 2004; Lacour and Landini, 2004) and the need for intact enolase in degradasome function (Morita *et al.*, 2004). Considering that Hsp31 chaperone activity is negatively regulated by ATP (Sastry *et al.*, 2002) and that there is a precipitous drop in cellular ATP content upon transition from the exponential to the stationary phase of growth (Tran and Uden, 1998), Hsp31 may be a particularly effective holdase in starved cells.

Bulk protein degradation increases from 1–2% per hour in growing cells to 4–5% under starvation conditions and is an important contributor to the survival of carbon-starved *E. coli* (Goldberg *et al.*, 1976; Reeve *et al.*, 1984). Because Hsp31 contains a catalytic triad similar to that of *Pyrococcus horikoshii* protease I (Quigley *et al.*, 2003) and exhibits aminopeptidase activity (Lee *et al.*, 2003; Malki *et al.*, 2005), it is also possible that it functions as a peptidase in stationary phase cells. The observations that the ClpA remodelling component of the ClpAP protease interacts with Hsp31 (Malki *et al.*, 2005) together with the fact that ClpAP and ClpXP contribute to cell viability maintenance during extended stationary phase growth (Weichart *et al.*, 2003) raise the possibility that Hsp31 hydrolyses peptides generated by Clp proteases into individual amino acids. Given that the release of ammonia stimulated by amino-acid catabolism exposes cells to basic growth conditions (McFall and Newman, 1996) and that the optimal pH for Hsp31 aminopeptidase activity *in vitro* is 8 (Malki *et al.*, 2005), Hsp31 should be fully functional as a peptidase in stationary phase cells.

Genes belonging to the σ^S regulon encode proteins that act in a preventive manner, allowing cells to avoid damage rather than to deal with its consequences (Hengge-Aronis, 2000). As a result, stationary phase cells tolerate a wide range of adverse environmental conditions including high osmolarity, acidic pH, oxidative stress and suboptimal growth temperatures. Because of its diverse structural features and membership in the general stress regulon, we anticipate that, in addition to functioning as a holdase under heat shock conditions, Hsp31 performs a general protective function that enables *E. coli* to cope with a wide range of environmental perturbations.

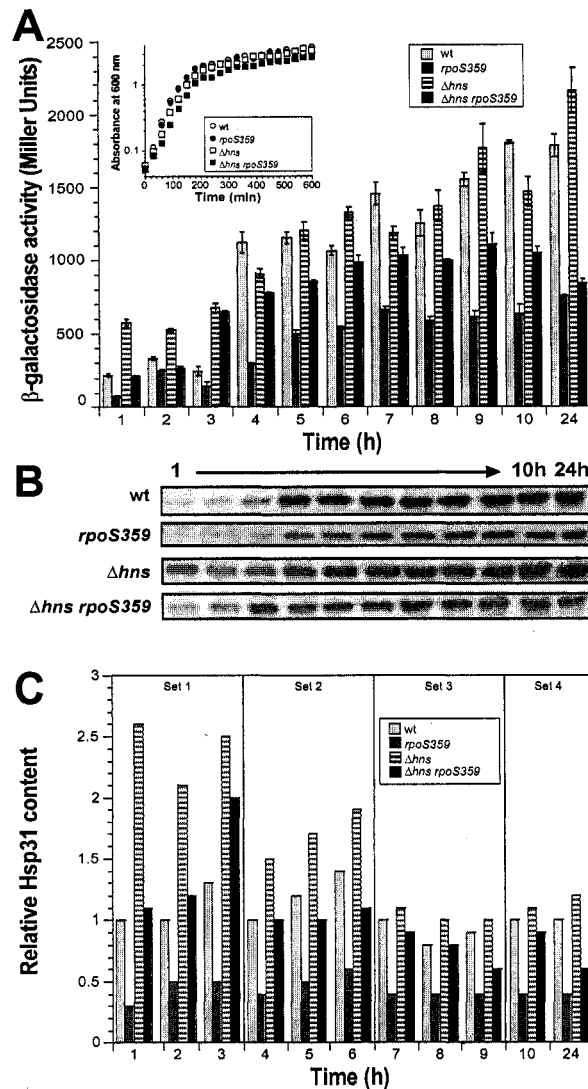


Figure 3.1. *hchA* is a stationary phase-inducible gene.

A. The growth of MIR510 (wt), MIR512 (*rpoS359*), MIR514 (Δhns) and MIR516 ($\Delta hns rpoS359$) cells in LB medium at 37°C was monitored at 30 min intervals for 10 h (inset). Soluble protein fractions collected every hour for 10 h and 24 h after inoculation were assayed for β -galactosidase activity.

B. Whole cells samples corresponding to 1-to-10h and 24h time-points were subjected to Western blot analysis with antiserum raised against Hsp31.

C. Whole cells samples from the various strains were run side by side on SDS-PAGE and immunoblotted to quantify Hsp31 levels. Four sets of blots corresponding to 1-3h, 4-6h, 7-9h and 10-24h samples were generated. Relative protein amounts were obtained by videodensitometric analysis and are normalized to the wild type 1h, 4h, 7h and 10h samples for sets 1 to 4, respectively. Thus, results can be directly compared within each set but not between sets.

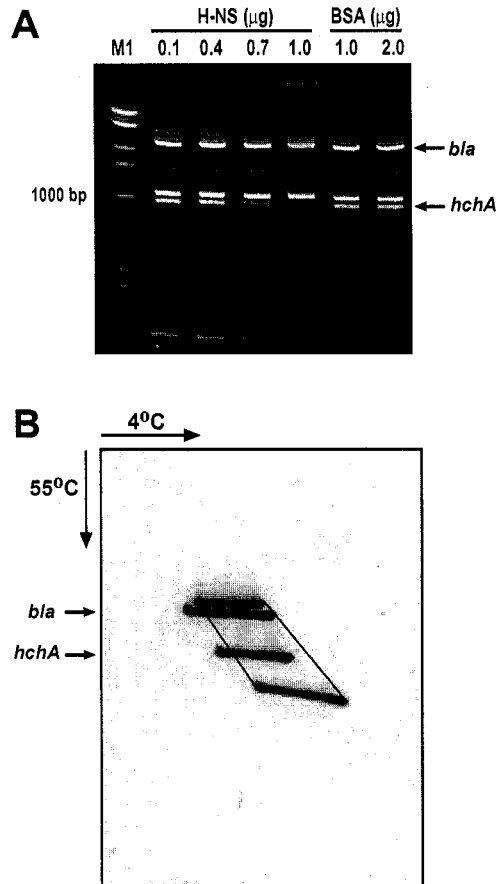


Figure 3.2. H-NS interacts with the curved *hchA* promoter region.

A. Plasmid pMM120 was digested with PvuII and BlnI to yield, among others, an 863 bp fragment spanning the *hchA* promoter region and a 2431 bp fragment containing the *bla* region. The DNA was incubated with increasing amounts of His₆-H-NS (0.1 to 1.0 µg) or with 1.0 or 2.0 µg of BSA, and samples were electrophoresed on a 6% acrylamide gel. Lanes M1 is a 1 kbp DNA ladder.

B. Plasmid pMM120 was digested with ApaLI and PstI and the resulting five DNA fragments (1325, 1246, 1167, 880 and 746 bp in length) were subjected to two-dimensional electrophoresis on a 4% acrylamide gel. The negative image of an ethidium bromide stained gel is shown. To facilitate identification of fragments deviating from the diagonal, parallel lines were drawn to connect the left and right ends of non-deviating fragments. Note that one of the non-curved fragments is partially masked by the *bla* fragment.

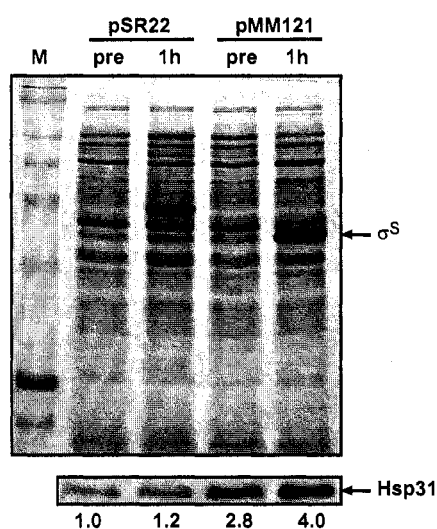


Figure 3.3. σ^S overproduction upregulates Hsp31 expression. Wild type (MIR510) cells harboring the *rpoS* expression plasmid pMM121 or the control vector pSR22 were grown at 37°C to mid-exponential phase and induced with 1 mM IPTG. Whole cell samples collected immediately before IPTG induction (pre lanes) or 1h thereafter (1h lanes) were analyzed by SDS-PAGE (top) and immunoblotting with antiserum raised against Hsp31 (bottom). Lane (M) contains molecular mass markers (250, 148, 98, 64, 50, 36, 22 and 16-kDa from top to bottom). Numbers below the blot correspond to the intensity of the Hsp31 band normalized to the pre-induction control sample.

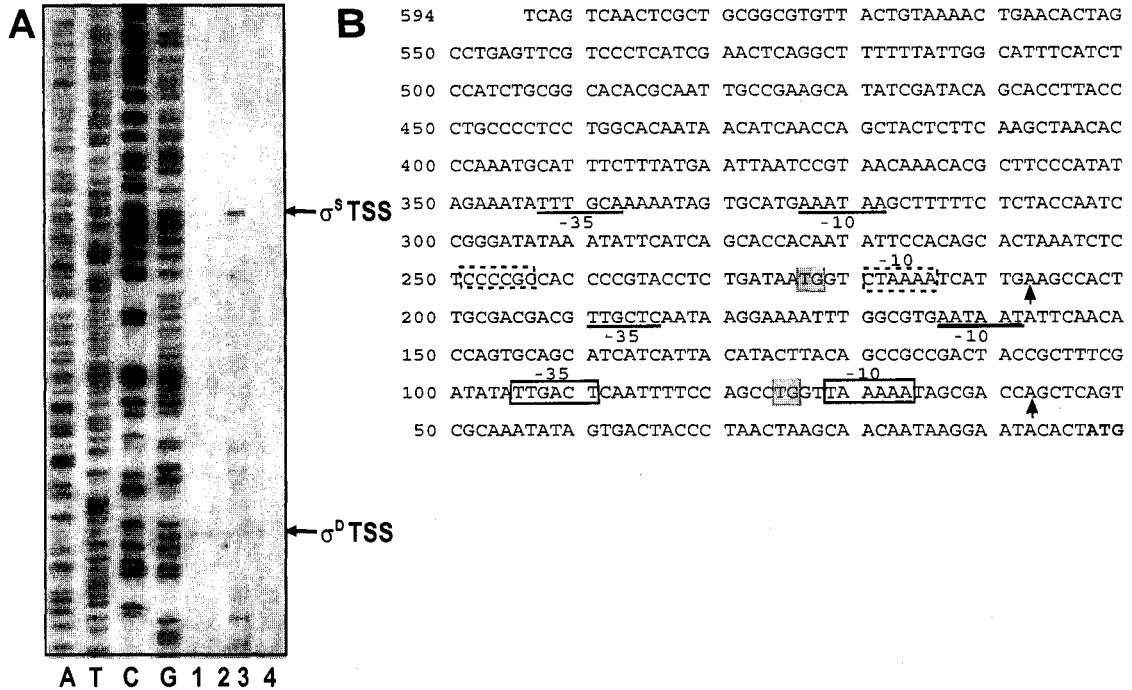


Figure 3.4. The growth phase influences *hchA* transcription start point.

A. RNA was extracted from mid-exponential (lanes 1 and 2) and stationary phase (lanes 3 and 4) cultures of wild type (lanes 1 and 3) or *rpoS359* (lanes 2 and 4) cells. Reverse transcription products were fractionated on a sequencing gel along with a dideoxy sequence ladder (A, T, C and G lanes). σ^D and σ^S -dependent transcription start sites are indicated by arrows. The faint low molecular mass bands in lane 3 may be caused by premature termination of primer extension. However, because they only appear in the stationary phase sample of wild-type cells and were also observed with a different primer (data not shown), they are more likely the result of an mRNA processing event.

B. Nucleotide sequence of the *yedS-hchA* intergenic region. σ^D and σ^S -dependent transcriptional start sites are shown with arrows. The proposed -10 and -35 hexamers of the σ^D -dependent promoter are identified by solid boxes. The proposed -10 hexamer of the σ^S -dependent promoter and the upstream CG-rich region are shown with dashed boxes. TG doublets are highlighted in gray and the start codon of *hchA* is in bold. Other putative σ^D promoters that may be active in run-off transcription experiments are underlined and labelled.

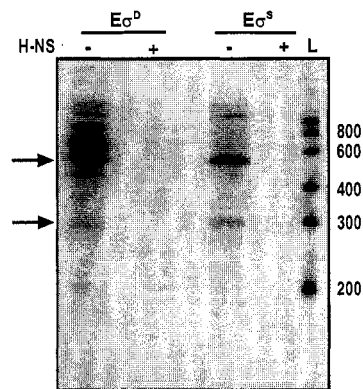


Figure 3.5. Reconstituted $E\sigma^S$ transcribes *hchA* in a reaction that is inhibited by H-NS. Run-off transcription assays were carried out with a blunted 878-bp *Pst*I pMM120 fragment carrying the complete intergenic region between *hchA* and its upstream neighbor *yedS*, as well as the first 270-nt of the *hchA* gene. Template DNA was incubated with either $E\sigma^S$ or $E\sigma^D$ in the absence (-) or presence (+) of 528 ng of His₆-H-NS. Lane L contains an RNA ladder.

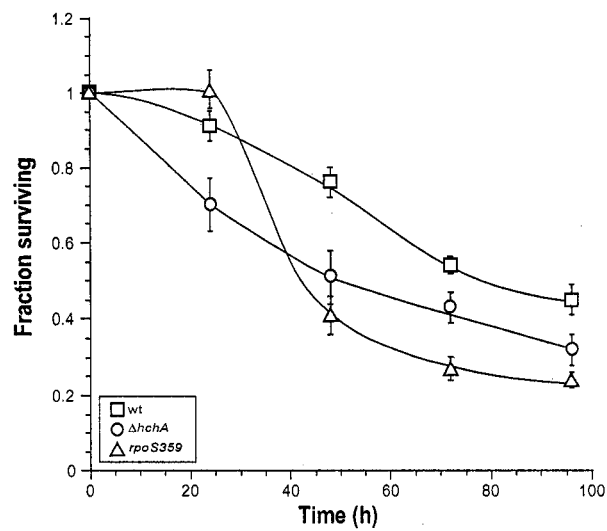


Figure 3.6. Hsp31 contributes to long-term survival under nutrient-limited conditions. MC4100 (wt), MIR401 ($\Delta hchA$), and RH90 ($rpoS359$) cells were grown at 37°C in W salts minimal medium supplemented with ammonium sulfate and glucose. Fraction of cells surviving at each time-point was determined as described in Material and Methods. The experiment was repeated on three different occasions and similar survival patterns were observed.

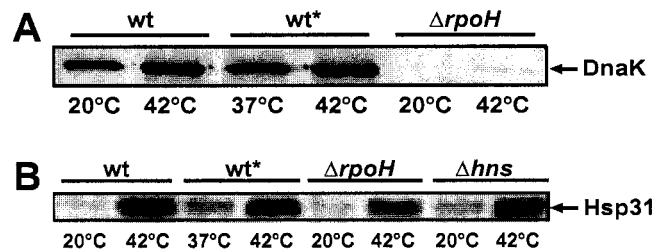


Figure 3.7. *hchA* remains heat-inducible in the absence of a functional *rpoH* allele. MC4100 (wt), KY1612 ($\Delta rpoH$) and MIR517 (Δhns) cells were grown to mid-exponential phase at 20°C or 37°C (wt* lanes) and subjected to a 30 min heat shock at 42°C. Whole cell samples collected before (20°C and 37°C lanes) and after heat-shock (42°C lanes) were subjected to immunoanalysis with antiserum raised against DnaK (**A**) or Hsp31 (**B**). Twice as much material was loaded in the pre-stress samples to facilitate Hsp31 detection.



Figure 3.8. *hchA* is primarily transcribed from its $E\sigma^D$ -dependent promoter upon heat shock. Wild-type (lane 2) and $\Delta rpoH$ cells (lane 3) were grown at 20°C to mid-exponential phase and transferred to 42°C for 30 min. RNA extracted from heat-shocked cells was subjected to primer extension analysis as described in the legend of Figure 3.4A. The product of a primer extension reaction conducted with wild-type stationary phase RNA was run in lane 1 to facilitate detection of the σ^S -dependent transcription start point.

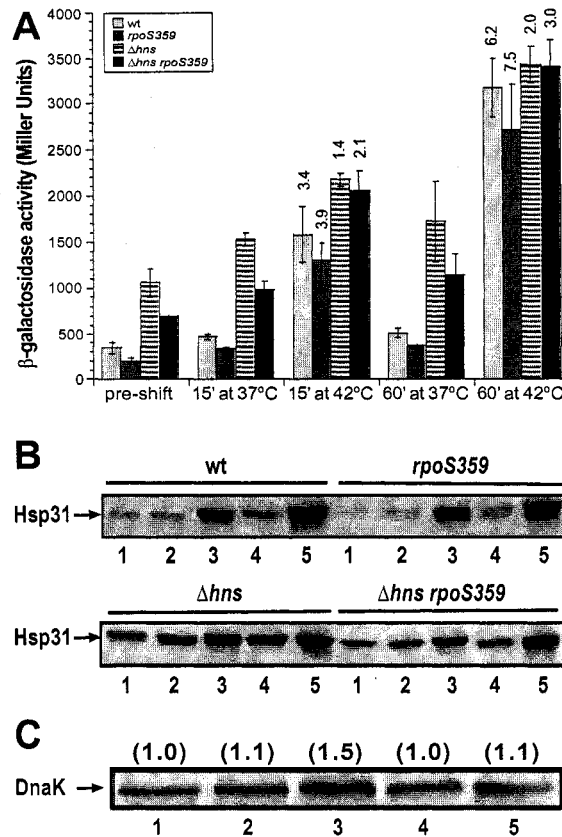


Figure 3.9. Influence of H-NS and σ^S on the heat induction of *hchA*.
A. MIR510 (wt), MIR512 (*rpoS359*), MIR514 (Δhns) and MIR516 ($\Delta hns rpoS359$) cultures were grown to mid-exponential phase in LB medium at 37°C (pre-shift) and either held at 37°C or transferred to 42°C. Samples were harvested after 15 and 60 min and assayed for β -galactosidase activity. Numbers above the bars show the fold increase in β -galactosidase activity in heat-shocked samples relative to samples held at 37°C. Error bars correspond to three independent experiments.
B. Whole cells samples collected immediately before heat shock (lanes 1), after 15 or 60 min incubation at 37°C (lanes 2 and 4, respectively), and after 15 or 60 min incubation at 42°C (lanes 3 and 5, respectively) were analyzed by immunoblotting with antiserum raised against Hsp31.
C. Whole cells samples collected from MIR510 (wt) cultures immediately before heat shock (lanes 1), after 15 or 60 min incubation at 37°C (lanes 2 and 4, respectively), and after 15 or 60 min incubation at 42°C (lanes 3 and 5, respectively) were immunoblotted with antiserum raised against DnaK. Numbers shown in parentheses above each lane correspond to the DnaK cellular content normalized to the pre-stress sample.

Table 3.1. *Escherichia coli* strains and plasmids used in Chapter 3 studies

Strain or plasmid	Genotype or description	Source or reference
<i>Strain</i>		
BSN1	M182 <i>hns::cat</i>	Forsman <i>et al.</i> (1992)
BL21(DE3)	<i>E. coli</i> B F- <i>dcm ompT hsdS</i> (τ_B - m_B -) <i>gal</i> λ (DE3)	Novagen
MC4100	<i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Laboratory stock
KY1612	MC4100 Δ <i>rpoH30::kan zhf50::Tn10</i> [λ pF13-(<i>groEp-lacZ</i>)]	Zhou <i>et al.</i> (1988)
MIR401	MC4100 Δ <i>hchA</i>	Mujacic <i>et al.</i> (2004)
RH90	MC4100 <i>rpoS359::Tn10</i>	Lange and Hengge-Aronis (1991)
MIR510	MC4100 $\lambda\phi$ (<i>hchA::lacZ</i>)	This work
MIR512	MC4100 <i>rpoS359::Tn10</i> $\lambda\phi$ (<i>hchA::lacZ</i>)	This work
MIR514	MC4100 <i>hns::cat</i> $\lambda\phi$ (<i>hchA::lacZ</i>)	This work
MIR516	MC4100 <i>rpoS359::Tn10 hns::cat</i> $\lambda\phi$ (<i>hchA::lacZ</i>)	This work
MIR517	MC4100 <i>hns::cat</i>	This work
<i>Plasmid</i>		
pTBGM	pBR322 derivative encoding a promoterless <i>lacZ</i> gene, Amp ^R	Vasina and Baneyx (1996)
pMM110	pTBGM derivative encoding a <i>hchA::lacZ</i> fusion, Amp ^R	This work
pMM120	pCR2.1TOPO derivative encoding <i>hchA</i> gene and its promoter, Amp ^R , Neo ^R	This work
pSR22	pET22b(+) derivative containing <i>trc</i> promoter followed by MCS, Amp ^R	Chow and Baneyx (2005)
pMM121	pSR22 derivative encoding <i>rpoS</i> gene under the control of <i>trc</i> promoter, Amp ^R	This work
pET28a	T7 expression vector, Kan ^R	Novagen
pMM122	pET28a derivative encoding N-terminally His-tagged <i>rpoS</i> gene under the control of T7 promoter, Kan ^R	This work
pTAC4632	Contains His-tagged <i>hns</i> gene under the control of T7 promoter, Amp ^R	Tove Atlung

CHAPTER 4: Expression Patterns of *hchA* and the Effect of Its Absence on Cell Survival under Various Stress Conditions

4.1. Objectives

As Chapter 3 results have shown, *hchA* is a member of the σ^S regulon, members of which are involved in a wide range of stress responses. To further explore a possible role of Hsp31 in the general stress response, we investigated its expression patterns under a variety of environmental stress stimuli. Particularly, we focused on ethanol, osmotic, oxidative, acidic, and electron transport chain inhibiting stresses. Once again, we utilized *hchA-lacZ* fusion in wild-type, *rpoS359*, Δhns and Δhns *rpoS359* backgrounds in order to delineate regulation hierarchy of *hchA* under the imposed stress conditions. Since Hsp31 accumulates in the stationary phase of growth and its absence impairs fitness of stationary phase cells, we also decided to examine whether Hsp31 is involved in promoting stress resistance of stationary phase cells. For this set of experiments, we utilized our $\Delta hchA$ mutant and compared its ability to survive exposure to the above-mentioned stress stimuli to that of the wild-type cells during the stationary phase of growth.

4.2. Materials and Methods

4.2.1. Strains and plasmids

Escherichia coli MC4100 derivatives RH90, MIR401, MIR510, MIR512, MIR514 and MIR516 have been previously described (Lange and Hengge-Aronis, 1991; Table 2.1; Table 3.1). To generate Hsp31 expression vector pMM114, *hchA* gene was PCR amplified from MC4100 chromosomal DNA with a forward (5'-CCCTAACTAAGCTTCAATAAGGAATAC-3') and reverse (5'-TTCAAACGTAAGCTTGATTAA CCGCG-3') primer pair, which introduced a 5' HindIII site upstream of the gene's ribosomal binding site and a 3' HindIII site downstream of the gene's stop codon. The gene was placed under the control of anhydrotetracycline-inducible *tetA* promoter following the ligation of HindIII-digested PCR product into the dephosphorylated

HindIII backbone of ptetA3, a chloramphenicol resistant derivative of pACYC184 with the *tetA* promoter insert from pSKAP/S (Griep *et al.*, 1999). DNA sequencing was used to confirm the integrity of *hchA* gene in pMM114 construct.

Bacterial cells were grown in one of the following media: Luria Bertani (LB), LB supplemented with 20 mM glucose (LBG), LB buffered at a pH of 5.5 with 100 mM morpholinethanesulfonic acid (LB-MES pH 5.5), brain-heart infusion supplemented with 0.4% glucose (BHIG), and minimal E medium supplemented with 0.4% glucose (EG; Vogel and Bonner, 1956).

4.2.2. Ethanol, hydrogen peroxide, sodium azide and sodium chloride induction experiments

Overnight cultures of various strains carrying *hchA-lacZ* fusion grown at 37°C in 5 ml of LB medium supplemented with 50 µg/ml streptomycin were used to inoculate 250 ml flasks containing 60 ml of LB/streptomycin at a 1:50 dilution. Cells were grown at 37°C to mid-exponential phase and were then split into two 25-ml aliquots. One aliquot was left untreated, while the other aliquot was treated with either 4% absolute ethanol (ETOH), 0.005% (~1.5 mM) hydrogen peroxide (H₂O₂), 2 mM sodium azide (NaN₃), or 500 mM sodium chloride (NaCl). Induction of the *hchA* promoter and levels of Hsp31 1h after stress administration were measured via β-galactosidase enzymatic activity assays and Western blotting, respectively.

To obtain soluble protein fractions for β-galactosidase assays, 2 ml culture samples were collected at indicated timepoints, sedimented by centrifugation at 6500 g for 10 minutes, resuspended in the equal volume of 50 mM monobasic potassium phosphate (pH 6.5) and lysed by French pressing at 10,000 psi. After centrifugation at 20,000 g for 20 minutes, soluble protein fractions were recovered as supernatants, triplicate aliquots of which were assayed for β-galactosidase activity using the chromogenic substrate *o*-nitrophenyl D-galactopyranoside (ONPG) as has been described (Miller, 1972). Whole cells samples used for Western blot analysis were obtained by resuspending the pellets, obtained after centrifugation of 1 ml cell samples at 10,000 g for 10 min, in 1X SDS-

DTT loading buffer. Buffer volumes were adjusted so that each sample corresponded to the identical amount of cells. Proteins were first separated on 12.5% SDS-PAGE mini-gels and then transferred onto nitrocellulose membranes. Hsp31 levels were determined using 1:600 dilution of rabbit-raised polyclonal Hsp31 antibody.

4.2.3. Acidic induction

Cells grown overnight at 37°C in 5 ml of LB supplemented with 50 µg/ml streptomycin were used to inoculate 125 ml flasks containing 25 ml of antibiotic-supplemented pH 7 LB. The corresponding cultures were grown at 37°C to late-exponential phase ($A_{600} \sim 1.0$) and were then inoculated at 1:5 ratio into pH 7 and pH 4 (adjusted with HCl) LB medium. Soluble protein fractions needed for the measurement of β -galactosidase enzymatic activity and whole cells samples for Western blot analysis were collected prior to and 1h after inoculation into acidic medium and were analyzed as described above.

4.2.4. Protein aggregation studies

For the experiments shown in Figure 4.2, wild-type and $\Delta hchA$ cells grown overnight in 5 ml of LB/streptomycin were inoculated into 500 ml flasks containing 150 ml of antibiotic-supplemented LB. Cultures were grown at 37°C to mid-exponential phase and were split into four 25-ml aliquots, two of which received 2 mM NaAz and the other two 0.005% H₂O₂. One of the NaAz and one of the H₂O₂ aliquots were kept at 37°C, while the remaining aliquots were shifted to 45°C. Insoluble protein samples were collected prior to and 1h after the stress treatment. The experiments with the Hsp31 expression plasmid pMM114 (Figure 4.3) were conducted in the same manner except for the use of chloramphenicol instead of streptomycin and the addition of 0.05 µg/ml anhydrotetracycline prior to inoculation of cells into 150 ml of LB.

Similarly, to determine the amount of aggregation under acidic conditions (Figure 4.7A), overnight cultures of wild-type and $\Delta hchA$ cells were inoculated into pH 7

LB/streptomycin in which they were grown until late exponential phase, followed by 1:5 inoculation into 25 ml of pH 7 and pH 4 LB. To observe the effects of Hsp31 overexpression on aggregation in $\Delta hchA$ cells under acidic conditions (Figure 4.7B), the experiment was conducted in the same manner except for the inoculation of overnight cultures into LB/chloramphenicol supplemented with 0.05 $\mu\text{g}/\text{ml}$ anhydrotetracycline. Once again, samples were collected prior to and 1h after the inoculation into the acidic medium.

To isolate insoluble protein fractions, 2 ml culture samples were collected, sedimented by centrifugation at 10,000 g for 10 min, resuspended in 2 ml of 50 mM potassium phosphate monobasic (pH 6.5), and disrupted with a French press at 10,000 psi. Insoluble protein fractions were recovered via centrifugation at 20,000 g for 20 minutes. Pellets corresponding to aggregated and membrane proteins were resuspended in 1X SDS-DTT loading buffer. Buffer volumes were adjusted based on A_{600} values so that each sample would correspond to an identical amount of cells. Aliquots were fractionated on 12.5% SDS-PAGE mini-gels and proteins were visualized by Coomassie blue staining. The gels were digitized, and protein content in each lane was determined by densitometric analysis using the NIH IMAGE software 1.61 for PowerPC. Numbers indicated below certain lanes represent the amount of aggregation in the lane normalized to the intensity of the OmpF band (identified in the figures with an asterisk).

4.2.5. Survival of stationary phase cells in the presence of various environmental stresses

Cells were grown for two days at 37°C in 5 ml of LB/streptomycin. Each culture was divided into 600 μl aliquots which were incubated for 1h at 37°C and 45°C in either the presence or the absence of one of the following compounds: 4% ETOH, 2 mM NaAz, and 0.005% H₂O₂. Samples collected after 1h were plated onto LB-agar-streptomycin plates, and the percent survival was calculated by counting the number of colonies on the plates and dividing them by the number of colonies present at the beginning of the

experiment. Each sample was plated in triplicate, and the experiment was repeated on three different occasions.

4.2.6. Cell survival under severe acidic conditions

Acidic resistance (AR) assays were performed in a manner similar to the previously described methods (Castanie-Cornet *et al.*, 1999). Cells were cultured overnight in a medium suitable for the expression of a specific acid resistance system. For the glucose-repressed oxidative acid resistance system (AR1), cells were grown in LB-MES pH 5.5, while for the glutamate-dependent acid resistance (AR2) and arginine-dependent acid resistance (AR3), LBG and BHIG were used, respectively. Overnight cultures of cells grown at 37°C in the appropriate medium supplemented with 50 µg/ml streptomycin were inoculated in a 1:40 ratio into 2 ml of unsupplemented pH 2.5 EG (AR1) and into pH 2.5 EG supplemented with either 1.5 mM glutamate (AR2) or 0.6 mM arginine (AR3). Cells were incubated at 37°C for 1h after which they were plated onto LB-agar-streptomycin plates. To determine percent survival, the number of colonies obtained after 1h incubation in pH 2.5 medium was divided by the number of colonies subjected to the acid stress. Each sample was plated in triplicate, and the experiment was repeated on three different occasions.

4.3. Results

4.3.1. Expression of Hsp31 is induced by a variety of environmental stresses

To determine whether Hsp31 exhibits characteristics typical of the members of σ^S regulon, we monitored its expression patterns in the presence of various environmental stresses. We decided to focus on four different stress stimuli: ethanol, sodium azide, hydrogen peroxide, and sodium chloride (Figure 4.1). Ethanol, hydrogen peroxide and sodium chloride are known inducers of σ^S regulon members

(Hengge-Aronis, 2000; Storz and Imlay, 1999; Hengge-Aronis *et al.*, 1993). Sodium azide is a toxin which inhibits ATP production by blocking the respiratory oxidation cascade. We were prompted to investigate its effects on *hchA* expression based on the observation that *in vitro*, under heat shock conditions, ATP inhibits chaperoning activity of Hsp31 (Sastry *et al.*, 2002). Thus, we postulated that Hsp31 might play an important role under limited ATP conditions when the function of the ATP-dependent workhorse chaperone systems DnaK-DnaJ-GrpE and GroEL-GroES might become impaired.

In order to gain insight into regulation of *hchA* expression under the imposed stress conditions, we used the previously described MC4100 derivative harboring a single copy of the translational *hchA-lacZ* fusion at the *att λ* site, as well as the corresponding *rpoS359*, Δhns and Δhns *rpoS359* mutants (Chapter 3, Table 3.1). In addition to monitoring *lacZ* transcription from the *hchA* promoter with β -galactosidase assays, we also measured corresponding Hsp31 levels via immunoblotting.

Several features of Hsp31 expression patterns are shared between the four employed stress stimuli. In all four cases in a wild-type background, stress treatment lead to a clear increase in β -galactosidase activity and Hsp31 protein levels. Beta-galactosidase activity levels were 1.9-2.5-fold higher in stressed wild-type cells compared to the cells that received no stress treatment (Figure 4.1 bar graphs). Corresponding increases were observed in the cellular Hsp31 pool (Figure 4.1 blots). Stress-induced increase in β -galactosidase activity and Hsp31 levels was also observed in the *rpoS359* mutant. However, the amount of post-stress Hsp31 produced in this background was lower by 18% (ethanol), 46% (sodium azide), 39% (hydrogen peroxide) and 70% (sodium chloride) than the amount produced in the wild-type cells (compare wt and *rpoS359* lanes 3 in Figure 4.1). As we previously observed (Figure 3.1B), the absence of a functional *rpoS* allele caused an

approximate 45% decrease in Hsp31 levels even under basal conditions (i.e. prior to any stress treatment). Since this difference actually decreased in ethanol-stressed cells and remained about the same in sodium azide treated cells, we can speculate that RpoS is not the main sigma factor involved in the induction of *hchA* expression under these conditions (Figure 4.1A-B). On the other hand, the difference in Hsp31 protein levels between wild-type and *rpoS359* cells further increased under oxidative and osmotic shock conditions (Figure 4.1C-D), indicating that *hchA* induction under these stress conditions is primarily RpoS-dependent.

While *rpoS359* mutants exhibited across the board lower levels of β -galactosidase activity and Hsp31, the opposite was true in the cells lacking the global regulator H-NS (Figure 4.1A-D). This is not surprising considering that H-NS acts as a negative regulator of *hchA* expression by directly interacting with its promoter region (Chapter 3). Overall, basal β -galactoside activities and Hsp31 protein levels were about 3-6-fold higher in Δhns cells compared to the wild-type cells. These fold differences decreased by about 50% in stress-treated cells suggesting that relief of H-NS silencing is one of the steps involved in induction of the *hchA* gene in the presence of the examined stress stimuli (compare wild-type and Δhns lanes 3 in Figure 4.1A-D). Similar pattern was exhibited by the double Δhns *rpoS359* mutant. However, the fold differences in Hsp31 protein levels between the wild-type and the double mutant under basal and stress conditions were ~50% of those observed in the Δhns mutant (Figure 4.1A-D). Furthermore, the absence of a functional *rpoS* allele in the double mutant lead to Hsp31 protein levels that were consistently 25-30% lower than those observed in the single Δhns mutant under basal and stress conditions (compare Δhns and Δhns *rpoS359* lanes 1-3 in Figure 4.1A-D). These observations further attest to the contribution of σ^S to *hchA* transcription. Finally, owing to such high basal levels of Hsp31 and β -galactosidase in the single and double mutants, the addition of various stresses resulted in much lower or non-existent fold inductions (Figure 4.1A-D).

In summary, *hchA* expression is induced in the presence of various stress stimuli suggesting its role as a general stress protein. To be fully induced under these conditions, *hchA* appears to rely on the removal of promoter silencing by H-NS and is dependent on the presence of a functional *rpoS* allele, especially in the cases of oxidative and osmotic shocks.

4.3.2. *The role of Hsp31 in in vivo protein aggregation under ATP-limiting and oxidative stress conditions*

Since we observed induction of Hsp31 under ATP-depleting and oxidative stress conditions (Figure 4.1B-C), we decided to investigate whether Hsp31 might play a role in maintaining structural integrity of cellular proteins under such conditions. Thus, we subjected mid-exponential phase MC4100 cells and their $\Delta hchA$ derivatives to 1h treatment with either 2 mM sodium azide (Figure 4.2A) or 0.005% hydrogen peroxide (Figure 4.2B) at 37°C and 45°C. As we noted in our previous study (Mujacic *et al.*, 2004), $\Delta hchA$ cells exhibited slightly higher levels of insoluble proteins even prior to the stress treatment. Milder stress consisting of 1h incubation in the presence of 2 mM sodium azide at 37°C lead to modest 11% (wt) and 20% ($\Delta hchA$) increases in aggregation when compared to pre-stress samples. However, sodium azide administration at 45°C resulted in a 49% and a 67% increase in insoluble protein accumulation in wild-type and *hchA* null cells, respectively (Figure 4.2A). Furthermore, the level of aggregation in $\Delta hchA$ cells under these conditions was ~40% higher than the level observed in wild-type cells. Similar trends were observed when cells were treated with hydrogen peroxide (Figure 4.2B). No significant increase in protein aggregation was observed in the wild-type sample collected after 1h incubation at 37°C in the presence of 0.005% hydrogen peroxide, while only an 11% increase was observed in the $\Delta hchA$ mutant. Combination of heat and oxidative shocks, however, lead to a 49% increase in aggregation in wild-type

cells and a 60% increase in mutant cells, with mutant cells containing ~26% more insoluble proteins than the cells harboring an intact *hchA* allele (Figure 4.2B).

Next, we wanted to determine whether overexpression of Hsp31 is capable of alleviating the observed increase in aggregation in $\Delta hchA$ cells subjected to either sodium azide or hydrogen peroxide treatment at 37°C and 45°C. We constructed a pACYC184 derivative containing the *hchA* gene under the transcriptional control of the anhydrotetracycline-inducible *tetA* promoter. MC4100 cells lacking their chromosomal *hchA* copy and harboring either the empty expression vector, ptetA3, or Hsp31 expression vector, pMM114, were incubated in LB medium in the presence of 0.05 µg/ml anhydrotetracycline until the mid-exponential growth phase at which point they were treated with either 2 mM sodium azide or 0.005% hydrogen peroxide and were left at 37°C or shifted to 45°C (Figure 4.3). As is evident from the corresponding gels, overexpression of Hsp31 resulted in a decrease in aggregation of a subset of cellular proteins marked by arrows in Figure 4.3. A large fraction of the affected proteins is less than 36 kD in size, and their migration agrees well with the migration of proteins exhibiting increased aggregation in $\Delta hchA$ background in the presence of sodium azide and hydrogen peroxide (Figure 4.2) as well as under severe heat shock conditions (Figure 2.4). Thus, Hsp31 appears to be required for maintaining structural integrity of a subset of cellular proteins under ATP-depleting and oxidizing conditions, especially when these stresses occur in a heat shock environment when other chaperones might become overburdened or dysfunctional.

4.3.3. Stationary phase cells lacking Hsp31 are impaired in their ability to survive exposure to various environmental stresses

In lieu of the above-presented data and in addition to our previous observation that Hsp31 levels increase dramatically during the stationary phase of growth in an RpoS-dependent manner (Figure 3.1), we investigated the role of Hsp31 in resistance of stationary phase cells to various environmental stimuli. Wild-type and $\Delta hchA$ cells were grown in LB medium at 37°C for two days, after which they were split

into aliquots and either left at 37°C and 45°C for one hour or exposed to 4% ethanol at 37°C, 2 mM sodium azide at 37°C and 45°C, and 0.005% hydrogen peroxide, likewise at 37°C and 45°C. Samples were plated and the number of surviving colony forming units was counted and compared to the number present prior to the stress treatment. The addition of ethanol, sodium azide and hydrogen peroxide to the wild-type cells at 37°C did nothing to diminish their survival, which ranged from 102% in the presence of ethanol to 111% in the presence of hydrogen peroxide (Figure 4.4). Even under elevated temperature conditions these reagents were incapable of making a significant impact on the survival of wild-type cells, which hovered at around 94-98%. On the other hand, the number of surviving $\Delta hchA$ mutants decreased either by 10% under oxidative stress conditions or by ~20% during ethanol and sodium azide stresses at 37°C. Even more drastic survival impairment was observed at 45°C where 76% of $\Delta hchA$ colony forming units survived the exposure to heat shock alone, while an additional 10-11% drop in survival was observed when heat shock treatment was combined with either hydrogen peroxide or sodium azide (Figure 4.4). Therefore, we conclude that Hsp31 plays a role in maintaining stress resistance of stationary phase cells.

4.3.4. Hsp31 is strongly induced by acidic stress and plays an important role in the ability of Escherichia coli to survive exposure to severe acid stress

As previously mentioned, acid resistance is one of the hallmarks of *E. coli*'s stationary phase cells with three main acid resistance systems - oxidative or glucose-repressed (AR1), glutamate-dependent (AR2), and arginine-dependent (AR3) - identified to date (Chapter 1; Richard and Foster, 2003). To determine whether Hsp31 is involved in acid stress response, we first aimed to discern whether acidic stress promotes accumulation of *hchA* gene product. To do this, we grew wild-type, Δhns and $\Delta hns rpoS359$ cells harboring the chromosomal *hchA-lacZ* fusion at 37°C in pH 7 LB until the late exponential phase after which we inoculated the cells in a

1:5 ratio into pH 4 and pH 7 LB. A strong ~3-fold acidic induction in β -galactosidase activity and the level of Hsp31 was observed in a wild-type background (Figure 4.5A). As a comparison, the level of molecular chaperon DnaK increased only by 20% under acidic conditions when compared to its level in pH 7 LB (Figure 4.5A). Acidic induction of Hsp31 was significantly diminished in *rpoS359* cells. A slight 30-40% increase in β -galactosidase activity and Hsp31 was observed in acid-stressed *rpoS359* cells with the amount of post-acid stress Hsp31 reaching only 36% of the amount present in wild-type cells (compare wild-type and *rpoS359* Hsp31 lanes 3 in Figure 4.5A). Despite the already high basal Hsp31 and β -galactosidase levels in Δhns background, acidic stress prompted an even further increase in the activity and protein levels (Figure 4.5A). However, when *rpoS359* allele was placed into the Δhns background, the extent of acidic induction decreased (compare Δhns and Δhns *rpoS359* lanes and bars in Figure 4.5A). Overall, these data suggest that the removal of H-NS-mediated silencing of the *hchA* promoter and the concomitant recruitment of RpoS to the promoter region contribute to Hsp31 induction under acidic conditions in a glucose-free rich medium. However, RpoS is not absolutely required for the observed acidic induction of *hchA* since Hsp31 is capable of accumulating in acid-stressed *rpoS359* cells, albeit at a level much lower than that obtained in the wild-type background.

To further elucidate the role of Hsp31 in acid stress response, we decided to investigate its involvement in the ability of *E. coli* to withstand exposure to severe acidic stress. Wild-type, $\Delta hchA$ and *rpoS359* cells were incubated overnight at 37°C in three different media, each optimal for activation of one of the three acid resistance systems. LB-MES pH 5.5 was used to induce the expression of AR1, while LBG and BHIG were used to activate AR2 and AR3, respectively (Castanie-Cornet *et al.*, 1999). Aliquots of overnight cultures were then exposed to 1h acidic challenge in pH 2.5 EG medium that was either left unsupplemented for the AR1

test, or was supplemented with 1.5 mM glutamate or 0.6 mM arginine for AR2 and AR3 tests, respectively. Percent survival was determined by comparing the number of colony forming units present at the end of the acid challenge to the number present prior to the administration of acid stress

Glutamate-dependent acid resistance system proved to be the most efficient defense system in wild-type cells, allowing an 85% survival rate in glutamate-supplemented pH 2.5 EG (Figure 4.6). Activation of AR3 resulted in 67% survival of wild-type cells, while, at 19%, AR1 provided the lowest survival rate under severe acidic conditions. When compared to wild-type cells, acidic survival of *rpoS359* mutants was significantly impaired under the conditions in which AR1 was activated. This is not surprising because RpoS is one of the key regulators of AR1 and its absence results in the blockage of this system (Castanie-Cornet *et al.*, 1999). Conversely, RpoS is not a major regulator of AR2 and AR3 systems (Foster, 2004), and therefore percent survival of *rpoS359* cells in pH 2.5 EG in the presence of either glutamate or arginine was somewhat lower but in the same order of magnitude as that of the wild-type cells tested under the identical conditions (Figure 4.6). While the absence of *rpoS* allele severely impacted the performance of on only one of the three AR systems, the same was not true for $\Delta hchA$ cells in which all three AR systems exhibited markedly diminishment function (Figure 4.6). The biggest impact of the *hchA* deletion was observed on the performance of the glutamate-dependent acid resistance system. When the cells lacking *hchA* were subjected to an acidic challenge in pH 2.5 EG supplemented with glutamate, only 0.003% of them survived, compared to 85% and 57% survival of wild-type and *rpoS359* mutants, respectively. Acidic survival of $\Delta hchA$ mutants in the arginine-supplemented pH 2.5 EG was higher by an order of a magnitude compared to the AR2 survival, while the absence of Hsp31 under AR1 activating conditions resulted in a survival rate (1.2%) that was ~70-fold lower than that observed in wild-type cells, but was not as severe as the rate obtained in the absence of a functional *rpoS* allele (Figure 4.6). Clearly,

Hsp31 contributes greatly to the performance of all three acid resistance systems in *E. coli* with special emphasis on the glutamate-dependent system.

4.3.5. The absence of Hsp31 under acidic stress conditions leads to an increase in protein aggregation

As a consequence of incubation in an acidic environment, carboxyl groups of amino acids become protonated, leading to a decrease in the number of positive charges in a protein and a concurrent reduction in protein stability governed by electrostatic interactions. Therefore, acidic challenge tends to promote protein unfolding and aggregation. The majority of Hsp31 produced under acidic stress conditions is soluble (Figure 4.5B) and presumably functional and capable of molecular chaperoning activity. To investigate the involvement of Hsp31 in maintaining the folding fidelity of cellular proteins in a low pH environment, we compared the amount of aggregation in wild-type and $\Delta hchA$ cells exposed to pH 4 LB for one hour. The level of protein aggregation in $\Delta hchA$ cells was 73% higher than the level observed in the wild-type (Figure 4.7A). The pattern of aggregated protein was very similar to that observed in $\Delta hchA$ cells subjected to ATP-depleting and oxidative conditions (Figure 4.2). Once again, overexpression of Hsp31 in the $\Delta hchA$ background from the anhydrotetracycline-inducible *tetA* promoter in pMM1114 was marked by a reduction in aggregation of a subset of proteins (Figure 4.7B). Taken together with the acid survival data (Figure 4.6), these results further attest to the importance of Hsp31 in the ability of *E. coli* to cope with severe acid stress.

4.4. Discussion

Based on the strong σ^S -dependent stationary phase induction of Hsp31, its heat shock inducibility and the lessened ability of $\Delta hchA$ cells to survive under conditions of prolonged stasis, we postulated that Hsp31 might play an important role in

E. coli's general stress response. Data presented in this chapter confirm our hypothesis. Here we have shown that ethanol, sodium azide, hydrogen peroxide, acidic and osmotic shocks promote cellular accumulation of Hsp31 (Figure 4.1, Figure 4.55A). The removal of H-NS silencing and increased transcription from *hchA*'s $E\sigma^S$ -dependent promoter appear to be the major factors affecting *hchA* induction under oxidative, hyperosmotic and acidic conditions (Figure 4.1C-D, Figure 4.5A). However, based on the observations that even in the absence of RpoS these stresses are capable of eliciting a modest increase in Hsp31, we can conclude that $E\sigma^D$ -dependent transcription also makes a contribution to *hchA* expression during oxidative, hyperosmotic and acidic stresses. On the other hand, ethanol and sodium azide-stimulated increases in Hsp31 appear to be mainly driven by the relief of the association of H-NS with *hchA*'s promoter and subsequent transcription by $E\sigma^D$ (Figure 4.1A-B). Ethanol is a known inducer of heat shock response (Van Bogelen *et al.*, 1987), with misfolded membrane protein pre-cursors acting as the most likely cytoplasmic heat shock triggers (Chaudhuri *et al.*, 2006). As we have shown previously, heat shock induction of *hchA* is mainly driven by $E\sigma^D$ (Chapter 3). Therefore, it is not surprising that in the presence of ethanol major contributor to *hchA* transcription is $E\sigma^D$. The involvement of multiple transcription regulators is a feature shared by many members of various stress resistance systems. For example, *gadABC* genes of the glutamate-dependent acid resistance system are subject to a complex regulatory circuit governed by σ^D and σ^S , as well as by a handful of transcription factors, among them H-NS (Masuda and Church, 2003; Giangrossi *et al.*, 2005). Similar to our *hchA* acidic induction data, removal of H-NS allele in a $\Delta rpoS$ background results in $E\sigma^D$ -driven expression of *gadAB* (Waterman and Small, 2003).

In agreement with the *hchA* expression patterns, the absence of Hsp31 under the examined stress conditions leads to an increase in the accumulation of aggregated proteins (Figure 4.2, Figure 4.7A). This phenotype is most pronounced when cells

subjected to oxidative or electron transport chain uncoupling stresses are further exposed to heat shock conditions (Figure 4.2). *In trans* addition of Hsp31 can, on the other hand, alleviate the extent of misfolding under these circumstances (Figure 4.3). Why might the contribution of Hsp31 under such conditions be important? As the electron transport chain uncoupler, sodium azide decreases cellular ATP pool. Similarly, it has been recently shown that exposure of *E. coli* cells to reactive oxygen species leads to a decline in intracellular ATP levels under physiological and heat shock conditions (Winter *et al.*, 2005). A similar effect of hydrogen peroxide stress on ATP levels was observed in *Saccharomyces cerevisiae* (Osorio *et al.*, 2003). In addition to a drop in ATP pools, oxidative stress damages major molecular chaperone DnaK by carbonylating its amino acid residues (Tamarit *et al.*, 1998), while a combination of oxidative and heat stresses reversibly inactivates DnaK by rendering its N terminus thermolabile (Winter *et al.*, 2005). One can postulate that under such conditions in which DnaK and possibly other ATP-dependent chaperones become incapacitated, the role of ATP-independent molecular chaperones in maintaining structural integrity of cellular proteins becomes prominent. Indeed, a recent study has shown that redox-regulated and ATP-independent chaperone holdase Hsp33 protects numerous cellular proteins from irreversible aggregation in an oxidizing environment at elevated temperatures (Winter *et al.*, 2005). Similarly, Hsp31 has been shown to function as an ATP-independent molecular holdase *in vitro* under heat shock conditions (Sastry *et al.*, 2002). Additionally, we have shown that its absence in a *dnaK* mutant background further aggravates temperature-sensitive phenotype of these cells and causes a concomitant increase in protein aggregation (Chapter 2), further suggesting its importance under the conditions in which DnaK becomes dysfunctional. It is possible that Hsp31's role under these stress conditions is to prevent structural damage of proteins involved in elimination of the imposed stress. In our initial study with the $\Delta hchA$ mutant, we have identified AhpC, a hydrogen peroxide scavenger and a member of OxyR regulon, as a substrate of Hsp31 (Table 2.2). Furthermore, association of Hsp31 with elongation factor EF-

Tu, one of the proteins susceptible to oxidation-induced damage (Dukan and Nyström, 1998), has been reported (Malki *et al.*, 2005). Finally, another clue as to a possible role of Hsp31 under oxidative stress conditions comes from the observations made with its human ortholog, DJ-1. The absence of human DJ-1 enhances cell death by oxidative stress (Yokota *et al.*, 2003)). *In vitro*, DJ-1 eliminates hydrogen peroxide by oxidizing itself (Taira *et al.*, 2004). Therefore, it is possible that in addition to acting as a molecular chaperone under oxidative stress conditions, Hsp31 provides relief from oxidizing damage by directly eliminating reactive oxygen species.

In addition to impacting the folding fidelity of a subset of cellular proteins under ATP-depleting and oxidative conditions, Hsp31 also plays an important role in the fitness of cells exposed to a number of stress stimuli during stasis. Its absence leads to a diminished capacity for survival of stationary phase cells in the presence of elevated levels of ethanol, hydrogen peroxide and sodium azide under physiological and heat shock conditions (Figure 4.4). Because stationary phase cells exhibit a drop in ATP levels (Tran and Uden, 1998) and an increase in production of aberrant protein chains combined with a stasis-induced increase in oxidative carbonylation (Ballesteros *et al.*, 2001), Hsp31 might have a dual purpose in stationary phase cells: first, as a sort of a house-keeping molecular chaperone, whose role is to make sure that the structural integrity of the members of various stress responses remains intact and well primed for dealing with stresses that cells might encounter, and second, as the alleviator of the protein folding damage resulting from the exposure of stationary phase cells to any particular stress they may encounter. The most drastic effect of Hsp31 on stress-coping abilities of stationary phase cells was observed upon the exposure of cells to a severe acidic challenge (Figure 4.6). Acid stress triples cellular supply of Hsp31 (Figure 4.5A) in a mainly RpoS-dependent manner, while the level of major molecular chaperone DnaK increases by only 20%. In concurrence with such a significant increase in Hsp31, the removal of the *hchA* allele has a dramatic impact on the survival of stationary phase cells in pH 2.5 medium. In

its absence, the performance of all three acid resistance systems is severely impacted (Figure 4.6), with the biggest drop in function exhibited by the glutamate and arginine-dependent systems. In a wild-type background, glutamate-dependent system affords the highest level of acid protection to the cells, while in $\Delta hchA$ cells its performance is completely attenuated. The precise role of Hsp31 under acidic conditions has yet to be elucidated, but it is of interest to note that acidic induction seems to be a conserved characteristic of Hsp31 homologs as it has been reported that expression of YDR533C, yeast ortholog of *hchA*, is induced 2-fold following the exposure of yeast cells to 0.9 mM sorbic acid at pH 4.5 (de Nobel *et al.*, 2001). Based on our aggregation data (Figure 4.7) and due to its effect on all three acid resistance systems, we postulate that Hsp31 most likely promotes acidic stress survival of cells by ensuring that proteins directly involved in removal and neutralization of excess proton charges retain their function. In other words, Hsp31 might act as the cytoplasmic counterpart of HdeA, molecular chaperone whose main role is alleviation of periplasmic aggregation in acid-stressed cells (Gajiwala *et al.*, 2000). However, due to its diverse structural features, including a putative catalytic triad (Quigley *et al.*, 2003), one cannot exclude a possibility that, in addition to its role as a molecular chaperone, Hsp31 might exhibit additional functional capabilities in an acidic environment.

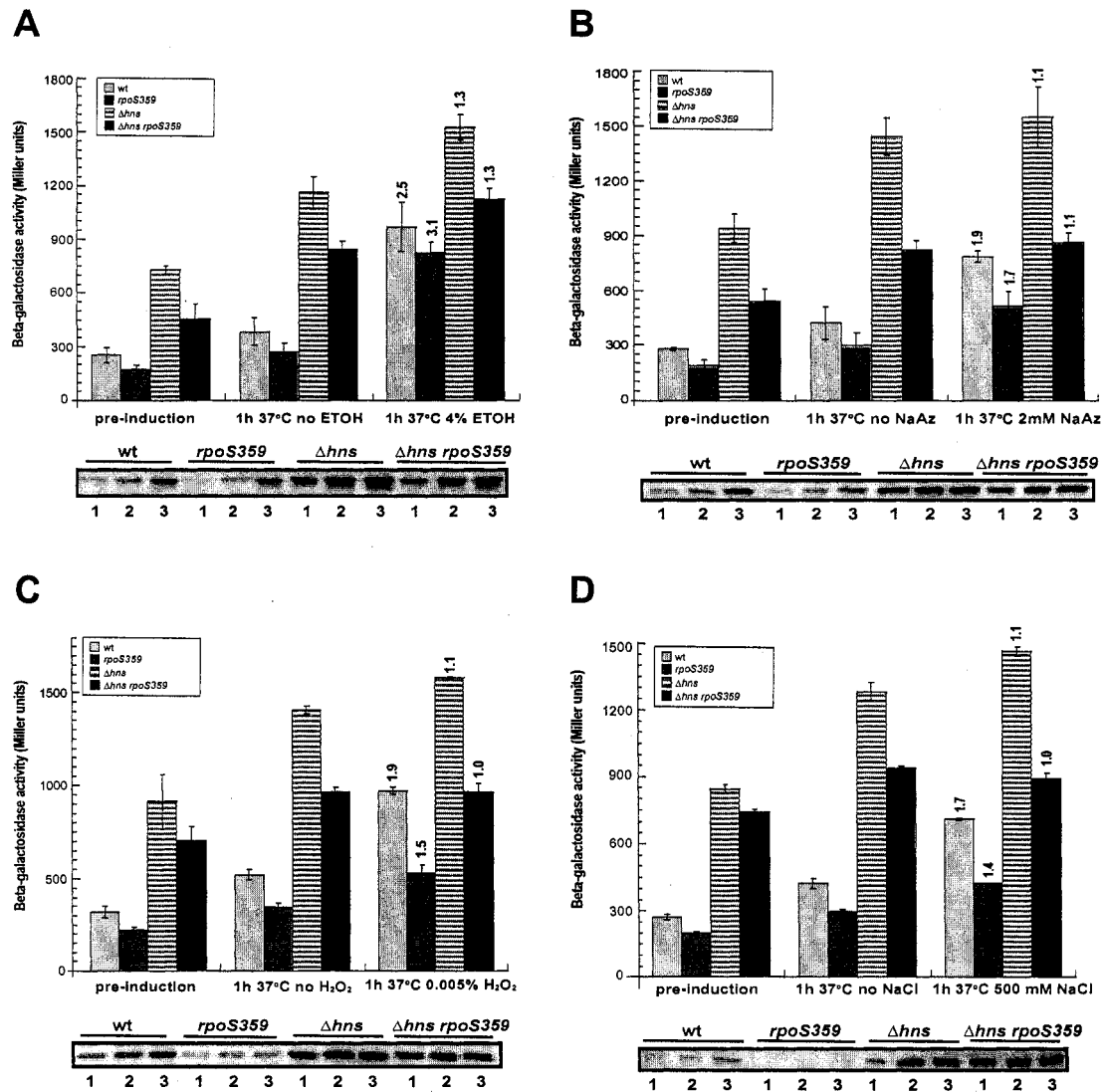


Figure 4.1. *hchA* expression is induced under a variety of environmental stresses. *hchA-lacZ* fusion strains MIR510 (wt), MIR512 (*rpoS359*), MIR514 (Δhns) and MIR516 ($\Delta hns rpoS359$) were grown to mid-exponential phase in LB/streptomycin medium at 37°C (pre-induction) and were then either left at 37°C without any stress treatment or were treated with either 4% ETOH (**A**), 2 mM sodium azide (NaAz; **B**), 0.005% hydrogen peroxide (H₂O₂; **C**), or 500 mM sodium chloride (NaCl; **D**). Samples were collected after 1h and β -galactosidase activities of soluble protein fractions were measured. Fold increases in the activity are shown above the columns, with error bars representing three independent experiments. Corresponding whole cells samples collected immediately before stress administration (lanes 1), 1h after incubation at 37°C in the absence of stress (lanes 2), and 1h post-stress treatment (lanes 3) were analyzed via immunoblotting with antiserum raised against Hsp31 and are shown below each graph.

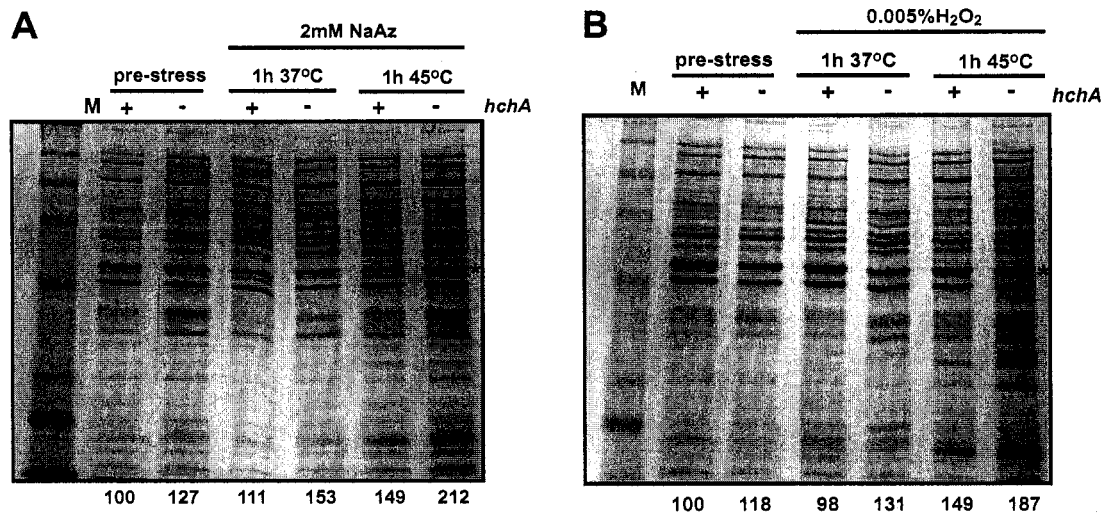


Figure 4.2. The effect of Hsp31's absence on *in vivo* protein aggregation in the presence of oxidative and ATP-depleting stresses. Wild-type (+) and $\Delta hchA$ (-) cells were grown to mid-exponential phase at 37°C in LB/streptomycin medium, treated with either 2 mM NaAz (**A**) or 0.005% H₂O₂ (**B**) and left either at 37°C or shifted to 45°C. Insoluble protein fractions were collected prior to (pre-stress lanes) and 1h after stress treatment. Numbers at the bottom of the gel correspond to the amount of insoluble proteins normalized to the intensity of the OmpF band (asterisk) and to the levels of insoluble proteins in wild-type cells prior to the stress treatment (arbitrarily set at 100). Markers (lane M) are 250, 148, 98, 64, 50, 36, 22 and 16-kDa from top to bottom.

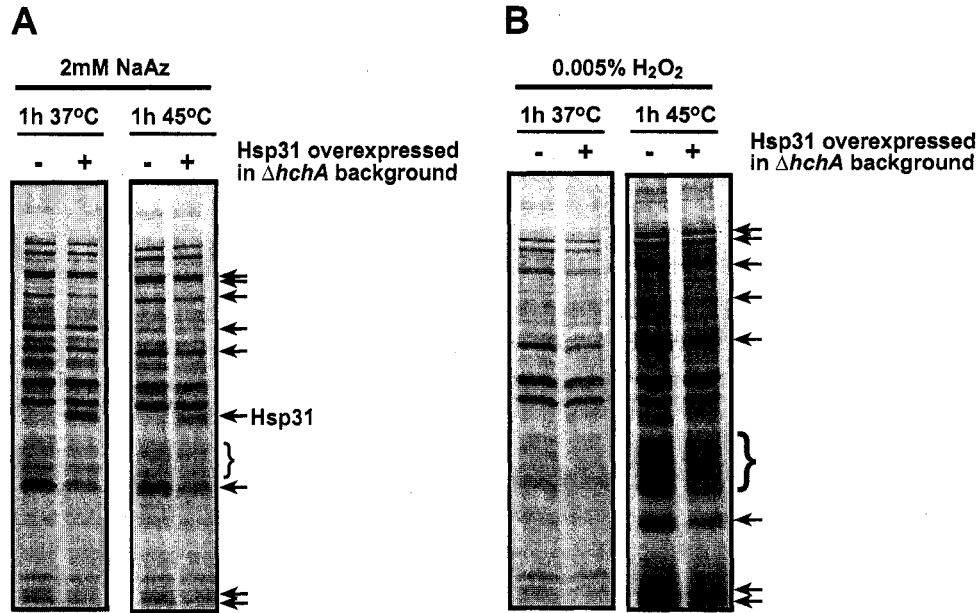


Figure 4.3. The effect of Hsp31's overexpression in the *hchA* null background on *in vivo* protein aggregation in the presence of oxidative and ATP-depleting stresses. $\Delta hchA$ cells carrying either empty expression vector ptetA3 (-) or Hsp31 expression vector pMM114 (+) were grown to mid-exponential phase at 37°C in LB/chloramphenicol medium supplemented with 0.05 μ g/ml anhydrotetracycline and were then treated with either 2 mM NaAz (**A**) or 0.005% H₂O₂ (**B**) and left either at 37°C or shifted to 45°C. Insoluble protein fractions were collected 1h after stress treatment. The arrows show migration positions of proteins that are present at lower levels in the insoluble fraction of the mutant cells overexpressing Hsp31. Markers (lane M) are 250, 148, 98, 64, 50, 36, 22 and 16-kDa from top to bottom.

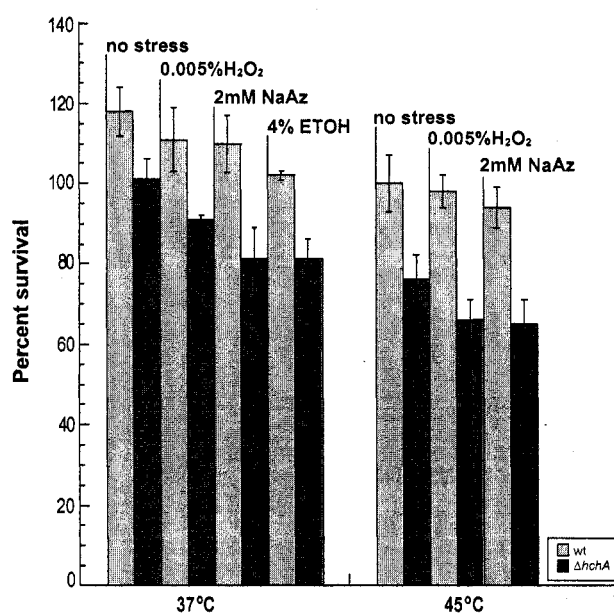


Figure 4.4. The effect of Hsp31's absence on the stress survival of stationary phase cells. Wild-type and *hchA* null mutants incubated at 37°C for two days were either left untreated for 1h at 37°C and 45°C or were subjected to 1h stress treatment at the same temperatures in the presence of either 4% ETOH, 2 mM NaAz, or 0.005% H₂O₂. Survival following 1h exposure to various stresses was determined by counting the number of colony forming units and comparing them to the number present at the beginning of the experiment. Error bars are based on three independent experiments.

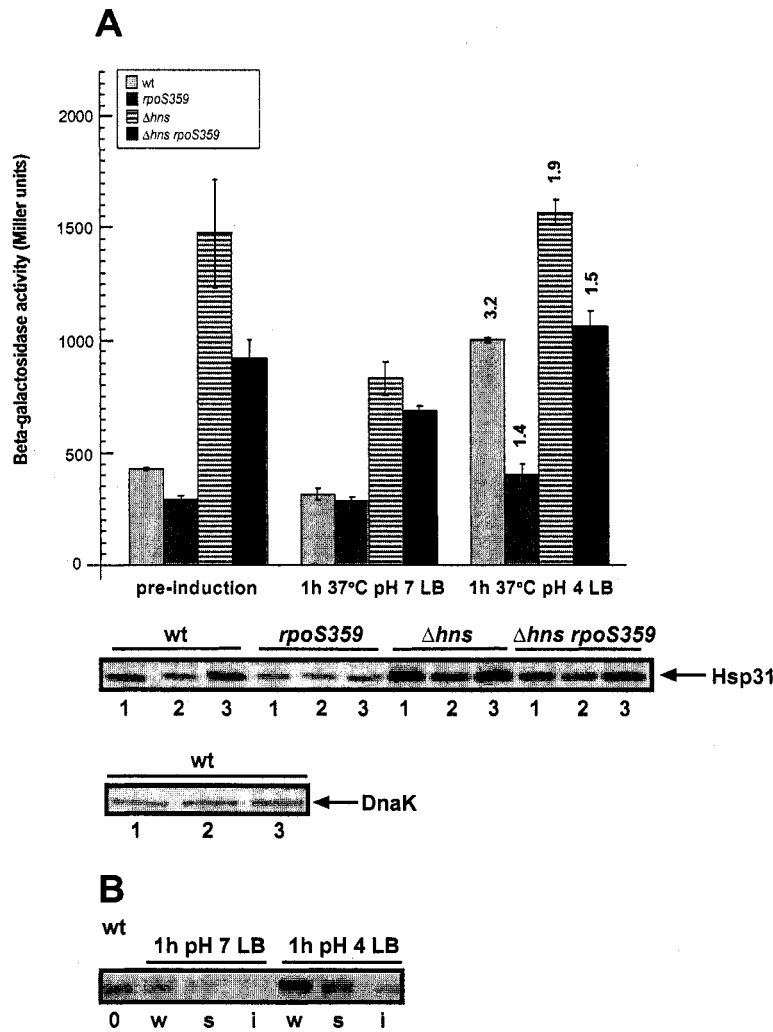


Figure 4.5. Induction of Hsp31 under acidic conditions.

A. *hchA-lacZ* fusion strains MIR510 (*wt*), MIR512 (*rpoS359*), MIR514 (Δhns) and MIR516 ($\Delta hns rpoS359$) were grown to late exponential phase at 37°C in pH 7 LB/streptomycin medium (pre-induction) and were then inoculated into pH 7 and pH 4 LB medium. Samples were collected after 1h and β -galactosidase activities of soluble protein fractions were measured. Fold increases in the activity are shown above the columns, with error bars representing three independent experiments. Corresponding whole cells samples collected immediately before inoculation into acidic LB (lanes 1) and 1h after inoculation into pH 7 (lanes 2) and pH 4 (lanes 3) LB were analyzed via immunoblotting with antiserum raised against Hsp31 or DnaK and are shown below the graph.

B. MC4100 cells grown in LB/streptomycin to late exponential phase were inoculated into pH 7 and pH 4 LB. Pre-inoculation whole cell sample (0), as well as whole cell (w), soluble (s), and insoluble (i) samples collected 1h post-inoculation into one of the two media were analyzed via immunoblotting with antiserum raised against Hsp31.

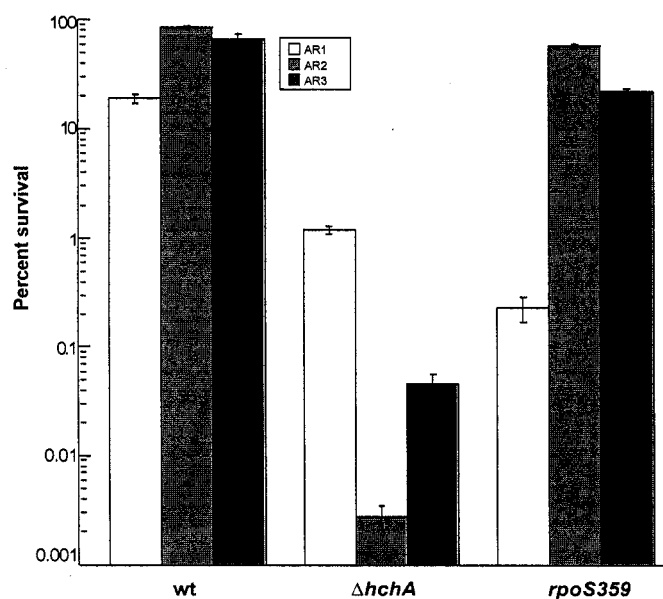


Figure 4.6. The role of Hsp31 in acid resistance. Wild-type (wt), $\Delta hchA$ and *rpoS359* mutants grown overnight in LB-MES pH 5.5 (AR1), LBG (AR2), and BHIG (AR3) were inoculated into either 2 ml of unsupplemented pH 2.5 EG (AR1) or into pH 2.5 EG supplemented with 1.5 mM glutamate (AR2) or 0.6 mM arginine (AR3). After 1h incubation at 37°C, percent survival was determined by counting the number of colony forming units and comparing them to the number present at the beginning of the experiment. Error bars are based on three independent experiments.

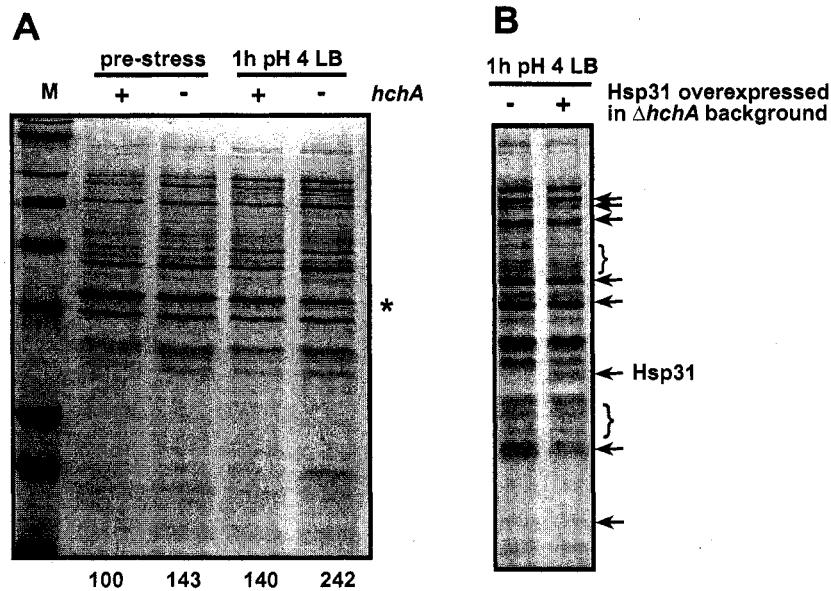


Figure 4.7. The effect of Hsp31 on *in vivo* protein aggregation under acidic conditions. **A.** Wild-type (+) and $\Delta hchA$ (-) cells were grown to late exponential phase at 37°C in pH 7 LB/streptomycin medium and were inoculated into pH 4 LB medium. Insoluble protein fractions were collected before acid-stress (pre-stress) and 1h after inoculation into pH 4 LB. Numbers at the bottom of the gel correspond to the amount of insoluble proteins normalized to the intensity of the OmpF band (asterisk) and to the levels of insoluble proteins in wild-type cells prior to the stress treatment (arbitrarily set at 100). **B.** *hchA* null mutants harboring either the empty expression vector ptetA3 (-) or Hsp31 expression vector pMM114 (+) were grown to late exponential phase at 37°C in LB/streptomycin supplemented with 0.05 $\mu\text{g/ml}$ anhydrotetracycline and were then inoculated pH 4 LB medium. Insoluble protein fractions were collected 1h after stress treatment. The arrows show migration positions of proteins that are present at lower levels in the insoluble fraction of the mutant cells overexpressing Hsp31. Markers (lane M) are 250, 148, 98, 64, 50, 36, 22 and 16-kDa from top to bottom.

CHAPTER 5: Summary and Future Directions

The data described in this dissertation represent the first *in vivo* characterization studies of the *E. coli hchA* gene and its protein product Hsp31. We have shown that Hsp31 is an abundant stationary phase protein, whose absence negatively impacts the ability of cells to survive exposure to severe heat and acidic stresses. Additionally, its absence decreases folding integrity of proteins under a variety of stress conditions, several of which are known to cause a depletion of cellular ATP pools and presumably reduce the activity of ATP-dependent molecular chaperones. In terms of its transcription, *hchA* employs a unique regulatory network consisting of σ^D , σ^S and H-NS. Namely, we have found that an increase in Hsp31 protein levels under various growth and stress conditions can be attributed to the relief of H-NS association with the *hchA* promoter region in conjunction with an increased transcription from its σ^D and/or σ^S -dependent promoters.

Our Hsp31 studies were focused on a broad characterization of Hsp31 and its expression patterns. Consequentially, our data have allowed us to identify the most interesting phenotypes that warrant further in depth investigation. First, to confirm our *in vivo* data suggesting cooperation between Hsp31 and DnaK-DnaJ-GrpE under heat shock conditions, a series of biochemical assays could be performed with model substrates such as luciferase or alcohol dehydrogenase, or with one of the identified Hsp31 substrates, such as DeoA or DeoB, whose folding also depends on DnaK-DnaJ-GrpE. Second, it would also be of interest to investigate whether Hsp31 can on its own or in combination with DnaK-DnaJ-GrpE facilitate *in vivo* folding of proteins not native to *E. coli*, which would make it an extremely useful tool for recombinant protein expression. Third, we have shown that Hsp31 is crucial for optimal performance of all three acid resistance systems in *E. coli*, with an especially pronounced importance in the glutamate-dependent system. To date, no cytoplasmic molecular chaperones specific to acid stress have been identified. Therefore, it would be interesting to employ immunoprecipitation in combination with 2D PAGE and MALDI analyses to identify

proteins which associate with Hsp31 during acid stress. Experiments could also be performed to assess the activity of glutamate-dependent acid resistance system in the absence of Hsp31 to elucidate whether Hsp31 directly impacts the performance of various components of this system or whether it is involved in some as-of-yet unidentified pathway. Finally, a series of genetic studies aimed at further elucidation of mechanisms involved in *hchA* transcription could be carried out. We have found that both acidic and oxidative stresses cause a clear induction in *hchA* expression. Both of these stress pathways involve a complex network of transcriptional factors. Therefore, further studies could be employed to determine whether transcription of *hchA* is also governed by some of these stress-specific factors. Additionally, the *hchA* promoter region could be mutagenized to identify the precise location of the σ^S -dependent -10 promoter box, while DNase footprinting could be employed to identify regions of the *hchA* promoter with which H-NS associates.

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