EFFECT OF HEAT SHOCK ON *hilA* EXPRESSION IN *Salmonella* Typhimurium

A Thesis

by

ASAWARI CHURI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Food Science and Technology

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ABSTRACT

Effect of Heat Shock on *hilA* Expression in *Salmonella* Typhimurium. (December 2004)

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The effect of heat shock was observed on the expression of *hilA* in *Salmonella* Typhimurium by creating a fluorescence-based reporter strain of *Salmonella* and by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

The *hilA* gene in *Salmonella* is known to play an important role in its pathogenesis. *hilA* is known to be activated when the bacteria encounter stress-inducing conditions. A number of factors have been identified that affect *hilA* expression, such as, pH, osmolarity, oxygen tension. When *Salmonella* enter their warm-blooded hosts, they encounter an increase in temperature. Therefore, heat is another stressor that is encountered by *Salmonella* during infection of their hosts.

A fluorescence-based strain of *Salmonella* was created to study the effect of heat shock. The gene for green fluorescent protein (*gfp*) was placed under the control of the promoter of *hilA* on a plasmid. This plasmid was used to transform *Salmonella* cells to create a fluorescent strain. In this strain, when the *hilA* promoter is activated, *gfp* is transcribed, which encodes the green fluorescent protein. This protein can be measured by a fluorescence assay. The results of this study indicated that at 45ºC, *hilA* is activated.

RT-PCR was used to look at *hilA* expression at different temperature. The results of this study indicated that, compared to 37ºC, higher temperatures like 45ºC and 55ºC significantly activate *hilA*.

To

Mum and Papa,

I am because you are.

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CHAPTER I

INTRODUCTION

During infection of its hosts, *Salmonella enterica* serovar Typhimurium enters the epithelial cells of the small intestine. This process requires a number of invasion genes encoded on *Salmonella* pathogenicity island-1 (SPI-1), a 40 kb stretch of DNA located near minute 63 of the *S. typhimurium* chromosome. Expression of *S. typhimurium* SPI1 invasion genes is activated by the transcription factor HilA. *hilA* is tightly regulated in response to many environmental conditions, including oxygen, osmolarity and pH. Regulation of *hilA* expression may serve to limit invasion gene expression to the appropriate times during *Salmonella* infection (5). Another factor encountered by the pathogen upon entry into a warm blooded host is an increase in the temperature. The heat shock proteins, expressed as a part of the heat shock response, include chaperones and proteases that are presumably essential for overcoming heat-induced changes that involve protein denaturation as well as other genes, such as transcriptional regulators (4,11). In several bacterial species heat shock proteins have been shown to play a direct role in pathogenesis including heat shock proteins required for binding of *Salmonella* Typhimurium to mucosal cells (34). This induction of the heat shock response probably affects pathogenesis indirectly by increasing bacterial resistance to host defenses or regulating virulence genes (52).

This thesis follows the style and format of Journal of Bacteriology.

Transcriptional fusions have been widely used in gene expression and regulation studies. The construction of such fusions is greatly facilitated by the use of promoter-probe vectors. These vectors have a common motif in which a promoterless reporter gene, encoding an easily assayable protein, is present downstream of one or more restriction sites. Known promoter sequences or uncharacterized sequences of genomic DNA can be ligated into these restriction sites, and the expression of the reporter gene can then be quantified under various conditions. Most of the early promoter-probe vectors that were constructed used the *lacZ* reporter gene. Such a vector conferred β-galactosidase activity in cells that were β-galactosidase negative. Thus, gene expression could be quantified by introducing a β-galactosidase substrate in the medium and carrying out a β-galactosidase assay. These vectors cannot be used in strains with native β-galactosidase activity. For such strains, another reporter gene would have to be used.

In the recent years, green fluorescent protein has come to be widely used for creating such promoter-probe fusions. The green fluorescent protein (GFP) from *Aequoria victoria* is an excellent tool for monitoring gene expression, because it is naturally fluorescent without any exogenous cofactor or substrate (18). Plasmid-borne *gfp* fusions have already been used to monitor gene expression in *Salmonella* strains (12, 130), as well as in other gram-negative or gram-positive bacteria, including *Escherichia coli, Serratia liquefaciens, Erwinia herbicola, Staphylococcus aureus, Listeria monocytogenes,* and *Streptococcus gordonii*. Such fusions have proven to be useful for identifying bacterial genes induced during infection of eukaryotic cells or animal hosts

and have allowed some assessment of bacterial gene expression levels in such complex environments (58).

Currently there are a number of techniques used to evaluate the amount of mRNA expression including Northern Blotting, cDNA arrays, *in situ* hybridization, RNase protection assays, and reverse transcription polymerase chain reaction (RT-PCR) (51). Reverse transcription along with the polymerase chain reaction has proven to be a powerful method to quantify gene expression (97). Of these methods RT-PCR is the method for quantification known to be the most discerning and faultless in its use (51). Real-Time RT-PCR is rapidly becoming the new method for determining mRNA expression (13) due to its capacity to use up to 1000 times less RNA than other known methods (56).

The objective of this research was to evaluate the effect of heat shock on the expression of *hilA* in *Salmonella*. Toward this end, a fusion strain was created where the gene for green fluorescent protein was placed under the control of the *hilA* promoter. Activation of the *hilA* promoter in this strain leads to expression of GFP. This expression can be detected by a fluorescence assay. We also looked at *hilA* expression due to heat shock by using real-time RT-PCR.

CHAPTER II

LITERATURE REVIEW

Salmonella

Salmonella is a rod-shaped, motile, nonsporeforming and Gram-negative bacterium (nonmotile exceptions *S*. gallinarum and *S*. pullorum). Daniel E. Salmon, U.S. veterinary surgeon, discovered the first strain of *Salmonella* in 1885. There is a widespread occurrence of the bacteria in animals, especially in poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafoods, to name only a few (CFSAN). The infection caused by *Salmonella* is called salmonellosis. Symptoms of salmonellosis include diarrhea, fever, and abdominal cramps that develop 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. However, in some persons the diarrhea may be so severe that the patient needs to be hospitalized. In these patients, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics. The elderly, infants, and those with impaired immune systems are more likely to have a severe illness (CDC). The bacterial genus *Salmonella* is divided into two species, *Salmonella bongori* and *S. enterica*. *S. enterica* itself is comprised of six subspecies: they are *S. enterica* subsp. enterica, *S. enterica* subsp. salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. indica, and *S. enterica* subsp. houtenae, or I, II, IIIa, IIIb, IV, and VI, respectively

(105). Of these six subspecies, only subspecies I is associated with disease in warm-blooded animals. To date, there are over 2,300 serovars identified within subspecies I. However, only a small fraction of the thousands of described subspecies I serovars frequently cause disease in humans and domestic animals.

Incidence of *Salmonella* in the United States

The annual report of the Centers for Disease Control and Prevention (CDC) for the year 2001 registered 360 different serovars in human infections in the U.S. Approximately 50% of these infections were caused by only three *Salmonella* serovars, specifically Typhimurium, Enteritidis, and Newport. The 12 most prevalent *Salmonella* serovars were responsible for >70% of all human *Salmonella* infections. Every year, approximately 800,000 to 4 million cases of *Salmonella* result in 500 deaths in the United States. It is more commonly seen in young children.

The three most common serotypes of *Salmonella* in 2002 (Typhimurium, Enteritidis, and Newport respectively) accounted for 51% of isolates. Compared with 1992, the frequency rank of *S*. Typhimurium and *S*. Enteritidis in 2001 remained first and second respectively, though in 1994-1996 their rank was temporarily reversed. A large proportion of *S*. Typhimurium isolates were resistant to multiple antimicrobial drugs; in a 2001 national survey, 53% were resistant to one or more drugs and 30% had a five-drug resistance pattern characteristic of a single phage type, DT104 (16).

***Salmonella* pathogenesis**

During a long-standing association with its hosts, *Salmonella* have evolved a sophisticated mechanism to modulate host cell functions. Many virulence-associated genes are acquired during the evolution of pathogenic bacteria via horizontal gene transfer. These genes are often clustered on plasmids or on chromosomal 'pathogenicity islands' and 'pathogenicity islets' (PIs). The emerging concept is that different horizontally acquired plasmids and PIs influence different phases of infection. *Salmonella* possesses both a large virulence plasmid and a variety of chromosome-encoded PIs. Although it has been established that the virulence plasmid-encoded genes are needed for systemic virulence in mice (54), the key factors affecting enteropathogenicity are encoded on the chromosome. The evidence for this is that isogenic plasmid cured strains are fully enteropathogenic (134) and also that plasmid-free strains were associated with outbreaks of enteritis in humans.

The type III secretion system

Several Gram-negative animal and plant pathogenic bacteria possess highly homologous protein secretion systems, known as type III secretion systems (TTSS) (68). The main function of TTSS is to co-ordinately secrete and translocate a set of effector proteins into eukaryotic target cells. All mutations in the secretory apparatus and associated regulatory genes appear to be pleiotropic because they block the translocation of a range of proteins to their site of action. *Salmonella* possess at least two TTSS secreting virulence-associated effector proteins, TTSS-1 and TTSS-2. The emerging concept is that the TTSS-1 secreted proteins are the key effectors of *Salmonella* invasion

and enteritis. TTSS secreted proteins could be considered to be toxins as almost all of them affect eukaryotic target cells in one or another way. However, unlike 'classical' toxins, TTSS secreted proteins generally lack receptor-binding domains and thus are unable to gain access to the site of their action without the active contribution of viable bacteria. TTSS secreted proteins can be loosely divided into two groups: the translocators and the translocated effectors. The translocator proteins are essential for translocation of secreted protein effectors across the eukaryotic cell plasma membrane. A set of effector proteins is simultaneously delivered into the cytoplasm of the eukaryotic cell. The effector proteins do not participate in the process of translocation. However, some translocators are delivered into the eukaryotic cells as well.

Salmonella *pathogenicity island-1*

The *Salmonella* pathogenicity island-1 (SPI-1) chromosomal region encodes for more than 30 proteins, which include regulatory proteins and structural components of TTSS-1, and several TTSS-1 secreted proteins and their chaperones. In addition, a number of proteins encoded by the genes located elsewhere on the *Salmonella* chromosome are secreted by TTSS-1. Several translocated effector proteins from *Salmonella* have been identified to date. Originally these were designated Sop proteins (<u>*Salmonella*</u> <u>O</u>uter <u>P</u>roteins) (144). Genes encoding several Sops have been cloned using reverse genetic techniques and the corresponding proteins have generic names (SopA, SopB, SopD and SopE). Other TTSS-1 translocated effector proteins have been identified and named individually, based on structural or functional features (AvrA, SptP and SspH1). In addition, some translocators may have a direct effector function.

Invasion

Intestinal invasion is a characteristic feature of *Salmonella* pathogenesis. Within minutes of injecting *Salmonella* into ligated ileal loops in calves, *Salmonella* can be seen to invade both M cells and enterocytes that overlie domed villi associated with lymphoid follicles and absorptive villi respectively (41). This is in marked contrast to what is seen in mice where the major portal of entry appears to be only through M cells (72). Accordingly, foci of infection develop in murine but not bovine Peyer's patches (15, 141). The significance of these differences in the apparent route of invasion in mice and cattle is not clear. It is tempting to speculate that the route of intestinal invasion may influence the nature of the subsequent infection because only calves, but not mice develop overt diarrhoeal disease. Upon interaction with host cells, *Salmonella* elicit membrane ruffles in the apical membranes of both M cells and enterocytes, which results in the uptake of bacteria into membrane bound vesicles. Thereafter, *Salmonella* can be seen associated with reticulo-endothelial cells and PMNs, which are recruited to the focus of infection.

The molecular basis of *Salmonella* invasion of eukaryotic cells has been intensively studied in vitro using cultured cells (43). Similar molecular genetic approaches are now being applied to study intestinal invasion in vivo. TTSS-1 has a profound influence on *Salmonella* invasiveness. Mutations in genes encoding structural proteins of TTSS-1 block bacterial invasion of cultured cells in vitro (43) and invasion of bovine and murine intestines in vivo (141, 103). In addition to cell invasion, TTSS-1 also has a major role in the induction of intestinal secretory and inflammatory responses. Mutations of *invH*, *hilA*

or *sirA*, which encode structural and regulatory proteins of TTSS-1, abolish induction of fluid secretion and the recruitment of PMNs in bovine ligated ileal loops (141, 2). Consistent with this observation, *invH*, *hilA* and *prgH* mutations markedly reduce severity of enteritis in orally infected calves (141, 126). Thus, a common finding in such experiments is that mutations affecting entire TTSS-1 functions almost uniformly abrogate or greatly reduce enteropathogenicity. The results of recent studies provide the evidence that key virulence genes are associated with TTSS (type III secretion system) and their secreted proteins. Many aspects of the interaction of *Salmonella* with host cells are dependent on the function of TTSS-1 and TTSS-2 encoded within SPI-1 and SPI-2 respectively. TTSS-1 plays a major role in enteropathogenesis whereas the influence of TTSS-2 on enteropathogenesis is less clear. TTSS-2 influences net intracellular growth (64), however, first reports suggest that TTSS-2 does not influence enteropathogenesis to any great extent (37, 126).

It has been postulated that the acquisition of the SPI-1 by horizontal gene transfer represented a quantum leap in *Salmonella* evolution by enabling the pathogen to exploit the host intestinal environment. TTSS-1 secreted proteins play a central role during the intestinal phase of infection affecting invasion of intestinal epithelial cells, induction of inflammatory cell recruitment and fluid secretion. Nevertheless, TTSS-1 has little impact on systemic virulence. Together, these facts have implications for the understanding of the evolution of *Salmonella* natural history. It is likely that the primary function of TTSS-1 and the associated secreted effector proteins is to enable the pathogen to induce self-limiting diarrhoeal disease in host animals without causing their death. This ability

is likely to be of great significance for *Salmonella* dissemination, because cross-infection occurs mainly through a faecal-oral route. Diarrhoeal disease results in the prolonged shedding of large numbers of bacteria into the host's environment. Such bacteria reside in a relatively moist and nutrient rich niche, until the next suitable host consumes the contaminated food or water. Thus, the possession of TTSS-1 increases *Salmonella* dissemination.

Role of *hilA* in pathogenesis

Expression of invasion genes is controlled by HilA, a member of the OmpR/ToxR-type transcriptional regulator family due to similarities in its DNA binding domain. HilA activates the transcription of SPI-1 invasion genes and effector proteins (5,84). Importantly, expression of *hilA* is modulated by the same conditions that regulate the invasive phenotype. In addition, overexpression of *hilA* confers a hyperinvasive phenotype and over-expression also counteracts the effects of repressing signals. Therefore, modulation of *hilA* expression by environmental signals appears to be a primary method of regulating the invasive phenotype of *Salmonella* (5). Using lacZY fusions to chromosomal invasion genes, HilA has been shown to be required for the optimal expression of three essential invasion genes: *invF*, *sspC*, *orgA* (5). *invF* codes for a transcriptional regulator (33). *sspC* codes for a secreted invasion protein (69, 75). *orgA* codes for a component of the export machinery (43).

Heat shock response

Since their initial recognition in *Drosophila* cells following hyperthermia, the stress proteins have been the subject of great scientific interest. Their gene expression and protein accumulation following heat and a variety of other stresses, coupled with their ability to confer tolerance to subsequent stresses, led to an interest in their role in the cellular adaptive response. From these initial studies, investigators have now demonstrated that the stress proteins are essential in many cellular housekeeping functions such as protein synthesis and transport across biologic membranes. The stress proteins include families of proteins termed heat shock proteins (HSPs), glucose-regulated proteins (GRPs), and ubiquitin. These proteins range in size from ubiquitin's 8 kD to the 110 kD HSP 110 and are found in various cellular compartments (92). The HSPs and GRPs share structural and functional similarities, and ubiquitin's role in protein transport and degradation mirrors that of the HSPs and GRPs. Cellular accumulation of HSPs results in thermotolerance, defined as the cell's ability to withstand an otherwise lethal thermal challenge. Thermotolerance occurs when the cell undergoes prior stress sufficient to cause HSP accumulation or when at least one HSP family member is overexpressed through gene manipulation. A variety of stresses in addition to heat can induce an HSP response, and the induction of an HSP response by one stress confers tolerance to a rechallenge with the same stress as well as to other stresses that cause HSP accumulation. For example, cellular HSP accumulation resulting from whole body hyperthermia renders the cell or organ tolerant to ischemia/reperfusion injury. Whereas the stresses capable of inducing an HSP response are variable, they share

a common mechanism of protein alteration and/or interference with protein translation. An important aspect of this HSP response is that upon awakening from the stress-induced translational arrest, the cell preferentially translates the HSP message, initially to the exclusion of other messages (90). This preferential translation likely reflects both transcriptional (146, 76) and post-transcriptional (95, 49) regulatory mechanisms. The result of this preferential translation is that during cellular recovery, the cell's protein content is biased towards HSPS.

Temperature increase constitutes one of the major environmental stresses encountered by bacteria. It is, therefore, not surprising that a large number of HSPs are induced. The bacterial heat shock response is not limited to changes in temperature and is often a general stress response. Thus, many of the heat shock proteins are also induced by other environmental changes such as the addition of ethanol, heavy metals, high osmolarity, pollutants, starvation or interaction with eukaryotic hosts (8, 59, 96, 131, 132). The genes belonging to this regulon are transcriptionally activated by specific heat shock regulators. The heat shock proteins include chaperones and proteases that are presumably essential for overcoming changes that involve protein denaturation as well as other genes, such as transcriptional regulators (4,11).

Induction of the heat shock response improves thermotolerance and tolerance to heavy metals (70, 78, 79). Furthermore, in several bacterial species heat shock proteins have been shown to play a direct role in pathogenesis including heat shock proteins required for binding of *Salmonella* Typhimurium to mucosal cells (34). Pathogens of warm blooded animals encounter temperature shifts upon contact with their hosts. The

contact also involves exposure to additional stresses such as changes in pH or oxidative stress. All these types of stress are denaturing for many proteins and are therefore expected to elicit the heat shock response. This induction of the heat shock response probably affects pathogenesis indirectly by increasing bacterial resistance to host defenses or regulating virulence genes (52).

Role of DnaK in Salmonella

DnaK forms a chaperone machinery with the cochaperones DnaJ and GrpE and is involved in many cellular processes, such as DNA replication of the bacterial chromosome, RNA synthesis, protein transport, cell division, and autoregulation of the heat shock response (48, 63). Furthermore, this chaperone machinery nonspecifically interacts with many unfolded and misfolded proteins and therefore assists in proper folding, helps in refolding, prevents aggregation, and mediates the degradation of misfolded proteins (55, 92). During the course of infection in mice, serovar Typhimurium colonizes many different organs, including the Peyer's patches of the small intestine, mesenteric lymph nodes, spleen, and liver, and causes a severe systemic infection, which can be fatal. The virulence assay after infection of BALB/c mice by oral and subcutaneous routes demonstrated that the DnaK/DnaJ-depleted mutant apparently lost the ability to colonize and cause systemic disease in mice. Of further interest is the finding that the DnaK/DnaJ-depleted mutant could not invade cultured epithelial cells (intestine-407) (120). It is known that the invasion of epithelial cells by S. *enterica* serovar Typhimurium is mediated by the SPI1-encoded invasion proteins (31). It was previously demonstrated that the SPI1-encoded proteins and flagellum-related proteins,

which are also secreted by a type III secretion system, are the predominant proteins secreted into culture media (120). Proteome analysis of the secreted proteins from DnaK/DnaJ-depleted mutant cells revealed that none of the SPI1-encoded proteins was secreted into the culture medium. Among the SPI1-encoded proteins are SipA to SipD, which are translocated into the mammalian cell cytosol and trigger host signal transduction pathways, resulting in profuse actin cytoskeletal rearrangements and membrane ruffles at the point of bacterial cell-host cell contact that ultimately lead to bacterial uptake (38, 44). Therefore, the inability of the DnaK/DnaJ-depleted mutant to invade intestine-407 cells could have been due to the loss of Sip proteins, which are essential for bacterial entry. The synthesis of the SPI1-encoded type III secretion apparatus and most of its secreted effectors is controlled by a complex regulatory system. Many studies have shown that the SPI1-encoded transcriptional regulators HilD, HilC, HilA, and InvF act in an ordered fashion to coordinately activate the expression of SPI1 (84). HilD and HilC derepress *hilA* transcription. HilA activates *invF* as well as other SPI1 genes and therefore plays a key role in coordinating the expression of the SPI1-encoded type III secretion system. Since none of the SPI1-encoded proteins was secreted from the *dnaK*::Cm mutant, it is likely that the DnaK/DnaJ chaperone machinery is involved in the expression of SPI1 through interactions with regulators such as *hilD*, *hilC*, *hilA*, and/or *invF*. Alternatively, the type III secretion apparatus may not function in DnaK/DnaJ-depleted cells.

Role of opdA in Salmonella

The *Salmonella enterica* serovar Typhimurium *opdA* gene encodes the metalloprotease oligopeptidase A (OpdA) (133). *opdA* is a homolog of the *Escherichia coli prlC* gene, a site of suppressors of the localization defect conferred by certain signal sequence mutations (24,124,125). OpdA is also required for the proteolytic processing of a phage P22 protein in vivo (24), and it can degrade the cleaved *lpp* signal peptide in vitro (100). Comparison of the OpdA amino acid sequence with other protein sequences indicated that it is a member of a subfamily of Zn metalloproteases with representatives in both animals and fungi (23). The *opdA* operon is a σ^{32}-dependent heat shock operon (25). Several other protease genes in *E. coli* are known to be part of the heat shock regulon, and it seems likely that the degradation of irremediably misfolded proteins is a major component of the heat shock response (53). Only one natural macromolecular substrate for OpdA has been identified (phage P22 gp7), and this protein is not degraded but rather is specifically processed by OpdA. It is not clear whether OpdA participates directly in the degradation of misfolded proteins or whether it plays a more specialized role in the heat shock response.

The green fluorescent protein

Green Fluorescent Protein was discovered by Shimomura et al. (118) as a companion protein to aequorin, the famous chemiluminescent protein from the jellyfish *Aequorea victoria*. Morise et al. (94) purified and crystallized GFP, measured its absorbance spectrum and fluorescence quantum yield, and showed that aequorin could efficiently transfer its luminescence energy to GFP when the two were coadsorbed onto a cationic

support. Prendergast & Mann (108) obtained the first clear estimate for the monomer molecular weight. Shimomura (117) proteolyzed denatured GFP, analyzed the peptide that retained visible absorbance, and correctly proposed that the chromophore is a 4-(p-hydroxybenzylidene) imidazolidin-5-one attached to the peptide backbone through the 1- and 2-positions of the ring. The *gfp* gene was first cloned by Prasher et al. (107). Chalfie et al. (18) and Inouye and Tsuji (71) demonstrated that expression of the gene in other organisms creates fluorescence. Therefore the gene contains all the information necessary for the posttranslational synthesis of the chromophore, and no jellyfish-specific enzymes are needed.

Green fluorescent proteins exist in a variety of coelenterates, both hydrozoa such as Aequorea, Obelia, and Phialidium, and anthozoa such as Renilla (93,140). Aequorea and Renilla GFPs were later shown to have the same chromophore (139); and the pH sensitivity, aggregation tendency (141), and renaturation (138) of Aequorea GFP were characterized. Other than Aequorea GFP, only Renilla GFP has been biochemically well characterized (140). Despite the apparent identity of the core chromophore in Renilla and Aequorea GFP, Renilla GFP has a much higher extinction coefficient, resistance to pH-induced conformational changes and denaturation, and tendency to dimerize (139).

Structure

Although GFP was first crystallized in 1974 (94) and diffraction patterns reported in 1988 (105), the structure was first solved in 1996 independently by Ormö et al. (101), Protein Data Bank accession number 1EMA, and by Yang et al (148), accession number 1GFL. Both groups relied primarily on multiple anomalous dispersion of

selenomethionine groups to obtain phasing information from recombinant protein. Subsequent structures of other crystal forms and mutants (10, 102, 134) have been solved by molecular replacement from the 1EMA coordinates. GFP is an 11-stranded _-barrel threaded by an α-helix running up the axis of the cylinder (Fig. 1). The chromophore is attached to the _-helix and is buried almost perfectly in the center of the cylinder, which has been called a _-can (148, 134). Almost all the primary sequence is used to build the _-barrel and axial helix, so that there are no obvious places where one could design large deletions and reduce the size of the protein by a significant fraction.

The chromophore is a p-hydroxybenzylideneimidazolinone (107, 21) formed from residues 65–67, which are Ser-Tyr-Gly in the native protein. First, GFP folds into a nearly native conformation, then the imidazolinone is formed by nucleophilic attack of the amide of Gly67 on the carbonyl of residue 65, followed by dehydration. Finally, molecular oxygen dehydrogenates the _-_ bond of residue 66 to put its aromatic group into conjugation with the imidazolinone. Only at this stage does the chromophore acquire visible absorbance and fluorescence. This mechanism is based on the following arguments: (a) Atmospheric oxygen is required for fluorescence to develop (62,17). (b) Fluorescence of anaerobically preformed GFP develops with a simple exponential time course after air is readmitted (61,62), which is essentially unaffected by the concentration of the GFP itself or of cellular cofactors. (c) Analogous imidazolinones autoxidize spontaneously (77). (d) The proposed cyclization is isosteric with the known tendency for Asn-Gly sequences to cyclize to imides (146). Glycine is by far the best nucleophile in such cyclizations because of its minimal steric hindrance, and Gly67 is

conserved in all known mutants of GFP that retain fluorescence. One predicted consequence of oxidation by O_2 is that hydrogen peroxide, H_2O_2, is presumably released in 1:1 stoichiometry with mature GFP. This byproduct might explain occasions when high-level expression of GFP can be deleterious.

GFP mutants

Wild-type GFP folds fairly efficiently when expressed at or below room temperature, but its folding efficiency declines steeply at higher temperatures. Presumably this natural temperature sensitivity is of no consequence to the jellyfish, which would never encounter warm water in the Pacific Northwest. Temperature sensitivity is restricted to the folding process. GFP that has matured properly at low temperature is stable and fluorescent at temperatures up to at least 65°C. The poor ability of GFP to mature in warm temperatures has been used in pulse-chase experiments in which the fate of fluorescent protein made at low temperatures is followed after restoration of normal warmth and simultaneous suppression of new fluorescence (73, 82). However, for other applications it would be desirable to have a GFP that works well at 37°C. The most extensive attempt to develop such a mutant while preserving the complex wild-type spectrum utilized DNA shuffling (29), a technique for recombining various mutations while creating new ones. This approach produced a triple mutant, F99S, M153T, V163A, which improved 37°C-folding, reduced aggregation at high concentrations, and increased the diffusibility of the protein inside cells (149).
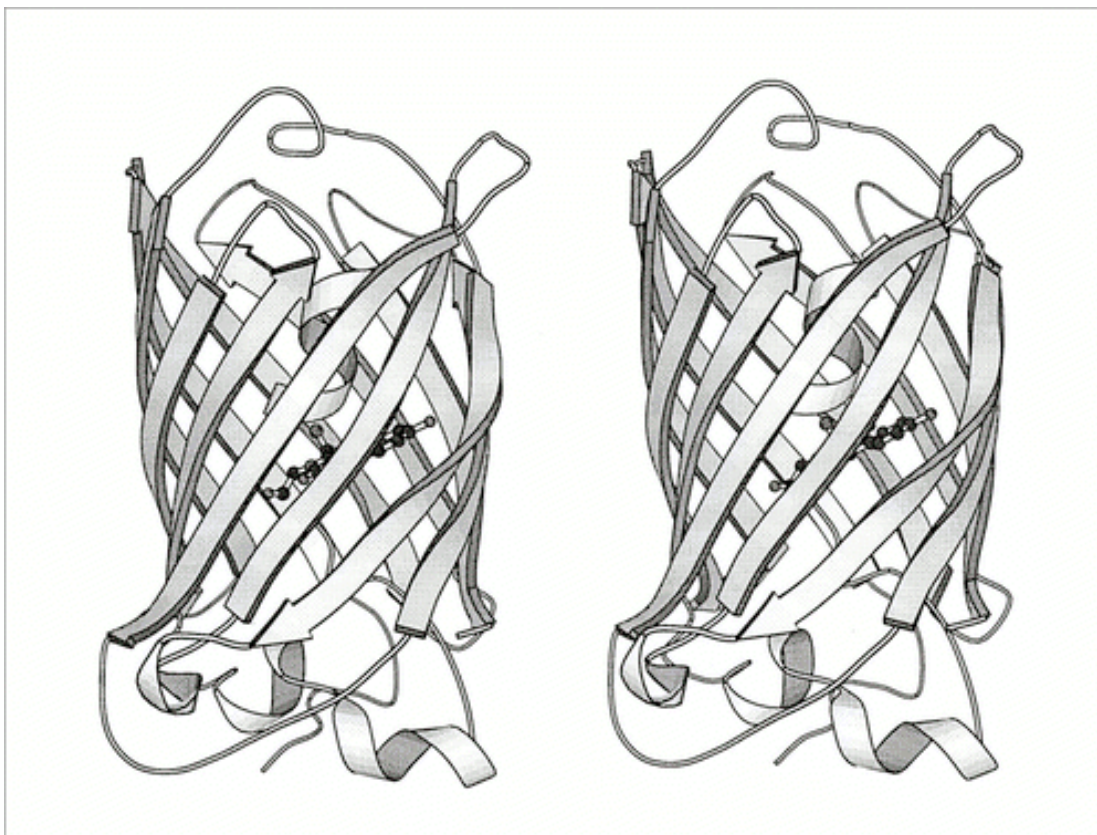
FIG. 1. Stereoview of the three-dimensional structure of GFP. 11 β-strands form a hollow cylinder through which is threaded a helix bearing the chromophore, shown in ball-and-stick representation (126).

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Wild type GFP is an extremely stable protein. Cells containing this protein show no decrease in fluorescence over at least 24h (3). This may be favorable in some studies but in gene regulation studies where the expression varies over time or in response to various environmental conditions, a shorter half life is preferable. A number of GFP mutants have been created to this end. An addition of an AANDENYALAA tag to the C-terminal end of wild-type GFP reduced the half life of the protein to approximately 40 min in *E. coli* (3), while retaining a maturation time of 45 min that was seen in the case of the variant Gfpmut3b (28) from which it was derived.

Real-time quantitative polymerase chain reaction for studying gene expression

Many cellular functions are regulated by changes in gene expression. Thus, quantification of transcription levels of genes plays a central role in the understanding of gene function and of abnormal alterations in regulation that may result in a disease state. The innovation of the real-time polymerase chain reaction (PCR) technique played a crucial role in molecular medicine and clinical diagnostics. Examples are the quantitation of relative gene expression, cancer diagnostics (7), pathogen detection (99), and quantitation of viral load (98). Other applications include detection of genetically modified organisms in food samples (1), measurements of DNA or transgene copy number, and allelic discrimination (116). Real-time quantitative reverse transcriptase PCR (RT-PCR), the latest innovation in the field of PCR technology, provides a sensitive, reproducible, and accurate method for determining mRNA levels in tissues or cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without post-PCR processing (60).

The first real-time PCR assay developed (the TaqMan assay) combined the principles of two important findings: one, the Taq polymerase has, apart from its polymerase activity, a 5'–3' exonuclease activity (67), and the second, dual-labeled fluorogenic oligonucleotide probes had been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer (14). In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and reverse primers. The probe is dually labeled, with a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at one end and a quencher dye (e.g., 6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3' end. Importantly, in its intact form, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe has a melting temperature (m) approximately 10°C higher than the m of the primers, in order to anneal to the amplicon during the extension phase of the PCR process (which is performed at 60°C). Consequently, the probe will be degraded during the extension phase by the 5'–3' exonuclease activity of the Taq polymerase. This will result in an increase in reporter fluorescence emission because reporter and quencher are separated. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

As the technology has become more commonly used, other sophisticated chemistries have been developed to directly measure PCR product accumulation by fluorescence emission. Examples include molecular beacons, Scorpions, and minor groove binder (MGB) probes (e.g., Eclipse, TaqMan MGB). Molecular beacons are single-stranded

oligonucleotide hybridization probes that are not fluorescent when present in free form in solution. When they hybridize to a nucleic acid strand containing a complementary sequence, they undergo a conformational change that enables them to fluoresce brightly (128). With Scorpion probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed. Minor groove binders are a potent class of naturally occurring antibiotics that bind to duplex DNA specifically in the minor groove. Minor groove binders are long, flat molecules that can adopt a "crescent shape" that fits snugly into the minor groove to form close atomic contacts in the deep, narrow space formed between the two phosphate-sugar backbones in the double helix. MGB probes bind to duplex DNA and increase the melting temperature due to increased stability. Incorporating an MGB probe allows other probes, like TaqMan, to be shorter, allowing for greater sequence specificity. Other new technologies include ResonSense probes, light-up probes, and Hy-Beacon probes (51). Finally, the use of double-stranded DNA minor groove binding dyes, such as SYBR Green I, is a cheaper, widely used alternative, where the need for an expensive probe can be avoided.

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. SYBR Green I has an excitation and emission maxima of 494 nm and 521 nm, respectively. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles (www.ambion.com).

The accumulation of fluorescence is read at the end of every cycle via the target amplification. The threshold cycle values are determined by the cycle at which the fluorescent emission rises above the threshold (51). Since SYBR green is not sequence specific, melting curves can be used to analyze different products. By this method primer dimers can be distinguished by their lower melting temperature (Tm) (111, 143). The cycle at which the threshold was overcome (Ct) decreases as the amount of target increases (51).
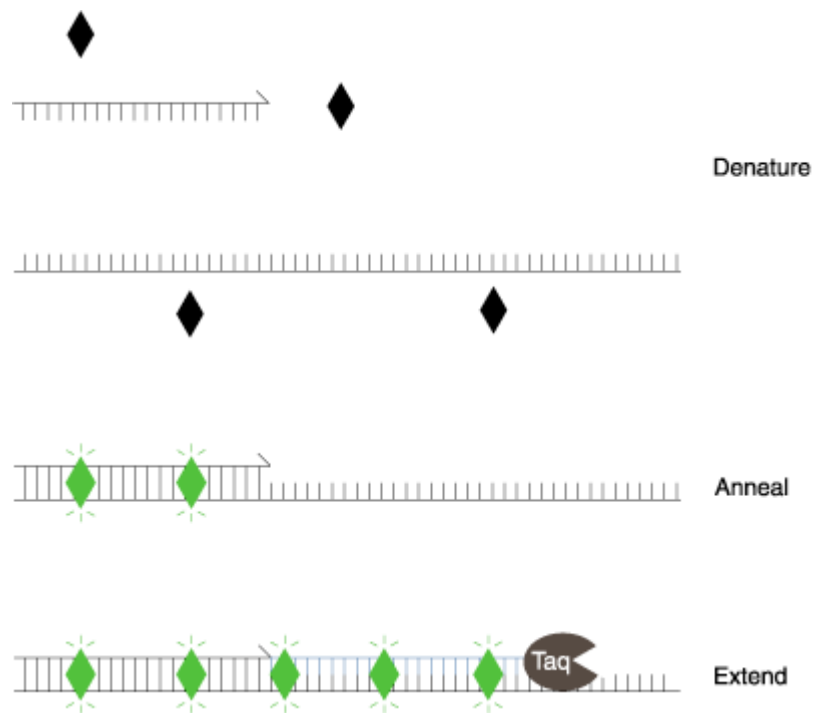
FIG. 2. Schematic representation of real-time PCR with the SYBR Green I dye. SYBR Green I dye (black diamonds) becomes fluorescent (green diamonds) upon binding to double-stranded DNA, providing a direct method for quantitating PCR products in real time (www.probes.com).

A method for determining relative quantification is the comparative Ct method. This method does not require the construction of cDNA plasmids nor does it depend on techniques where the variations in transcription efficiency can not be controlled. It involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping-gene.

The comparative Ct method is also known as the $2^{-\Delta\Delta\ Ct}$ method. The determination of the relative expression that a treatment causes can be sufficient to assess its effects and the absolute expression level is not necessarily needed (114). Relative expression is determined by calculating $2^{-\Delta\Delta\ Ct}$, where $\Delta\Delta\ Ct = \Delta\ Ct\ (sample)-\Delta\ Ct\ (control)$. $\Delta\ Ct$ is calculated by subtracting the Ct of the target gene from the Ct of the housekeeping gene (51, 81, 83).

In each quantitative RT-PCR method, specific errors will be introduced due to minor differences in starting amount of RNA or differences in efficiency of cDNA synthesis and PCR amplification. Therefore, a reliable quantitative RT-PCR method requires correction for these experimental variations. At present this is most often performed by normalization to a housekeeping gene. Finding a suitable housekeeping gene (i.e., a gene that is constant during the experimental conditions) is, however, not always straightforward. Therefore, different housekeeping genes should be tested in each experimental setup to find the most suitable one (not influenced by the experimental treatment).

Many recent articles have discussed the problem of housekeeping genes, and it turns out that the housekeeping genes most commonly used are subject to variation in numerous experimental conditions, bringing into question the reliability of the results obtained. In particular, the use of GAPDH and of ß-actin has been severely criticized (13, 114). Ribosomal RNA (rRNA) may be a more valuable alternative, because its expression is less likely to vary under conditions that change the levels of mRNA expression. Drawbacks of using rRNA, however, are that it cannot be used for quantification of samples when starting from mRNA, or when oligo d(T)16 is used for cDNA synthesis. Another drawback is its very abundant expression relative to mRNA expression levels of target genes.

CHAPTER III

CONSTRUCTION OF A P*hilA*-*gfp* FUSION STRAIN IN *Salmonella* Typhimurium

Introduction

Role of hilA *in invasion*

During infection of its hosts, *Salmonella* enter enterocytes and M cells of the terminal ileum (72, 121). This invasive property requires the expression of a large repertoire of invasion genes located on a 40 kb 'pathogenicity island' near minute 59 of the *Salmonella* Typhimurium chromosome (22, 74, 89, 103). *hilA*, a gene in SPI1, encodes a transcriptional regulator and is essential for *S*. typhimurium invasion (5). The amino terminus of HilA is homologous to the DNA-binding and transcriptional activation domain of the OpmR/ToxR family of transcriptional activators. Using *lacZY* fusions to chromosomal invasion genes, HilA has been shown to be required for the optimal expression of three essential invasion genes: *invF*, *sspC* and *orgA* (5). *invF* codes for a transcriptional regulator (33). *sspC* codes for a secreted invasion protein (69, 75). *orgA* codes for a component of the export machinery (43). Because *hilA* lies in SPI1 and is required for the optimal expression of these essential and diverse invasion functions, HilA appears to play a key role in the regulation of invasiveness.

Transcriptional fusions for studying gene expression

Transcriptional fusions have been widely used in gene expression and regulation studies. The construction of such fusions is greatly facilitated by the use of promoter-probe vectors. These vectors have a common motif in which a promoterless reporter

gene, encoding an easily assayable protein, is present downstream of one or more restriction sites. Known promoter sequences or uncharacterized sequences of genomic DNA can be ligated into these restriction sites, and the expression of the reporter gene can then be quantified under various conditions. Most of the early promoter-probe vectors that were constructed used the *lacZ* reporter gene. Such a vector conferred β-galactosidase activity in cells that were β-galactosidase negative. Thus, gene expression could be quantified by introducing a β-galactosidase substrate in the medium and carrying out a β-galactosidase assay. These vectors cannot be used in strains with native β-galactosidase activity. For such strains, another reporter gene would have to be used.

Use of gfp *as a reporter gene*

In the recent years, green fluorescent protein has come to be widely used for creating such promoter-probe fusions. The green fluorescent protein (Gfp) from *Aequoria victoria* is an excellent tool for monitoring gene expression, because it is naturally fluorescent without any exogenous cofactor or substrate (18). Variants of Gfp that have different spectral characteristics, folding properties, levels of chromophore brightness, or half-lives have broadened the spectrum of possible applications for Gfp (9). Measurement of fluorescence by flow cytometry has revolutionized the use of fluorescent reporter genes in bacteria (57) and now allows observation of gene expression at the level of individual bacterial cells. Plasmid-borne *gfp* fusions have already been used to monitor gene expression in *Salmonella* strains (12, 130), as well as in other gram-negative or gram-positive bacteria, including *Escherichia coli, Serratia*

liquefaciens, Erwinia herbicola, Staphylococcus aureus, Listeria monocytogenes, and *Streptococcus gordonii.* Such fusions have proven to be useful for identifying bacterial genes induced during infection of eukaryotic cells or animal hosts and have allowed some assessment of bacterial gene expression levels in such complex environments (58).

Wild-type Gfp is an extremely stable protein: cells containing this protein show no decrease in fluorescence over at least 24h (3). This is not particularly suitable for studying the expression of genes which decreases over time or under different conditions. For such studies, Gfp mutants have been created where the half-life varies from 40 minutes to 110 minutes.

Here, we describe the construction of a transcriptional fusion between the promoter of *hilA* and the gene for Gfp (*gfp*). We used this fusion product to observe the effect of heat as a stressor in activating *hilA* in *Salmonella*.

Materials and methods

Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 1. A poultry isolate of *Salmonella* Typhimurium (151) was the recipient strain for the plasmid pPROBE'gfp[LVA]. *Escherichia coli* DH5α was used to propagate the plasmid construct. The plasmid, pPROBE'gfp[LVA] contains a promoter-probe cassette that consists of a multicloning site, containing several unique restriction sites and *gfp* as a reporter gene. The gene encodes for a Gfp variant with a half-life of 40 min. *Salmonella* Typhimurium EE658 was used for the β-galactosidase assay.

Construction of the fusion plasmid

The promoter region of *hilA* was amplified by polymerase chain reaction (PCR) using the Gene-Amp® PCR System 2400 (PE Applied Bio-systems, Foster City, CA, USA). The primers used in this study were designed using the software, Primer3, available on a WWW server at the Whitehead Institute, MIT and obtained from IDT, Inc. (Coralville, IA, USA). The forward primer (5' GAA TTC TGT CCG GAA GAC AGC TT 3') contained a restriction site for the enzyme *EcoRI* and the reverse primer (5' AAG CTT GCC CTG TCC GTA CAG TGT TT 3') contained a restriction site for the enzyme *HinDIII*. The primers amplified 940 bp upstream of *hilA* and 300 bp into the gene. The PCR product was purified using Qiagen PCR purification kits (Qiagen, Valencia, CA, USA) and its size was confirmed in a 1.5% agarose gel. DNA molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Taq DNA polymerase was purchased from Promega (Madison, WI, USA). The product was then digested with restriction enzymes *EcoRI* and *HinDIII* (Sigma-Aldrich, USA).

The vector pPROBE'gfp[LVA] was digested with the same restriction enzymes, *EcoRI* and *HinDIII* (Sigma Chemicals). The amplified promoter region of *hilA* was ligated to the vector using T4 DNA Ligase (Fig. 3). The construct was introduced into *E.coli* DH5α (Invitrogen) for propagation. Confirmation was obtained by agarose gel electrophoresis, PCR analysis and sequencing. It was then isolated from *E.coli* with a plasmid miniprep kit (Qiagen, Valencia, CA, USA).

Preparation of electrocompetent cells and cell transformation

The procedures for preparation of electrocompetent cells and cell transformation are described by Yu et al. (150). The plasmid construct (2 μl) isolated from *E. coli* was mixed with 40 μl electrocompetent *Salmonella* Typhimurium cells and incubated on ice for 2 min. Electroporation was performed using a MicroPulser Electroporation Apparatus (Bio-Rad, Hercules, CA, USA) set at 1.8 kV, 25 μF with 200 ohms. The electroporated cells were immediately diluted with 1ml of LB medium and incubated for 1 h at 37 C with shaking before selecting on LB agar plates with 50 μg ml^{-1} of kanamycin. The plates were incubated at 37 C for 24 h and kanamycin resistant colonies representing successfully transformed cells were selected.

TABLE 1. Strains and plasmids used in this study.

| (a) Strains and plasmids	(b) Description	(c) Source
Salmonella Typhimurium	Poultry isolate, wild type	USDA-ARS College, Station, TX.
Salmonella Typhimurium EE658	*hilA*::Tn5*lacZY* fusion strain	Catherine Lee, Harvard Medical School, Boston, MA.
E. coli DH5α		Invitrogen, Carlsbad, CA.
pPROBE'gfp[LVA]	Plasmid with *gfp* mutant (half-life 40 min)	S.E. Lindow, University of California, Berkeley, CA.
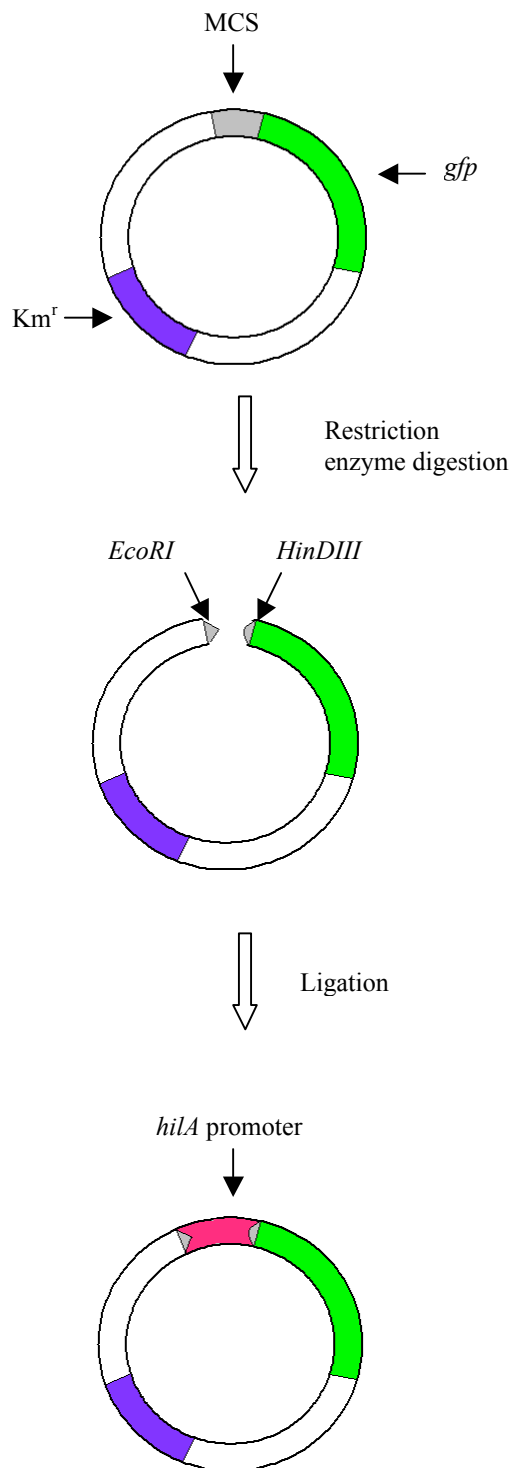
FIG. 3. Construction of fusion plasmid. The plasmid pPROBE'gfp[LVA] and the promoter of *hilA* were digested with *EcoRI* and *HinDIII*. The *hilA* promoter was ligated to the plasmid. MCS- multi cloning site. Km^r- kanamycin resistance gene.

Confirmation of transformation

After electroporation, the colonies resistant to kanamycin were checked for transformation by sequencing and PCR analysis using the same primers used for generating the PCR product. The construct size was confirmed in a 1.5% agarose gel.

Fluorescence assay

All fluorescence measurements were taken on a spectrofluorimeter (Tecan SPECTRAFluor Plus, Maennedorf, Switzerland). The excitation wavelength was kept at 485nm and the emission wavelength at 535nm. 300 µl of the culture was dispensed into each well of a 96-well microtiter plate. LB was used as control. To calculate fluorescence per cell, initial fluorescence readings were divided by cfu/ml from each sample. This yielded a better estimate of live cells as compared to OD readings.

β-Galactosidase assay

Salmonella Typhimurium EE658 was used to confirm the results of our study. This strain has a chromosomal *hilA-lacZY* fusion where *laczy* is under the control of *hilA* promoter. The cells were subjected to heat shock at 45 C for one hour and *hilA* expression was measured by quantifying β-galactosidase activity. β-galactosidase assays were performed as per Miller, 1972.

Statistical analysis

Relative expression values were analyzed by the least square means separation of the GLM procedure in the SAS Statistical Analysis Software (SAS Institute, Cary, NC). Significance determination for all statistical analyses was calculated at p<0.05.

Results

Plasmid fusion

The *gfp* gene on the vector was placed under the control of the promoter region of *hilA*. *Salmonella* Typhimurium cells were transformed with this construct and the new strain was designated as *Salmonella* Typhimurium AC. The plasmid was isolated from *S*. Typhimurium AC was subjected to sequencing and PCR, which confirmed the presence of the *hilA* promoter upstream of the *gfp* gene and in the correct orientation (data not shown).

Fluorescence assay

Fluorescence per cell was measured at different time point when *Salmonella* Typhimurium AC cells were incubated at 37ºC and 45ºC. At zero time, i.e. before heat shock, the tubes kept at 37ºC exhibited the same response with respect to fluorescence as that of tubes kept at 45ºC. One hour after heat shock, the cells incubated at 45ºC showed higher fluorescence per cell values. A similar difference was seen at 2 hours after heat shock (Fig. 4). At three hours, however, no fluorescence was detected. The same experiment was carried out in BHI broth (Fig. 5). In this medium, no fluorescence was

detected at 37ºC. At 45ºC, maximum fluorescence per cell was seen at one hour after heat shock.

β-galactosidase assay

To confirm the results obtained with the new strain, we subjected the *hilA-lacZY* fusion strain to heat shock. After one hour of heat shock at 45ºC, the β-galactosidase activity was greater than that seen in the control kept at 37ºC (Fig. 6).
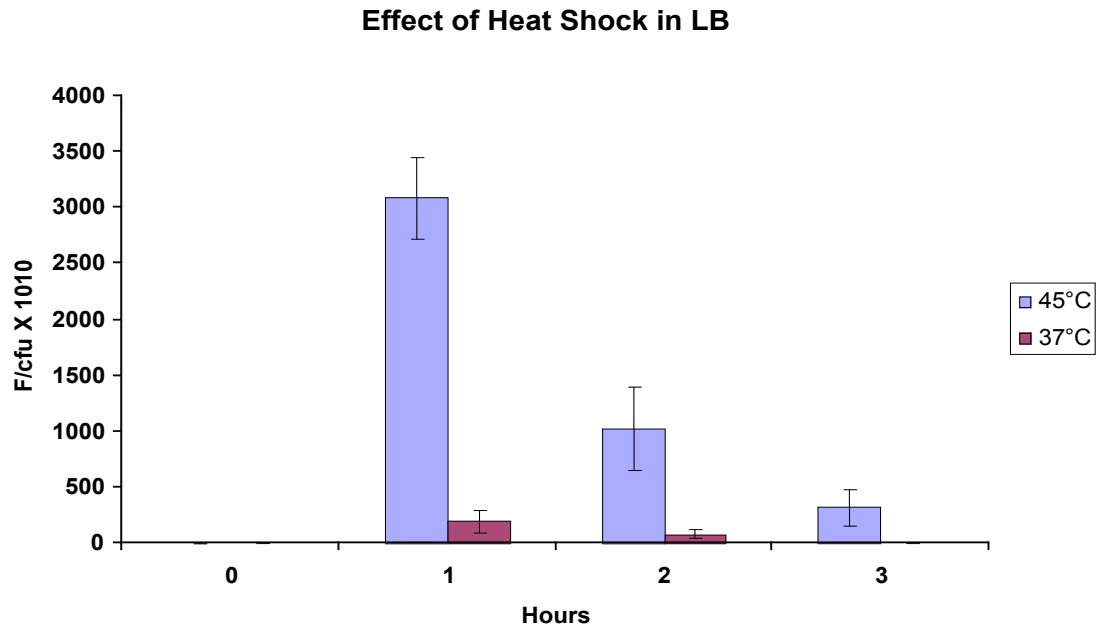
Effect of Heat Shock in LB

FIG. 4. Effect of heat shock on *hilA* in LB medium. An overnight culture of *Salmonella* Typhimurium AC grown in LB was used to inoculate fresh LB. The cells were grown to OD 0.5 at 37ºC in a shaking water bath. One set of tubes was kept at 37ºC, while another set was transferred to 45ºC for one hour, and then transferred back to 37ºC. Fluorescence measurements were taken up to 3 hours after heat shock. Averages were calculated by using four or more values from two different experiments (n=2). Error bars represent the standard deviations.
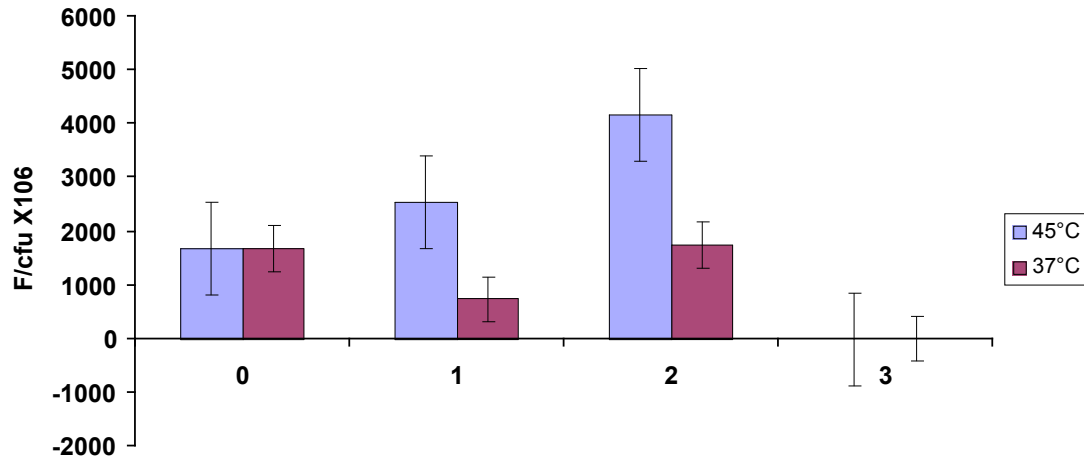
Effect of Heat Shock in BHI

FIG. 5. Effect of heat shock on *hilA* in BHI medium. One loop of an overnight culture of *Salmonella* Typhimurium AC in BHI was used to inoculate 5 ml fresh BHI. The cells were grown to OD 0.5 at 37ºC in a shaking water bath. One set of tubes was kept at 37ºC, while another set was transferred to 45ºC for one hour, and then transferred back to 37ºC. Fluorescence measurements were taken up to 3 hours after heat shock. Averages were calculated by using four or more values from two different experiments. Error bars represent the standard deviations.

Beta-Galactosidase Assay

FIG. 6. Beta-galactosidase assay. An overnight culture of *Salmonella* Typhimurium AC was used to inoculate fresh LB. The cells were grown to OD 0.5 in a shaking water bath. One set of tubes was kept at 37 C throughout. The other set was transferred to 45ºC after OD 0.5 was reached. Aliquots were drawn after 1 hour from both sets and β-galactosidase activity was measured using the Miller assay (Miller, 1972). Averages were calculated by using four or more values from two different experiments (n=2). Error bars represent the standard deviations.
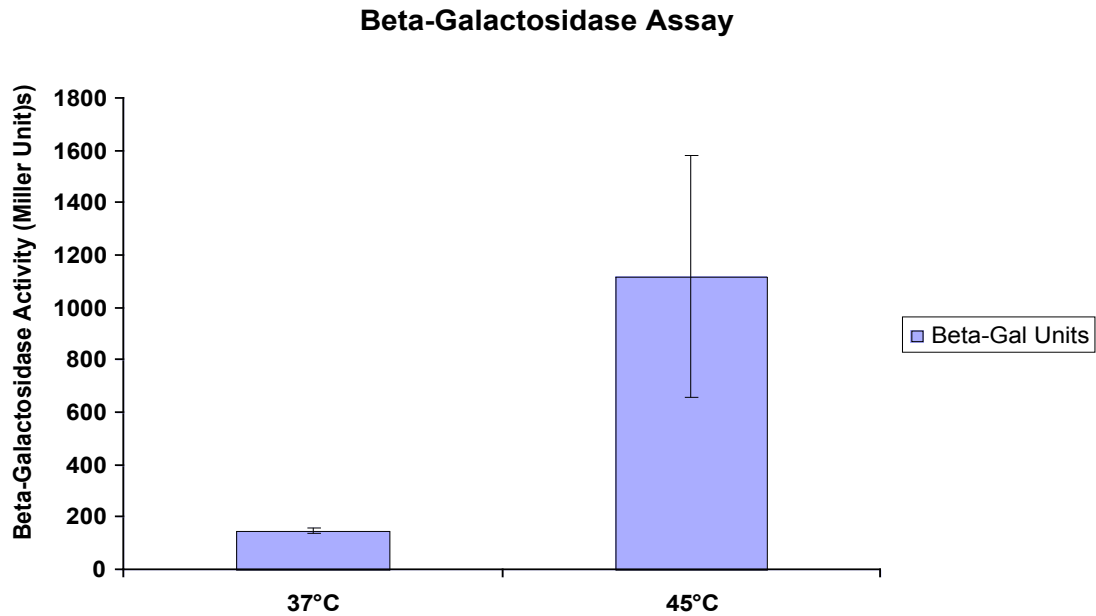
Discussion

During the course of infection, *Salmonella* is exposed to different extracellular and intracellular environments. In response to their particular environment, bacteria appear to express virulence genes required for pathogenicity specifically at that host site (87). It has been proposed that when *Salmonella* are present in the intestinal lumen, several relevant environmental conditions regulate the expression of invasion factors required for bacterial entry into intestinal epithelial cells. Bacterial growth state, osmolarity and oxygen levels are all conditions that affect the ability of *Salmonella* to enter cells (35, 45, 80, 85). It has been shown that when *Salmonella* encounter stressful conditions, *hilA* expression is turned on.

Entry of a pathogen into a warm-blooded host is accompanied by an increase in temperature. It is therefore expected that heat shock genes play and important role in bacterial infection. In several bacterial species, pathogenesis has been shown to be affected by the expression of heat shock proteins. In *Salmonella* Typhimurium, heat shock proteins are shown to be required for binding of bacterial cells to mucosal cells (34). Based on the results of this study, it appears that when *Salmonella* cells are subjected to heat shock, there is an expression of *hilA*. As reviewed by Gophna and Ron (52), heat shock genes and virulence genes are concurrently regulated. Since *hilA* plays an important role in the pathogenicity of *Salmonella*, it appears that heat shock directly activates virulence genes in *Salmonella*. Whether HilA interacts with heat shock proteins in the course of virulence activation is not known.

CHAPTER IV

DETECTION OF *hilA* RESPONSE TO HEAT SHOCK USING REAL-TIME

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Introduction

Currently there are a number of techniques used to evaluate the amount of mRNA expression including Northern Blotting, cDNA arrays, *in situ* hybridization, RNase protection assays, and reverse transcription polymerase chain reaction (RT-PCR) (51). Reverse transcription along with the polymerase chain reaction has proven to be a powerful method to quantify gene expression (99). Of these methods RT-PCR is the method for quantification known to be the most discerning and faultless in its use (51). Real-time RTPCR is rapidly becoming the new method for determining mRNA expression (13) due to its capacity to use up to 1000 times less RNA than other known methods (56).

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. SYBR Green I has an excitation and emission maxima of 494 nm and 521 nm, respectively. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR

product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles (www.ambion.com).

The accumulation of fluorescence is read at the end of every cycle via the target amplification. The threshold cycle values are determined by the cycle at which the fluorescent emission rises above the threshold (51). Since SYBR green is not sequence specific, melting curves can be used to analyze different products. By this method primer dimers can be distinguished by their lower melting temperature (Tm) (111, 143). The cycle at which the threshold was overcome (Ct) decreases as the amount of target increases (51). A method for determining relative quantification is the comparative Ct method. This method does not require the construction of cDNA plasmids nor does it depend on techniques where the variations in transcription efficiency can not be controlled. The comparative Ct method is determined by normalizing the target to a housekeeping gene which is then compared to the non treated sample or control. The determination of the relative expression that a treatment causes can be sufficient to assess its effects and the absolute expression level is not necessarily needed (114). Relative expression is determined by calculating $2^{-\Delta\Delta\ Ct}$, where $\Delta\Delta\ Ct = \Delta\ Ct\ (sample)-\Delta\ Ct\ (control)$. $\Delta\ Ct$ is calculated by subtracting the Ct of the target gene from the Ct of the housekeeping gene (51, 81, 83).

In each quantitative RT-PCR method, the expression of the gene under study has to be compared with the expression of a control gene. This is because the amount of assayed mRNA will vary with differences in cell number, experimental treatment or

various efficiency of RNA preparation. Specific errors will be introduced due to minor differences in starting amount of RNA or differences in efficiency of cDNA synthesis and PCR amplification. Therefore, a reliable quantitative RT-PCR method requires correction for these experimental variations. At present this is most often performed by normalization to a housekeeping gene. Finding a suitable housekeeping gene (i.e., a gene that is constant during the experimental conditions) is, however, not always straightforward. Therefore, different housekeeping genes should be tested in each experimental setup to find the most suitable one (not influenced by the experimental treatment).

Many recent articles have discussed the problem of housekeeping genes, and it turns out that the housekeeping genes most commonly used are subject to variation in numerous experimental conditions, bringing into question the reliability of the results obtained. In particular, the use of GAPDH and of ß-actin has been severely criticized (13, 115). Ribosomal RNA (rRNA) may be a more valuable alternative, because its expression is less likely to vary under conditions that change the levels of mRNA expression. Drawbacks of using rRNA, however, are that it cannot be used for quantification of samples when starting from mRNA, or when oligo d(T)16 is used for cDNA synthesis. Another drawback is its very abundant expression relative to mRNA expression levels of target genes.

Materials and methods

Inoculation for RT-PCR

Three different temperatures were used to evaluate the *hilA* expression of *Salmonella* Typhimurium using RT-PCR. An overnight culture of *Salmonella* Typhimurium was used to inoculate fresh Luria Bertini medium. The cells were grown to OD 0.4 at 37 C in a shaking water bath (Barnstead, MA, USA) and then transferred to an incubator with the required temperature for one hour. Two aliquots of 400ul each were taken from each sample at different time points for RNA extraction.

RNA extraction

After the different media were inoculated with the bacterial suspension following the inoculation phase, the samples were placed in a shaking water bath at 37°C for the desired duration. At these specific time points 400ul of the bacterial suspension were removed and placed in a microcentrifuge tube with 800ul of RNAprotect (Qiagen, Valencia, CA). Samples were stored at -4°C overnight prior to RNA extraction. RNA was extracted from *Salmonella* Typhimurium cultures according to the RNeasy Mini Kit from Qiagen.

Primer design

Primers were designed using sequences obtained from the GenBank website for the *hilA* and 16srRNA gene sequences and then primers were designed using Primer Express 1.0 Software from Perkin-Elmer Applied Biosystems (Foster City, CA). Sequences were

then blasted on the NCBI website in order to determine if they were compatible with any other species of bacteria. The primers used in this study are listed in Table 2.

Reverse transcription reactions

Reverse transcription reactions were performed using the reagents from the QIAGEN OneStep RT-PCR Kit. Each reaction contained 5X QIAGEN OneStep RT-PCR Buffer, 400 µM of each dNTP Mix , 5U/reaction RNase Inhibitor, 2.0ul of QIAGEN OneStep RT-PCR Enzyme Mix, 200 ng of RNA, 6.0ul of each Primer (0.6µM), and RNase-free water to a final volume of 20 µl. A positive RT reaction was run in order to ensure that the procedure was working properly. The positive reaction contained all of the same components except that in place of a RNA sample template a DNA sample was used as the template. In order to determine if RNA samples were contaminated with DNA, two (-) RT reactions were run on each RNA sample. One (-) RT reaction contained the same components as the (+) RT reactions, except it lacked the RNA sample template and contained more water to ensure that the final concentration of the remaining components remains the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no DNA contamination. All one step RT reactions were performed on a Gene Amp PCR System from Perkin Elmer (Wellesley, MA) under the following conditions: reverse transcription at 30 minutes at 50°C, initial PCR activation at 15 minutes at 95°C, three step cycling 1 minute at 94°C, 1 minute at 53°C and 1 minute at 72°C for 40 cycles, followed by a final extension period of 10 minutes at 72°C. The samples were then held at 4°C until the samples could be removed.

Three RT reactions were performed, one for each RNA sample. Samples were subsequently run on 1.5% agarose gel in 1X TAE buffer for 30 minutes at 100V.

Real-time PCR reactions

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Each 20 µl SYBR Green PCR reaction contained 2µl RNA, 0.2µl (5µM) of each primer, 10µl 1X SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 0.1µl MultiScribe Reverse Transcriptase (Perkin-Elmer Applied Biosystems), 0.4µl RNase Inhibitor (Perkin-Elmer Applied Biosystems), and water. Thermal cycling conditions were as follows: 48°C for 30 minutes, 95°C for 10 minutes, and 40 repeats of 95° C for 15 seconds and 60°C for 1 minute. A dissociation curve was run following the real-time reaction to determine if the primers used in each reaction generated a specific product. The same (+) and (-) RT reactions were run for the Real-Time PCR as for RT reactions.

TABLE 2. Primers used in this study.

Target gene	Primer name	Sequence (5'-3')	Position on gene	Product size (bp)
16S rRNA	rsmC Fwd	TGCGCGACCAGGCTAAA	243-263	108
	rsmC Rev	CGAATTATGGACGGTCACCACTT	275-296	
hilA	hilA Fwd	TATCGCAGTATGCGCCCTT	443-461	40
	hilA Rev	GACGGCCACTGGTAATGCT	475-493	
dnaK	dnaK Fwd	TATGGATGGAACGCAGGCA	54-72	105
	dnaK Rev	GGCTGACCAACCAGAGTTTCAC	158-179	
opdA	opdA Fwd	ATCCACAGCAAGGAGCGAAA	1710-1729	105
	opdA Rev	GGCTGAACGCATGTGGAAA	1814-1932	

Data analysis

Real-time PCR reactions were run to analyze the expression of two genes, each gene was analyzed in replicate three times for each sample. Each sample was run in triplicate for each trial. Data were analyzed using the CT value for each sample, or the cycle at which each samples amplification curve crosses a specified threshold. First, the CT of the replicated samples for each gene was averaged. The relative induction of each target gene of the three samples was then determined using the Comparative CT (ΔΔCT) method. The Δ CT value of the target gene (*hilA*) was determined by normalizing to the endogenous control gene (*rsmC*). The gene rsmC was chosen since the gene product it encodes for, 16S RNA, is constitutively expressed by the bacterium. These samples were then subtracted from the baseline sample. The resulting ΔΔ CT was then used to calculate relative expression using the formula 2^-ΔΔ CT. Dissociation curves were also run to determine gene melting temperatures. Amplification curves were also run to determine the cycle at which the threshold was overcome and corresponds to the CT value.

Statistical analysis

Relative expression values were analyzed by the least square means separation of the GLM procedure in the SAS Statistical Analysis Software (SAS Institute, Cary, NC). Significance determination for all statistical analyses was calculated at $p<0.05$. Significantly different means were further separated using the Tukey test.

Results

The expression of both *dnaK* and *opdA* was greater at 55ºC than at 45ºC (Fig. 7 and 8). Both *dnaK* and *opdA* showed expression at 10 and 20 minutes at 45ºC and this declined after 20 minutes. At 55ºC however, the expression does not appear to subside after 20 minutes. At that temperature, it is likely that a high rate of protein denaturation requires the presence of DnaK and OpdA.

In the case of *hilA*, expression was seen at both 45ºC and 55ºC, thus clearly indicating that *hilA* responds to heat stress (Fig. 9).

Discussion

Heat treatment is one of the most widely used methods for destroying microorganisms in foods. The inactivation of foodborne pathogens using a heat treatment is a critical control point in the safe preparation of many foods. The prediction of microbiological safety of mild heat treatments given to foods is a special problem for the food industry and needs to be defined with greater precision and accuracy (36). This is because insufficient cooking, reheating and/or subsequent cooling are often contributing factors in food-poisoning outbreaks.

A survey performed by the WHO (World Health Organization), 1995, in Europe indicated that almost 25% of the food-borne outbreaks could be traced back to recontamination. The most important factors contributing to the presence of pathogens in prepared foods were insufficient hygiene (1.6%), cross-contamination (3.6%), processing or storage in inadequate rooms (4.2%), contaminated equipment (5.7%) and contamination by personnel (9.2%) (110).

Processed foods, which are presumed to be safe, are not generally subjected to heat treatment with the intention of killing microorganisms. However, the simple act of warming them before consumption could very well lead to activation of virulence in any bacteria that might have been introduced by recontamination. Similarly, entry of a pathogen into a warm-blooded host subjects it to a shift in temperature. Therefore, temperature increase is one of the major environmental stresses encountered by bacteria. It is proposed that *Salmonella*, when confronted with environmental stresses that compromise its survivability, react by increasing virulence expression (39).

The major adaptive response to elevation in temperature involves the induction of many heat shock proteins (132). In several bacterial species, heat shock proteins have been shown to play a direct role in pathogenesis including heat shock proteins required for binding of *Salmonella* Typhimurium to mucosal cells (34). Since *hilA* appears to play a central role in virulence in *Salmonella*, it would not be surprising to see a *hilA* response to heat stress.

We looked at the expression of two heat shock genes, *opdA* and *dnaK* at heat shock temperatures. The *opdA* gene of *Salmonella* Typhimurium encodes a metallopeptidase, oligopeptidase A (OpdA). It was originally identified by Vimr and Miller (133) as one of two activities in extracts of *Salmonella* Typhimurium which hydrolyzed N-acetyl-L-Alanine. The *opdA* gene (also known as *prlC*) has been cloned and sequenced (26). The *opdA* promoter is part of a σ^{32} –dependent heat shock operon (25). Expression of the gene has been shown to be induced by incubation for 1h at 39 C (133).

DnaK, the product of the *dnaK* gene, forms a chaperone machinery with the cochaperones DnaJ and GrpE and is involved in many cellular processes, such as DNA replication of the bacterial chromosome, RNA synthesis, protein transport, cell division, and autoregulation of the heat shock response. Furthermore, this chaperone machinery nonspecifically interacts with many unfolded and misfolded proteins and therefore assists in proper folding, helps in refolding, prevents aggregation, and mediates the degradation of misfolded proteins (120). The DnaK/J chaperone system binds and promotes degradation of σ^{32} at lower temperatures. At heat shock temperatures, the DnaK/J-mediated degradation of σ^{32} is largely abolished by a mechanism that is not fully understood (4). This interaction is completely abolished at 42 C (19). The DnaK/J chaperone machinery could also be involved in the expression of SPI1 through interactions with regulators such as *hilD*, *hilC, hilA* and/or *invF*. (120).

Both these genes show an increase in expression at 45 C and at 55 C as compared to 37 C. Based on the results of this research, it appears that when *Salmonella* cells are subjected to heat shock, there is an expression of *hilA*. As reviewed by Gophna and Ron (52), heat shock genes and virulence genes are concurrently regulated. Since *hilA* plays an important role in the pathogenicity of *Salmonella*, it appears that heat shock directly activates virulence genes in *Salmonella*. Whether HilA interacts with heat shock proteins in the course of virulence activation is not known.
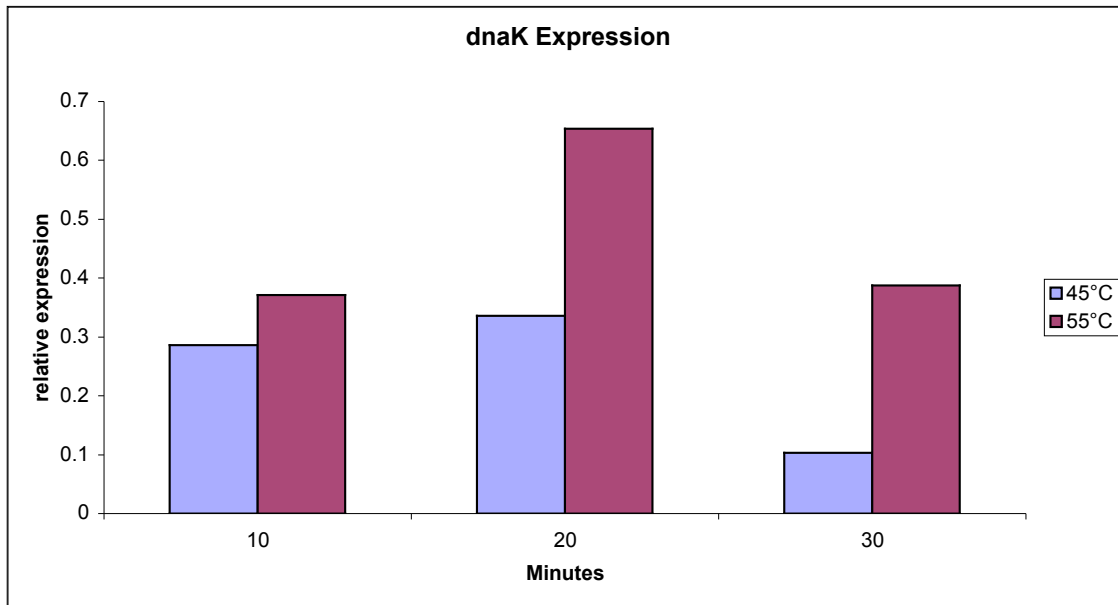
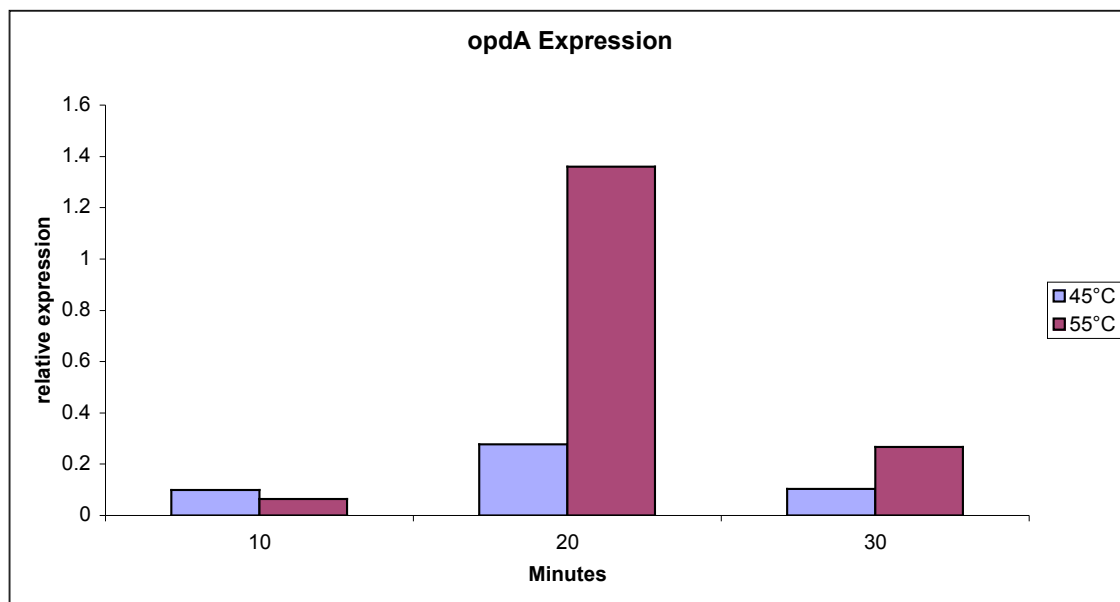FIG. 7. Relative expression of *dnaK* at 45ºC and 55ºC.

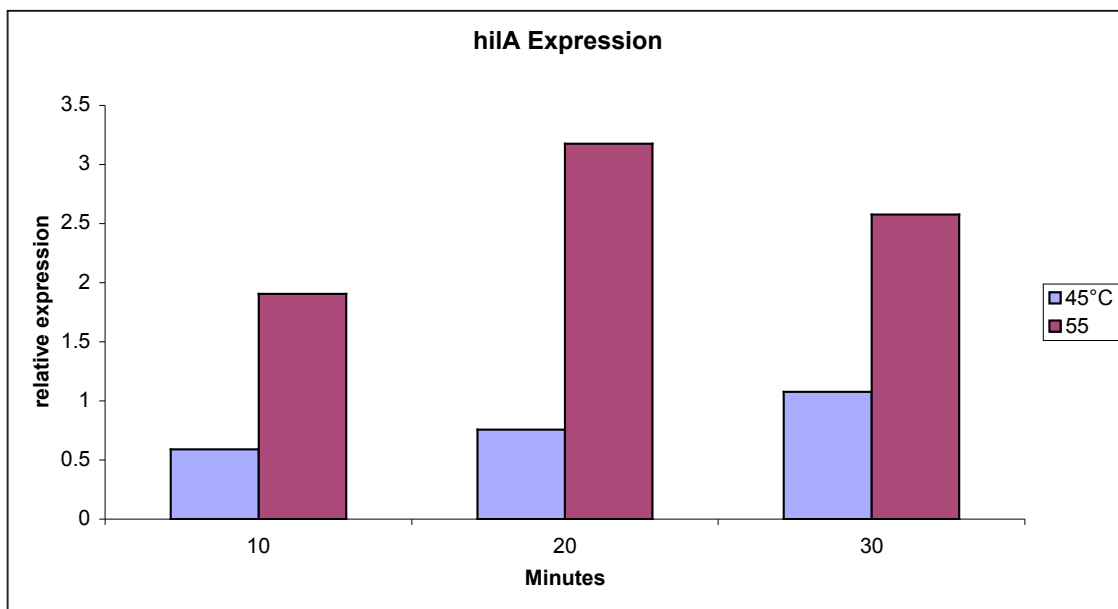FIG. 8. Relative expression of *opdA* at 45ºC and 55ºC.

FIG. 9. Relative expression of *hilA* at 45ºC and 55ºC.

CHAPTER V

CONCLUSIONS

The *Salmonella* pathogenicity island-1 (SPI-1) chromosomal region encodes for more than 30 proteins, which include regulatory proteins and structural components believed to be involved in virulence. The *hilA* gene is present on SPI-1. HilA is thought to coordinate the regulation of SPI1 genes in response to environmental and regulatory factors. Previous studies have concluded that the regulation of SPI1 genes is primarily controlled by changes in *hilA* transcription (6). *hilA* is known to be activated when the bacteria encounter stress-inducing conditions. A number of factors have been identified that affect *hilA* expression, such as, pH, osmolarity, oxygen tension (6). When *Salmonella* enter their warm blooded hosts, they encounter an increase in temperature. Therefore, heat is another stressor faced by *Salmonella* during infection of their hosts.

Based on the results of this research, it appears that when *Salmonella* cells are subjected to heat shock, there is an expression of *hilA*. As reviewed by Gophna and Ron (52), heat shock genes and virulence genes are concurrently regulated, however, it is not known whether one of them controls the other or if both are independently affected by the same regulator or signal. Since *hilA* plays an important role in the pathogenicity of *Salmonella*, it appears that heat shock directly activates virulence genes in *Salmonella*. Whether HilA interacts with heat shock proteins in the course of virulence activation is not known.

The fusion strain, *Salmonella* Typhimuirum AC, created during this research can be used as a reporter strain to evaluate the effect of temperature shifts on *Salmonella* pathogenesis. Since *Salmonella* is a foodborne pathogen, the exposure of food products to heat not sufficient to kill any present *Salmonella*, could subject the pathogen to a temperature shock. On the basis of the results of this study, those bacterial cells that survive the heat shock, could possibly be more virulent. A sudden decrease in the ambient temperature could also serve as a temperature shock (cold shock). Whether this has any effect on *hilA* expression can be studied using this strain.

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