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Role of DksA and Hfq in *Shigella flexneri* Virulence

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Role of DksA and Hfq in *Shigella flexneri* Virulence

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Dedication

To Anil and Parents

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Role of DksA and Hfq in *Shigella flexneri* Virulence

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Hfq is a post-transcriptional regulator playing an important role in virulence and cellular physiology by regulating the expression of several genes either directly or indirectly through interaction with small regulatory RNAs (sRNA). Hfq is highly abundant and its synthesis in *E. coli* is subject to auto-repression at the level of translation. My studies with *Shigella flexneri* showed that *hfq* gene expression is regulated at the transcriptional level by a pleiotropic regulatory protein, DksA. I compared the gene expression profiles of wild type and *dksA* mutant *S. flexneri* by microarray and real time PCR analyses and determined that *hfq* expression was reduced in the *dksA* mutant. Significantly reduced Hfq levels in the *dksA* mutant were restored to wild type levels in the *dksA* mutant complemented with wild type *dksA*. Characterization of an *hfq* mutant in *S. flexneri* showed several phenotypes in common with the *dksA* mutant including reduced ability to survive in stress conditions and formation of elongated cells within cultured epithelial cells. Because DksA is required by *S. flexneri* to form plaques in cultured epithelial cell monolayers, a measure of virulence, the role of Hfq in the *dksA* virulence phenotype was assessed. Inducing expression of *hfq* in the *dksA* mutant restored plaque formation, and an *S. flexneri hfq* mutant failed to form wild type plaques. These data suggest that DksA plays a role in regulating *hfq* gene expression and

that this regulation is important for *S. flexneri* virulence. In an *in vitro* transcription assay, addition of purified DksA increased transcription of *hfq* and this effect was greater with one of the two known *hfq* promoters. Addition of ppGpp, a stringent response molecule, along with DksA in the *in vitro* transcription assay resulted in a further increase in transcription of *hfq*, indicating that DksA is required for maximal transcription of *hfq* during both exponential and stringent response growth conditions. Real time PCR analysis showed reduced mRNA levels of the three major transcriptional activators of *S. flexneri* virulence genes, VirF, VirB and MxiE in the *hfq* mutant providing an explanation for its reduced ability to invade and form plaques in cultured monolayers.

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I. INTRODUCTION

A. Overview of disease caused by *Shigella* species

Shigella spp. are responsible for causing bacillary dysentery, or shigellosis, in humans. Shigellae are Gram-negative, facultative intracellular bacteria that are divided into four species, *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei*. Shigellosis is a condition characterized by bloody diarrhea, fever and severe abdominal cramps. The global burden of shigellosis was estimated by reviewing the literature published on *Shigella* infection between years 1966 and 1997. This diarrheal disease is estimated to be responsible for approximately 164.7 million cases annually, of which about 163.2 million are in developing countries (with 1.1 million deaths) and about 1.5 million in industrialized countries. A total of 69% of all episodes and 61% of all deaths attributable to shigellosis involved children less than 5 years of age (Kotloff *et al.*, 1999). Also, shigellosis in the immunocompromised humans results in more severe manifestations of *Shigella* infection, including persistent and recurrent intestinal diseases and bacteremia (Angulo and Swerdlow, 1995).

S. sonnei is the causative agent of most Shigellosis in industrialized nations (median 77%), whereas *S. flexneri* is endemic in developing nations (median 60%) and is the most frequently isolated species worldwide. *S. dysenteriae* type 1 is the causative agent for most extensive outbreaks of *Shigella* infections in south Asia and sub-Saharan Africa (Kotloff *et al.*, 1999). *S. dysenteriae* causes a severe form of dysentery mediated by production of an

exotoxin, Shiga toxin, whereas *S. flexneri* produces *Shigella* enterotoxins and the secreted autotransporter toxin (Niyogi *et al.*, 2004). Because of their prevalence and the severity of infection caused by them, *S. dysenteriae* and *S. flexneri* are considered the most important *Shigella* species medically and have been the focus of a great deal of research (Weekly Epidemiological Record 2005). Most of the studies have been conducted on *S. flexneri* and a model of the mechanism of pathogenesis, thought to be common to the other *Shigella* spp., has been derived from these studies.

B. Pathogenesis of *S. flexneri*

Humans are the only known natural host of *Shigella*. However, *in vit*ro and *in vivo* studies have used various cell types (epithelial cells, macrophages, monocytes, fibroblasts and red blood cells) (Oaks *et al.*, 1985) and animal models of infection, such as guinea pigs, rabbit ligated ileal loops and mouse lungs, to study various aspects of the molecular basis for pathogenesis of *Shigella*. *Shigella* has a very low infectious dose, requiring as few as 10-200 bacteria to establish infection in a healthy adult (DuPont *et al.*, 1989). This is mainly due to the ability of *Shigella* to survive the low pH of the human stomach (Gorden and Small, 1993). After passing through the acidic conditions in the stomach, *Shigella* ultimately reaches the colon. Experiments using polarized cell lines have demonstrated that *S. flexneri* invades colonic epithelial cells from the basolateral side (Mounier *et al.*, 1992). Invasion of epithelial cells requires the invasion plasmid antigens (Ipa) secreted by the virulence plasmid encoded type three secretion system in *S. flexneri*.

There are three known mechanisms by which *Shigella* gains access to the sub-mucosal layer of epithelial cells. First, *S. flexneri* can disrupt the tight junction proteins on epithelial cells, allowing paracellular movement of bacteria into the sub-mucosa (Sakaguchi *et al.*, 2002). Second, polymorphonuclear leukocytes recruited by interleukins produced in response to *S. flexneri* invasion create gaps between epithelial cells, through which the bacteria can transmigrate into the sub-mucosa (Beatty and Sansonetti, 1997). Third, in the

colonic mucosa, *Shigella* crosses the epithelial layer barrier by invading the M cells overlying the lymphoid follicles (Wassef *et al.*, 1989) (Figure 1, step 1). From the M cells, the bacteria are released into an intraepithelial pocket filled with lymphocytes and macrophages. Macrophages phagocytose the bacteria but *S. flexneri* escapes the phagosome and induces apoptosis in the macrophage (Figure 1 steps 2 and 3) with the help of a type three secretion system effector protein, IpaB, which can bind and activate caspase-1 induced apoptosis (High *et al.*, 1992). After release from the macrophage, bacteria enter the sub-mucosal layer and invade the epithelial cells from the basolateral side. The uptake of bacteria by the epithelial cells involves membrane ruffling due to rearrangement of the eukaryotic cell cytoskeleton, which is induced by the Ipa proteins secreted by the bacteria.

Once the bacterium comes in contact with the host cell, the pre-formed Ipa proteins are secreted from the type three secretion apparatus (a flagella like structure) made from Mxi-Spa proteins (Menard *et al.*, 1994). IpaB and IpaC translocators insert a pore (translocon) in the host cell membrane, allowing other effector molecules to gain access to host cytoplasm through this pore (Blocker *et al.*, 1999). IpaC activates host cell Rho GTPases, triggering actin polymerization and filopodial extensions in the vicinity of the bacteria (Tran Van Nhieu *et al.*, 1999). IpaA is secreted into the host epithelial cell cytosol where it binds the cytoskeleton-associated protein vinculin, resulting in depolymerization of actin filaments and formation of entry foci around the bacterium (Bourdet-Sicard *et al.*, 1999). Another effector protein, IpgD is secreted into the epithelial cell by the *Shigella* type three secretion system, where it acts as a phosphoinositide phosphatase, uncoupling the

plasma membrane from the actin cytoskeleton and forming membrane extensions around the bacterium (Niebuhr *et al.*, 2002). Additionally, VirA, an effector protein also secreted into the epithelial cell by the type three secretion system, interacts with tubulin and destabilizes microtubules around the bacterial entry site (Yoshida *et al.*, 2002).

After internalization (Figure 1, step 4), the bacteria rapidly lyse the membrane of the entry vacuole (Figure 1, step 5), with the help of membranolytic properties of IpaB invasin, and gain access to the host cytoplasm where they can multiply with a generation time of ~40 min (Sansonetti *et al.*, 1986). *Shigella* induces actin polymerization (Figure 1, step 6), with the help of a bacterial polar effector protein, IcsA. IcsA is present on the bacterial surface, with the greatest concentration at the old pole of the bacterium. This unipolar localization of IcsA leads to formation of an actin tail at one end of the bacterium that propels the bacterium, facilitating its intracellular and intercellular movement (Goldberg *et al.*, 1993). This movement results in cellular protrusions containing a single bacterium, which are engulfed by the adjacent epithelial cell, resulting in intercellular spread of shigellae. Bacteria released from M cells (after initial uptake) or from epithelial cells (after intracellular multiplication) interact with macrophages, escape phagocytosis and induce apoptosis of the infected macrophage. This results in induction of the host inflammatory response and further disruption of the epithelial barrier (Figure 1).

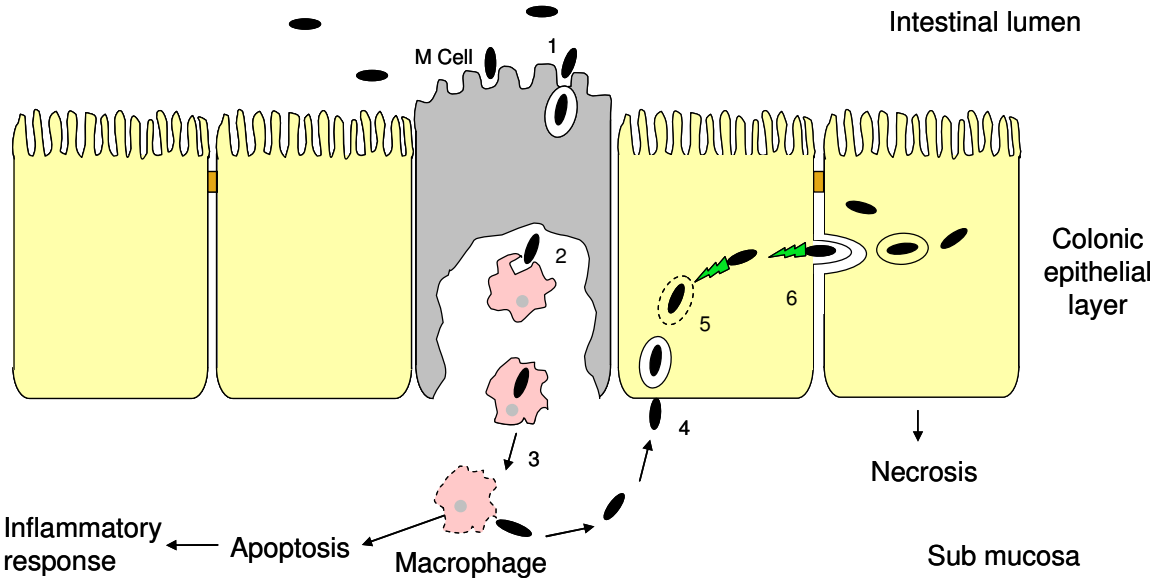


Figure 1. Pathogenesis of *Shigella flexneri*

1. In the intestinal lumen, *S. flexneri* enters into the M cells (gray). 2. The bacteria are delivered in the intraepithelial pocket of M cells and are phagocytosed by the resident macrophages (pink). 3. *S. flexneri* induces apoptosis of the macrophage by secreting type three secretion system effector proteins. 4. *S. flexneri* reaches the basolateral side of the epithelial cells (yellow) in which they induce their entry using the type three secretion system. 5. The vacuole surrounding the bacterium is lysed and shigellae are released into the cell cytoplasm. 6. *S. flexneri* polymerizes actin, which facilitates the intracellular movement of the bacteria and formation of protrusions. These protrusions lead to dissemination of bacteria to adjacent cells and epithelial disruption. Epithelial cell necrosis and apoptosis of macrophages elicits a strong host inflammatory response. Modified from (Jennison and Verma, 2004).

C. *Shigella* virulence genes

Shigella strains carry a large virulence plasmid (230 Kb) that encodes genes that are required and sufficient for invasion in epithelial cells (Venkatesan *et al.*, 2001). The genome sequence of an *S. flexneri* 2a strain was recently determined and compared to *E. coli* K12 strain MG1655 (Wei *et al.*, 2003). There are 195 genes that are specific to *S. flexneri* and they are carried by islands that encode an integrase, are located at tRNA sites, or are associated with an insertion sequence or prophage remnants. Several genes within these islands are involved in pathogenicity. Pathogenicity islands are characterized as chromosomal regions which encode one or more virulence determinants and are foreign to the host bacterium (Hacker *et al.*, 1997).

Three pathogenicity islands have been identified on the *S. flexneri* chromosome (for a review see Hale, 1991). Some genes present on these islands have a role in pathogenesis, including *sit* genes encoding the iron uptake system required for intercellular spread in Henle cells (Runyen-Janecky *et al.*, 2003), *sigA* encoding an extracellular protease involved in intestinal fluid accumulation (Al-Hasani *et al.*, 2000), *gtr* genes encoding proteins for glucosylation of O antigen (West *et al.*, 2005), *shiA* encoding a protein of unknown function, *stx* encoding the Shiga toxin (in *S. dysenteriae* type 1) (Unkmeir and Schmidt, 2000), and *set* and *sat*, in *S. flexneri* encoding *Shigella* entertoxin-1 and the secreted autotransporter toxin, respectively (Niyogi *et al.*, 2004). Other chromosomal genes that are not specific to *Shigella* spp. are also required for its virulence.

1. Virulence plasmid encoded virulence genes

The 230 Kb virulence plasmid present in the *S. flexneri* 2a strain, pCP301, carries a 30 Kb entry region essential for entry of *Shigella* into epithelial cells (for a review, see Le Gall *et al.*, 2005) (Figure 2). The genes present on the entry region encode components of the Type three secretion apparatus (TTSA) (*mxi* and *spa*), translocators (*ipaB* and *ipaC*) and some effectors (*ipaA-D*, *ipgB1*, *ipgD*, *icsB*, *virA*), chaperones (*ipgA*, *ipgC*, *ipgE*, *spa15*), and transcriptional activators (*virF*, *virB*, *mxiE* and *orf81*) (Figure 2). When *Shigella* contacts the host cell, IpaB and IpaC translocators are secreted through the type three secretion apparatus, which is a flagella-like structure made from Mxi and Spa structural proteins used to deliver proteins. IpaB and IpaC generate a pore (translocon) on the host cell membrane with the help of two hydrophobic regions that facilitate interaction with the host lipid membrane. Through the translocon, effector proteins are injected into the cell via the type three secretion apparatus, where they affect cellular functions. In most cases, the exact function of the effectors, i.e. enzymatic or binding activities, have not been elucidated. In general, effectors facilitate the internalization of bacteria in epithelial cells (IpaC function), uncoupling of the plasma membrane from the cellular cytoskeleton (IpgD function), depolymerization of actin filaments (IpaA function), formation of membrane ruffles during entry (IpgB1 function), destabilization of microtubules (VirA function), and avoidance of an autophagy protein (IcsB function) (Ogawa *et al.*, 2005). After cell uptake, the movement of *Shigella* is facilitated by an asymmetrically distributed surface protein, IcsA, which is present on the old pole of the

bacterium. IcsA binds the neural Wiskott-Aldrich syndrome protein (N-WASP), leading to a complex formation with Arp2/3, which induces actin polymerization and both intracellular and intercellular movement of *Shigella* (Suzuki *et al.*, 2002).

The virulence plasmid encodes three transcriptional activators: VirF and MxiE, members of the AraC family of transcription activators, and VirB, a member of the ParB family of partition proteins (Figure 2). Another transcription activator of the AraC family, Orf81 is encoded by the virulence plasmid, but since its inactivation does not affect expression of any virulence plasmid genes under inducing conditions (37ºC), it is believed to be a remnant of a regulatory circuit that no longer exists (Le Gall *et al.*, 2005). *S. flexneri* virulence gene expression is primarily induced by an environmental stimulus, which is a temperature of 37°C. Optimum induction also requires a moderate level of osmotic pressure, similar to that of the physiological saline, and a pH of 7.4 (Bernardini *et al.*, 1993; Nakayama and Watanabe, 1995). The type three secretion apparatus is assembled, and the effector proteins are synthesized when *Shigella* is grown at 37°C, but the activity of the system resulting in secretion of effector proteins is very poor in these conditions. Activity is stimulated when *Shigella* comes in contact with the host epithelial cells. Secretion activation also can be induced *in vitro* conditions by growing *Shigella* in the presence of a dye, Congo red (Payne and Finkelstein, 1977) or by exposure to bile salts (Pope *et al.*, 1995).

At 30°C, binding of H-NS (heat-stable nucleoid structuring protein) to the *virF* promoter prevents transcription, and at 37°C, the change in DNA topology releases H-NS,

facilitating the binding of FIS (factor for inversion stimulation) and direct positive transcriptional activation of the *virF* promoter (Falconi *et al.*, 2001). H-NS also directly represses *virB* expression by binding to the *virB* transcriptional start site. VirF activates transcription of *virB* by binding to the upstream sequence of the *virB* promoter in a DNA-topology-dependent manner (Tobe *et al.*, 1993). VirF also induces transcription of *icsA*, which encodes a protein located at the old pole of bacteria facilitating actin tail polymerization for intracellular movement of *Shigella* within the host epithelial cell (Sakai *et al.*, 1988). In turn, activated VirB transcriptionally activates genes of the entry region encoding the type three secretion effectors and approximately 15 proteins that are part of the type three secretion system (Figure 2) (Le Gall *et al.*, 2005).

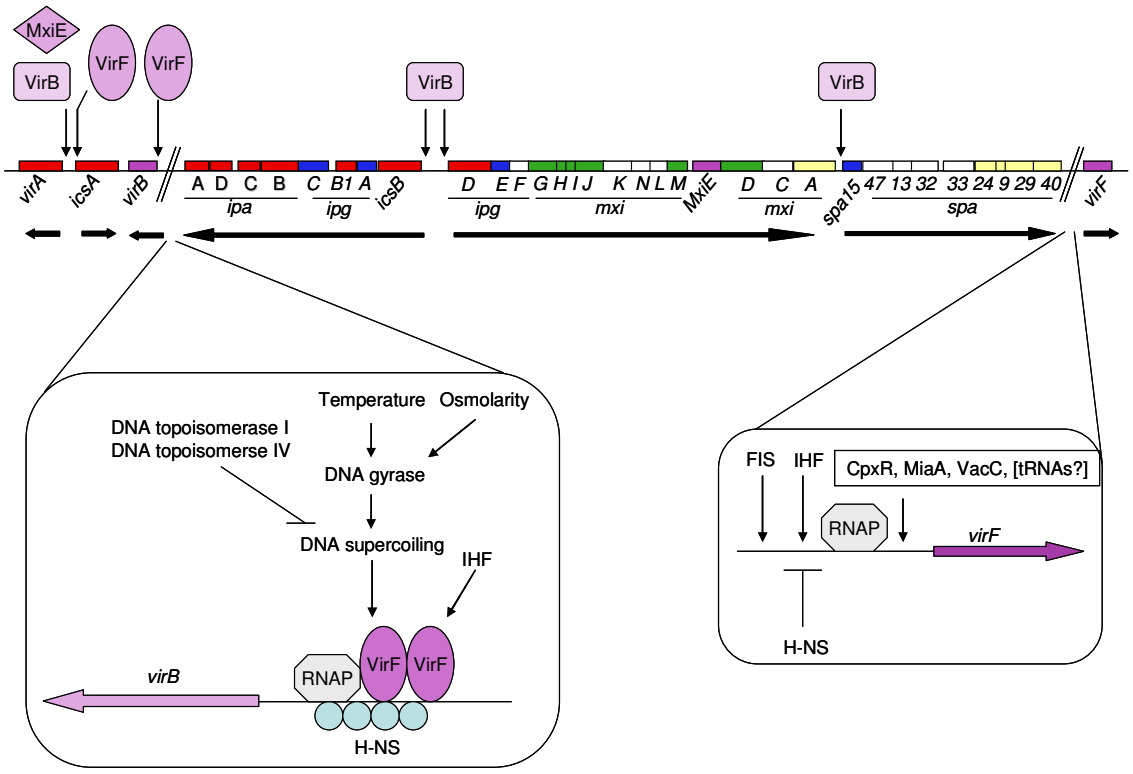


Figure 2. *S. flexneri* virulence gene regulatory cascade

The upper portion of the figure summarizes the virulence gene regulon located within the 31 Kb entry region on the 230 Kb virulence plasmid (not drawn to scale). Genes encoding transcription activators are in purple, effectors in red, chaperones in blue, components of the needle complex in green, and inner-membrane proteins in yellow. Positive transcriptional regulation by VirB and VirF is indicated by vertical arrows. Lower left shows the regulatory region of the *virB* promoter showing overlapping binding sites of the positively acting VirF protein and the negatively acting H-NS protein. Positive effect of IHF and DNA supercoiling are indicated by arrows, with supercoiling being promoted by DNA gyrase (probably under environmental control) and antagonized by the DNA-relaxing enzymes topoisomerases I and IV. Lower right shows the regulatory region of *virF* promoter showing IHF, FIS and CpxR acting as positive transcriptional regulators and H-NS as a negative regulator. At the level of translation, MiaA and VacC proteins have modulatory roles and similarly certain tRNA species may be modulatory. Modified from (Dorman and Porter, 1998).

VirB also activates the transcription of another transcriptional activator, MxiE. In addition to transcriptional activation by VirB, MxiE requires the IpgC chaperone for its activation. IpgC, a chaperone for the translocators IpaB and IpaC, is titrated by its association with IpaB and IpaC during non-secretion conditions. IpaB and IpaC are released during secretion conditions, releasing IpgC chaperone for MxiE activation (Mavris *et al.*, 2002) (Figure 3). Thus, IpaB and IpaC function as coactivators of MxiE. In addition, MxiE is associated with the type three secretion apparatus substrate OspD1 and its chaperone Spa15 during non-secretion conditions, which prevents MxiE from being activated by free IpgC (Parsot *et al.*, 2005). However, during secretion conditions, OspD1 is secreted into the host cell, releasing MxiE for activation by IpgC (Figure 3). MxiE activates genes required during secretion conditions, after the activation of the type three secretion apparatus, whereas VirB activates genes that do not depend on type three secretion apparatus activity (Le Gall *et al.*, 2005) (Figure 4).

Genes whose expression is regulated by type three secretion apparatus activity (*mxiE* regulon) are induced upon entry into epithelial cells and are no longer transcribed in bacteria growing in the intracellular compartment, suggesting that type three secretion activity is turned on during entry and off in the cell cytoplasm (Kane *et al.*, 2002). However, the involvement of the IpaB and IpaC in lysis of the cell membrane that surrounds bacteria in protrusions during cell-to-cell spread suggests that the type three secretion apparatus is reactivated in protrusions (Page *et al.*, 1999). MxiE regulated genes, *virA* and *ipaH* are activated only after activation of the type three secretion apparatus and are likely to

correspond to a second wave of effectors that are involved in cell to cell spread during *Shigella* infection (Demers *et al.*, 1998). This differential regulation of genes encoding the type three secretion effectors suggests that different effectors might be required at different times following contact of bacteria with the host cells. It is challenging to elucidate the function of these effectors and their role(s) at various stages of infection.

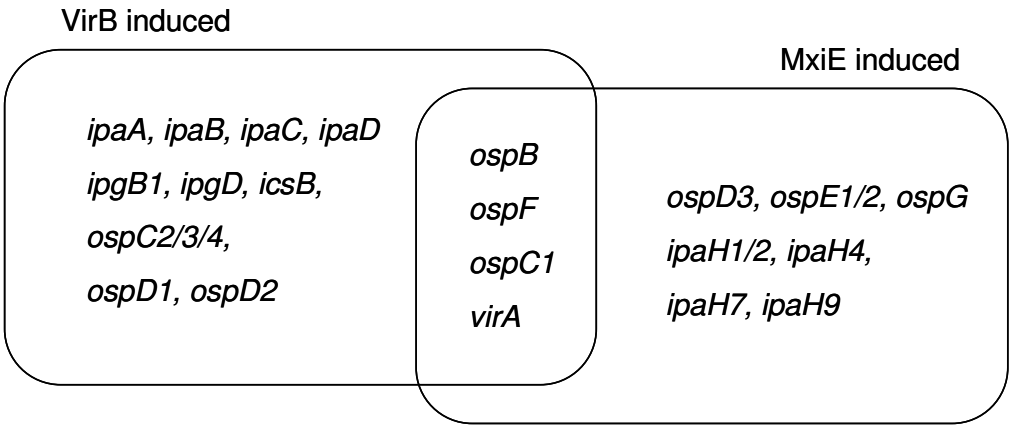


Figure 3. Repertoires of the TTS effector genes controlled by VirB and/or MxiE

Transcription of genes controlled by VirB is independent of the TTSA activity, whereas transcription of genes controlled by MxiE is activated (or increased) under secretion conditions. Adapted from (Le Gall *et al.*, 2005).

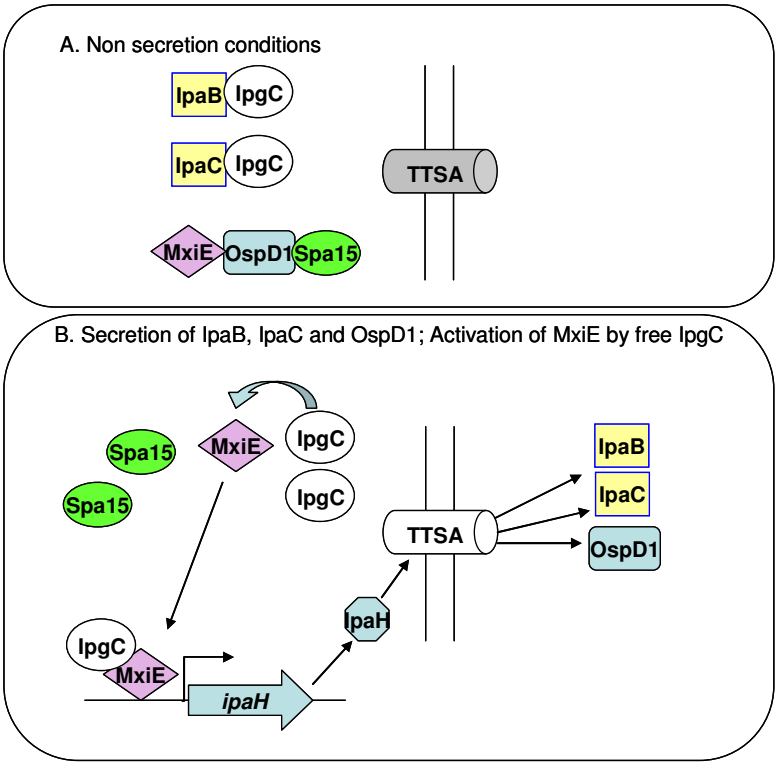


Figure 4. Regulation of MxiE activity by type three secretion activity in *S. flexneri*

During non-secretion conditions, the chaperone IpgC is titrated out by its association with the effector proteins IpaB and IpaC. Effector protein, OspD1 is associated with another chaperone, Spa15, which stabilizes OspD1 and probably maintains it in a secretion competent state. MxiE is associated with the Spa15-OspD1 complex, which both stabilizes MxiE and prevents it from being activated by any free IpgC. Upon secretion activation through the type three secretion apparatus, due to contact of bacteria with host epithelial cells, secretion of IpaB and IpaC liberates IpgC. Subsequent secretion of OspD1 releases MxiE, which can interact with IpgC and activate transcription from the MxiE-regulated promoters. Only one MxiE-regulated gene (*ipaH*), encoding the late effector IpaH is shown here. IpaH is synthesized and secreted through the type three secretion apparatus. Adapted from (Parsot *et al.*, 2005).

2. Chromosomally encoded virulence genes

Many chromosomally encoded factors that are required for virulence or regulation of virulence genes in *Shigella* include those encoding the DNA topoisomerase I, nucleoid associated proteins (H-NS), integration host factor (IHF) and factor for inversion stimulation (Fis). Optimal transcription of the *Shigella* virulence genes occurs at 37ºC, and they are activated by the virulence plasmid encoded VirF and VirB transcriptional activators. Chromosomally encoded heat stable nucleoid-structuring protein H-NS represses transcription from *virF* and *virB* genes by binding the promoter region at non-permissible temperature (30ºC) in a DNA conformation dependent manner, whereas at permissible temperature (37ºC), changes in DNA conformation facilitates the binding of IHF and Fis, which can destabilize the H-NS-repression complex and activate transcription from *virF* and *virB* promoters (Beloin and Dorman, 2003). Mutation in the transcription terminator protein encoding gene, *rho*, results in increased transcription of *virB* and expression of invasion genes. A *rho* mutant was less responsive to changes in DNA superhelicity compared to that in wild-type *S. flexneri* and the DNA conformation prevents transcription repressor H-NS from binding and repressing *virB* expression. Moreover, addition of inhibitor of DNA gyrase (introduces negative DNA supercoiling) to a *rho* mutant strain resulted in diminished expression of *virB* at both 30ºC and 37ºC (Tobe *et al.*, 1994). Other evidence supporting the role of DNA superhelicity in regulation of *virF* and *virB* includes experiments with a *topA* mutant, encoding DNA topoisomerase I (relaxes DNA supercoiling). A *topA* mutant shows reduced expression of virulence genes, and this phenotype can be partially restored by

overexpressing the *parC* and *parE* genes (encoding DNA topoisomerase IV) on a plasmid (McNairn *et al.*, 1995).

tRNA modifying enzymes including MiaA and VacC are also required for efficient production of VirF and IcsA, indicating post-transcriptional regulation of *virF* and *icsA* (Durand *et al.*, 1994; Durand *et al.*, 1997). However, it is not clear how the lack of these tRNA modifications results in altered efficiency of *virF* mRNA translation. Reduced expression of virulence related genes IpaB, IpaC, IpaD, IcsA and VirF was seen in a *miaA* and a *vacC* mutant strain, accounting for reduced contact hemolytic activity and delayed formation of plaques in cultured epithelial cells.

Superoxide dismutase (SodB) is required for resistance to oxidative stress, and mutation in *sodB* results in decreased survival of *S. flexneri* in phagocytic cells (Franzon *et al.*, 1990). *Shigella* also shows sensitivity to osmotic stress in regulating virulence gene expression. Higher osmolarity increases expression of virulence genes (*mxiC*), whereas at lower osmolarity, repression of transcription of *mxiC* and *icsB* was mediated by H-NS (Porter and Dorman, 1994). Mutation in the EnvZ-OmpR two component system, which senses osmolarity, and the loss of expression of outer membrane protein (OmpC), which is regulated by the EnvZ-OmpR regulatory system, results in significant loss of virulence gene expression in *S. flexneri* (Bernardini *et al.*, 1990; Bernardini *et al.*, 1993).

A periplasmic disulfide bond catalyst, DsbA, facilitates the oxidative folding of Spa32, an outer membrane protein constituent of the *Shigella* type three secretion needle structure. In the absence of proper folding of Spa32 and subsequent aberrant needle structure formation in a *dsbA* mutant, secretion of Ipa proteins specifically within epithelial protrusions is inhibited, which in turn prevents cell-to-cell spread of *S. flexneri* (Yu and Kroll, 1999). Chaperone/protease DegP is also required for intercellular spread of *S. flexneri*. DegP utilizes its chaperone activity for polar localization of IcsA, which is required for efficient cell to cell spread of *S. flexneri* in cultured epithelial cells (Purdy *et al.*, 2002). IspA (intracellular septation) is required for efficient intercellular spread and intracellular septation of *S. flexneri* (Hong *et al.*, 1998; Mac Siomoin *et al.*, 1996). Spread of the *ispA* mutants intercellularly occurs in the cultured epithelial cells at the same rate as wild-type, but gradually, the mutant slows down and fails to spread, resulting in filamentous intracellular bacteria that fail to polymerize actin. The mechanism of action of IspA is not yet identified. Proteins encoded by *vacB* (RNaseR) and by *vacJ* are also required for intercellular spread of *S. flexneri* in cultured epithelial cells and show reduced effector protein secretion by an undetermined mechanism (Cheng *et al.*, 1998; Suzuki *et al.*, 1994).

Genes required for lipopolysaccharide biosynthesis (*rfa*, *rfb*, *rol*) and the O-antigen chain-length regulator encoding gene, *cld*, are important for *Shigella* virulence (Hong and Payne, 1997). *rfaL*, *rfaX* and *rfb* loci involved in lipopolysaccharide biosynthesis are required for complement-mediated serum resistance and intercellular spread, but not for invasion. cld_{pHS2} (plasmid encoded O-antigen chain-length regulator gene) is required for resistance to

serum killing and for full inflammation in the Sereny test, but not for invasion or intracellular spread, while *rol* (chromosomally encoded chain-length regulator gene) is required for invasion and plaque formation. The presence of lipopolysaccharide O-antigen side chain is necessary for *S. flexneri* resistance to complement-mediated serum killing. Additionally, inefficient acylation of lipid A endotoxin results in decreased capacity to induce TNF-α production by human monocytes and to cause rupture and inflammatory destruction of the intestinal epithelial barrier. These results indicate that lipid A plays an important role in aggravating inflammation that results in epithelial destruction during Shigellosis (D'Hauteville *et al.*, 2002). Moreover, lipopolysaccharide is also required for polar localization of IcsA (Van den Bosch *et al.*, 1997). The O-antigen masks IcsA, but this effect is greater at the lateral regions than at the old pole of the bacteria (Morona and Van Den Bosch, 2003).

D. Role of *dksA* in *S. flexneri* virulence

The ability of *Shigella* to invade epithelial cells and spread from one cell to another is the key determinant of establishment of the disease. Several genetic and biochemical studies have identified the role of several chromosomal and virulence plasmid encoded genes in invasion, intracellular multiplication or spread during *S. flexneri* pathogenesis. Previously in our lab, a mutant screen to identify genes required for virulence in *S. flexneri* was performed. Random Tn*phoA* mutagenesis was followed by selection of mutants showing a $PhoA^+$ phenotype on phosphate containing plates. These mutants were tested for their ability to invade and form plaques in cultured epithelial cells (Hong and Payne, 1997). This study led to the identification of one chromosomal gene, *dksA*, which contained a Tn*PhoA* insertion and had low alkaline phosphatase activity. A *dksA* mutant invaded and multiplied within the cultured cells with an efficiency similar to wild type, but was defective in plaque formation. This defect was restored by introducing the wild type *dksA* gene on a plasmid. These results suggested that *dksA* is required for intercellular spread of *S. flexneri*, but not for invasion and intracellular multiplication (Mogull *et al.*, 2001). The *dksA* mutant was also shown to efficiently escape the phagocytic vacuole and gain access to the host cell cytoplasm. Furthermore, the *dksA* mutant showed aberrant IcsA localization on the cell surface and elongation in the intracellular environment of cultured epithelial cells.

1. The *rpoS* regulon

In *E. coli* and other enteric bacteria, *rpoS* encodes an RNA polymerase sigma subunit, σ^s/ RpoS also known as σ^{38} (molecular mass is 38 kDa). RpoS synthesis is induced during stationary growth phase and under many stress conditions and can partially replace the vegetative sigma factor σ^{70} encoded by *rpoD* during these conditions (Hengge-Aronis, 2002b). RpoS is the master regulator of the general stress response in *E. coli*. Many gene products regulated by RpoS are required for protection against severe environmental conditions including starvation, hyperosmolarity, low pH, ethanol, suboptimal temperature, oxidative damage and UV irradiation in *E. coli*. *rpoS* mutants in *E. coli* show changes in cell envelope and overall cell morphology (Lange and Hengge-Aronis, 1991). Microarray and *lacZ* fusion studies have shown that the regulatory roles of RpoS are not restricted to stress response genes. RpoS dependent genes encode proteins whose functions include DNA repair, protein synthesis, transport, biosynthesis and metabolism of sugars, amino acids and fatty acids (Rahman *et al.*, 2006; Weber *et al.*, 2005). This multi-functional role is consistent with the observation that RpoS is important under stationary growth phase conditions where cells switch from a metabolism directed toward maximal growth to a maintenance metabolism. Additionally, RpoS is absolutely required for virulence in *Salmonella typhimurium* by inducing expression of virulence plasmid genes (*spv*) which are required for persistence of growth of *S. typhimurium* in human host tissues (Norel *et al.*, 1992). In *S. flexneri*, RpoS is required for both survival in acid conditions (pH 2-3) and in base conditions (pH 10.2) (Small *et al.*, 1994).

2. Regulation of *rpoS* by DksA

Regulation of RpoS is differentially affected by various stress conditions. Increases in cellular RpoS levels can be obtained either by stimulating RpoS synthesis at the levels of *rpoS* transcription or *rpoS* mRNA translation or by inhibiting RpoS proteolysis (Hengge-Aronis, 2002b). In an *E. coli dksA* mutant, a two-fold decrease in transcription from the *rpoS* promoter was observed, and Northern blot analysis revealed the presence of 70-80% less *rpoS* mRNA in the *dksA* mutant compared to that in wild type cells (Webb *et al.*, 1999). However, a 15-fold decrease in *rpoS* translation was observed in a *dksA* mutant, suggesting that DksA has a modest effect on *rpoS* transcription, but significantly increases *rpoS* translation of *rpoS*. Additionally, the region between codons 8 and 73 on the *rpoS* mRNA was important for DksA control of *rpoS* translation (Webb *et al.*, 1999). Another pleiotropic regulatory protein, Hfq is also required for efficient *rpoS* translation, but it targets the upstream untranslated region of *rpoS* mRNA via the small RNA, DsrA, which is different from the effect noted with DksA. These results suggest that DksA and Hfq both increase *rpoS* translation by two different mechanisms (Muffler *et al.*, 1996).

A *dksA* mutant in *S. flexneri* was more sensitive to oxidative stress conditions compared to wild type. This defect could be restored by inducing the expression of *rpoS* in the *dksA* mutant, whereas the decreased sensitivity of the *dksA* mutant to low pH could only partially be restored by *rpoS* induction in the *dksA* mutant. Thus, for resistance to stress conditions, but not for resistance to acid conditions in *S. flexneri*, *dksA* is required upstream

of *rpoS*. Thus, *dksA* mediates resistance to acid conditions in *S. flexneri* by an *rpoS*-independent unidentified pathway.

E. Overview of *dksA*

DksA was originally identified in *E. coli* as a multicopy suppressor of the temperature-sensitive growth and filamentation phenotype of a strain carrying a mutation in *dnaKJ*, which encodes heat shock chaperone protein (Kang and Craig, 1990). Studies with *dksA* mutants and DksA over-expressing constructs have identified pleiotropic effects on the cell. YhhP is a ubiquitous protein required for normal cell growth and formation of the FtsZ ring during cell division in *E. coli*. Multicopy expression of *dksA* suppressed the filamentous phenotype of the *yhhP* mutation in *E. coli*, suggesting that DksA is required for cell division in *E. coli* (Ishii *et al.*, 2000). The increased sensitivity of *dksA* mutant to stress conditions is mainly due to the requirement of DksA for translation of *rpoS*, which is the regulator of stress response genes (Hirsch and Elliott, 2002; Webb *et al.*, 1999). However, DksA also participates in the regulation of genes other than *rpoS*, as demonstrated using a two-dimensional gel electropheresis by the production of eight other proteins in lower amounts in a *S. typhimurium dksA* mutant in (Webb *et al.*, 1999). However, the mode of regulation of these targets by DksA is not yet identified. DksA is also required for post-transcriptional activation of extracellular quorum sensing associated virulence factors, including rhamnolipids and LasB elastase, produced by *Pseudomonas aeruginosa* (Branny *et al.*, 2001; Jude *et al.*, 2003), suggesting a role of *dksA* in quorum sensing.

1. Stringent response

During stationary growth phase conditions, or stress conditions when the cell experiences amino acid and nutrient deprivation, a down-regulation of rRNA biosynthesis and ribosome production and an up-regulation of amino acid biosynthesis are observed. This cell response is known as the stringent response (Magnusson *et al.*, 2005). When the ribosomal A-site is occupied by an uncharged tRNA due to insufficient amounts of amino acids present in the cell, a ribosomal-associated protein, RelA is activated to produce ppGpp, the effector molecule of the stringent control modulon (Figure 5). ppGpp is a small nucleotide guanosine tetraphosphate derived from pppGpp (guanosine 3'-diphosphate 5'-triphosphate) by hydrolysis (Lagosky and Chang, 1980). ppGpp is also synthesized by SpoT, but less is known about the mechanism behind SpoT-dependent production of ppGpp and how SpoT senses starvation conditions. In addition, SpoT is also responsible for hydrolyzing ppGpp (Murray and Bremer, 1996). ppGpp binds in the secondary channel close to the RNA polymerase active site, with each of the ppGpp diphosphates coordinating one Mg^{2+} ion in the active site (Artsimovitch *et al.*, 2004). This binding affects transcription of several target genes (Figure 5) (Magnusson *et al.*, 2005).

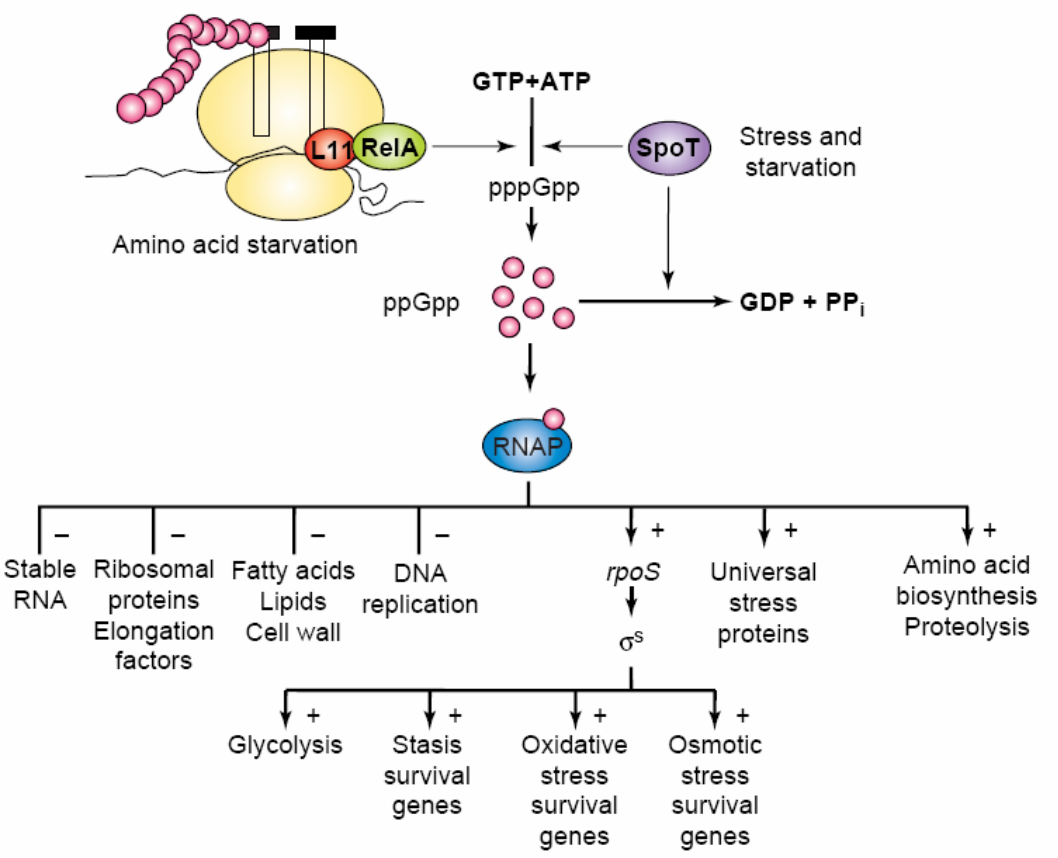


Figure 5. Stringent response regulation by ppGpp

RelA associated with the ribosomes produces ppGpp from GTP and ATP in response to starvation and stress. SpoT dependent production of ppGpp is less understood. SpoT is also responsible for hydrolyzing ppGpp. ppGpp binds RNA polymerase and redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival. Figure taken from (Magnusson *et al.*, 2005).

2. ppGpp-mediated gene regulation

Transcription of genes such as amino acid biosynthesis genes is increased and that of rRNA biosynthesis genes is decreased by RNA polymerase-associated ppGpp to combat the stringent conditions experienced by the cell (Paul *et al.*, 2004; Paul *et al.*, 2005; Perron *et al.*, 2005). Several models have been proposed for negative regulation of transcription by ppGpp binding to RNA polymerase. ppGpp destabilizes the RNA polymerase-promoter open complex resulting in direct negative regulation at the promoters that form intrinsically unstable open complexes, for example, the rRNA promoters. At the λp_R promoter, the RNA polymerase forms a stable complex, but the binding of ppGpp to RNA polymerase decreases the rate of promoter escape of this complex, thus inhibiting transcription at the λp_R promoter (Potrykus *et al.*, 2002). The rate of open complex formation and pausing of the open complex during transcriptional elongation are mediated by ppGpp bound to RNA polymerase, resulting in decreased transcription from rRNA promoters (Wagner, 2002). ppGpp and the nucleoside triphosphate (NTP) substrate compete for access to the active center of RNA polymerase, where binding of NTP substrates results in increase in transcription from rRNA promoter, contrary to the effect seen with ppGpp binding (Jores and Wagner, 2003). Thus, ppGpp can also interact with the cytosines on the non-template strand of the promoter DNA and may be responsible for ppGpp-mediated inhibition of transcription elongation (Artsimovitch *et al.*, 2004).

ppGpp also acts as a positive regulator of expression of different classes of genes upon growth arrest. Many of the positively regulated promoters are dependent on alternative sigma factors, for example *rpoS* (σ^s) regulon genes (Kvint *et al.*, 2000) and σ^{54}-dependent genes (Carmona *et al.*, 2000). In addition some promoters are dependent on the housekeeping sigma factor σ^{70}, for example, promoters controlling genes encoding the universal stress proteins (Gustavsson *et al.*, 2002) and proteins involved in amino acid biosynthesis and uptake (Paul *et al.*, 2005). In general, genes involved in cell proliferation and growth are negatively regulated by ppGpp, whereas those implicated in maintenance and stress defence are positively regulated by the alarmone, ppGpp.

3. Requirement of DksA for ppGpp-mediated regulation

In vitro experiments to determine the effect of ppGpp in highly purified systems on the transcription of target promoters yielded only a small, albeit reproducible, effect. Moreover, activation of amino acid biosynthesis genes by ppGpp has been demonstrated only in crude extracts (Choy, 2000). This suggested the requirement for cellular factor(s) that modulate ppGpp activity in vivo. This modulator was identified as DksA (Paul *et al.*, 2004). In *E. coli*, DksA is required for regulating transcription during stringent growth conditions in response to increasing concentrations of ppGpp. Paul *et al*. (2004) have shown that DksA binds to RNA polymerase, stabilizing binding of ppGpp to RNA polymerase.

DksA is 151 amino acids long and an all α-helical protein containing a globular domain, a central coiled coil domain and a C-terminus α-helix (Figure 6). The 151 amino acid sequence is well conserved, especially within the C-terminal half, which contains a potential coiled coil motif with several invariant residues, and a C4-type Zn finger motif. Binding of zinc to the Zn^{++} finger domain stabilizes the protein and confers diverse functions, including protein-nucleic acid interactions, protein folding and protein-protein interaction (Berg and Shi, 1996). The structure of DksA resembles that of the bacterial transcription factor GreA. However, there is no sequence homology between DksA and GreA. Once bound to RNA polymerase, DksA extends its coiled coil domain into the secondary channel of RNA polymerase towards the ppGpp binding site. Using an invariant acidic side chain (Asp) at the tip of the coiled coil domain, DksA coordinates with one of the ppGpp bound

Mg^{2+} ions, thereby stabilizing the RNA-ppGpp complex (Perederina *et al.*, 2004). The concerted binding of ppGpp and DksA reduces the open complex half-life, thereby inhibiting rRNA promoter activity during stationary growth phase when ppGpp concentration rises. The synergistic binding of DksA and ppGpp has also been demonstrated to significantly increase the rate of transcription initiation from certain amino acid biosynthetic gene promoters by accelerating the rate of an isomerization step involved in formation of the open complex (Paul *et al.*, 2005). Thus, DksA exerts both positive and negative effects on gene regulation by binding RNA polymerase and enhancing the effect of ppGpp. My studies with DksA have shown that it is also required for efficient transcription of *hfq* in *S. flexneri*, and this activation is enhanced by ppGpp.

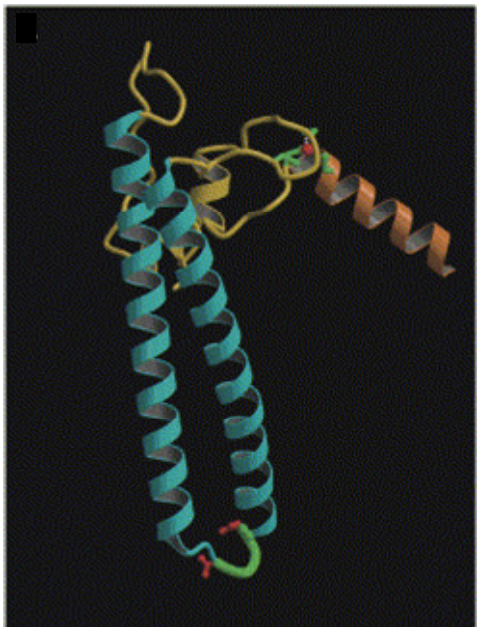


Figure 6. Structure of DksA

The coiled coil domain (blue), globular domain (yellow) and the α turns (green) between the coiled coil domain and the C terminal helix (orange) in the DksA protein are shown. Zn^{2+} ion (red sphere) is coordinated by four cysteine residues (green ball-and-stick models). The red ball-and-stick model shown at the tip of the coiled coil domain represents the conserved Asp residues, which coordinate a ppGpp bound Mg^{2+} ion when the coiled coil region of the DksA protrudes into the RNA polymerase secondary channel. Thus, DksA binding to RNA polymerase stabilizes the ppGpp-RNA polymerase complex (Perederina *et al.*, 2004).

F. Overview of *hfq*

Hfq is an RNA-binding protein originally discovered in *E. coli* as a bacterial host factor (also known as HF-1) required for initiation of plus-strand synthesis by the Qβ RNA bacteriophage replicase (Franze de Fernandez *et al.*, 1968). Binding of Hfq to the 3' end of the phage RNA plus strand results in melting of the RNA, which facilitates its interaction with Qβ replicase and initiation of the synthesis of the minus strand (Schuppli *et al.*, 1997). Further studies in other bacterial systems have identified Hfq as a pleiotropic, post-transcriptional regulator that acts as an RNA chaperone, controlling stability of several mRNAs and small regulatory RNAs. Hfq affects a variety of cellular functions including decreased growth rate, increased cell length, reduced biofilm formation, decreased nitrogen fixation, reduced glycogen synthesis, decreased RpoS synthesis, increased sensitivity to survival in several stress conditions and decreased virulence (for a review, see Niyogi *et al.*, 2004; Valentin-Hansen *et al.*, 2004). *hfq* mutants show reduced virulence in several bacterial pathogens, including *Listeria monocytogenes* (Christiansen *et al.*, 2004), *Vibrio cholerae* (Ding *et al.*, 2004), *P. aeruginosa* (Sonnleitner *et al.*, 2003), *S. typhimurium* (Brown and Elliott, 1996) and *Brucella abortus* (Robertson and Roop, 1999).

The crystal structure of Hfq has been determined for both the *E. coli* and *Staphylococcus aureus* proteins (Sauter *et al.*, 2003; Schumacher *et al.*, 2002). The full length *S. aureus* of 77 amino acids forms a hexameric ring with a central pore around which RNA binds in a circular unwound manner. The Hfq RNA binding motif is an A/U rich

region, with no single consensus motif, preceded or followed by a stem-loop structure (Moll *et al.*, 2003).

1. The *hfq* operon

The *hfq* gene is part of the *amiB-mutL-miaA-hfq-hflX-hflK-hflC* operon (Tsui *et al.*, 1996) (Figure 7). *amiB* encodes N-acetylmuramoyl-L-alanine amidase II, which is involved in cell wall hydrolysis (Tsui *et al.*, 1994b). *mutL* encodes a highly conserved protein involved in at least three major DNA repair pathways (Spampinato and Modrich, 2000), whereas *miaA* encodes the tRNA modification enzyme tRNA dimethylallyl diphosphate transferase (Caillet and Droogmans, 1988). The *hflA* locus (high frequency of lysogenization) consists of *hflX*, *hflK* and *hflC* genes. *hflX* encodes a putative GTP-binding protein, and *hflK* and *hflC* encode a membrane localized protease that mediates bacteriophage lambda lysogeny and expression of some *E. coli* proteins (Noble *et al.*, 1993). All seven genes are cotranscribed, and transcription initiation primarily occurs from four promoters P_{mutL}, P_{miaA}, $P2_{hfq}$ and $P3_{hfq}$ (σ^{70} specific promoters) during exponential growth in L broth under non-stress conditions and from P_{mutL}HS and P_{miaA}HS and $P1_{hfq}$HS (σ^{32} specific promoters) during heat shock conditions (Tsui *et al.*, 1996). *hfq* is cotranscribed with an upstream gene, *miaA*, giving rise to a *miaA-hfq* transcript under the control of another σ^{70}-dependent promoter, P_{miaA}. However, this transcript is also seen to be processed further by endonucleolytic cleavage at the transcription processing site, PT1, within *miaA* and is not abundant. In *E. coli*, a plasmid expressing *hfq* only from the $P2_{hfq}$ and $P3_{hfq}$ promoters complemented an *hfq* mutant for *mutS*

transcription (Tsui *et al.*, 1997). These data suggested that in *E. coli*, *hfq* transcription occurs from $P1_{hfq}$HS during heat shock conditions, but the gene can be transcribed from $P2_{hfq}$ and $P3_{hfq}$ promoters under other conditions. Moreover, the transcription of *hflA* region is also dependent on the $P2_{hfq}$ and $P3_{hfq}$ promoters during exponential growth phase and from the $P1_{hfq}$HS during heat shock conditions.

E. coli Hfq regulates its own expression by destabilization of its transcripts (Tsui *et al.*, 1997). Hfq autoregulation occurs by RNaseE-dependent degradation of *hfq* mRNA (Vecerek *et al.*, 2005). Hfq binds at two different sites on the 5' untranslated region of the *hfq* mRNA, and this binding leads to inhibition of translation initiation complex formation and exposure of the RNaseE binding sites on the *hfq* mRNA. However, autoregulation of *hfq* occurs at a modest level; binding of Hfq to the 5'-untranslated region of *hfq* mRNA leads to approximately a 2-fold repression of translation (Vecerek *et al.*, 2005). Since Hfq levels have been reported to increase during stationary growth phase and in bacteria growing at slower growth rates (Tsui *et al.*, 1997; Vytvytska *et al.*, 1998), it is possible that other unidentified factors, required for stimulating expression of *hfq* also exist. Here I show that *hfq* expression is positively regulated by DksA and this regulation is important for virulence in *S. flexneri*.

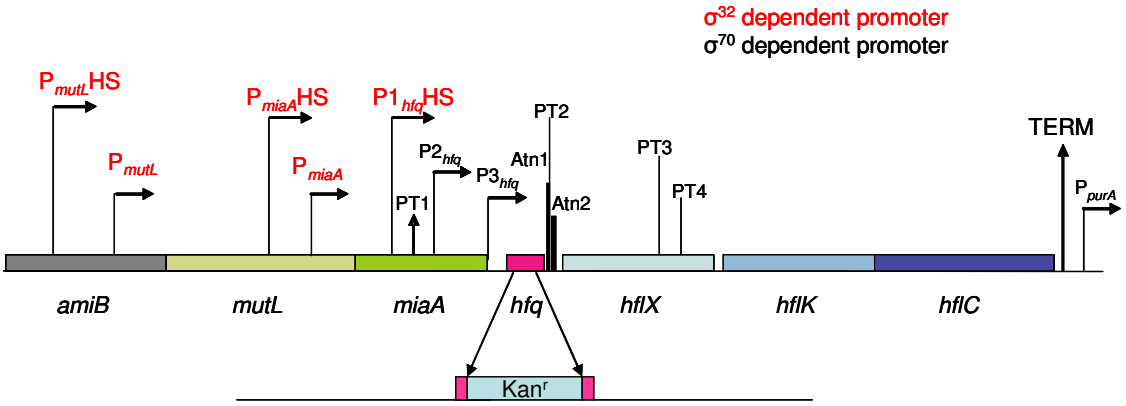


Figure 7. *hfq* operon

The positions of the following sites are indicated (not to scale): σ^{32} promoters P_{mutL}HS, P_{miaA}HS and $P1_{hfq}$HS; σ^{70} promoters P_{mutL}, P_{miaA}, $P2_{hfq}$ and $P3_{hfq}$; attenuators Atn1 and Atn2; PT1, PT2, PT3 and PT4 transcript processing sites; Rho independent terminator (TERM) (modified from (Tsui *et al.*, 1997). The gene segment deleted and replaced with a non-polar kanamycin resistance gene to make the *hfq* mutant in this study is shown below the map.

2. Hfq-mediated gene regulation and involvement of sRNAs

Early studies with *hfq* mutants showed pronounced pleiotropic phenotypes including decreased growth rate, increased cell length, sensitivity to several stress conditions, lower negative supercoiling of plasmids, more rapid carbohydrate oxidation and reduced virulence (for a review, see Valentin-Hansen *et al.*, 2004). Some of the phenotypes of an *hfq* mutant are also seen in an *rpoS* mutant. *rpoS* encodes the stationary phase sigma factor RpoS/σ^{38}/σ^{s}, a global regulator of various stress situations in bacteria (Hengge-Aronis, 2002a). Further studies done in *E. coli* and *S. typhimurium* showed that Hfq is essential for efficient translation of the *rpoS* mRNA (Brown and Elliott, 1996; Muffler *et al.*, 1996). However, not all the phenotypes of an *hfq* mutant can be attributed to its effects on *rpoS* expression, indicating the presence of additional targets of Hfq besides RpoS (Muffler *et al.*, 1997).

Hfq levels increase in cells that are grown at slower growth rates (Vytvytska *et al.*, 1998). However, the effect of growth phase on Hfq levels is unclear. In *E. coli*, Hfq levels have been reported both to decrease in stationary phase to about one-third the levels in logarithmic growth phase (Ali Azam *et al.*, 1999; Kajitani *et al.*, 1994) and to increase about two fold during stationary phase (Sonnleitner *et al.*, 2006; Tsui *et al.*, 1997). Consistent with the latter observation, in *P. aeruginosa*, Hfq protein levels increased about two fold during stationary phase as compared to those during exponential growth phase (Sonnleitner *et al.*, 2006). Increase in the Hfq levels during stationary phase is consistent with increased

expression of a significant number of non-coding small regulatory RNAs (sRNA), which require Hfq for their function during stationary phase in *E. coli* (Argaman *et al.*, 2001).

In *E. coli*, more than 70 sRNAs have been identified and about 35 sRNAs have been demonstrated to bind Hfq (Zhang *et al.*, 2003). Hfq is a key player in mRNA translational control by sRNAs where binding of Hfq to the sRNA increases stability of the sRNA and facilitates the binding of the sRNA to its target mRNA, resulting in either repression or activation of translation from the target mRNA or mRNA degradation (Figure 8) (for a review see Valentin-Hansen *et al.*, 2004). Four Hfq binding sRNAs, DsrA, OxyS, Spot42 and RyhB, have been studied in some detail. DsrA and another small RNA, RprA, are complementary to the upstream stem of the hairpin and both activate translation or *rpoS* by pairing (Majdalani *et al.*, 2002; Sledjeski *et al.*, 2001). Binding of Hfq protects the small RNA from cleavage by RNase E, the major endonuclease involved in mRNA decay as well as in processing of tRNA and rRNA precursors.

The OxyS sRNA is induced in response to oxidative stress conditions in *E. coli* and down regulates the translation of *rpoS* by an uncharacterized mechanism (Zhang *et al.*, 1998). Translation of *fhlA*, encoding a transcriptional activator, is also repressed by OxyS-mediated base-pairing interactions (Figure 8). FhlA is required for activating formate hydrogenlyase, which is required for metabolizing formate during anaerobic growth conditions (Maupin and Shanmugam, 1990). OxyS binds at two sites on *fhlA* mRNA, one site overlaps the ribosome binding site, and the other one resides within the coding sequence of

fhlA mRNA. OxyS-*fhlA* mRNA base-pairing prevents ribosome binding resulting in translation repression (Altuvia *et al.*, 1998). Spot42 sRNA regulates the *galK* gene, which is the third gene in the galactose operon. Spot42 binds to the translation initiation region of *galK* mRNA and represses translation initiation by inhibiting ribosome binding (Moller *et al.*, 2002). Similarly, Hfq also mediates down-regulation of iron proteins including SodB (iron superoxide dismutase) during iron deprived conditions in the cell. Binding of Hfq to the *sodB* mRNA alters the mRNA structure such that sequences complementary to RyhB and encompassing the translation initiation codon are exposed from an otherwise unexposed region of the mRNA. RyhB binding blocked the translation initiation codon of *sodB* and triggered the degradation of both RyhB and *sodB* mRNA in an RNase E dependent manner (Geissmann and Touati, 2004) (Figure 8). Hfq also negatively regulates the synthesis of iron transporters FepA and FhuE when iron is in excess, to prevent hydroxyl radical production during aerobic growth conditions (Wachi *et al.*, 1999). However, the mechanism of regulation of FepA and FhuE by Hfq is not yet identified. Thus Hfq plays a role in iron uptake and in switching off genes encoding iron scavenger proteins.

Hfq also targets some mRNA for degradation by binding to their poly(A) tails and stimulating polyadenylation (Mohanty *et al.*, 2004). Hfq can also directly repress translation of *ompA* mRNA during stationary growth phase. Hfq was shown to directly bind the *ompA* mRNA and modify the mRNA structure, resulting in inhibition of ribosome binding and exposure of the otherwise sequestered RNaseE sites for degradation of the mRNA by RNase (Vytvytska *et al.*, 2000). However, recent data also show involvement of a sRNA, SraD in

mediating decay of *ompA* mRNA, in an Hfq dependent manner in *E. coli* (Rasmussen *et al.*, 2005). SraD (MicA) interacts with the ribosome binding site on *ompA* mRNA, which exposes the RNase E binding site for degradation.

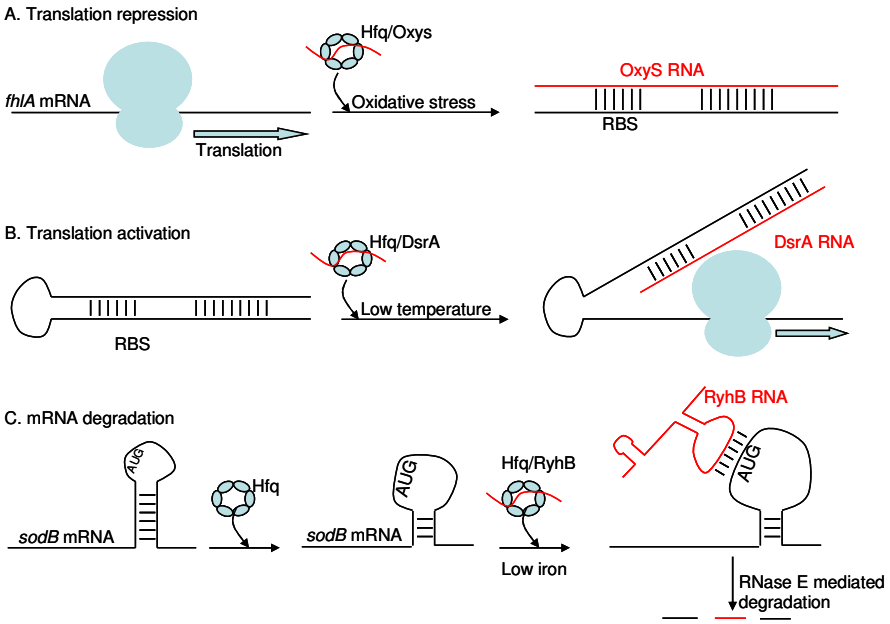


Figure 8. Hfq and small RNA-mediated post-transcriptional regulatory effects

A. OxyS sRNA expression is induced during oxidative stress conditions in *E. coli*. OxyS RNA base pairs with a short sequence overlapping the ribosome binding site (RBS) on *fhlA* mRNA, thereby blocking ribosome binding and repressing translation initiation (Altuvia *et al.*, 1998).

B. DsrA sRNA, stabilized by Hfq binding, activates translation of *rpoS* mRNA at low temperature by exposing the RBS on the *rpoS* mRNA and unfolding the hairpin structure on the 5' untranslated region of *rpoS* mRNA that traps the RBS (Lease and Woodson, 2004).

C. Binding of Hfq to *sodB* mRNA opens the loop containing the translation start codon and sequence complementary to RyhB sRNA. During iron deficiency, RyhB sRNA is stabilized by Hfq binding and can base pair with the region present on the *sodB* mRNA loop containing the translation start codon. The block of translation and structural change render the RNA molecules susceptible to RNase E cleavage (Geissmann and Touati, 2004). Figure adapted from (Storz *et al.*, 2004).

II. MATERIALS AND METHODS

A. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Table 1 and 2, respectively. Plasmid DNA was isolated using either QIAprep Spin Miniprep Kits (Qiagen) or GenElute plasmid miniprep kit (Sigma) following the manufacturer's instructions. Plasmids were eluted in double distilled water and stored at -20℃.

B. Media, reagents and growth conditions

All strains were stored at -80℃ in tryptic soy broth plus 20% glycerol. *E. coli* strains were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or on LB agar at 37℃. *S. flexneri* was cultured either in LB broth or in EZ Rich Defined Medium (EZ RDM) (http://www.genome.wisc.edu/functional/protocols.htm), a modification of the supplemented MOPS Defined Medium described by Neidhardt *et al*. (Neidhardt *et al.*, 1974). Glucose (0.2% final concentration) was added to the EZ RDM as the carbon source. *Shigella* was incubated routinely at 37℃. Incubation at 30℃ was performed whenever the expression of virulence genes was not desired. *S. flexneri* strains were grown on Tryptic Soy broth (TSB) agar plates (Becton, Dickinson and Company, Sparks, MD) containing 0.01% Congo red (w/v) at 37℃ to screen colonies that bind Congo red. Antibiotics were used at the

following concentrations; carbenicillin, 250 μg/ml; kanamycin, 50μg/ml; streptomycin 300 μg/ml.

C. Restriction digestion and ligation

DNA molecular weight markers such as λDNA-BstEII, λDNA-HindIII and ΦX174 DNA-HaeIII and restriction enzymes were purchased from New England Biolabs (Beverly, MA). Restriction digestions and DNA ligations were carried out as described by Sambrook and Russell, 2001.

Table 1. Bacterial strains used in this study

Strains	Relevant characteristics	Reference or source
DH5α (λpir)	*E. coli* cloning strain; host strain for pGP704 derivatives	J. Kaper
SM10 (λpir)	λpir *recA* Kan^r; host and mobilizing strain for pGP704 derivatives	(Miller and Mekalanos, 1988)
SA100	*S. flexneri* wild-type serotype 2a	(Payne *et al.*, 1983)
SM100	SA100 Str^r	S. Seliger
SA200	SA100 carrying pAKS200	This study
SA5287	SA100 *dksA*::Tn*phoA*	(Mogull *et al.*, 2001)
AKS5	SM100 *hfq*::AphA3	This study
Δ*hfq*	SA100 *hfq*::AphA3	This study

Table 2. Plasmids used in this study

Plasmids	Relevant characteristics	Reference or source
pQE2	N-terminal His-tag protein expression, IPTG inducible expression vector, Cb^r	Qiagen
PQF50	Promoterless *lacZ* reporter plasmid, Cb^r	(Farinha and Kropinski, 1990)
pWKS30	Low-copy-number cloning vector; Cb^r	(Wang and Kushner, 1991)
pHM5	Suicide vector pGP704 carrying *sacB*; Cb^r Suc^s	(Friedman *et al.*, 1982)
pSAM1	SA100 *dksA* in pWKS30	(Mogull *et al.*, 2001)
pAKS200	SA100 *hfq* in pQE2	This study
pAKS10-2	*hisG*::*lacZ* transcriptional fusion on pQF50; Cb^r	This study
pAKS700	SA100 *hfq*, including the $P1_{hfq}$, $P2_{hfq}$ and $P3_{hfq}$ promoters, in pWKS30	This study
pAKS701	SA100 *hfq* operon in pWKS30	This study
pAKS101	WT DksA tagged N-terminally with 6 histidine residues in pQE2; Cb^r	This study
pQF-$P2_{hfq}$	Transcriptional fusion of $P2_{hfq}$ promoter to *lacZ* in pQF50	This study
pQF-$P3_{hfq}$	Transcriptional fusion of $P3_{hfq}$ promoter to *lacZ* in pQF50	This study

Plasmids	Relevant characteristics	Reference or source
pQF-*virF*	Transcriptional fusion of *virF* promoter to *lacZ* in pQF50	This study
pQF-*virB*	Transcriptional fusion of *virB* promoter to *lacZ* in pQF50	This study
pWKS*virF*	*virF* under the control of IPTG inducible promoter in pWKS30, also carrying *lacI* from pQE2	This study
pWKS*virB*	*virB* under the control of IPTG-inducible promoter in pWKS30, also carrying *lacI* from pQE2	This study
pWKS*mxiE*	*mxiE* under the control of IPTG-inducible promoter in pWKS30, also carrying *lacI* from pQE2	This study

D. Transformation of bacterial strains

1. Transformation of $CaCl_2$-competent *E. coli*

Plasmid DNA or ligation mixtures were introduced into $CaCl_2$-competent DH5α, DH5α*λpir*, SM10*λpir* or RM1602 cells by heat-shock transformation. To prepare $CaCl_2$-competent *E. coli* cells, an overnight culture started from a single colony was diluted 1:100 in fresh L broth and grown to mid-logarithmic growth phase (OD650 of 0.4-0.6). The culture was then chilled on ice for 30 to 60 minutes. The cells were pelleted by centrifugation at 6000 x g for 10 minutes at 4ºC and resuspended in 10 ml ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol and 10 mM PIPES, pH7). The cells were washed with $CaCl_2$ solution one more time and resuspended in 2 ml $CaCl_2$ solution and divided into 150 µl aliquots for storage at -80 ºC. Plasmid DNA (100-200 ng) was transformed into these competent cells by first incubating on ice for 45 minutes followed by heat shock at 42 ºC for 1 minute 45 seconds, chilling on ice for 2 minutes and incubating in 1.5 ml L broth at 37 ºC for 1 hour. Transformed bacteria were selected by plating on L agar supplemented with the appropriate antibiotics.

2. Electroporation of *Shigella*

Plasmid DNA was transformed in *S. flexneri* strains by electroporation. *Shigella* strains were made electrocompetent as follows. Overnight cultures from a single colony were

diluted 1:100 in L broth and grown to mid-logarithmic growth phase at 37 ºC in the presence of antibiotics where appropriate. Cultures were chilled on ice for one hour, and the cells were washed twice with the same volume, and then again with half the volume of the starting culture using ice cold sterile dH_2O. Cells were resuspended in ice cold 10% glycerol. Aliquots (100µl) of electrocompetent cells were transformed as described by Sambrook and Russell (Sambrook, 2001a).

3. Conjugation

Plasmids were transferred by conjugation by washing One ml of overnight cultures of the donor strain, recipient strain and the mobilizing strain was washed with L broth and was resuspended in 100 µl L broth. Twenty µl of each strain were mixed, and the mixture was spotted onto an L agar plate and incubated at 37ºC for 6-8 hours. The cells were swabbed off the plate and resuspended in 1 ml of L broth. Serial dilutions were plated on medium selective for the recipient strain and for the transferred plasmid.

E. Oligonucleotides

All the primers used in this study are listed in Table 3. DNA oligonucleotides for polymerase chain reactions were designed using Clone Manager Professional Software Suite (Sci Ed Central) and purchased from either Integrated DNA Technologies, (Corallville, IA) or Sigma Genosys, (The Woodlands, TX). The primers were received in lyophilized form

and were resuspended in double distilled water to a final concentration of 100μM and stored at -20℃.

F. Polymerase chain reactions

PCRs were performed using *Taq* DNA polymerase (QIAGEN), *Pfu* polymerase (Stratagene) or Expand High Fidelity enzyme mix (Roche Applied Science) containing *Taq* DNA polymerase and *Tgo* DNA polymerase, according to the manufacturer's instructions. Bacterial genomic DNA preparations or bacterial cultures grown overnight in L broth or on L agar or TSB agar plate were used as the template. Unless otherwise specified, 50μl PCRs contained template DNA, 200μM of each dNTP, 2μM of each primer, Taq reaction buffer and 1-5 units of enzyme. Conditions for PCR amplification using Taq DNA polymerase were 30 sec at 95℃, 30 sec at the appropriate annealing temperature for the primer pair, elongation at 72℃ for the appropriate amount of time (one minute per 1000 base pairs) followed by a final elongation step at 72℃ for 12 min. When using the Expand High Fidelity amplification, the elongation time after the first 10 cycles was extended 5 seconds for every subsequent cycles. All clones derived from PCR fragments were verified by sequencing.

Table 3. Oligonucleotides used in this study

Primer name	Primer sequence (5′ - 3′)
Hfqa	GGGTGGTCTGTTACAATCTGTTG
Hfqb	TGTTGGATTCGCGTTCAGGAACGGATCTTG (*Eco*RI)
Hfqc	ACGCGATTCCAACAGGACAGCGAAGAAAC (*Eco*RI)
Hfqd	CGGTTTGTTCTCTTCGTCCC
hfqFor	GTAGAATTCAGCATATAAGGAAAAGAGAG (*Eco*RI)
hfqRev	ACGAAGCTTCGCTGGCTCCCC (*Hind*III)
HisG3	CTCCCCATGGCGCTCATTCATTAAACAAATCC (*Nco*I)
HisG4	CATGGATCCCTCTGTGAATCTTTATTCAACTG (*Bam*HI)
dksA-His1	GGGGTACCAACATGCAAGAAGGGCAAAACCG (*Kpn*I)
dksA-His2	TTCAAGCTTGTGGTAAACGTGATGGAACGGC (*Hind*III)
hfqRT1	AAGCTGCAAGGGCAAATCG
hfqRT2	TGGCTGACCGTGTTTTTCAA
hfqRT probe	6FAMTTTGATCAGTTCGTGATCC
virFfor	TCGATAGCTTCTTCTCTTAGTTTTTCTG
virFrev	GAAAGACGCCATCTCTTCTCGAT
virF Probe	6FAMTCAGATAAGGAAGATTGTTGAAA
WTvirBfor	CCAGAAAACTAGCAAACGATATACAAA
WTvirBrev	CAGAAAATTAAGACCAATACCAAGTTCTC
WTvirBprobe	6FAMAGCAAAAGAGCATAGCAT
Hfq10	CGCGTGACGAAGTATTACAG

Primer name	**Primer sequence (5′ - 3′)**
NcoI Hfq13	GTAGCCATGGTTGCGGCAGCAAGGATCC (*Nco*I)
BamHI Hfq14	CGAGGATCCGCACCTGATACGGTAGAG (*Bam*HI)
NcoI VirF3	AGAGCCATGGGAAGCTGCATAAGCTCTTTC (*Nco*I)
BamHI VirF4	TTCAGGATCCGAGCTAACCGTCATTGAACACC (*Bam*HI)
VirBNcoI	ATGGCCATGGCACATCAGAGCTCCACAAG (*Nco*I)
VirBBamHI	ATTGGGATCCGTTAAATGCAGTGTGAGCAAG (*Bam*HI)
virFSacII	CCCCCGCGGCAATAGCGATGGGCTATG (*Sac*II)
virFNdeI	TGTTGAACATATGATGGATATGGGACATAAA (*Nde*I)
virBBseRI	TGAAGAGGAGGGGTGTGATATGGTGGATTTG (*Bse*RI)
virBHindIII	ATCAAGCTTGGGCAGTTTACATCAGTGTTCG (*Hind*III)
mxiESacII	ATCCCGCGGAGCAACATATTTCGCATTGTC (*Sac*II)
mxiEBseRI	AATGAGGAGGGCTCCAGTATGGAAGGGTT (*Bse*RI)

Relevant recognition sites for restriction enzymes (listed in parentheses) are underlined.

G. DNA Sequencing

DNA sequencing was performed by the University of Texas at Austin Institute for Cellular and Molecular Biology DNA Core Facility using the automated dye termination procedure and analyzed on an ABI 377A DNA sequencer. Analysis of DNA sequences was carried out using Clone Manager Professional Suite.

H. Construction of recombinant plasmids and strains

1. Construction of chromosomal mutants in *S. flexneri*

hfq gene deletion and replacement with a non polar kanamycin resistance gene was performed using allelic exchange as described by Sambrook and Russell (Sambrook, 2001b). An overlap extension PCR with primer pairs hfqa – hfqb and hfqc – hfqd to amplify overlapping fragments was followed by amplification of the overlap extension product with primer pair hfqa – hfqd to create the final *hfq*Δ allele containing a unique EcoRI site. The PCR product was subjected to restriction digestion using *Sac*II and *Cla*I enzymes and ligated to *Sac*II and *Cla*I digested vector pWKS30 to give pAKS20. The AphA3 kanamycin resistance gene was removed from pUCK18K by digestion with *Bsr*BI and *Hinc*II. The ends were made blunt by treatment with Klenow exo$^-$ polymerase and were ligated to *Eco*RI digested and Klenow exo$^-$ polymerase treated pAKS20 to produce pAKS21. pAKS21 was digested with *Sac*II and *Xho*I to release the insert carrying *hfq* deleted with a non polar

kanamycin resistance gene. This fragment was subjected to Klenow exo$^-$ polymerase to produce blunt ends and was ligated into *Eco*RV digested pHM5 suicide vector to produce pAKS22. pAKS22 was transformed by heat shock into DH5αλpir cells and subsequently transferred to SM100 using MM294/pRK2013 as the helper strain on L agar plate for 8 hours at 37℃. Primary integrants were screened on TSB agar plates containing carbenicillin, streptomycin and kanamycin. To select for secondary cross-overs carrying the *hfq* deletion, the primary integrants were grown in L broth in the presence of 1% sucrose at 30℃ for 4 hours and plated on L agar plates carrying streptomycin, kanamycin and 10% sucrose. Resulting colonies were patched on plates with or without carbenicillin, and the carbenicillin sensitive, kanamycin resistant colonies were checked by PCR and sequencing to confirm the substitution of kanamycin resistance gene for the *hfq* gene and loss of the integrated plasmid.

The *hfq* deletion in SM100 was reconstructed in SA100 by P1 transduction as previously described (Sambrook, 2001b). P1 lysates were prepared following growth in the SM100 *hfq*::AphA3 mutant. The SM100-*hfq*::AphA3 P1 lysate was used to transduce *hfq*::AphA3 into SA100. Selection of *hfq*::AphA3 was performed by plating the recipient cells on TSB agar plates with Congo red and kanamycin. Insertional inactivation of the *hfq* gene in SA100 and SA200 was confirmed by PCR using a primer internal to the AphA3 kanamycin resistance gene and another primer in the region flanking the *hfq* gene.

2. Construction of plasmids

The predicted promoter region for *E. coli hisG* was amplified by PCR using primers HisG3 and HisG4 (Table 2). The PCR fragment was digested with *Nco*I and *Bam*HI and cloned into pQF50 digested with *Nco*I and *Bam*HI to yield pAKS10-2. Similarly, the predicted promoter regions for the *S. flexneri* $P2_{hfq}$ and $P3_{hfq}$ promoters were amplified by PCR using primers *hfq* 13 and *hfq* 14 for P2*hfq* and *hfq* 10 and *hfq* 1 for P3*hfq* promoter. The resulting PCR products were digested with *Nco*I and *Bam*HI and ligated to pQF50 digested with *Nco*I and *Bam*HI to yield $pQFP2_{hfq}$ and $PQFP3_{hfq}$, respectively. Additionally, *virF* and *virB* promoters were cloned into *Nco*I and *Bam*HI double digested pQF50 after restriction digestion of the two PCR products obtained from primers, VirF-*Nco*I and VirF-*Bam*HI (for *virF* promoter) and primers, VirB-*Nco*I and VirB-*Bam*HI (for *virB* promoter) with *Nco*I and *Bam*HI.

3. Construction of *S. flexneri hfq*, *virF*, *virB* and *mxiE* expression plasmids

The full-length *S. flexneri hfq* gene excluding the promoter region was amplified by PCR using primers hfqFor and hfqRev. The PCR product was digested with *Eco*RI and *Hind*III and cloned into pQE2 digested with *Eco*RI and *Hind*III to produce pAKS200 carrying the *hfq* gene with an IPTG inducible promoter.

The full length *virF*, *virB* and *mxiE* genes, excluding the promoter region, were amplified by PCR using primer pairs, VirFSacII-VirFNdeI, VirBHindIII-VirBBseRI and MxiESacII-MxiEBseRI, respectively. The PCR products were digested with the indicated restriction enzymes and cloned into pQE2 digested with the same restriction enzymes to yield pQE*virF*, pQE*virB* and pQE*mxiE* carrying *virF*, *virB* and *mxiE*, respectively, expressed from an IPTG inducible promoter. pQE*virF*, pQE*virB* and pQE*mxiE* constructs were digested using restriction enzymes, *Xho*I and *FspI*, to release the *lacI* gene. The full length *virF*, *virB* and *mxiE* genes were cloned down stream of the IPTG inducible promoter, which was cloned into *Xho*I and *Eco*RV digested pWKS30 to produce pWKS*virF*, pWKS*virB* and pWKS*mxiE*, respectively.

I. β galactosidase Assay

Strains carrying transcriptional fusions with the promoter-less *lacZ* in the pQF50 vector were grown overnight in L broth at 37 ℃ followed by sub-culturing 1:100 in L broth and growing them to the indicated growth phase. β galactosidase assays were performed using the chloroform lysis method as previously described (Sambrook, 1989).

J. Acid resistance assays

Acid resistance assays were performed as described by (Lin *et al.*, 1995) with modifications. Strains were grown overnight at 30℃ with shaking in L broth, pH 7, plus carbenicillin as indicated and then diluted in L broth at pH 2.5. These acid shock cultures were incubated for 2 hours at 37℃ with shaking, and percent survival was determined by plating serial dilutions of the acid shock cultures at the start and after 2 hours of incubation in L broth at pH 2.5.

K. Oxidative stress assay

Resistance to oxidative stress conditions was measured by growing bacterial strains in the presence of 3 mM H_2O_2. Overnight cultures grown in L broth at 37℃ were diluted 1:50 in L broth containing 3 mM H_2O_2 and the cultures were incubated for 2 hours at 37℃ with shaking. Percent survival was determined by plating serial dilutions of the oxidative stressed cultures at the start and after 2 hours of incubation in the presence of 3 mM H_2O_2. Cultures were also allowed to incubate overnight in the presence of 3 mM H_2O_2, and the OD_{650} was measured for end-point growth analysis.

L. Microarray analysis

1. Construction of microarrays

Hybrid *E. coli*/*Shigella* microarrays were constructed using 70-mer oligonucleotides from *E. coli* and enterohemorrhagic *E. coli* sequenced genomes purchased from Qiagen (Santa Clarita, CA). *Shigella*-specific genes, including oligonucleotides specific for genes located on the *S. flexneri* virulence plasmid, several *S. dysenteriae* specific genes and putative sRNA sequences previously identified in *E. coli* (Hershberg et al., 2003) were custom designed and synthesized by Qiagen. Glass microscope slides were poly-lysine coated (www.microarray.org) prior to spotting of each oligonucleotide using a robotic arrayer and Array Maker 2.4 (MGuide at smgm.stanford.edu/pbrown/mguide). Hydration and post-processing were performed as published by MGuide (Hershberg *et al.*, 2003)

2. Isolation of RNA and cDNA synthesis

The wild-type *S. flexneri*, SA100, and the *dksA* mutant, SA5287, were grown in L broth at 37ºC to mid-logarithmic phase (an optical density at 650 nm of 0.6) for comparison, whereas the *hfq* mutant, SA100-*hfq*::AphA3 (Δ*hfq*), and the wild type were cultured to late-logarithmic growth phase (an optical density at 650 nm of 0.9). Total RNA was isolated using the RNeasy® Midi kit (Qiagen, Valencia, CA). cDNA was generated by reverse transcription of the isolated RNA as follows. Fifteen µg RNA of total RNA per sample was

used to synthesize cDNA. pd(N)6 randomized hexamer primers (5 µg) (Amersham Biosciences, Piscataway, NJ) were hybridized to 15 µg RNA for 10min at 65°C. Primed RNA was mixed with deoxy-nucleotides [0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.2 mM dTTP and 0.3 mM amino allyl modified dUTP (Sigma-Aldrich, St. Louis, MO), 0.01 M dithiothreitol (DTT), 120 units of RNasIn (Promega, Madison, WI), 800 units SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 1X SuperScript RT buffer and incubated for 2 hours at 42°C. An additional 400 units of SuperScript II was added to the reaction mix after 1 hour of incubation at 42°C. Template RNA in each reaction was hydrolyzed by incubation with 50 mM NaOH for 10 min at 65°C and the reaction was neutralized using 50 M HCl. Samples were washed and concentrated to 9 µl on a Microcon-30 column (Millipore, Billerica, MA).

3. Labeling and hybridization

Cy3 and Cy5 dyes (Amersham Biosciences) were used to couple cDNA samples incorporated with amino allyl-dUTP. Concentrated cDNA samples were buffered using 1 µl of 10 M sodium bicarbonate (pH 10) and combined with either Cy3 or Cy5 reactive dye. Cy3 fluorescent dye was coupled to the reference sample, and Cy5 was coupled to the test sample. The coupling reaction was incubated for 1 hour in the dark. Following purification of the two labeled cDNA samples using Qiagen QiaQuick columns (QIAGEN), the Cy3 and Cy5 labeled cDNA samples were mixed and combined with 3X SSC and 0.25% SDS and applied

to the array surface for hybridization at 65℃ for 3 hours. After hybridization, the arrays were washed and dried as described (http://chipmunk.icmb.utexas.edu/ilcrc/protocols/index.html).

4. Analysis of microarray data

Hybridized microarrays were scanned using a GenePix 4000b scanner (Axon Instruments). The fluorescence intensities were determined using the GenePix Pro 5.1 software. The Longhorn Array Database (powered by the Stanford Microarray Database) (http://chipmunk.icmb.utexas.edu/ilat/) was used to normalize the microarray data and perform data filtering and analysis (Killion *et al.*, 2003). Only gene features ("spots") that passed certain quality control filters, including minimum intensity and pixel consistency and a regression correlation higher than 0.6, were included for further analysis. Hierarchial clustering and expression map generation of data were performed using the Cluster and TreeView programs (Eisen et al., 1998). Differential expression was considered significant if the $|\log_2 R|$ was ≥ 1, where R is the normalized red/green ratio. Fold change in gene expression was calculated by using the formula, $2^{|\log 2R|}$.

M. Real-time polymerase chain reaction

RNA was isolated using the RNeasy® Midi or Mini kits (QIAGEN, Valencia, CA), as per the manufacturer's directions, from the same number of bacteria cultured to the indicated growth phase at 37℃ in L broth or on TSB agar plates supplemented with Congo red and the indicated antibiotic. Each RNA sample was then treated with 16 units of amplification grade DNase I (Invitrogen, Carlsbad, CA), ethanol precipitated and dried. The RNA pellet was resuspended in DEPC-treated water, and the nucleic acid was quantitated using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). No more than 10 μg of total RNA was used to generate cDNA with the High Capacity cDNA Archival Kit (Applied Biosystems, Foster City, CA) as per the product directions. Each cDNA sample was diluted 1:10 in water, and 5 μl was used as the template for each reaction. For all the reactions, minor groove binding (MGB) primers and FAM labeled probes were designed using Primer Express (Applied Biosystems, Foster City, CA) (Table 2). Each sample was normalized to *rrsA* mRNA levels and all values were calibrated to the value obtained from the parental strain, SA100. All reactions were performed in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) under standard reaction conditions.

N. Western blot analysis

Cell lystes were prepared using 10^{10} bacteria resuspended in 100µl of 10X sample buffer (100 mM Tris pH 8.0 and 100 mM $MgCl_2$) and 100 µl 2X Laemmli buffer (10% β-mercaptoethanol, 6% w/v SDS, 20% glycerol, 0.2 mg/ml Bromophenol blue) and the resulting samples were boiled for 10 minutes. Twenty µl samples were electroporated on an SDS-containing 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was probed with polyclonal rabbit antibodies raised against Hfq protein (from Andrew Feig, Indiana University, Bloomington, In) and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Perkin Elmer life sciences, Inc.). Hfq was detected using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc.).

O. Cell Culture

Henle cell monolayers were cultured in 6-well polystyrene tissue culture plates (Corning Inc. Costar, Corning, NY) in Gibco Minimum Essential Medium (MEM) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. Plates were incubated at 37℃ in an atmosphere of 5% CO_2.

1. Plaque assays

Plaque assays were performed essentially as published (Oaks *et al.*, 1985). Bacterial strains were grown overnight at 37℃ on TSB agar plates containing Congo red and appropriate antibiotics. A single red colony was inoculated in L broth plus carbenicillin, if indicated, and incubated at 30℃ overnight, and then diluted 1:100 in LB broth containing carbenicillin, if indicated. Cultures were grown to mid-logarithmic phase at 37℃ (optical density at 650 nm of 0.6). Strains carrying IPTG inducible genes on a plasmid (pAKS200/pQEvirF/pQEvirB/pQEmxiE) were grown in the presence of carbenicillin to an optical density at 650 nm of 0.5 followed by addition of 25 μM IPTG and incubation at 37℃ for 30 min. Bacteria were diluted in phosphate buffered saline (PBS), and 10^4 bacteria were used to infect the Henle cell monolayer in each tissue culture well containing 2 ml MEM supplemented with 250 μg/ml carbenicillin and 25 μM IPTG. Plates were centrifuged for 10 minutes at 1000 rpm in a Centra GP8 (International Equipment Company, Needham Heights, MA). Plates were incubated at 37℃ for 1.5 hours, washed with 2 ml PBS, then covered with a 2 ml overlay composed of MEM supplemented with 0.3% glucose, 250 μg/ml carbenicillin and 20 μg/ml gentamicin. For strains carrying an IPTG inducible gene, 25 μM IPTG was added. Following incubation for 72 hours at 37℃, the plates were washed with 2 ml PBS, stained with Wright-Giemsa stain (Camco, Ft. Lauderdale, FL), washed with distilled water and air-dried.

2. Invasion Assays

For invasion assays, the Henle cell monolayer was infected with 2 x 10^9 bacteria per well as detailed above. Plates were incubated at 37℃ for 30 minutes prior to the addition of 2 ml MEM supplemented with 0.3% glucose and 20 μg/ml gentamicin. Where indicated, 250 μg/ml carbenicillin and 25 μM IPTG was added only to the indicated strains. Plates were then incubated for an additional 90 minutes and washed with 2 ml PBS. Next, the monolayer was stained with Wright-Giemsa stain (Camco, Ft. Lauderdale, FL), washed with distilled water and air dried. Henle cells were visualized under 600X magnification and scored positive for invasion if they contained 3 or more bacteria.

P. DksA purification

The full length *dksA* gene was amplified by PCR using primers dksA-his1 and dksA-his2. The amplified PCR product was digested with KpnI and HindIII and ligated to pQE2 vector digested with KpnI and HindIII giving rise to pAKS101, which expressed IPTG-inducible N-terminally histidine-tagged DksA. pAKS101 was introduced into DH5α as well as the *S. flexneri dksA* mutant strain, SA5287. DH5α cells carrying pAKS101 were grown to an OD_{650} of 1 in LB broth supplemented with 250 μg/ml carbenicillin and 100 μM IPTG. His-DksA purification was performed using Ni-NTA Fast Start kit (Qiagen) and following the manufacturer's instruction for purification of 6xHis-tagged proteins under native conditions. The eluted purified His-DksA protein was then dialyzed against His-DksA

storage buffer (25mM Tris (pH 8), 100 mM NaCl, 50% glycerol and 2 mM β-mercamptoethanol) and quantitated by Bradford assay.

Q. *In vitro* transcription assays

Multiple rounds of *in vitro* transcription assays were performed using RNAP from *E. coli* ($E\sigma^{70}$) (Epicentre Technology). Linear templates were generated by PCR amplification of promoter regions of genes as indicated (Figure 14 legend). Each reaction contained linear DNA template (2 nM), ppGpp (100 μM) (TriLink Biotechnologies) and N-terminally His-tagged DksA (5 μM). The reaction buffer contained 40 mM Tris-HCl, pH 7.9, 55 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 0.1 μg/μl BSA, 500 μM ATP, 200 μM CTP and GTP, 10 μM UTP and [α-^{32}P] UTP (2.5 μCi; 1Ci = 37GBq) (Amersham). Transcription reactions were initiated by the addition of 10 nM RNAP, incubated at 30℃ for 10 min, and terminated by the addition of an equal volume of formamide loading buffer. Samples were were subjected to electrophoresis on 7M urea-6% polyacrylamide gels and visualized and quantified by phosphorimaging.

III. RESULTS

A. Identify targets of *dksA* potentially mediating virulence in *S. flexneri*

Our previous studies had shown a role for *dksA* in virulence of *Shigella* (Mogull *et al.*, 2001). DksA is a pleiotropic regulator required for virulence in several different bacterial pathogens. For example, enterohaemorrhagic *E. coli* DksA regulates two virulence regulatory genes, *ler* and *pch*, which control the expression of the genes involved in attachment and effacement lesion formation on the intestinal epithelial cells (Nakanishi *et al.*, 2006). DksA controls the secretion of extracellular virulence factors rhamnolipids and LasB elastase in *P. aeruginosa* (Jude *et al.*, 2003) and the production of RpoS (σ^S) in *S. typhimurium*, which is required for expression of virulence genes (*spv*) present on a *Salmonella* virulence plasmid (Webb *et al.*, 1999). Because *Shigella* pathogenesis is distinct from these pathogens and involves different processes from those identified in these species, the targets of DksA affecting virulence in *S. flexneri* are likely to be different.

1. Gene expression analysis of the *dksA* mutant in *S. flexneri*

In *S. flexneri*, DksA is required for intercellular spread of the bacteria in epithelial cell monolayers (Mogull *et al.*, 2001). These experiments suggested a direct or indirect role of DksA on regulation of genes required for cell-to-cell spread of *S. flexneri*. Since DksA is a known pleiotropic transcriptional regulator, it was likely that the genes required for *S.*

flexneri pathogenicity are down-regulated in a *dksA* mutant. In order to investigate this further, a global gene expression profile of wild-type *S. flexneri* strain was compared with that of the *dksA* mutant by microarray analysis to screen for known or potential virulence genes that are regulated by DksA. RNA isolated from both the *dksA* mutant and its parent strains grown to mid-logarithmic growth phase, was used to generate cDNA. Differentially labeled cDNA from both strains was hybridized to microarrays. These microarrays are composed of oligonucleotides specific for *E. coli* K12 or additional *S. dysenteriae* open reading frames. Multiple hybridizations with RNA isolated from different cultures were performed to identify genes that were consistently down-regulated (≥2 fold) in the *dksA* mutant. *rpoS*, a known target of DksA regulation in *E. coli* (Brown *et al.*, 2002) was down-regulated in the *dksA* mutant compared to wild-type *S. flexneri* (Figure 9), indicating that DksA-regulated genes could be identified by this screen. A number of virulence factors genes were also down-regulated in the *dksA* mutant identifying new putative targets of DksA affecting virulence in *S. flexneri* (Figure 9). The complete results of the microarray analysis are shown in Tables 6 and 7 (Appendix).

2. Virulence gene regulated by DksA

Several known virulence genes were down-regulated in the *S. flexneri dksA* mutant (Figure 9). Target genes include those that are present on the virulence plasmid that encode the structural components of the type three secretion system (*mxi*, *spa*), translocators and effectors (*ics*, *ipa*, *ipg*), chaperone (*spa15*) and a transcriptional activator (*virF*). Two chromosomal genes required for pathogenicity of *S. flexneri*, *rfb* and *vacB*, also showed reduced expression in the *dksA* mutant compared to wild-type cells. Thus, there were several virulence plasmid genes from the VirF-VirB regulon, as well as chromosomal virulence genes that are not part of the VirF-VirB regulon, which showed reduced expression in the *dksA* mutant compared to that in wild type. The large number of genes affected by DksA suggested that another pleiotropic regulator required for *S. flexneri* virulence could be affected by DksA.

3. Genes of the *hfq* operon

Among potential regulators affected by DksA, *hfq* and one of the three genes (*hflK*) located downstream in the *hfq* operon (*hflA* region) consistently showed reduced expression in the *dksA* mutant compared to wild-type *S. flexneri* in the arrays (Figure 9). Since *hfq* is required for virulence in other, related bacterial species, and the mode of regulation of *hfq* has not yet been extensively investigated. Further experiments were performed to confirm this negative regulatory effect of DksA on *hfq* in *S. flexneri* and to investigate a role for this

regulation on virulence of *S. flexneri*. Also, comparison of the phenotypes of the *S. flexneri dksA* mutant and *E. coli* and *Salmonella hfq* mutants revealed several similar phenotypes between the *dksA* and *hfq* mutants including reduced RpoS levels, increased sensitivity to stress conditions, elongated cell phenotype and reduced virulence (Mogull *et al.*, 2001; Sittka *et al.*, 2007; Tsui *et al.*, 1994a). These results suggested that these two genes could be functioning through a common pathway, where *dksA* acts upstream of *hfq* to affect virulence in *S. flexneri*.

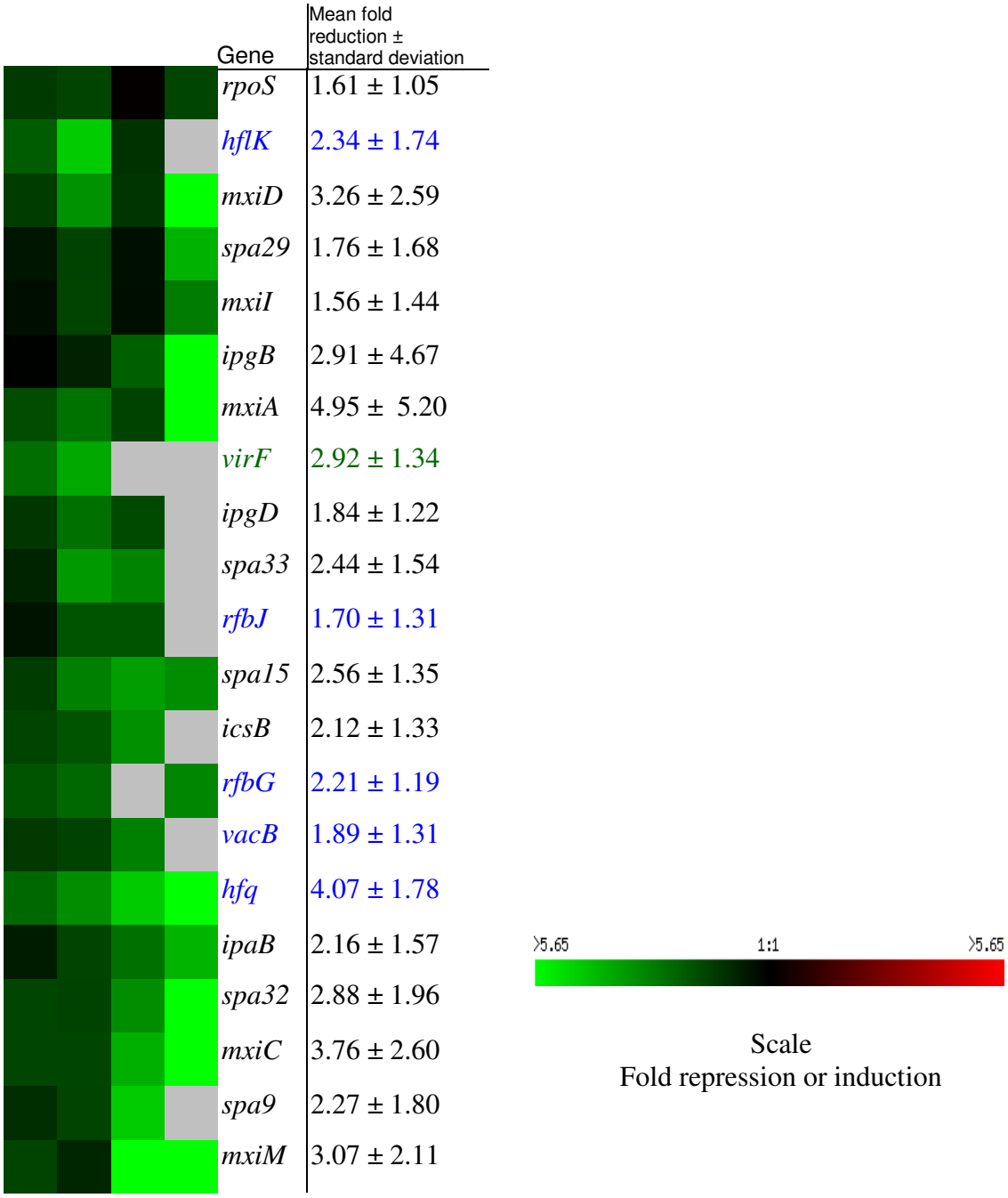


Figure 9. *dksA* microarray analysis showing *Shigella* virulence genes with reduced expression in an *S. flexneri dksA* mutant. *Shigella* virulence genes encoded on the chromosome are shown in blue and those on the virulence plasmid are in black. *virF*, encoding the major transcriptional activator of type three secretion system genes is shown in green. Only genes showing 1.5 fold or more average fold reduction in expression from four independent arrays are listed. The symbol ± indicates the standard deviation for the mean.

4. Reduced *hfq* mRNA levels in the *dksA* mutant

In order to confirm and quantitate reduced mRNA levels of *hfq* in the *dksA* mutant, real time PCR analysis was used to compare *hfq* mRNA levels in wild type and the *dksA* mutant. RNA was isolated from wild type cells and the *dksA* mutant grown to mid-logarithmic growth phase in either RDM (Figure 10) or L broth (data not shown). Real time PCR with primers and probe specific for *hfq* indicated that *hfq* expression was reduced approximately 50% in the *dksA* mutant as compared with wild-type *S. flexneri*.

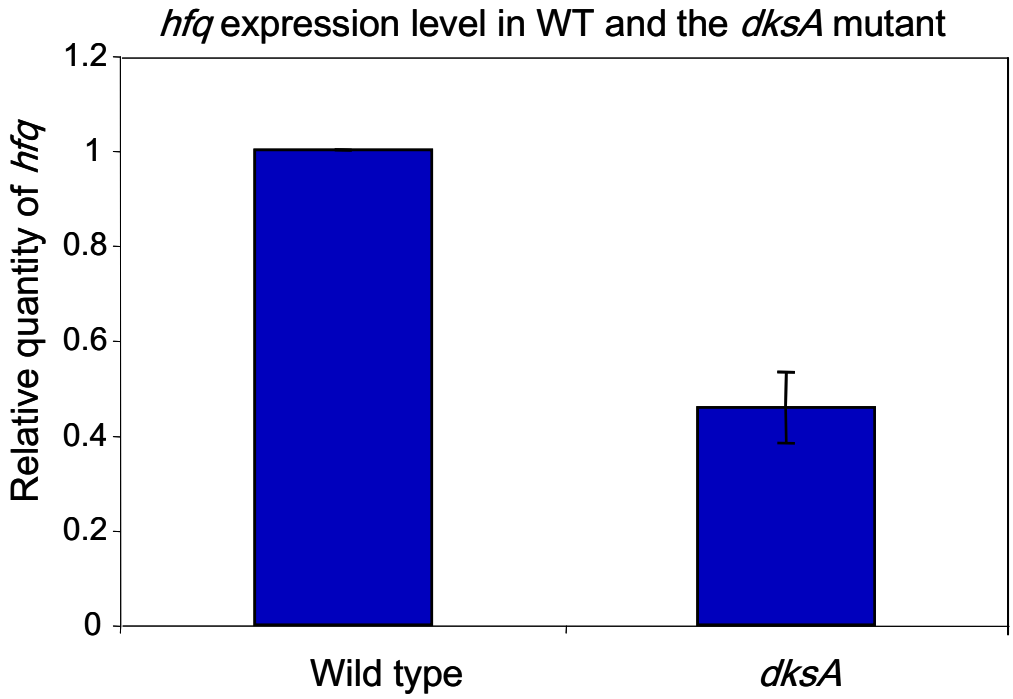


Figure 10. Reduced *hfq* mRNA levels in a *dksA* mutant

Real time PCR analysis showing *hfq* mRNA levels in wild type (SA100) and in the *dksA* mutant (SA5287) grown to mid-logarithmic growth phase in RDM at 37ºC. The amount of *hfq* mRNA in the *dksA* mutant is represented relative to the wild type strain. Data shown are mean values obtained from three independent experiments performed in triplicate. Error bar represent one standard deviation.

5. Reduced Hfq protein levels in the *dksA* mutant

To confirm that reduced *hfq* mRNA levels resulted in reduced levels of Hfq protein, the amount of Hfq in the *dksA* mutant was compared to that of the wild-type *S. flexneri* by Western blot assay of mid-logarithmic phase cultures. Hfq protein levels were significantly reduced in the *dksA* mutant compared to the wild-type parent strain (Figure 11). The Hfq protein levels were restored to wild-type levels in the *dksA* mutant complemented with the wild-type *dksA* gene on a plasmid (Figure 11). These data suggest that the mutation in *dksA* is responsible for reduced *hfq* expression and that DksA plays a role in *hfq* gene expression in *S. flexneri*.

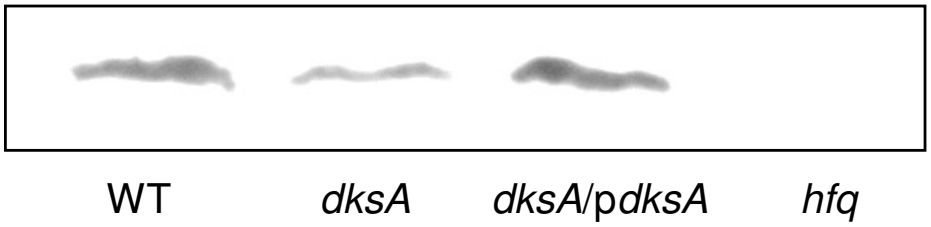


Figure 11. Western blot analysis showing reduced Hfq protein levels in the *dksA* mutant

Wild type SA100, *dksA* mutant SA5287 and *dksA* mutant complemented with wild type *dksA* in plasmid pSAM1 (*dksA*/p*dksA*) and an *hfq* deletion mutant, were grown to mid-logarithmic growth phase in L broth at 37ºC and total protein extracts, prepared from equal numbers of cells, were subjected to electrophoresis on an SDS 15% polyacrylamide gel and transferred to nitrocellulose membrane. The blot was probed with polyclonal rabbit anti-Hfq antiserum.

B. Determination of the mode of regulation of *hfq* by DksA

To determine whether DksA increases *hfq* expression by increasing the rate of transcription at σ^{70}-dependent promoters, as has been shown for certain *E. coli* amino acid biosynthesis genes (Paul *et al.*, 2005), I examined the effect of addition of purified DksA and ppGpp on *in vitro* transcription of *hfq* from the two known σ^{70}-dependent promoters, $P2_{hfq}$ and $P3_{hfq}$. The effect of DksA on *hfq* transcription at the two σ^{70}-dependent promoters, $P2_{hfq}$ and $P3_{hfq}$ and not the σ^{32}-dependent promoter, $P1_{hfq}$, was tested because transcription of *hfq* from $P1_{hfq}$ occurs when the cells are subjected to heat shock conditions, while transcription from $P2_{hfq}$ and $P3_{hfq}$ promoters occurs under other growth conditions (Tsui *et al.*, 1996) including conditions comparable to those under which virulence gene expression was affected. Purified DksA was prepared for the *in vitro* transcription assays by constructing a plasmid (pAKS101) encoding N-terminally tagged His-DksA, which was expressed from an IPTG inducible promoter. The plasmid was introduced into DH5α, and the His-tagged DksA was purified from the strain by Ni affinity chromatography of the lysate obtained from the IPTG induced culture (Figure 12). The *S. flexneri dksA* mutant carrying pAKS101 was tested in the Henle cell plaque assay and was found to be functionally indistinguishably from the *dksA* mutant complemented with wild type *dksA* (Figure 13).

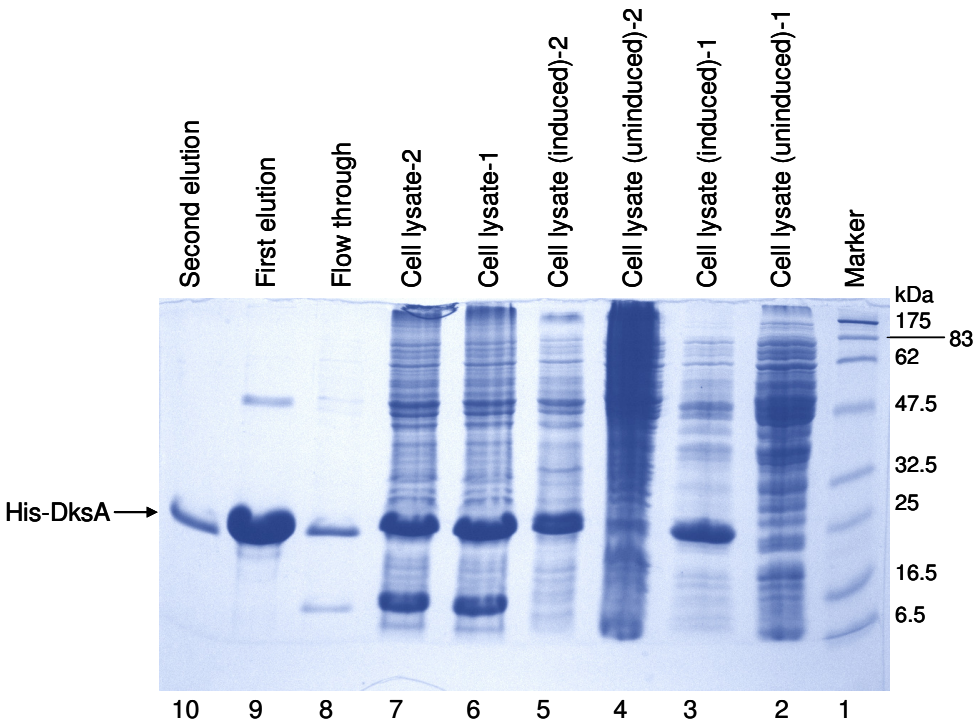


Figure 12. His-DksA purification

Overexpression of N-terminally His-tagged DksA (His-DksA) in DH5α cells was performed using pAKS101, which carries seven histidine encoding codons ligated upstream of the start codon of SA100 *dksA* under the control of an IPTG inducible promoter. DH5α/pHis*dksA* was incubated in the presence of carbenicillin to mid-logarithmic growth phase. Samples were tested before (lanes 2 and 4) and 4 hours after (lane 3 and 5) induction of His-DksA by 100μM IPTG. Induced cells were incubated in native lysis buffer, and the cell lysates obtained after removing cell debris by centrifugation were analysed in duplicates in lanes 6 and 7. A cell lysate containing His-DksA was tested on a Ni-NTA resin column and a sample of flow through from the column was loaded in lane 8. Purified His-DksA was eluted from the column using two aliquots of elution buffer, and samples after each elution step were analysed in lanes 9 and 10.

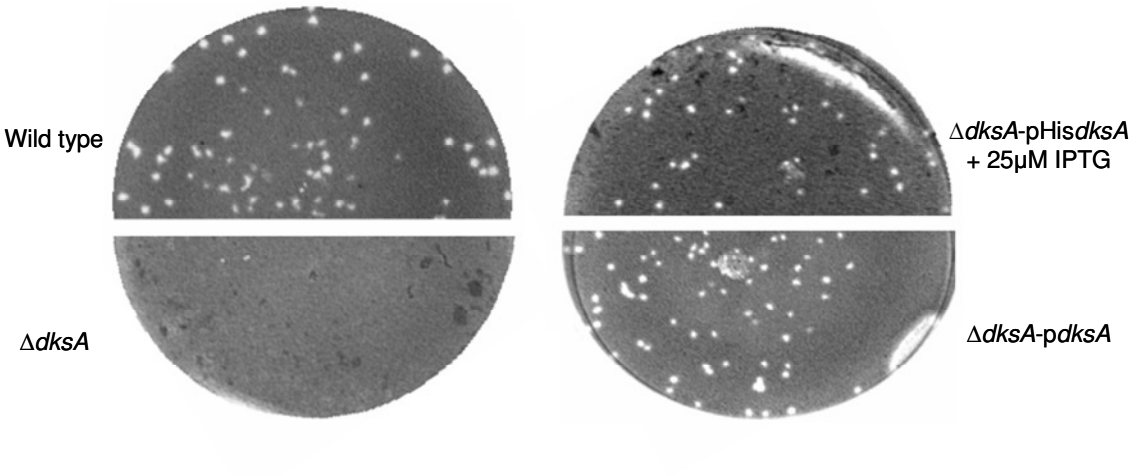


Figure 13. Complementation of *dksA* mutation by pHis*dksA* in a plaque assay

Wild type (SA100), Δ*dksA* (SA5287), Δ*dksA* expressing His-DksA from an IPTG inducible promoter from the plasmid, pHis*dksA* (SA5287/pHis*dksA*) and Δ*dksA* complemented with wild type *dksA* on a plasmid (5287-pSAM1) (10^4 cells) were used to infect Henle cell monolayers. After 48 hours of incubation at 37ºC, the cells were stained with Wright Giemsa stain to observe plaques.

1. DksA increases transcription of *hfq*

In vitro transcription assays were performed using linear templates obtained as PCR products and containing the indicated promoters. Promoters of the histidine biosynthesis gene *hisG* and the rRNA gene *rrsA* were included in the assay as control promoters whose activity has previously been shown in *E. coli* to be enhanced (*hisG*) (Paul *et al.*, 2005) or reduced (*rrsA*) (Paul *et al.*, 2004) in the presence of DksA and ppGpp (Figure 14). DksA had a small effect on transcription of *hfq* from the $P3_{hfq}$ promoter, and this effect was not further enhanced by ppGpp. However, transcription of *hfq* from the $P2_{hfq}$ promoter increased approximately four fold in the presence of DksA (Figure 14). This effect of DksA on *hfq* transcription from promoter $P2_{hfq}$ was further increased by the addition of ppGpp, resulting in a 16-fold increase in the level of *hfq* transcript. These results indicate that, in *S. flexneri*, DksA promotes *hfq* gene expression, and this effect is enhanced by ppGpp. This is the first report showing positive regulation of *hfq* at the transcriptional level by DksA.

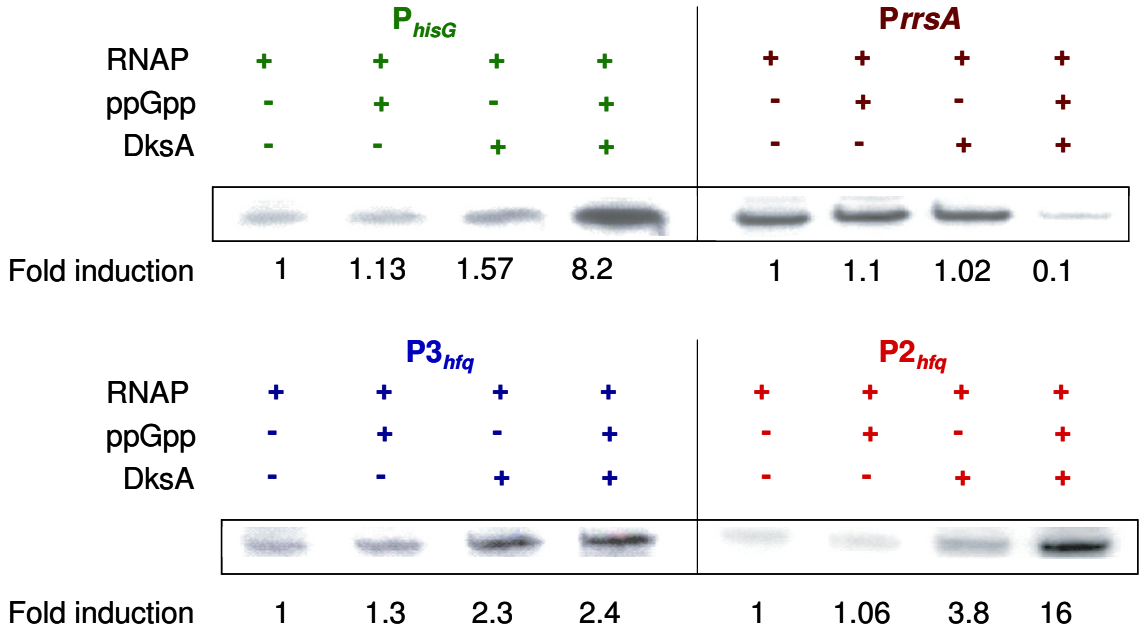


Figure 14. *hfq* transcription is increased by DksA and ppGpp

In vitro transcription was performed using 2 nM linear templates carrying the indicated promoters and RNAP alone or with the addition of either 100 μM ppGpp or 5 μM DksA, or both. The experiment was repeated twice and gave similar results; a representative experiment is shown.

2. Reduced expression from the $P2_{hfq}$ promoter in the *dksA* mutant

To determine the *in vivo* expression levels from the $P2_{hfq}$ and $P3_{hfq}$ promoters in the *dksA* mutant compared to that in wild type *S. flexneri*, both $P2_{hfq}$ and $P3_{hfq}$ promoters were fused upstream to a promoterless *lacZ* in the vector pQF50. The resulting constructs, pQF50-$P2_{hfq}$ and pQF50-$P3_{hfq}$, were introduced into wild type and the *dksA* mutant strains, and β-galactosidase assays were performed during mid-logarithmic, late-logarithmic and stationary growth phases of the cultures. These three growth phases were chosen to measure the effect of increasing concentrations of ppGpp on *hfq* transcription. Based on previous observations in *E. coli*, during mid-logarithmic phase, very low ppGpp levels are expected, and during late-logarithmic growth phase ppGpp levels are expected to start rising in the cell and reach a maximal level during the stationary growth phase (Fiil *et al.*, 1972). Additionally, an increase in the amount of Hfq protein was observed by Western blot analysis in *S. flexneri* during stationary growth phase when ppGpp levels are expected to increase (Figure 15A).

Since the results from *in vitro* transcription assays showed a very modest (2-fold) activation of the transcription from $P3_{hfq}$ promoter in the presence of DksA or DksA in combination with ppGpp (Figure 14), it was expected that the β-galactosidase levels from pQF50-$P3_{hfq}$ promoter would not decrease significantly in the *dksA* mutant compared to that in the wild type during late-logarithmic and stationary growth phase. β-galactosidase activity from pQF50-$P3_{hfq}$ confirmed this hypothesis by showing no significant decrease in β-galactosidase activity during stationary growth phase in the *dksA* mutant compared to that in

wild type cells (Figure 15B). However, surprisingly there was a 2-fold increase in β-galactosidase activity expression for $P3_{hfq}$ in the *dksA* mutant during mid-logarithmic growth phase.

Transcription from the $P2_{hfq}$ promoter was reduced to about 50% in the *dksA* mutant compared to that in the wild type during stationary growth phase, where the cell experiences nutrient deprivation conditions and ppGpp levels are high. Transcription from the $P2_{hfq}$ promoter in the *dksA* mutant during late-logarithmic growth phase was about 60% of wild type levels, whereas there was no significant reduction of expression from $P2_{hfq}$ promoter during mid-logarithmic growth phase in the *dksA* mutant compared to that in wild type *S. flexneri*. These results further confirm that DksA enhances *hfq* gene expression at the $P2_{hfq}$ promoter, and this effect is maximally seen during stationary growth phase conditions when ppGpp levels are high.

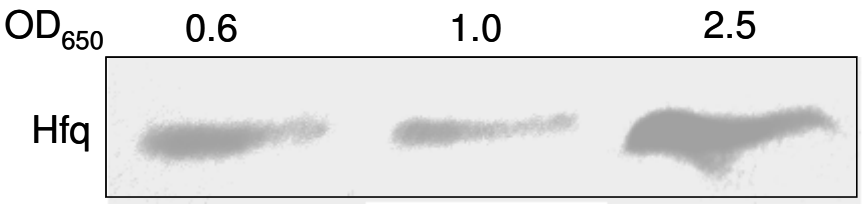


Figure 15A. Hfq abundance increases during stationary growth phase conditions in *S. flexneri*

Wild type *S. flexneri*, SA100 was grown in L broth at 37ºC, and samples were taken at an OD_{650} of 0.6 (mid-logarithmic), 1.0 (late-logarithmic) and 2.5 (stationary phase) as indicated. Total protein extracts prepared from equal numbers of cells were subjected to electrophoresis on an SDS-containing 15% polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with polyclonal rabbit anti-Hfq antiserum.

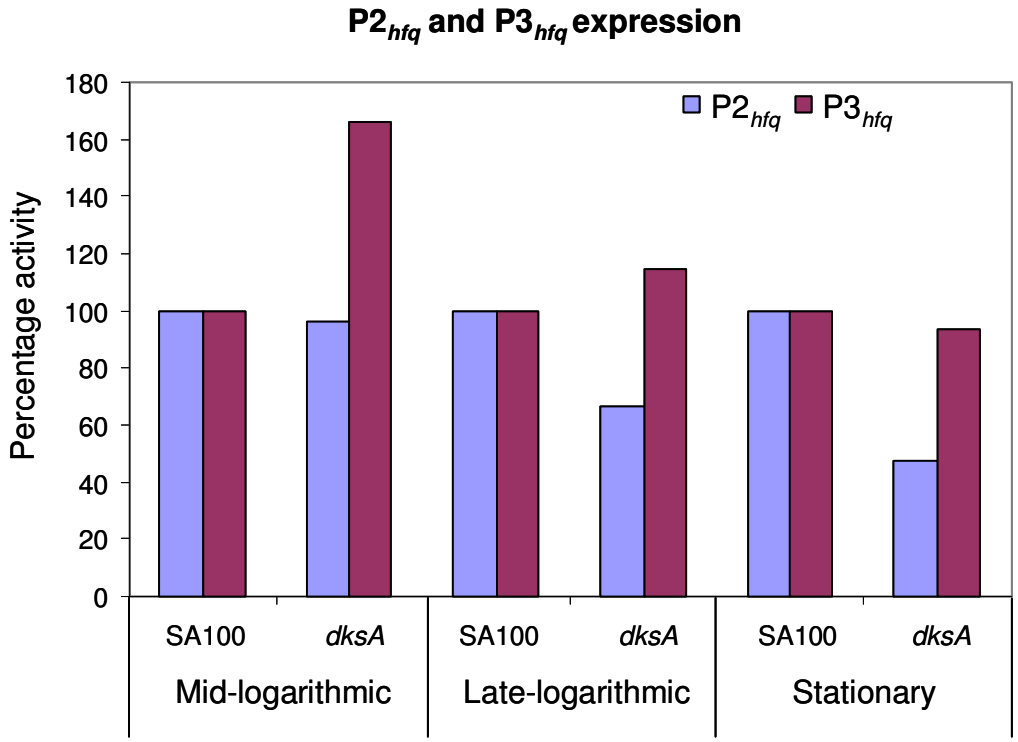


Figure 15B. Reduced expression from $P2_{hfq}$ promoter in the *dksA* mutant during stationary growth phase

Overnight cultures of SA100 and the *dksA* mutant transformed with pQF-$P2_{hfq}$ were subcultured 1:100 in L broth and grown to mid-logarithmic, late-logarithmic or stationary growth phases. Samples from each growth phase were tested for β-galactosidase activity by Miller's assay (Miller, 1972). Miller unit values obtained for SA100 in each growth phase were set to 100%, and the corresponding values for the *dksA* mutant were calculated and plotted accordingly.

C. Characterization of *S. flexneri hfq* mutant

Hfq is a highly abundant protein and its levels increase during stationary growth phase in *P. aeruginosa* (Sonnleitner *et al.*, 2006), consistent with its role in *rpoS*-mediated general stress responses in several bacterial systems. In *B. abortus*, *hfq* is required for resistance to hydrogen peroxide and for survival under acid conditions (pH 4) (Robertson and Roop, 1999). In the Gram-positive bacterium *L. monocytogenes*, *hfq* is required for resistance to osmotic and ethanol stress and long term survival in amino acid-depleted media, but not for resistance to acid and oxidative stress (Christiansen *et al.*, 2004). In an *E. coli hfq* mutant, increased synthesis of iron transport proteins FepA and FhuE resulted in increased incorporation of iron in the cell, leading to increased susceptibility to killing by hydrogen peroxide (Wachi *et al.*, 1999). *hfq* plays a role in thermotolerance, glycogen synthesis, cell division, resistance to several stress conditions in a RpoS (σ^{38})-dependent manner, generation of optimal σ^{E} (RpoE)-mediated envelope stress responses and in σ^{32} (RpoH)-mediated cytoplasmic stress response in *E. coli* (Guisbert *et al.*, 2007). In *Salmonella* spp., Hfq is required for efficient RpoS-mediated response to environmental stress (Brown and Elliott, 1996) and for RpoE dependent response to envelope stress (Figueroa-Bossi *et al.*, 2006). Since pathogenesis of *Shigella* requires survival in several stress conditions in the human host, the significance of *hfq* in *S. flexneri* stress resistance was analyzed.

To determine whether *hfq* is required for *S. flexneri* stress resistance, an *hfq* deletion mutant was prepared in the wild type *S. flexneri* strain SA100, in which the *hfq* gene was

replaced by a kanamycin resistance gene (Figure 7). A non-polar kanamycin cassette was used to avoid any polar effects on the expression of the downstream genes, *hflX*, *hflK* and *hflC*. Deletion of *hfq* and loss of Hfq synthesis was confirmed by PCR, DNA sequencing, real time PCR analysis (Figure 16) and Western blot analysis (Figure 11).

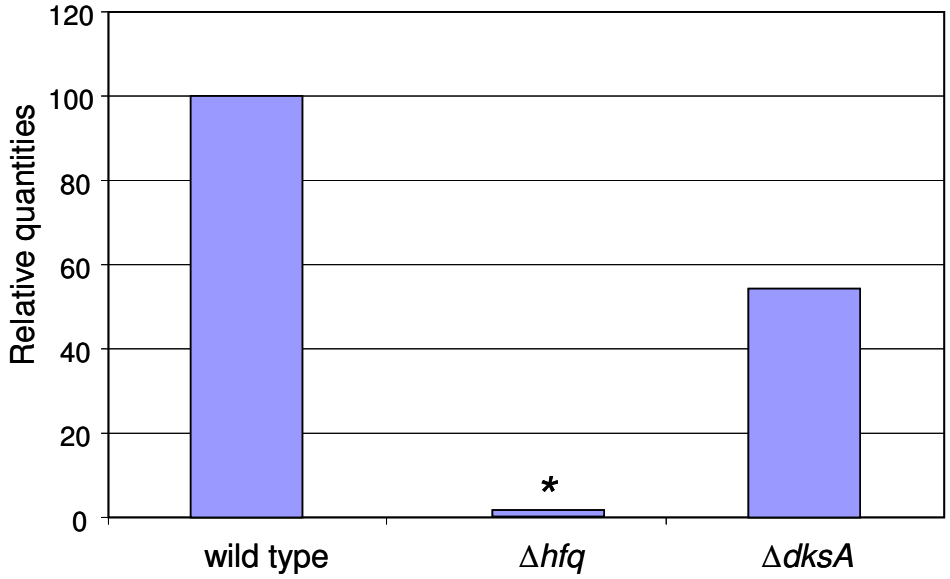


Figure 16. Loss of *hfq* mRNA in the *S. flexneri hfq* mutant

Real time PCR analysis showing *hfq* mRNA levels in wild type (SA100), *hfq* mutant (Δ*hfq*) and the *dksA* mutant (SA5287) grown to mid-logarithmic growth phase in L broth at 37ºC. The amount of *hfq* mRNA in the *hfq* mutant and the *dksA* mutant is represented relative to the wild type strain assigned a relative value of 100. An asterisk (*) indicates that the *hfq* mRNA levels were 0.02% in the *hfq* mutant, a value too low to be seen on the graph.

1. Role of *hfq* in survival in low pH medium

Shigella pathogenesis involves survival of the bacteria in the acidic conditions of human stomach. To determine if *hfq* is required for this virulence-associated phenotype in *S. flexneri*, survival of an *hfq* mutant was compared to that of wild type cells in L broth at pH 2.5. The *hfq* mutant failed to survive in L broth at pH 2.5 after two hours of incubation, whereas the wild-type and the *hfq* mutant complemented with wild type *hfq* on a plasmid, showed nearly 100% survival (Figure 17). These results indicate that *hfq* is required for acid resistance in *S. flexneri*.

2. Role of *hfq* in resistance to oxidative stress conditions

Within the host cells, particularly macrophages, *Shigella* encounters a variety of stress conditions e.g., oxidative stress (Babior, 1984). To examine whether *hfq* is required for oxidative stress resistance, resistance to hydrogen peroxide was determined for the wild type and *hfq* mutant. (Figure 18A) Only 2.5% of the *hfq* mutant cells survived after two hours of incubation in L broth in the presence of 3 mM hydrogen peroxide compared to nearly 100% survival of wild type. The *hfq* mutant complemented with wild type *hfq* on a plasmid had similar survival as wild type cells. Also, overnight growth of the *hfq* mutant in L broth containing 3mM hydrogen peroxide was significantly reduced compared to that of wild type or the *hfq* mutant complemented with wild type *hfq* (Figure 18B). These results indicate that *hfq* is required for resistance of *S. flexneri* under oxidative stress conditions.

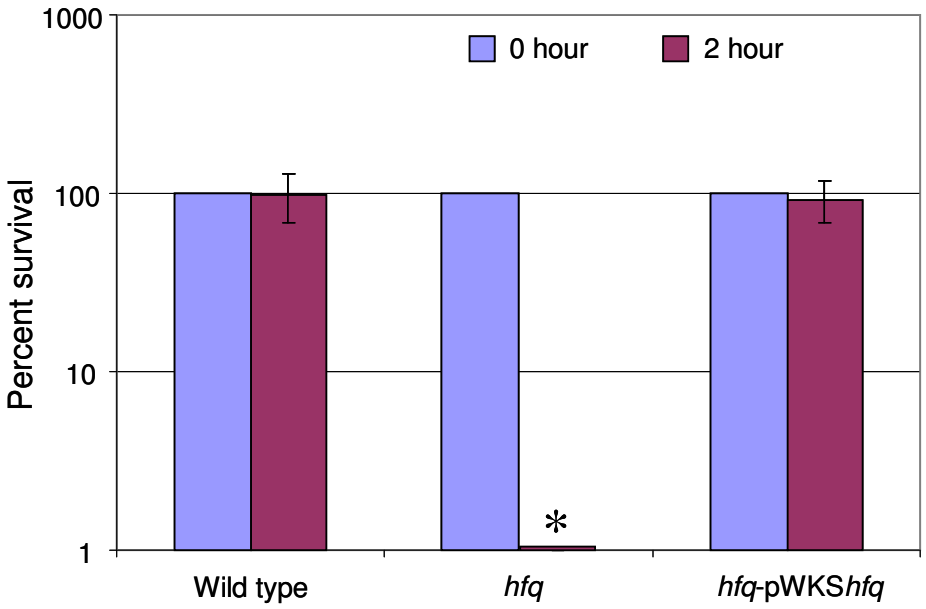


Figure 17. *hfq* is required for survival in acid conditions in *S. flexneri*

Mid-logarithmic cultures of wild type (SA100), *hfq* mutant (Δ*hfq*) and *hfq* mutant complemented with wild type *hfq* (*hfq*-pWKS*hfq*) were exposed to L broth, pH 2.5, for 2 hours, and the percent survival was determined by plating dilutions at 0 and 2 hours. Growth at time zero for each strain was set to 100%. An asterisk (*), corresponding to the growth of *hfq* after 2 hours at pH 2.5, indicates that percent survival at 2 hours was below detectable levels (< 1%). The average of three independent experiments is shown with one standard deviation.

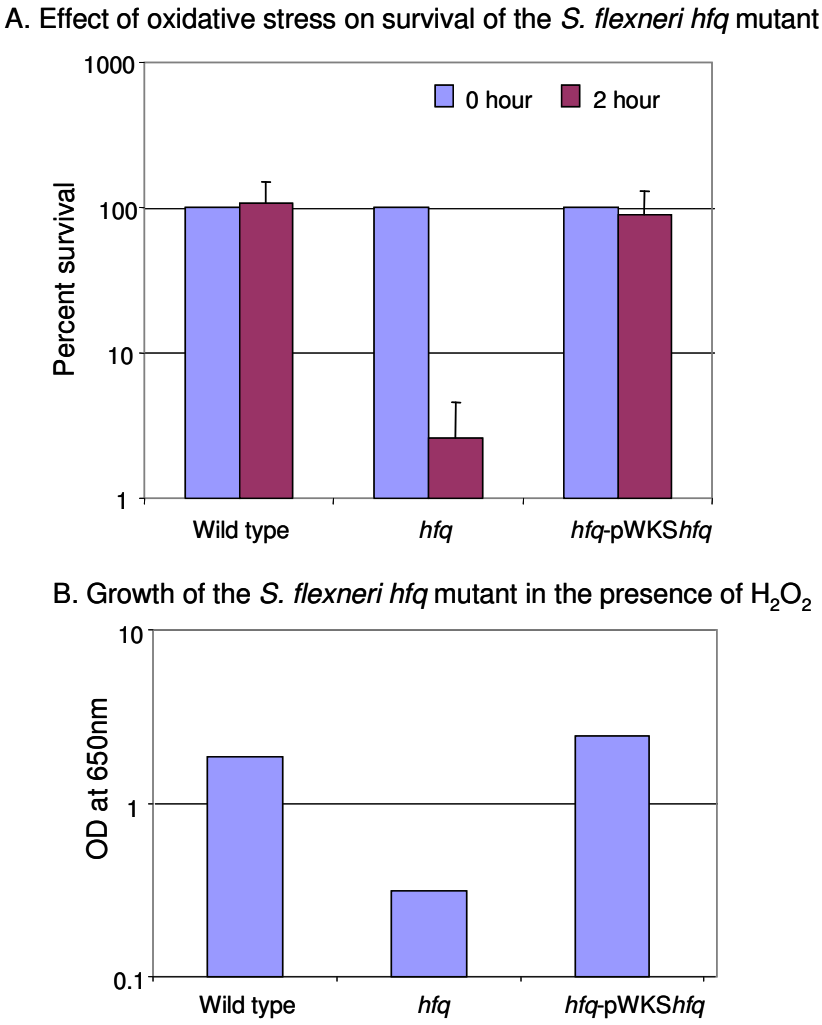


Figure 18. *hfq* is required for survival in oxidative stress conditions in *S. flexneri*

H_2O_2 (3 mM) was added to mid-logarithmic growth phase cultures (4 x 10^7 cells) of wild type (SA100), *hfq* mutant (Δ*hfq*) and Δ*hfq* complemented with wild type *hfq* (*hfq*-pWKS*hfq*).

A. Percent survival in 3 mM hydrogen peroxide was determined by plating at time 0 and after 2 hours. Growth at time zero for each strain was set to 100% and, after 2 hours was calculated and plotted accordingly. The average of three experiments is shown with one standard deviation.

B. The same cultures were grown for 15 hours in L broth containing 3 mM H_2O_2, and OD_{650} values were measured for each strain.

3. Role of *hfq* in growth during stationary growth phase

The composition of the eukaryotic cytosol is not well characterized. The cytosolic environment is reducing, and is low in sodium and calcium ions, and high in potassium and magnesium relative to the extracellular environment (Hwang *et al.*, 1992). In *S. flexneri*, several genes involved in metabolic processes including sugar metabolism, phosphate transport, and some in iron transport, lysine biosynthesis and biotin biosynthesis showed increased expression in the intracellular (Henle cell) environment (Runyen-Janecky and Payne, 2002). Intracellular induction of *S. flexneri* genes encoding phosphate utilization (*ptsS* and *phoA*) and iron uptake and metabolism systems (*sitA*, *fhuA* and *sufA*) indicated low-phosphate and iron-depleted conditions, respectively, in the cytosol of Henle cells. Such nutrient deprivation conditions are also experienced by a bacterium during stationary growth phase conditions (Chuang *et al.*, 1993). An *hfq* mutant showed reduced survival compared to wild type cells in L broth after 24 hours incubation at 37°C, when cells reach stationary growth phase and the nutrient levels in the medium decline; the *hfq* mutant complemented with the wild type *hfq* showed wild type phenotype (Figure 19). Poor survival of the *hfq* mutant in stationary growth phase conditions has been linked to decreased acid tolerance in *S. flexneri* (Bhagwat and Bhagwat, 2004). Moreover, the ability of *S. flexneri* in stationary growth phase to survive at low pH is responsible for a low infective dose in shigellosis (Small *et al.*, 1994). Additionally, stationary growth phase associated-expression of the *Shigella* virulence genes (*virF*, *virB*, *icsA*) is required for efficient expression of type three secretion effector proteins and is under the control of transcriptional activator, IHF (Porter

and Dorman, 1997b). Thus, reduced survival of the *S. flexneri hfq* mutant in stationary growth phase conditions is correlated with reduced virulence of the *hfq* mutant.

These results suggest that *hfq* is required for *Shigella* to survive in harsh conditions such as low pH, oxidative stress and nutrient deprivation that are encountered by the bacteria within the host and in the environment.

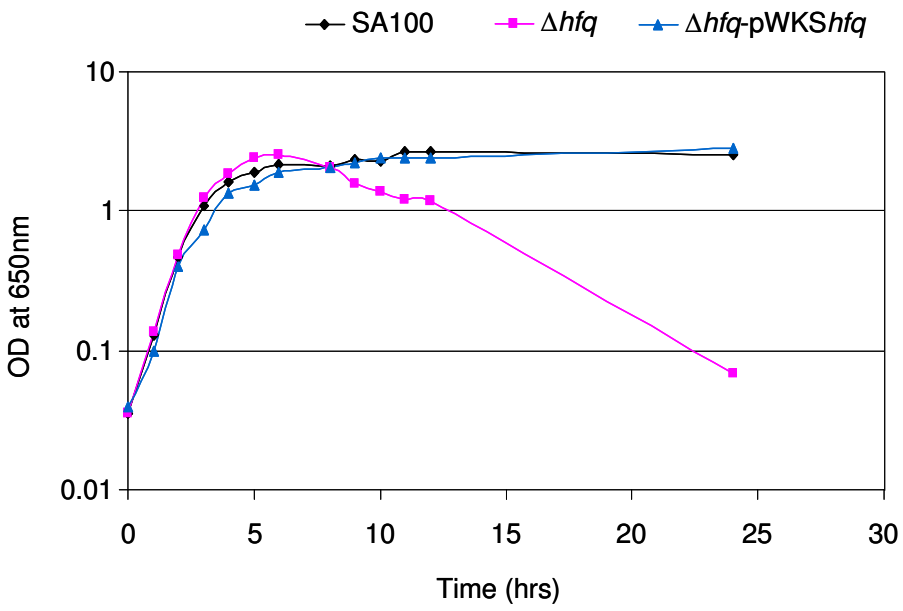


Figure 19. *hfq* is required for survival during stationary growth phase conditions

In vitro growth rates of *S. flexneri* wild type (SM100), *hfq* mutant (Δ*hfq*) and Δ*hfq* complemented with the wild type *hfq* gene under the control of an IPTG inducible promoter (Δ*hfq*-pAKS200) in L broth at 37ºC. *hfq* gene expression in *hfq*-pAKS200 was induced by the addition of 25 µM IPTG.

D. Role of *hfq* in *S. flexneri* virulence

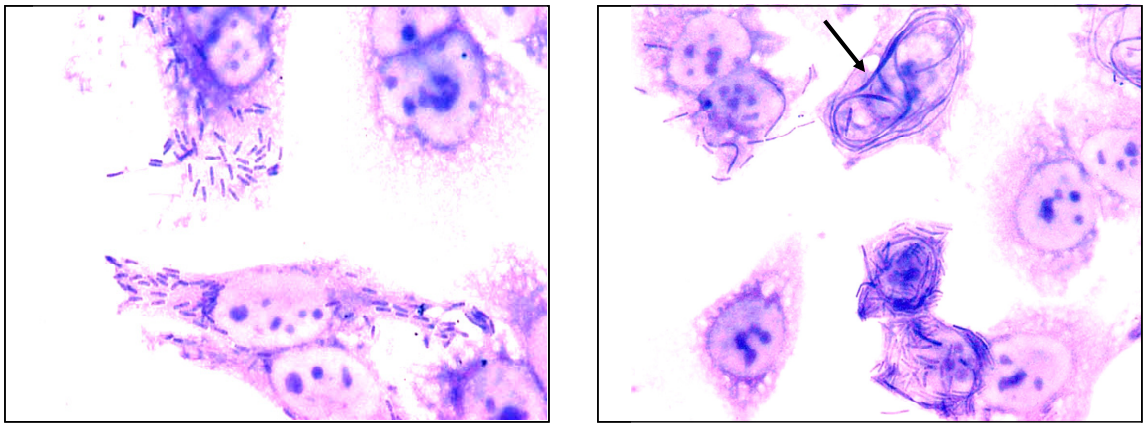
Hfq is a pleiotropic regulator required for several important physiological cell functions as well as virulence in several different bacterial species tested. For example, an *hfq* mutant showed attenuated virulence in *B. abortus* and significantly reduced survival in cultured murine macrophages due to reduced *rpoS* expression in the *hfq* mutant. RpoS is required for long-term intracellular survival of *B. abortus* in host macrophages. In *V. cholerae*, an *hfq* mutant is avirulent and fails to colonize suckling mouse intestine (Ding *et al.*, 2004). *hfq* also contributes to pathogenesis of the Gram positive bacterium, *L. monocytogenes*, in mice (Christiansen *et al.*, 2004) and to *Legionella pneumophila* survival in amoebae and macrophages (McNealy *et al.*, 2005). Furthermore, an *hfq* mutation reduces the virulence of the opportunistic human pathogen *P. aeruginosa* by affecting both cell-associated (flagellum, adhesion factors) as well as secreted elastases and pyocyanin virulence factors. In *S. typhimurium*, *hfq* mutation results in reduced *rpoS* expression, which mediates expression of *Salmonella* plasmid virulence genes (*spv*) required by the bacteria to cope with diverse stress conditions experienced in their mammalian hosts. Recently, the *hfq* mutation in *S. typhimurium* has been demonstrated to affect other virulence-associated phenotypes, such as cell motility, membrane morphology, adhesion and effector protein secretion, in a RpoS-independent manner. In most cases, the observed virulence defects were accompanied by reduced stress tolerance, likely reflecting a compromised ability to cope with the unfavorable environment in the host cell. Since an *rpoS* mutant in *S. flexneri* is fully virulent (Mogull *et al.*, 2001), there must be other targets of *hfq* that mediate virulence.

1. Reduced efficiency of invasion of epithelial cells by *hfq* mutant

The SA100 *hfq* mutant showed reduced binding of Congo red, a characteristic feature of virulence in *S. flexneri*. *S. flexneri hfq* mutant appears pink on Congo red agar, and the colonies had irregular margins as compared to the red and round SA100 colonies. Expression of *hfq* from a plasmid restored formation of red colonies with regular margins by the *hfq* mutant strain (data not shown). Reduced Congo red binding suggested a defect in virulence gene expression. Therefore the ability of an *S. flexneri hfq* mutant to infect cultured epithelial cells was investigated. The percentage of invasion of cultured epithelial cells by the *hfq* mutant was significantly reduced; about 50% of wild type *S. flexneri* (Figure 20). Furthermore the *hfq* mutant had an elongated cell phenotype when growing in the cytoplasm in epithelial cells (Figure 20). To determine whether this result was a general characteristic of *hfq* mutant cells or was specific to growth in the intracellular environments, cells grown *in vitro* were observed in exponential and stationary growth phases. Elongated cell length of the *hfq* mutant cells were also observed *in vitro* during exponential and stationary growth phases at 37ºC. The *hfq* mutant cells were longer than wild type cells during both the growth phases tested, but the difference was more pronounced during stationary growth phase (Figure 21). However, the *hfq* cell length in broth was significantly less than that seen in intracellular conditions in the cultured epithelial cells. These data suggest that compared to growth conditions in L broth, the intracellular epithelial environment further inhibits the ability of *hfq* mutant to carry out normal cell division, resulting in elongated cells in the *hfq* mutant.

2. Reduced efficiency of plaque formation by the *hfq* mutant

The *hfq* mutant was also assessed for its ability to form plaques in epithelial cell monolayers. The *hfq* mutant failed to form plaques or occasionally formed a smaller number of pinpoint plaques compared to wild type *S. flexneri* (Figure 22). Thus, an *hfq* mutant was impaired both in its ability to invade epithelial cells and to spread intercellularly.



Wild type (71%)

Δ*hfq* (30%)

Figure 20. *hfq* is required for epithelial cell invasion by *S. flexneri*

Henle cell monolayers were infected with 2 x 10^8 wild type (SA100) or the *hfq* mutant (Δ*hfq*) cells. Infected monolayers were incubated for 2 hours, washed and stained with Wright-Giemsa and observed using an oil emersion microscope. The numbers in parenthesis are the percentages of invasion calculated for each strain. The arrow indicates elongated *hfq* mutant cells inside the cultured epithelial cell.

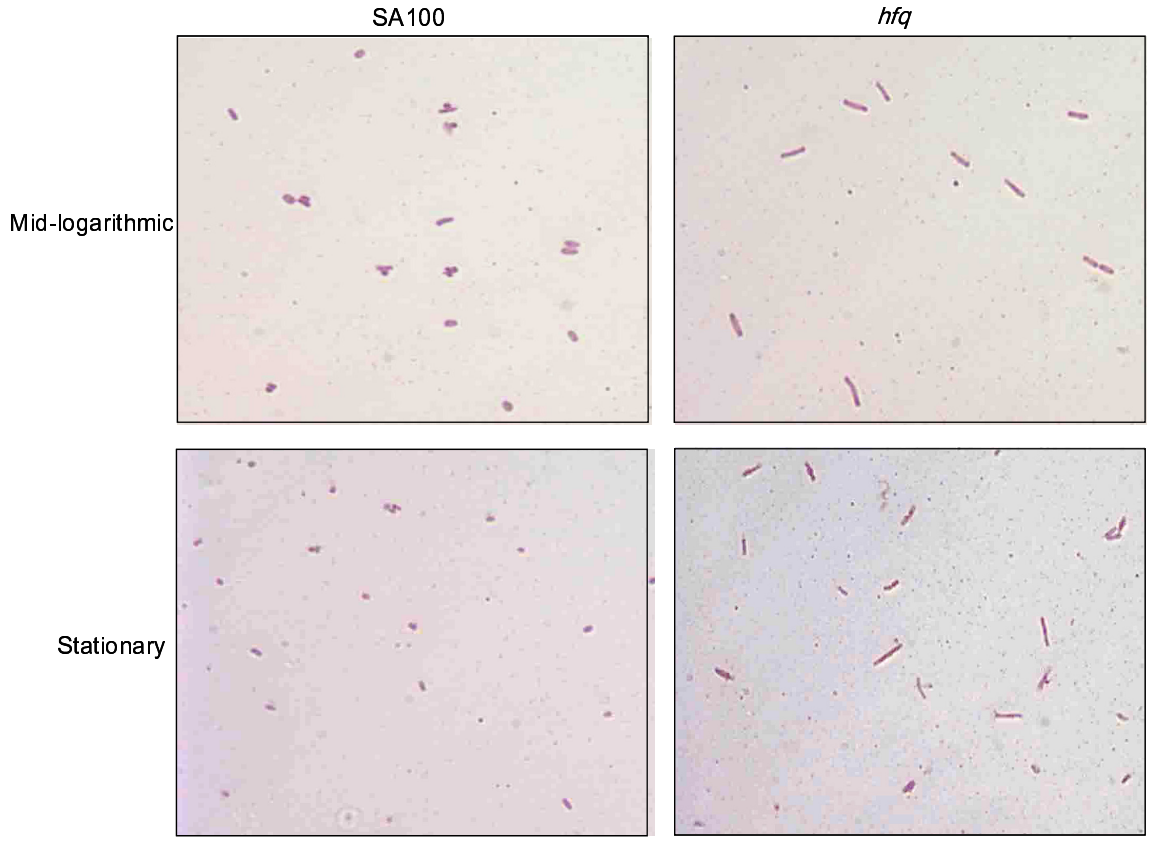


Figure 21. Cell length of a *S. flexneri hfq* mutant in L broth

An *hfq* mutant in *S. flexneri* has increased cell length in L broth during mid-logarithmic and stationary growth phases, compared to that in wild-type. Overnight and mid-logarithmically growing cultures of wild-type and *hfq* mutant were stained with Gram stain and observed under the microscope using an oil emersion lens.

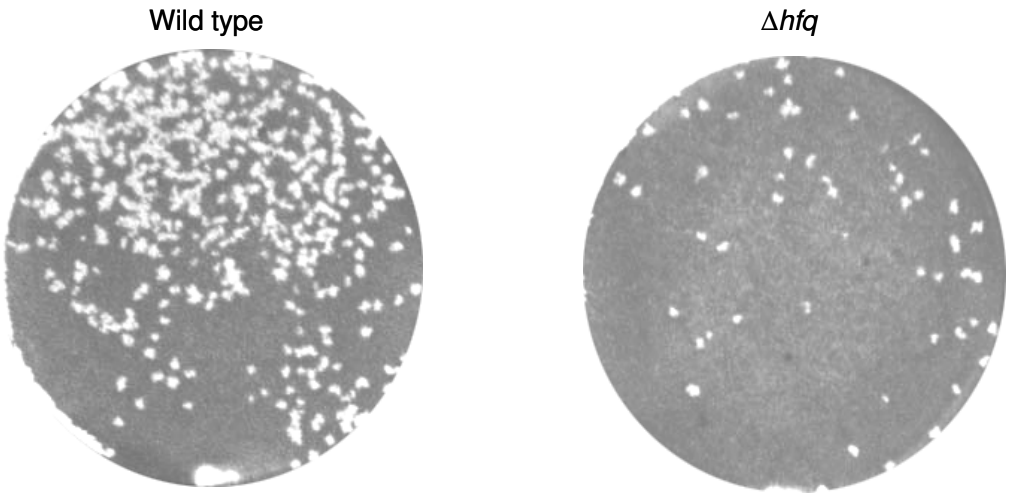


Figure 22. *hfq* is required for *S. flexneri* spreading in epithelial cells

Plaque assays were performed using 10^4 cells of wild type (SA100) and *hfq* mutant (Δ*hfq*) to infect Henle cell monolayers. The infected cells were incubated for 48 hours and stained with Wright-Giemsa stain to observe plaque formation.

E. Determination of the role of DksA regulation of *hfq* on virulence of *S. flexneri*

The *hfq* mRNA and protein levels were significantly reduced in a *dksA* mutant. Since *hfq* is required for virulence in *S. flexneri*, it is possible that reduced amounts of Hfq could be responsible for the plaque defect seen in the *S. flexneri dksA* mutant. To determine whether the plaque formation defect of a *dksA* mutant is due to reduced expression of *hfq*, the *S. flexneri hfq* gene was expressed independently of *dksA* from an IPTG-inducible promoter in pAKS200. Western blot assays were performed to detect and compare Hfq protein levels between wild type and *dksA*/pAKS200 expressing *hfq* in the presence of variable IPTG concentrations. *dksA*/pAKS200 grown in the presence of 25μM IPTG resulted in approximately the same level of Hfq as seen in the wild-type cells (Figure 23) whereas inducing *hfq* expression in the presence of 50μM IPTG resulted in higher than wild-type levels of Hfq (Figure 23), these levels may have direct effects on the virulence genes in addition to the effect of DksA mediated by Hfq.

Wild type, SA100 and the *dksA* mutant strain, SA5287, transformed with pAKS200, were subjected to plaque assays in the presence and absence of IPTG to control *hfq* expression. Inducing expression of *hfq* in the presence of 25 μM IPTG in the *dksA* mutant background (SA5287/pAKS200) restored plaque formation (Figure 24B). The same strain grown in the absence of IPTG did not form plaques, suggesting lack of any leakiness from the IPTG-inducible promoter driving *hfq* expression. As a control, wild-type *S. flexneri* expressing *hfq* from the plasmid pAKS200 in the presence of 25 μM IPTG resulted in wild-

type size plaque formation (Figure 24B). This result indicates that the reduced levels of Hfq may be responsible for the plaque-negative phenotype of the *dksA* mutant.

The effects of *hfq* over-expression on plaque formation were also determined. Inducing expression of *hfq* in the presence of ≥50 μM IPTG failed to restore plaque formation in the *dksA* mutant (Figure 24B). Plaque assays were also performed with wild-type *S. flexneri* over-expressing *hfq* in the presence of 50μM IPTG, and a significant reduction in the plaque size was observed in the wild-type *S. flexneri* (Figure 24A). However, the effect of *hfq* over-expression in the wild-type background was not as severe as that seen in the *dksA* mutant background. This could be due to other unknown direct effects of *dksA* on virulence of *S. flexneri* independent of *hfq*. There was no defect in growth of either the wild type *S. flexneri* or the *dksA* mutant in rich media when *hfq* was over-expressed at these levels (Figure 25). These experiments indicate that the plaque defect was not an effect of altered growth rate. These data indicate that optimum endogenous amounts of Hfq are required for plaque formation by *S. flexneri*; both reduced and increased amounts of the protein are deleterious to invasion and intercellular spread of the bacteria in cultured epithelial cells.

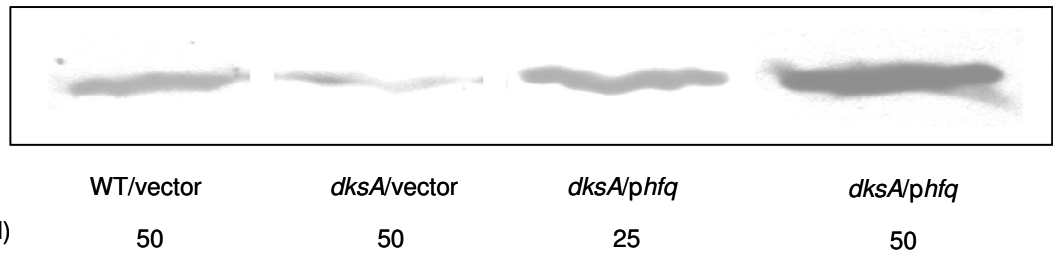


Figure 23. Hfq protein levels in the *dksA* mutant carrying the inducible *hfq* or vector

Wild type SA100 carrying vector pQE2 (SA100/pQE2), *dksA* mutant, SA5287 carrying vector pQE2 (SA5287/pQE2) and the *dksA* mutant carrying *hfq* in pAKS200 (SA5287/p*hfq*) were grown to mid-logarithmic growth phase in L broth at 37ºC. The concentration of IPTG added to induce expression of *hfq* from pAKS200 is shown below each lane. Total protein extracts, prepared from equal number of cells were separated on an SDS-containing 15% polyacrylamide gel and transferred to nitrocellulose membrane. The blot was incubated with polyclonal rabbit anti-Hfq antiserum.

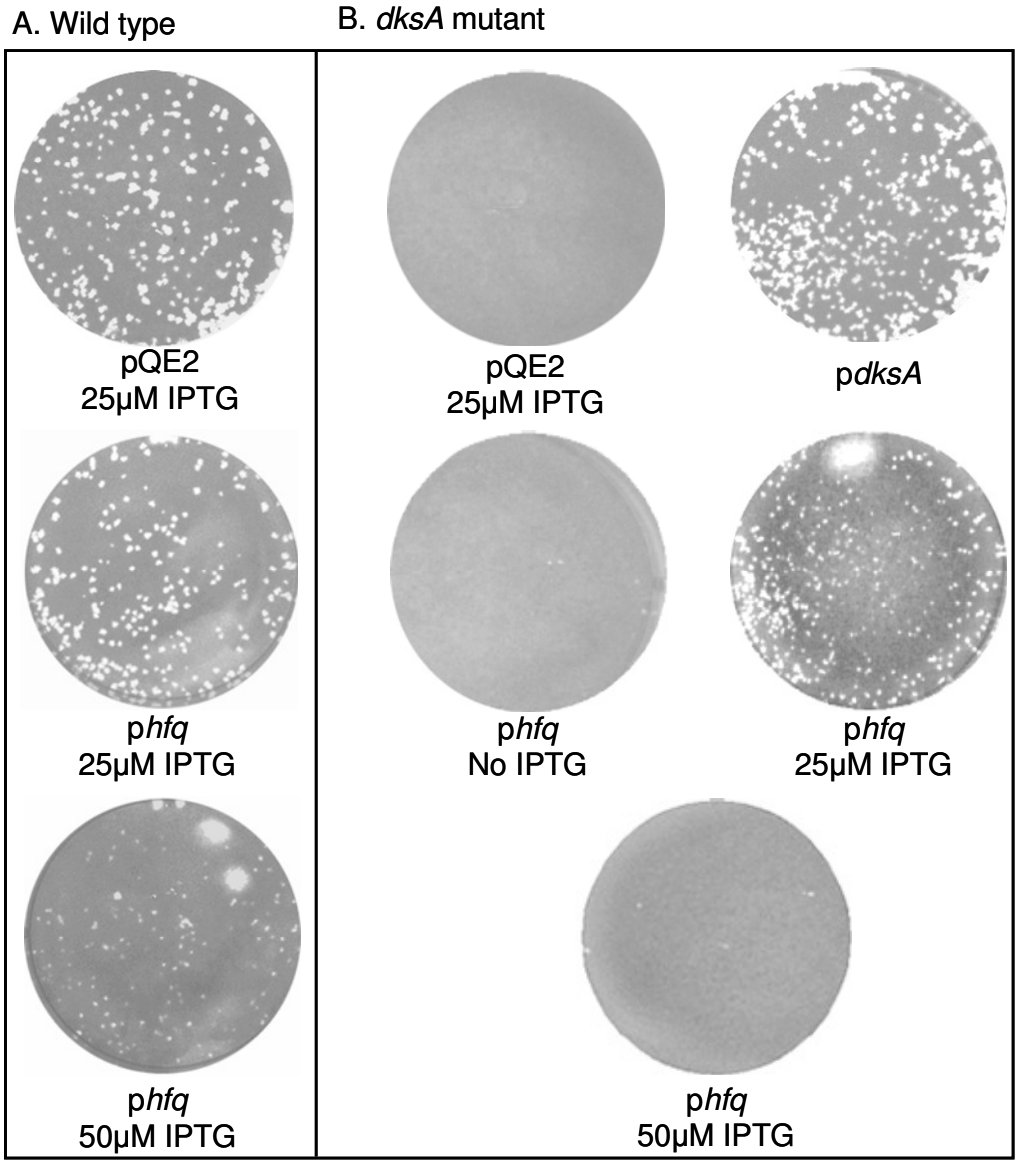


Figure 24. Hfq expression in a *dksA* mutant restores plaque formation

In (A), 10^5 cells of wild type *S. flexneri* SA100 carrying either vector pQE2 or pAKS200, the vector containing an inducible *hfq* (p*hfq*), were used to infect Henle cell monolayer. In (B) 10^5 cells of the *dksA* mutant (SA5287) carrying either vector pQE2, wild type *dksA* in pSAM1 (p*dksA*), or inducible *hfq* in pAKS200 (p*hfq*) supplemented with indicated concentrations of IPTG were used to infect Henle cell monolayers. All the infected monolayers were incubated for 48 hours and stained with Wright-Giemsa to visualize plaque formation.

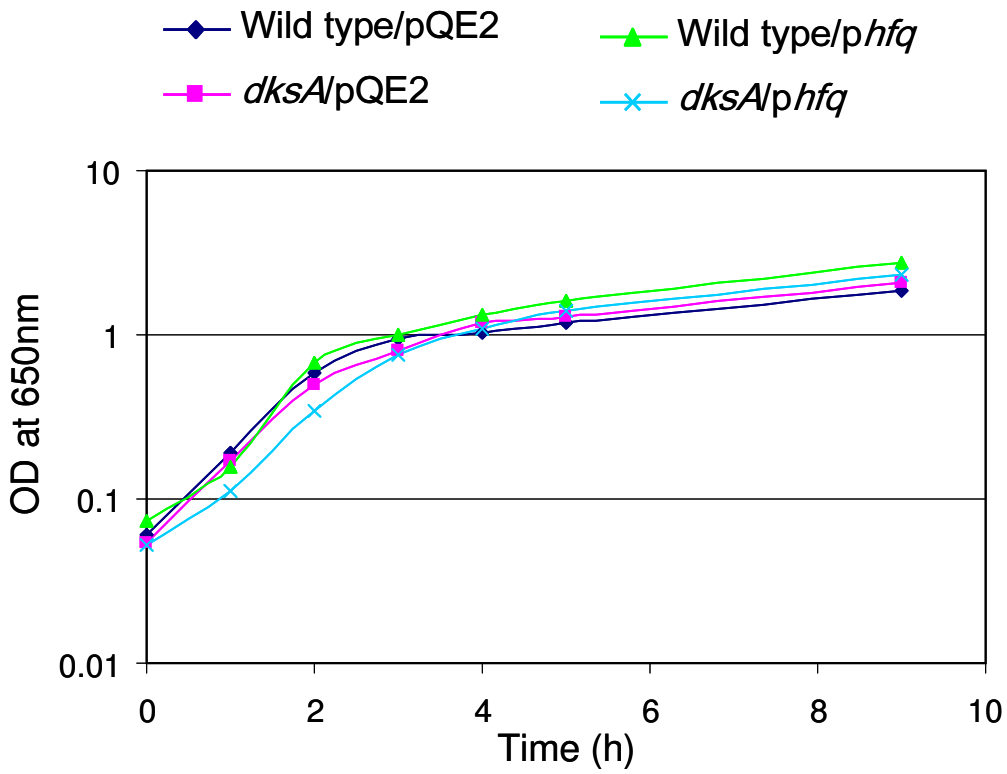


Figure 25. Over-expression of *hfq* does not affect growth of *S. flexneri* in L broth

Overnight cultures of wild type SA100 carrying vector pQE2 (SA100-pQE2) or *hfq* over-expression construct (SA100-pAKS200) and the *dksA* mutant carrying vector pQE2 (SA5287-pQE2) or *hfq* over-expression construct (SA5287-pAKS200) were subcultured by diluting 1:50 in L broth with carbenicillin and 50 µM IPTG. The optical density of each sample was read at 650 nm at the indicated times for 9 hours of incubation at 37ºC.

F. Identification of targets of *hfq* in *S. flexneri*

My model suggests that DksA-mediated virulence in *S. flexneri* occurs via Hfq where *dksA* acts upstream of *hfq*. *hfq* is required for efficient invasion, intracellular multiplication and intercellular spread of *S. flexneri* in cultured epithelial cells, as shown by my experiments (Figures 20 and 22). *hfq* is also required for survival of *S. flexneri* under stress conditions including low pH, oxidative stress and nutrient starvation. In other related bacterial pathogens, virulence defects in most cases were accompanied by reduced stress tolerance, likely reflecting a compromised ability to cope with the unfavorable environments encountered in the cultured epithelial cells or in the environment. Since an *rpoS* mutant in *S. flexneri* is virulent, there must be other targets of Hfq that mediate virulence. However, the virulence gene targets of Hfq have not been identified and studied in detail. In order to identify virulence gene targets of *hfq* in *S. flexneri*, a global gene expression profile of wild type *S. flexneri* and an *hfq* mutant and wild type were compared using microarray analysis.

1. *hfq* gene expression profile

Microarray analysis of the *hfq* mutant was performed using RNA isolated from wild type and the *hfq* mutant grown to early stationary growth phase, since Hfq has been identified as a regulator of stationary phase gene expression in *E. coli* (Muffler *et al.*, 1996). In order to identify *Shigella* virulence gene targets of *hfq*, the expression of the type three secretion system genes, and several *Shigella* virulence genes whose expression was reduced (≥ 2fold)

in the *hfq* mutant were examined (Table 4). These genes include those present on the *Shigella* virulence plasmid encoding the type three secretion system. *icsA* and *virB*, the only two genes under the control of the VirF transcriptional activator (Le Gall *et al.*, 2005), showed reduced expression in the *hfq* mutant. v*irA*, from the *mxiE* regulon and several *virB* regulon genes, including those that encode the type three secretion apparatus (*mxi-spa*), translocators (*ipaC*), effector proteins (*ipaA/D/J*, *ipgB*, *icsB*), chaperones (*ipgA*, *ipgC*) and transcriptional regulator (*mxiE*) showed reduced expression in the *hfq* mutant. Several previously identified *hfq* target genes, including those regulated by sRNAs (*acnA*, *gadA*, *galK*, *ompA*, *ompF*, *sdhD*, *yejE* and *rpoS*) as well as those regulated by Hfq by an unidentified mechanism (*htrA*, *oppA* and *hns*) also showed altered expression, confirming the validity of this screen (Table 5). The complete results of the microarray analysis are shown in Tables 8 and 9 (Appendix).

Table 4. Microarray analysis showing *Shigella* virulence genes with reduced expression in an *S. flexneri hfq* mutant [1]

Name	Average fold change[2]	Function
icsB	16.3*	Virulence protein secreted by TTSA
ipaA	4.9 ± 1.6	TTSS effector protein
ipaC	27.6*	TTSS translocator protein
ipaD	5.1 ± 1.2	TTSS effector protein
ipaJ	3.0 ± 2.2	TTSS effector protein
ipgA	29.2 ± 1.5	TTSS chaperone for IcsB
ipgB	25.2 ± 1.1	TTSS effector protein
ipgC	18.7 ± 1.8	TTSS chaperone for IpaB and *ipaC*
ipgE	43.9*	TTSS chaperone for IpgD
ipgF	34.9 ± 1.2	Invasion plasmid antigen
ipgH	2.3 ± 1.4	Invasion plasmid antigen
mxiA	9.2 ± 1.9	TTS apparatus
mxiC	8.3 ± 1.2	TTS apparatus
mxiE	**33.8***	**TTSS transcriptional activator**
mxiG	21.9*	TTS apparatus
mxiH	33.3 ± 1.3	TTS apparatus
mxiI	2.5 ± 2.2	TTS apparatus
mxiL	16.1*	TTS apparatus
mxiM	2.7 ± 1.0	TTS apparatus
spa13	5.0 ± 0.2	TTS apparatus
spa24	2.7 ± 0.9	TTS apparatus
spa29	2.2 ± 0.8	TTS apparatus
spa32	6.6 ± 2.2	TTS apparatus
spa33	2.5 ± 1.3	TTS apparatus
spa40	2.6 ± 2.1	TTS apparatus
spa47	7.1 ± 3.0	TTS apparatus
spa9	2.5 ± 1.4	TTS apparatus
spa-orf10	2.1 ± 1.4	TTS apparatus
spa-orf11	6.2*	TTS apparatus
virA	5.3 ± 1.2	TTSS effector protein
virB	**10.9 ± 2.1**	**TTSS transcriptional activator**

[1]Overnight cultures in L broth were diluted 1:100 into L broth and RNA was isolated from late-logrithmically-growing cultures (OD650 ≈ 0.9) at 37ºC.

[2]Ratio of expression in wild type vs. *hfq* mutant. Values in green indicate reduction in expression of the corresponding genes in the hfq mutant compared to that in wild type, respectively. The two transcriptional activators encoding genes, *virB* and *mxiE*, are shown in bold. Mean values for genes showing a two fold reduction or higher in the *hfq* mutant in one or more of the three arrays are shown. Mean of three independent experiments is shown; ± indicates the standard deviation from the mean.

*Fewer than three data points were available for these genes in these experiments

Table 5. Microarray analysis showing Hfq regulated genes with altered expression in an *S. flexneri hfq* mutant [1]

Name	Average fold change[2]	Function	sRNA
acnA	2 ± 1.4	aconitase hydratase I	RyhB
cyoA	3.2*	cytochrome O ubiquinol oxidase subunit II	
gadA	3.0 ± 2.1	glutamate decarboxylase isozyme	GadY
galK	4.4*	Galactokinase	Spot42
htrA	2.7 ± 2.6	periplasmic serine protease	
ompA	3.0 ± 1.2	Outer membrane protein A	MicA/SraJ
ompF	2.3 ± 1.2	Outer membrane protein 1a	MicF
oppA	2.1 ± 1.3	periplasmic oligopeptide binding protein	
sdhD	2.4 ± 2.0	succinate dehydrogenase	RyhB
yejE	2.0 ± 1.9	putative transport system permease	RydC
Hfq	11.2 ± 1.4	pleiotropic post transcriptional regulator	
Hns	2.3 ± 1.3	DNA binding protein, *virF*& *virF* repressor	
rpoS	2.6 ± 1.5	stationary phase sigma factor (RpoS)	DsrA & RprA

[1]Overnight cultures in L broth were diluted 1:100 into L broth and RNA was isolated from late-logrithmically-growing cultures (OD650 ≈ 0.9) in L broth at 37ºC.

[2]Ratio of expression in wild type vs. *hfq* mutant. Values in green and red indicate reduction or induction in expression of the corresponding genes in the *hfq* mutant compared to that in wild type cells, respectively. The regulatory small RNAs (sRNAs) of some genes are shown. Genes with no corresponding sRNA, are regulated by Hfq by an unidentified mechanism. Mean values for genes showing a two-fold change or higher in the *hfq* mutant in one or more of the three arrays are shown. Mean of three independent experiments is shown; ± indicates the standard deviation for the mean.

*Fewer than three data points were available for these genes in these experiments

G. Reduced expression of transcriptional activators in the *hfq* mutant

Shigella utilizes the type three secretion system to establish infection in the human epithelial cells, which involves three crucial steps; invasion, intracellular multiplication and intercellular spread. Most of the genes involved in carrying out these crucial infection processes are present on the *Shigella* virulence plasmid and encode the type three secretion system and its effectors. The transcriptional activators, VirF, VirB and MxiE, are required for increasing transcription of genes encoding this type three secretion system. Since two of these transcriptional activators showed reduced expression in the *hfq* mutant compared to wild type after microarray analysis, their down regulation was monitored using real time PCR analysis. Wild type *S. flexneri* and the *hfq* mutant were grown to late-logarithmic growth phase in L broth and RNA was isolated to determine *virF*, *virB* and *mxiE* mRNA levels using primers and probes specific to each gene. mRNA levels obtained from the wild type sample were set to 100% and the corresponding values for the *hfq* mutant were calculated accordingly. Results show significantly reduced expression of all three transcriptional activators in the *hfq* mutant compared to the level of expression in wild type *S. flexneri* (Figure 26). These data suggest a role of *hfq* in regulation of the transcriptional activators of *Shigella* virulence genes.

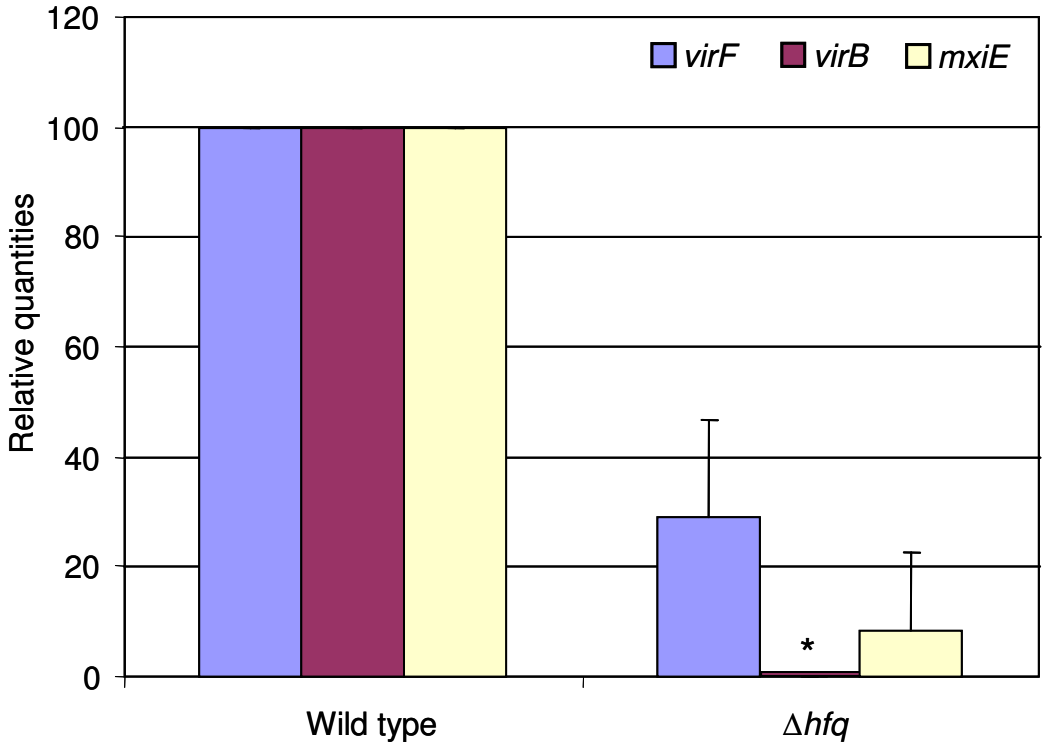


Figure 26. Reduced *virF*, *virB* and *mxiE* mRNA levels in a *hfq* mutant

Real time PCR analysis showing *virF*, *virB* and *mxiE* mRNA levels in wild type (SA100) and in the *hfq* mutant (Δ*hfq*). The amount of *virF*, *virB* and *mxiE* mRNA in the *hfq* mutant is represented relative to that in the wild type strain. Data shown are mean values obtained from three independent experiments performed in duplicate. Error bars represents one standard deviation. An asterisk (*) indicates that the *virB* mRNA levels were 0.025% in the *hfq* mutant, a value too low to be seen on the graph.

H. Hfq activates *virF* expression and suppresses *virB* and *mxiE* expression

Since the *Shigella* virulence regulatory cascade involves induction of VirF synthesis as the foremost transcriptional activator required for inducing transcription of *virB*, and VirB in turn induces transcription of *mxiE*, it is possible that Hfq is required for induction of *virF* only and the reduced levels of *virB* and *mxiE* mRNA seen in the *hfq* mutant are as a result of reduced VirF production in the *hfq* mutant. To test this hypothesis, the *virF*, *virB* and *mxiE* mRNA levels in *hfq* over-expressing conditions were compared using real time PCR analysis. If Hfq is required for up-regulating *virF* mRNA levels only and not the *virB* and *mxiE* levels, then in *hfq* over-expressing conditions, only *virF* mRNA levels may increase to a greater extend than *virB* and *mxiE*. Real time PCR analysis comparing *virF*, *virB* and *mxiE* mRNA levels in wild type, *hfq* mutant and wild type cells expressing *hfq* under the control of an IPTG inducible promoter showed a decrease of *virF* mRNA in the *hfq* mutant and a significant increase in the *hfq* over-expressing wild type strain (Figure 27). This result indicates a direct correlation between Hfq levels and the *virF* mRNA levels in the cell. However, the *virB* and *mxiE* levels in *hfq* over-expressing conditions did not significantly increase with the increasing *virF* mRNA levels. Since there is greater than wild type levels of Hfq in the *hfq* over-expressing wild type strain and the *virB* and *mxiE* mRNA levels are below wild type levels, these data suggest that increasing Hfq levels represses *virB* and *mxiE* expression in the cell, independent of the amount of VirF present in the cell.

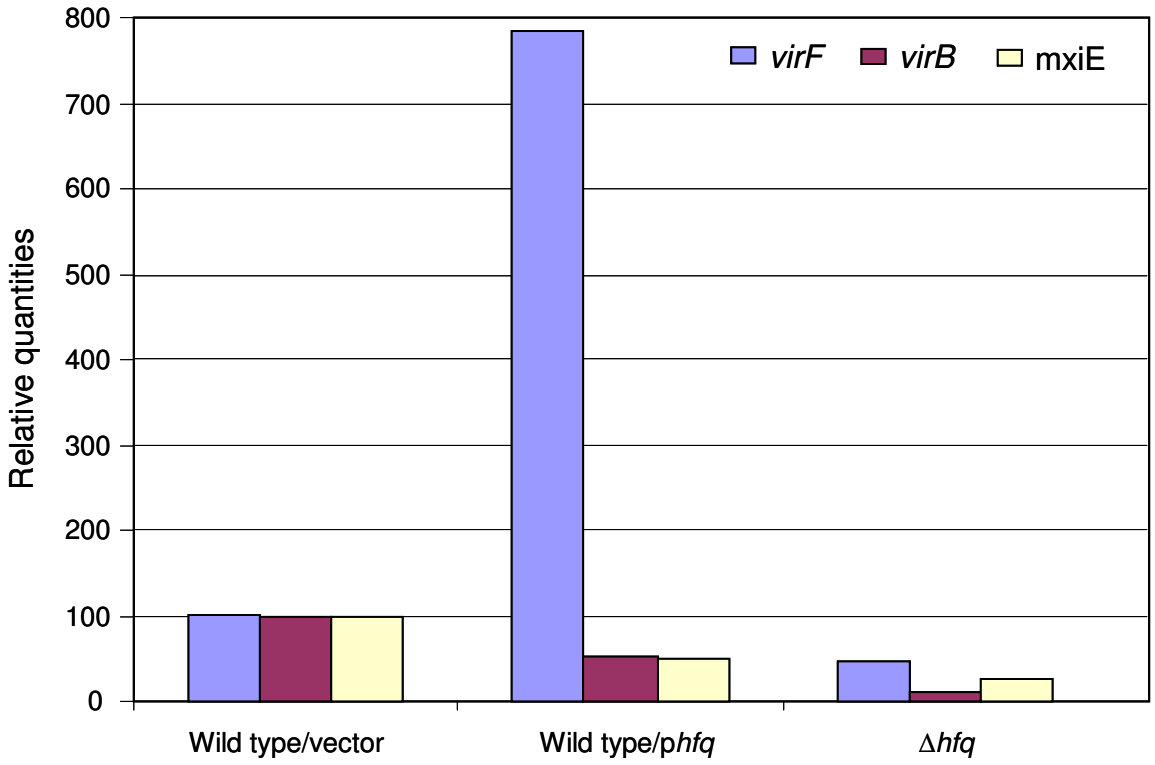


Figure 27. Increased *virF* and decreased *virB* and *mxiE* levels in wild type over-expressing *hfq*

Real-time PCR analysis showing *virF*, *virB* and *mxiE* mRNA levels in wild type SA100 carrying vector, pQE2 (wild type/vector), wild type inducing *hfq* expression from an IPTG-inducible promoter in pAKS200 (wild type/p*hfq*) and the *hfq* mutant (Δ*hfq*). All the three strains were grown on TSB agar plates containing Congo red for 14 hours at 37ºC. The wild type cells carrying pQE2 and those carrying pAKS200 were also supplemented with carbenicillin and 50µM IPTG. The level of *virF*, *virB* and *mxiE* mRNA in the *hfq* over-expressing strain and the *hfq* mutant is represented relative to that in the wild type strain. The experiment was performed in duplicate and the average values are plotted.

1. Suppression of *hfq* mutant intracellular phenotype by *mxiE*

Microarray and real-time PCR analysis results have confirmed reduced mRNA levels of all the three major *Shigella* virulence gene activators, VirF, VirB and MxiE in Δ*hfq* cells. During growth of bacteria in broth, expression of virulence gene encoding the type three secretion system is induced at 37°C, and the TTS apparatus is assembled in the bacterial envelope, but is maintained in an inactive state. Expression of invasion genes is coordinately induced in response to environmental stimuli by the transcriptional activator, VirF, which then activates transcription of *virB*, whose product is required for transcription of entry region genes. MxiE, with the chaperone IpgC acting as a co-activator, controls expression of several effectors that are induced under secretion conditions. Only a small portion of the effector and translocator proteins (IpaA-D) are secreted by *Shigella* growing in broth. However, efficient secretion is activated upon contact of *Shigella* with host cells. Down-regulation of *virF*, *virB* or *mxiE* genes could be responsible for the reduced efficiency of the *hfq* mutant to invade and spread in the human intestinal epithelial cells. To test this hypothesis, *virF*, *virB* or *mxiE* gene expression was induced from an IPTG inducible promoter on a plasmid in the *hfq* mutant strain during invasion and plaque assays. Invasion assay results showed that inducing expression of *mxiE*, but not *virF* or *virB*, in the *hfq* mutant strain abrogated the intracellular elongated cell phenotype of the *hfq* mutant (Figure 28). No effect on invasion or plaque formation was observed after expression of *mxiE* in the *hfq* mutant. Expression of *virF* and *virB* resulted in loss of invasion by the *hfq* mutant. This result could be due to non-optimal levels of VirF and VirB in the cell upon induction of *virF* and

virB with 25 μM IPTG. The imbalance in VirF and VirB levels may result in inefficient effector protein secretion, which prevents invasion of cultured epithelial cells by *S. flexneri*. These data suggest that reduced levels of *mxiE* were responsible for the elongated intracellular cell phenotype but were not solely responsible for the reduced invasion by the *hfq* mutant.

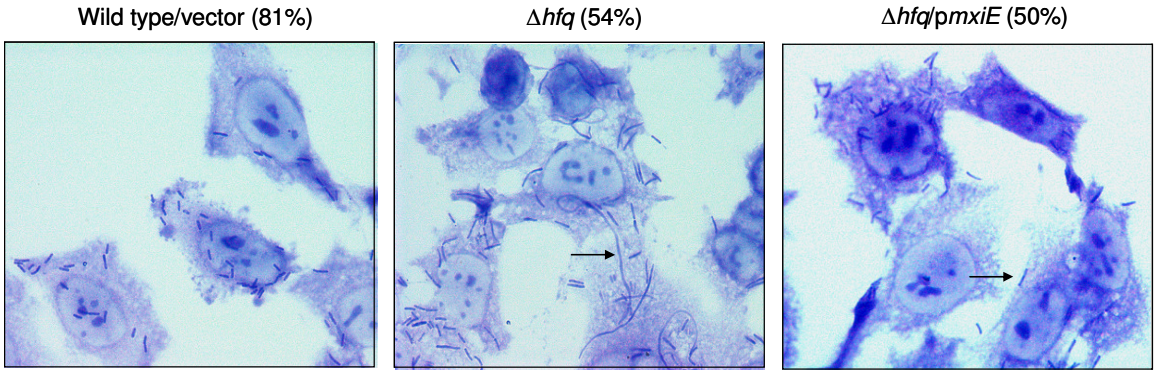


Figure 28. *mxiE* expression in the *hfq* mutant abrogates the elongated cell phenotype of the *hfq* mutant

Wild type, SA100 carrying vector, pQE2 (wild type/vector), *hfq* mutant (Δ*hfq*) and *hfq* mutant expressing *mxiE* (Δ*hfq*/p*mxiE*) from an IPTG inducible promoter in pWKS*mxiE* (2 x 10^8 cells) were incubated with Henle cell monolayer and incubated for 2 hours, washed, and stained with Wright-Giemsa and observed using an oil emersion microscope. Wild type/vector and Δ*hfq*/p*mxiE* strains were incubated in the presence of carbenicillin and 25 μM IPTG. The numbers in parentheses represent the percentages of invasion of each strain. The arrow in *hfq* mutant cells indicates elongated bacteria inside the cultured epithelial cell, and the arrow in the Δ*hfq*/p*mxiE* strain indicates abrogation of the elongated cell phenotype upon *mxiE* expression.

IV. DISCUSSION

To establish infection, *S. flexneri* must efficiently invade, multiply intracellularly and spread from one cell to another. A *dksA* mutant in *S. flexneri* was defective in intercellular spreading in cultured epithelial cells, and this phenotype correlated with aberrant IcsA localization and increased intracellular cell length (Mogull *et al.*, 2001). IcsA is a 120-kDa autotransporter protein whose carboxy-terminus forms a β-barrel in the outer membrane through which the amino-terminal portion of IcsA is transported and exposed on the bacterial surface (Goldberg *et al.*, 1993). Mutations in several genes have displayed aberrant IcsA localization including those in *icsP* encoding the protease required for cleavage of IcsA releasing the surface exposed N-terminal domain from the C-terminal domain (Egile *et al.*, 1997). This cleavage is required for the unipolar localization of IcsA on the bacterial surface. *degP*, encoding a chaperone/protease, is also involved in proper localization of IcsA in a protease independent manner (Purdy *et al.*, 2002). The chaperone activity of DegP may be responsible for proper folding in the periplasm or efficient migration of IcsA to the outer membrane. Mutations in the *Shigella* virulence plasmid located gene, *apy* which encodes apyrase, is also required for unipolar IcsA localization by an unidentified mechanism (Santapaola *et al.*, 2006).

Additionally, previous studies have shown that mutations of genes involved in the biosynthesis of the lipopolysaccharide (LPS) of *S. flexneri* affect the unipolar localization or the maintenance of IcsA, resulting in defective cell-to-cell spread (Van den Bosch and

Morona, 2003). Mutations in lipopolysaccharide biosynthesis genes results in distribution of IcsA on the entire bacterial cell surface instead of unipolar localization required for actin tail polymerization. The reduction in expression of some of the lipopolysaccharide biosynthesis genes in the *dksA* mutant could provide an explanation for the aberrant IcsA localization in the *dksA* mutant.

Mutations in several genes have been demonstrated to affect cell length of *Shigella* when present inside the cultured epithelial cells. For example, mutations in the chromosomal gene, *ispA*, show filamentous cell phenotype for intracellular *Shigella*, which affects its intracellular cell division by an unidentified mechanism (Mac Siomoin *et al.*, 1996). However, expression of *ispA* was not found altered in the *dksA* mutant compared to that in wild type cells by microarray analysis. Additionally, nucleotide auxotrophic mutants have been demonstrated to exhibit filamentous cell phenotype inside the epithelial cells during *Shigella* infection of cultured epithelial cells, suggesting the requirement for thymine in intracellular multiplication (Cersini *et al.*, 1998). An *E. coli* K12 *dksA* mutant showed multiple auxotrophic requirements for Leu, Val, Thr, Gly, Phe and Ile whereas an *S. flexneri dksA* mutant is also auxotrophic for amino acids. An *S. flexneri dksA* fails to grow in minimal medium that is not supplemented with all the twenty amino acids and vitamins (data not shown). However, it is not yet known which amino acids in particular is the *S. flexneri dksA* mutant auxotrophic for. An *S. flexneri* auxotrophic mutant for aromatic amino acids has previously been shown to be defective in intracellular multiplication and plaque formation (Cersini *et al.*, 1998). Thus, the intercellular spreading defect in the *dksA* mutant could be

attributed to its multiple amino acid auxotrophy. Mutations in *mxiE* encoding the transcriptional activator also showed increased cell length of *S. flexneri* within cultured epithelial cells, suggesting a role of MxiE and the effectors that require MxiE for their secretion (Kane *et al.*, 2002).

Mutations in several other genes have been reported to reduce the ability of *S. flexneri* to spread from one cell to another. These genes include plasmid encoded *mxiG*, which is an integral part of the *mxi-spa* encoded type three secretion apparatus, *icsB*, which is required for lysis of protrusion vacuolar membrane, *virA*, which is activated during secretion conditions, MxiE activated *ospE3* expression, which encodes for a protein that colocalizes with the focal contacts of infected epithelial cells, thus OspE3 facilitates the maintainance of an intact cell morphology and intercellular spread. Also, lack of expression from *mxiM*, *spa33*, *ipaB*, *ipaC*, *ipaD*, *virF* and *virB* during intracellular growth in the epithelial cells results in intercellular spread defects. The microarray analysis showed reduced expression of *mxiM*, *ipaB*, *spa33* and *virF*, which may contribute to the inability of the *dksA* mutant to spread from one cell to another. Chromosomal genes required for intercellular spread include *vpsA*, *-B* and *-C* (virulence protein secretion), *dsbA* (periplasmic thiol:disulfide oxidoreductase), *vacJ*, and *ipaH*. None of these genes showed altered expression in the *dksA* mutant compared to wild type cells in the microarray analysis.

DksA is a pleiotropic regulator mediating expression of several genes either at the level of transcription or post-transcriptional levels. Moreover, the presence of a zinc finger

motif in DksA confers the property of interaction with other proteins and nucleic acids. The significance of the DksA zinc finger motif for DksA regulatory activity has been shown in *P. aeruginosa* in which a *dksA* mutant lacking the zinc figer motif was unable to complement the production of virulence factors and failed to exhibit increased rRNA transcription (Perron *et al.*, 2005). The mechanism by which DksA mediates post-transcriptional regulation of genes including *rpoS*, *rhlAB* and *lasB* has not been identified. However, the mechanism by which DksA mediates transcription repression of rRNA and transcription induction of some amino acid biosynthesis genes during stringent response conditions is well understood and requires the presence of ppGpp. DksA and ppGpp are also required for virulence gene expression of enterohaemorrhagic *E. coli* (EHEC) during stationary growth phase by inducing the transcription of genes encoding the locus of enterocyte effacement (LEE) transcriptional regulators, Ler and Pch (Nakanishi *et al.*, 2006). Similar to the entry region present on the *S. flexneri* virulence plasmid, the LEE pathogenicity island encodes the type three secretion system and secreted proteins. Thus, DksA is a key regulator of several different physiological functions of the cell as well as virulence. However, the genes regulated by DksA in *S. flexneri* are not yet identified.

Microarrays were used to screen for possible DksA targets that would explain the loss of virulence in an *S. flexneri dksA* mutant. This analysis revealed that many of the known virulence genes showed reduced expression levels in a *dksA* mutant. Affected genes included those present on the virulence plasmid encoding the type three secretion system, invasion plasmid antigens and VirF, a transcriptional regulator of many of the virulence plasmid

encoded genes, as well as genes on the chromosome encoding lipopolysaccharide biosynthesis. None of the known genes affecting intracellular cell division (*ispA*, *thyA*) showed aberrant expression in the *dksA* mutant compared to that in wild type cells. Since virulence genes in addition to those in the *virF* regulon were down-regulated in the *dksA* mutant, it was likely that *dksA* affected a global regulator important for virulence in *S. flexneri*. Microarray analysis showed that expression of a gene encoding one such global regulator, *hfq*, was significantly reduced in the *dksA* mutant. The requirement of Hfq in virulence of several different bacterial systems, including *S. typhimurium*, *V. cholerae*, *E. coli*, *B. abortus*, *L. monocytogenes*, *P. aeruginosa* and *Y. enterocolitica* has been well established. Moreover, there are several common phenotypes reported for a *dksA* and an *hfq* mutant including increased cell length and decreased survival in stress conditions, which is due to their positive regulation on *rpoS* expression. These observations suggested that DksA and Hfq could be could be functioning through a common pathway where *dksA* acts upstream of *hfq* to affect virulence in *S. flexneri*. Genes involved in lipopolysaccharide bisoynthesis are downregulated in both the *dksA* and *hfq* mutants, and wild type lipopolysaccharide is required for efficient IcsA localization, which facilitates intracellular movement and intercellular spread of *S. flexneri* in host epithelial cells. These results also suggest that there are common gene targets of *dksA* and *hfq* that mediate cell morphology.

Comparing *hfq* mRNA levels in wild type and the *dksA* mutant showed about 50% reduction of *hfq* mRNA levels in the *dksA* mutant (Figure 10). Additionally, a significant decrease in the protein levels of Hfq in the *dksA* mutant compared to that in wild type cells

were observed. The reduced Hfq levels were restored to wild type levels in the *dksA* mutant complemented with the wild type *dksA* on a plasmid, suggesting that loss of *dksA* is responsible for reduced Hfq levels (Figure 11).

Although Hfq levels were decreased in the *dksA* mutant, none of the known Hfq target genes were affected in the microarray results. However, these arrays were performed with RNA isolated during exponential growth phase, and Hfq affects gene expression primarily during stationary growth phase conditions and during stress. For example, *ompA* mRNA is destabilized by Hfq only during stationary growth phase in *E. coli* (Vytvytska *et al.*, 2000). Hfq increases *rpoS* mRNA stability during stationary growth phase conditions and during intracellular survival of *S. typhimurium* in the host macrophages where they are exposed to oxidative stress (Sittka *et al.*, 2007). Additionally, Hfq is primarily a post-transcriptional regulator inhibiting or activating translation initiation via small regulatory RNAs, and in microarray analysis, no regulatory defects that affect mRNA levels would be detected.

If the loss of virulence in a *dksA* mutant was due to loss of Hfq, then inducing *hfq* expression in a *dksA* mutant should restore plaque formation in the *dksA* mutant, a hypothesis that was confirmed in this study. Decreased levels of Hfq in the *dksA* mutant appeared to be responsible for the observed lack of plaque formation, and expression of *hfq* in a *dksA* mutant restored plaque formation (Figure 24B). However, the sizes of plaques produced by the *dksA* mutant expressing Hfq from an inducible promoter were smaller than those produced by wild

type *S. flexneri*. Smaller plaques likely indicate that Hfq levels are not optimal when the gene is expressed from a plasmid but sufficient for partial complementation of the plaque defect in the *dksA* mutant. Further, over-expression of *hfq* in both the mutant and wild type *S. flexneri* adversely affected plaque formation even though there were no obvious effects on growth of the bacteria *in vitro*. Thus, the ability of *S. flexneri* to grow intracellularly and spread to adjacent cells is sensitive to both under and over-production of Hfq. Complete loss of Hfq, by deletion of the genes, also significantly reduced the ability of *hfq* mutant to form plaques (Figure 22). Interestingly, the *hfq* mutant invade inefficiently (Figure 20), a more severe phenotype than that seen with the *dksA* mutant. The residual level of Hfq in the *dksA* mutant may be sufficient for invasion, but not for efficient cell-to-cell spread.

hfq transcription during heat shock conditions occurs from the σ^{32} dependent promoter, $P1_{hfq}$, whereas during other conditions, it occurs from the σ^{70} dependent promoters $P2_{hfq}$ and $P3_{hfq}$. My data shows that DksA positively regulates transcription of *hfq* by acting primarily on one of the two σ^{70}-dependent *hfq* promoters. *hfq* transcription from $P2_{hfq}$ increased in the presence of DksA alone and was further enhanced in the presence of ppGpp, which is the stringent response regulator. ppGpp levels vary inversely with growth-rate and are induced further during stringent response growth conditions. Stringent response condition is encountered by the cell during amino acid deprivation. Thus, DksA not only positively regulates *hfq* transcription during exponential phase, but also during stringent conditions. Consistent with this data, by western blot analysis indicated, an increase in the amount of

Hfq protein in *S. flexneri* during stationary growth phase, when ppGpp levels are expected to increase (Figure 15A).

DksA increases transcription of *hfq*, and this effect may be similar to the effect of DksA on enhanced transcription of *hisG*, where binding of DksA and ppGpp increases the rate of an isomerization step towards the formation of the open complex during transcription initiation (Paul *et al.*, 2005). ppGpp binding to RNA polymerase during stringent response also has a negative effect on transcription from rRNA synthesis gene (*rrn*) promoters.. The negative effect of ppGpp on *rrn* promoters is dependent on the binding of DksA to the RNA polymerase and occurs by decreasing the half life of the open complex formed at the *rrn* promoters (Paul *et al.*, 2004). The DNA sequences required for the sensitivity of a promoter to the variable effects of ppGpp are unclear. However, promoters whose activities increase during a stringent response have A + T-rich discriminators (promoter DNA sequences between the -10 hexamer and the transcription start site), and promoters whose activities decrease have G + C-rich discriminators, suggesting that the discriminator sequence is one of the determinants for regulation by ppGpp (Josaitis *et al.*, 1995). Consistent with this observation, the $P2_{hfq}$ promoter also has an A + T-rich discriminator sequence affecting the increase in transcription from this promoter in the presence of ppGpp and DksA. DksA directly increases transcription of *hfq* ~ 4-fold in *S. flexneri* in the absence of ppGpp. Although this effect is not as large as that seen in the presence of ppGpp, it is consistent with a previous study by Paul *et. al*. (Paul *et al.*, 2005) where higher concentration of DksA alone increased the rate of isomerization of RNA polymerase from a closed complex state to an

open complex state promoting gene transcription. However, ppGpp significantly enhances the ability of DksA to promote this transition.

The effect of DksA and ppGpp, either alone or in combination, on the transcription from $P3_{hfq}$ was not as drastic as that seen with the $P2_{hfq}$. It is possible that $P3_{hfq}$ has different promoter kinetics and ppGpp/DksA are unable to overcome the energy barrier that is needed to facilitate the transition of RNA polymerase from a closed conformation to an open conformation. This effect could be due to the increased half-life of the closed RNA polymerase complex formed at the $P3_{hfq}$ promoter. A similar phenomenon has been observed at the *rrnB* P1 (dis) promoter with mutations that greatly increased the half-life of the closed RNA polymerase complex (Paul *et al.*, 2004). In general, ppGpp decreases the open complex half-lives of all the promoters tested, but at the *rrnB* P1 promoter, the open complex was longer-lived. In these longer-lived complexes, the RNA polymerase encounters the substrates for transcription and clears the promoter long before dissociation occurs. Promoters from which transcription is inhibited by ppGpp (e.g., *rrnB*), form a relatively short-lived open complex, which is further reduced by binding of ppGpp and DksA to the RNA polymerase, thereby destabilizing the open complex form before transcription initiations.

Both Hfq and DksA affect *rpoS* translation induction during stationary phase by acting on the upstream sequences of the *rpoS* mRNA leader region (Hirsch and Elliott, 2002). A *dksA* mutant fails to induce RpoS synthesis in *E. coli* upon entry into stationary phase or upon acid induction (Brown *et al.*, 2002). DksA is required for induction of *rpoS* post-

transcriptionally in a ppGpp dependent manner (Brown *et al.*, 2002). An *rpoS* mutant of *S. flexneri*, like those of *E. coli* and *S. typhimurium*, is sensitive to acid and oxidative stress. However, in contrast to *E. coli* and *S. typhimurium rpoS* mutants (Webb *et al.*, 1999), an *S. flexneri rpoS* mutant does not exhibit reduced virulence (Mogull *et al.*, 2001). The sensitivity to acid and oxidative stress in the *S. flexneri dksA* mutant, but not the loss of virulence, was rescued by inducing expression of *rpoS* in a *dksA* mutant (Mogull *et al.*, 2001).

The mechanism of post-transcriptional regulation of *rpoS* expression by DksA is not yet identified. DksA regulation requires a region downstream of the *rpoS* start codon, whereas Hfq binds at a region upstream to the start codon. The ribosome binding site of the *rpoS* mRNA leader region is trapped in a secondary structure that prevents the binding of ribosomes to the mRNA leading to repression of translation (Brown and Elliott, 1997). During stationary phase, binding of two small RNAs, DsrA and RprA frees the ribosome binding site and leads to activation of *rpoS* mRNA translation (Gottesman, 2004). Since DsrA and RprA require Hfq for their stability, and Hfq levels are reduced in a *dksA* mutant, it is possible that the sub-optimal levels of Hfq in the *dksA* mutant are responsible for the decreased expression of *rpoS* in the *S. flexneri dksA* mutant. Since the DNA sequences of *dksA* and *hfq* in *S. flexneri* are identical to those in *E. coli*, this regulatory mechanism is likely present in *E. coli* as well. Thus, it is possible that Hfq is functioning downstream of DksA in regulation of *rpoS*.

The mechanism by which Hfq promotes interactions between the sRNAs and their target mRNAs is being extensively investigated but is not fully understood. The changes introduced in the mRNA structure by Hfq opens up the RNA structure facilitating the base-pairing of the sRNA with this target mRNA. This mechanism is observed in the destabilization of *sodB* mRNA by RyhB sRNA during low iron conditions (Geissmann and Touati, 2004). Binding of Hfq to *sodB* mRNA opens the loop that contains sequences complementary to RyhB sRNA. Another observation is that Hfq binds both the sRNA and its corresponding target mRNA simultaneously, thereby increasing the local concentration (Arluison *et al.*, 2007). This result has been demonstrated for stabilization of *rpoS* mRNA by the DsrA sRNA during cold shock conditions. DsrA transcription increases during cold shock conditions and stabilizes the *rpoS* mRNA. RpoS is required for combating cold shock conditions. Hfq binds to both DsrA and *rpoS* mRNA melting the stem of *rpoS* in an irreversible manner. Increased local concentration of both RNAs and melting of *rpoS* facilitates the annealing between DsrA and *rpoS* mRNA, resulting in stabilization of the *rpoS* mRNA and enhanced translation. Hfq loses affinity to the melted form of *rpoS* mRNA and dissociates from the complex (Arluison *et al.*, 2007). Auto repression of *hfq* occurs by direct binding of Hfq to the 5' untranslated region of its own mRNA, which inhibits ribosome binding and thus subsequent transit of ribosomes through the coding sequence rendering the untranslated mRNA vulnerable to RNaseE cleavage (Vecerek *et al.*, 2005).

Taken together, these data indicate that, in *S. flexneri*, *hfq* is regulated at the transcriptional level, and this regulation is mediated in part by DksA. DksA positively

regulates *hfq* gene expression, and the level of Hfq in the cell plays an important role in controlling expression of S. *flexneri* virulence factors.

In *S. typhimurium*, Hfq-mediated regulation of *rpoS* is required for increasing expression of *Salmonella* plasmid virulence (*spv*) genes (Sittka et al., 2007). Hfq also regulates the expression of several other *spv* genes independent of *rpoS* by inducing expression of genes required for cell motility, membrane composition, adhesion and efficient effector protein secretion. Inducing expression of *hilA*, which encodes a major transcriptional activator responsible for most of the *Salmonella* pathogenicity island 1 (SPI1) type three secretion system and effector gene expression, in the *hfq* mutant restored gene expression but not protein secretion, probably due to inefficient assembly of the functional *Salmonella* pathogenicity island 1 type three secretion system (Sittka *et al.*, 2007). Thus, inducing a downstream gene in the *hfq* regulon bypasses the requirement of Hfq and partially restores initial steps of *S. typhimurium* virulence gene expression.

Shigella virulence genes encoding the type three secretion system are located on a plasmid that is believed to have been acquired by *Shigella* spp. from enteroinvasive *E. coli* by genetic mobility events (Yang *et al.*, 2007). *ipaBCD*, *virF* and *icsA* all exhibit G + C content less than the 50% G + C content of the *Shigella* chromosome, suggesting horizontal transfer of the *Shigella* virulence plasmid. Moreover the differences in the phylogenetic tree obtained for rRNA compared to that for type three secretion system tree leads to the conclusion that type three secretion systems were acquired by horizontal transfer. These gene

transfer events resulted in diversity of gene regulation. Homologs of type three secretion system encoded by the *mxi-spa* operons of *S. flexneri* are found in other pathogens and are either encoded on their chromosomes or present on a plamid. For example, in *P. aeruginosa* the genes encoding the type three secretion system are located together on the chromosome within five operons (Yahr and Wolfgang, 2006). The *S. typhimurium* chromosome possesses two virulence associated type three secretion systems encoded by the *Salmonella* pathogenicity islands 1 and 2 for intestinal and systemic phases of infection, respectively (Dieye *et al.*, 2007). In the enteropathogenic *E. coli*, the chromosomal locus encoding the type three secretion system is a 35 Kb locus designated LEE and is responsible for formation of attaching/effacing lesions in the human host (Ogino *et al.*, 2006). The *yop* virulon in *Yersinia* spp. encoding the type three secretion system on a 70 Kb virulence plasmid is the core of *Yersinia* pathogenicity (Cornelis, 2002). However, although the genes encoding the secretion apparatus are clustered, the genes encoding the secreted proteins and some transcriptional regulators are often located in unlinked positions, indicating that these secreted targets and regulators have evolved independently of the core secretion apparatus, resulting in functional type three secretion units. The *S. flexneri* VirF virulence regulon is subject to multiple regulatory inputs, resulting in a complicated regulatory cascade.

In *S. flexneri*, the *virF*, *virB* and *mxiE* regulatory genes are on the virulence plasmid along with the secretory system. The transcription of *virF* and *virB* is sensitive to environmental stimuli and DNA supercoiling and is regulated by chromosomally encoded nucleoid-associated proteins, H-NS, a general DNA binding protein, and IHF, which is

known to interact with mobile genetic elements, and Fis, a histone-like protein that controls expression of several genes by direct control of transcription initiation. Other chromosomal genes that influence the expression of plasmid-linked virulence genes have been described (Falconi *et al.*, 2001; Porter and Dorman, 1997a). For example, CpxA, the pH sensory protein of a two component system, is involved in virulence gene expression by post-transcriptional processing of VirB (Mitobe *et al.*, 2005). In this study Hfq, encoded by a chromosomal gene, known for post-transcriptional regulation of several target genes, is shown to be required for expression of *virF*, *virB* and *mxiE* virulence regulatory genes in *S. flexneri*.

In *S. flexneri*, *virF*, *virB* and *mxiE* appear as major targets of Hfq that mediate virulence. Expression of *virF* is induced at 37ºC by the DNA-binding proteins IHF and FIS, whereas expression of *virF* at lower temperatures e.g., 30ºC, is repressed by H-NS. VirF then activates the transcription of *icsA* and *virB*. VirB activates expression of the *ipa* genes and *mxiE*. My studies with an *hfq* mutant have shown reduced levels of *virF* mRNA in a *hfq* mutant compared to that in wild type *S. flexneri*. A complete loss of *virF* transcription was not observed in the *hfq* mutant. About 30% of wild type *virF* transcript levels were observed in the *hfq* mutant, which may reflect that *virF* transcription is also positively regulated by other proteins such as by the DNA binding proteins IHF and Fis during growth at 37ºC. *virB* and *mxiE* mRNA levels were also significantly reduced in the *hfq* mutant, probably because of inefficient induction of their transcription by the reduced VirF transcriptional activator levels present in the *hfq* mutant. Since an *hfq* mutant invades epithelial cells, albeit with a

lower efficiency, it is possible that the smaller amounts of VirB synthesized in the *hfq* mutant are still sufficient for some invasion in the epithelial cells. Additionally, since Hfq is a pleiotropic regulator affecting the proper functioning of cell physiology, it is possible that suppressor mutations occur in the *hfq* mutant and facilitate a limited invasion in the epithelial cells.

An *hfq* mutant also shows reduced efficiency to spread to adjacent cells, which could also be indirectly mediated by the positive effect of VirF on *icsA* expression. The absence of wild type levels of VirF results in sub-optimal IcsA synthesis and may be responsible for inefficient spread of an *hfq* mutant in plaque assays. Moreover, the *hfq* mutant showed reduced expression of several lipopolysaccharide biosynthesis genes. This result is consistent with the earlier observation with the *E. coli hfq* mutant showing aberrant cell membrane. Proper localization of IcsA requires the presence of wild type lipopolysaccharide and even small alterations of the cell membrane in the *hfq* mutant could be responsible for improper anchoring or localization of IcsA resulting in inefficient spread of the *hfq* mutant.

Increasing Hfq levels in the wild type background resulted in increased *virF* mRNA levels, suggesting a positive regulation of *virF* by Hfq. However, surprisingly the *virB* and *mxiE* mRNA levels did not increase with increasing *virF* mRNA levels. These results suggest that Hfq positively regulates *virF* mRNA expression and negatively regulates *virB* and *mxiE* expression. It is arguable that since *virB* and *mxiE* expression is known to be dependent on VirF levels, their expression should change according to the changes in VirF levels in the

cell. This result is contrary to previous data where increase in *virB* and *mxiE* levels is observed with increase in *virF* levels. One explanation is that lack of Hfq in the *hfq* mutant results in less *virF* mRNA which subsequently results in lower *virB* and *mxiE* mRNA levels in the cell. However, in Hfq over-expressing conditions, the effect of VirF on *virB* and *mxiE* expression is overcome by the repressing effect of Hfq on these mRNAs. It is not yet clear how Hfq mediates these opposite effects on *Shigella* transcription activators, but it appears that Hfq is required for maintaining optimal expression of these transcriptional activators in the cell, during different infection stages, for efficient invasion and spread in the host cell. Thus, over-expression of Hfq results in an imbalance in the levels of the major transcriptional activators of *Shigella* virulence genes. This observation is consistent with data showing reduction in plaque formation by the wild type *S. flexneri* in the presence of Hfq over-expression (Figure 24A).

mxiE expression and MxiE synthesis is initiated upon induction by VirB in *Shigella* when the bacteria are exposed to 37ºC. However, MxiE protein and its target genes are expressed only during active secretion conditions when the bacteria is present within the host cells. A *mxiE* mutant in *S. flexneri* retains the ability to invade mammalian cells, but has reduced efficiency for cell-to-cell spread compared to that of wild type cells (Kane *et al.*, 2002). Expression of *mxiE* partially abrogated the intracellular elongated cell phenotype of the *hfq* mutant, but it did not increase the overall percentage of invasion to wild type levels. This implies that reduced *mxiE* levels may cause the bacteria to form filaments inside host cells but are not solely responsible for the reduced invasion seen in the *hfq* mutant. Failure of

incomplete complementation of the *hfq* mutant by *mxiE* could be due to non-optimal levels of *mxiE* expression from the IPTG-inducible promoter, resulting in aberrant effector protein secretion intracellularly; alternatively, other genes, in addition to *mxiE* may be affected. Also, since several type three secretion structural genes, which are under the control of VirB, also showed reduced expression in the *hfq* mutant, this phenotype could result in inadequate or abnormal needle formation in the *hfq* mutant. Lack of wild type needle formation, would hinder efficient secretion of effector molecules during *mxiE* expression in the *hfq* mutant. Additionally, other factors/effectors that are part of the VirF or VirB regulon or other unidentified regulators affected by *hfq* may be playing a role in invasion, intracellular cell division and intercellular spread. The induction of VirF and VirB in the *hfq* mutant did not result in complementation of the *hfq* mutant phenotype. It is possible that these inducing conditions fail to restore the levels and secretion of the necessary effector molecules to wild type levels. Also, since the expression and secretion of the type three secretion effector molecules is concentration and time dependent, it is possible that the induction of *virF*, *virB* and *mxiE* is unable to induce optimal and timely secretion of the effector molecules to facilitate efficient invasion and spread of the *hfq* mutant in the cultured epithelial cells.

These studies have identified a *dksA* and *hfq*-mediated pathway leading to virulence in *S. flexneri* (Figure 29). Hfq mediates virulence in *S. flexneri* by increasing the expression of genes encoding the three major *Shigella* virulence gene transcriptional activators, VirF, VirB and MxiE. Inefficient expression of these virulence gene transcriptional activators results in reduced invasion and spread of the *hfq* mutant in cultured epithelial cells. *hfq* is

also required for survival in the stress conditions, such as low pH, which is experienced by shigellae within the host stomach and oxidative stress conditions in the host macrophages. An *hfq* mutant shows the elongated cell phenotype in broth as well as intracellularly, probably due to altered cell division. The presence of elongated intracellular shigellae is likely responsible for inefficient spread of the *hfq* mutant in cultured epithelial cells.

Regulation of *hfq* in *S. flexneri* is mediated by another pleiotropic regulator, DksA. The DksA protein enhances the transcription of *hfq* at the $P2_{hfq}$ promoter and is facilitated in the presence of ppGpp, suggesting that DksA enhances transcription of *hfq* during exponential growth and during stress conditions.

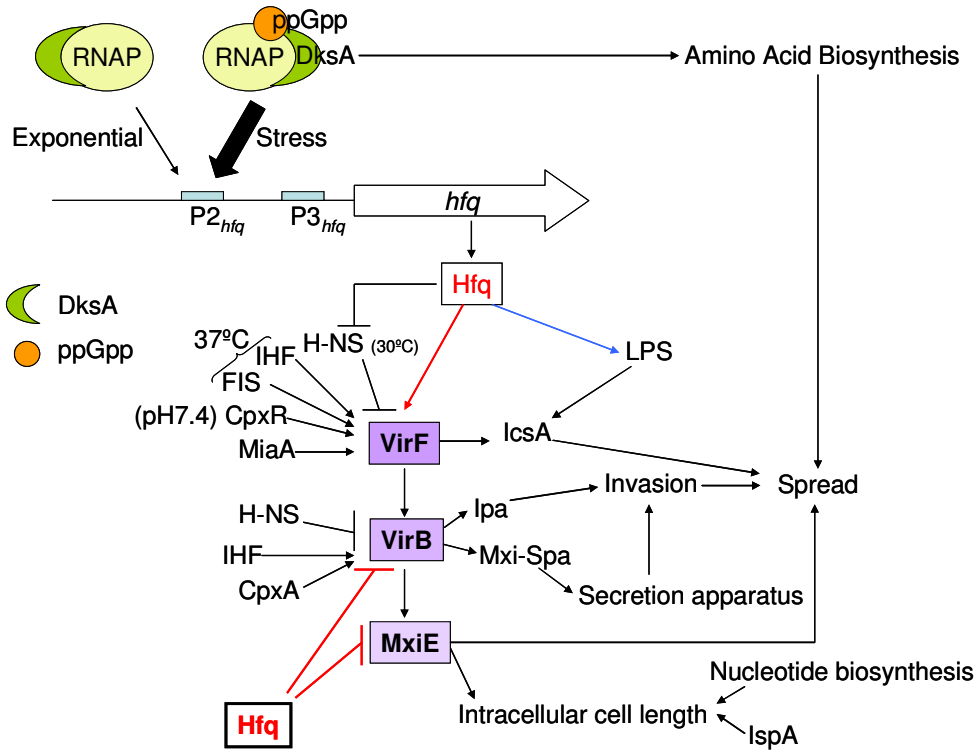


Figure 29. Summary of *dksA* and *hfq*-mediated virulence in *S. flexneri*

Positive regulation of VirF at 37ºC by IHF, FIS, CpxR and MiaA is shown by arrows and negative regulation by H-NS at 30 ºC is shown by a line. Results of this study, shown in red, indicate that Hfq also positively regulates VirF. VirF activates *virB* transcription. Synthesis of VirB, which activates *mxiE* transcription, is also negatively regulated by H-NS and positively regulated by IHF and CpxA. My studies show that high concentrations of Hfq repress *virB* and *mxiE* mRNA levels, independent of *virF* expression. Hfq was also required for expression of genes encoding lipopolysaccharide biosynthesis (blue arrow) [as shown previously by Guisbert *et al*. (2007) and by microarray data], which is required for efficient localization of IcsA and cell-to-cell spread of *S. flexneri* in cultured epithelial cells. VirB activates the *mxi-spa* that encode the needle apparatus of type three secretion system through which the VirB-activated Ipa proteins translocate and facilitate the entry of other effector molecules into the host cell, ultimately resulting in spread of shigellae from one cell to another. IspA, nucleotide biosynthesis, and MxiE are required for maintenance of intracellular cell length, which also is required for efficient cell to cell spread of *S. flexneri* in cultured cells. MxiE is required for secretion of effector molecules under post-secretion conditions resulting in efficient cell to cell spread. DksA increases transcription of *hfq* from $P2_{hfq}$ and $P3_{hfq}$, and this effect is enhanced by ppGpp at the $P2_{hfq}$ during stringent response conditions. Positive regulation of Hfq by DksA is required for virulence in *S. flexneri*. DksA and ppGpp-mediated amino acid biosynthesis is also required for efficient spread of *S. flexneri* in cultured monolayers.

APPENDIX

Table 6. Microarray analysis showing genes with reduced expression in *S. flexneri dksA* mutant

Genes with at least two-fold average repression in the *dksA* mutant are shown. The symbol ± indicates standard deviation from the mean of four independent experiments.

Number	Name	Average Fold Repression ± Standard Deviation
1	accB	2.64 ± 1.92
2	acnA	2.06 ± 1.45
3	acpP	2.06 ± 1.61
4	agp	2.13 ± 1.33
5	amyA	3.45 ± 1.53
6	arcA	2.03 ± 1.70
7	argT	2.10 ± 1.74
8	aroB	2.43 ± 1.50
9	artI	3.05 ± 2.24
10	asd	2.44 ± 1.23
11	asnS	3.14 ± 1.41
12	aspA	2.40 ± 2.25
13	atpC	2.19 ± 1.36
14	atpH	2.11 ± 1.51
15	b0919	2.40 ± 1.61
16	b1310	2.52 ± 1.38
17	b1397	2.77 ± 1.14
18	b1668	2.20 ± 1.73
19	b1725	2.17 ± 1.67
20	b1824	4.50 ± 2.24
21	b1832	4.06 ± 1.19
22	b1836	5.31 ± 1.46
23	b1837	3.42 ± 1.28
24	b1955	2.50 ± 1.05
25	b1976	2.93 ± 1.20
26	b2226	2.47 ± 1.99
27	b2463	2.46 ± 1.91
28	b2494	2.27 ± 1.46
29	b2495	3.19 ± 2.23
30	b2511	2.06 ± 1.48
31	b2659	2.83 ± 1.88
32	b2999	2.30 ± 1.71
33	b3042	2.10 ± 1.12
34	b3837	2.29 ± 1.79
35	blc	3.80 ± 2.38
36	bolA	3.77 ± 1.57
37	btuD	2.34 ± 1.12
38	clpP	2.53 ± 1.57
39	clpX	2.53 ± 1.25
40	crp	2.45 ± 1.35
41	cspC	3.71 ± 2.23
42	cspE	4.95 ± 2.92
43	cspG	2.24 ± 1.14
44	csrA	4.33 ± 2.95
45	csrB	2.84 ± 1.76
46	csrC	2.94 ± 1.39
47	cydA	2.50 ± 1.62
48	cysE	2.16 ± 1.34
49	damX	2.83 ± 2.11
50	dapD	2.61 ± 1.44
51	deaD	4.46 ± 2.47
52	dedA	2.78 ± 1.59
53	def	2.49 ± 1.45
54	deoB	2.54 ± 1.67
55	dnaG	2.31 ± 1.17
56	dnaJ	2.53 ± 1.27
57	dnaX	2.23 ± 1.11
58	dniR	2.42 ± 1.52
59	dppA	2.80 ± 1.63
60	dppB	2.44 ± 1.34
61	dut	2.10 ± 1.45
62	ecnB	3.51 ± 1.10

Number	Name	Average Fold Repression ± Standard Deviation
63	ECs1363	2.77 ± 1.03
64	elaB	7.05 ± 2.15
65	eno	4.63 ± 2.32
66	evgA	2.04 ± 1.52
67	fabF	2.29 ± 1.24
68	fadB	2.08 ± 1.41
69	fba	2.35 ± 1.29
70	fdoI	3.06 ± 2.40
71	fic	2.21 ± 1.25
72	fmt	4.06 ± 3.03
73	folE	2.74 ± 1.28
74	frdA	3.56 ± 1.82
75	frdB	2.32 ± 1.09
76	ftsJ	2.40 ± 1.35
77	ftsZ	2.82 ± 1.14
78	gadA	4.16 ± 1.99
79	galM	4.66 ± 2.20
80	galR	2.29 ± 1.92
81	gapC_1	2.64 ± 1.10
82	gapC_2	2.36 ± 1.16
83	gcpE	2.11 ± 1.18
84	gcvH	2.40 ± 1.30
85	glgA	2.88 ± 1.32
86	glgP	2.77 ± 1.37
87	gloA	2.29 ± 1.40
88	goaG	3.09 ± 1.41
89	gph	2.59 ± 1.69
90	gppA	2.21 ± 1.52
91	gpsA	2.66 ± 1.38
92	grxB	2.75 ± 1.59
93	gyrB	2.93 ± 2.16
94	hdeA	5.91 ± 2.58
95	hemC	2.38 ± 1.80
96	hemD	2.86 ± 2.00
97	hemM	3.04 ± 1.63
98	hflK	2.24 ± 1.74
99	hfq	3.64 ± 1.78
100	hha	3.48 ± 1.00
101	himA	2.51 ± 1.06
102	hisS	2.42 ± 1.38
103	hlpA	2.49 ± 2.26
104	hnr	2.06 ± 1.32
105	hns	2.73 ± 1.70
106	hupA	2.55 ± 1.44
107	icsB	2.00 ± 1.33
108	icsB	2.00 ± 1.33
109	ilvD	2.18 ± 1.30
110	imp	2.29 ± 2.08
111	infC	2.53 ± 1.48
112	insA_1	2.31 ± 1.66
113	insA_3	2.24 ± 1.25
114	ipaB	1.96 ± 1.57
115	ipgB	2.91 ± 4.67
116	ipgD	1.75 ± 1.22
117	ldcC	2.08 ± 1.16
118	lepA	2.29 ± 1.99
119	leuS	3.40 ± 1.93
120	lpp	3.87 ± 3.98
121	lpxA	2.65 ± 1.83
122	lysS	2.18 ± 1.38
123	manX	2.03 ± 1.38
124	menB	2.50 ± 1.36
125	metG	2.87 ± 2.80
126	mxiA	4.14 ± 5.20
127	mxiC	3.28 ± 2.60
128	mxiD	2.87 ± 2.59
129	mxiI	1.47 ± 1.44
130	mxiM	2.76 ± 2.11
131	oppD	2.22 ± 1.53
132	osmB	2.20 ± 1.27
133	osmB	2.55 ± 1.44
134	pabB	2.07 ± 1.86
135	parE	2.84 ± 1.58
136	pbpC	2.59 ± 1.40
137	pepP	2.12 ± 1.57
138	pflA	2.66 ± 2.30
139	pflB	4.77 ± 1.31
140	pgk	2.38 ± 1.38
141	pgm	2.00 ± 1.92
142	pheT	2.19 ± 1.57
143	poxB	2.37 ± 1.13
144	pqiB	2.03 ± 1.33
145	prlC	2.85 ± 1.40
146	proA	2.70 ± 1.86
147	pssA	2.50 ± 1.17
148	ptsH	2.20 ± 1.03

Number	Name	Average Fold Repression ± Standard Deviation
149	ptsI	3.87 ± 1.70
150	ptsN	2.81 ± 1.46
151	purA	2.09 ± 1.31
152	purU	2.09 ± 1.47
153	Rb0918b	2.19 ± 1.50
154	rcsF	2.12 ± 1.47
155	recF	2.40 ± 1.80
156	relF	2.19 ± 1.32
157	rfbG	2.11 ± 1.19
158	rfbJ	1.55 ± 1.31
159	rho	3.17 ± 1.83
160	rhoL	2.94 ± 1.61
161	ribB	3.03 ± 1.17
162	ribF	2.15 ± 2.12
163	rmf	6.57 ± 4.99
164	rpmG	3.11 ± 3.85
165	rpoB	2.37 ± 2.25
166	rpoH	3.08 ± 1.51
167	rpoS	1.58 ± 1.05
168	rpsT	2.23 ± 1.61
169	rpsU	2.69 ± 2.62
170	rseA	2.79 ± 1.64
171	rseC	2.02 ± 1.21
172	S0373	2.15 ± 1.63
173	S2711	2.12 ± 1.09
174	slyD	2.55 ± 1.86
175	smg	2.98 ± 1.76
176	smpA	2.29 ± 1.62
177	SP0003	2.37 ± 1.22
178	SP0088	2.34 ± 2.74
179	SP0176	2.18 ± 1.67
180	SP0205	3.08 ± 1.12
181	spa15	2.35 ± 1.35
182	spa29	1.64 ± 1.68
183	spa32	2.62 ± 1.96
184	spa33	2.12 ± 1.54
185	spa9	2.09 ± 1.80
186	sraJ	2.56 ± 1.44
187	sspB	2.04 ± 1.36
188	sulA	2.70 ± 1.06
189	sun	2.10 ± 1.14
190	tdh	2.04 ± 1.39
191	tktB	2.73 ± 1.21
192	tldD	2.30 ± 1.43
193	tpiA	4.64 ± 1.27
194	tpke11	3.11 ± 2.43
195	tpx	2.25 ± 1.68
196	trpA	3.28 ± 1.95
197	trpC	2.12 ± 1.87
198	truA	2.76 ± 1.86
199	tyrS	2.05 ± 1.21
200	ugd	3.21 ± 1.75
201	ushA	2.35 ± 1.24
202	uvrY	2.17 ± 1.47
203	uxaB	2.30 ± 1.96
204	vacB	1.79 ± 1.31
205	virF	2.63 ± 1.34
206	waaI	2.56 ± 2.00
207	wrbA	5.03 ± 1.30
208	yadB	2.16 ± 1.80
209	yadF	2.57 ± 1.62
210	yaeO	2.17 ± 1.62
211	ybaU	2.42 ± 1.48
212	ybcW	5.41 ± 2.90
213	ybeN	2.23 ± 1.69
214	ybgL	2.24 ± 1.49
215	ybiP	2.05 ± 1.58
216	ycbB	2.93 ± 1.11
217	ycbK	2.91 ± 1.29
218	ycbL	2.61 ± 1.30
219	yccA	2.69 ± 1.47
220	yccD	5.13 ± 3.09
221	yccJ	9.33 ± 3.24
222	yccV	2.40 ± 1.33
223	yceF	2.77 ± 1.61
224	ycfO	2.26 ± 1.21
225	ychB	2.05 ± 1.45
226	yciC	2.03 ± 2.05
227	yciG	2.03 ± 1.86
228	ydcC	3.04 ± 1.77
229	yddM	2.42 ± 1.55
230	yebA	2.34 ± 2.99
231	yebG	2.07 ± 1.28
232	yecC	2.05 ± 1.69
233	yehZ	2.48 ± 1.46
234	yejM	2.03 ± 1.07

Number	Name	Average Fold Repression ± Standard Deviation
235	yfcG	2.41 ± 1.46
236	yfiA	7.30 ± 1.37
237	ygaG	3.25 ± 1.71
238	ygaH	2.26 ± 1.81
239	ygdK	3.66 ± 3.12
240	ygiB	2.29 ± 1.51
241	ygiC	2.90 ± 1.21
242	ygiN	2.51 ± 1.81
243	ygjI	3.38 ± 1.50
244	yhbG	2.57 ± 1.28
245	yhbH	2.64 ± 1.46
246	yhbJ	3.47 ± 1.77
247	yhbM	2.29 ± 1.99
248	yhbN	2.44 ± 1.24
249	yhdE	2.80 ± 1.41
250	yheU	2.12 ± 1.44
251	yhfG	2.42 ± 1.53
252	yhhA	2.72 ± 1.62
253	yhiW	4.14 ± 1.41
254	yhiX	8.12 ± 1.22
255	yi21_1	3.20 ± 1.78
256	yi21_4	2.97 ± 1.52
257	yi21_5	2.44 ± 1.58
258	yi22_2	3.14 ± 1.85
259	yi22_3	2.07 ± 1.38
260	Yi22_4	2.49 ± 1.43
261	Yi22_5	2.37 ± 1.40
262	Yi22_6	2.66 ± 1.16
263	yiaG	2.27 ± 1.74
264	yibF	3.48 ± 1.19
265	yibO	2.82 ± 2.12
266	yieP	2.82 ± 2.07
267	yigL	3.25 ± 2.10
268	yihK	2.20 ± 1.27
269	yjaE	3.89 ± 1.48
270	yjaG	2.59 ± 1.52
271	yjbJ	2.03 ± 1.76
272	yjbQ	2.39 ± 1.81
273	yjjP	7.18 ± 5.95
274	yncB	2.18 ± 1.42
275	yqgE	2.07 ± 1.34
276	yqiB	2.57 ± 2.07
277	yqjC	2.22 ± 1.78
278	yqjD	3.33 ± 1.06
279	yqjE	2.28 ± 1.46
280	yraM	2.32 ± 1.58
281	yrbK	2.39 ± 1.71
282	yrdC	3.46 ± 1.57
283	yrdD	2.26 ± 1.78
284	yrfE	2.20 ± 1.29
285	ytfP	2.26 ± 1.47
286	Z1134	3.03 ± 1.74
287	Z1150	2.25 ± 1.93
288	Z1589	2.14 ± 1.84
289	Z4695	3.19 ± 1.16
291	Z5401	2.10 ± 1.73
292	zipA	2.18 ± 1.88
293	zwf	2.44 ± 1.88

Table 7. Microarray analysis showing genes with increased expression in *S. flexneri dksA* mutant

Genes with at least two-fold average induction in the *dksA* mutant are shown. The symbol ± indicates standard deviation from the mean of four independent experiments.

Number	Name	Average Fold Induction ± Standard Deviation
1	adiA	2.03 ± 1.28
2	Aer	4.99 ± 3.11
3	araF	3.78 ± 4.15
4	araJ	2.34 ± 1.49
5	arsR	2.23 ± 1.60
6	b0024	2.20 ± 1.63
7	b0309	3.15 ± 1.72
8	b0816	2.12 ± 1.48
9	b0817	2.36 ± 1.53
10	b0836	2.31 ± 1.87
11	b1152	2.20 ± 1.29
12	b1171	2.65 ± 1.69
13	b1462	2.07 ± 1.38
14	b1486	2.39 ± 1.96
15	b1522	3.07 ± 2.86
16	b1553	2.29 ± 1.52
17	b1565	2.51 ± 2.46
18	b1590	3.50 ± 2.80
19	b1722	2.82 ± 1.54
20	b1758	2.19 ± 1.42
21	b1964	2.19 ± 1.37
22	b2247	2.57 ± 1.42
23	b2291	3.29 ± 3.79
24	b2342	2.10 ± 1.51
25	b2363	2.18 ± 1.66
26	b2635	3.99 ± 3.80
27	b2639	3.16 ± 3.70
28	b2680	2.36 ± 1.59
29	b2710	2.09 ± 1.24
30	b2736	2.94 ± 2.19
31	b2792	2.15 ± 1.29
32	b2853	2.55 ± 1.28
33	b3051	2.78 ± 2.42
34	b4286	3.20 ± 3.05
35	betI	2.57 ± 1.72
36	bioC	5.03 ± 6.48
37	C0362	2.11 ± 1.24
38	C0719	2.06 ± 1.42
39	caiD	2.17 ± 1.47
40	caiT	3.19 ± 3.31
41	carA	3.14 ± 1.45
42	chuS	2.27 ± 1.42
43	chuW	2.21 ± 1.37
44	citB	2.91 ± 1.78
45	cmr	2.01 ± 1.06
46	cpdB	3.94 ± 2.22
47	crcB	2.01 ± 1.42
48	creB	3.06 ± 1.12
49	csgB	2.41 ± 1.34
50	cspB	2.03 ± 1.87
51	cyaY	2.98 ± 2.36
52	cynX	2.88 ± 2.19
53	cyoA	3.13 ± 2.10
54	cyoE	3.41 ± 2.05
55	cysJ	2.27 ± 1.50
56	dcp	2.34 ± 1.28
57	dicC	2.86 ± 2.81
58	ebgA	5.25 ± 3.27
59	ECs1585	2.25 ± 1.87
60	ECs1596	2.04 ± 1.23
61	ECs1644	2.24 ± 1.55
62	ECs4945	3.45 ± 2.10
63	ECs4949	5.36 ± 2.93
64	ECs4969	3.58 ± 1.96
65	ECs4973	2.54 ± 1.85
66	ECs4977	3.15 ± 2.09
67	ECs4993	2.58 ± 1.72
68	ECs4997	2.12 ± 1.69
69	fadR	3.47 ± 3.35
70	fdnG	3.23 ± 2.49
71	fimE	2.76 ± 2.33
72	fixX	3.57 ± 3.35
73	fruA	3.56 ± 1.16
74	ftn	2.08 ± 1.15

Number	Name	Average Fold Induction ± Standard Deviation
75	galK	2.42 ± 1.80
76	gamW	2.38 ± 1.44
77	glnQ	2.02 ± 1.73
78	hisF	2.33 ± 2.42
79	hofQ	2.46 ± 1.53
80	htgA	3.76 ± 2.99
81	hycB	2.04 ± 1.42
82	hycI	2.83 ± 2.58
83	hydN	3.39 ± 2.53
84	icdA	2.90 ± 2.46
85	intE	2.95 ± 1.72
86	kdpA	3.81 ± 2.38
87	malY	2.58 ± 1.37
88	mdlA	2.62 ± 2.08
89	mhpC	2.22 ± 1.64
90	Mob9	2.44 ± 1.64
91	Nac	2.48 ± 1.44
92	narI	2.79 ± 2.20
93	Nei	2.14 ± 2.15
94	nuoB	3.12 ± 2.33
95	pflC	2.32 ± 1.24
96	phoR	3.34 ± 2.50
97	potC	2.00 ± 1.43
98	ppdB	2.36 ± 1.44
99	pspA	4.50 ± 3.06
100	purN	2.15 ± 1.94
101	purT	3.17 ± 1.74
102	pyrB	3.81 ± 1.68
103	pyrH	2.30 ± 1.76
104	rimK	3.19 ± 3.09
105	Rne	2.16 ± 1.60
106	rplA	2.12 ± 1.39
107	S3185	2.97 ± 1.59
108	S4826	2.07 ± 1.54
109	sbcC	2.36 ± 1.27
110	sdhD	3.42 ± 1.36
111	sdiA	2.22 ± 1.40
112	sgcE	2.24 ± 1.23
113	slpA	2.16 ± 1.73
114	sucA	2.31 ± 1.46
115	tdcD	2.47 ± 1.49
116	thiJ	2.03 ± 1.82
117	treR	2.52 ± 1.97
118	trxB	2.04 ± 1.41
119	uidR	2.55 ± 1.54
120	wbdO	2.36 ± 1.83
121	xerC	2.14 ± 1.55
122	yahC	2.51 ± 1.40
123	yahK	2.30 ± 1.55
124	yahoo	2.33 ± 1.97
125	yaiC	4.54 ± 4.84
126	yaiN	2.83 ± 1.48
127	yajI	2.20 ± 1.56
128	ybaK	2.56 ± 2.34
129	ybaX	2.50 ± 1.47
130	ybbJ	2.15 ± 1.12
131	ybbM	2.51 ± 1.61
132	ybfD	2.06 ± 1.33
133	ybgI	2.41 ± 2.51
134	ybgQ	2.36 ± 1.57
135	ybiB	2.56 ± 2.30
136	ybiK	2.75 ± 2.49
137	ybiO	2.25 ± 1.61
138	ybiT	3.35 ± 2.43
139	ybiX	2.61 ± 2.16
140	ybiY	2.98 ± 2.17
141	ybjH	2.87 ± 1.12
142	ycfC	3.50 ± 2.06
143	ycjC	2.29 ± 1.41
144	ycjS	2.43 ± 1.34
145	ycjW	2.18 ± 1.21
146	ydeK	2.57 ± 1.34
147	ydeY	10.26 ± 1.15
148	ydfC	2.37 ± 1.66
149	ydfI	3.93 ± 2.11
150	ydfM	4.37 ± 2.40
151	yecK	3.00 ± 1.91
152	yedL	2.45 ± 1.42
153	yfbB	2.75 ± 2.90
154	yfbL	2.97 ± 2.48
155	yfcB	2.25 ± 1.32
156	yfeR	2.00 ± 1.46
157	yfjH	2.17 ± 1.39
158	yfjO	3.88 ± 3.48
159	ygaA	2.82 ± 1.78
160	ygaE	3.76 ± 2.08
161	ygaF	2.44 ± 2.91
162	ygbA	2.89 ± 1.73

Number	Name	Average Fold Induction ± Standard Deviation
163	ygcP	3.38 ± 1.29
164	ygcY	2.19 ± 1.40
165	ygjK	2.53 ± 1.32
166	yhdT	2.39 ± 1.48
167	yhhL	2.27 ± 2.52
168	yhhP	2.62 ± 2.61
169	yhhT	4.42 ± 2.08
170	yhhZ	2.94 ± 2.96
171	yhiH	3.75 ± 2.21
172	yhiL	4.14 ± 2.39
173	yhiO	2.52 ± 2.37
174	yhjH	2.08 ± 1.41
175	yi81_1	3.79 ± 3.22
176	yiaC	2.74 ± 1.59
177	yiaF	2.13 ± 1.15
178	yiaN	2.09 ± 1.70
179	yidI	3.11 ± 1.45
180	yihA	3.61 ± 2.88
181	yihT	2.12 ± 1.45
182	yihX	2.29 ± 2.38
183	yiiE	3.78 ± 3.70
184	yijF	2.20 ± 1.30
185	yjcO	2.60 ± 1.66
186	yjcZ	2.29 ± 1.72
187	yjfG	2.57 ± 1.69
188	yjgN	2.00 ± 1.40
189	yjiG	2.14 ± 1.48
190	yjiS	2.38 ± 1.27
191	ykgB	2.21 ± 1.59
192	ykgD	2.91 ± 1.63
193	ymfD	2.70 ± 1.88
194	ymgE	5.79 ± 3.66
195	yraR	2.41 ± 1.59
196	yrbE	2.38 ± 1.55
197	yrfD	2.13 ± 1.43
198	ytfE	2.39 ± 1.47
199	ytfK	2.46 ± 1.61
200	Z0251	2.18 ± 1.20
201	Z0254	2.13 ± 1.36
202	Z0255	2.57 ± 1.32
203	Z0312	2.18 ± 1.38
204	Z0336	2.21 ± 1.31
205	Z0340	2.30 ± 1.42
206	Z0346	2.18 ± 1.33
207	Z0366	2.06 ± 1.21
208	Z0415	2.22 ± 1.70
209	Z0418	2.18 ± 1.30
210	Z0638	3.05 ± 1.59
211	Z1184	2.26 ± 1.78
212	Z1190	2.43 ± 1.53
213	Z1197	2.11 ± 1.21
214	Z1225	2.10 ± 1.32
215	Z1350	2.51 ± 2.14
216	Z1355	2.32 ± 2.48
217	Z1382	2.65 ± 2.70
218	Z1443	3.05 ± 1.53
219	Z1447	2.91 ± 1.43
220	Z1467	3.18 ± 1.71
221	Z1479	2.24 ± 1.46
222	Z1486	2.00 ± 1.28
223	Z1576	2.68 ± 1.24
224	Z1580	2.18 ± 1.51
225	Z1600	2.50 ± 1.78
226	Z1629	2.34 ± 1.35
227	Z1800	2.21 ± 1.02
228	Z1806	3.24 ± 1.33
229	Z1810	2.41 ± 1.45
230	Z2070	2.22 ± 1.92
231	Z2108	2.53 ± 1.98
232	Z2115	2.35 ± 2.22
233	Z2132	2.46 ± 1.83
234	Z2136	3.31 ± 3.46
235	Z2140	3.48 ± 3.57
236	Z2148	2.19 ± 1.59
237	Z2200	2.90 ± 2.74
238	Z2255	2.11 ± 1.59
239	Z2346	3.98 ± 1.89
240	Z2351	2.39 ± 1.14
241	Z2804	2.28 ± 1.49
242	Z2979	2.05 ± 1.32
243	Z2988	2.11 ± 1.23
244	Z2991	2.20 ± 1.32
245	Z3269	3.11 ± 1.94
246	Z3307	3.69 ± 1.91
247	Z3316	4.27 ± 1.58
248	Z3354	2.18 ± 1.28
249	Z4064	2.48 ± 1.64
250	Z4068	2.23 ± 1.56

Number	Name	Average Fold Induction ± Standard Deviation
251	Z4313	2.09 ± 1.41
252	Z4322	3.67 ± 3.57
253	Z4384	3.67 ± 3.83
254	Z4474	2.46 ± 2.56

Number	Name	Average Fold Induction ± Standard Deviation
255	Z4853	2.35 ± 1.47
256	Z5618	4.10 ± 1.94
257	Z5886	2.34 ± 1.91

Table 8. Microarray analysis showing genes with reduced expression in *S. flexneri* *hfq* mutant

Genes with at least two-fold average repression in the *hfq* mutant are shown. The symbol ± indicates standard deviation from the mean of four independent experiments.

Number	Name	Average Fold Repression ± Standard Deviation	Number	Name	Average Fold Repression ± Standard Deviation
1	apyrase	7.09 *	39	mxiI	2.53 ± 2.22
2	argF	2.13 *	40	mxiL	16.07 *
3	atpC	2.05 ± 1.10	41	mxiM	2.68 ± 1.96
4	b0671	2.13 ± 1.13	42	nhaA	2.02 ± 1.25
5	b0725	4.55 ± 1.11	43	nuoK	2.28 ± 1.18
6	b1788	2.08 ± 1.05	44	nusA	2.11 ± 1.11
7	b3472	2.53 ± 1.43	45	proA	2.05 ± 1.28
8	crcA	2.10 ± 1.32	46	proV	2.01 ± 1.47
9	dksA	2.11 ± 1.17	47	Rb0918b	9.20 *
10	dnaJ	2.20 ± 1.07	48	rfbU	3.33 ± 1.18
11	ECs1062	2.08 ± 1.08	49	rnhA	2.00 ± 1.25
12	ECs3851	2.08 ± 1.49	50	rpoS	2.57 ± 1.56
13	ECs4472	2.13 ± 1.61	51	Rv0919b	7.26 *
14	entE	2.02 ± 1.23	52	S3211	2.00 ± 1.37
15	fumB	2.72 *	53	sdhC	2.10 ± 1.41
16	fur	2.10 ± 1.84	54	selD	2.96 ± 1.36
17	gst	2.22 ± 1.89	55	sepA	5.68 ± 1.61
18	hfq	11.24 ± 1.37	56	shf	7.09 ± 1.13
19	icsB	16.27 *	57	sigA	2.26 ± 1.33
20	infC	2.02 ± 1.86	58	slyD	2.03 ± 1.24
21	ipaA	4.91 ± 1.59	59	SP0007	4.21 ± 2.28
22	ipaC	27.55 *	60	SP0016	6.51 ± 2.50
23	ipaD	5.13 ± 1.23	61	SP0066	5.36 ± 1.51
24	ipaJ	3.02 ± 2.20	62	SP0122	3.13 ± 1.21
25	ipgA	29.21 ± 1.53	63	SP0176	3.13 ± 1.48
26	ipgB	25.19 ± 1.12	64	SP0205	2.25 ± 1.16
27	ipgC	18.75 ± 1.82	65	SP0290	2.31 ± 1.01
28	ipgE	43.87 *	66	spa13	5.00 ± 1.15
29	ipgF	34.91 ± 1.21	67	spa24	2.66 ± 1.85
30	ipgH	2.34 ± 1.40	68	spa29	2.20 ± 1.77
31	kefB	2.01 ± 1.29	69	spa32	6.65 ± 2.24
32	leuS	2.06 ± 1.27	70	spa33	2.45 ± 1.34
33	mvpT	7.77 ± 1.07	71	spa40	2.61 ± 2.08
34	mxiA	9.24 ± 1.89	72	spa47	7.07 ± 3.02
35	mxiC	8.35 ± 1.21	73	spa9	2.47 ± 1.42
36	mxiE	33.75 *	74	spa-orf10	2.11 ± 1.40
37	mxiG	21.89 *	75	spa-orf11	6.17 *
38	mxiH	33.30 ± 1.33	76	spf	3.27 ± 1.26

Number	Name	Average Fold Repression ± Standard Deviation	Number	Name	Average Fold Repression ± Standard Deviation
77	sraC	2.18 ± 1.31	94	yecI	3.30 ± 1.31
78	sucD	3.46 ± 1.17	95	yfjD	2.67 ± 1.32
79	thrL	2.11 ± 1.66	96	ygiN	2.18 ± 1.27
80	trcA	3.68 ± 1.20	97	yhjQ	2.20 ± 1.57
81	uspA	2.44 ± 1.29	98	yi21_1	2.54 ± 1.38
82	virA	5.28 ± 1.19	99	yi21_4	2.41 ± 1.63
83	virB	10.94 ± 2.13	100	yjfN	2.67 ± 1.65
84	yafA	2.15 ± 1.40	101	yjfR	2.00 ± 1.19
85	ybeM	2.05 ± 1.28	102	ykfD	2.14 ± 1.27
86	ybeW	2.08 ± 1.00	103	ynaC	2.06 ± 1.50
87	ybgR	2.39 ± 1.35	104	yqfE	2.05 *
88	ybjG	2.12 ± 1.34	105	yqiB	2.04 ± 1.42
89	ycdO	2.02 ± 1.42	106	Z0391	2.17 ± 1.26
90	yceB	2.08 ± 1.25	107	Z0950	2.03 ± 1.21
91	ydaW	2.06 *	108	Z1567	2.03 ± 2.64
92	ydbH	2.12 ± 1.31	109	Z2080	2.08 ± 1.06
93	yeaH	2.06 ± 1.35	110	Z2085	2.01 ± 1.43

* Fewer than three data points were available for these genes.

Table 9. Microarray analysis showing genes with increased expression in *S. flexneri* *hfq* mutant

Genes with at least two-fold average induction in the *hfq* mutant are shown. The symbol ± indicates standard deviation from the mean of four independent experiments.

Number	Name	Average Fold Induction ± Standard Deviation
1	adhP	2.23 ± 1.46
2	aefA	2.86 ± 2.24
3	aer	2.30 ± 1.17
4	afuB	2.66 *
5	agaD	2.28 ± 1.71
6	agaZ	2.11 ± 1.69
7	alaS	2.97 *
8	alkB	2.26 ± 1.70
9	amn	2.40 ± 1.94
10	araC	2.01 ± 1.31
11	araF	2.13 ± 1.11
12	arcA	2.26 ± 1.64
13	argH	2.39 ± 1.47
14	arsR	2.48 ± 1.39
15	artP	2.40 ± 1.08
16	b0024	4.49 ± 2.39
17	b0309	2.97 ± 2.23
18	b0373	3.42 *
19	b0392	2.32 ± 1.62
20	b0669	2.11 ± 2.13
21	b0753	4.26 *
22	b0816	3.47 ± 2.69
23	b0832	2.98 ± 3.70
24	b0836	2.90 ± 3.17
25	b0844	2.11 ± 1.20
26	b0872	2.38 ± 1.49
27	b1152	2.12 ± 1.04
28	b1171	2.97 ± 1.29
29	b1284	2.17 ± 1.36
30	b1337	5.02 *
31	b1341	6.69 *
32	b1367	2.03 ± 1.85
33	b1486	2.33 ± 1.75
34	b1498	2.37 ± 1.79
35	b1502	2.79 ± 1.85
36	b1506	2.30 ± 2.64
37	b1518	2.23 *
38	b1522	2.35 *
39	b1565	2.55 ± 1.87
40	b1586	2.69 ± 2.23
41	b1590	2.59 ± 1.65
42	b1598	2.70 ± 2.05
43	b1726	2.24 ± 2.17
44	b1731	2.46 ± 2.33
45	b1841	2.40 ± 1.43
46	b1953	2.03 ± 1.16
47	b1956	3.05 ± 2.58
48	b1964	2.07 ± 1.19
49	b1972	5.64 ± 1.27
50	b1978	2.80 ± 1.87
51	b2001	2.70 ± 2.23
52	b2072	2.17 ± 1.52
53	b2080	2.06 ± 1.76
54	b2247	2.16 ± 1.03
55	b2255	3.50 ± 1.96
56	b2275	5.02 *
57	b2291	2.44 ± 1.77
58	b2295	2.53 ± 1.69
59	b2299	3.31 *
60	b2326	2.25 ± 1.83
61	b2334	3.15 ± 2.73
62	b2342	3.01 ± 2.14
63	b2351	2.53 ± 2.08
64	b2359	2.88 ± 1.94
65	b2363	3.25 ± 1.63
66	b2375	2.46 ± 1.62
67	b2383	3.25 ± 1.94
68	b2387	5.76 *
69	b2391	2.40 ± 2.07
70	b2431	3.05 ± 2.65
71	b2444	2.32 ± 1.74
72	b2460	2.71 ± 2.62
73	b2635	2.45 ± 2.27
74	b2651	3.28 ± 2.00
75	b2656	2.05 ± 1.07
76	b2736	2.70 ± 3.12

Number	Name	Average Fold Induction ± Standard Deviation
77	b2740	3.01 ± 2.66
78	b2756	4.41 ± 2.03
79	b2772	2.57 ± 1.31
80	b2792	2.04 ± 1.59
81	b2931	2.06 ± 1.69
82	b3015	6.26 *
83	b3023	2.44 ± 2.67
84	b3027	2.26 ± 2.17
85	b3051	2.31 ± 1.86
86	b3100	2.42 ± 1.99
87	b3914	2.18 ± 1.17
88	betI	5.23 ± 1.39
89	bioC	2.72 ± 1.04
90	blc	2.44 ± 1.09
91	btuE	2.56 *
92	C0293	2.42 ± 1.94
93	C0362	3.06 ± 1.13
94	C0465	4.66 *
95	caiD	2.46 ± 1.00
96	caiT	2.07 ± 1.30
97	carA	2.13 ± 1.88
98	ccmA	2.35 ± 1.75
99	ccmE	3.05 *
100	cdd	3.29 ± 1.37
101	chpB	2.38 *
102	clpB	2.43 ± 1.75
103	cls	2.46 ± 2.76
104	cobB	2.99 *
105	codA	3.97 ± 1.83
106	cpdB	2.00 ± 1.06
107	cpsG	2.11 ± 1.57
108	cpxR	2.82 ± 1.52
109	crr	2.37 ± 2.36
110	csrB	3.13 ± 1.16
111	csrC	4.53 *
112	cyaY	2.78 ± 1.60
113	cynX	2.04 ± 1.27
114	cyoA	3.21 *
115	cyoE	3.24 ± 1.08
116	cysD	2.64 ± 2.07
117	cysJ	3.56 ± 2.06
118	cysM	2.68 ± 1.82
119	cysZ	2.96 ± 2.23
120	dcp	2.46 ± 1.08
121	ddlA	2.67 ± 1.82
122	ddlB	2.25 ± 1.28
123	dicC	3.01 ± 1.46
124	dnaC	2.53 ± 1.21
125	dppD	2.62 ± 1.53
126	dps	2.70 ± 1.49
127	dxs	3.65 ± 1.85
128	eaeH	2.52 *
129	ECs0810	2.01 ± 1.63
130	ECs1170	2.81 ± 2.76
131	ECs1226	2.87 ± 2.52
132	ECs1231	2.25 ± 2.04
133	ECs1234	2.48 ± 2.05
134	ECs1315	2.47 ± 2.04
135	ECs1418	2.44 ± 1.38
136	ECs1562	2.06 *
137	ECs1569	2.28 *
138	ECs1579	2.30 *
139	ECs3003	4.12 ± 1.60
140	ECs3237	3.80 ± 1.69
141	ECs4548	3.25 ± 1.55
142	ECs4656	3.49 ± 2.15
143	ECs4865	4.08 ± 1.53
144	ECs4949	3.18 ± 1.05
145	ecs4957	3.04 ± 1.86
146	ECs4961	2.65 ± 1.79
147	ECs4965	2.87 ± 1.34
148	ECs4981	3.14 ± 1.45
149	ECs4985	2.97 ± 1.82
150	ECs4989	2.77 ± 1.85
151	ECs4992	2.02 ± 1.32
152	ECs4997	2.03 *
153	ECs5247	2.82 ± 1.06
154	emrR	2.55 ± 1.28
155	emrY	3.93 ± 2.34
156	escT	2.06 ± 2.53
157	espF	2.37 ± 2.55
158	eutC	2.43 ± 2.18
159	eutI	2.10 ± 1.90
160	fabB	2.96 ± 1.06
161	fabD	3.75 *
162	fadR	2.73 ± 1.26
163	fdnG	2.09 *
164	fepB	2.10 ± 1.92
165	fimD	2.18 ± 2.09
166	fimE	2.69 ± 1.41
167	finO	2.21 ± 1.01
168	fixX	3.89 ± 1.07
169	flgE	2.73 ± 1.40
170	flgI	3.79 *

Number	Name	Average Fold Induction ± Standard Deviation
171	fliG	4.28 ± 1.77
172	fliK	2.95 ± 1.69
173	fliO	3.35 ± 1.48
174	fliQ	2.04 ± 1.51
175	fliY	4.34 ± 1.16
176	folC	2.06 ± 1.10
177	folX	5.31 *
178	frdB	2.47 ± 2.96
179	fruR	2.50 ± 1.45
180	frvA	2.47 ± 1.83
181	ftsX	2.45 ± 1.44
182	fucU	2.78 ± 2.12
183	gadA	2.98 ± 2.09
184	galK	4.38 *
185	gcvA	2.96 ± 1.77
186	gcvH	3.27 ± 1.41
187	gcvP	2.49 ± 1.18
188	glgC	3.03 ± 1.44
189	glpD	2.26 ± 2.82
190	glpQ	2.16 ± 2.24
191	gltL	2.08 ± 2.17
192	gnd	3.12 ± 1.31
193	gntP	2.46 ± 1.58
194	gntU_1	2.11 ± 1.04
195	greB	3.39 ± 1.24
196	gsk	3.00 *
197	gyrA	3.83 ± 2.17
198	gyrB	2.23 ± 1.14
199	hdeB	2.46 ± 1.19
200	hemB	2.37 ± 1.39
201	hemX	2.42 ± 1.23
202	hisC	2.61 ± 1.90
203	hisF	3.70 ± 1.90
204	hisJ	2.33 ± 1.55
205	hisM	4.56 *
206	hns	2.07 ± 1.25
207	hns	2.11 ± 1.52
208	hofQ	2.05 ± 1.66
209	hpaX	2.08 ± 1.06
210	hslV	6.07 ± 2.02
211	htgA	3.17 ± 1.30
212	htpG	2.98 *
213	htrA	2.72 ± 2.58
214	hupB	2.07 ± 1.23
215	hycB	2.27 ± 2.59
216	hycI	3.01 ± 1.68
217	hydN	2.12 ± 1.72
218	hypC	3.48 ± 1.50
219	hypD	2.11 ± 1.24
220	icdA	2.44 ± 1.26
221	infB	2.70 ± 1.25
222	insB_5	3.23 ± 1.54
223	intE	2.14 ± 1.45
224	iroC	2.17 ± 1.49
225	kdpA	3.37 ± 1.43
226	kdpE	3.22 ± 1.46
227	lacI	2.55 ± 1.31
228	leuC	2.46 ± 1.35
229	livF	3.50 ± 1.84
230	livK	4.69 ± 1.63
231	lomW	2.01 ± 1.86
232	lpxD	2.98 ± 1.34
233	malG	2.10 ± 2.16
234	malM	2.69 ± 2.60
235	malY	2.57 ± 1.47
236	manC	2.16 ± 1.76
237	manY	2.25 ± 2.37
238	marR	6.29 *
239	mdlA	2.64 *
240	metH	2.81 *
241	metL	2.96 ± 1.96
242	mhpC	2.43 ± 1.38
243	mhpT	3.35 ± 1.27
244	miaA	2.64 ± 2.04
245	mlc	2.80 ± 2.19
246	mltB	4.77 *
247	moaA	2.59 ± 1.44
248	mobB	3.84 ± 1.43
249	modC	2.76 *
250	modE	2.26 *
251	motB	5.40 *
252	mukB	2.01 ± 1.42
253	murB	2.39 ± 1.29
254	murD	2.32 ± 1.05
255	nac	2.19 ± 1.29
256	napF	4.49 ± 1.50
257	napH	9.12 ± 2.43
258	narI	2.23 ± 1.78
259	narK	3.19 ± 2.89
260	narW	2.13 ± 1.02
261	nei	2.75 ± 1.06
262	nfo	2.62 ± 1.72
263	nhaR	2.21 *
264	nikC	2.45 ± 1.64

Number	Name	Average Fold Induction ± Standard Deviation
265	nrdB	3.34 ± 2.73
266	nrdF	3.84 ± 1.73
267	nrdG	2.19 ± 1.28
268	nrfB	2.56 ± 1.74
269	ntpA	2.59 ± 1.03
270	nuoB	2.55 ± 1.08
271	nuoG	3.44 *
272	nuoK	3.36 *
273	nusB	3.21 ± 2.65
274	ompA	2.97 ± 1.25
275	ompF	2.30 ± 1.16
276	oppA	2.11 ± 1.33
277	oppC	3.45 ± 1.30
278	oppD	2.04 ± 1.38
279	osmB	2.23 ± 1.87
280	osmE	3.40 ± 1.79
281	osmY	2.16 ± 1.05
282	pabC	3.60 *
283	parC	6.34 *
284	pdxA	2.11 ± 1.17
285	pdxH	2.98 ± 1.68
286	pepP	2.52 ± 2.58
287	pepQ	3.02 *
288	perA	2.09 ± 1.91
289	pfkA	2.38 ± 1.16
290	pflC	2.11 ± 1.18
291	pgsA	3.83 ± 1.21
292	phnQ	2.17 ± 1.90
293	phoR	2.42 ± 1.02
294	pmrD	2.09 ± 1.10
295	pntB	2.04 ± 2.23
296	polB	2.27 ± 1.19
297	potC	4.77 *
298	potH	3.40 ± 1.03
299	ppc	3.02 ± 1.55
300	pqqL	2.15 ± 1.15
301	prfA	2.33 ± 1.86
302	priB	2.73 ± 1.90
303	prpC	4.62 ± 1.58
304	prsA	2.10 ± 1.79
305	ptrB	2.89 ± 1.10
306	pyrB	2.50 ± 1.22
307	pyrG	3.13 ± 1.13
308	rarD	2.21 ± 1.95
309	rcsA	3.28 ± 2.29
310	relA	3.57 ± 1.66
311	rem	2.59 ± 1.47
312	rfbB	2.65 *
313	rfbC	2.08 ± 1.41
314	rhaB	2.12 ± 1.82
315	rhsB	3.13 ± 1.60
316	rimK	2.32 ± 1.98
317	rne	3.78 ± 1.30
318	rnhB	2.39 ± 1.69
319	rplB	2.07 ± 1.80
320	rplC	4.06 ± 1.17
321	rplD	3.92 ± 1.41
322	rplV	2.96 ± 1.59
323	rpmE	2.07 ± 1.29
324	rpoD	3.84 ± 1.73
325	rpoN	2.83 ± 1.66
326	rpsC	3.19 ± 2.74
327	rpsD	2.53 ± 1.42
328	rpsJ	2.19 ± 1.34
329	rpsK	2.28 ± 1.03
330	rpsS	3.32 ± 1.03
331	rsuA	2.19 ± 1.53
332	ruvA	3.00 ± 1.56
333	S0237	2.79 ± 1.19
334	S0559	2.07 ± 1.37
335	S1245	2.03 ± 1.84
336	S2215	2.21 ± 1.39
337	S3212	2.98 ± 2.92
338	S3228	3.59 *
339	S3871	2.17 ± 1.33
340	S4255	2.07 *
341	S4547	2.80 *
342	S4561	2.18 ± 1.84
343	S4635	2.39 ± 1.23
344	sbmA	3.65 ± 1.24
345	sdaA	2.28 ± 1.93
346	sdhD	2.41 ± 2.04
347	sdiA	2.88 ± 1.03
348	secD	2.03 ± 1.12
349	serB	2.07 ± 1.22
350	serC	2.00 ± 2.36
351	sgbU	2.90 ± 2.36
352	sgcE	2.24 ± 2.19
353	sgcX	2.39 ± 2.24
354	slyA	2.76 ± 1.30
355	SP0031	2.54 ± 1.10
356	spy	2.15 ± 1.30
357	srlD	4.21 ± 1.33
358	surE	2.87 ± 1.57

Number	Name	Average Fold Induction ± Standard Deviation
359	t150	3.67 ± 3.81
360	talA	4.46 ± 3.15
361	talB	3.68 ± 2.66
362	tap	2.65 ± 1.67
363	tauA	2.51 ± 1.47
364	tbpA	2.67 ± 1.24
365	tdcD	3.10 ± 1.16
366	tdcR	3.37 ± 2.17
367	terF_2	2.15 ± 1.17
368	tesB	2.61 *
369	thiF	2.84 ± 1.10
370	thiJ	2.24 ± 1.11
371	thrC	3.67 ± 1.79
372	tolC	4.38 ± 1.04
373	treR	2.26 ± 1.14
374	trkA	2.57 *
375	trpB	2.75 ± 1.44
376	trs5_11	2.22 ± 1.21
377	trs5_3	2.34 ± 1.97
378	tufA	2.72 ± 1.34
379	tufB	2.52 ± 1.48
380	tus	2.84 ± 1.83
381	udp	3.22 ± 2.01
382	ugpC	2.74 ± 1.47
383	uidR	2.96 ± 2.25
384	umuD	2.71 ± 2.11
385	uvrA	2.65 ± 2.77
386	uxaC	3.85 ± 1.84
387	vacJ	2.37 ± 2.10
388	vsr	2.60 ± 1.95
389	wbbJ	3.89 ± 3.66
390	wcaG	2.45 ± 2.19
391	wrbA	4.12 ± 1.71
392	wza	2.35 ± 1.61
393	wzxC	2.81 ± 1.06
394	xerC	2.33 ± 1.85
395	xylF	2.00 ± 1.54
396	yabP	4.03 *
397	yadL	2.08 ± 1.00
398	yadN	2.06 ± 1.10
399	yaeR	2.78 ± 1.98
400	yagW	3.39 ± 3.01
401	yahC	2.21 ± 1.21
402	yahG	3.16 *
403	yahO	3.81 ± 1.61
404	yaiC	2.66 ± 1.76
405	yaiN	3.27 ± 1.30
406	yajI	2.83 ± 2.75
407	ybaK	3.50 ± 1.08
408	ybbP	2.01 ± 1.12
409	ybfB	3.88 ± 1.12
410	ybfD	2.63 ± 1.64
411	ybfF	2.36 ± 2.12
412	ybfG	2.33 ± 1.85
413	ybgF	3.53 ± 1.17
414	ybgI	2.44 ± 1.40
415	ybgQ	2.99 ± 1.35
416	ybhB	2.60 ± 1.48
417	ybhH	4.08 *
418	ybhN	2.24 *
419	ybhR	2.07 *
420	ybiB	2.79 ± 1.12
421	ybiH	3.42 ± 1.39
422	ybiT	2.57 ± 1.20
423	ybiY	2.91 ± 1.18
424	ybjD	2.41 ± 1.36
425	ybjM	4.44 ± 1.89
426	ycaO	2.24 ± 2.15
427	ycdS	2.57 ± 1.47
428	yceD	2.38 ± 1.37
429	ycfC	2.63 ± 1.05
430	ycfH	2.47 ± 1.66
431	ycfL	3.86 ± 1.14
432	ycfP	2.19 ± 1.22
433	ycfR	3.45 ± 1.43
434	ycfU	3.32 ± 1.34
435	ychF	2.66 ± 2.16
436	yciA	3.03 *
437	yciE	2.44 ± 1.53
438	ydaJ	3.14 *
439	ydbL	2.14 ± 1.66
440	ydeF	5.90 *
441	ydeK	2.44 ± 1.04
442	ydfI	3.38 *
443	ydfM	2.36 ± 1.45
444	ydgA	3.06 ± 1.68
445	ydgO	2.77 ± 2.15
446	ydgR	4.61 *
447	ydiR	2.02 ± 1.94
448	ydjJ	2.03 ± 2.18
449	yebE	2.84 ± 1.58
450	yebL	2.32 ± 1.53
451	yecN	2.32 *
452	yeeV	2.88 ± 2.17

Number	Name	Average Fold Induction ± Standard Deviation
453	yefM	2.58 ± 2.51
454	yehD	2.26 ± 1.54
455	yehL	2.29 ± 1.42
456	yehW	2.79 ± 2.67
457	yeiL	2.08 ± 2.66
458	yfaO	3.40 ± 2.96
459	yfbB	2.11 ± 1.47
460	yfbL	2.08 ± 1.50
461	yfcU	3.36 ± 2.06
462	yfdE	2.16 ± 1.87
463	yfdL	2.91 ± 2.00
464	yfeR	2.03 ± 1.45
465	yffG	4.63 ± 3.15
466	yfjH	3.07 ± 2.26
467	yfjK	3.60 ± 1.91
468	yfjX	2.70 ± 2.17
469	ygaM	2.98 ± 2.51
470	ygaP	2.71 ± 2.49
471	ygbA	2.82 ± 2.79
472	ygcE	2.27 ± 1.33
473	ygcP	2.47 *
474	ygcY	3.13 ± 1.65
475	ygdL	3.72 ± 1.30
476	ygeW	2.13 ± 1.55
477	ygfA	2.03 ± 2.36
478	yggG	2.00 ± 1.66
479	yggM	2.10 ± 1.67
480	yggW	2.15 ± 1.39
481	ygiB	2.07 ± 1.67
482	ygiD	5.55 *
483	ygiH	3.10 ± 1.80
484	ygiL	5.01 ± 1.48
485	ygjE	4.73 ± 1.52
486	ygjK	2.10 ± 1.66
487	ygjO	3.35 ± 2.30
488	ygjT	3.70 ± 2.49
489	ygjV	2.44 ± 1.02
490	yhaD	2.46 ± 2.22
491	yhaG	2.65 ± 2.06
492	yhaI	2.50 ± 1.80
493	yhaP	2.21 ± 1.14
494	yhbS	2.81 ± 1.51
495	yhbW	2.37 ± 1.82
496	yhbX	2.87 ± 2.26
497	yheM	2.71 ± 1.99
498	yhgG	6.36 ± 1.47
499	yhgN	2.90 ± 2.50
500	yhhP	2.24 ± 1.62
501	yhhT	3.22 ± 1.58
502	yhiL	2.18 ± 2.00
503	yhiO	2.44 ± 2.13
504	yhiU	3.07 ± 1.65
505	yhjC	2.76 ± 1.38
506	yhjD	2.15 ± 2.08
507	yhjO	3.44 ± 2.45
508	yhjT	2.39 ± 1.83
509	yhjW	2.12 ± 1.28
510	yi22_1	3.67 ± 1.27
511	yi81_1	2.75 ± 1.55
512	yiaA	2.49 ± 1.44
513	yiaG	2.04 ± 1.35
514	yiaN	2.63 ± 1.47
515	yiaV	2.27 ± 2.14
516	yifK	3.75 ± 1.12
517	yigC	2.14 *
518	yigE	2.71 ± 1.83
519	yigJ	2.58 ± 1.14
520	yigM	2.10 ± 1.36
521	yihM	3.56 *
522	yihP	6.74 ± 1.08
523	yihT	2.55 ± 1.25
524	yijF	2.38 ± 1.64
525	yijI	2.07 ± 1.99
526	yjaB	2.45 ± 1.59
527	yjaD	2.13 ± 1.53
528	yjaI	2.04 ± 1.54
529	yjbG	2.28 ± 1.82
530	yjgB	2.79 ± 2.25
531	yjgI	2.89 ± 1.39
532	yjgL	6.11 *
533	yjgP	2.20 *
534	yjgW	3.51 *
535	yjhG	2.18 ± 1.90
536	yjhS	2.63 ± 1.44
537	yjiG	2.85 ± 1.83
538	yjiK	2.18 ± 1.43
539	yjiO	2.53 ± 1.68
540	yjiS	2.42 ± 1.03
541	yjiX	2.72 ± 1.35
542	yjjI	3.09 ± 1.23
543	yjjQ	2.21 *
544	yjjU	2.18 ± 1.69
545	ykgB	2.22 ± 1.24
546	ykgD	2.45 ± 1.93

Number	Name	Average Fold Induction ± Standard Deviation
547	ylaB	2.44 *
548	ylbF	2.04 ± 1.53
549	ylcC	2.11 ± 1.74
550	yliI	2.38 ± 1.75
551	ymcD	2.39 ± 1.75
552	ymgE	2.12 ± 1.04
553	yneJ	5.54 *
554	ynhE	2.34 ± 1.34
555	yohC	2.56 ± 1.80
556	ypjA	4.92 ± 1.29
557	ypjF	2.06 *
558	yqeA	2.01 ± 1.72
559	yqiA	2.17 ± 1.43
560	yqjB	2.12 ± 1.34
561	yqjC	2.11 ± 1.24
562	yquE	2.16 ± 1.24
563	yraJ	3.98 ± 2.82
564	yraN	2.82 ± 2.09
565	yraR	2.86 ± 2.60
566	yrbE	2.09 ± 1.34
567	yrbI	2.94 ± 1.47
568	yrfH	2.21 ± 2.17
569	yrhB	2.00 ± 1.64
570	ytfB	2.65 ± 1.43
571	ytfE	2.42 ± 1.49
572	ytfK	2.39 ± 1.62
573	ytfN	2.48 ± 1.39
574	ytfS	2.55 ± 1.39
575	Z0057	2.63 ± 1.83
576	Z0250	2.15 ± 1.61
577	Z0259	2.08 ± 1.31
578	Z0327	2.28 ± 1.25
579	Z0366	2.07 ± 1.38
580	Z0377	2.24 ± 1.17
581	Z0394	2.73 ± 1.89
582	Z0414	2.66 ± 2.11
583	Z0416	2.07 ± 1.73
584	Z0418	2.98 ± 1.94
585	Z0419	2.32 ± 2.40
586	Z0510	2.01 ± 2.32
587	Z0976	2.43 ± 1.62
588	Z1336	2.37 ± 1.59
589	Z1340	2.17 ± 1.37
590	Z1350	2.28 ± 1.73
591	Z1355	2.20 ± 1.12
592	Z1359	2.28 ± 1.41
593	Z1370	3.44 ± 3.74
594	Z1374	2.37 ± 1.16
595	Z1379	2.59 ± 1.49
596	Z1428	2.76 ± 1.73
597	Z1451	2.11 ± 1.50
598	Z1459	2.12 *
599	Z1460	2.58 ± 1.38
600	Z1467	2.32 ± 1.81
601	Z1494	2.34 ± 1.35
602	Z1501	2.09 ± 1.29
603	Z1545	2.29 ± 1.26
604	Z1549	2.31 ± 1.30
605	Z1553	2.46 ± 1.01
606	Z1640	3.87 *
607	Z1652	2.30 ± 2.71
608	Z1662	2.32 ± 1.80
609	Z1773	2.43 ± 1.92
610	Z1777	2.38 ± 1.81
611	Z1779	2.12 ± 1.65
612	Z1803	2.03 ± 1.80
613	Z1857	2.23 ± 2.18
614	Z2065	2.12 ± 1.34
615	Z2089	2.03 ± 2.14
616	Z2099	2.39 ± 2.86
617	Z2103	2.06 ± 1.62
618	Z2119	2.20 ± 1.42
619	Z2312	2.04 *
620	Z2385	2.26 ± 1.16
621	Z2501	2.10 ± 1.15
622	Z2967	2.09 ± 1.10
623	Z2971	2.58 ± 1.68
624	Z2992	3.02 ± 1.07
625	Z3069	2.19 ± 1.26
626	Z3091	2.22 ± 2.19
627	Z3107	3.12 ± 1.92
628	Z3108	2.06 ± 1.13
629	Z3123	2.02 ± 2.48
630	Z3307	2.24 ± 2.54
631	Z3312	2.39 ± 2.32
632	Z3974	2.42 *
633	Z4045	3.05 ± 1.04
634	Z4092	2.61 ± 1.08
635	Z4177	2.85 ± 1.04
636	Z4282	2.66 ± 2.34
637	Z4322	2.14 ± 2.17
638	Z4906	2.30 ± 1.42
639	Z4966	2.01 ± 1.09
640	Z4971	2.12 ± 1.15

Number	Name	Average Fold Induction ± Standard Deviation
641	Z5000	2.09 ± 1.08
642	Z5088	2.16 ± 1.08
643	Z5098	2.01 ± 1.18
644	Z5102	2.35 ± 1.29
645	Z5142	2.22 ± 2.48
646	Z5220	2.18 ± 2.03
647	Z5224	2.20 ± 2.07
648	Z5331	3.37 ± 3.51
649	Z5401	2.63 ± 1.04
650	Z5430	2.28 ± 2.60
651	Z5619	2.97 *

* Fewer than three data points were available for these genes.

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