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Identification of RNA targets of the global virulence regulator Hfq in the enteropathogen *Yersinia enterocolitica*

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1 Introduction

1.1 Current challenges in microbiology

Antimicrobial resistances have drastically increased over the past years. Often bacteria do not respond to the standard regimen anymore. There have even been case reports about bacteria that are resistant against all available substances (Ventola, 2015). This has become one of the greatest threats to global public health and is recognized as such by the WHO (WHO, 2018). Should the spread of resistances continue, we might enter a 'post-antibiotic era' and common infections and small wounds could once again be lethal. The problem itself is not new: Alexander Fleming, who discovered penicillin, warned as early as 1945 in an interview with the *New York Times*, that overuse of penicillin could accelerate the development of resistance ("Penicillin's Finder Assays its Future", 1945).

Several strategies have been proposed to battle the spread of resistant microbes: First, transmission of germs and bacterial infections should be prevented, hospital admissions and invasive measures like IV catheters avoided. Second, antibiotics use should be restricted, eliminating use to promote livestock growth and limiting the prescription of antibiotics to a minimal amount while shortening the length of antibiotic regimens if possible (WHO, 2015, Spellberg, 2013, Ventola, 2015).

While these strategies can slow down the spread of resistances, the uncomfortable truth is that resistances will develop anyway. Antibiotics are mostly derived from microorganisms found in nature. Throughout billions of years of evolution, microorganisms have developed antibiotics against a plethora of possible targets, and necessarily also defense mechanisms against those antibiotics. Therefore, resistances can already exist even before the discovery of a new drug (Spellberg, 2013). In a cave in Lechuguilla, New Mexico, that had been isolated from the surface for over 4 million years, bacteria were found to carry several distinct types of resistance mechanisms against a wide range of different antibiotics (Bhullar, 2012).

Therefore, the development of new treatment options is inevitable. At the moment, too few pharmaceutical companies tackle this challenge. Developing new antibiotic drugs bears financial risks many companies are not prepared to take (Ettel, 2020). This issue needs to be addressed urgently in order to prevent an antibiotic crisis. Ideally, strategies

with diminished potential to drive antibiotic resistance should be pursued. These could include therapies that do not kill bacteria but attenuate their virulence, mitigate their pathogenicity and dampen the inflammatory response. Another strategy could be to increase bacterial sensitivity to antibiotics. To be able to identify possible targets and strategies against pathogenic bacteria, a thorough understanding of their physiology and virulence as well as their regulation of pathogenicity mechanisms is crucial.

Many antibiotics have already been discovered that interact with different RNA species like rRNA, mRNA and tRNA (Hong et al., 2014). Riboswitches have also been identified as possible targets (Howe et al., 2015). In recent years, regulatory sRNAs as well as RNA chaperones have come into the spotlight as potential drug targets. They often regulate global stress responses in bacteria and mediate resistance to antibiotics (Dersch et al., 2017). Metabolites inhibiting the RNA-binding protein CsrA in vitro by preventing its interaction with RNA have already been identified (Maurer et al., 2016). Similarly, a cyclic peptide was found to inhibit the interactions between the RNA chaperone Hfq and sRNAs (El-Mowafi et al., 2014). The protein is not yet a proper candidate for *in vivo* usage, but the study shows that Hfq is a druggable target. However, the posttranscriptional regulatory networks are complex and effects of their inhibition on antibiotic resistance are often difficult to predict (Dersch et al., 2017). It is therefore essential to gain a thorough understanding of the interplay in these networks, for instance between Hfq and its RNA partners.

To study pathomechanisms in bacteria, the enteropathogen *Yersinia enterocolitica* is a suitable model. First, it exhibits a plethora of virulence factors, like the type III secretion system Ysc, which it shares with many other Gram-negative pathogenic bacteria like *Pseudomonas aeruginosa*, *Salmonella*, *Shigella* and enteropathogenic *Escherichia coli* (EPEC) (Wagner et al., 2018). In *Yersinia* species, the interplay between the secretion system and other factors, like adhesins, is comprehensively understood (Bohn et al., 2019). Second, like many other Gram-negative bacteria, *Y. enterocolitica* possesses the RNA chaperone Hfq, which plays an important role in regulating its pathogenicity factors and was shown to be required for its virulence (Kakoschke et al., 2016). Third, *Y. enterocolitica* causes the same gastrointestinal disease in mice as it does in humans, which allows us to examine effects of alterations in its pathogenicity factors and regulators on the overall virulence (Galindo et al., 2011).

1.2 The genus *Yersinia*

Yersinia are a genus of bacteria within the order Enterobacterales (Adeolu, 2016). They are Gram-negative, non-spore forming, facultative anaerobic coccobacilli (Suerbaum et al., 2016). *Yersinia* are psychrophilic organisms. Their optimal growth temperature is 28°C, but they are also able to survive and grow at +4°C, which is rare among the usual enteropathogenic suspects and can be used for diagnostic procedures (Greenwood et al., 1975; Pawlowski et al., 2011, Prentice and Rahalison, 2007). There are 26 *Yersinia* species (Parte, as of 07/2022) of which three are known to be pathogenic for humans: The plague agent *Y. pestis* and the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. enterocolitica* and *Y. pseudotuberculosis* are peritrichously flagellated and therefore motile at lower temperatures (below 30°C), but nonflagellated and nonmotile at 37°C, while *Y. pestis* is in general nonflagellated (Suerbaum et al., 2016).

1.2.1 Yersinia pestis

The first *Yersinia* species to be discovered was *Y. pestis*, which had developed from *Y. pseudotuberculosis* about 1,500-20,000 years ago (Achtman et al, 1999; Achtman et al, 2004, Rasmussen et al., 2015). It was identified as the agent of the plague by Alexandre Yersin in 1894 during an outbreak in Hong Kong (Yersin, 1994). Throughout history several devastating outbreaks of the plague occurred, accounting for example for the death of at least one third of the European population between 1347 and 1353 (Howard, 2019). While recurrent plague infections still emerge to this day, nowadays outbreaks are much more contained. Over the last six years cases have only been reported in Asia, America and Sub-Saharan Africa with larger outbreaks in the Democratic Republic of Congo and Madagascar, the latter suffering from an epidemic in 2017 with 661 infected and 87 casualties (WHO, 2019). The main reservoir for *Y. pestis* is rats and other rodents. It usually infects other hosts through fleas as vectors. The bacteria disseminate from the site of the flea bite to regional lymph nodes, causing them to swell, which is why this condition is referred to as bubonic plague. The infection can then spread through blood vessels and affect lungs, liver, spleen and occasionally the meninges (WHO, 2019). If the lungs are affected, the disease can also spread airborne

between humans and cause primary pulmonary plague. The systemic infection can lead to disseminated intravasal coagulation (DIC) causing purpura and gangrene, which brought it its epithet, the black death (Suerbaum et al., 2016). Diagnosis can be made through microscopic examination and culturing of blood, sputum or lymph node aspirate. The bacterium shows its typical safety-pin shape in Wayson staining (Prentice and Rahalison, 2007). If treatment starts early, the disease usually responds well to Aminoglycosides and Tetracyclines, although multidrug-resistant (MDR) isolates have been described (Galimand, 1997; Guiyoule et al., 2001).

1.2.2 Yersinia enterocolitica and Y. pseudotuberculosis

The enteropathogenic species *Y. pseudotuberculosis* and *Y. enterocolitica* are the causal agents of yersiniosis. *Y. enterocolitica* is more frequently isolated in human infections (Galindo et al., 2011). They are encountered all over the world, although mostly in zones of moderate climate. Their reservoir comprises a variety of animals. Pigs are most likely the primary concern with regard to human infection. In a recent study, birds were discovered as carriers as well. Especially migratory birds were carrying strains with significant antimicrobial resistances, raising concerns for the contamination of public drinking water sources (Odyniec et al., 2020). The most common source of yersiniosis in humans is undercooked pork, but unpasteurized milk, tofu, water or unwashed vegetables can be contaminated too (Fredriksson-Ahomaa, 2006). Due to their psychrophilic properties, storing food in the refrigerator does not prevent yersinia from growing. Most infections occur sporadically, and many are probably overlooked. However, there are sometimes epidemic outbreaks. In 1976 over 200 children in the state of New York reported yersiniosis-like symptoms, which resulted in 36 hospitalizations and 16 appendectomies. The source of infection could be traced back to a batch of chocolate milk contaminated with *Y. enterocolitica* (Black et al., 1978). A similar outbreak happened in Finland in 2003, which could be linked to grated carrots contaminated with *Y. pseudotuberculosis* (Jalava et al., 2006).

Upon ingestion, the bacteria invade the gastrointestinal wall via the M cells – specialized epithelial cells of the mucosa-associated lymphatic tissue (MALT), which transport antigens from the lumen to the underlying immune cells (Gebert et al., 1996). The bacilli replicate extracellularly in Peyer's patches and disseminate to mesenteric lymph nodes,

liver and spleen, where they can form extracellular monoclonal microcolonies and microabscesses (Oellerich et al., 2007; Autenrieth and Firsching, 1996; Handley et al., 2005).

The most common presentation, which occurs mostly in children and infants, is gastroenteritis with fever, abdominal pain, diarrhea and occasionally vomiting (Mäki et al., 1980). In older patients, it presents more often as terminal ileitis with mesenterial lymphadenitis (Galindo et al., 2011). Due to the affection of the Peyer's patches in the terminal ileum and the resulting pain in the right lower abdomen, this can mimic an appendicitis, which is why this condition is also referred to as pseudoappendicitis. The infection can lead to sepsis, although this has mostly been described in immunocompromised patients, patients in an iron-overload state like hemochromatosis, or during infections through transfusions with contaminated blood (Galindo et al., 2011). There also have been case reports of mycotic aneurysms (Prentice et al., 1993; Tame et al., 1998). Chronic sequelae of a *Yersinia* infection may include erythema nodosum, reactive arthritis, uveitis, myocarditis and glomerulonephritis, especially in HLA-B27 positive individuals (RKI, 2019; Fredriksson-Ahomaa 2006). Diagnosis is made through stool cultures and PCR detection. Yersiniosis is usually self-limiting. Should the symptoms persist or the presentation be acute, antibiotic therapy can be warranted with Ciprofloxacin or Cotrimoxazole, in severe cases also with Ceftriaxone (AWMF, 2015) or according to antibiogram.

Y. enterocolitica can be divided into six biogroups (1A, 1B and 2-5) of which 5 (1B, 2-5) are considered pathogenic (Bottone, 1999). They can also be classified based on the O-antigen of their lipopolysaccharide into more than 70 serotypes. While the serotypes O:3 and O:9 are more frequently found in Europe, serotype O:8 usually occurs in North America. In this study, I used the highly pathogenic strain serotype O:8, biogroup 1:B, whose virulence factors are well studied, as a model for a Gram-negative, extracellular enteropathogen.

1.3 Virulence factors of *Yersinia enterocolitica*

Bacteria have developed a sophisticated arsenal of mechanisms to overcome a host's defense. These mechanisms often affect the outer bacterial envelope since this is where

they get in contact with host cells. But they also include other systems like iron homeostasis and metabolism. Fig. 1) gives an overview over the most important virulence factors, which are described in the following chapters.

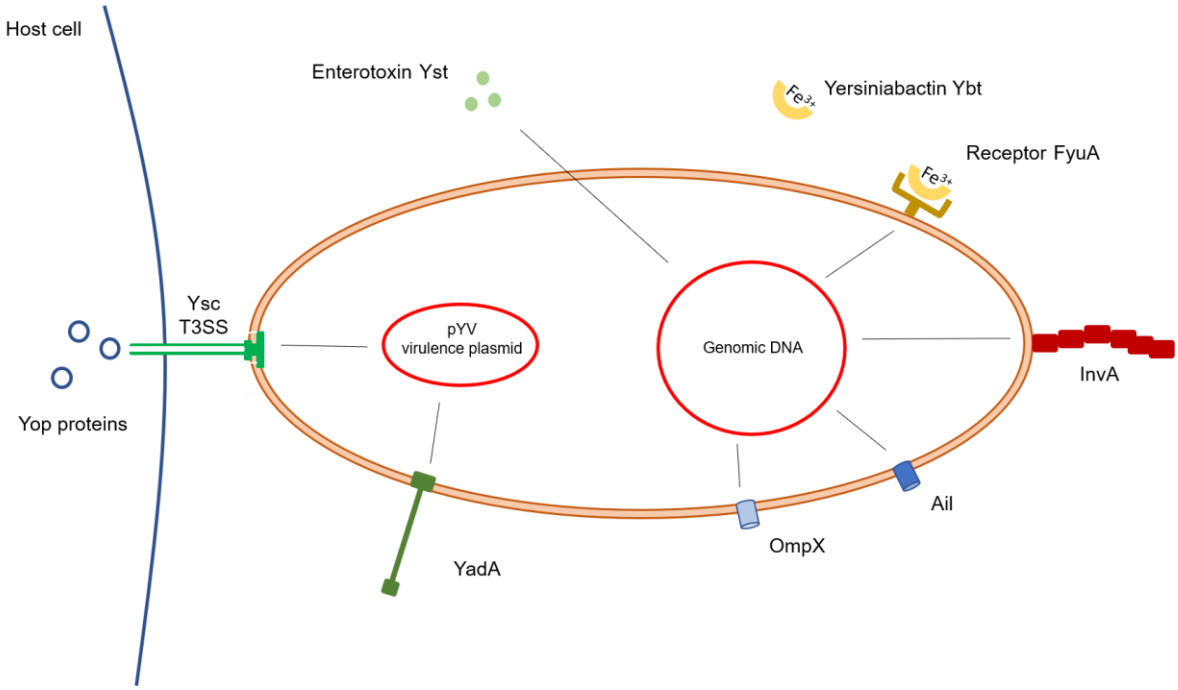


Figure 1) Overview over Yersinia virulence factors. While the adhesin YadA and the Ysc type 3 secretion system (T3SS) are encoded on the pYV virulence plasmid, other virulence factors including the enterotoxin Yst, the adhesins InvA, OmpX and Ail, as well as yersiniabactin and its receptor FyuA are encoded on the genomic DNA. Adhesins are mediating adhesion to the host cells and/or serum resistance. The siderophore yersiniabactin with its receptor FyuA provide an iron scavenging mechanism. The Ysc T3SS is a needle-like structure used to inject virulence factors inside host cells.

1.3.1 Metabolism

1.3.1.1 Iron acquisition

A crucial determinant of virulence for *Yersinia* is the acquisition of iron. Both the host and the microorganism require iron since it is a co-factor in many enzymatic processes. By limiting its accessibility, the host tries to starve bacteria from iron, e.g. by increasing the synthesis of iron binding proteins like transferrin and ferritin. However, some bacteria are able to produce siderophores, strong iron-chelating agents that allow them to

scavenge the iron from those proteins (Weinberg, 1978). Yersiniabactin (Ybt) is such a siderophore, which is produced by many Enterobacteriaceae, including *Y. enterocolitica*. The genes involved in its biosynthesis and uptake are located on the genomic high pathogenicity island (HPI). The proteins Irp 1-5 and Irp 9 are part of the biosynthesis of yersiniabactin (Pelludat et al., 2003). The outer membrane protein FyuA acts as a receptor for iron-loaded yersiniabactin, while Irp 6 and 7 transport the molecule through the inner membrane (Schubert et al., 2004). This system of iron acquisition is essential for *Y. enterocolitica*. Studies have shown that it is upregulated during infection (Bent et al., 2015) and required for virulence in the mouse model (Heesemann et al., 1993; Rakin et al., 1994, Pelludat et al., 2002). *Y. enterocolitica* also has a receptor for the siderophore ferrichrome (Koebnik et al., 1993).

1.3.1.2 Carbon metabolism

Interestingly, many genes whose expression changes during the course of infection are related to the metabolism (Bent et al., 2015). Although not directly involved in the invasion of the host, managing resources seems to be a crucial virulence determinant. In *E. coli,* loss of the *glg* operon, which carries genes involved in glycogen synthesis and metabolism, leads to decreased ability of colonizing the intestines of mice (Jones et al., 2008). In a transcriptomic study on *Y. enterocolitica* biotype B1, the *glg* operon was upregulated during infection, along with genes involved in inositol metabolism (Bent et al., 2015). The authors suggested, that *Y. enterocolitica* might use the inositol on host cell membranes either as a carbon source, or to disrupt cell signaling or membrane function of macrophages (Bent et al., 2015).

1.3.2 Enterotoxin Yst

Pathogenic *Y. enterocolitica* biotypes produce the heat-stable enterotoxin YstIA (Bancerz-Kisiel et a., 2018). It is a chromosomally encoded, short polypeptide of 30 amino acids, that is produced and secreted at 37°C, pH 7.5 and at temperatures below 30°C (Singh and Virdi, 2004). It is similar to the enterotoxin produced by ETEC. Both activate the host guanylate cyclase, leading to rising levels in cGMP, which decreases absorption of sodium and chloride ions and increases secretion of bicarbonate and chloride ions, ultimately leading to accumulation of fluid in the intestinal lumen, which

causes watery diarrhea (Uzzau and Fasano, 2001; Revell and Miller, 2001, Bancerz-Kisiel et al., 2018).

1.3.3 The *Yersinia* bacterial envelope, a "loaded gun" with many virulence factors

1.3.3.1 Adhesins: Outer membrane proteins that mediate attachment to host cells

Y. enterocolitica produces several adhesins, outer membrane proteins or protein complexes that help *Yersinia* attach to intestinal and other cells, which is crucial during infection.

The chromosome-encoded invasin (InvA or Inv) is a non-fimbrial adhesin, a high affinity-ligand to β_1-integrin and is essential for transcytosis across the epithelium (Kakoschke et al., 2016; Mikula et al., 2013; Grassl et al., 2003). It binds integrins on the host-cell surface, which leads to internalization of the bacterium (Mikula et al., 2013). It has also proinflammatory effects by triggering the secretion of cytokines like IL-8 and TNF, activating the host's defence (Mikula et al., 2013). It has a high similarity to the intimin protein family found in some pathogenic *E. coli* strains. The *invA* gene is maximally expressed at 25°C pH 8.0 or 37°C pH 5.5, but not at 37°C pH 8.0, and is therefore expressed before entering the intestine, which is probably enough for the protein to be sufficiently abundant in the early steps of infection (Mikula et al., 2013). *invA* is under control of the transcriptional activator RovA, which has less regulatory function and gets degraded at 37°C (Uliczka et al., 2011), as well as OmpR (Brzostek et al., 2007; Nieckarz et al., 2016) and H-NS (nucleoid structuring protein) which downregulate it (Chauhan et al., 2016; Kakoschke et al., 2016).

Another OMP that is crucial for *Y. enterocolitica* virulence is the trimeric YadA adhesin (Chauhan et al., 2016). It is encoded on the pYV virulence plasmid, that also contains the genes of the type III secretion system Ysc (Ysc-T3SS) (Mikula et al., 2013). Expression of *yadA* is induced at 37°C. It binds mostly to collagen, laminin and fibronectin and forms stable connections. YadA also binds to β_1-integrin on eukaryotic cells, e.g. epithelial cells or immune cells like neutrophils and macrophages, through extracellular matrix (ECM) protein bridges. YadA has the same length as the injectisome needle of the T3SS. This allows the injection of effector proteins into the cell (Chauhan et al., 2016). Furthermore, YadA promotes autoaggregation of bacterial cells, blocks the complement system and provides protection from phagocytosis (Mikula et al., 2013;

Chauhan et al., 2016). It is also required for the invasion of deeper tissues. Mutants in *yadA* are able to invade the mucosa, but cannot persist for longer than 2 days, rendering the strains avirulent (Chauhan et al., 2016). Its collagen-binding properties seem to promote the development of reactive arthritis in hosts (Gripenberg-Lerche et al., 1995). *yadA* is regulated by the activator VirF, the histone-like protein YmoA, that allows transcription only at host-temperature, and the transcriptional repressor OmpR (Chauhan et al., 2016, Kakoschke et al., 2016).

Ail (attachment invasion locus) is similar to YadA in function. It is also expressed at 37°C. Ail is relatively small and usually masked by LPS O-antigen, so that it only comes into effect in strains with rough LPS like *Y. pestis*. However, since the LPS O-antigen is altered at host temperature, Ail might be unmasked during infection in *Y. enterocolitica* as well (Chauhan et al., 2016). Ail can bind ECMs like laminin, fibronectin, heparan sulfate proteoglycans and host cells. It also provides serum resistance and blocks the complement system (Mikula et al., 2013).

The role of the adhesin OmpX in *Y. enterocolitica* remains untested, however the protein has 37% identity and 56% similarity to Ail (Kakoschke et al., 2016). In *Y. pestis*, *ompX* has 70% identity with *Y. enterocolitica ail* and seems to be involved in adherence to and internalization by host cells, as well as serum resistance (Kolodziejek et al., 2007). OmpX is preserved in many bacterial species, e.g. *E. coli* and *E. cloacae*, although their role in virulence might differ (Mecsas et al., 1995). Regarding its regulation, expression of *Y. enterocolitica ompX* is like *invA* under the control of RovA and OmpR (Kakoschke et al., 2016).

While the importance of Myf fimbriae for pathogenicity in *Y. enterocolitica* is unclear, MyfA, the major subunit, is similar to the Psa (ph6 antigen) in *Y. pestis* and *Y. pseudotuberculosis*, which is involved in adhesion to host cells and resistance to phagocytosis (Kakoschke et al., 2016; Rastawicki and Gierczynski, 2009). It is also similar to CS3 fimbriae in ETEC (Enterotoxigenic *E. coli*), which allows adhesion to cells and triggers an immune response (Bancerz-Kisiel et al., 2018; Levine et al., 1984; Knutton et al., 1985). They are expressed at 37°C at an acidic pH (Iriarte et al., 1993; Kakoschke et al., 2016) and during infection (Bent et al., 2015) which can be used for serological diagnostic (Rastawicki and Gierczynski, 2009).

1.3.3.2 Secretion systems

Y. enterocolitica has several protein secretion systems that play a role in virulence. The most essential one is the type III secretion system (T3SS) Ysc, which is encoded by the 70-kb virulence plasmid pYV along with the adhesin YadA. When introduced to a temperature of 37°C and a low calcium concentration, conditions similar to a mammalian host, transcription is induced. Ysc proteins form a needle-like structure that injects effector proteins called *Yersinia* outer proteins (Yops) into the cell's cytosol. Once inside the cell, the Yops interfere with the cell's signaling pathways, ultimately blocking phagocytosis and cytokine production and inducing apoptosis (Trosky et al., 2008; Atkinson and Williams, 2016). Taken together, the T3SS Ysc and the effector Yop proteins elaborate a sophisticated cell reprogramming to attenuate the innate immune response.

Y. enterocolitica biovar B1 has another T3SS called *Yersinia* secretion apparatus Ysa. It is chromosomally encoded within the YSA pathogenicity island. It is induced at 26°C in a nutrient-rich medium at high NaCl concentrations (Venecia and Young., 2005). While this implies a function for Ysa outside the host, a more recent study could show that it is expressed during infection as well and plays a role in rapid colonization of the gut and during the systemic phase (Bent et al., 2013). It is most highly expressed in bacteria that have been internalized by macrophages (Bent et al., 2015). Like Ysc, the Ysa secretion system promotes virulence (Venecia and Young, 2005).

Interestingly, a third T3SS-like platform, that elaborates assembly of the flagellum, also facilitates the transport of proteins and enzymes from the cytoplasm of *Y. enterocolitica* to the outer surface (Young et al., 1999). Thus, depending on the strain, *Y. enterocolitica* may use up to three T3SS to secrete proteins into the extracellular milieu or inject proteins into host cells.

Furthermore, *Y. enterocolitica* has two type two secretion systems (T2SS), Yts1 and Yts2. While Yts1 was important for colonizing the spleen and liver of infected mice in the strain WA-314 (Iwobi et al., 2003), it was not differently expressed during infection by the strain JB580v (Bent et al., 2015). Similar to Ysa, Yts2 seems to have a part in intracellular infection (Bent et al., 2015). Thus *Y. enterocolitica* possesses several secretion systems that rely on elaborate multiprotein complexes spanning the bacterial envelope.

1.4 Stress resistance

1.4.1 Stress responses through transcription regulatory pathways

The virulence attributes and metabolic pathways described in the previous chapters help bacteria withstand various harsh conditions. However, a constitutive expression of these traits is impractical. Some of them require a lot of energy, while others are highly immunostimulatory. Therefore, a tight regulation and quick adaptation to a changing environment is mandatory. To orchestrate all the changes within the bacterium a variety of transcriptional regulators are necessary. They affect the transcription rate of genes in different ways, for instance by binding in the vicinity of the promoter and activating or repressing transcription. LysR-type transcriptional regulators, like RovM, function in this manner. Bacteria often utilize two-component systems (TCS) like OmpR/EnvZ, PhoB/PhoR or PhoP/PhoQ to rapidly respond to changes in the environment. They consist of a membrane-bound histidine kinase and a cytosolic response regulator. Signals perceived by the histidine kinase trigger - through one or several steps – the phosphorylation and activation of the response regulator, which then acts as a transcription factor (Nguyen et al., 2015).

Another way of transcriptional regulation are sigma-factors, which bind to the RNA-polymerase, initiating transcription of a distinct set of genes, often depending on the environmental conditions. One such sigma factor is RpoE, which will be presented in paragraph 1.4.4. Another example is RpoS, which has been thoroughly studied in *E. coli* as a regulator of a variety of genes (Patten et al., 2004). *rpoS* mutants were more sensitive to carbon starvation, high temperature, low pH, osmotic and oxidative stress and showed altered biofilm formation (Gottesman, 2019). In *Y. enterocolitica*, RpoS is necessary for the expression of the enterotoxin *yst* (Iriarte et al., 1995). Similar to *E. coli*, it is also critical for survival to diverse environmental stresses like high temperature, low pH, osmolarity and oxidative stress at 37°C. However, it is dispensable for host cell invasion and virulence in the mouse model (Iriarte et al., 1995; Badger and Miller, 1995).

1.4.2 Expecting protection: Defense against diverse threats

In the environment and the host, bacteria are exposed to a variety of different stresses. Resistance against those threats is an important factor in bacterial virulence. Physical or chemical stresses often lead to protein misfolding or chemical modifications, so that chaperones are needed to maintain their functional form. Many of them are involved in response to multiple different triggers.

Resistance to acidic pH is crucial for *Y. enterocolitica* virulence, considering that they must withstand the acidity of the stomach before being able to invade the intestine of their host (Koning-Ward and Robins-Browne, 1995; Gripenberg-Lerche et al., 2000). Urease is an enzyme that splits urea into ammonium and carbon dioxide. This allows the bacterium to use urea as a source of nitrogen, but the production of ammonium also elevates the pH in the vicinity. Interestingly, urease is more abundant at 27°C than at 37°C (Nieckarz et al., 2020). Acid stress resistance is also provided by chaperones, that restore structure and function of proteins denatured by acid. Examples of such chaperones are the proteins HdeA and HdeB (Carter et al., 2012). Finally, acid stress resistance is also provided by decarboxylation of amino acids, e.g. glutamate to γ-aminobutyric acid (GABA), which consumes protons. An antiporter system can then exchange GABA with another glutamate (Hong et al., 2012).

Besides acid stress, oxidative stress can possibly damage proteins, nucleic acids and cell membranes. In fact, all aerobic organisms require scavenging enzymes, freeing them from reactive oxygen species, which form when oxygen oxidizes electron carriers (Storz and Imlay, 1999). Superoxide dismutase (SOD) converts the highly reactive superoxide (O_2^-) with two protons (H^+) to oxygen (O_2) and hydrogen peroxide (H_2O_2). Hydrogen peroxide can subsequently be converted to oxygen and water by catalase or alkyl hydroperoxide reductase (Ahp) (Seaver and Imlay, 2001). The model organism *E. coli* has two transcription factors to sense and react to oxidative stress. SoxRS responds primarily to O_2^- and promotes expression of many genes, among others the superoxide dismutase *sodA* and the iron uptake regulator *fur* (Storz and Imlay, 1999), increasing the reducing power of the cell and its resistance to oxidative stress. SodB and SodC are thought to be differentially regulated. *sodB* transcription for instance is upregulated by Fur and downregulated by H-NS and IHF (Dubrac and Touati, 2000). OxyS responds

more to H_2O_2 and upregulates for example the abovementioned catalase (*katG*) and alkyl hydroperoxide reductase (*ahpCF*) and also *fur* (Storz and Imlay, 1999).

Y. enterocolitica is a psychrotrophic organism, capable of growing at cooler temperatures even around the freezing point. Cold shock proteins (Csp) are short nucleic acid binding proteins, which bind ssRNA weakly and with low-sequence specificity. They destabilize low temperature-induced secondary structures, which allows continued transcription and translation (Keto-Timonen et al., 2016). In *Y. enterocolitica*, the cold shock proteins CspA1 and CspA2 are expressed when exposed to a cold temperature after two hours until 24 hours of continued exposure (Annamalai and Venkitanarayanan, 2005). Regulation of Csps is often temperature dependent. Csp mRNA undergoes structural changes at low temperatures resulting in stabilization and more efficient translation (Keto-Timonen et al., 2016).

Similar to low temperatures, high temperatures can misfold proteins and trigger a heat shock response. Chaperons are necessary like the heat shock proteins Hsp90 and Hsp70/DnaK. DnaK requires the co-chaperone DnaJ/CpbA and the nucleotide exchange factor (NEF) GrpE. DnaJ/CpbA stimulates hydrolysis of ATP, while GrpE facilitates ADP release and ATP uptake (Genest et al., 2019). Another important heat shock protein is the periplasmic DegP/HtrA, which has both chaperone and protease function, or Clp, which forms heterooligomeric complexes with separate subunits with either chaperone or proteolytic function (Spiess et al., 1999). DegP is also involved in osmotic, pH and oxidative stress resistance (Spiess et al., 1999). Many chaperones are either directly under the regulation of the sigma factor RpoE or controlled by RpoH, which in turn is regulated by RpoE.

1.4.3 Periplasmic space: ensuring the proper folding of newly translocated proteins and envelope homeostasis

The bacterial envelope is the barrier and contact between a bacterial cell and the environment, where the bacterium perceives changing conditions, faces threats or invades neighboring cells. It is therefore not surprising that many virulence factors are found here. The envelope of Gram-negative bacteria consists of an inner and an outer membrane, encompassing the periplasmic space, which contains the peptidoglycan cell wall. Most envelope proteins are exported in an unfolded state through the secretory

(Sec) pathway across the inner membrane. The periplasmic space comprises a plethora of proteins and different enzymes that ensure correct protein folding in the periplasm (Raivio and Silhavy, 2001).

Disulfide bond oxidoreductases (Dsb) ensure the correct formation and isomerization of disulfide bonds. They work in pairs, for instance DsbA/DsbB in which one protein catalyzes the disulfide bond formation, whereas its partner is required for the regeneration of the initial redox state of the system. Some Dsb proteins also have chaperone function (Raivio and Silhavy, 2001).

Another class of periplasmic enzymes are peptidyl-prolyl-isomerases (PPI) like FkpA or PpiA. They catalyze cis-trans isomerization in peptide bonds with the imino acid proline, and thus assist in envelope protein folding (Raivio and Silhavy, 2001). The aforementioned DegP/HtrA is located in the periplasmic space as well. It acts as a chaperone at lower temperatures and as a protease at higher temperatures (Spiess et al., 1999). Most importantly these chaperones, isomerases and proteases participate in the homeostasis of envelope proteins within what are known as envelope stress responses (ESRs) that will be described in the following paragraphs.

1.4.4 The RpoE envelope stress response

Many bacteria have developed envelope stress responses (ESR) to survive a plethora of threats to the integrity of the bacterial envelope, like changes in temperature and pH, but also surfactants and alcohol. It is therefore not surprising that ESRs play a major role for bacterial virulence (Flores-Kim & Darwin, 2014). Gram-negative bacteria have five important ESRs. The *E. coli* sigma factor σ^E is one of the most extensively studied ESRs.

The sigma factor σ^E or RpoE (extracytoplasmic RNA polymerase sigma factor), is activated upon detection of stress (Fig. 2).

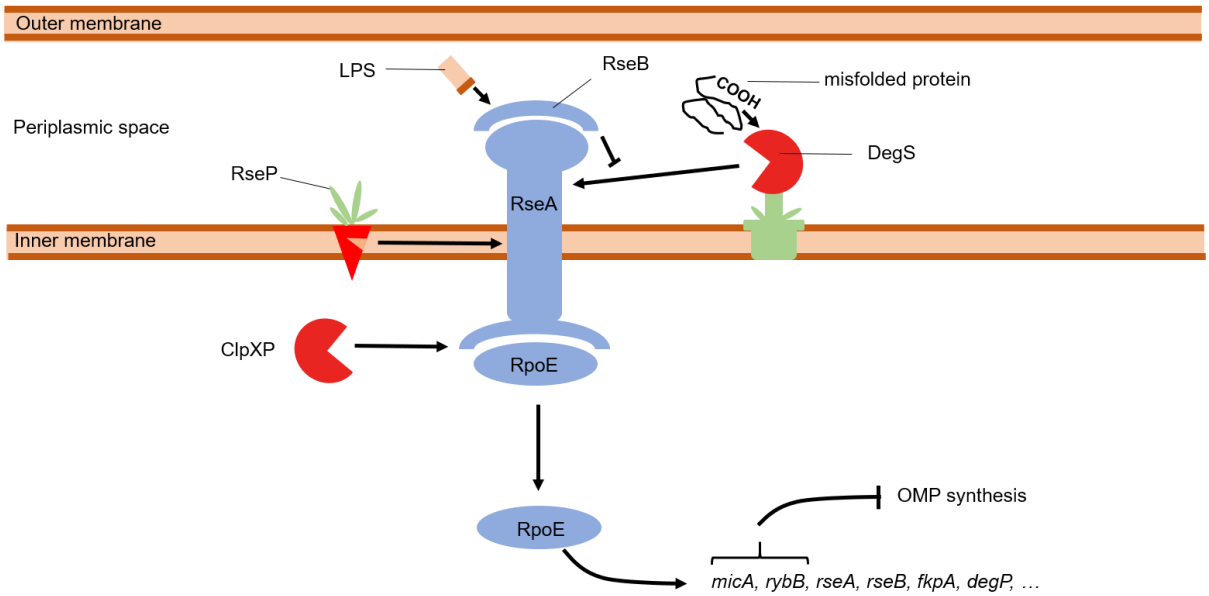


Figure 2) Model for the regulation of RpoE in E. coli. RseA binds RpoE on the cytoplasmic site and RseB on its periplasmic site. When RseB interacts with LPS intermediates, it is released by RseA and enables DegS to cleave the periplasmic domain of RseA. DegS needs to interact first with misfolded proteins in order to be activated. After that, RseP cleaves the transmembrane region of RseA, which releases the RseA-RpoE-complex into the cytoplasm. The ClpXP protease degrades RseA and enables RpoE to interact with RNA polymerases to initiate the transcription of different genes. The figure is modified after Rowley et al. (2006), Raivio and Silhavy (2001) and Grabowicz and Silhavy (2017).

In *E. coli*, the activating stress was found to be e.g. heat or ethanol, but not in *Y. enterocolitica*, for which *rpoE* was even downregulated at 37°C compared to 26°C (Heusipp et al., 2003). It is however activated by osmotic stress (through high sugar concentrations) and is an essential sigma factor both during infection and standard laboratory conditions (Heusipp et al., 2003). Two proteins, which are encoded in the same operon as RpoE are important for the initiation of the cascade: RseA and RseB (Fig. 2). RseA is an inner membrane protein with a cytoplasmic and a periplasmic domain. In absence of misfolded OMPs, the anti-σ-factor RseA binds RpoE with its cytoplasmic domain, thereby sequestering it and inhibiting its function. On its periplasmic site, RseA is bound and stabilized by RseB (Missiakis and Raina, 1998; Raivio and Silhavy, 2001). Two signals are necessary to release σ^E. The first signal are intermediates in LPS transport and assembly, which bind to RseB and cause it to release RseA making it accessible for cleavage (Lima et al., 2013). The second signal is contact between the periplasmatic protease DegS and the C-terminal residues of

misfolded OMPs, mostly porins. This activates the proteolytic activity of DegS, which then cleaves the periplasmic domain of RseA (Walsh et al., 2003). After that, RseA is cleaved in the transmembrane region by the RIP (regulated intramembrane proteolysis) protease RseP. This finally releases the RseA-σ^E- complex into the cytoplasm, where RseA is degraded by cytoplasmic proteases and σ^E is finally free to associate with the core RNA polymerase and initiate transcription (Chaba et al., 2007). It was suggested that this regulation enables a rapid response to outer-membrane dysfunctions, because it incorporates two stress signals, and at the same time provides a buffer for transient fluctuations in signal abundance (Lima et al., 2013). Although it was proposed as a positive regulator of RpoE (Missiakas et al., 1997), the role of *rseC*, which is encoded in the same operon and codes for an inner membrane protein, is not entirely clear. Another study suggested that RseC is involved in keeping the regulator SoxR in a reduced and inactive state (Koo et al., 2003).

Consequently, when the biogenesis of LPS and OMP is disrupted, σ^E is released into the cytoplasm, binds to the RNA polymerase complex and promotes the expression of a plethora of different genes, aiming to increase production of chaperones and proteases and to decrease new OMP synthesis to maintain a level of functioning OMPs (Mogensen, 2005; Mitchell and Silhavy, 2019). In *E. coli*, the σ^E regulon includes *rseA* and *rseB* from its own operon, which creates a negative feedback mechanism, but also other sigma factors like the heat shock sigma factor *rpoH*, the PPIase *fkpA*, chaperones and proteases like *degP*, the lipid A biosynthesis enzymes *lpxD* and *lpxA* and many more (Dartigalongue et al., 2001; Rowely et al., 2006). RpoE also upregulates the sRNAs RybB and MicA, which repress OMP production in *E. coli*, and MicL, which decreases the level of the lipoprotein Lpp (Grabowicz and Silhavy, 2017; Mitchell and Silhavy, 2019).

In contrast to *E. coli*, the *Y. enterocolitica rpoE* has shown a decreased expression level at 37°C (Heusipp et al., 2003). This is surprising for a heat shock sigma factor and hints at other regulatory mechanisms. RpoE has been shown to be important for virulence in many organisms, like *S. typhimurium* (Humphreys et al., 1999), *Vibrio cholerae* (Kovacikova and Skorupski, 2002) and *Y. pseudotuberculosis* (Palonen et al., 2013).

1.4.5 The Cpx envelope stress response

While RpoE reacts to misfolded OMPs, the Cpx (conjugative plasmid expression) ESR is primarily activated upon contact with misfolded inner membrane and periplasmic proteins (Raivio and Silhavy, 1999; Raivio, 2014). In *E. coli*, the Cpx ESR is activated by alkaline pH, high salt concentration, altered inner membrane lipid composition and misfolded proteins associated with the inner membrane and peptidoglycan defects (Raivio and Silhavy, 2001; Raivio, 2014).

The activation of these two ESR pathways follows a similar pattern, in which a cytosolic transcription factor is released or activated by a membrane bound regulator, mediated by a periplasmic protein (Raivio and Silhavy, 2001) (Fig. 3).

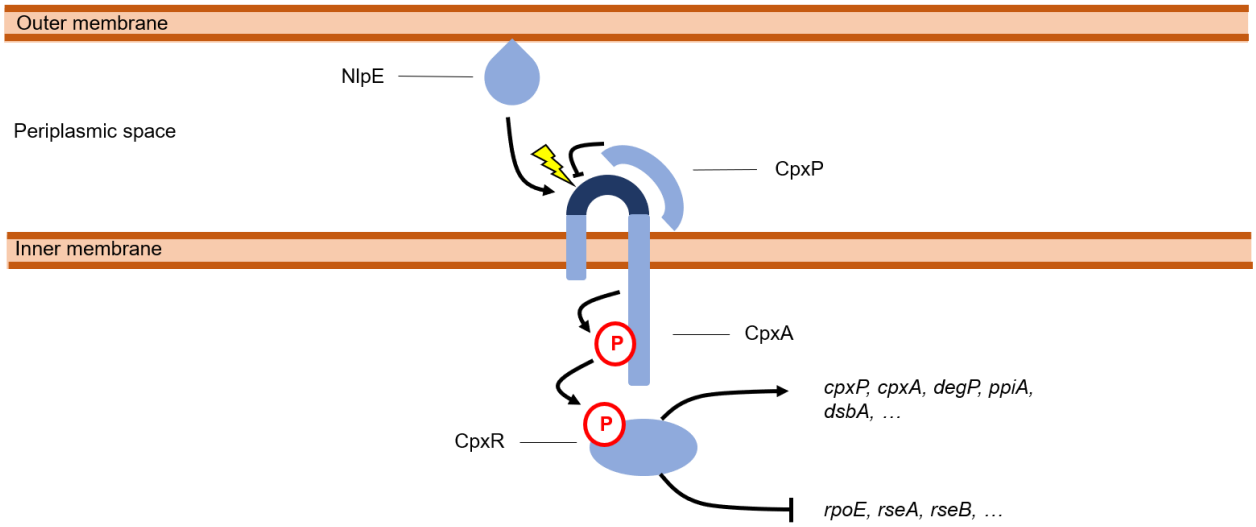


Figure 3) Model for the regulation of CpxR in E. coli. When stress is detected by the periplasmic loop of the transmembrane protein CpxA, CpxA autophosphorylates its histidine kinase domain, which then phosphorylates and activates the transcriptional regulator CpxR. CpxA is controlled by the negative regulator CpxP, providing a negative feedback mechanism, and by the positive regulator NlpE, a lipoprotein that activates CpxA upon contact to hydrophobic surfaces. The figure is modified after Grabowicz and Silhavy (2017) and Flores-Kim and Darwin (2014).

The regulatory transmembrane protein of the Cpx ESR is the histidine kinase CpxA, which consists of two transmembrane domains. The periplasmic loop acts as the stress sensing domain (Grabowicz and Silhavy, 2017). Upon activation of CpxA, it autophosphorylates its histidine kinase domain, which in turn phosphorylates and activates CpxR. The transcriptional regulator CpxR can bind to specific DNA sequences

(Grabowicz and Silhavy, 2017). CpxA has not only a kinase, but also a phosphatase activity, which allows for regulatory fine-tuning of CpxR activation (Raivio and Silhavy, 1997). Activation of CpxA is modulated by two additional proteins: the positive regulator NlpE and the negative regulator CpxP. NlpE is an outer membrane lipoprotein that directly activates CpxA upon adhesion to abiotic surfaces (Otto and Silhavy, 2002; Hirano et al., 2007; Grabowicz and Silhavy, 2017). The negative regulator CpxP binds to the sensory domain of CpxA and - being upregulated by CpxR - provides a negative feedback mechanism (Grabowicz and Silhavy, 2017).

In *E. coli*, CpxR was shown to increase expression of genes encoding periplasmic chaperones, isomerases and proteases, e.g. *degP*, *ppiA* and *dsbA* (De Wulf et al., 1999). In *Y. enterocolitica*, a crosstalk with the RpoE ESR was also observed, since *rpoE*, *rseA* and *rseB* were downregulated by CpxR (Rönnebäumer et al., 2009).

1.4.6 The Psp envelope stress response

Contrary to what its name implies, the Psp (phage shock protein) regulates not only phage-related, but various other threats to the integrity of the cell envelope. The Psp responds to extracytoplasmic stress perturbating the inner membrane, like mislocalized outer membrane secretins and overproduced IM proteins, exposure to ethanol or to extremes in osmolarity and temperature (Maxson and Darwin, 2004; Flores-Kim and Darwin, 2016). The transcriptional regulator PspF is bound by PspA in the cytosol (Fig. 4).

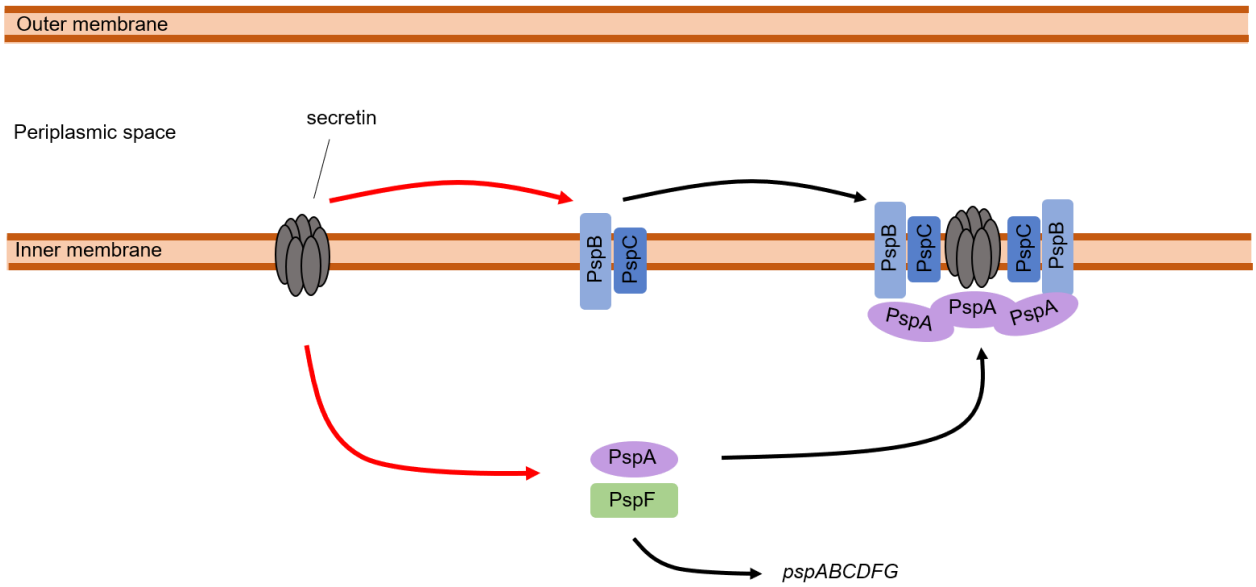


Figure 4) Model for the regulation and function of Psp. Mislocalized secretin proteins and other stressors in the inner membrane can be sensed (red arrows) by either PspA or PspBC. PspA is recruited to the inner membrane or the PspBC complex (black arrows). PspABC can stabilize the membrane through unknown mechanisms. PspF is released into the cytosol and positively regulates expression of genes of the psp operon. The Psp system is an autonomous system that has an important role in membrane function and integrity. The figure is modified after Flores-Kim and Darwin (2016).

The stress signal is detected either by the PspB-PspC-complex in the inner membrane, or directly by PspA, which is thought to be able to sense stored curvature elastic stress in the membrane (Flores-Kim and Darwin, 2016). This causes PspA to release PspF and interact with either the PspB-PspC complex or the inner membrane itself. PspA and PspBC are thought to stabilize compromised areas in the membrane. The mechanism for this stabilization is so far unknown. Unlike other ESRs, PspF does not affect global gene expression, but only upregulates genes of the Psp operon (Flores-Kim and Darwin, 2016). While *E. coli* shows only moderate changes in its phenotype, in *Y. enterocolitica* strains with a T3SS, loss of *pspC* was shown to reduce virulence and impair growth (Darwin and Miller, 2001, Flores-Kim and Darwin, 2016). Many questions about the Psp remain unanswered, e.g. the exact signal detection, the mechanism that allows the Psp proteins to preserve cell integrity or the function of other proteins of the Psp operon.

1.4.7 The Bae envelope stress response

Another envelope stress response that was discovered in 2002 is the Bae (bacterial adaptive response) (Raffa and Raivio, 2002). It increases resistance to toxins and antibiotics by inducing expression of multi-drug efflux pumps (Raivio, 2005). The Bae ESR is a two-component system. Upon exposure to toxic molecules, like ethanol, indole, nickel chloride, zinc, or by pilin subunit overproduction, the inner membrane histidine kinase BaeS autophosphorylates and then transfers the phosphate group to the cytosolic response regulator BaeR, activating it (Mitchell and Silhavy, 2019). This leads to the upregulation of the periplasmic chaperone *spy,* several efflux pumps, the regulators *baeR* and *baeS* themselves as well as genes of yet unknown function (Mitchell and Silhavy, 2019). Some of the regulated genes overlap with those upregulated by the Cpx ESR. It was proposed that Cpx facilitates BaeR binding to the promoter region of the multidrug resistance locus *mdt*. Both ESRs are involved in drug resistance and a *baeR cpxR* double mutant is more sensitive to envelope stress than either single mutant (Macritchie and Raivio, 2009).

1.4.8 The Rcs envelope stress response

The Rcs (regulation of capsular synthesis) ESR reacts to stress like osmotic shock, desiccation and overproduction of envelope proteins, alterations in LPS charge and fluidity and changes in peptidoglycan biosynthesis (Flores-Kim and Darwin, 2014; Mitchell and Silhavy, 2019). In an inactive state, the IMP IgaA binds the histidine kinase RcsC. The outer membrane lipoprotein RcsF can sense stress and interact with IgaA, disinhibiting RcsC. This initiates a cascade of phosphorylations, beginning with RcsC, which phosphorylates the inner membrane RcsD, which in turn phosphorylates the cytoplasmic protein RcsB (Flores-Kim and Darwin, 2014). The phosphorylated RcsB can then bind DNA either as a homodimer or as a heterodimer with accessory proteins like RcsA (Flores-Kim and Darwin, 2014; Mitchell and Silhavy, 2019).

The activated RcsB can promote expression of different envelope related targets: in encapsulated *E. coli* it promotes expression of *galF*, which enhances capsule production (Rahn and Whitfield, 2003); in *K. pneumoniae* RcsB activates the *cps* operon increasing capsular polysaccharide production and in *Salmonella* it promotes expression of *ugd*, which is needed for LPS modifications (Mouslim et al., 2003; Flores-Kim and Darwin,

2014). In *Y. pseudotuberculosis* and *Y. enterocolitica*, RcsB was shown to be involved in the activation of the T3SS (Flores-Kim and Darwin, 2012; Li et al., 2014).

1.5 Post-transcriptional regulation

1.5.1 Regulatory RNAs

In the previous section, we have presented an overview of the transcriptional regulatory pathways that govern the response to stress in bacteria, including *Y. enterocolitica*. The transcriptional regulation represents the 'classical' way of gene expression regulation. The past two decades have revealed that post-transcriptional regulation and RNA-mediated regulation also play a significant role in bacteria (Chakravarty and Massé, 2019; Westermann, 2018).

One type of RNA-mediated regulator is found in the 5'-UTR of the regulated mRNA itself. Riboswitches are secondary RNA structures, that undergo conformational changes upon contact to metal ions or metabolites. These changes can influence expression of their downstream gene either by transcription elongation or termination or by modulating translation, for example by making the start codon or the ribosome binding site (RBS) sequence accessible (Breaker, 2012). Other examples for this type of regulation are RNA thermometers and 5'-UTR regulatory elements that respond to a changing pH (Chakravarty and Massé, 2019). Both metabolite and temperature sensitive 5'-UTR elements can indicate the switch to a mammalian host environment and are able to induce the regulation of virulence factors in several bacterial organisms (Westermann, 2018). In *Yersinia* for instance, the T3SS and the Yop proteins are regulated by the transcriptional activator LcrF/VirF, which is under the control of an RNA thermometer. At 37°C the destabilization of a hairpin in *virF* mRNA allows translation and therefore subsequent VirF-dependent transcription at host temperature (Hoe and Goguen, 1993; Böhme et al., 2012). Production of cold shock proteins described above are also often regulated by such a structure (Mega et al., 2010).

Another type of RNA regulators are *cis*-encoded anti-sense RNAs (asRNA), meaning they are transcribed from the same genomic site as their target, but from the opposite DNA strand. They can influence gene expression at different stages, (i) on the transcriptional level by transcription interference, (ii) on the posttranscriptional level by binding to its complementary mRNA and leading to degradation of the double stranded

RNA, or (iii) on the translational level by obstructing the RBS (Westermann, 2018; Chakravarty and Massé, 2019). An example for this mechanism is the regulation of urease genes in *Helicobacter pylori*. The *ureAB* mRNA is bound by an asRNA and subsequently degraded, inhibiting urease production at a neutral pH. In an acidic environment, expression of this asRNA is downregulated, leading to a stable *ureAB* transcript and increased urease production (Wen et al., 2011; Wen et al., 2013).

The third type of regulatory RNA are *trans*-encoded small regulatory RNAs (sRNA). They are transcribed from a different genomic site than their target and, contrary to asRNAs, have a limited complementarity to their mRNA partners. They are small RNA molecules between roughly 40 and 500 nucleotides (nt) long (Santiago-Frangos and Woodson, 2018). Although mostly non-coding, there are also examples of sRNAs encoding proteins (Vanderpool et al., 2011). They are often found in intergenic regions, between protein coding sequences, but also in the 3' or 5' UTR of mRNAs, where they can additionally regulate genes in *cis* as riboswitches, and even within the coding sequence of genes (Chao et al., 2012; Miyakoshi et al., 2015; Wagner and Romby, 2015, Guo et al., 2014). These trans-acting regulatory RNAs can bind to mRNA, mostly close to the 5'-UTR, and affect the stability and translation rate of the target RNA. They can have a downregulating effect, by obstructing the RBS and/or facilitating degradation. They can also upregulate genes, by altering the secondary structure of mRNAs, making their RBS accessible and/or inhibiting degradation. A single sRNA can have multiple mRNA targets and vice versa, and an sRNA can have opposite effects on different mRNAs (Santiago-Frangos and Woodson, 2018). Due to their limited complementarity, many of them also rely on proteins, that assist their interaction with their mRNA target, like ProQ or Hfq (Olejniczak and Storz, 2017). Another form of *trans*-encoded sRNAs primarily binds and titrates RNA-binding proteins, making them inaccessible for other targets (Westermann, 2018; Chakravarty and Massé, 2019). The sRNAs CsrB and CsrC, sequestering the regulatory protein CsrA, are examples for such a mechanism, that is preserved in many bacterial species (Heroven et al. 2012).

Trans-encoded sRNAs are not necessarily assigned exclusively to one of the two groups. A single sRNA can interact with multiple proteins through different mechanism. The *E. coli* McaS for instance, is involved in biofilm formation via interaction with Hfq as

a classical mRNA-interacting sRNA but also interacts with the CsrA regulatory protein (Jorgensen et al., 2013; Kavita et al., 2018).

As much as sRNAs regulate other genes, they are also subject to regulation. Besides transcriptional control, sRNA abundance also depends on their stability. While some sRNAs are reused, others are degraded after binding to their target. For 'reusable' sRNAs, 'decoy' mRNAs have been described as a way of downregulation, that bind sRNA only to lead to their degradation (Figueroa-Bossi et al., 2009; Kavita et al., 2018). Another mechanism of sRNA regulation is through RNA sponges. Often fragments of tRNA precursors, which are constitutively expressed, bind sRNAs, reducing any 'regulatory noise', by lowering the basal level. The sRNA RyhB, involved in iron regulation, is regulated in this way (Lalaouna et al., 2015).

1.5.2 The RNA-binding protein CsrA

CsrA (Carbon storage regulator A) is a dimeric RNA binding protein. Each identical monomer is composed of five β-strands, an α-helix and a flexible C-terminus (Gutiérrez et al. 2005). It binds to GGA-containing sequences in single stranded loop regions in 5'-UTRs of its target RNAs (Gutiérrez et al., 2005; Kusmierek and Dersch, 2018). Through its homodimeric structure, CsrA can bind two GGA sites in a transcript separated by 10 to about 63 nucleotides, preferably within hairpins in the 5'-UTR of mRNAs (Mercante et al., 2009). Through this bridging mechanism, CsrA can affect specific mRNAs in different ways.

On one hand, CsrA can inhibit translation by obstructing the ribosome binding site, when the GGA motif is part of the RBS, or by stabilizing a stem loop structure that obstructs the RBS. Once the ribosome is blocked off, mRNAs are often degraded by RNases. On the other hand, CsrA can also have the opposite effect on mRNA translation and stability. It can open up stem loops and make the RBS accessible to the 30S ribosomal subunit, hence activating translation, or stabilize an mRNA by inhibiting cleavage by RNase E. In addition, there are also examples of CsrA promoting Rho-dependent transcription termination (Vakulskas et al., 2015; Kusmierek and Dersch, 2018).

The complex autoregulation of CsrA is best studied in *E. coli*. CsrA is controlled by the small non-coding RNAs CsrB and CsrC (Müller et al, 2019). They possess multiple GGA-containing stem loops and can therefore sequester several CsrA proteins at the

same time, reducing its availability in the cell. The protein CsrD/YhdA increases RNase E-dependent degradation of CsrB and CsrC. CsrA increases CsrB and CsrC expression and inhibits expression of CsrD/YhdA, creating a negative feedback loop. Carbon sources like glucose activate CsrD/YhdA. Additional to the sRNAs CsrB and CsrC, CsrA can also bind to other sRNAs, mRNAs and proteins, which are involved in feedback-loops, regulatory fine-tuning and decreasing the intrinsic noise in the regulatory system (Dersch et al., 2017). Other regulatory systems are linked with the Csr system, for instance the global regulator RpoS or the PhoPQ system, which has been shown to increase the CsrC level in *Y. pseudotuberculosis* (Nuss et al., 2016).

The Csr regulatory system was first discovered in *E. coli* in 1993 (Romeo et al., 1993), but it has since been described in numerous other bacterial species (White et al., 1993). In many pathogenic bacteria, especially enterobacteria, CsrA is involved in the regulation of carbon metabolism, stress resistance, iron homeostasis, motility and cell division. It also controls several pathogenic traits and is indispensable for virulence (Dersch et al., 2017). It promotes for instance the production of pili, that are important for adhesion to host cells in *P. aeruginosa*, *V. cholerae S. typhimurium* and EPEC (Dersch et al., 2017; Brencic and Lory, 2009; Sterzenbach et al, 2013; Katsowich et al., 2017). In *Yersinia* species, CsrA has similar effects. In *Y. pseudotuberculosis*, it promotes expression of the Psa adhesin (Bücker et al., 2014). For both enteropathogenic *Yersinia* species, CsrA is important for the T3SS (Ozturk et al., 2017; Nuss et al., 2017). In *Y. enterocolitica*, CsrA also represses the expression of T2SS *ysa* genes and diminishes Ysps protein secretion (Walker and Miller, 2009; Ozturk et al., 2017). The Ysc T3SS has a similar regulatory cascade as Ysa, with *virF* on top, which regulates *yscB*, which induces effector proteins like *yopE*. While Ysc genes were repressed by CsrA as well, the corresponding protein secretion of Yops was promoted by CsrA (Ozturk et al., 2017). The authors proposed that YopE might be processed by a protease, that is downregulated by CsrA, and hence the overall effect of CsrA on Yop protein secretion is positive, despite it repressing Ysc gene expression (Ozturk et al., 2017). Additionally, CsrA affects motility by activating the master motility regulator *flhDC*, which regulates the sigma factor *fliA*, which in turn regulates *yplA* and other genes involved in flagellar assembly and function (LeGrand et al., 2015). Loss of *csrA* also leads to increased sensitivity to osmotic stress and growth inhibition at low (+4°C) and high (+42°C)

temperatures, as well as increased sensitivity to the antibiotics ampicillin and spectinomycin (LeGrand et al., 2015).

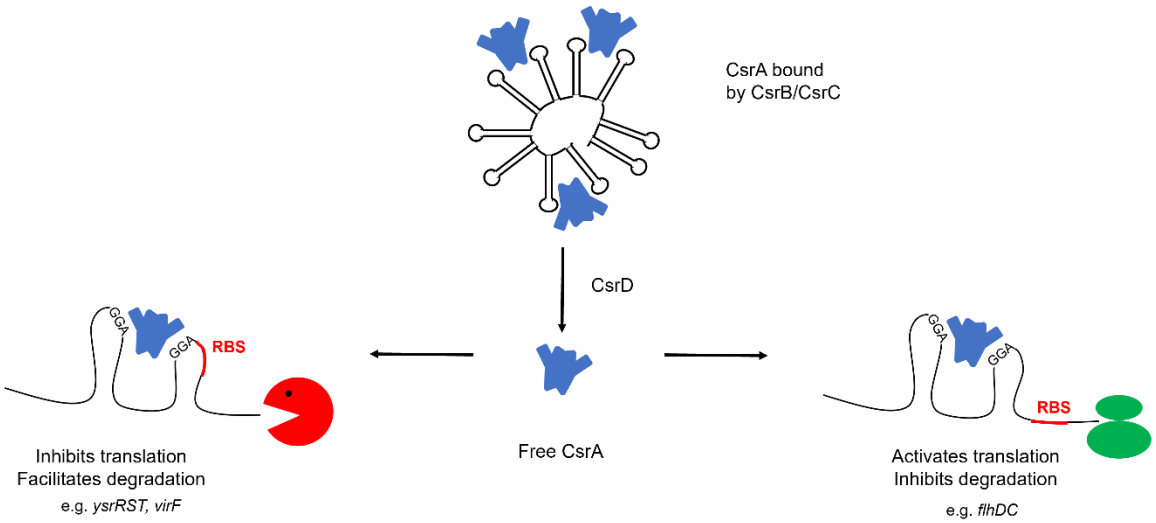


Figure 5) Regulation of CsrA in enteropathogenic Yersinia. The sRNAs CsrB and CsrC bind multiple CsrA molecules, sequestering them from their targets. Upon digestion of CsrB and CsrC by CsrD and RNase E, the level of free CsrA rises. The homodimeric CsrA binds two GGA motifs in the 5'-UTR of mRNAs, either obstructing the RBS, which inhibits translation and makes the RNA susceptible for degradation (left) or disrupting secondary structures that inhibit the RBS, therefore increasing translation (right). CsrA governs many important systems, e.g. the T3SS Ysc and Ysa, presumably via their regulators VirF, YsrRST and FlhDC. The figure is modified after Ozturk et al. (2017) and Vakulskas et al. (2015).

1.5.3 The RNA chaperone Hfq

1.5.3.1 Structure of Hfq

Hfq is an RNA chaperone that belongs to the Lsm protein family (Wilusz and Wilusz, 2013). It protects sRNAs from ribonuclease degradation and facilitates the interaction between sRNAs and their target mRNAs (Vogel and Luisi, 2011). It was originally discovered in *E. coli* as the host factor required for replication of the RNA bacteriophage Qβ (Franze de Fernandez et al., 1968). Hfq has a homohexameric ring structure consisting of six equal protomers, each of which is composed of one α-helix and five β-strands (Fig. 6).

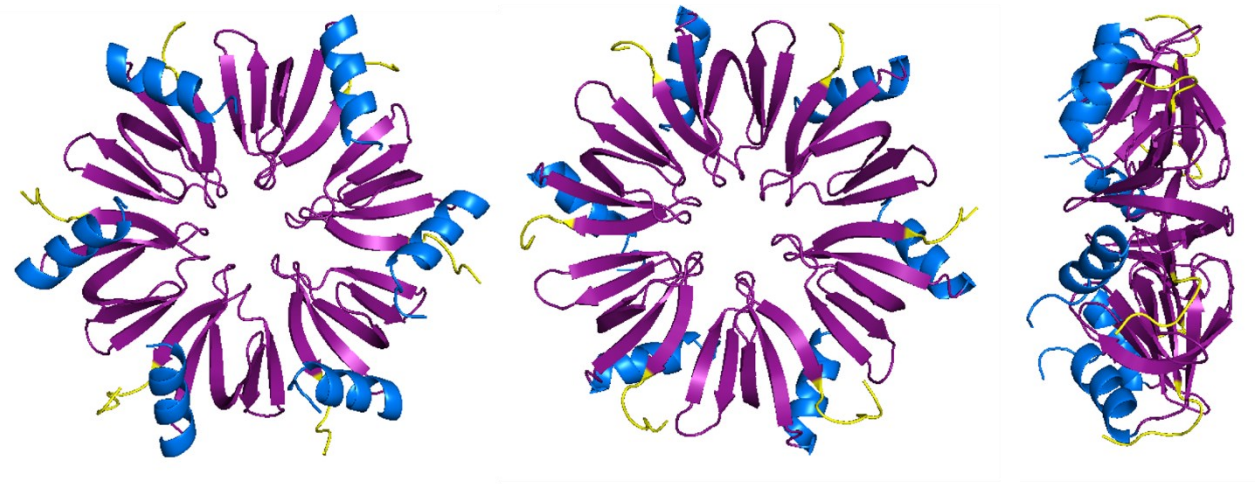


Figure 6) Structure of the E. coli Hfq. View from the proximal, distal and lateral side. The beta-sheets are purple, the alpha-helices blue, the C-terminal region is yellow. This graphic was made with PyMOL (PDB 1HK9).

While the β-strands line up as antiparallel sheets, the β_2-strand is curved, forcing the sheets into a doughnut-like structure. The protomer is linked via the β_4- and β_5-strand to its neighbouring protomers, connecting them into a ring (Vogel and Luisi, 2011). Hfq has four sites that can interact with RNA: the proximal face, the distal face, and according to newer studies also the rim and the C-terminal tail (Updegrove et al. 2016; Sauer et al., 2012). The proximal face with the amino-terminal α-helix binds to single-stranded poly-U sequences, common in Rho-independent transcription terminators in the 3' ends of sRNAs (Sauer & Weichenrieder, 2011; Morita et al., 2017). While the poly-U 3'-end wraps around the inside of the proximal core, with each monomer binding to a single uridine base, the dsRNA of its hairpin interacts independently of its sequence with residues across the proximal face (Orans et al., 2020). The distal face of Hfq has an affinity to polyA-sequences, commonly in the form of 2 to 4 ARN repeats (A = adenosine, R = purine, N = any base), while Genomic SELEX, along with structure analysis and modelling studies identified that Hfq binds the sequence AAYAAYAA (Y = pyrimidine) (Link et al., 2009; Lorenz et al., 2010). It should be noted that the proximal and distal face are not exclusive to sRNA and mRNA and some RNAs are able to interact with both sites (Fender et al., 2010). The positively charged rim exhibits three arginine residues. It provides an additional binding site especially for UA-rich sRNA or U-U dinucleotides within a sRNA, and also for some mRNAs (Updegrove et al. 2016,

Orans et al., 2020). Mutations in the arginine residues of the rim do not revoke the RNA binding ability of Hfq. However, even though ternary complexes between Hfq and its RNA partners can still be formed, sRNA-mRNA pairs cannot be released, leading to the assumption that the rim is crucial for the actual chaperone activity of Hfq (Panja et al., 2013). The flexible C-terminal region (CTR) takes part in diverse functions. It can assist the interaction with some RNAs and is involved in the recognition of sRNAs (Dimastrogiovanni et al., 2014) as well as the rapid release of RNA targets (Santiago-Frangos et al., 2016). A recent study in *E. coli* and the alphaproteobacterium *Caulobacter crescentus* also found the CTR to be involved in selecting sRNAs, by occluding the positively charged rim with its acidic residues and only unblocking it upon encounter of a preferred Hfq-dependent sRNA (Santiago-Frangos et al., 2019).

1.5.3.2 Functions of Hfq

Hfq is mostly described as an RNA chaperone, it has multiple functions such as

Chaperone activity: facilitating sRNA-mRNA interactions

As an RNA chaperone, Hfq binds mRNA on one side and regulatory sRNA on the other, facilitating their interaction (Vogel and Luisi, 2011). sRNAs have been grouped in two classes, which differ in the way they bind to Hfq and their targets (Schu et al., 2015). Class I sRNAs bind the proximal core with their U-rich 3'-end and the rim with their AU-rich region and a stem-loop. Their targets bind the distal face of Hfq. Class II sRNAs bind the proximal core too, but also the distal face of Hfq, while their mRNA targets interact with the rim (Schu et al., 2015; Santiago-Frangos et al., 2018). The order in which sRNA and mRNA bind to Hfq is random. Although the intrinsic dissociation rate of a single sRNA is slow, sRNAs bound to Hfq exchange rapidly, driven by the high concentration of free RNA molecules (Wagner, 2013). When an mRNA and a cognate sRNA are bound by Hfq, helix nucleation begins at the arginine residues of the rim, followed by the other complementary sequences. The mRNA-sRNA double strand is released rapidly from Hfq by the CTR, which reduces chances of a reverse reaction (Santiago-Frangos and Woodson, 2018). Hfq does not require ATP for its chaperone activity (Hämmerle et al., 2012).

Modulating mRNA stability or translation

Through the modulation of mRNA stability or translation, Hfq chaperone activity may have a negative or positive impact on protein synthesis (Fig. 7). An RBS can be obstructed by an sRNA, thus inhibiting translation. At the same time degradation can be facilitated either through presenting the mRNA in a way that facilitates ribonuclease cleavage, often through RNaseE, or by stimulating the polyadenylation of an mRNA by poly(A) polymerase which then triggers 3'-to 5' degradation by an exoribonuclease (Fig. 7-1) (Vogel and Luisi, 2011; Santiago-Frangos and Woodson, 2018). On the other hand, an Hfq-bound sRNA can disrupt a secondary structure of an mRNA, making an RBS accessible and therefore facilitate translation. It can also protect an mRNA from degradation by masking a ribonuclease cleavage site (Fig. 7-2). Hfq also seems to directly associate with proteins like RNase E and PNPase, protecting sRNAs from degradation.

Hfq-mediated post-transcriptional regulation without sRNAs

Mechanisms of mRNA control by Hfq that do not require its chaperone activity were recently discovered. For instance, Hfq can bind an mRNA, resolving secondary structures that impede sRNA binding, therefore remodeling mRNA structure rather than acting as a chaperone for mRNA-sRNA pairs (Hoekzema et al., 2019). Hfq can also repress translation without sRNAs, by binding at the RBS or close to it, creating a secondary structure inhibiting ribosome access (Ellis et al., 2015; Chen and Gottesman, 2017). In *P. aeruginosa*, Hfq was found to build complexes with the regulatory protein Crc (Catabolite repression control) and bind to mRNA targets to repress their translation (Pei et al., 2019). Although Enterobacteriaceae do not produce Crc, this shows yet another way of post-transcriptional regulation mediated by Hfq.

Hfq-mediated regulation of transcription

Hfq and sRNAs can also affect the transcription of genes by altering Rho-dependent transcription termination. Usually, the RNA helicase Rho binds to a cytosine-rich Rho utilization site (*rut*), moves along the mRNA strand and removes the RNA polymerase (Mitra et al., 2017). Hfq-bound sRNAs can either block ribosome entry, allowing Rho-

dependent transcription termination (Fig. 7-3) or block Rho access, allowing transcription of downstream genes (Fig. 7-4) (Kavita et al., 2018).

Hfq also interacts with the RNA polymerase (RNAP) and assists the assembly of the multi-subunit core of the RNAP (Sukhhodolets and Garges, 2003) and may affect the elongation step of transcription (Le Derout et al., 2010; Dos Santos et al., 2019).

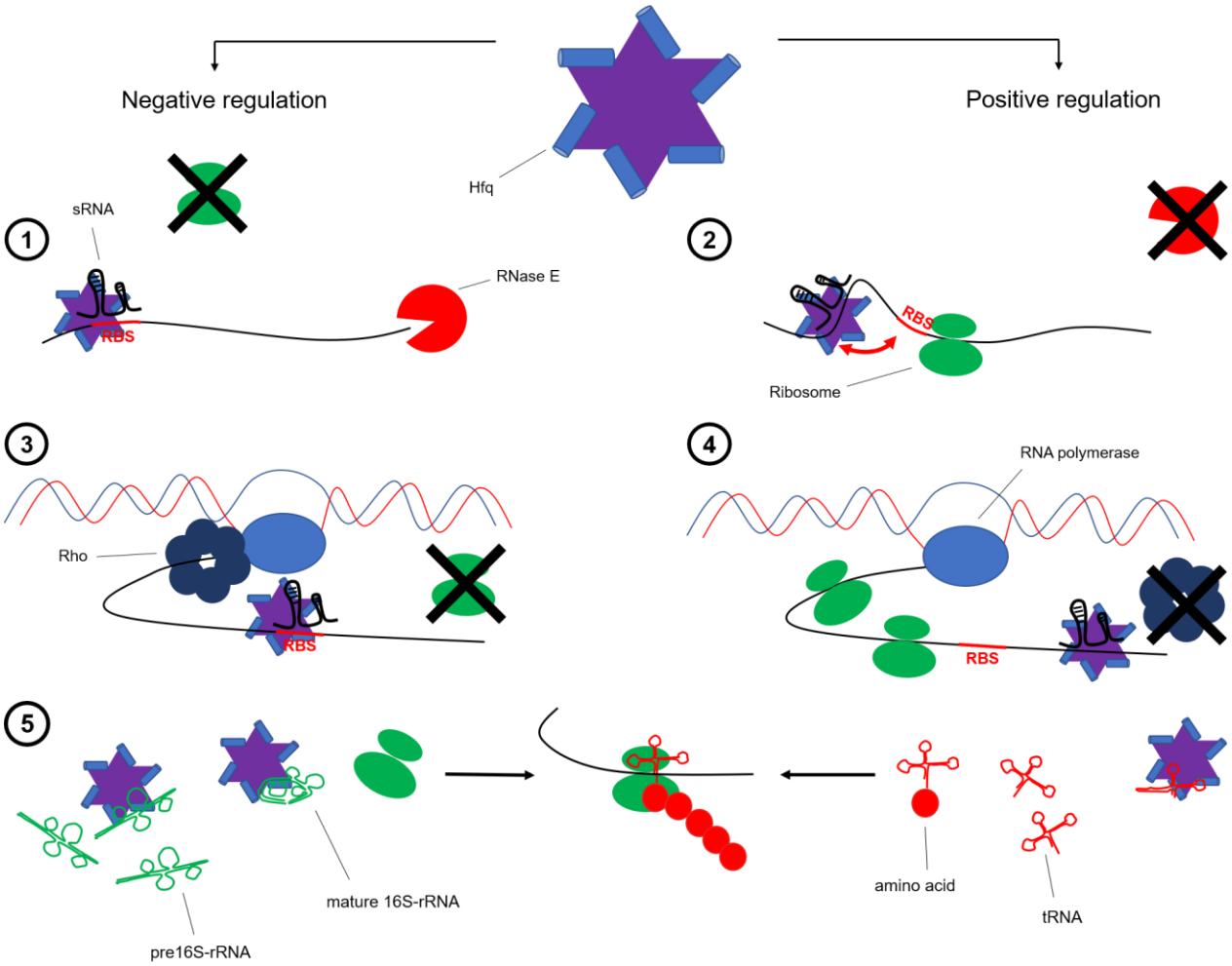


Figure 7) Regulation of genes via Hfq and sRNAs. Hfq and its associated sRNAs can upregulate or downregulate genes on different levels. ***1)*** *The RBS can be obstructed, inhibiting translation and degradation by RNases can be facilitated, resulting in negative regulation. Hfq can also inhibit translation without sRNA (not shown).* ***2)*** *By opening secondary structures, the RBS can also be made accessible, allowing translation, and RNases can be blocked, resulting in positive regulation.* ***3)*** *By blocking the RBS, Rho-dependent transcription termination can be promoted.* ***4)*** *Rho access can also be blocked, inhibiting transcription termination and allowing the transcription and translation of downstream genes.* ***5)*** *Hfq is also involved in tRNA and rRNA maturation and ribosome assembly. The figure is modified after Kavita et al. (2018).*

Hfq-mediated ribosome assembly and tRNA maturation

Additional to these functions of Hfq in gene regulation, newer studies have also shown its significance in ribosome biogenesis and assembly. Hfq is involved in processing and folding of 17S rRNA to mature 16S rRNA and also binds to pre30S particles (Andrade et al., 2018). Lack of Hfq is associated with disturbed, error-prone ribosome function, similar to the phenotypes produced by mutations in other ribosome assembly factors (Andrade et al., 2018; Dos Santos et al., 2019). Hfq also binds to tRNAs with its proximal face. It is thought to be involved in tRNA maturation as well, which would provide another explanation for the decreased translation fidelity observed in *hfq* mutants (Dos Santos et al., 2019).

Hfq interaction with DNA

Hfq can also interact with DNA through its C-terminal tail (Updegrove et al., 2010; Malabirade et al., 2018). The CTR seems to be important in the self-assembly of Hfq into amyloid-like fibrillar structures, which might have a role in directing Hfq cellular localization and compacting DNA (Fortas et al., 2015). Although the relation between Hfq and DNA has yet to be fully uncovered, Hfq seems to be directly involved in compaction and condensation and indirectly in supercoiling (Jiang et al., 2015; Malabirade et al., 2018). Hfq seems to bind dsDNA independent of its sequence at the phosphate backbone (Orans et al., 2020).

1.5.3.3 Distribution of Hfq-like proteins

Hfq and Hfq-like proteins are a widespread mediator of gene expression. Even archaea and eukaryotes have similar proteins, like the Sm proteins and the Sm-like (LSm) proteins (Mura et al. 2013; Wilusz and Wilusz, 2013), suggesting that this family of RNA binding proteins and their general role in posttranscriptional regulation has an ancient common ancestor (Vogel and Luisi, 2011; Møller et al., 2003; Zhang et al, 2002). Their mechanisms have sometimes developed in different directions over time, for example Sm-LSm proteins are involved in mRNA-splicing (Wilusz and Wilusz, 2005).

Regulatory sRNAs and their RNA chaperone Hfq are especially common among many bacterial lineages. About 50% of all sequenced bacterial species contain at least one copy of the RNA chaperone (Sun, 2002), including the pathogenic *Yersinia* species *Y. pseudotuberculosis* and *Y. pestis* (Schiano et al., 2010; Geng et al, 2009), other Gram-negative pathogenic bacteria, noteably enteropathogens, like enterohemorrhagic *Escherichia coli* (EHEC) (Shakhnovich et al., 2009), *Salmonella enterica* (Sittka et al., 2007), the abovementioned *Vibrio cholerae* (Ding et al., 2004) and others, like *Pseudomonas aeruginosa* (Sonnleitner et al., 2003), *Francisella tularensis* (Meibom et al., 2009) and *Neisseria meningitidis* (Fantappiè et al. 2009). Hfq is also found in Gram-positive pathogens like *Listeria monocytogenes* (Christiansen et al., 2004), but it is often not required for the interaction of sRNAs with their targets (Vanderpool et al., 2011). In these species, Hfq might be more important for rRNA and tRNA maturation (dos Santos et al., 2019).

The mechanism of Hfq-mediated posttranscriptional control is so crucial for quick adaptation, that mutants of *Y. enterocolitica, V. cholerae and P. aeruginosa* and other species lacking *hfq* are strongly attenuated in mice (Kakoschke, 2016; Ding et al., 2004; Sonnleitner et al., 2003). Other studies showed that loss of Hfq for instance in *E. coli* and *P. aeruginosa* can lead to an increased sensitivity to a variety of antibiotic classes, e.g. by altering influx and efflux, changes in energy metabolism or cell wall and LPS composition (Yamada et al., 2010; Pusic et al., 2018). Post-transcriptional regulation and sRNA play a major role in the regulation of antibiotic resistance (Dersch et al., 2017). Since regulatory RNA have been shown to be druggable targets, attenuating those regulatory networks could therefore re-sensitize resistant bacteria or weaken their virulence (Dersch et al., 2017).
The regulatory effects of Hfq in different bacteria can vary. A hypothetic drug targeting Hfq could therefore have very variable effects. Even seemingly similar mechanisms in closely related bacterial species can be acquired independently or evolve differently after acquisition (Reuter et al., 2014). Therefore, the role of Hfq has to be examined separately in every single species.

1.5.3.4 The pleiotropic role of Hfq in Yersinia enterocolitica

Prior to the beginning of this thesis, the role of Hfq in the three pathogenic *Yersinia* ssp. had been investigated by reverse genetics (Nakao et al. 1995; Geng et al., 2009, Bai et al., 2010, Schiano et al., 2010, Kakoschke et al. 2014) and further studies were reported in the course of this work (Kakoschke et al., 2016, Leskinen et al., 2017). Loss of Hfq was associated with changes in growth and metabolism, production of surface-associated pathogenicity factors, as well as reduced resistance to stress.

Loss of Hfq had a considerable impact on bacterial growth and cell morphology. *hfq* mutants of *Y. enterocolitica* exhibited a slower growth as wild-type strains and entered stationary phase earlier (at a lower OD_{600}) (Kakoschke et al., 2014; Leskinen et al., 2017), similar to *Y. pseudotuberculosis* and *Y. pestis* (Geng et al., 2009; Schiano et al., 2010 and Bai et al., 2010). The mutant cells were elongated, approximately twice as long as wild-type cells (Leskinen et al., 2017). Additionally, *hfq* mutants did not form bacterial cell aggregates and were more dispersed (Leskinen et al. 2017).

Hfq contributes greatly to the remodeling of the cell envelope. For instance, *lpxR,* which controls lipid A deacylation was overexpressed at 37°C in the *hfq* mutant in both serotypes (Kakoschke et al., 2014; Leskinen et al., 2017). Additionally, in serotype O:8, the LPS O-antigen was significantly altered, an effect that was not seen in serotype O:3 (Kakoschke et al., 2016, Leskinen et al., 2017). Hfq also impacted OMP expression, downregulating Ail, OmpX and the pilin MyfA, while upregulating YadA and InvA (Kakoschke et al., 2016). Finally, Hfq negatively impacted flagellin production, thereby impairing motility and biofilm production in *Y. enterocolitica* O:3 *hfq* mutants (Leskinen et al., 2017). Hfq did not seem to influence the function of the Ysc type III secretion system in *Y. enterocolitica* in vitro, as opposed to *Y. pseudotuberculosis* (Schiano et al., 2010; Kakoschke et al., 2014). Nevertheless, in vivo Yop protein secretion was still affected, most likely because the adhesins InvA and YadA were downregulated in *hfq* mutants, resulting in less contact between the bacterial and the host cells and a less effective protein translocation (Kakoschke et al., 2016).

Hfq also regulates bacterial metabolism. It repressed carbohydrate metabolism in *Y. enterocolitica* (Kakoschke et al., 2014; Leskinen et al., 2017), downregulated proteins involved in amino acid metabolism and peptide transport, and upregulated proteins involved in lipid metabolism and transport as well as ATP synthesis (Kakoschke et al.,

2014). In contrast to other species, in *Y. enterocolitica* Hfq also seemed to negatively impact iron metabolism by downregulating yersiniabactin and its receptor FyuA as well as the ferrichrome siderophore receptor FcuA (Kakoschke et al., 2014; Salvail and Massé, 2012; Prévost et al., 2007).

Loss of Hfq has been associated with reduced resistance to heat, oxidative stress, and acidic environments, most likely caused by the downregulation of chaperones and other stress resistance genes (Leskinen et al., 2017, Kaskoschke et al., 2014). *Y. enterocolitica hfq* mutants have shown an increased level of *rpoE* expression, which could reflect envelope stress (Zeuzem, 2018).

Hfq has a huge effect on the pathogenicity of *Yersinia* spp. (Geng et al., 2009 and Schiano et al., 2010). Mice infected intraperitoneally with *Y. enterocolitica* O:8 *hfq*-mutants showed less symptoms than those infected with wild-type bacteria, while loss of *Hfq* also impacted the ability to colonize liver and spleen (Kakoschke et al., 2016). *Y. enterocolitica* O:3 lacking Hfq were also considerably less virulent and invasive upon intragastric infection than wild-type strains (Leskinen et al., 2017). However, after intraperitoneal infection, mice infected with *hfq* mutant strains died early, while wild-type infected mice did not die at all. It was hypothesized, that Hfq deficiency is associated with a fragile cell envelope and that those bacteria would release more LPS than wild-type strains, causing an endotoxic shock syndrome (Leskinen et al., 2017).

In summary, Hfq has a profound impact on *Y. enterocolitica*. It influences growth and cell morphology, alters the cell envelope, induces changes in carbon, nitrogen and lipid metabolism and impairs iron uptake. Furthermore, it promotes resistance to a variety of stressors and is overall aggravating virulence and invasiveness.

1.6 Goals of this dissertation

Throughout their billions of years of evolution, bacteria have evolved a sophisticated arsenal of virulence factors that help them survive in the environment and during host infection. Since most virulence factors are not constitutively expressed, bacteria must respond quickly to changes in surrounding conditions. Changes in gene transcription might not always come into effect quickly enough and furthermore do not influence the

fate of mRNAs that have already been synthesized. While transcription takes only a few seconds, translation might take several minutes. If genes are only influenced on the transcriptional level, previously produced mRNA would still be translated, and the newly synthesized proteins would need to be degraded. Therefore, stoping the translation of unwanted proteins and conducting the entire gene expression machinery in the right direction might be an effective way to save time and resources.

Hfq is known as an RNA chaperone assisting in the posttranscriptional regulation of numerous genes in a wide variety of bacterial species, including many pathogens. At the beginning of this dissertation, the impact of Hfq on *Y. enterocolitica* had been assessed using reverse genetics, unveiling its role in the deployment of several virulence factors as well as in metabolism and stress resistance. Many pathogenicity-factors are already known or suspected to be Hfq-dependent, but the nature of their dependence – be it direct through interaction with Hfq and a regulatory sRNA or indirect through other regulatory factors – is in many cases still unknown. In *Y. enterocolitica* O:3, *rpoS*, *rovA* and genes belonging to their regulon are downregulated in the *hfq* mutant, whereas *rovM* is overexpressed. Many of the observed effects might be mediated by effects of Hfq on those regulatory proteins. In *Y. enterocolitica* O:3 overexpression of RovM at least partially accounted for the growth defect and the different colony morphology as well as the decreased flagellin production, impaired motility and reduced biofilm production observed in the *hfq*-mutant (Leskinen et al., 2017). Indeed, Hfq could similarly affect gene expression in *Y. enterocolitica O:8* at different stages, either directly through the binding of mRNA (thereby mediating post-transcriptional regulation) or indirectly through its effect on transcriptional regulators or on the production of proteases (post-translational regulation).

The goal of this dissertation is to shed light on the regulatory pathways controlled by Hfq in *Y. enterocolitica*. We aimed to:

1) identify mRNAs and sRNAs whose abundance depends on Hfq
2) analyze their interaction with Hfq.

We first performed a transcriptome analysis of the wild-type strain JB580v and of the *hfq*-negative strain SOR17. Gene expression at different temperatures (environment vs.

host) was compared between wild-type and *hfq* mutant strains. This allowed us to gain an overview over the influence of Hfq on transcript abundance for a number of different functional systems and virulence factors. We confirmed the differential expression of some genes using Northern blots.

Second, the ability of identified Hfq-dependent RNAs to directly interact with Hfq was examined. To achieve this, we performed co-immunoprecipitation of Hfq, extracted RNA bound to Hfq and analyzed it by Northern blotting. This allowed us to assess whether candidate mRNAs bind to Hfq and are most likely subject to its chaperone function.

Detailed knowledge of bacterial pathophysiology is necessary to identify possible targets for antimicrobial drugs. This study together with other research in this field can help with future efforts to develop alternative strategies in the struggle against infections and their resistance to established treatment options.

2 Material and Methods

2.1 Material

2.1.1 Devices

Table 1) Devices used in the study

Device	Model	Brand
Analytical balance	1702MP8 Typ 440-33, Typ 2500-2	Sartorius (Göttingen) Kern (Balingen)
Automated Electrophoresis System	Experion ™ vortex, priming and electrophoresis station	Bio-Rad (Hercules, CA)
Blotter	V10-SDB Semi-Dry Blotter	Scie-Plas (Cambridge)
Centrifuge • Tabletop • Refrigerated • Minicentrifuge • Microcentrifuge	 100VAC 5417R, 5417C 3-30K, 4K15 Sprout Qik spin	 Roth (Karlsruhe) Eppendorf (Hamburg) Sigma-Aldrich (St. Louis, MO) Heathrow Scientific (Vernon Hills, IL) Edwards Group (Narellan, NSW)
Crosslinker	CL-508	Techne (Cambridge)
Electrophoresis cell • For proteins (PAGE) • For nucleic acids (RNA and DNA)	 Mini-PROTEAN® Tetra Cell, Power Pac 200 Owl™ Easy Cast™ B1A, B1, B2	 Bio-Rad (Hercules, CA) Thermo Fisher Scientific (Waltham, MA)
Freezer / Refrigerator • +4°C • -20°C • -80°C	 Profi Line Profi Line Ultima II	 Liebherr (Bulle) Liebherr (Bulle) Revco Technologies (Twinsburg, OH)
Gel documentation system	GelDoc EQ System	Bio-Rad (Hercules, CA)
Heating block	TR-L 288 Dri Block ® DB-2D	Liebisch (Bielefeld) Techne (Cambridge)
Imaging System	ChemiDoc XRS+	Bio-Rad (Hercules, CA)
Incubator Incubator (rotating)	B20 PersonalHyb ™	Heraeus (Hanau) Stratagene (San Diego, CA)
Laminar flow	Herasafe HS 12 BDK-S 1200 Safe 2020	Heraeus (Hanau) BDK Luft- und Reinraumtechnik GmbH (Sonnenbühl) Thermo Fisher Scientific

		(Waltham, MA)
Magnetic Stirrer	RCT basic	Ika (Staufen)
pH meter	SG2	Mettler Toledo (Columbus, OH)
Pipet	Pipetman 2µl, 10µl, 100µl, 200µl, 1000µl 0,5-10 µl, 2-20 µl, 10-100 µl,20-1000 µl	Gilson (Middleton, WI) Eppendorf (Hamburg)
Pipet aid	accu-jet ® pro	Brand (Wertheim)
Platform shaker	Duomax 1030 T	Heidolph (Schwabach)
Shaking incubator	Multitron Pro	Infors HT (Bottmingen)
Sonicator	Sonifier 250 CE	Branson (Danbury, CT)
Spectrophotometer	Nanodrop 1000	Thermo Fisher Scientific (Waltham, MA)
Spectrophotometer	Ultrospec 3100 pro	Amersham Biosciences (Little Chalfont)
Thermocyler	Veriti ™ Applied Biosystems ™	Thermo Fisher Scientific (Waltham, MA)
Vortex	Vortex Genie 2 G-560E Reax top	Scientific Industries (Bohemia, NY) Heidolph (Schwabach)
Waterbath (unstirred)	W350	Memmert (Schwabach)

Unlisted devices are standard laboratory equipment

2.1.2 Consumables (Specific material)

Table 2) Consumables used in the study

Item	**Supplier**
Cryogenic tubes	Nalgene (Rochester, NY)
Cuvettes for photospectrometer (1.5 ml)	Brand (Wertheim)
Filter (0.22 µm, sterile)	Merck (Darmstadt)
Glass pipets (1 ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Hirschmann (Eberstadt)
Hybridization bags	Roche (Basel)
PCR tubes (0.2 ml)	VWR (Radnor, PA)
Petri dishes	Greiner Bio-One (Kremsmünster)
Pipet tips • White (0.5 – 20 µl) • Yellow (2 – 200 µl) • Blue (50 – 1000 µl)	Brand (Wertheim)

• SafeSeal	Biozym Scientific (Hessisch Oldendorf)
Plastic tubes (PCR grade, 0.2 ml)	Eppendorf (Hamburg)
Plastic tubes (1.5 ml, 2 ml)	Eppendorf (Hamburg)
Plastic tubes (15 ml, 50 ml)	BD Biosciences (Franklin Lakes, NJ) Greiner Bio-One (Kremsmünster)
PVDF membrane ROTI®PVDF 0.45, 375 × 26.5 cm	Roth (Karlsruhe)
Whatman® cellulose filter paper	Thermo Fisher Scientific (Waltham, MA)

Unlisted consumables are standard laboratory equipment

2.1.3 Chemicals

Table 3) Chemicals used in the study

Item	Supplier
6-amino-n-caproic acid	Sigma-Aldrich (St. Louis, MO)
Acetic acid	Roth (Karlsruhe)
Acrylamide	Serva (Heidelberg)
Agarose	Invitrogen (Waltham, MA) Cambrex (East Rutherford, NJ)
Ammonium persulfate (APS)	Roth (Karlsruhe)
Bacto Agar	BD Biosciences (Franklin Lakes, NJ)
Bromophenol Blue	Sigma-Aldrich (St. Louis, MO)
Cetyltrimethylammoniumbromid (CTAB)	Roth (Karlsruhe)
Chloroform	Roth (Karlsruhe)
Coomassie Brilliant Blue	Merck (Darmstadt)
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich (St. Louis, MO)
DNase (Rnase free)	Qiagen (Hilden)
Ethanol	Roth (Karlsruhe)
Ethidium bromide	Sigma-Aldrich (St. Louis, MO)
Ethylenediaminetetraacetic acid (EDTA)	Roth (Karlsruhe)
EDTA 0.5M, pH 8.0	Invitrogen (Waltham, MA)
Formaldehyde 37%	Sigma-Aldrich (St. Louis, MO)

Formamide	Sigma-Aldrich (St. Louis, MO)
Gene ruler 1 kb DNA Ladder	Thermo Fisher Scientific (Waltham, MA)
Glycerol	Sigma-Aldrich (St. Louis, MO)
H_2O_{dest} (Ampuwa)	Fresenius Kabi (Bad Homburg)
H_2O_{dest} used for PCR reactions	Gibco, Thermo Fisher Scientific (Waltham, MA)
H_2O, DEPC-treated	Ambion, Thermo Fisher Scientific (Waltham, MA)
HCl	Roth (Karlsruhe)
HRP Substrate Luminol Reagent Immobilon®	Merck Millipore (Darmstadt)
HRP Substrate Peroxide solution Immobilon®	Merck Millipore (Darmstadt)
Isopropanol	Sigma-Aldrich (St. Louis, MO)
Lysozyme	AppliChem (Darmstadt)
Magnesiumchloride ($MgCl_2$)	Roth (Karlsruhe)
Mercaptoethamol	OLS Omni Life Science (Bremen)
Methanol	Roth (Karlsruhe)
Milk powder	Sigma-Aldrich (St. Louis, MO)
MESA (MOPS EDTA Sodium Acetate)	Sigma-Aldrich (St. Louis, MO)
Parafilm	Benis (Neenah, Wisconsin)
Phenol:chloroform:isoamylalcohol (25:24:1)	Roth (Karlsruhe)
Phosphate buffered saline (PBS) tablet	Sigma-Aldrich (St. Louis, MO)
Potassium chloride (KCl)	Merck (Darmstadt)
Proteinase K	Sigma-Aldrich (St. Louis, MO)
Protein ladder	Thermo Fisher Scientific (Waltham, MA)
RNAprotect ® Cell reagent	Qiagen (Hilden)
Rnase Zap ®	Ambion, Thermo Fisher Scientific (Waltham, MA)
Saline Sodium Citrate (20xSSC)	Thermo Fischer Scientific (Waltham, MA)
SDS 10% (v/v)	Serva (Heidelberg), Sigma-Aldrich (St. Louis, MO)
Sodium acetate	Roth (Karlsruhe)
Sodium chloride (NaCl)	Roth (Karlsruhe)
Sodium hydroxide (NaOH)	Roth (Karlsruhe)
Sodium hydroxide (NaOH) pellets	Sigma-Aldrich (St. Louis, MO)

Tetramethylethylenediamine (TEMED)	Biomol (Hamburg)
Tris(hydroxymethyl)aminomethane (TRIS)	MP Biomedicals (Irvine, CA), Roth (Karlsruhe)
TRIS 1.0 M, pH 7.5	Merck (Darmstadt)
Tryptone	Roth (Karlsruhe)
Tween	Serva (Heidelberg)
Q5® Hot Start High-Fidelity 2X Master Mix	New England Biolabs (Ipswich, MA)
Qiazol Lysis Reagent	Qiagen (Hilden)
Yeast Extract Powder	MP Biomedicals (Irvine, CA)

Unlisted chemicals are standard laboratory equipment

2.1.4 Solutions, buffers and media

Table 4) Solutions, buffers and media used in the study

Buffer/Solution	Preparation		Source
Agarose gel (1%)	1 g 100 ml	agarose TAE buffer	
Anode Buffer I	36.3 g 100 ml	(300 mM) Tris (10% (v/v)) Methanol ad 1000ml H_2O_{dest} and adjust pH to 10.4 with HCl	
Anode Buffer II	3.0 g 100 ml	(25 mM) Tris (10% (v/v)) Methanol ad 1000ml H_2O_{dest} and adjust pH to 10.4 with HCl	
Blocking solution (Western Blot)	5 g 100 ml	Milk powder PBS-T	
Cathode Buffer	3.0 g 5.2 g 100 ml	(25 mM) Tris (40 mM) 6-amino-n-caproic acid (10% (v/v)) Methanol ad 1000ml H_2O_{dest} and adjust pH to 9.4 with HCl	
Coomassie stain solution	2 g 360 ml 360 ml	Coomassie brilliant blue Methanol H_2O	

	80	Acetic acid	
DEPC-treated water	1ml	DEPC ad 1000ml H_2O_{dest}, stirr for at least 4 h, autoclave twice	
Gel (running gel, 2x)	4 ml 3.32 ml 2.5 ml 0.1 ml 0.1 ml 0.004 ml	H_2O_{dest} 30% acrylamide 1.5 M Tris HCl pH 8.8 10% SDS 10% APS TEMED	
Gel (stacking gel, 2x)	2 ml 0.51 ml 0.39 ml 0.03 ml 0,03 ml 0.003 ml	H_2O_{dest} 30% acrylamide 1.5 M Tris HCl pH 8.8 10% SDS 10% APS TEMED	
IP-buffer	6.057 g 11.183 g 0.095 g	Tris-HCl (pH 7.4) (50 mM) KCl (150 mM) $MgCl_2$ (1 mM) ad 1000 ml H_2O_{dest}	Derived from Pfeiffer et al., 2007
KCl (1.5M)	111.825g	KCl ad 1000 ml H_2O_{dest}	
KCl (1 M)	74.55 g	KCl ad 1000 ml H_2O_{dest}	
Laemmli-buffer (2x)	5 ml 8 ml 4 ml 2 ml 1 ml 0.02 g	Tris-Cl (pH 6.8) SDS 10% Glycerol mercaptoethanol 1 ml H_2O_{dest} to dissolve Bromphenol blue	
LB (lysogeny broth) agar	6 g 400 ml	Agar LB medium	
LB (lysogeny broth) medium	5 g 5 g	NaCl Yeast Extract	

	10 g 1 ml	Tryptone Sodium hydroxide ad 1000 ml H_2O_{dest}	
LB (lysogeny broth) freezing medium	30 ml 70 ml	Glycerol LB medium	
MgCl2 (1M)	203.3 g	$MgCl_2$ ad 1000 ml H_2O_{dest}	
NaCl (5M)	292.2 g	NaCl ad 1000 ml H_2O_{dest}	
PBS solution (phosphate buffered sline)	1 tab 200 ml	PBS Sigma P4417 H_2O_{dest}	
PBS-Tween (PBS-T)	1 ml	Tween ad 1000 ml PBS solution	
Running buffer (10x) (protein gels)	30.2 g 188 g 10 g	Tris Glycine SDS ad 1000 ml H_2O	
Running buffer (10x) (RNA gels)	1 bottle 26.302 g	MESA buffer (Sigma-Aldrich) EDTA (pH 8.3) (90 mM) ad 1000 ml H_2O_{dest} the final buffer contains: • 40 mM MOPS • 10 mM sodium acetate • 100 mM EDTA (pH 8.3)	
Stripping buffer	10 ml 10 ml 1 ml	Formamide (99.7%) SDS (10%) 1 M Tris/HCl pH 7.5	
TBS (pH 7.4)	6.057 g 8.766 g	Tris-HCl (50 mM) NaCl (150 mM) ad 1000 ml H_2O_{dest} Adjust to pH 7.4	
TAE buffer	242 g 57.1 ml	Tris Acetic acid	

	37.2 g	EDTA ad 1000 ml H_2O_{dest}	
TE buffer	10 ml 2 ml	1M Tris-Cl, pH 8.0 500mM EDTA, pH 8.0 ad 1000 ml H_2O_{dest}	

2.1.5 Antibodies

Table 5) Antibodies used in the study

Target	Raised in	Source
FLAG	Mouse 1:5000	Sigma-Aldrich (St. Louis, MO)
Horseradish peroxidase-conjugated anti-mouse immunoglobulin G	Goat (dilution 1:10,000)	GE Healthcare (Chicago, IL)

2.1.6 Kits

Table 6) Kits used in the study

Application	Kit	Supplier
RNA purification	miRNeasy ® Mini Kit	Qiagen (Hilden)
Northern blots	DIG Northern Starter Kit DIG Wash and Block Buffer Set	Roche (Basel) Roche (Basel)
DNA purification	NucleoSpin ® Gel and PCR Clean-up	Macherey-Nagel (Düren)
Experion	RNA StdSens Analysis	Bio-Rad (Hercules, CA)

2.1.7 Primers

Table 7) Primers used in the study

Primer	Sequence	T_m
OR298-T7-hfq	**TAATACGACTCACTATAGGG**TCATCGCTATCCTGCT	63°C
OR299-hfq	TGGCTAAGGGGCAATCTTTG	55°C
forward primer SB1-rseB	TTGGTTCTCCGTCTGTTTAATGGC	58°C
reverse primer SB2-T7-rseB	**TAATACGACTCACTATAGGG**GCAGACTCCAAAACGA	63°C
forward primer SB3-	CGGAGAAACTCTTGATAGTGAGCTGA	58°C

rseA		
reverse primer SB4-T7-rseA	**TAATACGACTCACTATAGGG**AGCGGCTTGTGTAGAT	63°C
forward primer SB5-rpoE	TCGGATGAGCGAGCAGTT	57°C
reverse primer SB6-T7-rpoE	**TAATACGACTCACTATAGGG**CGGCTGAACTTTG	62°C
forward primer SB11-ail	AGCCATGTCAGTGATATGGTTATTGT	56°C
reverse primer SB12-T7-ail	**TAATACGACTCACTATAGGG**CTGCACCAAGCATC	63°C
forward primer SB13-ompX	TAATTTCATGGTGTTTTAGTTTCACTTAAA	53°C
reverse primer SB14-T7-ompX	**TAATACGACTCACTATAGGGG**AAAGTGTAACCAAC	60°C
forward primer SB15-YE3262	AAACTTTTCCAGGAGGATTACTGTGC	57°C
reverse primer SB16-T7-YE3262	**TAATACGACTCACTATAGGG**TCACTCATCGACATAA	60°C

TAATACGACTCACTATAGGG (bold) is the sequence of the T7 promoter.

2.1.8 Strains

Table 8) Strains used in the study

Organism	Strain	Description	Reference
Y. enterocolitica	JB580V	Wildtype, derivative of clinical isolate 8081, restriction endonuclease-negative (R-), methyltransferase-positive (M+), carrying virulence plasmid pYVO8	Kinder et al., 1993
Y. enterocolitica	SOR17	JB580v derivative with a deletion of *hfq* marked with a Km^R cassette	Kakoschke et al., 2014
Y. enterocolitica	SOR35	JB580v derivative with an unmarked chromosomal fusion of *hfq* with sequences encoding the 3xFLAG epitope (Hfq-FLAG)	Kakoschke et al., 2014
Y. enterocolitica	SOR43	JB580v derivative with a chromosomal fusion of *csrA* with sequences encoding the 3xFLAG epitope (CsrA-FLAG)	Fischbach, 2012

2.1.9 Software

Primer design

Primers were designed with CLC DNA Workbench and analyzed with the OligoAnalyzer Tool from Integrated DNA Technologies - IDT (https://eu.idtdna.com/pages). The genomic sequence for the analysis was downloaded from the website of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

Transcriptomic analysis

Results of the RNA-seq analysis were performed using CLC Genomic Workbench 7.0. For the transcriptomic analysis, functional annotation clustering was performed with DAVID Bioinformatics (https://david.ncifcrf.gov/). Further information on clustered pathways was gained from the KEGG PATHWAY database (https://www.genome.jp/kegg/pathway.html). Information on particular genes was also derived from the website of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). CopraRNA and IntraRNA by Freiburg RNA tools were used for binding predictions between sRNA and mRNA molecules (http://rna.informatik.uni-freiburg.de/). For analysis of RNAs and similarity to homologes in other species Rfam (https://rfam.xfam.org/) and BLAST (Basic local alignment search tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used.

Blot analysis

Images from Northern and Western Blots were taken with ChemiDoc XRS+ and analyzed with Image Lab by BioRad.

Figures and images

Figures and images were created with Microsoft PowerPoint, PyMOL and CLC Genomics Workbench.

2.2 Methods

2.2.1 Growth of bacterial cultures

Cultures were grown at 27°C or 37°C under aerobic conditions either on solid LB agar or in liquid LB medium on a shaking incubator at 180 rpm (rounds per minute). By measuring the optical density of the cultures at a wavelength of 600nm (OD_{600}) their growth could be assessed. Liquid LB medium was used as a reference. To ensure an

accurate measurement, the cultures were diluted if their OD_{600} was higher than 0.8. Bacteria were precultured overnight and then diluted to an OD_{600} of 0.1 to ensure an equal starting point.

2.2.2 Storage of bacterial cultures

For long term storage bacteria were grown overnight on LB agar and subsequently suspended in LB medium with 50% glycerol. The cultures were filled in cryotubes and stored at -80°C. For shorter storage periods of a few days, bacteria streaked out on agar plates were kept at +4°C.

2.2.3 RNA purification

Total RNA was purified from bacterial cultures using miRNeasy kit. Culture OD_{600} was measured and a volume containing an estimated $5*10^8$ cells (~1-1.5 ml) was mixed together with twice the volume of RNA protect reagent. Following centrifugation, the cell pellet was stored at -80°C until further processing. Pellets were resuspended in 100 µl of TE buffer containing 1 mg lysozyme, incubated for 5 min at room temperature, ensuring a quick lysis and minimizing the duration of RNA degradation. The lysis was immediately followed by RNA preparation, beginning with the addition of Qiazol Lysis Reagent. Qiazol contains the chaotropic agent guanidinium thiocyanate that denatures proteins including RNases.

140 µl chloroform were added and the mixture was shaken vigorously for 15 seconds. The tubes were centrifuged at 12,000 g for 15 min at +4°C, separating the mixture into three phases: The lower organic phase containing lipids and cellular debris, the interphase containing the proteins, and the upper aqueous phase containing the nucleic acids. 350 µl of the upper, aqueous phase were carefully transferred to a new RNase-free collection tube. 525 µl pure ethanol were added and mixed by pipetting up and down. The solution was pipetted on an RNeasy Mini spin column and centrifuged at 8,000 g for 15 s at room temperature. 350 µl Buffer RWT were pipetted on the columns and centrifuged again with the same settings. The buffer contains guanidine salt and ethanol and provides optimal conditions for the RNA to bind to the silica membrane of the column. 80 µl DNase I, containing about 27 Kunitz units, were pipetted on top of the column and then incubated for 15 min at room temperature to digest any co-purified

DNA. The columns were washed with 500 µl Buffer RWT and twice with Buffer RPE, a mild washing buffer that removes remaining salts from the column. The column was then centrifuged for 1 min to drain any excess fluid. The collection tubes were replaced, and the columns were centrifuged with 30 µl RNase-free water for 1 min. The eluted RNA was split in different tubes and stored at -80°C for later analysis.

In co-IP experiments both the 'input-samples' taken immediately after the lysis and centrifugation and the 'output-samples' that had been incubated with the beads were prepared at the same time. The addition of Qiazol stopped RNase activity which allowed me to store the samples until after the incubation and then perform the RNA extraction with all samples at the same time. This was according to manufacturer's information and was confirmed in experiments that showed no difference in RNA quality between samples prepared immediately and samples stored for one hour.

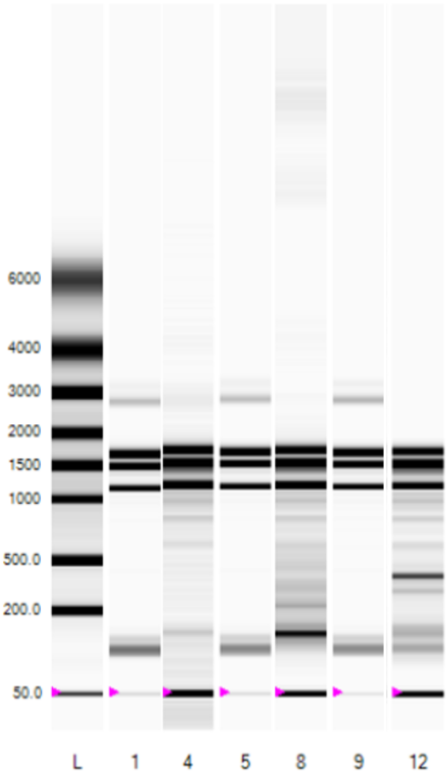
2.2.4 Determination of RNA quality

To determine RNA quantity and quality, the Nanodrop spectrophotometer was used. It measures the absorption of light at a spectrum between 220 nm and 750 nm and then calculates the concentration of the sample based on the Beer-Lambert equation (absorbance = extinction coefficient * path length * concentration) as well as quotients of the absorption at different wavelengths. For purified RNA, a A_{260nm}/A_{280nm} ratio of around 2.0 and a slightly higher A_{260nm}/A_{230nm} ratio is typical. A considerably lower ratio can be indicative of protein, phenol or other contaminants that absorb strongly at 280 nm and 230 nm, respectively (Thermo Fisher Scientific, NanoDrop User Manual).

As a second determinant of RNA quality the Experion RNA StdSens Analysis Kit by BioRad was used. The Experion is an automated electrophoresis system that allows the separation, staining and detection of small amounts (1 µl) of samples on a microfluidic chip. For the analysis the program aligns the ladder to the samples using the lower marker, that was contained in the loading buffer and was therefore visible in every sample. Besides the lower marker, the electropherogram showed two other peaks that related to the 16S and 23S rRNA. The RQI (RNA quality index) was developed for eukaryotic cells. It therefore takes into account the 18S region, the 28S region and the region below the 18S band. An algorithm is used to compare these regions to a set of

degradation standards (Denisov et al., 2008). The regions the program would use for its calculations sometimes had to be adjusted to the prokaryotic rRNA manually.

a)



b)

well	sample	ng/µl	260/280	260/230
1	Wildtype (JB580v)	2472.4	2.07	2.44
4	Wildtype (JB580v)	19.6	1.79	0.35
5	Hfq-FLAG (SOR35)	2276.0	2.07	2.46
8	Hfq-FLAG (SOR35)	30.9	2.08	0.37
9	CsrA-FLAG (SOR43)	2425.4	2.08	2.29
12	CsrA-FLAG (SOR43)	18.7	2.00	1.39

c)

Well ID	Sample Name	RNA Area	RNA Concentration (ng/µl)	Ratio [28S:18S]	RQI
L	Ladder	582.58	160.00		
1	Sample 1	1,262.25	346.66	0.04	10.0
4	Sample 4	31.19	8.57	0.01	N/A
5	Sample 5	1,193.25	327.71	0.04	10.0
8	Sample 8	104.02	28.57	0.00	8.5
9	Sample 9	1,407.83	386.64	0.05	10.0
12	Sample 12	75.50	20.73	0.00	8.3

d)

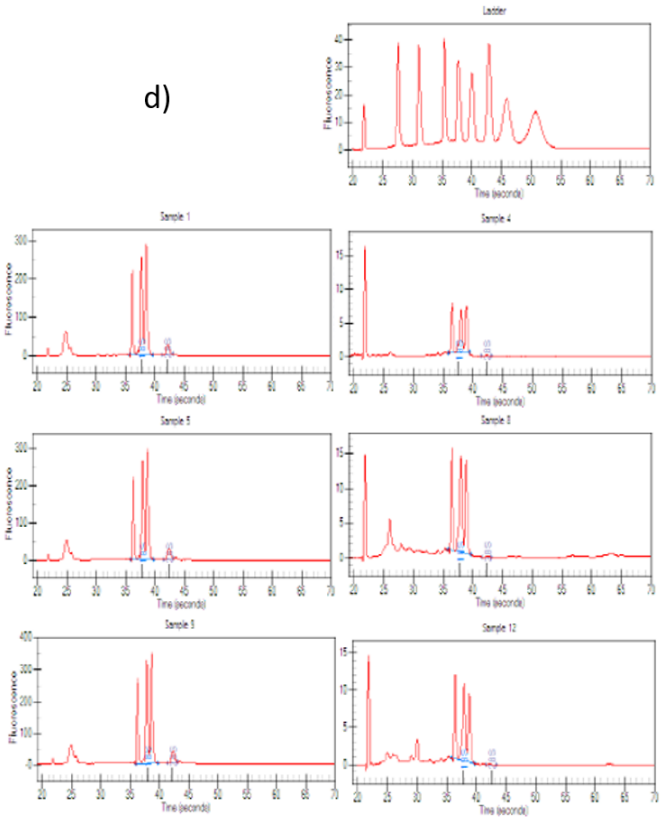


Figure 8) Analysis of the RNA prepared before and after co-IP

samples 1, 5, 9: before co-IP
samples 4, 8, 12: after co-IP
b) photospectrometric measurement with the NanoDrop, taken directly after the RNA preparation. A 260/280 quotient of about 2.0 is usually seen as pure for RNA. Measurements below this value can be indicative of contamination e.g. with DNA. The 260/230 quotient is usually between 1.8 and 2.2 and should be higher than the 260/280 quotient. In the samples taken after co-IP the 260/230 quotient was consistently lower, which was not indicative of lower quality results.
c) Estimation of the quality of the RNA with the RQI on a scale between 0.0 and 10.0. The samples were diluted to values between 300 and 400 ng/µl.
a), d) gels from the Experion BioRad gel electrophoresis showing samples before (1, 5, 9) and after (4, 8, 12) co-IP. The image intensity of the lanes after co-IP is adjusted to

make them visible due to their lower concentration. After the co-IP additional bands in the area between 100 – 500 bases are visible in the Hfq-FLAG (8) and CsrA-FLAG (12) samples, which is not visible in the wild-type sample (4). Those additional bands could correspond to mRNA or sRNA that was bound to Hfq during the co-IP and therefore protected from degradation through RNases. Due to the adjusted visibility they might not be visible in the lanes before co-IP.

2.2.5 RNA sequencing

RNA samples were prepared like described in chapter 2.2.3. The total RNA was sent to Vertis Biotechnology AG, Freising. Vertis prepared the strand-specific cDNA libraries and sequenced them using an Illumina HiSeq 2000.

2.2.6 Co-Immunoprecipitation

For the co-immunoprecipitation I used a strain SOR35 that carries a modified chromosomal *hfq* gene encoding Hfq protein tagged with 3 FLAG peptides at the C-terminus. A FLAG-tag is a short sequence of eight amino acids (AspTyrLysAspAspAspAspLys) that can be genetically added to either end of a protein (Hopp et al., 1988). Specific antibodies bind to this tag and hence allow to precipitate it, and co-precipitate everything that binds to it.

I always performed the experiment with a wild-type strain in comparison to distinguish RNA bound by Hfq from any co-purified or background RNA. In some experiments I also used a strain that has a FLAG-tag on its CsrA protein, to distinguish unspecifically bound RNA from molecules that interact specifically with Hfq.

Bacteria from the stock at -80°C were streaked out on LB agar plates and then incubated overnight at 27°C. The following day one colony was picked, streaked out again on an LB agar plate and incubated under the same conditions. From those plates precultures from one colony were subsequently grown overnight at 27°C under constant agitation on a shaking incubator at 180 rpm in 20 ml LB fluid medium in Erlenmeyer flasks. A small sample of 100 µl was collected and diluted 1:10 with LB medium. The OD_{600} nm was measured with a sample of LB medium as reference value. The necessary dilution to achieve an OD_{600} of 0.1 was calculated. 100 ml of the calculated dilutions were made, and the new OD_{600} was measured. The cultures were incubated again at 27°C or 37°C for 4h or 12h. Samples from those cultures were streaked out on a plate and incubated at 37°C to exclude contaminations. Another sample was taken

after the incubation to measure the OD_{600}. The bacterial cells were harvested by centrifuging at 5,000g at +4°C for 10 min in Falcon tubes.
For co-Immunoprecipitation, cells were disrupted via sonication. This seemed to be the better option than disruption with lysozyme for this type of experiment. First, there were larger amounts of bacterial culture to be lysed (5ml vs. ~1-1.5ml). Second, I tried to avoid any possible contamination that could hypothetically interact with the co-immunoprecipitation process as well as the further analysis. I had experimented with the French press as well, but overall sonication proved to be quicker, cleaner and less prone to malfunctions so that I reserved the French press solely for the purpose of brewing coffee.

The bacterial pellets were resuspended in 25 ml cold IP-buffer, pooled in one tube and centrifuged again. The supernatant was discarded and the tube with the pellet was weighed. The pellet was again resuspended in 5 ml IP-buffer and lysed through sonication for a total of 6 cycles with 30s sonication, sonicating for half a second every second, and 30s cooling time. The cells were cooled during the procedure through a cooling bath with 200g NaCl, 500g ice and cold water using a magnetic stirring system. The lysed cells were centrifuged at 20,172g at 4°C for 7 min. 200 µl supernatant were taken from the surface and 700 µl Qiazol Lysis Reagent immediately pipeted on top of it and vortexed for 10s. Another 12 µl supernatant were added to 12 µl Laemmli buffer and stored at -20°C. Those samples were used to compare the total RNA and proteins of the different strains before the immunoprecipitation (‘input samples’). One sample was taken to measure the absorbance at 280 nm (A280). The concentration, calculated based on the Beer-Lambert-equation, varied between 20 and 25 mg/ml. 1 ml supernatant was incubated with 50 µl anti-FLAG antibody coated ferromagnetic beads at 4°C for 1 h under gentle agitation. The beads were stored in glycerol at -20°C and had been washed three times with IP-buffer before. After the incubation the tube was placed in a magnetic separator. The supernatant was discarded, and the beads were washed two to three times with 1 ml cold IP-buffer until the A280 was less than 0.05, to ensure that the background was low. The measurements were made with the Nanodrop, using IP-buffer as reference. 12 µl of the beads were then mixed with an equal volume of Laemmli buffer for protein analysis. The remainder was used for RNA extraction and mixed with

700 µl Qiazol Lysis Reagent, which denatured the proteins and eluted the RNA. Those samples represent the RNA and proteins that were enriched through co-IP ('output samples'), namely the FLAG-tagged proteins (Hfq-FLAG and CsrA-FLAG) and the RNA that was bound to them and hence protected from degradation. Fig. 9) gives an overview over the main steps.

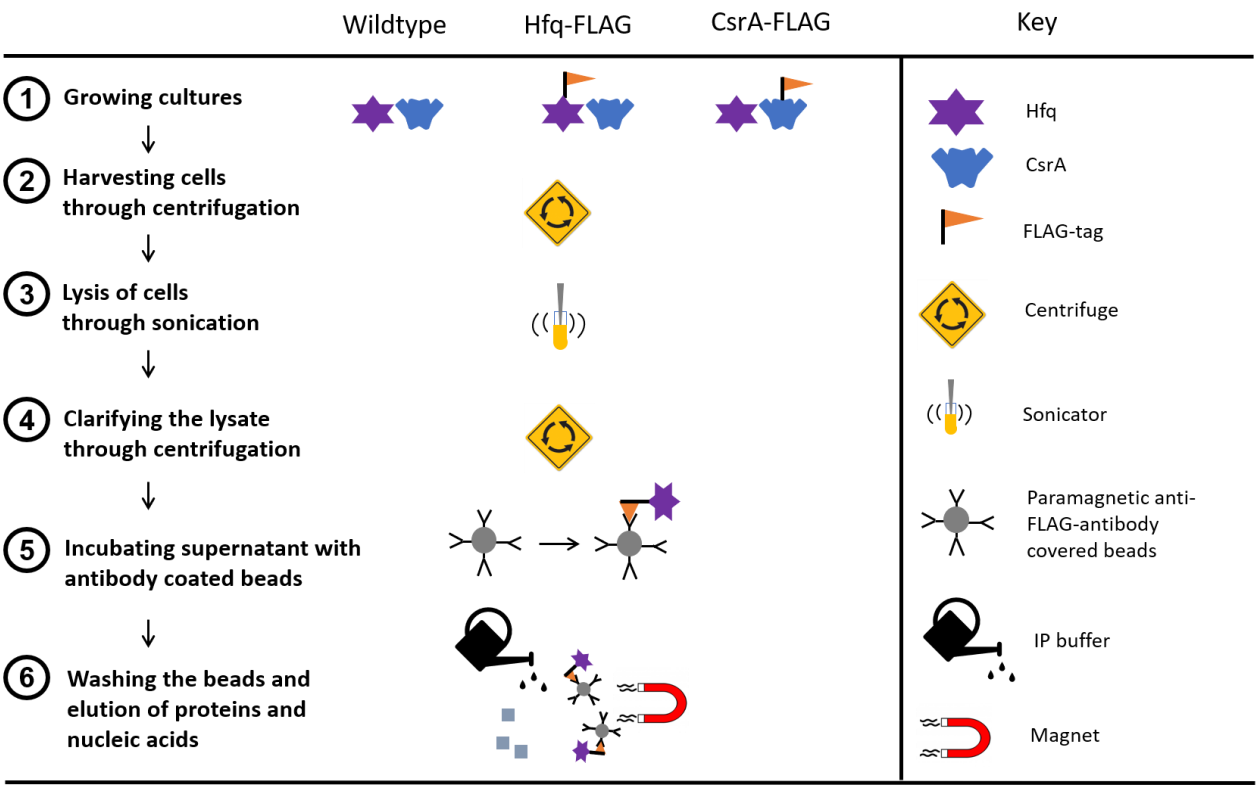


Figure 9) Workflow of the co-IP. ***1)*** *Precultures of the wild-type strain JB580v, the Hfq-FLAG strain SOR35 and the CsrA-FLAG strain SOR43 were grown overnight at 27°C. They were used to grow cultures at 27°C or 37°C for 4h.* ***2)*** *Cells were harvested through centrifugation.* ***3)*** *The pellets were resuspended in 5 ml of IP-buffer and lysed through sonication.* ***4)*** *The lysate was clarified through centrifugation.* ***5)*** *The supernatant was incubated with anti-FLAG-antibody coated paramagnetic beads at +4°C for 1h.* ***6)*** *The beads were washed repeatedly with IP-buffer. Proteins were eluted with Laemmli-buffer, while RNA was eluted with Qiazol.*

2.2.7 Protein gel electrophoresis

To allow further protein analysis, an SDS-polyacrylamide gel electrophoresis was performed with proteins extracted before and after co-immunoprecipitation. The gels were prepared with and without the addition of 2,2,2-trichloroethanol for stain free gels

and Coomassie stains respectively. An SDS- PAGE was run with a 12-15% polyacrylamide gel at 180 V for 30-40 min.

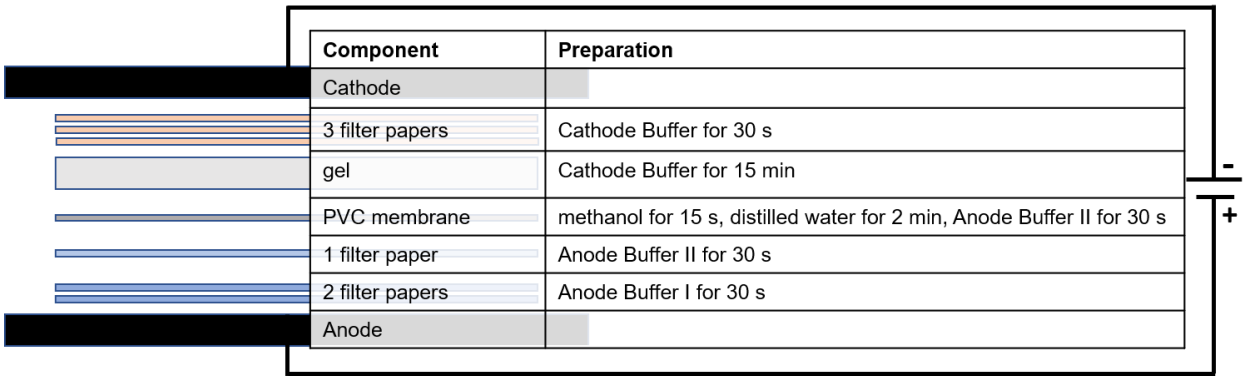
2.2.8 Coomassie stain

The gel was incubated shaking with Coomassie stain for 4 hours at room temperature. The dye was drained, and the gel was destained with water. It was incubated overnight or up to several days, while the water was changed regularly. A picture was taken with the ChemiDoc.

2.2.9 Western Blot

The stain free gels allow the visualization of proteins without the necessity of staining them and at the same time permits the use of the gels for further analysis such as Western blots. A trihalo compound such as 2,2,2-trichloroethanol was added to the gel. Upon exposure to UV light, it is covalently crosslinked to aromatic amino acid residues, especially tryptophan. After a brief activation of 2.5 min under UV light, the tryptophan adducts emit light that can be detected with an imaging system like the ChemiDoc.

The gel was immersed in Cathode buffer for 15 min. The membrane was wetted in methanol for 15 s, soaked with distilled water for 2 min and then put in Anode Buffer II for 30 sec. Two filter papers were soaked in Anode Buffer I, one filter paper was soaked in Anode Buffer II and three in Cathode Buffer for 30 sec. The transfer stack was set up as shown in the figure below (Fig. 10). The two filter papers soaked in Anode Buffer I were put on the anode, topped with one filter paper soaked in Anode buffer II followed by the membrane, the gel and finally three pieces of filter paper soaked in Cathode buffer. A glass pipet was rolled over the stack to remove air bubbles, that could impair an even transfer. The Cathode electrode was put in place and a potential of 180 mA was applied for 30-45 min (2.5mA/cm^2). Fig. 10 gives an overview over the transfer stack setup.



Component	Preparation
Cathode	
3 filter papers	Cathode Buffer for 30 s
gel	Cathode Buffer for 15 min
PVC membrane	methanol for 15 s, distilled water for 2 min, Anode Buffer II for 30 s
1 filter paper	Anode Buffer II for 30 s
2 filter papers	Anode Buffer I for 30 s
Anode	

Figure 10) Transfer stack set up

Afterwards the membrane was cut to an appropriate size, the gel was removed, and the membrane was rinsed with PBS-T. To check if the transfer was complete, a picture of the gel was taken. To block the membrane, it was incubated with 3% dry skim milk in PBS-T for at least one hour. The membrane was rinsed again afterwards with PBS-T. The primary antibody was added (see table 5) and incubated for 1 h at room temperature under constant agitation. The membrane was washed three times with PBS-T for 10 min. The secondary antibody was added and again incubated for 1 h. The secondary antibody binds to the primary antibody and is attached to a horseradish peroxidase (HRP). The washing steps were repeated. The detection solution was prepared with equal volumes of HRP substrate luminol reagent and HRP substrate peroxide solution and left to adjust to room temperature for 10 min. The membrane was put on a transparent plastic film and 2 ml of the detection solution were applied evenly. It was incubated for 5 min at room temperature and then covered with another piece of plastic film. The HRP catalyzes the oxidation of luminol by peroxide, creating the excited state product 3-aminophthalate, which decays to the ground state under the emission of light with a wavelength of 425 nm. This can be detected, for example with a cooled CCD-camera (charge-coupled device) like the ChemiDoc MP Imaging system (Alegria-Schaffer et al., 2009).

2.2.10 DNA extraction

Bacterial cultures were grown in LB fluid medium at 27°C overnight. 1 ml of this culture was transferred to an Eppendorf tube and centrifuged for 2 min. The pellet was resuspended in 150 µl TE buffer, 10 µl 10% SDS, 40 µl lysozyme (10mg/ml) and

incubated for 1.5 h at 37°C. 417 µl TE buffer, 30 µl 10% SDS and 3 µl Proteinase K (20 mg/ml) were added and briefly vortexed. The mixture was incubated for 1 h at 55°C. 100 µl of 5M NaCl and 80 µl CTAB were added and incubated for 10 min t 65°C. An equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) was added, mixed and then centrifuged for 5 min. Analogous to the RNA extraction this leads to the formation of three phases: Since phenol and chloroform have a higher density than water, they are found at the bottom and bind the unpolar organic compounds like lipids and cellular debris. The proteins accumulate at the interphase. The polar nucleic acids remain in the upper, aqueous phase, which was carefully transferred to a new tube. 0.7 volumes isopropanol and 0.1 volumes 3M NaAcetate were added. The tube was centrifuged for 30 min at +4°C and the supernatant was removed. 500 µl ice cold 70% ethanol were added and mixed. The sample was again centrifuged for 15 min and the supernatant removed. The extracted DNA was resuspended in 50 µl TE buffer and stored at -20°C.

2.2.11 PCR

For the creation of the probes that later bind to the RNA and can be detected on the Northern blot membranes, a DNA template has to be created. This template is later used by the T7 RNA polymerase. The *Yersinia enterocolitica* genomic sequence was downloaded from https://www.ncbi.nlm.nih.gov/. Primers were designed with (CLC DNA Workbench) and analysed with the OligoAnalyzer Tool from Integrated DNA Technologies - IDT (https://eu.idtdna.com/pages). An initiating sequence for the T7 RNA polymerase was added to the reverse primer. Primers had therefore to be chosen carefully and the different annealing temperatures due to the added sequence considered. Often several test PCRs were necessary in order to define appropriate conditions. The primers were ordered from Metabion.

2.5 µl forward primer, 2.5 µl reverse primer, 2 µl cDNA, 25 µl master mix and 18 µl nuclease-free water were added to a PCR tube. The thermocycler was put to settings appropriate for the primers and the PCR was run (see table 9).

Table 9) Thermocycler settings

Step	Temperature	Time
Initialization	98°C	30 s

35 cycles:		
Denaturation	98°C	10s
Annealing	50-60°C, depending on the primers' T_m	30s
Elongation	72°C	30s
Final elongation	72°C	2 min
Hold	8°C	

A 1% gel with TAE buffer and Ethidium bromide was prepared. The samples were mixed with the loading dye and then, together with the DNA ladder, loaded on the gel. The gel was run at 100 V for 40 min. A picture was taken with the GelDoc.

To purify the PCR product, it was mixed with 2 volumes of buffer NTI, pipetted on a NucleoSpin column and centrifuged for 30 s at 11,000 g. Afterwards the column was centrifuged twice with 700 µl buffer NT3 and then centrifuged again for 1 min to dry the membrane. The column was put in a new collection tube. 30 µl buffer NE were pipetted on top and incubated for 1 min, then centrifuged for 1 min at 11,000g. The purified PCR product was stored at -20°C.

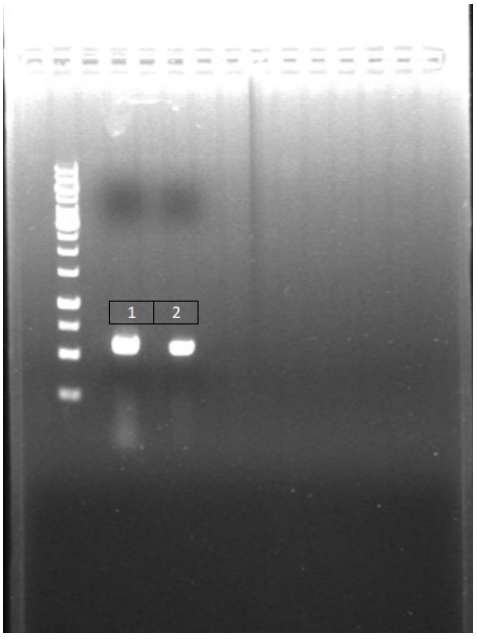


Figure 11*) Picture of the PCR performed with the primers designed for the rpoE probe.*
1) shows the PCR product before and **2)** after the cleanup. The slight smear in the first sample has vanished.

2.2.12 Probe labeling and determination of labeling efficiency

The non-radioactive RNA detection is based on enzyme induced light emission. The PCR product is incubated with an RNA polymerase and ribonucleotides including

Digoxigenin-11-UTP (DIG-UTP). This UTP is bound to digoxigenin (DIG), which is a steroid isolated from digitalis plants. The DIG-labeled UTP is incorporated by the RNA polymerase into its RNA product approximately every 25-30 nucleotides. These RNA probes can later be detected using anti-digoxigenin antibodies coupled to an alkaline phosphatase (AP). When a chemiluminescent substrate (CDP-star) for the AP is added, the resulting light emission can be detected with an imaging system.

For the formation and labeling of the probes, 4 µl PCR product and 6 µl DEPC-treated water were added to an Rnase free tube and put on ice. 4 µl labeling mix, 4 µl transcription buffer and 2 µl T7 RNA polymerase were added, mixed, and briefly centrifuged. The mixture was incubated for 1 h at 42°C. 2 µl Dnase I was added and incubated for 15 min at 37°C. The reaction was stopped by adding 2 µl 0.2 M EDTA (pH 8.0).

To determine labeling efficiency and calculate the appropriate amount of material to be used for the following Northern blots a dilution series with dilutions between 10 pg/µl and 0.3 pg/µ was prepared with Dilution Buffer. 1 µl spots of the dilutions were applied to a membrane and crosslinked with UV-light (0.120 J/cm^2) The membrane was washed in washing buffer for 2 min and incubated for 30 min in Blocking Solution. It was then incubated for 30 min with the anti-digoxigenin-AP diluted 1:10,000 in Blocking Solution and washed afterwards with Washing Buffer twice for 15 min to remove any traces of the antibody binding to the membrane nonspecifically. The membrane was then equilibrated in Detection Buffer for 2-5 min before being covered evenly with 1 ml CDP-star substrate and put between two plastic sheets. Light emission was detected for up to 30 min with the GelDoc. Fig. 12) shows the dilution series for the *hfq* and *rpoE* probe as an example.

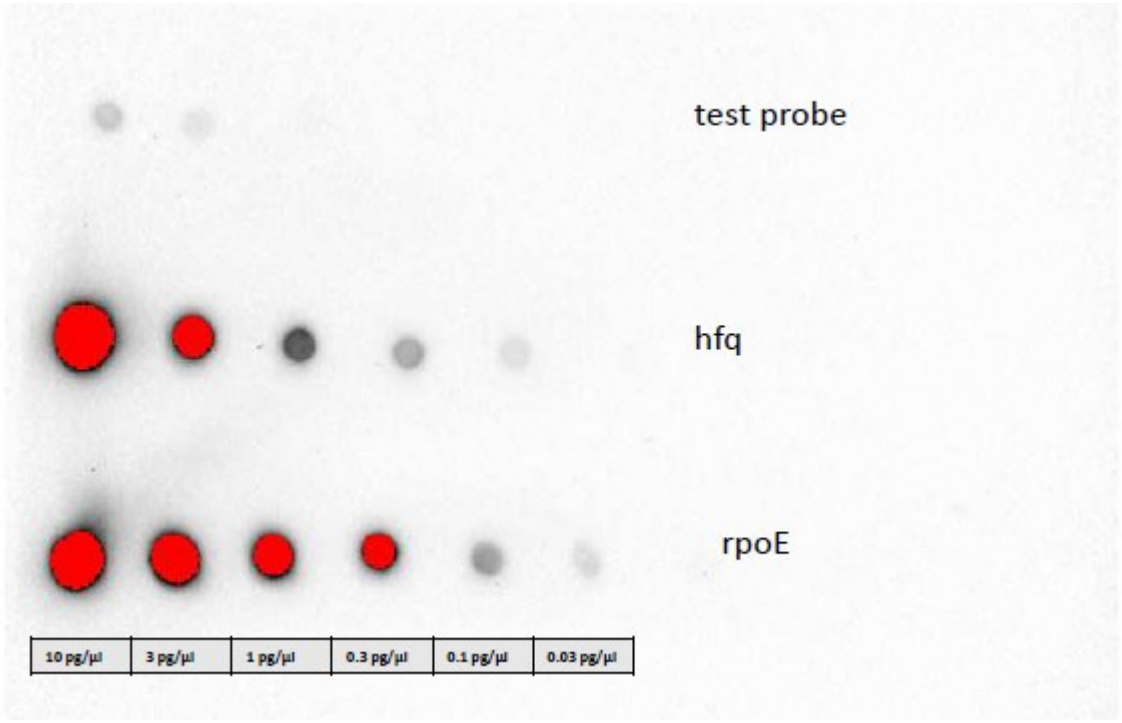


Figure 12) Testing of the Hfq- and rpoE probe. The top lane represents a standardized test probe, the concentration of the test probe is indicated at the bottom of the blot. The second lane is the Hfq-probe and the bottom lane the rpoE probe. The generated probes have a much stronger signal than the standardized probe, corresponding to a higher concentration and therefore have to be diluted accordingly for the Northern blot.

2.2.13 Northern Blot

A 12% agarose gel containing 2% formaldehyde was prepared. The gel chamber had previously been sprayed with Rnase Zap and rinsed with DEPC-treated water. RNA samples were thawed, and their concentration was again measured with the NanoDrop. The samples were diluted to 250 ng/µl. When the concentration was considerably below 250 ng/µl, as was the case for the samples prepared from the co-immunoprecipitation, a lower concentration was chosen that would still allow a comparison of the samples. The concentration of the new dilutions was controlled again. A loading buffer was added to the samples and they were denatured at 65°C for 10 min and chilled on ice before being pipetted in the gel pouches. The gel was run for at least 12 h between 15 V and 25 V.

The gel was rinsed twice with 20x SSC for 15 min on a shaking plate. The gel was placed on a filter paper with its ends dipping in 20xSSC. The membrane was placed on top, followed by three filter papers and a stack of paper towels. The blot was weighed down with 5 kg of heavy literature to ensure a tight contact between the layers (Fig. 13).

The 20xSSC gets drawn through the gel towards the paper stack on top through capillary forces taking the RNA along and transferring it to the membrane. The process took at least 6h or overnight.

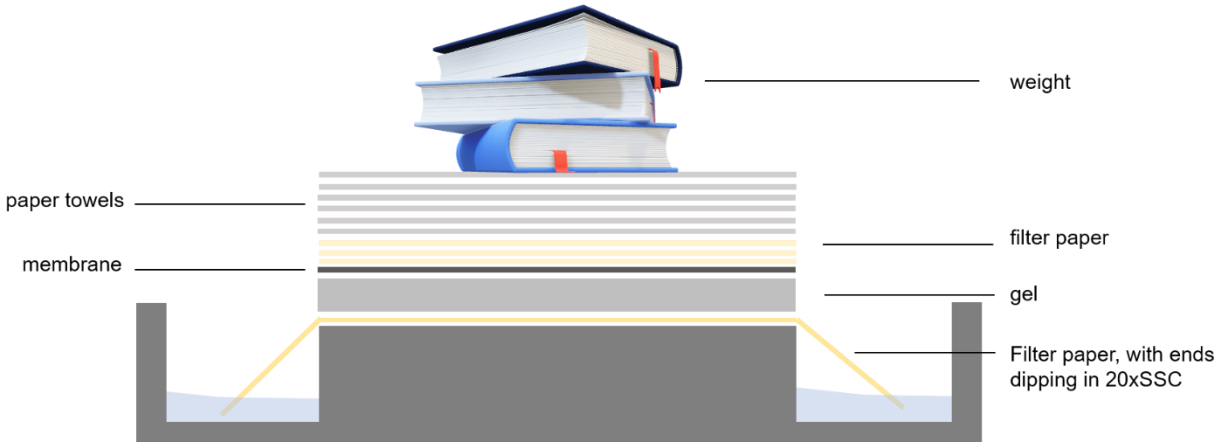


Figure 13) Set up for the transfer of the RNA from the gel to the membrane.

The membrane was crosslinked with UV light (0,12 J), rinsed with DEPC-treated water and incubated for 30 min with hybridization solution at 68 °C. The probe was denatured at 65°C for 10 min and a dilution with hybridization solution based on the results from testing the labeling efficiency was prepared (see chapter 2.2.12), usually between 1:10.000 and 1: 15:000. The membrane was hybridized in this solution at 68°C for at least 6 h or overnight under constant agitation.

The membrane was washed three times with 2xSSC, 0.1xSSC and Washing Buffer. The following steps for the immunological detection are the same as those for determining the labeling efficiency: after blocking the membrane and incubating with the antibody, the CDP-star substrate is applied, and a picture is taken with exposure times between 5 – 30 min until the signal is clearly visible in all the samples.

2.2.14 Membrane stripping

To strip Northern Blot membranes from the antibody and the attached enzyme, the membrane was incubated twice for 1 hour at 80°C in stripping buffer, consisting of 50% formamide, 5% SDS and 50 mM Tris/HCl, pH 7.5. The membrane was washed afterwards in 2xSSC. To control the success of the stripping process the membrane was then equilibrated in detection buffer and covered with CDP-star substrate to check for any remaining signals that would interfere with the detection of another probe. After

washing the membrane, it can be hybridized with another probe. The stripping buffer was stored at 4°C and used up to three times.

2.2.15 Inactivating RNases using diethyl pyrocarbonate (DEPC)

1 ml diethyl pyrocarbonate (DEPC) was dissolved in 1 l deionized water over at least 4 h at room temperature using a magnetic stirring system. This inactivates RNase enzymes by covalently modifying histidine, lysine, tyrosine and cysteine residues. The DEPC-treated water was then autoclaved twice before being used for RNA preparation. This process ensured the hydrolysis of DEPC to carbon dioxide and ethanol to prevent any interference with other chemicals.

3 Results

3.1 Comparison of gene expression in wild-type and *hfq* mutant strains

3.1.1 Transcriptomic analysis

3.1.1.1 RNA-seq and differential gene expression analysis

For the transcriptomic analysis, wild-type and *hfq* mutant cultures were grown in duplicate at 27°C and 37°C. Total RNA was prepared from cells in exponential phase and sent to company Vertis AG for cDNA library preparation and next generation sequencing. A global RNA-seq approach was employed by comparing mapped sequence reads from strand-specific bar-coded cDNA libraries. Mapping was performed against the sequence of the isogenic virulent strain 8081 (RefSeq number NC_008800.1) and 4171 different RNAs could be identified. When mapping was performed using the sequence of the virulence plasmid pYV, it became apparent that one of the two wild-type samples did not contain reads that could be mapped on the plasmid, suggesting that the colony that was used for the preculture of this biological replicate probably lost the plasmid early during replication. Therefore, I will only report here the analysis of chromosomal gene expression. The global gene expression profiles of the two strains were distinct (Fig. 14). Moreover, the expression profiles of bacteria grown at 27°C and 37°C were also distinct, consistent with the temperature-dependent gene regulation described in *Y. enterocolitica*.

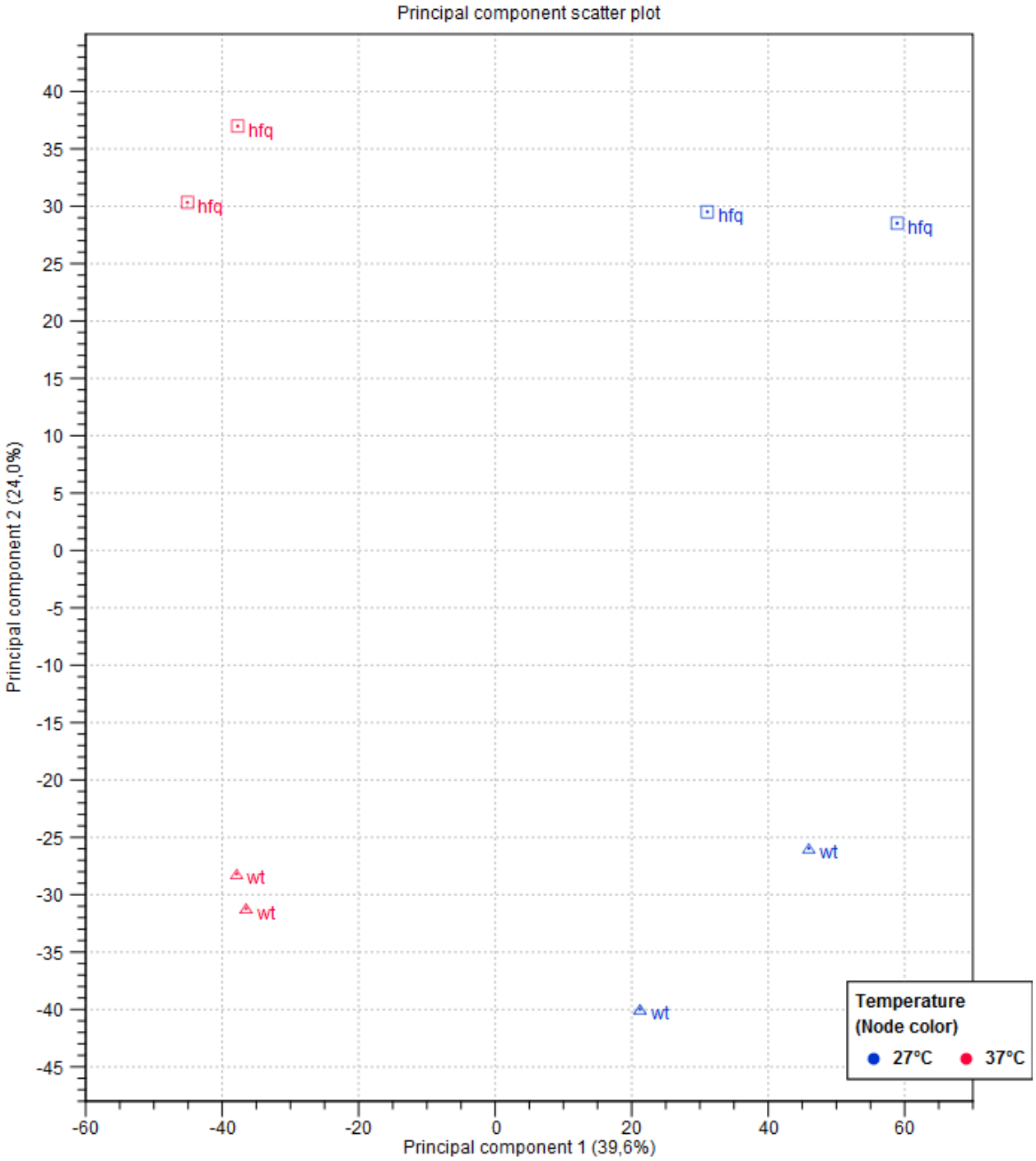


Figure 14) Principal-component analysis of RPKM counts of the RNA-seq data for wild-type (wt) and hfq-negative strain (hfq) grown at 27°C (blue) or 37°C (red). RPKM (reads per kilobase (of transcript), per Million (mapped reads)) is a measuring unit, that accounts for the fact that during sequencing more reads are generated from longer transcripts. It is calculated by dividing the RPM (reads per million) by the length of the gene in kilobases.

For the comparison between wild-type and *hfq*-negative strain, we focused on genes whose expression varied by an absolute fold change of 2 or more with a $p < 0.001$ in order to minimize false positives (Fig. 15).

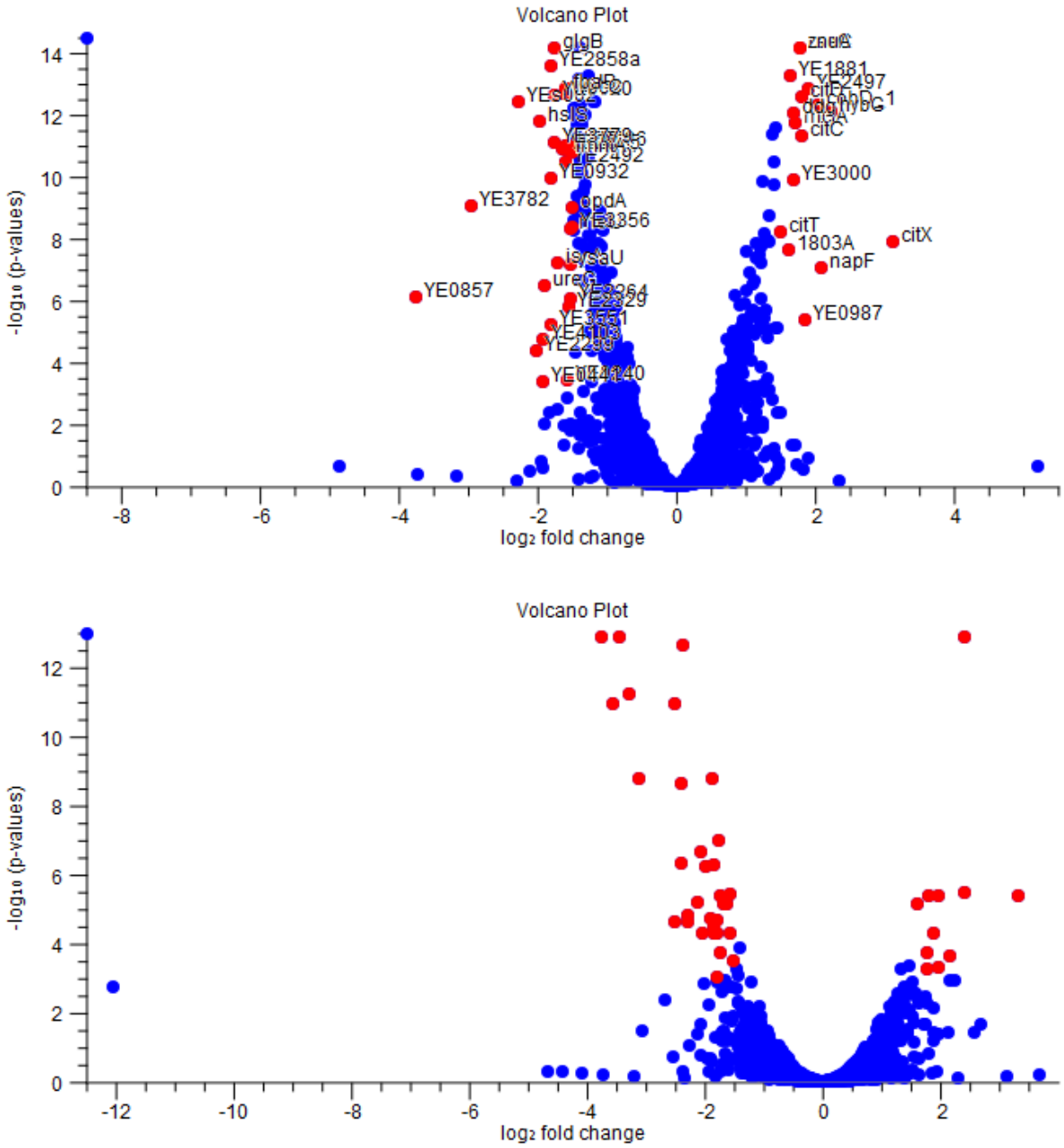


Figure 15). Volcano plot representing the statistical significance (-Log$_{10}$ P value) versus magnitude of change (Log$_2$ fold change). Differentially expressed genes between hfq mutant vs. wild type at 37°C (top panel) and 27°C (bottom panel). Red dots indicate genes within our threshold (p < 0,001; fold change ≥ 2), while blue dots do not meet these criteria.

A total of 355 genes were diffentially expressed (see appendix for complete list) between the wild-type and the *hfq* mutant (including *hfq*), which is approximately 8.5% of the 4171 gene transcripts, that were identified through sequencing. More genes were differentially expressed at 37°C than at 27°C (282 at 37°C and 145 genes at 27°C), which is in line with recent results reported for *Y. enterocolitica* serotype O:3 (Leskinen et al., 2017). These genes were further examined for potential Hfq-binding motifs (i.e. (ARN)4- or AAYAAYAA) within 50 bp of the start codon. The results are summarized in Fig. 16 and Table 10.

hfq naturally appears as the most differently expressed in the transcriptomic analysis. Seven genes had at least one putative binding site for Hfq and had a significant difference between the wildtype and the mutant at both temperatures (including *hfq* itself). Six had also at least one binding site but had only significant differences at 27°C and 19 only at 37°C. 65 genes had changes at both temperatures, but no putative binding site. Finally, 191 genes showed only differences at 37°C, and 67 only at 27°C, without any predicted binding sites (Fig. 16). We also noted that 259 genes with putative Hfq-binding sequences did not show significant difference in their expression at either temperature (Fig. 16). 133 RNAs (3.2% of all genes) were more abundant in the mutant (21 at both temperatures, 36 at 27°C and 76 at 37°C), therefore probably being downregulated by Hfq. 222 RNAs (5.3% of all genes) were less abundant in the mutant (including *hfq*), 51 at both temperatures, 37 at 27°C and 134 at 37°C), hence being upregulated by Hfq. Compared to the large number of genes with a putative RNA binding site (291), only a small proportion (32, ~11%) seemed to be affected by Hfq. Although these genes, with a putative binding site, but without significant difference between wild-type and mutant, are less likely to be a target of Hfq-mediated regulation, it does not exclude them automatically either, since Hfq could for example inhibit their translation without altering their stability and hence their abundance in the RNA-sequencing results would not change.

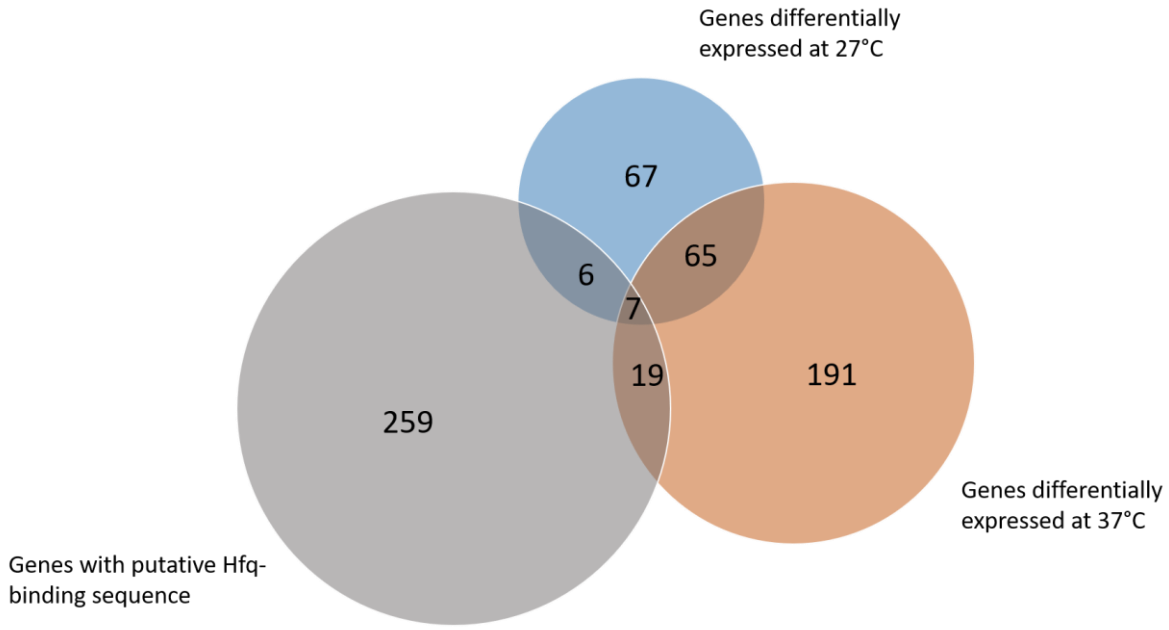


Figure 16) Venn diagram showing the number of differently expressed genes at 27°C and 37°C and the number of genes with putative a Hfq-binding sequence.

Table 10) Overview over the number of differently expressed genes, up- and downregulation and putative Hfq binding sites.

	Upregulated genes in *hfq* mutant	**Downregulated genes in *hfq* mutant**	**Total genes with differential expression**
At 27°C only	36 (1)*	37 (5)	73 (6)
At 37°C only	76 (9)	134 (10)	210 (19)
At both temperatures	21 (2)	51 (5)	72 (7)
Total	**133 (12)**	**222 (20)**	**355 (32)**

* In parentheses: number of genes with putative Hfq-binding site within 50 bp of start codon

3.1.1.2 Functional annotation clustering

To get an overview over the 354 differently regulated genes (additional to *hfq*), we first clustered them using the Functional Annotation Tool 6.8 by David Bioinformatics (Huang et al., 2008 & Huang et al., 2009). The gene list was compared to the list of functional annotated genes from the whole genome of *Y. enterocolitica* 8081. It then groups the genes based on GO terms (gene ontology) and many other annotation categories, for instance KEGG pathways. Because many of the terms overlap and generate redundant groups with the same or similar genes, the software then clusters the annotated groups, providing a clearer overview. The clusters are sorted by their enrichment score, which is

calculated based on the EASE score. EASE is a “conservative adjustment” of Fishers exact probability, that highlights clusters with multiple genes over small groups or single genes (Hosack et al., 2003).

The analysis found 17 clusters covering 246 differentially expressed genes between wild type and *hfq* mutant, while 108 genes were not clustered. Interestingly, the first two clusters were comprised of iron and heme related terms. Other clusters were formed around cell redox homeostasis, membrane proteins and transporters, LPS biosynthesis, carbon metabolism (especially citrate cycle), amino-acid biosynthesis, cysteine-methionine metabolism, phosphotransferases and methyltransferases, translation and ribosomal proteins, and DNA binding and regulation. Among the unclustered terms were stress response and chaperons, which comprised i.a. many heat-shock proteins. We further grouped the terms, including the unclustered genes into 10 categories (Fig. 17).

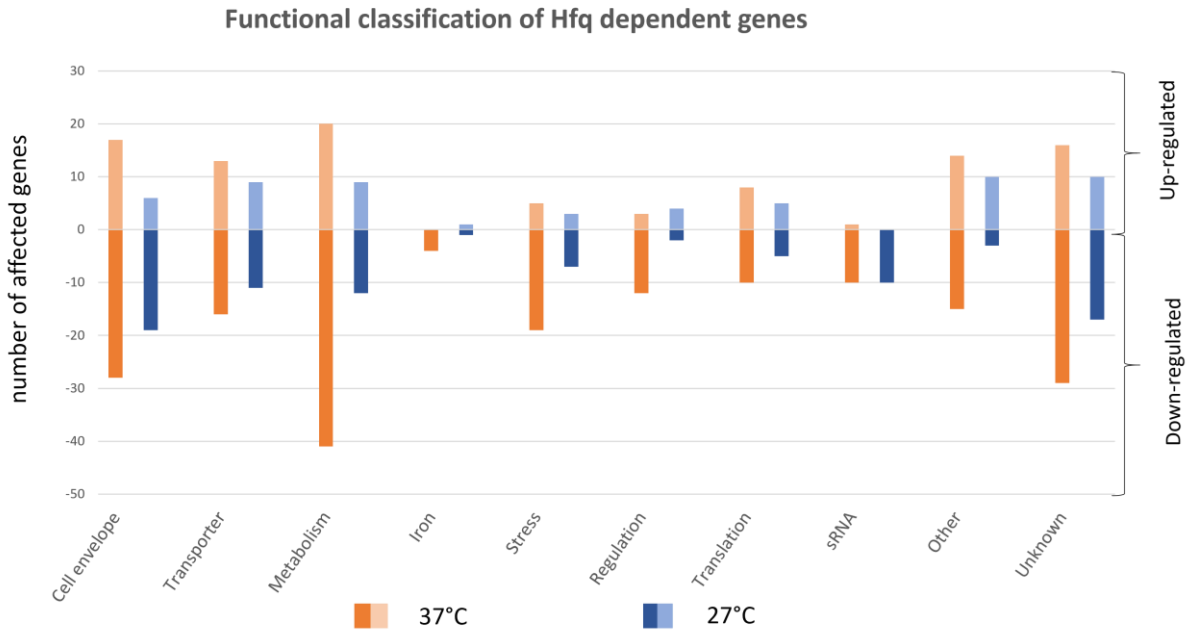


Figure 17) Functional classification of Hfq-dependent genes. Shown are absolute numbers of up-regulated (top half) and down-regulated genes (bottom half) in the hfq mutant compared to the wild-type at 27°C and 37°C.

3.1.1.3 Cell envelope: Adhesins, transporters and outer membrane proteins

Several genes encoding inner and outer membrane proteins were differently expressed in the mutant. None of the genes encoding adhesins had any predicted Hfq-binding

sequences. For the chromosomally-encoded invasin *invA*, we observed a downregulation (-2.2, i.e., a 2.2-fold decrease) in the *hfq*-negative strain at 27°C, the temperature at which it is maximally expressed. This result was consistent with our previous analysis (Kakoschke et al. 2016). The transcriptional regulator H-NS, which downregulates *invA,* was downregulated at 37°C (and also at 27°C, but only with a p ~ 0.01) in the mutant as well. Concerning the genes encoding the adhesin *ail* and the Ail-like *ompX*, we observed a decrease in the transcript abundance in both genes: *ail* appeared downregulated (-2.5) in the mutant at 37°C, and *ompX* was strongly downregulated (-6.8) in the mutant at 37°C as well. Our previous analysis had failed to show an effect of Hfq on post-transcriptional regulation of *ail* and *ompX* by Hfq in exponential phase but indicated a clear inhibition during stationary phase (Kakoschke et al. 2016). The abundance of the transcript encoding the pilin MyfA did not change significantly. However, only few transcripts were detected, since *myfA* expression depends on low pH (Iriarte et al., 1995), which we did not use in this study. Therefore, these results might not reflect the actual influence of Hfq on *myfA* under expression inducing conditions.

Products of some of the differentially expressed genes we found are not only part of the membrane but influence its structure. The lipid A biosynthesis palmytoleoyltransferase gene *ddg* for instance, which is part of the lipopolysaccharide biosynthesis pathway was upregulated in the mutant at both temperatures. The peptidoglycan transpeptidase MrdA (*pbpA/YE3002*) cross-linking the peptidoglycan was upregulated in the mutant at 37°C. The lipoprotein gene *nlpD* was downregulated at 37°C in the mutant. NlpD is preserved in other Gram-negative species, and is involved in surface remodeling and cell separation (Uehara et al., 2009; Ercoli et al., 2015)

Among the transporter genes that were downregulated in the mutant, were many ABC transporters (e.g., *YE2875*, *YE2492*), sugar (e.g., *YE2606*), aminoacid (e.g., *yecS*, *tdcC*) and peptide transporters (e.g., *YE1609*) as well as cobalt (*cbiQ*) and zinc transporters (*zntB*). Some transporter genes were upregulated in the mutant, although this was the case for fewer genes, for example the potassium transporter gene *trkD* and the fructuronate transporter gene *gntP*. Two efflux transporters of the major facilitator

superfamily (MFS), *YE2160/ydhC* and *YE0097/emrD*, that could provide resistance against antibiotics, were downregulated in the mutant.

Furthermore, we found differential expression of many genes encoding hypothetical proteins with a transmembrane domain, whose function and significance has yet to be investigated.

As one of our wild-type replicates had lost the pYV plasmid, we were not able to analyze the abundance of transcripts encoding the pYV-encoded adhesin YadA or the T3SS Ysc and the Yop proteins. However, *ysaH* and *ysaU*, two possible T3SS proteins as well as *outE,* a T2SS protein, were downregulated in the *hfq* mutant at 37°C. Finally, two genes, *flgL* and *flhB*, predicted to be involved in flagellar biosynthesis, were upregulated in the mutant at 37°C and 27°C, respectively.

3.1.1.4 Hfq-dependent expression of genes involved in stress resistance

Resistance to acid stress

The acid shock resistance gene *asr*, which promotes growth in *E. coli* at moderate acidity (pH 4.5) and induces acid tolerance for extreme acidity (pH 2.0) (Seputiene et al., 2003) was highly diminished in the *hfq*-mutant (-23.0-fold at 37°C, -15.8-fold at 27°C), showing one of the highest observed fold-changes among protein coding genes. *asr* is conserved in many Enterobacteria. Its mechanism is unknown, although it has been suggested to sequester protons in the periplasm (Seputiene et al., 2004). Its function in *Y. enterocolitica* is unclear.

As mentioned before, the *Yersinia* urease provides acid resistance. One urease subunit gene (*ureC*) was downregulated in the mutant at 37°C (-2.1-fold) and 27°C (-3.8-fold). Two urease accessory genes were less abundant in the mutant as well, one at 37°C (*ureG*) and one at 27°C (*ureD*). While the *ureF* gene that encodes an urease accessory protein did initially not show up in our analysis, with less stringent criteria, we could see that it was downregulated in the *hfq*-mutant at both temperatures (37°C: -1.7-fold, $p < 0.05$, 27°C -2.3-fold, $p < 0.05$). Genes immediately upstream of the urease genes were slightly downregulated at 37°C in the mutant as well, e.g. the urease transporter

yut/YE0958 and the voltage gated potassium channel *kch*/YE0961. Two copies of the acid activated chaperone *hdeB* were detected in the transcriptomic analysis. One of them, *hdeB_1*, was in the immediate vicinity of the urease genes and was downregulated in the mutant at both temperatures (-4.4 at 37°C, -4.0 at 27°C). *YE3696*, a HdeD family protein, which in *E. coli* is involved in acid resistance (Mates et al., 2007), was downregulated in the *hfq* mutant at both temperatures (-2.0-fold at 37°C and -3.6-fold at 27°C). Finally, the glutamate decarboxylase *gadA*, which decarboxylates glutamate to GABA and thereby consumes protons, was downregulated -3.2-fold in the *hfq* mutant at 27°C.

Resistance to oxidative stress

Expression of genes that provide oxidative stress resistance was also promoted by Hfq in this study. At 37°C, the alkyl hydroperoxide reductase subunit *ahpC* was 3.4-fold downregulated in the *hfq* mutant, consistent with the proteomic results published by our lab (Kakoschke et al., 2014). Just below our threshold, the catalase *katA*, which is dependent on *Hfq* in *Y. pestis* (Geng et al., 2009), was 1.8-fold downregulated. Both of them carry an Hfq-binding sequence. The superoxide dismutases genes *sodB* and *sodC* were also slightly downregulated at 37°C in the *hfq* mutant (-1.7-fold and -1.9-fold respectively).

Chaperones

Many genes encoding heat shock proteins were downregulated in the *hfq* mutant at 37°C. The most striking change was seen in *dnaK*/Hsp70, which experienced a -7.4-fold decrease. Its co-chaperone gene *dnaJ/cbpA* (YE3356) was also downregulated at 37°C (-2.8). The nucleotide exchange factor *grpE*, which is involved in DnaJ/DnaK function, was not differentially expressed, but carries an Hfq-binding sequence. Other genes with lower expression in the mutant at 37°C were *htpG*/Hsp90 (-2.5-fold), *groEL*/Hsp60 (-2.0-fold) and its co-chaperone *groES* (-2.2-fold), *hslU* (-2.1-fold), *hslT/ibpA* (-3.9-fold) and *hslS/ibpB* (-3.9-fold), the latter one was also downregulated at 27°C (-2.6-fold). Furthermore, genes encoding ClpP and ClpB, which are part of a protease complex, were downregulated at 37°C (-2.1 and -2.7-fold respectively).

In contrast, most cold shock proteins showed no significant difference between wildtype and *hfq* mutant at either temperature. Only *cspC1* was 2.2-fold downregulated in the mutant at 27°C.

Taken together, our results indicate that Hfq promotes the expression of many genes involved in stress responses, including acid and oxidative stress, and many cytoplasmic chaperones involved in protein folding. They confirm and extend our phenotypic observations that Hfq facilitates resistance to acidic and oxidative stress in *Y. enterocolitica* (Kakoschke et al., 2014).

Envelope stress response systems

Few genes encoding periplasmic proteins were differently expressed in the mutant. Only the peptidyl-prolyl cis-trans isomerase gene *ppiA* was -2.1-fold down-regulated while *fkpA* showed only a 1.4-fold up-regulation at 37°C in the mutant. Although our previous proteomic analysis showed that the protease/chaperone DegP was more abundant in *hfq* mutants (Kakoschke et al., 2014), slight but not significant changes (considering our threshold) in *degP* transcript abundance could be detected in the mutant (at 27°C ~2.1-fold upregulated in the mutant with p~0.011). As stated before, this discrepancy could be due to post-transcriptional regulation mechanisms targeting translation rather than mRNA decay.

Several genes involved in envelope stress responses RpoE, Cpx and Psp (Fig. 2-4) appeared differentially regulated in the *hfq*-negative strain. Four genes involved in the RpoE-dependent ESR and belonging to the same operon, *rpoE*, *rseA*, *rseB* and *rseC* were upregulated in the *hfq*-negative strain at 37°C (2.6-, 3.3-, 4.7- and 3.4-fold respectively). *rseB* was also upregulated 2.7-fold at 27°C. Both RpoE-regulating factors, *rseA* and *rseB* had a predicted Hfq-binding sequence (4x ARN motives). The increased expression of the extracytoplasmic stress response in absence of Hfq is compatible with what has been observed in other pathogens like *Salmonella* and *Vibrio* (Figueroa-Bossi et al., 2006 & Ding et al., 2004).

cpxP was upregulated in the mutant at 27°C (2.9-fold). For *cpxA* and *cpxR* there was no significant difference between wild type and mutant, consistent with previous studies (Zeuzem, 2018). *cpxR* has a putative Hfq-binding motif. Since CpxP inhibits the Cpx-

ESR (Fig. 2), an upregulation in the mutant could indicate an Hfq-mediated upregulation of the Cpx-ESR by decreasing CpxP activity, which binds and inactivates CpxA. However, the observed changes in *degP* were not highly significant and the decreased *ppiA* expression occurred only at 37°C and is therefore more likely to be caused by RpoE, which inhibits its expression (Zeuzem, 2018).

pspC was downregulated in the mutant at 37°C (-2.5-fold) and carries an Hfq-binding sequence. *pspB* and *pspA* did not change significantly. Using RT-qPCR, Zeuzem (2018) showed that *pspA* expression remained unchanged in *hfq*-negative *Y. enterocolitica*. Since the *pspABC* genes are in an operon, but we only saw changes for *pspC*, it might be possible that Hfq finetunes the expression of a subset of the *psp* genes.

Finally, genes encoding the two-component BaeS-BaeR system or the Rcs relay were not significantly changed at either temperature, consistent with previous RT-qPCR results (Zeuzem, 2018).

3.1.1.5 Metabolism

Iron acquisition and homeostasis

The first two clusters found by David functional annotation were comprised of iron and heme related terms. Both iron storage proteins bacterioferritin (bfr) and ferritin (ftn) were downregulated in the *hfq* mutant at 37°C. Furthermore, the yersiniabactin biosynthesis thioesterase *ybtT*/*irp4* was upregulated in the mutant at 37°C, while the genes *irp1*, *irp2*, *irp7* and *fyuA* were upregulated slightly in the mutant, but did not reach our significant thresholds of 2-fold change and p-value < 0.001 (fold change between 1.6 and 1.9, $p < 0.05$). *irp2* carries an Hfq-binding sequence. *Y. enterocolitica* is predicted to possess two Fur-repressed sRNAs homologous to RyhB (YEs023 and YEs040 as predicted by Rfam, retrieved from https://www.genome.jp), analogous to RyhB1 and RyhB2 in *Y. pestis*, as well as their equivalents in *Y. pseudotuberculosis*, Ysr146.1 and Ysr146.2 (Koo et al., 2011). Only one of them was slightly downregulated in the mutant (YEs023), however only with a significance of $p < 0.05$. Both of them were lowly expressed. RyhB1 was shown to be dependent on Hfq in *Y. pestis* (Deng et al., 2012).

The effect of Hfq in *Y. enterocolitica* on iron metabolism seems to differ from other organisms. The siderophore yersiniabactin has been shown to be downregulated by Hfq along with FyuA and FcuA (Kakoschke et al., 2014). In another study in *E. coli*, it was observed, that RyhB upon release of Fur-mediated repression, downregulates bacterioferritin and ferritin, as well as other genes like the superoxide dismutase *sodB*, that use iron. It was hypothesized, that by decreasing these proteins, bacterial cells do not use scarce iron for nonessential genes (Massé and Gottesman, 2002).

However, in this study cultures were not grown under iron-limiting conditions and genes thought to be involved in siderophore production were expressed at very low levels. This, of course, makes it difficult to interpret the results.

Carbon metabolism

In this study, genes of the glycolysis and gluconeogenesis pathway seemed to be affected by Hfq. The fructose-bisphosphate aldolase gene *fba* was downregulated in the mutant at both temperatures. In our previous proteomic study, the pyruvate kinase PykF had been shown to be more abundant in the mutant than in the wild-type strain (Kakoschke et al, 2014). We did not see any changes in the abundance of *pykF* mRNA, however the phosphoenolpyruvate synthase (*pps*), which catalyzes the inverse reaction from pyruvate to phosphoenolpyruvate, was downregulated in the mutant at 27°C.
The glycogen branching protein *glgB* and the glycogen phosphorylase *glgP* were both downregulated in the mutant at 37°C, while alpha-amylase *amyA* was downregulated at 27°C.

The pentose phosphate pathway did not show many changes. Transcripts encoding the transketolase TktA and the transaldolase TalB, which had been shown to be downregulated by Hfq (Kakoschke et al., 2014), were not significantly altered. While *talB* did not show any difference, *tktA* was slightly upregulated in the mutant at 37°C, but only 1.7-fold, missing our threshold.

Our previous proteomic study had revealed repression of propanediol utilization (Pdu) by Hfq on the transcriptional level (Kakoschke et al., 2014). In this study *pduK* was downregulated in the mutant at 27°C, which also carries a putative Hfq-binding sequence. However, we did not grow bacteria on agar containing 1,2-propanediol and Vit. B12, and hence the *pdu* genes were only lowly expressed, leading to only few reads in the transcriptional analysis. The gene *YE2751*, which is a putative sugar binding and transport protein close to he *pdu* region, was upregulated in the mutant at 27°C, consistent with the proteomic results found by Kakoschke et al. (2014).

Many genes involved in the citrate cycle were upregulated by Hfq. The oxaloacetate-decarboxylating malate dehydrogenase (*maeA*), which converts malate directly to pyruvate, the dihydrolipoamide acetyltransferase (*aceF*), involved in pyruvate decarboxylation which links glycolysis to the TCA cycle, as well as citrate synthase (*gltA*), dihydrolipoamide succinyltransferase (*sucB*), succinate dehydrogenase (*sdhACD*) and malate dehydrogenase (*mdh*), which are all part of the TCA cycle, were all downregulated in the *hfq* mutant at 37°C. Conversely, genes encoding the citrate lyase (*citCDEFX*), which converts citrate to oxaloacetate and acetate and is involved in the anaerobic metabolism of citrate, were upregulated in the mutant at 37°C. Additionally, *citF* has a putative Hfq-binding sequence. Citrate synthase and citrate lyase are antagonisitic enzymes and should therefore not be active simultaneously to avoid loss of energy (Subramanian & Sivaraman, 1984).

Some genes involved in phosphotranspherase systems (PTS) were affected: the PTS mannose-specific transporter gene *gptB* and the glucose specific transporter gene *crr* were downregulated in the mutant at 37°C.
Fructose and mannose metabolism were also affected: The phosphomannomutase gene *manB*, the fructose-bisphosphate aldolase gene *fba* and the GDP-fucose synthetase gene *fcl* were down-regulated in the mutant at both temperatures.

Fatty acid metabolism

Two genes involved in oxidation of fatty acids were down-regulated in the mutant at 37°C, i.e. the acetyl-CoA acyltransferase *fadA* and the acyl-coA dehydrogenase *fadE*

genes. The 2,4-dienoyl-CoA reductase gene *fadH* was up-regulated in the mutant at 37°C. This enzyme is not part of any known pathways in *Y. enterocolitica* according to KEGG Orthology but could be involved in the oxidation of polyunsaturated fatty acids. The transcription factor *fadR* was 1.6-fold more abundant in the mutant. In *E. coli* FadR represses fatty acid oxidation genes while activating synthesis genes (Cronan, 2020).

Nitrogen metabolism

Compared to carbon metabolism, we found fewer genes involved in nitrogen metabolism. Among them was the asparagine synthetase gene *asnA*, which promotes production of asparagine from ammonia (Reitzer and Magasanik, 1982). *asnA* was downregulated in the mutant at both temperatures and carries a putative Hfq-binding sequence. Furthermore, while the nitrate reductase subunit *napB*, converting nitrate to nitrite, was upregulated in the mutant at 37°C, the nitrite reductase subunits *nirB* and *nirD*, converting nitrite to ammonia, were upregulated in the mutant at 27°C. The genes *mtnA*, *mtnB* and *mtnC*, that are in immediate vicinity of each other and are all involved in L-methionine biosynthesis were downregulated in the mutant at 37°C. We did not see any significant changes in transcripts for the tryptophanase (*tnaA*), the periplasmic oligopeptide binding protein precursor (*oppA*) or the ornithine decarboxylase (*speC*), despite proteomic and functional changes which were shown before in our lab (Kakoschke et al., 2014). The sRNA *gcvB* (YEs033, *gcvB* as predicted by Rfam), which is involved in regulation of peptide transport by repressing *oppA* in other species was also not regulated by Hfq in our study (Urbanowski et al., 2000; Sharma et al., 2007). However, we saw the downregulation of some peptide (*tatE)* or amino acid transporters (*yecS*, *tdcC)* in the mutant at 37°C or both temperatures (*yecS*).

Cell energetics

At 37°C the ATP synthetase genes *atpC* and *atpD* were downregulated in the mutant 1.8- and 2.6-fold respectively. Additionally, the genes *cyoA*, *cyoB*, *cyoC* and *cyoE* coding for subunits of the cytochrome o ubiquinol oxidase as well as *cydB*, encoding a subunit of the cytochrome d ubiquinol oxidase, were all downregulated in the mutant at 37°C. Furthermore, as mentioned above, the succinate dehydrogenase subunit genes

sdhA, *sdhC* and *sdhD* that are not only part of the TCA but also of the electron transport chain were all downregulated in the mutant as well.

3.1.1.6 Hfq-dependent sRNAs

Twelve sRNAs were identified in the transcriptomic analysis as being influenced by Hfq. Eleven of them were downregulated in the *hfq* mutant. Most of them experienced fold changes far higher than those of mRNAs. Half of them are over 10-fold less abundant in the *hfq* mutant, even up to a fold change of 290. Although change in mRNA abundance does not prove interaction with Hfq, such a strong fold change could very well be due to the strong contribution of Hfq to sRNA stability, rather than to gene expression.

The sRNAs CsrB and CsrC (which target the RNA-binding protein CsrA) were downregulated in the mutant at both temperatures. The transcript abundance for the protein CsrA was not significantly changed at either temperature. However, *csrD*, which is involved in RNase E-dependent degradation of the sRNAs CsrB and CsrC, was 2.3-fold upregulated in the *hfq* mutant at 37°C.

Table 11) sRNAs that were differentially expressed in the hfq mutant.

sRNA	37°C[1]	27°C[2]	description
RprA (YEs024)	-2	-4	Binds *rpoS* mRNA with Hfq and increases translation in *E. coli* (Updegrove et al., 2008)
SraC/RyeA (YEs020)	-3	-9	*cis*-acting, complementary to SdsR/RyeB, acts as an RNA decoy, controlled by σ70 and induced under low pH conditions, not regulated by Hfq in *E. coli* (Gupta et al., 2019)
SdsR/RyeB (YEs021)	-5	-10	*trans*-acting sRNA, RpoS-regulated, Hfq-dependent, represses *mutS* and *tolC* in *E. coli* and *ompD*, *crp*, *stpA*, *hupB*, *tolC* and *rtsA/B* in Salmonella, toxic in high concentrations during exponential phase, mediated through the repression of inner membrane protein *yhcB* (Gupta et al., 2019; Fröhlich et al., 2016; Choi et al., 2018)
CsrB (YEs032)	-3	(-2)[3]	Binds to and sequesters carbon storage regulator CsrA (Ozturk et al., 2017 & Vakulskas et al., 2015)
CsrC (YEs002)	-5	-5	CsrC-family RNA, as predicted by Rfam, binds and sequesters CsrA, downregulating its activity (Ozturk et al., 2017 & Vakulskas et al., 2015)
Spot42 (YEs001)	-7	-13	Regulator in carbohydrate uptake and metabolism, activated by glucose, inhibited by the cAMP-CRP-complex, Hfq-dependent, inhibits translation of *galK* and *sdhC* in *E. coli* (Görke and Vogel, 2008; Desnoyers and Massé, 2012)

YEs034	-18	-19	SraE/RygA/RygB-family RNA as predicted by Rfam, Hfq-binding in *E. coli*, regulation of OMP (Guillier and Gottesman, 2006)
MicF (YEs016)	-24	-11	*trans*-acting sRNA, downregulates e.g. *ompF* and *lpxR* expression posttranscriptionally in *E. coli* (Corcoran et al., 2012)
MicM (SroB, RybC, YEs029, ChiX)	-60	-56	SroB as predicted by Rfam, Hfq binding in *E. coli*, negatively regulates the OMP gene *ybfM* (Rasmussen et al., 2009)
RybB (YEs017)	-67	-290	Hfq binding in *E. coli*, induced by RpoE, downregulates *ompA* expression (Thompson et al., 2007)
YEs005	(-)[4]	-4	TPP riboswitch as predicted by Rfam
YEs013	2	3	repeat structure of the *tyrT* operon in *E. coli*, function unknown, in *Y. enterocolitica* from a locus within tRNAs

Criteria: fold change ≥ 2 and $p < 0.001$.

[1] fold change at 37°C, - means less abundant mRNA in the *hfq* mutant

[2] fold change at 27°C. - means less abundant mRNA in the *hfq* mutant

[3] $p \sim 0.002$, FDR < 0.05

[4] no significant fold change

Although the transcriptional analysis shows changes in RNA abundance for both protein coding genes as well as sRNA genes, we cannot deduce whether the effects are direct or indirect, or which sRNA interacts with which mRNA. To get an idea of possible mRNA targets we performed an in-silico analysis of sRNA binding predictions within the *Y. enterocolitica* O:8 genome, using *CopraRNA* by *Freiburg RNA tools* (Busch et al., 2008; Mann et al., 2017; Raden et al., 2018; Wright et al., 2013; Wright et al., 2014). We compared the results to our transcriptional analysis, looking for genes that are a predicted sRNA target and at the same time differently expressed in the *hfq* mutant. Table 12 gives an overview over the results. Out of 355 genes that show an Hfq-dependent abundance, 90 were predicted to be sRNA targets (~ 25%). Several of them were predicted to interact with multiple sRNAs.

Table 12) Predicted targets of sRNAs

sRNA	**Predicted targets (also Hfq-dependent)**	**Annotation**
RprA	rpoS*	sigma factor
	fliZ	flagella biosynthesis protein
	araC	DNA-binding transcriptional regulator
	trkD	potassium transport protein Kup

	ychH	YE2436, involved with CRP
	fdX	[4Fe-4S] ferredoxin
	hflX	GTPase HflX
	citE	citrate lyase subunit beta
	sdhA	succinate dehydrogenase flavoprotein subunit
	YE2035	membrane transport protein
	cysG	siroheme synthase
	rplP	50S ribosomal protein L16
	YE4066	insulinase family protease
RyeB	YE1160*	Similar to *Escherichia coli* hypothetical RelE protein or b1563
	outJ	general secretion pathway protein J
	ihfA	integration host factor subunit alpha
	cyoA	cytochrome o ubiquinol oxidase subunit II
	groEL	chaperonin
	sucB	dihydrolipoamide succinyltransferase
	arsR2	arsenical resistance operon trans-acting repressor
	yfgG	hypothetical protein
	YE3931	sulfur transfer complex subunit TusB
	glgB	glycogen branching protein
	rpoS	sigma factor
	YE0524	Aldolase
	YE1569	sulfur relay protein TusC
	yfeY	hypothetical protein
	glnB	nitrogen regulatory protein P-II 1
	YE3094a	Similar to *Yersinia pestis* putative membrane protein y1058
	gltA	type II citrate synthase
	YE0495	hypothetical protein
	cyoC	cytochrome o ubiquinol oxidase subunit III
	glgP	glycogen phosphorylase
RybB	aceF*	dihydrolipoamide acetyltransferase
	YE0524	Aldolase
	rpoS	sigma factor
	YE0495	hypothetical protein
	znuA	high-affinity zinc transporter substrate-binding protein
	phoH	hypothetical protein
	rpoE	sigma factor
	atpC	F0F1 ATP synthase subunit epsilon
	YE0402	hypothetical protein
	YE2299	phage tail assembly protein
	yfgG	hypothetical protein
	rseA	anti-RNA polymerase sigma factor SigE
	ppk	polyphosphate kinase
	bolA	transcriptional regulator BolA
	YE0706	hypothetical protein
	ftsQ	cell division protein FtsQ
MicF	**trkD**	potassium transport protein Kup
	ye0452	acetyltransferase
	ye1569	hypothetical protein
	wbcA	epimerase
	rseA	anti-RNA polymerase sigma factor SigE

	zntB	zinc transporter
	nirB	nitrite reductase
	manB	phosphomannomutase
	cydB	cytochrome D ubiquinol oxidase subunit II
	putA	trifunctional transcriptional regulator/ proline dehydrogenase/ pyrroline-5-carboxylate dehydrogenase
	ye2299	phage tail assembly protein
	bolA	transcriptional regulator BolA
	ye0402	hypothetical protein
	ye3001	23S rRNA (pseudouridine(1915)-N(3))-methyltransferase RlmH
	ye2436	YchH; transcription activated by CRP (cyclic AMP receptor protein) a global transcription factor involved in regulation of metabolism in enteric bacteria; ychH presents a class II promoter to bind CRP; unknown function
	ye0706	hypothetical protein
	ye3788	cytochrome
	hypB	hydrogenase nickel incorporation protein HybF
	ygiW	Predicted periplasmic protein YdeI
	lamB	maltoporin
	glnB	nitrogen regulatory protein P-II 1
	dapA	4-hydroxy-tetrahydrodipicolinate synthase
	rpsH	30S ribosomal protein S8
	ye3931	sulfur transfer complex subunit TusB
SroB	ye3063*	putative uncharacterized protein, regulation of transcription
	ye1738*	hypothetical protein
	ye0402	hypothetical protein
	arsR2	arsenical resistance operon trans-acting repressor
	dkgA	2 5-diketo-D-gluconate reductase A
	corE	hypothetical protein
	degQ	protease
	rseC	SoxR reducing system protein RseC
	cyoB	cytochrome O ubiquinol oxidase subunit I
	fadH	2 4-dienoyl-CoA reductase
	rplB	50S ribosomal protein L2
	ye0084	bifunctional regulatory protein/DNA repair protein
	ye3853	zinc uptake transcriptional repressor
	sdhD	succinate dehydrogenase cytochrome b556 small membrane subunit
	ihfA	integration host factor subunit alpha
	glgB	glycogen branching protein
	ye1145	YpfN family protein
	ye2995	hypothetical protein
	phnH	carbon-phosphorus lyase complex subunit
Yes034	ccmA*	cytochrome c biogenesis protein CcmA
	ye3094a*	Similar to *Yersinia pestis* putative membrane protein y1058
	ye1881	hypothetical protein
	hemD	uroporphyrinogen-III synthase
	ye2995	hypothetical protein
	ye2705	LuxR family regulatory protein
	ftsQ	cell division protein FtsQ

	dga	glutamate racemase
	yecS	amino-acid ABC transporter permease
	nirC	nitrite transporter NirC
Spot42	sdhC*	succinate dehydrogenase cytochrome b556 large membrane subunit
	pps	phosphoenolpyruvate synthase
	gltA	type II citrate synthase
	ye3931	sulfur transfer complex subunit TusB
	cysG	siroheme synthase
	rpsQ	30S ribosomal protein S17
	rpsH	30S ribosomal protein S8
	ye3936	hypothetical protein
	gptB	PTS system mannose-specific transporter subunit IID
	cyoA	cytochrome o ubiquinol oxidase subunit II
	groEL	chaperonin
	fliZ	flagella biosynthesis protein FliZ
	ye0523	autoinducer-2 (AI-2) modifying protein LsrG
	rovM	LysR family transcriptional regulator
	ihfA	integration host factor subunit alpha
	mrdA	penicillin-binding protein 2
	ssiC	taurine transporter subunit

* $p < 0.001$, all others $p < 0.05$ (calculated p-values by *CopraRNA*). All genes met the criteria of $p < 0.001$ and fold change ≥ 2 in our sequencing analysis. The **bold** genes carry a predicted Hfq-binding sequence.

3.1.1.7 Transcriptional regulators

Next, we assessed the influence of Hfq on transcriptional regulators. *ompR* and *rovA* showed no significant differences between wildtype and mutant. However, *rovM* (~ 2.5-fold), *phoB* (~1.8-fold) and *rpoS* (~2.0 fold) were downregulated in the mutant at 37°C.

Among the genes within the PhoB-regulon in *E. coli* (Gardner & McCleary, 2019), only few were affected in the *hfq* mutant of *Y. enterocolitica*: *phoH*, which encodes an ATP-binding protein of unknown function was downregulated, while *phnHFGI*, which are involved in phosphonate uptake and catabolism were upregulated in the mutant.

When looking for OmpR-regulated genes in our transcriptomic analysis, we found that about half of the genes under the control of OmpR in *Y. enterocolitica* O:9 in one study (Nieckarz et al., 2020) were *hfq*-dependent in our analysis (e.g., *dgkA, clpB, groEL, htpG,* and genes encoding ribosomal proteins). The *flhDC* genes that had also been shown to be OmpR-dependent in O:9 were not altered, while *ompC* and *ompF*, which are also thought to be part of the OmpR-regulon, were slightly down-regulated (the latter one only -1.7-fold), as well as *invA* and *ail* (Skorek et al., 2013 & Kakoschke et al., 2016).

The TCS PhoP/PhoQ was not differently expressed in our analysis. However, the gene *lpxR*, which is part of its regulon, was upregulated, in accordance with our previous proteomic study (Kakoschke et al., 2016).

At 27°C *rpoS* showed no significant difference between wildtype and mutant. At 37°C it was downregulated in the mutant with a fold change of almost -2.0 and a p-value < 0.001. Several sRNAs are associated with the regulation of *rpoS*: ArcZ, RprA and DsrA, which enhance translation of *rpoS* and OxyS, which inhibits its translation. RprA was less abundant in the mutant at both temperatures, while ArcZ, OxyS and DsrA were not detected. In *E. coli*, *rpoS* expression is under control of the *nlpD* promoter, a gene that is located immediately upstream of *rpoS* in *E. coli* and *Y. enterocolitica* as well (Gottesman, 2019). Interestingly, *nlpD* was downregulated at 37°C, too. Many genes that are part of the RpoS regulon in *E. coli* (Patten et al., 2004) were downregulated in the *hfq* mutant along with *rpoS* itself, e.g. the superoxide dismutase *sodC*, the DNA-binding protein *dps* and the periplasmic protein gene *ydeI.*

3.1.1.8 Translation

Three rRNAs showed significant differences between wildtype and mutant. Two of them were 23S-rRNA (YEr008 and YEr011, which were downregulated in the mutant) and one 16S-rRNA (YEr018, upregulated). Seven ribosomal proteins were identified, of which three belonged to the 30S subunit and four to the 50S subunit. Six of them were downregulated in the mutant (*rplA*, *rplB*, *rplL*, *rplP*, *rpsC*, *rpsQ*), while only one was upregulated (*rpsH*). One of them had a predicted Hfq-binding sequence (*rplP*, ARN type, four repeats). Eight other ribosomal proteins were found to have an ARN motif as well but were not significantly up- or downregulated (*rpsM*, *rplF*, *rplJ*, *rplO*, *rplR*, *rplS*, *rplT*, *rplV*). In addition, two methyltransferase genes associated with 23S rRNA were changed (*YE3001* upregulated, *YE0385* downregulated).

Five tRNAs were found to be differently expressed in the transcriptional analysis, two were upregulated (*YEt011*, *YEt039*) and three downregulated (*YEt022*, *YEt038*; *YEt041*) in the mutant. Three genes involved in tRNA synthesis or processing were more abundant in the mutant as well (*hisS*, *tusB*, *tusC*). Four more enzymes involved in tRNA

synthesis carried ARN motifs but without significant fold changes (*cysS*, *truB*, *pheS*, *ttcA*).

3.1.1.9 Overall conclusion about the transcriptomic analysis

Loss of *hfq* caused a variety of alterations in the transcriptome of *Y. enterocolitica* O:8 (355 genes). We saw transciptomic changes for some, but not all genes, that had previously been shown to be Hfq-dependent through proteomics (Kakoschke et al., 2014). Moreover, our analysis revealed many differences that are in accordance with growth and survival characteristics described for *hfq* mutants (Kakoschke et al., 2014): many genes related to stress resistance, metabolism and cell envelope were significantly altered in the mutant in this analysis. Most importantly, this study showed that not only protein coding genes changed, but that multiple sRNAs, potentially binding to a third of all Hfq-dependent transcripts, were strongly downregulated in the *hfq* mutant as well. Taken together, this study indicates that Hfq is a global regulator in *Y. enterocolitica*.

3.1.2 Validation of a subset of transcriptomic results by Northern blots

To confirm the results seen in the transcriptomic analysis, we tested the expression of selected genes with Northern blots. Although this technique only allows a semiquantitative evaluation of RNA abundance, it can also shed light on the structure and integrity of the RNA molecules and can serve as a quality control.

Before this study, our former mutational analysis uncovered the role of Hfq in the deployment of several OMPs involved in adhesion to host cells or iron acquisition, suggesting that Hfq participates in pathways that remodel the bacterial envelope (Kakoschke et al., 2014 & Kakoschke et al., 2016). Since my transcriptomic analysis suggested that the RpoE envelope stress response might be upregulated in the *hfq*-negative strain, I investigated whether I could confirm that the transcripts of *rpoE*, *rseA* and *rseB* were detectable in *Y. enterocolitica* and whether they were up-regulated in the *hfq* mutant. Northern blots were performed with digoxigenin-labelled RNA probes complementary to the transcripts that were tested.

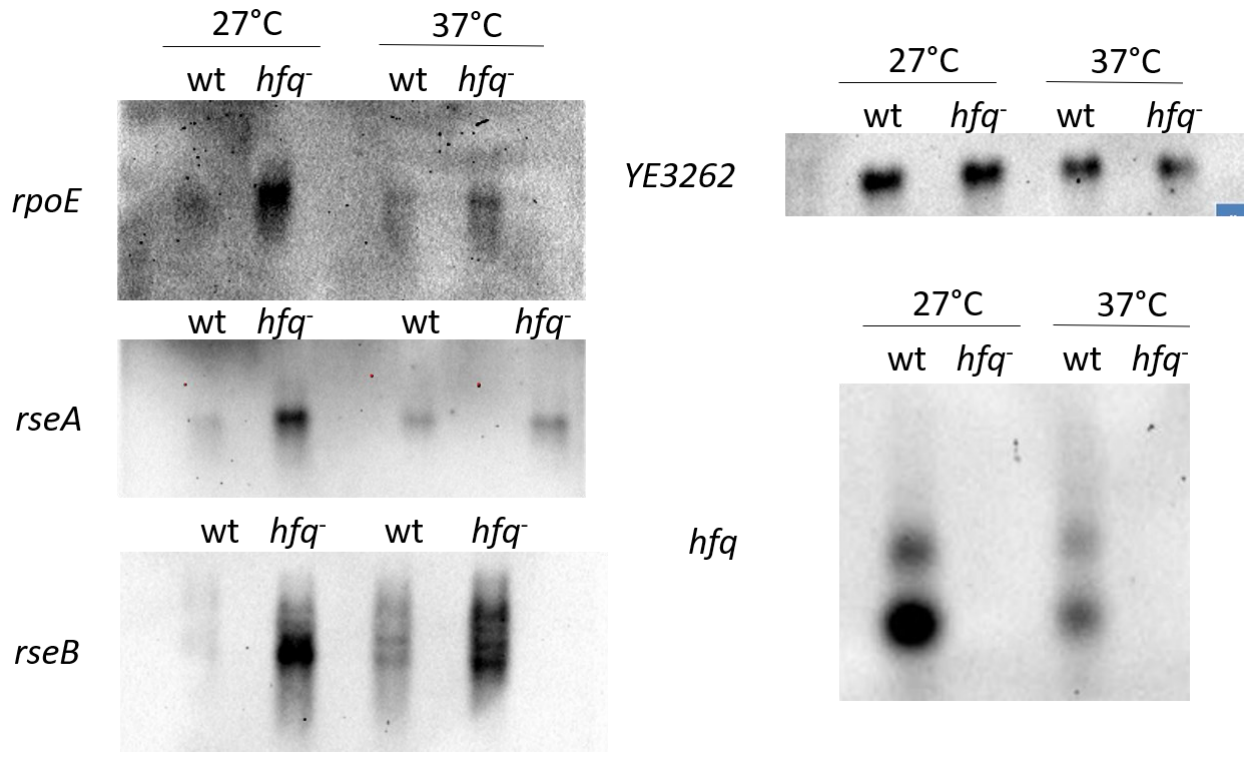


Figure 18) Northern blots comparing wt and hfq-. Total RNA (1 µg) prepared from wild-type and hfq-negative strains grown in LB at 27°C or 37°C was separated by agarose gel electrophoresis. Following transfer, the membranes were reacted with digoxigenin-labelled RNA probes complementary to rpoE, rseA, rseB, YE3262 or hfq.

Equal amounts of total RNA prepared from wild type and *hfq* mutant grown at 27°C and 37°C were tested by Northern blotting. As a negative control, we used the hypothetical protein gene *YE3262* because the RNA-seq analysis indicated that its transcript was abundant, and its expression was independent of *hfq*. Indeed, we observed equivalent signal in all the samples tested (Fig. 18). Our second control was the *hfq* gene: as expected, no transcript could be detected in the *hfq* mutant (Fig. 18), confirming that the knockout of *hfq* was successful. The signal is stronger in the 27°C sample than in the 37°C one. This is in accordance with the transcriptomic analysis, which show more reads at 27°C than at 37°C in the wildtype. Another interesting observation is that there appears to be two bands, a stronger, smaller one and a fainter one of bigger molecular size. This could mean, that there are two promoters for *hfq*, which would result in two differently sized transcripts, as has been observed in *E. coli* (Tsui et al., 1994). Another

reason for this might be that there is a sequence which is recognized by an RNase, therefore leading to cleavage of the mRNA.

Regarding the genes involved in RpoE-dependent ESR, we observed that the transcripts for *rpoE*, *rseA* and *rseB* were more abundant in the *hfq*-negative strain than in the wild-type (Fig. 18). Compared to other Northern blots, the signal for *rpoE* was fainter and longer exposure was necessary (as can be observed with the higher background). The Northern blot could confirm the results from the deep sequencing analysis. The transcriptomic analysis showed that *rpoE* was more abundant at 37°C than at 27°C and was upregulated in the mutant at 37°C (37°C fold change 2.64, $p < 0.001$) and only weakly at 27°C (27°C fold change 1.85, $p < 0.05$, missing our threshold). In the Northern blot *rpoE* was also more abundant in the *hfq*-mutant than in the wild-type samples. This was not only the case at 37°C, but also at 27°C. In the transcriptomic analysis *rseB* was more abundant in the *hfq*-mutant at both temperatures and was overall more abundant at 37°C. This could be confirmed in the Northern blots (Fig. 18). *rseA* seemed to be equally abundant at both temperatures in the transciptomic analysis, while the influence of Hfq was stronger at 37°C (37°C: fold change 3.31, $p < 0.001$; 27°C: fold change 1.96, $p < 0.05$). The higher abundance of *rseA* mRNA in the mutant was consistently observed in the Northern blots, although the effect was not always stronger at 37°C (Fig. 18 and data not shown). However, we should bear in mind that Northern blots are semiquantitative and therefore we should be careful when drawing conclusions on the magnitude of the effect. The crucial finding is that the Northern blots could confirm the Hfq-dependence of *rpoE*, *rseA* and *rseB* transcripts.

3.2 Assessing the direct interaction of Hfq and mRNA by co-immunoprecipitation

Next, we assessed whether Hfq interacts directly with some of the Hfq-dependent mRNAs. For this study we used a strain in which the chromosomal copy of *hfq* is replaced by a modified *hfq* gene encoding a functional Hfq tagged with the three copies of the FLAG epitope (SOR35) (Kakoschke et al., 2014). As a control for the specificity of the co-immunoprecipitation (co-IP), we used the wild-type strain, and in some

experiments, a strain modified to produce the RNA-binding protein CsrA tagged with FLAG (Fischbach, 2012).

3.2.1 Protein analysis following co-IP

Bacteria were grown at 37°C in exponential phase, lysed and the co-IP was performed as described in chapter 2.2.6. A sample was taken right after bacterial cell lysis. Another sample was taken after incubating the lysed cells with beads coated with the anti-FLAG antibody by pipetting a small portion of the beads in Laemmli sample buffer, which denatures the antibody and elutes the proteins. Proteins were separated by SDS-PAGE and stained with Coomassie blue (Fig. 19).

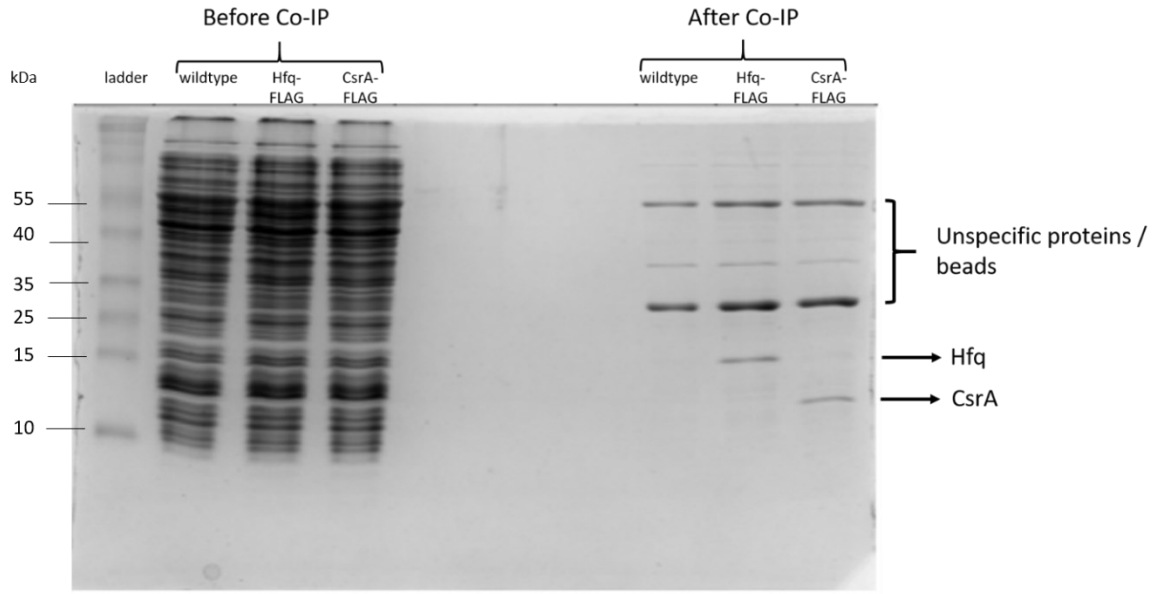


Figure 19) Coomassie stain, comparing the protein profiles before and after the co-IP using the wildtype strain (negative control), and strains producing the RNA-binding proteins Hfq and CsrA tagged with 3 FLAG epitopes.

Fig. 19 shows that there is an equal amount of proteins in the three samples before the co-IP. As anticipated, there is a great variety of bands of different sizes, since this depicts the entirety of the proteome. After the co-IP two strong bands can be seen in all three samples, which correspond to the light and heavy chains of the antibody (25 and 50 kDa respectively). In the samples prepared from strains producing proteins with the FLAG-tag, an additional band is visible that corresponds to the molecular size of Hfq (~ 11 kDa) and CsrA (~ 7 kDa) with the additional 3xFLAG-tag (~3 kDa).

To further confirm whether the additional bands were FLAG-tagged proteins, I also did a Western blot with the same samples, using an anti-FLAG-antibody (Fig. 20). After incubating with an anti-FLAG-antibody, there were strong signals in the samples from strains encoding the Hfq-FLAG and the CsrA-FLAG. The bands were at the same molecular size as seen in the Coomassie stain. They were present before and after the co-IP. There is one additional high molecular size band in the Hfq-FLAG input sample that is also slightly visible in the output sample. This could be due to Hfq forming hexamers that were not fully denatured. It is also possible that this is Hfq bound to another protein. An even bigger, very faint band is also seen in the CsrA input, but not in the output sample. Additionally, there is one band of medium size (~40 kDa) in all three input samples. Since they are equally visible even in the sample without any FLAG-tagged proteins, this most likely reflects unspecific binding of the antibody to another protein.

Taken together our results show that we were successful in purifying proteins with a FLAG epitope by immunoprecipitation.

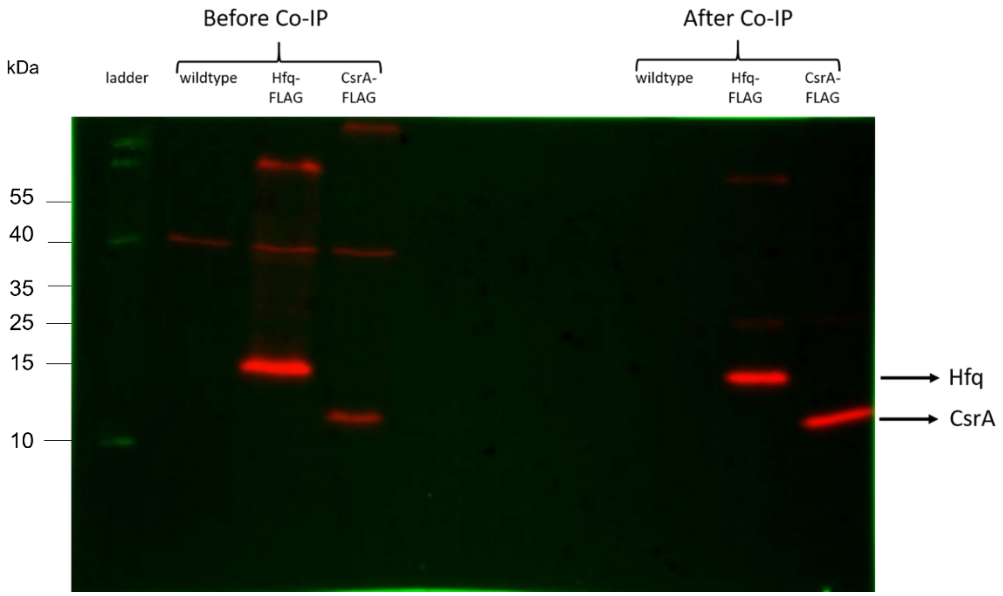


Figure 20) Western blot using an antibody specific for the FLAG epitope, comparing the protein samples before and after the co-immunoprecipitation (co-IP).

3.2.2 RNA analysis following co-IP using Northern blots

Next, using Northern blotting, we tested whether some mRNAs were present in the samples following co-immunoprecipitation with Hfq.

Interaction of Hfq with *rseA* and *rseB* mRNA

Since *rpoE*, *rseA* and *rseB* all appeared to be more abundant in the *hfq* mutant (Fig. 18) and since *rseA* and *rseB* have a predicted Hfq-binding sequence (4x ARN motifs), we first analyzed the transcripts linked to the RpoE-dependent ESR. As expected, *rpoE* could barely be detected in the input samples (data not shown), and we therefore concentrated on *rseA* and *rseB* transcripts (Fig. 22). In the co-IP samples, *rseA* and *rseB* mRNA were equally abundant in both 'input' samples before the pulldown. In the 'output' samples, the transcripts were only detectable in samples with Hfq-FLAG and not in the negative control (wildtype). Whereas the recovered *rseA* mRNA gave a faint signal, the recovered *rseB* transcript appeared processed following the incubation of the cell lysate with the antibody. Our results suggest that Hfq binds to *rseB*, and maybe also *rseA* mRNAs.

Interaction of Hfq with *hfq* mRNA

In *E. coli*, Hfq exerts an autoregulatory inhibition through binding its own mRNA (Morita & Aiba, 2019) and previous work in the lab suggested that Hfq exerts a negative regulation on its own production in *Y. enterocolitica* as well (O. Rossier, unpublished data). To test the interaction of Hfq with its own mRNA, we performed a Northern blot with RNA from the co-IP and an *hfq*-specific probe. In the input samples, bands of equal intensity were observed in the wild-type and Hfq-FLAG samples (Fig. 22). The transcripts encoding Hfq-FLAG reflected a larger molecular size, due to the added sequence encoding the FLAG-tag, that naturally not only increases the protein size, but also elongates the mRNA by several nucleotides (Fig. 22). As described in chapter 3.1.2, there were two bands in each sample: they most likely correspond to transcripts originating from alternative promoters for *hfq*, similar to *E. coli hfq* (Tsui et al., 1996). In the output samples only the Hfq-FLAG sample shows a band, that is of lower molecular size and appears more smeared due to partial degradation that inevitably happens

during the incubation period (Fig. 22). It can therefore be concluded that the Hfq protein binds to *hfq* mRNA.

Interaction of Hfq with *YE3262* mRNA

As a negative control for RNA bound to Hfq, we used the gene YE3262 again. This gene codes for a hypothetical protein with so far unknown function. It showed equally strong bands in the input samples. In the output samples a very faint smear was barely visible after coIP using the Hfq-FLAG sample (Fig. 22), suggesting abundant mRNAs could not be completely eliminated despite multiple washing steps or that YE3262 mRNA interacts very slightly with Hfq. In any case, compared to the other Northern blots with *hfq* or *rseB*, the band is considerably fainter. YE3262 does most likely not interact with Hfq.

Interaction of Hfq with *ompX* and *ail* mRNA

Finally, we tested the interaction of Hfq with transcripts encoding adhesins. To add another control, we conducted experiments with a strain producing the RNA-binding protein CsrA tagged with the FLAG epitope. Preliminary evidence obtained in the lab suggested that CsrA negatively regulated the expression of *ail* but does not have any influence on that of *ompX*. While the wild-type sample showed no *ompX* and *ail* mRNA in the output sample, a strong band was detectable in the Hfq-FLAG output sample (Fig. 23). It shows the usual characteristics of slightly degraded RNA, appearing smeared and of lower molecular size. The Hfq-FLAG strain remains the only output sample that showed a band for *ompX,* however *ail* mRNA also showed up in the CsrA-FLAG output samples. This shows that the results obtained with the co-IP are due to specific interactions between Hfq or CsrA and RNA and not due to unspecific interactions that take place with any RNA binding protein. Furthermore, we took additional samples from the IP-buffer that was used to wash the beads after the incubation (washout). Faint bands can be seen in all three samples, proving that there is actually sufficient and intact RNA in the samples, but it cannot be pulled down by the co-IP (Fig. 23). Taken together, these results indicate that Hfq binds to transcripts encoding the adhesin Ail or OmpX, while CsrA binds only *ail* mRNA.

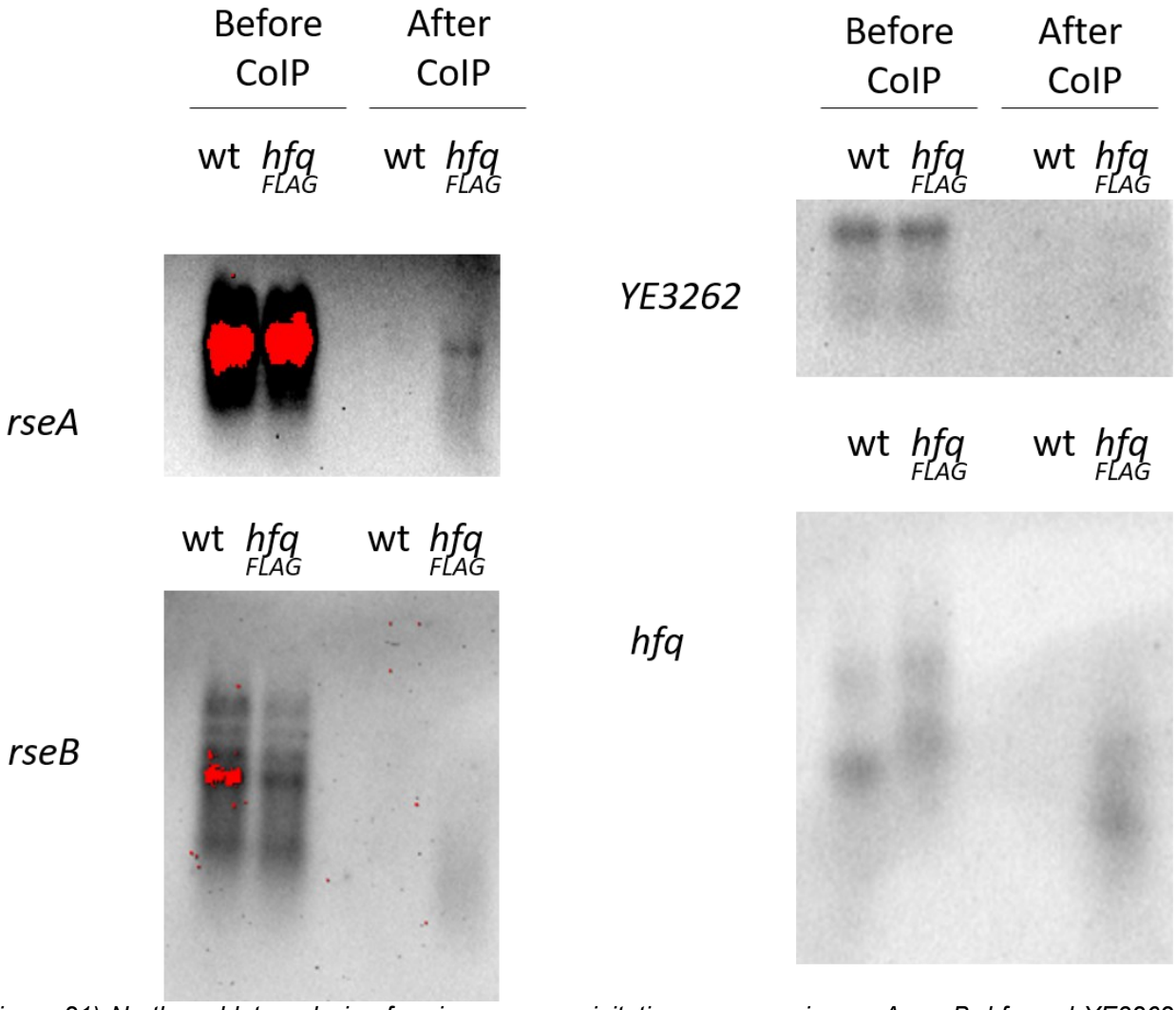


Figure 21) Northern blot analysis of co-immunoprepcipitation assays using rseA, rseB, hfq and YE3262 probes, comparing wild-type (wt) and hfq-FLAG strains. The detection period was extended to make the signal visible. The red areas correspond to saturation of the signal.

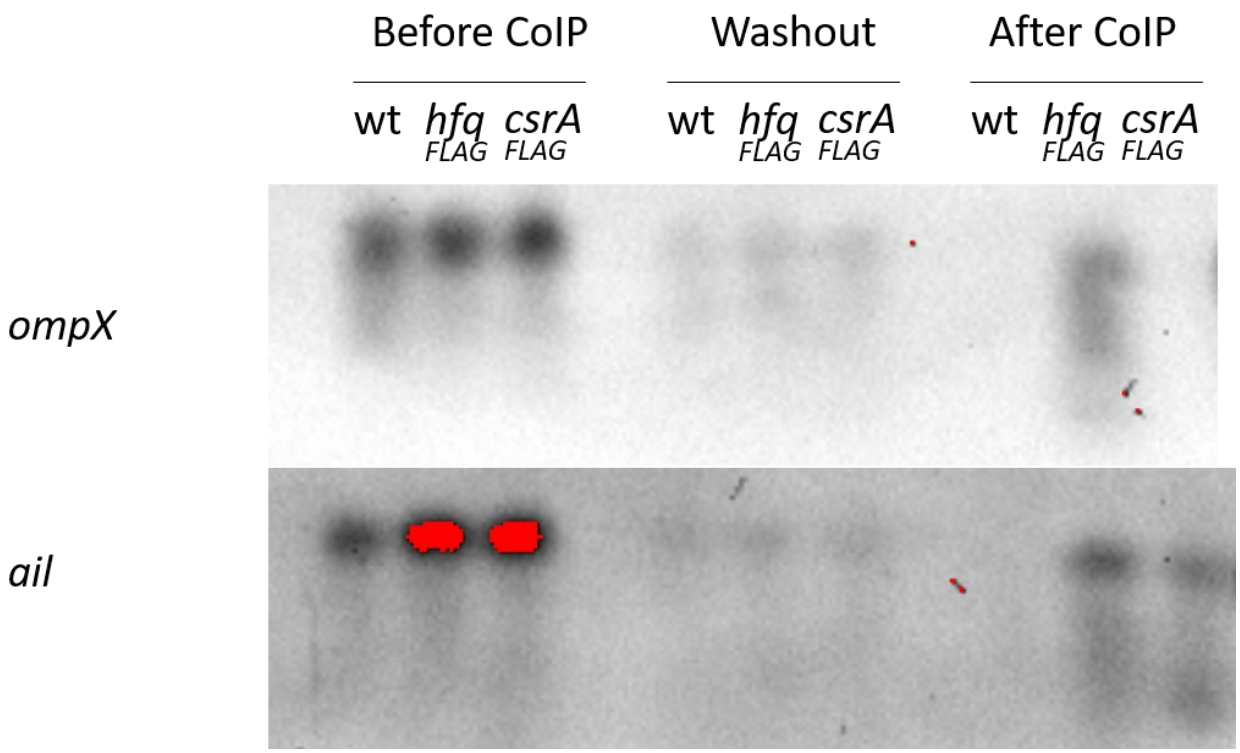


Figure 22) Northern blots analysis of co-immunoprepcipitation assays using ompX and ail probes, comparing wild-type (wt), hfq-FLAG and csrA-FLAG strains.

4 Discussion

Through its versatile function as an RNA chaperone and its role in posttranscriptional regulation, Hfq was found to be a crucial determinant of virulence in a variety of bacterial species (Chao & Vogel, 2010). Especially in Gram-negative bacteria, Hfq has a profound influence on metabolism, resistance to stress and virulence factors. Indeed, *hfq* mutants often show growth defects, decreased mobility or increased sensitivity to environmental stressors, which could in many cases be linked to attenuated virulence in *in vivo* studies (Chao & Vogel, 2010).

Previous to this thesis, our lab performed a phenotypic characterization of an *hfq*-negative strain in *Y. enterocolitica*, that included a proteomic analysis, stress resistance and virulence assays. The analysis showed a slowed growth rate, altered cell morphology, increased sensitivity to acidic pH and oxidative stress and a variety of metabolic changes in *hfq* mutants (Kakoschke et al., 2014 and 2016).

In this study we went on to further characterize the influence of Hfq in *Y. enterocolitica*, by performing transciptomic studies and assessing direct interactions with some of the Hfq-dependent mRNAs.

4.1 General considerations in the interpretation of transcriptomic and co-immunoprecipitation data

A decrease of the absolute fold change in the transcriptomic analysis means that the respective gene was found to have less reads in the *hfq* mutant compared to the wild-type. Therefore, the gene is downregulated in the absence of Hfq and upregulated when Hfq is present. There are several possible relationships between a gene and Hfq that would explain those changes.

1. Hfq interacts directly with the mRNA. If the RNA is less abundant in the mutant, it most likely means that Hfq upregulates the gene by protecting it from degradation through RNases.
2. Hfq promotes or inhibits the transcription factor of the respective gene, thus influencing it indirectly.

3. The observed fold change is random, which even with a small p-value, still remains within possibilities. Indeed, this transcriptomic analysis was performed with biological duplicates only.

For the co-IP it can be said that an RNA that co-purifies with Hfq most likely binds to it and interacts directly with it, which leaves two possibilities:

1. Hfq upregulates the respective gene by stabilizing the RNA and/or increasing translation
2. Hfq downregulates the gene by destabilizing the RNA and/or decreasing translation.

In any case, the results should be related to other available data. It may seem paradoxical that it is possible to co-precipitate RNAs, when Hfq has such a high RNA turnover. However, contrary to mRNA-sRNA duplexes, that dissociate quickly from Hfq, complexes between Hfq and single RNAs are actually quite stable and have very low dissociation rates, with a half-life of about 100 min (Santiago-Frangos and Woodson, 2018). To find a cognate sRNA-mRNA pair, single sRNAs bound to Hfq need to exchange rapidly. A model was proposed, in which sRNAs ‘actively cycle’ on the multiple RNA binding surfaces of Hfq (Wagner, 2013; Sanitago-Frangos and Woodson, 2018). This happens however only with high RNA concentrations. Due to the degradation through RNases following cell lysis, RNA concentrations decline quickly, and the residual RNA remains bound to Hfq.

4.2 Regulation of Hfq

Since Hfq regulates the virulence of pathogenic bacteria, its own regulation is a matter of interest. Both, the transcriptomic analysis as well as the Northern blot showed that *hfq* mRNA is more abundant at 27°C than at 37°C. Furthermore, we found that *hfq* mRNA co-immunoprecipitates with the Hfq protein, showing that the two interact with each other. Possible explanations for the nature of this interaction can be found upon closer examination of the regulation of Hfq in other organisms:

In *E. coli* the regulation of *hfq* has been studied in greater detail already. In early studies, Hfq was found to destabilize its own mRNA, already hinting at a post-transcriptional autoregulation (Tsui et al., 1997). In later studies, Hfq was found to bind to two regions in the 5’-UTR of its own mRNA with its distal face and obstruct the ribosome binding site

(Vecerek et al. 2005; Morita and Aiba, 2019). This interaction alone could inhibit translation, and so far, no sRNAs have been identified to regulate *hfq* post-transcriptionally. The authors already noted that the suggested autoregulation mechanism would help to retain appropriate levels of Hfq: The more RNAs with a high affinity to Hfq are present, the more they compete with the *hfq* mRNA, ultimately leading to less inhibition of *hfq* translation when the demand for Hfq is high (Morita and Aiba, 2019).

This concept might also account for the lower abundance of *hfq* mRNA at 37°C: When other mRNAs are present and interact with Hfq, the *hfq* mRNA might be less inhibited and get translated at a higher rate, thus leading to a lower mRNA concentration. This would be in line with the fact that we found more differently regulated genes at 37°C than at 27°C (145 at 27°C vs. 282 at 37°C), suggesting that more RNAs interact with Hfq at host temperature. On the other hand, when the translation of mRNA is blocked by Hfq, it might be expected that it is rapidly degraded by RNases and not accumulate.

Another study investigated a similar idea. Sagawa et al. (2015) found that an optimal concentration of Hfq was necessary to allow a maximum interaction between mRNA and sRNA. The order by which mRNA and sRNA bind to Hfq is random. This means, that either of them can bind Hfq first. If Hfq is highly abundant, it might bind only one RNA without finding an available cognate RNA to interact. Thus, a high concentration of Hfq can sequester the RNAs and make an interaction between them impossible. A ‘set-point’ of optimal concentration is required for a smooth interaction (Sagawa et al., 2015; Adamson and Lim, 2011). This shows that a reduced Hfq concentration cannot be equated with a reduced function of the protein. Therefore, it might be possible that the decrease of RNA at 37°C actually reflects a decrease in Hfq protein concentration as well, but at the same time an improved function.

How Hfq is regulated in *Y. enterocolitica* has not been studied as thoroughly so far. The co-IP experiment shows that *hfq* mRNA binds to the protein it encodes. It does not reveal however, which face of the protein Hfq is involved, neither to which part of the mRNA it binds. Given that Hfq in *Y. enterocolitica* exhibits the same ARN motif as the *E. coli* Hfq (a known Hfq-binding site), it seems likely that the mechanism is similar to the one already described in *E. coli* (the *hfq* mRNA binding to the distal face of Hfq (Morita

and Aiba, 2019)). Further studies are necessary to uncover the nature of this mRNA-protein interaction.

4.3 Regulation of OMPs and the bacterial envelope

OMPs and the bacterial envelope provide adhesion to as well as invasion of host cells, serum resistance and adaptation to environmental conditions. They are therefore important determinants of virulence. The effect of Hfq on OMPs in *Y. enterocolitica* has been studied before in our laboratory: Hfq promoted the transcription of *invA*, while inhibiting the expression of *ompX* and *ail* on the posttranscriptional level (Kakoschke et al., 2016).

In this transcriptomic study, *ompX*, *ail*, as well as *invA* transcripts were less abundant in the *hfq*-negative strain. Since the study was performed with cells in exponential phase, our results suggests that Hfq promotes expression of *ompX* and *ail* in this growth phase, a conclusion we were not able to draw beforehand, maybe because the abundance of mRNA varied greatly between experiments (Kakoschke et al., 2016). In contrast to its positive effect in log phase, Kakoschke et al. established that Hfq represses *ompX* and *ail* expression at the post-transcriptional level during stationary phase (Kakoschke et al., 2016). In this work, we showed, using co-IP, that Hfq binds to the *ail* and *ompX* mRNA transcripts, and therefore showed, that Hfq influences production of these OMPs directly by interacting with their mRNA.

invA was downregulated in the mutant, while its repressor H-NS was downregulated as well, and its other regulatory genes *ompR* and *rovA* were not significantly changed. In another study, *invA* was found to be downregulated by overexpression of *rpoE* and *cpxR* (Zeuzem, 2018). However, there was a discrepancy between transcript and protein abundance, suggesting a post-transcriptional regulation of *invA*. Taken together, earlier results and our transcriptomic data suggest an interplay of regulatory mechanism for InvA synthesis.

It remains to be seen if these interactions between OMPs and Hfq are mediated by sRNAs and if they are, by which sRNAs. We can take some suggestions from other studies. For instance, *ompF* was slightly (-1.7-fold) downregulated in the mutant at 37°C. In *E. coli*, *ompF* mRNA is a target of the sRNA MicF (Corcoran et al., 2012). MicF also seemed to be Hfq-dependent in this study.

The observed downregulation of flagellar genes by Hfq fits with previous results, that showed an upregulation of *flhDC* by OmpR, and a downregulation of *ompR* by Hfq (Kakoschke et al., 2014 & Raczkowska et al., 2010). The downregulation of putative T3SS genes (*ysaU, ysaH*) and T2SS (*outE*) in the mutant could also be of interest. The YSA secretion system was linked in different studies to colonization of the host and systemic infection (Bent et al., 2013 & Bent et al., 2015).

Some of the transporters downregulated in the mutant are putative drug resistance efflux pumps (*emrD, ydhC*). Additionally, Hfq-regulated genes involved in cell envelope synthesis could be antibiotic targets, like the penicillin-binding protein (*pbpA/mrdA*). While in *E. coli* loss of *hfq* was associated with increased drug sensitivity, this has so far not been the case in *Y. enterocolitica* (Yamada et al., 2010 & Kakoschke et al., 2014).

Apart from OMPs we also saw changes in lipoproteins and LPS altering proteins, which could potentially have a great impact on the bacterial surface. NlpD for instance, which was downregulated at 37°C in the mutant in our study, was linked in *Y. pestis* to the translocation of folded proteins across the membrane, iron acquisition, and was found to be an important virulence factor for the development of the plague (Tidhar et al., 2009; Tidhar et al., 2019).

4.4 Regulation of the bacterial stress response

Acid resistance

Many genes involved in acid resistance were expressed at a lower level in the *hfq* mutant in this study, suggesting that they are promoted by Hfq, especially at 37°C, the temperature of the mammalian host. This observation is in accordance with previous studies, that showed decreased resistance to acid stress in *Y. enterocolitica hfq* mutants (Kakoschke et al., 2014). In this study, transcripts promoted by Hfq encoded the chaperone HdeB, the glutamate decarboxylase GadA, the putative acid shock resistance protein Asr, the HdeD family acid-resistance protein YE3696. We also saw that Hfq promotes expression of several genes important for urease production: the urease subunit gene *ureC* and the urease accessory protein genes *ureD, ureF* and *ureG*. These results are consistent with those of Kakoschke et al. (2014), who showed that Hfq promoted *ureB* expression. The authors hypothesized, that Hfq either increased

ureABC transcript stability directly, since it carries an Hfq-binding sequence, or indirectly through its influence on OmpR, which controls urease production in *Y. pseudotuberculosis* (Hu et al., 2009). Nieckarz et al. (2020) showed recently, that the effect of OmpR differs between biotypes. While in 2/O:9 strains urease was promoted by OmpR, this was not the case in 1B/O:8 strains, which we used in this study. However, OmpR still provided acid resistance for 1B/O:8 strains at 37°C through so far unknown mechanisms. Furthermore, Nieckarz et al. (2020) found an AraC-like transcriptional regulator in *Y. enterocolitica*, UreR, that activates *ure* in other species. While OmpR upregulated UreR in serotype O:9, it downregulated UreR in serotype O:8. In our transcriptomic analysis, we did not see any significant changes between wildtype and *hfq* mutant in *ompR* expression, nor in expression of the new transcriptional regulator UreR/YE2527, which does not suggest that Hfq influences the urease genes through any of these regulatory proteins. The gene cluster is sorted into three operons, *ureABC*, *ureEF* and *ureGD*. It is noteworthy, that many genes in the *ure* gene cluster were slightly downregulated in the *hfq* mutant, including genes downstream of urease and its accessory proteins. Therefore, Hfq might facilitate transcription of the entire region. However, these genes were not always influenced to the same extent by Hfq. This on the other hand could indicate that Hfq directly interacts with single mRNAs. It is possible that Hfq could act on *ure* genes through both mechanisms.

Oxidative stress resistance

At least two genes involved in oxidative stress resistance were promoted by Hfq, *ahpC* and *katA*. Both were carrying an Hfq-binding sequence and hence are candidates for a direct regulation through interaction with Hfq. Furthermore, we saw a slight downregulation of the superoxide dismutases *sodA* and *sodB* in the *hfq* mutant. Loss of Hfq was associated with diminished resistance to oxidative stress in several bacterial species, including *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Kakoschke et al., 2014; Geng et al., 2009; Schiano et al., 2010). In *Y. enterocolitica*, increased sensitivity of the *hfq* mutant to oxidative stress was associated with decreased amounts of AhpC (Kakoschke et al., 2014), while in *Y. pestis*, it correlated with a decreased transcription of *katA* (Geng et al., 2009).

Heat shock resistance

Transcripts encoding many chaperones involved in heat shock resistance were downregulated in the *hfq* mutant at 37°C, including *dnaK*, *dnaJ*, *groEL*, *groES* and *htpG*. Interestingly, in a proteomic study, loss of Hfq lead to an increase in HtpG protein abundance at 37°C (Kakoschke et al., 2014). Therefore, Hfq might promote the degradation of HtpG on the protein level. Loss of Hfq lead to decreased resistance to heat in *Y. pestis* (Geng et al., 2009). Surprisingly though, mRNA levels of many heat shock chaperones were upregulated in the *hfq* mutant (Geng et al., 2009). These results show, although Hfq is crucial for resistance to heat, loss of *hfq* does not always correlate with a decrease in heat shock chaperones, and levels of heat shock chaperones do not necessarily correlate with sensitivity to heat, suggesting additional crucial mechanisms. A study in *Klebsiella pneumoniae* found that loss of *rpoE* produced the same results as loss of *hfq* with respect to heat resistance, whereas for instance resistance to oxidative stress was preserved. This led the authors to suggest that the observed loss of heat resistance was due to the decreased RpoE levels in *hfq* mutants (Chiang et al., 2011). Since RpoE regulates many stress resistance genes in *Y. enterocolitica* as well, it is worth taking a closer look at regulatory genes like RpoE and other ESRs.

The interplay of Hfq and the RpoE envelope stress response

There was no strong differential expression for many ESR except RpoE. Before this dissertation it was already observed that the ESR sigma factor RpoE plays a crucial role in Hfq-mediated changes in several Gram-negative bacteria. Kulesus et al. noted that many phenotypes of *rpoE* and *hfq* mutants were similar in *E. coli* (Kulesus et al., 2008). Ding et al. found that *rpoE* controls about half of all genes that are downregulated by Hfq in *Salmonella* (Ding et al., 2004) and that loss of *hfq* leads to an increase in transcription of RpoE-dependent genes (Figueroa-Bossi et al., 2006; Vogt and Raivio, 2014). However, the exact nature of the relationship of these two genes was not clear. It is possible that *rpoE* was upregulated because of envelope stress caused by the loss of *hfq*, but it is also possible that there is a direct interaction between them.

In this study, *rpoE* was more abundant at 27°C than at 37°C in the Northern blots, although the deep sequencing analysis showed more reads for *rpoE* at 37°C. However,

other studies have already shown a decrease of *Y. enterocolitica rpoE* upon host temperature (Heusipp et al., 2003), consistent with our Northern blots.

Our transcriptomic data found that *rpoE* was more abundant in the *hfq* mutant than in the wild-type at 37°C. In another dissertation from our lab, Zeuzem (2018) could show, using gfp-reporter fusions and quantitative RT-PCR, that *hfq* mutants have an increase in expression of *rpoE* and of *fkpA* and *degP*, two RpoE-dependent genes*,* as well as a decrease in expression of *ppiA.* In this study, we saw similar changes for *rpoE*, *fkpA* and *ppiA* at 37°C. While we did see the same increase for *degP,* with a p of ~0.01 it did not reach our significance threshold of $p < 0.001$. Hfq might influence *degP* translation rather than mRNA abundance, or possibly these results are due to low expression of *degP* in this study.

In *E. coli* and *Salmonella,* it was shown that RpoE induces expression of the sRNAs RybB and MicA, which repress the translation of several OMP (Johansen et al., 2006; Papenfort et al., 2006). This reduces the number of OMP precursors in the periplasm and alleviates the stress put on the periplasmic chaperones (Vogt and Raivio, 2014). Since Hfq provides stability and supports their function, loss of *hfq* leads to decreased stability of sRNAs like RybB and MicA. The resulting increase in OMP production could overwhelm the protein folding chaperons in the periplasmic space, and ultimately lead to increased envelope stress in *hfq*-mutants (Vogt and Raivio, 2014).

In this study we could show, that (i) *hfq* mutants have a higher expression of genes induced by the RpoE-ESR, like *fkpA*, *rseA*, *rseB* and *rpoE*, confirming previous results (Zeuzem, 2018). Additionally, our co-IPs with an Hfq-FLAG strain indicate that (ii) Hfq directly interacts with the mRNA of *rseA* and *rseB*. For both of them, the in-silico analysis had shown a possible Hfq binding motive. Unfortunately, attempts to discover interactions between *rpoE* mRNA and Hfq were unsuccessful due to very low *rpoE* transcripts. Northern blots with *rpoE* did not show sufficient quality to draw conclusions (data not shown).

The fact that *rseA* and *rseB* bind to Hfq show that a direct interaction between the transcript encoding the RpoE sigma factor and Hfq is very likely. It is possible that Hfq binds to the polycistronic transcript of the *rpoE-rseABC* operon and inhibits translation of *rpoE*, like it was suggested by Figueroa-Bossi et al. (2006). It is also possible that Hfq

binds to *rseA* and *rseB* mRNA and stabilizes them and promotes their expression, which would increase the signal threshold at which RpoE is freed and active. This would explain why we see an increase in RpoE-regulated genes in *hfq* mutants. However, these two hypotheses are not mutually exclusive. It is possible that *rpoE* directly interacts with Hfq, and at the same time loss of Hfq indirectly causes envelope stress, which then activates the RpoE-ESR.

4.5 Regulation of the bacterial metabolism

Carbon metabolism

While there was conflicting evidence for the regulation of glycolysis/gluconeogenesis and glycogenesis/glycogenolysis with antagonistic enzymes being regulated in the same way (pps/fba, glgB/glgP), the TCA cycle seems to be overall upregulated by Hfq, while antagonistic enzymes involved in anaerobic citrate metabolism were downregulated. This is a surprising finding since other studies have found that Hfq repressed glycolysis and the TCA cycle, e.g. in *N. meningitidis*, or downregulated the carbon metabolism in general in *Y. enterocolitica* (Huis In ‘t Veld et al., 2017; Kakoschke et al., 2014).

With all the observed changes in carbon metabolism, the question remains how Hfq influences them. Only few of them, like *citF*, have a putative Hfq-binding sequence. This of course, does not prove that there is no direct interaction with the other genes, however the observed changes might as well be mediated through a transcription factor or other regulatory genes. Interestingly, *cra* (catabolite repressor activator), which encodes a transcriptional regulator for carbon metabolism in *E. coli*, was upregulated at both temperatures in the *hfq* mutant in this study (Kim et al., 2018; Shimada et al., 2011). Some of the genes that are known to be part of the *cra* regulon in *E. coli* were differently expressed in our study as well (*aceF*, *fbaA*, *nirBD*, *pps*, *cyoABC*), although they did not always correspond to the expected change, considering that *cra* was upregulated in the mutant (e.g. *cyoABC* were downregulated in the mutant, although their expression is promoted by *cra* in *E. coli* (Kim et al., 2018)). It Is worth noting, that Cra was shown to attenuate acid resistance in *Y. pseudotuberculosis* (Hu et al., 2011). If this is the case for *Y. enterocolitica* as well is unknown. Another carbon metabolism regulator, CRP (cAMP receptor protein), which noteably affects the TCA in *E. coli* (Kim et al., 2018) did not show any significant changes in our study.

Fatty acid metabolism

Before this study, Kakoschke et al. (2014) had shown that Hfq promotes lipid metabolism and transport. Two genes involved in beta-oxidation of fatty acids were downregulated in the mutant, suggesting that Hfq activates this pathway. At the same time, *fadR*, which encodes a transcription regulator conserved in gammaproteobacteria (Cronan, 2020) was slightly but significantly upregulated. In a very recent study on *V. cholerae* Huber et al. (2020) used a similar approach with RNA co-IP with an Hfq-FLAG strain and deep sequencing. They showed that *fadE* was downregulated by Hfq and the sRNA FarS. FarS is derived from the 3'-UTR of the fatty acid synthesis gene *fabB* which in turn is under the control of FadR (Huber et al., 2020). Whether similar regulatory patterns exist in *Y. enterocolitica* would certainly be an interesting subject for future research.

Nitrogen metabolism

Previously we knew that Hfq has an overall negative effect on nitrogen metabolism (Kakoschke et al., 2014). Ornithine decarboxylase activity, the OppA peptide transporter and the tryptophanase TnaA were increased. In this study we did not see corresponding changes on the transcriptional level. The sRNA GcvB, which represses *oppA* expression in *E. coli* and *S. typhimurium* (Urbanowski et al., 2000; Sharma et al., 2007) did not show any differences between wildtype and mutant as well. This is in contrast to findings in *Y. enterocolitica* O:3, in which *gcvB* was downregulated at 37°C in the mutant (Leskinen et al., 2017). Alongside some aminoacid and peptide transporters, we found the asparagine synthetase *asnA* to be upregulated in the mutant at 27°C and 37°C, which also carries a putative Hfq-binding sequence. AsnA produces asparagine using ammonia. It is interesting that urease also seems to be upregulated by Hfq, which hydrolyzes urea into carbon dioxide and ammonia, which would – apart from raising the pH in the vicinity, which was discussed earlier – provide additional substrates for the asparagine synthetase. Together with the downregulation of nitrate and nitrite reductases, this could counteract an accumulation of toxic levels of ammonia and also keep the urease active by removing its product, as to not reach an equilibrium.

Cell energetics

A considerable number of genes involved in oxidative phosphorylation are downregulated in the mutant, consistent with the proteomic study by Kakoschke et al. (2014), who found AtpD to be less abundant in the mutant strain. The same has been found to be the case in *E. coli* (Guisbert et al., 2006). As Guisbert et al. already mentioned, this could either be a direct effect of Hfq or an indirect effect, owing to the reduced growth rate in *hfq* mutants. *Vibrio alginolyticus hfq* mutants show a growth defect as well, while genes of the electron transfer chain are upregulated in the mutant, along with genes of the TCA cycle (Kakoschke et al., 2014; Deng et al., 2016), suggesting that slowed growth rate does not necessarily coincide with similar changes in transcripts encoding components of the electron transfer chain.

4.6 sRNAs

Twelve sRNAs had a different abundance in the *hfq* mutant as compared to the wild-type strain, nearly all of them were less abundant in the mutant. As mentioned before, the difference in abundance could be either from interaction with Hfq, which protects them from degradation, or from Hfq mediated changes in their transcription. Alternatively, induction of RNases in the *hfq* mutant could also lead to these changes.

Interestingly we saw a downregulation of the CsrA-sequestering sRNAs CsrB and CsrC in the mutant, while *csrD*, which is involved in the downregulation of said sRNAs, was upregulated. We did not see any changes in mRNA levels of *csrA*. However, given that CsrB and CsrC interact with the protein CsrA and sequester it, the observed changes could indeed mean that Hfq attenuates CsrA activity. Other studies showed no interaction between Hfq and CsrB/CsrC in *E. coli* and *Salmonella* (Zhang et al., 2003 and Sittka et al., 2008). Although this might be different in *Y. enterocolitica*, there is also the possibility that Hfq does not directly interact with these sRNAs and that the observed changes are mediated through downregulation of *csrD*. Furthermore, in *L. pneumophilia*, transcript levels of *csrA* were reduced in an *hfq* mutant (McNealy et al., 2005), an effect we did not observe. The influence of CsrA on Hfq has also been studied. It has been reported, that in *E. coli,* CsrA binds *hfq* mRNA and inhibits its translation as well as *hfq* transcription (Baker et al., 2007).

Many of the putative mRNA targets found through in-silico analysis were differentially expressed in our transcriptomic analysis. Additionally, some genes carried on the virulence plasmid pYV were predicted to bind to the analyzed sRNAs, especially to MicF. Unfortunately, loss of the plasmid in one of the cultures prevented us from studying these genes in our transcriptomic data. We noticed that some mRNAs are predicted to interact with several sRNAs (e.g. *rpoS*). This raised the question if they would bind to a common region on the mRNA, however the predicted target regions differ between sRNAs. The in-silico results should always be taken with a pinch of salt, as long as they are not confirmed by experimental data. However, they can be the starting point for future research on the subject. Especially genes that additionally show an Hfq-binding sequence in the in-silico analysis are promising candidates for further studies. For the detection of sRNAs it would be preferable to turn to radioactive probes for Northern blots as they are more sensitive than the digoxigenin labelled probes used in this study.

4.7 Hfq regulation of transcriptional regulators: One gene to rule them all?

The impact of Hfq on gene expression could partially be explained by its effect on transcription and transcriptional regulators. This was shown to be the case in other species. However, extrapolation from other species has some limitations. Transcriptional regulons are not always similar between *Yersinia* species. For instance, the transcriptional regulator RovA is a determinant of virulence in both *Y. pestis* and *Y. enterocolitica.* In *Y. enterocolitica*, RovA positively regulates *invA* (Kakoschke et al., 2016). While *invA* is not a functional gene in *Y. pestis*, *rovA* promotes expression of the *psa* genes, which are important for the development of the bubonic plague (Cathelyn et al., 2006). Even between serotypes of the same species, there can be major differences between transcriptional regulons. This is for example the case between the RovA-mediated expression of *invA*. While in *Y. enterocolitica* O:3 *invA* is expressed at 25°C as well as 37°C, in serotype O:8 *invA* is only expressed at 25°C, due to H-NS mediated repression of its activator *rovA* (Uliczka et al., 2011). Furthermore, while OmpR upregulates urease in *Y. enterocolitica* O:9, it does not do so in serotype O:8 (Nieckarz et al., 2020). In a recent analysis comparing transcriptomes of *Y. enterocolitica* O:3 and

O:8 found major differences between the two serotypes. Among many other genes, *invA* transcripts were more abundant in O:3, which was partly caused by a higher production of its activator RovA, while *ail* transcripts were far more abundant in O:8 (Schmühl et al., 2019).

Leskinen et al. (2017) found that in *Y. enterocolitica* O:3 *rovM* and *ompR* were upregulated, while *phoB*, *rpoS* and *rovA* were downregulated in the *hfq* mutant. In this study conducted with *Y. enterocolitica* O:8, *phoB* and *rpoS* were also downregulated in the mutant, while *ompR* and *rovA* showed no significant changes. Interestingly, *rovM* was downregulated in the mutant, indicating a significant difference in the effects of Hfq between these two serotypes. Leskinen et al. (2017) also describe that the downregulation of *rovA* and *rpoS* is caused by the overexpression of *rovM* in the mutant. This might explain why we do not see any changes in *rovA* and only a slight downregulation of *rpoS.*

Many genes known to be in the regulon of RpoS were downregulated in the *hfq* mutant, along with *rpoS* itself. Since the transcriptional regulator *rovM* was not significantly changed in our study, this could indicate, as Leskinen et al. (2017) already noted, that additionally to RovM-mediated control, Hfq also promotes *rpoS* expression directly, for instance through enhanced translation. The sRNA RprA which is thought to positively affect translation of RpoS, was less abundant in the mutant as well. In *E. coli* the regulation of *rpoS* is mostly believed to be translational (Updegrove et al., 2008). The observed Hfq associated increase in *rpoS* mRNA and RprA could therefore reflect an improved stability of RprA by binding to Hfq and an enhanced translation and stability of *rpoS* mRNA either by binding to Hfq as well or by independently binding to RprA, like it was observed with DsrA in *E. coli* (Kim et al., 2019). It is also possible, that Hfq upregulates *rpoS* on the transcriptional level, since *nlpD*, the gene upstream of *rpoS*, was equally downregulated at 37°C in the mutant.
Using transcriptional fusions, Kakoschke et al. (2016) showed that in *Y. enterocolitica* O:8 *rovA* is downregulated in the *hfq* mutant, however only about 30%. Therefore, it is not surprising that it is not significantly changed in the transcriptomic analysis.

While we did not see significant changes in *ompR* RNA, Kakoschke et al. (2016) already showed that in fact *ompR* is upregulated in the *hfq* mutant in *Y. enterocolitica* O:8. We therefore compared our transcriptomics to the genes that are regulated by OmpR in *Y. enterocolitica* O:9 (Nieckarz et al., 2020). About half of them appeared in our transcriptional analysis. However, only few (*groEL*, *htpG*) were influenced in the same direction as in serotype O:9 (assuming that OmpR was more abundant in the mutant on the protein level in our study and/or activated) while most of them were either only slightly changed or inversely correlated (e.g. *dkgA*, *clpB*) to the observed change in serotype O:9 (Nieckarz et al., 2020).

For *phoB,* which was downregulated in the mutant, we found – similar to Leskinen et al. (2017) - only few genes that were affected and the data was conflicting. While *phoH* was downregulated, corresponding to the downregulation of *phoB*, *phnHFGI* were upregulated in the mutant.

It remains unclear why *rovM* was downregulated in the *hfq* mutant in our study. Most genes that Leskinen et al. (2017) found to be under the control of RovM were in our study not significantly changed (e.g. *rovA, glk, srlAB*) or inversely correlated with a downregulated *rovM* (e.g. *ompX*), so that the data remains inconclusive. In *Y. pseudotuberculosis rovM* was more induced during growth on minimal media, as opposed to LB medium (Heroven and Dersch, 2006). Since we only saw a relatively low abundance of *rovM* mRNA*,* further studies during growth on *rovM* inducing medium would be an interesting approach. Additionally, proteomic studies should be made in the future.

4.8 Translation

While previously being considered background noise due to it being the most abundant type of RNA, rRNA has come into focus in recent years. Several studies suggested an interaction between Hfq and rRNA (Andrade et al., 2018). Hfq could be shown to be important for processing and folding of 16S rRNA, as well as for biogenesis of the small ribosomal subunit. In *E. coli*, inactivation of Hfq not only led to a reduction in the total number of assembled and functioning ribosomes, but it also resulted in the formation of defective ribosomes and accumulation of translation errors. The interaction of Hfq with

rRNA is independent of the sRNA mediated regulation. The rRNA seems to interact directly with the distal face of Hfq (Dos Santos et al. 2019).
We found that two 23S rRNAs (downregulated at 27°C) and one 16S rRNAs (upregulated at both temperatures) were affected by the deletion of *hfq*. Additionally, some ribosomal proteins were found to be under the influence of Hfq and were predominantly downregulated in the mutant. Many more rRNAs than the one found in this study might be affected. Since Hfq is involved in the processing, folding and function of ribosome subunits, changes might not be apparent from the number of transcripts. In another study in *E. coli*, an accumulation of 17S rRNA, the precursor of 16S rRNA, was found along with reduced levels of 70S ribosomes (Andrade et al. 2018). However, the sequencing analysis in this study might not have picked up present 17S rRNA but instead counted it as 16S rRNA. To safely make this distinction, probes for Northern blots could be created that reliably detect 17S rRNA, like it was done by Andrade et al. (2018).

We found significant fold changes in five tRNAs. In a similar study in *E. coli,* tRNA, tRNA precursors and intergenic regions between tRNA genes were co-immunoprecipitated with Hfq. The authors proposed Hfq could be involved in tRNA biogenesis (Bilusic et al. 2014). In another study Hfq was found to bind to the 3' external transcribed spacer of tRNA in *E. coli*. It was suggested that those and other tRNA derived fragments act as sRNA sponges, creating a threshold and reducing sRNA noise (Lalaouna et al., 2015). We did not test the enrichment of similar regions through co-IP and intergenic regions would not have shown up in the transcriptional analysis, so that additional studies are necessary to investigate the role of tRNAs in sRNA regulation and the interaction with Hfq.

Altogether it seems that genes involved in translation, especially ribosomal genes, are mostly downregulated in the mutant. Thus, overall Hfq seems to promote translation in *Y. enterocolitica* O:8, which confirms earlier studies (Kakoschke et al. 2014). This might enhance its transcriptional and post-transcriptional effects and fits with the idea that Hfq plays a role in quickly changing gene expression.

4.9 Limitation of results and future necessary experiments

The data shown provides an overview over possible relationships between Hfq and mRNAs and describes some distinct genes in greater detail. However, to get a complete picture of the role of Hfq in *Y. enterocolitica* serotype O:8, additional studies are warranted.

Subsequent functional studies are necessary to truly understand the influence of Hfq. Since the regulation of Hfq is often post-transcriptional, transcript levels do not correlate necessarily with protein levels. Kakoschke et al. (2016) showed for instance, that expression of *yadA* was increased in an Hfq mutant, but protein levels were nevertheless decreased. The authors suggested that YadA was processed by an Hfq-dependent protease. Leskinen et al. (2017) too showed that transcriptomics and proteomics are not necessarily congruent. At room temperature, many of the affected genes were downregulated in transcriptomics, while being upregulated in proteomic studies. Other proteins, that had significantly changed in the *hfq* mutant strain showed no change at all in transcriptomics, proving that as a posttranscriptional regulator, effects of Hfq cannot solely be determined by mRNA levels.

Finally, to connect sRNAs to their mRNA targets, it is not sufficient to show that they bind to Hfq. It would also be interesting to see, that they bind each other. Through conventional co-immunoprecipitation however, it is only possible to enrich single RNAs, it is not possible to directly precipitate mRNA-sRNA pairs. For this, confirmation could be done by mutational analysis of sRNA and compensatory mutation in the target mRNA, or other techniques could be used, like the recently developed RIL-seq (RNA interaction by ligation and sequencing) which includes UV-crosslinking RNA pairs before immunoprecipitation (Melamed et., al, 2016).

Ultimately, this study together with future research can provide a thorough understanding of the regulation of virulence genes in Gram-negative bacteria. These findings, with Hfq as a key player of pathogenesis and all its binding partners, can then be the basis for the development of new antimicrobial agents. There is an ongoing rise of antibiotic resistance and further innovations are sorely needed.

5 Summary

Yersinia enterocolitica is a species of Gram-negative bacteria, which can cause gastrointestinal symptoms or even severe systemic infections. They are close relatives of *Y. pseudotuberculosis* and *Y. pestis* and share many virulence factors with other Gram-negative bacteria. Virulent bacteria possess an arsenal of 'weapons', allowing them to adapt to different environments and overcome their host's defence mechanisms. In recent years, it has become apparent, that these virulence factors are meticulously regulated. When presented with a new environment, bacteria not only transcribe a different set of genes, but they also manipulate already existing mRNA. This post-transcriptional regulation has turned out to be crucial for a quick adaptation and therefore determines if bacteria can successfully infect their host. As an RNA chaperone, Hfq is a central hub of post-transcriptional regulation. It facilitates interaction between mRNAs and regulatory sRNAs, which determine if an mRNA is either degraded or quickly translated into proteins. In this study, we investigated the role of Hfq in the regulation of genes in *Y. enterocolitica* O:8. In the first part, we analyzed differences in the abundance of mRNAs between a wild-type and an *hfq* mutant at different temperatures by whole transcriptome sequencing. We saw that 8% of genes were regulated by Hfq, affecting mostly the cell envelope, resistance to stress and the metabolism. We confirmed these changes for a subset of genes, using Northern blots. In the second part, we examined whether the divergences in gene regulation we found had occured as a direct result of interaction between Hfq and mRNA. We used a strain with an Hfq that carries a FLAG-tag, allowing us to isolate Hfq with an antibody and co-immunoprecipitate any RNA molecules bound by Hfq. Here we saw that Hfq binds to the mRNAs encoding the outer membrane proteins OmpX and Ail, the envelope stress response proteins RseA and RseB, as well as to its own *hfq* mRNA.

In summary, we show that Hfq has a profound effect on gene expression and binds directly to several mRNAs in *Y. enterocolitica*. Hfq occupies a central position in the regulation of virulence in various bacterial species. This makes Hfq an attractive target for antimicrobial drugs. Innovations are sorely needed in the field of microbiology in order to combat the ongoing rise of antimicrobial resistance. This study, together with

further research in this area can help us understand the exact role of Hfq which can ultimately lay the foundation for the development of new antibiotics.

Zusammenfassung

Yersinia enterocolitica ist eine Spezies Gram-negativer Bakterien, die gastrointestinale Symptome bis hin zu schweren systemischen Infektionen verursacht. Sie sind enge Verwandte von *Y. pseudotuberculosis* und *Y. pestis* und haben viele gemeinsame Virulenzfaktoren mit anderen Gram-negativen Bakterien. Virulente Bakterien besitzen ein regelrechtes „Waffenarsenal“, das es ihnen erlaubt sich an verschiedene Umweltbedingungen anzupassen und die Abwehrmechanismen ihrer Wirte zu überwinden. Solche Virulenzfaktoren werden durch teils komplexe Mechanismen reguliert. Wenn Bakterien mit einer neuen Umgebung konfrontiert sind, transkribieren sie nicht nur andere DNA-Abschnitte, sie verändern auch bereits bestehende mRNA. Diese post-transkriptionelle Regulation ist entscheidend für eine schnelle Anpassung und ausschlaggebend dafür, ob Bakterien einen Wirt infizieren können. Das RNA-Chaperon Hfq ist ein zentraler Knotenpunkt der post-transkriptionellen Regulation. Es vereinfacht die Interaktion zwischen mRNA und regulatorischer sRNA, die an ihre mRNA-Partner bindet und dadurch entweder zu deren Abbau oder einer schnelleren Translation führt.

In dieser Arbeit wurde die Rolle von Hfq in der Genregulation in *Y. enterocolitica* O:8 untersucht. Im ersten Teil wurde das gesamte Transkriptom von Wildtyp und *hfq*-Mutante bei verschiedenen Temperaturen sequenziert und die Anzahl der mRNA-Kopien der einzelnen Gene verglichen. Wir stellten fest, dass 8% der Gene von Hfq beeinflusst wurden. Die meisten der Gene standen im Zusammenhang mit der Zellhülle, Stressresistenz und dem Metabolismus. Durch Northern Blots bestätigten wir die Ergebnisse für einig ausgewählte Gene. Im zweiten Teil wurde untersucht, ob die beobachteten Veränderungen der Genregulation auf direkter Interaktion zwischen Hfq und mRNA beruhen. Wir benutzten einen Bakterienstamm mit einem Hfq-FLAG, wodurch wir Hfq und daran bindende RNA-Moleküle mittels Co-Immunpräzipitation isolieren konnten. Es zeigte sich, dass Hfq an die mRNA der äußeren Membranproteine OmpX und Ail, der Zellhüllstress-Regulatoren RseA und RseB sowie an seine eigene *hfq* mRNA bindet.

5 Summary

Zusammenfassend konnte gezeigt werden, dass Hfq einen tiefgreifenden Einfluss auf die Genexpression von *Y. enterocolitica* hat und einige mRNAs direkt bindet. Da Hfq in zahlreichen bakteriellen Spezies ein zentraler Regulator virulenter Eigenschaften ist, stellt es einen vielversprechenden Angriffspunkt für die Entwicklung neuer antibiotischer Therapeutika dar. Innovationen in der Mikrobiologie sind dringend notwendig, um die Zunahme antibiotischer Resistenzen zu bekämpfen. Die vorliegende Arbeit zusammen mit weiterer Forschung kann dabei helfen die genaue Rolle von Hfq zu verstehen und letztlich die Grundlage für die Entwicklung neuer Antibiotika sein.

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9 Abbreviations

1,2-PD	1,2-propanediol
3’-UTR	3’ untranslated region
5’-UTR	5’ untranslated region
µl	micro liter
A	Adenosine
Ahp	Alkyl hydroperoxide reductase
Ail	Attachment invasion locus
AP	Alkaline phosphatase
asRNA	anti-sense RNA
Bae	Bacterial adaptive response
Bfr	bacterioferritin
Cas	CRISPR associated proteins
cGMP	cyclic guanosine monophosphate
co-IP	Co-Immunoprecipitation
Cpx	conjugative plasmid expression
CRISPR	clustered regularly interspaced short palindromic regions
Csp	Cold shock protein
CTR	C-terminal region
DEPC	Diethyl pyrocarbonate
DIC	Disseminated Intravasal Coagulation
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA

9 Abbreviations

ECM	extracellular matrix
e.g.	Exempli gratia – for example
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESR	Envelope stress response
ETEC	Enterotoxigenic *Escherichia coli*
FDR	false discovery rate
Fe	iron
Ftn	ferritin
Fur	ferric uptake regulator
GABA	γ-aminobutyric acid
Gfp	green fluorescent protein
GO	gene ontology
h	Hour(s)
Hfq	host factor required for bacteriophage Qβ in *E. coli*
HPI	high pathogenicity island
HRP	Horse radish peroxidase
Hsp	heat shock protein
i.a.	inter alia, among others
i.g.	intragastric
IL	interleukin
IMP	inner membrane protein
inv	invasion
i.p.	intraperitoneal
kDa	kilo Dalton

9 Abbreviations

l	liter
LB	Lysogeny broth
LPS	lipopolysaccharide
MALT	Mucosa-associated lymphatic tissue
MDR	Multidrug-resistant
MFS	Major facilitator superfamily
min	Minute(+s)
ml	Milliliter
mRNA	Messenger RNA
N	Any base (purine or pyrimidine)
ncRNA	non-coding RNA
Nt	nucleotides
OD600	Optical density at a wavelength of 600 nm
OMP	Outer membrane protein
PCR	Polymerase chain reaction
Pdu	Propanediol utilization
PNPase	Polynucleotide phosphorylase
Psp	Phage shock protein
PTS	Phosphotransferase system
R	Purine
RBS	ribosome binding site
Rcs	regulation of capsular synthesis
RIP	regulated intramembrane proteolysis
RNA	Ribonucleic acid
RNAP	RNA Polymerase
ROS	reactive oxygen species
Rov	regulator of virulence

RPKM	reads per kilobase transcript length per million mapped reads
RpoE	extracytoplasmic RNA polymerase sigma factor
rpm	Rounds per minute
rut	Rho utilization site
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SOD	Superoxide dismutase
sRNA	Small RNA
T2SS	type II secretion system
T3SS	type III secretion system
TCA	Tricarboxylic acid cycle
TCS	Two-component system
T_m	melting temperature
TNF	Tumour necrosis factor
V	Volt
wt	wild-type
Y	Pyrimidine
Ybt	yersiniabactin
Yop	Yersinia outer protein
Ysa	Yersinia secretion apparatus

10 Appendix

Table 13) List of genes under the influence of Hfq in Y. enterocolitica

Gene ID	Gene name	Region	fold change 37°C	p-value 37°C	fold change 27°C	p-value 27°C	no. of ARN repeats [3]	proteomics [4]
4716609	rseB	1131773..1132729	4,69	0,0000	2,72	0,0010	4	
4715251	trkD	6708..8435	3,23	0,0000	2,32	0,0003	4	
4713479	YdhC	2360403..2361620	-2,28	0,0000	-2,23	0,0003	4	
4715716	ygiW	complement(1323318..1323731)	-2,49	0,0000	-2,17	0,0004	4	
4714193	YE2966	3227908..3229359	-2,54	0,0000	-3,32	0,0000	6	
4713282	asnA	1449..2441	-2,73	0,0000	-4,19	0,0007	5	
4713087	hfq	446155..446460	-306,00	0,0000	-4192,14	0,0000	4	
4715084	YE2376	complement(2563680..2564231)	3,75	0,0000	1,71	0,0479	4	
4713726	citF	complement(2866683..2868200)	3,59	0,0000	1,24	0,4827	4	
4716608	mclA	1131117..1131773	3,31	0,0000	1,96	0,0204	4	
4714046	YE3648	3975920..3976582	2,36	0,0000	1,55	0,0506	5	
4715884	sfs1	complement(840914..841639)	2,35	0,0000	1,31	0,2759	4	
4713154	outJ	complement(3657072..3657665)	2,25	0,0002	-1,07	0,9021	4	
4714097	fadH	4037692..4039713	2,21	0,0000	-1,02	0,9366	5	
4716142	YE0792	916006..917181	2,11	0,0001	1,86	0,0679	4	
4714546	ybbJ	3324732..3325181	2,02	0,0000	-1,10	0,6523	4	
4715188	emrD	105845..107029	-2,04	0,0000	-1,15	0,6292	4	
4714347	tdcC	400972..402300	-2,24	0,0000	-3,28	0,0014	5	
4713518	pspC	complement(2321309..2321659)	-2,51	0,0001	-1,13	0,6535	4	
4713573	YE2705	complement(2942187..2942789)	-2,62	0,0006	-1,61	0,2011	4	
4714759	rplP	complement(4265189..4265599)	-2,73	0,0000	-1,30	0,3087	4	
4715229	ahpC	3444333..3444935	-3,39	0,0000	-2,24	0,0022	4	- at 37°C [1]
4716476	YE0932	complement(1060412..1060720)	-3,50	0,0000	-1,59	0,0783	5	
4713559	YE2691	complement(2923697..2924221)	-3,56	0,0006	-1,84	0,1700	4	
4715818	YE0441	513550..513822	-3,80	0,0000	-2,01	0,0562	5	
4715438	ybaY	complement(3388778..3389308)	-5,90	0,0000	-1,60	0,0582	4	
4716300	YE0863	988741..989850	1,09	0,6033	2,40	0,0003	4	
4715693	amyA	2141101..2142600	-1,77	0,0000	-2,07	0,0003	4	
4713690	YE2885	3137850..3138482	-1,81	0,0008	-2,10	0,0007	4	
4713405	b4376	650433..651047	-1,20	0,2013	-2,58	0,0001	4	
4716031	pduK	2972099..2972701	-1,02	0,9026	-3,31	0,0000	4	
4714551	YE3063	3329766..3330245	-1,40	0,0049	-3,48	0,0000	4	
4716306	gpN_1	995092..996258	7,83	0,0000	3,40	0,0000		
4716301	YE0864	989892..990536	7,63	0,0000	2,90	0,0000		
4714774	YE3931	complement(4275511..4275798)	4,40	0,0000	3,68	0,0000		
4716378	yfiA	complement(1012660..1013022)	3,96	0,0000	3,34	0,0001		
4712813	YE2497	complement(2688504..2690441)	3,72	0,0000	3,73	0,0002		
4714234	YE3001	3271878..3272348	3,52	0,0000	2,72	0,0000		
4713446	cra	764279..765283	3,29	0,0000	2,18	0,0005		
4714667	ddg	4173848..4174768	3,25	0,0000	2,53	0,0001		
4714665	YE3827	4172077..4173489	3,22	0,0000	4,44	0,0000		
4715947	phnH	complement(551600..552181)	2,75	0,0006	2,92	0,0006		
4713088	hflX	446559..447860	2,62	0,0000	2,63	0,0003		
4716283	YE0848	complement(974471..975814)	2,30	0,0000	2,43	0,0006		
4715965	YE0495	569932..570363	2,28	0,0000	2,54	0,0000		
4713110	YE0402	468378..468581	2,14	0,0000	2,80	0,0002		

4712532	YE2494	complement(2685062..2686354)	2,08	0,0000	3,48	0,0000
4714691	YE3853	4203328..4203837	2,07	0,0000	3,08	0,0000
4713345	YEr018	complement(3550757..3552245)	2,03	0,0000	3,09	0,0001
4716180	cysG_2	4312558..4313976	2,01	0,0000	3,35	0,0007
4716620	gntP	1141652..1142995	2,01	0,0000	3,92	0,0000
4713193	YE0515	585621..586928	-2,00	0,0000	-2,74	0,0000
4714481	YEt038	1573270..1573357	-2,02	0,0001	-2,45	0,0007
4714095	YE3696	4035918..4036490	-2,03	0,0000	-3,60	0,0000
4716494	ureC	1076597..1078315	-2,07	0,0000	-3,81	0,0001
4713498	YEs024	2382205..2382313	-2,10	0,0000	-4,22	0,0000
4714241	ylaC	2794337..2794870	-2,15	0,0000	-3,64	0,0000
4713952	YE3558	3872952..3873437	-2,35	0,0000	-2,71	0,0001
4714561	fcl	complement(3344007..3344972)	-2,41	0,0000	-2,21	0,0002
4713480	cdfA	2361928..2363079	-2,50	0,0000	-3,61	0,0000
4714394	rplA	327597..328301	-2,50	0,0000	-2,44	0,0008
4715587	YE0246	282591..283550	-2,53	0,0000	-4,34	0,0000
4714181	YE3783	complement(4128862..4129638)	-2,64	0,0000	-2,41	0,0001
4714278	YE2635	complement(2852375..2853514)	-2,67	0,0000	-2,98	0,0000
4715460	YE2187	complement(2396113..2396457)	-2,76	0,0000	-2,76	0,0000
4715333	YE2329	2531679..2532017	-2,91	0,0000	-3,15	0,0000
4713805	fba	complement(3726210..3727289)	-3,02	0,0000	-2,86	0,0003
4713680	YE2875	complement(3127508..3128413)	-3,12	0,0000	-3,43	0,0000
4712535	iscA	1180285..1180608	-3,29	0,0000	-5,27	0,0000
4714854	YE2436	complement(2627161..2627433)	-3,31	0,0000	-5,66	0,0000
4712767	YE1730	complement(1929451..1929711)	-3,39	0,0000	-3,48	0,0000
4712600	YEs020	complement(1991547..1991664)	-3,40	0,0000	-8,59	0,0000
4716248	corE	complement(968548..969339)	-3,47	0,0000	-3,06	0,0000
4716445	YE4103	4491149..4491463	-3,76	0,0000	-6,35	0,0001
4716557	hslS	complement(4561809..4562282)	-3,92	0,0000	-2,59	0,0001
4715617	YE1259	complement(1403195..1403854)	-4,12	0,0000	-2,09	0,0005
4714564	wbcH	complement(3347156..3348004)	-4,33	0,0000	-2,67	0,0000
4715660	YE1929	2105632..2105910	-4,38	0,0000	-4,92	0,0000
4716501	hdeB_1	complement(1083904..1084236)	-4,45	0,0000	-4,04	0,0000
4714567	wbcE	complement(3349831..3350850)	-4,45	0,0000	-3,92	0,0000
4714369	ecnB	424699..424830	-4,62	0,0000	-3,19	0,0000
4715272	YEs002	31382..31582	-4,81	0,0000	-5,16	0,0000
4712601	YEs021	1991571..1991671	-5,04	0,0000	-9,75	0,0000
4715462	YE2189	complement(2398118..2399020)	-5,18	0,0000	-11,79	0,0000
4712446	yohJ	3030901..3031308	-5,52	0,0000	-3,38	0,0000
4714518	YE0254	293567..294097	-6,84	0,0000	-10,32	0,0000
4715270	YEs001	30017..30134	-7,47	0,0000	-13,42	0,0000
4714180	YE3782	complement(4128190..4128843)	-7,73	0,0000	-2,68	0,0001
4712831	yecS	complement(2710477..2711139)	-8,32	0,0000	-12,06	0,0000
4712636	yfgG	complement(1257091..1257282)	-9,76	0,0000	-10,64	0,0000
4715175	YE0084	92988..93986	-10,87	0,0000	-5,31	0,0000
4712893	ycfL	1897655..1898044	-16,26	0,0000	-33,65	0,0000
4713137	YEs034	complement(3638306..3638400)	-17,85	0,0000	-18,58	0,0000
4716477	asr_1	1061138..1061536	-23,03	0,0000	-15,75	0,0000
4714479	micF	complement(1571499..1571589)	-23,86	0,0000	-10,89	0,0000
4714550	YEs029	complement(3329500..3329583)	-60,41	0,0000	-55,51	0,0000
4713289	YEs017	complement(1660972..1661052)	-67,36	0,0000	-289,62	0,0000
4713727	citE	complement(2868204..2869100)	10,46	0,0000	2,05	0,0281
4713725	citX	complement(2866131..2866679)	8,73	0,0000	1,30	0,5156
4714775	YE3932	complement(4275818..4276183)	5,75	0,0000	2,34	0,0178
4713999	hybG	complement(3926179..3926463)	4,67	0,0000	3,11	0,0244

4713998	hypD	complement(3925089..3926192)	4,51	0,0000	2,30	0,0961
4712670	napF	1294605..1295108	4,26	0,0000	6,00	0,0015
4713593	cobD_1	complement(2959472..2960428)	4,10	0,0000	1,62	0,0738
4714001	hybF	complement(3927575..3927916)	4,08	0,0000	2,52	0,0510
4716583	YE0987	complement(1108214..1108765)	3,64	0,0000	1,43	0,1337
4715952	nrdG	complement(556986..557522)	3,56	0,0000	1,90	0,0861
4713728	citD	complement(2869097..2869390)	3,53	0,0000	2,53	0,0034
4713729	citC	complement(2869491..2870567)	3,52	0,0000	2,54	0,0029
4714809	znuA	complement(2576699..2577661)	3,44	0,0000	1,00	0,9978
4712674	napC	1298422..1299021	3,44	0,0000	3,93	0,0016
4716610	rseC	1132726..1133187	3,42	0,0000	1,97	0,0086
4712745	menC	1537214..1538185	3,39	0,0000	1,62	0,1114
4714233	YE3000	3271557..3271874	3,23	0,0000	1,85	0,0079
4715993	YE1881	complement(2063815..2064513)	3,10	0,0000	1,04	0,8955
4714002	hybE	complement(3927909..3928427)	3,07	0,0000	2,94	0,0040
4712607	1803A	complement(1994790..1994930)	3,06	0,0000	1,43	0,1330
4713723	citT	complement(2863701..2865164)	2,85	0,0000	-1,11	0,7486
4713701	moaD	complement(3149906..3150151)	2,81	0,0005	2,29	0,0580
4715650	trp1400A_3	2099704..2099994	2,73	0,0000	1,53	0,1881
4712486	YE2444	2634101..2634670	2,72	0,0000	1,27	0,3845
4716355	YE4066	complement(4434877..4436382)	2,70	0,0000	1,75	0,0253
4714848	YE2430	complement(2620620..2621474)	2,65	0,0000	1,05	0,8652
4716607	rpoE	1130517..1131092	2,64	0,0000	1,85	0,0431
4715949	phnF	complement(552658..553383)	2,62	0,0002	2,33	0,0392
4715050	dga	159212..160075	2,54	0,0000	1,55	0,0593
4712661	YE1145	complement(1284224..1284418)	2,53	0,0001	1,37	0,2199
4713730	citA	2870925..2872580	2,52	0,0000	1,68	0,0621
4713703	moaA	complement(3150659..3151639)	2,51	0,0000	1,31	0,3095
4714700	lamB	complement(4214265..4215551)	2,48	0,0000	1,31	0,3973
4713913	YE3521	complement(3832233..3833827)	2,48	0,0000	1,26	0,3663
4713097	YE0387	complement(458229..458537)	2,47	0,0000	1,14	0,5689
4712550	hisS	1202445..1203719	2,45	0,0000	1,39	0,2158
4716158	ssiC	complement(4287659..4288462)	2,43	0,0001	-1,31	0,3526
4715948	phnG	complement(552181..552657)	2,42	0,0000	1,92	0,0117
4714144	degQ	4089861..4091234	2,39	0,0000	1,44	0,1707
4713447	YE0662	complement(765339..765785)	2,38	0,0000	2,17	0,0187
4716519	YE4124	4513765..4514859	2,35	0,0000	1,56	0,1605
4714235	mrdA	3272423..3274318	2,34	0,0000	2,06	0,0135
4716156	YE3944	4285808..4286785	2,33	0,0000	1,78	0,0126
4715908	flgL	complement(2742771..2743751)	2,33	0,0000	1,32	0,2402
4714646	yhdA	complement(4152561..4154480)	2,31	0,0000	1,55	0,1239
4712464	hycI	3048740..3049213	2,31	0,0000	1,22	0,4833
4713464	YE0679	complement(784809..785339)	2,28	0,0000	1,59	0,0930
4714066	YE3668	complement(4000693..4001475)	2,27	0,0000	1,27	0,3209
4712673	napB	1297939..1298394	2,25	0,0000	4,38	0,0015
4716605	YE1008	1127808..1128563	2,22	0,0000	1,22	0,3561
4715889	fhuD	848599..849381	2,22	0,0001	1,23	0,5231
4716586	YE0990	complement(1111058..1112893)	2,21	0,0000	1,22	0,4451
4716067	fdx_1	complement(1143061..1143321)	2,21	0,0005	1,20	0,5297
4712654	dapA	1276675..1277556	2,20	0,0000	1,31	0,2172
4713020	YE1443	1618976..1619230	2,19	0,0001	-1,08	0,7887
4715325	YE2321	complement(2524224..2525429)	2,18	0,0000	1,47	0,1479
4713059	YEs013	1356689..1356852	2,17	0,0005	2,71	0,0017
4714941	tolR	complement(3190197..3190625)	2,15	0,0000	1,20	0,4319
4714000	hypB	complement(3926454..3927554)	2,14	0,0000	1,65	0,2266

4716157	tauD	complement(4286814..4287662)	2,11	0,0000	1,62	0,0449
4716593	YE0996	1117719..1117952	2,11	0,0000	1,76	0,0118
4716556	yidE	complement(4559886..4561544)	2,09	0,0000	2,26	0,0063
4713460	ftsQ	780244..781101	2,06	0,0000	1,25	0,3397
4716006	YE1894	complement(2077751..2078536)	2,06	0,0004	1,78	0,0798
4714752	rpsH	complement(4262583..4262975)	2,05	0,0000	1,12	0,7177
4715717	yfeY	1324114..1324725	2,05	0,0000	1,76	0,0148
4714808	YE2387	complement(2575368..2576621)	2,03	0,0000	1,56	0,0970
4714557	cld	complement(3339012..3340103)	-2,00	0,0000	-1,49	0,1113
4716504	ccrB	complement(1086013..1086417)	-2,00	0,0000	-1,75	0,0356
4712676	YE1160	1299423..1299710	-2,01	0,0002	-1,02	0,9397
4715736	crr	complement(1344156..1344665)	-2,01	0,0000	-1,97	0,0146
4713694	YE2890	3142308..3143015	-2,01	0,0000	-1,52	0,0809
4713309	YE1506	complement(1681290..1682177)	-2,02	0,0001	-1,59	0,1065
4713158	outE	complement(3659722..3661203)	-2,03	0,0000	-1,18	0,4775
4714763	rplB	complement(4266968..4267792)	-2,03	0,0000	-1,60	0,0725
4714699	malM	complement(4213110..4214045)	-2,04	0,0001	-1,69	0,0399
4714365	groEL	420434..422086	-2,04	0,0000	-1,69	0,0757
4714956	gltA	3208007..3209287	-2,05	0,0000	-1,82	0,0325
4713635	YE2062	complement(2257016..2257690)	-2,06	0,0001	1,02	0,9355
4715612	YE1254	1397537..1398115	-2,06	0,0000	-2,06	0,0057
4715752	araC	2193899..2194831	-2,06	0,0000	-1,35	0,1741
4712806	gptB_1	complement(1967791..1968654)	-2,06	0,0000	-1,91	0,0204
4715025	metF	127374..128258	-2,06	0,0000	-1,51	0,1071
4715195	hslU	complement(113424..114755)	-2,07	0,0000	-1,55	0,0410
4713925	acpY	complement(3847625..3847876)	-2,08	0,0000	-1,26	0,3482
4715423	YE3102	3377642..3378004	-2,09	0,0004	-1,66	0,0378
4716124	nlpD	896518..897501	-2,09	0,0000	-1,72	0,0468
4714628	YE3788	complement(4131672..4132058)	-2,10	0,0001	-1,56	0,0609
4713657	YE2085	2282083..2283576	-2,10	0,0000	-1,57	0,0242
4714768	bfr	complement(4270078..4270551)	-2,11	0,0000	-1,71	0,0133
4715858	pdhR	808535..809299	-2,12	0,0000	-1,34	0,2739
4714297	dps	3091166..3091675	-2,13	0,0000	-1,88	0,0172
4716227	YE0823	948857..949801	-2,13	0,0000	-1,50	0,0352
4714760	rpsC	complement(4265612..4266310)	-2,13	0,0000	-1,46	0,1935
4716172	ppiA	complement(4302067..4302636)	-2,13	0,0000	-1,31	0,2507
4713708	bioD_2	complement(3157143..3157790)	-2,13	0,0000	1,05	0,8509
4714050	dkgA	3981740..3982573	-2,14	0,0000	-1,03	0,8909
4715455	clpP	complement(3409738..3410361)	-2,14	0,0000	-1,14	0,6742
4712638	ppk	complement(1259121..1261190)	-2,14	0,0000	-1,44	0,2255
4714363	fxsA	419304..419831	-2,14	0,0000	1,23	0,4303
4712516	phoH	complement(2668285..2669073)	-2,14	0,0000	-1,10	0,6755
4715124	fadE	3509424..3511871	-2,14	0,0000	-1,79	0,0109
4716503	YE0962	complement(1085778..1086008)	-2,15	0,0000	-2,14	0,0065
4714559	manB	complement(3341221..3342591)	-2,19	0,0000	-1,12	0,7143
4715200	cyoB	3417723..3419714	-2,19	0,0000	-1,15	0,6509
4715533	YE1985	2167117..2168151	-2,19	0,0004	-3,09	0,0033
4715203	cyoE	3420676..3421566	-2,21	0,0000	-1,09	0,7644
4712450	maeA	3033911..3035608	-2,21	0,0000	-2,02	0,0048
4714364	groES	420084..420377	-2,21	0,0000	-1,31	0,3234
4716574	YE0977	1099313..1100518	-2,21	0,0001	-2,87	0,0034
4714137	YE3737	4084540..4085025	-2,22	0,0000	-1,64	0,0347
4715649	ihfA	2099211..2099507	-2,24	0,0000	-1,73	0,0305
4716194	nudE	complement(4327043..4327600)	-2,24	0,0000	-1,50	0,0933
4716083	glnB	1162428..1162766	-2,26	0,0000	-1,55	0,0198

4715545	YE0202	complement(238765..239235)	-2,26	0,0000	-1,55	0,0263	
4715434	YE3114	3386958..3387326	-2,28	0,0000	-2,44	0,0082	
4714566	wbcF	complement(3348866..3349834)	-2,29	0,0000	-1,83	0,0100	
4713263	YE1569	1766941..1767498	-2,29	0,0000	-1,10	0,7377	
4713512	tpx	2315091..2315594	-2,30	0,0000	-1,63	0,1160	
4713095	YE0385	455489..456229	-2,31	0,0000	-1,85	0,0055	
4714757	rpsQ	complement(4264744..4264998)	-2,32	0,0000	-2,12	0,0333	
4713198	YE0520	complement(590196..590498)	-2,33	0,0000	-2,12	0,0248	
4714579	tatE	complement(3280576..3280791)	-2,34	0,0000	-1,56	0,0607	
4714480	meoA	1571842..1573016	-2,34	0,0000	-2,34	0,0029	
4714843	YE2425	complement(2614957..2616072)	-2,37	0,0000	-1,99	0,0089	
4714779	YE3936	4278667..4278882	-2,38	0,0000	-1,64	0,0571	
4715042	YE0132	complement(151070..151801)	-2,38	0,0000	-1,39	0,1509	
4714396	rplL	329248..329616	-2,39	0,0000	-1,99	0,0104	
4714433	ail	2007722..2008258	-2,45	0,0000	-1,09	0,7669	+ at 37°C [2]
4712711	hexA/rovM	1495357..1496289	-2,46	0,0000	-1,52	0,0215	
4715410	htpG	complement(3360566..3362440)	-2,48	0,0000	-1,95	0,0078	+ at 37°C [1]
4713182	YEt022	579198..579282	-2,49	0,0000	-1,18	0,6027	
4715131	mntC	complement(3526077..3526766)	-2,55	0,0000	-1,54	0,0591	
4715201	cyoC	3419704..3420318	-2,56	0,0000	-1,27	0,3746	
4715415	YE3094A	complement(3366599..3366979)	-2,56	0,0000	-1,49	0,1108	
4714392	nusG	326426..326971	-2,56	0,0000	-1,69	0,0524	
4714950	sucB	complement(3199736..3200959)	-2,57	0,0000	-1,81	0,0367	
4714954	sdhD	complement(3206627..3206974)	-2,57	0,0000	1,10	0,7779	
4712704	atpC	complement(4605243..4605665)	-2,57	0,0000	-1,74	0,0364	
4715509	bglY	2453110..2453520	-2,59	0,0000	-2,02	0,0148	
4714563	rfpB	complement(3346093..3347169)	-2,61	0,0000	-1,15	0,6108	
4714955	sdhC	complement(3206968..3207357)	-2,62	0,0000	1,01	0,9627	
4716108	cysI	882055..883785	-2,63	0,0000	1,02	0,9184	
4715639	YE1280	1423360..1424292	-2,63	0,0000	-1,44	0,1114	
4715435	ymoA	3387372..3387575	-2,65	0,0000	-1,36	0,2911	
4715860	aceF	812153..814030	-2,66	0,0000	-1,45	0,2211	
4713122	mdh	484611..485546	-2,69	0,0000	-1,62	0,1047	
4716382	clpB	1016096..1018669	-2,70	0,0000	-1,54	0,0951	+ at 37°C [1]
4712774	YE1738	complement(1936070..1936927)	-2,71	0,0000	-1,93	0,0220	
4712628	YE1110	complement(1244217..1244483)	-2,76	0,0000	-2,29	0,0059	
4715132	mntB	complement(3526763..3527452)	-2,77	0,0000	-1,33	0,2487	
4715199	cyoA	3416762..3417718	-2,78	0,0000	1,12	0,7036	
4714531	fadA	complement(305990..307153)	-2,80	0,0000	-2,66	0,0016	
4716345	opdA	complement(4419613..4421655)	-2,83	0,0000	-1,68	0,0276	
4715129	mntA	3524356..3525396	-2,84	0,0000	-1,76	0,0230	
4713166	YE3356	complement(3667426..3668379)	-2,84	0,0000	-1,98	0,0048	
4713931	ysaU	complement(3855090..3856214)	-2,89	0,0000	-1,15	0,6239	
4715021	metJ	complement(120521..120838)	-2,89	0,0000	1,18	0,5300	
4715377	YE2264	complement(2472916..2473329)	-2,89	0,0000	-1,16	0,5463	
4714251	YE2606	complement(2804804..2805805)	-2,94	0,0000	-1,67	0,0188	
4714572	wbcA	complement(3355330..3355854)	-2,98	0,0002	-2,08	0,0020	
4714571	prt	complement(3354419..3355297)	-2,99	0,0000	-1,65	0,0458	
4715581	YE0240	complement(276430..276906)	-2,99	0,0000	-1,78	0,0133	
4714945	cydB	complement(3192297..3193436)	-2,99	0,0000	-1,19	0,6328	
4712530	YE2492	complement(2683198..2683911)	-3,00	0,0000	-1,37	0,1906	
4714984	YE1609	complement(1810022..1811557)	-3,01	0,0000	-1,67	0,0658	
4713027	myfE	1628085..1628750	-3,02	0,0000	-1,85	0,0080	
4714570	wbcC	complement(3353349..3354353)	-3,07	0,0000	-1,89	0,0013	
4714896	hemD	complement(221720..222466)	-3,13	0,0000	-1,26	0,2984	

4713951	ysaH	3872301..3872777	-3,27	0,0004	-4,19	0,0138	
4714974	YEt041	1796717..1796804	-3,27	0,0000	-2,13	0,0270	
4714177	YE3779	complement(4124986..4125726)	-3,35	0,0000	-1,80	0,0133	
4716273	glgB	complement(4367589..4369772)	-3,36	0,0000	-1,88	0,0600	
4713389	csrB	complement(3595392..3595708)	-3,41	0,0000	-2,15	0,0018	
4712657	YE1141	1280059..1280823	-3,43	0,0000	-1,58	0,0348	
4714953	sdhA	complement(3204860..3206626)	-3,48	0,0000	-1,65	0,1184	
4713945	YE3551	3869209..3869517	-3,48	0,0000	-1,91	0,0299	
17503114	YE2858a	3109785..3109985	-3,50	0,0000	-2,18	0,0019	
4714569	rfbX	complement(3352027..3353316)	-3,65	0,0000	-2,01	0,0112	
4716497	ureG	1080067..1080672	-3,75	0,0000	-2,69	0,0056	
4716269	glgP	complement(4360202..4362649)	-3,88	0,0000	-2,08	0,0217	
4716558	hslT	complement(4562407..4562820)	-3,91	0,0000	-1,71	0,0475	
4715306	YE2299	complement(2511244..2511846)	-4,05	0,0000	-3,14	0,0120	
4712596	ftn	1988793..1989302	-4,11	0,0000	-1,86	0,0523	
4715663	putA	complement(2108495..2112484)	-4,17	0,0000	-1,80	0,0482	
4714939	tolB	complement(3187528..3188820)	-4,38	0,0000	-1,66	0,0926	
4716356	dctA	complement(4436866..4438158)	-5,34	0,0000	-1,29	0,4368	
4714289	ompX	complement(3085714..3086241)	-6,81	0,0000	-1,05	0,7949	+ at 37°C [1]
4712914	dnaK	700187..702094	-7,38	0,0000	-2,28	0,0049	
4714228	YE2995	complement(3265493..3265975)	-8,44	0,0000	-2,12	0,0030	
4716292	YE0857	983455..983820	-13,46	0,0000	-8,41	0,0013	
4716178	nirD	4311030..4311356	1,35	0,2576	9,98	0,0000	
4714266	irp4	2837033..2837836	2,00	0,0839	6,42	0,0007	
4714031	flp	3962020..3962214	1,29	0,3322	5,34	0,0000	
4714014	YE3615	complement(3939448..3941022)	1,29	0,0690	5,29	0,0000	
4714482	YEt039	1573364..1573439	1,13	0,3711	4,76	0,0000	
4714385	YEt011	323856..323931	1,09	0,5378	4,52	0,0000	
4716179	nirC	4311547..4312353	1,80	0,0000	3,95	0,0000	
4712528	YE2490	complement(2682406..2682720)	1,69	0,0255	3,43	0,0001	
4715623	ccmB	1407271..1407930	1,19	0,1965	3,41	0,0000	
4716177	nirB	4308487..4311033	1,06	0,6398	3,28	0,0007	
4715625	ccmD	1408835..1409080	1,17	0,6047	3,08	0,0001	
4714607	YE3039	3307405..3308364	1,86	0,0000	2,97	0,0000	+ at 37°C [1]
4712675	YE1159	1299175..1299426	1,30	0,1218	2,92	0,0000	
4715702	YE1171	1310337..1311557	-1,49	0,0012	2,89	0,0000	
4715178	cpxP	96348..96812	1,50	0,0164	2,89	0,0000	
4712529	YE2491	complement(2682720..2683169)	1,48	0,1085	2,87	0,0003	
4712800	YE1769	complement(1960600..1960878)	1,17	0,2523	2,75	0,0000	
4713860	arsR2	3783946..3784299	-1,52	0,0154	2,64	0,0002	
4712531	YE2493	complement(2683915..2685036)	1,67	0,0001	2,62	0,0001	
4713089	YE0379	447958..449241	1,50	0,0020	2,61	0,0001	
4713702	moaC	complement(3150148..3150627)	1,59	0,0053	2,61	0,0000	
4716352	YE4063	complement(4432402..4433178)	-1,47	0,0374	2,51	0,0001	
4715110	YE3210	complement(3496015..3496647)	-1,19	0,2119	2,45	0,0001	
4715622	ccmA	1406645..1407271	1,17	0,1907	2,41	0,0000	
4715828	YE0452	527379..527882	1,36	0,0221	2,39	0,0009	
4715024	YE0113	complement(124892..127000)	1,98	0,0000	2,37	0,0001	
4715827	YE0451	526864..527385	1,24	0,1421	2,34	0,0007	
4714055	mda66	3990153..3990746	1,16	0,3816	2,32	0,0001	
4712834	fliZ	complement(2713429..2713938)	1,47	0,0116	2,29	0,0009	
4714769	bfd	complement(4270631..4270825)	1,74	0,0002	2,27	0,0010	
4716046	YE2751	2984805..2985788	1,32	0,0511	2,26	0,0003	+ at 27°C [1]
4715925	flhB	complement(2760810..2761961)	1,36	0,1867	2,22	0,0001	
4715865	YE0706	823034..823396	1,44	0,0112	2,20	0,0007	

4714439	tnpB_3	complement(2011853..2012602)	1,45	0,0267	2,17	0,0007	
4713906	dnaB2	complement(3825720..3827087)	1,91	0,0001	2,00	0,0005	
4713619	YE3313	complement(3622973..3623359)	-1,93	0,0000	-2,07	0,0006	
4713499	pps	complement(2382512..2384893)	-1,31	0,0382	-2,07	0,0008	
4715458	bolA	complement(3412538..3412870)	-1,10	0,5725	-2,10	0,0001	
4714539	YEr008	316294..319287	-1,58	0,0005	-2,15	0,0005	
4712562	YE1086	1217941..1219194	-1,20	0,1728	-2,18	0,0008	
4712796	cspC1	1956541..1956750	-1,20	0,1313	-2,19	0,0008	
4715922	invA	complement(2755292..2757799)	-1,11	0,4090	-2,23	0,0004	- at 27°C [2]
4713201	YE0523	complement(592004..592294)	-1,84	0,0000	-2,26	0,0003	
4714418	YEr011	356873..359866	-1,61	0,0000	-2,31	0,0000	
4714565	wbcG	complement(3347994..3348860)	-1,84	0,0000	-2,37	0,0002	
4712998	YE1324	1475876..1476574	-1,56	0,0008	-2,39	0,0002	
4715331	YE2327	2530999..2531142	-1,08	0,6960	-2,40	0,0002	
4714279	YE2825	3072406..3073065	-1,68	0,0003	-2,43	0,0004	
4713581	cbiQ	complement(2949762..2950439)	-1,22	0,3314	-2,52	0,0006	
4714780	YE3937	4278942..4279106	-1,18	0,1984	-2,54	0,0009	
4716498	ureD	1080669..1081652	-1,84	0,0001	-2,55	0,0002	
4713291	grxA	complement(1663249..1663512)	1,09	0,6005	-2,56	0,0002	
4716468	proW	complement(1051114..1052295)	-1,43	0,0607	-2,59	0,0001	
4713202	YE0524	complement(592356..593231)	-1,42	0,0053	-2,63	0,0000	
4714459	YE1845	complement(2026785..2027630)	-1,64	0,0002	-2,84	0,0000	
4716422	dppC	complement(4460544..4461446)	-1,73	0,0071	-2,91	0,0000	
4715304	YE2297	2510655..2510954	-1,83	0,0028	-2,91	0,0005	
4713678	zntB	complement(2309579..2310562)	-1,69	0,0001	-2,95	0,0000	
4715780	YE2035	complement(2224870..2226117)	-1,90	0,0000	-3,07	0,0000	
4714092	gadA	complement(4032296..4033696)	-1,46	0,0749	-3,16	0,0004	
4715715	YE1184	1322982..1323164	-1,67	0,0059	-3,26	0,0000	
4713522	ytxB	complement(2324044..2324400)	-1,96	0,0000	-3,48	0,0000	
4713675	arbF	complement(2305196..2307097)	-1,15	0,4039	-3,58	0,0000	
4714406	YEs005	complement(345526..345651)	-1,12	0,6026	-3,75	0,0000	
4714792	YE1652	complement(1857369..1857551)	-1,99	0,0006	-4,09	0,0000	
4714186	ybfA	complement(3218478..3218684)	-1,92	0,0000	-4,91	0,0000	
4715332	YE2328	complement(2531348..2531527)	-2,33	0,0044	-5,65	0,0000	

[1] Kakoschke et al., 2014
[2] Kakoschke et al., 2016
[3] putative Hfq binding site
[4] (+ more, - less abundant in *hfq* negative strain)
All genes have a fold change of > 2 and a $p < 0.001$ at 27°C or 37°C or both temperatures.