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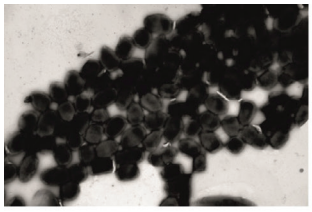
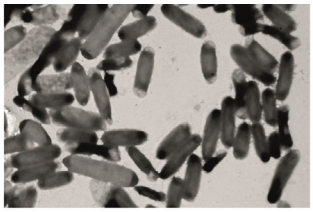
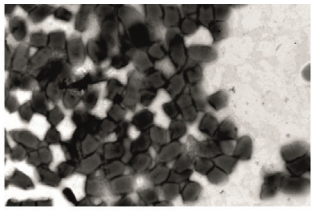
KATARZYNA LESKINEN

The Roles of YbeY, RfaH, and Hfq in Gene Regulation and Virulence of *Yersinia enterocolitica* O:3

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The roles of YbeY, RfaH, and Hfq in gene regulation and virulence of *Yersinia enterocolitica* O:3

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"Keep me incomplete"

-Liam Cormier

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I. Leskinen K, Varjosalo M, Skurnik M. 2015. Absence of YbeY RNase compromises the growth and enhances the virulence plasmid gene expression of *Yersinia enterocolitica* O:3. Microbiology 161:285-99.

II. Leskinen K, Varjosalo M, Li Z, Li CM, Skurnik M. 2015. The expression of the *Yersinia enterocolitica* O:3 lipopolysaccharide O-antigen and outer core gene clusters is RfaH-dependent. Microbiology 161: 1282-1294.

III. Leskinen K, Varjosalo M, Fernández-Carrasco H, Bengoechea JA, Skurnik M. Several Hfq-dependent alterations in physiology of *Yersinia enterocolitica* O:3 are mediated by derepression of the transcriptional regulator RovM. Submitted.

ABBREVIATIONS

Ail	Attachment invasion locus
AHL	*N-Acyl* Homoserine Lactone
asRNA	antisense RNA
BHI	Brain Heart Infusion
bp	base pair
CFU	Colony Forming Unit
Clm	Cloramphenicol
ECA	Enterobacterial Common Antigen.
DMEM	Dulbecco's Modified Eagle's Medium
DOC	2,5-Dimethoxy-4-chloroamphetamine
DOC-PAGE	2,5-Dimethoxy-4-chloroamphetamine Polyacrylamide Gel Electrophoresis
FC	Fold Change
GMP	Guanosine monophosphate
GTP	Guanosine-5'-triphosphate
HIS	Heat Inactivated Serum
IC	Inner Core
Inv	Invasin
kbp	kilo base pair
Km	Kanamycin
LB	Lysogeny Broth
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LPS	Lipopolysaccharide
Man*p*NAcA	D-mannopyranuronic Acid
mRNA	messenger RNA
MS	Mass Spectrometry
ncRNA	non-coding RNA
NHS	Normal Human Serum
O-Ag	O-Antigen
OC	Outer Core
PAPI	Poly(A) Polymerase I

PBS	Phosphate-Buffered Saline
PTM	Post-Translational Modifications
PTS	Phosphotransferase system
pYV	Yersinia virulence plasmid
RBP	RNA-binding protein
RBS	Ribosome-binding site
RNA-seq	RNA sequencing
RT	Room Temperature
SD	Shine-Dalgarno
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Spec	Spectinomycin
sRNA	small RNA
Strep	Streptomycin
T3SS	Type III Secretion System
TCEP	Tris(2-carboxyethyl)phosphine
TCS	Two-Component regulatory System
TIR	Translation Initiation Region
UTR	Untranslated Region
YadA	Yersinia adhesin A
Yops	Yersinia outer membrane proteins

ABSTRACT

Understanding the molecular mechanisms of bacterial virulence has broad implications. In addition to just academic interest many practical applications can be foreseen emerging from virulence research: identification of novel antimicrobial drug targets, potential vaccines, and diagnostics of infectious diseases. Different virulence factors are responsible for the initiation of the disease and others for the disease symptoms. Consequently, elimination of a single virulence factor can severely attenuate or even completely abrogate virulence. Due to the increasing antibiotic resistance world-wide there is an urgent need for new antimicrobial agents. The virulence factors and their eukaryotic interaction partners are recognized as potential targets for vaccine and antibacterial drug development and therefore highly prioritized research topics internationally.

Genus *Yersinia* consists of 17 species of which *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are human pathogens. *Y. pestis* causes bubonic plague while *Y. pseudotuberculosis* and *Y. enterocolitica* cause mostly food-borne yersiniosis, usually a diarrheal disease sometimes followed by post-infectious reactive arthritis. The pathogenic potential of these bacteria resides on many essential virulence factors some of which are encoded by genes located on a 70 kb virulence plasmid of *Yersinia* (pYV) and others by chromosomal loci. Yersiniosis is considered to be the third most common cause of gastroenteritis in Europe. In Finland both *Y. pseudotuberculosis* and *Y. enterocolitica* cause hundreds of human infections annually.

The aim of the study is to characterize the intricate regulatory networks of *Yersinia* especially those that control the expression of the virulence factors. To achieve that goal three regulators were initially selected. The first gene studied, *ybeY*, was selected based on the literature due to the fact its protein product is believed to affect the sRNA regulation similar to Hfq. YbeY was recently recognized as an endoribonuclease playing an important role in the process of ribosome biosynthesis. The absence of *ybeY* gene in *Y. enterocolitica* serotype O:3 resulted in misprocessing of 16S rRNA and in severe decrease of growth rate with complete growth arrest at elevated temperatures. Interestingly, the lack of YbeY disturbed severely the regulation of the *Yersinia* virulence plasmid genes and affected the expression of regulatory small RNA species. Furthermore, the *ybeY* mutant displayed impairment of many virulence-related features, and decreased infectivity in the cell infection model.

The gene *rfaH* was selected as RfaH is implicated in regulation of different virulence factors in pathogenic bacteria, where it is required for the expression of lipopolysaccharide (LPS), capsule, hemolysin, exotoxin, hemin uptake receptor, and F pilus. This study revealed that RfaH of *Y. enterocolitica* O:3 acts as a highly specific regulator that enhances the transcription of the operons involved in biosynthesis of LPS O-antigen and outer core but does not affect the expression of enterobacterial common antigen. Furthermore, the transcriptome of the Δ*rfaH* strain showed high similarity with the transcriptome of the O-antigen negative mutant, what indicated that the some changes seen in the *ΔrfaH* strain were actually due to indirect responses to the loss of O-antigen. Moreover, the lack of RfaH resulted in attenuated stress response and lower resistance to compounds such as sodium dodecyl sulfate and polymyxin B. Conversely, the Δ*rfaH* strain displayed higher resistance to complement-mediated killing by normal human serum.

Due to an established role of non-coding RNAs in the gene regulation of bacteria, the small RNA chaperone gene *hfq* was chosen for further study. Previous studies recognized the role of Hfq in bacterial virulence. However, the effects of Hfq-deficiency differ between the bacterial species. In *Y. enterocolitica* O:3 loss of Hfq caused impairment in growth, elongation of the bacterial cells, and decreased the resistance of bacteria to heat, acid and oxidative stresses, as well as attenuation in mouse infection experiments. Moreover, this study revealed that several alterations typical for the *hfq*-negative phenotype were due to derepression of the transcriptional factor RovM. The inactivation of the *rovM* gene of the *hfq* mutant reversed the motility and biofilm formation defects, mannitol utilization changes, and partially complemented the growth defect of the *hfq* mutant.

In conclusion, all the studied proteins affected the gene regulation of *Y. enterocolitica* O:3 in different manner causing changes in gene and protein expression. The conducted experiments demonstrated that all the studied mutations compromised the bacterial virulence. The studied mutants showed significant decrease in resistance to different environmental conditions that are normally encountered during the course of infection. Furthermore, the loss of studied proteins resulted in such effects as growth defect, impairment of motility and biofilm formation, changes in carbohydrates metabolism, and alterations in production of different virulence factors that also contributes to vitality and ability to establish infection in host organism.

1. INTRODUCTION

Yersiniosis is currently the third most common food-borne gastroenteritis in Europe after *Salmonella* and *Campylobacter* infections, with the *Yersinia enterocolitica* subsp. *palearctica* serobiotype O:3/4 being most frequently isolated from humans and slaughter pigs. In Finland, the number of *Yersinia* incidence is among the highest in European Union, with approximately 500-700 cases per year (ca. 10 cases per 100 000 population). Moreover, as a psychrotrophic microorganism, *Y. enterocolitica* is able to proliferate at temperatures as low as 0^0C, which makes it a substantial concern for the public health.

Understanding the molecular mechanisms of bacterial gene regulation can bring many practical applications: identification of novel antimicrobial drug targets, development of novel vaccines, and improvements in diagnostics of infectious diseases. Due to the fact that different virulence factors are needed during different stages of infection, elimination of a single factor can severely attenuate the virulence. Therefore these factors are recognized as potential targets for vaccine and antibacterial drug development. In this respect *Yersinia* makes an exceptionally good model because it possesses tens of recognized virulence factors, there are good animal models for the disease, and the genomic sequences of several *Yersinia* species are known. In the face of rapidly emerging resistance it is vital that there is no diminish in the search for new antimicrobial agents, particularly of new lines (e.g. the inhibitors of bacterial virulence).

2. REVIEW OF THE LITERATURE

2.1. Gene regulation in bacteria

A large reservoir of genetic information increases the versatility of a bacterium by allowing it to adapt to variety of environmental conditions. The sequenced bacterial genomes contain from 700 up to 9 000 genes, although only approximately 600 – 800 are needed at a certain time point (Dale & Park, 2010). Furthermore, both gene expression and protein synthesis is an energy-consuming process. Therefore, in order to respond adequately to the external stimuli and conserve the energy, the gene expression undergoes tight regulation.

Regulation of gene expression in bacterial cell takes place at different levels (Fig. 3). The most general control occurs at the level of transcription. The expression is further controlled at the level of translation and subsequently undergoes the postranslational control. Following the scheme presented in the Figure 3 a number of potential regulatory factors can be mentioned. First of all, higher number of copies of the gene can increase the expression. In general, if the genome harbors more copies of a certain gene, there are more sites available for the transcription process to take place. However, most of the genes on the bacterial chromosome exist only in one copy, excluding genes coding for such molecules as rRNA. Therefore, this type of regulation is restricted to only several genes. Secondly, the promoter activity determines the efficiency with which the gene is transcribed by affecting the level of initiation of transcription by RNA polymerase. Promoter activity is considered to be the most important cue in control of expression of individual genes in bacteria. Next step of regulation focuses on the stability of mRNA molecules, which serve as the templates for translation. Most of the bacterial mRNAs are short-lived and are typically degraded with half-lives below 2 minutes, whereas other forms of RNA (rRNA or tRNA) are considerably more stable. All the above mentioned steps focus on providing and maintaining the proper amount of mRNA available in bacterial cell. The process of translation is controlled by the efficiency of initiation (ribosome binding) and the rate of translation (codon usage). Furthermore, the abundance of protein reflects both the rate of production and its stability. The last step of regulation involves different posttranscriptional effects that may include events like protein folding, covalent modifications, as well as activation and inhibition by other proteins. (Birge, 2006 189, Dale & Park, 2010 188).

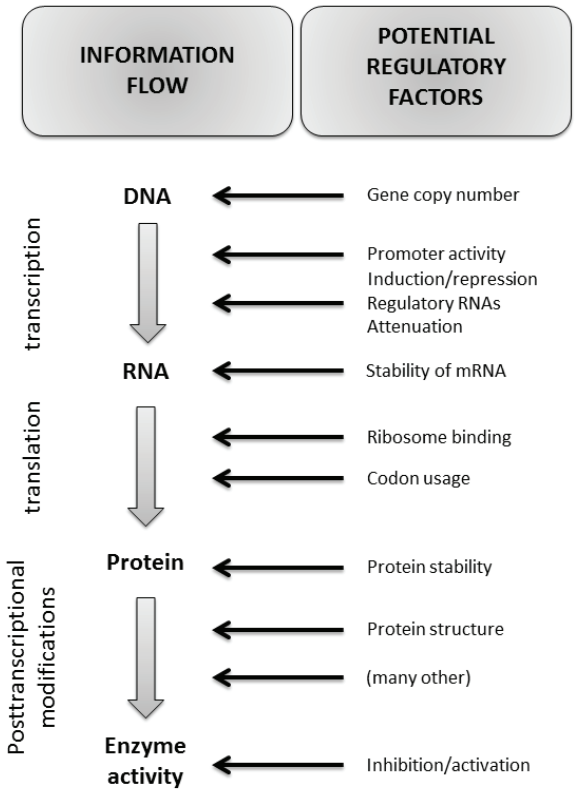

Figure 3. Model of information flow and potential regulatory factors affecting the gene expression in bacteria (modified from (Dale & Park, 2010)).

2.1.1. Regulation of transcription

In prokaryotes, the most common way to regulate gene expression is by influencing the rate at which transcription is regulated. This part of the gene expression regulation allows controlling the amount of mRNA produced from the coding gene. Although, this step is characterized by lowest sensitivity, it provides bacterial cell with possibility to stop the gene expression at earliest stage and thus preserve energy. (Birge, 2006 189, Dale & Park, 2010 188).

2.1.1.1. Promoters

Promoter is a sequence located upstream of a gene which acts as a specific recognition site for RNA polymerase and regulatory DNA binding proteins. Generally, promoters contain two highly conserved regions that are placed approximately 33 and 10 bp upstream from the start of the gene and are termed -35 and -10 boxes. In *Escherichia coli* the highly conserved regions

TTGACA and TATAAT constitute the consensus sequences for -35 and – 10 boxes, respectively. The sequence separating these highly conserved regions is variable and for major of promoters it is 16 to 18 bp long. The variations of the promoter sequence reflect both its strength and its capacity to bind different classes of RNA polymerases. Promoters that have sequence close to the ideal consensus are considered to be strong and they can direct the initiation of the transcription of the gene every 2 seconds. Changes in the conserved boxes or in the separating sequence lead to decrease in the promoter activity. Despite the diversity of the promoters, the TATA motif of the -10 box is common for all classes of promoters, including eukaryotic and archeal promoters. Therefore promoter constitutes a fixed level of control that determines the potential level of expression of a certain gene. (Travers, 1987, Dale & Park, 2010).

2.1.1.2. Sigma factors and anti-sigma factors

RNA polymerase that conducts transcription is composed of four subunits ($\alpha_2\beta\beta'$) and additional dissociable element called σ-factor. The σ-factor allows recognition of the conserved -10 and -35 boxes in the promoter region determining the specificity of the enzyme. Since regulons of σ-factors can be comprised of hundreds of genes, this mechanism is frequently used to respond to such stimuli as environmental stresses, nutritional downshifts, and variations in pH and osmolarity (Kazmierczak *et al.*, 2005, Dale & Park, 2010). The most common σ-factor, σ70, is responsible for transcription of housekeeping genes required for the essential cellular functions. The presence of alternative sigma factors allows bacterium to redirect the transcription into set of a smaller number of genes linked to a specific function. In *E. coli* seven different σ-factors have been identified (Table 3) (reviewed in (Landini *et al.*, 2014)).

The general stress response alternative sigma factor RpoS (σ38) is responsible for transcription of genes contributing to bacterial survival under unfavorable environmental conditions. Its expression is activated during starvation, oxidative damage, reduced pH, as well as during stationary phase of growth. The *rpoS* gene is not essential for growth of *E. coli* and its deletion does not affect the growth rate in neither rich nor minimal medium. However, strains lacking RpoS display high sensitivity to a variety of environmental stresses. Approximately 140 genes are induced directly by the increase in σ38 levels, regardless of growth conditions and environmental cues. Moreover, up to 500 genes together can be affected directly or indirectly by the activity of RpoS, indicating interplay with additional regulators (reviewed in (Landini *et al.*, 2014)). Many studies have shown that in some species like *S. enterica* serovar Typhimurium RpoS is needed for full virulence. The RpoS mutant of this species displayed alterations in

transcription of several chromosomal and plasmid-carried virulence genes (reviewed in (Kazmierczak *et al.*, 2005)). The function of other alternative sigma factors is briefly described in Table 3.

Table 3. The function of the seven different σ-factors present in *E. coli* (modified from (Dale & Park, 2010)).

σ-factor	Function of regulated genes	Number of genes regulated in *E. coli*
σ70 / RpoD	Housekeeping genes	~1000
σ38 / RpoS	Stationary phase / general stress response	~140
σ32 / RpoH	Heat shock response	~40
σ28 / RpoF *	Chemotaxis and flagellum synthesis	~40
σ54 / RpoN	Nitrogen assimilation	~15
σ24 / RpoE	Periplasmic and surface proteins	~5
σ18 / FecI	Ferric citrate transport	~5

**no homologue annotated in the genome of Y. enterocolitica*

The shift from one sigma factor to another requires removal or inactivation of the expressed sigma factor the function of which is no longer needed. Such conversion is often assisted by anti-sigma factors, proteins that are defined by the ability to prevent its cognate sigma factor to compete for core RNA polymerase. The anti-sigma factors utilize various mechanisms, ranging from enzymatic modifications to export of the sigma factor out of the bacterial cell (reviewed in (Brown & Hughes, 1995, Hughes & Mathee, 1998)).

2.1.1.3. Operons and regulons

In bacteria, set of genes can be coordinately regulated through grouping in organizational unit called operon. The operon consists of a group of genes encoding for related functions, e.g. enzymes from a certain biochemical pathway. Such organization allows coordinate transcription and translation of all the genes in a sequential manner and ensures similar mRNA yields (Birge, 2006). Moreover, the expression of the genes from the operon undergoes either positive or negative regulation that is maintained by the presence of a repressor or an activator, commonly co-functioning with a metabolite or a catabolite from the biochemical pathway (Jacob, 1997). Such a mechanism of regulation allows an adequate response to changes in the environment, for example the appearance of different nutrients. In molecular terms, the operon contains certain genetic elements as: (i) structural genes that are transcribed into RNA, (ii) operator that serves as a repression/activation site, and (iii) a promoter from which the

transcription starts. Furthermore, there is a regulator gene that codes for a regulatory molecule which interacts with the operator (Birge, 2006). A regulon constitutes another form of organized gene regulation in bacteria, where a set of genes is regulated by the same regulatory gene product (Snyder & Champness, 2007).

2.1.1.4. Transcriptional regulators

Transcription process starts downstream from the promoter region, where the polymerase begins the elongation of the RNA. Termination site designates the location at which the polymerase is being released from the DNA and thus the elongation of the RNA molecule stops. That occurs in some distance from the translation termination codon, leaving the 3' untranslated region in between (Snyder & Champness, 2007). The dissociation of polymerase can be caused by two types of mechanisms: Rho-dependent (factor-mediated) or intrinsic (Rho-independent). Rho-independent termination occurs in the absence of auxiliary factors at locations where the RNA forms a stable hairpin structure, whereas Rho-mediated termination results from the action of Rho protein, which binds to specific sequences present in the RNA (Boudvillain *et al.*, 2013). Both the beginning and the termination of the transcription process can be influenced by the activity of transcriptional factors.

The initiation of transcription can be positively or negatively influenced by the recruitment of a specific activator or repressor, respectively. A repressor binds to an operator changing the conformation of the upstream region and prevents the polymerase from binding and/or advancing on the DNA template. An activator typically binds to an upstream activator sequence located upstream of the promoter and promotes the initiation of transcription. Transcription factors can either work solely as activators or repressors, or as both (dual regulators) depending on the target promoter (Snyder & Champness, 2007). A computational analysis of *E. coli* genome estimated presence of 314 transcriptional factors, out of which 35% were activators, 43% repressors, and 22% dual regulators (Perez-Rueda & Collado-Vides, 2000).

Distinct group of transcriptional regulators comprises global transcriptional regulators, which have the ability to regulate large numbers of genes belonging to different functional classes. The action of these factors can be complex, as they not only directly affect the expression of certain genes, but also indirectly regulate various cellular pathways by controlling different regulators. In *E. coli* it has been estimated, that seven global transcriptional regulators (CRP, FNR, IHF, Fis, ArcA, NarL and Lrp) control 50% of all regulated genes (Martinez-Antonio & Collado-Vides, 2003).

The rate of transcription can be also altered by changes in the topology of the bacterial chromosome. H-NS is a nucleid-associated protein that affects the DNA topology at specific loci and therefore modulates gene transcription by selective supercoiling of the promoter regions. The regulatory effects of H-NS are linked to metabolic and environmental conditions. The primary direct effect of H-NS is repression. (Fang & Rimsky, 2008).

Additionally, the process of transcription can be influenced at the stage of termination. Several proteins act to prevent transcriptional termination by utilizing two different mechanisms. The non-processive transcription antitermination factors bind a specific RNA sequence preventing the RNA from forming a transcription termination structure and allowing the polymerase to continue the elongation beyond this sequence. The processive elongation factors modify the polymerase so that it becomes resistant to the termination signal and it reads through the termination site (Rutberg, 1997).

Two-component regulatory systems (TCRs) and phosphorelay systems constitute a mechanism of sensing and responding to external stimuli by bacterial cells. Generally, this system comprises of two elements: (i) a histidine protein kinase, an integral membrane protein, and (ii) a cytoplasmic response regulator. Upon occurrence of a certain external stimulus, the histidine protein kinase undergoes a conformational change, autophosphorylates and subsequently transfers the phosphate group to the response regulator. The phosphorylation activates the regulator and enables it to bind to target DNA sequences and thus to regulate the expression of controlled genes. (Dale & Park, 2010).

Additionally, cyclic di-GMP signaling is implicated in regulation of wide range of bacterial features, including adhesion to surfaces, biofilm formation, aggregation and the virulence. The concentration of cyclic di-GMP inside of the cell results from the balance between the synthesis and degradation. The GGDEF protein domain catalyzes the synthesis of cyclic di-GMP from two GMP molecules, while EAL and HD-GYP domains catalyze the hydrolysis back to GMP. Cyclic di-GMP functions through binding to different receptors or effectors with the pilZ domain, as well as different transcription factors and riboswitches. Therefore, the regulation exerted by the cyclic di-GMP can occur not only at the level of transcription, but also at the post-transcription or post-translation level. (Ryan, 2013).

An exceptional situation, where bacterial cell requires nimble and adequate response to changes in external environment occurs during the infection. The expression of virulence

factors is tightly and coordinately regulated during different stages of infection. Precise control of virulence gene expression is ensured by the virulence-related transcriptional factors, which can sense host signals such as changes in temperature, osmolarity, pH, iron levels, nutrient availability, antimicrobial agents and oxygen levels. Disruption of these virulence factors leads to reduced virulence or complete attenuation of the pathogen (Cotter & Miller, 1998, Zhou & Yang, 2006).

2.1.1.5. RfaH antiterminator

RfaH is a bacterial transcriptional antiterminator that enables polymerase to overcome the intrinsic termination signals and prevent the polarization of long operons. Genetic polarity is caused by a failure in transcription of mRNA of particular part of the operon or reduced translation of a certain region (Birge, 2006). In case of long operons genetic polarity leads to reduced production of enzymes encoded by genes distal to the promoter. Loss of RfaH increases transcription polarity of limited long operons without affecting the transcription initiation from the operon promoters. In *E. coli* and *Salmonella* RfaH controls the transcription of a specialized group of operons that direct the synthesis, assembly and export of the lipopolysaccharide core, exopolysaccharide, F conjugation pilus and hemolysin toxin (Bailey *et al.*, 1997).

For its activity RfaH requires a non-coding 8 bp motif 5'-GGCGGTAG-3' termed *ops* (**o**peron **p**olarity **s**uppressor). In order to function, the ops element must be located downstream of an active promoter in the correct orientation. The importance of the *ops* element was further proven by the discovery, that the deletion of the *ops* sequence increased transcriptional polarity within the operon in a similar way as *rfaH* null mutation. Moreover, insertion of this 8 bp motif downstream of a non-native promoter resulted in increase of distal gene transcription (Nieto *et al.*, 1996). Further research showed that RfaH-dependent transcription elongation occurs upon recruitment of RfaH into a transcription complex and that this recruitment is specifically directed by the *ops* element (Bailey *et al.*, 2000). Moreover, the examination of genomic sequences revealed that *ops* element is conserved among gamma-proteobacteria, being present and functional in such species as *Shigella flexneri*, *Vibrio cholerae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Nieto *et al.*, 1996, Rahn *et al.*, 1999, Carter *et al.*, 2004, Carter *et al.*, 2007).

The RfaH protein consists of two domains connected by a flexible linker. The N-terminal domain is structurally similar to NusG and mediates the RNA polymerase binding and anti-pausing functions. The C-terminal domain is a short α-helical hairpin. In a free RfaH molecule the two

domains interact and are tightly associated. The contact with the *ops* element triggers the conformational change that separates the domains and allows the RfaH to bind to the polymerase. Moreover, the C- terminal domains refolds into a β-barrel. The association of the two domains in the free state restricts the RfaH actions to *ops*-containing operons and thus helps avoiding interference with NusG (Belogurov *et al.*, 2007). After binding to the ops-element RfaH delays the transcription, but after the escape from the *ops* element, it enhances the elongation by suppressing pausing and Rho-dependent termination (Artsimovitch & Landick, 2002). Moreover, the RfaH protein shows high level of conservation between the species. Orthologues of RfaH were also proven to complement an *E. coli rfaH* deletion suggesting high level of functional homology (Carter *et al.*, 2004).

RfaH was first described as a component of LPS synthesis machinery in *Salmonella* and believed to function as an enzyme (Wilkinson & Stocker, 1968). Later it was shown that RfaH acts as a positive regulator of the expression of a gene cluster involved in the lipopolysaccharide biosynthesis pathway ((Lindberg & Hellerqvist, 1980) and displays homology with *E. coli sfrB*, a gene required for the expression of F-factor functions (Sanderson & Stocker, 1981). Further studies revealed that RfaH is also needed for the synthesis and secretion of haemolysin (Bailey *et al.*, 1992) and the expression of the type II capsule K5 antigen in *E. coli* (Stevens *et al.*, 1994). In addition, RfaH enhances the expression of *kps* operons necessary for the synthesis of group 2 polysialic acid capsules (Navasa *et al.*, 2014) and hemin receptor molecule ChuA (Nagy *et al.*, 2001). Recent research demonstrated that RfaH selectively controls *fimB* expression at the post-transcriptional level by suppression of small RNA MicA inhibition (Moores *et al.*, 2014).

Due to the fact that RfaH promotes the expression of components that are required for bacterial virulence, loss of RfaH usually leads to decrease in pathogenicity (Nagy *et al.*, 2002, Nagy *et al.*, 2006). The decrease in virulence was observed for example for *Salmonella enterica* serovar Typhimurium, uropathogenic and avian pathogenic *E. coli*. The absence of RfaH in *E. coli* results in downregulation of several virulence factors (LPS, K15 capsule, alpha-hemolysin and hemin receptor ChuA) and subsequently to reduced urovirulence in the mouse model (Nagy *et al.*, 2002). The *rfaH* deletion mutant of *Salmonella* showed decreased intracellular net growth in epithelial and macrophage cells. Similarly, the mutant was deficient in production of outer membrane structures. In this case it was shown that the absence of *rfaH* results not only in changes that are a result of polarization of long operons with *ops* sequence, but also leads to indirect changes caused by rough-phenotype (Nagy *et al.*, 2006).

2.1.1.6. The LysR-family transcriptional regulators

The LysR-type transcriptional regulators are widely distributed among the prokaryotes. They are involved in the regulation of metabolic functions like sugar catabolism, amino-acid synthesis, aromatic compound degradation, antibiotic resistance and virulence. Typically they consist of two domains: N-terminal DNA binding domain with a helix-turn-helix motif and a C-terminal regulatory domain that binds an effector. Classically, these regulators oligomerize to form tetramers that bind to DNA to activate or repress transcription upon binding to one or more effectors. The two domains are connected by a linker helix that together with N- terminal domain plays also a role in oligomerization. (Schell, 1993).

In *E. coli* the LysR homologue A (LrhA) functions as a global transcriptional regulator of genes related to motility, chemotaxis and flagella synthesis. In other bacteria, the LrhA homologs are known under diverse names and functions. The PecT of *Erwinia chrysanthemi* and HexA of *Erwinia carotovora* are 75-79% identical to LrhA, and were implicated to regulate several virulence determinants (Surgey *et al.*, 1996, Mukherjee *et al.*, 2000). The *Yersinia pseudotuberculosis* LrhA homolog RovM (ca. 70% identical to LrhA) represses the invasin regulator RovA (Heroven & Dersch, 2006). The RovM of *Y. enterocolitica* O:3 is 88% identical to RovM of *Y. pseudotuberculosis* and ca. 70 % identical to LrhA of *E. coli*.

The structure analysis of RovM from *Y. pseudotuberculosis* revealed that it most likely adopts a tetrameric arrangement with two distant DNA-binding domains. Such a conformation would cause the target DNA to bend around the regulator. Additionaly, it was shown that RovM possesses a cavity that could bind small inducer molecules (Quade *et al.*, 2011).

Y. pseudotuberculosis RovM was shown to recognize a 50 bp region upstream of promoters that contains two palindromic sequences. Moreover, hyper-reactive bases were detected in the RovM-binding sequence suggesting that RovM bends its binding site upon interaction (Heroven & Dersch, 2006). LrhA is known to interact directly with the promoter of the flagellar *flhDC* genes, and thereby affects indirectly the genes that are under the control of the FlhDC master regulon (Lehnen *et al.*, 2002). In *E. coli*, it was shown that LrhA protein binds directly to the promoter region upstream of the *lrhA* gene and thus the expression of *lrhA* is subject to positive autoregulation (Lehnen *et al.*, 2002). Although, unlike the other orthologues, RovM does not bind directly to its own promoter, it is positively autoregulated through an unknown mechanism (Heroven & Dersch, 2006). In addition, the expression of *lrhA* in *E. coli* is repressed by the RcsCDB phosphorelay system (a cell-envelope stress-sensing pathway) and induced by

mutations in the FtsK DNA motor protein (Peterson *et al.*, 2006) and in *Pantoea stewartii* by the regulatory protein EsaR (Ramachandran & Stevens, 2013, Ramachandran *et al.*, 2014). In *Y. pseudotuberculosis* the expression of *rovM* is medium-dependent and mediated by CsrC (Heroven *et al.*, 2008). It was also shown that the expression of *lrhA* in *Salmonella* is growth phase-dependent (Mouslim & Hughes, 2014).

2.1.2. Post-transcriptional regulation

Expression of a gene can also be regulated at later stages, when the transcription process has already happened and mRNA was produced. In both prokaryotes and eukaryotes, the messenger RNA can be translated into a protein with different efficiency depending on its sequence, structure and presence of different factors. Such type of control of expression is called post-transcriptional or translational regulation. Generally, the post-transcriptional control of expression involves interactions of different molecules with the mRNA transcripts that affect the process of translation or may be based on different accessibility or stability of the mRNA transcript.

2.1.2.1. Translation initiation efficiency

Commonly, bacterial mRNA contains a translation initiation region (TIR) composed of the initiation codon and the Shine-Dalgarno sequence (SD). The SD site is located 4 to 15 bp upstream of the initiation codon and contains a sequence of nucleotides with variable complementarity to the 3' end of the 16S rRNA. Such complementarity allows the 30S ribosomal to bind to the mRNA upstream of the initiation codon. The level of complementarity is one of the factors contributing to the efficiency of translation (Jacob *et al.*, 1987). Moreover, TIRs can also harbor a short U- or A/U-rich sequence that binds the ribosomal protein S1. The presence of this sequence upstream of the initiation codon enhances the efficiency of translation initiation (Boni *et al.*, 1991). Another important factor that determines the efficiency of translation is the structure of the mRNA transcript. Occasionally, the conformation of the mRNA can inhibit the translation through hampering the access of the 30S ribosomal subunit to the ribosomal binding site (de Smit & van Duin, 1990). Moreover, it has been recently suggested that the synonymous mutations within the first 40 nt of the transcript can significantly affect the abundance of the protein through alterations in the mRNA folding (Kudla *et al.*, 2009).

2.1.2.2. Stability of mRNA

The stability of mRNA transcript comprises another level of post-transcriptional regulation. Generally, the concentration of mRNA within the bacterial cell is a result of balance between the synthesis and the degradation of the mRNA molecules. Therefore, not only the transcription, but also the degradation of mRNA directly affects the synthesis of protein by decreasing the concentration of mRNA available for translation. In many bacterial species, mRNA degradation is modulated in response to changes in the environment and to stress conditions (Redon *et al.*, 2005, Shalem *et al.*, 2008). This mechanism allows quick disposal of unnecessary mRNA and thus prevents the cell from producing the proteins that are no longer required under changed conditions.

In *E. coli* mRNA degradation is generally initiated by endoribonucleolytic cleavages induced by single-stranded RNA-specific endoribonucleases (e.g., RNase E and RNase G) or double-stranded RNA-specific endoribonucleases (e.g., RNase III). This initial nucleolytic step generates primary decay intermediates which are further degraded by a combination of endo- and exonucleases (PNPase, RNase II, RNase R). This stage yields short nucleotides which are later converted to mononucleotides by oligoribonuclease. In addition to major ribonucleases, bacteria possess a number of ancillary mRNA-modifying enzymes that assist the mRNA degradation process (reviewed in (Kaberdin *et al.*, 2011)).

Gene expression can be also influenced by the secondary structure of the RNA molecule. The RNA helicases are proteins that are able to change the secondary structure of RNA molecules by unwinding the RNA or DNA-RNA duplexes and by performing local strand separation. The most well-known group of the RNA helicases are DEAD-box proteins that induce a local strand separation. Such separation can be further used for either protein or regulatory RNA binding to one of the strands. The activity of different bacterial DEAD-box proteins has been implicated in ribosome biogenesis, RNA decay, and translation initiation. (Reviewed in (Khemici & Linder, 2016)).

2.1.2.3. Non-coding RNAs

Non-coding RNA (ncRNA) molecules are widely spread among all kingdoms of life and recently have become recognized as a novel class of gene expression regulators. These molecules encompass a large and diverse group of RNA species that do not encode for proteins and thus do not undergo the translation process. Instead, they present regulatory functions. Regulatory ncRNAs can be divided into several classes: (i) *cis*-encoded base-pairing RNAs (antisense RNAs,

asRNAs), (ii) *trans*-encoded base-pairing RNAs, (iii) riboswitches, (iv) ribozymes, (v) RNAs modulating protein activity, and (vi) CRISPRs (clustered regulatory interspaced short palindromic repeats) (Liang *et al.*, 2011). The asRNAs are encoded on the DNA strand opposite to the coding mRNA and they typically share over 75 nt complementarity with their target mRNA. The *trans*-encoded RNAs are typically encoded in the intragenic regions away from their target mRNA and they share only limited complementarity with the mRNA species. Moreover, unlike cis-encoded RNAs, trans-acting RNAs can regulate more than one mRNA molecule (Michaux *et al.*, 2014). Small RNA molecules (sRNAs) are also known to interact directly with proteins and alter their activity by sequestration (e.g. 6*S* RNA in E. *coli*) (Wassarman, 2007). The ribozymes are catalytic RNAs which typically catalyze cleavage or ligation of another RNA particle through a phosphodiester cleavage reaction (Serganov & Patel, 2007). Riboswitches are metabolite-sensing RNA structures that response to such environmental cues as cations or temperature shifts. Upon a change in the environment they change their conformation leading to activation or inhibition of gene expression (Serganov & Patel, 2007). The last group, CRISPRs, are highly variable regions of 24-47 bp, separated by a series of 2-249 repeat-spacer units. The whole CRISPR region, preceded by a ~550 bp leader sequence, determines resistance to bacteriophages and foreign plasmids (Michaux *et al.*, 2014).

Trans-encoded sRNAs range in size from 50 to 500 nt in length and present various secondary structures. Base-pairing with the target mRNA is usually imperfect and based on 7-10 nt seed sequence. Moreover, for proper functionality trans-encoded sRNAs often require the presence of Hfq chaperone that simultaneously bind to both the sRNA and mRNA and facilitate the interaction (Gottesman & Storz, 2011). The sRNAs regulate the gene expression by base-pairing with the target mRNAs, which leads to changes in mRNA translation or stability. sRNAs can be both activators and repressors of gene expression depending on the location of pairing with the target mRNA. They can act positively by changing the mRNA conformation and preventing formation of an inhibitory structure that sequesters the ribosome-binding site (Fig. 4A). sRNAs can act negatively by base pairing with 5' untranslated region (UTR) and inferring with the ribosome binding (Fig. 4C) or by targeting the sRNA-mRNA duplex for degradation by ribonucleases (Fig. 4D) (Waters & Storz, 2009).

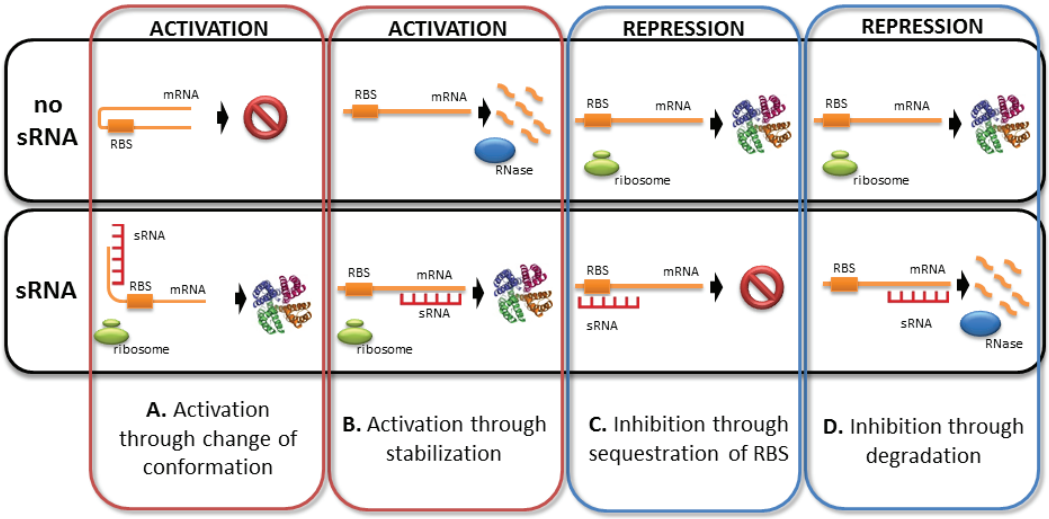

Figure 4. Different regulatory functions of base-pairing regulatory RNAs (modified from (Waters & Storz, 2009)).

The various biological functions of sRNA elements encompass the regulation of metabolism, growth, quorum sensing and biofilm formation, as well as adaptation to stress conditions. Furthermore, recent studies showed that non-coding RNAs play an important role in microbial pathogenesis. Among other functions, during infection process sRNAs serve as signal transducers of external cues and thus allow fast and efficient adaptation to changing environmental conditions. Moreover, several sRNAs have been implicated in regulation of synthesis of virulence factors (Toledo-Arana *et al.*, 2007, Gong *et al.*, 2011, Koo *et al.*, 2011). There are also instances pointing to the existence of cross-kingdom gene silencing between pathogens and their hosts. Even though this system has been mainly described for plant pathogens (reviewed in (Weiberg *et al.*, 2015)), recently two sRNAs from *E. coli*, OxyS and DsrA, were shown to suppress protein-coding genes in *Caenorhabditis elegans* (Liu *et al.*, 2012).

The sizes of *cis*-encoded sRNAs vary considerably. The smallest ones can be as short as 100 nt, but frequently they are substantially longer, ranging from 700 up to 3,500 nt (Opdyke *et al.*, 2004, Stazic *et al.*, 2011). Based on their location relative to the sense strand transcriptional unit asRNAs can be classified as (i) 5'-overlapping, (ii) 3'-overlapping or (iii) internally located (Georg & Hess, 2011). It is generally believed, that the *cis*-acting sRNA species hybridize to their sense mRNA counterparts causing alterations in the secondary structures of both interacting molecules and resulting in changes in the stability and half-life of the mRNA species (Wagner,

1994). Eventually, asRNAs can influence the abundance of mRNA through regulation of transcription, degradation of sense transcripts or their stabilization. Moreover, antisense species can also regulate the translation process through binding to the SD sequence of their target mRNA (reviewed in (Georg & Hess, 2011) and (Sesto *et al.*, 2013)). Recently, the transcriptome analysis revealed that in *Mycoplasma pneumonie, Sinorizobium meliloti, Vibrio cholerae* and *Staphylococcus aureus* antisense transcription rates reach approximately 13%, 11%, 4.7% and 1.3%, respectively (Guell *et al.*, 2009, Liu *et al.*, 2009, Beaume *et al.*, 2010, Schluter *et al.*, 2010). In addition, a recent RNA sequencing-based study conducted on the transcriptome of *E. coli* identified about 1,000 different asRNA species (Dornenburg *et al.*, 2010).

Several recent studies led to identification of various non-coding sRNAs among *Yersinia*-species. A deep RNA-sequencing approach resulted in identification of 150 sRNAs in *Y. pseudotuberculosis* and 31 in *Y. pestis* (Koo *et al.*, 2011, Beauregard *et al.*, 2013). Another approach based on cDNA-cloning allowed verification of 43 novel sRNA from *Y. pestis* (Qu *et al.*, 2012). Recently, several studies focused on their expression under different conditions and their role in bacterial virulence (Koo *et al.*, 2011, Yan *et al.*, 2013). Moreover, it was shown, that some sRNAs, although conserved in both *Yersinia* display different function, suggesting evolutionary changes in sRNA regulation network between these two species (Koo *et al.*, 2011).

2.1.2.4. Usage of rare codons

The efficiency of translation is strongly influenced by the codon bias. The same amino acid can be coded by different triplets of nucleotides, and therefore different tRNAs have to be drawn during the translation. Due to the fact that different tRNA species show different abundance, the synonymous mutation can significantly affect the efficiency of translation (Parmley & Hurst, 2007, Tuller *et al.*, 2010).

2.1.2.5. RNA-binding proteins

Bacterial post-transcriptional regulators typically influence RNA degradation, translation initiation efficiency or transcript elongation. The RNA-binding proteins (RBPs) can use different mechanisms to exert their regulatory functions: (i) change in the mRNA susceptibility to RNases, (ii) modulation of mRNA RBS accessibility, or (iii) acting as a chaperone to facilitate the interaction between mRNA and other factors (Van Assche *et al.*, 2015).

Apart from RNases that are directly involved in mRNA decay (described in 2.2.2.2.), bacteria also possess a number of mRNA modifying enzymes that can facilitate mRNA degradation. For example in *E. coli*, the pyrophosphate removal at the 5' end by pyrophosphate hydrolase (RppH) and addition of a single-stranded poly(A) extension to the 3' end of mRNA facilitated by poly(A) polymerase I (PAPI) both enhance the mRNA degradation. Additionally, the degradation of highly structured RNA molecules can be assisted by the RhlB, which unwinds the RNA structures in an ATP-dependent manner. (Kaberdin *et al.*, 2011, Van Assche *et al.*, 2015).

Regulatory RBPs can alter the efficiency of translation initiation directly by competing with ribosomes for the RBS or indirectly by altering the secondary structure of the mRNA near the ribosome interaction region (Van Assche *et al.*, 2015). One example of RBP that is conserved among many bacterial species is CsrA, a central component of the global carbon storage regulatory system. CsrA binds to GGA-motifs in the 5' UTR near the Shine-Dalgarno region and represses translation by competing with the 30S ribosomal subunit (Baker *et al.*, 2007). Another group of RBPs can affect RNA stability or translation initiation efficiency by assisting in the interactions with other molecules. These proteins typically bind simultaneously the mRNA target and its co-effector molecule that can be a sRNA or another protein. A well-known example of such function is Hfq, RNA-chaperone implicated in global post-transcriptional regulation (Geng *et al.*, 2009, Schiano *et al.*, 2010, Kakoschke *et al.*, 2014). The selected examples of RBPs are presented in Table 4.

Table 4. Selected examples of RNA-binding proteins involved in post-transcriptional gene expression regulation in *Yersinia* species.

Regulator	Target	Function	References
CsrA	GGA-motifs in the 5' UTR	global carbon storage regulation; in *Y. enterocolitica* CsrA activates expression of genes encoding the master motility regulator *flhDC*, enhances resistance to osmolytes and allows growth at 4°C and 42°C	(LeGrand *et al.*, 2015)
Hfq	AU-rich regions	sRNA chaperone that stabilizes the sRNA-mRNA interactions; highly pleiotropic; affects growth, metabolism and virulence	(Geng *et al.*, 2009, Schiano *et al.*, 2010, Kakoschke *et al.*, 2014)
SmpB	Small stable RNA A	in *Y. pseudotuberculosis* affects pathogenesis, resistance to environmental stresses, and motility; enables proliferation in macrophages, affects Yop-mediated cytotoxicity	(Okan *et al.*, 2006)
YopD	5' UTR of T3SS genes	represses expression of T3SS genes, shows highest affinity to effector Yops; prevents ribosome binding and accelerates degradation.	(Chen & Anderson, 2011)

2.1.2.6. RNA chaperone Hfq as a global post-transcriptional regulator

Hfq, an RNA chaperone required for maintaining the stability and function of many sRNAs, has been recognized as a central component of global post-transcriptional regulation network (reviewed in (Vogel & Luisi, 2011)). Hfq was first identified as a host bacterium factor required for the replication of bacteriophage Qβ RNA (Franze de Fernandez *et al.*, 1968). Subsequent research revealed that it is widely distributed in the bacterial kingdom, present in many different pathogenic species. Hfq is a bacterial homolog of the eukaryotic and archeal Sm/LSm proteins, with a characteristic ring-like multimeric quaternary architecture supporting interactions with other macromolecules. In eukaryotes, many different functions were implicated for the Sm/LSm proteins, including role in mRNA splicing, RNA decapping and RNA stabilization (reviewed in (Wilusz & Wilusz, 2005)).

The Hfq of *E. coli* is a 102 amino acid residue (11.2 kDa) highly abundant protein with an estimated 50 000 to 60 000 copies per cell, of which 80 – 90% are found in association with ribosomes (Brennan & Link, 2007). Hfq has a ~25Å thick toroidal structure with an outer diameter of around 70 Å and a 8-12 Å wide central pore. The protein is characterized by an N-terminal α helix followed by β strands displaying the topology β5α1β1β2β3β4. Like other proteins from Sm family Hfq contains Sm1 and Sm2 motives, two highly conserved regions. The Sm1 motif encompasses the first three β strands, whereas the Sm2 motif is located in fourth and fifth β strand. The hexamer structure is formed through the interactions between the residues of β4 and β5 of pairing subunits. The α helix is located on the top of the β sheet and constitutes the distal side of the protein (Sauter *et al.*, 2003).

In *E. coli*, AU-rich sequences of sRNAs typically bind to the proximal surface of the Hfq protein and A-rich sequences of mRNAs bind to the distal surface (Mikulecky *et al.*, 2004). The structure of *S. aureus* Hfq showed that the Sm1 and Sm2 motifs play an important role in RNA binding. The sRNA molecule expands and fills the central pore on the proximal side binding to Hfq through AU-rich regions in a circular manner. The Hfq structure possesses six AU nucleotide binding pockets, yet as many of the sRNAs contain stretches shorter than six U or A, it is unlikely that all the pockets are filled simultaneously (Schumacher *et al.*, 2002). The motif required for binding of A-rich sequences of mRNA is located on the distal side of the Hfq protein, opposite to the AU-binding side (Mikulecky *et al.*, 2004).

There are different mechanisms through which Hfq exhibits its regulatory functions. First, Hfq can suppress protein synthesis by allowing the sRNA to bind to the 5' region of the mRNA

sequestering the translation initiation site. It can also display opposite function by promoting sRNA binding to the 5' region of mRNA in order to disrupt a secondary structure that initially inhibited the translation. By binding to sRNAs Hfq can also protect them from the ribonuclease cleavage or promote the degradation. The mechanism of action depends on the RNA molecules (reviewed in (Vogel & Luisi, 2011)). Recent studies using co-immunoprecipitation and subsequent detection of sRNAs and mRNAs led to identification of a large number of Hfq targets (Zhang *et al.*, 2003, Sittka *et al.*, 2008, Chao *et al.*, 2012, Bilusic *et al.*, 2014).

The regulation of Hfq expression is growth phase dependent. The study showed that the level of Hfq protein is higher during the log phase and decreases when bacteria enter the stationary phase (Kajitani *et al.*, 1994). It is also known, that in *E. coli* CsrA can bind to *hfq* mRNA and inhibit its synthesis by blocking the ribosome binding (Baker *et al.*, 2007).

In most of the studied bacterial species the absence of Hfq results in pleiotropic phenotypic alterations that compromise the fitness and the responses to external cues. Due to its pleiotropic nature, many different defects were observed among Hfq-deficient strains: impaired growth, inability to cope with different types of environmental stresses, higher susceptibility to antimicrobial agents, defects in quorum sensing and host invasion. However, the effects of Hfq-deficiency seemed to be always unique for each bacterial species. Moreover, the virulence of many pathogenic species was attenuated upon depletion of Hfq. The highest levels of attenuation were observed for Gram-negative pathogens like *Brucella abortus, Salmonella spp, Vibrio cholerae,* uropathogenic *E. coli, Neisseria meningitis* and *Y. pestis* (reviewed in (Chao & Vogel, 2010)). Considering the high levels of attenuation observed in many pathogens, it is believed that the Hfq-deficient strains may serve as live attenuated vaccines (Geng *et al.*, 2009, Chao & Vogel, 2010, Schiano *et al.*, 2010, Hayashi-Nishino *et al.*, 2012).

Previous studies showed that Hfq has a profound influence on the fitness of *Y. enterocolitica* O:8 including the metabolism of carbohydrates, nitrogen, iron, fatty acids and ATP synthesis. Moreover, the depletion of Hfq led to slower bacterial growth, decreased resistance to stress and impaired synthesis of urease and yersiniabactin. In addition, the role of Hfq in biofilm formation was implicated for that species (Kakoschke *et al.*, 2014). The *hfq* mutant of *Y. pseudotuberculosis* presented hypermotility and increased production of a biosurfactant-like substance. Furthermore, it showed decreased survival in macrophages, affected biofilm formation, impaired production of T3SS effector proteins and high attenuation rate in mouse

model infection (Schiano *et al.*, 2010, Bellows *et al.*, 2012). Also in *Y. pestis* Hfq was implicated in the persistence inside of macrophages and resistance to stress. Similarly, the loss of *hfq* led to attenuation (Geng *et al.*, 2009).

2.1.2.7. Ribonuclease YbeY

YbeY is a 17-kDa highly conserved protein from the UPF0054 family that was discovered during the global transcriptional analysis as a product of a heat-shock gene. At first, due to the sequence similarity to metal-dependent hydrolases, YbeY was believed to possess a hydrolytic function (Oganesyan *et al.*, 2003, Zhan *et al.*, 2005). Although the structure homology analysis showed similarity to eukaryotic extracellular proteinases such as collagenase and gelatinase, *in vitro* studies failed to detect any hydrolase activity (Rasouly *et al.*, 2009). Only recently it was discovered that YbeY is a ribonuclease that plays a critical role in rRNA maturation, as well as in late-stage 70S ribosome quality control (Jacob *et al.*, 2013).

The structural study revealed that the overall protein structure of YbeY consists of six α helices and four β strands in a βααβαββααα fold. It harbors a conserved domain, characteristic for the UPF0054 family. Moreover it contains a metal ion, most probably a Ni^{2+} ion that is coordinated by the residues of His114, His118 and His124 (Zhan *et al.*, 2005). YbeY shows high level of conservation between the bacterial species, as *ybeY* genes from four distantly related pathogens can fully complement *ybeY* mutant strain of *E. coli* (Vercruysse *et al.*, 2014).

In *E. coli* the YbeY functions as a single strand-specific endoribonuclease that in its purified form effectively degrades total rRNA and mRNA, yet is unable to degrade double-stranded RNA. Moreover, the ribonuclease activity is manifested at 37°C and at 45°C, though it significantly decreases at 65°C, in line with previous findings about YbeY playing a role in heat-shock response. It was also proposed that YbeY cleaves the 17S rRNA precursor generating a 3' phosphate terminus. Furthermore, under standard growth conditions as well as under stress YbeY together with RNase R removes defective 70S ribosomes from the cellular pool allowing effective translation. It was proposed that YbeY acts as a sensor of defective ribosomes by recognizing defects in 30S subunits, which subsequently initiates degradation of complete 70S ribosomes by introducing endonucleolytic cuts in the rRNA. Such damage in rRNA leads to its misprocessing and misfolding and further destruction by other ribonucleases (Jacob *et al.*, 2013). Moreover, YbeY plays a role in transcriptional antitermination of rRNA synthesis, that is also critical for ribosome biogenesis (Grinwald & Ron, 2013). Recent studies show that in *S.*

meliloti, E. coli, as well as in *V. cholerae* YbeY has an impact on small non-coding RNAs (sRNAs) (Pandey *et al.*, 2011, Pandey *et al.*, 2014, Vercruysse *et al.*, 2014).

YbeY belongs to the 206 genes postulated to comprise the minimal bacterial genome set (Gil *et al.*, 2004), but is essential only in some bacteria like *Vibrio cholerae, Haemophilus influenzae* and *Bacillus subtilis* (Akerley *et al.*, 2002, Kobayashi *et al.*, 2003) (Vercruysse *et al.*, 2014). In other species like *E. coli* and *Shinorhizobium meliloti* its loss causes increase in the sensitivity to environmental stresses and in addition in *S. meliloti* its loss abrogates intracellular infection necessary for the symbiosis (Davies & Walker, 2008, Rasouly *et al.*, 2009). In *E. coli* the *ybeY* deletion mutant presented severe translational defects caused by very low level of functional polysomes and accumulation of free ribosomes and ribosomal subunits. Translational defects were mostly manifested at elevated temperatures and resulted in growth failure (Rasouly *et al.*, 2009). In *V. cholerae* loss of YbeY resulted in complete loss of mouse colonization and biofilm formation, reduced cholera toxin production, as well as alterations in expression of virulence-related sRNAs (Vercruysse *et al.*, 2014). Additionally, it was implicated that in *E. coli* YbeY plays a role in apoptosis-like death (Erental *et al.*, 2014).

An YbeY endoribonuclease homolog was also observed in eukaryotes. The *ybeY* null mutants of *Arabidopsis thaliana* are seeding lethal, suggesting an important role of this protein in plant growth. The *ybeY* mutant displayed slow growth, impaired photosynthesis, defective chloroplast development and alterations in rRNA maturation (Liu *et al.*, 2015).

2.1.3. Post-translational regulation

The last step of gene expression regulation is based on post-translational modifications (PTM) that alter the structure or the function of a synthetized protein. PTMs can alter the activity of a protein by having an impact on protein complex formation, enzyme catalysis or interactions with other biomolecules. The possible modifications in prokaryotes include phosphorylation, acetylation, methylation, carboxylation, glycosylation, lipidation, adenylation, ribosylation, nitrosylation, oxidation, pupylation and deamination (reviewed in (Cain *et al.*, 2014)).

It was previously shown that PTMs contribute significantly to bacterial adaptability and cell cycle control. Protein phosphorylation, the attachment of phosphate onto the functional groups of amino acid side chains, is probably the most extensively studied type of modification. Different bacterial kinases are involved in signal trafficking in regulatory networks (reviewed in (Cain *et al.*, 2014)). PTMs have also a profound impact on bacterial physiology and virulence.

Campylobacter jejuni, impaired in protein glycosylation, showed decreased ability to adhere and invade eukaryotic cells and lost ability to colonize intestinal tracts of mice (Szymanski *et al.*, 2002). Although single PTM can already change the function of a protein, in some cases a protein can undergo several modifications at competing sites. Interplay between acylation and phosphorylation was previously observed in bacteria (Liarzi *et al.*, 2010).

2.2. The genus Yersinia

Yersiniae are Gram-negative, rod-shaped, facultative anaerobic, non-spore-forming bacteria, about 2 μm long and 0.6 μm in diameter. They are named after Alexandre Yersin, a bacteriologist who discovered *Yersinia pestis* in 1894. *Yersinia* species are widely distributed in nature and adapted, depending on the species, to survive in the outside environment and/or within a specific animal host.

The genus Yersinia belongs to the family *Enterobacteriaceae*, within the class *Gammaproteobacteria* of the phylum Proteobacteria (Garrity, 2005). Currently, this genus consists of 17 species of which three, *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, are infect humans, and one, *Y. ruckeri*, fish (Ewing *et al.*, 1978). *Y. pestis* is a causative agent of plaque, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens that usually cause self-limited gastroenteritis, yersiniosis, typically restricted to the intestinal tract and lymph nodes. *Y. ruckeri* causes enteric red mouth disease, which affects mainly salmonid fish (Ewing *et al.*, 1978). Other species belonging to this genus are considered to be environmental bacteria appearing mainly in fresh water and terrestrial ecosystems and include *Y. aldovae, Y. alecksiciae, Y. bercovieri, Y. entomophaga, Y. fredriksenii, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretti, Y. nurmii, Y. pekkanenii, Y. rohdei,* and *Y. similis* (Garrity, 2005, Sprague & Neubauer, 2005, Merhej *et al.*, 2008, Sprague *et al.*, 2008, Hurst *et al.*, 2011, Murros-Kontiainen *et al.*, 2011, Murros-Kontiainen *et al.*, 2011)

2.2.1. Yersinia enterocolitica

Currently *Y. enterocolitica* is the most common pathogenic *Yersinia* infecting humans. Based on DNA-DNA hybridization and differences in 16S rRNA sequences, *Y. enterocolitica* was divided into two subspecies: *enterocolitica* and *palearctica* (Neubauer *et al.*, 2000). A further division of the species is based on the biochemical heterogeneity and it distributes the strains into six

biotypes: 1A, 1B, 2, 3, 4 and 5 (Wauters *et al.*, 1987). Generally, the biotype 1B is considered to be pathogenic, biotypes 2-5 are characterized by low pathogenicity, and biotype 1A includes non-pathogenic strains (Bottone, 1997). Furthermore, *Yersinia* species are additionally divided into different serotypes based on the variability of O-antigen structure. *Y. enterocolitica* subsp. *enterocolitica* comprises the biotype 1B strains formerly termed North American and is represented by the serotype O:8 ATCC 9610 strain, while *Y. enterocolitica* subsp. *palearctica* comprises the former European strain from biotypes 1A and 2-5 with the serotype O:3 strain Y11 being the postulated representative (Neubauer *et al.*, 2000).

Typically *Y. enterocolitica* causes self-limiting gastroenteritis with non-specific diarrhea, fever, abdominal pain, and sometimes nausea and vomiting. Normally symptoms of acute illness appear after 1-11 days of incubation and last for about 5-14 days (Cover & Aber, 1989). The most common post-infectious sequelae include reactive arthritis and erythema nodusum, more rarely erythema multiforme, uveitis, and conjunctivitis (Saari *et al.*, 1980, Bottone, 1997). Moreover, *Y. enterocolitica* can lead to such extraintestinal complications as liver and spleen abscesses, pneumonia, meningitis, empyema and endocarditis (Zheng *et al.*, 2008). In immunocompromised patients yersiniosis can result in septicemia (Zheng *et al.*, 2008). Additionally, asymptomatic carriage of *Y. enterocolitica* is has been reported (Van Ossel & Wauters, 1990).

Most of the yersiniosis cases are sporadic, however, rare outbreaks have also been reported, with sources tracked to contaminated water, dairy and porcine products (Thompson & Gravel, 1986, Marjai *et al.*, 1987, Ackers *et al.*, 2000). The incidence of yersiniosis is hard to estimate as it is rarely reported, and usually only the genus name is recorded. Nevertheless, vast majority (around 91%) of the reported cases are due to *Y. enterocolitica*, with the 4/O:3 being the most common bio/serotype worldwide (Long *et al.*, 2010, EFSA, 2014). Currently, *Y. enterocolitica* is the third most commonly reported cause of enteric zoonosis in European Union, after *Salmonella* and *Campylobacter,* with 6 471 confirmed cases of yersiniosis reported in 2013 (EFSA, 2014). Finland is among the countries with the highest incidence rate in EU, with the number of reported cases between 500 and 700 per year. Even though a decreasing trend in the number of cases was observed, the incidence rate of yersiniosis is still higher than salmonellosis. Moreover, also in Finland the bio/serotype 4/O:3 is the most predominant one. (EFSA, 2014).

Raw pork meat has been shown to be the most important reservoir of enteropathogenic *Yersiniae* (Tauxe *et al.*, 1987, Rosner *et al.*, 2012). In 2013 positive findings were reported mainly for the pig meat and products thereof, but also for bovine meat and raw cow milk. In addition, *Y. enterocolitica* was found in other animal species, including cattle, sheep, goat, dogs and cats (EFSA, 2014).

2.2.1.1. General characteristics

Y. enterocolitica is capable of anaerogenic fermentation of glucose and other carbohydrates, produces urease, and lacks lysine decarboxylase and phenylalanine deaminase activities. Additionally, a distinctive characteristic of temperature dependent motility is observed, as bacteria display motility when grown at 27°C, but not when grown at 37°C. Despite high similarity, *Y. enterocolitica* can be differentiated from *Y. pseudotuberculosis* by indole and acetyl methyl carbinol production, ornithine decarboxylation, sucrose fermentation and incapability to ferment rhamnose (Sedgwick & Tilton, 1971). The biochemical characteristics of *Y. enterocolitica* are summarized in Table 1.

Table 1. Biochemical reactions of *Y. enterocolitica* (modified from (Sedgwick & Tilton, 1971)).

Substrate or test	Reaction	Substrate or test	Reaction
Triple sugar iron butt	A	Catalase	+
Slant	A	Dextrose	A
H_2S	-	Lactose	-
Urease production	+	Sucrose	A
Indole production	+	Salicin	-
Nitrate reduction	+	Maltose	A
Citrate utilization	-	Mannitol	A
Voges-Proskauer at 35°C	-	Rhamnose	-
similar reaction at 27°C	+	Arabinose	A
Lysine decarboxylase	-	Raffinose	-
Arginine decarboxylase	-	Inositol	A
Ornithine decarboxylase	+	Xylose	A
Oxidase	-	Sorbitol	A

+, positive; -, negative; A, production of acid.

The optimal temperature for growth of *Y. enterocolitica* is 28°C-30°C, but it is able to grow in a temperature range from 0°C to 44°C (Garrity, 2005). At temperatures below 5°C the growth is

significantly slowed down and the bacterium undergoes cold adaptation that includes alterations in the compositions of lipids and proteins in the cell membrane (Goverde *et al.*, 1994). Moreover, *Y. enterocolitica* can survive in frozen food product for extended period of time as well as withstand repeated freezing and thawing (Asadishad *et al.*, 2013). *Y. enterocolitica* can grow in pH range between 4.0 and 10.0, with the optimum around 7.2 - 7.4, and tolerate sodium chloride concentrations up to 5% (Garrity, 2005). It is able to grow under both aerobic and anaerobic conditions, though the presence of carbon dioxide affects the growth rate by prolonging the lag phase of the organism (Pin *et al.*, 2000).

2.2.1.2. Virulence factors

2.2.1.2.1. Plasmid encoded virulence factors

Virulence factors of *Y. enterocolitica* are encoded both on the bacterial chromosome and on the 70-kb virulence plasmid (pYV). The latter one encodes, among the others, the proteins of the type III secretion system (T3SS), effector proteins (Yops) and adhesion protein YadA (Table 2). The presence of pYV is strictly necessary for virulence, yet not sufficient (Gemski *et al.*, 1980, Heesemann & Laufs, 1983, Heesemann *et al.*, 1984). The injectosome of T3SS of *Y. enterocolitica* consists of 29 Ysc proteins (schematic model with the major proteins presented in Fig. 1). Its main function is to translocate the effector proteins from the bacterial cells into the eukaryotic host cytosol without extensive leakage. Ten of these Ysc proteins (YscD, -J, -L, -N, -Q, _R, -S, -T, U, and –V) are conserved, typical for all T3SSs and constitute the proximal part attached to the membrane (Cornelis, 2002). The injectosome is anchored by the basal body in the peptidoglycan and membranes with a ring-shaped structure with external diameter of about 200 Å and a central pore of about 50 Å. Basal body constitutes a base for a needle that is 600-800 Å in length and 60-70 Å in width (Koster *et al.*, 1997, Hoiczyk & Blobel, 2001). The needle tip consists of a hypothetical structure called the pore complex, which connects the needle with the target host cell and forms a pore within its membrane (Dewoody *et al.*, 2013). Based on the size of the molecules that can be transported through the pore complex, its size is estimated to be about 16 – 23 Å (Neyt & Cornelis, 1999). T3SS was generally believed to translocate the effector proteins from the bacterial cytosol to the target-cell cytoplasm in one step process, but recent research showed that the translocation of Yops can also occur through a different mechanism that involves an intermediate extracellular step (Akopyan *et al.*, 2011).

Table 2. Overview of the major virulence factors of *Y. enterocolitica*.

Factor	Function	Reference
pYV encoded		
Ysc (29 proteins)	components of the T3SS injectosome	reviewed in (Cornelis, 2002, Dewoody *et al.*, 2013)
LcrV	interacts with YopB and YopD, required for translocation of the effector proteins, regulatory role in Yop synthesis	(Nilles *et al.*, 1998, Sarker *et al.*, 1998)
YopN	regulatory function involved in calcium response (part of calcium plug complex)	(Forsberg *et al.*, 1991)
YopB **YopD**	together form a pore complex within the host membrane, responsible for translocation of other effector proteins from bacterium to the cytosol of the host cell	(Montagner *et al.*, 2011)
YopE	cytotoxic, antiphagocytosis activity, inactivates Rho family of GTPases and disrupts actin microfilament of the target cell	(Rosqvist *et al.*, 1991, Mejia *et al.*, 2008)
YopH	phosphotyrosine phosphatase, dephosphorylates host proteins ($p130^{Cas}$, paxillin, FAK), exerts a negative role on the cytoskeleton dynamics thus contributes to resistance to phagocytosis by macrophages	(Black & Bliska, 1997, Persson *et al.*, 1997, Black *et al.*, 1998)
YopT	depolymerizes actin by modifying a GTPase that regulates the formation of stress fibers (RhoA), contributes to resistance to phagocytosis by macrophages	(Zumbihl *et al.*, 1999)
YopO	serine-threonine kinase, disrupts actin cytoskeleton, interacts with RhoA and Rac-1, contributes to resistance to phagocytosis by macrophages	(Barz *et al.*, 2000, Dukuzumuremyi *et al.*, 2000)
YopP **YopJ**	anti-inflammatory activity, induce apoptosis in phagocytic cells, interfere with MAPK signaling pathways, inhibit activation of NF-κB by preventing the phosphorylation of IκB, reduce the release of TNF-α and IL-8, reduce the presentation of ICAM-a and E-selectin on the surface of endothelial cells	(Mills *et al.*, 1997, Boland & Cornelis, 1998, Schesser *et al.*, 1998, Orth *et al.*, 2000, Denecker *et al.*, 2002)
YopM	strongly acidic, leucine-rich protein, is delivered to the target cell and traffics to the nucleus by a vesicle-associated pathway, documented contribution to pathogenesis in *Y. pestis*, its action in the nucleus still remains unknown	(Leung *et al.*, 1990, Boland *et al.*, 1996, Skrzypek *et al.*, 1998)
YopQ	regulatory function, controls translocation fidelity	(Dewoody *et al.*, 2013)
YadA	adhesion to epithelial cells and inhibition of receptor – mediated phagocytosis, binds collagen, fibronectin and laminin, mediates invasion into tissue culture cells, binds to neutrophils, crucial for serum resistance by binding factor H and C4Bp	(El Tahir & Skurnik, 2001, Biedzka-Sarek *et al.*, 2005)
Chromosome encoded		
Inv	Invasin; binds to β1-integrins and promotes translocation of bacteria across the intestinal epithelium, required for colonization of the lymph nodes	(Miller & Falkow, 1988, Isberg & Leong, 1990, Pepe & Miller, 1993)
Ail	Attachment invasion locus; mediates attachment and invasion into eukaryotic cells, contributes to serum resistance	(Miller & Falkow, 1988, Biedzka-Sarek *et al.*, 2005)
LPS	Lipopolysaccharide; required for effective colonization of host tissues in early stage of infection, allows invasion of deeper tissues, renders resistance to cationic antimicrobial peptides, contributes indirectly to serum resistance	(al-Hendy *et al.*, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999, Biedzka-Sarek *et al.*, 2005, Reines *et al.*, 2012)
Yst	heat-stable enterotoxin; associated with fluid secretion and diarrheal symptoms	(Delor & Cornelis, 1992)

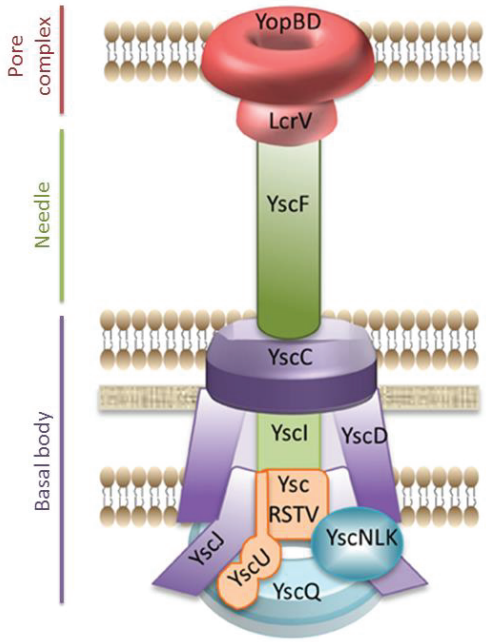

Figure 1. Model of the *Y. enterocolitica* T3SS injectosome (modified from (Dewoody *et al.*, 2013)).

The expression of T3SS is regulated by temperature and calcium, being expressed maximally at 37°C in absence of calcium (Straley *et al.*, 1993). In medium containing calcium, secretion of effector proteins is prevented by forming of the YopN-TyeA-YscB-SycN complex, known as the calcium plug (Forsberg *et al.*, 1991, Dewoody *et al.*, 2013). Under favorable conditions, at 37°C in the low calcium medium or upon the contact with host cells, effector proteins are released. The Yop effector proteins serve different functions; some are directly involved in attacking the host cells while other bear regulatory functions. Four of them, YopE, YopH, YopT and YopO interfere with different signal transduction pathways, disturb the host cytoskeleton dynamics and thus contribute to the resistance to macrophage phagocytosis (reviewed in (Fallman *et al.*, 1997)). The functions of the Yop effector proteins are listed in Table 2.

Yersinia adhesion A (YadA) is expressed as a homotrimeric 200-240 kDa, lollipop-shaped, outer membrane protein (Skurnik *et al.*, 1984). It mediates adherence to epithelial cells, professional phagocytes and proteins of the extracellular matrix. Furthermore, during the course of infection, YadA protects the bacterium from complement mediated lysis (Biedzka-Sarek *et al.*, 2005). It also mediates the autoagglutination of bacteria that is most likely another means of

protection against host defenses (Skurnik *et al.*, 1984). It was shown, that YadA is an essential virulence factor for *Y. enterocolitica* (reviewed in (El Tahir & Skurnik, 2001)).

2.2.1.2.2. Chromosomally encoded virulence factors

Even though pYV is necessary for virulence of *Y. enterocolitica*, the chromosomally encoded virulence factors are needed for full infectivity (Revell & Miller, 2001) (Table 2). One important aspect of virulence is the ability of these bacteria to invade intestinal epithelial cells, which is mediated by invasin (Inv) and Ail (attachment and invasion locus) (Miller & Falkow, 1988). Invasin is a 92 kDa outer membrane protein, the expression of which is usually highest at ambient temperature, prior to initiation of infection in the eukaryotic host. It serves as a primary invasion factor and is necessary for efficient penetration of the intestinal barrier. In a mouse infection model, Inv negative strains are unable to colonize Peyer's patches early after infection and showed 3-4 day delay in colonization when compared with the wild type (Pepe & Miller, 1993). *In vivo*, invasin promotes the penetration of *Y. enterocolitica* into M cells by adhesion to β1-integrins located on the surface of epithelial cells (Isberg & Leong, 1990). In *Y. enterocolitica* serotype O:3 the synthesis of invasin is highly activated and nearly constitutive, with no temperature dependence due to acquisition of IS1667 insertion sequence in the *invA* regulatory region (Uliczka *et al.*, 2011). Ail is a 17 kDa integral membrane protein with four surface-exposed loops. In *Y. enterocolitica* it mediates the attachment and invasion into the host cells, as well as serum resistance (Miller *et al.*, 2001, Biedzka-Sarek *et al.*, 2005). Previously, the presence of attachment and invasion locus in the genome has been associated exclusively with pathogenic *Y. enterocolitica* strains (Miller *et al.*, 1989), yet recent study detected Ail in some biotype 1A strains (Sihvonen *et al.*, 2011).

One important virulence factor encoded chromosomally is lipopolysaccharide (LPS), a major component of Gram-negative bacterial outer membrane. It consists of three main structural components: the most external O-specific polysaccharide (O-antigen, O-Ag), the core region (inner and outer cores) and the lipid A, which anchors the whole structure into the membrane (Fig. 2) (Muszynski *et al.*, 2013). The O-Ag of the O:3 serotype is a homopolymer composed of 6-deoxy-L-altrose repeating units, linked together by 1,2 linkages (Hoffman *et al.*, 1980, Skurnik *et al.*, 1999). The inner core (IC) is composed of seven sugar residues, while the outer core (OC) includes six residues (Radziejewska-Lebrecht *et al.*, 1994, Skurnik *et al.*, 1995). Untypically for Gram-negative bacteria, in *Y. enterocolitica* O:3, there are two distinctive types of LPS molecules present on the bacterial surface. One form constitutes of molecules where the O-Ag is linked to

the IC, whereas in the other form is the OC attached to the IC (Pinta *et al.*, 2012, Muszynski *et al.*, 2013). The enterobacterial common antigen (ECA) is uniquely present in *Enterobacteriaceae* and constitutes of a polysaccharide build of →3)-α-D-Fuc*p*4NAc-(1→4)-β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→ repeats. The ECA polymer is either covalently anchored to the outer membrane (via its own L-glycerophospholipid or via the core of LPS) or is found in cyclic form in the periplasmic space (Lugowski *et al.*, 1983, Kuhn *et al.*, 1988, Kajimura *et al.*, 2005). The role of ECA is not fully known, it is assumed it has a protective function against environmental stresses, confers resistance to low pH, bile salts, host defense mechanisms, and is needed for motility (Danese *et al.*, 1998, Barua *et al.*, 2002, Ramos-Morales *et al.*, 2003, Castelli *et al.*, 2008).

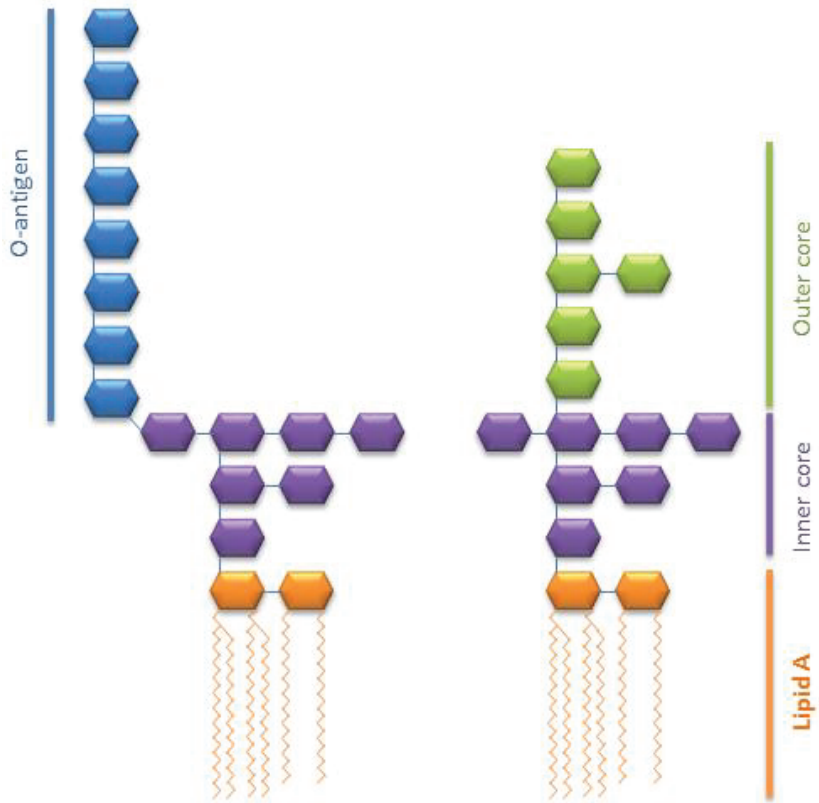

Figure 2. A schematic structure of two *Y. enterocolitica* O:3 LPS forms (modified from (Skurnik *et al.*, 1995, Pinta *et al.*, 2012, Muszynski *et al.*, 2013)).

In *Yersinia enterocolitica* serotype O:3 the OC and O-ag biosynthetic genes are organized into two distinct gene clusters. The OC gene cluster consists of nine genes (*wzx*, *wbcKLMNOPQ* and *gne*) and is located between the *hemH* and *gsk* genes (Skurnik *et al.*, 1995), whereas the O-antigen gene cluster consists of two operons and eight genes (*wbbSTU*, *wzm*, *wzt*, *wbbVWX*) (Zhang *et al.*, 1993, Skurnik & Bengoechea, 2003). The expression of the O-ag is regulated at transcriptional level and dependent on the temperature of growth. The optimal expression occurs at 25°C, while at 37°C the number of repeating units per LPS molecule is much lower. However, temperature seems to affect the transcription only in the stationary phase, whereas in the exponential phase the O-ag is expressed constitutively (Lahtinen *et al.*, 2003). The detailed mechanism of this regulation is still unknown.

LPS confers to the virulence of Gram-negative bacteria and their resistance to antimicrobial compounds (Al-Hendy *et al.*, 1992, Biedzka-Sarek *et al.*, 2005, Reines *et al.*, 2012). It has been shown previously that the O-antigen and OC are essential for full virulence of *Y. enterocolitica* O:3 and O:8 and loss of either of them leads to severe attenuation in murine models (Al-Hendy, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999) . The O-ag is required for effective colonization of host tissues during the first hours of infection, while the OC allows colonization of the deeper organs and prolonged persistence of the bacteria in the Peyer's patches (Al-Hendy, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999). Moreover, it was shown that LPS contributes to serum resistance indirectly, by blocking the other factors present on the surface of bacterial cell (Biedzka-Sarek *et al.*, 2005).

In addition, another gene located in the chromosome showed important involvement in the virulence of *Y. enterocolitica*. The heat-stable enterotoxin Yst appears in three different variants: YstA, YstB and YstC. It was shown in young rabbit model, that Yst plays an important role in fluid secretion and development of diarrhea (Delor & Cornelis, 1992).

2.2.1.2.3. Virulence-related features

In addition to typical virulence factors, *Y. enterocolitica* possesses other metabolic features that contribute to virulence. One of these features is production of urease, an enzyme that catalyzes the hydrolysis of urea to ammonia and carbamic acid, which subsequently spontaneously hydrolyzes to carbonic acid and ammonia. During the growth in external environment this enzyme enables bacteria to utilize urea as a source of nitrogen. However, as the hydrolysis of urea causes local increase in pH, urease is necessary for *Y. enterocolitica* to survive the passage through the acidic stomach of the host (De Koning-Ward & Robins-Browne, 1995). Unlike in

some other enteropathogens, acid tolerance of *Y. enterocolitica* depends only on production of urease. The regulation of urease expression in *Y. enterocolitica* is regulated by the growth phase, with maximal activity during the stationary phase, and temperature, with the activity being higher at 28°C than at 37°C. However, urease activity is not regulated by nitrogen limitation (de Koning-Ward & Robins-Browne, 1997).

Iron is an essential nutrient for almost all bacterial species. While this chemical element is easily available in natural environments and most of culture media, the concentration of free iron within the host tissues is remarkably low. Usually in mammalian tissues iron is bound to carrier proteins like transferrin and lactoferrin that further restricts its availability for bacteria. Many pathogens acquire iron from the host by secreting high-affinity iron-binding siderophores. Production of siderophores, mainly yersiniobactin, was reported for many strains of pathogenic *Yersinia*, including *Y*. *enterocolitica* biotype 1B. Therefore, without available iron, the low-pathogenic strains of *Y. enterocolitica* can cause only moderate intestinal syndromes, while patients with iron overload a may develop a systematic yersiniosis (Chambers & Sokol, 1994, Carniel, 2001). Moreover, patients treated with Desferal (commercially available form of iron-chelating deferoxamine) have been reported to be more prone to develop septicemia caused by *Y. enterocolitica* (Boelaert *et al.*, 1987). The virulence-enhancing effect of Desferal depends on the production of outer membrane protein FoxA that acts as a receptor for deferoxamine (Baumler & Hantke, 1992). Morover, Desferal was also shown to have a potential immunosuppressive effect on the host (Autenrieth *et al.*, 1994, Autenrieth *et al.*, 1995).

Another feature with implicated role in virulence is motility. It was shown, that flagellum-dependent motility in *Y. enterocolitica* ensures migration of the bacteria to host cells. In HEp-2 cell culture model non-motile strain displayed impairment in invasion. However, the same strain showed no difference in invasion when bacteria were brought into contact with host cells by centrifugation (Young *et al.*, 2000). Moreover, it was previously implicated, that some virulence genes can be regulated as part of the flagellar regulon (Young *et al.*, 1999). In *Y. enterocolitica ylpA*, a gene required for survival in Peyer's patches and stimulation of the acute inflammatory response, is a part of flagellar regulon (Schmiel *et al.*, 1998). Therefore the alterations in motility may have both direct and indirect effect on the virulence of this enteropathogen. Due to high similarity of flagellar regulon it is likely that many members of *Enterobacteriaceae* share the same type of regulation, which follows a hierarchical cascade with three major gene classes I, II, and III. The *flhDC* operon (class I) is at the top of the hierarchy

and it regulates the expression of class II genes. Class II consists of structural and accessory proteins, as well as sigma factor, which allows the transcription of the class III genes (proteins involved in maturation of flagellar system) (Young, 2004). Moreover, it has been demonstrated that motility is regulated by AHL-dependent quorum sensing in *Y. enterocolitica* (Atkinson *et al.*, 2006).

2.3. Coordination of gene regulation in Yersiniae

Pathogenic *Yersinia* species are well adapted for survival in various external environments and persistence in a variety of host organisms. During the invasion process, bacteria must adjust rapidly to several extremely different environments, such as gastric acidity, altered osmolarity, changing nutrient availability, and competition with the microbiota colonizing the host organism. In order to establish infection several virulence factors must be employed. That process involves precise regulation of expression that ensures that these virulence factors and adequate metabolic pathways are produced at the correct spatiotemporal conditions. The regulation is achieved at transcriptional, post-transcriptional and post-translational levels through the employment of various transcription factors, nucleoid-associated proteins, ncRNAs and ribonucleases (Heroven & Dersch, 2014, Erhardt & Dersch, 2015).

Although, the majority of the mechanisms and factors involved in gene regulation in *Yersiniae* are highly conserved, yet the network composition and architecture may vary between the species (Erhardt & Dersch, 2015). During the first stages of host colonization bacteria have to survive the acidic environment of the stomach. For this the bacteria use urease, an enzyme that counteracts low pH by neutralizing it with ammonia generated from urea (Young *et al.*, 1996). The regulators as TCS OmpR/EnvZ and Hfq chaperone have been implicated in the regulation of urease biosynthesis in *Yersinia* species (Hu *et al.*, 2009, Kakoschke *et al.*, 2014). The gastrointestinal tract is a rich source of food, yet the availability of nutrients depends on the host and there is a constant competition with the resident microbiota for resources. This phase of infection depends to a large extend on the ability to sense the available nutrients and to response adequately by changing the expression of enzymes of the metabolic pathways. This stage involves such regulators as the PhoP/PhoQ TCS, the global carbon storage regulator (Csr), cAMP receptor protein Crp, the UvrY/BarA TCS, and the sRNA regulatory network in cooperation with the Hfq chaperone (reviewed in details in (Heroven & Dersch, 2014)).

Subsequently, bacteria attach to and invade the M cells overlaying the Peyer's patches in the ileum (Grutzkau *et al.*, 1990). The key virulence factors of this stage are the outer membrane proteins invasin, Ail, and YadA, that are regulated by the MarR-type regulator RovA, H-NS, and YmoA. Moreover, the ability of bacterium to internalize is also enhanced by the flagella-mediated motility, which is controlled by the master operon *flhDC* (reviewed in (Erhardt & Dersch, 2015)). From the Peyer's patches bacteria disseminate to the mesenteric lymph nodes, liver and spleen (Cornelis & Wolf-Watz, 1997). This phase of infection requires the expression of *ysc* genes encoding the T3SS and the Yop effector proteins. These genes are encoded on the pYV and their transcription is activated by the LcrF(VirF) regulator (Bolin *et al.*, 1988). The ability of *Yersinia* spp. to avoid the host defense mechanisms depend also on the expression of LPS, YadA and Ail, due to their involvement in the resistance against the host serum complement system (Biedzka-Sarek *et al.*, 2005).

The sensing of the environment occurs through different cues, yet the changes in temperature is the most important signal received by the pathogenic *Yersiniae*. Temperature-dependent regulation of the gene expression happens through several different regulators. The expression of T3SS and associated Yop effector proteins is allowed only at body temperatures and controlled by LcrF/VirF, YmoA and levels of supercoiling of the pYV (Lambert de Rouvroit *et al.*, 1992, Hoe & Goguen, 1993, Rohde *et al.*, 1999, Bohme *et al.*, 2012). The temperature-dependent regulation of Inv expression is governed by the RovA regulator, an intristic temperature-sensing protein (Revell & Miller, 2000, Herbst *et al.*, 2009, Quade *et al.*, 2012). The loss of motility at 37°C occurs through σ^{28}/FliA without the involvement of the *flhDC* master operon (Kapatral *et al.*, 2004). Several virulence genes of *Yersinia* are expressed in response to changes in the environmental concentration of Ca^{2+} in a process referred to as low calcium response (Goguen *et al.*, 1984, Straley *et al.*, 1993). The changes in temperature and osmolarity are sensed by the TCS OmpR/EnvZ that controls the expression of the outer membrane porins (Dorrell *et al.*, 1998, Brzostek *et al.*, 2012). Another transcriptional regulator, IscR, responds to oxidative stress, oxygen limitation and iron availability and controls the expression of T3SS through the LcrF(VirF) (Miller *et al.*, 2014). In *Yersinia* the response to different carbon sources is mediated by BarA/UvrY, Crp and Csr systems (Poncet *et al.*, 2009, LeGrand *et al.*, 2015). The low pH and low levels of magnesium in the environment are the trigger factors affecting the expression of the PhoP/PhoQ TCS (Groisman, 2001). In addition, the CpxAR system is responsible for responses to the extra-cytoplasmatic stress in *Yersinia* (Liu *et al.*, 2012). The

regulatory networks that control the expression of virulence-related factors and motility in response to different environmental cues in *Yersinia* are presented in Figure 5. The details regarding the function of selected regulators controlling the gene expression in *Yersinia* are summarized in Table 5.

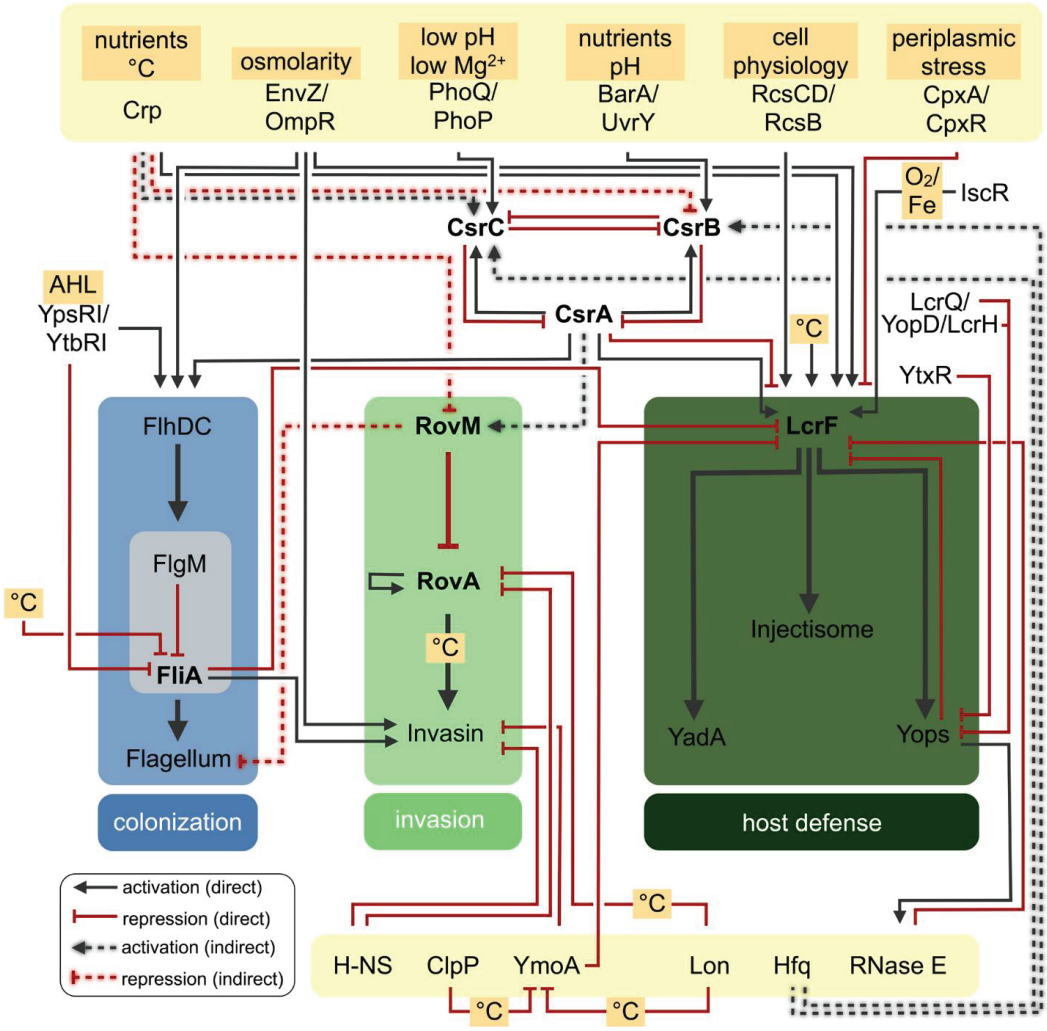

Figure 5. Regulatory networks controlling *Yersinia* virulence factors (reproduced from (Erhardt & Dersch, 2015) with the permission of the author).

Table 5. Selected examples of transcriptional regulators present in *Yersinia* species.

Regulator	Activity	References
RovA	positive regulation of invasin (*inv*) gene expression; important virulence factor; probably regulates genes in addition to *inv*	(Revell & Miller, 2000, Ellison *et al.*, 2004)
RovM	repression of RovA expression; elimination of *rovM* increases the virulence of *Y. pseudotuberculosis* in the mouse model;	(Heroven & Dersch, 2006)
PhoP/ PhoQ	in *Y. enterocolitica* affects the expression of *rovA* and *lpxR*; regulates lipid A deacylation; allows intracellular survival of *Y. pestis*; elimination of *phoP* in *Y. pestis* results in partial attenuation; shows positive and negative regulation of number of proteins	(Oyston *et al.*, 2000, Reines *et al.*, 2012, Reines *et al.*, 2012) }
OmpR/ EnvZ	control of the expression of outer membrane porins in response to changes in the osmolarity of the environment; adaptation to high osmolarity, oxidative stress and low pH; control of motility and biofilm; negative regulation of invasin (*inv*) gene expression	(Brzostek & Raczkowska, 2007, Raczkowska *et al.*, 2011, Raczkowska *et al.*, 2011, Brzostkowska *et al.*, 2012)
FlhDC	control of motility, flagellation and biofilm formation; the active heteromultimeric form ($FlhD_4C_2$) directs the σ70-RNA polymerase to transcribe genes with flagellar Class 2 promoters; repressed by OmpR in response to extracellular osmolarity; affects the expression of T3SS	(Young *et al.*, 1999, Bleves *et al.*, 2002, Raczkowska *et al.*, 2011)
LcrF	in a temperature-dependent manner activates the transcription of T3SS and associated Yop effector genes; AraC-type regulator; repressed by YmoA-H-NS complex; affected also by different environmental cues through different regulators	(Bohme *et al.*, 2012, Hoe & Goguen, 1993, Lambert de Rouvroit *et al.*, 1992, Rohde *et al.*, 1999)
Crp	cAMP receptor protein; by binding to cAMP signal molecule forms the active complex cAMP-Crp, that controls genes of different metabolic pathways; required for growth on different carbon sources and under limited carbon, nitrogen and phosphate sources.	(Petersen & Young, 2002, Heroven *et al.*, 2012, Lathem *et al.*, 2014)
CsrA	controls the translation of a large number of metabolic genes by binding to the 5'-untranslated regions of their mRNAs; inhibited by two sRNA species CsrB and CsrC, that have higher affinity to CsrA than other targets and thus sequester the binding sites of CsrA; affects the expression of RovA	(Liu & Romeo, 1997, Heroven *et al.*, 2008, Heroven *et al.*, 2012, Heroven *et al.*, 2012, LeGrand *et al.*, 2015)
BarA/UvrY	activated by metabolic end products and alterations in the citric acid cycle; controls the metabolism by affecting the Csr system; regulates the expression of adhesion and invasion genes	(Chavez *et al.*, 2010, Dahiya & Stevenson, 2010, Heroven *et al.*, 2012)
CpxAR	responds to extra-cytoplasmic stress, important for the bacterial envelope integrity; affects the expression of T3SS elements and RpoE	(Ronnebaumer *et al.*, 2009, Hunke *et al.*, 2012, Liu *et al.*, 2012)

3. AIMS OF THE STUDY

During the course of infection bacterial cell passes through different niches. Sensing properly environmental cues allows it to respond accordingly, survive and establish infection. Therefore for pathogens, the gene regulation also means adaptation to the host and coordination of expression of virulence factors, either directly or indirectly by affecting global regulators.

The details of bacterial gene expression regulation are not fully understood. This thesis work was undertaken to increase the knowledge of the regulatory networks and involvement of different regulators in bacterial physiology and virulence. In this study, I aimed at using two high-throughput methods, RNA-sequencing (RNA-seq) and quantitative proteomics (LC-MS/MS), to identify mRNAs and proteins of *Yersinia enterocolitica* O:3 dependent on selected regulatory proteins, and to elucidate their role in the physiology and virulence of the pathogen.

The specific objectives of this thesis work were:

I. To identify the genes regulated directly or indirectly by Hfq, YbeY and RfaH in *Y. enterocolitica.*

II. To investigate the role of Hfq, YbeY, RfaH and RovM in the physiology of *Y. enterocolitica*

III. To investigate the role of Hfq, YbeY, RfaH and RovM in the virulence and virulence-associated phenotypes of *Y. enterocolitica.*

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Bacterial strains and bacteriophages

All bacterial strains and bacteriophages used in this thesis are listed in Table 6. The detailed descriptions of the strains, including the growth conditions, are available in the original publications I, II and a manuscript (III).

Table 6. Bacterial strains and bacteriophages used in this study.

Bacterial strain	Description	Reference	Used in
***Y. enterocolitica* O:3 strains**			
YeO3-wt	6471/76, serotype O:3, patient isolate, wild type	(Skurnik, 1984)	I, II, III
YeO3-Δ*ybeY*	YeO3-wt, *ybeY::* pKNG101-*ybeY*; $Strep^R$	this study	I
YeO3-Δ*ybeY*/pMMB207-*ybeY*	YeO3-Δ*ybeY* strain transformed with pMMB207-*ybeY;* $Strep^R$, Clm^R	this study	I
YeO3-c	Virulence plasmid cured derivative of YeO3-wt	(Skurnik, 1984)	II
YeO3-c-R1	Spontaneous rough derivative of YeO3-c	(al-Hendy *et al.*, 1992)	II
YeO3-Δ*rfaH*	YeO3-wt, *rfaH::*pSW25T-*rfaH*; $Spec^R$	this study	II
YeO3-c-Δ*rfaH*	YeO3-c, *rfaH::*pSW23T-*rfaH*; Clm^R	this study	II
YeO3-Δ*rfaH*/pTM100-*rfaH*	YeO3-Δ*rfaH* strain transformed with pTM100-*rfaH;* $Spec^R$, Clm^R	this study	II
YeO3-c-Δ*rfaH*/pLux232oT-P_{OC}	YeO3-c-Δ*rfaH* strain carrying OC gene cluster promoter reporter vector pLux232oT-P_{OC}, Clm^R, Km^R	this study	II
YeO3-c-Δ*rfaH*/pLux232oT-P_{ECA}	YeO3-c-Δ*rfaH* strain carrying ECA gene cluster promoter reporter vector pLux232oT-P_{ECA}, Clm^R, Km^R	this study	II
YeO3-c-Δ*rfaH*/pLux232oT-P_{OP1}	YeO3-c-Δ*rfaH* strain carrying O-antigen gene cluster promoter 1 reporter vector pLux232oT-P_{OP1}, Clm^R, Km^R	this study	II
YeO3-c-Δ*rfaH*/pLux232oT-P_{OP2}	YeO3-c-Δ*rfaH* strain carrying O-antigen gene cluster promoter 2 reporter vector pLux232oT-P_{OP2}, Clm^R, Km^R	this study	II
YeO3-*hfq*::Km	YeO3-wt, *hfq*::Km-GenBlock, Km^R	this study	III
YeO3-*hfq*::Km/p*hfq*	YeO3-*hfq*::Km transformed with pTM100-*hfq*; Km^R, Clm^R	this study	III
YeO3-*rovM*	YeO3-wt, *rovM*::pKNG101, $Strep^R$	this study	III
YeO3-*rovM-hfq*::Km	YeO3-wt, Δ*hfq* Δ*rovM* double mutant; Km^R, $Strep^R$	this study	III
YeO3-*rovM*/pMMB207-rovM	YeO3-wt, Δ*rovM* strain with pMMB207-*rovM* plasmid to overexpress *rovM* under the IPTG induced promoter; $Strep^R$, Clm^R	this study	III
YeO3/ pLux232oT-*rovM*	YeO3-wt carrying *rovM* promoter reporter vector pLux232oT-*rovM,* Km^R	this study	III

YeO3-*rovM*/ pLux232oT-*rovM*	YeO3-*rovM* carrying *rovM* promoter reporter vector pLux232oT-*rovM,* Km^R, $Strep^R$	this study	III
YeO3/pMMB207-*rovM*/ pLux232oT-*rovM*	YeO3-*rovM*/pMMB207-*rovM* strain carrying *rovM* promoter reporter vector pLux232oT-*rovM,* Km^R, $Strep^R$, Clm^R	this study	III
***E. coli* strains**			
ω7249	(F^-) RP4-2-Tc::Mu *ΔdapA::(erm-pir) Δnic35*, *E. coli* strain for suicide vector delivery, requirement for diaminopimelic acid, $Km^R Erm^R$	(Babic *et al.*, 2008)	I, II, III
SY327λpir	*recA56*(*λpir*), *E. coli* strain for suicide vector delivery	(Miller & Mekalanos, 1988)	III
S17-1λpir	*recA*, *λpir*, *E. coli* strain for suicide vector delivery	(Simon *et al.*, 1983)	I, II, III
S17-1λpir/ pLux232oT-*rovM*	*E. coli* S17-1*λpir* strain carrying *rovM* promoter reporter vector pLux232oT-*lrhA,* Km^R	this study	III
Bacteriophages			
ϕR1-37	YeO3 OC-specific	(Skurnik *et al.*, 1995, Kiljunen *et al.*, 2005, Skurnik *et al.*, 2012)	II
ϕYeO3-12	YeO3 O-antigen-specific	(al-Hendy *et al.*, 1991, Pajunen *et al.*, 2000, Pajunen *et al.*, 2001)	II

4.1.2. Plasmids

All plasmids used in this thesis, are listed in Table 7. Detailed descriptions and primer sequences are available in the original publications.

Table 7. Plasmids used in this study.

Plasmid	Description	Reference	Used in
pTM100	Mobilizable vector, pACYC184-oriT of RK2; Clm^R	(Michiels & Cornelis, 1991)	II, III
pUC18	Cloning vector; Amp^R	(Yanisch-Perron *et al.*, 1985)	III
pUC-4K	Origin of the Km-GenBlock cassette; Amp^R, Km^R	(Taylor & Rose, 1988)	III
pKNG101	Suicide vector; $Strep^R$	(Kaniga *et al.*, 1991)	I, III
pSW23T	Suicide vector; Clm^R	(Demarre *et al.*, 2005)	II
pSW25T	Suicide vector; $Spec^R$	(Demarre *et al.*, 2005)	II
pMMB207	Cloning vector derived from RSF1010; Clm^R	(Morales *et al.*, 1991)	I, III
pLux232oT	Promoterless reporter plasmid	II	II, III
pKNG101-*ybeY*	Internal 286 bp fragment of the *ybeY* gene cloned into pKNG101; $Strep^R$	I	I

pMMB207-*ybeY*	Overexpression plasmid; the complete *ybeY* gene cloned into pMMB207; Clm^R	I	I
pSW23T-*rfaH*	Internal 201 bp fragment of the *rfaH* gene cloned into pSW23T; Clm^R	II	II
pSW25T-*rfaH*	Internal 201 bp fragment of the *rfaH* gene cloned into pSW25T; $Spec^R$	II	II
pTM100-*rfaH*	Full *rfaH* gene with the upstream promoter cloned into pTM100; Clm^R	II	II
pLux232oT-P_{OC}	OC gene cluster promoter fragment cloned into the promoter reporter vector pLux232oT; Km^R	II	II
pLux232oT-P_{ECA}	ECA gene cluster promoter fragment cloned into the promoter reporter vector pLux232oT; Km^R	II	II
pLux232oT-P_{OP1}	O-antigen gene cluster promoter 1 fragment cloned into the promoter reporter vector pLux232oT; Km^R	II	II
pLux232oT-P_{OP2}	O-antigen gene cluster promoter 2 fragment cloned into the promoter reporter vector pLux232oT; Km^R	II	II
pUC18-*hfq*	The *hfq* gene with flanking regions cloned into pUC18; Amp^R	III	III
pUC18-*hfq*::Km	pUC18-*hfq* derivative with the internal part of *hfq* gene replaced with Km-GenBlock; Km^R, Amp^R	III	III
pKNG101-*hfq*::km	Δ*hfq*::Km-GenBlock fragment of pUC18-*hfq*::Km cloned into BamHI site of pKNG101; Km^R,, $Strep^R$	III	III
pKNG101-*rovM*	Internal *rovM* gene fragment cloned into pKNG101; $Strep^R$	III	III
pMMB207-*rovM*	Overexpression plasmid; the complete *rovM* gene cloned into pMMB207; Clm^R	III	III
pTM100-*hfq*	Full *hfq* gene with the upstream promoter cloned into pTM100; Clm^R	III	III
pLux232oT-*rovM*	*rovM* promoter fragment cloned into the promoter reporter vector pLux232oT; Km^R	III	III

4.1.3. Antibodies and antisera

Table 8. Antibodies and antisera used in this study.

Antibody/Antiserum	Description	Source or reference	Used in
	Primary antisera and antibodies		
α-YopM	Polyclonal rabbit anti-YopM antiserum	(Trulzsch *et al.*, 2004)	I
α-YopH	Polyclonal rabbit anti-YopH antiserum	(Trulzsch *et al.*, 2004)	I
α-YopE	Polyclonal rabbit anti-YopE antiserum	(Trulzsch *et al.*, 2004)	I
α-YopD	Polyclonal rabbit anti-YopD antiserum	(Trulzsch *et al.*, 2004)	I
α-LcrV	Polyclonal rabbit anti-LcrV antiserum	(Trulzsch *et al.*, 2004)	I
2A9	Mouse anti-YadA monoclonal antibody	(Skurnik *et al.*, 1994, Biedzka-Sarek *et al.*, 2008)	I
α-Inv	Mouse anti-Invasin monoclonal antibody	Petra Dersch	I
Mab 898	Mouse anti-ECA monoclonal antibody	(Meier-Dieter *et al.*, 1989)	II

TomA6	Mouse anti- *Y. enterocolitica* O:3 O-antigen monoclonal antibody	(Pekkola-Heino *et al.*, 1987)	II
2B5	Mouse anti- *Y. enterocolitica* O:3 OC monoclonal antibody	(Pekkola-Heino *et al.*, 1987)	II
15D8	Mouse anti-flagellin monoclonal antibody	(Feng *et al.*, 1990)	III
α-RpoS	Polyclonal rabbit anti-RpoS antiserum	(Coynault *et al.*, 1996)	III
Secondary antisera			
P0447	Polyclonal goat peroxidase-conjugated anti-mouse immunoglobulin antibodies	Dako	I, II, III
P0217	Polyclonal swine peroxidase-conjugated anti-rabbit immunoglobulin antibodies	Dako	I, III

4.1.4. Bioinformatics resources used

The following web resources were used to obtain reference sequences and annotation information: NCBI National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), UniProt (http://www.uniprot.org) and RCSB PDB Protein Data Bank (http://www.rcsb.org/pdb).

4.2. Methods

4.2.1. Bacterial cultivation

Cultures were grown aerobically in Lysogeny Broth (LB) (Bertani, 2004), in brain heart infusion (BHI) (Fluka) or on *Yersinia* selective agar (cefsulodin, irgasan and novobiocin supplemented; CIN-agar, Oxoid, UK) at either 37°C or at room temperature (RT, ca. 22°C). LB plates were prepared by adding 15 g of bacto agar to 1 L of LB. For electron microscopy, flagellin production and motility evaluation bacteria were grown in tryptone broth (TB) (1% tryptone, 0.5% NaCl) and on motility trypton agar plates (0.3% TB plates) (1% tryptone, 0.5% NaCl, 0.3% bacto agar) at 22°C. Additionally, for biofilm formation test M9 (2mM $MgSO_4$, 0.1 mM $CaCl_2$, 1x M9 salts, 0.4% glucose) and MedECa (Skurnik, 1985) media were used. Antibiotics were used when needed at the following concentrations: streptomycin (Str) 50 μg/ml, chloramphenicol (Clm) 20 μg/ml, kanamycin (Km) 100 μg/ml, ampicillin (Amp) 50 μg/ml. Expression from plasmid pMMB207 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

4.2.2. Growth curves.

Bacteria were grown overnight at RT. Subsequently, cultures were diluted in fresh medium to an OD_{600} of 0.2 and 200 µl aliquots were distributed into honeycomb plate wells (Growth Curves Ab Ltd). The growth experiments were carried out at selected temperatures using the Bioscreen C incubator (Growth Curves Ab Ltd) with continuous shaking. The OD_{600} values were measured at 10 or 15 min intervals. The average values were calculated from 9 or 10 parallel results.

4.2.3. SDS-PAGE.

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking and 12% separating gels. After the electrophoresis the material was either visualized using InstantBlue (Expedon), by silver staining (Mortz *et al.*, 2001), or transferred onto nitrocellulose membrane (Protran, Whatman, pore size 0.45 µm). Transfer of the proteins from the SDS-PAGE gel onto the membrane was done using the semi-dry apparatus (Thermo Scientific Owl, USA).

4.2.4. Preparation of LPS samples.

LPS samples were prepared using the modified protocol of Hitchcock and Brown (Hitchcock & Brown, 1983) as described previously (Pinta *et al.*, 2012).

4.2.5. Detection of O-antigen in culture supernatants.

Bacteria were grown in 5 ml LB overnight at 22°C or at 37°C. Subsequently the overnight cultures were pelleted and 50 µl of the supernatant was mixed with 50 µl of DOC lysis buffer. The mixture was boiled for 10 min, cooled down and incubated for 4 h at 55˚C with proteinase K. Altogether 3, 6, 9 and 12 µl of the samples in 3 µl portions were spotted onto nitrocellulose membranes and left at RT to air-dry. The membrane was blocked and incubated with the O-antigen specific mAb TomA6 as described below (see immunoblotting).

4.2.6. DOC-PAGE.

LPS was separated using deoxycholate–polyacrylamide gel electrophoresis (DOC-PAGE) with 4% stacking and 12% separating as described previously (Krauss *et al.*, 1988, Zhang & Skurnik, 1994, Skurnik *et al.*, 1995). After the run the material was either visualized by silver staining (Al-Hendy *et al.*, 1991) or transferred for immunoblotting onto nitrocellulose membrane (Protran, Whatman, pore size 0.45 µm) using the semi-dry apparatus (Thermo Scientific Owl,

USA). For dot-blotting 5 µl aliquots of serial 1:2 dilutions of the LPS samples were applied directly to the membrane and left at room temperature to air-dry.

4.2.7. Immunoblotting

The membrane was blocked using 5% fat-free milk in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH7.6) for 1h at RT. Subsequently, the membrane was incubated for 16h at 4°C with primary antibodies diluted in blocking buffer. After washing 3 times with TBST buffer, the membrane was incubated for 1h at RT with suitable peroxidase-conjugated secondary antibodies (dilution 1:2000 in blocking buffer). Subsequently membrane was washed in TBST as before and incubated in ECL solution (0.1M Tris-HCl pH 8.5, 1.25 mM luminol, 0.2 mM cumaric acid, 5.3 mM hydrogen peroxide). After draining excess fluid, the membrane was exposed to light sensitive film (Kodak, USA).

4.2.8. Total RNA extraction.

The total RNA of bacteria was isolated using the SV Total RNA Isolation System (Promega) following the instructions provided by the manufacturer. The quality of the isolated RNA was verified using the Bioanalyzer (Agilent).

4.2.9. RNA-sequencing.

The RNA-seq was performed at the FIMM Technology Centre Sequencing Unit (www.fimm.fi). The ribosomal RNA was removed using Ribo-ZeroTM rRNA Removal Kit for Gram-negative Bacteria (Epicentre). Paired-end sequencing was completed on Illumina HISeq2000 sequencer (Illumina) producing the read length of 90 nucleotides. The obtained sequencing reads were filtered for quality and aligned against the *Y. enterocolitica* strain Y11 genome (accession number FR729477) using the TopHat read aligner (Langmead *et al.*, 2009). Subsequently, the Cufflinks program (Trapnell *et al.*, 2013) was used to obtain the fragments per kilobase of gene per million aligned fragments (FPKM) values for differential expression. The genes were considered differentially expressed if the fold change (FC) of the average values was >2, and the Student's T-test p-value was <0.01. The data for RfaH project was analyzed using the *edgeR* differential expression analysis package (Robinson *et al.*, 2010). The RNA sequence data has been deposited to Gene Expression Omnibus (Acc. no GSE66516 and GSE62601).

4.2.10. Quantitative RT-PCR.

The extracted total RNA was diluted to the final concentration of 25 ng/µl. The quantitative RT-PCR was performed using the GoTaq 1-step RT-qPCR System (Promega). The results were calculated using the Unit Mass method. The gene-specific primers used are listed in the respective studies.

4.2.11. Quantitative proteomics.

After growing the bacteria overnight at RT in 3 ml of LB cultures were diluted 1:10 in fresh LB and incubated for another 4h at either RT or 37°C Afterwards, bacterial cells were pelleted by centrifugation at 3000 x g, washed with sterile phosphate-buffered saline (PBS) and adjusted to 2.5 x 10^8 cfu/ml. Subsequently, 1 ml of each culture was pelleted, resuspended in lysis buffer (100 mM ammoniumbicarbonate, 8M urea, 0.1% RapiGest™), sonicated for 3 min (Branson Sonifier 450, pulsed mode 30%, loading level 2) and stored at -70°C. Samples were prepared in 3 parallels. The proteins in the samples were reduced with Tris(2-carboxyethyl)phosphine (TCEP), alkylated with iodoacetamide and digested with trypsin. The obtained digests were purified by C18 reversed-phase chromatography columns. Afterwards, the MS analysis was completed on an Orbitrap Elite ETD mass spectrometer (Thermo Scientific), with Xcalibur version 2.7.1 coupled to an Thermo Scientific nLCII nanoflow HPLC system. Both the peak extraction and the protein identification was performed using Proteome Discoverer software (Thermo Scientific). Calibrated peak files were used to search against the *Y. enterocolitica* O:3 strain Y11 proteins (Uniprot) using a SEQUEST search engine. Error tolerances on the precursor and fragment ions were ±15 ppm and ±0.6 Da, respectively. A stringent cut-off (0.5 % false discovery rate) was used for peptide identification. Spectral counts for each protein in each sample were extracted and used in relative quantitation of protein abundance alterations for label-free quantification.

4.2.12. Resistance assays

Thermotolerance was tested by diluting the overnight cultures to obtain ca. 1,000 bacterial cells in 10 µl of PBS. The dilutions were incubated in a thermoblock heated to 55°C. Serial 10-fold dilutions were prepared before the incubation and after 5 min of incubation. The number of viable bacteria was determined by plating the 10-fold dilutions.

Acid tolerance was tested as described earlier (De Koning-Ward & Robins-Browne, 1995) with modifications. Briefly, the overnight cultures were diluted to obtain ca. 1,000 bacterial cells in 10 µl of PBS. Aliquots of 10 µl of freshly prepared bacterial cultures were added to 90 µl

of PBS pH 2.5 or pH 2.0 supplemented with 1.4 mM urea. The mixtures were incubated for 2h at 37°C. 10-fold dilutions were prepared before and after the incubation and plated on LA plates to determine the number of bacteria.

4.2.13. Urease test.

The production of urease was verified in urea broth (0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% KH_2PO_4, 0.00012% phenol red, 2% urea) (Stuart *et al.*, 1945). The broth was inoculated using the overnight cultures (1:20) and incubated at RT or 37°C with shaking. The test result was considered negative if the final color was yellow and positive if the medium changed the color from orange to red.

4.2.14. Sugar utilization.

The production of acid from different carbohydrates was determined in phenol red broth (10 g peptone, 5 g NaCl and 0.018 g phenol red per liter supplemented with the selected carbohydrate to a final concentration of 1%) (Iversen *et al.*, 2006). Bacterial overnight cultures were pelleted and diluted in phenol red broth. Dilutions 1:10 were used to inoculate 3 ml of medium supplemented with selected sugars. Following carbohydrates were studied: glucose, sucrose, maltose, mannitol, arabinose, galactose, mannose, and myo-inositol.

4.2.15. Motility and biofilm assays.

After growing bacteria overnight in 5 ml of tryptone broth at RT with gentle shaking, 5 µl of each culture was applied in the middle of the tryptone motility plates (1% tryptone, 0.5% NaCl and 0.35% agar). Motility plates were incubated for 24h at RT. Subsequently, the radius of the bacterial growth was measured and images of the motility plates were taken (GelLogic 200 Imaging System, Kodac).

Biofilm formation was tested as described earlier (Blumer *et al.*, 2005) with modifications. Overnight cultures of bacteria grown in TB, M9 or MedECa were diluted 1:10 into the same medium. The 200 µl aliquots of the cultures were transferred to the wells of 96-well polystyrene microtiter plate (Nunc). After 72h of incubation at RT the wells were emptied, washed three times with sterile PBS and drained. Subsequently, wells were washed with 200 µl of methanol and left overnight to dry in order to fix the biofilm. Adhered cells were stained using 200 µl of 0.1 % crystal violet solution. Non-bound dye was removed by rinsing the wells three times with distilled water. To solubilize the crystal violet the wells were filled with 200 µl of 96% ethanol

and incubated 30 min at RT. Subsequently, the absorbance of the dye was measured at 560 nm using the Labsystems iEMS Reader MF.

4.2.16. Luminescence assay.

The activities of the promoters cloned into the luciferase reporter plasmid pLux232oT were measured using a Chameleon microplate reader (Hidex). Bacteria were grown in black 96-well plates (PerkinElmer) in a total volume of 200 µl of LB per well with continuous shaking. The luminescence emission was measured at indicated time points. The relative light unit (RLU) values were normalized using the obtained OD_{600} values of the cultures.

4.2.17. Bacteriophage sensitivity.

Bacterial cultures were grown overnight at either RT or 37˚C with vigorous shaking. Afterwards, 100 µl of saturated culture was mixed with 3 ml of 0.3% soft agar (LB broth with 0.3% agar, solubilized by boiling and cooled to 50°C) and poured over a sterile LB plate. After solidification of soft agar, 5 µl aliquots of indicated phage dilutions prepared in LB broth were applied on the plate. The bacteria were considered sensitive if a clear lysis zone was visible after 24 h of incubation. Bacteriophages used in this experiment are listed in the Table 6, study II.

4.2.18. Electron microscopy.

Bacteria grown overnight on tryptone motility plates were collected from the migration area, washed with sterile PBS, pelleted and resuspended in 0.1 M ammonium acetate. Subsequently, the bacterial cells were allowed to sediment on carbon coated grids for 1 min and stained negatively using 1% uranyl acetate. Samples were examined with JEOL JEM1400 transmission electron microscope. Pictures were taken using the Olympus Morada CCD camera with the iTEM software.

4.2.19. Serum killing assay.

The serum was prepared and the killing assay was performed as described previously (Biedzka-Sarek *et al.*, 2005). Normal human serum (NHS) was obtained from healthy individuals lacking anti-*Yersinia* antibodies (Biedzka-Sarek *et al.*, 2005). Overnight bacterial cultures were diluted to obtain ca. 1,000 bacterial cells in 10 µl. 10 µl bacterial aliquots were incubated for 30 minutes at 37°C with (i) 20 µl of NHS, (ii) 20 µl of heat-inactivated serum (HIS) or (iii) 20 µl of EGTA serum (10 mM EGTA, 5 mM $MgCl_2$). Afterwards, 70 µl of ice-cold brain heart infusion broth (BHI) was added to stop the activity of complement and the tubes were

incubated on ice. Subsequently, the mixtures were plated on LB plates supplemented with proper antibiotics. The bactericidal effect of the serum was calculated as the survival percentage taking the CFU values obtained for bacteria incubated with HIS as 100%. The experiment was carried out in triplicates, starting from individual colonies. Two separate experiments were carried out on two separate occasions.

4.2.20. Cell culture infection assay.

The experiments were performed as described earlier (Schulte *et al.*, 2000). HeLa cell cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/ml of streptomycin and 100 µg/ml of penicillin. The cells were kept at 37°C in a humidified 5% CO_2 atmosphere. After incubation with trypsin and harvesting, cells were diluted 1:3 in fresh DMEM and transferred into the 6-well plates. Following the 24 h incubation the medium was changed into DMEM with 10% FBS and 2 mM L-glutamine without the antibiotic supplementation. The cells were incubated at 37°C for further 2 h. Meanwhile bacterial overnight cultures were diluted in LB and incubated for 3h at RT. Subsequently, bacterial cells were washed in PBS, pelleted and resuspended in DMEM. The dilutions of bacterial cultures were adjusted to infect the eukaryotic cells with 100:1 bacterium-to-cell ratio. The number of bacteria was assessed by plating and the host cell number was estimated using a hemacytometer. Following the inoculation of the plates, bacteria were centrifuged down onto the cells (5 min, 750 rpm) and incubated at 37°C in a humidified 5% CO_2 atmosphere for 1h. After the incubation monolayers were washed with PBS. Subsequently, either DMEM or DMEM supplemented with 100 µl/ml gentamycin was added to the wells to determine total cell-associated bacteria and the intracellular bacteria, respectively. Following the 1h incubation, HeLa cells were lysed using 1% Triton X-100 in PBS. 100 µl aliquots of the serial 10-fold dilutions were plated on LB plates in order to determine the number of released viable bacteria.

4.2.21. Mouse experiments.

Animal experiments were performed under the permit from the Animal Experiment Board in Finland (permit no ESAVI/5893/04.10.03/2012). The 35 inbred 6-8 week old female BALB/c mice were purchased from Envigo (Blackthorn, UK). Upon arrival the mice were allowed to adjust to the new housing conditions for 1 week.

Bacteria were prepared as described earlier (Skurnik *et al.*, 1999) with modifications. Briefly, bacteria were grown overnight at RT in LB supplemented with appropriate antibiotics (Km, Clm, Strep). Afterwards, the bacterial cells were centrifuged down, resuspended in 10 ml of PBS, and mixed thoroughly. Three 1 ml aliquots were pelleted and after removal of the supernatant the weight of wet pellet mass was determined. Based on assumption that 100 mg (300 mg for YeO3-*hfq*::Km bacteria) of the bacterial pellet contains about 10^{11} bacterial cells, the bacterial suspensions were diluted to 10^{10} or 10^{8} CFU per ml. The number of viable bacteria was further confirmed by plating. For the coinfection experiments the suspensions of wild type and YeO3-*hfq*::Km bacteria were mixed at the ratio of approximately. 1:1. Samples from subsequent 10-fold dilutions were plated in order to determine the exact bacterial counts.

Mice were kept without food for 4-h before the infections. The bacterial suspensions (100 µl for the single infection and a total of 200 µl for the co-infection model) were administered either intraperitoneally using a 25G needle or intragastrically using a 20 gauge stainless-steel ball-tipped catheter. After mice were sacrificed, the Peyer's patches, spleen, and liver were removed, weighted and homogenized in 0.5, 0.5 and 1 ml of PBS, respectively, using the Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). The number of viable bacterial cells was determined by plating serial dilutions on CIN agar plates without antibiotics. Afterwards, for the co-infection experiments the ratio of wild type to YeO3-*hfq*::Km colonies was determined by patching the colonies on CIN agar plates supplemented with kanamycin.

5. RESULTS

5.1. YbeY, Hfq and RfaH are involved in the gene expression and protein synthesis

In order to detect genes and proteins that are affected by the inactivation of the *ybeY*, *hfq* and *rfaH* genes the transcriptomes and proteomes of the wild type bacteria and mutant strains grown to logarithmic phase at RT and 37°C were determined.

5.1.1. Transcriptomics

The transcriptomic profiling revealed that the *hfq* mutation had the highest impact on gene expression. At RT and 37°C resulted in differential expression of respectively 364 and 541 genes (Tables 2, S2 and S4, study III). The mutation in *ybeY* led to a similar profound change in the transcriptomic profile, affecting the expression of over 300 genes at growth in both of the studied temperatures (Table S2 and S3, study II). In contrast, loss of *rfaH* had less pronounced effect on the transcriptomic profile, as the *rfah* mutation led to alteration of expression of 102 genes at either RT or 37°C (Table 1, study II). The number of genes affected directly or indirectly by the selected mutations is presented in Table 9.

Table 9. Number of genes differentially expressed in *ybeY*, *rfaH* and *hfq* mutants at RT and 37°C.

	Mutant strain		
	YeO3-Δ*ybeY*	YeO3-Δ*rfaH*	YeO3-*hfq*::Km
RT	350	77	364
	• 229 down-regulated • 121 up-regulated	• 32 down-regulated • 45 up-regulated	• 216 down-regulated • 132 up-regulated
37°C	334	44	541
	• 286 down-regulated • 48 up-regulated	• 30 down-regulated • 14 up-regulated	• 96 down-regulated • 445 up-regulated

The RNA sequencing analysis furthermore revealed that the YeO3-Δ*ybeY* and YeO3-*hfq*::Km strains had alterations in abundance of sRNAs indicating the role of both Hfq and YbeY in sRNA regulatory network. The conducted analysis showed that five sRNAs were affected by the loss of YbeY under at least one studied condition (Fig. 4, study I). Two of them, CsrB and CsrC were downregulated, whereas GcvB, RtT and 4.5S RNA were up-regulated. The analysis of sRNA abundance in YeO3-*hfq*::Km showed that six RNA species were affected by the lack of the Hfq RNA chaperone. Similarily to *ybeY* mutant, CsrB and CsrC were downregulated, and GcvB, FnrS, RrpA and SroB were upregulated under at least one condition (Fig. S2, study III).

Moreover, both the YeO3-Δ*ybeY* and YeO3-*hfq*::Km displayed down-regulation of urease gene cluster.

Interestingly, the transcriptome of YeO3-Δ*ybeY* strain grown at RT revealed global upregulation of the pYV genes, which normally are repressed under these conditions (Fig. 2C, study I). Moreover, the inactivation of *ybeY* gene resulted at both temperatures in gene expression pattern typical for the cold shock response (Table 2, study I).

The analysis of YeO3-*hfq*::Km transcriptome showed that differentially expressed genes were scattered among different functional classes (Fig. 1, study III). In all functional classes, except for the motility and biofilm class where all the genes were down-regulated, both up- and down-regulation patterns were observed. In addition, the study revealed that the loss of Hfq resulted in changes in expression of several other transcriptional regulators (Table 3, study III), including a pronounced overexpression of RovM regulator (Fig. 2A, study III).

The comparison of YeO3-*rovM* and YeO3/pMMB207-*rovM* transcriptomes showed that in *Y. enterocolitica* 24 genes are under the regulation of RovM (Table 4, study III). The elevated levels of RovM resulted in higher levels of several transcript including outer membrane protein X, L-asparaginase and two members of the phosphotransferase system (PTS). Moreover, the overexpression of RovM regulator caused significant reduction of *rovA* transcript abundance.

The functional analysis of genes differentially expressed in YeO3-Δ*rfaH* mutant showed that most of the affected genes belong to the following functional classes: membrane, metabolism, cytoplasm and LPS biosynthesis (Fig. 3, study II). Moreover, the RNA sequencing data showed that the transcription of O-antigen and OC gene clusters was down-regulated up to 10-fold (Table 1 and Fig. 4, study II). The comparison of transcriptomic data of YeO3-Δ*rfaH* and YeO3-c-R1 strains cultivated at RT revealed that 22 out of 102 genes were similarly differentially expressed in both the strains (Table 1, study II).

5.1.2. Proteomics

Altogether, 2631 proteins could be reliably identified in the proteomics study, accounting for ca. 60% of all proteins annotated for the *Y. enterocolitica* Y11. The biggest impact on the proteome was observed for the YeO3-Δ*ybeY* and YeO3-*hfq*::Km strains, where abundance of around 100 proteins was affected at both the temperatures. The *rfaH* mutation resulted in alterations in expression of 31 and 14 proteins at RT and 37°C, respectively (Table 10).

Table 10. Number of proteins differentially expressed in *ybeY*, *rfaH* and *hfq* mutants at RT and 37°C.

	Mutant strain		
	YeO3-Δ*ybeY*	YeO3-Δ*rfaH*	YeO3-*hfq*::Km
RT	128	31	110
	• 78 down-regulated • 50 up-regulated	• 14 down-regulated • 17 up-regulated	• 41 down-regulated • 69 up-regulated
37°C	98	14	119
	• 44 down-regulated • 54 up-regulated	• 6 down-regulated • 8 up-regulated	• 18 down-regulated • 101 up-regulated

The analysis of YeO3-Δ*ybeY* proteome demonstrated significant stimulation of the pYV-encoded proteins, especially at RT (Fig. 2D, study I). Among the most highly overexpressed proteins were virulence effector proteins YopD and YopH, LcrG. Moreover, the loss of YbeY led to decrease in expression of urease proteins (Table 3, study I).

The analysis of LC-MS/MS data of YeO3-Δ*rfaH* strain showed that most of the affected proteins belonged to the functional classes of membrane, metabolism and cytoplasm (Fig. 3, study II). Furthermore, a significant decrease in abundance of dTDP-4-dehydrorhamnose 3,5-epimerase (WbbV), which plays a role in LPS biosynthesis was observed in the proteome of YeO3-Δ*rfaH* (Table 1, study II).

The analysis of YeO3-*hfq*::Km proteomic data revealed increase in the abundance of some outer membrane proteins (EnvZ, OmpX, OmpW), gene regulators (OmpR, RovM), members of Cpx signaling pathway (CpxA, CpxP, CpxR) and the decrease in abundance of urease proteins and RovA regulator (Table 3, study III).

5.2. The role of *ybeY, hfq, rfaH* and *rovM* in the physiology of *Y. enterocolitica* O:3

5.2.1. Bacterial growth

The mutation in *ybeY* and *hfq* genes resulted in retardation of bacterial growth. Growth of YeO3-Δ*ybeY* strain was affected more at 37°C than at RT, and completely abrogated at 42°C. The decrease in growth at 37°C was less pronounced when the *ybeY* mutant was cured of its virulence plasmid. The growth of the mutant at 4°C was significantly slower, yet continuous. The growth speed of the *ybey* mutant was partially relieved or fully restored by the *in trans* complementation (Fig. 1, study I).

In line with previous findings in *Yersinia* species (Schiano *et al.*, 2010, Kakoschke *et al.*, 2014), inactivation of *hfq* gene in *Y. enterocolitica* O:3 resulted in growth defect at all tested temperatures (4, 22, 37 and 42°C). The growth retardation was most prominent at 4 and 42°C. The *in trans* complementation restored the growth almost completely. Moreover, the knock-out of *rovM* gene of the YeO3-*hfq*::Km strain resulted in improved growth under all studied conditions (Fig 3, study III). The YeO3-Δ*rfaH,* YeO3-Δ*rovM* and YeO3/pMMB207-*rovM* strains showed no growth defects (data not shown).

5.2.2. Cell and colony morphology

The inactivation of the *hfq* gene in *Y. enterocolitica* caused alterations in colony morphology. After 48h of incubation on CIN agar plates at RT the YeO3-*hfq*::Km mutant strain formed small, dry and dark colonies surrounded by dark violet halo. Similar effect was observed upon overexpression of *rovM* in YeO3/pMMB207-*rovM* strain. Moreover, this phenotype was revoked after the knock-out of *rovM* gene in *hfq* mutant, indicating the role of RovM regulator in alterations of colony morphology.

The analysis of cell morphology conducted using the electron microscopy (EM) showed that the YeO3-*hfq*::Km bacterial cells were significantly elongated (Fig. 4A and S5, study III). Moreover, the same bacteria were visibly less aggregated when compared with wild type (data not shown).

5.2.3. Susceptibility to environmental stresses

All the mutations introduced to *Y. enterocolitica* in this study resulted in decreased resistance to environmental stresses. The YeO3-Δ*ybeY* strain presented highly impaired thermotolerance presented by decreased survival upon incubation at 50°C. While 95.26 ± 0.86% of YeO3-wt bacteria survived 5 min incubation under this condition, only 2.38 ± 3.78% of *ybeY* mutant bacteria survived. The *in trans* complementation restored the survival to the level of 87.17 ±6.44%. Moreover, the loss of YbeY decreased the ability of bacteria to survive in acidified environment, an ability of *Y. enterocolitica* that depends on the degradation of urea. The YeO3-Δ*ybeY* strain was killed upon incubation in PBS pH=2.5 with 1.4 mM urea, while 112.42 ± 8.78% of YeO3-wt bacteria survived these conditions. *In trans* complementation resulted in 78.75 ± 9.31% survival rate.

Similarly to *ybeY* mutation, the depletion of Hfq caused decrease in survival rates during incubation at 55°C and in the acidified medium with 1.4 mM urea (Fig. 7, study III). Only 3.39 ±

1.41% of YeO3-*hfq*::Km bacteria survived 5 min incubation at 55°C, while the survival rate of YeO3-wt under same conditions reached 41.12 ±8.12%. The ability to survive in PBS pH=2.0 was decreased from 26.35 ± 2.06% recorded for wild-type bacteria to 4.17 ± 1.03%. The *trans*-complementation restored both the heat- and acid-resistance.

The analyses of YeO3-Δ*rfaH* mutant revealed increased susceptibility to SDS and polymyxin B (Fig. 6, study II), while no significant difference between the *rfaH* mutant and wild type strains were observed in tolerance to H_2O_2 nor to osmotic stress (data not shown).

5.2.4. Motility and biofilm formation

In line with previous studies (reviewed in (Chao & Vogel, 2010)) the loss of Hfq resulted in impaired swimming motility. Our study showed, that the deterioration of motility is due to decrease in synthesis of flagellin. The flagellation defect was abolished by both the *in trans* complementation and *rovM* knock-out. Moreover, the YeO3-*hfq*::Km strain formed significantly less biofilm that the wild type counterpart. Inactivation of *rovM* in the *hfq* negative background increased the production of biofilm. Additionally, the *trans*-complemented strain YeO3-*hfq*::Km/p*hfq* showed overproduction of biofilm, presumably due to the *hfq* copy number effect (Fig. 5, study III).

Interestingly, unlike in some other species the *rfaH* mutant of *Y. enterocolitica* did not show any alterations in motility (data not shown). Also the YeO3-Δ*ybeY* strain presented same pattern of motility as the wild type bacteria.

5.2.5. Other physiological features affected by the mutations

In prokaryotes the 16S rRNA undergoes maturation process. The original 17S rRNA transcript is cleaved from both 5' and 3' ends by different endonucleases (RNase E, RNase G, RNase R and YbeY) with help of ribosome maturation factors (i.e. Era, KsgA, RbfA and RsgA) (Jacob *et al.*, 2013). The comparison of rRNA profiles of wild type and mutant strains grown at RT and 37°C revealed remarkable differences in the structure of *ybeY* mutant rRNA profile. A strong decrease in the amount of 16S rRNA species produced by YeO3-Δ*ybeY* was observed (Fig. 3, study I). The impairment in rRNA processing was more pronounced when YeO3-Δ*ybeY* bacteria were incubated at 45°C (data not shown). The other mutant strains, YeO3-*hfq*::Km and YeO3-Δ*rfaH*, presented normal rRNA patterns.

The transcriptomics and proteomics data indicated that the mutations in *ybeY* and *hfq* genes altered the bacterial metabolism (Fig. S2 and S3, study I; Table S2-S5, study III). Further research revealed a decreased ability of YeO3-Δ*ybeY* to utilize arabinose and galactose. Moreover, the same strain was not able to grow in the medium containing 1% *myo*-inositol as a carbon source (Table S5, study I). The YeO3-*hfq*::Km strain presented alteration in expression of several genes from the PTS, that optimize the utilization of carbohydrates present in the environment. The analysis of *hfq* mutant transcriptome showed up-regulation of β-glucoside, fructose, glucitol/sorbitol, glucose, mannitol, mannose and *N*-acetylgalactosamine-specific enzymes, whereas cellobiose and chitobiose-specific PTS systems were down-regulated under at least one incubation condition (Table S2 and S3, study III). Furthermore, both YeO3-*hfq*::Km and YeO3/pMMB207-*rovM* when grown on CIN agar plates formed dark colonies surrounded by dark violet halo indicating alterations in metabolism (Fig. S4B, study III). The same strains, when grown on mannitol plates displayed larger halo surrounding the bacterial growth suggesting higher rates of acidification of the medium (Fig S3B, study III).

5.3. The effect of *ybeY, hfq*, and *rfaH* mutations on the virulence and virulence-associated traits of *Y. enterocolitica* O:3

5.3.1. Production of Yop effector proteins

The analyses of YeO3-Δ*ybeY* transcriptome and proteome showed a significant upregulation of factors encoded on pYV (Fig. 2, study I) at RT. Among the overexpressed genes were *yopE, yopH, yopO, yopM*, as well as the injectisome genes *yscN, yscO, yscP, yscB* and *yscD* (Fig. 2C, study I). Consistently, the proteomic study showed the overexpression of pYV-encoded factors, especially at RT. Among the most highly expressed proteins were YopD, YopH and the secretion chaperone SycE (Fig 2D, study I). The overproduction of the effector proteins was examined in three different ways. First, the overnight incubation of *ybeY* mutant in liquid LB at 37°C resulted in strong aggregation, an effect known to be caused by Yop secretion (Fig 2A, study I). Secondly, the amount of proteins secreted by the wild type and YeO3-Δ*ybeY* bacteria was assessed on SDS-PAGE gel (Fig. 2B, study I) revealing increased secretion levels. In addition, the abundance of virulence plasmid encoded proteins was evaluated with immunoblotting (Fig. 5A, study I). Also this experiment indicated the overexpression of YopE and YopH at RT, as well as increase in expression of YopH and YadA in DMEM medium at 37°C. Finally, immunoblotting of samples prepared from the growth media showed the presence of Yops only after incubation at 37°C and did not allow detection of any Yops in the supernatants of bacteria grown at RT (data not

shown). Therefore, it is likely that the mutation in *ybeY* in *Y. enterocolitica* O:3 leads to a significant upregulation of factors encoded on pYV.

In line with previous results obtained for *Y. enterocolitica* O:8 (Kakoschke *et al.*, 2014), we did not observe changes in the expression of Yops in YeO3-*hfq*::Km strain (Table S8, study III). Similarly, no significant changes in Yops expression was observed in YeO3-Δ*rfaH* mutant.

5.3.2. LPS synthesis

Both silver staining and immunobloting of DOC-PAGE gels showed a significant decrease in production of O-antigen and OC in YeO3-Δ*rfaH* mutant strain at both RT and 37°C. The *in trans* complementation resulted not only in full restoration, but also slight overproduction of O-antigen and OC (Fig. 1, study II). The possibility that the decrease in amount of these LPS structures was due to shedding of the O-antigen and OC from the bacterial surface was excluded (Fig. 2, study II). Thus, these results indicated impairment in biosynthesis of O-antigen and OC structures upon loss of RfaH. The *rfaH* mutation did not cause any significant changes in the amount of ECA. These results were supported by findings from transcriptomic and proteomic study (described earlier), and further verified by the analysis of promoter activities (Fig. 5, study II). The conducted analyses of LPS structures in YeO3-Δ*ybeY* and YeO3-*hfq*::Km did not reveal any significant differences (data not shown).

5.3.3. Production of other virulence factors

The transcriptomics and quantitative proteomics analyses indicated decrease in the expression of urease genes in both YeO3-Δ*ybeY* (Table 3, study I) and YeO3-*hfq*::Km strains (Table 3, study III). The urease test showed gave negative result for *hfq* mutant strain (Fig. 7B, study III), while *ybeY* strain presented positive results in urea broth, but only after prolonged incubation. In coherence with reduced urease activity, both the strains presented increased sensitivity to low pH medium (described in more details in 5.2.3.).

In addition, the YeO3-Δ*ybeY* strain presented a significant decrease in production of Ail, a protein encoded by the attachment invasion locus. This result was verified using the RNA sequencing, quantitative proteomics, as well as quantitative RT-PCR (Fig. 5B and Table S3, study I).

Similarly, both YeO3-*hfq*::Km and YeO3-Δ*ybeY* strains showed alterations in expression of OmpR and RovA regulators (Table 3, study I; Table 3, study III). Furthermore, the loss of YbeY

resulted in decrease in abundance of such virulence factors as Hfq, Inv and H-NS, and increase of VirF, YmoA (Table 3, study I). The knock-down of *hfq* gene caused alterations in expression of several regulators implicated in bacterial virulence (i.e. PhoB, RovM, RpoS) as well as changes in the structure of bacterial outer membrane (Cpx signaling pathway and outer membrane structures) (Table 3, study III).

5.3.4. Resistance to normal human serum

Due to the contribution of LPS, Ail and YadA for serum resistance was previously shown (Biedzka-Sarek *et al.*, 2005), the susceptibility of the mutants to NHS was assessed. The test conditions probed for the classical, lectin and alternative complement activation pathways.

The loss of *ybeY* resulted in significantly (p=0.023) increased resistance to NHS under studied conditions. The *ybeY* mutation increased the resistance to NHS by over 3-fold (Fig. 7). Also the loss of *rfaH* resulted in a slight but statistically significant (p=0.048) increase in resistance to NHS killing (Fig. 5; Fig. 6C, study II). No significant difference was observed in resistance of YeO3-Δ*ybeY* and YeO3-Δ*rfaH* strains to EGTA/Mg^{2+} serum (data not shown), where only the alternative pathway can be activated. On the other hand, the lack of Hfq chaperone caused significant (p=0.018) decrease in resistance to NHS (Fig. 7).

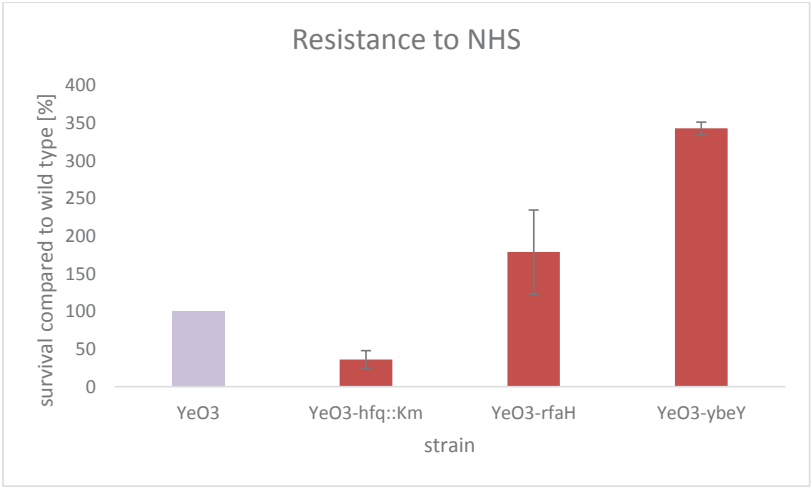

Figure 7. Resistance to normal human serum (NHS). The bacteria were incubated for 30 min in 66% NHS and in heat inactivated serum (HIS). The bactericidal effect was calculated as the survival percentage taking the bacterial counts obtained with bacteria incubated in HIS as 100 %. The columns show the mean ± SD serum bactericidal effect when compared with the wild type strain. The experiments were performed in triplicates.

5.3.5. Virulence in cell and animal models

The importance of YbeY for the virulence of *Y. enterocolitica* O:3 was evaluated in HeLa cell infection model. The experiment showed decrease in the ability of the bacteria to infect the eukaryotic cells (Fig. 5C, study I). The most prominent effect was visible during the stage of adhesion (Fig. 5D - association, study I). The *ybeY* mutant bacteria displayed no apparent alterations during the stage of invasion and the rate of invasion of associated bacteria was even higher than the wild type bacteria (Fig. 5E, study I). Due to highly impaired growth and increased susceptibility to environmental stresses observed for YeO3-Δ*ybeY*, the virulence was not evaluated in any animal model.

The impact of *rfaH* mutation on the intracellular growth of *Y. enterocolitica* in human intestinal epithelial and mouse macrophage cell lines has been studied previously (Nagy *et al.*, 2006). Moreover, the role of O-antigen and OC in virulence of *Y. enterocolitica* was also assessed in mouse experiments, showing 50- to 100-fold decrease in LD50 for the LPS mutant strains (al-Hendy *et al.*, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999).

The virulence of YeO3-*hfq*::Km was tested in experimental mouse infection models, where both oral and intraperitoneal routes of infection were investigated. In the co-infection model mice were infected with a mixture of wild type and YeO3-*hfq*::Km bacteria, and after mice were killed, the bacterial counts in different organs were performed, and the percentage of Km-resistant (Km^R) colonies was determined (Table 5, study III). The presence of Km^R bacteria was detected only in Peyer's patches. Moreover, the average percentage of Km^R bacteria decreased over time, from 50 % to 5.9 % two days post infection, and to 0.5 % nine days post infection. Among the mice that were orally infected with one strain at time, a statistically significant (p=0.0064) reduction in the number of bacteria recovered from mouse organs five days post infection was observed (Fig. 7 and Table S8, study III) for the YeO3-*hfq*::Km strain. The infection with the complemented strain YeO3-*hfq*::Km/p*hfq* increased the number of recovered bacteria, while the infection with the double mutant YeO3-*rovM-hfq*::Km had no significant effect on the bacterial counts. Interestingly, infected intraperitoneally, both the single *hfq* and double *rovM-hfq* mutants caused the death of two out of three mice within two days post infection (Table S9, study III), whereas none of the mice infected with the wild-type strain died prematurely. The number of bacteria recovered from organs of mice that were infected with YeO3 and YeO3-*hfq*::Km and survived till the fifth day post infection did not present any significant changes. Further investigation revealed that the amount of LPS released to the medium is increased in

YeO3-*hfq*::Km and YeO3-*rovM-hfq*::Km cultures (Fig S7, study III). Moreover, the *hfq* mutants were characterized by increased susceptibility to SDS (Fig. S8, study III) indicating that the structure of the outer membrane is compromised in the *hfq* and *rovM-hfq* mutant strains. Furthermore, the analysis of the lipid A revealed alterations in the structure of this molecule in the *hfq* negative bacteria grown at 37°C (Fig. S9, study III).

6. DISCUSSION

In this thesis two global approaches, RNA sequencing and quantitative proteomics were combined in order to better understand the role of selected factors in gene regulation and their impact on physiology and virulence of *Y. enterocolitica*. Two of the studies showed that the deletion of *hfq* and *ybeY* genes caused highly pleiotropic effects (study I and III). The mutants presented significant growth defects and impairment of many physiological functions, as well as deterioration of features related to virulence and survival inside of the host organism. Moreover, this thesis demonstrates that RfaH acts as a highly specific antiterminator in *Y. enterocolitica* O:3 and affects the expression of LPS O-antigen and OC gene clusters (study II).

6.1. The impact of studied mutations on the gene expression

The most profound alterations in gene expression were caused by *hfq* and *ybeY* mutations. A very high percentage of genes being under Hfq regulation have also been previously observed in numerous other bacterial species, including the closely related was *S. enterica* and *Y. pestis* (Sittka *et al.*, 2008, Geng *et al.*, 2009). A significant impact of *ybeY* mutation on the gene expression has also been reported for *E. coli* (Pandey *et al.*, 2014). On the other hand, the loss of RfaH do cause downregulation of gene clusters harboring the *ops* element located downstream in the *cis*-regulated promoter region (Bailey *et al.*, 1997). In this study, the comparison of wild type and YeO3-Δ*rfaH* transcriptomes revealed low number of differentially expressed genes, an indication of the narrow specificity of this regulator.

The comparison of results obtained through RNA-sequencing and LC-MS/MS showed general coherence. In the case of YeO3-*hfq*::Km mutant a large number of proteins with altered abundance while the corresponding genes did not show differential expression in transcriptomic analyses. That likely originates from the role of Hfq in post-transcriptional regulation.

The analyses of the transcriptome of the mutants indicated that the mutations caused changes in the gene expression. First of all, RfaH is known to bind to specific *ops* sequences present upstream of long operons. The main function of RfaH is to allow the polymerase to overcome the terminators (Bailey *et al.*, 1997). In this study the *rfaH* mutation caused decrease in abundance of transcripts preceded by *ops* sequence, indicating the direct effect on these genes. Subsequently, the changes in LPS biosynthesis was most likely reflected as indirect response to

rough phenotype. Another indication of indirect effect was observed for Hfq. The *hfq* mutation changed the expression pattern of several other regulators that affected the expression of their target genes. In this study it was shown that the overexpression of RovM in the YeO3-*hfq*::Km mutant was responsible for several of the phenotypic changes observed in the *hfq* mutant. Moreover, some changes in gene expression might be a response to changes in metabolism. This study showed also that both *hfq* and *ybeY* mutations presented alterations in carbohydrate metabolism as well as in abundance in Csr-RNAs.

6.2. YbeY

This study shows, that *ybeY* is not an essential gene in *Y. enterocolitica* O:3, but its loss affects the growth and sensitizes the bacterium to multiple environmental stresses. In line with previous results and the fact that *ybeY* was first discovered as a heat shock gene, the effect of the *ybeY* mutation was most profound at elevated temperatures (Rasouly *et al.*, 2009, Grinwald & Ron, 2013). Similar to *E. coli* (Jacob *et al.*, 2013), in *Y. enterocolitica* YbeY plays a role in rRNA maturation process. In this study, the electropherograms revealed misprocessing of 16S rRNA with the biggest impact observed when bacteria were incubated at 45°C. The impairment in maturation may be caused by a defect in 17S rRNA cleavage and/or its degradation, as immature form of 16S rRNA shows low stability (Jacob *et al.*, 2013). The changes in rRNA pattern can be also caused by a polarized expression of *rrn* operons that was previously observed in *ybeY* mutants (Grinwald & Ron, 2013).

Results obtained in study I indicated overexpression of virulence plasmid genes under all studied conditions. This happens plausibly due to the increase in plasmid copy number, alterations in sRNA regulation network or changes in the supercoiling of the pYV. The latter one can be a result of changes in expression of histone-like proteins and/or indirectly through alterations in stability of sRNAs that play a role in virulence gene regulation.

The loss of YbeY affected the abundance of several sRNA species. Two of them, CsrB and CsrC, are Hfq-independent and belong to the carbon storage regulatory system (Liu & Romeo, 1997, Schiano & Lathem, 2012). In *Y. pestis* GcvB binds to Hfq and represses *dppA*, a component of the dipeptide transport system (Koo *et al.*, 2011). These results indicate, that both the Hfq-dependent and Hfq-independent sRNA regulation pathways were affected in the YeO3-Δ*ybeY* mutant.

Moreover, the YeO3-Δ*ybeY* mutant revealed the presence of transcripts typical for cold shock response (Annamalai & Venkitanarayanan, 2005). It is known that changes in temperature result in dissociation of polysomes, accumulation of free ribosomes and their subunits and the state of ribosomes is one of the main sensors of environmental conditions that in *E. coli* elicit the heat and cold shock responses (VanBogelen & Neidhardt, 1990, Jones *et al.*, 1992, Golovlev, 2003). Moreover, cold shock response can be induced artificially using different antibiotics like chloramphenicol, erythromycin, spiramycin and tetracycline, all inhibitors of protein synthesis (VanBogelen & Neidhardt, 1990). It was previously noticed that mutation in *ybeY* gene results in significant alterations in the structure of ribosomes (Rasouly *et al.*, 2009, Rasouly *et al.*, 2010, Jacob *et al.*, 2013). Therefore it is highly possible, that the cold-shock like response in YeO3-Δ*ybeY* mutant occurs due to accumulation of defective ribosomes. The defect in the translation machinery together with accumulation of *cspA* transcript are factors decreasing the growth rate of the mutant.

In *Y. enterocolitica* urease is considered to be an important virulence factor that allows bacteria to survive in low pH environment and therefore to pass through the stomach during the course of infection (De Koning-Ward & Robins-Browne, 1995, Gripenberg-Lerche *et al.*, 2000). The decrease in urease production observed in YeO3-Δ*ybeY* mutant resulted in reduced ability to survive in acidic conditions. Previous studies showed that the loss of acid resistance leads to severe decrease in bacterial virulence in mouse models (De Koning-Ward & Robins-Browne, 1995, Gripenberg-Lerche *et al.*, 2000). Moreover, *ybeY* mutant presented decreased ability adhere to the HeLa cells that was most probably caused by alterations in expression of Ail (Felek & Krukonis, 2009, Kolodziejek *et al.*, 2010). It is known, that the ability of *Y. enterocolitica* to invade eukaryotic cells *in vitro* correlates with its virulence (Lee *et al.*, 1977, Une, 1977).

Taken together, the mutation in *ybeY* gene resulted in significant alterations in gene expression of *Y. enterocolitica* O:3. The results obtained in study I showed that YbeY is required for proper functioning of sRNA regulation network, the control of virulence gene expression and its loss induce the expression of cold-shock genes. Such profound changes in the control of gene expression resulted in a pleiotropic effect including an impairment of many virulence-related features.

6.3. RfaH

In study II of this thesis it was shown that RfaH serves as a functional antiterminator in *Y. enterocolitica* O:3. In coherence with results obtained for other bacterial species, the loss of RfaH resulted in downregulation of long operons harboring the *ops* element located in the *cis*-regulated promoter region (Bailey *et al.*, 2000). The same study showed the importance of the correct location of the *ops* element for the RfaH recruitment, as the ops element located far upstream of the ECA gene cluster promoter region was not functional (Fig. 4, study II). Disruption of *rfaH* caused decrease in abundance of transcripts of several genes from the LPS biosynthesis pathway and resulted in loss of O-antigen and OC structures.

The comparison of YeO3-Δ*rfaH* and wild type transcriptome profiles revealed general similarity, indicating a narrow specificity of the regulator. Moreover, a correlation between the transcriptome pattern of *rfaH* mutant and spontaneous rough strain was detected. Several RfaH-dependent changes observed in study II were likely to be the indirect responses to the loss of LPS. Both the YeO3-Δ*rfaH* and YeO3-c-R1 strains showed changes in the expression of Cpx envelope stress system indicating a response to changes in the cell wall integrity (Hunke *et al.*, 2012, Vogt & Raivio, 2012). Moreover, the alterations in amount of transport ATPase proteins, sialic acid permease and phosphate ABC transporters were observed (Table 1, study II) that was previously correlated with a response to decreased LPS production and consequential reduced demand for sugars and energy (Nagy *et al.*, 2006).

In line with previous results (Biedzka-Sarek *et al.*, 2005), loss of RfaH resulted in increased resistance to NHS. It is believed that the loss of LPS structure from the surface exposed the YadA and Ail, two other factors conferring to the complement resistance (Biedzka-Sarek *et al.*, 2005). Similarly as in *E. coli* and *S. enterica* (Yethon *et al.*, 2000, Nagy *et al.*, 2006), reduction in O-antigen and OC expression resulted in decreased resistance to SDS and polymyxin B (Fig. 6, study II), but no impairment in resistance to hydrogen peroxide was observed in this study.

Previously it was demonstrated that the O-antigen and OC are essential for full virulence of *Y. eneterocolitica* serotype O:3 (al-Hendy *et al.*, 1992, Skurnik *et al.*, 1999). Experiment conducted on mice showed that the oral LD_{50} decreased 50- to 100 fold for the LPS mutant strains (al-Hendy *et al.*, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999). The impairment in virulence occurs due to the importance of the O-antigen for the early stages of infection, the resistance to environment of gastrointestinal track, and for the immune system evasion (al-Hendy *et al.*,

1992, Biedzka-Sarek *et al.*, 2005). Furthermore, the impact of *rfaH* mutation on intracellular net growth of *Y. enterocolitica* has been already evaluated by Nagy *et al*. (Nagy *et al.*, 2006). In their study the comparison of the wild type and YeO3-Δ*rfaH* strain yield was determined in cultured human intestinal epithelial (INT407) and mouse macrophage (RAW264.7) cell lines . The study revealed that *rfaH* mutant exhibits reduced intracellular yield when compared to the wild type bacterium.

It was previously demonstrated that a rough mutant of *Y. enterocolitica* O:8 exhibited alterations in YadA function, loss of Ail and downregulation of *inv* expression. Moreover, due to upregulation of *flhDC*, the flagellar master regulatory operon, the mutant presented hypermotility (Bengoechea *et al.*, 2004). In study II no significant changes in the production of these virulence factors were observed. Furthermore, the wild type and YeO3-Δ*rfaH* strains presented similar motility. These discrepancies may be explained by differences underlying the serotypes (differences in motility and flagellation between the serotype O:8 and O:3 were described previously (Uliczka *et al.*, 2011)). Furthermore, the *rfaH* mutation does not cause full abrogation of O-antigen and OC production and thus some functional LPS is still present on the surface of bacterial cell what may result in less severe phenotype than the one of rough mutant.

Taken together, study II of this thesis showed that RfaH serves as a highly specific regulator that most likely affects only genes preceded by a functional *ops* element. Despite the small number of affected genes, the disruption of *rfaH* gene led to profound changes in the bacterial physiology. Decrease in the amount of LPS on the cell surface results in indirect changes in the transcriptome, stress response, and lower resistance to some environmental cues. Moreover, based on the previous results (al-Hendy *et al.*, 1992, Zhang *et al.*, 1997, Skurnik *et al.*, 1999, Nagy *et al.*, 2006), it can be assumed that YeO3-Δ*rfaH* has decreased virulence.

6.4. Hfq

The study III of this thesis demonstrated the importance of Hfq in the post-transcriptional regulation in *Y. enterocolitica* O:3. The results showed, that similar as in the case of *S. enterica* and *Y. pestis*, a high percentage of genes stay under the regulation of Hfq. Moreover, the fact that numerous proteins displayed alterations in abundance, even though their coding genes did not show differential expression affirms the importance of Hfq for post-transcriptional regulation.

Due to the fact that Hfq is a crucial element of the sRNA regulation network, it is assumed that apart from its direct effect, it can also affect the expression of genes through changes in

abundance and activity of other regulators. The study III reported differential expression of several regulators (Table 3, study III). In accordance with changes in the abundance of these regulators, the alteration in expression of their target genes were also observed. Analysis revealed differential expression of genes belonging to the RpoS, RovA and OmpR regulons (Patten *et al.*, 2004, Cathelyn *et al.*, 2007, Hu *et al.*, 2009). This study also revealed a significant derepression of RovM regulator in YeO3-*hfq*::Km mutant (Fig. 2, study III).

Study III also affirmed the requirement of Hfq presence for proper expression of the RpoS sigma factor in *Y. enterocolitica*. The decrease in abundance of RpoS factor was also observed previously in different bacterial species like *E. coli* and *S. enterica* upon the Hfq depletion (Brown & Elliott, 1996, Muffler *et al.*, 1996). RpoS confers the optimal growth during the stationary phase and under stress conditions (Tanaka *et al.*, 1993, Badger & Miller, 1995) and therefore reduction in its activity can contribute to increased susceptibility of YeO3-*hfq*::Km mutant to environmental stresses. Out of four sRNA species known to regulate the translation of RpoS in *E. coli*, namely DsrA, RprA, ArcZ and OxyS (Sledjeski *et al.*, 1996, Zhang *et al.*, 1998, Majdalani *et al.*, 2002, Mandin & Gottesman, 2010), study III identified two homologues in the genome of *Y. enterocolitica* (RprA and ArcZ). sRNAs are known to enhance the translation of RpoS by binding to the 5' leader region of the mRNA what results in better availability of the Shine-Dalgarno site (Brown & Elliott, 1997). The analysis of sRNA expression in YeO3-*hfq*::Km mutant revealed alterations in abundance in RprA. Moreover, it is known that these sRNA species require binding to Hfq for full functionality (Sledjeski *et al.*, 1996, Zhang *et al.*, 1998, Majdalani *et al.*, 2002, Mandin & Gottesman, 2010). Therefore, it is assumed that the loss of Hfq in *Y. enterocolitica* disrupts the sRNA regulation pathway and results in lower abundance of RpoS protein.

In line with the results obtained for other pathogenic *Yersinia* species, the loss of *hfq* in *Y. enterocolitica* resulted in a growth defect. The growth curves of YeO3-*hfq*::Km showed similar pattern as serotype O:8, displaying an intermediate phenotype between strongly impaired characteristics of *Y. pestis* and the marginally affected *Y. pseudotuberculosis* (Geng *et al.*, 2009, Schiano *et al.*, 2010, Kakoschke *et al.*, 2014). Furthermore, in coherence with results obtained for the serotype O:8, alterations in the bacterial metabolism, including changes in the utilization of mannitol that resulted in different growth on CIN agar and mannitol plates were observed (Kakoschke *et al.*, 2014). Moreover, study III showed that Hfq of *Y. enterocolitica* O:3 is involved in resistance to environmental stresses, including elevated temperature and acidic

environment. Similar to the case of serotype O:8, alterations in expression of urease genes were observed (Kakoschke *et al.*, 2014). In addition, YeO3-*hfq*::Km displayed impairment in motility and biofilm formation.

The mouse infection experiments conducted in study III showed that the YeO3-*hfq*::Km bacteria, similarly to other *Yersinia hfq* mutants (Geng *et al.*, 2009, Schiano *et al.*, 2010) were characterized by decreased virulence. Virulence is a remarkably multifactorial feature of *Y. enterocolitica* and therefore it is believed that different alterations caused by the loss of *hfq* can contribute to impairment in virulence. Although *rpoS* mutant was not attenuated in mouse infection experiments (Badger & Miller, 1995), the repression of *rpoS* may have a synergistic effect. In order to pass through the stomach during the course of infection, *Y. enterocolitica* must be able to survive in low pH environment (De Koning-Ward & Robins-Browne, 1995, Gripenberg-Lerche *et al.*, 2000), therefore reduction in the urease activity is likely one of the shortcomings that attenuates YeO3-*hfq*::Km in the oral infection model. Furthermore, the motility was also implicated in bacterial virulence (Young *et al.*, 2000). Interestingly, two out of three mice infected intraperitoneally with the YeO3-*hfq*::Km or YeO3-*rovM-hfq*::Km mutant died prematurely. The decreased resistance to SDS, increased release of the LPS molecule to the culture supernatant together with alterations in the abundance of membrane proteins indicate that the structure of the bacterial outer membrane of *hfq* mutant might be compromised. The importance of Hfq and sRNAs regulation for the biogenesis of the outer membrane was previously established (Guillier *et al.*, 2006, Van Puyvelde *et al.*, 2013). Therefore in the study III it is suggested, that the death observed in mice infected intraperitoneally with either YeO3-*hfq*::Km or YeO3-*rovM-hfq*::Km occurred due to the septic shock caused by the alterations in the structure of lipid A molecule and/or the increased release of the LPS from the mutant bacterial cells.

Taken together, study III demonstrated that the loss of Hfq in *Y. enterocolitica* leads to profound changes in the transcriptome and proteome affirming the importance of Hfq for gene regulation control. The YeO3-*hfq*::Km mutant showed alterations in abundance of several transcriptional regulators and sRNA species. Moreover, the loss of Hfq resulted in highly pleiotropic changes in the phenotype including abrogation of virulence.

6.5. RovM

In study III of this thesis it was shown that some of the phenotypic features of YeO3-*hfq*::Km mutant are due to derepression of RovM regulator. Inactivation of the *rovM* gene in the *hfq* mutant resulted in full or partial restoration of several characteristics.

Knocking out *rovM* gene in YeO3-*hfq*::Km mutant resulted in increased growth rate, even though did not restore it to the level presented by the wild type bacteria. Moreover, the colony morphology observed before by Kakoschke *et al*. (Kakoschke *et al.*, 2014), was fully reversed after the loss of RovM. In turn, overexpression of *rovM* in YeO3/pMMB207-*rovM* resulted in the same colony morphology on CIN agar and mannitol plates, strongly implicating the role of RovM in the regulation of mannitol metabolism in *Y. enterocolitica*.

Study III showed also that in contrast to majority of the LysR-type regulators, but similar to *rovM* of *Y. pseudotuberculosis* and *hexA*, the YeO3 *rovM* was also under positive autoregulation (Harris *et al.*, 1998, Heroven & Dersch, 2006). Moreover, most of the LysR-type regulators interact with effectors that ultimately steer their regulatory propensities. Therefore it is possible that RovM in *Y. enterocolitica* also requires additional cofactors for its full function (Schell, 1993). Additionally, the expression of *rovM* in *Y. pseudotuberculosis* is regulated by the Csr system (Heroven *et al.*, 2008).

Furthermore, study III demonstrated that RovM functions as a motility regulator that represses the flagella biosynthesis. It is likely, that in line with the results obtained in *E. coli*, this takes place through binding of RovM to *flhDC* promoter region (Blumer *et al.*, 2005). Study III showed that RovM influences the biosynthesis of flagellin and the YeO3-Δ*rovM* mutant is hypermotile. However, the knock-out of *rovM* in *hfq* negative background did not restore full motility, and the overexpression in pMMB207-*rovM* did not result in total abrogation of motility. Therefore it is believed that other factors still influence the motility in this system. In *Y. enterocolitica* YenS sRNA was found to be a positive regulator of motility that acts through the modulation of YenI production. Interestingly, the interplay between the levels of *yenI* and *yenS* may lead to either hypo- or hypermotility. (Tsai & Winans, 2011).

In contrast to previous findings in *E. coli* (Peterson *et al.*, 2006), overexpression of RovM in YeO3 increased the RpoS levels in the stationary phase cells. Yet, the overexpression of RovM did not increase the abundance of RpoS in the YeO3-*hfq*::Km strain (Fig. 6, study III). Therefore, in study III it was concluded that in *Y. enterocolitica* RovM positively regulates the levels of RpoS

in Hfq-dependent manner. It was previously observed that sRNAs allow proper translation of RpoS (described in subchapter 6.4), therefore the positive regulation of RovM might be visible only when certain sRNA species are functional.

In line with previous results obtained for *Y. pseudotuberculosis* and *Y. enterocolitica O:8*, RovM represses the expression of *rovA* also in *Y. enterocolitica O:3* (Heroven & Dersch, 2006, Lawrenz & Miller, 2007). In *Y. enterocolitica* serotype O:8 it controls the expression of *inv*, however, in serotype O:3 this regulation is prevented due to presence of an insertion sequence in the *inv* regulatory region (Uliczka *et al.*, 2011).

Taken together, in the study III of this thesis the effect of RovM overexpression in the *hfq* negative background was analyzed. Inactivation of *rovM* in the YeO3-*hfq*::Km mutant prevented the subsequent overexpression of this regulator and allowed to elucidate the role of RovM in the Hfq deficient strain. This study demonstrated that the growth defect, mannitol utilization and motility alterations observed for YeO3-*hfq*::Km were mediated by the overexpression of RovM.

7. CONCLUSIONS AND FUTURE CONSIDERATIONS

In conclusion, all four studied genes, *ybeY*, *rfaH*, *hfq* and *rovM*, had a profound impact on gene regulation of *Y. enterocolitica* O:3. The deletion of *hfq* and *ybeY* genes caused wide disturbances in gene expression and highly pleiotropic effects. Both the mutants presented significant growth defects and impairment of many physiological functions, as well as deterioration of features related to virulence and survival inside of the host organism. On the other hand, RfaH was shown to function as a highly specific antiterminator in *Y. enterocolitica* O:3 that affects only the expression of LPS O-antigen and OC gene clusters and most likely leads to an indirect response to rough phenotype. The RovM regulator was characterized by highest specificity, and affected the expression of 24 genes in *Y. enterocolitica* O:3.

The conducted experiments demonstrated that the studied mutations (*ybeY*, *rfaH* and *hfq*) compromised the bacterial virulence. The mutants showed significant decrease in resistance to different environmental conditions that are normally encountered during the course of infection. Moreover, the loss of studied genes resulted also in growth defect, impairment of motility and biofilm formation, changes in carbohydrates metabolism, and alterations in production of different virulence factors that also contributes to vitality and ability to establish infection in host organism.

Due to the fact that all the mutant strains demonstrated compromised virulence, the studied proteins might be regarded as potential targets for new drug development. Furthermore, the strains with established avirulence might be used as sources for vaccines. This thesis revealed the general function of RfaH in *Y. enterocolitica* O:3, yet the role of RfaH in the *in vivo* regulation of LPS expression is still unknown. The potential role of RfaH in *in vivo* controlling of tissue- and temperature-specific expression of LPS should be further investigated. The future experiments should be also aimed at explaining the details of the mechanism behind the overexpression of pYV genes in YeO3-Δ*ybeY* mutant.

Moreover, the results of high-throughput analyses can be further used in studies aiming at deciphering the role of yet unknown genes and proteins. This study revealed a large number of uncharacterized genes that stay under the YbeY, RfaH, Hfq and RovM regulation. They involvement in the regulatory pathways, as well as potential role is an interesting research question.

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