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Role of the RNA Chaperone Hfq in the Virulence of *Yersinia enterocolitica*

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München, 5. Februar 2021
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LIST OF ABBREVIATIONS

Ail	Attachment and invasion locus protein
BamA	Outer membrane protein assembly factor BamA
CDC	Centers of Disease Control and Prevention of the United States
CFU	Colony Forming Unit
CIN-Agar	Cefsulodin-Irgasan-Novobiocin-Agar
ClpP	Caseinolytic protease P
CS3	Coli Surface antigen 3
d	day
DD	Differential Diagnosis
DGVS	Deutsche Gesellschaft für Verdauungs- und Stoffwechselerkrankungen
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
e. g.	example gratia = for example
et al.	et alii / aliae
ETEC	Enterotoxic *Escherichia coli*
Fe^{2+}	Ferrous iron (iron with oxidation number of +2)
Fe^{3+}	Ferric iron (iron with oxidation number of +3)
Fur	Fe-uptake regulation
FyuA	Ferric-yersiniabactin uptake
g	gram
GALT	Gut Associated Lymphoid Tissue
Hfq	Host factor of bacteriophage Q beta
HPI	High Pathogenicity Island
IfSG	Infektionsschutzgesetz
IM	Inner Membrane
InvA	Invasin A
IS	Insertion Sequence
i. v.	intravenous
kb	kilobase
kDa	kilo Dalton
KDO	Keto-deoxyoctulosonate
lac	lactose
LB	Luria-Bertani medium

lcr	low calcium response
LBP	LPS binding protein
LPS	Lipopolysaccharide
LpxR	Lipid A 3′-O-deacylase, produces tetra-acylated LPS
MAPK	Mitogen-Activated Protein Kinase
MarR	Multiple antibiotic resistance Regulator
M cells	Microfold cells
mg	milligram
mRNA	messenger Ribonucleic Acid
Myf	Mucoid yersinia factor
nm	nanometer
nt	nucleotide
O-Ag	"Hitzestabiles Oberflächenantigen" of Gram-negative bacteria, exposed repetitive oligosaccharide units
OD_{600}	Optical Density (600nm)
OM	Outer Membrane
OmpX	Outer membrane protein X
OmpR	Outer membrane protein Regulator
PBS	Phosphate Buffered Saline
PhoP	Transcriptional regulatory protein of the phosphate regulon
PmrA	Polymyxin-resistance-associated response regulator A
p. o.	per os
pYV	Plasmid of *Yersinia* Virulence
RBS	Ribosome Binding Site
RNA	Ribonucleic Acid
RovA	Regulator of virulence
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sec	general Secretion System
spp.	species pluralis
sRNA	small Ribonucleic Acid
T3SS	Type Three Secretion System/ Type III Secretion System
T5SS/TVSS	Type Five Secretion System (comprising 5 subtypes: TVaSS – TVeSS)
TLR4	Toll-like Receptor 4
VirF	Virulence regulon transcriptional activator
VYE-Agar	Virulent *Yersinia enterocolitica* Agar
wt	wildtype

Ybt	Yersiniabactin
YadA	Yersinia adhesin A
Yop	Yersinia outer protein
Ysc	Yersinia secretion system
Yst	Yersinia heat-stable toxin

LIST OF PUBLICATIONS

Kakoschke TK, Ehrenfeld M, Mast G. Severe bacterial soft tissue infections in the head and neck region: Overview of the causes, pathogenesis, diagnostics, treatment and possible sequelae. Chirurg. 2020 Jan 29. doi: 10.1007/s00104-020-01121-x. [Epub ahead of print]

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* **Kakoschke T**, Kakoschke S, Magistro G, Schubert S, Borath M, Heesemann J, Rossier O. The RNA chaperone Hfq impacts growth, metabolism and production of virulence factors in *Yersinia enterocolitica*. PLoS One. 2014 Jan 15;9(1):e86113. doi: 10.1371/journal.pone.0086113. eCollection 2014.

*** published results of this dissertation**

1. Introduction

Bacterial pathogens have developed sophisticated strategies to infect mammalian host, to replicate extracellularly or intracellularly, to be released from the infected host to the environment or to be transmitted indirectly or directly to the next host. These complex life cycles require different survival strategies, which are determined by a specific set of genes and an adequately tuned gene regulation in terms of transcriptional, post-transcriptional and post-translational regulation.
This thesis is focusing on the post-transcriptional regulation of virulence of *Yersinia enterocolitica*.

1.1. The Genus *Yersinia*

In June 1894 in Hong Kong bacteriologist Alexandre Émile Jean Yersin (*1863 in Aubonne, Switzerland; † 1943 in Nha Trang, Vietnam) discovered, *Yersinia pestis*, the agent of the plague (Bibel and Chen, 1976), later named in his honour. He isolated the pathogen from pestilential buboes of infected persons and initially described "the pulp of the bubo, in every case, (…) filled with a thick puree of short, thick bacilli with rounded ends (…)" (Yersin, 1894). He showed the transmission of the disease to mice, rats and guinea pigs and set with these characterising studies, a weighty milestone in understanding and fighting a fatal disease.
The genus *Yersinia* (old name: *Pasteurella*) belongs to the *Enterobacteriaceae* family (Bercovier and Mollaret, 1984) and presently comprises more than 18 species (listed in Appendix 7.1) of which three species are pathogenic to mammals (*Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*) and one for salmonids (*Y. ruckeri*) (Wren, 2003, Kumar et al., 2015). The other species are widespread in the environment and are not obligatory pathogenic. *Yersinia* is a Gram-negative, facultative anaerobic, non-sporulating, rod- or coccobacillus-shaped bacterium. *Yersinia* can easily adapt itself to different environmental conditions. The environmental species are ubiquitous and persist in soil, water, plants, animals, food and infected humans (Heroven and Dersch, 2014).
Besides *Yersinia pestis*, which causes bubonic and pneumonic plague, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Koornhof et al., 1999, Smego et al., 1999) both cause gastrointestinal infections known as yersiniosis. The evolutionary path of *Y. pseudotuberculosis* and *Y. enterocolitica* is believed to have diverged in the last 200 million years (Heroven and Dersch, 2014). Strikingly, by gene loss and gene gain *Y. pestis* evolved from *Y. pseudotuberculosis* as recently as 4,000 - 20,000 years ago. (Achtman et al., 1999, Bos et al., 2016, Andrades Valtuena et al., 2017, Spyrou et al., 2018). Despite this recent evolution of this pathogen the clinical pictures and disease patterns caused by *Y. pestis* differ significantly from those associated with infections by enteropathogenic *Y. pseudotuberculosis* or *Y. enterocolitica* (Chain et al., 2004, Derbise et al., 2010).
The essential requirement of pathogenicity of the three *Yersinia* species is the presence of a 70-kilobase virulence plasmid, called pYV (plasmid of *Yersinia* virulence). It encodes a sophisticated

nanomachine, the Type 3 Secretion System (T3SS), that injects *Yersinia* anti-host proteins into host cells (Biot and Cornelis, 1988, Cornelis et al., 1989, Hammerl et al., 2012).

The next paragraphs describe the pathogenicity and clinical manifestations of the three human pathogenic yersiniae.

1.2. Clinical and Epidemiological Description of *Yersinia pestis* Caused Plague

Yersinia pestis, the causative agent of plague, is transmitted by fleas (e.g. *Xenopsylla cheopis*) from infected animals, mostly rodents, to other animals and at times to humans (Perry and Fetherston, 1997). By biting infected animals fleas incorporate blood containing the plague bacilli. Transmission of the infection is favoured through the formation of bacterial clumps (biofilm) that obstruct the flea's foregut and prevent a proper intake of food. This mechanism promotes hunger in the insect and leads to an increased search for blood meal (Bacot and Martin, 1914). By its next bite the flea passes along the pathogen, by regurgitating it with the infected blood, to another host. Within its mammalian hosts, *Y. pestis* invades the adjacent lymphatics, becomes filtered in regional lymph nodes and proliferates there forming the typical buboes (swollen, painful, haemorrhagic lymph glands). Subsequently it can affect the bloodstream, lead to bacteraemia and finally sepsis with a high mortality rate in humans and black rats (Wren, 2003). Apart from the bubonic and septicaemic plague the third form of *Y. pestis* infection is the pneumonic plague (**Figure 1**). Following systemic spread, the bacilli colonize the lungs. In addition the disease can also be communicated directly from person to person by droplet infection (Perry and Fetherston, 1997). Pneumonic plague is highly contagious and without any treatment mostly fatal (Apangu et al., 2017).

Throughout history three pandemics have been described (Spyrou et al., 2016). First the Justinian plague, which spread from 541 AD onwards around the late antique Mediterranean world and took millions of lives in nearly two centuries (Wagner et al., 2014). In the High Middle Ages the Black Death swept over Europe from 1347-1353 with outbreaks into the 18th century. The Black Death is said to have killed nearly one-third of the population in Europe (Zietz and Dunkelberg, 2004). The third wave started in China in the second half of the 19th century and disseminated from Hong Kong throughout the world (Kupferschmidt, 1993, Morelli et al., 2010). Recently the earliest known *Y. pestis* strain harbouring the plague causing genes has been identified by genomic analysis in 3800 years old human remains (Spyrou et al., 2018). It is considered to be a progenitor of the strains that cause the three above mentioned plague pandemics. To this day there are still isolated outbreaks of the plague for example in Madagascar, the South-Western United States, Myanmar and China (Wren, 2003, Carniel, 2008, Butler, 2013, Kugeler et al., 2015, Li et al., 2016, Gracio and Gracio, 2017, Kmietowicz, 2017, Tsuzuki et al., 2017, Spyrou et al., 2018).

The three historical pandemics are associated with three biovars, which can be differentiated based on their nitrate reduction and glycerol fermentation: Antiqua, Mediaevalis, Orientalis (Perry and

Fetherston, 1997, Wren, 2003). *Y. pestis* produces rough lipopolysaccharides without O-chains. Thus no specific O-serotypes exist.

Today the treatment of choice is streptomycin, gentamicin, tetracycline/doxycycline, chloramphenicol or fluorquinolones like ciprofloxacin and moxifloxacin (Oyston and Williamson, 2013).

A satisfying vaccine that protects against all forms of plague with a broad applicability is not available (Wren, 2003, Bubeck and Dube, 2007). Newly discovered antibiotic-resistant *Y. pestis* (Galimand et al., 1997, Guiyoule et al., 2001, Galimand et al., 2006) fans the fear of misuse in bioterrorism. Indeed, the CDC (Centers for Disease Control and Prevention of the United States) ranks *Y. pesitis* as a Category A high risk pathogen (Grundmann, 2014, Centers for Disease Control and Prevention, 2017, August 17).

The detection of an infection with *Y. pestis* is notifiable to the responsible public health department (IfSG, 2000).

1.3. Clinical and Epidemiological Description of Yersiniosis, a Gastrointestinal Infection

1.3.1. *Yersinia pseudotuberculosis*

Y. pseudotuberculosis, the ancestor of *Y. pestis*, is a biochemically homogenous group that is divided into 21 serotypes (Wren, 2003, De Castro et al., 2009). The most commonly isolated serotypes in Europe are O:1, O:2 and O:3 (Aleksic et al., 1995). All strains are virulent to humans and several animals. Wild animals (whether sick or just colonized) and in particular brown rats are considered to be the main reservoir. Nevertheless *Y. pseudotuberculosis* can also be found in abiotic environment such as natural water or in food crops likely contaminated by wildlife faeces (Fredriksson-Ahomaa et al., 2009, Heroven and Dersch, 2014, Jaakkola et al., 2015). Despite the fact that *Y. pseudotuberculosis* is genetically closer to *Y. pestis* (> 97% nucleotide identity) it shows similarity in pathomechanism and symptomatology of gastrointestinal infections to *Y. enterocolitica* (< 60% nucleotide identity) (Chain et al., 2004, Derbise et al., 2010, Heroven and Dersch, 2014). This will be described in section 1.3.3..

1.3.2. *Yersinia enterocolitica*

In contrast to the very homogeneous group of *Y. pseudotuberculosis*, *Y. enterocolitica* comprises 6 biotypes and about 60 serotypes, that differ from each other not only biochemically, but also genetically and in terms of pathogenicity (Wauters et al., 1987, Garzetti et al., 2012). **Table 1** summarizes important features, phenotypes and characteristics of *Y. enterocolitica* in association with biovars and serovars.

Table 1: Classification of *Y. enterocolitica* strains

	Yersinia enterocolitica		
6 biovars	**1A**	**1B**	**2-5**
Pathogenicity	**non-enteropathogenic/ facultative pathogenic** [1] (e.g. wound infection)	**enteropathogenic** [2] Yersiniabactin (Ybt) producer [3]	**enteropathogenic** [2]
Mouse infection model	non-virulent	highly virulent (mouse-lethal)	moderate virulent (non-mouse-lethal)
60 serovars (H- and O-antigens) most frequently serovars are listed here	O:5, O:6,30, O:6,31, O:7,8, O:10	O:4, **O:8**, O:13, O:18, O:20, O:21	O:3, O:9, O:5,27
Regional prevalence	environmental	North America = "new-world strains"	Europe and Japan = "old-world strains"
Subspecies differentiation based on 16S rRNA sequence	*Y. enterocolitica* subsp. *parlearctica*	*Y. enterocolitica* subsp. *enterocolitica*	*Y. enterocolitica* subsp. *parlearctica*
	[1] lack of pYV plasmid; not obligatory pathogenic; might cause (local) infection preferentially in immunocomprimised patients [2] systemic infection in immunocompromised patients [3] Ybt chelates Fe^{3+} and Cu^{2+} (Heesemann et al., 1993, Koh et al., 2017)		

Composed in accord with: (McNally et al., 2004, Schubert et al., 2004, Trebesius et al., 1998, Bottone, 1999, Neubauer et al., 2000b, Neubauer et al., 2000a, Batzilla et al., 2011b, Batzilla et al., 2011a)

1.3.3. Epidemiology and Pathogenesis

In Western Europe yersiniosis is most commonly caused by *Y. enterocolitica* serotype O:3 (Valentin-Weigand et al., 2014). Pigs are considered to be a main reservoir (infected pharyngeal tonsils) but also other domestic and wild animals such as wild boars (> 90% colonized), sheep and goats can be colonized or infected (Fredriksson-Ahomaa et al., 2009, Joutsen et al., 2016, European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2018). *Y. pseudotuberculosis* is more often isolated in Eastern Europe and Russia, but *Y. pseudotuberculosis* infections are generally less frequent (Galindo et al., 2011, Herold, 2014). Enteropathogenic yersiniae can still multiply at temperatures of 4°C, which means the pathogen can grow on foods stored in refrigerators for example. The main source of infection in humans is contact with animals bearing the pathogen or by consuming contaminated food, especially milk products and raw or undercooked pork for *Y. enterocolitica* (Bottone, 1999) and contaminated vegetables and lettuce (e.g. by rats) for *Y. pseudotuberculosis* (Heroven and Dersch, 2014). The typical route of infection is the fecal-oral route, but also severe *Y. enterocolitica* infections have been reported through blood transfusions, following bacterial growth in refrigerated blood stocks of blood donors suffering from recent yersinae infection (Arduino et al., 1989, Strobel et al., 2000, Guinet et al., 2011). After ingestion of

Y. enterocolitica the pathogen reaches the small intestine and invades the mesenteric lymph nodes after passage through the M cells of the Peyer's patches. The M cells of the Peyer's patches belong to the GALT-system (gut associated lymphoid tissue). In course of bacterial infiltration and infection the Peyer's patches get damaged because of abscess formation (Autenrieth and Firsching, 1996). Unlike other enteropathogenic bacteria such as salmonellae (Leung and Finlay, 1991) or shigellae (Sansonetti, 1993, Parsot and Sansonetti, 1996), *Y. enterocolitica* multiplies extracellularly and by using predominantly the lymphatic and occasionally the vascular system (Autenrieth and Firsching, 1996) it not only leads to micro-abscesses in the regional lymph nodes, but also in other organs, mainly the liver and spleen (Autenrieth and Firsching, 1996, Autenrieth et al., 1996, Hopfner et al., 2001, Oellerich et al., 2007). In some rare instances *Y. enterocolitica* can lead to systemic disease (Yersinia sepsis) (Moon et al., 2015, Sauter et al., 2017).

Figure 1 shows a summary how the different *Yersinia* species invade a human host.

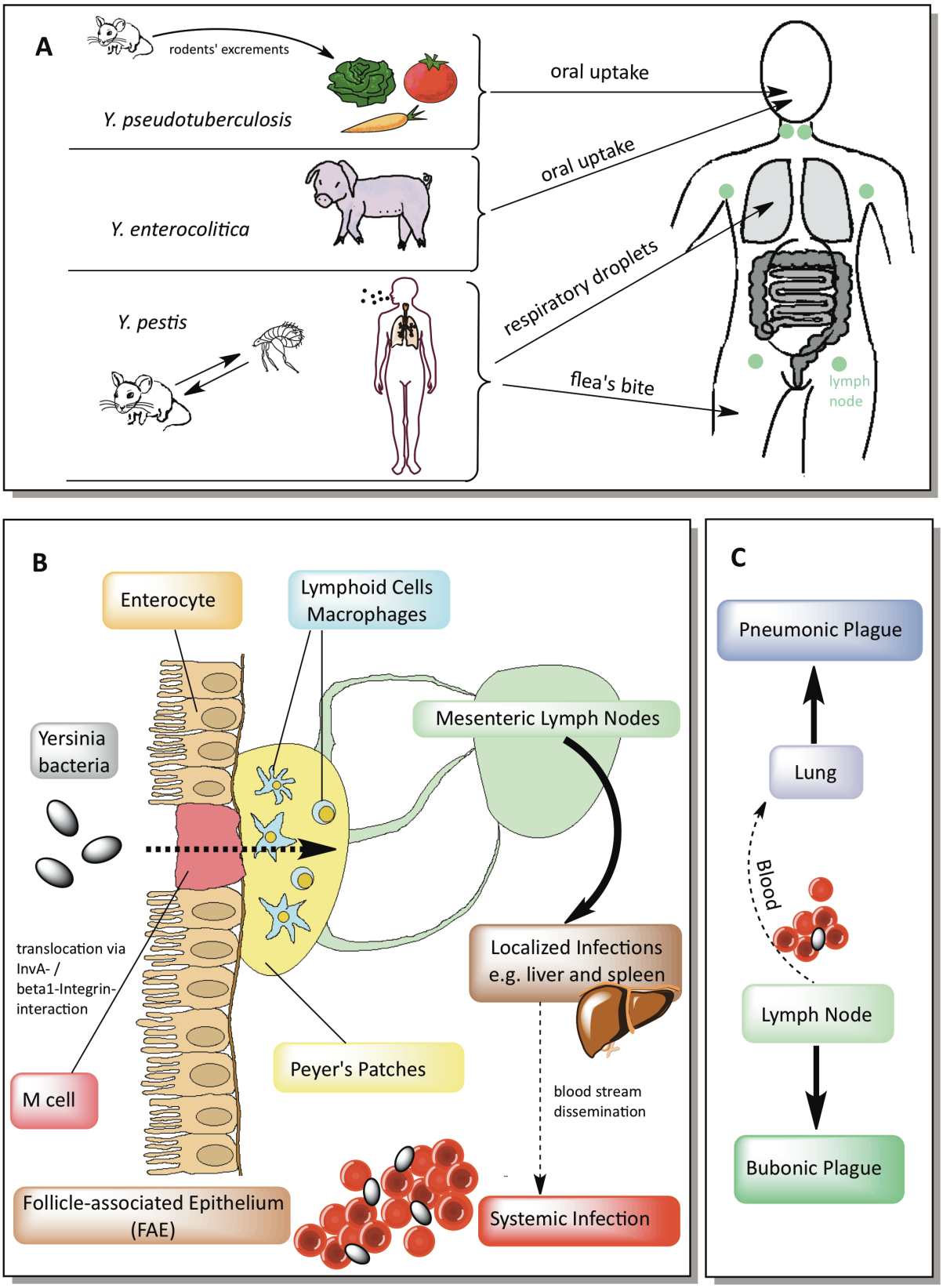

Figure 1: Routes of infection of the three human-pathogenic *Yersinia* species

(A) Transmission routes, (B) Invasion and spread of *Y. enterocolitica* and *Y. pseudotuberculosis*, (C) Infection pathways of *Y. pestis* and the three clinical forms of plague

1.3.4. Clinical Presentation, Diagnosis and Treatment of Yersiniosis

The average incubation time is three to ten days. Depending on the age and immune status of the patient yersiniosis leads to different clinical manifestations that are described in **Table 2**.

Table 2: Clinical Pictures of Yersiniosis (Herold, 2014)

	Symptoms	Mainly Affected
Gastroenteritis	1-2 weeks fever, vomiting, diarrhea usually self-limiting (Cave: Dehydration!)	infants/children
Pseudoappendicitis	acute lymphadenitis mesenterica and ileitis terminalis (DD: Appendicitis)	older children/ young adults
Enterocolitis	1-2 weeks diarrhea, colicky pain in the lower abdomen, at times chronic diarrhea (DD: Morbus Crohn)	young adults/ adults
Sequelae	reactive arthritis and/or erythema nodosum Reiter's disease: triad of arthritis, conjunctivitis/uveitis, urethritis	HLA-B27 associated
Systemic infection, dissemination	septicaemia	immunocompromised, diabetes mellitus, iron storage defects, liver cirrhosis

The diagnostics include direct cultivation of yersiniae from stool, extirpated mesenteric lymph nodes (e.g. in case of appendectomy), intestinal biopsies or blood as well as the detection of *Yersinia*-DNA and antibody titre. Yersiniae are selectively grown on CIN-Agar (= *Yersinia* Selectiv Agar, Cefsulodin-Irgasan-Novobiocin-Agar (Schiemann, 1979)) or VYE-Agar (= virulent *Yersinia enterocolitica* agar, (Fukushima, 1987)) or enriched by incubation of stool sample in PBS at 4°C for three weeks, because of their growth advantage at 4°C compared to other bacteria (Eiss, 1975, Greenwood et al., 1975).
Yersiniosis is treated mostly symptomatically by substituting fluids and electrolytes. Antimicrobial therapy should be used when the patient is a

- risk patient (i.e. immunocompromised)
- when the patient does not recover or his/her condition gets worse,
- when systemic infection occurs

(Herold, 2014, Hagel et al., 2015)

In these cases the S2k-guidelines from the DGVS (Deutsche Gesellschaft für Verdauungs- und Stoffwechselkrankheiten) from the 20 February 2015 recommend to use the in **Table 3** listed antibiotic regimen:

Table 3: Antibiotic treatment of Yersiniosis (Hagel et al., 2015)

Enterocolitis	Ciprofloxacin (1g/d p.o. or 0,8 g/d i.v. for 5-7 days) or Cotrimoxazol (1,92 g/d p.o. or i.v. for 5-7 days)
Bacteremia	Ceftriaxon (2g/d i.v. for 7-14 days) or Ciprofloxacin (1g/d p.o. or 0,8 g/d i.v. for 7-14 days)

According to the German Infection Protection Law the laboratory detection of enteropathogenic Yersiniae spp. have to be reported to the responsible public health department (IfSG, 2000).

Yersiniosis is a self-limiting disease in most cases, but infection of an immunocompromised patient can become life-threatening with a mortality rate of up to 50 percent (Cover and Aber, 1989, Galindo et al., 2011).

1.4. Yersiniae – Model Bacteria for Infection Biological Research

"The varied ecology, pathogenicity and host range of these related species, together with the availability of mouse models for both gastroenteritis and plague that mimic human disease, and the relative ease of constructing defined mutants, make the yersiniae a model genus in which to study the genetics and evolution of bacterial pathogens." This is how Wren describes yersiniae in his 2003 review (Wren, 2003).

As enteropathogenic yersiniae are also pathogenic to rodents (e.g. mice, rats and rabbits) they are used as model organisms to unravel the pathomechanisms on a cellular and molecular level.
In this study we used strains of the mouse-virulent *Yersinia enterocolitica* biotype 1B serovar O:8 from two different lineages, the wildtype (wt) strain WA-314 and JB580v, a derivative of the wt strain 8081 (Garzetti et al., 2012).

1.5. Pathogenicity Factors of *Yersinia enterocolitica* Serotype O:8

As yersiniae are transferred from contaminated food stored in a refrigerator to the location where invasion and infection take place, the human small intestine, they undergo different temperature changes from around 4°C to 37°C. To adapt to the alternating environmental conditions, and

continue to survive, multiply, penetrate the epithelial barrier of the host and trigger disease, many *Yersinia* pathogenicity factors are regulated by temperature (Heroven and Dersch, 2014, Chen et al., 2016).

Table 4 highlights known pathogenicity factors of *Y. enterocolitica* with their optimal expression temperatures and **Figure 2** summarizes functions and targets towards host cells.

Table 4: Overview of main pathogenicity factors of *Y. enterocolitica* (al-Hendy et al., 1991, Mikulskis et al., 1994, Iriarte and Cornelis, 1995, de Koning-Ward and Robins-Browne, 1997, Bottone, 1999, Jacobi et al., 2001, Holst, 2003, Heesemann et al., 2006, Kolodziejek et al., 2010, Matsuura, 2013, Atkinson and Williams, 2016, Bancerz-Kisiel et al., 2018).

Virulence Factor	Function	Optimal Expression Temperature *in vitro*
pYV-plasmid encoded		
T3SS	Type III Secretion System: injection of Yersinia outer proteins (Yops) into host cells tranquilizing phagocytosis	37°C
YadA	cell adhesion; serum resistance	37°C
chromosomally encoded		
Ail	adhesion, invasion; serum resistance	37°C
InvA	cell adhesion, invasion through interaction with β1-integrin	26°C and neutral pH 37°C and pH ↓
LPS (O-antigen)	endotoxin, antigenicity recognized by TLR4	21-25°C (S-form) 37°C (R-form)
MyfA	fimbriae, putative adhesion	37°C (acidic pH)
OmpX	Ail-like protein; unknown function	28 °C and 37°C
Urease	improved survival to acidity	28°C
Ybt	yersiniabactin (biovar 1B); uptake of Ybt-chelated Fe^{3+} and Cu^{2+}	iron deficiency
Yst	Yersinia heat-stable enterotoxin	< 30°C

At temperatures of 30°C or less *Y. enterocolitica* are usually flagellated, while at higher temperatures the bacteria lose motility (no flagella production at 37°C) and tend to autoaggregate through the production of adhesin YadA (Kapatral et al., 1996). A rise in temperature not only leads to a reduction in flagellum synthesis but also to an activation of virulence genes encoded by the *Yersinia* virulence plasmid pYV (Lambert de Rouvroit et al., 1992).

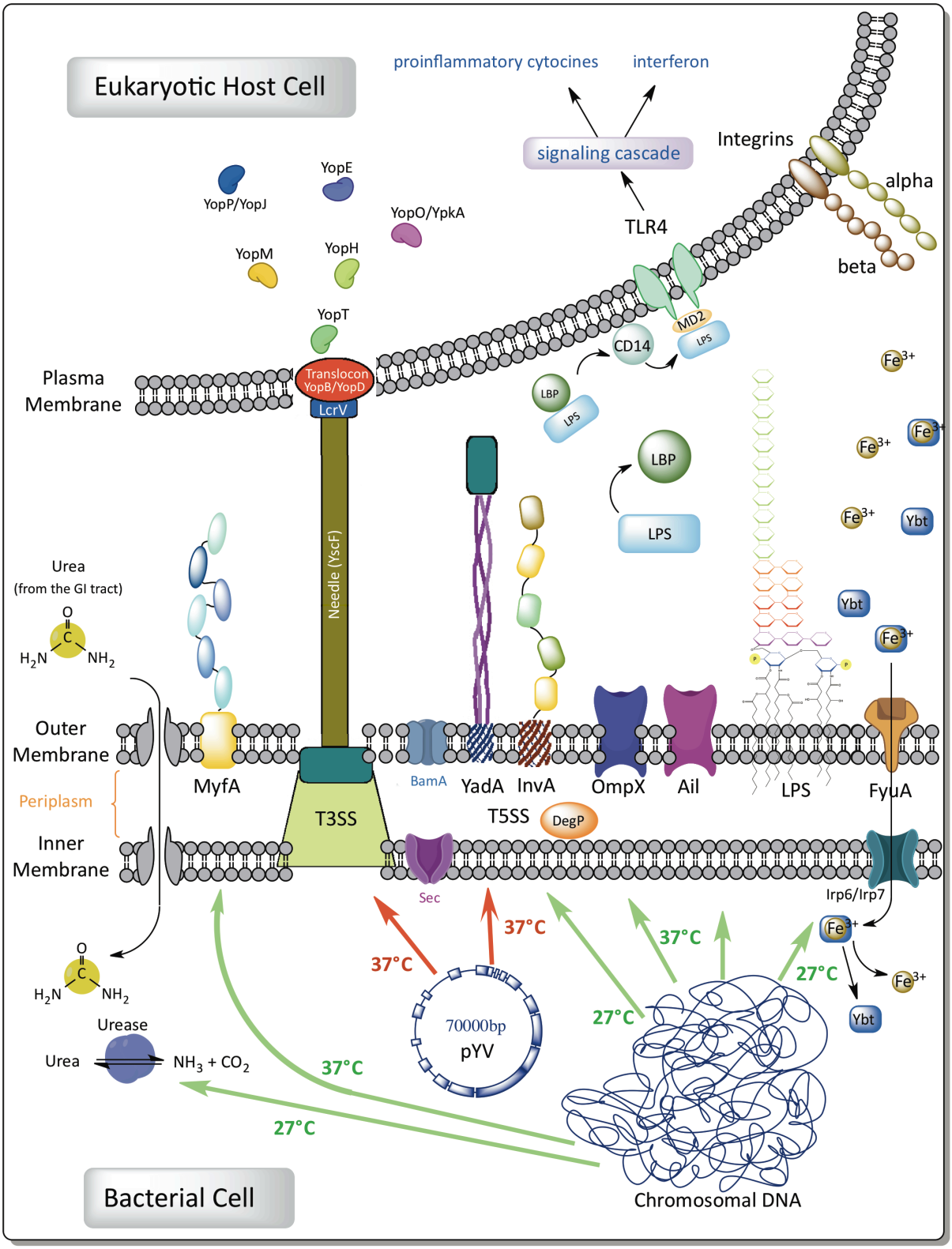

Figure 2: Summary of important virulence factors of *Yersinia enterocolitica* serotype O:8. T3SS modified from (Dewoody et al., 2013), Invasin and YadA modified from (Mikula et al., 2012), MyfA modified from (Bao et al., 2013).

1.5.1 Plasmid Encoded Virulence Factors

1.5.1.1. The Type III Secretion System

The pYV plasmid (Gemski et al., 1980, Zink et al., 1980) harbours the genes (*ysc* for Yop secretion and *lcr* for low calcium response, respectively) encoding the Type III Secretion System (T3SS) and secreted proteins in particular Yersinia outer proteins (Yops) (Heesemann et al., 1984, Aepfelbacher et al., 1999, Galan and Waksman, 2018).

According to current knowledge the T3SS acts as an injection-apparatus to translocate Yops from the bacterium into the target cell (**Figure 2**). About 25 Ysc-Lcr proteins build the molecular structure of this injectisome, including a basal part, that spans the bacterial membrane and a distal needle (needle protein YscF), which is 60-80 nm in length and 6-7 nm in width (Hoiczyk and Blobel, 2001, Cornelis, 2002a, Cornelis, 2002b, Cornelis, 2002c).

After Yersinia-cell-contact three proteins (YopB, YopD and LcrV) are secreted which are required for pore formation (YopB/YopD) in the host membrane and function as translocators in the delivery of the six effector Yops into the target cells (Hakansson et al., 1996, Mueller et al., 2005, Edgren et al., 2012). Once in the cytosol of macrophages or neutrophils Yops reveal their biochemical activities resulting in the inhibition of phagocytosis and downregulation of the inflammatory response (Cornelis, 2002a, Heesemann et al., 2006). The molecular function of the six effector Yops of *Y. enterocolitica* have been intensively studied (Young, 2007, Zhao and Shao, 2015):

- YopE, a Rho-family GTPase activator (inactivating Rho function) (Black and Bliska, 2000, Mohammadi and Isberg, 2009),
- YopH, a protein tyrosine phosphatase (inhibition of signal transduction pathways) (Guan and Dixon, 1993, de la Puerta et al., 2009),
- YopM, a nuclear localized protein (acts immunosuppressively by regulating host kinases such as ribosomal S6 kinase RSK1) (Berneking et al., 2016, Chung et al., 2016),
- YopO/YpkA, a serine-threonine kinase that binds to Rho GTPases (Barz et al., 2000), YopO phosphorylates gelsolin (Lee et al., 2017),
- YopP/YopJ, an acetyltransferase targeting MAPK kinases (Orth et al., 1999, Ruckdeschel et al., 2001, Menon et al., 2017),
- YopT, a cysteine protease that inactivates the Rho GTPases by C-terminal cleavage (Shao et al., 2002, Shao et al., 2003, Aepfelbacher et al., 2005).

Expression of *ysc*, *lcr* and *yop* genes is controlled by VirF (LcrF), an AraC-like transcriptional activator that is also encoded on the pYV-plasmid (Cornelis et al., 1989). The translation of *lcrF* m-RNA is temperature-dependent regulated (see 1.6.) (Schwiesow et al., 2015, Nuss et al., 2017). The physiological role of T3SS is believed to act mainly as a needle for Yop injection into host cells without the release of Yop effectors into the extracellular milieu (Rosqvist et al., 1994, Cornelis and Wolf-Watz, 1997, Mota et al., 2005). Nevertheless the secretion of Yop proteins by the T3SS can

artificially be induced in vitro culturing bacteria at 37°C in the absence of calcium (Heesemann et al., 1984, Biot and Cornelis, 1988).

In summary, this Yop-virulon constitutes an essential virulence determinant of *Yersinia* spp. that enables the extracellular bacteria to manipulate host cells in a contact-dependent manner.

1.5.1.2. The Adhesin YadA

Another crucial protein encoded by the pYV-plasmid is the *Yersinia* adhesin A (YadA), a trimeric autotransporter protein (monomer = 41-44 kDa) belonging to the Type Vc Secretion System. Type V secretion systems (TVSS) comprise five subtypes: TVaSS, TVbSS, TVcSS, TVdSS and TVeSS (Meuskens et al., 2019). The C-terminal domains form a 12-stranded β-barrel in the outer membrane (translocator). The three surface-exposed N-terminal domains form a homo-trimeric coiled-coil rod with an oval head domain (lollipop-like structure) that can be detected as a heat-stable protein band of about 160-250 kDa in SDS-PAGE (see **Figure 2**) (Skurnik and Wolf-Watz, 1989, Tamm et al., 1993, Gripenberg-Lerche et al., 1995, Hoiczyk et al., 2000, El Tahir and Skurnik, 2001, Leo et al., 2012, Meuskens et al., 2019).

YadA is produced mainly at 37°C but can be detected in lesser amounts at 25°C (Bottone, 1999). Electronic microscopy has revealed that this abundant protein covers nearly the entire bacterial surface explaining the increased hydrophobic property of enteropathogenic yersiniae cells grown at 37°C (Bolin et al., 1982, Hoiczyk et al., 2000, El Tahir and Skurnik, 2001). YadA binds to extracellular matrix (ECM) proteins (collagen, laminin, fibronectin), the intestinal submucosa, mucus and also to hydrophobic surfaces, for example polystyrene (Lachica and Zink, 1984a, Lachica and Zink, 1984b, Schulze-Koops et al., 1993, Tamm et al., 1993, Pepe et al., 1995, El Tahir and Skurnik, 2001). In stationary phase cultures YadA causes bacterial autoagglutination (Balligand et al., 1985, Skurnik et al., 1994). It plays a major part in adherence to host cells (Heesemann and Grüter, 1987) by binding indirectly to β1-integrin through an ECM protein-mediated bridging mechanism (Bliska et al., 1993). YadA also plays a crucial role in serum resistance of *Y. enterocolitica* through prevention of the activation of the classical complement pathway (Heesemann and Laufs, 1983, Heesemann and Grüter, 1987, Roggenkamp et al., 1996, Biedzka-Sarek et al., 2008).

Moreover YadA is made responsible for chronification of Yersiniosis and the occurrence of sequelae such as reactive arthritis (Gripenberg-Lerche et al., 1994, Gaede and Heesemann, 1995, Skurnik, 1995).

Expression of *yadA* is positively controlled by pYV-encoded VirF at 37°C and negatively regulated by the two-component regulatory system EnvZ/OmpR (Cornelis et al., 1989, Skurnik and Toivanen, 1992, Rohde et al., 1999, Nieckarz et al., 2016).

1.5.2. Chromosomally Encoded Virulence Factors

1.5.2.1 Invasin InvA and Its Transcriptional Regulator RovA

Another major adhesin in enteropathogenic yersiniae is invasin InvA (or Inv) (Isberg et al., 1987), a 92kDa OM protein encoded on the *Yersinia* chromosome by the *inv* locus. InvA is expressed optimally at 28°C but can also be detected at 37°C, but preferentially under acidic pH-conditions (Pepe and Miller, 1993, Pepe et al., 1994).

InvA binds directly to β1-integrin and promotes M cell penetration and translocation in early stage disease of oral infection (see **Figure 2**) (Isberg and Leong, 1990). In contrast to YadA, InvA is a monomeric adhesin, which forms an N-terminal 12-stranded β-barrel in the outer membrane (OM) with a surface-exposed C-terminal domain. Therefore InvA is assigned to the Type Ve Secretion System (Leo et al., 2012, Meuskens et al., 2019). InvA expression is controlled by RovA (Regulator of virulence), a MarR-type transcriptional regulator (Multiple antibiotic resistance Regulator), that autoregulates its own production (Revell and Miller, 2001). RovA binds to regulatory regions of the *rovA* and *inv* gene (Nagel et al., 2001). Its binding sites overlap with the binding sites of the repressing histone-like protein H-NS. The preponderance of RovA at 28°C leads to the displacement from DNA-binding of the antagonistic H-NS. Thus the less H-NS blocks *rovA* and *inv*, the higher their transcription. The binding affinity of the antirepressor RovA decreases at 37°C and RovA additionally gets proteolysed under in vitro conditions (Heroven et al., 2004, Cathelyn et al., 2007, Heroven et al., 2007, Uliczka et al., 2011).

In contrast to *Y. enterocolotica* and *Y. pseudotuberculosis*, there is no expression of a functional InvA in *Y. pestis* because of an insertion sequence (IS) integration disrupting *inv* gene expression (Simonet et al., 1996). This could be the result of positive selection due to an evolutionary change in lifestyle and infection pathway (Simonet et al., 1996). However the transcriptional activator RovA is conserved in *Y. pestis*. Mouse infections of *rovA* mutants in *Y. pestis* as well as in *Y. enterocolitica* showed a clear attenuation in virulence compared to the wildtype or regarding the latter compared to an *inv* mutant. This suggests RovA to be a global transcription regulator that controls a larger set of genes required for virulence than currently known (Cathelyn et al., 2006).

1.5.2.2. The Adhesin Ail and the Ail-like Protein OmpX

Together with InvA and YadA, Ail (*Attachment and invasion locus*) is another member in the series of adhesins. The 17-kDa surface-exposed protein is encoded chromosomally and consists of eight β-sheets spanning the bacterial membrane and exposing four loops to the surface (Miller et al., 1990, Beer and Miller, 1992, Miller et al., 2001, Mikula et al., 2012). The loops initiate attachment and invasion and are also indispensable for serum resistance (Miller et al., 2001, Biedzka-Sarek et al., 2005). Ail binds laminin, fibronectin and heparan sulfate proteoglycan (Yamashita et al., 2011) and is only found in pathogenic *Yersinia* species (Miller and Falkow, 1988, Pierson and Falkow, 1993). Ail is

maximally produced at 37°C (Atkinson and Williams, 2016). One study reported on modulation of Ail production by the ClpP protease complex (Pederson et al., 1997). Pathogenic yersiniae produce at least one other Ail-like protein called OmpX. Although the function of OmpX has not yet been examined in *Y. enterocolitica*, analysis in *Y. pestis* shows remarkable similarities to Ail in structure, properties and role in pathogenicity (Kolodziejek et al., 2007, Kolodziejek et al., 2010).

1.5.2.3. The Myf Fimbriae

The Myf fimbriae (Mucoid *Yersinia* factor) are produced by *Y. enterocolitica* at 37°C at acidic pH (Iriarte and Cornelis, 1995). They are fimbriae-like polymers of the 21-kDa MyfA subunits. The *myf* locus is located on the bacterial chromosome and comprises five genes organized in two operons (*myfABC* et *myfEF*) (Iriarte and Cornelis, 1995, Bialas et al., 2012):

- *myfA* encodes the major component of the surface fibrillae
- *myfB* encodes a putative periplasmic chaperone
- *myfC* encodes an outer membrane usher protein
- *myfE* and *myfF* encode transcriptional activators that act as a two-component system at the inner membrane and are necessary for expression of *myfABC* genes (Iriarte et al., 1993, Yang and Isberg, 1997).

MyfB and MyfC incorporate MyfA subunits to the growing pilus that protrudes through the outer membrane (Iriarte et al., 1993, Iriarte and Cornelis, 1995).

MyfA is only found in *Y. enterocolitica* strains that are virulent (Iriarte et al., 1993). The particular functions of MyfA in the pathogenicity of *Y. enterocolitica* have not been identified yet. Nevertheless it can be detected during infection and can be taken for serum tests to diagnose yersiniosis (Niefnecker, 2009, Kakoschke et al., 2016). MyfA shows structural similarities to other fimbriae of enterotoxic bacteria such as the CS3 pilin of *E. coli* pathotype ETEC (Levine et al., 1984, Iriarte and Cornelis, 1995). MyfA also shares 44% identity to the pH6 antigen (Psa) of *Y. pestis* (Lindler et al., 1990) and is homologue to the pH6 antigen in *Y. pseudotuberculosis* (Yang and Isberg, 1997). Moreover, there are clear similarities between the optimal conditions for expression of these two proteins; both are maximally produced at 37°C under acidic conditions. However the resulting fimbriae have different receptors: for example the pH6 antigen causes haemagglutination in *Y. pseudotuberculosis* (Yang and Isberg, 1997), whereas Myf does not in *Y. enterocolitica* (Mikula et al., 2012).

1.5.2.4. Lipopolysaccharides

Lipopolysaccharides (LPS) are essential components of the OM of Gram-negative bacteria. They consist of three basic modules (**Figure 3**; also see **Figure 2**):

(1) Lipid A represents the inner part of the LPS-molecule. It is a disaccharide consisting of glycosaminophosphates linked to four to six fatty acids (Skurnik and Toivanen, 1993, Reines et al., 2012a) that are anchored to the outer lipid leaflet of the OM. When released during destruction of the pathogen and in this free form lipidA functions as an endotoxin, inter alia causing fever, inflammation, apoptosis. In the blood circulation LPS binding protein (LBP) and CD14 interact with LPS. These complexes induce an inflammatory response of leukocytes by activation of the TLR4 complex (Park and Lee, 2013). LPS is relatively heat-stable and can only be eliminated with special sterilization methods such as dry heat at 250°C for at least 30 minutes (Tsuji and Harrison, 1978, Miyamoto et al., 2009).

(2) The core is composed of oligosaccharides linked to the lipid A by keto-deoxyoctulosonate (KDO) that can be divided into inner and outer core (Skurnik and Toivonen, 2011).

(3) The **O-antigen** structure is a variable, one or two-linked, polysaccharide chain. While lipid A and the core oligosaccharides are relatively conserved within different bacteria genera and species, the O-antigen shows many structural variations and is highly heterogeneous even within different strains of the same species (Jansson et al., 1981, Di Genaro et al., 2000), a feature used as the basis of serological serotyping.

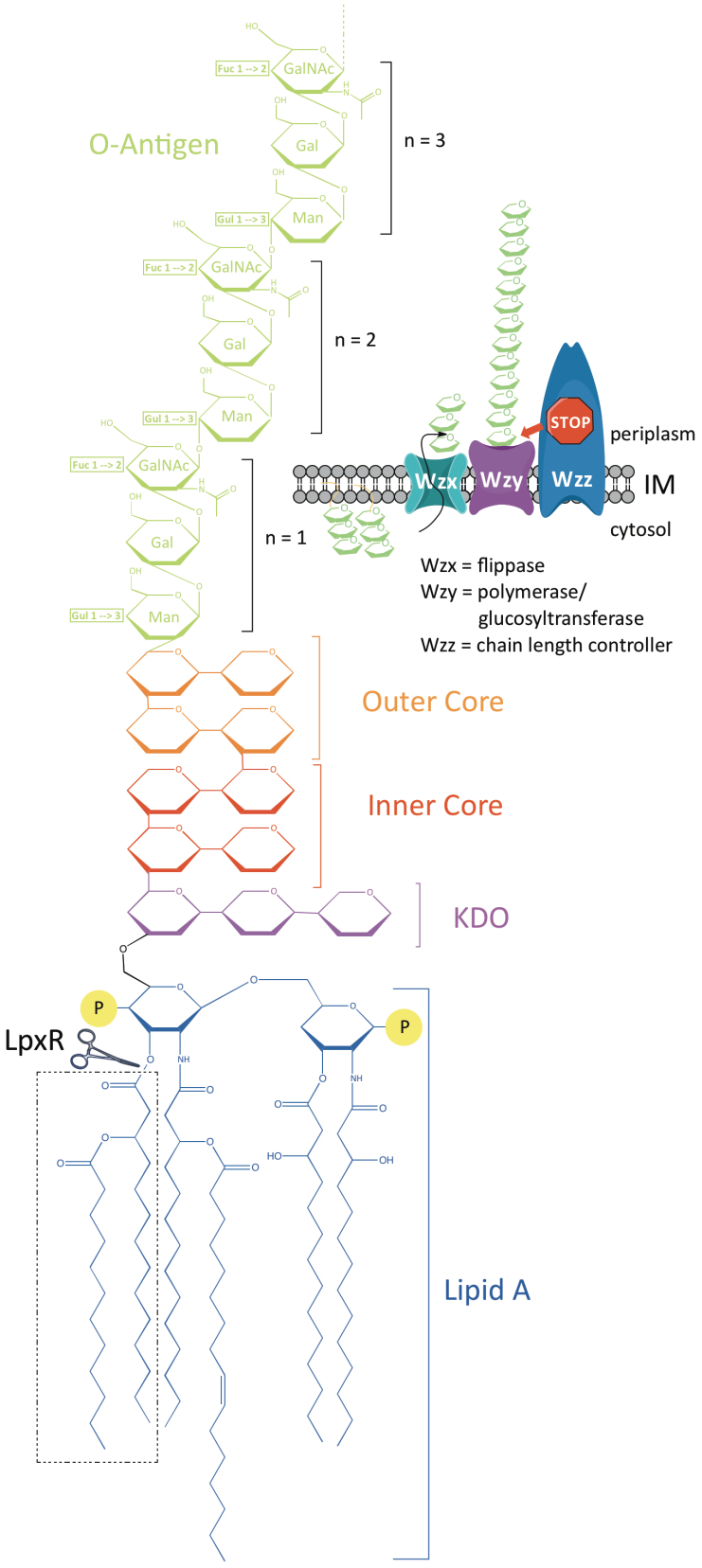

Figure 3: Schematic representation of hexa-acylated LPS structure with the O-Ag of *Y. enterocolitica* O:8.

Y. enterocolitica produces a high number of O-antigen repeats (n=7 to n=10) at room temperatures (22-25°C) (al-Hendy et al., 1991, Brubaker, 1991, Bengoechea et al., 2002b, Skurnik and Bengoechea, 2003). Single O-units are transferred from the cytoplasm via the inner membrane to the periplasm by the flippase Wzx. The polymerase Wzy polymerizes them at the periplasmic side of the inner membrane and Wzz controls the chain length (Bengoechea et al., 2002a). The O-Ag is then ligated to the lipid A and then exported to the bacterial surface on the outer membrane (Skurnik and Bengoechea, 2003).

Importantly, the O-antigen plays a significant role in the virulence of *Y. enterocolitica* serotype O:8 (Zhang et al., 1997, Darwin and Miller, 1999, Bengoechea et al., 2004). Accordingly, it promotes resistance to serum and to antimicrobial peptides and also modulates the function and/or abundance of the adhesins YadA, Ail and InvA (Bengoechea et al., 2004).

In addition to the O-antigen, other LPS modifications are linked with virulence and resistance to antimicrobial peptides (Reines et al., 2012a, Reines et al., 2012b, Simpson and Trent, 2019). Thus the acylation grade plays an important role. Hexa-acylated lipid A (possibly in addition with aminoarabinose or palmitate) causes a strong activation of the TLR4-pathway, while tetra-acylated lipid A does not function as an endotoxin anymore (Reines et al., 2012a, Reines et al., 2012b, Maeshima and Fernandez, 2013). Responsible for the deacylation especially at 37°C is LpxR, a 3'-O-deacylase (Reines et al., 2012a). At 21°C lipid A of *Y. enterocolitica* O:8 is mainly hexa-acylated with four C_{14} units, one C_{12} unit and one $C_{16:1}$ unit (**Figure 3**), whereas at 37°C most lipid A is tetra-acylated with four C_{14} units (Perez-Gutierrez et al., 2010).

The LPS modifications are controlled by the transcriptional regulators RovA, PhoP and PmrA (Reines et al., 2012a, Reines et al., 2012b), while OmpR appears to promote the overall amounts of LPS produced by *Y. enterocolitica* (Skorek et al., 2013).

1.5.2.5. High Pathogenicity Island

Some bacteria carry so-called pathogenicity islands, genomic regions acquired by horizontal gene transfer and that carry genes for virulence factors (Hacker et al., 2003). The *Yersinia* High Pathogenicity Island (HPI) is a chromosomal gene cluster governing siderophore biosynthesis, regulation and transport. As the name implies, within yersiniae it is only found in the highly pathogenic strains: mouse-virulent *Y. enterocolitica* biotype 1B, *Y. pseudotuberculosis* (most serotypes) and *Y. pestis* (Schubert et al., 1998). Interestingly HPI can be also present in other species of the family of *Enterobactericeae* causing systemic infections in humans, such as *E. coli*, *Klebsiella oxytoca*, *Citrobacter koseri* (Schubert et al., 2004).

Iron is essential for almost all living organisms and for many microbes (inter alia: respiratory chain, DNA synthesis). Although the element iron is abundant on our planet it is not readily available for

microorganisms as it is usually found chelated to specific compounds or in poorly soluble form. Moreover, in the case of pathogens, the host restricts the concentration of free iron by binding to transferrin, lactoferrin and ferritin as a mechanism to limit bacterial growth. Siderophores (Greek: iron carrier) are low-molecular weight molecules (about 500 – 1500 Da) produced and exported by a large number of bacteria and fungi to scavenge the surroundings for free iron (Hider and Kong, 2010). They have a great affinity for Fe^{3+} (ferric iron) and chelate these ferric ions. The resulting complexes are specifically imported by the microorganisms thanks to the interaction with surface receptors and then the iron can be freed and utilized e.g. by reduction to the soluble Fe^{2+} (ferrous iron) (see **Figure 2**) or stored intracellularly by bacterioferritin (Neilands, 1995, Rivera, 2017).

Yersiniabactin (Ybt) is a low molecular weight siderophore and closely related to the siderophore pyochelin of *Pseudomonas aeruginosa*. Ybt is responsible for the iron-uptake in highly virulent *Yersinia* species (Rakin et al., 2012). In *Y. enterocolitica* the six genes *irp1-5* and *irp9* (irp for iron-regulated protein) are needed for Ybt synthesis. The gene *fyuA* encodes the 71-kDa outer membrane protein FyuA (**F**erric-**y**ersiniabactin **u**ptake) which operates as the receptor for the uptake of the Fe^{3+}-Ybt-complex. It also binds pesticin, a bacteriocin produced by *Y. pestis*. Due to this dual function FyuA is also responsible for the pesticin-sensitivity of highly pathogenic *Yersinia* strains (Heesemann et al., 1993, Rakin et al., 1994, Pelludat et al., 1998). Irp6 and Irp7 are inner membrane proteins mediating the iron import. Expression of *irp* and *fyuA* genes is repressed by high iron concentrations due to the global repressor protein Fur (**F**e-**u**ptake **r**egulation). On the other hand, transcriptional activation of genes involved in Ybt production is regulated by the AraC-type protein YbtA, which inhibits its own production (Schubert et al., 2004, Perry and Fetherston, 2011).

In the mammalian host, transporting proteins like transferrin bind to free iron that is essential for the bacteria. To survive and grow in these iron deficient environmental conditions, yersiniae need siderophore production. Besides iron Ybt is also able to bind copper ions Cu(I) and Cu(II) providing an additional tool to evade the host's immune defence and copper toxicity (Koh and Henderson, 2015).

Significantly, Ybt is an essential virulence factor and necessary for mouse lethality in *Y. enterocolitica* (Heesemann et al., 1993).

1.5.2.6. Urease

The gastric acid has a pH varying between 1 and 4, depending on the fasting status. *Y. enterocolitica* has the highest growth rate at about a pH of 7,2 to 7,4 but generally can multiply between a constant pH ranging from 4,2 to 9,0 and even remains viable in pH 3,6-3,8 (Sutherland and Bayliss, 1994). A normal gastric passage takes place in about 2 hours. In fact *Y. enterocolitica* can survive at pH-values of 2, when exposed within this time interval, but only in the presence of urea (De Koning-Ward and Robins-Browne, 1995). As various other microbes, *Y. enterocolitica* employs the enzyme urease to

resist acidity (Mobley and Hausinger, 1989). Urease catalyses the hydrolysis of urea to ammonia and carbon dioxide (see **Figure 2**):

$$H_2N - CO - NH_2 \rightarrow 2\ NH_3 + CO_2$$

This multisubunit metalloenzyme (requires nickel in its active centre) is highly conserved among many bacteria species, but shows some specific characteristics for *Y. enterocolitica*. Within *Y. enterocolitica* urease reaches its top reaction rate at pH 3,5 – 4,5 and is more active at 28°C than at 37°C (de Koning-Ward and Robins-Browne, 1997). With a small amount of substrate available this cytoplasmic protein can convert at maximum rate of hydrolysis, demonstrating a strong affinity to environmental urea. In contrast to other ureolytic bacteria it is not regulated by urea (as for example in *Proteus mirabilis* (Jones and Mobley, 1988) or nitrogen availability (as in *Klebsiella aerogenes* (Friedrich and Magasanik, 1977)), or acidic surrounding conditions, but by growth phase. Urease activity is highest in stationary phase (de Koning-Ward and Robins-Browne, 1997). Integrating this knowledge into the life cycle of enteropathogenic yersiniae, we speculate that food-borne bacteria are likely to be in stationary phase and also at low temperature and therefore are likely to contain urease, which then facilitates the bacterial survival within the acidic gastric juice after oral incorporation.

1.5.2.7. The Heat-stable Toxin Yst

The *Yersinia* stable toxin (Yst) is a heat-stable enterotoxin that is found in all virulent *Y. enterocolitica* strains and is not present in non-pathogenic biovars (Delor et al., 1990). Resembling the *E. coli* enterotoxin STI, Yst also leads to a fluid secretion towards the lumen by stimulating the guanylate cyclase (Revell and Miller, 2001). Thus this 30-amino acid peptide (Takao et al., 1985) is assumed to contribute to the induction of diarrhea, which was shown in a peroral young rabbit infection model (Delor and Cornelis, 1992). The chromosomally encoded Yst is mainly expressed in late exponential growth phase (Delor and Cornelis, 1992, Amirmozafari and Robertson, 1993). In standard culture media Yst is expressed below 30°C, but *yst* transcription can also be detected at 37°C upon growth in conditions of increased osmolarity and pH that resemble physiological conditions of the small intestinal lumen (Mikulskis et al., 1994).

1.6. Post-transcriptional Regulation by sRNAs and the RNA Chaperone Hfq

During its life cycle, *Y. enterocolitica* continuously adapts to changes in its environment which enables it to survive and multiply, evade the host's defence system and cause disease. All of these processes require an efficient and reactive fine-tuned regulation of gene expression. But how is the gene expression and protein synthesis regulated and coordinated in a fine-tuned fashion? How does the bacterium produce the right virulence factors during the course of invasion and infection and repress others before it is killed by the host?

Until fifteen years ago, it was believed that bacterial gene expression was exclusively or mainly regulated at the transcriptional level. In a simplified scheme this means special proteins (so-called transcription factors) either repress or activate gene transcription into an mRNA by binding to defined DNA regions that are associated or often located next or near the promotor sequence. The binding of an activator protein to the activator binding site facilitate the RNA polymerase assembly and thus induces transcription of a gene or a group of genes (positive regulation), while the binding of a repressor at the repressor binding site (= operator) leads to a negative regulation of transcription (Nelson and Cox, 2008). **Figure 4** illustrates this "on and off switching system" of gene expression. The functional unit of the regulated genes, the promotor and the regulatory DNA regions (activator binding site, repressor binding site/operator) is collectively called an operon. The operon model was first introduced in 1960 with the operating principle of the *lac* operon (in *E. coli*) that is reagulated by the presence of lactose in the bacterial cell (Jacob et al., 2005).

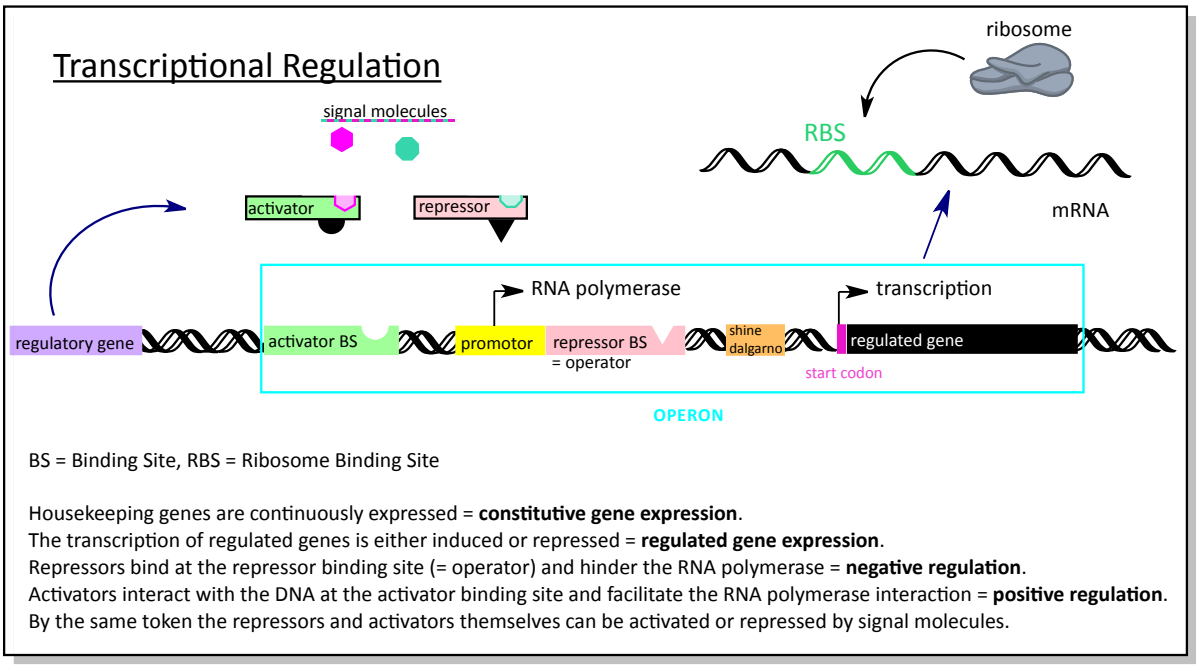

Figure 4: Simplified illustration of transcriptional regulation in prokaryotes in accordance with "Lehninger Principles of Biochemistry" (Nelson and Cox, 2008).

In the past decade, the discovery of small non-coding RNAs in bacteria has led to a paradigm shift similar to the shift that took place with the discovery that RNA molecules can possess catalytic qualities, subsequently named "ribozymes" (ribonucleic acid enzymes) (Cech and Steitz, 2014). Post-transcriptional regulation is now assumed to contribute significantly to the rapid adaptation of bacteria to changes in the environment.

As the name already indicates "post-transcriptional regulation" is the collective term for all regulatory processes after DNA has been transcribed into mRNA, but before the mRNA is translated into a protein product. Recently researchers revealed a broad network of post-transcriptional

regulation in microbes, which has been most studied in Gram-negative bacteria, primarily *E. coli* and *Salmonella enterica* (Wassarman et al., 1999, Papenfort et al., 2006, Padalon-Brauch et al., 2008, Sittka et al., 2008, Vogel, 2009, Waters and Storz, 2009, Papenfort and Vogel, 2010, Storz et al., 2011, Erhardt and Dersch, 2015)

Different classes of regulators have been identified, i.e. riboswitches, thermoswitches, small RNAs and RNA-binding proteins, also in yersiniae (Schiano and Lathem, 2012, Nuss et al., 2017, Knittel et al., 2018). Riboswitches are sequences within the mRNA that can bind to specific ligands, leading to changes in secondary conformation of the mRNA. Thus if the ribosome binding site (RBS) for example is concealed or revealed by this structural rearrangements, translation can be prevented or supported (Mandal and Breaker, 2004, Waters and Storz, 2009, Schiano and Lathem, 2012). Thermosensors, sometimes called thermoswitches, are important for the bacteria to respond quickly to shifts in temperature. In *Yersinia*, production of the transcriptional activator VirF is post-transcriptionally thermoregulated (see paragraph 1.5.1.) by a stem-loop motif in the mRNA formed immediately upstream of the *virF* coding sequence at 25°C. This thermosensor RNA region hides the sequence of the ribosome binding site and thus prevents the ribosome from binding. An upshift in temperatures leads to a conformational change and a disappearance of the stem-loop, thereby enabling the ribosome to dock and start translation (Bohme et al., 2012, Schiano and Lathem, 2012).

The most rapidly growing class of post-transcriptional regulators is the heterogeneous group of small regulatory RNAs (sRNAs). With exceptions (Friedman et al., 2017) sRNAs are non-coding RNAs, i.e. they are not translated into proteins. Most sRNAs modify gene expression and protein production by modulating mRNA stability and translation (Waters and Storz, 2009) (see **Figure 5**).

There are sRNAs that directly bind to proteins and influence their activity. For example the sRNAs CsrB and CsrC bind the **c**arbon **s**torage **r**egulator **A** (CsrA), an mRNA-binding protein that facilitates carbon usage, motility and sensitivity to antibiotics. CsrA allow the cell to perform a series of metabolic adjustments in order to account for environmental challenges (Babitzke and Romeo, 2007, Romeo et al., 2013, LeGrand et al., 2015).

The major known group of sRNAs, however, act by base-pairing to their target mRNAs. They are typically between 50-250 nt long (Michaux et al., 2014) and generally divided into two subgroups:

(1) The *cis*-acting antisense sRNAs are encoded directly on the opposite DNA strand of the mRNA they influence. Therefore their defining feature is precise base-pairing because of their complete complementary to parts of the mRNA (Brantl, 2007).

(2) In general, the gene loci of *trans*-acting sRNAs are not located close to the genes that they regulate. These sRNAs show limited and imperfect complementarity to the mRNA that they regulate, usually around 10-15 nucleotides (Waters and Storz, 2009). Moreover these *trans*-acting sRNAs have the ability to base-pair with more then one mRNA, thus possessing lower specificity and having more flexibility in their effects (Gottesman, 2005, Storz et al., 2011).

Figure 5 gives an overview of the mechanisms of action of small non-coding RNAs:

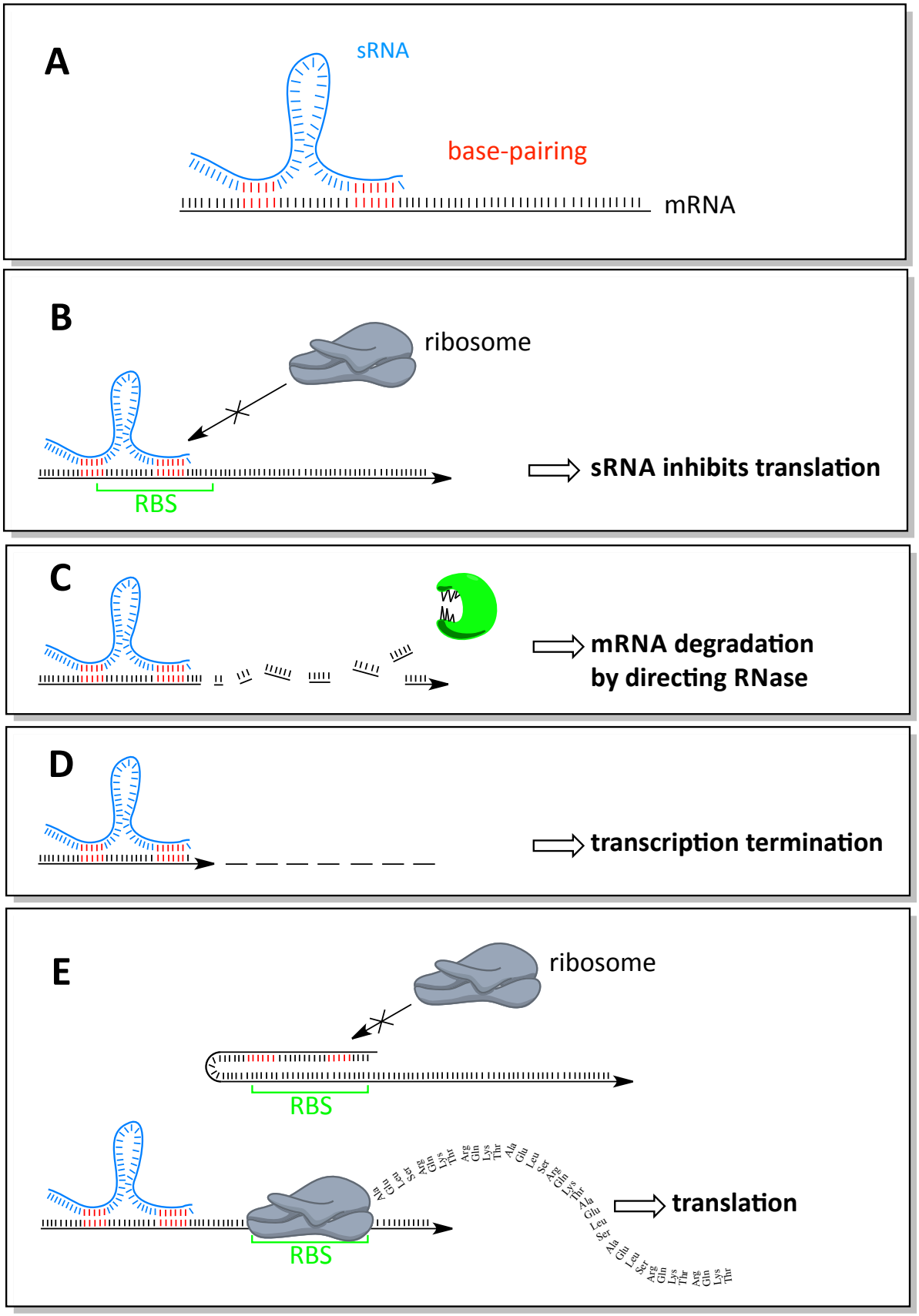

Figure 5: Small non-coding RNAs affecting/targeting mRNA: (A) Base-paring of an sRNA to an mRNA, (B) Inhibition of translation, (C) Degradation of mRNA, (D) Termination of transcription, (E) Support of translation. RBS = Ribosome Binding Site.

In order to be fully functional many of the *trans*-encoded sRNAs in Gram-negative bacteria need the RNA-binding chaperone Hfq. First described over half a century ago in *E.coli* as a host factor required for the replication of the RNA bacteriophage Qβ (Franze de Fernandez et al., 1968), Hfq became a focus of interest with the onset of discoveries of new sRNAs and the awareness of its crucial role in the mediation of post-transcriptional regulation (Vogel and Luisi, 2011). Hfq is a highly conserved protein within a great number of bacterial species and has orthologues in eukaryotes and archaea, i.e. the so-called Sm proteins or Sm-like (LSm) proteins (Wilusz and Wilusz, 2005, Rabhi et al., 2011). Hfq is a small protein (in *Yersinia*: 101 amino acids or 11kDa) and forms a homohexameric toroid structure. Each subunit is composed of an α-helical domain (proximal face) followed by five twisted anti-parallel β-strands (distal face) (Link et al., 2009).

The ring-shaped protein has four different sites to interact with single-stranded RNAs (Updegrove et al., 2016, Holmqvist and Vogel, 2018). According to the current state of research the Hfq-RNA-interaction can be described in a simplified scheme as followed: the proximal face of Hfq-protein binds sRNA at U-rich sequences (Otaka et al., 2011, Sauer and Weichenrieder, 2011), whereas the distal face binds mRNA at A-rich regions (Link et al., 2009, Robinson et al., 2014, Updegrove et al., 2015). The rim area brings together the AU-rich regions of the simultaneously bound sRNA and mRNA and facilitates the pairing of these two RNAs (imperfect RNA base pairing) (Panja et al., 2013, Holmqvist and Vogel, 2018). The C-terminus of Hfq can also interact with RNAs. It is considered that it plays a role in the clearance of nonspecific RNA binding and in enabling a rapid RNA turnover rate (Fender et al., 2010, Holmqvist and Vogel, 2018, Santiago-Frangos and Woodson, 2018).

Hfq-binding sRNAs, are composed of three components: an mRNA base-pairing region, a rho-independent transcription terminator and an Hfq-binding site (Otaka et al., 2011).

A great variety of sRNAs excists differing in size, shape and sequence and in the way they interact with Hfq, not always strictly following the general model of Hfq-RNA-interaction just described above (Zhang et al., 2013, Schu et al., 2015, Holmqvist and Vogel, 2018).

In Gram-negative bacteria, the RNA-chaperone Hfq is involved in different regulatory mechanisms. By interacting with sRNA and/or mRNA it can stabilise sRNA, but can also lead in other cases to RNA degradation. It can also modulate translation by facilitating sRNA-mRNA-interaction (Vogel and Luisi, 2011, Kavita et al., 2018). In some cases Hfq can modulate post-transcriptional regulation independently of sRNA binding (Kavita et al., 2018) or by direct protein-protein interactions (Sobrero and Valverde, 2012).

Hfq is considered an important and global player in post-transcriptional regulation. A summary of major regulatory mechanisms of Hfq is shown in **Figure 6** (Vogel and Luisi, 2011, Sobrero and Valverde, 2012, Kavita et al., 2018).

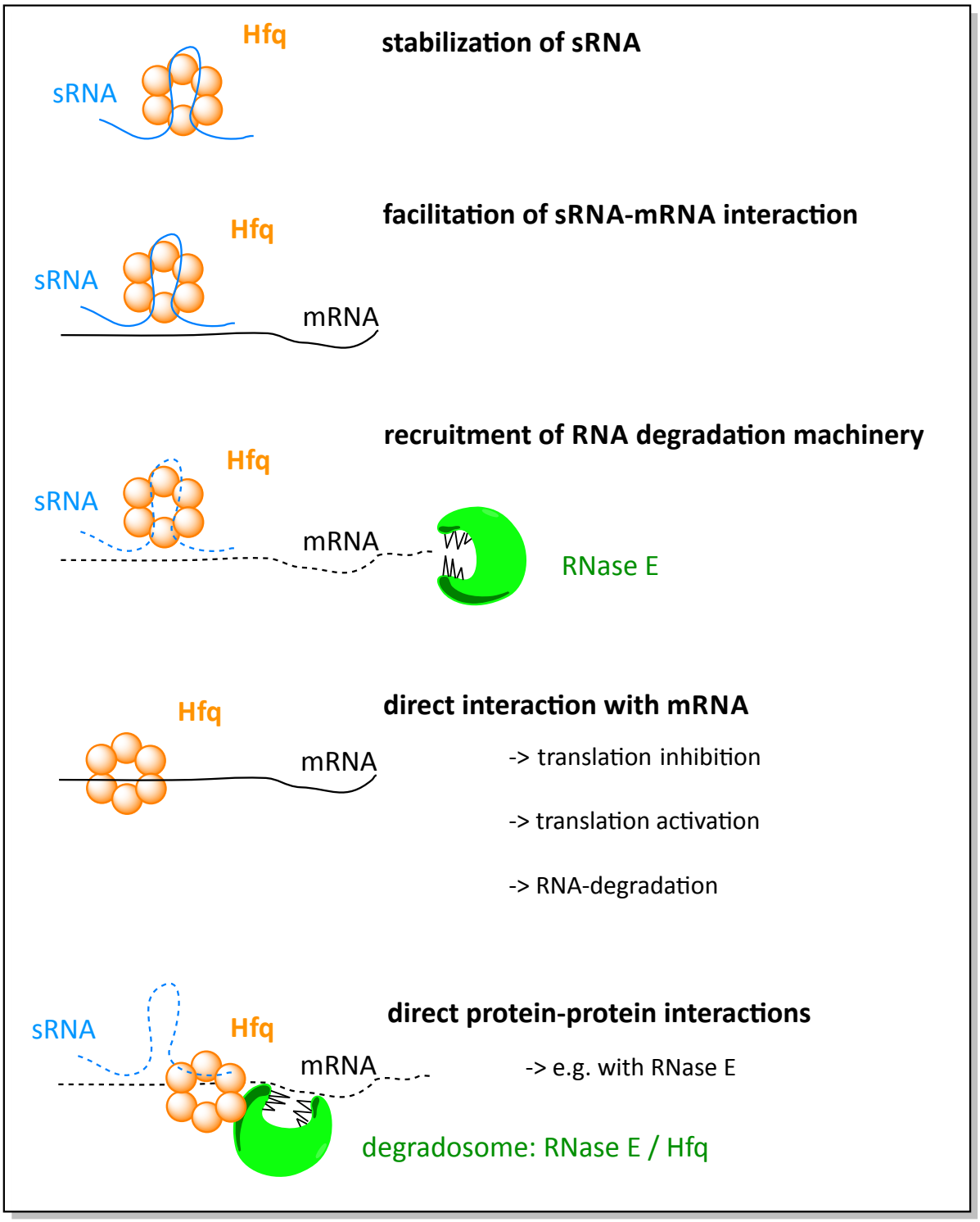

Figure 6: Depending on the mode Hfq interacts with sRNAs, mRNAs or proteins, it can lead to different effects: By binding sRNAs and/or mRNAs it can stabilze them and their interaction with each other. But it can also ease their degradation. By interacting directly with mRNAs it can also regulate translation by either blocking or exposing the RBS or leading to mRNA degradation. Hfq can also interact directly with proteins. For instance an Hfq-RNaseE complex can lead again to a recruitment of the degradation machinery (degradosome).

Through its interaction with many distinct RNA molecules, Hfq impacts a variety of cellular processes, including pathogenicity and fitness in several Gram-negative bacteria. However, there are many differences between bacterial species in what Hfq influences and to what extent (Chao and Vogel, 2010).

In *Y. pestis* and *Y. pseudotuberculosis* Hfq is essential for virulence (Geng et al., 2009, Schiano et al., 2010). However Hfq does not always have identical effects in these two closely related organisms. For example Hfq promotes growth in *Y. pestis*, but no growth defect can be seen in *Δhfq* mutants of *Y. pseudotuberculosis* (Bai et al., 2010). Further the T3SS seems differently affected in these two *Yersinia* species (Schiano and Lathem, 2012).

In 1995 Nakao et al. showed that *Y. enterocolitica* experiences a decrease in expression of the enterotoxin Yst due to a mutation in the *hfq* gene (Nakao et al., 1995). Since then no further results of Hfq effects have been published for *Y. enterocolitica*.

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3. Aims of this Study

The chaperon Hfq is a widely conserved RNA-binding protein and involved in the regulation of many genes in Gram-negative bacteria (**Figure 6**, page 31). Prior to the start of this study it was known that Hfq has an influence on the stress regulation and virulence of *Y. pestis* (Geng et al., 2009). For *Y. enterocolitica* only an impact of Hfq on the expression of a single virulence factor, the enterotoxin Yst, was reported in 1995 by Nakao et al (Nakao et al., 1995). No information about other pathogenicity factors, growth and metabolic behaviour, stress response or virulence in *Y. enterocolitica* with regard to Hfq had been published at the beginning of the experimental phase of this doctoral thesis.

The aim of this work was to investigate the role of Hfq in the virulence of *Y. enterocolitica* serotype O:8. By comparing *hfq*-negative mutants with their parental strains it should be assessed whether Hfq affects the bacterial growth, metabolism and resistance to stress.

Moreover the impact of Hfq on production of important virulence factors (T3SS/Yop, adhesins, siderophores, mouse virulence) should be examined.

Special emphasis should be put on the role of Hfq in post-transcriptional regulation.

The production of Hfq-protein in different growth phases should be examined and finally comparative mouse infection experiments with isogenic strains (wildtype versus Δ*hfq* mutant) should prove, if Hfq is involved in pathogenicity of *Y. enterocolitica in vivo*.

The essential results of this study will be summarized and the own contributions as well as those of the co-authors will be indicated.

4. Summary of Published Results

4.1. The RNA Chaperone Hfq Impacts Growth, Metabolism and Production of Virulence Factors in *Yersinia enterocolitica*

Kakoschke T, Kakoschke S, Magistro G, Schubert S, Borath M, Heesemann J, Rossier O

PLoS ONE 2014; 9(1): e86113. https://doi.org/10.1371/journal.pone.0086113

Genomic analysis of *Y. enterocolitica* wildtype strain 8081 (serotype O:8, biotype IB) revealed a gene sequence with high similarity and identity (both > 80%) to the known *hfq* gene of *E. coli*. To study the role of the protein Hfq in *Y. enterocolitica* serotype O:8 three independent mutants with *hfq* gene deletions were generated by homologeous gene replacement from two strains of different genotype (WA-314 and JB580v; latter a derivative of wildtype strain 8081). In the strains SOR3 (derived from strain WA-314) and SOR17 (derived from JB580v) a gene cassette with kanamycin resistance was put in place of the *hfq* gene. The third mutant strain SOR4 was derived from WA314 by unmarked deletion of *hfq* (deletion of kanamycin cassette).

Macroscopically on most agar plates bacterial colonies of wildtype and mutant strains appeared very similar. However light microscopy revealed that *hfq* mutants are enlarged in length and width compared to their wildtype.

Growth of *hfq* mutants in exponential phase was reduced in rich and minimal media. In stationary phase the number of bacteria was significantly reduced compared with that of their parental strains, indicated by a lower optical density (OD_{600nm}) in liquid culture and in reduced colony forming units (CFU) recovered on agar after plating. All mutants could be complemented with a pACYC184 plasmid carrying *hfq* gene. Complementation experiments also revealed that higher copy numbers of *hfq* can raise physiological growth of *Y. enterocolitica* wild-type strains.

A two-dimensional electrophoresis of soluble (supernatant) and total membrane proteins (sediment) of JB580v and its mutant strain showed differences in the abundance of various proteins. Mass spectrometry allowed the identification of several proteins (listed in **Table 3** and **Table 4** in Kakoschke et al. 2014). Deletion of *hfq* resulted in a decreased production of proteins playing a role in the lipid metabolism and transport, cell homeostasis, chaperone modulation, anaerobic respiration, translation or ATP-synthesis. Increase of protein production could be assigned to different classes comprising chaperones and proteins involved in stress response, carbon and amino acid metabolism, peptide transport and tRNA synthesis as well as outer membrane proteins, including siderophore receptors such as FyuA. All together these results suggest that Hfq is involved in regulation of metabolism, production of surface proteins and stress response in *Y. enterocolitica*.

To get a first evidence for the impact of Hfq on metabolism, the utilization of common saccharides and cyclic polyalcohols (sugar alcohols) was examined. Indirect biochemical tests and growth tests in defined minimal media suggested that Hfq affects the catabolism of mannitol, inositol and 1,2-propanediol. Moreover further results showed that Hfq inhibits tryptophanase, indole production and ornithine decarboxylase activity and therefore also impacts nitrogen metabolism.

The *hfq* mutant strains showed less survival to acidic stress. Western blot analysis revealed a lower presence of the enzyme urease in the strains that lack *hfq*. Hence Hfq promotes the production of urease and subsequently enhances the resistance of *Y. enterocolitica* to low pH-environment. Whereas Hfq is also necessary for the bacteria's ability of *Y. enterocolitica* to withstand oxidative stress (shown by higher survival of the parental strains in the presence of hydrogen peroxide) it has no influence on antibiotic resistance (no difference in minimal inhibitory concentration of different antibiotics tested).

Using a pesticin sensitivity assay, immoblotting and a luciferase-based reporter assay, it could be shown that the presence of Hfq attenuates the production of the siderophore yersiniabactin and its receptor FyuA, which are major virulence factors of *Y. enterocolitica* serotype O:8. In contrast to *Y. pseudotuberculosis* (Schiano et al., 2010) Hfq had no impact on the production or secretion of Yops in vitro in *Y. enterocolitica*.

To monitor the growth state dependence of Hfq production in *Y. enterocolitica*, the protein was tagged with a FLAG epitope for immunodetection of whole cell lysates. Late exponential and stationary growth phase favoured production of Hfq.

Taken together Hfq is differentially involved in the regulation of metabolic pathways and pathogenicity factors, which appears to be *Yersinia* species-specific.

4.1.1. Author Contributions:

Ombeline Rossier and **Tamara Kakoschke** planned and designed the experiments. Sara Kakoschke performed stress tests and the experiments on indole production. Giuseppe Magistro did the FyuA immunodetection experiment and the luciferase reporter assay measuring yersiniabactin production. Marc Wirth (née Borath) performed mass spectrometry. Marc Wirth (née Borath) and Sören Schubert analysed mass spectrometry data. The deletion mutants were generated by Ombeline Rossier. Transformation and complementation experiments as well as overexpression experiments were done by **Tamara Kakoschke**. Growth experiments were performed by **Tamara Kakoschke**. Morphological analysis including light microscopy was done by **Tamara Kakoschke**. Ombeline Rossier did the proteomic analysis of the 2-DE gel. She also analysed the Yop-secretion and production of Hfq protein. **Tamara Kakoschke** performed the immunoblottings and experiments on urease production. Sören Schubert and Jürgen Heesemann contributed reagents, materials and analysis tools. Jürgen Heesemann gave intellectual support. Ombeline Rossier wrote the paper.

Author Assignment of the Shown Figures:

Table 1/Table 2/Table 3/Table 4 (Ombeline Rossier), Figure 1 (**Tamara Kakoschke**), Figure 2 (Ombeline Rossier), Figure 3 a, b (Ombeline Rossier, **Tamara Kakoschke**), Figure 4 a, b (Sara Kakoschke), Figure 4 c (Ombeline Rossier), Figure 5 a, b (**Tamara Kakoschke**), Figure 6 a, b (Sara Kakoschke), Table 5 (Ombeline Rossier, **Tamara Kakoschke**), Figure 7 a, b (Giuseppe Magistro), Figure 8 (Ombeline Rossier), Figure 9 (Ombeline Rossier).

4.2. The RNA Chaperone Hfq Is Essential for Virulence and Modulates the Expression of Four Adhesins in *Yersinia enterocolitica*

Kakoschke TK, Kakoschke SC, Zeuzem C, Bouabe H, Adler K, Heesemann J, Rossier O

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This publication reports on the role of Hfq in the production of surface-exposed pathogenicity factors inserted in the outer membrane that promote adherence of *Y. enterocolitica* to host cells. Using Northern blotting, translational fusions of the gene encoding green fluorescent protein with virulence-associated outer membrane proteins and immunoblotting, the expression of a subset of adhesin genes and production of the proteins in the wild-type and isogenic *hfq*-negative strain were compared. To get evidences whether Hfq has a transcriptional or post-transcriptional effect, the endogenous promotor controlling the expression of the target gene was replaced with the Plac promotor.

Eventually Hfq has an effect on the production of the major adhesins Ail, Ompx, InvA and YadA and the fimbriae-like MyfA.

Ail and OmpX production are decreased by Hfq post-transcriptionally likely by destabilizing *ail* and *ompX* transcripts in stationary phase. Production of invasin is rather increased by Hfq on the transcriptional level. A closer examination revealed that Hfq has a slight influence on expression of *rovA* and *ompR* (translational *gfp*-reporter fusions), encoding a transcriptional activator and a transcriptional repressor of *inv*, respectively.

A more complex role of Hfq in regulating the expression of *yadA* was uncovered thanks to complementation experiments and morphological analysis of microcolonies (grown in 3D-collagen gels imitating a physiological environment for forming microabscesses) and the production of YadA-dependent collagen binding. A fine-tuned amount of Hfq seems to be important in the regulation of YadA production: the lack of Hfq lead to a reduction of YadA production, but on the other hand additional copies of *hfq* expressed from plasmid pACYC184 resulted in reduced amounts of YadA within the OM. Moreover depending upon growth conditions and bacterial growth phases Hfq can on the one hand promote YadA production independently of the transcriptional activator VirF but can also lead to a reduction of *yadA* transcripts. The discrepancy in *yadA* expression (reporter fusions) and the amount of surface-exposed YadA protein might result from post-translational mechanisms, such as *hfq*-dependent secretion and/or degradation of YadA protein in the periplasmic space. This hypothesis is supported by the increase of the YadA-cleaving protease DegP in the periplasmic space in the *hfq* mutant (observed in the proteomic analysis in Kakoschke et al. 2014 (Kakoschke et al., 2014). Future work has to show whether Hfq indirectly affects the transport of

YadA through the periplasmic space, its folding and correct insertion into the outer membrane as well as the stability of *yadA* mRNA. Taken together, these results suggest that Hfq has an impact on YadA regulation by using different mechanisms on the transcriptional, post-transcriptional and likely post-translational level.

By analysing supernatant and total cell protein compositions it could be shown that Hfq represses the production of the fimbriae-like adhesin MyfA and *gfp*-reporter gene fusion experiments revealed that Hfq inhibits *myfA* expression. Both down-regulations happen in acidic media, but not at neutral pH, indicating that the environmental pH can also play a role in Hfq-dependent regulation. Expression of *myfA* seems to be regulated transcriptionally in stationary phase at 27°C (transcriptional regulators: MyfE/MyfF (Iriarte and Cornelis, 1995, Yang and Isberg, 1997)) and post-transcriptionally in exponential phase at 27°C and 37°C.

Besides InvA, Ail, OmpX and YadA, Hfq also had an impact on lipopolysaccharide biosynthesis. The amount and length of the O-Ag of LPS (involved genes: *wzx*, *wzy*, *wzz*; see **Figure 3**) are decreased by Hfq under certain conditions. The previous proteomic analysis (see Kakoschke et. al. 2014) revealed more LpxR, an LPS-modifying enzyme (3'-O-deacylase), in the *hfq* mutant. The expression of *lpxR* as well as remodelling processes of LPS are known to be influenced by RovA and PhoP. As already shown for *rovA* transcriptional reporter fusion, the values for the phoP reporter fusion were also slightly decreased in the *hfq*-negative strain. Consequently Hfq favours the production of LPS molecules with truncated O-antigen and hexa-acylated LPS (LpxR↓). Whether such structural changes of LPS results in changes towards the O-antigen specific immune response remains worth to be studied.

To clarify if Hfq not only influences the composition of the bacterial envelope and intracellular processes, but also plays a role in "injecting" virulence factors into host cells, a Yop-translocation assay was performed. Therefore isolated mice splenocytes were infected with yersiniae by using a YopH-β-lactamase reporter fusion (YopH-Bla-fusion). It could be shown that translocation of YopH-Bla is reduced in the absence of *hfq*, which is in line with the observed decrease of adhesins like YadA and InvA.

Finally the mouse virulence of isogenic pairs of yersiniae (wt versus *hfq* mutant) was tested. In an intraperitoneal mouse infection model Balb/c mice significantly showed fewer symptoms when inoculated with the same amount of mutants lacking *hfq* in comparison to the isogenic parent strains. The bacterial loads of *hfq* mutants in spleens and livers were clearly reduced in comparison to those of parent-strain mice. The impact of Hfq on virulence is likely to stem from a complex interplay of different functions that include metabolism, resistance to stress and the regulation of virulence factors.

4.2.1. Author Contributions:

Ombeline Rossier and **Tamara Kakoschke** designed the study. Clonings were done by **Tamara Kakoschke** and Kristin Adler. **Tamara Kakoschke** and Sara Kakoschke performed immunoblottings and flow cytometry of *gfp*-reporter constructs. LPS staining experiments were done and analysed by **Tamara Kakoschke**. Sara Kakoschke performed the experiments with the 3D-collagen gels. Kristin Adler performed the Northern Blottings and prepared the RNA for the RT-qPCR. Catharina Zeuzem performed the RT-qPCR. Hicham Bouabe generated the YopH-Bla reporter and executed the translocation assay. **Tamara Kakoschke** and Hicham Bouabe did the animal experiments. Jürgen Heesemann provided reagents as well as financial and intellectual support. Ombeline Rossier wrote the manuscript.

Author Assignment of the Shown Figures:

Figure 1 a, b (**Tamara Kakoschke**), Figure c, d, f, g (**Tamara Kakoschke**, Sara Kakoschke), Figure 1 e (Kristin Adler), Figure 2 a (**Tamara Kakoschke**), Figure 2 b, c, d, e (**Tamara Kakoschke**, Sara Kakoschke), Figure 3 a, b, c, d (**Tamara Kakoschke**), Figure 3 e (Ombeline Rossier), Figure 3 f (Sara Kakoschke), Figure 3 g, h, i (**Tamara Kakoschke**, Sara Kakoschke), Figure 4 a, b (**Tamara Kakoschke**), Figure 4 c, d (Sara Kakoschke, **Tamara Kakoschke**), Figure 5 a, b (**Tamara Kakoschke**), Figure 6 (Hicham Bouabe), Figure 7 a, b (**Tamara Kakoschke**, Hicham Bouabe), Figure 8 (Ombeline Rossier), Table 1 (**Tamara Kakoschke**, Kristin Adler), Table S1/S2 (Ombeline Rossier), Table S3 (Catharina Zeuzem, Ombeline Rossier), Table S4 (Ombeline Rossier, **Tamara Kakoschke**, Sara Kakoschke), Figure S1 a, b (Kristin Adler), Figure S1 d, e (Catharina Zeuzem), Figure S2 a, b (Sara Kakoschke, **Tamara Kakoschke**), Figure S2 c, d (Ombeline Rossier), Figure S3 a, b, c (**Tamara Kakoschke**), Figure S4 (Hicham Bouabe).

While in the former publication it could be shown that the RNA-chaperone Hfq influences bacterial growth, morphological cell shape, metabolism and stress resistance in *Y. enterocolitica*, the second report demonstrates its impact on virulence factors (mainly components of the bacterial outer membrane) and thus on mouse pathogenicity.

5. Zusammenfassung der publizierten Ergebnisse

5.1. Das RNA Chaperon Hfq hat Einfluss auf das Wachstum und den Stoffwechsel von *Yersinia enterocolitica* und auf die Produktion von Virulenzfaktoren

Kakoschke T, Kakoschke S, Magistro G, Schubert S, Borath M, Heesemann J, Rossier O

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Mittels eines Genomvergleichs konnte in dem *Y. enterocolitica*-Stamm 8081 (Serovar O:8, Biovar IB) eine Gensequenz identifiziert werden, die eine über 80-prozentige Sequenzähnlichkeit und Sequenzübereinstimmung mit dem bekannten *hfq* Gen von *E. coli* Bakterien aufwies. Um Kenntnisse über die Rolle des Proteins Hfq bei *Y. enterocolitica* Bakterien zu erhalten, wurden drei voneinander unabhängige Mutanten generiert. Dazu wurde bei zwei genotypisch unterschiedlichen *Y. enterocolitica* Stämmen des Serotyps O:8, WA-314 und JB580v (letzterer ein Derivat des Stammes 8081), die *hfq* Gensequenz deletiert. Dadurch erhielt man vom WA314 abstammend die *hfq*-negativen Stämme SOR3 (*hfq*::KanR) und SOR4 (Δ*hfq*) und von JB580v abstammend SOR17 (*hfq*::KanR).

Auf Agarplatten unterschieden sich die Kolonien dieser Stämme, makroskopisch betrachtet, nicht wesentlich von denen ihrer Ausgangsstämme. Mikroskopisch stellten sich allerdings die einzelnen Bakterienzellen der Mutanten gegenüber ihren Ausgangsstämmen vergrößert und elongiert dar.

Ihr Wachstumsverhalten (getestet in reichhaltigen sowie in Minimalmedien) zeigte sich in der exponentiellen Phase verzögert. In der stationären Phase von Flüssigkulturen wurde letztendlich eine geringere optische Dichte (OD_{600}) erreicht und auf Agarplatten wurden weniger koloniebildende Einheiten gezählt. Komplementationsversuche zeigten, dass über ein Plasmid zusätzlich in die Ausgangsstämme eingeführte *hfq*-Genkopien zu einem Wachstumsanstieg über das physiologische Maß hinaus führten. Insgesamt lässt sich schlussfolgern, dass Hfq das Wachstum von *Y. enterocolitica* fördert.

Eine zweidimensionale Gelelektrophorese zeigte Unterschiede zwischen JB580v und SOR17 im Vorkommen von Membranproteinen. Massenspektrometrische Analysen konnten einige dieser Proteine identifizieren, von denen bekannt war, dass sie entweder eine Rolle im bakteriellen Stoffwechsel oder der Stressresistenz von *Y. enterocolitica* spielten oder Bestandteile der Bakterienwand waren. Insgesamt wies dieses Ergebnis auf einen breiten Einfluss von Hfq auf den Metabolismus und die Produktion von Virulenzfaktoren hin. So waren in der Δ*hfq*-Mutante Proteine herunterreguliert, die beispielsweise eine Rolle im Lipidstoffwechsel und –transport spielen bzw. an der Zellhomöostase, der Modulation von Chaperonen, der anaeroben Atmung oder der ATP-

Synthese beteiligt sind. In größeren Mengen vertreten waren hingegen bestimmte Chaperone bzw. Proteasen, die im Rahmen der bakteriellen Stressantwort von Bedeutung sind oder wichtige Funktionen im Stoffwechsel, Peptidtransport oder tRNA-Synthese einnehmen. Andere wiederum sind Virulenzproteine der äußeren Bakterienmembran, wie beispielsweise der Siderophorrezeptor FyuA. Die einzelnen Proteine und ihre jeweilige Beschreibung sind in Tabelle 3 und Tabelle 4 in Kakoschke et al. 2014 aufgeführt.

Weitere molekularbiologische Untersuchungen und Wachstumsversuche auf definierten Minimalmedien zeigten einen Einfluss von Hfq auf den Abbau von Kohlehydraten und Zuckeralkoholen, insbesondere Mannitol, Sorbitol und 1,2-Propandiol. Zudem konnte gezeigt werden, dass Hfq die Tryptophanase, Indolproduktion und die Ornithin-Dekarboxylase Aktivität hemmt. Folglich hat dieses regulatorische Protein sowohl auf den Kohlehydrat- als auch den Stickstoffhaushalt von *Y. enterocolitica* Einfluss.

Die Säureresistenz war in den *hfq*-negativen Stämmen geringer. Ebenso konnten in diesen Mutanten deutlich kleinere Mengen an Urease nachgewiesen werden. Somit befähigt Hfq die Bakterien mit einem besseren Überleben in einer Umgebung mit niedrigem pH-Wert. Zudem konnte eine höhere Überlebensrate auch gegenüber oxidativem Stress nachgewiesen werden. In Bezug auf Antibiotikaresistenzen ergaben sich keine signifikanten Unterschiede.

Durch Messung der Pesticin-Sensitivität, Western-Blot-Analysen und Verwendung eines Luciferase-Tests konnte gezeigt werden, dass Hfq die Produktion des Siderophors Yersiniabactin und dessen Rezeptor FyuA inhibiert. Allerdings konnte, im Gegensatz zu *Y. pseudotuberculosis* (Schiano et al., 2010), bei *Y. enterocolitica* kein Einfluss auf die Menge und Sekretion der Yop-Proteine nachgewiesen werden.

Um herauszufinden in welcher bakteriellen Wachstumsphase die Produktion des Hfq-Proteins am größten ist, wurde es mit einem FLAG-Epitop markiert. Immunoblots von Ganzzelllysaten wiesen die größte Menge an Hfq in der exponentiellen und stationären Phase auf.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass Hfq sowohl Einfluss auf das Wachstumsverhalten und die Morphologie als auch auf eine Reihe von Stoffwechselprozessen und Pathogenitätsfaktoren von *Y. enterocolitica* hat und es scheint als sei seine Wirkung im Wesentlichen *Yersinia*-spezifisch.

5.1.1. Beitrag der Autoren:

Ombeline Rossier und **Tamara Kakoschke** konzipierten und planten die Versuche. Sara Kakoschke führte Stressresistenztests und die Experimente zur Indolproduktion durch. Giuseppe Magistro führte den FyuA-Immunodetektionsversuch und den Luziferase-Reporter-Assay durch. Marc Wirth (geb. Borath) führte die Massenspektrometrie aus. Marc Wirth (geb. Borath) und Sören Schubert werteten die Daten der Massenspektrometrieanalyse aus. Ombeline Rossier generierte die

Deletionsmutanten. **Tamara Kakoschke** führte die Transformationen sowie die Komplementations- und Überexpressionsversuche durch. Die Wachstumsversuche wurden von **Tamara Kakoschke** durchgeführt. Die Analysen zur Bakterienmorphologie inklusive Lichtmikroskopie übernahm **Tamara Kakoschke**. Ombeline Rossier wertete die 2-DE Gele, die Yop-Sekretion sowie die Produktion des Hfq-Proteins aus. **Tamara Kakoschke** führte die Westernblots und Experimente zur Ureaseproduktion durch. Sören Schubert und Jürgen Heesemann stellten Reagenzien, Materialien und Analyseinstrumente und –geräte zur Verfügung. Jürgen Heesemann unterstützte durch intellektuellen Rat sowie geistigen Austausch und Anregungen. Ombeline Rossier verfasste das Manuskript.

Autoren-Zuordnung der abgebildeten Graphiken:
Tabelle 1/Tabelle 2/Tabelle 3/Tabelle 4 (Ombeline Rossier), Bild 1 (**Tamara Kakoschke**), Bild 2 (Ombeline Rossier), Bild 3 a, b (**Tamara Kakoschke**, Ombeline Rossier), Bild 4 a, b (Sara Kakoschke), Bild 4 c (Ombeline Rossier), Bild 5 a, b (**Tamara Kakoschke**), Bild 6 a, b (Sara Kakoschke), Tabelle 5 (Ombeline Rossier, **Tamara Kakoschke**), Bild 7 a, b (Giuseppe Magistro), Bild 8 (Ombeline Rossier), Bild 9 (Ombeline Rossier).

5.2. Das RNA Chaperon Hfq wird für die Virulenz von *Yersinia enterocolitica* benötigt und reguliert die Expression von vier Adhäsinen

Kakoschke TK, Kakoschke SC, Zeuzem C, Bouabe H, Adler K, Heesemann J, Rossier O

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Der Einfluss von Hfq auf die Produktion von Adhäsinen und auf das Virulenzverhalten von *Y. enterocolitica* ist wesentlicher Gegenstand dieser Publikation. Adhäsine sind Pathogenitätsfaktoren, die ein Anhaften der Bakterien an Wirtszellen ermöglichen. Mittels Northern Blots, translationalen Fusionen mit dem *gfp*-Gen und Immunoblots wurde die Produktion einer Reihe von Adhäsinen und die Expression ihrer Gene in den Wildtypstämmen und in den *hfq*-negativen Stämmen untersucht und verglichen. Um zu untersuchen, ob Hfq auf transkriptioneller oder posttranskriptioneller Ebene agiert wurde der jeweilige Promotor einer translationalen Genfusion durch einen Plac Promotor ersetzt.

Es konnte gezeigt werden, dass Hfq die wichtigen Adhäsine Ail, OmpX, InvA, YadA und MyfA reguliert. Ail und OmpX werden posttranskriptional (wahrscheinlich durch eine Destabilisierung ihrer Transkripte in der stationären Phase) durch Hfq gehemmt. Invasin wird durch Hfq eher positiv und auf Ebene der Transkription beeinflusst. Nähere Untersuchungen zeigten eine Wirkung von Hfq auf die Expression des Gens *rovA*, welches einen aktivierenden Transkriptionsfaktor von InvA kodiert, und auf die Expression des Gens *ompR*, welches den transkriptionellen Repressor kodiert.

Mithilfe von Komplementationsversuchen und Experimenten mit 3D-Kollagen-Gelen zur morphologischen Beschreibung von Mikrokolonien und ihrer Interaktion mit dem umliegenden Kollagen konnte eine komplexere Funktion von Hfq bei der Regulation von YadA erschlossen werden. Hierbei scheint zunächst, die vorhandene Menge an Hfq Protein ausschlaggebend zu sein: Wenn Hfq in *Y. enterocolitica* fehlt resultiert dies in einer geringeren Menge an YadA-Protein. Allerdings führt auch eine Überexpression von *hfq* (über das Plasmid pACYC184) zu einer reduzierten Produktion von YadA. Es konnte zudem gezeigt werden, dass Hfq je nach Wachstumsbedingungen und -phasen die YadA-Produktion unabhängig von dem Aktivator VirF fördern kann, aber unter bestimmten Bedingungen auch die Menge an *yadA*-Transkripten reduzieren kann. Diese Diskrepanz bezüglich der *yadA*-Expression und der nachweisbaren Menge an YadA-Protein könnte ein Ergebnis post-translationaler Modifikationen sein. Hierbei könnten eine *hfq*-abhängige Sekretion und/oder der Abbau von YadA-Protein im periplasmatischen Raum in Frage kommen. Diese Annahme wird dadurch unterstützt, dass die YadA-spaltende Protease DegP in der *hfq*-Mutante vermehrt nachgewiesen wurde (siehe Proteomanalyse in Kakoschke et al. 2014). Letztendlich müssen zukünftige Arbeiten zeigen, ob Hfq indirekt den Transport von YadA über den periplasmatischen

Raum, die Proteinfaltung und die korrekte Insertion von YadA in der äußeren Membran sowie die Stabilität der *yadA* mRNA beeinflusst. Was man aber bereits sagen kann: Hfq scheint mit unterschiedlichen Mechanismen auf transkiptioneller, posttranskriptioneller und vermutlich auch posttranslationaler Ebene auf die Regulation von YadA zu wirken.

Durch Untersuchungen von Überstandsproteinen und Ganzzelllysaten konnte gezeigt werden, dass Hfq die Produktion von MyfA unterdrückt und *gfp*-Reportergen-Fusions-Experimente wiesen ebenso auf eine Inhibition der *myfA* Expression hin. Beide Effekte passierten allerdings nur in saurem Milieu und nicht in neutralem Medium. Der pH-Wert der Umgebung scheint also auch einen Einfluss auf Hfq-abhängige Regulation zu haben. Die Regulation der *myfA* Expression zeigte sich am ehesten auf transkriptioneller Ebene während der stationären Phase bei 27°C und auf posttranskriptioneller Ebene während der exponentiellen Phase, hier sowohl bei 27°C als auch 37°C.

Neben Ail, OmpX, YadA und MyfA hat Hfq auch einen Einfluss auf das Lipopolysaccharid von *Y. enterocolitica*. Es konnte nachgewiesen werden, dass unter bestimmten Bedingungen die Menge und die Länge des O-Antigens von LPS (involvierte Gene: *wzx*, *wzy*, *wzz*; siehe **Figure 3**) durch Hfq reduziert werden. Die vorangegangene Proteomanalyse (siehe Kakoschke et. al. 2014) zeigte zudem in der *hfq*-Mutante eine höhere Menge von LpxR, einem LPS-modifizierenden Enzyms (Acetylierung von LPS). Es ist bereits bekannt, dass die Expression des Gens *lpxR* sowie Umbauprozesse des LPS durch RovA und PhoP beeinflusst werden. Wie bereits für *rovA* gezeigt, ist auch die Expression von *phoP* in Abwesenheit des *hfq*-Gens in *Y. enterocolitica* leicht reduziert. Folglich begünstigt Hfq die Produktion eines hexa-acetylierten LPS-Moleküls (LpxR↓) mit verkürztem O-Antigen. Ob die strukturellen Veränderungen des LPS-Moleküls die O-Antigen vermittelte Immunantwort beeinflussen sollte Gegenstand weiterer Untersuchungen werden.

Abgesehen von der Bakterienwandzusammensetzung und intrazellulären Prozessen wurde auch die „Injektion“ von Virulenzfaktoren in Wirtszellen in Abhängigkeit von Hfq untersucht. In einem Translokationsversuch unter Verwendung eines β-Laktamase Reportersystems (YopH-Bla-Fusion) wurden hierzu murine Splenozyten mit Yersinien infiziert. In der *hfq*-Mutante zeigte sich eine geringere Translokation von YopH-Bla in die Zielzellen, was auch vereinbar mit der beobachteten Abnahme der Adhäsine YadA und InvA ist.

Schließlich wurden Balb/c Mäuse intraperitoneal mit Yersinien (*hfq*-Mutante und jeweiliger Ausgangsstamm) infiziert, um den Einfluss von Hfq auf die Virulenz von *Y. enterocolitica* im Tiermodell zu untersuchen. Die mit den Ausgangsstämmen infizierten Mäuse (bei gleicher Menge an injizierten Bakterien) zeigten eine stärkere Ausprägung der Krankheitssymptome und eine erhöhte Bakterienbelastung der Organe Leber und Milz als die mit der *hfq*-Mutante infizierten Tiere. In Zusammenschau der bisherigen Ergebnisse scheint der Einfluss von Hfq auf die Virulenz von *Y. enterocolitica* in einem komplexen Zusammenspiel verschiedener Funktionen begründet. Darunter

fallen regulatorische Prozesse des Stoffwechsels, der Stressantwort und der Produktion von Virulenzfaktoren.

5.2.1. Beitrag der Autoren:

Ombeline Rossier entwarf die Studie. Ombeline Rossier und **Tamara Kakoschke** planten die Experimente und ihr Design. Die Klonierungen wurden von **Tamara Kakoschke** und Kristin Adler durchgeführt. **Tamara Kakoschke** und Sara Kakoschke führten die Immunoblots und die FACS-Analysen der hergestellten *gfq*-Reporter-Stämme durch. Die LPS-Färbeversuche wurden von **Tamara Kakoschke** geplant, durchgeführt und analysiert. Sara Kakoschke führte die Experimente zur Kollagen-YadA-Interaktion in 3D-Kollagen-Gel-Kammern durch. Kristin Adler machte die Northern Blots und bereitete die RNA für die RT-qPCR vor. Catharina Zeuzem führte die RT-qPCR aus. Hicham Bouabe generierte den YopH-Bla-Reporter und führte die Translokationsversuche durch. **Tamara Kakoschke** und Hicham Bouabe führten die Mausexperimente durch. Jürgen Heesemann stellte Reagenzien und Material zur Verfügung und unterstützte finanziell und intellektuell.

Autoren-Zuordnung der abgebildeten Graphiken:

Bild 1 a, b (**Tamara Kakoschke**), Bild c, d, f, g (**Tamara Kakoschke**, Sara Kakoschke), Bild 1 e (Kristin Adler), Bild 2 a (**Tamara Kakoschke**), Bild 2 b, c, d, e (**Tamara Kakoschke**, Sara Kakoschke), Bild 3 a, b, c, d (**Tamara Kakoschke**), Bild 3 e (Ombeline Rossier), Bild 3 f (Sara Kakoschke), Bild 3 g, h, i (**Tamara Kakoschke**, Sara Kakoschke), Bild 4 a, b (**Tamara Kakoschke**), Bild 4 c, d (Sara Kakoschke, **Tamara Kakoschke**), Bild 5 a, b (**Tamara Kakoschke**), Bild 6 (Hicham Bouabe), Bild 7 a, b (**Tamara Kakoschke**, Hicham Bouabe), Bild 8 (Ombeline Rossier), Tabelle 1 (**Tamara Kakoschke**, Kristin Adler), Tabelle S1/S2 (Ombeline Rossier), Tabelle S3 (Catharina Zeuzem, Ombeline Rossier), Tabelle S4 (Ombeline Rossier, **Tamara Kakoschke**, Sara Kakoschke), Bild S1 a, b (Kristin Adler), Bild S1 d, e (Catharina Zeuzem), Bild S2 a, b (Sara Kakoschke, **Tamara Kakoschke**), Bild S2 c, d (Ombeline Rossier), Bild S3 a, b, c (**Tamara Kakoschke**), Bild S4 (Hicham Bouabe).

Während in der ersten Publikation gezeigt wurde, dass das RNA Chaperon Hfq regulatorisch auf das Wachstum, die Zellmorphologie, den Metabolismus und die Widerstandsfähigkeit von *Y. enterocolitica* wirkt, belegt die zweite Publikation den Einfluss auf weitere Virulenzfaktoren (hauptsächlich Bestandteile der äußeren Membran der Bakterien) und die Pathogenität im Mausmodell.

6. Publications

6.1. Publication 1 (First Authorship)

Kakoschke T., Kakoschke S., Magistro G., Schubert S., Borath M., Heesemann J., Rossier O. (2014) **The RNA Chaperone Hfq Impacts Growth, Metabolism and Production of Virulence Factors in *Yersinia enterocolitica***. PLoS ONE 9(1): e86113. doi:10.1371/journal.pone.0086113

6.2. Publication 2 (First Authorship)

Kakoschke T. K., Kakoschke S. C., Zeuzem C., Bouabe H., Adler K., Heesemann J., Rossier O. (2016) **The RNA Chaperone Hfq Is Essential for Virulence and Modulates the Expression of Four Adhesins in *Yersinia enterocolitica***. Sci. Rep. 6, 29275; doi: 10.1038/srep29275

7. Appendix

7.1. List of Different *Yersinia* Species; as at March 2020 (https://lpsn.dsmz.de/genus/yersinia; accessed March 1st 2020)

Yersinia species	Reference
Yersinia aldovae	Bercovier H, Steigerwalt AG, Guiyoule A, Huntley-Carter G, Brenner DJ. *Yersinia aldovae* (formerly *Yersinia enterocolitica*-like group X2): a new species of *Enterobacteriaceae* isolated from aquatic ecosystems. *Int. J. Syst. Bacteriol.* 1984; **34**:166-172.
Yersinia aleksiciae	Sprague LD, Neubauer H. *Yersinia aleksiciae* sp. nov. *Int J Syst Evol Microbiol* 2005; **55**:831-835.
Yersinia bercovieri	Wauters G, Janssens M, Steigerwalt AG, Brenner DJ. *Yersinia mollaretii* sp. nov. and *Yersinia bercovieri* sp. nov., formerly called *Yersinia enterocolitica* biogroups 3A and 3B. *Int. J. Syst. Bacteriol.* 1988; **38**:424-429.
Yersinia enterocolitica	Frederiksen W. A study of some *Yersinia pseudotuberculosis*-like bacteria (Bacterium enterocoliticum and Pasteurella X). Proceedings of the XIV Scandinavian Congress of Pathology and Microbiology, Oslo 1964. Norwegian Universities Press, Oslo, 1964.
Yersinia enterocolitica subsp. *enterocolitica* *Yersinia enterocolitica* subsp. *palearctica*	Neubauer H, Aleksic S, Hensel A, Finke EJ, Meyer H. *Yersinia enterocolitica* 16S rRNA gene types belong to the same genospecies but form three homology groups. Int. *J. Med. Microbiol.* 2000; **290**:61-64.
Yersinia entomophaga	Hurst MR, Becher SA, Young SD, Nelson TL, Glare TR. *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub Costelytra zealandica. *Int J Syst Evol Microbiol* 2011; **61**:844-849.
Yersinia frederiksenii	Ursing J, Brenner DJ, Bercovier H, Fanning GR, Steigerwalt AG, Brault J, Mollaret HH. *Yersinia frederiksenii*: a new species of *Enterobacteriaceae* composed of rhamnose-positive strains (formerly called atypical *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Curr. Microbiol.* 1980; **4**:213-217.

Yersinia hibernica	Nguyen SV, Muthappa DM, Hurley D, Donoghue O, McCabe E, Anes J, Schaffer K, Murphy BP, Buckley JF, Fanning S. *Yersinia hibernica* sp. nov., isolated from pig-production environments. *Int J Syst Evol Microbiol* 2019; **69**:2023-2027.
Yersinia intermedia	Brenner DJ, Bercovier H, Ursing J, Alonso JM, Steigerwalt AG, Fanning GR, Carter GP, Mollaret HH. *Yersinia intermedia*: a new species of *Enterobacteriaceae* composed of rhamnose-positive, melibiose-positive, raffinose-positive strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Curr. Microbiol.* 1980; 4:207-212
Yersinia kristensenii	Bercovier H, Ursing J, Brenner DJ, Steigerwalt AG, Fanning GR, Carter GP, Mollaret HH. *Yersinia kristensenii*: a new species of *Enterobacteriaceae* composed of sucrose-negative strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Curr. Microbiol.* 1980; **4**:219-224.
Yersinia massiliensis	Merhej V, Adekambi T, Pagnier I, Raoult D, Drancourt M. *Yersinia massiliensis* sp. nov., isolated from fresh water. *Int J Syst Evol Microbiol* 2008; **58**:779-784.
Yersinia mollaretii	Wauters G, Janssens M, Steigerwalt AG, Brenner DJ. *Yersinia mollaretii* sp. nov. and *Yersinia bercovieri* sp. nov., formerly called *Yersinia enterocolitica* biogroups 3A and 3B. *Int. J. Syst. Bacteriol.* 1988; **38**:424-429.
Yersinia nurmii	Murros-Kontiainen A, Fredriksson-Ahomaa M, Korkeala H, Johansson P, Rahkila R, Bjorkroth J. *Yersinia nurmii*sp. nov. *Int J Syst Evol Microbiol* 2011; **61**:2368-2372.
Yersinia pekkanenii	Murros-Kontiainen A, Johansson P, Niskanen T, Fredriksson-Ahomaa M, Korkeala H, Bjorkroth J. *Yersinia pekkanenii* sp. nov. *Int J Syst Evol Microbiol* 2011; **61**:2363-2367.
Yersinia pestis	Van Loghem JJ. The classification of plague-bacillus. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* 1944; **10**:15-16.
Yersinia philomiragia (current name: *Francisella philomiragia)*	Jensen WI, Owen CR, Jellison WL. *Yersinia philomiragia* sp. n., a new member of the *Pasteurella* group of bacteria, naturally pathogenic for the muskrat (Ondatra zibethica). *J Bacteriol* 1969; **100**:1237-1241.

Yersinia pseudotuberculosis	Smith JE, Thal E. A taxonomic study of the genus *Pasteurella* using a numeral technique. *Acta Pathologica et Bacteriologica Scandinavica* 1965; **64**:213-223.
Yersinia rohdei	Aleksic S, Steigerwalt AG, Bockemühl J, Huntley-Carter GP, Brenner DJ. *Yersinia rohdei* sp. nov. isolated from human and dog feces and surface water. *Int. J. Syst. Bacteriol.* 1987; **37**:327-332.
Yersinia ruckeri	Ewing WH, Ross AJ, Brenner DJ, Fanning GR. *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. *International Journal of Systematic Bacteriology* 1978; **28**:37-44.
Yersinia similis	Sprague LD, Scholz HC, Amann S, Busse HJ, Neubauer H. *Yersinia similis* sp. nov. *Int J Syst Evol Microbiol*2008; **58**:952-958.
Yersinia wautersii	Savin C, Martin L, Bouchier C, Filali S, Chenau J, Zhou Z, Becher F, Fukushima H, Thomson NR, Scholz HC, *et al.* The *Yersinia pseudotuberculosis* complex: characterization and delineation of a new species, *Yersinia wautersii*. *Int J Med Microbiol* 2014; **304**:452-463.

7.2. Further Methods and Materials

7.2.1. Computer Programmes

Programs used in this work for diagrams and figures:

- Paintbrush Version 2.1.1. (Copyright 2007 – 2010 SoggyWaffles)
- BioChemDraw Ultra Version 12.0.3.1216 (CambridgeSoft)
- Word 2008 Version 12.3.6 (Microsoft)

7.2.2. Animal Experiments

The animal experiments were approved by the Regierung von Oberbayern (Gz. 55.2-1-54-2532-65-09) and were performed between May 2010 and September 2010 under the supervision and responsibility of Dr. Hicham Bouabe.

8. Danksagung

Ein außerordentlicher Dank gilt meinem Doktorvater Prof. Dr. Dr. Jürgen Heesemann für meine Aufnahme in den Promotionsstudiengang “Molekulare und Systembiologische Medizin” des Förderprogramms für Forschung und Lehre (FöFoLe) der Medizinischen Fakultät der LMU, für das interessante Forschungsthema und die Bereitstellung des Arbeitsplatzes, für die Geduld, Präsenz und leichte Erreichbarkeit sowie unsere Gespräche auf intellektueller und persönlicher Ebene. Vielen Dank für die intensive Unterstützung und Förderung während meiner Zeit am Max-von-Pettenkofer Institut und darüber hinaus.

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