

Persistent infection

by *Yersinia pseudotuberculosis*

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To my parents...

Table of contents

Abstract	i
List of Abbreviations	ii
Papers in This Thesis	iv
1 Introduction	1
1.1 Emergence of Bacterial Pathogens	3
1.1.1 Emergence of pathogenic properties	3
1.1.1.1 Bacteria–protozoa interactions.....	3
1.1.1.2 Genome plasticity.....	5
1.2 Bacterial Pathogenesis	6
1.2.1 Bacterial Pathogenic Mechanisms.....	6
1.2.1.1 Adherence.....	7
1.2.1.2 Invasion.....	8
1.2.1.3 Bacterial Camouflage.....	8
1.2.1.4 Damage to the Host.....	10
1.3 Host Defense Against Bacterial Infection	12
1.3.1 Barriers	12
1.3.1.1 Skin and Mucosal Surfaces.....	12
1.3.1.2 Antimicrobial Proteins	13
1.3.1.3 Microbiota.....	14
1.3.2 Cellular Innate Immune Response	15
1.3.2.1 Recognition of Bacteria	16
1.3.2.2 Cytokines and Chemokines	17
1.3.2.3 Complement System	17
1.3.2.4 Phagocytosis and Killing of Bacteria.....	18
1.3.3 From the Innate to the Adaptive Immune System	19
1.4 Bacterial Adaptation to Host Stresses	19
1.4.1 Sensing the Stress.....	19
1.4.2 General Stress Response.....	20
1.4.3 Temperature	20
1.4.4 Acidic Stress	21
1.4.5 Oxidative and Nitrosative Stress.....	21
1.4.6 Osmotic Stress	22
1.4.7 Oxygen Stress	22
1.5 Persistent Bacterial Infections	23
1.5.1 <i>Helicobacter pylori</i>	24
1.5.2 <i>Mycobacterium tuberculosis</i>	24
1.5.3 <i>Salmonella</i>	25
1.6 Yersinia as a New Model for Persistence	26
1.6.1 <i>Yersinia</i> pathogens.....	27
1.6.1.1 Pathogenesis and Adaptation of Enteric <i>Yersinia</i>	27
1.6.1.2 Adherence.....	28
1.6.1.3 T3SS	29
1.6.1.4 T6SS	30
1.6.1.5 LPS	30
1.7 Identification of Pathogenicity Factors	31
1.7.1 Biochemical Approaches	31
1.7.2 Molecular Genetic Approaches.....	32
1.7.2.1 Transposon Mutagenesis.....	32
1.7.2.2 Identification of <i>In Vivo</i> –Expressed Genes	32
1.7.2.2.1 Signature-tagged Mutagenesis.....	33
1.7.2.2.2 <i>In Vivo</i> Expression Technology	33
1.7.3 Genomic Approaches	33

1.7.3.1	Microarrays	33
1.7.3.2	Next-generation Sequencing Technology	34
1.7.3.2.1	Genome Sequencing by NGS.....	34
1.7.3.2.2	Metagenomics	35
1.7.3.2.3	Transposon Insertion Sequencing	35
1.7.3.2.4	ChiP-seq	35
1.7.3.2.5	RNA-seq	36
2	Objectives of This Thesis	41
3	Results and Discussion	42
3.1	Monitoring <i>Y. pseudotuberculosis</i> Infection in Mice.....	42
3.2	Persistent Infection of <i>Y. pseudotuberculosis</i> in Cecum	42
3.2.1	A Mouse Model for Persistent <i>Y. pseudotuberculosis</i> Infection.....	43
3.2.2	Cytokine Expression During Persistency.....	44
3.3	Transcriptome of Persistent <i>Y. pseudotuberculosis</i>	44
3.3.1	Transcriptome of Persistent <i>Y. pseudotuberculosis</i> is Similar to 26°C Growth	44
3.3.2	Transcriptional Reprogramming for Persistence	45
3.3.3	Persistent <i>Y. pseudotuberculosis</i> is Influenced by Environmental Cues	45
3.4	Complex RNA-Populations can be Resolved by RNA-seq	46
3.5	ArcA, Fnr, FrdA, and WrbA are Required for Persistent Infection.....	47
3.6	<i>Yersinia</i> Infections Alter Microbial Composition in Cecum.....	47
3.7	RfaH is Required for Establishment of Infection	48
4	Main Findings in This Thesis.....	49
5	Future Perspectives	50
1.1	Search for Novel Targets for Antimicrobials.....	50
5.1	Switch for Reprogramming	51
5.2	Possible Contribution of T6SS	51
5.3	Adaptation of <i>Y. pseudotuberculosis</i> to Host Stress Conditions	52
6	Acknowledgments	53
7	References	55

Abstract

Enteropathogenic *Yersinia* species can infect many mammalian organs such as the small intestine, cecum, Peyer's patches, liver, spleen, and lung and cause diseases that resemble a typhoid-like syndrome, as seen for other enteropathogens. We found that sublethal infection doses of *Y. pseudotuberculosis* gave rise to asymptomatic persistent infection in mice and identified the cecal lymphoid follicles as the primary site for colonization during persistence. Persistent *Y. pseudotuberculosis* is localized in the dome area, often in inflammatory lesions, as foci or as single cells, and also in neutrophil exudates in the cecal lumen. This new mouse model for bacterial persistence in cecum has potential as an investigative tool for deeper understanding of bacterial adaptation and host immune defense mechanisms during persistent infection. Here, we investigated the nature of the persistent infection established by *Y. pseudotuberculosis* in mouse cecal tissue using *in vivo* RNA-seq of bacteria during early and persistent stages of infection. Comparative analysis of the bacterial transcriptomes revealed that *Y. pseudotuberculosis* undergoes transcriptional reprogramming with drastic down-regulation of T3SS virulence genes during persistence in the cecum. At the persistent stage, the expression pattern in many respects resembles the pattern seen *in vitro* at 26°C. Genes that are up-regulated during persistence are genes involved in anaerobiosis, chemotaxis, and protection against oxidative and acidic stress, which indicates the influence of different environmental cues. We found that the Crp/CsrA/RovA regulatory cascades influence the pattern of bacterial gene expression during persistence. Furthermore, we show that ArcA, Fnr, FrdA, WrbA, RovA, and RfaH play critical roles in persistence. An extended investigation of the transcriptional regulator *rfaH* employing mouse infection studies, phenotypic characterizations, and RNA-seq transcriptomics analyses indicated that this gene product contributes to establishment of infection and confirmed that it regulates O-antigen biosynthesis genes in *Y. pseudotuberculosis*. The RNA-seq results also suggest that *rfaH* has a relatively global effect. Furthermore, we also found that the dynamics of the cecal tissue organization and microbial composition shows changes during different stages of the infection. Taken together, based on our findings, we speculate that this enteropathogen initiates infection by using its virulence factors in meeting the innate immune response in the cecal tissue. Later on, these factors lead to dysbiosis in the local microbiota and altered tissue organization. At later stages of the infection, the pathogen adapts to the environment in the cecum by reprogramming its transcriptome from a highly virulent mode to a more environmentally adaptable mode for survival and shedding. The *in vivo* transcriptomic analyses for essential genes during infections present strong candidates for novel targets for antimicrobials.

List of Abbreviations

(p)ppGpp	Guanosine pentaphosphate
AMP	Antimicrobial peptide/protein
BWT	Burrows–Wheeler transform
BLI	Bioluminescent imaging
c-di-GMP	Cyclic-di-GMP
CFU	Colony-forming unit
ChiP	Chromatin immunoprecipitation
DCs	Dendritic cells
Fnr	Fumarate-nitrate reductase
GAP	GTPase-activating proteins
GI	Gastrointestinal
GIT	Gastrointestinal tract
IgA	Immunoglobulin A
IgM	Immunoglobulin M
iNOS	Inducible nitric oxide
IVIS	<i>In vivo</i> imaging system
kb	Kilo-base
KEGG	Encyclopedia of Genes and Genomes
LBP	LPS-binding protein
LOS	lipo-oligosaccharide
LPS	Lipopolysaccharide
M cells	Microfold cells
Mb	Megabase
MBL	mannose-binding lectin
MHC	Major Histocompatibility Complex
NET	Neutrophil extracellular trap
NGS	Next-generation sequencing
nt	Nucleotide
O-PS	O-antigenic LPS
ORF	Open reading frame
p.i.	post-infection
PAI	Pathogenicity island

PAMP	Pathogen associated molecular pattern
pg	picogram
PMN	Polymorphonuclear leukocyte
PP	Payer's patches
ppGpp	guanosine tetraphosphate
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
R-LPS	rough LPS
RNAP	RNA polymerase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S-LPS	smooth LPS
SI	Small intestine
SI	Small intestine
SOS	Superoxide dismutase
T3SS	Type III secretion system
T6SS	Type VI secretion system
TB	Tuberculosis
TLR	Toll-like receptor
Tn-seq	Transposon insertion sequencing
TNF - α	Tumor necrosis factor alpha
Yops	<i>Yersinia</i> outer membrane protein

Papers in This Thesis

- I. Fahlgren A, Avican K, Westermark L, Nordfelth R, Fallman M. (2014) Colonization of cecum is important for development of persistent infection by *Yersinia pseudotuberculosis*. *Infect Immun* 82: 3471–3482
- II. Avican K, Fahlgren A, Huss M, Heroven AK, Beckstette M, Dersch P, Fällman M. (2015) Reprogramming of *Yersinia* from virulent to persistent mode revealed by complex in vivo RNA-seq analysis. *PLoS Pathog* 11: e1004600
- III. Avican K, Nilsson K, Fällman M. Transcriptomic characterization of RfaH linked to persistent infection of *Yersinia pseudotuberculosis*. (Manuscript)

1 Introduction

Earth is full of bacteria that have existed at least since the middle of the Precambrian time, about 3.5 billion years ago. Their widespread appearance on earth together with Archaea gave rise to the formation of various types of organisms. The blooming of eukaryotic life forms occurred after the formation of an ozone layer with the addition of oxygen, produced by *Cyanobacteria*, to the atmosphere. Formation of the ozone layer made different types of life possible by preventing the harmful effects of radiation on the Earth's surface. Since their initial appearance, bacteria have been evolving new mechanisms/strategies to adapt to a wide range of environmental conditions to survive. Hence, they are an exceedingly diverse group of organisms that differ in size, shape, niche, and metabolism. This level of bacterial diversity comes from their DNA plasticity that allows for mutations, acquisition of new genomic material, and rearrangement of existing DNA. The strength of the plasticity of adaptation to different environmental conditions has resulted in formation of more complex organisms with the combination of more than one single bacterial cell by endosymbiosis [1]. For example, cells eventually became plants by acquiring cyanobacteria, which harbor chloroplasts, conferring the ability to photosynthesize.

The first relatively more complex organisms are called protozoa, and some of them, such as amoebae, have developed properties similar to human phagocytic cells. The superfast nature of bacterial adaptation to the environment made them capable of evolving new strategies against killing by these newly formed complex organisms. This evolution of new strategies for survival in eukaryotic cells has generated new rich niches and led to the appearance of today's disease-causing bacteria. These new niches, such as

humans and animals, which are very well isolated from the external unpredictable environmental conditions, are good habitats for bacteria. For example, the human mouth, intestinal tract, and intragenital tract have more bacterial cells combined than the total cells in the human body. In a healthy person, the bacterial flora in different organs behaves as an organ and is beneficial to humans. However, some bacteria use eukaryotic organisms (mostly called ‘hosts’) as a growth medium and have developed toxic properties to overcome the immune defense of the host, eventually causing serious health problems or death. Such strategies that end with the death of the host are not very advantageous for pathogenic bacteria in the long run because of niche loss. Therefore, some pathogenic bacteria have evolved to use the host as a reservoir without causing serious damage. This strategy enables bacteria to stay in the host for a long time and for the colony members to find new niches by shedding from the body, described as persistent infection. Persistent bacterial infections are a major source for spread of infectious diseases caused by bacteria.

The success of human efforts to prevent bacterial infections peaked with the use of antibiotics. However, the strength of bacterial adaptation has meant the capacity to develop resistance against any antibiotics and remains a big problem in global health. Curing persistent bacterial infections is exceptionally more complicated than treating other acute infections, not only because of resistance to antibiotics at the gene level but also because of the structural organization in the region of infection. For example, *Mycobacterium tuberculosis* induces calcified granulomas in the infection site [2], and *Helicobacter pylori* forms biofilm on the gastric epithelium and produces persistent cells that are highly tolerant to antimicrobials [3].

We have found that the enteropathogen *Yersinia pseudotuberculosis* is a good choice for a mouse infection model to investigate the nature of bacterial persistent infections. We have used sophisticated technologies such as IVIS (*in vivo* imaging systems) and next-generation RNA-seq to investigate the nature of *Y. pseudotuberculosis* persistent infection. We employed transcriptional and translational approaches to understand the adaptation strategies of the pathogen towards persistent infection under *in vivo* and *in vitro* conditions. The approaches we used have provided information about the

environmental conditions during persistent infection and the role of other players such as gut microbiota in addition to important genes in the establishment of persistent infection. Here, I have focused on the results from our studies on persistent infection of *Y. pseudotuberculosis* combined with the background information on the related subjects. I believe the conclusions that we draw from this work with *Y. pseudotuberculosis* persistent infection hold promise for the development of strategies to control and treat persistent infections.

1.1 Emergence of Bacterial Pathogens

Multicellular eukaryotes evolved one billion years ago, and mammals proliferated past 65 million years ago [4]. Human-restricted pathogens such as *Streptococcus pyogenes*, and *Shigella spp.* and human-adapted *Salmonella* species must have adapted to their host one million years ago [5]. Limited resources and adaptation to new conditions in different environments are the major forces that promote emergence of bacterial pathogens. They adapted motility to search for nutrients, produce antibiotics to compete with others, and synthesize adhesins to stay in favorable environments. However, the successful individuals that survived through selective adaptations are defined not only by growth and reproduction but also by their abilities to defend themselves against any threat [6]. The interaction of environmental bacteria with both protozoans and phages is thought to be the driving force in the emergence of bacterial pathogens, development of different pathogenic strategies, and fitness of a pathogen for its host environment.

1.1.1 Emergence of pathogenic properties

1.1.1.1 Bacteria–protozoa interactions

The defense mechanisms of bacteria against protozoans such as amoebae, which have phagocytic properties, resulted in the emergence of new genotypes and phenotypes for the environmental bacteria and today's pathogenic bacteria. The interactions with protozoa mostly occur in prey–predator relations that begin with contact followed by trapping the prey and ingestion (phagocytosis) [7]. The resistance of bacteria to the ingestion can start before (pre-ingestional or extracellular) or after (post-ingestional or

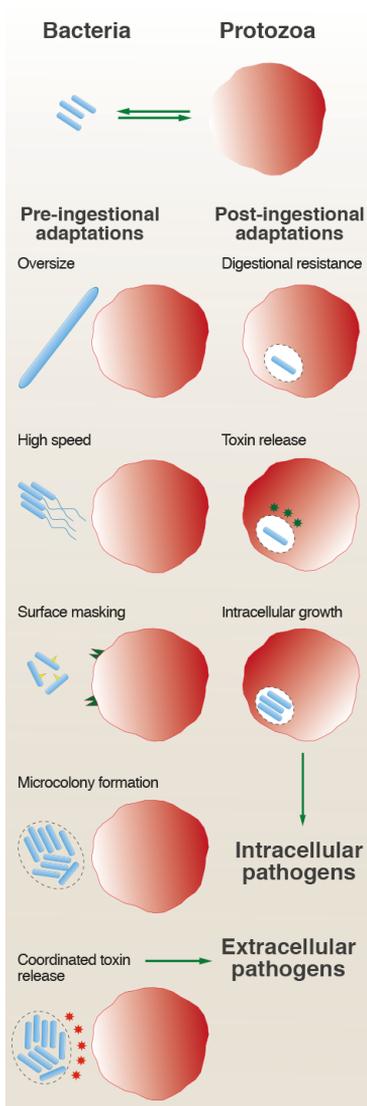


Figure 1. Potential bacterial adaptations against predations and emergence of pathogenesis. Pre-ingestional (on the left) and post-ingestional adaptations (on the right) lead to emergence of extracellular and intracellular pathogens, respectively. Figure is adapted from Matz and Kjelleberg, 2005 [6].

intracellular) ingestion (Figure 1). The strong similarities between bacterial defense against protozoans and professional phagocytes suggest a link to the emergence of pathogenic bacteria and evolution of the virulent strains.

The bacteria that could release toxins that cause lysis or death of predators are the progenitors of today's extracellular pathogens [8] while the bacteria that could successfully survive and replicate inside the vacuole gave rise to obligate or facultative intracellular pathogens [9]. Because amoebae and human macrophages have common phagocytic mechanisms [10] and killing mechanisms using oxygen radicals, intracellular pathogens use similar processes to survive in both [11,12].

The genetic variability of the successful individuals that could survive under the selective conditions created by the presence of protozoan predators forms the basis for the generation of new

phenotypes. Mutations and horizontal gene transfer are the two main mechanisms mediating this genetic variability. Evidence indicates involvement of bacterial conjugations and transduction by bacteriophages in the shaping of bacterial genomes, bacterial fitness, and host-pathogen interactions [5,13].

1.1.1.2 Genome plasticity

The rapidly adaptive genomic and physiological changes are the result of a short generation time and genomic plasticity, which are important for the emergence of different pathogenic properties. Several mechanisms contribute to the genetic changes and development of novel pathogenic properties. Point mutations provide genetic variations by gain or loss of function, which result in the gain of new pathogenic properties. Moreover, the inter/intraspecies distribution of DNA elements encoding for pathogenic properties and the adaptation to the environment can be employed by homologous recombination, conjugation, transformation, and transduction, giving rise to genomic rearrangements, mobilization of plasmids, and integration of large DNA regions, prophages, and transposons [14]. Many genes coding for toxic proteins or gene products playing important roles in bacterial pathogenicity are present in clusters known as pathogenicity islands (PAIs) [15]. These specific regions mostly have different G+C content and codon usage, indicating that this region might have been horizontally transferred from another strain [16]. The presence of direct repeats at their ends, the close distance to tRNAs, and the presence of integrase determinants and prophages are other clues that the generation of PAIs was by horizontal gene transfer. Plasmids and transposons can also carry genes important for the pathogenicity of bacteria, as in *Shigella* and *Yersinia spp.* [17]. It is very clear that phages play an important role in the evolution and virulence of pathogens by being important vehicles for horizontal gene transfer between different species and within the same bacterial species [5]. For example, β -phage encodes the diphtheria toxin of *Corynebacterium diphtheria* [18] and Phage C1 encodes the neurotoxin of *Clostridium botulinum* [19]. Comparative genomics has provided a lot of information regarding acquisition of genomic properties in the pathogenic bacterial world. To understand the origins of pathogenic properties, more comparisons of pathogenic strains and their close relatives and phages are needed. Next-generation DNA sequencing provides great opportunities for comparative genomic studies as the genomes of many other bacteria are sequenced every day.

1.2 Bacterial Pathogenesis

Bacterial pathogenesis is often defined as the chemical mechanisms by which microbial organisms cause disease in hosts [20]. For a microbial organism to be considered a pathogen, it must have competence to change the behavior and health of another organism, its host [21]. In host–pathogen interactions, some pathogens can infect a broad range of host species while others infect only a specific host species [22]. Host specificity of pathogens originates from their genetic repertoire, which provides the source for the different pathogenic mechanisms and lifestyle. According to their pathogenic lifestyle, they are commonly defined as either extracellular or intracellular pathogens (Figure 1). However, it should be noted that there is no precise distinction of these two because some pathogens have both extracellular and intracellular lifestyles in the host. For example, *M. tuberculosis* is an intracellular pathogen that also must survive the host defense in the extracellular milieu before invading host cells [23]. Similarly, *Staphylococcus aureus* and *Escherichia coli* are extracellular pathogens but can invade intracellular environments in the human host [24,25]. Even though bacterial pathogens have different host specificities and different pathogenic lifestyles in the host, almost all bacterial pathogens have some basic steps in common in bacterial pathogenesis. Those basic steps are as follows:

1. Attachment or the entry to the host
2. Evasion of the host defense
3. Reproduction at the site of the infection and/or to spread to other sites
4. Damage to the host, directly or indirectly, through specific or nonspecific host response to the pathogen
5. Transmission from the infected host to another

1.2.1 Bacterial Pathogenic Mechanisms

Different pathogenic bacteria use similar pathogenic mechanisms that are dynamically regulated in different phases of infection for successful pathogenesis in the host. For instance, many common mechanisms involve adhering to, invading, and damaging host cells and tissues, surviving host defenses, and establishing infection (Figure 2) [26].

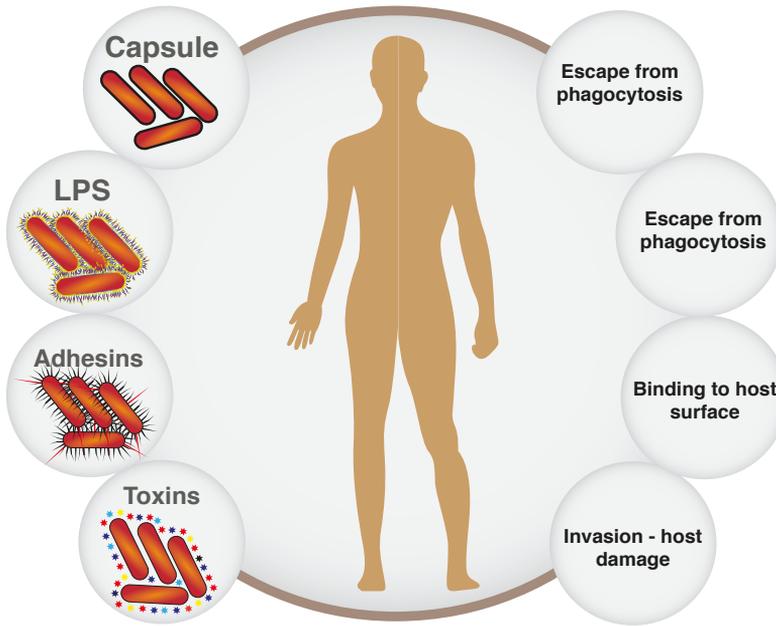


Figure 2. Bacterial pathogenic mechanisms. Interaction of bacterial components with the host include capsules and LPS (lipopolysaccharide) that protect bacteria from phagocytosis, adhesins that help bacteria to attach to host surfaces, and toxins that lead damage to the host. Figure adapted from Wilson et al., 2002 [26].

1.2.1.1 Adherence

One of the prerequisite processes in the host–pathogen interaction is successful adherence of the pathogen to host surfaces, such as skin, mucous membranes, and other tissues (lymphoid tissue, gastric and intestinal epithelia, alveolar lining, endothelial tissue). The first contact between host and pathogens is usually accomplished by adhesins, such as fimbria (pili) or afimbrial adhesins [27]. Many Gram-negative bacterial pathogens such as *E. coli*, *Vibrio cholera*, and *Pseudomonas aeruginosa* rely on fimbria for adherence [28–30]. Afimbrial adhesins that generally make more close contacts with host cells are also common and are produced by, for example, *Y. pseudotuberculosis*, enteropathogenic *E. coli*, *Staphylococcus spp.*, *Streptococcus spp.*, and mycobacterial pathogens [31,32]. Binding of the adhesin molecules may result in extracellular colonization or internalization of the pathogen. Adhesins can exhibit very specific binding properties and bind only to specific host cell receptors, thereby

providing the specificity of a pathogen regarding type of host, organ, and cell. For example, *Listeria monocytogenes* binds to and is internalized by human and rabbit epithelial cells only; its adhesion molecule internalin A (InlA) binds the surface receptor E-cadherin expressed by these species, but fails to bind, for example, mouse E-cadherin [33].

1.2.1.2 Invasion

Upon association of pathogenic bacteria with host surfaces, some pathogens gain access to the deeper tissues to evade host defenses and multiply to sufficient numbers for a successful infection. This pathogenic strategy, called invasion, can be either intracellular or extracellular. Extracellular invasion is a way for pathogenic bacteria, such as some *Streptococcus* species and *S. aureus*, to break down tissue barriers to disseminate in the host while remaining outside the host cells. This strategy allows bacteria to disseminate and access niches where they can proliferate. For this purpose, the bacteria secrete several enzymes to digest host cell molecules, such as streptokinase and staphylokinase that degrade fibrin clots, hyaluronidase that cleaves proteoglycans in connective tissue, lipases that degrade host oils, and nucleases that digest released DNAs and RNAs [26]. Intracellular invasion occurs when a pathogen penetrates into the cells of a tissue. Some Gram-negative and Gram-positive bacteria use intracellular invasion strategies to disseminate in the host and enter both phagocytic and non-phagocytic host cells [34-37]. However, many bacterial species use both extracellular and intracellular invasion during infection. An excellent example of that is adherence and invasion of M (microfold) cells by *Y. enterocolitica* and *Y. pseudotuberculosis*. The outer membrane protein invasin (Inv) binds to β 1-integrin on the M cell surface and mediates uptake in a zipper-like internalization process [38]. This process enables bacteria to reach the lymphoid tissue and draining lymph nodes, where they are thought to be extracellular.

1.2.1.3 Bacterial Camouflage

After the first contact with the host, bacterial pathogens need to evade recognition and/or activation of immune responses by hiding their externally exposed pathogenic

properties. They can conceal those properties by mechanisms such as modified LPS biosynthesis, capsule production, and biofilm formation.

LPS molecules are a family of glycolipids produced by Gram-negative bacteria. They have important roles in the integrity of the outer membrane and in host-pathogen interactions. The characterization of LPS is based on a highly conserved lipid moiety known as Lipid A. Few bacteria biosynthesize LPS as only Lipid A; in the majority, Lipid A is glycosylated with a core oligosaccharide that contains an attachment site for a long-chain O-antigenic polysaccharide [39] (Figure 3). LPS (also known as endotoxin) is generally considered to be a critical component for induction of septic

shock [40]. Lipid A constitutes the toxic portion of the LPS molecule and can trigger release of a number of proinflammatory cytokines and activate the complement cascade [26]. LPS is also associated with resistance to complement mediated bacterial killing [41].

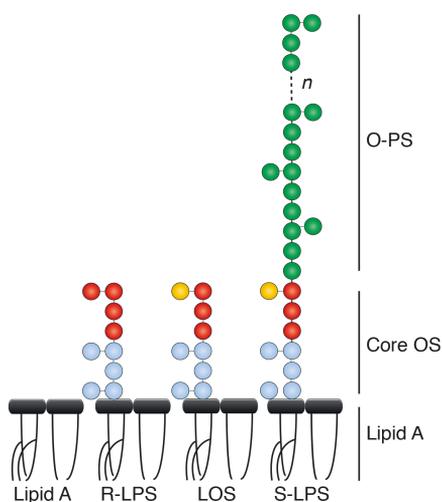


Figure 3. LPS structure. Abbreviations: O-PS, O-antigenic LPS; R-LPS, rough LPS (lacking O-PS); LOS, lipo-oligosaccharide; S-LPS, smooth LPS (containing O-PS). Figure adapted from Whitfield and Trent, 2014 [42].

Capsules are typically a surface layer of high molecular-weight extracellular polysaccharides produced by both Gram-positive and Gram-negative bacteria covering bacterial structures and that can allow pathogens to evade recognition by the host innate immune system [43]. This ability is quite striking for certain bacterial pathogens, such as *S. pyogenes* and *E. coli*, which produce capsules that mimic the host extracellular matrix to evade the host immune response [44,45].

Microbial biofilms are complex surface-attached bacterial cell groups that develop organized communities. Bacteria in biofilm live in an environment formed by hydrated extracellular polymeric substances, which is mainly composed of polysaccharides,

proteins, nucleic acids, and lipids [46]. Biofilm structures provide protection for the community members against a wide range of challenges, such as UV exposure, metal toxicity, acid exposure, dehydration and salinity, phagocytosis, and several antibiotics and antimicrobial agents [47]. This inherent resistance capacity of bacteria in biofilms provides roots for persistent and chronic bacterial infections [48].

Bacterial pathogens may develop strategies to modify their surface structures to avoid adaptive and cellular responses from the host. Antigenic variation of surface proteins permits bacteria to avoid recognition and thereby retain their infectivity or re-infect previously infected hosts. One example here is antigenic variation of the surface lipoprotein VlsE, encoded by *Borrelia burgdorferi*, and PilE, encoded by *Neisseria gonorrhoeae* [49]. Antigenic variation is a result of recombination events in the coding region of these surface components [50]. Another strategy is phase variation, high-frequency, reversible on–off switching gene expression of surface proteins [51].

1.2.1.4 Damage to the Host

Toxins are major players that help pathogens gain access to niches for colonization and to obtain nutrients. Some purified toxins, such as the cholera toxin of *V. cholera*, can cause disease symptoms by themselves but still require cholera adhesin to reach full bacterial virulence [52]. Toxins can be seen as analogous to biological weapons that destroy host tissues or cells [26]. They can be classified into two groups: surface-associated toxins that are released upon bacterial lysis into the extracellular milieu (e.g., endotoxins) and toxins that are actively secreted into the extracellular environment or directly translocated into the eukaryotic host cell (e.g., exotoxins).

The term ‘endotoxin’ is mostly used to refer to LPS. Release of endotoxins activates the innate immune system [42,53], which in turn can cause damage to the host cells; for example, by local production of toxic and degrading proteins and activated by activated immune cells. Bacterial mutants with defects in the early steps of LPS biosynthesis are usually not viable, indicating an essential role of LPS in bacterial survival [54]. Therefore, LPS biosynthetic enzymes are seen as potential antimicrobial targets [55]. Exotoxins that are secreted from pathogens can be classified into three main groups:

intracellular exotoxins, extracellular toxins, and superantigens. A-B toxins are a large group of intracellular exotoxins, such as the diphtheria, anthrax, Shiga, and cholera toxins. They consist of two components: an A-subunit with enzymatic activity on the host cell, and a B-subunit that binds to host cell receptors and assists in the transport of the toxic A-subunit into the host cell [56]. The enzymatic activity of the A-subunit can, for example, be ADP-ribosylating or proteolytic activity. A-subunits from different strains are usually well conserved while the B-subunits often vary, and this variation confers the host and tissue specificity on the pathogen [34]. Direct delivery of intracellular exotoxins into the host cell cytoplasm via the type III secretion system (T3SS) is another mechanism used by many Gram-negative pathogens, such as members of the *Yersinia*, *Shigella*, *Salmonella*, and *Pseudomonas* genera. T3SS effectors interfere with signal transduction, leading to cell death and/or modulation of host antimicrobial functions [57].

The extracellular toxins are exclusively associated with interference with the stability of the host cell membrane via pore formation or enzymatic activity. Pore-forming extracellular toxins bind to the host cell membrane by specific interaction with the cell surface receptors and form pores [58]. One such example is the uropathogenic *E. coli* pore-forming exotoxin α -hemolysin [59]. An example of an extracellular exotoxin causing membrane damage through enzymatic activity is the *Clostridium perfringens* α -toxin, a membrane-disrupting phospholipase C [60].

Superantigens are conceptually categorized as analogous to endotoxins because they do not directly mediate damage to host cells. Similar to endotoxins, they induce inflammatory responses in the host that lead to damage. Superantigens that can cause a massive non-specific activation of naïve T cells are mostly produced by Gram-positive bacteria [61,62].

In addition to direct action of bacterial products on host tissue, bacterial pathogens can produce degradative enzymes that contribute to pathogenesis by degrading important components of the immune response, including immunoglobulins, extracellular matrix, basement membrane, and the fibrin network. One example here is *S. pyogenes*, which can inhibit opsonization by immunoglobulins using an immunoglobulin-degrading

enzyme [63]. Another example is *Y. pestis* and its Pla protease, which inactivates plasminogen activator inhibitors to overcome fibrin-mediated physical entrapment and other inflammatory reactions in the host [64,65].

1.3 Host Defense Against Bacterial Infection

The defense mechanisms in most mammalian host systems are very effective, and most infection attempts from bacterial pathogens can be kept out of the tissues, blood stream, and skin. The defense mechanisms and barriers (skin and mucosa with associated microbiota), innate immune system (production of antimicrobial peptides, phagocytosis, complement cascade, and inflammation), and adaptive immune system (antibodies and cytotoxic T lymphocytes) are the main obstacles that pathogenic bacteria encounter in mammalian hosts.

1.3.1 Barriers

1.3.1.1 Skin and Mucosal Surfaces

Most mammalian hosts are covered with skin and mucosa, cellular barriers that isolate the internal milieu from the non-sterile external environment. In addition to their physical roles, the host barriers provide a first line of defense against pathogenic bacteria. The blood–brain barrier, blood barrier, intestinal barrier, and placental barrier are types of barriers that provide protected niches within the host [66]. Internal surfaces of the host are covered with epithelial cell layers. The external surface, the skin is composed of living cells, dermis, and a dry outer layer with dead cells, the epidermis. Epidermis also contains keratinocytes, cells that produce the protein keratin, which cannot be degraded by most bacteria; in addition, the dead cells in epidermis are continually shed so that attached bacteria are constantly removed [67].

The respiratory, gastrointestinal (GI), and urogenital tracts are constantly exposed to foreign substances. Mucosal epithelial cells are replaced very rapidly, giving rise to elimination of bacteria attached to mucosal surfaces. Mucus is an important protection barrier and is a mixture of heavily O-glycosylated glycoproteins (mucin) that form homo-oligomers, which give mucus its viscous properties [68]. The mucosal barrier is

very dynamic because it is consistently produced and secreted by the specialized Goblet cells. Infection of mucosal surfaces can trigger release of mucin granules, and pathogens trapped in the mucus consequently are eliminated from the site [69]. Mucus provides a binding matrix for lysozyme, an enzyme that degrades the peptidoglycan layer of bacterial cell walls, causing them to lyse [70]. Lactoferrin, an iron-sequestering protein in the mucus, depletes the iron that is essential for pathogens [71].

1.3.1.2 Antimicrobial Proteins

Host epithelial surfaces are substantially exposed to microorganisms, and they produce different antimicrobial proteins/peptides (AMPs) to kill or inhibit growth of microorganisms [72]. AMPs are evolutionarily ancient innate immune system components synthesized by almost all animal and plants [73]. Many varieties of AMPs are produced by the skin and epithelial linings of the gut and respiratory tract [72].

Some AMPs such as lysozyme and phospholipase A2 (PLA2) that are highly expressed by Paneth cells have enzymatic activity acting on cell wall structures [74,75]. Most AMPs, however, kill bacteria with non-enzymatic activity. Defensins are a major family of membrane-disrupting peptides in vertebrates. Cationic residues in most of the defensins and cathelidins, another family of cationic peptides, interact with negatively charged phospholipid groups in bacterial membranes and eventually cause formation of pores that initiate lysis of the targeted microorganisms [76,77]. RNase7, calprotectin, psoriasin, and dermcidin are other antimicrobial proteins that have been implicated in membrane disruption [72]. The characteristics of some major AMP families are summarized in Table 1.

Table 1 Major antimicrobial protein families

Family	Mechanism of action	Tissue sites of expression	Target organisms
α -defensins (cryptdins in mice)	Membrane disruption	Small intestine	Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, protozoa
β -defensins	Membrane disruption	Large intestine, skin, respiratory tract	Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, protozoa
Calprotectin	Metal chelation	Abscesses	<i>Staphylococcus aureus</i> [78]
Cathelicidins	Membrane disruption	Large intestine, skin, lung, urinary tract	Gram-positive bacteria, Gram-negative bacteria, viruses, fungi
C-type lectins	Peptidoglycan recognition; killing mechanism unknown	Small intestine	Gram-positive bacteria
Galectins	Unknown	Intestine	Bacteria bearing blood group antigens
Lipocalin	Sequestration of iron-laden siderophores	Intestine and lung	<i>Escherichia coli</i> [79]
Lysozyme	Enzymatic attack on bacterial cell wall peptidoglycan	Intestine, eye, and more; secretions, including tears, saliva	Gram-positive bacteria; some activity against Gram-negative bacteria

1.3.1.3 Microbiota

Symbiotic bacteria (microbiota) occupy a wide range of environmentally exposed surfaces such as the skin, mouth, intestines, and vagina in mammalian hosts. The microbiota protects the host from the invasion of pathogenic or harmful bacteria, virus, fungi, and protozoans. Beyond a primary role as a physical barrier against pathogens, the microbiota also have a role in priming systemic immune effector cells, providing benefits to the host through supplying essential nutrients, and metabolizing indigestible compounds [80,81]. The composition of the microbiota changes in different parts of the body. 16S ribosomal RNA metagenomic sequencing of human skin microbiota has defined four phyla: *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* [82]. Although all of these are present in the inner mucosal surfaces, their proportions differ remarkably. *Actinobacteria* members dominate the skin microbiota while *Firmicutes* and *Bacteroidetes* members are most abundant in the GI tract (GIT) [80]. GIT is the largest home for bacterial communities in mammalian hosts. It harbors over 100 trillion bacteria with thousands of different species [83]. The GI microbiota also protects the

host from invading pathogens by preventing their colonization through competition for nutrients and attachment spaces on epithelium and by production of bacteriocins. Although the vast majority of bacteria in human GITs are from the *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* phyla [84], the composition at the genus level varies remarkably among different individuals. The diversity continues in different parts of the GIT: The stomach and upper small intestine (SI) have higher levels of aerobic and facultative anaerobic bacteria while the lower SI, cecum, and colon have higher levels of bacterial diversity [85]. Most knowledge about the effects of the microbiota on host protection against bacterial pathogens comes from studies in gnotobiology. Studies on germ-free mice have shown that most of the immune system components are less developed or have extensive deficits until bacterial colonization occurs. Colonization of a single bacterial species can revert many of these defects, which shows that interaction with the microbiota triggers a postnatal phase of immune system development [86]. Germ-free animals are more susceptible to infections [87,88].

1.3.2 Cellular Innate Immune Response

When pathogens make it past the initial physical and chemical barriers, they trigger the cellular innate immune response in the host. The induction occurs by recognition of conserved molecular components of pathogens by surface or intracellular recognition receptors on host cells, usually residential macrophages, dendritic cells (DCs), and mast cells. This recognition triggers production of proteins and substances that activate other immune cells, have protective roles or antimicrobial activities, and stimulate recruitment of different immune cells to the site of infection. Polymorphonuclear leukocytes (PMNs), and later on monocytes/macrophages recruited from the blood stream, are rapidly activated to engulf and destroy bacteria by a process called phagocytosis. Beyond their phagocytic activity, these cells release a set of innate-immunity components that induce a complex cascade of events known as the inflammatory response, involving release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin (IL)-1, and IL-6; chemokines; prostaglandin; and histamine and other components. This cascade of production and release of substances with subsequent immune cell activation leads to physiological changes.

Another arm of the innate defense is the complement system, blood proteins that initiate a proteolytic cascade upon activation, resulting in potentiated immune cell recruitment, recognition, and inflammatory response and eventual lysis of bacteria. Together, these systems are very effective in eliminating most invading pathogens from the host.

1.3.2.1 Recognition of Bacteria

The recognition of bacterial pathogens by the immune cells is mediated by pathogen recognition receptors (PRRs) (such as Toll-like receptors (TLRs), mannose receptors, and NOD-like receptors) binding to pathogen-associated molecular patterns (PAMPs) (such as flagellin, nucleic acids, LPS of Gram-negative bacteria, and peptidoglycan and lipoteichoic acid from Gram-positive bacteria). The major PRRs are TLRs, a gene family conserved in vertebrates and invertebrates; to date, 11 mammalian TLRs have been identified. The various TLRs can act in different combinations and provide a wide range of ligand specificity for responding to different types of invading microorganisms. For example, TLR4 is the receptor for LPS, but TLR1/TLR2 and TLR2/TLR6 combinations can be the receptor for modified LPS of certain bacterial pathogens. NOD1 and NOD2 are intracellular PRRs that recognize peptidoglycan. NODs and TLRs activate host cell inflammatory responses, such as production of cytokines (Table 2) [67,89,90]. Other types of recognition occur via binding of soluble host proteins to the surface of invading microorganisms, known as opsonization. Many different soluble proteins can function as opsonins; mannose-binding lectin (MBL), C-reactive protein, the complement system components C1q and C3b, and IgA and IgG antibodies. The different opsonins are recognized by different PRRs, and the outcome of the receptor–ligand interactions can facilitate complement activation, phagocytosis, and activation of inflammatory responses [67].

Table 2 Recognition of bacterial components by PRRs

Receptor	Cellular localization	Ligand
TLR1/TLR2	Cell surface	Triacyl lipopeptides
TLR2/TLR6	Cell surface	Diacyl lipopeptides, lipoteichoic acid
TLR2	Cell surface	Lipoproteins, peptidoglycan, porins, lipoarabinomannan, modified LPS
TLR4	Cell surface	LPS
TLR5	Cell surface	Flagellin
TLR9	Endosome	CpG DNA
NOD1	Cytoplasm	Diaminopimelic acid
NOD2	Cytoplasm	Muramyl dipeptide

1.3.2.2 Cytokines and Chemokines

Cytokines are glycoproteins that act as messengers and form an integrated network involved in regulation of immune responses. Epithelial cells, monocytes, macrophages, natural killer cells, endothelial cells, lymphocytes, and fibroblasts all produce cytokines. Chemokines, as the name indicates, are chemotactic cytokines and induce directed chemotaxis of nearby responsive cells. They are relatively smaller peptides and also involved in regulation of the immune response. Both cytokines and chemokines recognize specific receptors on target cells and induce changes in their immune functions. In a case of infection, different cytokines and chemokines are produced by different cells depending on the phase of the infection [89].

1.3.2.3 Complement System

The complement system is composed of serum proteins produced by the liver and whichever cleavage products facilitate and potentiate bacterial clearance. The complement proteins are inactive until a proteolytic cleavage cascade is induced. This process, known as complement activation, can be induced as a consequence of bacterial infections. The cleavage cascade can be triggered by bacteria in three ways: by bacteria bound to MBL; by bacterial surface components such as LPS and teichoic acid, the so-called alternative pathway; and by bacteria bound to IgG or IgM, the so-called classical pathway. The contribution of the cleavage products to bacterial clearance can be direct or indirect. As an opsonin facilitating phagocytosis, the cleavage product C3b is recognized by the phagocytic CR3 receptor. C5a, which is a soluble product, is a powerful chemoattractant for PMNs, contributing to recruitment of immune cells. C5b

makes a complex with C6, C7, C8, and C9, which is known as the membrane-damaging complex and kills bacteria by punching holes in their membranes [67,89,91].

1.3.2.4 Phagocytosis and Killing of Bacteria

Phagocytosis is the process by which a cell engulfs particles and forms an internal vesicle called a phagosome. PMNs, monocytes, macrophages, and DCs are the major phagocytic cells. By dynamic rearrangements of actin in the cell cytoskeleton, induced through activation of various surface receptors, they protrude their plasma membrane to engulf foreign particles into a phagosome that matures by fusing with endosomes/granules harboring antimicrobial proteins and also membrane ATPases, resulting in phagosomes with a low internal pH of 5. The mature phagosomes finally fuse with lysosomal granules/lysosomes, resulting in a phagolysosome. The lysosomal proteases are activated by the low pH and contribute to bacterial killing along with nucleases and defensins together with produced reactive oxygen species (ROS) and reactive nitrogen species (RNS) [67].

Production of ROS by phagocytotic cells is called an oxidative burst and is generated by the nicotinamide adenine dinucleotide phosphate oxidase in the phagosomal membrane, yielding superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). Granules of PMNs contain an additional ROS-generating protein, myeloperoxidase, which generates hypochlorite (OCl^-) from H_2O_2 . The toxic effect of ROS molecules is due to oxidizing activity on the amino acid side chain of proteins, and oxidative conditions can also cause nucleic acid damage due to the presence of Fe^{3+} and H_2O_2 together, which forms hydroxyl radicals ($HO\bullet$) [67,89]. Microbial components can induce transcription of inducible nitric oxide synthase (iNOS), which generates RNS such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) in combination with O_2^- . RNS inhibits bacterial respiration and reversibly inhibits DNA replication by mobilization of zinc from metalloproteins [92,93]. Another type of bacterial killing performed by PMNs is a process called neutrophil extracellular traps (NETs), the release of a net-like structure composed of granulosome proteins and chromatin, which binds to both Gram-positive and Gram-negative bacteria, degrades virulence factors, and kills bacteria [94].

1.3.3 From the Innate to the Adaptive Immune System

When the innate immune system is not sufficient to clear bacterial infections, the antigen-specific defense mechanism known as the adaptive immune system, which involves B and T lymphocytes, is needed. The innate immune defenses slow down the infection and also bring the pathogens to the attention of lymphocytes. DCs engulf bacteria and migrate via lymphatic vessels to secondary lymphoid tissues such as draining lymph nodes, where they present antigens to activate naïve T cells. Activated T-helper 2 cells can activate B cells to become plasma cells that secrete antibodies.

1.4 Bacterial Adaptation to Host Stresses

Environmental conditions and interactions between host and pathogens are key features for bacterial pathogenesis. As a response to different environmental conditions encountered in the host, bacterial pathogens modulate their metabolic pathways and fluxes to adapt [95]. These conditions are commonly defined as stress conditions, which are derived from the natural host environment and components and host defense systems. Examples of such stress conditions are high temperature and nutrient limitation in the body, low pH in the stomach and inside macrophages, high osmolarity in the intestine, oxidative/nitrosative stress, and membrane-disrupting agents as a consequence of innate immune responses. Thus, invading pathogens may have damage to their cell wall, cell membrane, proteins, and nucleic acids depending on the level of stress exposure [96]. However, bacteria are equipped with several systems that enable them to sense the environment and modulate their metabolism by reprogramming the transcriptome in a way that favors survival.

1.4.1 Sensing the Stress

Adaptation to stress conditions in the environment involves several distinct steps: generation of a stress signal in the bacteria, registration of the signal by a sensor, and changes in the expression pattern of a subset of genes. The stress factor itself, such as ROS, can be the signal or it can trigger formation of signals inside the bacteria such as denatured proteins, ribosome instability, or generation of cyclic-di-GMP (c-di-GMP) [97] and of guanosine phosphate, guanosine tetraphosphate (ppGpp), and guanosine

pentaphosphate ((p)ppGpp), also known as alarmones [98]. The stress sensors are DNA, RNA, and proteins. Sensing mechanisms of DNA and RNA are usually similar, such as sensing heat through a conformational change in the structure [99]. Similarly, protein sensors such as molecular chaperones, proteases, sensor kinases, and transcriptional regulators can sense changes in temperature [100]. Protein sensors can also be activated by formation of disulfide bonds in case of oxidative stress [101].

1.4.2 General Stress Response

Because different stress conditions may have similar effects on bacterial cells, bacteria have developed a general stress response, which includes many proteins that are specific to different stress responses providing protection to multiple stress conditions. One of the well-characterized general stress responses is regulated by alternative sigma factors, which bind to the RNA polymerase and change its specificity during stress conditions, thus changing gene expression patterns [102]. RpoS in Gram-negative bacteria and SigF in Gram-positive bacteria are key factors regulating stress responses. They are involved in adaptation to low pH, high osmolarity, temperature, bacteriocins, antibiotics, ethanol, and starvation and in formation of biofilm formation and sporulation [103].

1.4.3 Temperature

Mild changes in temperature for most of the mesophilic bacteria that are pathogenic to mammals are not considered a stress response because they can grow in the body. However, for some pathogens, such as food-borne pathogens, the rapid change in temperature during transition from the environment to the mammalian host induces stress responses. The heat shock response enables bacteria to adapt to the change in temperature, and mutants deficient in heat shock response are usually attenuated in virulence [104]. For example, the periplasmic heat shock protease HtrA (also known as DegP) is crucial for survival of *Salmonella spp.* and *Brucella spp.* mouse infection models and for *Yersinia spp.* within macrophages [105]. Furthermore, virulence factors can be regulated by temperature, such as temperature-regulated virulence in *Listeria*

monocytogenes, *Shigella spp.*, and *Bordetella pertussis* [106-108], and in *Yersinia spp.* through *lcrF* and *rovA* genes [109,110].

1.4.4 Acidic Stress

A very challenging environment for a food-borne pathogen is the passage through the low level of pH (about 1–2) in the stomach. Pathogens use different mechanisms to tolerate this extremely low pH level. Examples of such mechanisms are action of enzymes resulting in increased pH, such as urease activity resulting in production of ammonium ions used by *Helicobacter pylori* colonizing stomach mucosa [111], and acid shock proteins involved in protection of proteins and DNA, such as periplasmic chaperones HdeB/HdeA and Dps in *E. coli* [112-114]. Bacteria can also maintain pH homeostasis by consumption of protons through the activity of amino acid decarboxylase systems that use protons (lysine decarboxylase (CadA), converting lysine to cadaverine, and arginine decarboxylase (AdiA), converting arginine to agmatine) [115,116].

1.4.5 Oxidative and Nitrosative Stress

Oxidative stress, produced as a result of the oxidative burst, is a common challenge that bacterial pathogens must overcome to survive in the host. To overcome the deleterious effects of oxidative stress and also nitrosative stress, pathogenic bacteria have developed detoxification and repair mechanisms. One such mechanism is pigmentation, such as carotenoid pigments in *S. aureus* that quench reactive oxygen derivatives whereas non-pigmented mutants of *S. aureus* have increased sensitivity to oxidative stress [117-119]. Other protective mechanisms arise through the enzymatic activities of proteins such as superoxide dismutases (SODs), catalases, and hemoglobins. SODs are metalloenzymes that catalyze dismutation of O_2^- to oxygen and H_2O_2 , which can be reduced to water and oxygen by catalase or alkyl hydroperoxide reductase [120,121]. The catalase family proteins are divided into typical catalases, bifunctional catalase peroxidases, and manganese-containing catalases [122]. In *E. coli*, the alkyl hydroperoxide reductase (AhpC) detoxifies low levels of H_2O_2 whereas KatA is the primary scavenger of H_2O_2 [123]. Catalases in different bacteria may be under regulation of different

transcriptional regulators such as OxyR, Fur, PerR, and σ^S [122]. Another protective protein family is flavohemoglobins (Hmp). Hmp family proteins commonly have three enzymatic activities: NO-reductase, NO-dioxygenase, and alkyl hydroperoxide reductase [124]. *E. coli* Hmp uses NAD(P)H and O₂ to convert •NO to nitrate; however, under anaerobic conditions, it converts •NO to N₂O [125,126].

Other responses involved in repair mechanisms are to oxidative damage of nucleic acids and proteins. Insoluble Fe³⁺ has a toxic effect on DNA, lipids, and proteins through formation of oxygen radicals, which bacteria can cope with by synthesis of Fe-binding proteins [127].

1.4.6 Osmotic Stress

Pathogenic bacteria can distinguish external environments from host-associated environments by sensing changes in levels of osmolarity. The osmolarity in aqueous environments outside the host is thought to be no more than 0.06 M NaCl while it is higher in the intestinal lumen (0.3 M NaCl) and blood stream (0.15 M NaCl) [128]. Therefore, changes in osmolarity can have a critical role in influencing the virulence of many pathogenic bacteria. In *S. flexneri*, the expression of plasmid-located *vir* genes, necessary for invasion of epithelial cells, is induced under high osmolarity conditions via a mechanism involving the known osmolarity-responsive signal transduction system, the OmpR-EnvZ two-component system [129]. Similarly, expression of invasion genes (*invABC*) of *S. typhimurium* is also induced during high osmolarity conditions [130]. Other examples of osmolarity-regulated functions are osmolarity-induced expression of the type six secretion system (T6SS) in both *Y. pseudotuberculosis*, which involves OmpR-EnvZ, and in *V. cholera*, involving OsmR, another osmolarity-responsive regulator [131,132].

1.4.7 Oxygen Stress

Like osmotic stress, oxygen stress can influence the expression of genes involved in adherence and invasion [128]. The switch from aerobic to anaerobic growth conditions leads to a dramatic change in bacterial gene expression profiles. Fumarate-nitrate reductase (Fnr) is one of the regulatory proteins that controls the response to low

oxygen levels [133]. Fnr activates expression of several respiratory genes such as fumarate reductase (*frd*) and nitrate-reductase (*nar*) and also represses expression of respiratory genes such as cytochrome d (*cyd*) [134-136]. Another regulatory system operates through the ArcA-ArcB two-component regulatory system, which senses the level of oxygen in the environment and represses expression of many genes involved in aerobic respiration [137].

1.5 Persistent Bacterial Infections

Bacterial infections commonly cause disease symptoms leading to death of the host or clearance of the infection at the early stages with the help of the innate immune response or later on with both innate and adaptive immune responses. However, in some cases, bacteria can reside in the host for a prolonged time without producing obvious disease symptoms. Colonization of commensal bacteria is a model for studying bacterial persistent infections and provides information increasing our understanding of how some pathogenic bacteria survive for a long time within a host [138,139]. Nevertheless, persistent infections by true commensal bacteria of the host normal flora differ from persistent infections by pathogenic bacteria that can cause disease in certain conditions. Persistent infections by pathogenic bacteria can be divided into two groups, which have distinct characteristics from commensal bacteria. The first group includes pathogens such as *M. tuberculosis*, *H. pylori*, and *S. enterica* serovar Typhi, that can create an initial disease state controlled by the host immune responses without being completely cleared and that can persist in the host-specific niche for a long time. *M. tuberculosis* can establish persistent infection that can be acute, chronic, or clinically asymptomatic with a possibility of being reactivated [140,141]. *H. pylori* colonizes human gastric mucosa, and the host can be a life-long carrier of this persistent infection [142]. *S. enterica* serovar Typhi can cause systemic infections and in some individuals can be life-long [143]. The second group consists of pathogens such as *S. pneumonia*, *Neisseria meningitidis*, and *Haemophilus influenza* type B that can colonize asymptotically in the nasopharynx in most humans but can cause disease in immuno-

incompetent individuals [139]. I have focused on the first group of persistent infection in the following.

1.5.1 *Helicobacter pylori*

H. pylori is a human pathogen that colonizes approximately half of the world's population. It can be transmitted orally during childhood and persist for years in the gastric mucosa, causing chronic gastritis [142]. Chronic gastritis is asymptomatic in most carriers but represents a risk factor for development of gastric and duodenal ulcers and mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma [144]. The very acidic environment in the stomach is a challenging factor for colonization, but *H. pylori* has developed strategies to quickly reach the gastric mucosa for initiation of colonization. Its urease activity contributes to bringing the pH close to neutral and shifts the mucus layer toward viscous properties, allowing the bacteria to swim with flagellar motility [145,146]. In addition, *H. pylori* has developed different mechanisms to resist challenges by immune cells. It uses proteins such as catalase and arginase to detoxify ROS and NOS [147,148] and various DNA recombination and repair pathways [149]. *H. pylori* also modifies its LPS by reducing its negative charge, which enables it to avoid binding by antimicrobial peptides and recognition by TLR4 [150]. Neither is *H. pylori* recognized by TLR5 because, in contrast to most Gram-negative bacteria, it does not produce FlaC that can be recognized by TLR5; instead, it expresses FlaA, which is 1000-fold less potent as a TLR5 stimulus [151]. Also, subversions of the adaptive immune response are expected to play a critical role for *H. pylori* persistence. One player here is VacA, a pore-forming toxin that disrupts cell polarity and induces apoptosis of epithelial cells but that also inhibits T cell proliferation by disruption of the T cell receptor signaling pathway [152,153]. In addition, the *H. pylori* γ -glutamyl transpeptidase inhibits T cell proliferation through inhibition of cyclin-dependent kinase activity in the G1 phase of the cell cycle [154].

1.5.2 *Mycobacterium tuberculosis*

M. tuberculosis, the causative agent of tuberculosis (TB), is a human pathogen that infects one-third of the world's population [155]. Initial infection is at pulmonary sites

but can later disseminate to extrapulmonary regions by migration of infected cells [139]. *M. tuberculosis* infections can stay asymptomatic for many years, sometimes throughout life. *M. tuberculosis* is generally found in macrophages within granulomas consisting of differentiated macrophages, T lymphocytes, DCs, PMNs, fibroblasts, and extracellular matrix components [156]. *M. tuberculosis* can remodel phagosomal progression, allowing survival within the macrophages. [157]. The granuloma state is a balance between the pathogen and the host immune system. Although persistent bacteria generally are known to be in a non-replicative dormant form, a low level of replication occurs in the center of the granuloma. It is believed that bacteria keep the balance with replication to maintain bacterial number against loss with the bacterial killing by the immune response [158]. However, reactivation with dissemination of the bacteria can occur if the balance is destroyed, which is common in immune-compromised individuals, such as HIV patients.

Persistent *M. tuberculosis* infections are mostly studied in mouse models, such as the Cornell mouse model (drug-induced model) and low-dose model latent TB (chronic or plateau model). The low-dose model involves aerosol or intravenous infection, resulting in long-term residence of bacteria in the lungs while animals remain healthy [159]. One factor that has been implicated as important for the development of infection is isocitrate lyase, which enables the bacteria to use fatty acids as a carbon source [160]. The transcriptional regulator MprA regulates several important genes such as *sigB* and *sigE* during persistent infection, and PcaA, Mkl, and MmpL4 are some important proteins for the development of persistent infections [161-164].

1.5.3 *Salmonella*

S. enterica causes diseases in humans from gastroenteritis to systemic infections [139]. *S. enterica* serovar Typhi (*S. Typhi*) causes human typhoid fever whereas *S. enterica* serovar Typhimurium (*S. Typhimurium*) causes self-limiting gastroenteritis and sometimes systemic infections in humans [143]. For typhoid fever, the most common infection sites are the SI, liver, spleen, bone marrow, and gall bladder. The bacteria infect Peyer's patches (PPs) and lymphoid-associated tissues by invasion of M cells in the SI. A portion of asymptomatic typhoid patients (1–6%) can be carriers for decades and serve

as reservoirs by periodically shedding in feces and urine [165]. *S. typhimurium* causes typhoid-like disease in mice and has been used as a mouse model for persistent infections. Many laboratory mouse strains, such as C57BL/6 and BALB/c, carry point mutations in the *Nramp1* gene (encoding an ion transporter expressed in macrophages), making them sensitive to intracellular pathogens [166,167]. However, in contrast to many pathogens, *S. Typhimurium* strains cause persistent infection in *Nramp1^{wt/wt}* mice, residing within macrophages in mesenteric lymph nodes [143]. Bacterial factors such as the fibronectin-binding proteins ShdA and MisL and surface components with possible adhesive properties encoded on the fimbrial operon have been suggested to contribute to establishment of persistent infection in the intestine [168,169]. Furthermore, the two-component system PhoPQ, which senses the presence of membrane-damaging antimicrobial peptides, acidic pH, and changes in metal ion concentrations, is crucial for persistent infection by this pathogen by regulating components of virulence-associated secretion systems, flagella, transport systems, and structural components of the outer membrane [170,171]. Here, the latter contributes to protection against antimicrobial peptides and to avoidance of recognition, such as the Vi-capsule that prevents recognition by TLR4, through masking the LPS structure [172]. Furthermore, TviA-mediated repression of flagellin during infection evades detection by TLR5 [173].

1.6 *Yersinia* as a New Model for Persistence

Enteropathogenic *Yersinia* infections are self-limited in immunocompetent humans. However, they can lead to development of persistent infections in some cases with or without symptoms [174]. Long-term infections of enteropathogenic *Yersinia* species have been reported in patients with chronic ileitis and arthritis within intestinal mucosa and gut-associated lymphoid tissues [175,176]. Furthermore, they have been isolated from the cecums of farm pigs [177,178] and wild rodents [179], indicating competence for enteropathogenic *Yersinia* species for a long-term residence in different hosts.

1.6.1 *Yersinia* pathogens

Yersinia members are classified into the Enterobacteriaceae family, which are facultative, oxidase-negative, Gram-negative rod- or coccobacilli-shaped and glucose-fermenting bacteria that form single colonies in laboratory conditions within 2–3 days. *Yersinia* can cause a variety of diseases in mammals, birds, and fish [180]. In the genus *Yersinia*, there are 11 species divided into serotypes based on the reactions of antibodies to LPS structures. *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are the only three species that are pathogenic to humans. *Y. pseudotuberculosis* and *Y. enterocolitica* are enteric pathogens causing GI syndromes from mild diarrhea to systemic infections [181]. *Y. pestis*, the causative agent of bubonic and pneumonic plague, depends on two hosts: the invertebrate flea (*Xenopsylla cheopis*) and a mammalian host. Transmission to humans is through a bite from a flea that acquired *Y. pestis* from a blood meal of an infected animal such as a rat. In humans and rodents, *Y. pestis* spreads to lymph nodes from the site of a flea bite and forms the characteristic buboes (swollen lymph nodes) of bubonic plague [182]. Bacteria then can spread to the blood stream and reach the spleen, liver, and lung. Colonization in the lung leads to development of pneumonic plague, which then constitutes the source for transmission via aerosols. *Y. pestis*, which is closely related to *Y. pseudotuberculosis* at the genomic level, evolved from the *Y. pseudotuberculosis* serotype Ib strain 1500–20,000 years ago [183] whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are thought to have evolved from the same ancestor about 5 million years ago. Transmission of these two enteric pathogens usually occurs with ingestion of contaminated food or water. From the lumen of the SI, they can cross the intestinal barrier by passing through M cells (specialized epithelial cells that sample intestinal contents and release them at their basolateral side of the epithelial barrier). The pathogens proliferate extracellularly in lymphoid follicles in underlying tissue from where they, upon acute infection, disseminate to local MLNs and later on break the barrier and become systemic, reaching other organs such as liver and spleen [184].

1.6.1.1 Pathogenesis and Adaptation of Enteric *Yersinia*

Like other pathogens, enteric *Yersinia* pathogens must rapidly adapt to host environments, attach to and invade host tissues, proliferate, avoid both innate and

immune responses, and access further environments in the host. Both *Y. pseudotuberculosis* and *Y. enterocolitica* can employ such strategies, for example, where changes in temperature and pH lead to changes in gene expression patterns for both pathogens [185]. Initially, while passing through the stomach, urease is believed to lower the negative effects of the acidic environment, as the *ure* mutant survives less during passage through the mouse stomach [186,187]. Enteropathogenic *Yersinia* also express a number of adhesin factors such as Inv, Ail, and YadA that contribute to attachment and invasion of intestinal tissues [188].

Enteropathogenic *Yersinia* gene expression is dynamically regulated upon sensing changes in diverse environmental cues by transcriptional regulators [189,190] and other regulatory components. *Yersinia* express a set of global regulators such as RovA, Crp, CsrA, and YmoA that are involved in environmental adaption [191]. The RovA regulon contains several transcriptional regulators, outer membrane proteins, two-component system proteins, and many hypothetical proteins involved in adaptation to different environmental conditions. RovA expression is indirectly regulated by CsrA, the major player in the global carbon storage regulatory system [192]. CsrA directly/indirectly regulates expression of approximately 500 genes involved in virulence, motility, stress responses, and metabolism in different environmental conditions [193]. The RNA chaperone Hfq is another regulatory component that contributes to adaption to new environments [194]. Similar to many other enteric pathogens, *Yersinia* expresses SODs, catalases, and peroxidases to diminish bacterial killing by NOS and ROS [195]. Furthermore, responses to osmolarity are regulated by the EnvZ/OmpR two-component system [196], and FNR and ArcA are involved in adaption to low oxygen [197].

1.6.1.2 Adherence

Yersinia species express a number of adhesion factors such as Inv, Ail, and YadA. Inv is a chromosomally encoded outer membrane protein present in enteric *Yersinia* but inactivated with an insertion in *Y. pestis* [198-202]. Inv enhances adherence and invasion of host cells, most importantly the M cells, by binding $\beta 1$ integrin receptors on their surface [203]. Expression of Inv is positively regulated by RovA and is co-

regulated with flagellar genes [204,205]. However, although its expression is highest at 26°C and not at host temperature, Inv is expected to be expressed during the initial invasive phase of the infection since its expression increases under *in vivo*-mimicking conditions, at 37°C in the presence of sodium and low pH [185,206]. Ail is another adhesin expressed by enteric *Yersinia* species but not in *Y. pestis*. Expression of Ail is induced at 37°C during the stationary phase [207]. Virulent *Yersinia* also express an adhesin encoded on the virulence plasmid-encoded gene *yadA*, which is important for virulence of *Y. enterocolitica* but not for *Y. pseudotuberculosis* [208,209].

1.6.1.3 T3SS

Pathogenic *Yersinia* species are armed with an arsenal of virulence factors and mechanisms to survive in host environments by manipulating host immune responses. *Yersinia* deliver virulence effector proteins called Yops (*Yersinia* outer membrane proteins) into host cells through specialized T3SS encoded on a 70-kb virulence plasmid, pYV (pCD1 in *Y. pestis*), which is shared by all three pathogenic *Yersinia* species and is a prerequisite for their virulence [184,210]. A number of other Gram-negative pathogens such as *Salmonella enteritidis*, *Bordetella*, and *Pseudomonas* and plant pathogens contain T3SS [211]. The system was initially discovered in *Yersinia* [212], and the *Yersinia* pYV-encoded T3SS has been extensively studied. Secretion of Yops by T3SS is induced at 37°C with depletion of Ca²⁺ under *in vitro* conditions. During infection, it is the contact between bacteria and host cells that triggers secretion and also translocation of Yops into interacting host cells. Yops such as YopE, YopH, YopJ, YopK, YopM, and YopT are exotoxins, some mimicking activities of host cell enzymes such as kinases, acetylases, phosphatases, proteases, guanine nucleotide exchange factors, and GTPase-activating proteins (GAPs) to modulate cytoskeleton and immune signaling pathways. Actions of the Yops inside the host cells lead to inhibition of phagocytosis and proinflammatory cytokine production, induction of apoptosis, and pyroptosis [213]. YopH is a protein tyrosine phosphatase that inhibits bacteria-induced signaling in host cells by dephosphorylating signaling proteins such as FAK, p130-CAS, paxillin, and Fyb, resulting in inhibition of phagocytosis [214]. In addition, YopE, which exhibits GAP activity, affects actin dynamics by inhibiting the small G-

proteins RhoA, Rac1, and Cdc42 [215-217]. Similarly, YopT (cysteine protease) and YopO (serine/threonine kinase) also act on RhoA and Rac1 [218]. YopJ/P is a protein acetylase that can induce apoptosis/pyroptosis in macrophages and also inhibit inflammatory signaling [219,220].

1.6.1.4 T6SS

Another relatively recently discovered secretion system is the T6SS, which has been suggested to be involved in virulence [221], immunomodulation [222], competitive growth in mixed bacterial populations [223], and adaptation of bacteria to different environmental conditions [224]. T6SS is a complex macromolecular machinery that translocates proteins to the extracellular milieu or into prokaryotic or eukaryotic cells [225]. T6SS is present in almost 25% of all sequenced Gram-negative bacteria, ranging from pathogenic to environmental species [226]. It consists of 13 core component proteins and accessory elements that vary in number among species. Hcp and VgrG are major structural components, and ClpV and IcmF are conserved ATPases that confer dynamic properties on the machinery for secretion of effector proteins [227].

Most bacteria (including *Y. enterocolitica*) contain one T6SS gene cluster whereas *Y. pseudotuberculosis* harbors four clusters [228]. Among those four, only features of T6SS-4 (cluster 4) have been reported. T6SS-4 has been reported to be expressed at 26°C during the stationary phase, regulated by the quorum-sensing system and in acidic conditions by OmpR. Very recently, it was reported that it functions to acquire Zn²⁺ through translocation of the zinc-binding protein YezP during oxidative stress conditions under regulation of the OxyR regulatory protein [228-230]. The other T6SS gene clusters in *Y. pseudotuberculosis* are expected to have different functions for adaptation in different niches; however, those functions and their effects on virulence remain to be elucidated.

1.6.1.5 LPS

LPS, which is the major component of the outer membrane, is important for *Y. pseudotuberculosis* virulence [231], and mutations in genes involved in O-antigen biosynthesis result in attenuation of virulence [232,233]. The O-antigens comprise the

outermost domain of the LPS molecule and contribute to resistance to lipophilic agents and antimicrobial peptides [234]. Biosynthesis of O-antigens starts with building of an O-antigen repeat unit, where the composition and number of O-antigen repeats differ in different species and in serotypes of the same species. The O-antigen repeat is translocated to the periplasm where it is ligated to lipid A and finally translocated to the outer membrane [235]. Genes involved in O-antigen synthesis and processing are in an operon flanked by *hemH* and *gsk* genes in *Yersinia* [236], the transcription of which is enhanced by the transcriptional anti-terminator RfaH [237].

Initial studies on RfaH described it as a component of LPS biosynthesis in *S. typhimurium* [238], but it was later identified as a NusG homolog, a transcriptional anti-terminator that enhances transcription of distal parts in large operons in *E. coli* [239]. Although NusG has a global effect on gene expression by binding RNA polymerase (RNAP), transcribing almost all genes, and being essential for viability, RfaH is dispensable [240]. RfaH recognizes a 12-nt conserved *ops* (operon polarity suppressor) sequence (5'-GGCGGTAGNNTN-3') located in the 5' untranslated regions of operons [241] and binds to the non-template DNA strand facing the RNAP, paused at the *ops* site [242].

1.7 Identification of Pathogenicity Factors

For a complete understanding of host-pathogen interactions, identification and detailed characterization of pathogenicity factors are necessary. There are several biochemical, immunological, and genetic approaches to identifying pathogenicity factors. Recently, many genomic approaches such as next-generation RNA/DNA sequencing and chromatin immunoprecipitation (ChiP)-seq have been developed and applied successfully.

1.7.1 Biochemical Approaches

Identification of pathogenicity factors such as toxins with biochemical methods includes isolation of the toxins from bacterial cultures and purification with filtration, centrifugation, selective precipitation, chromatography, and other similar methods. The

purified toxins can then be tested for their ability to reproduce disease symptoms upon reintroduction to the host. Once the protein is purified, the amino acid sequence can be determined with mass spectroscopy, and the gene encoding the toxin can be identified if the genome of the pathogen is available [21].

1.7.2 Molecular Genetic Approaches

One of the basic molecular genetic approaches to identifying pathogenicity factors is to clone genes of interest from pathogenic species, introduce them into avirulent *E. coli*, and assay for new recombinant virulent *E. coli* clones. An example can be the search for factors involved in adherence and invasion of a pathogen. In such a strategy, the genes of interest are inserted into *E. coli* that do not adhere to or invade tissue culture monolayers. Selecting the new *E. coli* clones that can adhere to or invade can then lead to identification of potential adhesins and invasins.

1.7.2.1 Transposon Mutagenesis

Transposon mutagenesis has been widely used as a global approach to identifying virulence genes for a pathogenic bacterial species. The approach involves random insertion of transposons into the genome of the pathogen, resulting in a pool of mutant strains, a transposon library. Successful insertion of transposons into genes important for a specific function is screened using suitable reporter systems, such as an antibiotic resistance gene, *lacZ* (β -galactosidase), *lux* (luciferase), or *phoA* (alkaline phosphatase) [243]. The inserted transposon serves as a marker to locate the mutation responsible for the observed phenotype.

1.7.2.2 Identification of *In Vivo*-Expressed Genes

Because no laboratory conditions completely mimic host conditions, efforts to identify virulence traits under *in vitro* conditions are always limited. Therefore, researchers have developed several molecular genetic strategies for identification of *in vivo*-expressed virulence genes. This approach commonly involves libraries of mutants that are used for infection, which has limitations in animal models with so-called “bottlenecks,” allowing only few bacterial clones to initiate infection.

1.7.2.2.1 Signature-tagged Mutagenesis

Signature-tagged mutagenesis is a combination of *in vitro*-generated transposon mutants with *in vivo* selection using an animal model to screen for the mutants that cannot grow in the host [244].

1.7.2.2.2 *In Vivo* Expression Technology

In vivo expression technology uses positive selection for genes expressed during infection in the host. It is based on a promoterless *in vivo* selection reporter system, which allows survival of the bacteria in the host only when the gene is expressed. The selection gene is usually on a plasmid that can integrate into the chromosome where a critical biosynthetic gene such *purA* is located. A deletion mutant of the critical biosynthetic gene will grow *in vitro* on a medium containing end products such as purins but will not grow in the host unless the fused promoter is activated [245]. Because the reporter system is based on on/off state of the promoter, it is impossible to obtain the expression level for a gene from this technique.

1.7.3 Genomic Approaches

1.7.3.1 Microarrays

DNA microarrays have been commonly used to profile global gene expression patterns of whole transcriptomes of many different organisms, in both *in vivo* and *in vitro* settings. It consists of an array of regularly ordered DNA oligonucleotides corresponding to all genes in the genome of the target organism. Fluorescently labeled cDNAs (generated by reverse transcription of bacterial mRNA samples) or DNAs (from different bacterial strains) isolated from different conditions are hybridized to complementary DNA probe spots on the array [246]. Gene expression is determined based on the fluorescent intensity of each corresponding probe hybridization. In addition to expression profiling, microarray technology has been used for bacterial species identification based on 16S rRNA, 23S rRNA, *katG*, or *gyrB* [247,248] and also for detection of methicillin-resistant *S. aureus* in clinical samples [249]. However, this technology is not suitable for global transcriptome profiling of bacteria in complex

bacterial populations because of cross-hybridization of probes with homologous genes in different species.

1.7.3.2 Next-generation Sequencing Technology

The automated Sanger method is considered a ‘first-generation’ technology, and newer methods are referred to as next-generation sequencing (NGS). These newer technologies constitute various applications that rely on a combination of template preparation, sequencing, and imaging and genome alignment and assembly methods. NGS has revolutionized the genomic research field by producing accurate and massive amounts of data at a relatively low cost [250]. The original scope of this new technology has been broadened to a more diverse range of utility-based applications, which has yielded more comprehensive analyses of the structure and content of microbial genomes, transcriptomes, molecular interactions, and interactions with their environments [251]. Microarrays for global transcriptome analysis studies are being replaced by NGS-based methods, which can identify and quantify even rare and homologous transcripts and provide information about alternative splicing and sequence variation of genes [252,253]. Various applications based on NGS technologies have contributed a lot to the current understanding of host–microbe interactions and development of infectious diseases.

1.7.3.2.1 Genome Sequencing by NGS

The number of completed microbial genome–sequence projects in 2009, the beginning of the NGS era, was 921; today, that number is 7624. Sequencing the whole genome of an organism was feasible only for large genome centers until the NGS era began. Today, many individual laboratories can sequence their own particular organisms. One of the first organisms subjected to NGS was the soil bacterium *Myxococcus xanthus*. Using Roche 454 GS20 technology, approximately 2.5 million reads (with an average read length of 100 nucleotides) were produced, and the *de novo* assembly resulted in 104 contigs, which left 104 gaps in the sequence, of which 88 were covered with capillary sequencing [254]. With development in NGS, today’s sequencing technologies, such as PacBio, can produce reads longer than 10 kb, which decreases the number of gaps in whole genome sequencing projects. However, for many applications, draft genome

sequencing can be enough to obtain particular information such as identification of differences in antibiotic resistance or virulence genes [255].

1.7.3.2.2 Metagenomics

Another advantage of NGS is sequencing of complex biological samples such as soil or gut microbiota samples that contain uncultivable microorganisms [256,257]. Enrichment of representative genes such 16S rRNA with PCR combined with high-throughput sequencing by NGS provides information about the composition of the samples and abundance of the particular organism. This application has shown that the abundance of *Ruminococcus obeum*, a species in fecal microbiota, significantly increases upon *V. cholera* infection to restrict the colonization of the pathogen [258]. However, 16S rRNA enrichment sequencing does not give much insight about metabolic activities in the samples. NGS has offered the capability in metagenomics studies of sequencing total microbial DNA in a complex sample and searching for a variety of genes involved in different metabolic processes with functional genomic annotations [259].

1.7.3.2.3 Transposon Insertion Sequencing

Transposon insertion sequencing (Tn-seq) is a technique in which random transposon mutant libraries are employed to study gene functions and the genetics of microbial physiology [260]. Several different approaches of Tn-seq are applied for various purposes. In general, DNA extracted from transposon libraries of bacteria treated under different conditions is digested, and small sequencing adapters are attached to the DNA fragments. The resulting transposon–chromosome junctions are sequenced with NGS, and the reads are aligned to the reference genomes to determine the location of the transposon insertions [261]. Tn-seq had been successfully employed to identify T6SS-dependent effectors and immunity proteins in *V. cholera* [262] and essential genes for adaptation to conditions mimicking host stress conditions in *S. enterica* Serotype Typhimurium [263].

1.7.3.2.4 ChiP-seq

ChiP followed by high-throughput sequencing (ChiP-seq) is used to identify DNA sequences that bind transcriptional factors. The method is based on sequencing of the

DNA region that is bound to the transcriptional factor, which is protected from the enzymatic cleavage performed before immunoprecipitation of the protein–DNA complex. Identification of transcription factors binding sequences by ChiP-seq has been done for many pathogenic bacteria [264]. A similar strategy using protection from enzymatic cleavage has been used to identify sRNAs and mRNAs bound to the mRNA chaperone Hfq [265,266]. Co-immunoprecipitation of Hfq–sRNA complexes with antibodies against Hfq itself or a tag fused to Hfq allows enrichment of bound RNA molecules. Subsequent cDNA synthesis followed by high-throughput sequencing with alignment of the sequencing reads enables identification of Hfq-dependent sRNAs or mRNAs [267].

1.7.3.2.5 RNA-seq

Development of NGS has provided many opportunities for studies of functional genomics of host–pathogen interactions, including identification of novel pathogenicity factors, bacterial evolution and emergence of pathogenic clones, molecular regulation of virulence capacity, and quantitative gene expression studies of pathogens both *in vivo* and *in vitro*. RNA-seq is high-throughput sequencing of RNA (in fact, the corresponding cDNA) and is different in principle from other quantitative gene expression methods such as quantitative real-time polymerase chain reaction (qRT-PCR), Northern blotting, and microarray [268]. One of the advantages is that it does not require any specific probe; therefore, the experimental design does not have to be adjusted to a specific genome sequence. It also provides a lot of information such as identification of operons and untranslated regions and improves sequence annotations. Mapping of the reads is more precise than with hybridization methods because the exact location of the reads can be determined to single nucleotide resolution [269]. This resolution allows transcriptomics analysis of repetitive regions and also determination of species-specific transcripts in complex samples such as infected tissues or gut microbiota, by discrimination of closely homologous transcripts. Furthermore, RNA-seq is free of the saturation problems that can be encountered with hybridization methods based on detection of fluorescence or radioactivity. The expression level for each open reading frame (ORF) is calculated with the amount of data matched to each

ORF, typically quantified with reads per kilobase ORF length per million reads (RPKM, see the calculation below) [270].

$$RPKM = \frac{\text{Total gene reads}}{\text{mapped reads (millions)} \times \text{gene length (KB)}}$$

Preparation of cDNA. Total RNA can be extracted from different types of samples with appropriate methods, commonly with organic solvents or commercially available kits. Because of a short half-life and the unstable nature of bacterial RNA molecules, the samples have to be processed very quickly to maintain RNA integrity. For instance, if the samples are taken from infected animal tissue in an animal facility, where the laboratory is usually not dedicated for RNA extraction, the samples should be treated with RNAlater or a similar treatment to preserve total RNA integrity and composition in the samples [271,272]. Special handling should be employed for homogenization of bacterial cells depending on the type of samples. Homogenization of bacterial RNA from host tissues may not be as straightforward as from *in vitro*-grown cultures because of the hard structure of tissues. Extracted total bacterial RNA from infected tissues is naturally saturated with host eukaryotic RNA, which will require more deep sequencing of the samples. This issue can be partly resolved by depleting poly(A)-RNAs using commercially available kits such as MICROBEnrich (Ambion), which uses hybridization of magnetic bead-linked oligonucleotides complementary to poly(A) in eukaryotic RNAs. Because ribosomal RNAs and tRNAs comprise the vast majority of the total RNA, it is necessary also to deplete them for enrichment of mRNAs and non-coding RNAs. In addition, these can be depleted using commercially available kits such as MicroExpress (Ambion) and Ribo-Zero (Epicentre) that are based on hybridization of magnetic bead-linked oligonucleotides, or use of terminator exonucleases that specifically degrade transcripts with a 5' monophosphate group [273-275]. After all depletion and DNase treatment steps, the RNA is converted to cDNA by reverse transcription followed by second DNA strand synthesis. However, generation of double-stranded cDNA leads to loss of information in the direction of transcripts. Therefore, Illumina strand-specific RNA-seq that avoids generation of second strand

cDNA synthesis is preferable [276]. Strand-specific RNA-seq has been critical for improving the annotations and for characterizations of sRNAs.

Sequencing and sequencing depth. Illumina, Roche 454, and SOLID sequencing platforms have been used in bacterial RNA-seq studies. Different platforms offer different read length and sequencing depth, and Illumina is most extensively used for transcriptomics studies. In contrast to genome sequencing analysis, RNA-seq does not require long reads specifically for bacterial transcriptomes that lack exon-intron junctions. Initial bacterial RNA-seq studies used ~36–40 nt long reads, resulting in ~5 million reads per sample [277,278]. Today's technologies can generate reads >300 nt with up to 1 billion reads per run. The coverage of the whole transcriptome, which is directly related to sequencing depth and genome size of the particular species, is one of the most important points to consider in the experimental design of RNA-seq. Typically, a minimum of 2–5 million reads from rRNA-depleted libraries generated from pure bacterial cultures is required for an accurate coverage of the bacterial transcriptome [277–279]. However, the minimum depth of sequencing for full coverage of pathogenic bacteria from mixed samples such as infected cell culture monolayers or infected host tissues is dramatically higher. Human and mouse genomes are ~3000 Mb in size whereas genomes of pathogens such as *E. coli* and *Y. pseudotuberculosis* are ~4.4–4.5 Mb. Furthermore, a eukaryotic cell typically contains 10–20 pg of total RNA, which is 100–200 times more than in a bacterial cell. For full coverage of human or mouse transcriptomes by RNA-seq, ≥100 million reads are required whereas ~2–5 million reads are required for bacteria. For complicated samples such as bacteria in infected host tissues, the required number of genes is much higher. The transcriptome of *V. cholera* in mouse intestine has been revealed with *in vivo* RNA-seq by >100 million reads per sample, of which only ~0.061–0.284 million reads were from *V. cholera*, which was not enough for full coverage [273]. Consequently, a sequencing depth of billions of reads would be required a full coverage of a pathogen transcriptome in the host.

Data analysis. Data analysis is a challenging issue because of sequencing errors or biases introduced during library preparation steps such as amplification, fragmentation, and reverse transcription [280–282]. Therefore, careful data quality control and

normalization procedures are necessary to obtain accurate RNA-seq data analyses. A typical data processing and analysis pipeline for differential gene expression analysis of a pathogen, if the annotated genome is available, consists of: (i) read mapping, (ii) counts computation, (iii) counts normalization, and (iv) detection of differentially expressed genes [283]. Read mapping is the alignment of the reads to matching genes in the reference genome or transcriptome. Several alignment tools are available for read mapping (Table 3). These can be divided into two groups based on the methodology for building the index: hash tables or Burrows–Wheeler transform (BWT) that have different memory usage, speed, read length support, and alignment accuracy [284]. In addition to different indexing, alignment algorithms play a critical role for read mapping. Because of sequencing errors, the alignment parameters should allow imperfect matching; in other words, it should tolerate a certain number of mismatches. Not only sequencing errors but also single nucleotide polymorphisms or insertions and deletions force a degree of mismatch tolerance for an accurate mapping [285]. However, the number of mismatches allowed for alignment parameters is difficult to determine for complex samples such as gut microbiota or infected cecal or intestinal tissues. The presence of many closely related bacterial species with homologous genes, which makes it difficult to discriminate species-specific reads, is challenging, and optimization trials are required to obtain accurate mapping of specific reads to the targeted genome.

Table 3 Short read mapping tools

Name	Website	Strategy	Ref.
Bowtie	http://bowtie.cbcb.umd.edu	BWT-based	[286]
BWA	http://bio-bwa.sourceforge.net/	BWT-based	[287]
Soap2	http://soap.genomics.org.cn/soapaligner.html	BWT-based	[288]
Map	http://maq.sourceforge.net/	Hash-based	[289]
RMAP	http://rulai.cshl.edu/rmap/	Hash-based	[290]
SeqMap	http://www-personal.umich.edu/~jianghui/seqmap/	Hash-based	[291]
SHRIMP	http://compbio.cs.toronto.edu/shrimp/	Hash-based	[292]

After mapping, the reads mapped to a matching region (transcript or gene) are used to compute counts to calculate expression levels as RPKM (defined earlier). Particular attention should be paid to overlapping genes, a problem that is partially solved with

strand-specific sequencing. Furthermore, normalization of count data is needed because of the presence of different biases such as sequencing depth and gene length. Normalization of sequencing depth is done through scaling read counts in each experiment with sequencing depth. Gene length bias in which longer genes produce more reads than shorter ones is normalized with the RPKM equation [283]. Differential expression analysis methods are based on statistics to identify genes that are characterized with statistically significant differences in the expression level. Because of the low replicate numbers in RNA-seq studies, parametric methods are preferred [293]. The models used and implemented in most differential expression analysis tools (Table 4) are based on the Poisson and negative binominal distributions.

Table 4 Differential expression analysis tools

Name	Website	Ref.
Cuffdiff	http://cole-trapnell-lab.github.io/cufflinks/	[294]
DEGseq	http://bioinfo.au.tsinghua.edu.cn/software/degseq/	[295]
DESeq	http://www-huber.embl.de/users/anders/DESeq/	[296]
EdgeR	http://www.bioconductor.org/packages/release/bioc/html/edgeR.html	[297]

In addition to transcriptomic profiling, RNA-seq has been extensively used for other types of applications such as identification of transcriptional start sites [275,298,299], improving bacterial annotations [300], and computing activities of transcriptional features, such as operon structures and expressions [301].

2 Objectives of This Thesis

- To study the effects of low-dose infections by *Y. pseudotuberculosis* in mice for development of a mouse infection model for persistent infections
- To perform *in vivo* RNA-seq of *Y. pseudotuberculosis* in mouse cecal tissue during early versus persistent stages of infection
- To identify key players/pathways in *Y. pseudotuberculosis* that are important for establishment and maintenance of persistent infection

3 Results and Discussion

3.1 Monitoring *Y. pseudotuberculosis* Infection in Mice

To monitor *in vivo* progression of *Y. pseudotuberculosis* infection in a mouse model, we employed bioluminescent imaging (BLI) using an *in vivo* imaging system, IVIS. We performed oral infections of BALB/c mice using different bacterial loads (10^7 to 10^9 CFU/ml) of bioluminescent *Y. pseudotuberculosis* YPIII (pIBX), which has the *lux* operon from *Photobabidus luminescens* inserted into the pYV-virulence plasmid. By monitoring anesthetized mice and dissected organs with BLI at different time points, the progression of infection and localization of bacteria could be revealed (Paper I, Figure 1A and 1B). We showed that mice infected with a high dose (10^8 and 10^9 CFU/ml) of bacterial load showed signs of disease from day 3 post infection (p.i.) in correlation with bacterial signals captured in the cecum, PPs, and MLN at 24 hours p.i. and in some cases in liver and spleen from day 3 p.i. (Paper I, Figure 1B, upper panel). However, no signs of disease were observed for mice infected with a low dose (10^7 CFU/ml) of bacterial load. In this group, bacteria colonized the cecum and PPs (Paper I, Figure 1B, lower panel). Altogether, in accordance with previous results [233,302,303], we found that *Y. pseudotuberculosis* bacteria, after oral infection of mice, initially colonize the cecum and PPs and may spread systemically depending on the dose of infection.

3.2 Persistent Infection of *Y. pseudotuberculosis* in Cecum

Prolonged monitoring of low-dose infections (10^6 and 10^7 CFUs/ml) in BALB/c mice for up to 24 days p.i. suggested that infection using sublethal doses can result in three possible scenarios: acute infection, clearance of infection, or an asymptomatic carrier state. Monitoring the bacterial localization at the organ level at later time points (day 24 and day 51 p.i.) showed that discrete areas of cecum were preferred niches for the bacteria (Paper I, Figure 2B, Figure S1B). In analogy, enteropathogenic *Yersinia* species have been detected in the cecum of pigs [177,178] and wild rodents [179], as has

persistent colonization of *Chlamydia muridarum* in mouse cecum [304], suggesting that this organ is a preferred niche for long-term residence. The bacterial counts in the cecums of persistently infected asymptomatic mice were similar, varying between 10^4 to 1.3×10^6 per cecum. Bacteria were also found in feces of persistently infected mice throughout the infection period, with bacterial counts of 10^3 and 10^6 CFU/g feces (Paper I, Table S1), while fecal pellets from mice that had cleared the infection did not contain any *Y. pseudotuberculosis*. This result suggests that bacteria might be consistently shed into feces from their location in the cecum where they multiply in a protected niche. Immunohistochemistry and immunofluorescent staining revealed that *Y. pseudotuberculosis* localizes in cecal lymphoid aggregates, mostly in the dome area facing the luminal site (Paper I, Figure 5A to C; Paper II, Figure 1A and B). The bacterial foci were surrounded by PMNs during both the early and late phases of the infection (Paper I, Figure 5K to L; Paper II, Figure 1C and D). Of interest, the long-term residence of persistent bacteria in proximity to PMNs, which may prevent the spread of bacteria to deeper tissues, indicates that *Y. pseudotuberculosis* has very efficient mechanisms to inhibit/resist antimicrobial functions of these innate immune cells, which normally rapidly eliminate bacteria.

3.2.1 A Mouse Model for Persistent *Y. pseudotuberculosis* Infection

We further showed that the capacity of *Y. pseudotuberculosis* to establish asymptomatic cecal colonization upon low-dose infection observed in BALB/c mice also was true for other mouse strains. Experiments using different mouse strains showed that FVB/N mice, compared to C57BL/6 and BALB/c animals, carried bacteria in the cecum for the longest periods of time. The C57BL/6 mice were most resistant to a low infection dose of *Y. pseudotuberculosis* with lower initial infection efficiency, lower susceptibility to systemic infection, and faster clearance of the infection (Paper I, Figure S3A). Consistent with previous findings, infection in BALB/c mice lasted until 48 days p.i. while the infection stayed up to 115 days p.i. in 2 out of 13 infected FVB/N mice. Different degrees of susceptibility among mouse strains have been observed for many bacterial infections, including pneumococci [305], which can trace to various factors, including the difference in MHC haplotypes [306]. C57BL/6 has H-2^b, BALB/c H-2^d,

and FVB/N H-2^a. Of note, FVB/n mice carrying transgene H-2^b are more resistant to persistent infection with encephalomyelitis virus, suggesting that the MHC haplotype of FVB/N may influence its susceptibility to infectious agents [306].

3.2.2 Cytokine Expression During Persistency

The presence of PMNs close to persistently colonized bacteria in asymptomatic mice indicates an active immune response. Measuring of 11 cytokines in mice infected with a low-dose bacterial load throughout the infection period showed that the production of most of the cytokines had increased already at day 1 p.i. This increase suggests an initial cytokine storm as previously has been reported for *Y. enterocolitica* infections in mice [307]. Compared to non-infected mice, the levels of IL-1 β , IL-4, IL-17, KC, and TNF- α were increased in sera from persistently infected mice at days 21 to 42 p.i. (Paper I, Figure 6). Of interest, the PMN chemokine KC was increased from day 1 p.i. and remained high in persistently infected mice throughout the entire infection period while it decreased in mice that cleared infection (Paper I, Figure 6). This finding correlates with the observed continuous infiltration of PMNs to the infection site. Overall, the increased levels of cytokines during persistency indicate an active host response likely involving Th2, Th1, and Th17 cells, PMNs, activated macrophages, and DCs.

3.3 Transcriptome of Persistent *Y. pseudotuberculosis*

To reveal bacterial mechanisms that promote and maintain the persistent infection, we performed comparative *in vivo* transcriptome analysis using RNA-seq of bacterial total RNAs isolated from cecums of infected FVB/N mice at early and persistent infection stages. In parallel, we analyzed the transcriptome of bacteria grown *in vitro* at 26°C and under T3SS-inducing conditions at 37°C.

3.3.1 Transcriptome of Persistent *Y. pseudotuberculosis* is Similar to 26°C Growth

One striking result of the transcriptome analyses was that the global expression profile of persistent *Y. pseudotuberculosis* is more similar to the profile of bacteria grown to the

stationary phase *in vitro* at 26°C whereas the expression profile during early infection is similar to that in bacteria grown *in vitro* under T3SS-inducing conditions (Paper II, Figure 5A and Table S2). This surprising similarity between persistent bacteria and *in vitro*-grown bacteria suggests a switch between two rather distinct expression patterns, which likely is a result of different environmental cues and not only the temperature. Additional distinct gene expression patterns observed are expected to be the result of adaptation to new environmental conditions.

3.3.2 Transcriptional Reprogramming for Persistence

The analysis of the transcriptome of persistently colonized bacteria revealed that the expression of T3SS components that were highly up-regulated during early infection were severely down-regulated during persistent infection (Paper II, Figure 3A, 4A, and Table S3). This finding was unexpected because T3SS is induced at 37°C [308]. Up-regulation of T3SS at the early stages suggests that T3SS components are necessary for initial infection to break down the epithelial barrier and defend against innate immune cells. This assumption is further supported by the fact that the *yopH* and *yopE* mutant strains are defective in establishment of initial infection and persistent infection (Paper I, Figure 3A–C). The observed repression of T3SS components during persistent infection suggests that the pathogen uses other genetic resources to adapt to the environment at the infection site. Another conspicuous finding is the up-regulation of genes involved in flagellar assembly in persistent bacteria (Paper II, Figure 3A, 4B, and Table S3), because flagella are known to be positively regulated at 26°C *in vitro* [309]. The surprising expression profiles of T3SS and flagella and the high similarity of the global transcriptome during persistency with that of bacteria grown *in vitro* at 26°C suggest that *Y. pseudotuberculosis* reprograms its transcriptome to adapt to the harsh environment for the prolonged residence in the host.

3.3.3 Persistent *Y. pseudotuberculosis* is Influenced by Environmental Cues

Functional gene clustering of differentially expressed genes *in vivo* with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [310] provided hints about the state of the persistent bacteria (Paper II, Figure 5B). Up-regulation of genes

involved in ribosome biogenesis, amino-acyl-tRNA biosynthesis, and RNA degradation indicates an active metabolic state during persistency. Furthermore, up-regulation of genes involved in DNA replication and repair, as well as in purine and pyrimidine biosynthesis, indicates that some degree of replication occurs. This implication in turn suggests that a replicative form of bacteria rather than a dormant form maintains bacterial load during persistent infection, which also correlates with the consistent bacterial shedding throughout the infection. It is also possible that up-regulation of flagella contributes to shedding for spread to other hosts, as shown for avian pathogenic *E. coli* in a chick persistence model [311]. The up-regulation of genes associated with different stress conditions such as acidic, oxidative, general, and other forms of stress suggests that bacteria actively sense and respond to a variety of environmental cues during persistency (Paper II, Table 2). Up-regulation of the two-component system *arcA-arcB*, the fumarate-nitrate reductase *fnr*, and other functional genes involved in microaerophilic/anaerobic respiration indicates a microaerophilic/anaerobic respiration for persistent bacteria. When comparing the differentially expressed genes in bacteria during persistent infection with those of bacteria grown under *in vitro* anaerobic conditions, we found significant similarities (42.5%) (Paper II, Figure 5C and Tables S4, S5). Thus, a substantial part of the expression profile of persistent bacteria is the result of limited oxygen availability. Taken together, the transcriptional profiles of persistent bacteria suggest an adaptation to an environment with limited oxygen, the presence of oxidative and acidic stress, need for motility/chemotaxis, and modulation of the bacterial surface.

3.4 Complex RNA-Populations can be Resolved by RNA-seq

Mapping species-specific transcripts to the target reference genome for RNA samples isolated from complex environments such as cecum where it harbors large number of bacterial species is challenging. We optimized a protocol for filtering only *Y. pseudotuberculosis*. Mapping the RNA-seq reads to 16SMicrobial database identified the other bacterial species in each RNA samples. The available bacterial genomes were then used as reference genome together with *Y. pseudotuberculosis* and different

alignment parameters were employed. The alignment parameters that led only *Y. pseudotuberculosis* specific reads to map its genome with the highest number were found to be optimal. Accordingly, 5% mismatch tolerance within the 95% of aligned read was set for the alignment of reads for all the samples. Thus, we could retrieve as much as possible *Y. pseudotuberculosis* specific reads from a complex environment where it may contain many homologous transcripts. This methodology can easily be employed for other complex biological samples such as soil, fecal samples, seawater and others.

3.5 ArcA, Fnr, FrdA, and WrbA are Required for Persistent Infection

Based on the gene expression data, we selected 10 genes encoding proteins potentially important for persistent infections. These genes were then mutated, and the resulting strains were used to infect mice. The infection experiments showed that *arcA*, *fnr*, *frdA* (involved in anaerobic respiration), and *wrbA* (involved in oxidative stress) were required for establishment of persistent infection (Paper II, Figure 6A and B).

3.6 *Yersinia* Infections Alter Microbial Composition in Cecum

The mapping of RNA-seq library reads from tissue biopsies of FVB/N cecums that were uninfected or at day 2 p.i. or day 42 p.i. to the NCBI 16SMicrobial database revealed the presence of other bacterial species in the tissues. This finding was not surprising because the samples originated from intestinal tissues. Relative comparisons of mapped reads to the database showed increased numbers of bacterial species in infected tissues (5.2 fold higher for early infection and 3.7 fold higher for persistent infection) in comparison to un-infected tissues (Paper II, Figure 2B, Table S1). Moreover, the composition and abundance of bacterial phyla differed in infected tissues compared to un-infected tissue (which is similar to what has been reported for other bacteria [312,313] (Paper II, Figure 2C, Table S1). It can be speculated that the higher abundance of other bacteria in the infected cecal tissues is the result of migration of luminal bacteria to the deeper tissue. This migration would be expected to be facilitated by the tissue disruption observed by histochemistry.

3.7 RfaH is Required for Establishment of Infection

Because we found the expression of the transcriptional anti-terminator RfaH to be up-regulated during persistent infection (Paper II, Table S3), we next investigated the possible contribution of RfaH to adaptation of *Y. pseudotuberculosis* during persistent infection. We show that deletion of the *rfaH* gene leads to complete attenuation in virulence in the mouse infection model with low-dose bacterial load. We found that *rfaH* is induced under different stress conditions such as osmolarity, temperature, and bile stress, partly mimicking *in vivo* conditions. Phenotypic characterization assays showed bacterial surface disturbance in *rfaH* deletion mutants. Further investigations showed that these effects were mainly related to defective O-antigen biosynthesis, known to be positively regulated by RfaH. We used RNA-seq to compare the gene expression in wild-type and *rfaH* mutant strains at 26°C and after osmolarity, bile, and temperature stress. Here, we could confirm the importance of RfaH for expression of the genes in the operon encoding O-antigen biosynthesis proteins. In addition, many other genes were affected by the deletion of *rfaH*, of which some of them located within downstream *ops* regions. Those gene products are involved in different metabolic pathways such as ribosome biogenesis, motility, bacterial chemotaxis, DNA repair, and homologues recombination. Although the changes in expression in some of the genes likely are indirect effects of defective O-antigen, our results imply a more global effect than was suggested in a recent study in *Y. enterocolitica*. [237]. That study used both an *rfaH* mutant strain and an O-antigen negative strain and concluded that RfaH mainly regulates expression genes involved in O-antigen biosynthesis. However, this can be questioned as out of 102 differentially expressed genes in *rfaH* mutant strain only 22 genes had similar differential expression in the O-antigen negative strain. Hence, there were 80 additional differentially expressed genes in the *rfaH* mutant, and it can not be excluded that some of these are regulated directly by RfaH. In addition, in that study RNAs from both strains were extracted in different temperature. Our data suggest that several additional genes can be directly regulated by RfaH, suggesting a global role for RfaH on the regulation of transcription in *Y. pseudotuberculosis*.

4 Main Findings in This Thesis

- Infection of FVB/N mice with low dose of *Y. pseudotuberculosis* provides a suitable model system for studies of persistent infections.
- Persistent *Y. pseudotuberculosis* localizes in foci surrounded by PMNs in the dome area of cecal lymphoid aggregates where it replicates with consistent shedding to feces, implicating cecum as a reservoir.
- *Y. pseudotuberculosis* reprograms its transcriptome for persistence, involving repression of T3SS and induction of flagella and genes encoding proteins involved in anaerobiosis, chemotaxis, and protection against oxidative and acidic stress, indicating the influence of and adaptation to different environmental cues.
- *arcA*, *fnr*, *frdA*, and *wrbA* are important for establishment of persistent infection.
- RfaH is global transcriptional regulator in *Y. pseudotuberculosis* important for establishment of infection, and which in addition to regulating O-antigen biosynthesis genes, influences expression of many other genes .
- *Y. pseudotuberculosis* infection alters the bacterial composition in cecum.
- Complex RNA populations can be resolved with RNA-seq.

5 Future Perspectives

4.1 Search for Novel Targets for Antimicrobials

In our studies, we present the *Y. pseudotuberculosis* mouse model for persistent infections and provide information about genes expressed during different phases of infection (early and late infection phases). The expression levels of the different genes are expected to give hints about the need for and use of different pathways and functions during different stages of the infection. The expression profile of *Y. pseudotuberculosis* during persistent infection, in which the gene expression pattern resembled to a great extent the pattern seen *in vitro* at 26°C, was surprising; flagellar genes, for example, are never expressed at 37°C *in vitro*. This result demonstrates that bacterial *in vivo* gene expression patterns can be totally different from the pattern seen in laboratory conditions, underscoring the importance of *in vivo* studies. This important finding has significant implications for strategies for identifying potential bacterial targets for new antimicrobials. In light of this knowledge, one can, for example, question if classical virulence genes are suitable as targets for eliminating bacterial infections. Instead, genes that are important for infection maintenance should have higher potential as novel targets for antimicrobial drugs. Our *in vivo* transcriptomic profiling therefore provides a basis for further investigations. Another important aspect is that many of the genes that are important for maintenance of the infection in the host are expected to have similar roles in other bacterial species. In other words, candidate genes identified using the *Y. pseudotuberculosis* mouse model of persistent infections have a high probability of being suitable target genes for elimination of other bacteria. Here, it is of course important to consider genes specifically relevant to the maintenance of bacteria at the particular infection site and not genes critical for bacterial survival per se, to avoid elimination of the natural microbiota.

5.1 Switch for Reprogramming

The unusual expression profiles of T3SS components and flagellar genes was a surprising finding. This switch is potentially important for the long-term residence of *Y. pseudotuberculosis* in the host, where initial virulence is replaced by adaptation. The exact mechanism behind this switch is, however, unknown, and identification of the gene(s) involved in this novel regulation would be important for understanding the pathogenic strategies employed for invasion and survival in the host. It also is a putative target for treatment of bacterial persistent infections. A transposon library screen might be suitable for identifying this mechanism.

5.2 Possible Contribution of T6SS

We found that some genes such as *hcp* and *icmF* encoded in T6SS gene clusters in *Y. pseudotuberculosis* were up-regulated during persistent infection, suggesting a role for this secretion system and its secreted effectors (Paper II, Table S3). The presence of other bacteria in the infected tissue suggests possible bacterial interactions with other species, which is one function of T6SS [226]. To reveal a role for T6SS in interbacterial competition, deletion mutants of ClpV ATPase in each T6SS cluster of *Y. pseudotuberculosis* can be tested for growth competition with the bacterial species found in infected tissues during the early and persistent infection stages (Paper II). *Akkermansia muciniphila* is a perfect candidate, as it was found with high abundance in persistently infected tissues, and 75% of its transcriptome could be retrieved with RNA-seq. In addition to T6SS toxins, other possible T6SS effectors of importance for establishment and maintenance of persistent infection are small immunity proteins commonly encoded in T6SS clusters. These proteins also are examples of putative suitable targets for new antibiotics. It is possible that a drug that efficiently inhibits the neutralization effect of immunity proteins would lead to self-poisoning of the pathogen causing the persistent infection, which in turn could lead to clearance of the infection.

5.3 Adaptation of *Y. pseudotuberculosis* to Host Stress Conditions

The expression acid resistance chaperone HdeB was up-regulated during the persistent phase, suggesting a possible important role in establishment of persistent infection. Surprisingly, the deletion mutant of *hdeB* had a more aggressive profile than the wild-type strain, causing acute but not persistent infection. It can be hypothesized that this unexpected phenotype is the result of a failure in reprogramming of the transcriptome for the persistent infection. However, we found that the upstream region of *hdeB* contains a gene coding for a hypothetical gene (up-regulated during persistent infection) not annotated in the *Y. pseudotuberculosis* YPIII strain. Because the hypothetical gene is encoded on the opposite strand, the deletion mutant of *hdeB* may affect the expression of the hypothetical gene. Further investigation is required to reveal the roles of HdeB and the neighboring hypothetical protein in establishment and maintenance of persistent infection and their possible contributions to transcriptional reprogramming.

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