Infectious disease studies in zebrafish : the fish pathogen Edwardsiella tarda as a model system

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1 General introduction

Bacteria represent one of the most ancient life forms on this planet and have evolved into the most diverse kingdom in the phylogenetic tree of life. Their habitats range from icy conditions to hot springs, from nutrient-rich environments to situations of severe nutrient limitation, and from aerobe to anaerobe surroundings. In addition, many other characteristics, such as shape and growth rate, also differ extensively between bacterial species. Since many bacteria in general have the capacity for a high rate of replication, species are able to rapidly evolve and adjust to new conditions, making bacteria not only the most diverse, but also the most diversifying kingdom. In this abundance of bacterial species, it is not surprising that some are harmful to other organisms, for example to other bacteria, which might compete for nutrients, but also to multicellular organisms such as plants and animals. Although the majority of all bacterial species is assumed not to be harmful, there are many different pathogenic bacteria that are known to infect plants or animals. In the course of evolution, pathogens developed many specific properties, called virulence factors, which enhance their ability to survive in multicellular hosts. Examples of such virulence factors are toxins, capsules, flagella, and secreted proteins that manipulate host cellular processes to the advantage of the pathogen. Due to the harmful nature of pathogens, the host species developed specific defence mechanisms to counter infections. In turn, this caused new adjustments in the virulence factors of pathogens to avoid the host defence mechanisms. Consequently, intricate mechanisms of host-pathogen interaction have evolved, which are still poorly understood. Knowledge of these mechanisms is the basis for development of novel therapies to combat infectious diseases. In this thesis, we set up a model to study host-pathogen interactions using the zebrafish embryo as a model host and the bacterial species *Edwardsiella tarda*, a known fish pathogen, as the infectious agent.

Regulation of virulence by quorum sensing

An important characteristic of many bacteria is their ability to coordinate behaviour by quorum sensing mechanisms. Quorum sensing is a phenomenon that in pathogens is often involved in regulating virulence factors such as the secretion systems. Since the production of virulence factors costs energy and since these virulence factors are often not effective in low concentrations, it is only advantageous for a relatively high number of bacteria to produce them.

This requires communication between bacteria to measure their cell density, which involves the quorum sensing systems. By means of quorum sensing a single bacterial cell is able to determine the number of other bacteria in its surroundings through the accumulation of signalling molecules. There are several quorum sensing systems, of which the *luxI/R*-system is the best studied. First discovered in *Vibrio fischeri*, homologues of this system are found in many other Gram-negative bacteria [1-4]. In the *luxI/R* system, the protein LuxI produces acyl homoserine lactones (AHLs), also called autoinducers, which diffuse freely over the cell membranes into the environment. AHLs bind to the protein LuxR, which then binds to the operator region of the target genes. A low concentration of AHLs does not bind a sufficient amount of LuxR protein to induce the target genes. However, when the population of bacteria reaches a certain density, the concentration of AHLs reaches a threshold, where sufficient LuxR is bound to induce or repress its target genes. Among the target genes is *luxI*, enabling the bacteria to rapidly increase the concentration of AHLs and thereby strongly induce or repress other target genes [5, 6]. These targets include several virulence factors.

The contribution of secretion systems to bacterial virulence

In many cases, virulence factors need to be secreted to be able to exert their effect on the host. The mechanisms by which these virulence factors are secreted differ and dependent partly on whether the pathogen is Gram-positive or Gram-negative. Since many compounds can diffuse across the cell wall, but not across the cell membranes, it is obvious that differences in secretion mechanisms exist between Gram-positive bacteria, which have a single membrane, and Gram-negative bacteria, which have an inner and an outer membrane. Gram-positive bacteria use the Sec-pathway and the twin-arginine translocation (TAT) system to transport most proteins, containing typical signal sequences, across the cytoplasmic membrane [7, 8]. In addition, a secretion system for proteins without the classical signal sequences was recently discovered in Gram-positives, and named type VII secretion system (T7SS) [9]. Like Gram-positive bacteria, Gram-negative bacteria also use the Sec- and TAT-pathway to transport proteins across the cytoplasmic membrane, but several Sec-independent secretion systems exist as well. However, the proteins also need to be transported across the outer membrane and several secretion

systems are involved in this transportation step. So far, six secretion systems have been discovered in Gram-negative bacteria, called type I to VI secretion systems (T1SS – T6SS). The T2SS and T5SS are Sec-dependent, while T1SS, T3SS, T4SS and T6SS are Sec-independent [10-15]. The T3SS, T4SS and possibly the T6SS are able to pierce the host cell membrane and inject proteins directly into the host cytoplasm [10, 12, 16, 17].

The best studied secretion system is the T3SS, which was found to be an important contributor to virulence among many different species. Its basal structure is evolutionarily related to the bacterial flagellum. Rings of proteins form a tube spanning the inner and outer membrane and the periplasm. The flagellum is attached by a hook to this type of basal ring structure, while the T3SS has an extracellular needle which can penetrate the host cell membrane and deliver effector proteins directly into the cytosol [18, 19]. As the name suggests, the effector proteins are the active components with regard to altering host behaviour. The effector proteins from different pathogens have specific effects on the host, but some common features are present. *Pseudomonas*, *Salmonella*, *Shigella*, and *Yersinia* species all inject effectors into host cells that can change actin arrangement. However, where *Salmonella* and *Shigella* rearrange actin filaments to gain entry into host cells, *Pseudomonas* and *Yersinia* do this to prevent phagocytosis [20-23]. Another common feature is that many T3SS effectors function to alter the host innate immune response. In some cases, bacteria use T3SS effectors to promote inflammation, for example causing diarrhea, thereby facilitating the spreading of bacteria to new hosts [24]. In other cases, T3SS effectors inhibit innate immune signalling pathways and suppress inflammation in order to facilitate replication and longer term survival in the host [20, 22, 25].

Another secretion system which is an important contributor to virulence is the T6SS. It was first discovered as a set of genes involved in temperature-dependent protein secretion in the symbiont *Rhizobium*, and was subsequently found to be present in several animal pathogens such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Edwardsiella ictaluri* [26]. In *Edwardsiella tarda* it was recognized as a putative secretion system, and following studies in *V. cholerae* it was finally named T6SS [27, 28]. Although much less is known about the T6SS than about the T3SS, this secretion system is now being studied intensively and more knowledge is rapidly gained. Like the T3SS, the

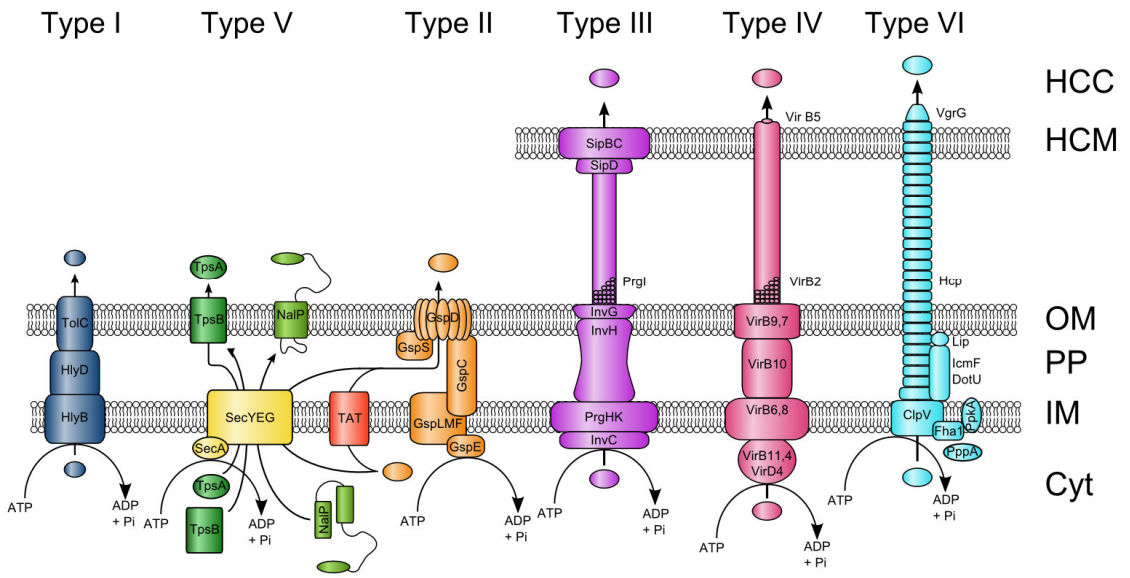


Figure 1. Secretion systems of Gram-negative bacteria. In Gram-negative bacteria six types of secretion systems have been identified, the type I – VI secretion systems (T1SS – T6SS). The T2SS and T5SS use the Sec- and/or Tat-dependent-pathway to transport substrates across the inner membrane (IM), similar to secretion over the single membrane of Gram-positive bacteria. The T2SS is a protein-complex anchored in both the inner and outer membrane that transports substrates from the periplasm across the outer membrane (OM). The T5SS include two-partner secretion systems and autotransporters. In two partner secretion systems the translocator protein transports the effector protein across the OM, whereas autotransporters are multi-domain proteins that insert themselves into the OM and transport the passenger domain across it. The T1SS is a simple transportation complex crossing both the IM and OM and secreting substrates in one step across both membranes. The T3SS, T4SS and T6SS are secretion systems that cross both the IM and OM and are also capable of penetrating the host cell membrane, enabling injection of effectors directly into the host cell cytoplasm. The structures of the T3SS and T4SS are well studied, while that of the T6SS is highly speculative.

Secretion systems exemplified by: T1SS, haemolysin secretion system in *Escherichia coli*; T2SS, the general secretion pathway (Gsp) system; T3SS, *Salmonella enterica* subsp. *enterica* serovar Typhimurium system; T4SS, *Agrobacterium tumefaciens* VirB/D system; T5SS, NalP from *Neisseria meningitides* (autotransporter) and tpsAB system; T6SS, hypothetical structure.

Abbreviations: Cyt - cytoplasm; IM - inner membrane; PP - periplasm; OM - outer membrane; HCM - host cell membrane; HCC - host cell cytoplasm.

Figure based on: Fronzes, Christie & Gabriel Waksman, Nature Reviews Microbiology, 2009; [138] M. Saier, Microbial Genetics, http://saier-144-51.ucsd.edu/~saier/bimm122/ (http://saier-144-51.ucsd.edu/~saier/bimm130/reading130/week9/Sec_Sys_Gram_nt.png); and KEGG pathway (www.genome.jp).

T6SS is thought to form a needle complex that punctures the host cell and to transport effectors directly into the host cell cytosol. However, whereas the T3SS is based on the structure of the bacterial flagellum, the T6SS is hypothesized to be similar to the cell-puncturing device of bacteriophages [16, 17, 29]. Direct evidence of both the structure of the T6SS and the delivery of effectors into the host cell is still lacking. Some of the genes that were initially thought to be secreted, such as Hcp and VgrG, are now thought to be part of the needle like structure [16].

Unravelling evolution of bacterial genomes and virulence factors

Since the T3SS and T6SS are widespread among the different pathogens, comparisons between the encoding DNA-sequences of different strains and species can give much information. The comparisons can reveal if the secretion systems and especially their effectors originate from horizontal gene transfer or from vertical descent with modifications [30]. In closely related species that have the same gene clusters, differences in amino acid sequences of proteins involved in virulence might determine different host specificities. In recent years, investigations into the evolution of bacterial genomes and virulence systems have been boosted by novel technologies for whole genome sequencing [31]. The increased speed and highly reduced price of whole genome sequencing, has made it possible not only to compare specific gene clusters of several strains or species, but also to compare complete bacterial genomes on a large scale. The number and type of single-nucleotide polymorphisms (SNPs) or insertions and deletions (indels) between large numbers of different strains may unravel the evolutionary history at high resolution and identify genomic regions with high selective pressure [31]. It can be predicted that virulence factors are subject to high selective pressure, for example positive selection (the generation and spread of new favourable alleles in the population) diversifying selection (the selective maintenance of multiple alleles in the population), or purifying selection (selection that acts to eliminate selectively deleterious mutations). Differences in pathogenicity or host specificity may be explained by studying which selective pressures act on different components of virulence factors [30].

Virulence factors of *Edwardsiella tarda*

Edwardsiella tarda is a Gram-negative, rod-shaped bacterium, able to cause infection in a wide range of animal hosts, such as birds, amphibians, reptiles and mammals, including humans [32, 33]. Its most common hosts, however, are fish, where it causes disease in many species like carp, tilapia, eel, catfish, mullet, salmon, trout and flounder. Not all strains of *E. tarda* are pathogenic. There are four different serotypes (A, B, C and D) and only one, serotype A, is highly virulent. It is also the most predominant serotype [34, 35]. Analysis of *E. tarda* strains isolated from different geographical locations shows that random amplified polymorphic DNA profiles differ between these locations [36]. This finding, combined with differences found in PCR-restriction fragment length polymorphism of 16S rDNA of *E. tarda* strains from different habitats [37], indicates that this bacterium is highly plastic and phenotypically polymorphic, and therefore capable of adapting to its broad range of hosts. In contrast, the close relative *Edwardsiella ictaluri*, has a more narrow host range, mainly restricted to catfish [38].

The disease caused by *E. tarda* is called edwardsiellosis, which can lead to mass mortality in fish populations. While the prevalence of the disease in ponds is seldom above 5%, in tanks it can reach 50% [39]. Fish are infected by waterborne contact with *E. tarda*, with multiple routes of entry: through the skin, gills or oral route [40]. Symptoms and gross signs include lesions on the skin, pale gills, tumefaction of the eye, excessive mucus secretion, scale erosion, distended abdomen, pigment loss, enlarged kidney, abscesses on internal organs and swelling and bleeding of the anus leading to reddening. In mild infections, small cutaneous lesions (3-5 mm in diameter) appear on the posterolateral parts of the fish [39, 41].

Much research into *E. tarda* has already been done and several virulence factors have been identified, although their precise functions are still being unravelled. To enter the host, the bacteria adhere to the host using the *fimA* gene. Once inside the host, *E. tarda* is able to resist complement activity and to survive within macrophages. The genes involved in macrophage survival are *sodB*, *katB*, *gadB*, *isor*, *astA*, *pstS*, *pstC*, *ompS2* and *ssrB* [42]. From these genes *sodB* (superoxide dismutase) and *katB* (catalase) have been studied in most detail, revealing that *sodB* differs between virulent and non-virulent strains and that *katB* is absent in non-virulent strains [43, 44].

The type III and type VI secretion systems are also present in *E. tarda* [42, 45-47]. The T3SS secretes EseB, EseC and EseD, which after secretion form a protein complex [48]. Knockout mutants of these proteins were unable to replicate in murine macrophages, indicating an essential role in intracellular replication [46]. The T6SS of *E. tarda* is one of the best studied secretion systems of this class. In one study, 16 known genes of this secretion system were knocked out separately. Among these genes, 13 were found to be essential for secretion. Three proteins are secreted, EvpC, EvpI and EvpP [47]. EvpC and EvpI, which are mutually dependent for secretion, are likely to be part of the bacteriophage injectisome-like needle [16, 47, 49]. The third secreted protein, EvpP, has so far only been found in *E. tarda*, and in addition in one *Aeromonas hydrophila* strain, which has been hypothesized to have obtained it by horizontal gene transfer [49]. EvpP is suggested to play a critical role in the cell invasion mechanism, since, compared to a wild type, an EvpP mutant of *E. tarda* was attenuated in infection of adult zebrafish and Japanese flounder [49]. In addition to these virulence factors, *E. tarda* possesses the classical quorum sensing systems homologous to the LuxI/R system and LuxS system [50, 51]. These systems regulate several virulence factors such as the T3SS and the flagellin protein FliC [51, 52].

The strong virulence of *E. tarda* in fish makes it an interesting pathogen for studies into host-pathogen interactions. However, its known common hosts are not convenient for studies in the laboratory. Therefore, the zebrafish was developed as a model host to study *E. tarda* pathogenicity [53].

Zebrafish as a model host organism

Originally applied as a model organism to study development, the zebrafish was subsequently recognized as a useful addition to mammalian models to study infectious diseases, cancer and many types of genetic disorders [54-57]. Many traits of the zebrafish make it a very practical vertebrate model organism. In comparison with rodent models, zebrafish care and maintenance is less costly. The adult fish are small (3 – 5 cm) and one pair of fish can lay several hundreds of eggs per week. The eggs are fertilized externally and the embryo remains transparent during the first days of development. For the study of host-pathogen interactions, the value of the zebrafish lies in the facts that *in vivo* imaging of infection is easily accomplished, advanced genetic tools and the genome

sequence are available, and the zebrafish and human immune systems are highly similar [55, 58-60].

Due to their transparency and small size, living zebrafish embryos are highly suitable to be studied with microscopic techniques. With the use of fluorescent markers, specific cell types of the immune system can be labelled, and their behaviour studied. In addition, fluorescently labelled bacteria can be injected and their spreading or proliferation followed in time. Several transgenic lines have been constructed with fluorescent markers under control of different leukocyte specific promoters. For example, in a transgenic line that labels early myeloid cells green, *gfp* was cloned under the control of the *spi1*-promoter [61-64]. In another transgenic line GFP is under the control of the neutrophil-specific *mpx*-promoter, labelling all neutrophils green [65, 66]. Macrophage-specific lines have also recently been reported [67, 68]These transgenic lines, especially in combination with fluorescently labelled pathogens, make *in vivo* imaging of host-pathogen interactions possible at a detail that is unmatched in mammalian models.

In addition to the possibility of constructing transgenic lines, the zebrafish is a powerful model for forward and reverse genetics approaches. Forward genetics, the induction of random mutations and subsequent screening for interesting phenotypes, can be performed at high throughput levels. Classical approaches such as retroviral- or transposon-mutagenesis are possible to introduce germline mutations in zebrafish, but the use of ethylnitrosurea (ENU) on male zebrafish is most common [69]. ENU introduces random point mutations in the genome that can be identified by positional cloning. In zebrafish, conventional targeted knockout techniques as used in mice are not available, but randomly generated mutants can also be used for reverse genetic screening. By sequencing a gene of interest in DNA pools from zebrafish mutant populations, zebrafish with mutant alleles in that gene can be identified. The high throughput sequencing methods make this approach, called TILLING (targeting induced local lesions in genomes), very effective [70]. At present, the use of zinc finger knock out technologies is under development to expand the toolbox for reverse genetics approaches.

A different approach towards reverse genetics is the use of morpholinos [71, 72]. Morpholinos are stable synthetic oligonucleotides that can bind to mRNA or pre-mRNA to prevent translation or splicing respectively. The

sequence of the morpholino determines which mRNAs are blocked, enabling gene specific targeting. Morpholinos injected in the yolk of 1-2 cell stage zebrafish embryos can lead to transient gene knockdown for up to the larval stage, depending on the dose and the sequence of the morpholino. The advantage of morpholinos is that for example in the case of genes involved in both embryo development and immunity, the knockdown effect can be titrated in order to avoid developmental phenotypes, so that the immune function can be studied under these partial knockdown conditions. Disadvantages of the morpholino approach are possible non-specific effects that need to be properly controlled for, and the transient nature of the technique.

The immune system of zebrafish

Zebrafish, like all jawed vertebrates, possess an innate and an adaptive immune system [59, 60, 73, 74]. Most of the cell types active in the mammalian immune system have been identified in the zebrafish or in other teleost fish [55, 73-75]. Both in mammals and in zebrafish, these cell types develop in distinct waves of primitive and definitive hematopoiesis [76-79]. Although the sites of hematopoiesis differ between mammals and zebrafish, the cellular and regulatory processes are highly conserved [75, 77, 78]. However, a useful feature of the zebrafish embryo model is that the innate and adaptive branches of the immune system are temporally separated. In the developing embryo, active macrophages are already present at 1 day post fertilization (dpf) and differentiated neutrophils at 2 dpf [80-82]. In contrast, immature T-cell precursors appear only by 4 dpf and the adaptive immune system is not fully active until 2 - 4 weeks post fertilization [79, 83, 84]. This separation makes it possible to study functions of the innate immune system without interference of the adaptive immune system. The innate immune system makes use of cellular and soluble components. Macrophages and neutrophils are able to phagocytose and kill invading microbes. Macrophages are also able to phagocytose cellular debris and apoptotic cell corpses. Neutrophils are able to release antimicrobials and proteases, enabling extracellular killing of microbes. Examples of soluble components of the innate immune system are acute phase proteins such as complement and antimicrobial peptides. Acute phase proteins are proteins with pro- and anti-inflammatory effects. Some, like the complement components, can bind to microbes either marking them for phagocytosis or

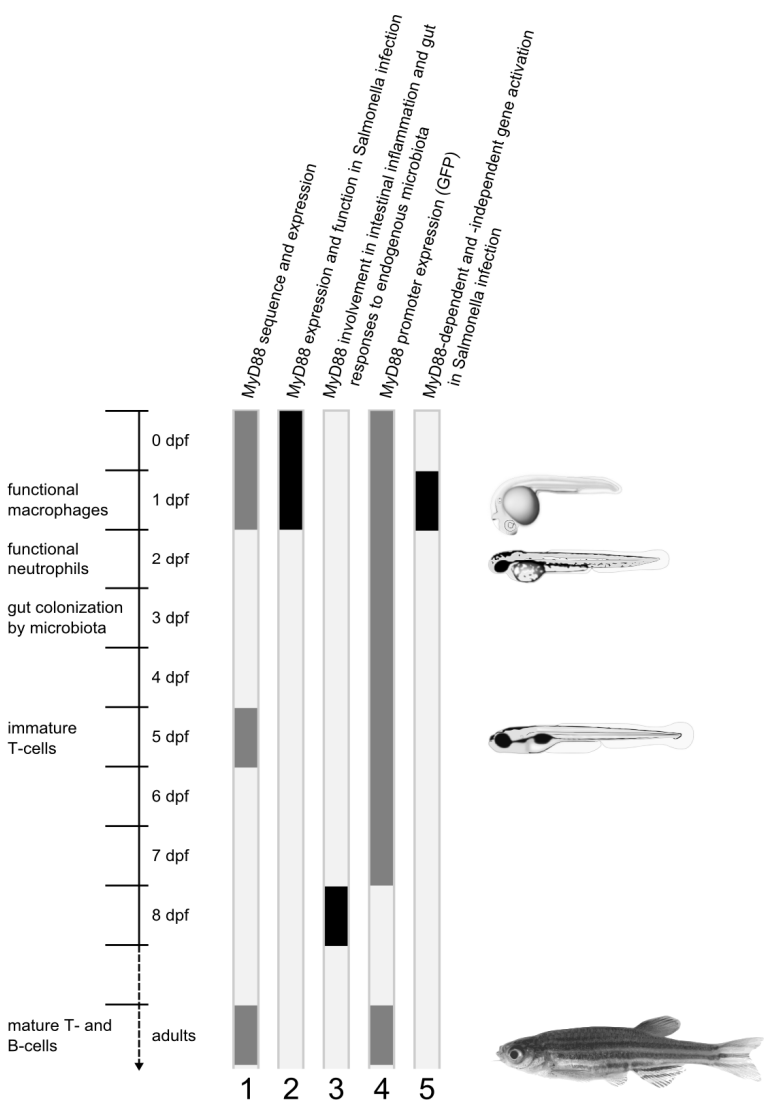
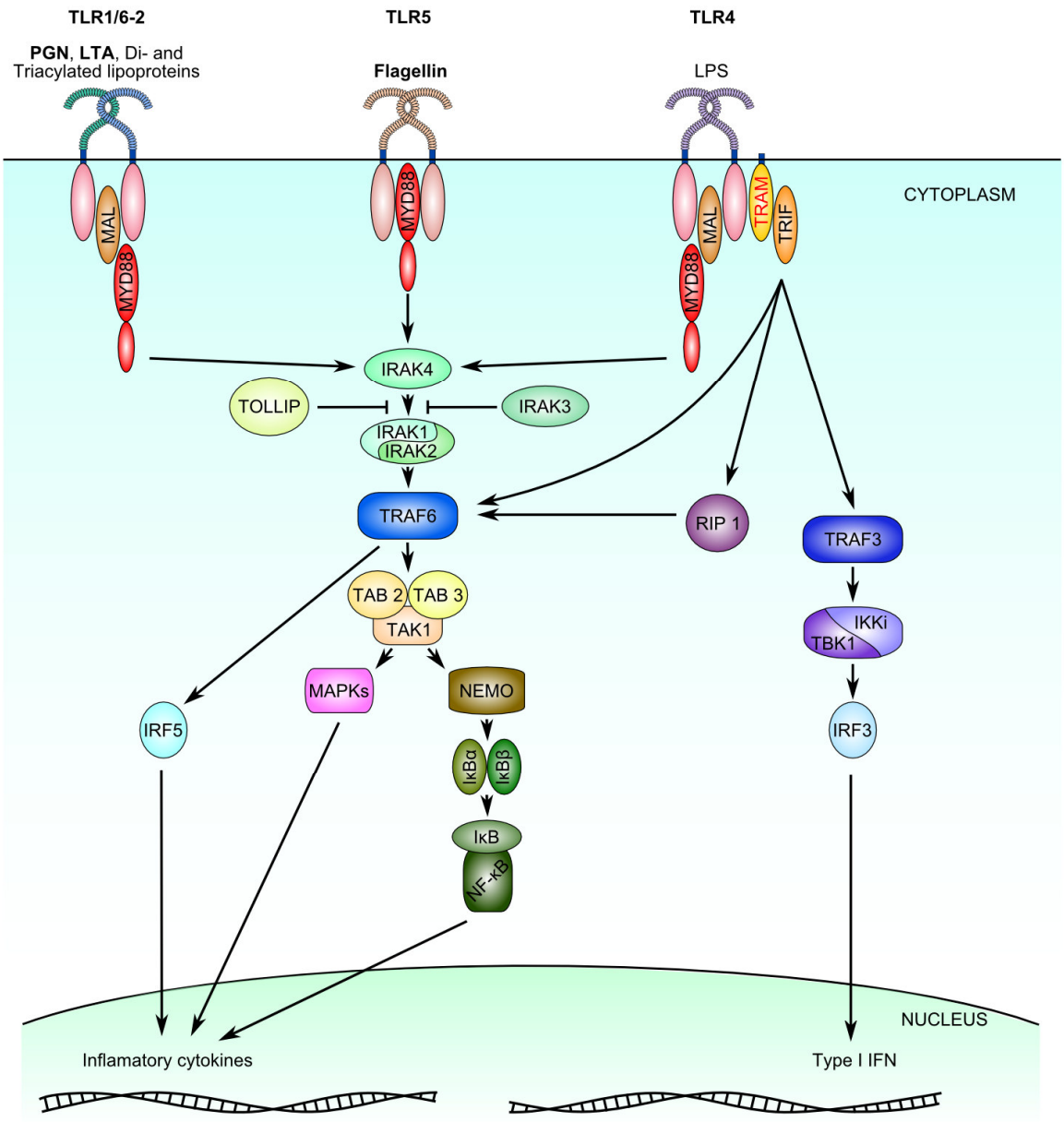


Figure 2. Schematic overview of the development of the zebrafish immune system and previous research into zebrafish Myd88. At 1 dpf two populations of innate immune cells can already be distinguished by expression of specific macrophage and neutrophil marker genes and both populations are capable of migrating to infection sites [64]. However, based on the presence of neutrophilic granules and myeloperoxidase enzyme activity, functional neutrophils appear only by 2 dpf [134]. At 3 dpf the mouth of the embryo opens and microbiota start colonizing the gut [122]. T-cell precursors arise at 4-5 dpf and adult zebrafish possess a fully mature adaptive immune system with T- and B-cell responses [55]. Mammalian MYD88 is known as the essential adaptor molecule for most of the Toll-like receptors and the IL-1 receptor. Research into Myd88 in the zebrafish model started with its simultaneous discovery and initial expression analysis by two different groups (column 1) [91, 92]. Morpholino knockdown was used to study the function of Myd88 in Salmonella infection (columns 2 and 5) [96, 115] and in responses of the gut to the endogenous microbiota (column 3) [114, 135, 136]. Loss of Myd88 function impaired the ability of embryos to clear an otherwise non-pathogenic Salmonella LPS-mutant infection [115]. Induction of inflammatory genes such as *il1b* and *mmp9* was shown to require Myd88-dependent signalling, while the interferon response was Myd88-independent similar as in mammalian models [96, 137]. Zebrafish larvae deficient in the LPS-detoxifying enzyme intestinal alkaline phosphatase were shown to display Myd88-dependent hypersensitivity to LPS and excessive neutrophil infiltration of the gut mucosa [114]. In addition, Myd88 signalling in zebrafish larvae was shown to be required for TNBS-induced intestinal inflammation [136] and for microbial-dependent intestinal epithelial cell proliferation [135] To analyse expression of the *myd88* gene in different tissues and immune cell types, GFP was coupled to the *myd88* promoter (column 4) [98]. The bars on the right-hand side of the figure indicate at which stage of zebrafish development the studies were performed. Dark grey bars refer to studies with wild type zebrafish and black bars to morpholino knockdown studies. Zebrafish embryo and larva drawings are courtesy of Oliver Stockhammer.

killing them directly by forming pores in the microbial membrane.

In order to raise a response to invading microbes, they must be recognized as such. Most microbes have features that differ substantially from host cells. Since microbes such as bacteria rapidly evolve, the features used for recognition by the innate immune system are often essential and therefore less prone to changes. These features are referred to as microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). Gram-negative bacteria for example, have LPS on their outer membrane and Gram-positive bacteria have their cell wall of peptidoglycan exposed to the host. These MAMPs or PAMPs are recognized by several different pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), the RIG-I-like receptors (RLRs), the scavenger receptors and lectins [85]. Different PRRs are located on the cell membrane, in endosomes, and in the cytosol of cells of the innate immune system. The best studied PRRs are the TLRs. TLRs are named due to their high homology to the *Drosophila* Toll receptor, which was discovered in 1985 as a receptor involved in determining dorsal-ventral polarity [86]. Later it was found to be involved in the anti-fungal immune response of *Drosophila* [87]. Soon after, TLR4 was discovered in mammals and shown to be involved in the response to LPS [88]. TLRs are germline-encoded type I transmembrane receptors, spanning either the cell membrane or an endosomal membrane. The TLRs consist of a leucine-rich repeat (LRR) domain and a Toll/Interleukin-1 receptor (TIR) domain. The LRR domain is the extracellular domain involved in ligand recognition and highly varies between different TLRs. The TIR domain is more conserved and is the intracellular domain responsible for signal transduction. Interestingly, some bacterial species, including *Escherichia coli*, *Brucella melitensis*, and *E. tarda*, also contain TIR-domain containing proteins, which they may use to interfere with the host TLR-signalling response [89, 90].

In humans, 10 TLRs have been found, each of which recognizes different ligands. Zebrafish have several homologues to the human TLRs, but in addition have some fish specific TLRs [91, 92]. For some human TLRs, there are two homologues in the zebrafish, like *tlr4a/b* and *tlr5a/b*, probably due to a duplication event. It is not yet known whether these duplicated TLRs differ in ligand specificity. Conserved ligand specificity has been shown between some mammalian and zebrafish TLRs. Flagellin is recognized by TLR5 in both



mammals and zebrafish and there are indications of viral RNA recognition by Tlr3 in zebrafish, like in mammals [93-96]. There are however also differences in ligand specificity between homologous TLRs in zebrafish and mammals. LPS, which is recognized by TLR4 in mammals, fails to activate the homologues Tlr4a/b in zebrafish [95, 97]. Consistent with this finding is the absence of co-receptors CD14 and MD2 in zebrafish, which are necessary for LPS recognition in mammals. Upon ligand binding, TLRs activate a signal transduction pathway consisting of several adaptors and other signalling proteins. The TLR adaptor proteins Myd88, Mal/Tirap, Trif/Ticam1 and Sarm are

Figure 3. Signalling by cell-surface TLRs. Mammalian cell surface TLRs can be activated by bacterial compounds such as PGN, LTA, di- and triacylated lipoproteins (TLR2 heterodimers with TLR1 or 6), flagellin (TLR5), and LPS (TLR4). Other members of the mammalian TLR family (TLR3/7/8/9) that are involved in recognition of nucleic acids are located on endosomal compartments (not shown). Upon ligand binding TLR1 and 6 form heterodimers with TLR2, while TLR4 and TLR5 form homodimers. These hetero- or homodimeric TLRs all signal through MYD88, either directly (TLR5) or via MAL, also known as TIRAP (TLR1/6-2 heterodimers and TLR4). MYD88 recruits IRAK4, which recruits IRAK1 and IRAK2 that subsequently activate TRAF6. TRAF6 interacts with IRF5 and with the TAB2, TAB3, TAK1 complex, which activates MAPK signalling and the IKK complex, consisting of NEMO and IKKαβ. This leads to phosphorylation and subsequent degradation of the IκB inhibitor, enabling transcription factor NFκB to translocate to the nucleus. The IRF5, MAPK and NFκB pathways all induce the expression of pro-inflammatory cytokine genes. The activation of IRAK1/2 can be inhibited by TOLLIP and IRAK3. In addition to the MYD88/MAL-dependent pathway, TLR4 also signals through a MYD88-independent pathway that uses TRIF (also known as TICAM1) and TRAM (also known as TICAM2). TRIF recruits TRAF6, either directly or through RIP1, and TRAF3. Signalling through TRAF6 continues as described for the MYD88-dependent pathway, with exception of the interaction with IRF5. TRAF3 activates TBK1 and IKKι, which in turn activate IRF3, inducing Type I interferons.
With the exception of TLR6, the mammalian cell surface TLRs are well conserved in zebrafish. As in mammals, zebrafish TLR5 has been shown to respond to flagellin [96], but zebrafish TLR4 does not appear to recognize LPS [95, 97]. The TLR2 protein of carp, a close relative of zebrafish, has been shown to respond to LTA and PGN [139]. The adaptor proteins Myd88, Mal and Trif are also conserved in zebrafish, but the zebrafish genome does not contain a homologue of TRAM, which is therefore shown in red in the figure. All other signalling intermediates shown in the figure are also present in zebrafish, but it remains to be determined to what extent the signalling pathways are similar. Ligands in bold have been shown to be recognized by the same TLRs in fish as in mammals.
Figure based on Kawai & Akira, Nature Immunology, 2010 [140].

all expressed in zebrafish embryonic leukocytes [98], but in contrast to the TLRs, only single copies of each adaptor protein are present in the genome. A fifth adaptor in mammals, Tram/Ticam2 has so far not been found in fish [92, 99, 100]. The TLR signal transduction pathways in zebrafish are not yet fully known. In mammals TLR3 is the only TLR that does not signal through myeloid differentiation factor 88 (MYD88), but instead utilizes TRIF. Similarly, the involvement of TRIF in zebrafish TLR3 signalling has been demonstrated, although there are also differences between the mammalian and zebrafish viral innate immune responses [100]. For all other TLRs in mammals, MYD88 is required for downstream signalling either by direct interaction with the TLR (TLR5, -7, -8 and -9), or by interaction with MAL, which in turn interacts with the

TLRs (TLR2 in heterodimers with TLR1 and -6). TLR4 also has direct interaction with MYD88, but it also has a MYD88-independent signalling pathway, making use of the adapter proteins TRAM (absent in zebrafish) and TRIF, leading to the production of type I interferons [101]. SARM is suggested to be a negative regulator of TRIF, but it has also been shown to be a positive regulator of the viral response in brain cells [102, 103].

Since Myd88 is used by all TLRs except for TLR3, it is one of the key adaptors in the TLR signalling pathway. In addition, it has been found to be an adaptor downstream of the interleukin-1 receptor (IL-1R) and has been associated with IFN-γ signalling [104, 105]. The Myd88 protein consists of two signalling domains, a TIR domain that interacts with the TIR-domain of the TLRs and IL-1R, and a death domain that interacts with the downstream IL-1 receptor associated kinases (IRAKs). This signalling leads to the production of pro-inflammatory cytokines and proteins involved in anti-microbial defence [106-108]. The key function of Myd88 in innate immune signalling was shown in mice deficient in this adaptor. In wild type mice LPS stimulation results in endotoxic shock, but the Myd88-deficient mice were hyporesponsive after stimulation. Not only LPS, the ligand for TLR4, but also the ligands for TLR2, -5, -7 and -9 failed to induce a response [104, 109]. It is therefore not surprising that Myd88-deficient mice were more susceptible to several pathogens [110-113]. In the zebrafish, Myd88 knockdown led to susceptibility towards an otherwise harmless *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) LPS-mutant and a lack of response towards LPS, similar as in mammals [114, 115]. Furthermore, it was shown that the zebrafish also has a Myd88-dependent and -independent pathway, inducing pro-inflammatory cytokines and type I interferons respectively [96]. In addition to this seemingly conserved pathway, there are fish specific pathways as well, like the Trif-dependent pathway that activates interferon in an IRF3/7-independent way and without interaction with Traf6 [100].

Zebrafish infection models

Recent years have seen an explosion of zebrafish infection models, especially with bacteria as the pathogenic invader [54, 56, 57, 116-119]. Both the bacterial virulence factors and the zebrafish immune constituents, in embryos as well as adults, have been the subject of interest in these models. Some examples of

human pathogens that are studied in the zebrafish are the Gram-negative bacteria *Pseudomonas aeruginosa* and *Salmonella enterica* and the Gram-positive *Streptococcus pyogenes* [96, 120-123]. *P. aeruginosa* is known as a broad host range pathogen, capable of infecting both plants and animals, and in humans is a primary cause of concern in cystic fibrosis. In the zebrafish, the role of the T3SS, quorum sensing and other virulence factors in the pathogenicity of *P. aeruginosa* have been studied, as well as the role of the early myeloid cells in battling the infection [120, 121]. Injection of the wild type *P. aeruginosa* strain PAO1 caused lethal infection in zebrafish embryos. Strains with mutations in the T3SS or the LasR quorum sensing system were attenuated in 50 hpf zebrafish embryos, but not in 28 hpf zebrafish embryos [120, 121]. Additionally, morpholino-induced manipulation of the number of phagocytic cells present in the zebrafish embryo showed that the T3SS of *P. aeruginosa* protects the bacterium from phagocytes [120]. *P. aeruginosa* strain PAO1 has also been used to study the interactions of gut microbiota with the zebrafish embryo in a gnotobiotic system [122]. Interestingly, the zebrafish readily ingested the bacteria when exposed by static immersion, but no infection occurred. The immune response towards PAO1 in the gut was similar to that in the mouse upon establishment of the gut microbiota [122]. *S. typhimurium* is often ingested with contaminated food and causes symptoms such as vomiting and diarrhea. In the zebrafish, it was used to study the innate immune response during systemic infection in the blood, in which it was shown to activate both the Myd88-dependent and -independent pathways [96]. One of several studies using *S. pyogenes* as the infecting agent compared *S. pyogenes* in an *in vitro* biofilm with *S. pyogenes* in zebrafish tissue using transcriptional profiling. This study showed that in *in vitro* biofilm *S. pyogenes* expresses only a small portion of the virulence factors that it expresses in live tissue [123].

The most extensively studied bacterial pathogen in zebrafish is *Mycobacterium marinum*. This bacterium is a natural fish pathogen, which can also cause mild skin infections in humans. What makes it interesting is the fact that its pathogenic hallmarks are similar to those of the human pathogen *Mycobacterium tuberculosis*, the causative agent of tuberculosis [124]. *M. marinum* induces the formation of granulomatous aggregates of infected and non-infected immune cells in zebrafish that highly resemble the granulomas formed during *M. tuberculosis* infection in humans [124, 125]. Studying this

pathogen in zebrafish has already led to new insights in the human disease. For example, through its newly discovered T7SS *M. marinum* secretes ESAT-6, a virulence factor that, when the bacterium is phagocytosed, stimulates nearby epithelial cells to express *mmp9* [126]. This *mmp9* induction was shown to be required for recruitment of macrophages into granulomas. Whereas granuloma formation was previously thought to function solely as a host-protective mechanism, studies in zebrafish demonstrated that mycobacteria actively exploit the early stages of granuloma formation for their expansion and dissemination in the host [127].

Many more examples of bacteria used to study host-pathogen interactions in zebrafish can be given, but most share at least one characteristic, the need to be injected to cause infection. Injection in adults is mostly done intraperitoneally or intramuscularly, while the most common injection sites used in embryos are the posterior blood island at 1 dpf and the Duct of Cuvier at 2-3 dpf [119]. Injection in the yolk of embryos is also possible, but only for slow growing bacteria, such as M. marinum [128], since fast growing bacteria rapidly expand in the nutrient-rich environment of the yolk, thereby causing early lethality of the embryos [82, 129]. For high throughput screening injection is a bottleneck, since automated injection is so far impossible for adults and only possible for yolk injections in the embryo [128]. It would be useful to identify pathogenic bacteria that are able to infect the zebrafish by static immersion only. Though some bacteria are able to infect the zebrafish through the oral route or after dermal abrasion [53, 54, 122, 130-132], only two bacterial species have been reported to infect 1 day old embryos by external exposure [53, 133]. It was found that static immersion of 1 dpf embryos in a suspension of *E. tarda* for 5 hours was enough to cause a cumulative lethality of 31% after 14 days, compared to 11% in control embryos. In addition, immune response markers *tnf* and *il1b* were found to be up-regulated in the zebrafish at 2 and 4-8 hpi (hours post infection) respectively [53]. In a later study also *Flavobacterium columnare* was successfully used to infect 1 dpf embryos by static immersion [133]. Like *E. tarda*, *F. columnare* is a highly contagious natural fish pathogen, causing disease in both wild and cultured fish populations. These bacteria might prove useful for high throughput mutant and drug screening.

Outline of this thesis

At the beginning of this research project, *E. tarda* was the only pathogen found to be able to lethally infect one day old zebrafish embryos by static immersion. Since static immersion would allow high throughput screening, we chose to work with this bacterium. In this thesis, we used *E. tarda* to establish a basis for comparison of virulence between different strains and for studying the contribution of different virulence factors. In addition, we used it to study the function of the embryonic innate immune system in the response to *E. tarda* infection.

In **Chapter 2** we determined the genome sequence of *E. tarda* FL6-60 with deep sequencing. We compared our genome sequence with that of the recently sequenced *E. tarda* EIB202 and *E. ictaluri* 93-146 and showed that single nucleotide polymorphisms can be identified with 50 nucleotide reads. We also compared in detail the T3SS and T6SS of *E. tarda* strain FL6-60 with the T3SSs and T6SSs of other *E. tarda* strains and an *Edwardsiella ictaluri* strain of which these secretion systems had been sequenced, where we showed a high number of polymorphisms that might cause differences in virulence.

Chapter 3 focuses on the method of infection. We set out to test the ability of *E. tarda* to establish a reproducible lethal infection in zebrafish by immersing the embryos in a suspension of this pathogen. In addition, we tested whether a similar system for *P. aeruginosa* was feasible. Comparison of mortality rates and an analysis of markers for the innate immune response at single embryo level demonstrated that caudal vein injection of bacteria led to more reproducible infections than the immersion method. Transcriptome profiling of embryos infected by the injection method showed a strong induction of inflammatory and defence genes, while only few immune-related genes were induced in embryos subjected to *E. tarda* immersion. Based on our microarray studies we distinguished markers that most likely result from epithelial responses in the immersion system and markers that are only induced upon systemic infection.

Finally in **Chapter 4** we studied the role of the embryonic host immune system to *E. tarda* infection. To this extent we used a zebrafish knockout mutant in the

myd88 gene, which codes for an essential adaptor protein in Toll-like receptor signalling. This is the first zebrafish knockout mutant of a central innate immune signalling component. The results of microarray expression profiling show that *myd88* plays a crucial role in the induction of many immune response genes, for example encoding cytokines and chemokines, and in the induction of transcriptional regulators of the immune response. The study shows the advantages of knockout mutant analysis over morpholino knockdown technologies.

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