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

Soest, J.J. van

Citation
Soest, J. J. van. (2011, November 29). *Infectious disease studies in zebrafish : the fish pathogen Edwardsiella tarda as a model system*. Retrieved from https://hdl.handle.net/1887/18149

Version: Not Applicable (or Unknown)
License: Leiden University Non-exclusive license
Downloaded from: https://hdl.handle.net/1887/18149

Note: To cite this publication please use the final published version (if applicable).

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

Joost J. van Soest

Cover: Scanning electron microscopy image by Gerda Lamers of *Edwardsiella tarda* bacteria on the skin of a zebrafish embryo

Printed by: Ridderprint, Ridderkerk, The Netherlands

ISBN: 978-90-5335-488-9

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

Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 29 november 2011
klokke 13.45 uur

Door

Joost. J van Soest

Geboren te ’s-Gravenhage, Nederland
in 1983

Promotiecommissie:

Promotor:	Prof. dr. H.P. Spaink
	Prof. dr. C.A.M.J.J. van den Hondel
Co-Promotor:	Dr. A.H. Meijer
Overige leden:	Prof. dr. C.J. ten Cate
	Dr. G.V. Bloemberg (Universität Zürich, Switzerland)
	Dr. A.C. Vergunst (INSERM, France)

The printing of this thesis was sponsored by ZF-screens B.V.

“Always look on the bright side of life”

Eric Idle, Monty Python

Voor mijn ouders

Contents

Chapter 1	General introduction	9
Chapter 2	Genome comparisons of *Edwardsiella bacteria* analysed using deep sequencing technology	37
Chapter 3	Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen *Edwardsiella tarda*	57
Chapter 4	Expression profiling of the response to *Edwardsiella tarda* infection in a zebrafish myd88 knockout mutant	87
Chapter 5	Summary and discussion	117
Samenvatting		131
Curriculum vitae		147
Publications		149

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

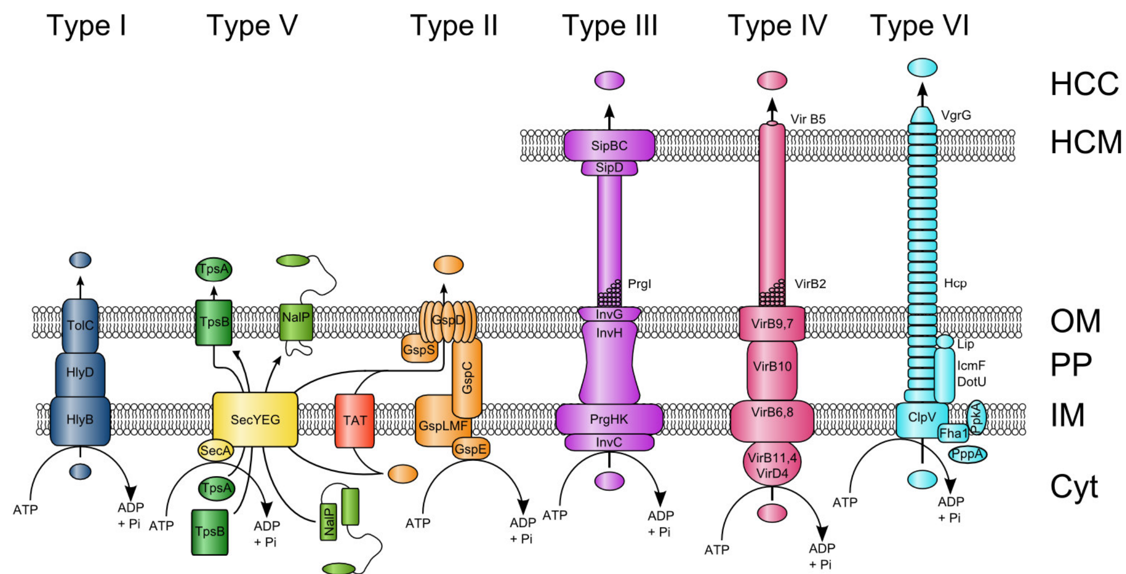


Figure 1. Secretion systems of Gram-negative bacteria. In Gram-negative bacteria six types of secretion systems have been identified, the type I – VI secretion systems (T1SS – T6SS). The T2SS and T5SS use the Sec- and/or Tat-dependent-pathway to transport substrates across the inner membrane (IM), similar 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

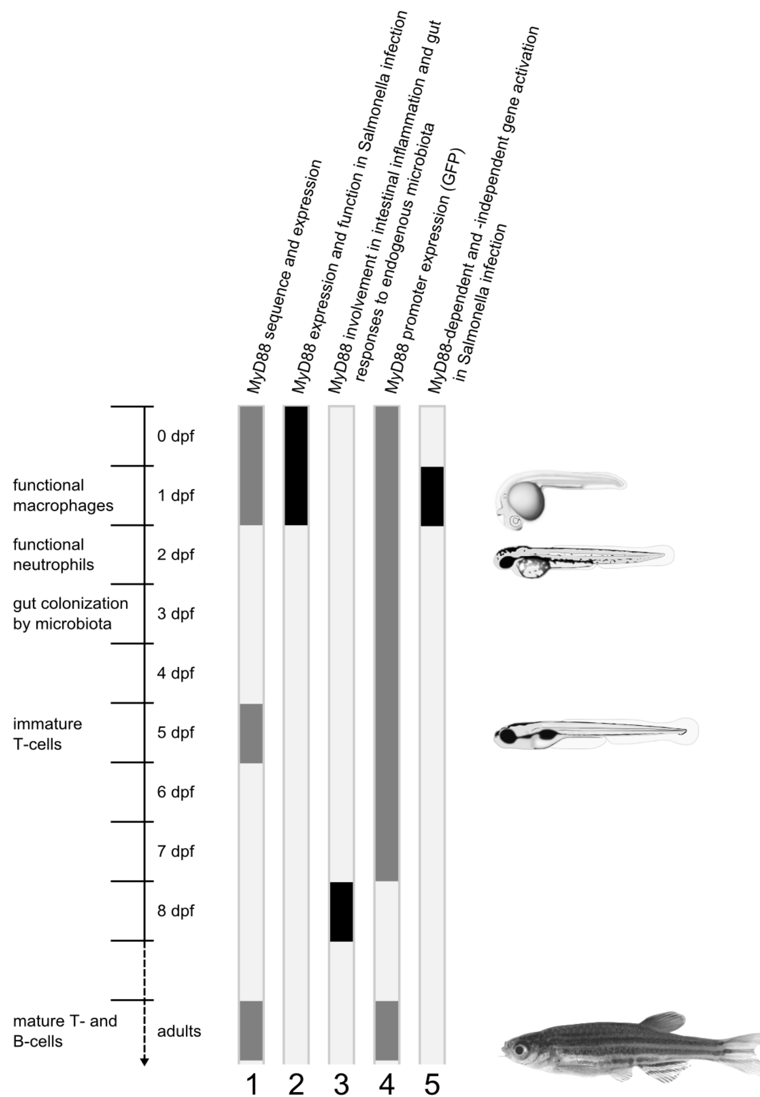
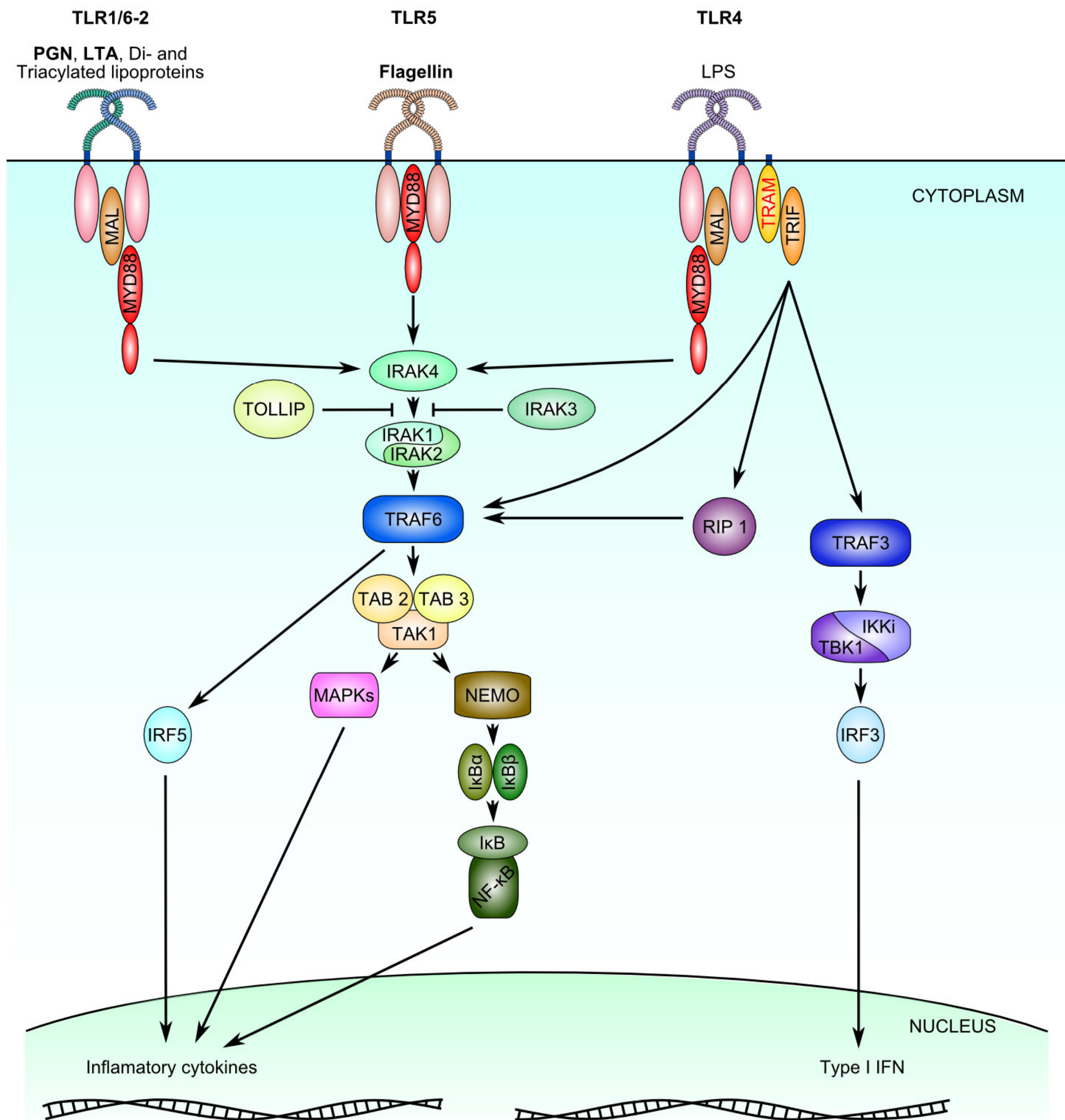


Figure 2. Schematic overview of the development of the zebrafish immune system and previous research into zebrafish Myd88. At 1 dpf two populations of innate immune cells can already be distinguished by expression of specific macrophage and neutrophil marker genes and both populations are capable of 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.

References

1. Nealson, K.H., T. Platt, and J.W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J Bacteriol 104:313-322.
2. Davies, D.G., M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, and E.P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-298.
3. de Kievit, T.R. and B.H. Iglewski. 2000. Bacterial quorum sensing in pathogenic relationships. Infect Immun 68:4839-4849.
4. Parsek, M.R. and E.P. Greenberg. 2000. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signalling mechanism involved in associations with higher organisms. Proc Natl Acad Sci USA 97:8789-8793.
5. Engebrecht, J., K. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fischeri*. Cell 32:773-781.
6. Engebrecht, J. and M. Silverman. 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc Natl Acad Sci USA 81:4154-4158.
7. de Keyzer, J., C. van der Does, and A.J.M. Driessen. 2003. The bacterial translocase: a dynamic protein channel complex. Cell Mol Life Sci 60:2034-2052.
8. Palmer, T., F. Sargent, and B.C. Berks. 2005. Export of complex cofactor-containing proteins by the bacterial Tat pathway. Trends Microbiol 13:175-180.
9. Abdallah, A.M., N.C. Gey van Pittius, P.A. Champion, J. Cox, J. Luirink, C.M. Vandenbroucke-Grauls, B.J. Appelmelk, and W. Bitter. 2007. Type VII secretion - mycobacteria show the way. Nat Rev Micro 5:883-891.
10. Alvarez-Martinez, C.E. and P.J. Christie. 2009. Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev 73:775-808.
11. Bönemann, G., A. Pietrosiuk, and A. Mogk. 2010. Tubules and donuts: a type VI secretion story. Mol Microbiol 76:815-821.
12. Cornelis, G.R. and F. Van Gijsegem. 2003. Assembly and function of type III secretory systems. Annu Rev Microbiol 54:735-774.
13. Henderson, I.R., F. Navarro-Garcia, M. Desvaux, R.C. Fernandez, and D. Ala'Aldeen. 2004. Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68:692-744.
14. Omori, K. and A. Idei. 2003. Gram-negative bacterial atp-binding cassette protein exporter family and diverse secretory proteins. J Biosci Bioeng 95:1-12.
15. Voulhoux, R., G. Ball, B. Ize, M.L. Vasil, A. Lazdunski, L.F. Wu, and A. Filloux. 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J 20:6735-6741.
16. Leiman, P.G., M. Basler, U.A. Ramagopal, J.B. Bonanno, J.M. Sauder, S. Pukatzi, S.K. Burley, S.C. Almo, and J.J. Mekalanos. 2009. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. Proc Natl Acad Sci USA 106:4154-4159.
17. Pukatzki, S., A.T. Ma, D. Sturtevant, and J.J. Mekalanos. 2007. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proc Natl Acad Sci USA 104:15508-15513.
18. Enninga, J. and I. Rosenshine. 2009. Imaging the assembly, structure and activity of type III secretion systems. Cell Microbiol 11:1462-1470.
19. Galan, J.E. and H. Wolf-Watz. 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature 444:567-573.
20. Engel, J. and P. Balachandran. 2009. Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr Opin Microbiol 12:61-66.
21. Hayward, R.D. and V. Koronakiss. 2002. Direct modulation of the host cell cytoskeleton by *Salmonella* actin-binding proteins. Trends Cell Biol 12:15-20.
22. Matsumoto, H. and G.M. Young. 2009. Translocated effectors of *Yersinia*. Curr Opin Microbiol 12:94-100.
23. Parsot, C. 2009. *Shigella* type III secretion effectors: how, where, when, for what purposes? Curr Opin Microbiol 12:110-116.
24. Layton, A.N. and E.E. Galyov. 2007. *Salmonella*-induced enteritis: molecular pathogenesis and therapeutic implications. Expert Rev Mol Med 9:1-17.
25. McGhie, E.J., L.C Brawn, P.J. Hume, D. Humphreys, and V. Koronakis. 2009. *Salmonella* takes control: effector-driven manipulation of the host. Curr Opin Microbiol 12:117-124.
26. Bladergroen, M.R., K. Badelt, and H.P. Spaink. 2003. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. Mol Plant Microbe Interact 16:53-64.
27. Pukatzki, S., A.T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W.C. Nelson, J.F. Heidelberg, and J.J. Mekalanos. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci USA 103:1528-1533.
28. Srinivasa Rao, P.S., Y. Yamada, Y.P. Tan, and K.Y. Leung. 2004. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. Mol Microbiol 53:573-586.

29. Pell, L.G., V. Kanelis, L.W. Donaldson, P.L. Howell, and A.R. Davidson. 2009. The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. Proc Natl Acad Sci USA 106:4160-5.
30. Stavrinides, J., H.C. McCann, and D.S. Guttman. 2008. Host-pathogen interplay and the evolution of bacterial effectors. Cell Microbiol 10:285-292.
31. MacLean, D., J.D.G. Jones, and D.J. Studholme. 2009. Application of 'next-generation' sequencing technologies to microbial genetics. Nat Rev Micro 7:287-296.
32. Janda, J.M. and S.L. Abbott. 1993. Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. Clin Infect Dis 17:742-8.
33. Kourany, M., M.A. Vasquez, and R. Saenz. 1977 Edwardsiellosis in man and animals in Panama: clinical and epidemiological characteristics. Am J Trop Med Hyg 26:1183-1190.
34. Park, S., H. Wakabayashi, and Y. Watanabe. 1983. Serotype and virulence of *Edwardsiella tarda* isolated from eel and their environment. Fish Pathol 18:85-89.
35. Rashid, M.M., K. Honda, T. Nakai, and K. Muroga. 1994. An ecological study of *Edwardsiella tarda* in flounder farms. Fish Pathol 29:221-227.
36. Nucci, C., W.D. da Silveira, S. da Silva Corrêa, G. Nakazato, S.Y. Bando, M.A. Ribeiro, and A.F. Pestana de Castro. 2002. Microbiological comparative study of isolates of *Edwardsiella tarda* isolated in different countries from fish and humans. Vet Microbiol 89:29-39.
37. Acharya, M., N.K. Maiti, S. Mohanty, and M. Samanta. 2007. Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system. Comp Immunol Microbiol Infect Dis 30:33-40.
38. Panangala, V.S., C.A. Shoemaker, S.T. McNulty, C.R. Arias, and P.H. Klesius. 2006. Intra- and interspecific phenotypic characteristics of fish-pathogenic *Edwardsiella ictaluri* and *E. tarda*. Aquacult Res 37:49-60.
39. Meyer, F.P. and G.L. Bullock. 1973. *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). Appl Microbiol 25:155-6.
40. Ling, S. H., X.H. Wang, T.M. Lim, and K.Y. Leung. 2001. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol Lett 194:239-243.
41. Plumb, J. 1999. *Edwardsiella* septicaemias, in Fish diseases and disorders, viral, bacterial and fungal infections, P. Woo and D. Bruno, Editors. CAB International 479–521.
42. Srinivasa Rao, P.S., T.M. Lim, and K.Y. Leung. 2003. Functional genomics approach to the Identification of virulence genes Involved in *Edwardsiella tarda* pathogenesis. Infect Immun 71:1343-1351.
43. Han, H. J., D.H. Kim, D.C. Lee, S.M. Kim, and S.I. Park. 2006. Pathogenicity of *Edwardsiella tarda* to olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). J Fish Dis 29:601-609.
44. Srinivasa Rao, P. S., Y. Yamada, and K.Y. Leung. 2003. A major catalase (KatB) that is required for resistance to H2O2 and phagocyte-mediated killing in *Edwardsiella tarda*. Microbiology 149:2635-2644.
45. Tan, Y. P., Q. Lin, X.H. Wang, S. Joshi, C.L. Hew, and K.Y. Leung. 2002. Comparative proteomic analysis of extracellular proteins of edwardsiella tarda. Infect Immun 70:6475-6480.
46. Tan, Y. P., J. Zheng, S.L. Tung, I. Rosenshine, and K.Y. Leung. 2005. Role of type III secretion in *Edwardsiella tarda* virulence. Microbiology 151:2301-2313.
47. Zheng, J. and K.Y. Leung. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol 66:1192-1206.
48. Zheng, J., N. Li, Y.P. Tan, J. Sivaraman, Y.K. Mok, and K.Y. Leung. 2007. EscC is a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon components EseB and EseD. Microbiology 153:1953-1962.
49. Wang, X., Q. Wang, J. Xiao, Q. Liu, H. Wu, L. Xu, and Y. Zhang. 2009. *Edwardsiella tarda* T6SS component evpP is regulated by esrB and iron, and plays essential roles in the invasion of fish. Fish Shellfish Immunol 27:469-477.
50. Morohoshi, T., T. Inaba, N. Kato, K. Kanai, and T. Ikeda. 2004. Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. J Biosci Bioeng 98:274-281.
51. Zhang, M., K. Sun, and L. Sun. 2008. Regulation of autoinducer 2 production and luxS expression in a pathogenic *Edwardsiella tarda* strain. Microbiology 154:2060-2069.
52. Morohoshi, T., Y. Yokoyama, M. Ouchi, N. Kato, and T. Ikeda. 2009. Motility and the expression of the flagellin protein FliC are negatively regulated by quorum sensing in *Edwardsiella tarda*. J Biosci Bioeng 108:314-318.
53. Pressley, M. E., P.E. Phelan III, P.E. Witten, M.T. Mellon, and C.H. Kim. 2005. Pathogenesis and inflammatory response to *Edwardsiella* tarda infection in the zebrafish. Dev Comp Immunol 29:501-513.
54. Kanther, M. and J.F. Rawls. 2010. Host-microbe interactions in the developing zebrafish. Curr Opin Immunol 22:10-19.
55. Meeker, N.D. and N.S. Trede. 2008. Immunology and zebrafish: Spawning new models of human disease. Dev Comp Immunol 32:745-757.
56. Sullivan, C. and C.H. Kim. 2008. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol 25:341-350.
57. Mione, M., A.H. Meijer, B.E. Snaar-Jagalska, H.P. Spaink, and N.S. Trede. 2009. Disease modeling in zebrafish: cancer and immune responses - a report on a workshop held in Spoleto, Italy, July 20-22, 2009. Zebrafish 6:445-451.

58. Lieschke, G.J. and N.S. Trede. 2009. Fish immunology. Curr Biol 19:R678-R682.
59. Traver, D., P. Herbomel, E.E. Patton, R.D. Murphey, J.A. Yoder, G.W. Litman, A. Catic, C.T. Amemiya, L.I. Zon, and N.S. Trede. 2003. The zebrafish as a model organism to study development of the immune system. Adv Immunol 81:253-330.
60. Trede, N.S., D.M. Langenau, D. Traver, A.T. Look, and L.I. Zon. 2004 The use of zebrafish to understand immunity. Immunity 20:367-379.
61. Hsu, K., D. Traver, J.L. Kutok, A. Hagen, T.X. Liu, B.H. Paw, J. Rhodes, J.N. Berman, L.I. Zon, J.P. Kanki, and A.T. Look. 2004. The pu.1 promoter drives myeloid gene expression in zebrafish. Blood 104:1291-1297.
62. Peri, F. and C. Nüsslein-Volhard. 2008. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion *in vivo*. Cell 133:916-927.
63. Ward, A.C., D.O. McPhee, M.M. Condron, S. Varma, S.H. Cody, S.M. Onnebo, B.H. Paw, L.I. Zon, G.J. Lieschke. 2003. The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. Blood 102:3238-3240.
64. Zakrzewska, A., C. Cui, O.W. Stockhammer, E.L. Benard, H.P. Spaink, and A.H. Meijer. 2010. Macrophage-specific gene functions in Spi1-directed innate immunity. Blood 116:e1-11.
65. Mathias, J.R., B.J. Perrin, T.X. Liu, J. Kanki, A.T. Look, and A. Huttenlocher. 2006. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol 80:1281-1288.
66. Renshaw, S.A., C.A. Loynes, D.M.I. Trushell, S. Elworthy, P.W. Ingham, and M.K.B. Whyte. 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108:3976-3978.
67. Ellett F, L. Pase, J.W. Hayman, A. Andrianopoulos, and G.J. Lieschke. 2011. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117:e49-56.
68. Gray, C., C.A. Loynes, M.K.B. Whyte, D.C. Crossman, S.A. Renshaw, and T.J. Chico. 2011. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. Thromb Haemost 105:811-819.
69. Amsterdam, A. and N. Hopkins. 2006. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. Trends Genet 22:473-478.
70. Moens, C.B., T.M. Donn, E.R. Wolf-Saxon, and T.P. Ma. 2008. Reverse genetics in zebrafish by TILLING. Brief Funct Genomic Proteomic 7:454-459.
71. Hogan, B.M., H. Verkade, G.J. Lieschke, and J.K. Heath. 2008. Manipulation of gene expression during zebrafish embryonic development using transient approaches. Methods Mol Biol 469:273-300.
72. Shestopalov, I.A. and J.K. Chen. 2010. Oligonucleotide-based tools for studying zebrafish development. Zebrafish 7:31-40.
73. Balla, K.M., G. Lugo-Villarino, J.M. Spitsbergen, D.L. Stachura, Y. Hu, K. Bañuelos, O. romo-Fewell, R.V. Aroian, and D. Traver. 2010. Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. Blood 116:3944-3954.
74. Lugo-Villarino, G., K.M. Balla, D.L. Stachura, K. Bañuelos, M.B. Werneck, and D. Traver. 2010. Identification of dendritic antigen-presenting cells in the zebrafish. Proc Natl Acad Sci USA 107:15850-15855.
75. Carradice, D. and G.J. Lieschke. 2008. Zebrafish in hematology: sushi or science? Blood 111:3331-3342.
76. Bertrand, J.Y., J.L. Cisson, D.L. Stachura, and D. Traver. 2010. Notch signalling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. Blood 115:2777-2783.
77. Bertrand, J.Y. and D. Traver. 2009. Hematopoietic cell development in the zebrafish embryo. Curr Opin Hematol 16:243-248.
78. Chen, A.T. and L.I. Zon. 2009. Zebrafish blood stem cells. J Cell Biochem 108:35-42.
79. Davidson, A.J. and L.I. Zon. 2004. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. Oncogene 23:7233-7246.
80. Davis, J.M., H. Clay, J.L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of *Mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17:693-702.
81. Herbomel, P., B. Thisse, and C. Thisse. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126:3735-3745.
82. van der Sar, A.M., R.J.P. Musters, F.J.M. van Eeden, B.J. Appelmelk, C.M.J.E. Vandenbroucke-Grauls, and W. Bitter. 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell Microbiol 5:601-611.
83. Lam, S.H., H.L. Chua, Z. Gong, T.J. Lam, and Y.M. Sin. 2004. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28:9-28.
84. Willett, C.E., A. Cortes, A. Zuasti, and A.G. Zapata. 1999. Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214:323-336.
85. Mogensen, T.H. 2009. Pathogen recognition and inflammatory signalling in innate immune defenses. Clin Microbiol Rev 22:240-273.
86. Anderson, K.V., G. Jürgens, and C. Nüsslein-Volhard. 1985. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: Genetic studies on the role of the Toll gene product. Cell 42:779-789.
87. Lemaitre, B., E. Nicolas, L. Michaut, J,M. Reichhart, and J.A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86:973-983.

88. Poltorak, A., X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085-2088.
89. Cirl, C., A. Wieser, M. Yadav, S. Duerr, S. Schubert, H. Fischer, D. Stappert, N. Wantia, N. Rodriguez, H. Wagner, C. Svanborg, and T. Miethke. 2008. Subversion of Toll-like receptor signalling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. Nat Med 14:399-406.
90. Wang, Q., M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, L. Xu, H. Zeng, S. Wang, G. Zhao, Q. Liu, Y. Zhang. 2009b. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. PLoS One 4:e7646.
91. Jault, C., L. Pichon, and J. Chluba. 2004. Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol Immunol 40:759-771.
92. Meijer, A.H., G.S.F. Krens SF, I.A. Medina Rodriguez, S. He, W. Bitter, B.E. Snaar-Jagalska, and H.P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol 40:773-783.
93. Novoa, B., A. Romero, V. Mulero, I. Rodríguez, I. Fernández, and A. Figueras. 2006. Zebrafish (*Danio rerio*) as a model for the study of vaccination against viral haemorrhagic septicemia virus (VHSV). Vaccine 24:5806-5816.
94. Phelan, P.E., M.T. Mellon, and C.H. Kim. 2005. Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). Mol Immunol 42:1057-1071.
95. Sepulcre, M.P., F. Alcaraz-Pérez, A. López-Muñoz, F.J. Roca, J. Meseguer, M.L. Cayuela, and V. Mulero. 2009. Evolution of lipopolysaccharide (LPS) recognition and signalling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J Immunol 182:1836-1845.
96. Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H.P. Spaink, and A.H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to salmonella infection. J Immunol 182:5641-5653.
97. Sullivan, C., J. Charette, J. Catchen, C.R. Lage, G. Giasson, J.H. Postlethwait, P.J. Millard, and C.H. Kim. 2009. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. J Immunol 183:5896-5908.
98. Hall, C., M.V. Flores, A. Chien, A. Davidson, K. Crosier, and P. Crosier. 2009. Transgenic zebrafish reporter lines reveal conserved Toll-like receptor signalling potential in embryonic myeloid leukocytes and adult immune cell lineages. J Leukoc Biol 85:751-765.
99. Stein, C., M. Caccamo, G. Laird, and M. Leptin. 2007. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol 8:R251.
100. Sullivan, C., J.H. Postlethwait, C.R. Lage, P.J. Millard, and C.H. Kim. 2007. Evidence for evolving Toll-IL-1 receptor-containing adaptor molecule function in vertebrates. J Immunol 178:4517-4527.
101. Yamamoto, M., K. Takeda, and S. Akira. 2004. TIR domain-containing adaptors define the specificity of TLR signalling. Mol Immunol 40:861-868.
102. Carty, M., R. Goodbody, M. Schröder, J. Stack, P.N. Moynagh, and A.G. Bowie. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signalling. Nat Immunol 7:1074-1081.
103. Szretter, K.J., M.A. Samuel, S. Gilfillan, A. Fuchs, M. Colonna, and M.S. Diamond. 2009. The immune adaptor molecule SARM modulates tumor necrosis factor alpha production and microglia activation in the brainstem and restricts West Nile Virus pathogenesis. J Virol 83:9329-9338.
104. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143-150.
105. Sun, D. and A. Ding. 2006. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol 7:375-381.
106. Burns, K., F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J.L. Bodmer, F. Di Marco, L. French, and J. Tschopp. 1998. MyD88, an adapter protein involved in interleukin-1 signalling. J Biol Chem 273:12203-12209.
107. Muzio, M., J. Ni, P. Feng, and V.M. Dixit. 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signalling. Science 278:1612-1615.
108. Wesche, H., W.J. Henzel, W. Shillinglaw, S. Li, and Z. Cao. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 7:837-847.
109. Akira, S. and K. Takeda. 2004. Toll-like receptor signalling. Nat Rev Immunol 4:499-511.
110. Scanga, C.A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E.Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol 168:5997-6001.
111. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol 165:5392-5396.
112. Edelson, B.T. and E.R. Unanue. 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. J Immunol 169:3869-3875.
113. Ryffel, B., C. Fremond, M. Jacobs, S. Parida, T. Botha, B. Schnyder, and V. Quesniaux. 2005. Innate immunity to mycobacterial infection in mice: Critical role for toll-like receptors. Tuberculosis 85:395-405.

114. Bates, J.M., J. Akerlund, E. Mittge, and K. Guillemin. 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe 2:371-382.
115. van der Sar, A.M., O.W. Stockhammer, C. van der Laan, H.P. Spaink, W. Bitter, and A.H. Meijer. 2006. MyD88 innate immune function in a zebrafish embryo infection model. Infect Immun 74:2436-2441.
116. Phelps, H.A. and M.N. Neely. 2002. Evolution of the zebrafish model: from development to immunity and infectious disease. Zebrafish 2:87-103.
117. Trede, N., A.H. Meijer, B.E. Snaar-Jagalska, and H.P. Spaink. 2008. Model systems for infectious disease and cancer in zebrafish: a report on an EMBO workshop held at the Lorentz Center, Leiden, The Netherlands, July 16-18, 2007. Zebrafish 4:287-292.
118. van der Sar, A.M., B.J. Appelmelk, C.M. Vandenbroucke-Grauls, W. Bitter. 2004. A star with stripes: zebrafish as an infection model. Trends Microbiol 12:451-457.
119. Meijer, A.H. and H.P. Spaink. 2011. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets 12: 1000-1007
120. Brannon, M.K., J.M. Davis, J.R. Mathias, C.J. Hall, J.C. Emerson, P.S. Crosier, A. Huttenlocher, L. Ramakrishnan, and S.M. Moskowitz. 2009. *Pseudomonas aeruginosa* Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. Cell Microbiol 11:755-768.
121. Clatworthy, A.E., J.S-W. Lee, M. Leibman, Z. Kostun, A.J. Davidson, and D.T. Hung. 2009. *Pseudomonas aeruginosa* infection of zebrafish involves both host and pathogen determinants. Infect Immun 77:1293-1303.
122. Rawls, J.F., M.A. Mahowald, A.L. Goodman, C.M. Trent, and J.I. Gordon. 2007. In vivo imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. Proc Natl Acad Sci USA 104:7622-7627.
123. Cho, K.H. and M.G. Caparon. 2005. Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. Mol Microbiol 57:1545-1556.
124. Tobin, D.M. and L. Ramakrishnan. 2008. Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*. Cell Microbiol 10:1027-1039.
125. Russell, D.G. 2007. Who puts the tubercle in tuberculosis? Nat Rev Micro 5:39-47.
126. Volkman, H.E., T.C. Pozos, J. Zheng, J.M. Davis, J.F. Rawls, and L. Ramakrishnan. 2010. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science 327:466-469.
127. Davis, J.M. and L. Ramakrishnan. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell 136:37-49.
128. Carvalho, R., J. de Sonneville, O.W. Stockhammer, N.D.L. Savage, W.J. Veneman, T.H.M. Ottenhoff, R.P. Dirks, A.H. Meijer, and H.P. Spaink. 2011. A high-throughput screen for tuberculosis progression. PLoS ONE 6:e16779.
129. Prajsnar, T.K., V.T. Cunliffe, S.J. Foster, and S.A. Renshaw. 2008. A novel vertebrate model of *Staphylococcus aureus* infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cell Microbiol 10:2312-2325.
130. Harriff, M.J., L.E. Bermudez, and M.L. Kent. 2007 Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection. J Fish Dis 30:587-600.
131. O'Toole, R., J. Von Hofsten, R. Rosqvist, P.E. Olsson, and H. Wolf-Watz. 2004. Visualisation of zebrafish infection by GFP-labelled *Vibrio anguillarum*. Microb Pathog 37: 41-46.
132. Szabady, R.L., M.A. Lokuta, K.B. Walters, A. Huttenlocher, and R.A. Welch. 2009. Modulation of Neutrophil Function by a Secreted Mucinase of *Escherichia coli* O157:H7. PLoS Pathog 5:e1000320.
133. Chang, M.X., P. Nie. 2008. RNAi suppression of zebrafish peptidoglycan recognition protein 6 (zfPGRP6) mediated differentially expressed genes involved in Toll-like receptor signaling pathway and caused increased susceptibility to *Flavobacterium columnare*. Vet Immunol Immunopathol 124:295-301.
134. Le Guyader, D., M.J. Redd, E. Colucci-Guyon, E. Murayama, K. Kissa, V. Briolat, E. Mordelet, A. Zapata, H. Shinomiya, and P. Herbomel. 2008. Origins and unconventional behavior of neutrophils in developing zebrafish. Blood 111:132-141.
135. Cheesman, S.E., J.T. Neal, E. Mittge, B.M. Seredick, and K. Guillemin. 2010. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. Proc Natl Acade Sci USA 108:4570-4577.
136. Oehlers, S.H., M.V. Flores, K.S. Okuda, C.J. Hall, K.E. Crosier, and P.S. Crosier. 2011 A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents. Dev Dyn 240:288-298.
137. Liu, Y., M. Li, S. Fan, Y. Lin, B. Lin, F. Luo, C. Zhang, S. Chen, Y. Li, and A. Xu. 2010. A unique feature of Toll/IL-1 receptor domain-containing adaptor protein is partially responsible for lipopolysaccharide insensitivity in zebrafish with a highly conserved function of MyD88. J Immunol 185:3391-3400.
138. Fronzes, R., P.J. Christie, and G. Waksman. 2009. The structural biology of type IV secretion systems. Nat Rev Micro 7:703-714.

139. Ribeiro, C.M.S., T. Hermsen, A.J. Taverne-Thiele, H.F. Savelkoul, and G.F. Wiegertjes. 2010. Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. J Immunology 184:2355-2368.
140. Kawai, T. and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373-384.

Genome comparisons of *Edwardsiella* bacteria analysed using deep sequencing technology

Joost J. van Soest, Christiaan V. Henkel, Hans J. Jansen,
Cees A.M.J.J. van den Hondel, Guido V. Bloemberg,
Annemarie H. Meijer and Herman P. Spaink

Abstract

Edwardsiella bacteria are well-known pathogens of a wide variety of animals, including humans. We have used shotgun deep sequencing technology to get a completely saturated (171 times coverage) genome sequence of *E. tarda* strain FL6-60, a common fish pathogen which is also used in zebrafish infection models. Bioinformatics analyses revealed interesting technical details of Illumina deep sequencing technology, such as a bias against regions with high CG content. We have compared our genome sequence with those of *Edwardsiella* strains already available (*E. tarda* EIB202 and *E. ictaluri* 93-146). These comparisons show that the current deep sequencing using ~50 nucleotides reads is very well suited to identify single nucleotide polymorphisms with high accuracy. Furthermore, we were able to identify all highly diversified regions that are related to transposon and viral sequences, thereby giving new insights in genome dynamics of closely related bacterial strains. The great depth of sequencing made it even possible to identify a novel circular single copy prophage that has not been found in other *Edwardsiella* strains. In addition, we show that with this technology it is possible to show with high confidence that a plasmid present in *E. tarda* strain EIB202 is not present in strain FL6-60. As an example of the usefulness of these datasets we compared single nucleotide polymorphisms in several gene clusters that are associated with virulence, also using other published incomplete *Edwardsiella* sequences. The results showed that several virulence genes, including those coding for type III and type VI secretion systems, have a remarkable high number of polymorphisms. The identification of these variations will be important for further comparisons in pathogenicity and virulence of different *Edwardsiella* strains.

Introduction

Edwardsiella tarda is a gram negative pathogen capable of infecting a wide range of host species, such as fish, amphibians, reptiles, birds and mammals, including humans [1, 2]. Edwardsiellosis, a generalized septicemia caused by *E. tarda*, is an often occurring disease in many fish species in aquaculture, which leads to extensive losses [3]. In humans, *E. tarda* is also a cause of

gastrointestinal and extra-intestinal infections [1] and is able to invade non-phagocytic cells in culture [4, 5].

E. tarda strain FL6-60 was shown to be able to infect zebrafish embryos by static immersion [6]. Adult zebrafish were susceptible to intraperitoneal injection and could also be infected by static immersion, but only in combination with skin wounding. With the zebrafish being increasingly used as a model system for infectious diseases [7], this makes this strain a valuable test organism for disease modelling. The zebrafish *E. tarda* infection model is now also used for detailed innate immune transcriptome analysis (this thesis, chapters 3 and 4). Recently the complete genome sequence of *Edwardsiella tarda* strain EIB202 has been published [8]. EIB202 was shown to be pathogenic in swordfish, turbot and adult zebrafish, but this strain has not been tested yet in zebrafish embryo infection models. Furthermore, the genome of its close relative *E. ictaluri* strain 93-146 has been completely sequenced and submitted to the Genbank database (accession number CP001600). In addition, a collection of unassembled short fragment reads of a shotgun sequencing attempt of *E. tarda* strain ATCC 23685 is available (SRA accession numbers SRX001436 and SRX001437).

The development of new sequencing techniques has rapidly reduced the time and cost of whole-genome sequencing making it attractive to compare many bacterial genomes [9]. The next-generation sequence platforms, provided by Illumina, ABI and 454 Life Sciences (Roche), generate large amounts of nucleotide sequence reads [10]. These techniques were primarily developed for resequencing of closely related individuals, but are with the increase of the read sequence lengths and the availability of assembly software specialized for short reads gradually becoming used for *de novo* genome sequencing [11-14]. We sequenced *E. tarda* strain FL6-60 with the Illumina Genome Analyzer II, which provided us with reads of a length of 51 bp that were assembled into large contigs and compared to the EIB202 sequence. We have used the FL6-60 genome sequence for comparison of virulence genes with the known sequences of other *E. tarda* strains to see if there are genetic differences between strains that can be a basis for differences in pathogenic behaviour. Several virulence factors, common in Gram-negative pathogens, have been identified as being important in *E. tarda* virulence. The three major systems involved are the protein secretion systems Type III Secretion System (T3SS)

[15] and Type VI Secretion System (T6SS) [16] and the regulatory quorum sensing system [17, 18].

The virulence determinants of *E. tarda* have been identified by functional genomics approaches such as transposon mutagenesis [19] and proteomics [20]. Of several *E. tarda* strains some of the genes found to be associated with virulence were sequenced, for instance the complete sequences are available for the T3SS gene cluster [15], the T6SS gene cluster [16], the quorum sensing genes LuxIR and LuxS, and some genes involved in toxicity [19] and resistance to phagocyte-mediated killing [19, 21, 22].

Our sequence comparisons show that the used current deep sequencing technology is highly suited to identify single nucleotide polymorphisms with high accuracy. For two highly related bacterial strains, we have mapped genome dynamic regions and identified all single nucleotide polymorphisms in the above mentioned loci and the presence or absence of plasmids or circular prophage-related sequences.

Results and discussion

Genome assembly strategy

The genome of *E. tarda* FL6-60 was assembled using a combination of automated *de novo* assembly, reference assembly against the recently published genome of *E. tarda* EIB202 and manual inspection of highly variable regions (see table 1). Using *de novo* assembly alone, 98.2% of the sequencing reads could be assigned to contigs of at least 200 bp. In a reference assembly using the *E. tarda* EIB202 genome, 92.4% of reads could be mapped. On average, every position on this genome is covered by 171 reads; however in total 145443 bp (3.9% of the genome) could not covered by any read. A reference assembly using plasmid pEIB202 of strain EIB202 did not yield a relevant number of matching reads, thereby demonstrating the absence of this plasmid in strain FL6-60.

In the reference assembly, many regions of the EIB202 genome are uniquely matched by sequencing reads with at least twice the average coverage (figure 1), indicating possible genomic duplications. However, when comparing

Table 1. Genome statistics of *Edwardsiella tarda* strain FL6-60.

Assembly	Reference	Accession	Length	%CG	ORFs*	Assembled reads	Average coverage
De novo	-	-	3801681 bp in 623 contigs (N50 = 12020 bp)	ND	ND	145153318 (98.2%)	180.16x
Reference	*E. ictaluri* 93-146 genome	NC_012779	3812315 bp	57.4%	3935	5234702 (35.4%)	63.47x
Reference	*E. tarda* EIB202 genome	NC_013508	3760463 bp	59.7%	3664	13661894 (92.4%)	171.01x
Reference	*E. tarda* pEIB202 plasmid	NC_013509	43703 bp	57.3%	53	2 (0.0%)	0.00x
Reference	*E. tarda* FL6-60 genome		3684607 bp	59.8%	3448	14250618 (96.4%)	182.38x
Reference	*E. tarda* FL6-60 phage-like element		44194 bp	51.4%	63	268143 (1.8%)	293.07x

* The number of ORFs in the reference sequence as found in Genbank or determined by Glimmer

local coverage to GC-content, a clear correlation was found (figure 2), suggesting a technical artefact that we have further analysed. A bias against AT-rich regions has been previously reported for Illumina sequencing [23]. In apparent contrast, we find a strong correlation between GC-content and sequencing depth: the lower the GC-content of the reference, the higher the number of matching reads. However, the *E. tarda* EIB202 genome contains very few AT-rich regions even near the resolution of single sequencing reads (100 bp windows), so any bias against AT-rich sequences would be difficult to observe. In fact, the observed apparent linear relationship between GC% and coverage (figure 1) makes it possible to predict sequencing depth for the whole reference genome, allowing several outliers to be quickly identified.

A complete draft genome of strain FL6-60 was then constructed by manually integrating *de novo* assembled contigs into the gaps of the reference assembly using matching flanking sequences (figure 2). One large contig could

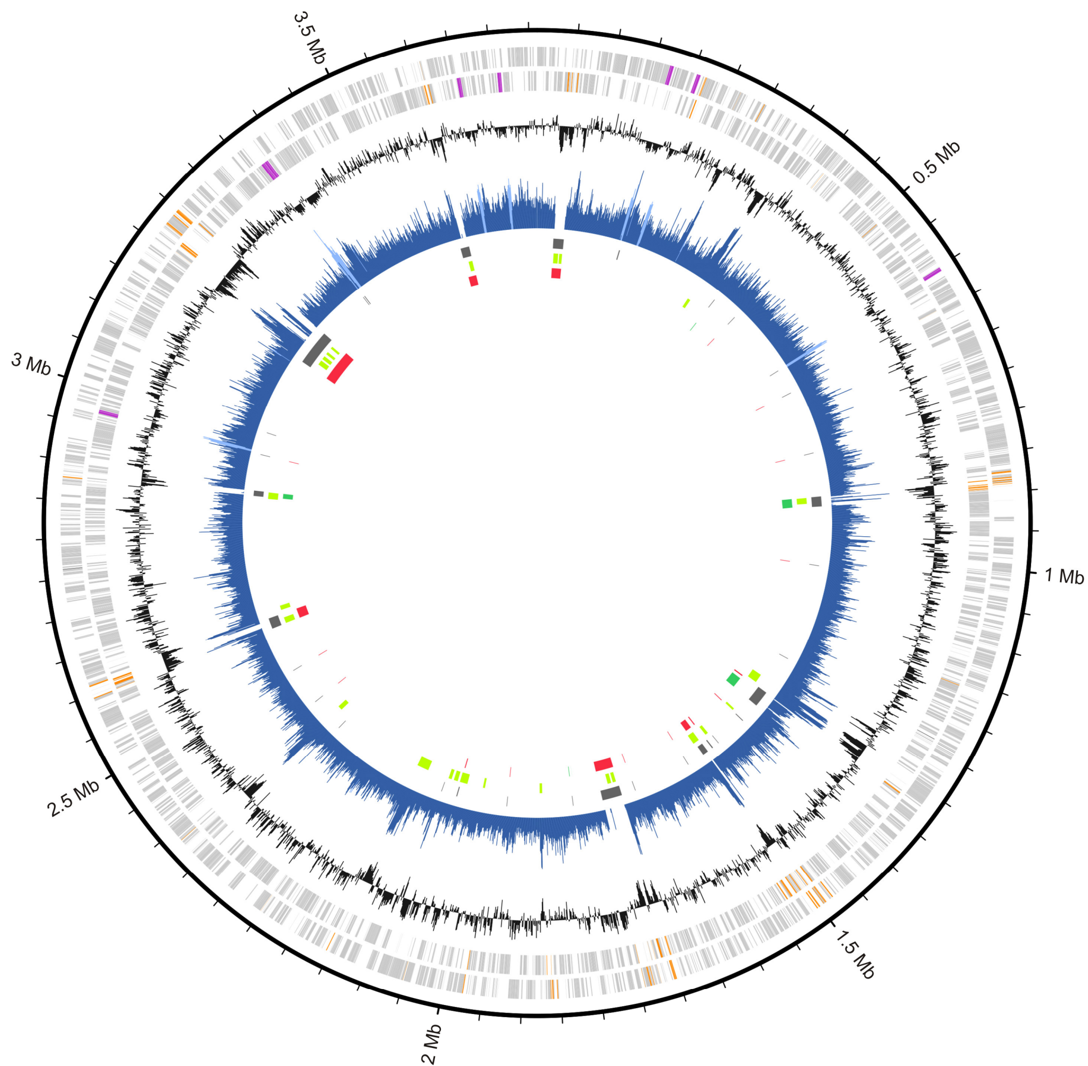


Figure 1. Overview of the genome of *Edwardsiella tarda* strain EIB202 excluding one circular phage-like element. The circles from outside to inside represent: Annotated genes plus strand (grey, protein coding; black, tRNA; purple, rRNA; orange, phage-related/integrase/transposase). Annotated genes minus strand (colour coding same as plus strand). CG content above or below the median (calculated for 1000 bp windows). Sequencing depth (coverage) for the reference assembly of strain FL6-60 (1000 bp windows), dark blue are unique matches, light blue are non-unique matches of sequencing reads. Regions masked in the second iteration of the reference assembly of strain FL6-60 (dark grey). Genomic islands as determined by IslandViewer (lime). Net insertions (green) and deletions (red) in strain FL6-60 with regard to strain EIB202, genes affected can be found in supplementary table S1.

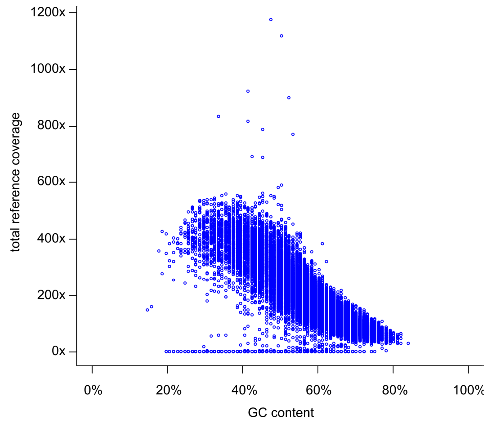


Figure 2. GC bias of Illumina sequencing. GC content and average coverage were calculated for 100 bp windows of the EIB202 genome. For 40-80% GC, the relationship with read coverage can be described by a straight line, allowing easy identification of regions with aberrant coverage. As there are few 100 bp windows with less than 25% GC, this relationship does not contradict reports of a bias against AT-rich regions.

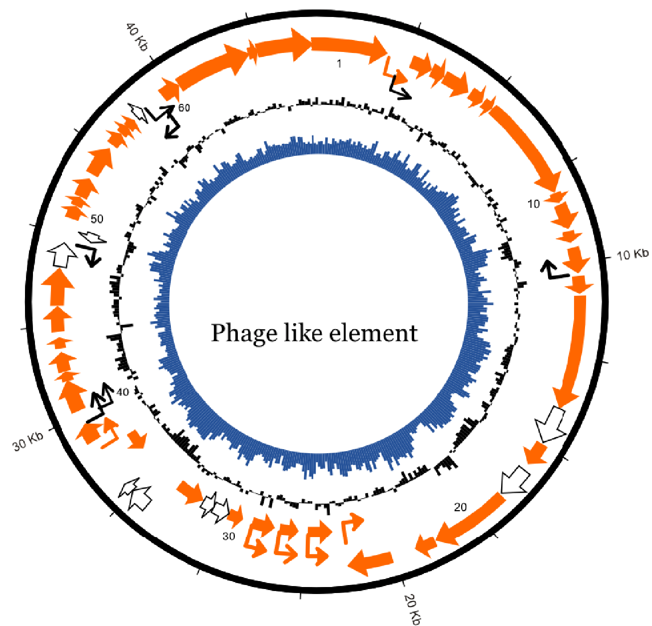


Figure 3. Map of a 44194 bp circular phage-like element of *Edwardsiella tarda* strain FL6-60. The circles from outside to inside represent: Scale; Annotated genes (orange is phage related), numbers with the genes indicate the ORF numbers, which correspond to those in table 2, a more detailed table can be found in supplementary table S2; GC content above or below median (1000 bp windows); Sequencing depth (coverage, 1000bp windows).

Table 2. Reading frame annotation of a circular phage-like element of *Edwardsiella tarda* FL6-60

Predicted functions based on sequence similarity	Number of open reading frame*
Phage tail components	4-11, 13, 15, 20, 21
Phage membrane proteins	12, 18, 50
Phage methyltransferases	30, 43
Other phage related proteins (i.e. nucleases, integrases, repressors)	1, 16, 22, 23, 26, 33, 36, 37, 41, 44, 51-55, 60, 61, 63
Phage hypothetical proteins	2, 24, 25, 28, 29, 33, 38, 42, 45, 46, 56, 62
other proteins	35, 58
hypothetical proteins	3, 17, 19, 27, 31, 32, 34, 39, 40, 47, 49, 57,
no blast hit	14, 48, 59

* as indicated in figure 3

not be assigned to the genome, and indeed appears to be a separate circular entity (figure 3). In a new reference assembly, 98.2% of reads (99.6% using less stringent settings) matched either the new draft genome or the circular element. Since slightly more reads match the drafts than can be assembled *de novo* (table 1), and the remaining 1.8% could not be further assembled *de novo* into significant contigs because of sequence repeats, we accepted the draft as an essentially correct representation of the *E. tarda* FL6-60 genome.

Comparison of *E. tarda* FL6-60, *E. tarda* EIB202 and *E. ictaluri* 93-146

The genome of *E. tarda* strain FL6-60 is highly similar to that of strain EIB202 as previously described by Wang et al [8]. Genomic differences due to insertions and deletions can be found in the supplementary data but these are limited to areas containing many phage-related sequences (table S1). Nearly 3300 reading frames annotated for the genome of EIB202 by Wang et al [8] could also be identified in the genome of strain FL6-60. Automatic sequence annotation did not reveal any particular gene differences with the published sequence of Wang et al [8] (data not shown). We have looked in detail to several gene clusters not discussed in detail by Wang et al [8] such as the genes involved in exopolysaccharide production and found these genes to be highly conserved as well (99.9-100 % nucleotide sequence identity) (data not shown). Differences in the genome represent recent evolutionary changes related to phage integrations and shuffling of repetitive sequences. The largest differences between the strains appear to be concentrated around predicted genomic islands, which harbour phage-related genes and transposons (figure 1).

A major difference is the absence of a plasmid that is present in strain EIB202. In contrast strain FL6-60 contains an additional circular element (figure 3). This element has a similar length as representatives of the P22 podoviridae and contains many predicted transcripts encoding proteins that are homologous to phage proteins (table 2). This element possibly represents a novel viral element. Because of the correlation between sequencing depth and GC-content, the higher coverage and lower GC-content in comparison with the genome, indicates that this element is present in a copy number of 1 per cell,

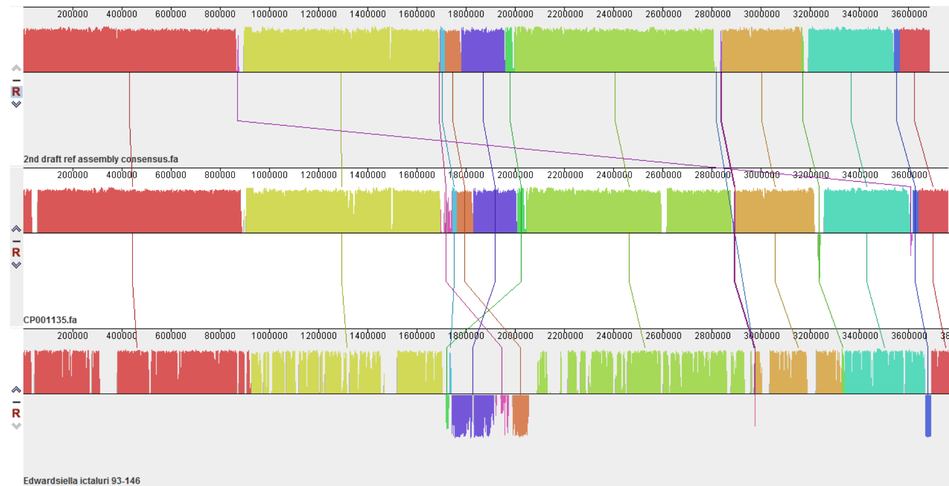


Figure 4. Comparison of the genomes of *Edwardsiella tarda* strains FL6-60 and EIB202 with *E. ictaluri* strain 93-146. The genomes of *Edwardsiella tarda* strains FL6-60 and EIB202 were compared with *E. ictaluri* strain 93-146 with Mauve. The graphs indicate the conservation. Conserved areas are connected with lines between the different genomes, identifying inversions and displacements.

indicating a strict co-replication with the chromosome. We have also compared the genome of strain FL6-60 with that of *E. ictaluri* strain 93-146 (figure 4). The results show there is remarkable overall structural similarity with 35.4 % of identity (table 1) and only two regions with a different orientation as confirmed by manual inspection of the variable regions (figure 4).

Comparison of type III and type VI secretion systems between different *Edwardsiella* strains

It was found that the pathogenicity of different natural isolates of *E. tarda* was corresponding with the presence of the T6SS since strains lacking the T6SS were not pathogenic [16]. Likewise, *E. tarda* strains with mutations that partly or completely shut down the T3SS were less virulent then their wild types [15].

Since type III and VI secretion systems are important virulence factors, knockdown of a single gene can already cause a decrease in pathogenicity. Small changes in these secretion systems might lead to loss of virulence or changing the specialization for a specific host. We have therefore compared these secretion systems in detail for five type III and four type VI secretion systems of *Edwardsiella*. The results show that the secretion systems of strains

FL6-60 and EIB202 are more similar than those of the other *E. tarda* strains (figure 5 and 6). Even though in *E. ictaluri* the T3SS and T6SS clusters are present and in the same order of genes as in *E. tarda*, there are large differences in the encoded protein sequences between the two *Edwardsiella* species. This might be explained by the fact that *E. ictaluri* is primarily pathogenic to catfish and therefore might have specialized for this host, in contrast to *E. tarda* that appears to be less host-specific. It would therefore be

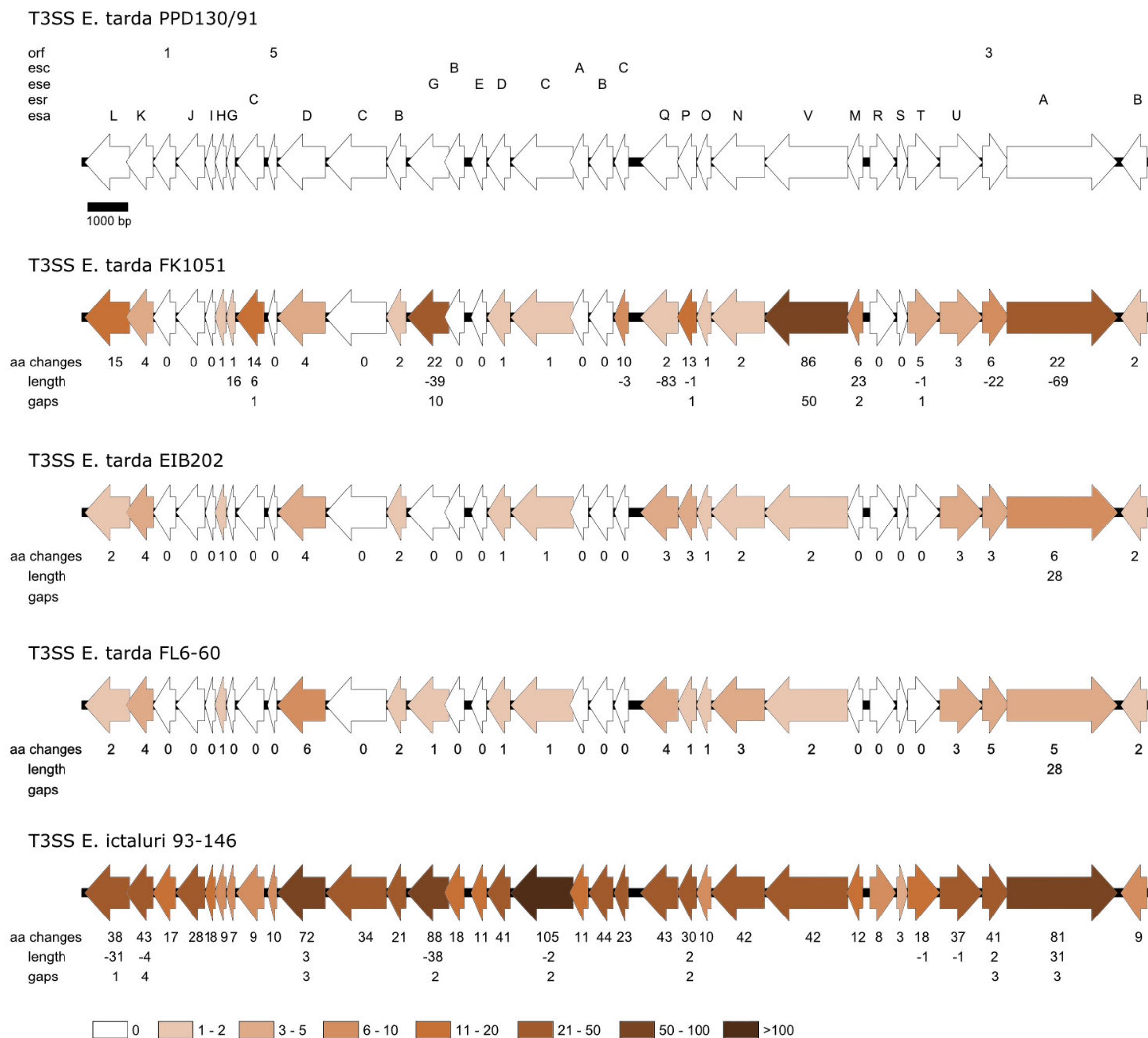


Figure 5. Comparisons of the type III secretion systems of five different *Edwardsiella* strains. The T3SS of *E. tarda* PPD130/91 [15] was used as the reference for comparison. The differences in amino acids in comparison with this strain are notated underneath the clusters. A darker colour represents a larger difference. To determine the difference in length, the nucleotide sequences were compared first, to exclude differences in length due to different ORF-predictions. The order of the genes is the same in all strains compared, with the largest differences found in *E. ictaluri*.

interesting to compare these strains in the same test systems, along with several mutants in the genes that are differing between these strains. The gene that is most different in the type III secretion systems of strains FL6-60 and EIB202 is *esrA* which is a regulatory gene of a two component system together with *esrB*. Other genes that differ considerably are part of the secretion apparatus, such as *esaD* (figure 5). There are many differences with the type III secretion system of *E. tarda* strain FK1051, with notable large differences in the genes *eseG* and *esaV* that are secreted proteins or involved in the secretion machinery, respectively. It remains a possibility that many of the apparent polymorphisms detected are due to sequencing errors in the previously submitted sequences, however the degree of polymorphism is too much to

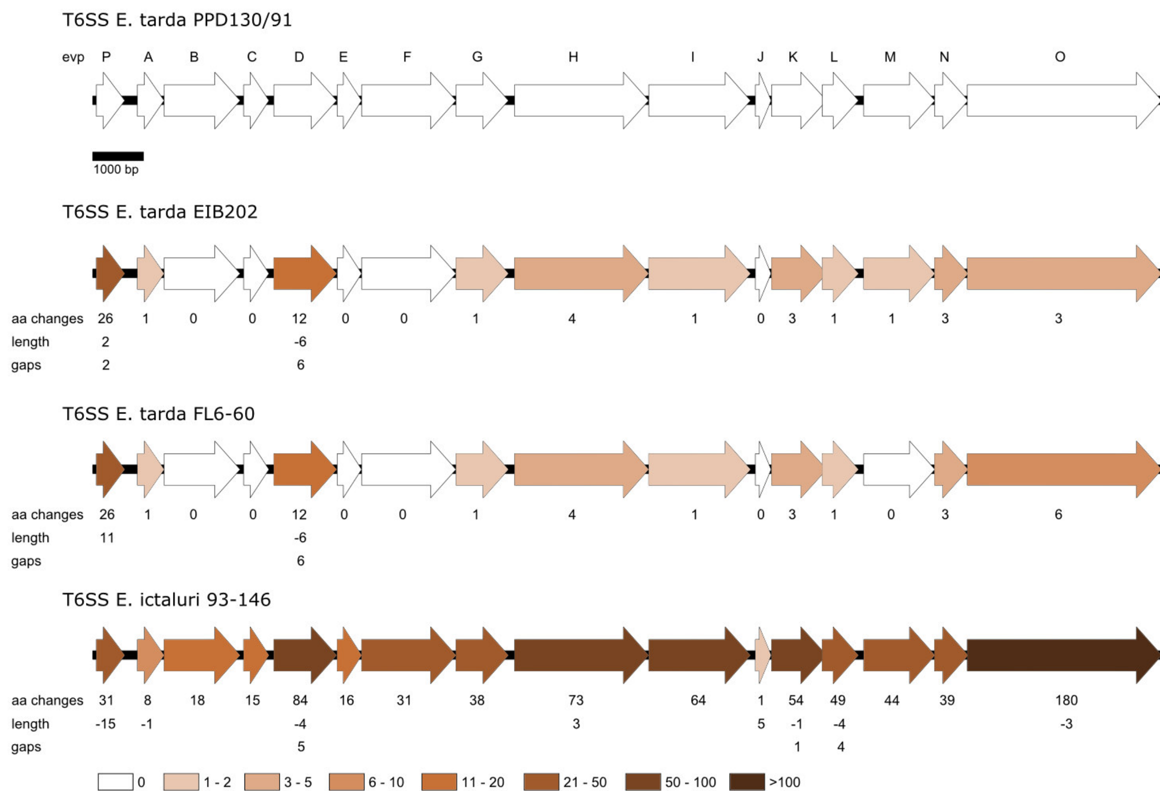


Figure 6. Comparisons of the type VI secretion systems of four different *Edwardsiella* strains. The T6SS of *E. tarda* PPD130/91 [16] was used as the reference for comparison. The differences in amino acids in comparison with this strain are notated underneath the clusters. A darker colour represents a larger difference. To determine the difference in length, the nucleotide sequences were compared first, to exclude differences in length due to different ORF-predictions. The order of the genes is the same in all strains compared, with the largest differences found in *E. ictaluri*.

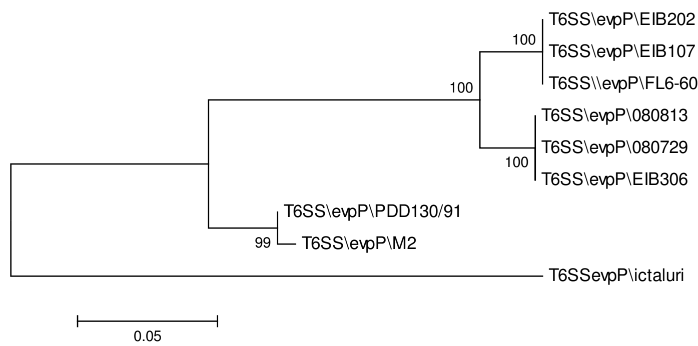


Figure 7. Evolutionary relationships of 9 EvpP genes. The evolutionary history was inferred using the Neighbour-Joining method [25]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [26]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

be accounted for by sequencing errors. The most obvious difference with the genes of *E. ictaluri* is in *eseC* for which 31 of the 105 predicted amino acid differences are localized in an area that has been shown to be important for secretion and accumulation of EseC in *E. tarda* [24]. Furthermore, the large differences between *eseB*, *eseC* and *eseD* are perhaps relevant since these genes have been shown to be important for virulence of *E. tarda* [15]. For the type VI secretion systems the difference between the *evpP* genes that are unique to *Edwardsiella* and are involved in pathogenicity [16] is shown in more detail for 9 *Edwardsiella* strains. The comparison (figure 7), shows that *evpP* of *E. ictaluri* is most different from the *E. tarda* strains and therefore just following the expected species divergence and giving no indications for recent horizontal gene transfer events between these species.

Comparison of non-essential genes showed that there is no clear correlation of the conservation in protein sequence with the essentiality of the gene for secretion as for instance *evpJ* is strongly conserved in contrast to *evpD*, whereas both have been shown not to be essential for secretion [16].

Concluding remarks

Our comparisons show that the current deep sequencing using 50 nucleotides reads is highly suited to identify an entire genome with high accuracy. Bioinformatics analyses also showed interesting technical details of deep sequencing technologies such as depletion of GC rich regions by the Illumina technology.

As a result we were able to compare the genome of *E. tarda* FL6-60 with previously published sequences of other strains and could identify all highly diversified regions with many repeats that are related to transposon and viral sequences. These data provide new insights in genomic variations of highly related bacterial strains. We have identified one novel circular phage-like element and could show with high confidence that a plasmid present in *E. tarda* strain EIB202 was not present in strain FL6-60. As an example of the usefulness of gene comparisons we investigated single nucleotide polymorphisms in several gene clusters that are associated with virulence also using other published incomplete *Edwardsiella* sequences. The results showed that several of the type III and type VI secretion genes, most notably *eseG*, *esaV*, *esrA* and *evpP* have a remarkable high number of polymorphisms not only between *E. tarda* and *E. ictaluri* species but also between different *E. tarda* strains. Other known virulence factors such as enzymes involved in resistance to phagocyte-mediated killing, like *katB* and *ankB* [19, 21, 22] showed only 11 SNPs difference between strains FL6-60 and EIB202. The quorum sensing system was found to be almost completely identical (2 SNPs) to those already published [17, 18]. The identification of these genomic variations in essential virulence genes provides a valuable basis for studying the pathogenic behaviour of different *Edwardsiella* strains.

Materials & methods

Sequencing

Bacterial strain *E. tarda* FL6-60 was grown on TSB medium at 28 °C. Bacterial DNA was isolated using the Qiagen DNeasy Blood and Tissue kit (Qiagen, San Diego, CA) according to the manufacturer’s instructions. To sequence the DNA, a single read library was made using the Illumina Genomic DNA Sample Preparation Kit (Illumina, Hilden, Germany) according to the manufacturer’s instructions. This library was sequenced, with a read length of 51 nt, on an Illumina GAII (Illumina, Hilden, Germany). Two lanes were sequenced, one lane with 3 pmol , and one lane with 4 pmol of library. A total of 16606657 sequencing reads was obtained.

Bioinformatics

Sequence reads were imported, trimmed to eliminate low quality sequences, and assembled using CLCbio Genomics Workbench 3.6.5 (www.clcbio.com). Putative genes were found using Glimmer3 (www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi) [27]. Genomic islands were predicted with IslandViewer (www.pathogenomics.sfu.ca/islandviewer) [28], which combines several methods based on sequence composition and comparative genomics.

Comparison of our assembled genome with the available genomes of *E. tarda* (accession number CP001135) and *E. ictaluri* (accession number CP001600) was done with Mauve 2.3.1 [29]. Gene analysis was done with Vector NTI and BLAST on the NCBI server.

Circos [30] was used for the construction of the maps of the genome and the circular phage-like element. Mega4 was used for the construction of the phylogenetic tree [31]. The evolutionary distances were computed using the Poisson correction method [32] and are given in the units of the number of amino acid substitutions per site.

Sequences used in comparisons were obtained from the NCBI-database with the following accession numbers excluding the ones already mentioned. T3SS: AY643478, AY850613. T6SS: AY424360. EvpP: FJ595672, FJ595674, FJ595675, FJ595676, FJ595677

Accession numbers

The genome sequence has been submitted under accession number CP002154 and the sequence of the phage-like element has been submitted under accession number CP002155.

Acknowledgements

We thank Dr. Philip Klesius (USDA, Auburn, AL) for providing us with *E. tarda* strain FL6-60. The research group of C.A.M.J.J.H. is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative.

Supplementary data

Supplementary tables can be found online at: http://tinyurl.com/ch2suptables

References

1 Janda, J. M. and S.L. Abbott. 1993. Infections associated with the genus Edwardsiella: the role of *Edwardsiella tarda* in human disease. Clin Infect Dis 17:742-748.

2 Kourany, M., M.A.Vasquez and R. Saenz. 1977. Edwardsiellosis in man and animals in Panama: clinical and epidemiological characteristics. Am J Trop Med Hyg 26:1183-1190.

3 Thune, R. L., L. Stanley, and R.K. Cooper. 1993. Pathogenesis of Gram-negative bacterial infections in warmwater fish. Fish Annu Rev Fish Dis 3:37-68

4 Ling, S. H., X.H. Wang, T.M. Lim, and K.Y. Leung. 2001. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol Lett 194:239-243.

5 Ling, S. H. M., X.H. Wang, L. Xie, T.M. Lim, and K.Y. Leung. 2000. Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology 146:7-19.

6 Pressley, M. E., P.E. Phelan III, P.E. Witten, M.T. Mellon, and C.H. Kim. 2005. Pathogenesis and inflammatory response to *Edwardsiella* tarda infection in the zebrafish. Dev Comp Immunol 29:501-513.

7 Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H.P. Spaink, and A.H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to salmonella infection. J Immunol 182:5641-5653.

8 Wang, Q., M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, L. Xu, H. Zeng, S. Wang, G. Zhao, Q. Liu, Y. Zhang. 2009b. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. PLoS One 4:e7646.

9 Wu, D., P. Hugenholtz, K. Mavromatis, R. Pukall, E. Dalin, N.N. Ivanova, V. Kunin, L. Goodwin, M. Wu, B.J. Tindall, S.D. Hooper, A. Pati, A. Lykidis, S. Spring, I.J. Anderson, P. D'haeseleer, A. Zemla, M. Singer, A. Lapidus, M. Nolan, A. Copeland, C. Han, F. Chen, J.F. Cheng, S. Lucas, C. Kerfeld, E. Lang, S. Gronow, P. Chain, D. Bruce, E.M. Rubin, N.C. Kyrpides, H.P. Klenk, and J.A. Eisen. 2009. A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. Nature 462:1056-1060.

10 Margulies, M., M. Egholm, W.E. Altman, S. Attiya, J.S. Bader, L.A. Bemben, J. Berka, M.S. Braverman, Y.J. Chen, Z. Chen, S.B. Dewell, L. Du, J.M. Fierro, X.V. Gomes, B.C. Godwin, W. He, S. Helgesen, C.H. Ho, G.P. Irzyk, S.C. Jando, M.L. Alenquer, T.P. Jarvie, K.B. Jirage, J.B. Kim, J.R. Knight, J.R. Lanza, J.H. Leamon, S.M. Lefkowitz, M. Lei, J. Li, K.L. Lohman, H. Lu, V.B. Makhijani, K.E. McDade, M.P. McKenna, E.W. Myers, E. Nickerson, J.R. Nobile, R. Plant, B.P. Puc, M.T. Ronan, G.T. Roth, G.J. Sarkis, J.F. Simons, J.W. Simpson, M. Srinivasan, K.R. Tartaro, A. Tomasz, K.A. Vogt , G.A. Volkmer, S.H. Wang, Y. Wang, M.P. Weiner, P. Yu, R.F. Begley, and J.M. Rothberg.. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376-380.

11 Hogg, J., F. Hu, B. Janto, R. Boissy, J. Hayes, R. Keefe, J.C. Post, and G. Ehrlich. 2007. Characterization and modelling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol 8:R103.

12 McCutcheon, J. P. and N.A. Moran. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci USA 104:19392-19397.

13 Reinhardt, J. A., D.A. Baltrus, M.T. Nishimura, W.R. Jeck, C.D. Jones, and J.L. Dangl. 2009. *De novo* assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae* pv. *oryzae*. Genome Res 19:294-305.

14 Worley, K. C. and R.A. Gibbs. 2010. Genetics: Decoding a national treasure. Nature 463:303-304.

15 Tan, Y. P., J. Zheng, S.L. Tung, I. Rosenshine, and K.Y. Leung. 2005. Role of type III secretion in *Edwardsiella tarda* virulence. Microbiology 151:2301-2313.

16 Zheng, J. and K.Y. Leung. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol 66:1192-1206.

17 Zhang, M., K. Sun, and L. Sun. 2008. Regulation of autoinducer 2 production and luxS expression in a pathogenic *Edwardsiella tarda* strain. Microbiology 154:2060-2069.

18 Morohoshi, T., T. Inaba, N. Kato, K. Kanai, and T. Ikeda. 2004. Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. J Biosci Bioeng 98:274-281.

19 Srinivasa Rao, P. S., T.M. Lim, and K.Y. Leung. 2003. Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis. Infect Immun 71:1343-1351.

20 Tan, Y. P., Q. Lin, X.H. Wang, S. Joshi, C.L. Hew, and K.Y. Leung. 2002. Comparative proteomic analysis of extracellular proteins of edwardsiella tarda. Infect Immun 70:6475-6480.

21 Srinivasa Rao, P. S., Y. Yamada, and K.Y. Leung. 2003. A major catalase (KatB) that is required for resistance to H2O2 and phagocyte-mediated killing in *Edwardsiella tarda*. Microbiology 149:2635-2644.

22 Han, H. J., D.H. Kim, D.C. Lee, S.M. Kim, and S.I. Park. 2006. Pathogenicity of *Edwardsiella tarda* to olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). J Fish Dis 29:601-609.

23 Hillier, L.W., G.T. Marth, A.R. Quinlan, D. Dooling, G. Fewell, D. Barnett, P. Fox, J.I. Glasscock, M. Hickenbotham, W. Huang, V.J. Magrini, R.J. Richt , S.N. Sander, D.A. Stewart, M. Stromberg, E.F. Tsung, T. Wylie, T. Schedl, R.K. Wilson, E.R. Mardis. 2008. Whole-genome sequencing and variant discovery in *C. elegans*. Nat Meth 5:183-188.

24 Wang, B., Z.L. Mo, Y.X. Mao, Y.X. Zou, P. Xiao, J. Li, J.Y. Yang, X.H. Ye , K.Y. Leung, P.J. Zhang. 2009. Investigation of EscA as a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon component EseC. Microbiology 155: 1260-1271.

25 Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

26 Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

27 Delcher, A., D. Harmon, S. Kasif, O. White, and S. Salzberg. 1999. Improved microbial gene identification with GLIMMER. Nucl Acids Res 27:4636-4641.

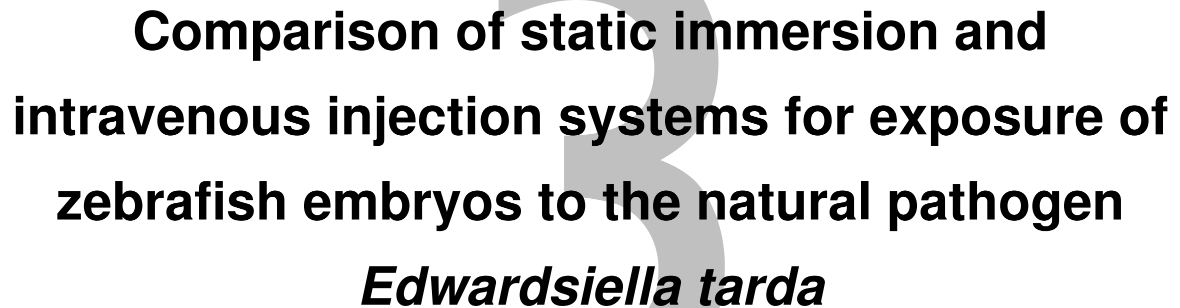
28 Langille, M. G. I. and F.S.L. Brinkman. 2009. IslandViewer: an integrated interface for computational identification and visualization of genomic islands. Bioinformatics 25:664-665.

29 Darling, A. C. E., B. Mau, F.R. Blattner, and N.T. Perna. 2004. Mauve: Multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14:1394-1403.

30 Krzywinski, M., J. Schein, Ä.n. Birol, J. Connors, R. Gascoyne, D. Horsman, S.J. Jones, and M.A. Marra. 2009. Circos: An information aesthetic for comparative genomics. Genome Res 19:1639-1645.

31 Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 24:1596-1599.

32 Zuckerkandl, E., L. Pauling, V. Bryson, and H.J. Vogel. 1965. Evolutionary divergence and convergence in proteins. In Evolving Genes and Proteins, pp. 97-166.



Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen *Edwardsiella tarda*

Joost J. van Soest, Oliver W. Stockhammer, Anita Ordas, Guido V. Bloemberg, Herman P. Spaink and Annemarie H. Meijer

Abstract

The zebrafish embryo is an important *in vivo* model to study the host innate immune response towards microbial infection. In most zebrafish infectious disease models, infection is achieved by micro-injection of bacteria into the embryo. Alternatively, *Edwardsiella tarda*, a natural fish pathogen, has been used to treat embryos by static immersion. In this study we used transcriptome profiling and quantitative RT-PCR to analyze the immune response induced by *E. tarda* immersion and injection.

Mortality rates after static immersion of embryos in *E. tarda* suspension varied between 25-75%, while intravenous injection of bacteria resulted in 100% mortality. Quantitative RT-PCR analysis on the level of single embryos showed that expression of the proinflammatory marker genes *il1b* and *mmp9* was induced only in some embryos that were exposed to *E. tarda* in the immersion system, whereas intravenous injection of *E. tarda* led to *il1b* and *mmp9* induction in all embryos. In addition, microarray expression profiles of embryos subjected to immersion or injection showed little overlap. *E. tarda*-injected embryos displayed strong induction of inflammatory and defence genes and of regulatory genes of the immune response. *E. tarda*-immersed embryos showed transient induction of the cytochrome P450 gene *cyp1a*. This gene was also induced after immersion in *Escherichia coli* and *Pseudomonas aeruginosa* suspensions, but, in contrast, was not induced upon intravenous *E. tarda* injection. One of the rare common responses in the immersion and injection systems was induction of *irg1l*, a homolog of a murine immunoresponsive gene of unknown function.

Based on the differences in mortality rates between experiments and gene expression profiles of individual embryos we conclude that zebrafish embryos cannot be reproducibly infected by exposure to *E. tarda* in the immersion system. Induction of *il1b* and *mmp9* was consistently observed in embryos that had been systemically infected by intravenous injection, while the early transcriptional induction of *cyp1a* and *irg1l* in the immersion system may reflect an epithelial or other tissue response towards cell membrane or other molecules that are shed or released by bacteria. Our microarray expression data provide a useful reference for future analysis of signal transduction pathways underlying the systemic innate immune response versus those

underlying responses to external bacteria and secreted virulence factors and toxins.

Introduction

In the last decade the zebrafish has been firmly established as a model for infectious diseases [1-4]. The increasing popularity of the zebrafish is due to its many useful characteristics. The embryos develop fast *ex utero* and are transparent, making it possible to follow infection *in vivo*. The real-time analysis of infection processes in this model is facilitated by the development of transgenic zebrafish lines with fluorescently marked immune cell populations that can be used in combination with differential fluorescently labelled pathogens [5-8]. In addition, reverse and forward mutagenesis screens are possible, as are antisense knock-down techniques using morpholinos.

Like all jawed vertebrates the zebrafish possesses an innate and adaptive immune system. Innate immunity forms the first line of defence against invading microorganisms. Humoral components of the innate immune system, such as complement and acute phase proteins, were shown to be expressed in embryos and larvae and could be induced by lipopolysaccharide (LPS) challenge or infection [9-10]. The major cell types required for cell-mediated innate immunity, macrophages and neutrophils, also develop during the first days of zebrafish embryogenesis [11-13]. An essential step in innate immunity is the recognition of invading microorganisms by pattern recognition receptor families, the most well studied being the Toll-like receptor (TLR) family. The TLRs activate a signalling pathway leading to a cytokine response and the activation of antimicrobial defence genes [14]. The TLR signalling components are highly conserved between zebrafish and humans [15, 16]. In adults the innate and adaptive immune systems are tightly connected, however in the zebrafish embryo there is a temporal segregation. Whereas innate immunity is functional as early as 1 day post fertilization (dpf) [11, 17, 18], adaptive immunity does not reach full maturity until 2 - 4 weeks post fertilization [13, 19, 20]. This makes the zebrafish embryo a useful *in vivo* model to study vertebrate innate immunity separate from adaptive immunity [3].

Bacterial infection models that have been developed in zebrafish differ in mode and time of infection, inoculum size, pathogenicity and host response [2-4]. The most common method of infection is injection, with the caudal vein as injection site at 1 dpf or the yolk circulation valley at 2 dpf [21]. *Salmonella typhimurium*, a mammalian pathogen, was shown to be lethal to zebrafish embryos after caudal vein injection of a low dose of 25-50 bacteria [22]. In contrast, injection of *E. coli* or an LPS-mutant of *Salmonella typhimurium* (Ra-mutant) was not lethal and the bacteria were cleared efficiently by the embryonic innate immune system [22]. *Pseudomonas aeruginosa*, a broad host range pathogen, capable of infecting plants, invertebrates, and vertebrates, was lethal after injection into the yolk circulation valley at 10-100-fold higher injection inocula than used for *S. typhimurium*, while *Burkholderia cenocepacia* was recently shown to cause a lethal infection upon intravenous injection at a dose of less than 10 bacteria [23-25]. At relatively high doses, also gram-positive bacteria such as *Streptococcus* and *Staphylococcus* species were shown to be capable of causing lethality upon injection in both adults and embryos [26-29]. Injection of embryos with *Mycobacterium marinum* does not lead to a lethal infection, but the immune system is unable to clear this bacterium, leading to a chronic infection. This chronic infection is characterized by aggregation of macrophages into granuloma-like structures similar to the tuberculous granulomas found in human tuberculosis patients [17]. The different infection models were useful to study bacterial virulence factors and the response of the host immune system [3, 9, 30, 31].

For experimental screening, intravenous injection of zebrafish embryos is a relatively low throughput method. For high throughput analysis, such as mutant or drug screens, it is highly desirable to have an easier method of infection like static immersion. Thus far, the only bacterial pathogens that were reported to be capable of infecting zebrafish embryos without the need of injection are *Edwardsiella tarda* and *Flavobacterium columnare* [32,33], which are Gram-negative naturally occurring fish pathogens. *E. tarda* is primarily known for infecting channel catfish, Japanese eel and flounder, in which it causes edwarsiellosis, a generalized septicemia. Pressley and colleagues showed that 24 hpf zebrafish embryos immersed for five hours in a suspension of *E. tarda* had a cumulative mortality rate of 31% after 14 days, compared to 11% in the control embryos [32]. In addition, the zebrafish embryos showed peaks in the

expression of *tnfa* and *il1b* at 2 and 4 hours post exposure, respectively. In adults, *E. tarda* is capable of causing infection by static immersion in combination with dermal abrasion [32].

The aim of this study was to compare the robustness of immersion and injection methods for treatment of 1-day-old zebrafish embryos with *E. tarda* and to identify marker genes that provide a reproducible read-out for the immune response. We set out with a microarray analysis of embryos subjected to immersion in *E. tarda*, and used *E. coli* and *P. aeruginosa*, both non-lethal in the immersion method, for comparison. Several markers were selected for a qPCR time-course analysis of the immersion method and for comparison with caudal vein injection. Marker expression analysis at single embryo level revealed high variation between individuals in response to static immersion. In contrast, qPCR and microarray analysis of single embryos that were systemically infected by caudal vein injection showed a consistent profile of strong activation of the proinflammatory marker genes *il1b* and *mmp9*. We conclude that the injection method is best suited for studying the innate immune response towards systemic infection, while the immersion system is useful for studying epithelial or other tissue responses towards cell membrane or other molecules that are shed or released by bacteria.

Results

Survival of zebrafish embryos after immersion in *E. tarda* suspension

In order to test the *E. tarda* immersion method for future screening applications, we set out to confirm the results obtained by Pressley *et al.* [32]. To this end, zebrafish embryos at 25 hpf were immersed for 5 h in 10^8 CFUs/ml of *E. tarda* and survival was monitored for four days. The ability of *E. tarda* to cause mortality by static immersion was confirmed, while exposure to heat-killed bacteria did not cause mortality (figure 1). However, the percentage of mortality following *E. tarda* exposure after 4 days was found to be quite variable, ranging from 25 % to 75 % between different experiments (figure 1). In addition to *E. tarda*, we also tested the ability of *P. aeruginosa* to establish a lethal infection

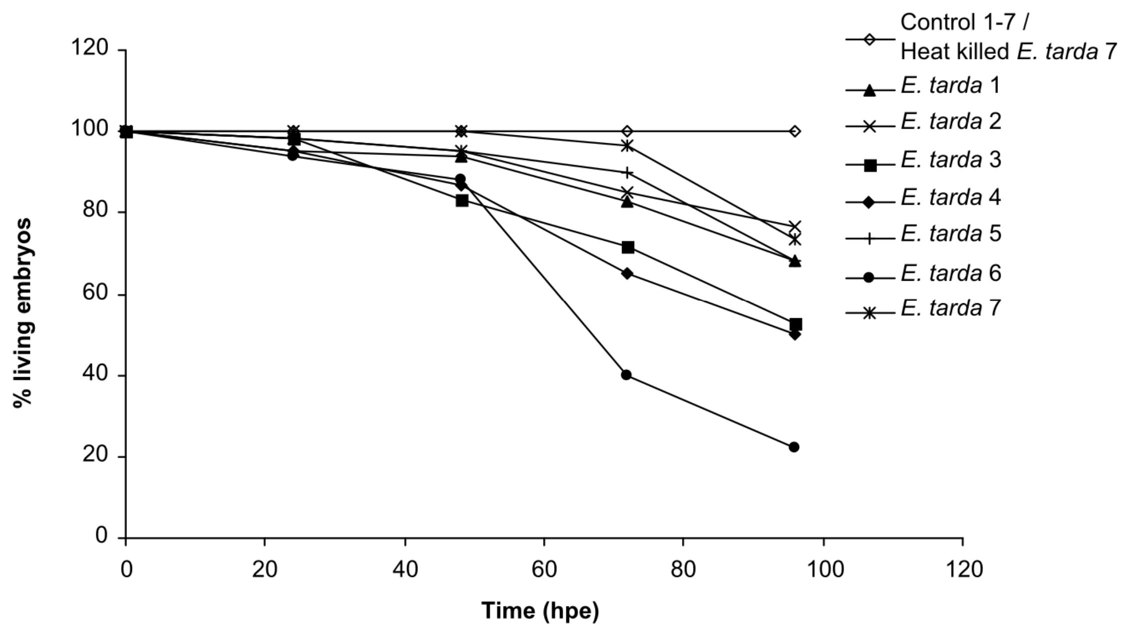


Figure 1. Survival curve of zebrafish embryos treated by immersion in *E. tarda* suspension. For each of the independent 7 experiments 20 embryos at 25 hpf were immersed for 5 h in 10^8 CFUs/ml of *E. tarda* or in clean egg water as a control. Subsequently embryos were washed and transferred to fresh egg water, and survival was monitored for 4 days. In one experiment, heat-killed bacteria (45 min at 95℃) were included as an extra control group. Survival varied between approximately 25 and 75%.

by static immersion, using strains PAO1 and PA14. However, even with concentrations up to 10^9 CFUs/ml, these strains were unable to cause mortality (data not shown).

Microarray analysis of embryos subjected to the immersion system

The variability of the mortality rate in the *E. tarda* immersion assay was high. Therefore, we performed microarray analysis on pools of 20 zebrafish embryos immersed at 25 hpf for 5 h in *E. tarda* to find markers for a reproducible readout of the immune response as alternative. To determine if we could differentiate between reactions towards pathogenic and non-pathogenic bacteria, *E. coli* DH5α and *P. aeruginosa* strains PAO1 and PA14 were tested in addition. Surprisingly, *E. tarda* immersed embryos showed the smallest signature set in terms of gene induction or repression (figure 2a). The number of differentially expressed genes after *E. coli* immersion was four times higher (figure 2b), with *P. aeruginosa* PAO1 immersion six times higher (figure 2c), and with *P.*

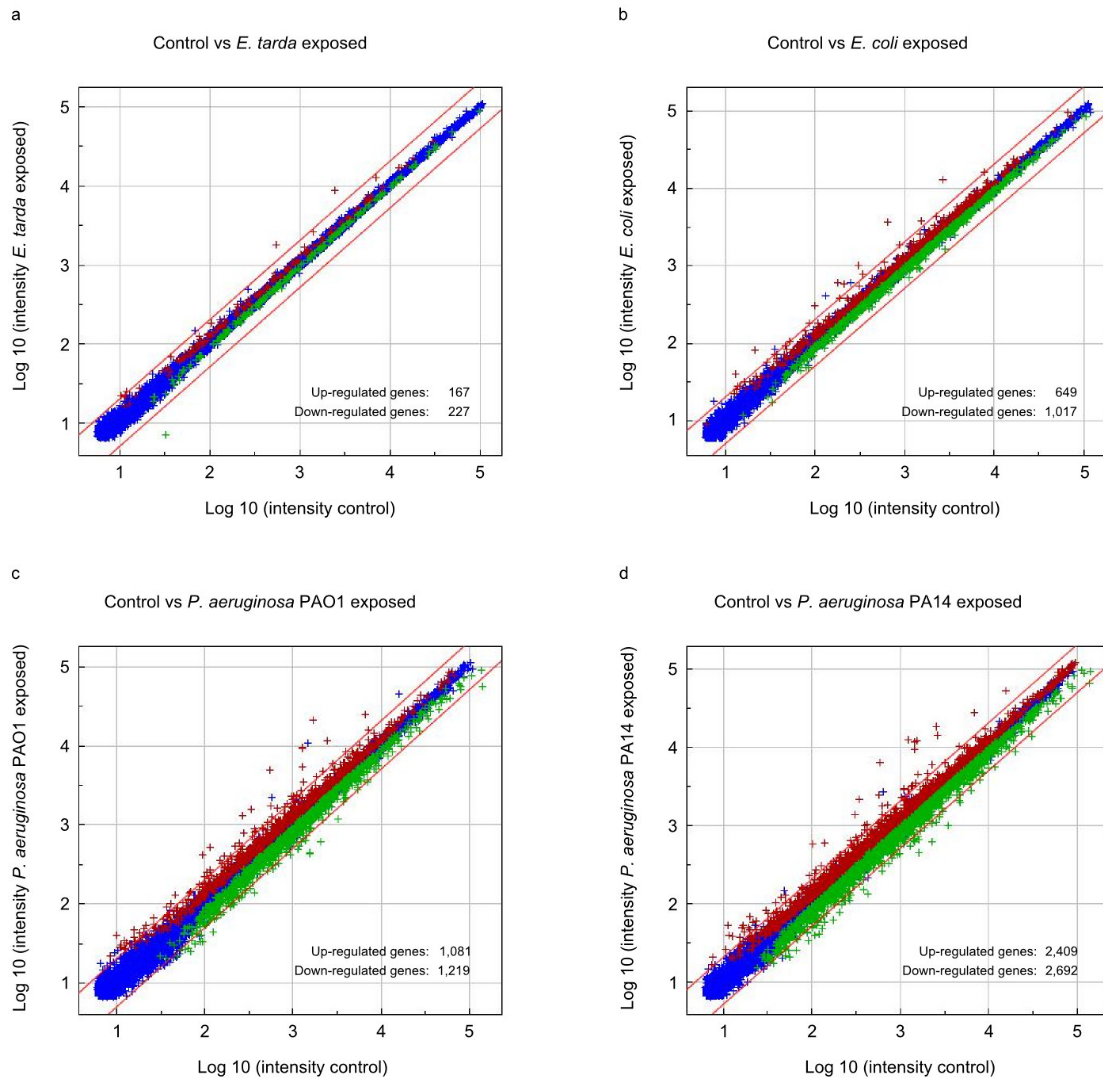


Figure 2. Intensity plots from microarrays of zebrafish embryos treated by immersion in *E. tarda*, *E. coli*, or *P. aeruginosa* suspensions. Embryos were immersed at 25 hpf in *E. tarda* (a), *E. coli* (b) and *Pseudomonas aeruginosa* PAO1 (c) and PA14 (d) suspensions, or in clean egg water as a control. RNA for microarray analysis was isolated from pools of 20 embryos at 5 h post exposure (hpe). RNA samples from embryos exposed to bacterial suspensions and control embryos were hybridized against a common reference from all treatment groups. The intensity plots show comparisons of treatment versus control groups derived from re-ratio analysis against the common reference. Significantly ($P < 0.0001$) up-regulated genes are shown in red, down regulated genes are shown in green and remaining genes in blue.

aeruginosa PA14 immersion 13 times higher (figure 2d) (supplementary table S1).

Surprisingly, very few of the genes up-regulated in the zebrafish embryo after exposure to *E. tarda* were immune related. Although transient induction of *il1b* and *tnfa* was previously observed by Pressley et al. [32], no induction of

these genes was detected in our microarray analysis. Furthermore, expression of *mmp9*, one of the most strongly induced markers after *Salmonella* infection [9], was only slightly up-regulated (1.4 times). In total only 21 genes showed 2-fold or higher levels of up-regulation (P<1.0 E-4) after *E. tarda* exposure (supplementary table S1). Some of these genes have a possible immune-related function. The highest induced gene after exposure to *E. tarda* was *cyp1a* (9.8-fold induction), which encodes a cytochrome P450 enzyme known to be involved in the toxic response [34, 35]. As shown in supplementary table S1, this gene is also highly induced after *P. aeruginosa* and *E. coli* exposure. The second highest induced gene was *zgc:154020* (6.8-fold), which shows 62.1 % identity with *immunoresponsive gene 1* (*irg1*) from *Mus musculus*, a gene with homology to bacterial methylcitrate dehydratase, which is up-regulated in murine macrophages after exposure to LPS, cytokines, and mycobacteria [36-39]. *Zgc:154020* will hereafter be referred to as *irg1-like* (*irg1l)*. Like *cyp1a*, *irg1l* was also highly up-regulated after *P. aeruginosa* and *E. coli* exposure. A third gene with a possible immune-related function is stanniocalcin 1 (*stc1*), which was only induced after *E. tarda* exposure (2.1-fold). Stanniocalcin is involved in Ca^{2+} homeostasis in fish [40, 41], but in humans has also been implicated in inflammatory responses [42-44].

To compare the responses of zebrafish embryos to immersion with the different bacterial strains, we performed a gene ontology analysis on all genes showing differential expression in the microarray analysis (supplementary table S2). In embryos immersed in *P. aeruginosa* PAO1 and PA14, and in *E. coli*, but not in embryos immersed in *E. tarda*, genes with the GO-term “response to stimulus” were significantly enriched. The largest group of up-regulated genes with this GO-term (61 genes) was observed in the case of immersion with *P. aeruginosa* PA14. Further analysis into the “response to stimulus” GO category revealed that in particular genes with the GO-term “response to stress” were up-regulated (41 genes in the case of PA14), while only few genes were associated with the GO-term “immune response” (6 genes in the case of PA14). An overview of the genes with the GO-term “response to stimulus” that were up-regulated in response to the different bacteria is given in supplementary table S3. The lack of induction of many of the known immune response genes after 5 hours of exposure to *E. tarda* suggests that at that time, tissue infection has not yet been established.

Time course analysis of marker gene expression in the immersion system

To determine whether a stronger immune response is induced at later time points after exposure to *E. tarda*, we performed a time-course qPCR analysis of several immune related genes. In addition to the putative immune markers *cyp1a*, *irg1l* and *stc1* found in the microarray analysis (supplementary table S1), the known immune markers *il1b*, *mmp9* and *tnfa* were chosen for the time course analysis of the *E. tarda* exposure. Embryos immersed in 10^8 CFUs/ml of *E. tarda* were snap-frozen in pools of 20 embryos at 5, 24 and 48 hours post exposure (hpe). RNA was isolated from pools of embryos collected at each time point and the expression of the chosen markers was analyzed. The results showed that *cyp1a* is primarily a marker for the early response towards *E. tarda*, showing 10 times higher expression in *E. tarda*-exposed than in untreated embryos at 5 hpe, but less than 3-fold induction at 24 hpe and no induction at

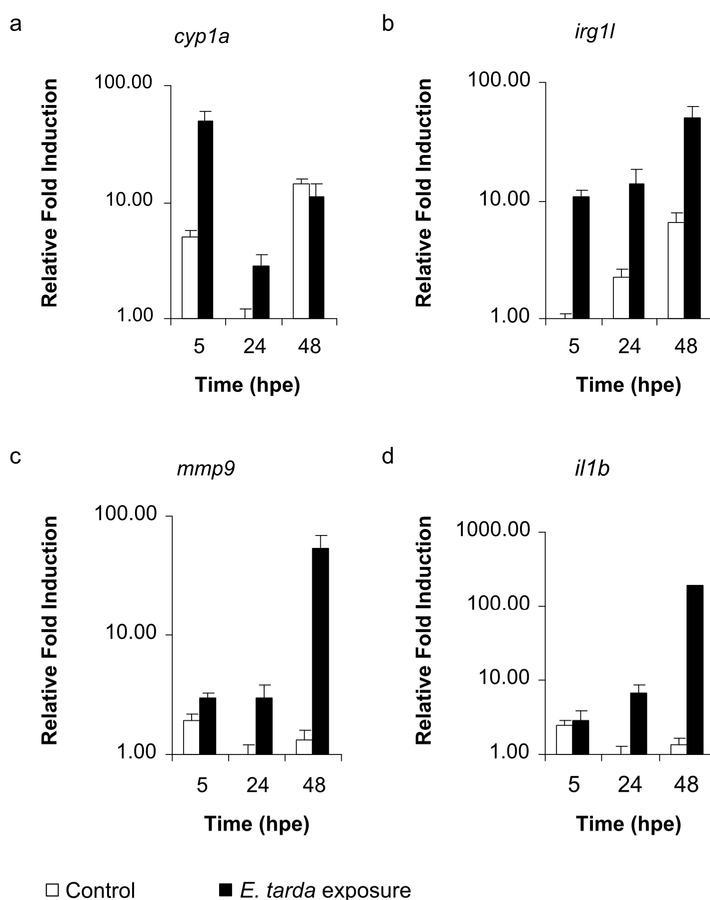


Figure 3. Time course analysis of marker gene expression in embryos treated by immersion in *E. tarda* suspension. Embryos were immersed at 25 hpf for 5 h in *E. tarda* suspension or in clean egg water as a control. Subsequently, embryos were washed and transferred to fresh egg water. RNA was isolated from pools of 20 embryos at 5, 24 and 48 h after the start of exposure (hpe) and the expression levels of *cyp1a* (a), *irg1l* (b), *mmp9* (c), and *il1b* (d) were quantified by qPCR. A representative example of three independent experiments is shown. Relative induction levels are shown with the lowest expression level set at 1.

48 hpe (figure 3a). This might suggest that *cyp1a* induction is the result of an epithelial response. *Irg1l* was induced between 10 and 50 fold at all time points tested (figure 3b).The *mmp9* (figure 3c) and *il1b* genes (figure 3d) showed little to no induction at 5 hpe, but induction started to increase at 24 hpe and reached 54 to 212-fold induction at 48 hpe. The induction of *tnfa* and *stc1* was highly variable between the different experiments and therefore excluded in further analyses (data not shown). To test the possibility that the early response in the immersion system might be elicited by cell membrane components or other molecules released by the bacteria, we separated the *E. tarda* suspension used for the immersion experiments into two fractions by centrifugation. Exposure of embryos either to the wash fluid obtained after centrifugation or to the resuspended bacterial pellet, showed that expression of *cyp1a* and *irg1l* was induced to higher levels by the wash fluid than by the washed bacteria, while the opposite was observed for the induction of *il1b* and *mmp9* (Figure 4). Therefore, the early transcriptional induction of *cyp1a* and *irg1l* appears not to be due to bacterial infection.

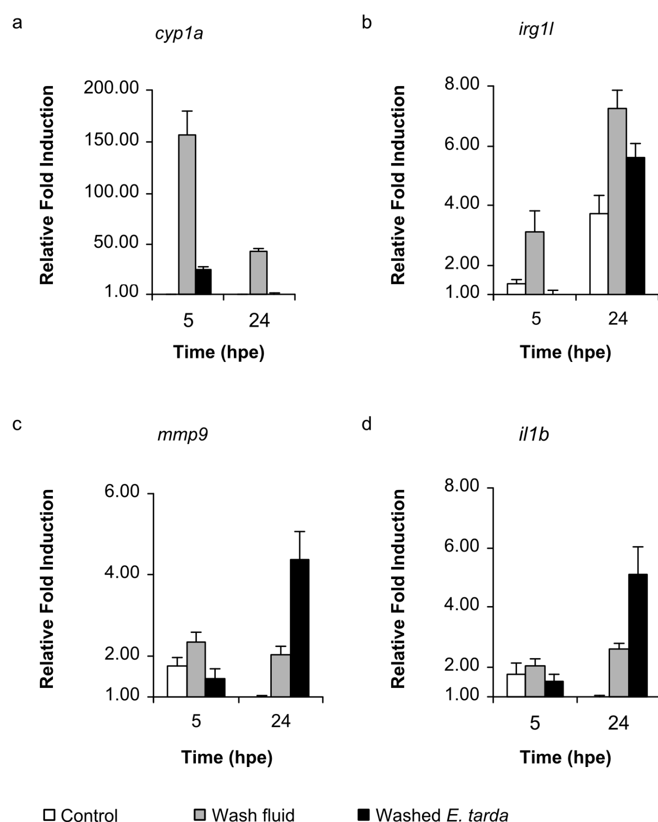


Figure 4. Marker gene expression in immersion tests after fractionation of *E. tarda* suspension.
The *E. tarda* suspension as used for the immersion experiments in Figure 1-3 was separated into two fractions by centrifugation. Embryos were immersed at 25 hpf for 5 h in the wash fluid obtained after centrifugation, or in the resuspended bacterial pellet (washed bacteria), or in clean egg water as a control. Subsequently, embryos were washed and transferred to fresh egg water. RNA was isolated from pools of 20 embryos at 5 and 24 h after the start of exposure (hpe) and the expression levels of *cyp1a* (a), *irg1l* (b), *mmp9* (c), and *il1b* (d) were quantified by qPCR. A representative example of two independent experiments is shown. Relative induction levels are shown with the lowest expression level set at 1.

Immune response in single embryos after static immersion in *E. tarda*

The variability in mortality rates in the static immersion system, led us to hypothesize that not all embryos become systemically infected with this method. At 4 days after *E. tarda* immersion, none of the surviving embryos, even those that were close to dying, showed clear fluorescence of the mCherry marker plasmid. Subsequently, we plated individual surviving embryos for CFU counting. From five surviving embryos, of which three showed a slow heart beat indicative of approaching death, we obtained CFU counts of 140 to 690 per individual embryo. In contrast, the egg water medium of these embryos, kept individually in well plates, contained between 80,000 and 300,000 CFUs. It cannot be ascertained from CFU plating if the surviving embryos were actually

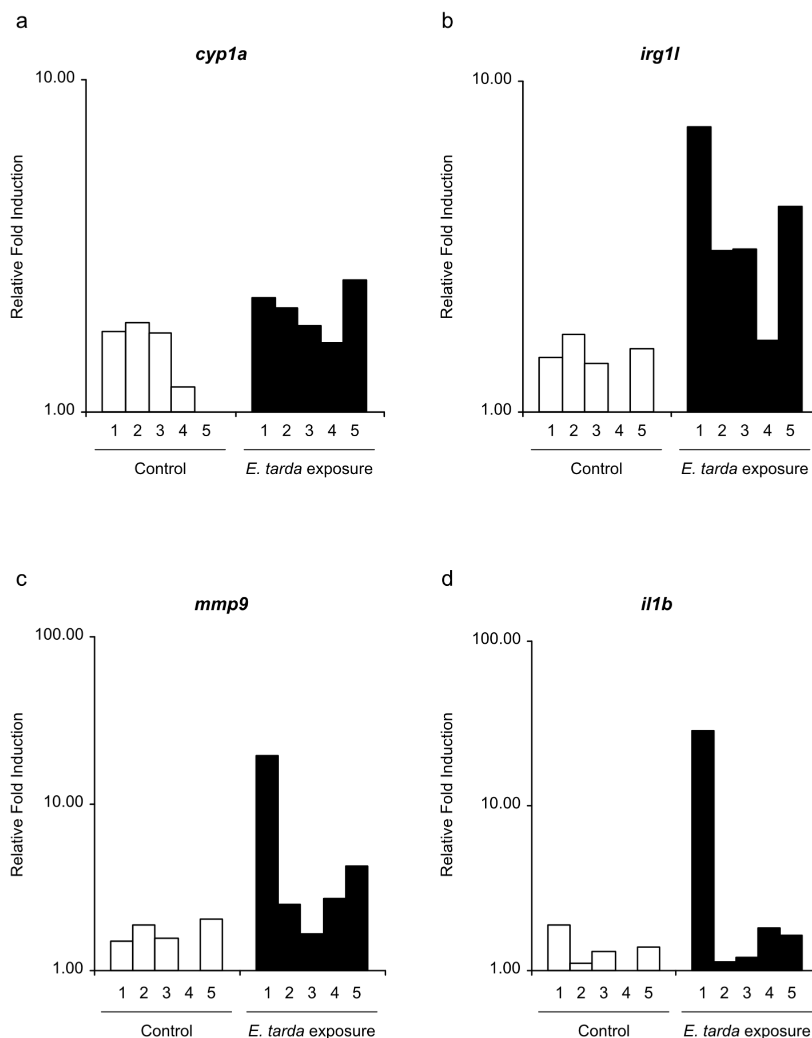


Figure 5. Marker gene expression in individual embryos treated by immersion in *E. tarda* suspension. Pools of 20 embryos were immersed at 25 hpf for 5h in *E. tarda* suspension or in clean egg water as a control. Subsequently, embryos were washed and transferred to fresh egg water. RNA was isolated from 5 single embryos at 48 h after the start of exposure (hpe) and expression levels of *cyp1a* (a), *irg1l* (b), *mmp9* (c) and *il1b* (d) were measured by qPCR. Relative induction levels are shown with the lowest expression level set at 1.

infected with low numbers of bacteria or that the low CFU counts resulted from bacteria sticking to the surface epithelium of these embryos. However, it is clear that the surviving embryos did not carry heavy infections.

To further test our hypothesis that not all embryos are systemically infected after immersion, we used an RNA-isolation protocol for single embryos [45]. Five single embryos exposed for 5 h to 10^8 CFUs/ml of *E. tarda* and five single embryos grown under non-inoculated circumstances were snap-frozen at 48 hpe. RNA was isolated from each embryo and qPCR analysis was done on *cyp1a*, *irg1l, mmp9*, and *il1b* (figure 5). Expression of *cyp1a* showed little to no induction, similar to what we observed in the analysis of pools of embryos at 48 hpe. The difference in induction of *il1b* and *mmp9* between individual embryos was much more pronounced than we initially expected. Out of the five embryos tested, only one showed a high induction of both markers compared to the control embryos. All embryos showed induction of *irg1l*, but a strong induction of this gene was only observed in the embryo that showed a high *il1b* and *mmp9* induction, which might indicate that *irg1l* is involved in both an initial response to bacterial components and a later systemic immune response.

Immune response in single embryos after caudal vein injection of *E. tarda*

Results of immersion experiments suggested that induction of *il1b* and *mmp9* expression may be specifically correlated with systemic infection. To exclude that the large variation in *il1b* and *mmp9* induction found after immersion might be due to individual variation in responsiveness of different embryos, we decided to compare the immersion system with intravenous infection. Embryos were injected in the caudal vein with 200 CFUs of *E. tarda* at 28 hpf and snap-frozen individually at 4 and 8 hours post infection (hpi) after which RNA was isolated. As before, qPCR analysis was done on *cyp1a*, *irg1l*, *mmp9*, and *il1b* (figure 6). The results show that the genes *irg1l, mmp9*, and *il1b* were induced at much higher levels than in the immersion system, whereas *cyp1a* showed similar induction (2-5-fold) as in the immersion system in some embryos or no induction in other embryos. Expression of *il1b* was clearly induced in all embryos at 4 hpi, while *mmp9* was induced only in two embryos at this time point and *irg1l* was not induced. Although induction of *mmp9* and *irg1l* at 8 hpi

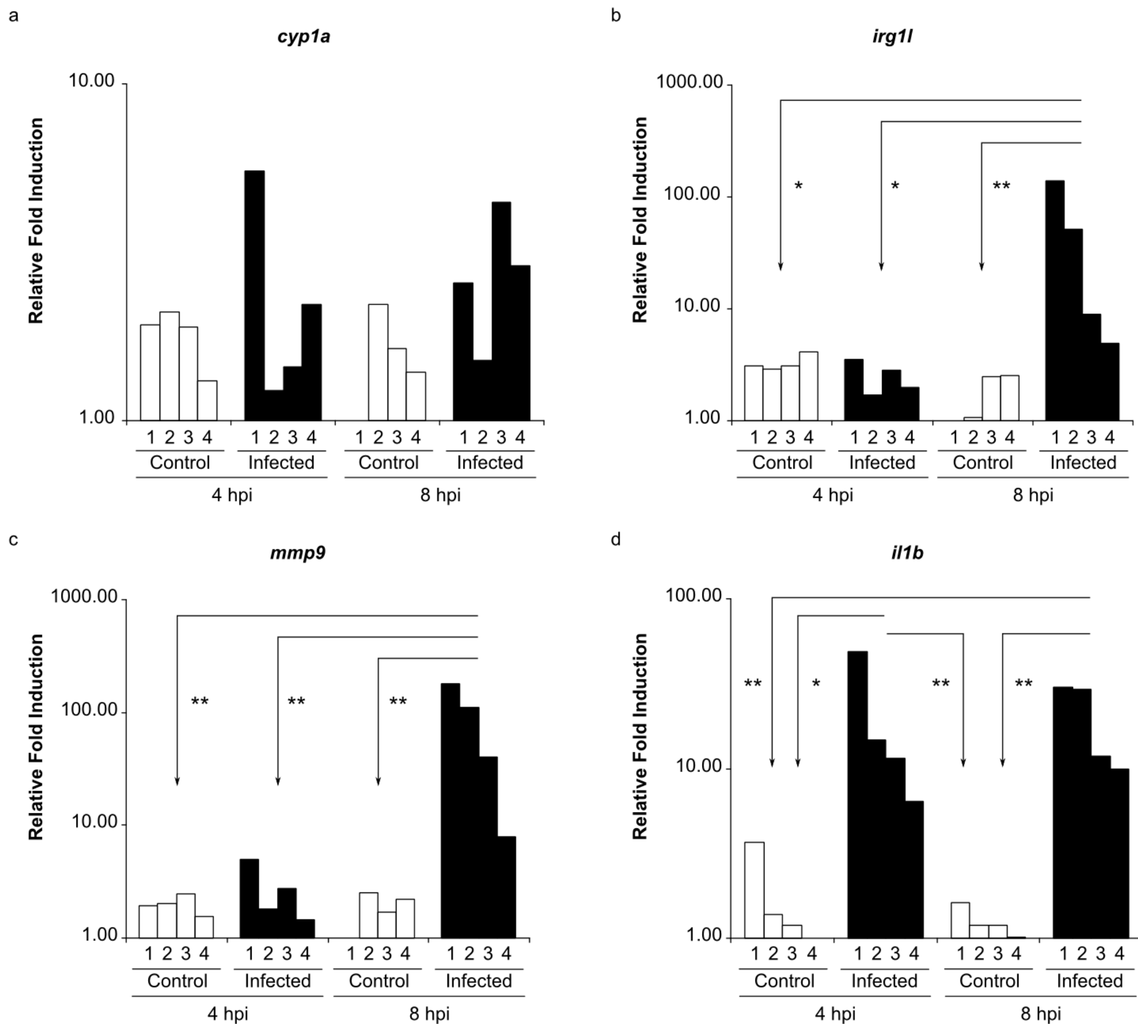


Figure 6. Marker gene expression in individual embryos in response to injection of *E. tarda*. Expression levels of *cyp1a* (a), *irg1l* (b), *mmp9* (c) and *il1b* (d) were measured by qPCR in 4 single embryos at 4 h and 8 h after injection (hpi) of approximately 200 CFUs of *E. tarda* into the caudal vein of embryos at 28 hpf. Control embryos were injected with PBS. Relative induction levels are shown with the lowest expression level set at 1. Lines with * indicate a significant difference of P < 0.05. Lines with ** indicate a significant difference of P <0.01 (tested by two-way ANOVA analysis of log-transformed data with the Bonferroni method as post-hoc test).

was consistent, the induction levels showed large variation, ranging between 7- and 180-fold for *mmp9* and between 4- and 140- fold for *irg1l*. Induction levels of *il1b* between individual embryos were the least variable, ranging between 5- and 50-fold at 4 hpi and between 10- and 30-fold at 8 hpi. Compared to injection of 200 CFUs, injection of 25 CFUs resulted in lower *il1b* and *mmp9* induction levels (supplementary figure S1). Furthermore, these genes were induced at much higher levels by 200 CFUs of live bacteria than by the same dose of heat-killed bacteria.

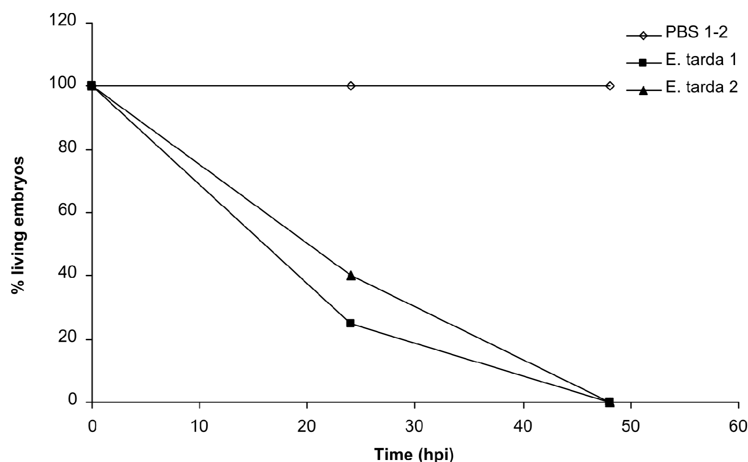
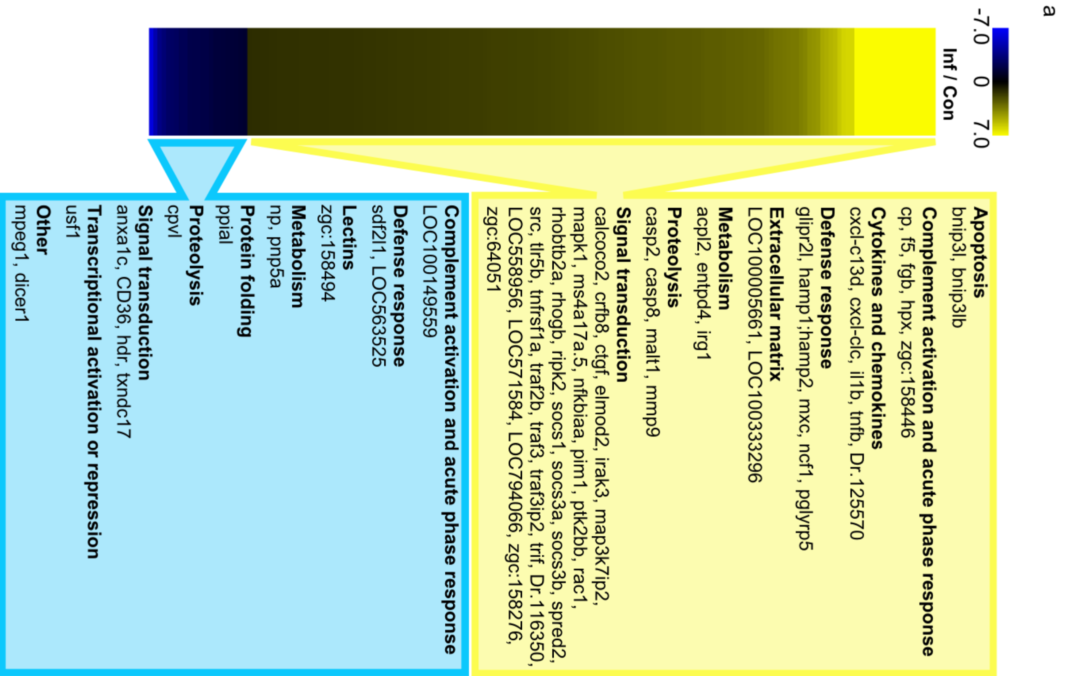


Figure 7. Survival curve of embryos infected by injection of *E. tarda*. Survival after injection of approximately 200 CFUs of *E. tarda* into the caudal vein of embryos at 28 hpf was monitored for two days, after which no embryos survived. Control embryos were injected with PBS. Experiments were performed in duplicate. In each experiment 20 embryos were used per treatment group.

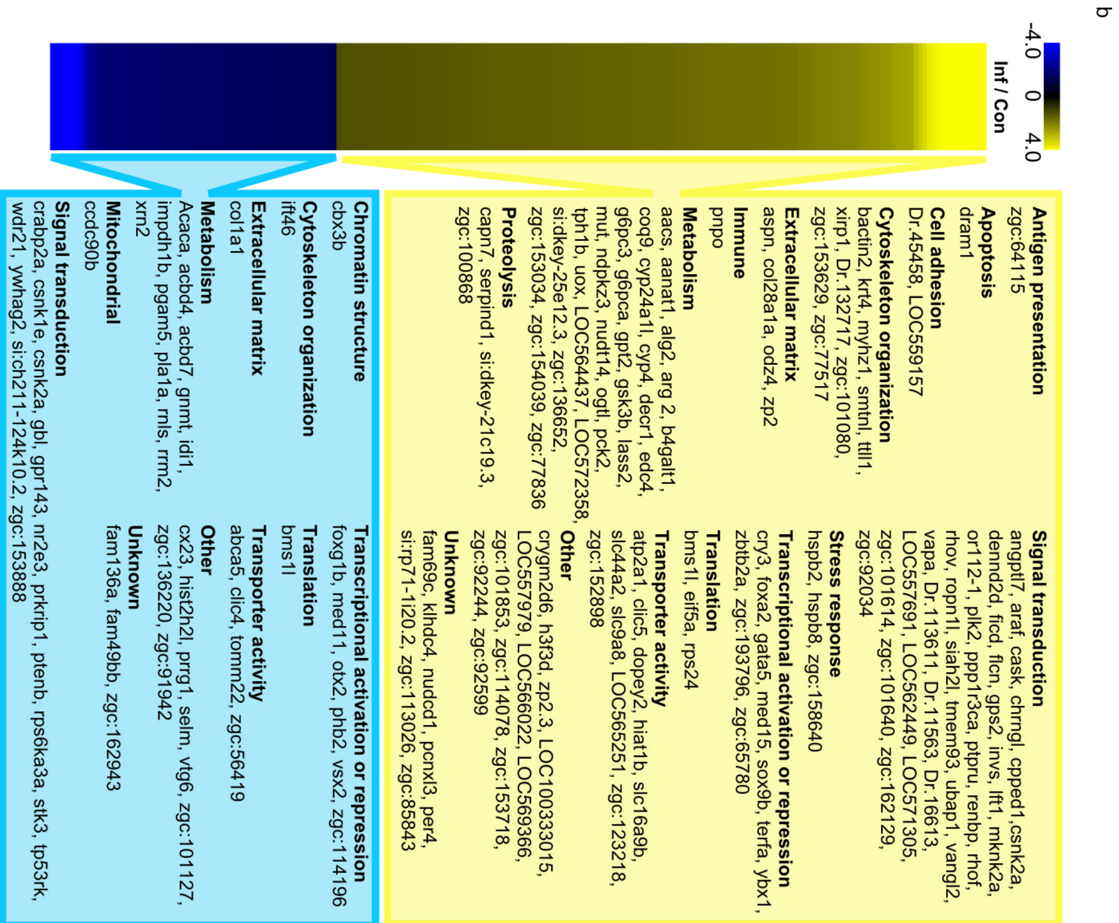
In addition to the analysis of *cyp1a*, *irg1l*, *mmp9* and *il1b* induction, we monitored the embryos for two days after injection for appearance of fluorescence from the mCherry-labelled *E. tarda* and for survival. In all injection experiments embryos showed fluorescence at 24 hpi (data not shown) and mortality after injection was very consistent, reaching 100% at 48 hpi (figure 7). Based on these results we conclude that reproducible systemic infection of zebrafish embryos can be achieved by microinjection of *E. tarda* bacteria, accompanied by induction of *il1b* and *mmp9* expression.

Microarray analysis of embryos infected by caudal vein injection

Microarray analysis was used to further characterize the immune response in response to microinjection of *E. tarda* bacteria and compare this with the previous microarray results of the immersion system and with our published data of the response to *Salmonella typhimurium* injection [9]. Single infected and mock-injected embryos were analyzed at 8 hpi in triplicate. In gene ontology analysis we observed significant enrichment of the GO-terms “immune system process” and “response to stimulus” (supplementary table S4), whereas these GO-terms were not enriched in results of the immersion method (supplementary table S2). In addition, functional annotation using DAVID [46] showed significant enrichment of the KEGG pathways for apoptosis and for Toll-like receptor, adipocytokine, NOD-like receptor, insulin, MAP kinase, RIG-I-like receptor, ErbB, and Jak-Stat signalling. Manual annotation of the induced



a
Inf / Con
-7.0 0 7.0
Apoptosis
bnip3l, bnip3lb
Complement activation and acute phase response
cp, f5, fgb, hpx, zgc:158446
Cytokines and chemokines
cxcl-c13d, cxcl-clc, il1b, tnfb, Dr.125570
Defense response
glipr2l, hamp1/hamp2, mxc, ncf1, pglyrp5
Extracellular matrix
LOC100005661, LOC100333296
Metabolism
acpl2, entpd4, irg1
Proteolysis
casp2, casp8, malt1, mmp9
Signal transduction
calcoco2, crfb8, ctgf, elmod2, irak3, map3k7ip2, mapk1, ms4a17a.5, nfkbiaa, pim1, ptk2bb, rac1, rhobtb2a, rhogb, ripk2, socs1, socs3a, socs3b, spred2, src, tlr5b, tnfrsf1a, traf2b, traf3, traf3ip2, trif, Dr.116350, LOC558956, LOC571584, LOC794066, zgc:158276, zgc:64051
Complement activation and acute phase response
LOC100149559
Defense response
sdf2l1, LOC563525
Lectins
zgc:158494
Metabolism
np, pnp5a
Protein folding
ppial
Proteolysis
cpvl
Signal transduction
anxa1c, CD36, hdr, txndc17
Transcriptional activation or repression
usf1
Other
mpeg1, dicer1



b
Inf / Con
-4.0 0 4.0
Antigen presentation
zgc:64115
Apoptosis
dram1
Cell adhesion
Dr.45458, LOC559157
Cytoskeleton organization
bactin2, krt4, myhz1, smtnl, ttll1, xirp1, Dr.132717, zgc:101080, zgc:153629, zgc:77517
Extracellular matrix
aspn, col28a1a, odz4, zp2
Immune
pnpo
Metabolism
aacs, aanat1, alg2, arg 2, b4galt1, coq9, cyp24a1l, cyp4, decr1, edc4, g6pc3, g6pca, gpt2, gsk3b, lass2, mut, ndpk3, nudt14, ogtl, pck2, tph1b, uox, LOC564437, LOC572358, si:dkey-25e12.3, zgc:136652, zgc:153034, zgc:154039, zgc:77836
Proteolysis
capn7, serpind1, si:dkey-21c19.3, zgc:100868
Signal transduction
angptl7, araf, cask, chmgl, cpped1, csnk2a, dennd2d, ficd, flcn, gps2, invs, lft1, mknk2a, or112-1, plk2, ppp1r3ca, ptpru, renbp, rhof, rhov, ropn1l, siah2l, tmem93, ubap1, vangl2, vapa, Dr.113611, Dr.11563, Dr.16613, LOC557691, LOC562449, LOC571305, zgc:101614, zgc:101640, zgc:162129, zgc:92034
Stress response
hspb2, hspb8, zgc:158640
Transcriptional activation or repression
cry3, foxa2, gata5, med15, sox9b, terfa, ybx1, zbtb2a, zgc:193796, zgc:65780
Translation
bms1l, eif5a, rps24
Transporter activity
atp2a1, clic5, dopey2, hiat1b, slc16a9b, slc44a2, slc9a8, LOC565251, zgc:123218, zgc:152898
Other
crygm2d6, h3f3d, zp2.3, LOC100333015, LOC557979, LOC566022, LOC569366, zgc:101853, zgc:114078, zgc:153718, zgc:92244, zgc:92599
Unknown
fam69c, klhdc4, nudcd1, pcnxl3, per4, si:rp71-1i20.2, zgc:113026, zgc:85843
Chromatin structure
cbx3b
Cytoskeleton organization
ift46
Extracellular matrix
col1a1
Metabolism
Acaca, acbd4, acbd7, gnmt, idi1, impdh1b, pgam5, pla1a, mls, rrm2, xrn2
Mitochondrial
ccdc90b
Signal transduction
crabp2a, csnk1e, csnk2a, gbl, gpr143, nr2e3, prkrip1, ptenb, rps6ka3a, stk3, tp53rk, wdr21, ywhag2, si:ch211-124k10.2, zgc:153888
Transcriptional activation or repression
foxg1b, med11, otx2, phb2, vsx2, zgc:114196
Translation
bms1l
Transporter activity
abca5, clic4, tomm22, zgc:56419
Other
cx23, hist2h2l, prrg1, selm, vtg6, zgc:101127, zgc:136220, zgc:91942
Unknown
fam136a, fam49bb, zgc:162943

Figure 8. Heat-map and annotations of genes differentially expressed at 8 h after injection of *E. tarda*. Genes were manually annotated and assigned to functional groups based on GO annotations of the zebrafish genes and their human homologues and on searching of PubMed abstracts. (a) Genes previously implicated to be involved in the immune response or novel genes with strong sequence similarity to those genes, (b) genes with known or predicted functions not previously linked to the immune response. Up-regulation is indicated by increasingly bright shades of yellow and down-regulation by increasingly bright shades of blue. The significance cut-off for the analysis was set at $P < 0.00001$.

gene group showed several representatives of the categories complement activation and acute phase response, immune-related transcription factors and signalling components, cytokines and chemokines, apoptosis, and defence response (figure 8a). In addition, many genes that were not previously linked to the immune response were differentially expressed, including genes involved in signal transduction, transporting activity and metabolism (figure 8b). Out of 498 significantly regulated probes at 8 hpi (supplementary table S5), only 2 down-regulated probes (for *vtg6* and an unannotated transcribed locus) and 1 up-regulated probe (for an unknown gene) were also significantly changed in the immersion system at 5 hpe. The microarray comparison supports that the transcriptional signatures of embryos subjected to immersion and injection are markedly different, although it should be noted that the immersion and injection data are not directly comparable due to a few hours difference in time to respond to the bacteria and in the developmental stage of assessment. The *E. tarda* injection microarray data were also compared with our previous microarray data set of intravenous *Salmonella typhimurium* infection of embryos at 2, 5 and 8 hpi [9]. This comparison showed an overlap of 141 probes with significantly changed expression in response to both pathogens (supplementary table S5). These probes represented among others *tnfb*, *il1b*, *cxcl-c1c*, *mmp9*, *ncf1*, *mxc*, *pglyrp5*, *hamp1* and several signal transduction (e.g. *tlr5b*, *irak3*, *nfkbiaa*, *pim1*, *socs1/3a/3b*) and transcription factor genes (e.g. *atf3*, *elf3, fos*, *junb*, *irf9*/*11*, *rel*, *stat1*) (supplementary table S5).

Discussion

Zebrafish is being established as an alternative vertebrate model to murine models for infection research. To enable large scale mutant and chemical screening the development of an easily applicable infection test system is highly desired. In this report we studied the effectiveness and variability of treatment of zebrafish embryos by static immersion in *Edwardsiella tarda*, a method previously described by Pressley et al. [32], in comparison with the caudal vein injection method.

In order to perform large scale screenings, a model test system should be optimized for a reproducible response. Our results confirmed the ability of *E. tarda* to cause mortality in zebrafish embryos after static immersion. However, the mortality rate was highly variable between different experiments, ranging from 25 – 75 %, comparable to the mortality rate of 31 % reported by Pressley *et al* [32]. In order to find a more reproducible readout, we performed microarray analysis on zebrafish embryos that had been exposed to *E. tarda* by static immersion. Surprisingly, only a small number of genes showed differential expression. In contrast, a much larger number of genes were regulated by immersion in bacterial suspensions of *E. coli* and *P. aeruginosa* strains PAO1 and PA14 that do not cause any mortality. In addition, very few immune-related genes were induced by immersion in *E. tarda* and we observed no induction of *il1b* and *tnfa*, which showed transient induction patterns between 2 and 12 hpi in the study of Pressley et al. [32].

Interestingly, *cyp1a* was highly induced by all tested bacteria. In *E. tarda* immersion experiments the induction of this gene preceded that of *il1b* and *mmp9* induction. Our results suggest that this gene is not induced by direct exposure to the bacteria, but by released cell membrane components or other molecules. Expression of *cyp1a* was most strongly induced by *P. aeruginosa*. *Cyp1a* is known to be induced by toxic chemicals in vascular endothelium, but also in the epithelium of the gills [34, 35]. *P. aeruginosa* PAO1 and PA14 are known to secrete large amounts of toxins and protein virulence factors [47-50]. Since *cyp1a* belongs to the cytochrome P450 family, its induction might be involved in a detoxification response. The observation that many of the genes regulated by *P. aeruginosa* are associated with the GO term “response to stress”, and the lack of enrichment of genes with the GO-term “immune system

process" is consistent with a response to toxins rather than an immune response to systemic infection.

A further time-course analysis by qPCR of pools of embryos subjected to *E. tarda* immersion showed strong induction of *il1b* and *mmp9* after 48 hours. In addition, the *irg1l* gene, one of the few immune-related genes identified in the microarray study, was also induced at later time points after exposure to *E. tarda*. The *irg1l* gene is homologous to mammalian *irg1*, expression of which in murine macrophages is induced by cytokines, agonists of TLR signalling, and by mycobacterial infections [36-39]. Sequence similarity of *irg1l* and mammalian *irg1* with bacterial methylcitrate dehydratases suggests an important role in metabolism, but the function in vertebrates remains unknown. When we analyzed gene expression at the level of single embryos we observed that *il1b* and *mmp9* were highly expressed in only one out of five treated embryos. Expression of *irg1l* was induced in all embryos, but only at a high level in the same embryos that also showed induction of *il1b* and *mmp9*. One possible explanation for the variable results of the static immersion assay is that embryos can individually differ in their resistance towards *E. tarda*. To test this, we compared the immersion system with intravenous injection of bacteria. In contrast to the relatively low and highly variable mortality rates that we observed with the immersion method, injection of bacteria resulted in a reproducible rate of 100% mortality within 2 days. Strong individual differences in levels of gene expression were also observed in the injection system, but nevertheless, induction of the proinflammatory marker genes *il1b* and *mmp9* was positive in all embryos and their induction levels correlated with the dose of live bacteria injected. Furthermore, microarray experiments with single injected embryos showed a consistent profile of strong activation of proinflammatory and defence genes and regulatory genes of the immune response. The observed gene expression profiles are concordant with those observed for intravenous *Salmonella typhimurium* infection of embryos at similar time periods after injection [9]. Detailed comparisons of the responses to *E. tarda* and *S. typhimurium* infections will be part of a follow-up study that will also address the function of essential immune regulators in these models.

Since only a subset of embryos in the immersion assay showed induction of immune response markers and mortality it is conceivable that only these embryos were systemically invaded by *E. tarda* bacteria or that non-responsive

embryos were invaded by a much lower number of bacteria. Neither fluorescence monitoring nor CFU plating indicated that embryos become heavily infected close before dying. On the contrary, bacteria were present in high abundance in the egg water medium and only few were associated with dying embryos. It therefore remains uncertain whether infection or toxic insult is the actual cause of mortality in the immersion system. It is possible that the variable immune gene inductions and mortality rates resulted from slight epithelial damage to embryos that occurred during dechorionating and washing procedures, providing sites of entry for bacteria. Instead of exposure at 1 dpf, we used the same immersion protocol on embryos of 3 dpf, which is the developmental stage when the mouth opens and the gut begins to be colonized by environmental bacteria [51]. We followed survival until 5 dpf, which is the time-point up to which larvae do not fall under the European animal experimentation law, but did not observe mortality within that time (data not shown).

Besides being more practical for high-throughput screening, an immersion system might be preferred as a more natural route of infection compared to injection methods. However, we conclude that the *E. tarda* immersion method as applied here on 1-day-old zebrafish embryos is not suitable to achieve reproducible systemic infection. Therefore, unless a more virulent strain can be identified, injection remains the preferred method of infection for screening purposes. On the other hand, the immersion system is shown to be useful for studying epithelial or other tissue responses towards cell membrane or other molecules that are shed or released by bacteria. An alternative solution for high-throughput screening of systemic infection is the use of robotic yolk injection system recently developed for *Mycobacterium marinum* infection [52]. However, the wild type *E. tarda* FL6-60 strain used here causes early lethality after yolk injection (data not shown). The use of less virulent (wild type or mutant) strains might provide a solution for this problem. In any case, our gene expression profiling data sets will be necessary for comparisons to the immune response in such alternative yolk infection methods.

Conclusions

Zebrafish embryos proved to be remarkably resistant to becoming systemically infected after immersion in bacterial suspensions of *E. tarda*, whereas they are strongly susceptible to intravenous injection of this pathogen. While the microarray expression profile of intravenously infected embryos indicates a strong inflammatory response, the transcriptional signature of embryos subjected to immersion was markedly different. Our data suggest that most of the early transcriptional responses in the immersion system may reflect an epithelial or other tissue response towards cell membrane or other molecules that are shed or released by bacteria. Therefore, our studies on the expression analysis in the bacterial immersion system will be useful for future analysis of signal transduction pathways underlying responses to external bacteria and secreted putative virulence factors and toxins. Transient induction of the cytochrome P450 gene *cyp1a* was specifically observed in immersion experiments but not when embryos were systemically infected by injection. In addition, our identification of the *irg1l* gene as a rapid response factor to externally added bacteria deserves further study of the underlying signal transduction pathway as compared to systemic tissue responses. Although *irg1l* is also up-regulated during systemic infection, its expression kinetics in embryos immersed in *E. tarda* is very different from that of well-known inflammation genes such as *il1b* and *mmp9*. Considering the important function of epithelial cells in cross talk with cells of the innate immune system, as recently underscored by studies in zebrafish [31], further analysis of infection modes using the identified marker genes will help to better understand the systemic response of tissues toward an infection in a whole organism context.

Materials & methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). An albino strain was used for all immersion and injection experiment, except for the microarray study

of injected embryos that was performed with wild type zebrafish. Embryos were grown at 28.5-30℃ in egg water (60 µg/ml Instant Ocean salts). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02 % buffered 3-aminobezoic acid ethyl ester (tricaine; Sigma-Aldrich).

Bacterial immersion and injection experiments

Edwardsiella tarda strain FL6-60 was grown over night on tryptic soy agar (Difco) at 28 ˚C and subsequently a liquid culture in tryptic soy broth (TSB, Difco) was inoculated and grown overnight at 28 ˚C with shaking at 150 rpm. *Pseudomonas aeruginosa* PAO1 and PA14 and *Escherichia coli* were grown over night in Luria-Bertani broth (LB) [53] at 37 ˚C. For immersion experiments bacterial cultures were centrifuged in 50 ml tubes and the pellet was subsequently suspended in egg water to a final 10^8 CFU/ml for *E. tarda* and *E. coli*, and 10^9 CFU/ml for *P. aeruginosa*. Embryos were dechorionated at 24 hpf by a 3-5 min pronase treatment (2 mg/ml in embryo medium prewarmed to 30 ˚C) and left to recover for one hour in egg water. Subsequently pools of 20 embryos in 6-well plates were immersed in 5 ml of the bacterial suspension and incubated for 5 hours at 28 ˚C. After 5 hours of incubation, the embryos were either snap-frozen in liquid nitrogen or transferred to a new 6-wells plate, washed 3 times in egg water, and incubated at 28 ˚C in 5 ml of egg-water. For CFU plating experiments, embryos were kept individually in 2.5 ml of egg water in 24-well plates.

For caudal vein injection experiments, *E. tarda* labelled with mCherry [54] was washed and subsequently suspended in PBS (phosphate-buffered saline) to a final 10^8 CFU/ml. Embryos were manually dechorionated at 24 hpf. Approximately 200 CFUs of *E. tarda* were injected into the blood island after the onset of blood flow at 28 hpf, or PBS was injected as a control. After injection, embryos were kept at 28 ℃ and snap-frozen in liquid nitrogen at the required time points.

RNA isolation from pools of embryos

Pools of 20 – 30 embryos were snap-frozen in liquid nitrogen and subsequently stored at –80℃. Embryos were homogenized in 1 ml of TRI reagent (Ambion),

and subsequently total RNA was extracted according to the manufacturer's instructions. The RNA samples were incubated for 20 min at 37°C with 10 U of DNaseI (Roche Applied Science) to remove residual genomic DNA before purification using the RNeasy MinElute Cleanup kit (Qiagen) according to the RNA clean-up protocol. The integrity of the RNA was confirmed by lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). Samples used for microarray analysis had an average RNA integrity number value of 9 and a minimum RNA integrity number value of 8.

RNA isolation from single embryos

The single embryo RNA isolation procedure was performed according to de Jong *et al.* [45]. Embryos were individually snap-frozen in liquid nitrogen and subsequently stored at -80°C. A frozen embryo was crushed with a chilled pestle and homogenized in 300 µl of TRI reagent (Ambion). 60 µl of chloroform was added and the mixture was transferred to a 1.5 ml reaction tube containing 50 mg phase lock gel (Eppendorf) and incubated at room temperature for 5 minutes. The mixture was centrifuged at 12000 g at 4°C for 15 minutes, after which the aqueous phase was transferred to a fresh tube. 1 volume of 70 % ethanol was added and the mixture transferred to a RNeasy MinElute Cleanup kit (Qiagen) column which was centrifuged 15 seconds at 8000 g. 500 µl RPE buffer from the kit was applied to the column and centrifuged 15 seconds at 8000 g. 500 µl 80 % ethanol was applied to the column and centrifuged 2 minutes at 8000 g. The collection tube was replaced and the column centrifuged 5 minutes at 14000 g. 14 µl H_2O was applied to the column and centrifuged 1 minute at 14000 g. The average amount of RNA isolated from a single embryo was 500 ng.

Microarray analysis

The microarray slides were custom-designed by Agilent Technologies as previously described [9]. The slides contained in total 43,371 probes of a 60-oligonucleotide length.

Amino-allyl-modified amplified RNA (aRNA) was synthesized in one amplification round from total RNA using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). The amount of total RNA used was 1 µg in

experiments using RNA from pooled embryos and 400 ng in experiments using RNA from single embryos. Subsequently, 6 µg of amino-allyl-modified aRNA was used for coupling of monoreactive Cy3 and Cy5 dyes (GE Healthcare) and column purified. Samples from embryos immersed in *E. tarda*, *E. coli*, or *P. aeruginosa* suspensions or untreated control embryos were labelled with Cy5 and hybridized against a Cy3-labeled common reference that consisted of a mixture of all samples from the immersion experiments. *E. tarda* and control immersions were analyzed in triplicate using pools of 20 embryos and compared with single experiments of *E. coli*, *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 immersion. For the *E. tarda* injection study, infected embryos and control embryos injected with the PVP-carrier solution were labelled with Cy5 and analyzed in triplicate against a Cy3-labeled common reference. Dual-color hybridization of the microarray chips was performed at ServiceXS according to Agilent protocol G4140-90050 version 5.7 (www.Agilent.com) for two-color microarray-based gene expression analysis.

Microarray data were processed from raw data image files with Feature Extraction Software 9.5.3 (Agilent Technologies). Processed data were subsequently imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modelling. The raw data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession no. GSE28486. To compare samples from treatment groups to the control samples re-ratio analyses were performed using the Rosetta built-in re-ratio with common reference application. Data were analyzed at the level of UniGene clusters (UniGene build no. 105) and at probe level. Significance cut-offs for the ratios were set at 1.5-fold change at $P < 10^{-4}$ for analysis at UniGene cluster level and $P < 10^{-5}$ for analysis at probe level.

Gene ontology (GO) analysis was performed using the GeneTools eGOn v2.0 web-based gene ontology analysis software (www.genetools.microarray.ntnu.no) [55]. KEGG pathway analysis was performed using DAVID tools for functional annotation (http://david.abcc.ncifcrf.gov/) [46]. In addition, genes were manually annotated based on information in the ZFIN (zfin.org) and NCBI Entrez Gene databases, and PubMed abstracts.

cDNA synthesis and quantitative reverse transcriptase PCR

For RNA samples from pooled embryos, cDNA synthesis reactions were performed in a 20 µl mixture of 500 ng of RNA, 4 µl of 5x iScript reaction mix (Bio-Rad Laboratories), and 1 µl of iScript reverse transcriptase (Bio-Rad Laboratories). For RNA samples from single embryos, cDNA synthesis reactions were performed in a 10 µl mixture of 100 ng of RNA, 2 µl of 5x iScript reaction mix (Bio-Rad Laboratories), and 0.5 µl of iScript reverse transcriptase (Bio-Rad Laboratories). The reaction mixtures were incubated at 25℃ for 5 min, 42℃ for 30 min, and 85℃ for 5 min.

Real-time PCR was performed using the Chromo4 Real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Each reaction was performed in a 25-µl volume comprised of 1 µl of cDNA, 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad Laboratories), and 10 pmol of each primer. Cycling parameters were 95℃ for 3 min to activate the polymerase, followed by 40 cycles of 95℃ for 15 s and 59℃ for 45 s. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were performed as technical duplicates. For normalization, peptidylprolyl isomerase A-like (*ppial*), which showed no changes over the infection time course series, was taken as reference. Results were analyzed using the $\Delta\Delta C_t$ method. Sequences of forward and reverse primers are described in supplementary table S6.

Acknowledgements

We thank Dr. Philip Klesius (USDA, Auburn, AL) for providing us with *E. tarda* strain FL6-60, Roel de Haan for help with qPCR, and Davy de Wit and Ulrike Nehrdich for fish care. This work was supported by the European Commission 6th framework project ZF-TOOLS (LSHG-CT-2006-037220).

Supplementary data

Supplementary tables can be found online at:

Supplementary table S1
http://www.biomedcentral.com/imedia/3650422161273884/supp1.xls

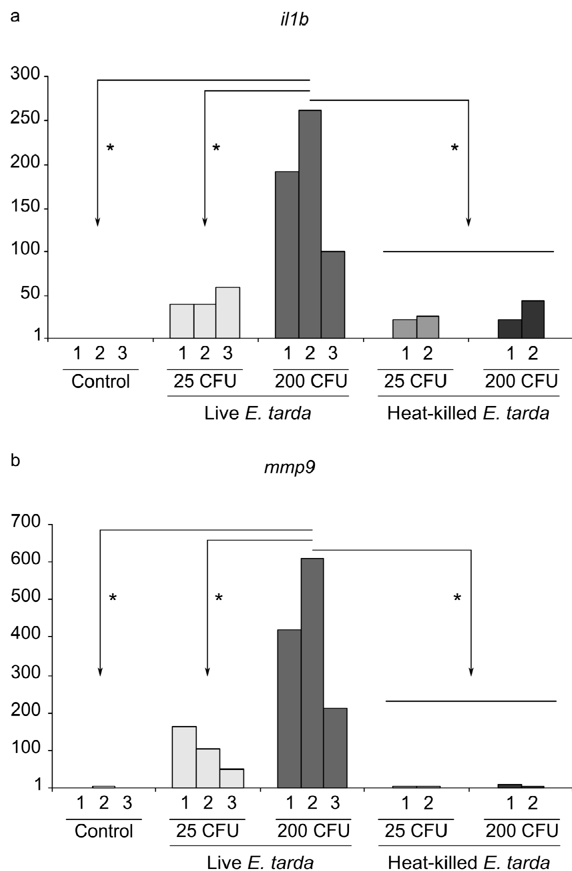
Supplementary table S2
http://www.biomedcentral.com/imedia/1629964913612738/supp2.xls

Supplementary table S3
http://www.biomedcentral.com/imedia/1084060966127380/supp3.xls

Supplementary table S4
http://www.biomedcentral.com/imedia/1241037959612738/supp5.xls

Supplementary table S5
http://www.biomedcentral.com/imedia/1457234230612739/supp7.xls

Supplementary table S6
http://www.biomedcentral.com/imedia/1174915202612739/supp8.xls



Supplementary figure S1. Marker gene expression in individual embryos in response to injection of different doses of live and heat-killed *E. tarda*. Expression levels of *mmp9* (a) and *il1b* (b) were measured by qPCR in single embryos at 8 h after injection (hpi) of approximately 25 or 200 CFUs of live or heat-killed (45 min at 95℃) *E. tarda* into the caudal vein embryos at 28 hpf. Control embryos were injected with PBS. Relative induction levels are shown with the lowest expression level set at 1. Lines with * indicate a significant difference of $P < 0.05$ (tested by one-way ANOVA analysis with the Bonferroni method as post-hoc test).

References

1. Kanther, M. and J.F. Rawls. 2010. Host-microbe interactions in the developing zebrafish. Curr Opin Immunol 22:10-19.
2. Meeker, N.D. and N.S. Trede. 2008. Immunology and zebrafish: Spawning new models of human disease. Dev Comp Immunol 32:745-757.
3. Meijer, A.H. and H.P. Spaink. 2011. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets 12: 1000-1007
4. Sullivan, C. and C.H. Kim. 2008. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol 25:341-350.
5. Hall, C., M.V. Flores, K. Crosier, and P. Crosier. 2009. Live cell imaging of zebrafish leukocytes. Methods Mol Biol 546:255-271.
6. Meijer, A.H., A.M. van der Sar, C. Cunha, G.E.M. Lamers, M.A. Laplante, H. Kikuta, W. Bitter, T.S. Becker, and H.P. Spaink. 2008. Identification and real-time imaging of a myc-expressing neutrophil population involved in inflammation and mycobacterial granuloma formation in zebrafish. Dev Comp Immunol 32:36-49.
7. Renshaw, S.A., C.A. Loynes, D.M.I. Trushell, S. Elworthy, P.W. Ingham, and M.K.B. Whyte. 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108:3976-3978.
8. Ellett F, L. Pase, J.W. Hayman, A. Andrianopoulos, and G.J. Lieschke. 2011. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117:e49-56.
9. Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H.P. Spaink, and A.H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to salmonella infection. J Immunol 182:5641-5653.
10. Wang, Z., S. Zhang, G. Wang. 2008. Response of complement expression to challenge with lipopolysaccharide in embryos/larvae of zebrafish Danio rerio: Acquisition of immunocompetent complement. Fish Shellfish Immunol 25:264-270.
11. Herbomel, P., B. Thisse, and C. Thisse. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126:3735-3745.
12. Le Guyader, D., M.J. Redd, E. Colucci-Guyon, E. Murayama, K. Kissa, V. Briolat, E. Mordelet, A. Zapata, H. Shinomiya, and P. Herbomel. 2008. Origins and unconventional behavior of neutrophils in developing zebrafish. Blood 111:132-141.
13. Willett, C.E., A. Cortes, A. Zuasti, and A.G. Zapata. 1999. Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214:323-336.
14. Mogensen, T.H. 2009. Pathogen recognition and inflammatory signalling in innate immune defenses. Clin Microbiol Rev 22:240-273.
15. Meijer, A.H., G.S.F. Krens SF, I.A. Medina Rodriguez, S. He, W. Bitter, B.E. Snaar-Jagalska, and H.P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol 40:773-783.
16. Stein, C., M. Caccamo, G. Laird, and M. Leptin. 2007. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. Genome Biol 8:R251.
17. Davis, J.M., H. Clay, J.L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of *Mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17:693-702.
18. Herbomel, P., B. Thisse, and C. Thisse. 2001. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol 238:274-88.
19. Davidson, A.J. and L.I. Zon. 2004. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. Oncogene 23:7233-7246.
20. Lam, S.H., H.L. Chua, Z. Gong, T.J. Lam, and Y.M. Sin. 2004. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28:9-28.
21. Cui, C., E.L. Benard, Z. Kanwal, O.W. Stockhammer, M. van der Vaart, A. Zakrzewska, H.P. Spaink, and A.H. Meijer. 2011. Infectious disease modelling and innate immune function in zebrafish. Methods Cell Biol 105:273-308.
22. van der Sar, A.M., R.J.P. Musters, F.J.M. van Eeden, B.J. Appelmelk, C.M.J.E. Vandenbroucke-Grauls, and W. Bitter. 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell Microbiol 5:601-611.
23. Brannon, M.K., J.M. Davis, J.R. Mathias, C.J. Hall, J.C. Emerson, P.S. Crosier, A. Huttenlocher, L. Ramakrishnan, and S.M. Moskowitz. 2009. *Pseudomonas aeruginosa* Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. Cell Microbiol 11:755-768.
24. Clatworthy, A.E., J.S-W. Lee, M. Leibman, Z. Kostun, A.J. Davidson, and D.T. Hung. 2009. *Pseudomonas aeruginosa* infection of zebrafish involves both host and pathogen determinants. Infect Immun 77:1293-1303.
25. Vergunst, A.C., A.H. Meijer, S.A. Renshaw, D. O'Callaghan. 2010. *Burkholderia cenocepacia* creates an intra-macrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. Infect Immun 78:1495-1508.
26. Lin, A., J.A. Loughman, B.H. Zinselmeyer, M.J. Miller, and M.G. Caparon. 2009 Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes Infection. Infect Immun 77:5190-5201.

27. Lin, B., S. Chen, Z. Cao, Y. Lin, D. Mo, H. Zhang, J. Gu, M. Dong, Z. Liu, and A. Xu. 2007. Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: Striking similarities and obvious differences with mammals. Mol Immunol 44:295-301.
28. Neely, M.N., J.D. Pfeifer, and M. Caparon. 2002. *Streptococcus*-zebrafish model of bacterial pathogenesis. Infect Immun 70:3904-3914.
29. Prajsnar, T.K., V.T. Cunliffe, S.J. Foster, and S.A. Renshaw. 2008. A novel vertebrate model of *Staphylococcus aureus* infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cell Microbiol 10:2312-2325.
30. Phelps, H.A. and M.N. Neely. 2002. Evolution of the zebrafish model: from development to immunity and infectious disease. Zebrafish 2:87-103.
31. Volkman, H.E., T.C. Pozos, J. Zheng, J.M. Davis, J.F. Rawls, and L. Ramakrishnan. 2010. Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science 327:466-469.
32. Pressley, M. E., P.E. Phelan III, P.E. Witten, M.T. Mellon, and C.H. Kim. 2005. Pathogenesis and inflammatory response to *Edwardsiella* tarda infection in the zebrafish. Dev Comp Immunol 29:501-513.
33. Chang, M.X., P. Nie. 2008. RNAi suppression of zebrafish peptidoglycan recognition protein 6 (zfPGRP6) mediated differentially expressed genes involved in Toll-like receptor signalling pathway and caused increased susceptibility to *Flavobacterium columnare*. Vet Immunol Immunopathol 124:295-301.
34. Guiney, P.D., R.M. Smolowitz, R.E. Peterson, and J.J. Stegeman. 1997. Correlation of 2,3,7,8-tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. Toxicol Appl Pharmacol 143:256-273.
35. Jönsson, M.E., B. Brunström, and I. Brandt. 2009. The zebrafish gill model: Induction of CYP1A, EROD and PAH adduct formation. Aquat Toxicol 91:62-70.
36. Basler, T., S. Jeckstadt, P. Valentin-Weigand, and R. Goethe. 2006. *Mycobacterium paratuberculosis*, *Mycobacterium smegmatis*, and lipopolysaccharide induce different transcriptional and post-transcriptional regulation of the IRG1 gene in murine macrophages. J Leukoc Biol 79:628-638.
37. Degrandi, D., R. Hoffmann, C. Beuter-Gunia, and K. Pfeffer K. 2009. The proinflammatory cytokine-induced IRG1 protein associates with mitochondria. J Interferon Cytokine Res 29:55-68.
38. Lee, C.G., N.A. Jenkins, D.J. Gilbert, N.G. Copeland, and W.E. O'Brien. 1995. Cloning and analysis of gene regulation of a novel LPS-inducible cDNA. Immunogenetics 41:263-270.
39. Shi, S., C. Nathan, D. Schnappinger, J. Drenkow, M. Fuortes, E. Block, A. Ding, T.R. Gingeras, G. Schoolnik, S. Akira, K. Takeda, and S. Ehrt. 2003. MyD88 primes macrophages for full-scale activation by Interferon-gamma yet mediates few responses to *Mycobacterium tuberculosis*. J Exp Med 198:987-997.
40. Tseng, D.Y., M.Y. Chou, Y.C. Tseng, C.D. Hsiao, C.J. Huang, T. Kaneko, and P.P. Hwang. 2009. Effects of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo. Am J Physiol Regul Integr Comp Physiol 296:R549-557.
41. Wagner, G.F., G.E. Dimattia, J.R. Davie, D.H. Copp, and H.G. Friesen. 1992. Molecular cloning and cDNA sequence analysis of coho salmon stanniocalcin. Mol Cell Endocrinol 90:7-15.
42. Chakraborty, A., H. Brooks, P. Zhang, W. Smith, M.R. McReynolds, J.B. Hoying, R. Bick, L. Truong, B. Poindexter, H. Lan, W. Elbjeirami, and D. Sheikh-Hamad. 2007. Stanniocalcin-1 regulates endothelial gene expression and modulates transendothelial migration of leukocytes. Am J Physiol Renal Physiol 292:F895-904.
43. Kanellis, J., R. Bick, G. Garcia, L. Truong, C.C. Tsao, D. Etemadmoghadam, B. Poindexter, L. Feng, R.J. Johnson, and D. Sheikh-Hamad. 2004. Stanniocalcin-1, an inhibitor of macrophage chemotaxis and chemokinesis. Am J Physiol Renal Physiol 286:F356-F362.
44. Wang, Y., L. Huang, M. Abdelrahim, Q. Cai, A. Truong, R. Bick, B. Poindexter, and D. Sheikh-Hamad. 2009. Stanniocalcin-1 suppresses superoxide generation in macrophages through induction of mitochondrial UCP2. J Leukoc Biol 86:981-988.
45. de Jong, M., H. Rauwerda, O. Bruning, J. Verkooijen, H.P. Spaink, and T. Breit. 2010. RNA isolation method for single embryo transcriptome analysis in zebrafish. BMC Res Notes 3:73.
46. Dennis, G. Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, and R.A. Lempicki. 2003. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol 4: P3.
47. Darby, C., C.L. Cosma, J.H. Thomas, and C. Manoil. 1999. Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 96:15202-15207.
48. Galloway, D.R. 1991. *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. Mol Microbiol 5:2315-2321.
49. Peters, J.E. and D.R. Galloway. 1990. Purification and characterization of an active fragment of the LasA protein from *Pseudomonas aeruginosa*: enhancement of elastase activity. J Bacteriol 172:2236-2240.
50. Wick, M.J., D.W. Frank, D.G. Storey, and B.H. Iglewski. 1990. Structure, function, and regulation of *Pseudomonas aeruginosa* exotoxin A. Ann Rev Microbiol 44:335-363.
51. Rawls, J.F., M.A. Mahowald, A.L. Goodman, C.M. Trent, and J.I. Gordon. 2007. In vivo imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. Proc Natl Acad Sci USA 104:7622-7627.

52. Carvalho, R., J. de Sonneville, O.W. Stockhammer, N.D.L. Savage, W.J. Veneman, T.H.M. Ottenhoff, R.P. Dirks, A.H. Meijer, and H.P. Spaink. 2011. A high-throughput screen for tuberculosis progression. PLoS ONE 6:e16779.
53. Sambrook, J.D.R. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor NY, USA: Cold Spring Laboratory Press.
54. Lagendijk, E.L., S. Validov, G.E.M. Lamers, S. de Weert, and G.V. Bloemberg. 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol Lett 305:81-90.
55. Beisvag, V., F. Junge, H. Bergum, L. Jolsum, S. Lydersen, C.C. Gunther, H. Ramampiaro, M. Langaas, A. Sandvik, and A. Laegreid. 2006. GeneTools - application for functional annotation and statistical hypothesis testing. BMC Bioinformatics 7:470.

4

Expression profiling of the response to *Edwardsiella tarda* infection in a zebrafish *myd88* knockout mutant

Joost J. van Soest, Michiel van der Vaart,
Herman P. Spaink and Annemarie H. Meijer

Abstract

The availability of knockout mutants has greatly assisted functional studies in many model organisms. However, zebrafish knockout mutants in genes of the innate immune system have thus far been lacking. Here we describe the functional analysis of a knockout mutant in the gene encoding Myd88, a central adaptor in Toll like and IL1 receptor signalling. We focus on the responsiveness of *myd88* mutant embryos to infection by the natural fish pathogen *Edwardsiella tarda*. The results of microarray expression profiling show that, as expected, *myd88* plays a crucial role in the response to infection. We have annotated the genes whose expression is highly dependent on *myd88*. Besides impaired induction of immune response genes in *myd88* mutants, we also observed a positive influence of *myd88* deficiency on the induction of several gene groups after *E. tarda* infection. The *myd88*-dependent gene set includes many genes that in previously published data were shown to be responsive to Salmonella infection in zebrafish embryos. In the Salmonella infection studies, we identified *il1b*, *mmp9* and *irak3* as *myd88*-dependent targets. Here we show the *myd88*-dependence of these genes in *E. tarda* infection and add many other immune response genes as *myd88*-dependent targets, for example genes encoding transcription factors, chemokines, and the flagellin receptor Tlr5. The *cxcl-c1c* chemokine gene is an example of a *myd88*-dependent gene that could not be identified in previous morpholino knockdown studies due to off target effects of these treatments. In addition, our analysis of the knockout mutant showed that *E. tarda* induction of *tnfa* was independent of the Myd88 pathway, whereas *tnfb* induction did require *myd88* function. The significantly impaired innate immune response to *E. tarda* infection demonstrates that the *myd88* knockout mutant represents an immunodeficient model that will be a valuable asset to immunological and cancer studies in zebrafish.

Introduction

The innate immune system is the first line of defence against invading microbes. Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), of which the Toll-like receptors (TLRs)

have been studied most extensively. There are to date 10 functional TLRs identified in humans, each of which recognizes distinct groups of PAMPs [1]. Following ligand binding, TLRs recruit one or more cytosolic adaptor molecules, thereby initiating a signalling cascade that ultimately leads to the activation of the innate immune response. Myeloid differentiation factor 88 (MYD88) is an important adaptor protein in the TLR signalling pathway, and is used by all TLRs except TLR3. It contains two domains, a C-terminal TIR-domain (Toll/IL-1 receptor-domain) separated with a short linker sequence from the N-terminal death domain. The TIR-domain enables interaction with the TLRs, while the death domain enables interaction with IRAKs (IL-1 receptor associated kinase), leading to an initiation of signalling to MAP-kinases (mitogen activated protein kinases) and NF-κB (nuclear factor κB) [2-4]. MYD88 is the only adaptor used by TLR5, -7, -8 and -9. TLR2, forming heterodimers with TLR1 and -6, requires an additional adaptor, Mal to bridge the interaction with MYD88. TLR4 signals through a MYD88-dependent and a MYD88-independent (TRIF/TRAM-dependent) signalling pathway, leading to the production of pro-inflammatory cytokines and type I interferons, respectively. In addition to its pivotal role in the TLR pathway, MYD88 is an adaptor molecule downstream of the interleukin-1 receptor (IL-1R) and it has been associated with IFN-γ receptor signalling, leading to p38 activation [5, 6].

Mice deficient in Myd88 were shown to be hyporesponsive to LPS, which in wild type mice induces endotoxic shock [7]. Furthermore they were shown to be unresponsive to PAMPs recognized by Tlr2, Tlr4, Tlr5, Tlr7 and Tlr9, as well as to Il-1 [5, 8]. Myd88-deficient mice were also found to be more susceptible to infection by *Toxoplasma gondii* parasites [9] and by a number of bacterial pathogens, including *Staphylococcus aureus* [10] , *Listeria monocytogenes* [11], *Chlamydia pneumoniae* [12], and *Mycobacterium tuberculosis* [13].

Elucidating pathways of the innate immune system in animal models is complicated by the interaction of the innate immune system with the adaptive immune system. To circumvent these interactions, *in vitro* studies using peripheral blood monocytes, polymorphonuclear cells or peritoneal macrophages as well as cultured cell lines have been used to elucidate innate immunity signalling. The zebrafish embryo model provides a useful *in vivo* alternative to study vertebrate innate immunity, since it has a temporal segregation of the development of the innate and adaptive immune system. At

one day post fertilization (dpf) the zebrafish embryo has a functional innate immune system, capable of mounting an effective immune response against different pathogens [14-16]. In contrast, the adaptive immune system does not reach maturity until 2 - 4 weeks post fertilization [17-19]. In addition, zebrafish embryos are transparent, facilitating the visual study of the immune response with the use of fluorescent protein expressing immune cells and bacteria [14, 16, 20-24].

In recent years several infection models have been developed which enabled validation of the zebrafish embryo as a model for vertebrate innate immunity [25-28]. The highly conserved TLR family was identified in zebrafish and its members were found to be highly expressed in one day old zebrafish embryos [29-31], at which stage functional macrophages are first present. As in mammals, Tlr3 was linked to the response against viral infections and Tlr5 to recognition of flagellin [32, 33]. However, zebrafish Tlr4 is not activated by LPS, indicating that there are also differences in TLR ligand recognition between fish and mammals [34, 35].

The TLR adaptor molecules, Myd88, Mal and Trif are conserved in zebrafish, while Tram is not found in the zebrafish genome [30, 36, 37]. Similar to the TLR receptors, the adaptor molecules are broadly expressed during zebrafish embryogenesis [31]. Using a transgenic reporter line, *myd88* expression was detected in adult immune cell lineages and its expression was also confirmed in embryonic and larval leukocytes that contribute to wound healing and phagocytosis of injected bacteria [38]. Morpholino knockdown of Myd88 in zebrafish embryos affected their ability to clear an infection with the *Salmonella enterica* serovar *Typhimurium* Ra mutant [31]. In addition, morpholino knockdown studies showed that, like in mammals, Myd88-dependent and Myd88-independent signalling pathways are involved in the response to Salmonella infection: the Myd88-dependent pathway leading to expression of pro-inflammatory genes such as *il1b* and *mmp9*, and the Myd88-independent pathway to expression of *ifn1* [33]. Induction of *il1b* by TLR ligands, such as peptidoglycan and lipoteichoic acid, was also impaired under Myd88 knockdown conditions in zebrafish embryos [37]. Furthermore, knockdown analysis demonstrated the role of Myd88 in LPS- or TNBS-induced intestinal inflammation and microbial-dependent intestinal epithelial cell proliferation in zebrafish larvae [39-41].

A limitation of morpholino knockdown is that it can only achieve a temporal and incomplete block of gene activity. For further analysis of *myd88* in the zebrafish model, it is therefore desirable to have a knockout mutant available. In this report we describe a *myd88* mutant allele that was identified by resequencing of an ENU (N-ethyl-N-nitrosourea)-mutagenized population and contains an early stop codon in the N-terminal death domain. This leads to a truncated protein, unable to signal, since it lacks most of its death domain, necessary for downstream interaction with IRAK, and the complete TIR-domain, necessary for the interaction with the TLRs. We used a natural fish pathogen, *Edwardsiella tarda*, to compare the immune response of individual *myd88*-knockout embryos to that of their wild-type siblings. Microarray analysis revealed that a large set of known immune response genes as well as many genes not previously associated with the immune response are dependent on *myd88* function during *E. tarda* infection.

Results

Identification of a zebrafish knockout mutant in myd88

Previous morpholino knockdown studies indicated a central role for *myd88* in the innate immune response of zebrafish embryos and larvae [31, 33, 37, 39-41]. A stable knockout mutant of this gene is therefore highly useful. Resequencing of an N-ethyl-N-nitrosourea (ENU)- mutagenized zebrafish library resulted in the identification of a *myd88* mutant allele, *myd88*^{hu3568}, which carries a T to A point mutation creating an early stopcodon (figure 1). The mutation is in the N-terminal death domain leading to a truncated protein lacking part of its death domain required for interaction with the downstream Irak1 kinase and lacking also the complete TIR-domain required for interaction with cytoplasmic domains of the upstream TLR receptors (figure 1).

Zebrafish embryos homozygous for the mutation (*myd88*$^{-/-}$) were found at Mendelian frequencies after crossing of heterozygous parents. *Myd88*$^{-/-}$ embryos and larvae showed no developmental differences with their wild type siblings and could only be distinguished by genotyping. Until 5 dpf we observed no differences in survival between mutants and wild type siblings. However,

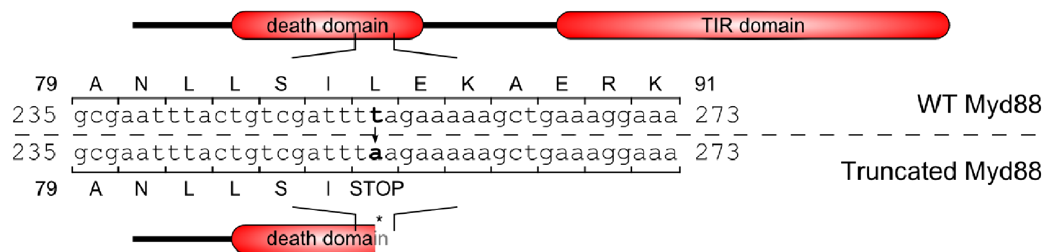


Figure 1. Wild type and mutant Myd88 sequence and protein structure. A random mutagenized zebrafish library was screened for *myd88* knockout mutants. One mutant had a T to A point mutation at nucleotide position 254 with respect to the ATG start codon, leading to a stopcodon that truncates the protein at amino acid position 85 (GenBank accession of wild type sequence: AY388401). The schematic drawing shows the resulting truncation of the death domain and lack of the TIR domain in the Myd88 mutant protein, with the DNA and amino acid sequences surrounding the mutation.

until now only a few zebrafish carrying the *myd88* mutation reached adulthood under our standard aquarium conditions. Similar as reported for *rag1*$^{-/-}$ zebrafish [42, 43], these few m*yd88*$^{-/-}$ fish died after finclips were taken for genotyping, suggesting a severely compromised immune system. Since we have not yet been able to obtain sufficient adults for further analysis, we concentrated this study on the characterization of *myd88*$^{-/-}$ embryos.

Gene expression profiles of myd88$^{-/-}$ embryos during development

To determine the effect of the *myd88*$^{-/-}$ mutation at the transcriptional level, we isolated RNA from wild type and *myd88*$^{-/-}$ sibling embryos at 1, 2, and 3 dpf for analysis on our previously described custom Agilent 44k microarray platform [33]. This platform is a combination of the commercial Agilent 22k and Sigma-Genosys 16k probe sets that we extended with probes for immune response genes. On this platform, a number of immune response genes are represented by multiple probes to increase reliability of the microarray data for this subset of genes. During the RNA isolation procedure, the chromosomal DNA fraction was retained for genotyping, based on which RNA samples from three individual wild type and three individual *myd88*$^{-/-}$ embryos were selected for microarray analysis.

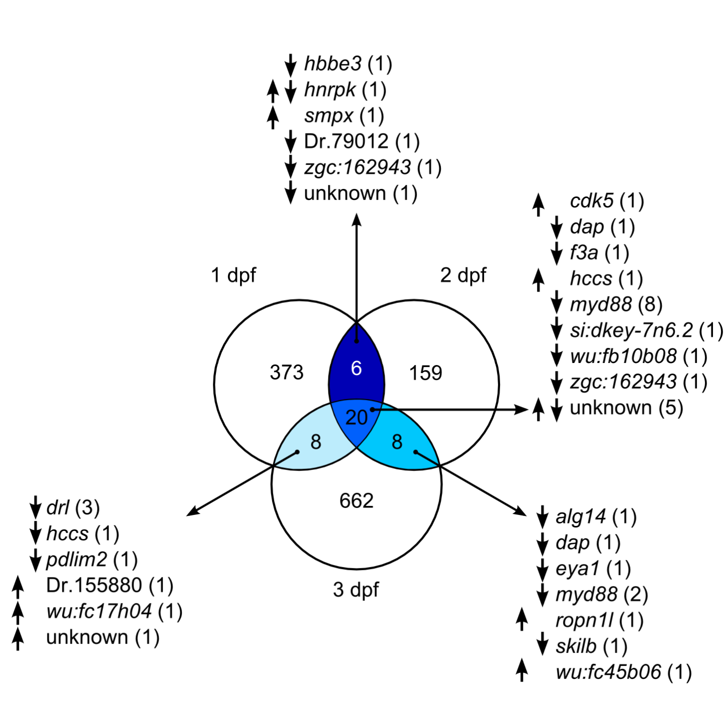


Figure 2. Comparison of *myd88*$^{-/-}$ transcriptomes at different development stages. RNA isolated from single embryos at 1, 2 and 3 dpf was analyzed by microarray. For each stage samples from three individual WT or *myd88*$^{-/-}$ embryos were hybridized and averaged. The Venn diagram indicates the genes that were significantly up- or down-regulated in *myd88*$^{-/-}$ embryos compared to WT siblings in samples of at least two developmental stages. The arrows in front of the gene names indicate whether the gene was up- or down-regulated. The numbers behind the gene name indicates the number of different probes sequences for that gene that showed up- or down-regulation.

Only 20 probes in total were consistently changed (P<0.00001) between *myd88*$^{-/-}$ and wild type embryos at all three developmental time points, and only 22 additional probes were changed at 2 out of 3 time points (figure 2, supplementary table S1). The probes changed at all time points included 8 probes for *myd88* itself, all showing down-regulated expression in *myd88*$^{-/-}$ embryos in comparison to wild type siblings. Two other probes for *myd88* additionally showed down-regulation at 2 out of 3 time points (2 and 3 dpf). None of the other probes that were differently expressed between wild type and *myd88*$^{-/-}$ at 2 or 3 time points corresponded to TLR signalling components. Besides the probes for *myd88*, 5 other probes corresponded to genes associated with the immune response and were down-regulated in *myd88-/-* embryos compared to wild type, including: *death associated protein* (*dap*, identified by 2 probes), identified in HeLa cells as a positive mediator of interferon-γ induced programmed cell death [44]; *coagulation factor 3a* (*f3a*); and *pdz and lim domain 2* (*pdlim2*), which has been shown to function as a negative regulator of transcription factor NF-κB [45].

Analysis of the immune response of myd88$^{-/-}$ embryos infected with *E. tarda*

To study the effect of the *myd88-/-* mutation on the immune response to infection, we injected *E. tarda* bacteria into the bloodstream of 28 hpf embryos from MyD88$^{+/-}$ parents. The inoculum was approximately 200 CFU per embryo embryo and to avoid clumping of the bacteria we used a carrier solution of

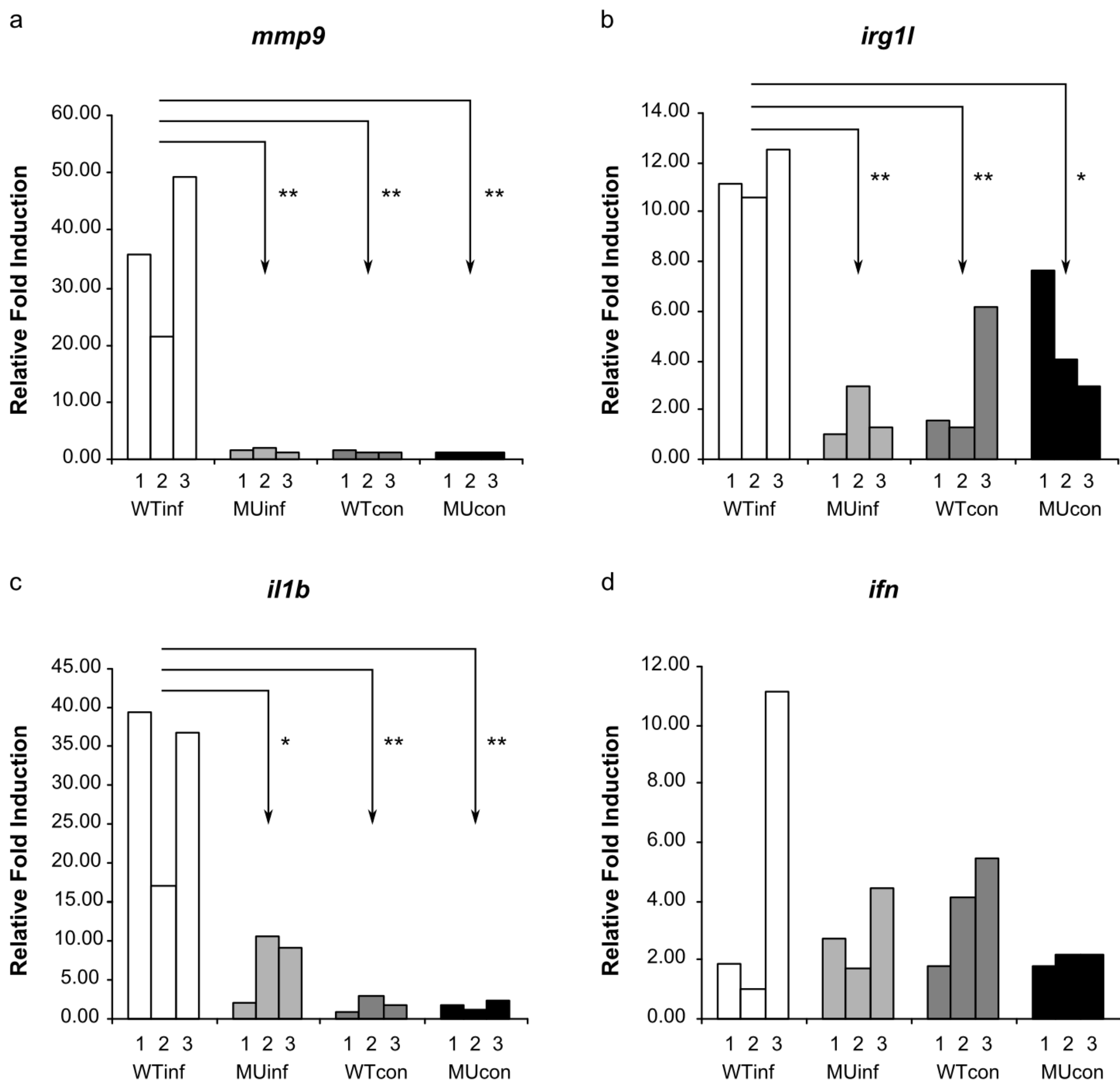


Figure 3. qPCR analysis of the response of wild type and *myd88*$^{-/-}$ embryos to *E. tarda*. Expression of *mmp9*, *irg1l*, *il1b* and *ifnphi1* was analysed by qPCR in uninfected (con) and infected (inf) wild type (WT) and mutant (MU) embryos. The bars represent the relative expression level with the lowest expression set at 1. Each bar represents a single embryo. Lines with * indicate a significant difference of $P < 0.05$. Lines with ** indicate a significant difference of $P < 0.01$ (tested by two-way ANOVA analysis with the Bonferroni method as post-hoc test).

polyvinylpyrrolidone (PVP) in PBS for the injections [49]. As a control, embryos from the same parents were injected with the carrier solution alone. DNA and RNA from control and infected embryos was isolated at 8 hpi and the DNA was used for genotyping. RNA from three individual embryos per group was then used for quantitative PCR (qPCR) analysis of three previously identified *E. tarda*-responsive genes, *matrix metalloproteinase 9* (*mmp9*), *interleukin 1b* (*il1b*), and *immunoresponsive gene 1 like* (*irg1l*) (Chapter 3). In addition, we analyzed the interferon *ifnphi1*gene, since the pathway to interferon induction is expected to be independent of *myd88*. *E. tarda* infection did not induce *mmp9* expression in *myd88*$^{-/-}$ embryos, while a 20 to 50 fold induction was observed in infected wild type siblings (Figure 3a). Expression of *il1b* was induced by *E. tarda* infection both in wild type and mutant embryos, but induction levels were significantly lower in the mutants (Figure 3b). Expression levels of *irg1l* showed a large variation between individual uninfected embryos, but clear induction by *E. tarda* infection was observed only in wild type embryos (Figure 3c). It was not possible to draw any conclusion on the *myd88*-dependence or -independence of *ifnphi1,* since the expression of this gene was increased in only one out of three wild type embryos compared to the controls. In summary, qPCR analysis showed that a functional *myd88* gene is required for the induction of *mmp9* and *irg1l* expression during *E. tarda* infection, and that *il1b* induction is partially dependent on *myd88*.

Expression profiling of the *E. tarda* response in myd88$^{-/-}$ embryos

To further characterize the immune response of *myd88*$^{-/-}$ embryos, the same samples as used for qPCR were subjected to microarray analysis. RNA samples from the individual embryos were hybridized against a common reference using the custom designed 44k Agilent microarray platform, as above. In the data analysis, the triplicate measurements of the *myd88*$^{-/-}$ embryos were combined and compared to the combined measurement of the wild type siblings. Next, four expression ratios were derived from re-ratio analysis of the sample data against the common reference: wild type control vs. wild type infected, *myd88*$^{-/-}$ control vs. *myd88*$^{-/-}$ infected, wild type infected vs. *myd88*$^{-/-}$ infected, and wild type control vs. *myd88*$^{-/-}$ control. Expression differences of the wild type control vs. *myd88*$^{-/-}$ control ratio were in most cases not significant, consistent with the earlier microarray analysis of uninfected embryos at three

developmental time points. In contrast, clear differences were observed between the infected wild type and mutant embryos.

Probes that showed a significant expression signature of at least 2-fold ($P <$ 0.00001) in the wild type control vs. wild type infected ratio (663 probes) and/or in the *myd88*$^{-/-}$ control vs. *myd88*$^{-/-}$ infected ratio (355 probes) were selected (912 different probes in total, supplementary table S2). The genes corresponding to these infection-responsive probes were then grouped into three annotation categories for further analysis. Category 1 (433 probes) contains annotated genes previously implicated in the vertebrate immune response based on GO annotations of the zebrafish genes and their human homologues and on searching of PubMed abstracts. Novel/hypothetical genes with sequence similarity to these immune response genes were also included in category 1. Category 2 (266 probes) contains all annotated or novel/hypothetical genes whose known or predicted functions could not be linked to the vertebrate immune response based on the same criteria. Category 3 (213 probes) contains genes with unknown functions or without any similarity to genes with known functions. A hierarchical cluster analysis was performed separately for each category (figure 4). For each annotation category two main clusters could be identified: the first containing probes that were mostly higher expressed in the *myd88*$^{-/-}$ infected embryos than in the wild type infected embryos (blue in figure 4), and the second (yellow in figure 4) showing the opposite response, i.e. a higher expression upon infection of wild type than of *myd88*$^{-/-}$ embryos. In category 1, this second (yellow) cluster was the largest, containing 81% of the total number of probes (figure 4a), while in categories 2 (figure 4b) and 3 (not shown) the two clusters were approximately equal in size. This difference is partially explained by the fact that the larger cluster of category 1 contains many immune response genes that are represented by multiple probes for the same gene. However, also at the gene level, the yellow cluster of category 1 was still larger than the first, i.e.73% of the total number of genes in category 1 showed higher expression in infected wild type than in *myd88*$^{-/-}$ embryos. This group of genes included substantial subgroups of complement and acute phase response genes, cytokine and chemokine genes, defence response genes, lectin genes, proteolytic genes, signal transduction genes and transcription factor genes. Smaller subgroups of each, except for the transcription factor gene subgroup, were present in the other (blue) cluster,

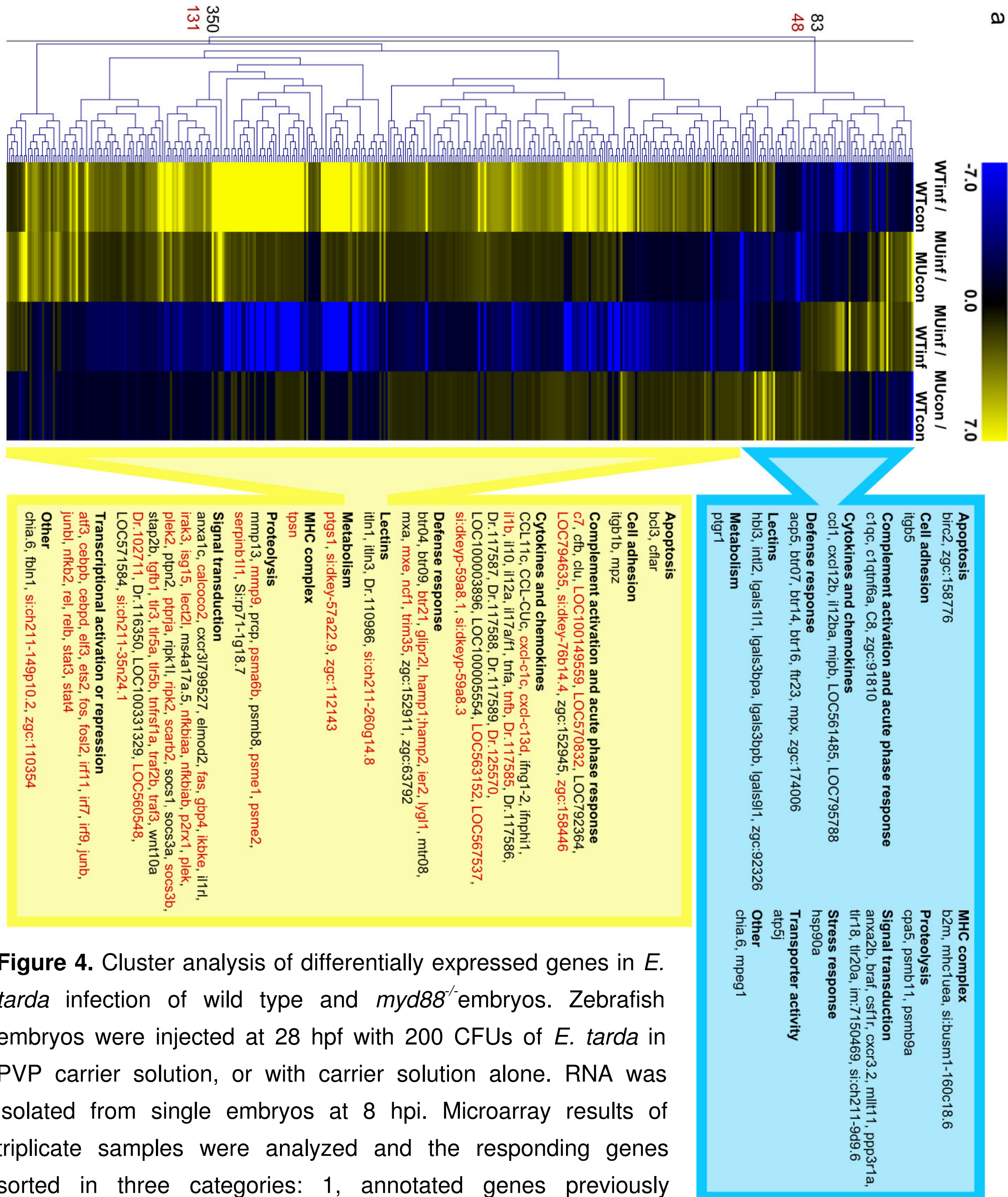


Figure 4. Cluster analysis of differentially expressed genes in *E. tarda* infection of wild type and *myd88*$^{-/-}$embryos. Zebrafish embryos were injected at 28 hpf with 200 CFUs of *E. tarda* in PVP carrier solution, or with carrier solution alone. RNA was isolated from single embryos at 8 hpi. Microarray results of triplicate samples were analyzed and the responding genes sorted in three categories: 1, annotated genes previously implicated in the immune response or novel genes with strong sequence similarity to those genes; 2, genes with known or predicted functions not previously linked to the immune response; 3, genes with unknown function. Hierarchical cluster analysis was performed separately for genes in category 1 (a) and in category 2 (b). In both cases two main clusters are distinguished: the first containing probes that show lower expression levels in wild type infected embryos than in mutant infected embryos, and the second containing probes that show higher expression levels in wild type infected embryos than in mutant infected embryos. The genes in these main clusters are indicated in the blue and yellow boxes and are sorted in functional annotation categories. The genes indicated in red are induced in a *myd88*-dependent manner based on the microarray data, and additional qPCR data for selected genes (Fig. 3). The black numbers in front of the clusters are the number of different probes in a cluster and the red numbers are the number of different genes represented by

b

-7.0 0.0 7.0

WTinf / WTcon | MUinf / MUcon | MUinf / WTinf | MUcon / WTcon

137
118

129
113

Cell adhesion
cntn2

Metabolism
aacs, acss1, amy2a, arg2, bcdo2l, cbr1, cryzl1, entpd2a.1, fn3krp, gbgt1l3, gygl, loxl2a, lpin1, lrata, mcee, metap2, ndufs7, oaz1, pck1, ywhae2, Im:7158043, LOC100002007, zgc:101071, zgc:101826, zgc:112282, zgc:171538, zgc:63546, zgc:92083

Proteolysis
LOC563795, wu:fc84d11

Signal transduction
ankle2, apbb1ip, cha, dlg5, drd3, dvl3, fbxo16, heg, limch1, lin7c, lrrn1, mast2, mknk2a, mtnr1br, mylipb, nedd8, nrarpb, pptc7, rnf13, stx5a, tmem2, tmem93, txlnba, Dr.113785, Dr.155759, Dr.6902, LOC559103, LOC561647, LOC567670, si:ch211-125a15.2, si:dkey-175m17.8, si:dkeyp-39e9.2, zgc:110695, zgc:110801, zgc:153888, zgc:153917, zgc:161973, zgc:73336, zgc:91880

Signaling
ahnak

Transcriptional activation or repression
coe2, eaf2, hesx1, hoxb1a, nono, npm2, pnrc2, pou1f1, Smarcc1, tfap2b, LOC568846, LOC792482, LOC792735, zgc:162349

Translation
zgc:92586

Transporter activity
ba2, hbaa1, kcnh2l, rbp1b, slc13a2, slc4a1ap, zgc:56136

Other
bcas2, ccdc53, col7a1l, emilin1a, env, fam69c, fxr2, myh6, nol6, papolb, prpf3, tnnt1, trmt6, ttna, Dr.75216, LOC100332905, si:ch211-113e8.10, si:ch211-14a17.10, zgc:114121, zgc:136220, zgc:158219, zgc:171663, zgc:55652, zgc:73265, zgc:91951

Apoptosis
dram1

Cell adhesion
cldn7a, Dr.45458, LOC792790

Metabolism
acbd3, carm1l, ch25h, d2hgdh, eno2, enpp6, glrx, nmnat1, Dr.107194, Dr.10826, Dr.140666, LOC100000026, LOC797909, zgc:136374

Proteolysis
agt, aph1b, pigk, timp2b, LOC563795, zgc:91963

Signal transduction
ap1s3b, arl3l1, arl3l2, aspn, atl2, cab39, cdon, dgcr2, fbxo16, gnrh2, guca1c, igf1rb, mtnr1a, or13.1, pde6h, plekhf1, ppfibp2b, rab25, rab6b, rem1, rgs14, rgs4, rln3, s100a10a, sfrp1b, stk35, ush1c, wnt5b, Dr.77571, im:7137300, LOC563864, LOC569664, si:ch211-219a4.3, si:ch211-281l24.1, si:rp71-4m17.1, zgc:101744, zgc:103685, zgc:113078, zgc:114170, zgc:64012, zgc:73189

Transcriptional activation or repression
cirh1a, smarcd3b, Dr.155984, LOC562071, si:ch211-57g18.1, si:dkey-46k9.12, zgc:162349

Transporter activity
apooa, clic2, slc22a7b, slc39a13, slc39a14, slc41a1, slc7a3, slc7a7, LOC556422, zgc:123218, zgc:123236

Other
brp16, csrp3, cx27.5, cx44.2, ddx39b, eif2c3, fam189a1, pllp, stmn4, vtg6, zc3h14, znf395, zp3, Dr.119676, LOC325449, LOC565309, LOC569366, si:dkey-25e12.3, si:dkeyp-35b8.5, wu:fb11a01, wu:fk76c08, zgc:171663, zgc:194449

these probes. Increasingly brighter shades of yellow indicate a higher up-regulation of the probe. Increasingly brighter shades of blue indicate a higher down-regulation.

WTinf/WTcon: infected vs uninfected (control) wild type embryos; MUinf/MUcon: infected vs uninfected (control) *myd88* knockout mutants; MUinf/WTinf: infected *myd88* knockout mutant vs infected wild type embryos; MUcon/WTcon: uninfected *myd88* knockout mutant vs uninfected wild type embryos.

containing the genes with higher expression in infected mutants than wild type. In category 2 (figure 3b), the majority of genes in both the blue and yellow clusters fitted in subgroups of metabolic genes, signal transduction genes, transcription factor genes, and genes for proteins with transporter activity.

A closer look at the blue and yellow clusters of categories 1 and 2 revealed further differences. In the blue cluster of category 1 most genes were down-regulated by infection both in wild type and in *myd88*$^{-/-}$ embryos, but to a lesser extent in the mutants than in the wild type. In the blue cluster of category 2 many genes are slightly up-regulated in the *myd88*$^{-/-}$ embryos, while down-regulated in the wild type embryos. In addition, approximately half of the blue cluster of category 2 represents a group of genes with weakly up-regulated expression in infected wild type embryos, but much stronger up-regulation in *myd88*$^{-/-}$ embryos. Such a group of genes is not observed in category 1. Most of the genes in the yellow cluster of category 1 were higher induced in wild type infected embryos than in *myd88*$^{-/-}$ infected embryos. Again there is a clear difference between the genes in category 1 and 2. In category 1, most genes are up-regulated both in wild type and *myd88*$^{-/-}$ embryos, while only a small number of genes is up-regulated in the wild type embryos and down-regulated in the mutant embryos. In category 2, however, there is a larger group of genes that is up-regulated by infection in wild type and down-regulated in *myd88*$^{-/-}$ embryos.

In conclusion, microarray analysis showed that the infection-induction of a large group of immune response genes was reduced in *myd88*$^{-/-}$ embryos compared to the induction in wild type siblings, as expected based on its role as a signalling adaptor of the TLR and IL1R pathways. In addition, the microarray data also indicated a positive influence of *myd88*-deficiency on other *E. tarda*-induced gene groups.

Myd88-dependency of transcription factor and signal transduction gene induction during *E. tarda* infection

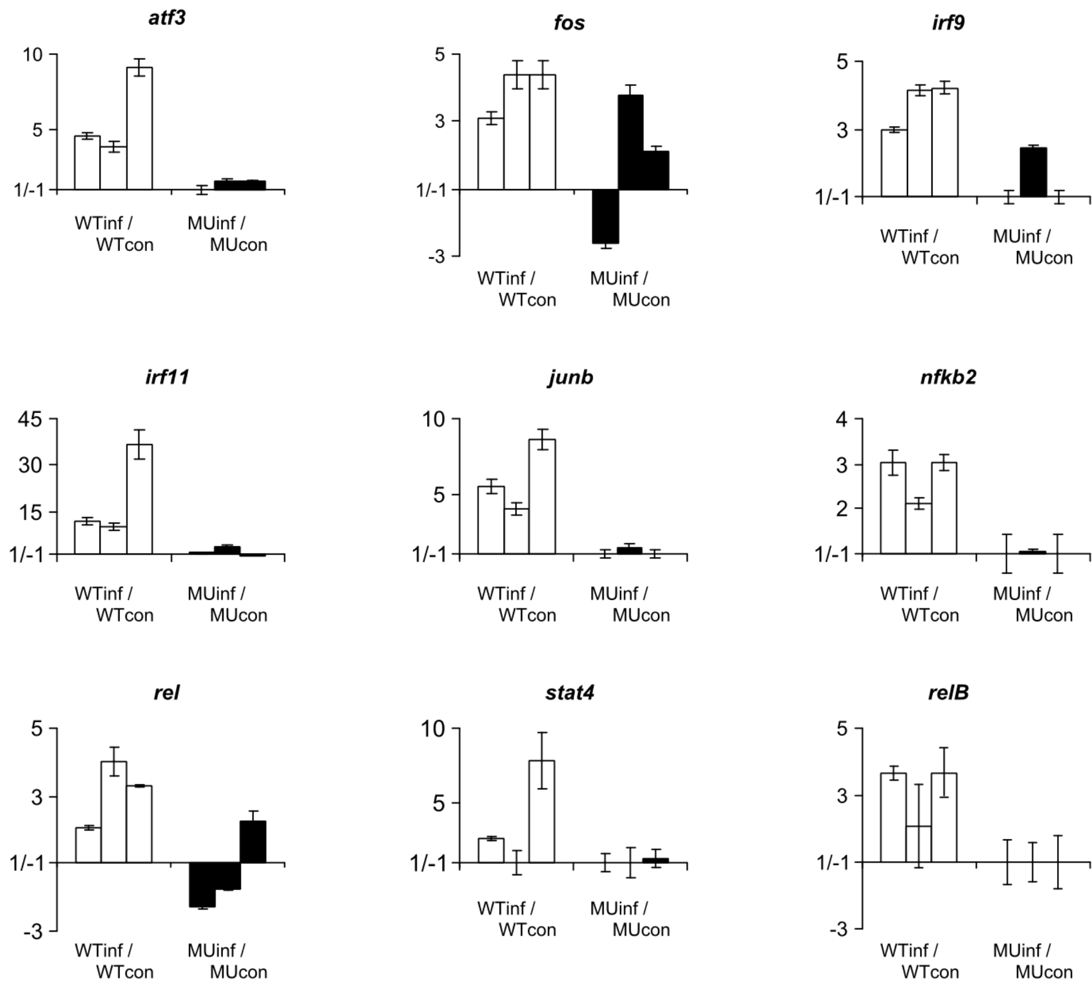
As shown in the cluster analysis of figure 4a, a number of known immune-related transcription factor and signal transduction genes were induced by *E. tarda* infection. Several of these genes are represented by more than one probe on our custom microarray. We selected these genes for further analysis of

myd88 function in the infection response. To this end, we re-analyzed the microarray data, this time comparing each of the individual *myd88*$^{-/-}$ embryos separately to a wild type sibling in order to show the biological variation (supplementary table S2). Subsequently, the induction levels (control vs. infected) of different probes for the same gene were averaged and compared between *myd88*$^{-/-}$ and wild type embryos (figure 5). This analysis showed that *E. tarda*-induction of immune-related transcription factor genes, including members of the ATF, AP-1, IRF, NFκB, and STAT families, was largely dependent on *myd88* function. In addition, this analysis showed *myd88*-dependency of the induction of *irak3*, a negative regulator of TLR signalling, *isg15*, encoding an interferon-activated ubiquitin-like protein, the receptor-interacting serine-threonine kinase gene *ripk2*, and the TLR family genes, *tlr3*, *tlr5a*, and *tlr5b*.

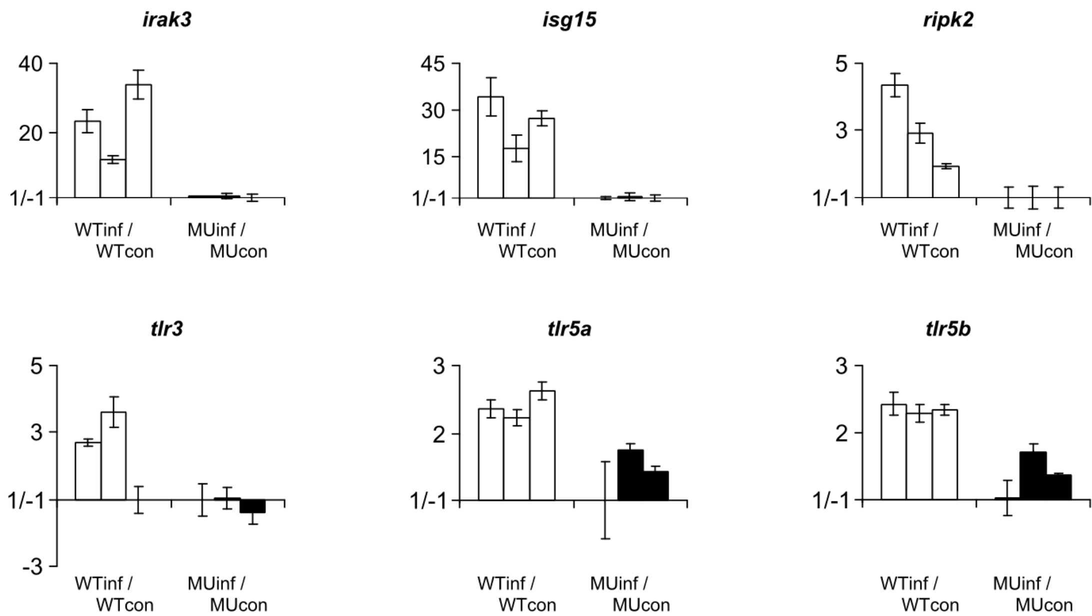
Role of myd88 in induction of other immune response genes

The same analysis as performed for the transcription factor and signal transduction genes was applied to other immune response genes that were induced by *E. tarda* infection in wild type embryos and represented by multiple probes with a significant expression signature on the microarray (figure 6, supplementary table S2). These included seven cytokine/chemokine genes and the genes for matrix metalloproteinase 9 (*mmp9*), neutrophil cytosolic factor 1 (*ncf1*) and the homologue of the human metalloreductase STEAP4, also known as TNFα-induced protein9 (*zgc:112143*). Seven of the ten investigated genes, including two CXCL chemokine genes (*cxcl-C1c*, *cxcl-C13d*), a CC chemokine gene (*si:dkeyp-59a8.1*), *tnfb*, *mmp9, ncf1* and *zgc:112143*, were not or barely induced in *myd88*$^{-/-}$ embryos, indicating their dependency on the *myd88* pathway during *E. tarda* infection. Consistent with the qPCR analysis described above, the *il1b* gene showed lower induction in *myd88*$^{-/-}$ embryos than in WT embryos, suggesting a partial dependence on *myd88*. The expression of *interleukin 10* (*il10*) also seemed to be partially dependent on *myd88*, but this result remains inconclusive since one of the wild type infected embryos showed a very low induction of this gene. In zebrafish, two genes, *tnfa* and *tnfb*, are homologous to the mammalian *TNF* gene, which encodes tumor necrosis factor alpha and is a known target of *MYD88*-dependent signalling. While our microarray data showed that *E. tarda*-induction of *tnfb* was *myd88*-dependent,

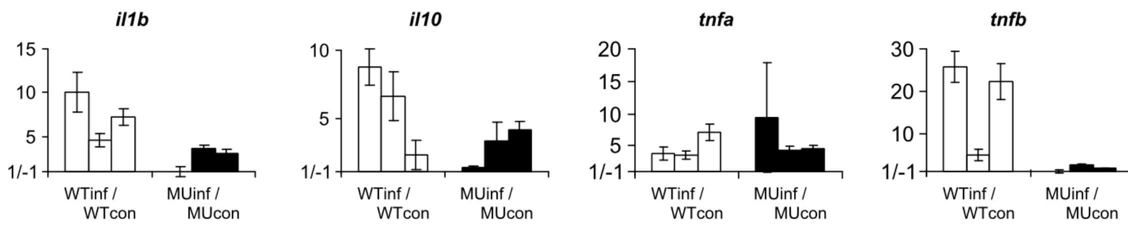
Transcription factor genes



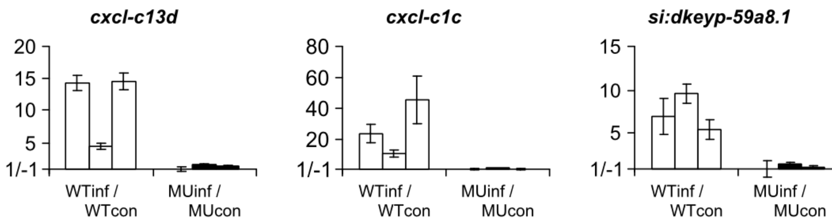
Signal transduction genes



Cytokine genes



Chemokine genes



Other immune response genes

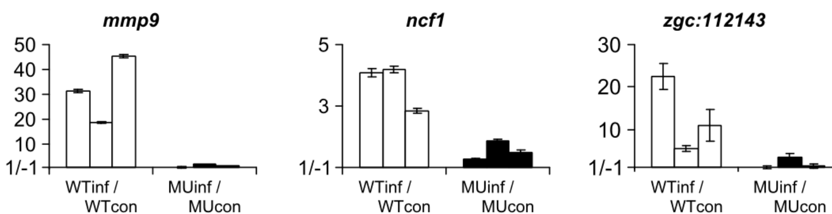


Figure 6. Induction of effector genes of the immune response by *E. tarda* infection of wild type and mutant embryos. Microarray induction levels of cytokine genes, chemokine genes and other immune response genes were compared for three individual wild type (WT) embryos and three individual *myd88* knockout mutant (MU) embryos infected with 200 CFUs of *E. tarda*. Gene expression levels of each infected embryo (WTinf and MUinf) are shown relative to a corresponding uninfected control embryo (WTcon and MUcon). For all genes shown in the figure at least three different probes on the microarray had a significant signature (*il1b*: 11 probes, *il10*: 3 probes, *tnfa*: 10 probes, *tnfb*: 11 probes, *cxcl-c13d* (*LOC100003911*): 7 probes, *cxcl-c1c* (*si:ch73-6k14.1*): 6 probes, *si:dkeyp-59a8.1*: 3 probes, *mmp9*: 15 probes, *ncf1*: 12 probes, *zgc:112143*: 11 probes). Induction levels of the different probes are averaged and the errorbars indicate the standard error.

Figure 5. Induction of transcription factor and signal transduction genes by *E. tarda* infection of wild type and mutant embryos. Microarray induction levels of transcription factor and signal transduction genes were compared for three individual wild type (WT) embryos and three individual *myd88* knockout mutant (MU) embryos infected with 200 CFUs of *E. tarda*. Gene expression levels of each infected embryo (WTinf and MUinf) are shown relative to a corresponding uninfected control embryo (WTcon and MUcon). For all genes shown in the figure at least three different probes on the microarray had a significant signature (*atf3*: 8 probes, *fos*: 14 probes, *irf9*: 13 probes, *irf11*: 3 probes, *junb*: 15 probes, *nfkb2*: 5 probes, *rel*: 10 probes, *relB* (*Wu:fj85b02*): 4 probes, *stat4*: 5 probes, *irak3*: 5 probes, *isg15*: 6 probes, *ripk2*: 10 probes, *tlr3*: 9 probes, *tlr5a*: 4 probes, *tlr5b*: 9 probes). Induction levels of the different probes are averaged and the error bars indicate the standard error.

surprisingly, *tnfa* was induced to similar levels in wild type and *myd88*$^{-/-}$ embryos, suggesting that expression of the two zebrafish *tnf* genes is controlled by different pathways. The complete *myd88*-dependency of *mmp9* induction and partial *myd88*-dependency of *il1b* induction was consistent between the results of qPCR (figure 3) and microarray analysis (figure 6).

Discussion

Many zebrafish models for infectious diseases have recently been developed and these have proved useful additions to mammalian models [27]. However, with the zebrafish still being a relative newcomer in immunological studies, there is a strong need to expand the toolbox of immunological reagents and mutants. Targeted knockout approaches in zebrafish, known as TILLING, allow the detection of point mutations in genes of interest in chemically mutagenized genomes. The success of this approach was first demonstrated in 2002 with the targeted inactivation of a gene central to the adaptive immune system, the recombination activating gene 1 (*rag1*) [47]. However, mutants disrupted in central pathways of the innate immune system, have not been available thus far. Here we describe a knockout mutant of the zebrafish homolog of human *MYD88*, a gene with a pivotal function in TLR and IL1R signalling. By transcriptome profiling analysis we show that zebrafish *myd88* is critically involved in the innate immune response of zebrafish embryos infected with the natural fish pathogen *E. tarda*.

An early stop codon in the *myd88*^{hu3568} mutant allele leads to deletion of the conserved C-terminal TIR domain, which is indispensible for interaction with the cytoplasmic face of the TLR and IL1R receptors. In addition, the mutation disrupts the N-terminal death domain of Myd88, required for interaction with downstream IRAK family kinases. We cannot exclude a possible effect of the presence of a partial death domain in the truncated Myd88 mutant protein. However, this is unlikely to play a major role, because we observed that *myd88* mutant mRNA was expressed at a significantly lower level than mRNA of the wild type allele. This may be explained by a lower stability of *myd88*^{hu3568} mRNA or by a possible feed-back mechanism by which wild type Myd88 might regulate its own expression level. In any case, based on the deletion of the TIR domain,

the truncation of the death domain and reduced mRNA expression level, the *myd88*^{hu3568} mutation can be considered a null allele.

While *myd88*$^{-/-}$ embryos and larvae showed no developmental abnormalities, it has been difficult to rear them to adulthood. We suspect that this problem can be attributed to an increased susceptibility to infections, since the few *myd88*$^{-/-}$ adults that we obtained appeared normal but died after fin clipping, similar to what has been reported for *rag1*$^{-/-}$ fish [42, 43]. Therefore, we kept heterozygous fish and analyzed individual wild type and mutant siblings from their offspring. To this end, we used a procedure for simultaneous DNA and RNA isolation from single embryos [48] and compared the gene expression profiles of embryos at 1, 2 and 3 dpf, i.e. before intestinal colonization might induce microbe-dependent differences. Besides the observed down-regulation of *myd88* mutant transcript levels, discussed above, our microarray analysis identified only a few other genes that showed consistent differences between wild type and mutant siblings at all developmental time points. Genes changed at only one developmental time point probably reflect individual variation rather than effects of the mutation. In previous studies we used triplicate pools of embryos to level out such variation (e.g. [33]), but here triplicate analyses were performed on single embryo level as our study required individual genotyping. Only four of the genes, other than *myd88*, that were differentially expressed between wild type and mutant siblings had an association with the immune system. Therefore, microarray analysis showed that, in the absence of infection, wild type and mutant siblings display largely similar basal levels of immune-related genes. In contrast, following *E. tarda* infection, the transcriptome profiles of mutants and wild type siblings were significantly different.

As expected, the induction of a large set of immune-related genes by *E. tarda* infection was found to be dependent on the presence of a functional *myd88* gene. For several of these genes, including a group of transcriptional regulators of the immune response and several effector genes of the innate immune system, the *myd88*-dependency was supported by multiple probes on our microarray platform that showed consistent expression differences between infected mutants and wild type embryos. In addition, we confirmed the *myd88*-dependency of *mmp9*, *il1b* and *irg1l* by qPCR. Of these three genes, *mmp9* and *il1b* show the highest induction levels upon *E. tarda* infection of wild embryos, in both cases in the range of 15-50-fold induction compared to uninfected controls.

Interestingly, the induction profiles of these two proinflammatory markers in mutants were different: *il1b* induction showed a partial dependence on *myd88,* while *mmp9* induction was completely absent in *myd88*$^{-/-}$ embryos. Several other genes also showed complete *myd88*-dependency, for example chemokine genes such as *cxcl-c1c* and *cxcl-c13d*, and *irak3*, a negative regulator of TLR signalling.

The *myd88*-dependency of *E. tarda* induced *mmp9*, *il1b,* and *irak3* expression was consistent with our earlier observations in *myd88* morpholino knockdown studies, where the induction of these genes by *S. typhimurium* infection was also found to be *myd88*-dependent. However, in the previous studies it was not possible to discriminate between complete or partial *myd88*-dependency because a morpholino knockdown effect in itself may be incomplete. Furthermore, for other genes analyzed in the previous study, including *cxcl-c1c* and *tnfa*, we could not draw conclusions due to non-specific morpholino effects on the expression of these genes. Therefore, we have not subjected *myd88* morpholino knockdown embryos to microarray analysis. The availability of a knockout mutant now solves these problems. Microarray analysis of *myd88*$^{-/-}$ embryos clearly demonstrates the *myd88* dependency of *cxcl-c1c*, whereas *tnfa* induction by *E. tarda* infection appears *myd88* independent. For the first time, we also show that induction of the flagellin receptor homologues, *tlr5a* and *tlr5b*, is directly dependent on *myd88*-mediated signalling. Several genes identified as (partially) dependent on *myd88* in the *E. tarda* infection study overlap with genes identified as *traf6*-dependent based on morpholino knockdown in the context of *S. typhimurium* infection, for example *mmp9*, *il1b*, *tnfb*, *ncf1*, zgc:*114032* (complement factor B), zgc:*112143* (TNFα-induced protein 9), and *tlr5b*. Therefore, these genes can now be placed as targets of a *myd88*-*traf6* signalling pathway.

In multiple studies investigating macrophages and spleen cells from *MyD88* knockout mice the expression of *Tnfα* in response to different stimuli (IL-1, LPS, *S. aureus* infection, Staphylococcal enterotoxin A) was found to be critically dependent on *MyD88* [5, 7, 10, 49-51]. Zebrafish contain two copies of the gene encoding Tnfα, designated *tnfa* and *tnfb*, both of which are induced by *E. tarda* infection in wild type embryos. Interestingly, we observed that only the induction of *tnfb* was dependent on *myd88*, whereas *tnfa* induction levels where similar between wild type and mutant siblings. The difference in Myd88 dependency of

zebrafish *tnfa* and mouse *Tnfα* may be linked to functional divergence between these cytokines in fish and mammals. Recombinant zebrafish Tnfa protein and recombinant Tnfα proteins from seabream and carp failed to exert classical proinflammatory effects on phagocytes [52, 53]. Instead, fish Tnfα is thought to be predominantly involved in the recruitment of phagocytes rather than in their activation and its main proinflammatory effects are thought to be mediated through the activation of endothelial cells [53].

In agreement with studies of cells and organs from *MyD88*-deficient mice the induction of several interleukin and chemokine genes was *Myd88* dependent in zebrafish embryos [5, 7, 10, 50, 51, 54]. In some cases induction was not completely abrogated in mutant embryos. For example, as mentioned above, *il1b* induction appeared to be mediated both by *Myd88*-dependent and *MyD88*-independent pathways. It is currently unknown if these pathways operate simultaneously or sequentially in the same cell type, or that distinct cell types or organs in the embryo induce *il1b* either in a *Myd88*-dependent or *Myd88*-independent manner. In this respect, it is of interest to note that microarray analysis of spleen and liver from septic wild-type and *Myd88*-deficient mice revealed striking differences. Whereas sepsis-induced gene expression in the liver was strongly dependent on Myd88, responses in the spleen were largely independent of *Myd88*, thus indicating an organ-specific contribution of *Myd88* to gene expression [54].

In previous *S. typhimurium* infection studies, we concluded that *ifnphi1* and *il8* were induced independently of *myd88* morpholino knockdown. In the present study we observed no induction of *il8* upon *E. tarda* infection and *ifnphi1* was induced only in one out of three wild type embryos, thus not permitting conclusions about the relationship to *myd88*. *E. tarda* infection also did not induce zebrafish homologues of interferon-responsive genes that were shown to be induced through a TRIF-dependent pathway in mice [50]. However, our microarray analysis indicated that approximately 61% of the genes induced by *E. tarda* infection are induced in a *myd88*-independent manner. This is much less than in a study of LPS stimulation, where approximately 75% of the transcriptional response was similar between macrophages taken from *Myd88* null mice and wild type mice [49].

In addition to the large set of genes whose induction during *E. tarda* infection was impaired by *myd88* deficiency, we also observed gene groups that

were repressed to a larger extent in *myd88* mutants than in wild type embryos and observed a positively influence of *myd88* on the expression of other gene groups. Similar observations were made in mice knockout mutants [49, 54, 55]. In particular, interferon-regulated genes were found to be overexpressed in septic *Myd88*-deficient organs compared to wild type organs [54]. In another study, overexpression of genes involved in post-transcriptional processes, including mRNA splicing and translation, was observed in LPS-stimulated macrophages from *Myd88* knockout mice [55]. The overexpression of these genes was suggested to act as a compensatory mechanism for the reduced transcriptional activation of many immune response genes. In our study, we did not observe increased expression of interferon-regulated genes or of a gene set involved in post-transcriptional processes, but many metabolic genes and genes involved in signal transduction were induced to higher levels in *E. tarda*-infected mutant embryos than in wild type siblings. These observations are of great interest for follow up studies.

Because *E. tarda* infection of zebrafish embryos is lethal within one to two days, this infection model is less useful to analyze the effect of *myd88* knockdown on bacterial proliferation or host survival. In future work, infection models using attenuated mutants of *E. tarda* or pathogens causing chronic infection, such as *Mycobacterium* marinum, will be more suitable to further study the *myd88* knockout effect. Preliminary results already point to a critical role of *myd88* in control of *M. marinum* infection. Currently, we are using this model to investigate the link between the *myd88*-dependent innate immune response and autophagy, a process recently shown to play a significant role in control of mycobacterial and other pathogenic infections by the host immune system. The *myd88* knockout mutant will also be highly useful to study the interplay with other components of TLR and IL1R signalling and for application as an immune-compromised zebrafish model in cancer studies.

Materials and methods

Zebrafish husbandry

Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos were grown at 28.5-30℃ in egg water (60 µg/ml Instant Ocean salts). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02 % buffered 3-aminobezoic acid ethyl ester (tricaine; Sigma-Aldrich).

Myd88 mutant and Genotyping

The *myd88*^{hu3568} mutant line was isolated in the TILLING programme of the Hubrecht Institute led by Dr. Edwin Cuppen. Heterozygous carriers of the mutation were outcrossed twice against wild type (AB strain), and subsequently were incrossed twice. Heterozygous fish of the resulting family were used to produce embryos for the infection experiments. Genotyping zebrafish embryos was done by restriction analysis after PCR. Genomic DNA was amplified using forward primer 5'-GAGGCGATTCCAGTAACAGC-3' and reverse primer 5'-GAAGCGAACAAAGAAAAGCAA-3' and the product of this reaction was digested with MseI (New England Biolabs). Fragments were analysed on 2% agarose (Difco) gel. The mutant allele can be distinguished from the wild type allele by the presence of an extra MseI site that cuts a fragment of approximately 300 bp into two fragments of 200 and 100 bp.

Infection conditions

Edwardsiella tarda labelled with mCherry [56] was grown over night in tryptic soy broth (TSB, Difco) supplemented with 8 µg/ml tetracycline at 28 ˚C. For caudal vein injection experiments, *E. tarda* was washed and subsequently suspended in PBS amended with 2% polyvinylpyrrolidone (PVP, Sigma) to a final concentration of 10^8 CFU/ml. Embryos were manually dechorionated at 24 hpf. Approximately 200 CFUs of *E. tarda* were injected into the blood island after the onset of bloodflow, or PBS amended with 2% PVP was injected as a control. After injection, embryos were kept at 28 ℃ and snap-frozen in liquid nitrogen at the required time points.

DNA-isolation, RNA isolation, labelling, and hybridization

The single embryo RNA isolation procedure was according to de Jong *et al.* [48]. Embryos for RNA isolation were snap-frozen in liquid nitrogen and subsequently stored at -80℃. Embryos were homogenized in 300 µl TRI reagent (Ambion). After chloroform extraction the aqueous phase was stored at -80℃ until the embryos were genotyped.

DNA isolation from the trizol phase was done according to the alternate DNA isolation protocol of the TRI reagent DNA/protein isolation protocol (Ambion), using glycogen as a co-precipitant.

RNA from the aqueous phase was purified using the RNeasy MinElute Cleanup kit (Qiagen) according to the RNA clean-up protocol.

Amino-allyl-modified amplified RNA (aRNA) was synthesized in one amplification round from 500 ng of total RNA using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion). Subsequently 6µg of amino-allyl-modified aRNA was used for coupling of monoreactive Cy3 and Cy5 dyes (GE Healthcare) and column purified. The dual-color hybridization of the microarray chips was performed at the Microarray Department of the University of Amsterdam according to Agilent protocol G4140-90050 version 5.7 (www.agilent.com) for two-color microarray-based gene expression analysis.

cDNA synthesis and quantitative reverse transcriptase PCR

cDNA synthesis reactions were performed in a 10-µl mixture of 100 ng of RNA, 2 µl of 5x iScript reaction mix (Bio-Rad Laboratories), and 0.5 µl of iScript reverse transcriptase (Bio-Rad Laboratories). The reaction mixtures were incubated at 25℃ for 5 min, 42℃ for 30 min, and 85℃ for 5 min.

Real-time PCR was performed using the Chromo4 Real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Each reaction was performed in a 25-µl volume comprised of 1 µl of cDNA, 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad Laboratories), and 10 pmol of each primer. Cycling parameters were 95℃ for 3 min to activate the polymerase, followed by 40 cycles of 95℃ for 15 s and 59℃ for 45 s. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were performed as technical duplicates. For normalization, peptidylprolyl isomerase

A-like (*ppial*), which showed no changes over the infection time course series, was taken as reference. Results were analyzed using the $\Delta\Delta C_t$ method. Sequences of forward and reverse primers are as in chapter 3.

Microarray analysis

The microarray slides were custom-designed by Agilent Technologies as previously described [33]. The slides contained in total 43,371 probes of a 60-oligonucleotide length.

Samples from *E. tarda* or control injections were labelled with Cy5 and hybridized against a Cy3-labeled common reference that consisted of a mixture of samples from the injection experiments. *E. tarda* and control injections were analyzed in triplicate. Microarray data were processed from raw data image files with Feature Extraction Software 9.5.3 (Agilent Technologies). Processed data were subsequently imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modelling. The raw data were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession no. …. To compare wild type control, mutant control, wild type infected and mutant infected samples a re-ratio experiment was performed using the Rosetta built-in re-ratio with common reference application. Data were analyzed at the level of probes. Significance cut-offs for the ratios were set at 2-fold change at $p < 10^{-5}$ for the probes.

One dimensional hierarchical cluster analysis was performed using MultiExperiment Viewer (MeV, v 4.5.1) [57] with the settings to Pearson correlation, average linkage and leaf order optimization.

Acknowledgements

We thank Dr. Edwin Cuppen for the *myd88*^{hu3568} mutant, Dr. Philip Klesius (USDA, Auburn, AL) for providing *E. tarda* strain FL6-60, and Davy de Wit and Ulrike Nehrdich for fish care. This work was supported by the European Commission 6th framework projects ZF-MODELS (LSHG-CT-2003-503496) and ZF-TOOLS (LSHG-CT-2006-037220).

Supplementary data

Supplementary tables can be found online at: http://tinyurl.com/ch4suptables

References

1. Mogensen, T.H. 2009. Pathogen recognition and inflammatory signalling in innate immune defenses. Clin Microbiol Rev 22:240-273.
2. Burns, K., F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J.L. Bodmer, F. Di Marco, L. French, and J. Tschopp. 1998. MyD88, an adapter protein involved in interleukin-1 signalling. J Biol Chem 273:12203-12209.
3. Muzio, M., J. Ni, P. Feng, and V.M. Dixit. 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signalling. Science 278:1612-1615.
4. Wesche, H., W.J. Henzel, W. Shillinglaw, S. Li, and Z. Cao. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 7:837-847.
5. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143-150.
6. Sun, D. and A. Ding. 2006. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol 7:375-381.
7. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11:115-122.
8. Akira, S. and K. Takeda. 2004. Toll-like receptor signalling. Nat Rev Immunol 4:499-511.
9. Scanga, C.A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E.Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol 168:5997-6001.
10. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol 165:5392-5396.
11. Edelson, B.T. and E.R. Unanue. 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. J Immunol 169:3869-3875.
12. Naiki, Y., K.S. Michelsen, N.W. Schröder, R. Alsabeh, A. Slepenkin, W. Zhang, S. Chen, B. Wei, Y. Bulut, M.H. Wong, E.M. Peterson, and M. Arditi. 2005. MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of *Chlamydia pneumoniae* pneumonia. J Biol Chem 280:29242-29249.
13. Ryffel, B., C. Fremond, M. Jacobs, S. Parida, T. Botha, B. Schnyder, and V. Quesniaux. 2005. Innate immunity to mycobacterial infection in mice: Critical role for toll-like receptors. Tuberculosis 85:395-405.
14. Davis, J.M., H. Clay, J.L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of *Mycobacterium*-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17:693-702.
15. Herbomel, P., B. Thisse, and C. Thisse. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126:3735-3745.
16. van der Sar, A.M., R.J.P. Musters, F.J.M. van Eeden, B.J. Appelmelk, C.M.J.E. Vandenbroucke-Grauls, and W. Bitter. 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell Microbiol 5:601-611.
17. Davidson, A.J. and L.I. Zon. 2004. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. Oncogene 23:7233-7246.
18. Lam, S.H., H.L. Chua, Z. Gong, T.J. Lam, and Y.M. Sin. 2004. Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28:9-28.
19. Willett, C.E., A. Cortes, A. Zuasti, and A.G. Zapata. 1999. Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214:323-336.
20. Mathias, J.R., B.J. Perrin, T.X. Liu, J. Kanki, A.T. Look, and A. Huttenlocher. 2006. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol 80:1281-1288.
21. Meijer, A.H., A.M. van der Sar, C. Cunha, G.E.M. Lamers, M.A. Laplante, H. Kikuta, W. Bitter, T.S. Becker, and H.P. Spaink. 2008. Identification and real-time imaging of a myc-expressing neutrophil population involved in inflammation and mycobacterial granuloma formation in zebrafish. Dev Comp Immunol 32:36-49.
22. Redd, M.J., G. Kelly, G. Dunn, M. Way, and P. Martin. 2006. Imaging macrophage chemotaxis in vivo: Studies of microtubule function in zebrafish wound inflammation. Cell Motil Cytoskeleton 63:415-422.
23. Renshaw, S.A., C.A. Loynes, D.M.I. Trushell, S. Elworthy, P.W. Ingham, and M.K.B. Whyte. 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood 108:3976-3978.
24. Ward, A.C., D.O. McPhee, M.M. Condron, S. Varma, S.H. Cody, S.M. Onnebo, B.H. Paw, L.I. Zon, G.J. Lieschke. 2003. The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. Blood 102:3238-3240.
25. Lesley, R. and L. Ramakrishnan. 2008. Insights into early mycobacterial pathogenesis from the zebrafish. Curr Opin Microbiol 11:277-283.
26. Meeker, N.D. and N.S. Trede. 2008. Immunology and zebrafish: Spawning new models of human disease. Dev Comp Immunol 32:745-757.
27. Meijer, A.H. and H.P. Spaink. 2011. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets 12: 1000-1007

28. Phelps, H.A. and M.N. Neely. 2002. Evolution of the zebrafish model: from development to immunity and infectious disease. Zebrafish 2:87-103.
29. Jault, C., L. Pichon, and J. Chluba. 2004. Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol Immunol 40:759-771.
30. Meijer, A.H., G.S.F. Krens SF, I.A. Medina Rodriguez, S. He, W. Bitter, B.E. Snaar-Jagalska, and H.P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol 40:773-783.
31. van der Sar, A.M., O.W. Stockhammer, C. van der Laan, H.P. Spaink, W. Bitter, and A.H. Meijer. 2006. MyD88 innate immune function in a zebrafish embryo infection model. Infect Immun 74:2436-2441.
32. Phelan, P.E., M.T. Mellon, and C.H. Kim. 2005. Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). Mol Immunol 42:1057-1071.
33. Stockhammer, O. W., A. Zakrzewska, Z. Hegedus, H.P. Spaink, and A.H. Meijer. 2009. Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to salmonella infection. J Immunol 182:5641-5653.
34. Sepulcre, M.P., F. Alcaraz-Pérez, A. López-Muñoz, F.J. Roca, J. Meseguer, M.L. Cayuela, and V. Mulero. 2009. Evolution of lipopolysaccharide (LPS) recognition and signalling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J Immunol 182:1836-1845.
35. Sullivan, C., J. Charette, J. Catchen, C.R. Lage, G. Giasson, J.H. Postlethwait, P.J. Millard, and C.H. Kim. 2009. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. J Immunol 183:5896-5908.
36. Fan, S., S. Chen, Y. Liu, Y. Lin, H. Liu, L. Guo, B. Lin, S. Huang, and A. Xu. 2008. Zebrafish TRIF, a golgi-localized protein, participates in IFN induction and NF-kappaB activation. J Immunol 180:5373-5383.
37. Liu, Y., M. Li, S. Fan, Y. Lin, B. Lin, F. Luo, C. Zhang, S. Chen, Y. Li, and A. Xu. 2010. A unique feature of Toll/IL-1 receptor domain-containing adaptor protein is partially responsible for lipopolysaccharide insensitivity in zebrafish with a highly conserved function of MyD88. J Immunol 185:3391-3400.
38. Hall, C., M.V. Flores, A. Chien, A. Davidson, K. Crosier, and P. Crosier. 2009. Transgenic zebrafish reporter lines reveal conserved Toll-like receptor signalling potential in embryonic myeloid leukocytes and adult immune cell lineages. J Leukoc Biol 85:751-765.
39. Bates, J.M., J. Akerlund, E. Mittge, and K. Guillemin. 2007. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe 2:371-382.
40. Cheesman, S.E., J.T. Neal, E. Mittge, B.M. Seredick, and K. Guillemin. 2010. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signalling via Myd88. Proc Natl Acade Sci USA 108:4570-4577.
41. Oehlers, S.H., M.V. Flores, K.S. Okuda, C.J. Hall, K.E. Crosier, and P.S. Crosier. 2011 A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents. Dev Dyn 240:288-298.
42. Jima, D.D., R.N. Shah, T.M. Orcutt, D. Joshi, J.M. Law, G.W. Litman, N.S. Trede,and J.A. Yoder. 2009. Enhanced transcription of complement and coagulation genes in the absence of adaptive immunity. Mol Immunol 46:1505-1516.
43. Traver, D., P. Herbomel, E.E. Patton, R.D. Murphey, J.A. Yoder, G.W. Litman, A. Catic, C.T. Amemiya, L.I. Zon, and N.S. Trede. 2003. The zebrafish as a model organism to study development of the immune system. Adv Immunol 81:253-330.
44. Deiss, L.P., E. Feinstein, H. Berissi, O. Cohen, and A. Kimchi. 1995. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. Genes Dev 9:15-30.
45. Tanaka, T., M.J. Grusby, and T. Kaisho. 2007. PDLIM2-mediated termination of transcription factor NF-kappaB activation by intranuclear sequestration and degradation of the p65 subunit. Nat Immunol 8:584-591.
46. Carvalho, R., J. de Sonneville, O.W. Stockhammer, N.D.L. Savage, W.J. Veneman, T.H.M. Ottenhoff, R.P. Dirks, A.H. Meijer, and H.P. Spaink. 2011. A high-throughput screen for tuberculosis progression. PLoS ONE 6:e16779.
47. Wienholds, E., S. Schulte-Merker, B. Walderich, R.H. Plasterk. 2002. Target-selected inactivation of the zebrafish rag1 gene. Science 297:99-102.
48. de Jong, M., H. Rauwerda, O. Bruning, J. Verkooijen, H.P. Spaink, and T. Breit. 2010. RNA isolation method for single embryo transcriptome analysis in zebrafish. BMC Res Notes 3:73.
49. Björkbacka, H., K.A. Fitzgerald, F. Huet, X. Li, J.A. Gregory, M.A. Lee, C.M. Ordija, N.E. Dowley, D.T. Golenbock, and M.W. Freeman. 2005. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signalling cascades. Physiol Genomics 19:319-330.
50. Hirotani, T., M. Yamamoto, Y. Kumagai, S. Uematsu, I. Kawase, O. Takeuchi, and S. Akira. 2005. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. Biochem Biophys Res Commun. 328:383-392.
51. Kissner, T.L., E.D. Cisney, R.G. Ulrich, S. Fernandez, and K.U. Saikh. 2010. Staphylococcal enterotoxin A induction of pro-inflammatory cytokines and lethality in mice is primarily dependent on MyD88. Immunology 130:516-526.
52. Forlenza, M., S. Magez, J.P. Scharsack, A. Westphal, H.F. Savelkoul, and G.F. Wiegertjes. 2009. Receptor-Mediated and Lectin-Like Activities of Carp (*Cyprinus carpio*) TNF-alpha. J Immunol 183:5319-5332.

53. Roca, F.J., I. Mulero, A. López-Muñoz, M.P. Sepulcre, S.A. Renshaw, J. Meseguer, and V. Mulero. 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol. 181:5071-5081.
54. Weighardt, H., J. Mages, G. Jusek, S. Kaiser-Moore, R. Lang, and B. Holzmann. 2006. Organ-specific role of MyD88 for gene regulation during polymicrobial peritonitis. Infect Immun 74:3618-3632.
55. Tsuchiya, M., V. Piras, S. Choi, S. Akira, M. Tomita, A. Giuliani, and K. Selvarajoo. 2009. Emergent genome-wide control in wildtype and genetically mutated lipopolysaccarides-stimulated macrophages. PLoS One 4:e4905.
56. Lagendijk, E.L., S. Validov, G.E.M. Lamers, S. de Weert, and G.V. Bloemberg. 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol Lett 305:81-90.
57. Saeed, A.I., N.K. Bhagabati, J.C. Braisted, W. Liang, V. Sharov, E.A. Howe, J. Li, M. Thiagarajan, J.A. White, and J. Quackenbush. 2006. TM4 microarray software suite. Methods Enzymol 411:134-193.

5

Summary & discussion

In a world where bacterial infections are becoming harder and harder to fight due to rising antibiotic resistance, disease models that allow high throughput screening are needed more than ever. Such models will allow the discovery and study of novel and existing virulence factors of infectious agents by mutant screens, as well as discovery of novel antimicrobial compounds and compounds that enhance the immune system of the host. Model organisms used in disease studies range from invertebrates to mammals, for example the nematode *Caenorhabditis elegans* and the mouse. Of these two examples the mouse allows a fairly high extrapolation of the results to human, however it is not suitable for high throughput screening. In contrast *C. elegans* is suitable for high throughput screening. For example, it has been used to study *Pseudomonas aeruginosa* virulence, even to the extent of comparing the genomes of *P. aeruginosa* strains PAO1 and the more virulent PA14. However, for *C. elegans* extrapolation of the results to humans is difficult. Several other model organisms have similar shortcomings, thus, the need for a model organism that allows both extrapolation of the results and high throughput screening arose. This gap has now been successfully filled by the zebrafish.

The embryos and larvae of the zebrafish are highly suited for high throughput screening. A female can lay up to two hundred eggs, which are fertilized *ex utero*. Before the larvae start to be able to eat they are not considered an experimental animal under European guidelines for animal experimentation. At this stage the brain and neuronal system is still not fully developed and therefore large throughput screening for medical purposes at this stage can be considered broadly ethically acceptable. From a scientific perspective, the developing embryos are transparent, enabling real-time analysis of infection and the immune response. Several transgenic zebrafish lines with fluorescently labelled immune related cells are available. Like all jawed vertebrates, the zebrafish possesses the innate and adaptive immune system, however, in the developing zebrafish, these are temporally separated. The innate immune system is already functional at 1 day post fertilization (dpf), while the adaptive immune system does not reach full maturity until 2 - 4 weeks post fertilization. This allows the innate immune system to be studied separate from the adaptive immune system.

At the start of the research described in this thesis, several bacterial infection systems in the zebrafish had already been set up, both with Gram-

positive bacteria (e.g. *Streptococcus* sp.) and Gram-negative bacteria (e.g. *Salmonella typhimurium*). Additionally, extensive research was being done with *Mycobacterium marinum*, which forms granulomas in zebrafish just as its close relative *Mycobacterium tuberculosis* does in humans. However, almost all of the zebrafish infection models required intravenous injection of the infecting agent, laborious work that does not allow for high throughput screening. For high throughput screening it would be easier if infection could be achieved by immersion only within the first five days after infection and one bacterium capable of just that had been just described, *Edwardsiella tarda*.

E. tarda is a naturally occurring fish pathogen, capable of infecting many different hosts, not restricted to fish alone. This bacterium was found to be able to cause a lethal infection in zebrafish embryos after 5 hour immersion of these embryos in a bacterial suspension. Not much was known yet about this bacterium and we chose it to conduct our studies.

Genome sequence of *E. tarda*

With new sequencing techniques greatly reducing the cost and the time to sequence a whole genome, and with no available genome of *E. tarda*, we set out to be the first to sequence it and determine the virulence factors of the strain FL6-60 that was chosen for our study. Just after our first *de novo* construction of the genome however, the genome of another *E. tarda*, strain EIB202, was published. Therefore in **chapter 2** we used *E. tarda* EIB202 as a reference genome for the assembly of our *E. tarda* FL6-60 genome. This allowed us to almost completely assemble the genome, leaving only a small amount of *de novo* assembled contigs to be added manually. By matching flanking sequences of these contigs with the gaps in the reference assembly, we completed the genome. However, several contigs were left unassembled. *E. tarda* EIB202 contains a plasmid, but none of the reads generated in the sequencing could be matched to this plasmid, showing its absence in *E. tarda* FL6-60. The unassembled contigs however could be *de novo* assembled into a circular element with several overlapping reads. The coverage of this element, when corrected for the bias that we found towards the AT-rich regions, equalled that of the completed genome, showing that it is present in one copy per cell. When annotating the genes, they were primarily found to be phage related.

Moreover, the length of the element is similar to that of the P22-podoviridae, leading us to hypothesize that it is a novel phage-like element.

The genomes of the two *E. tarda* strains were compared and were found to be very similar. The gaps in the *E. tarda* FL6-60 genome that needed to be filled manually with *de novo* assembled contigs were all regions containing phage related genes. Other regions with high variation between the two strains were concentrated around predicted genomic islands, also containing phage-related genes and additionally transposons. These observations show that these two strains have rarely diverged in the none-phage-related genes. Comparison with *E. trade*'s close relative *Edwardsiella ictaluri* also showed a remarkable structural similarity. Only two regions had a different orientation and the identity between the two species was 35.4 %.

To look in more detail for differences between different *E. tarda* strains and *E. ictaluri*, we compared the T3SS and the T6SS of the different strains. This showed a high identity between the *E. tarda* strains EIB202 and FL6-60. The T3SS of *E. tarda* strain FK1051 showed the highest divergence from the other *E. tarda* strains, but this might be due to sequencing errors. This in depth comparison between the different strains of *E. tarda* shows that the deep sequencing technology used is highly suited to quickly identify differences between genomes.

Where *E. tarda* has quite a broad host range, its close relative *E. ictaluri* is a more specialized pathogen, mainly infecting catfish. Differences in hosts or host-range could be attributed to differences in the virulence factors. Therefore we took *E. ictaluri* along in the analysis of the secretion systems. We found that the order of the genes of both the T3SS and the T6SS is the same between *E. tarda* and *E. ictaluri*, but that there is a significant amount of divergence within the genes. It would be very interesting to study the effect of the differences between different *Edwardsiella* strains in a single model organism, as well as the contribution of other virulence-related genes with the help of mutants.

Exposure of zebrafish embryos to *E. tarda*

Having determined the presence of the virulence factors of *E. tarda* in our strain, we set out to set up the infection model by immersion in our lab, as described in **chapter 3**. In this chapter, we started out by attempting to confirm the ability of *E. tarda* FL6-60 to cause lethal infection in zebrafish embryos by

immersion. In addition to *E. tarda*, we also tested the pathogen *Pseudomonas aeruginosa* strains PAO1 and PA14, known to have a broad host range. Where *E. tarda* indeed caused mortality in zebrafish embryos, both strains of *P. aeruginosa* were unable to. The mortality of zebrafish embryos upon immersion in an *E. tarda* suspension was however very variable, ranging from 25 to 75 % after 4 days between different experiments. This kind of variation is too large when screening for attenuated mutants or antimicrobial compounds, so we wanted a different readout in stead of mortality.

In order to find a different readout of infection, microarray analysis was performed on zebrafish embryos immersed in an *E. tarda* suspension. Additionally, we wanted to see if *P. aeruginosa* PAO1 and PA14 did elicit a transcriptional response, since they did not cause mortality. As a non-pathogenic control *Escherichia coli* was taken along in the analysis. The microarray yielded some surprising results. *E. tarda*, the only bacterium among the four tested able to cause mortality after immersion, caused the smallest number of up- and down-regulated genes in the zebrafish, smaller even than the non-pathogenic *E. coli*. Interestingly, *P. aeruginosa* PAO1 and PA14 caused a very high response of the zebrafish. Further analysis of the different responses to the bacteria, revealed that barely any immune response gene was up-regulated. The highest up-regulated gene after exposure to *E. tarda* was *cyp1a*, a gene of the cytochrome p450 family, known to be induced by toxic chemicals in vascular endothelium and the epithelium of the gills. This gene was up-regulated by all four bacteria, and most strongly by *P. aeruginosa*, which is known to excrete large amounts of toxins and other virulence factors. Other genes that were found to be up-regulated upon exposure to *E. tarda* were *zgc:154020*, a gene with high identity to the mouse *immunoresponsive gene 1* (*irg1*) which we therefore named *irg1-like* (*irg1l*), and stanniocalcin 1 (*stc1*), which in humans has been implicated in the inflammatory response. Gene ontology analysis confirmed the lack of an immune response, showing only an enrichment of the genes with the GO-term “response to stimulus” and more specifically “response to stress”.

The lack of up-regulated immune-related genes at 5 hours post exposure (hpe) indicated that no tissue infection had been established yet, and that the regulated genes possibly reflected an epithelial response. Therefore we wanted to look at later time points after immersion in *E. tarda*. For this analysis, six

genes were chosen for qPCR analysis, *cyp1a*, *irg1l* and *stc1* from the microarray results and known immune markers *il1b*, *mmp9* and *tnfa*. The immune markers *il1b* and *mmp9* showed a response at 24 hpe which became much stronger at 48 hpe. *Irg1l* was induced at all time points. *Stc1* and *tnfa* had such variable results, that we excluded them from analysis, but these variations, in combination with the variation in mortality rate, led us to hypothesize that not all embryos were infected. Experiments fractionating the *E. tarda* suspension by centrifugation into a wash fluid and washed bacteria showed that the early response of *cyp1a* and *irg1l* could be explained as a response to shed cell membrane components or other released bacterial compounds. The response to these bacterial compounds could be epithelial, where that of *cyp1a* is relatively short lived and *irg1l* might be continued as an internal tissue response towards systemic infection or as a remaining response to the bacterial components. While it is possible that this is a purely epithelial response, one can imagine that shed bacterial compounds might diffuse into the embryo causing a general response. The response of *il1b* and *mmp9* showed to be primarily due to the bacterial fraction, suggesting that these are markers for systemic infection.

Using a single embryo RNA-extraction protocol, we tested the regulation of the genes *cyp1a*, *irg1l*, *il1b* and *mmp9* in individual embryos at 48 hpe with qPCR. The results confirmed our hypothesis stronger than expected. Where *cyp1a* was, as expected, not induced and *irg1l* was induced in all embryos as a possible result of a response to shed bacterial compounds, *mmp9* and *il1b* were only up-regulated in one out of five embryos, suggesting that only this embryo was systemically infected.

To check whether the found variation was due to individual differences between the embryos, we decided to inject zebrafish with *E. tarda*. The response of the same four genes as before was determined by qPCR at two time points, 4 hours post infection (hpi) and 8 hpi. *Cyp1a* showed little to no induction, consistent with our suggestion that it may be involved in a response of the skin epithelium upon external exposure to shed bacterial compounds. Of the other three genes *irg1l* and *mmp9* were not or only weakly induced at 4 hpi, but highly induced at 8 hpi in all embryos, while *il1b* was highly induced at both time points, also in all embryos. Not only the transcriptional response proved

reproducible in all embryos upon *E. tarda* injection, but also the mortality, since after 48 hpi all embryos had died.

Seeing that injection of embryos gave more reproducible results on the level of single embryos, we did a microarray analysis on *E. tarda* injected embryos. In contrast to the results of the microarray from the immersion experiment, this microarray showed up-regulation of many immune response genes other than *irg1l*, *mmp9* and *il1b*. A gene ontology analysis reflected this by a significant enrichment of the GO-term "immune system process" in addition to the " response to stimulus", the last one of which was the only one found after immersion.

The results described in this chapter raise some interesting points for discussion and further research. One of the most striking conclusions that can be drawn is that the zebrafish embryo is remarkably resistant to external infection. Of the many bacteria tested by different research groups, only two, *E. tarda* and *Flavobacterium columnare*, were reported to be able to infect 1 dpf zebrafish embryos by static immersion. However, as shown in this thesis, the ability of *E. tarda* to cause infection in the immersion assay remains doubtful. If infection occurs at all, this is with low efficiency, and it cannot be excluded that the embryos die as the result of a toxic response instead. A pilot with *E. tarda* immersion of 3 dpf embryos, embryos with open mouths, did not provide a suitable alternative, as all embryos survived the following 2 days and after this time frame the zebrafish embryo becomes less interesting for high throughput screening. These results would suggest a very strong immune system being present very early on, but the mortality rate of 100% after intravenous injection shows that this is not the case. Instead, the bacteria do not seem to be able to penetrate the zebrafish skin or gut epithelium, thereby unable to cause internal infection. While it might be possible that *E. tarda* and *F. columnare* are able to penetrate the skin, it is more likely that small unintended damage to the skin, caused during handling of the embryos, provides points of entry. In fact, at the beginning of this research, the *P. aeruginosa* strains PAO1 and PA14 seemed to cause mortality upon immersion, but when more experience was gained in working with zebrafish embryos, this mortality was reduced to none. Still, *E. tarda* and *F. columnare* are special, since they seem to be the only tested bacteria needing very little skin damage to be able to cause infection.

The resistance of zebrafish embryos to external exposure is likely to be by preventing entry. The question that remains is how this is achieved. Maybe the epithelium produces so far unidentified antimicrobial compounds. Another explanation is that the zebrafish epithelium is surrounded by mucus that traps the bacteria. More likely it is a combination of both, mucus invested with antimicrobials, but this should be studied in more detail. Maybe the ability of *E. tarda* and *F. columnare* to infect embryos arises from a resistance to possible antimicrobials.

The results of the microarray after immersion show a response of the zebrafish towards external bacteria and, as shown by qPCR, especially towards shed or excreted bacterial compounds, a response we think might be epithelial. The most interesting result was that *P. aeruginosa* strains PAO1 and especially PA14 elicited a much stronger response than *E. tarda*. Since *P aeruginosa* is known to excrete many compounds, it is interesting to use this bacterium in addition to *E. tarda* to further study this response and see whether it is epithelial. Differences between *E. tarda* and *P. aeruginosa* in the compounds involved in epithelial responses can be identified. To test if secretion systems are involved, bacterial mutants and additionally a trans-well assay could be used. In the trans-well assay, the bacteria and the embryos in a well are separated by a 0.2 μm filter mesh, barring the passage of bacteria, but allowing secreted compounds to pass and interact with the embryos. To test if the response was caused by LPS components (lipopolysaccharides) sheared of the bacterial membrane during the washing process, which is equally possible, studies with purified LPS and LPS-mutants can be done.

In conclusion to chapter 3 it can be said that the *E. tarda* immersion assay is not suitable for high throughput screening. Recently, a robotic injection system for high throughput screening has been described. In this system bacteria are automatically injected into the yolk of fertilized eggs, which slowly proliferate to cause an infection in a fully grown embryo. It is very useful for the study of *Mycobacterium marinum*. However, due to the pathogenicity of *E. tarda* it is not suitable for this bacterium, since it would kill the developing zygote before it becomes a full grown embryo. This may change if attenuated *E. tarda* mutants are found.

Analysis of a zebrafish mutant in Myd88

During this project the first knockout mutant in an essential immune-related gene was identified in a TILLING-screen. Therefore, in **chapter 4**, we used *E. tarda* to characterize this zebrafish with a knockout mutation in the gene encoding Myd88. Myd88 is an important adaptor protein in the Toll-like receptor (TLR) pathway. TLRs are one family of receptors of the innate immune system that recognize pathogen-associated molecular patterns. Several studies with *myd88*-morpholinos have already been conducted, but morpholinos have several downsides. They are only applicable during the first days of embryo development, since they will be diluted out or destroyed during further growth. Moreover, they can cause non-specific effects. The point mutation in *myd88* creates an early stop codon, leading to a truncated Myd88 protein, consisting only of a partial death domain, that has been cut off just before a residue essential for communication with downstream components, and completely lacking the TIR-domain, responsible for communication with the TLR. Comparison of transcriptional profiles between wild type and *myd88*$^{-/-}$ embryos at three different time points showed that only a small amount of genes was consistently changed, the changed genes including *myd88* itself. This latter observation suggests either a feedback loop of *myd88* to itself or a strongly reduced stability of the mutated *myd88* mRNA.

Raising the *myd88*$^{-/-}$ mutants to adulthood has proven difficult. When we started this investigation adults died as soon as they were fin clipped for genotyping. Therefore we used the wild type and mutant siblings from the offspring of heterozygous adults. Since the immersion assay was shown not to be suitable for analysis on the level of single embryos and did not produce a clear immune response, we used intravenous injection for characterization of the immune response of this mutant. qPCR analysis was performed on the genes *mmp9*, *irg1l*, *il1b* and *ifn*. This showed that *mmp9* is clearly dependent on Myd88 and *irg1l* and *il1b* are at least partially dependent. For *ifn* it was not possible to draw any conclusions, since this gene was up-regulated in only one of the wild type embryos.

The embryos used for the qPCR analysis were then subjected to microarray analysis. As expected, expression of many immune-related genes was shown to be dependent on Myd88. Making use of the presence of multiple probes for several immune-related genes on the array, we could confidently identify

several of them as being (partially) m*yd88*-dependent. Confirming previous results obtained with m*yd88* morpholino knockdown and *Salmonella* infection, we found *mmp9*, *il1b* and *irak3* expression to be *myd88*-dependent. Several other genes such as transcription factor genes (e.g. *junb*), signal transduction genes (e.g. *rip2k*), cytokine genes (e.g. *tnfb*), chemokine genes (e.g. *cxcl-c1c*) and other immune response genes (e.g. *ncf1*) were shown to be m*yd88*-dependent. These results are in line with findings in *MyD88*-deficient mice. Several genes that were shown to be (partially) m*yd88*-dependent, were previously shown to be *traf6*-dependent in a morpholino knockdown study. These included *mmp9*, *il1b*, *tnfb*, *ncf1*, zgc:*114032* (complement factor B), zgc:*112143* (TNFα-induced protein 9), and *tlr5b*. These genes can now be placed as targets of a *myd88-traf6* signalling pathway.

The most surprising observation was the difference in expression between *tnfa* and *tnfb* in the *myd88*$^{-/-}$ mutant. In contrast to *tnfb*, which was shown to be completely dependent on Myd88, *tnfa* was completely independent on Myd88. *Tnfa* and *tnfb* are two copies in the zebrafish genome of the gene encoding TNF in the mouse and human. While *tnfb* reflects the same dependence on Myd88 as human TNF, *tnfa* does not, possibly reflecting the functional divergence of these homologous genes.

The observation that several genes (such as *il1b*) are partially dependent on Myd88 may be due to tissue specific differences. Since in these experiments RNA was isolated from whole embryos, differences between different tissues are lost. In septic wild type and *Myd88*-deficient mice for example, striking differences were found between the transcriptional response of spleen and liver. The response of the liver showed a strong dependence on Myd88, while the response of the spleen was largely independent on Myd88.

In this chapter, we have shown that the *myd88*$^{-/-}$ mutant zebrafish is severely impaired in its innate immune response towards *E. tarda*. Studies using this zebrafish mutant with other infecting agents are already on its way. Initial results show that *myd88* plays an important role in the control of a *M. marinum* infection. Since the problems with raising the *myd88*$^{-/-}$ mutant zebrafish to adulthood are now resolved, the full scale possibilities of the zebrafish mutant, as opposed to a morpholino knockdown, can be taken advantage of. Furthermore, increased efficiency of the TILLING-screens due to high throughput sequencing techniques will lead to the identification of more

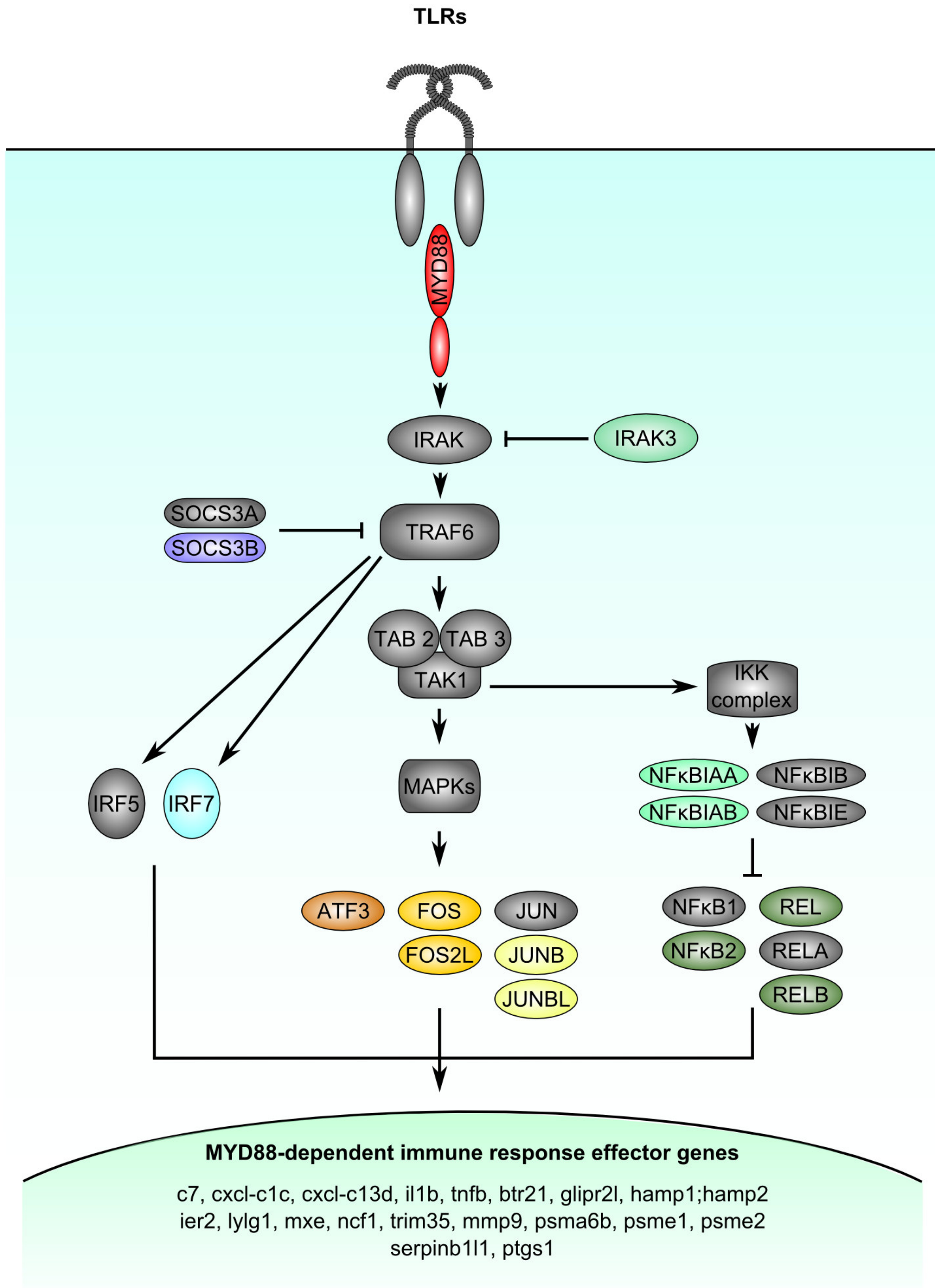


Figure 1. Schematic representation of the Tlr-Myd88 dependent pathway. Transcriptional regulation of components pictured in color was shown to be Myd88-dependent in this thesis, as well as all of the immune response effector genes shown. The pathway shown is based on knowledge of TLR signalling in mammalian species and it should be noted that most interactions remain to be experimentally confirmed in zebrafish.

immune-related mutants soon. In fact, a mutant in *tirap*, encoding another adaptor protein of the TLRs that cooperates with Myd88, has already been identified. The ambition of the Sanger Institute is that within a couple of years zebrafish mutants for each gene will be available. In addition to the TILLING-screens, zinc finger technology for the zebrafish is fast emerging, making targeted gene knock-out and gene knock-in possible. These developments will allow the zebrafish to quickly catch up with the mouse.

Conclusion

In conclusion, the research described in this thesis provides a strong basis for future research. The genome of *E. tarda* can provide insight in differences between pathogenic and non-pathogenic strains, as well as in broad host-range and more host-specific *Edwardsiella*-strains. The zebrafish embryo model will be especially suitable to study the interaction of *E. tarda* virulence factors with the host innate immune system, where comparison with the response to *E. ictaluri* infection will also be of interest. Unfortunately, we found that *E. tarda* is not completely suitable for high throughput screening, since the immersion assay is not reproducible enough, while on the other hand it is too pathogenic to use in automated yolk injection. Still, our studies into the immersion system gave many interesting leads for studying the epithelial response of the zebrafish, not only in response to *E. tarda*, but also with regard to the *P. aeruginosa* strains. Not only the epithelial response is of interest, but also the bacterial components that cause this response.

However, the characterization of the *myd88*$^{-/-}$ mutant will probably be the most important legacy of this thesis. It is the first zebrafish knock-out mutant in a gene central in the innate immune system. In the future, studies with many different pathogens and with different cancer models can be done, revealing the complete role of *myd88* in the zebrafish, with possible novel insights in the function of the innate immune system.

Nederlandse samenvatting

In een wereld waarin het steeds moeilijker wordt om bacteriële infecties te bestrijden vanwege de toenemende resistentie tegen antibiotica, zijn ziektemodellen die snelle screening van grote aantallen proefdieren mogelijk maken meer dan ooit nodig. Zulke modelsystemen kunnen helpen bij de ontdekking en studie van virulentiefactoren van ziekteverwekkers en bij de ontdekking van nieuwe antibiotica en stoffen die het immuunsysteem versterken. Modelorganismen die in de studie van ziekten worden gebruikt, lopen uiteen van invertebraten, zoals de nematode *Caenorhabditis elegans*, tot vertebraten, zoals de muis. De resultaten verkregen uit onderzoek met de muis zijn het beste te extrapoleren naar de mens, maar de muis is niet geschikt voor een snelle screening van grote aantallen, in tegenstelling tot *C. elegans. C. elegans* is bijvoorbeeld gebruikt om de virulentie van verschillende stammen van *Pseudomonas aeruginosa* te vergelijken. De extrapolatie van resultaten verkregen met deze nematode naar de mens is echter lastig. Verschillende andere modelorganismen hebben vergelijkbare tekortkomingen en dus rees de vraag naar een modelorganisme dat een goede extrapolatie naar de mens en snelle screening van grote aantallen combineerde. De zebravis als infectiemodel is hiervoor zeer geschikt.

De embryo's en larven van een zebravis zijn zeer geschikt voor snelle screening van grote aantallen. Een vrouwtje kan tot wel tweehonderd eitjes leggen die *ex utero* bevrucht worden. Zolang de larven niet zelfstandig kunnen eten, worden ze volgens de Europese wetgeving niet als proefdier beschouwd. In dit stadium zijn de hersenen en het zenuwstelsel nog niet volledig ontwikkeld en om die reden kan snelle screening van grote aantallen voor medische doeleinden als ethisch aanvaardbaar worden beschouwd. Belangrijk voor het wetenschappelijk onderzoek is dat de zich ontwikkelende embryo's transparant zijn, wat real-time analyse van de infectie en de reactie van het immuunsysteem mogelijk maakt. Hiervoor zijn verschillende transgene zebravislijnen met fluorescerende immuungerelateerde cellen beschikbaar. Zoals alle vertebraten, met uitzondering van primitieve kaakloze vissen zoals de prik en de lamprei, heeft de zebravis een aangeboren en een adaptief (verworven) immuunsysteem. Deze zijn tijdens de ontwikkeling van het embryo echter gescheiden in de tijd. Het aangeboren immuunsysteem is reeds één dag na bevruchting functioneel, terwijl het adaptieve immuunsysteem pas drie weken

na bevruchting volledig functioneel is. Dit maakt het mogelijk om het aangeboren immuunsysteem los van het adaptieve te bestuderen.

Bij aanvang van het in dit proefschrift beschreven onderzoek waren er al verscheidene bacteriële infectiesystemen met de zebravis opgezet, zowel met Gram-positieve bacteriën (bijvoorbeeld *Streptococcus pyogenes*) als met Gram-negatieve bacteriën (bijvoorbeeld *Salmonella typhimurium*). Tevens werd er uitgebreid onderzoek gedaan met *Mycobacterium marinum*, die granulomen vormt in de zebravis en nauw verwant is aan *Mycobacterium tuberculosis*, die granulomen vormt in de mens. Voor de meeste van deze infectiesystemen in zebravisembryo's was het echter noodzakelijk dat de ziekteverwekker in de bloedbaan werd geïnjecteerd: tijdrovend werk dat niet geschikt is voor snelle screening van grote aantallen. Hiervoor zou het makkelijker zijn als immersie van de zebravisembryo's in een oplossing met de ziekteverwekker binnen enkele dagen zou kunnen resulteren in een infectie. Eén bacterie met precies dit vermogen was net beschreven in de literatuur, *Edwardsiella tarda*.

E. tarda is een natuurlijke vispathogeen die behalve vissen tevens veel andere gastheren kan infecteren. Er werd ontdekt dat deze bacterie het vermogen heeft om een dodelijke infectie te veroorzaken in zebravisembryo's, wanneer deze op de leeftijd van 1 dag oud vijf uur aan een suspensie van deze bacterie worden blootgesteld. Over deze bacterie, die wij kozen voor ons onderzoek, was nog niet veel bekend.

Genoomsequentie *E. tarda*

Doordat nieuwe technieken de kosten en tijd van het bepalen van een genoomsequentie flink omlaag hadden gebracht en het genoom van *E. tarda* nog niet bekend was, wilden we als eerste het genoom van *E. tarda* bepalen, evenals de virulentiefactoren van de stam FL6-60, die we hadden gekozen voor ons onderzoek. Net na de eerste *de novo* samenstelling van het FL6-60-genoom werd het genoom van een andere *E. tarda* stam, EIB202, gepubliceerd. Daarom is in **hoofdstuk 2** het genoom van *E. tarda* EIB202 gebruikt als referentie voor het samenstellen van het *E. tarda* FL6-60 genoom. Dit maakte het voor ons mogelijk om het genoom bijna compleet samen te stellen, waarbij er slechts een klein aantal van *de novo* samengestelde contigs handmatig moesten worden toegevoegd. Door de flankerende sequenties van deze contigs te vergelijken met de gaten in het genoom, kon dit genoom

gecompleteerd worden. Een aantal contigs viel echter buiten het samengestelde genoom. Hoewel *E. tarda* EIB202 een plasmide bevat, kon geen van de reads op dit plasmide worden teruggevonden, wat laat zien dat dit plasmide ontbreekt in *E. tarda* FL6-60. De overgebleven contigs konden echter wel *de novo* worden samengesteld tot een circulair element met meerdere overlappende reads. Na correctie voor de grotere hoeveelheid reads van AT-rijke regio's bleek dat het element dezelfde dekking heeft als het genoom, wat betekent dat er slechts één kopie is per cel. Bij de annotatie van de genen van het element bleek dat deze voornamelijk gerelateerd zijn aan de genen van bacteriële virussen, de bacteriofagen of kortweg fagen. Tevens is de lengte van het element vergelijkbaar met dat van de P22-podoviridae, wat leidde tot de veronderstelling dat het een nieuw element is afkomstig van fagen.

De genomen van de twee stammen van *E. tarda* bleken zeer vergelijkbaar. De gaten in het genoom van *E. tarda* FL6-60 die handmatig gevuld moesten worden met *de novo* samengestelde contigs, waren stuk voor stuk regio's met faaggerelateerde genen. Andere regio's met grote variatie tussen de twee stammen, waren voornamelijk geconcentreerd rond voorspelde genomische eilanden die eveneens faaggerelateerde genen en ook transposons bevatten. Deze twee observaties laten zien dat de twee stammen nauwelijks veranderd zijn in de niet-faaggerelateerde genen. Vergelijking met *Edwardsiella ictaluri*, de nauwe verwant van *E. tarda*, liet eveneens een opmerkelijke structurele overeenkomst zien. Slechts twee regio's hadden een andere oriëntatie en de twee soorten waren voor 35,4 % identiek.

Om de verschillen tussen de verschillende *E. tarda* stammen en *E. ictaluri* meer in detail te bekijken, vergeleken we het type III secretiesysteem (T3SS) en het type VI secretiesysteem (T6SS) van de verschillende stammen. Deze vergelijking liet een grote mate van gelijkheid zien tussen de *E. tarda* stammen EIB202 en FL6-60. Het T3SS van de *E. tarda* stam FK1051 week het meest af van de andere *E. tarda* stammen, maar dit zou kunnen komen door fouten tijdens het sequencen. Deze dieptevergelijking tussen de verschillende stammen van *E. tarda* laat zien dat de gebruikte sequencingtechniek zeer geschikt is om snel verschillen tussen genomen te identificeren.

Terwijl *E. tarda* een groot aantal gastheren kan infecteren, is de nauw verwante *E. ictaluri* meer gespecialiseerd. Deze bacteriesoort infecteert voornamelijk meervallen. Verschillen in gastheren of in gastheerbereik kan

worden toegewezen aan verschillen in de virulentiefactoren. Om die reden hebben we *E. ictaluri* meegenomen in onze analyse van de secretiesystemen. Uit deze analyse bleek dat de volgorde van de genen van zowel het T3SS als het T6SS hetzelfde is tussen *E. tarda* en *E. ictaluri*, maar dat er significante verschillen zijn binnen de genen. Het zou erg interessant zijn om het effect van deze verschillen tussen *Edwarsiella* stammen te onderzoeken in een modelorganisme zoals de zebravis en om de bijdrage van andere virulentiegenen te onderzoeken met behulp van mutanten.

Blootstelling van zebravisembryo's aan *E. tarda*

Na bepaling van de aanwezigheid van virulentiefactoren van *E. tarda* in de gebruikte FL6-60-stam, hebben we getracht om een systeem op te zetten om zebravisembryo's door middel van immersie in *E. tarda*-suspensie te infecteren, zoals beschreven in **hoofdstuk 3**. Naast *E. tarda* zijn ook de stammen PAO1 en PA14 van de pathogeen *P. aeruginosa*, die bekend staat om zijn grote verscheidenheid aan gastheren, getest. Terwijl *E. tarda* in de immersieassay sterfte van zebravisembryo's veroorzaakte, deden beide stammen van *P. aeruginosa* dat niet. Het sterftepercentage van de zebravisembryo's na immersie in een *E. tarda*-suspensie was echter zeer wisselend tussen verschillende experimenten, variërend van 25 tot 75 % na vier dagen. Deze mate van variatie is te groot om te screenen op verzwakte mutanten of antimicrobiële stoffen, dus wilden we een andere methode voor het uitlezen van infectie vinden dan bepaling van het sterftepercentage.

Om genexpressiemarkers voor de mogelijke infectie te vinden werd een microarray-analyse uitgevoerd op zebravisembryo's na immersie in *E. tarda*-suspensie. Verder werd onderzocht of *P. aeruginosa* PAO1 en PA14 een reactie op transcriptieniveau zouden geven, aangezien ze geen sterfte veroorzaken. Als niet-pathogene controle werd ook *Escherichia coli* meegenomen in de analyse. De resultaten van de microarray-analyse waren verrassend. *E. tarda*, de enige van de vier geteste bacteriën die sterfte veroorzaakt na immersie, bracht de kleinste verandering in expressie van de genen in de zebravis teweeg, kleiner zelfs dan de niet-pathogene *E. coli*. Interessant was het dat *P. aeruginosa* PAO1 en PA14 voor een zeer grote reactie van de zebravis zorgden. Verdere analyse van de verschillende reacties op de bacteriën liet zien dat er nauwelijks immuungerelateerde genen tussen de

genen met een hogere expressie zaten. Het gen dat het meest omhoog was gegaan in expressie na blootstelling aan *E. tarda* was *cyp1a*, een gen van de cytochroom p450-familie, waarvan bekend is dat het door toxische stoffen in het vasculaire epitheel en het epitheel van de kieuwen wordt geïnduceerd. Dit gen had een hogere expressie na immersie in alle vier de bacteriën, het hoogst na blootstelling aan *P. aeruginosa*, waarvan bekend is dat het grote hoeveelheden toxines en andere virulentiefactoren uitscheidt. Andere genen waarvan een hogere expressie gevonden werd na blootstelling, waren *zgc:154020*, een gen met een grote overeenkomst met het muis-gen *immuno-responsive gene 1* (*irg1*) en die we daarom *irg1-like* (*irg1l*) hebben genoemd, en *stanniocalcin 1* (*stc1*), dat bij mensen in verband is gebracht met de ontstekingsreactie. Analyse van de ontologie van de genen bevestigde het gebrek aan een immuunrespons. Enkel bij genen met de GO-term “response to stimulus” en meer specifiek “response to stress” werd een significante verrijking gevonden.

De afwezigheid van een geïnduceerde expressie van immuungerelateerde genen 5 uur na immersie, wees erop dat er nog geen infectie van het weefsel had plaatsgevonden en dat de gevonden veranderingen in expressie mogelijk een reactie van het epitheel waren. Daarom hebben we naar latere tijdspunten na immersie in *E. tarda* gekeken. Voor deze analyse werden zes genen uitgekozen voor qPCR-analyse, *cyp1a*, *irg1l* en *stc1*, geselecteerd op basis van de microarray-analyse, en de bekende immuunmarkers *il1b*, *mmp9* en *tnfa*. De immuunmarkers *il1b* en *mmp9* vertoonden 24 uur na infectie een reactie, die 48 uur na infectie nog veel sterker was. *Irg1l* was op alle tijdspunten geïnduceerd. *Stc1* en *tnfa* gaven dermate wisselende resultaten, dat deze verder niet zijn meegenomen in de analyse. Echter, deze variatie in combinatie met de variatie van het sterftepercentage, leidde tot de hypothese dat niet alle embryo’s werden geïnfecteerd. Experimenten waarin de *E. tarda* suspensie werd gefractioneerd in de was-vloeistof en gewassen bacteriën lieten zien dat de vroege reacties van *cyp1a* en *irg1l* verklaard konden worden als een reactie op losgelaten delen van de celmembraan of andere losse bacteriële stoffen. De reactie op deze bacteriële stoffen kan van het epitheel zijn. Hierbij is de reactie van *cyp1a* relatief kort, terwijl die van *irg1l* misschien doorgezet wordt als een reactie op systemische infectie van interne weefsels of als een blijvende reactie op de bacteriële stoffen. Hoewel het mogelijk is dat dit puur een reactie is van het epitheel, kan het ook zijn dat de losgekomen bacteriële stoffen het embryo

in diffunderen, waar het vervolgens een algemene reactie veroorzaakt. De reacties van *il1b* en *mmp9* volgden voornamelijk op de blootstelling aan de bacteriële fractie, wat suggereert dat dit markers zijn voor een systemische infectie.

Door gebruik te maken van een protocol voor de isolatie van RNA uit een enkel embryo, konden we de regulatie van de genen *cyp1a*, *irg1l*, *il1b* en *mmp9* in individuele embryo's 48 uur na infectie testen. De resultaten gaven een sterkere bevestiging van onze hypothese. Terwijl *cyp1a* zoals verwacht niet was geïnduceerd en *irg1l* geïnduceerd was in alle embryo's, mogelijk als een reactie op losgelaten bacteriële stoffen, waren *mmp9* en *il1b* geïnduceerd in slechts één van de vijf embryo's. Het lijkt er dan ook op dat alleen dat ene embryo daadwerkelijk geïnfecteerd was.

Om te controleren of de gevonden variatie veroorzaakt werd door individuele verschillen tussen de embryo's, werd besloten om de zebravissen met *E. tarda* te injecteren. De reactie van dezelfde vier genen werd bepaald door middel van qPCR op twee tijdspunten, namelijk 4 en 8 uur na infectie. *Cyp1a* werd niet of nauwelijks geïnduceerd, wat in overeenstemming is met onze suggestie dat het een rol zou kunnen spelen in de reactie van het huidepitheel bij externe blootstelling aan bacteriële stoffen. Van de andere drie genen waren *irg1l* en *mmp9* 4 uur na infectie niet of zwak geïnduceerd, maar 8 uur na infectie zeer sterk geïnduceerd in alle embryo's, dit terwijl *il1b* sterk geïnduceerd was op beide tijdspunten, eveneens in alle embryo's. Niet alleen de reactie op transcriptieniveau bleek reproduceerbaar in alle embryo's na injectie met *E. tarda*, maar ook het sterftepercentage, aangezien 48 uur na infectie alle embryo's dood waren.

Nadat gebleken was dat injectie van *E. tarda* in embryo's beter reproduceerbare resultaten gaf op het niveau van individuele embryo's, hebben we hier microarray-analyse op uitgevoerd. In tegenstelling tot de resultaten van de microarray van het immersie-experiment, toonde deze microarray-analyse een hogere inductie van een groot aantal immuungerelateerde genen naast *irg1l*, *mmp9* en *il1b*. Een ontologie-analyse van de genen weerspiegelde dit met een significante verrijking van de GO-term "immune system process" naast "response to stimulus", die als enige werd gevonden na immersie.

De resultaten beschreven in dit hoofdstuk, zorgen voor enkele interessante discussiepunten en punten voor toekomstig onderzoek. Een van de meest

opvallende conclusies die getrokken kan worden, is dat het zebravisembryo verbazingwekkend resistent is tegen infectie van externe ziekteverwekkers. Van de diverse bacteriën die door verschillende onderzoeksgroepen zijn getest, zijn er slechts twee gevonden die één dag oude embryo's mogelijk kunnen infecteren door middel van statische immersie, namelijk *E. tarda* en *Flavobacterium columnare*. Bovendien, zoals aangetoond in dit proefschrift, is het zelfs twijfelachtig of *E. tarda* in immersieassays echt infectie kan veroorzaken. Als er al infectie optreedt dan is dat met een lage efficiëntie, maar het is ook niet uit te sluiten dat een deel van de embryo's dood gaat als gevolg van een toxische respons. Een test met statische immersie van drie dagen oude embryo's, waarvan de mond open is, was ook geen bruikbaar alternatief, aangezien er geen sterfte optrad in de daaropvolgende 2 dagen en na deze periode worden de zebravisembryo's minder interessant voor screeningsprojecten. Deze resultaten suggereren de vroege aanwezigheid van een zeer sterk immuunsysteem. Echter, het sterftepercentage van 100 % na intraveneuze injectie laat zien dat dit niet het geval is. In plaats daarvan lijkt het erop dat de bacteriën de huid of het darmepitheel niet kunnen penetreren en daarom geen interne infectie kunnen veroorzaken. Hoewel het mogelijk is dat *E. tarda* en *F. columnare* de huid wel kunnen penetreren, is het waarschijnlijker dat kleine, onbedoelde beschadigingen van de huid, veroorzaakt tijdens het werken met de embryo's, ervoor zorgen dat bacteriën binnen kunnen komen. Aan het begin van dit onderzoek leek het erop dat *P. aeruginosa* stammen PAO1 en PA14 sterfte veroorzaakten na immersie van de zebravisembryo's in suspensies van deze bacteriën. Naarmate meer ervaring in het werken met embryo's werd opgedaan, nam deze sterfte af tot het punt dat alle embryo's overleefden. Desalniettemin zijn *E. tarda* en *F. columnare* speciaal, aangezien zij de enige geteste bacteriën lijken te zijn die slechts weinig schade aan de huid nodig hebben om een infectie te kunnen veroorzaken.

De reden van de resistentie van zebravisembryo's tegen externe blootstelling aan bacteriën is waarschijnlijk dat het binnenkomen van bacteriën wordt voorkomen. De vraag is hoe dit wordt bereikt. Misschien produceert het epitheel tot nu toe ongeïdentificeerde antimicrobiële stoffen. Een andere mogelijke verklaring is dat het epitheel van zebravissen is omgeven door een slijmlaag die bacteŗïen tegenhoudt. Waarschijnlijker is dat het een combinatie van deze twee verklaringen is: een slijmlaag vol met antimicrobiële stoffen. De

aanwezigheid van resistentie tegen deze stoffen zou de virulentie van *E. tarda* en *F. columnare* in de immersieassay, kunnen verklaren. Dit zou meer in detail bestudeerd moeten worden. De resultaten van de microarray-analyse na immersie laten een reactie zien van de zebravis op uitwendig aanwezige bacteriën en voornamelijk, zoals aangetoond met qPCR, op losgelaten of uitgescheiden bacteriële stoffen. Deze reactie zou van het epitheel kunnen komen. Het meest interessante resultaat was dat *P. aeruginosa* stammen PAO1 en vooral PA14, een veel sterkere reactie veroorzaakten dan *E. tarda*. Aangezien bekend is dat *P. aeruginosa* veel stoffen uitscheidt, zou deze bacterie naast *E. tarda* gebruikt kunnen worden om deze reactie te onderzoeken en te bepalen of deze inderdaad van het epitheel afkomstig is. Verschillen tussen *E. tarda* en *P. aeruginosa* in de stoffen die een rol spelen in de respons van het epitheel, kunnen dan worden geïdentificeerd. Om te testen of de secretiesystemen een rol spelen, kunnen mutanten van bacteriën en “trans-well”-proeven worden gebruikt. In “trans-well”-proeven worden de bacteriën en de embryo’s gescheiden door een filter met poriën van 0,2 µm. Deze laat geen bacteriën, maar wel uitgescheiden stoffen door, die vervolgens het embryo kunnen beïnvloeden. Om te testen of de reactie veroorzaakt wordt door componenten van LPS (lipopolysacchariden), losgekomen van de buitenmembraan tijdens het wassen, kan geëxperimenteerd worden met gezuiverde LPS of met LPS-mutanten.

De conclusie van hoofdstuk 3 is, dat het *E. tarda* immersiesysteem niet geschikt is voor grote screeningsprojecten. Recentelijk is er een automatisch injectiesysteem voor snelle screening van grote aantallen embryo’s beschreven. In dit systeem worden bacteriën automatisch in de dooiers van bevruchte eieren geïnjecteerd, die vervolgens langzaam prolifereren om pas in een volgroeid embryo een infectie te veroorzaken. Dit systeem is zeer bruikbaar voor onderzoek met *Mycobacterium marinum*. Voor *E. tarda* is dit systeem echter niet geschikt vanwege de pathogeniciteit en snelle vermeerdering van de bacterie. Het zou de zich ontwikkelende zygote doden voordat het zich tot een volgroeid embryo kan ontwikkelen. Dit systeem kan mogelijk wel bruikbaar zijn als er verzwakte mutanten van *E. tarda* worden gevonden.

Analyse van een zebravismutant in Myd88

Tijdens dit promotieonderzoek werd, in een TILLING-screen, de eerste mutant geïdentificeerd met een knock-out-mutatie in een essentieel immuungerelateerd gen. Daarom hebben we in **hoofdstuk 4** *E. tarda* gebruikt om deze zebravis met een knock-out-mutatie in het gen coderend voor Myd88 te karakteriseren. Myd88 is een belangrijk adapter-eiwit in de Toll-like receptor (TLR) signaaltransductie. TLR's vormen één van de families van receptoren van het aangeboren immuunsysteem dat pathogeen-gerelateerde moleculen herkent. Er waren al eerdere studies met *myd88*-morpholino's uitgevoerd, maar morpholino's hebben een aantal nadelen. Ze kunnen alleen gebruikt worden voor de uitschakeling van genen tijdens de eerste paar dagen van de embryo-ontwikkeling, omdat ze te veel verdund of afgebroken worden tijdens latere stadia. Verder kunnen ze niet-specifieke effecten veroorzaken. De puntmutatie in *myd88* creëert een voortijdig stopcodon. Dit leidt tot een incompleet Myd88-eiwit, dat slechts bestaat uit een gedeeltelijk "death domain", dat net een deel mist dat essentieel is voor communicatie met verder in de signaaltransductieroute gelegen componenten. Dit eiwit mist eveneens het volledige TIR-domein, nodig voor communicatie met de TLR. Vergelijking van de transcriptieprofielen van de wild-type en de *myd88*$^{-/-}$ embryo's op drie verschillende tijdstippen liet slechts een klein aantal genen zien met een consistente verandering in expressie, inclusief *myd88* zelf. Deze laatste observatie suggereert een terugkoppeling van *myd88* naar zichzelf of een sterk verminderde stabiliteit van het gemuteerde *myd88*-mRNA.

Het opkweken van de *myd88*$^{-/-}$ mutanten tot volwassen vissen is moeilijk gebleken. Bij de start van dit onderzoek stierven de volwassen vissen zodra er een stukje vin geknipt werd voor genotypering. Daarom maakten we gebruik van wild-type en mutante zebravisembryo's van heterozygote volwassenen. Aangezien van het immersiesysteem in hoofdstuk 3 was aangetoond dat het niet geschikt is voor analyse op het niveau van individuele embryo's en het bovendien geen makkelijk meetbare immuunrespons opwekt, gebruikten we de intraveneuze injectiemethode voor de karakterisering van de immuunrespons van deze mutant. Er werd qPCR-analyse gedaan op de genen *mmp9*, *irg1l*, *il1b* en *ifn*. Dit liet een duidelijke *myd88*-afhankelijkheid zien van *mmp9* en een gedeeltelijke *myd88*-afhankelijkheid van *irg1l* en *il1b*. Voor *ifn* was het niet

mogelijk om conclusies te trekken, aangezien dit gen slechts in één van de wild-type embryo's was geïnduceerd.

De embryo's die voor de qPCR-analyse waren gebruikt, werden vervolgens geanalyseerd met microarrays. Zoals verwacht, liet dit zien dat veel immuungerelateerde genen afhankelijk zijn van Myd88. Omdat een aantal van deze immuungerelateerde genen op de array vertegenwoordigd is door meerdere probes, konden deze op een betrouwbare manier worden geïdentificeerd als *myd88*-afhankelijke genen. Onze resultaten lieten zien dat *mmp9*, *il1b* en *irak3* expressie *myd88*-afhankelijk zijn, wat eerdere resultaten verkregen met *myd88*-morpholino knockdown en *Salmonella* infectie, bevestigt. Ook verschillende andere genen zoals transcriptiefactoren (bijv. *junb*), signaaltransductiegenen (bijv. *rip2k*), cytokinegenen (bijv. *tnfb*), chemokinegenen (bijv. *cxcl-clc*) en andere immuunresponsgenen (bijv. *ncf1*) vertoonden afhankelijkheid van Myd88. Deze resultaten komen overeen met bevindingen uit onderzoek met knock-out-muizen van *MyD88*. Van een aantal genen, waarvan werd aangetoond dat ze *myd88*-afhankelijk zijn, was eerder in een morpholino-onderzoek ook aangetoond dat ze *traf6*-afhankelijk zijn. Dit waren onder andere *mmp9*, *il1b*, *tnfb*, *ncf1*, *zgc:114032* (complement factor B), *zgc:112143* (TNFα-induced protein 9) en *tlr5b*. Deze genen kunnen nu als doelwitgenen van de *myd88*-*traf6*-signaaltransductie worden beschouwd.

De meest verrassende observatie was het verschil in expressie tussen *tnfa* en *tnfb* in de *myd88*$^{-/-}$-mutant. In tegenstelling tot *tnfb*, dat volledig *myd88*-afhankelijk bleek te zijn, was *tnfa* totaal onafhankelijk van Myd88. *Tnfa* en *tnfb* zijn twee kopieën in het zebravisgenoom van het gen dat in de muis en in de mens codeert voor *TNFα*. Terwijl *tnfb* dezelfde afhankelijkheid van Myd88 laat zien als humaan *TNFα*, doet *tnfa* dat niet, wat mogelijk een divergentie van de functie van deze homologe genen weerspiegelt.

De inductie van verschillende genen (zoals *il1b*) bleek niet volledig maar gedeeltelijk afhankelijk zijn van Myd88, wat mogelijk verklaard kan worden door weefselspecifieke verschillen. Aangezien in deze experimenten het RNA geïsoleerd werd van hele embryo's, gaan de verschillen tussen de diverse weefsels verloren. Zo werden er bijvoorbeeld opvallende verschillen gevonden tussen de transcriptionele respons van de milt en de lever bij septische wild-type muizen en knock-out-muizen van Myd88. De reactie van de lever toonde

een sterke afhankelijkheid van Myd88, terwijl de reactie van de milt grotendeels Myd88-onafhankelijk was.

In dit hoofdstuk hebben we aangetoond dat de *myd88*$^{-/-}$ mutant van de zebravis een sterk verminderde aangeboren immuunrespons tegen *E. tarda* heeft. Momenteel worden experimenten met deze zebravismutant gedaan, waarin andere pathogenen worden onderzocht. De eerste resultaten wijzen erop dat *myd88* een belangrijke rol speelt bij het onder controle houden van een *M. marinum*-infectie. Aangezien de problemen met het opkweken van de *myd88*$^{-/-}$ zebravis mutant naar volwassenheid inmiddels zijn opgelost, kan het voordeel van de zebravismutant ten opzichte van de morpholino-knockdown nu volledig worden benut. Daarbij zullen nieuwe TILLING-screens, waarvan de efficiëntie door nieuwe sequencingtechnieken in de laatste jaren enorm is toegenomen, ertoe leiden dat er snel meer immuungerelateerde mutanten geïdentificeerd zullen worden. Het Sanger Instituut heeft de ambitie om binnen een paar jaar zebravismutanten voor ieder gen beschikbaar te hebben. Naast de TILLING-screens is ook het gebruik van de zinkvinger-technologie snel in opkomst, wat gerichte knock-out en knock-in van genen in de zebravis mogelijk maakt. Deze ontwikkelingen zullen ervoor zorgen dat de zebravis als genetisch testmodel snel op gelijke hoogte zal komen met de muis.

Conclusie

Concluderend legt het in dit proefschrift beschreven onderzoek een sterke basis voor toekomstig onderzoek. Het genoom van *E. tarda* kan inzicht geven in de verschillen tussen pathogene en niet-pathogene stammen en ook in de verschillen tussen gastheer-specifieke *E. tarda*-stammen en stammen met een breder gastheerbereik. Het zebravisembryomodel zal bij uitstek geschikt zijn om de interactie van virulentiefactoren van *E. tarda* met het aangeboren immuunsysteem van de gastheer te bestuderen. Hierbij zal het ook zeer interessant zijn om de reactie op *E. tarda* te vergelijken met de reactie op *E. ictaluri*. Helaas moeten we concluderen dat *E. tarda* weinig geschikt is voor grote screeningsprojecten, aangezien het immersiesysteem niet reproduceerbaar genoeg is en de bacterie een te sterke pathogeen is voor geautomatiseerde dooierinjecties. Toch heeft ons onderzoek naar het immersiesysteem veel interessante openingen gecreëerd voor het onderzoeken van de reactie van het zebravisepitheel, niet alleen in reactie op *E. tarda* maar

ook met betrekking tot de *P. aeruginosa* stammen. Niet alleen is de respons van het epitheel interessant, maar ook de bacteriële stoffen die deze respons veroorzaken. De karakterisering van de *myd88*$^{-/-}$-mutant is waarschijnlijk de belangrijkste contributie van dit proefschrift aan toekomstig onderzoek. Het is de eerste zebravismutant met een knock-out mutatie in een gen, dat centraal staat in het aangeboren immuunsysteem. In de toekomst kunnen er onderzoeksprojecten worden uitgevoerd met verschillende pathogenen en kankermodellen, waarbij de volledige rol van *myd88* in de zebravis kan worden blootgelegd, mogelijk leidend tot nieuwe inzichten in de functie van het aangeboren immuunsysteem.

Curriculum vitae

Joost Jeremy van Soest werd geboren op 26 januari 1983 te ’s-Gravenhage. Na het behalen van zijn VWO diploma aan het Christelijk College De Populier in 2001, startte hij de studie biologie aan de Universiteit Leiden, waar hij in 2006 *cum laude* afstudeerde. Tijdens deze opleiding werden door hem drie stages uitgevoerd. De eerste was een stage van drie maanden bij de afdeling Infectieziekten van het LUMC, alwaar hij onderzoek deed naar de antimicrobiële effecten van madenexcreet en het effect van dat excreet op humane monocyten. De tweede stage was bij Moleculaire Microbiologie in de groep van Prof. Dr. Lugtenberg, waar hij een flowcell-systeem opzette voor onderzoek naar bacteriële biofilms. De derde en laatste stage werd in de groepen Moleculaire Celbiologie van Prof. Dr. H.P. Spaink en Moleculaire Microbiologie uitgevoerd en was het voorwerk voor zijn latere promotie-onderzoek naar bacteriële infecties in de zebravis. Dit promotie-onderzoek werd in januari 2006 gestart met als einddoel het vinden van probiotica voor viskwekerijen en stond onder begeleiding van Dr. G.V. Bloemberg en Prof. Dr. E.J.J. Lugtenberg, wiens plaats als promotor later werd ingenomen door Prof. Dr. C.A.M.J.J. van den Hondel en Prof. dr. H.P.Spaink. Met het vertrek van Dr. Bloemberg naar Zwitserland in 2008 werd de directe begeleiding overgenomen door Dr. A.H. Meijer. Hiermee veranderde de insteek van het onderzoek. Het opgezette infectiesysteem met *Edwardsiella tarda* werd verder verfijnd en vervolgens gebruikt voor het karakteriseren van de eerste zebravismutant in een gen centraal in het aangeboren immuunsysteem, namelijk *myd88*. Dit onderzoek heeft tot dusverre geresulteerd in een publicatie en de tot standkoming van dit proefschrift. In augustus 2010 startte Joost met de lerarenopleiding aan de Universiteit Leiden met als doel het verkrijgen van een eerste graads lesbevoegdheid. Sinds augustus 2011 werkt hij aan het Maris College te ’s-Gravenhage als docent biologie op de locatie Belgisch Park.

Publications

van Soest, J.J., O.W. Stockhammer, A. Ordas, G.V. Bloemberg, H.P. Spaink, A.H. Meijer. 2011. Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen Edwardsiella tarda. BMC Immunol 12:58