Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections

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Summary

Bacterial virulence is best studied in animal models. However, the lack of possibilities for real time analysis and the need for laborious and invasive sample analysis limit the use of experimental animals. In the present study 28 h-old zebrafish embryos were infected with DsRed-labelled cells of Salmonella typhimurium. Using multidimensional digital imaging microscopy we were able to determine the exact location and fate of these bacterial pathogens in a living vertebrate host during three days. A low dose of wild-type S. typhimurium resulted in a lethal infection with bacteria residing and multiplying both in macrophage-like cells and at the epithelium of blood vessels. Lipopolysaccharide (LPS) mutants of S. typhimurium, known to be attenuated in the murine model, proved to be non-pathogenic in the zebrafish embryos and were partially lysed in the bloodstream or degraded in macrophage-like cells. However, injection of LPS mutants in the yolk of the embryo resulted in uncontrolled bacterial proliferation. Heat-killed, wild-type bacteria were completely lysed extracellularly within minutes after injection, which shows that the blood of these zebrafish embryos does already contain lytic activity. In conclusion, the zebrafish embryo model allows for rapid, non-invasive and real time analysis of bacterial infections in a vertebrate host.

Introduction

All multicellular eukaryotic organisms are continuously exposed to bacterial and viral threats. The selection pressure imposed by these microorganisms has resulted in the development of defence systems, such as the complex and ingenious immune system of mammals. This immune system is composed of many different cell types, which are spread throughout the organism to recognise and destroy foreign invaders. We are only beginning to understand what cell types are actually encountered by pathogens in the host. In order to study the full interaction of a bacterial pathogen with the host immune system we can therefore not stay within the confines of the tissue culture flask, but need in vivo infection models. A major disadvantage of animal models is that the interaction between invading bacteria and the individual host immune cells is difficult to follow in real time. Recently, lower eukaryotes have been introduced as model to study bacterial infections. The nematode Caenorhabditis elegans has proven useful for the study of virulence factors of the bacterial pathogens Pseudomonas aeruginosa and Salmonella typhimurium (Mahajan-Miklos et al., 1999; Aballay et al., 2000; Labrousse et al., 2000; Tan and Ausubel, 2000). These nematodes are translucent and bacteria can easily be followed by fluorescence microscopy. However, C. elegans is genetically not closely related to humans and its immune system appears quite different from the complex mammalian immune system. Caenorhabditis elegans lacks a complement system and macrophage-like cells able to phagocytose bacteria (Ewbank, 2002). Even the Toll-like receptors, which play an important role in the innate immune system of highly divergent organisms such as humans and insects, do not seem to have a similar function in C. elegans (Pujol et al., 2001). Therefore, we set out to find an alternative infection model to study bacterial infections in real time.

Teleost fish species, such as the zebrafish (Danio rerio), have a well-developed immune system, both innate and adaptive, which is quite similar to the mammalian immune system (Trede et al., 2001; Zarkadis et al., 2001). Furthermore, zebrafish embryos develop externally and are optically transparent during their development. These characteristics render the zebrafish a useful model for the study of vertebrate development, and as such zebrafish has become the preferred model for developmental biol-
ogists. As a consequence, important genetic tools to study zebrafish development have been developed (Nasevicius and Ekker, 2000; Wienholds et al., 2002), and the complete genome sequence of the zebrafish will be available in the near future (Sanger Institute). The same characteristics that make the zebrafish embryo an interesting model to analyse vertebrate development may prove useful for the study of bacterial diseases.

In the zebrafish embryo, macrophage-like cells appear at about 25 h post fertilization (hpf) in the circulation (Herbomel et al., 1999). These cells are able to phagocytose intravenously injected bacteria and, upon sensing the presence of bacteria, migrate into infected areas to eradicate the microbial invaders. Infection leads to the activation of the entire population of macrophage-like cells, similar to what occurs with mammalian macrophages (Herbomel et al., 1999). Granulocytes appear in zebrafish embryos at 48 hpf and have been shown to accumulate at sites of inflammation (Lieschke et al., 2001). In the present study zebrafish embryos were infected with *Salmonella typhimurium* for the real time analysis of a bacterial infection. This bacterium was able to establish a lethal infection in the embryos. In contrast, *S. typhimurium* mutants, known to be attenuated for virulence in the mammalian system (Roantree, 1967; Ohno et al., 1995; Tsolis et al., 1999), were avirulent in the zebrafish embryo. It has been suggested previously that the zebrafish may be used as a model for the analysis of a variety of human diseases (Dooley and Zon, 2000; Kalev-Zylinska et al., 2002). Here, we show that the zebrafish embryo is a useful model to study infectious diseases.

**Results**

**Infection of zebrafish embryos with *E. coli***

In general, bacteria can only be observed at high magnification (400× or 1000×). In order to follow bacteria in intact animals, even in zebrafish embryos, it is important to be able to visualize bacteria at low magnification. Therefore, we first isolated highly fluorescent bacteria. Bacteria that expressed green fluorescent protein (GFP) proved not very well suited for our purpose, because of a relatively low fluorescence of the bacteria, and the green fluorescent background of zebrafish embryos (results not shown). Instead, we used the red fluorescent protein (DsRed) from the sea anemone *Discosoma* sp. DsRed is a slowly maturing protein with a maturation half time of about 24 h, which is not in line with the division time of most bacteria. Recently, different fast maturing variants of DsRed, with maturation half times of about 1 h, have been isolated (Bevis and Glick, 2002). Upon adjusting the translation start site (see **Experimental procedures**) and by placing the DsRed variant T3 under control of the lac promoter we were able to create a construct that resulted in bright red fluorescent *E. coli* cells that could be discerned as single cells at 60× magnification.

To determine whether we were able to follow these bacteria in zebrafish embryos, DsRed expressing *E. coli* cells were injected in the axial vein of zebrafish embryos at 28 h post fertilization (hpf). The individual bacteria could be observed directly at low magnification (Fig. 1). Initially, the bacteria were seen circulating in the bloodstream, which led them throughout the entire embryo. After approximately half an hour, most bacteria were seen fixed in the blood vessels. At 30 h post infection (hpi) no more fluorescence was observed, which indicated that all bacteria had been cleared from the host. These results show that individual bacteria can be observed at low magnification in a living vertebrate host.

**Infection of zebrafish with *S. typhimurium***

To test the possibility to use the zebrafish embryo as a model for intracellular infections, embryos were challenged with *S. typhimurium*. Approximately 50 cells (high dose) of *S. typhimurium* wild type strain LT2, expressing DsRed, were injected in zebrafish embryos at 28 hpf. The zebrafish embryos were kept at 28°C and the bacterial infection was followed in time (Fig. 2A). This suboptimal

![Image](image-url)
growth temperature for S. typhimurium had only a limited effect on these bacteria: in culture, an increase in the doubling time of 20% was observed, whereas no influence on cell length was observed. As for E. coli, individual S. typhimurium cells were observed circulating in the bloodstream directly after injection. At 4 hpi, approximately all S. typhimurium cells were still present (Fig. 2A) and most of them were seen in a fixed position, whereas a single cell kept circulating in the bloodstream. In the infected embryos the amount of DsRed spots, representing S. typhimurium cells or the remains thereof, seemed to remain relatively constant between 4 hpi and 20 hpi. From this point onwards, a steady increase in the amount of DsRed containing spots was observed (Fig. 2A). Interestingly, at the same time we observed a continuous increase in the amount of free DsRed in the bloodstream. This DsRed fluorescence appeared as a diffuse staining and was not associated with bacteria (Fig. 2A and 26 hpi and 28 hpi). Also the number of freely circulating S. typhimurium cells increased in the latter stages of disease. Eventually, an infection dose of 50 wild-type S. typhimurium cells resulted in 100% lethality between 30 and 48 hpi.
and multiply within phagosomes of macrophages or a facultative intracellular bacterium, which is able to persist and multiply within phagosomes of macrophages or polymorphonuclear cells (Duniap et al., 1992; Salcedo et al., 2001). However, also extracellular bacteria can be isolated from infected murine tissues and extracellular bacterial replication has been reported within sinusoids or lesions (Wang et al., 1988). To determine the exact location of S. typhimurium replication within living zebrafish embryos, we used multidimensional digital imaging microscopy. With this technique we were able to analyse the cells in four dimensions (3D and time) in living zebrafish embryos. At 4 hpi, with a high dose of wild-type S. typhimurium (50 cells), the majority (approximately 60%) of the DsRed-loaded host-cells did not contain intact rods, but the red fluorescence was located in spheres of different sizes (Fig. 3, arrowhead and Movie 1 of Supplementary material). These structures represent (partially) degraded bacteria in lysosomes of macrophage-like cells, in which the fluorescent signal gradually faints upon prolonged observation. Apparently, not all S. typhimurium cells are able to prevent lysosomal killing upon phagocytosis by macrophage-like cells. Another group of fluorescent macrophage-like cells contained DsRed in the form of long rods of approximately 8–13 μm or short chains or rods (Figs 3 and 4, Movie 2 of Supplementary material). In vitro grown S. typhimurium cells at 28°C or 37°C, with

### Table 1. Relative number of DsRed containing spots per embryo in time.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type low dose A</th>
<th>low dose B</th>
<th>high dose</th>
<th>Ra mutant low dose</th>
<th>high dose</th>
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<tr>
<td>no of embryos</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 10</td>
<td>n = 6</td>
<td>n = 10</td>
</tr>
<tr>
<td>dcs at 30 mpi</td>
<td>24.4 ± 4.7</td>
<td>15.4 ± 10</td>
<td>55 ± 10</td>
<td>22.2 ± 6.0</td>
<td>58 ± 8.3</td>
</tr>
<tr>
<td>dcs at 90 mpi</td>
<td>29.2 ± 10.9</td>
<td>15.2 ± 10.4</td>
<td>nd</td>
<td>16.2 ± 5.2</td>
<td>nd</td>
</tr>
<tr>
<td>dcs at 26 hpi</td>
<td>&gt;100</td>
<td>12.4 ± 6.7</td>
<td>nd</td>
<td>5.3 ± 3.9</td>
<td>nd</td>
</tr>
<tr>
<td>no dead embryos at 2dpi</td>
<td>5</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
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Zebrafish embryos were injected in their axial vein at 28 hpf with either wild-type or Ra-LPS mutant (Ra) S. typhimurium cells expressing DsRed and grown at 28°C. Two doses were injected, a low dose of approximately 25 cells per embryo or a high dose of approximately 50 cells per embryo. The embryos injected with a low dose of wild-type cells were retrospectively divided in two groups based on the final outcome of the infection, i.e. lethal (A) or not (B). Given is the number of DsRed containing spots (dcs) after 30 min post infection (mpi), 90 mpi or 1 d post infection (dpi). These spots represent either single bacteria, partially lysed bacteria in macrophages or groups of bacteria. In addition the number of zebrafish embryos that died of the infection is given at 2 dpi. The embryos were followed till 5 dpi. No differences in lethality between 2 dpi and 5 dpi were observed. (dsc = DsRed containing spots; mpi = minutes post infection; hpi = hours post infection; nd = not done).

At the final stages of infection the amount of bacteria dramatically increased, although these bacteria still remained within the circulatory system (Fig. 2C).

When zebrafish embryos were injected with a lower dose (15–25 cells) of S. typhimurium cells, 50% of the embryos succumbed to the infection (Table 1). To be able to quantify disease development, 10 zebrafish embryos were injected with a low dose of bacteria and followed individually. Retrospectively, the zebrafish embryos that died of the infection were grouped in low dose A (Table 1), whereas the surviving embryos were grouped in low dose B. Disease development of the lethally infected embryos (group A) was identical to that observed for the high dose infected embryos. On the other hand, embryos of group B showed a slow, but steady, decline in the number of DsRed containing spots. At 3 dpi no more DsRed containing spots were observed. Together these experiments show that a small number of wild type S. typhimurium cells are able to raise a lethal infection in zebrafish embryos.

### Localization of S. typhimurium early in infection

Analysis of the S. typhimurium infection at low magnification showed that these bacteria are able to multiply within the zebrafish host. Salmonella typhimurium is known as a facultative intracellular bacterium, which is able to persist and multiply within phagosomes of macrophages or polymorphonuclear cells (Duniap et al., 1992; Salcedo et al., 2001). However, also extracellular bacteria can be isolated from infected murine tissues and extracellular bacterial replication has been reported within sinusoids or lesions (Wang et al., 1988). To determine the exact location of S. typhimurium replication within living zebrafish embryos, we used multidimensional digital imaging microscopy. With this technique we were able to analyse the cells in four dimensions (3D and time) in living zebrafish embryos. At 4 hpi, with a high dose of wild-type S. typhimurium (50 cells), the majority (approximately 60%) of the DsRed-loaded host-cells did not contain intact rods, but the red fluorescence was located in spheres of different sizes (Fig. 3, arrowhead and Movie 1 of Supplementary material). These structures represent (partially) degraded bacteria in lysosomes of macrophage-like cells, in which the fluorescent signal gradually faints upon prolonged observation. Apparently, not all S. typhimurium cells are able to prevent lysosomal killing upon phagocytosis by macrophage-like cells. Another group of fluorescent macrophage-like cells contained DsRed in the form of long rods of approximately 8–13 μm or short chains or rods (Figs 3 and 4, Movie 2 of Supplementary material). In vitro grown S. typhimurium cells at 28°C or 37°C, with

![Fig. 3. Zebrafish macrophage-like cells loaded with intact (arrows) and disrupted S. typhimurium cells (arrowhead). This zebrafish embryo was inoculated at 28 hpf by injection in the axial vein with approximately 50 cells of S. typhimurium expressing DsRed. The infected macrophages are located in the yolk sac circulation valley. An overlay photo of DIC image and Cy-3 fluorescent images (in red) is shown. Bar represents 10 μm.](image-url)
or without expressing DsRed, are generally 3 μm, which means that *S. typhimurium* cells are elongated within these host macrophages. Prolonged observation (hours) of these intracellular bacteria usually showed no obvious changes. However, in several occasions such an elongated intracellular bacterium divided simultaneously into multiple small daughter cells (Fig. 4A–C). This behaviour shows that these intracellular rods indeed represent viable bacterial cells. The macrophage-like host cells of these intact bacteria showed active movement, which means that these cells are not rapidly killed by *S. typhimurium* (Movie 2). However, upon prolonged presence of intact bacteria in macrophages these movements subsided (data not shown). In contrast, macrophages containing lysed bacteria always showed active cellular rearrangements in time-lapse recordings (Movie 1). Together these experiments show that *S. typhimurium* is able to survive and to replicate in macrophage-like cells. In addition to these two groups of fluorescent host-cells a DsRed-containing bacterium could sometimes be observed circulating in the bloodstream (seen as occasional red flashes in Movie 1).

These results were confirmed in bacterial plating experiments, groups of infected embryos were disintegrated at different time points and plated on selection plates. Directly after injection of 50 bacterial cells at average 23 colony forming units (cfu) could be isolated per embryo, which indicates a plating efficiency of approximately 50%. This number declined to 8 at 4 hpi (Fig. 5), which shows that more than half of the bacteria are killed by the immune system of the embryo in these first hours. The number of lysed bacteria observed inside macrophages could account for this percentage. After this bottleneck the number of cfu inside the zebrafish embryos increased rapidly (Fig. 5). More than 99% of the colonies at all time points were expressing DsRed, which indicates that nearly all living bacteria in the infected animals could be visualized. In order to confirm the location of the bacteria by another experiment, groups of infected embryos were disintegrated into free cells and subsequently treated with gentamycin to kill all extracellular bacteria. Shortly after the injection of bacteria only 4% of the colony-forming bacteria were protected against the antibiotic, which means that on average only one bacterium is phagocytosed at this time-point. This number increased to 45% at

![Fig. 4. Replicating wild-type *S. typhimurium* cells inside macrophage-like cells. The zebrafish embryo was inoculated at 28 hpf by injection in the axial vein of approximately 50 cells of *S. typhimurium* expressing DsRed. Overlay photographs of DIC images and Cy-3 fluorescent images (in red) of an intracellular bacterium within a macrophage in the axial vein are shown. The macrophage contours and the macrophage movements of this host-cell can be seen in Movie 2 (Supplementary material). This intracellular bacterium was followed for 4 h, starting from 4 hpi, and representative pictures of this period are shown in A–C. Bar represents 5 μm.](image)

![Fig. 5. Number of colony forming units (cfu) of wild-type *S. typhimurium* (■) and the isogenic Ra mutant (○) isolated per infected zebrafish embryo at different time points (hpi). Groups of 5 embryos were analysed at each time-point. The numbers are the average of two separate experiments.](image)
2 hpi and 70% at 4 hpi, as could also be observed with microscopy.

**Localization of *S. typhimurium* late in infection**

At 23 hpi, both types of DsRed-containing macrophages described above can still be observed in the infected zebrafish embryos. Interestingly, at the same time-point, increasing amounts of extracellular *S. typhimurium* cells can be observed. These cells are mostly attached to the epithelium of the smaller blood vessels (results not shown) or seen in the marginal regions of the yolk sac circulation valley (a large sinus which fans out across both sides of the yolk) (Fig. 6) and appear as short rods or coccoid-like cells of 0.8–1.5 μm. 3D stack analysis of these microcolonies showed that these bacteria were located extracellularly on the epidermis surrounding the yolk sac circulation valley or on the epithelium of blood vessels (results not shown). Prolonged observation of such individual microcolonies showed that these microcolonies are dynamic structures. We observed bacterial replication (Fig. 6A and D), and also the separation of a colony-segment (Fig. 6D). The detachment of these bacteria could explain the increase in the amount of free circulating bacteria that we observe at this time point. Eventually, phagocytic cells do appear at these remote sites and incorporate part of the microcolony (Movie 3 of Supplementary material).

Plating of lysed zebrafish showed that the number of cfu dramatically increased during this period (Fig. 5). This increase was substantially higher than the observed increase in DsRed containing spots, which means multiple bacteria are located at the same position. Gentamycin treatment of disrupted embryos showed that at 20 hpi 35% of the living bacteria are located intracellularly, whereas this percentage dropped to 20% at 28 hpi. This means that in the later stages of disease the increasing amounts of the bacteria is mainly due to extracellular bacteria in microcolonies, which was also observed in fluorescence microscopy (Fig. 2C). Together, these results show that there are two populations of replicating *S. typhimurium* cells, one formed by elongated bacteria in macrophage-like cells and the other by small microcolony-forming bacteria in macrophage-poor regions of the blood circulation system.

**LPS mutants of *S. typhimurium* are avirulent**

The infection with wild type *S. typhimurium* cells demonstrates that zebrafish embryos might be used as an infection model. To further substantiate this model, we tested the behaviour of different LPS mutants of *S. typhimurium* in zebrafish embryos. Defects in the synthesis of intact LPS are known to affect the virulence of *S. typhimurium* in the mammalian host (Roantree, 1967; Ohno et al., 1995; Tsolis et al., 1999), mainly due to the increased susceptibility of these mutant bacteria to complement lysis. Isogenic mutants affected in the synthesis of the LPS O-antigen (Ra) or of different residues of the LPS core region (Rc and Rd) were used. These LPS mutants, modified to express DsRed, were also injected in a high and a low dose. In contrast to wild-type *S. typhimurium* cells, a reduction in the number of mutant *S. typhimurium* cells was already observed in the first hour after infection. For instance, within 1 h after the injection of 50 cells of the Ra mutant their number was reduced by 34.6% ± 20 (n = 5). Similar results were obtained with the Rc and Rd mutants. A simultaneous slight increase in the diffuse DsRed fluorescence of the bloodstream suggested that the reduction of visible cells could be due to extracellular lysis of these bacteria. After an initial rapid reduction, the number of DsRed spots declined more slowly but steadily, until after 26 hpi almost all of the DsRed spots were cleared (shown in Fig. 2, right panels for the high dose and Table 1 for the low dose). Subsequent detailed microscopic analysis

![Fig. 6. Behaviour of wild-type *S. typhimurium* cells within a microcolony. The zebrafish embryo was inoculated at 28 hpf by injection in the axial vein of approximately 50 cells of *S. typhimurium* expressing DsRed. This microcolony is situated in the margins of the yolk sac circulation valley. Deconvoluted images of the Cy-3 channel are shown (A–E) and (F) a DIC image of the situation in panel E. This microcolony was followed for 3 h, starting from 23 hpi, and representative pictures of this period are shown. Replicating bacteria were observed (indicated with arrows), and also a small part of the microcolony segregated after 2 h (arrowhead). After this period, a phagocytic cell internalized part of this microcolony, as shown in Movie 3 (Supplementary material). Bar represents 2 μm.](image-url)
S. typhimurium infection in zebrafish embryos

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could not detect any Ra mutants as intracellular rods, in contrast to the wild-type S. typhimurium cells. After 20 hpi only red fluorescent spherical structures could be discerned in the macrophages (Fig. 7). Disintegration and plating of the infected embryos showed a similar picture: the number of Ra mutants gradually declined until at 40 hpi no more cfu’s could be isolated (Fig. 5). The challenge with the different S. typhimurium mutants did not lead to a lethal infection (shown for Ra in Table 1). This shows that Ra, Rc and Rd mutants are avirulent in the zebrafish embryo model.

As a control, also a high dose of heat-killed wild-type S. typhimurium cells was injected. Already at 12 minutes post infection (mpi) no bacteria were observed in the bloodstream anymore (Fig. 8). In addition, the blood of these embryos was also slightly red fluorescent, indicating that these cells were rapidly lysed in the bloodstream. Apparently, heat-killed bacteria are highly susceptible to extracellular lysis in the blood of zebrafish embryos.

Proliferation of LPS mutants in the yolk

The avirulent character of the S. typhimurium LPS mutants could be caused by the inability of this mutant to replicate in host tissue. However, S. typhimurium Ra mutant cells accidentally injected in the yolk of the embryo were able to survive for two days and even showed a massive proliferation (Fig. 9). This implies that infection of the yolk with S. typhimurium may be used as an in vivo growth control for bacterial mutants. The infected zebrafish embryos survived the Ra mutant infection of the yolk for two days. At that time the yolk did not contain the bacteria, which entered the embryo itself and rapidly killed it.

Fig. 7. Location of Ra-LPS mutant S. typhimu-
rium cells. Zebrafish embryos were inoculated at 28 hpf by injection of approximately 50 cells of Ra-LPS mutant S. typhimurium expressing DsRed in the axial vein. Overlay photographs of brightfield and Cy-3 fluorescent images (red) are shown.
A. Composition photograph of an infected embryo at 20 hpi, anterior side to the left and dorsal side up. Only some DsRed spots (red) are still present. Scattering (arrow) is due to a DsRed spot in a different focal plane.
B. Macrophage-like cells containing (partially) lysed S. typhimurium Ra cell(s). The dark cells are melanocytes (pigment cells). Bar indicates 20 μm.

Fig. 8. Rapid lysis of heat-killed bacteria. Zebrafish embryos were injected at 28 hpf with more than 500 cells of heat-killed S. typhimu-
rium expressing DsRed. Photos of DsRed fluorescence in the axial vein were recorded directly after injection (A), at 4 min post injection (mpi) (B), 8 mpi (C) and 12 mpi (D).
escaped from the macrophages and colonized the more remote regions of the blood circulation system, such as the small blood vessels and the marginal region of the yolk sac circulation valley. These regions are probably preferred by these extracellular bacteria because of the reduced blood flow, or because of the sparse presence of macrophages. At these sites, the bacteria were seen to form microcolonies, in which they actively replicated, and from which they were shed in the circulation. They are able to do so until macrophages are directed to these infected areas. From this time-point onwards the number of extracellular bacteria outnumber the bacteria residing inside host cells. These findings may shed new light on a long-standing question in the pathogenesis of *S. typhimurium* infections about the site of bacterial multiplication in the host. Different cell types, including macrophages and polymorphonuclear cells (Dunlap *et al.*, 1992; Salcedo *et al.*, 2001), and extracellular locations have been suggested as prime site of bacterial replication (Wang *et al.*, 1988). In zebrafish embryos a combination of both intracellular and extracellular replication can be seen, which suggests a dynamic situation.

Apart from the location of individual bacteria also their shape can be observed directly in this real time infection model. Whereas *S. typhimurium* cells grown in vitro are of moderate length (about 3 μm), these cells elongate to 8–14 μm upon phagocytosis by macrophages. These elongated bacteria were not seen in spacious phagosomes, as has been reported recently for *Salmonella arizonae* inside zebrafish macrophages (Davis *et al.*, 2002). The elongated bacteria occasionally divided into multiple daughter cells, which probably indicates that these bacteria are polyploid. Although cell elongation has not been reported for *S. typhimurium* in murine macrophages, this characteristic has been observed upon internalization in human melanocytes (Martinez-Lorenzo *et al.*, 2001). In addition *S. typhimurium* swarmer cells are also known to be elongated and polyploid (Harshey and Matsuyama, 1994). Within microcolonies in the zebrafish embryo, the *S. typhimurium* cells were significantly smaller (about 1 μm). Whereas at the final stages of infection most extracellular bacteria showed a ‘normal’ length of 2–3 μm. The nature of these differences is not known.

This infection model also allows the direct observation of lysing bacteria, as the disappearance of fluorescent rods and the release of red fluorescent protein into the bloodstream. Hardly any wild-type bacteria were lysed within the first hour in the bloodstream, whereas the different LPS mutants appeared susceptible to extracellular lysis: the number of mutant *S. typhimurium* cells generally reduced by more than 30% within one hour. These results are in line with what is observed in mammalian models (Ohno *et al.*, 1995; Tsolis *et al.*, 1999). Heat-killed, wild-type bacteria were even more prone to direct lysis in the

**Discussion**

In this study, we have demonstrated that a bacterial infection can be analysed in real time in a vertebrate host. The zebrafish embryo infection model proved both quick and non-invasive and offers a number of advantages over classical infection models. Results are obtained within days and the number of experiments can be scaled up relatively easy. Because the bacteria can be observed at low magnification the handling time is severely reduced. This means that large numbers of bacterial mutants may be studied. In addition, the fate of each single bacterium and the actual site of bacterial replication can be analysed. The suboptimal growth temperature did not prevent that low numbers of *S. typhimurium* were able to mount a rapid lethal infection.

In vivo analysis showed that after their initial flow in the bloodstream, macrophage-like cells captured most wild-type *S. typhimurium* cells. Although some bacteria were lysed in the macrophages, other bacteria seemed to resist degradation and were able to divide. The affected macrophages were neither killed rapidly, nor did the phagocytosed bacteria escape from these cells. A rather static interaction was observed, with an initially active macrophage whose movements gradually subsided. These data could indicate that, upon successful invasion by *S. typhimurium*, zebrafish macrophages are killed in a slow process, similar to the cytotoxicity mediated by the effectors of the SPI2 type III secretion system of this bacterium (Monack *et al.*, 2001). At 20 hpi some bacteria must have

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**Fig. 9.** Proliferation of Ra-LPS mutant *S. typhimurium* in the yolk at 48 hpi.
A. Zebrafish embryos were inoculated at 28 hpf by injection in the yolk with approximately 50 cells of Ra-LPS mutant *S. typhimurium* expressing DsRed.
B. Light microscopic image of A. Anterior side is to the left, dorsal side up.
bloodstream: 100% of the bacteria were lysed within 12 min. What could be the molecular basis of this rapid extracellular lysis of both S. typhimurium mutants and heat-killed bacteria in the blood of zebrafish embryos? The complement system is known to be present in fish (Sunyer and Lambris, 1998; Zarkadis et al., 2001), and also serum mannos-binding lectin (mbl) with binding activity to bacterial cell envelopes has been detected in Teleost species (Ewart et al., 1999). This means that S. typhimurium cells may be targeted for destruction both via the lectin-mediated and via the alternative pathway of the complement system in the zebrafish embryos. Moreover, Salmonella O-antigen mutants and, to a lesser extent, core mutants are known to be susceptible to complement killing, due to the increased binding of mbl (Devyatatarova-Johnson et al., 2000). However, at the 28 hpf stage zebrafish embryos do not have a fully functional liver. Therefore, both complement factors and acute-phase proteins, if present, will have to be synthesized at other sites in the embryo. Future experiments will be directed to answer these questions.

The translucent character of the zebrafish embryo is not the only advantage of this model. The genome sequence of the zebrafish will be available in the near future and together with the large-scale analysis of expressed sequence tags (ESTs) sequences (WashU-zebrafish genome resources project), this will enable the identification of mammalian homologues of the immune system. The use of the zebrafish embryo provides the possibility of genetic manipulation of the host. By using a knockdown approach with antisense morpholinos (Nasevicius and Ekker, 2000), and also serum mannos-binding lectin (mbl) with binding activity to bacterial cell envelopes has been detected in Teleost species (Ewart et al., 1999). This means that S. typhimurium cells may be targeted for destruction both via the lectin-mediated and via the alternative pathway of the complement system in the zebrafish embryos. Moreover, Salmonella O-antigen mutants and, to a lesser extent, core mutants are known to be susceptible to complement killing, due to the increased binding of mbl (Devyatatarova-Johnson et al., 2000). However, at the 28 hpf stage zebrafish embryos do not have a fully functional liver. Therefore, both complement factors and acute-phase proteins, if present, will have to be synthesized at other sites in the embryo. Future experiments will be directed to answer these questions.

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**Experimental procedures**

**General zebrafish methods**

Zebrafish embryos were collected from a laboratory-breeding colony kept at 24°C on a 12 : 12 h light/dark rhythm as previously described (van der Sar et al., 1999). Embryos were staged at 28.5°C according to hours post fertilization (hpf) and morphological criteria (Kimmel et al., 1995).

**Microinjection of zebrafish embryos**

Embryos were staged at 28 hpf dechorionated and anaesthetized in 0.02% buffered 3-aminobenzoic acid methyl ester (MS222, Sigma). Embryos were individually infected by microinjection of approximately 25 cells and 50 cells (2 nL) of E. coli or S. typhimurium bacteria in the axial vein near the blood island and the urogenital opening. To control the infections we used a stereoscopic dissecting microscope (SMZ1000, Nikon) with epi-fluorescence attachment (model P-FLA, Nikon); a pneumatic picopump (PV820, World Precision Instruments) and a micromanipulator with pulled microcapillary pipettes. As a control, a similar dose was spotted onto LB plates, counted and incubated overnight at 37°C. 80–90% of the red fluorescent cells formed colonies. Live infected embryos were analysed under a stereoscopic dissecting microscope (SMZ100, Nikon) and photographed using a Coolpix 995 digital camera (Nikon) or analysed using wide-field deconvolution microscopy.

**Bacterial strains and growth conditions**

*Escherichia coli strain* XL10 was grown in LB broth or on LB agar plates. *S. typhimurium* wild type strain SL1027 and its isogenic LPS derivatives SF1592 (Ra), SF1195 (Rc) and SF1567 (Rd) were obtained from Gunther Schmidt. Cells used for the infection of zebrafish embryos were freshly grown overnight on LB agar plates supplemented with 50 μg ml⁻¹ ampicillin and resuspended in phosphate-buffered saline. If necessary, this suspension was incubated for 5 min at 70°C to obtain heat-killed *S. typhimurium* cells.

**Isolation of DsRed variants**

The T3 variant of DsRed (Bevis and Glick, 2002) was obtained as a derivative of the pDsRed1-N1 construct (Clontech), which has a translation initiation site for mammalian cells. In order to optimize for high gene expression in bacteria, a new Shine/ Dalgarno sequence was introduced upstream of the original ATG initiation codon. This was achieved by a PCR reaction using pDsRed T3-N1 plasmid DNA as template and two degenerate primers. The first primer, WBDsRed1 (5'-GGGATCCAGAGGAGCGCCACCATGCGTC-3'), introduces the SD sequence (AGAGGA) in front of the ATG (shown in bold), and the second primer, WBDsRed2 (5'-CCGCTACAGGAACAGGTGGT-3'), overlaps with the stop codon of DsRed. The obtained PCR product was cloned in pGEM-T easy (Promega) including the manufacturers protocol. The ligation mixture was used to transform E. coli XL10 cells, which were prepared for transformation by the method of Inoue (Inoue et al., 1990). The resulting plasmid, which included expression of DsRed and yielded brightly red coloured *E. coli* colonies under normal light, was designated pGMDs3. This plasmid was introduced in the different *S. typhimurium* strains by transformation of cells prepared by the CaCl₂ method.

**Multidimensional digital imaging microscopy of zebrafish embryos**

Living zebrafish embryos were examined with a ZEISS Axiovert 200 Marianne™ inverted microscope, equipped with a motorised stage (stepper-motor z-axis increments: 0.1 μm), and both a Cy-3 as well as a DIC cube. A cooled CCD camera [Cooke Sensicam (Cooke, Tonawanda, NY), 1280 x 1024 pixels] recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4000) while dark-back-
ground currents are typically <100. Under the present set of chosen dyes and loading conditions, exposures ranged from 2 ms to 2 s. This ensures that genuine signals are at least >5 × higher than autofluorescence (typically about 200–500 in these embryo’s). Exposures, objective, montage and pixel binning were automatically recorded with each image stored in memory. The microscope, camera and all other aspects of data acquisition as well as data processing were controlled by SlideBook® software [SlideBook version 3.1 (Intelligent Imaging Innovations, Denver, CO)]. All live microscopy was performed with a custom 10× or 40× air lens (ZEISS). The data acquisition protocol included both timelapses and series of confocal optical planes to obtain 3D definition.

Isolation of bacteria from infected zebrafish embryos

*Salmonella typhimurium* wild-type and Rf mutant cells were isolated from infected zebrafish embryos in two different methods. For the analysis of total cfu groups of five embryos, injected with approximately 50 bacterial cells, were disintegrated by repeated (30 ×) pipetting in 100 µl PBS supplemented with 1% Triton X-100. Subsequently, this mixture was plated on LB-agar plates containing 100 µg/ml ampicillin. For the quantification of intracellular bacteria, groups of 10 infected zebrafish embryos were treated with trypsin and EDTA in order to obtain intact host cells (Westerfield, 2000). Subsequently, half of this mixture was incubated for 45 min with 100 µg/ml gentamycin and half of the mixture was used as a control. Both suspensions were centrifuged, washed with PBS and thoroughly resuspended in 100 µl PBS supplemented with 1% Triton X-100 and plated on selection plates. The results are the average of two separate experiments.

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**Supplementary material**

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/CMII/CMII303/CMII303sm.htm

**Fig. S1.** Time-lapse recording of a DsRed-loaded macrophage-like cell in the axial vein of zebrafish embryos, infected with a high-dose of wild-type *S. typhimurium* cells expressing DsRed. At the time of recording the zebrafish was infected for 4 h (hpi). Overlay frames of DIC images and Cy-3 fluorescent images (in red) are shown. Free circulating bacteria can be observed as occasional red flashes. Timelapse annotations 4.52 min, average timelapse interval 6167 ms, frames 44.

**Fig. S2.** Time-lapse recording of a bacteria-loaded macrophage-like cell in the axial vein of zebrafish embryos. The embryo was infected with a high-dose of wild-type *S. typhimurium* cells expressing DsRed. At the time of recording the zebrafish was infected for 4 h (hpi). Overlay frames of DIC images and Cy-3 fluorescent images (in red) are shown. The same macrophage-like cell was analysed for a prolonged period, which is shown in Fig. 4. Timelapse annotations 4.87 min, average timelapse interval 4864 ms, frames 60.

**Fig. S3.** Time-lapse recording of a microcolony, which is detected by a phagocytic cell. This microcolony is the same colony as the one observed in Fig. 6. The microcolony is observed at 26 hpi. Overlay frames of DIC images and Cy-3 fluorescent images (in red) are shown. Timelapse annotations 2.22 min, average timelapse interval 4596 ms, frames 26.

**References**


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