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Published paper
Zebrafish as a Novel Vertebrate Model To Dissect Enterococcal Pathogenesis

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Enterococcus faecalis is an opportunistic pathogen responsible for a wide range of life-threatening nosocomial infections, such as septicemia, peritonitis, and endocarditis. E. faecalis infections are associated with a high mortality and substantial health care costs and cause therapeutic problems due to the intrinsic resistance of this bacterium to antibiotics. Several factors contributing to E. faecalis virulence have been identified. Due to the variety of infections caused by this organism, numerous animal models have been used to mimic E. faecalis infections, but none of them is considered ideal for monitoring pathogenesis. Here, we studied for the first time E. faecalis pathogenesis in zebrafish larvae. Using model strains, chosen isogenic mutants, and fluorescent derivatives expressing green fluorescent protein (GFP), we analyzed both lethality and bacterial dissemination in infected larvae. Genetically engineered immunocompromised zebrafish allowed the identification of two critical steps for successful establishment of disease: (i) host phagocytosis evasion mediated by the Epa rhamnopolysaccharide and (ii) tissue damage mediated by the quorum-sensing Fsr regulon. Our results reveal that the zebrafish is a novel, powerful model for studying E. faecalis pathogenesis, enabling us to dissect the mechanism of enterococcal virulence.

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brafish model of infection is particularly suitable for studying both *E. faecalis*-induced lethality and pathogenesis.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** *E. faecalis* strains and plasmids are described in Table S1 in the supplemental material. Bacteria were grown in brain heart infusion (BHI) broth medium (Oxoid) at 37°C supplemented with antibiotics where appropriate at the following concentrations: tetracycline, 5 μg/ml; kanamycin, 2,000 μg/ml; and erythromycin, 30 μg/ml. *E. faecalis* strains were transformed with pMV158GFP (30) and pTEX5249 (31) by electroporation as previously described (31).

**Zebrafish maintenance and transgenesis.** London wild-type (LWT) inbred zebrafish embryos were provided by the aquarium staff of the MRC Centre for Developmental and Biomedical Genetics for zebrafish husbandry. The Tg(mpeg1:GFP;Gal4-VP-16)sh256 strain was generated as previously described (19). The Tg(mpeg1:Gal4-VP-16)sh256 strain was crossed to the Tg(UAS:Kaede);19995 strain to enable visualization of macrophages. Embryos were incubated in E3 medium at 28.5°C according to standard protocols (32).

**Morpholino knockdown of pu.1.** Morpholino-modified antisense oligomers against pu.1 were injected as previously described (25).

**Microinjections of *E. faecalis* into zebrafish embryos.** Bacteria were grown in BHI broth until they reached an optical density at 600 nm of about 0.7 and harvested by centrifugation (5,000 × g, 10 min). Bacteria were microinjected into the circulation of dechorionated zebrafish embryos at 30 h postfertilization (hpf) as previously described (25). Briefly, anesthetized embryos were embedded in 3% (wt/vol) methylcellulose and injected individually using micropipette pipettes filled with the bacterial suspension of known concentration. Larvae were observed frequently up to 90 h postinjection (hpi).

**Microscopic observations of larvae.** Live anesthetized larvae were immersed in 1% (wt/vol) low-melting-point agarose solution in E3 medium and mounted flat on a transparent slide. Images were acquired using the TE-2000U microscope (Nikon) with a Hamamatsu Orca-AG camera. Image acquisition and processing were performed with Volocity software (Improvision). A 4× Nikon Plan Fluor objective with a numerical aperture (NA) of 0.13 and a 60× Nikon Plan Apo oil objective with an NA of 1.4 were used.

**Determination of in vivo bacterial load.** At various times postinfection, six living zebrafish larvae were anesthetized and individually transferred with 100 μl of E3 medium into 0.5-ml Precellys tubes containing 1.4-mm ceramic beads (Peqlab) and homogenized using a Precellys 24-Dual homogenizer (Peqlab). The homogenates were serially diluted and plated on BHI agar to determine *E. faecalis* CFU numbers. Bacterial load was also determined for dead larvae at each time point.

**Phagocytosis assay.** Prior to infection, bacteria were labeled with pHrrodo red succinimidyl ester (Invitrogen) by mixing 200 μl of bacterial suspension with 0.5 μl pHrrodo red (2.5 mM) and incubating for 30 min in the dark. To remove the excess dye, bacteria were then washed with phosphate-buffered saline (PBS) followed by 50 mM Tris-HCl (pH 8.5) and subsequently resuspended in PBS. At 1.5 h postinfection (hpi), 18 larvae infected with each strain tested were mounted in 1% low-melting-point agarose. The total pixel number in the phagocytosis assay was compared between two groups (OG1RF and V583) by the Kaplan-Meier method. Comparisons between curves were made using the log rank test. The total pixel number in the phagocytosis assay was compared between two groups (OG1RF and epd) using a two-tailed, unequal Student's t test. Analysis was performed using Prism version 5.0 (GraphPad). Statistical significance was assumed at P values below 0.05.

**RESULTS**

**Virulence of *E. faecalis* isolates in a zebrafish embryo infection model.** We analyzed the virulence of three *E. faecalis* reference strains in a zebrafish embryo infection model: OG1RF and V583, two clinical isolates extensively used to study *E. faecalis* pathogenesis, and JH2-2, a nonvirulent laboratory strain used for genetic studies. Each strain was injected into the bloodstream of LWT zebrafish embryos at 30 h postfertilization (hpf). Embryos were maintained at 28.5°C, and the survival of larvae was monitored for 90 h postinfection (hpi). Injection of high numbers of CFU (10,500 to 12,000) showed that the three strains displayed radically distinct virulence phenotypes (Fig. 1A and B): while JH2-2 killed only 10% of larvae at 90 hpi, OG1RF killed 100% of larvae by 24 hpi. V583 displayed an intermediate virulence, killing 50% of larvae at 42 hpi. Inactivation of different numbers of CFU revealed that killing by *E. faecalis* was dose dependent. OG1RF was more virulent than V583, as 4,000 CFU of this strain were able to kill approximately 80% of larvae at 24 hpi, whereas 4,900 CFU of V583 killed only 10% of larvae. Remarkably, OG1RF was still vir-
ulent at relatively low doses (1,100 CFU induced 50% mortality at 42 hpi).

The striking differences between OG1RF and JH2-2 prompted us to enumerate bacteria within larvae following infection. Embryos infected with sublethal doses of either OG1RF or JH2-2 strain were homogenized, and the numbers of bacteria within individual larvae were counted. In larvae infected with OG1RF, the number of bacteria increased slightly during the first 7 hpi. In some hosts, numbers did not increase, and larvae survived. In several larvae, CFU increased exponentially, reaching approximately 10^6 CFU at 20 to 30 hpi, at which point death often occurred (Fig. 1C). The variable number of CFU from larva to larva may reflect the variability between individual hosts. Alternatively, we can speculate that high CFU numbers reflect a more advanced infection, leading to death when CFU numbers reach 10^5 to 10^6 per larva. In contrast, strain JH2-2 was unable to proliferate in vivo, and the bacterial numbers remained constant until at least 55 hpi (Fig. 1D).

Taking advantage of the embryonic optical transparency of developing zebrafish, we monitored bacterial dissemination during infection. For comparison purposes, both OG1RF and JH2-2 were transformed with a replicative plasmid constitutively expressing gfp. Zebrafish larvae were infected with one of the two E. faecalis-transformed strains and analyzed by fluorescence microscopy. Larvae infected with OG1RF developed lesions resembling necrotic tissues at sites of bacterial growth, referred to here as “tissue damage” (Fig. 1E, arrows). In contrast, the JH2-2-infected larvae remained apparently normal, with bacteria being restricted to small foci within the embryonic vasculature (data not shown). The OG1RF strain was able to disseminate systemically and proliferate within larvae and was often detected in the heart and central nervous system prior to embryo death (see Fig. S1A in the supplemental material). Zebrafish larvae that survived the infection were not able to completely clear bacteria from their bodies, and small foci of infection were seen until at least 90 hpi (see Fig. S1B).

Contribution of phagocytes to E. faecalis infection. At 30 hpf, embryo immune defenses rely on the innate response, primarily macrophages and neutrophils. To gain insight into the differences in virulence of OG1RF and JH2-2, we compared bacterial uptake by phagocytes at 2 hpi. In zebrafish larvae infected with 1,500 CFU of fluorescent E. faecalis OG1RF, a large proportion of fluorescent bacteria resisted phagocytosis and remained free in the bloodstream (Fig. 2A, arrows). In contrast, JH2-2 bacteria injected at a much higher dose (10,000 CFU) were all promptly phagocytosed (Fig. 2B).

To determine specific macrophage roles in engulfling injected enterococci, we used embryos expressing the macrophage-specific transgenes mpeg1:Gal4 and UAS:Kaede (19). At 29 hpf, embryos were subjected to 436 nm irradiation for 20 min to enable Kaede photoconversion from green to red. The embryos were then infected with gfp-expressing E. faecalis JH2-2. Similarly to previous studies with other bacteria (25, 33), the majority of fluorescent enterococci were detected within macrophages (Fig. 2C).

To further explore the role of zebrafish phagocytes in resistance to E. faecalis infection, phagocytes were depleted using antisense pu.1 morpholinos. pu.1 morphants were significantly more susceptible to both OG1RF and JH2-2 than their immunocompetent counterparts (Fig. 2D). However, while OG1RF rapidly killed all phagocyte-depleted larvae, JH2-2 required 3 to 4 days to kill immunocompromised larvae. Despite the distinct death kinetics associated with strains OG1RF and JH2-2, both were able to proliferate rapidly in pu.1 morphants, reaching approximately 10^6 CFU per embryo at 20 hpi (Fig. 2E and F). Imaging of pu.1 morphants when bacterial proliferation was maximal showed severe deformation and tissue damage in OG1RF-infected larvae (Fig. 2G), whereas larvae infected with JH2-2 showed no signs of disease despite heavy dissemination (Fig. 2H). This result suggested that in addition to being easily phagocytosed, the JH2-2 strain lacks virulence determinants that contribute to killing of the host. Interestingly, this work indicates that zebrafish larvae represent a tractable model for studying E. faecalis pathogenesis under conditions that mimic neutropenia, a major risk factor for E. faecalis infections.

Contribution of epa, fsr, gelE, and sprE to E. faecalis pathogenesis. To gain further insights into the zebrafish model of E. faecalis infection, we analyzed OG1RF mutants previously shown to be attenuated in other animal models. Based on our results, which suggested a role of phagocytosis and revealed substantial tissue damage following infection, we focused on two types of virulence factors: (i) the enterococcal polysaccharide antigen (encoded by the epa locus), a rhamnopolysaccharide that has previously been shown to play a role in resistance to neutrophil uptake in vitro (34), and (ii) two major exoproteases playing a role in virulence, a gelatinase (GelE) and a serine protease (SprE) that are regulated by the Fsr quorum-sensing system (7, 31, 35). Four OG1RF mutants (epaB, gelE, sprE, and fsr mutants) were tested in our infection model.

The epaB mutant was significantly attenuated compared to the parental strain (Fig. 3A) (P value of 0.0001). To investigate the role of Epa in phagocytosis in vivo, we carried out microscopic examination of larvae infected with epaB mutant expressing green fluorescent protein (GFP). Unlike the parental strain (Fig. 3B), the epaB mutant was not able to evade phagocytosis in vivo and was readily taken up by zebrafish phagocytes compared to the wild-type strain (Fig. 3C). The difference in phagocytic uptake between OG1RF and its epaB derivative was quantified following labeling of bacterial cells with pHrodo-S-ester, a pH-sensitive dye that enables us to visualize bacteria only in low-pH compartments of phagosomes (36). This assay confirmed that at 1.5 hpi, the epaB mutant is phagocytosed more efficiently than the parental strain (Fig. 3D). Bacterial counts following infection with the epaB mutant remained constant until 55 hpi (Fig. 3E), clearly showing that although growth was abolished in zebrafish larvae, epaB-deficient bacteria were still able to survive within phagosomes. Together, our results therefore indicate that the Epa rhamnopolysaccharide contributes to evasion of phagocytosis but does not contribute to survival in zebrafish phagosomes.

In our model, gelE, sprE, and fsr mutants were significantly attenuated compared to the parental strain (Fig. 4A and B) (P values of 0.04, 0.03, and 0.0001 for gelE, sprE, and fsr mutants, respectively). Both gelE and fsrB mutants were selected to be further analyzed in vivo, as two examples of intermediate or severe reduction of virulence, respectively. Unexpectedly, both attenuated strains were able to proliferate similarly to the parental strain (Fig. 4C and D), although this growth did not result in larva death. In agreement with this observation, time-lapse imaging of larvae infected with fsrB mutants expressing gfp did not reveal any morphological change, even when the majority of the developing zebrafish were overwhelmed with bacteria (Fig. 4E).
FIG 2 Differential uptake of OG1RF and JH2-2 strains by phagocytes plays an important but not exclusive role in killing. (A and B) In vivo images of the yolk circulation valley of embryos at 2 hpi following injection with OG1RF (A) and JH2-2 (B) strains expressing gfp. Arrows indicate examples of unphagocytosed bacteria. These appear as “smears” due to their movement in the bloodstream and the relatively long exposure time. (C) In vivo imaging of the yolk circulation valley of Tg(mpeg1:Gal4 × UAS:Kaede) transgenic embryos at 2 hpi injected with GFP-expressing JH2-2. Photoconverted Kaede-positive macrophages (M) and nonfluorescent neutrophils (N) containing fluorescent E. faecalis are indicated. (D) Survival of wild-type or phagocyte-depleted zebrafish larvae following injection with E. faecalis OG1RF or JH2-2 (n = 25). (E and F) Growth of E. faecalis within phagocyte-depleted zebrafish larvae upon injection with OG1RF (E) or JH2-2 (F) strain into the bloodstream. (G and H) In vivo images of phagocyte-depleted zebrafish larvae injected with GFP-expressing OG1RF (G) or GFP-expressing JH2-2 at 18 hpi (H). Bars, 10 μm (A, B, and C) and 500 μm (G and H). The numbers of CFU injected are indicated on each panel.
Figure 3: Enterococcal polysaccharide antigen (epa) mutants of OG1RF are unable to evade phagocytosis and fail to establish lethal infection. (A) Survival of zebrafish larvae following injection with E. faecalis OG1RF and epa derivatives (n = 25). In log rank tests for epaB versus wild type, P is 0.0001. (B and C) In vivo images of the yolk circulation valley of larvae at 2 hpi following injection with gfp-expressing OG1RF (B) or the epaB mutant (C). Arrows indicate examples of unphagocytosed bacteria. (D) Efficacy of phagocytosis by zebrafish embryos (n = 20) at 1.5 h following infection with pHrodo-labeled wild-type E. faecalis OG1RF or the epaB mutant. (E) Growth of the E. faecalis epaB mutant in zebrafish larvae. The numbers of CFU injected are indicated on each panel. Bars, 10 μm.

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To determine whether the attenuation of the mutants analyzed was caused by their inability to overcome the host cellular immune response, we followed the infection of phagocyte-depleted larvae (Fig. 5). As expected, the virulence of epaB mutant was fully restored in pu.1 morphants. In contrast, death of larvae infected with the gelE mutants was significantly delayed compared to that of the parental strain. For pu.1 morphants infected with the fsrB mutant, the time to death was severely delayed (Fig. 5A). Interestingly, bacterial growth in larvae was very similar for all mutants tested, reaching approximately 10^6 CFU at 20 hpi (Fig. 5B to D). However, different death rates indicated that in vivo proliferation is not sufficient to cause death. Altogether, these results indicate that zebrafish represent a particularly suitable model for following two steps crucial to the progression of E. faecalis pathogenesis: phagocytosis evasion and host killing, in part mediated by exoprotease genes and other fsrB-dependent genes.

Heterologous complementation of the fsr locus in JH2-2. Survival of pu.1 knockdown larvae infected with the OG1RF fsrB mutant (Fig. 5A) was very similar to that of immunocompromised larvae infected with JH2-2 (Fig. 2D). We hypothesized that the inactive fsr system in JH2-2 could at least in part account for the delay in mortality in immunocompromised larvae. To test this hypothesis, we complemented the JH2-2 strain with the OG1RF fsr locus using plasmid pTEX5249 (31). As anticipated, heterologous expression of the OG1RF fsr locus restored the production of exoproteases in JH2-2 (37) (see Fig. S2 in the supplemental material). JH2-2 fsr^- bacteria were avirulent when injected into immunocompetent larvae but rapidly killed phagocyte-depleted larvae (Fig. 6A). Analysis of bacterial load following infection revealed no growth of JH2-2 fsr^- bacteria in wild-type larvae (Fig. 6B), indicating that restoration of neither exoprotease production nor any other Fsr-dependent factor allowed JH2-2 to overcome phagocytosis. In pu.1 knockdown larvae, JH2-2 fsr^- bacteria proliferated rapidly and caused death of infected larvae (Fig. 6C), highlighting the important role of Fsr-dependent factors, including exoproteases, in later stages of infection, after the initial immune hurdle (i.e., phagocytes) has been overcome. These results show that the genetically manipulable strain JH2-2 can be used as a platform strain to investigate functions of virulence factors.

DISCUSSION

Several vertebrate models for studying specific E. faecalis infections have been described: endophthalmitis and endocarditis in rabbits and urinary tract infection, bacteremia, and peritonitis in mice (5). Although these models have been successfully used to characterize a large variety of E. faecalis virulence factors, they are not adapted for large-scale studies and remain costly. Over the past decade, zebrafish have been successfully used as a surrogate model host for human bacterial pathogens (38). In this study, we showed that zebrafish can be used as a host to study E. faecalis pathogenesis. Our results revealed that this infection model is particularly suitable for identifying E. faecalis virulence factors, dissecting the mechanisms of infection, and following the dissemination of bacteria in vivo.

Zebrafish as a model for studying E. faecalis virulence. This work showed that zebrafish larvae are susceptible to virulent E. faecalis strains (OG1RF and V583) in a dose-dependent manner but resistant to strain JH2-2, a nonvirulent laboratory strain. In agreement with previous studies comparing OG1RF and V583 in mouse and C. elegans models (6, 39), we also show that OG1RF was more virulent than V583 in the zebrafish lethality model (Fig. 1). This work also confirms the poor pathogenic potential of JH2-2. When high numbers of CFU of these bacteria were injected, they failed to induce lethality despite the fact that they were able to multiply and persist in vivo.

To demonstrate the relevance of the zebrafish model of infection, we focused on the analysis of mutations in the protease genes gelE and sprE and the fsr quorum-sensing system, which positively controls their expression (31). Mutation in the fsr locus has been associated with a significant decrease in virulence in all animal models tested (7, 31, 35, 40). Similarly to previous studies on various animal models, we found that fsrB deletion resulted in severe loss of enterococcal virulence in zebrafish larvae. However, we showed that the fsrB mutant was able to proliferate as well as its parent strain, OG1RF (Fig. 4), indicating that fsrB controls factors important for the late stages of infection, leading to killing. While GelE has been un-
ambiguously demonstrated to be important for enterococcal virulence in multiple models (31, 35, 41–43), the importance of SprE as an *E. faecalis* virulence factor is controversial. The *sprE* mutant was shown to be attenuated in mouse peritonitis in the nematode and the fly models (8, 31, 35). However, SprE was dispensable for rabbit infective endocarditis and had no lethal activity in *Galleria mellonella* (42, 43). The role of these exoproteases in promoting tissue damage is strongly supported based on histopathological examination of infected eyes using the endophthalmitis model (44) and their proteolytic activity over a wide substrate range (45). Here, we showed that both the gelatinase GelE and the serine protease SprE contribute to *E. faecalis* virulence in zebrafish larvae and appear to have additive effects in pathogenesis. Altogether, given that *E. faecalis* is able to proliferate and disseminate within zebrafish larvae, this infection model appears to be suitable for identifying a range of virulence factors associated with *E. faecalis* pathogenesis. One potential limitation of the zebrafish infection model is the temperature at which the larvae are kept (28.5°C). Thus, alternative infection models need to be considered to analyze virulence factors specifically expressed at 37°C, the temperature of the mammalian host.

**Zebrafish as a model system to study innate immune response against *E. faecalis***. In this study, we showed that zebrafish larvae can be used as a model to evaluate both uptake and survival of bacteria in phagocytes. Upon injection into the bloodstream, *E. faecalis* phagocytosis is mediated by macrophages. This result is in agreement with previous studies indicating that in zebrafish, bacteria in blood circulation are essentially phagocytosed by macrophages, neutrophils being able to engulf only surface-associated microbes (46). Although macrophages completely phagocytosed a large inoculum of JH2-2, a significant proportion of OG1RF cells escaped phagocytosis and remained in the bloodstream. *E. faecalis*-specific rhamnopolysaccharide encoded by the *epa* locus was previously shown to mediate resistance to uptake and killing by human neutrophils (11, 34). Using the Epa-deficient *epaB* mutant (47), we showed that the Epa rhamnopolysaccharide protects OG1RF from uptake by macrophages in zebrafish. In the phagocyte-depleted larvae, the *epaB* mutant was as virulent as
the parental OG1RF strain, indicating that during infection, the main role of the epa cluster is to prevent phagocytosis. However, our data indicate that the rhamnopolysaccharide encoded by the JH2-2 epa locus is not able to confer resistance to phagocytosis in vivo. Diversity of the epa locus within E. faecalis species was recently reported (48). The zebrafish model is particularly appropriate for evaluating whether Epa diversity impacts E. faecalis resistance to phagocytosis in vivo. E. faecalis persistence within zebrafish larvae is in agreement with the extreme resistance of this bacterium to various stresses (reviewed in reference 49). It will be interesting to test whether factors that have been shown to enhance survival in murine macrophages (50) or human neutrophils (51) play a role in resistance to zebrafish macrophages. Neutropenia is a major risk factor for enterococcal infections (2). The higher susceptibility of immunocompromised zebrafish larvae to E. faecalis infection suggests that this model is suitable for investigating E. faecalis pathogenesis under conditions mimicking disease onset.

Concluding remarks. Our study revealed that zebrafish larvae represent an attractive alternative model system for studying host-pathogen interaction during E. faecalis infections, allowing the identification of both host immune components and pathogen virulence factors. This novel vertebrate model enables spatial and temporal dissection of the complex mechanisms of E. faecalis infection in vivo, from initial interaction with host innate immune system to development of bacterial quorum sensing in vivo and degradation of host tissues, resulting in death. We showed that the degree of virulence can be readily measured by a simple lethality assay. More importantly, thanks to zebrafish optical transparency and genetic tractability, we also reveal that successful infection by E. faecalis relies

FIG 5 Protease-deficient mutants of OG1RF remain attenuated in phagocyte-depleted larvae. (A) Survival of pu.1 knockdown zebrafish larvae (n = 25) following infection with E. faecalis fsrB, gelE, and epaB mutants. In log rank tests, for gelE versus WT, P < 0.0001; for fsrB versus WT, P < 0.0001; for epaB versus WT, P = 0.97. (B to D) Growth of E. faecalis epaB (B), gelE (C), and fsrB (D) mutants in pu.1 knockdown larvae.

FIG 6 Heterologous expression of OG1RF fsr locus in JH2-2 restores virulence in phagocyte-depleted but not wild-type larvae. (A) Survival of wild-type or pu.1 knockdown zebrafish larvae (n = 25) following infection with E. faecalis JH2-2 with or without fsrB complementation. In log rank tests, for JH2-2 versus JH2-2 fsrB” in wild-type larvae, P = 0.56; for JH2-2 versus JH2-2 fsrB” in pu.1 knockdown larvae, P < 0.0001. (B and C) Growth of E. faecalis JH2-2 fsr” in wild-type (B) or pu.1 knockdown (C) larvae. The numbers of CFU injected are indicated on each panel.
on two major steps: phagocyte evasion and tissue damage. Based on these observations, we are confident that the model described here will be useful for large-scale screening of mutant libraries to identify new bacterial virulence determinants and host factors important in immunity against enterococci.

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