

# Susceptibility of zebra fish *Danio rerio* to infection by *Flavobacterium columnare* and *F. johnsoniae*

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**ABSTRACT:** *Flavobacterium columnare* is a serious pathogen in a wide range of fish species. *F. johnsoniae* is an opportunistic pathogen of certain fish. Both are gliding bacteria. These species were tested for their ability to infect the zebra fish *Danio rerio*. Both injection and bath infection methods were tested. The results indicate that *F. johnsoniae* is not an effective pathogen in *D. rerio*, but that *F. columnare* is an effective pathogen. *F. johnsoniae* did not cause increased death rates following bath infection, but did cause increased death rates following injection, with an LD<sub>50</sub> (mean lethal dose) of approximately  $3 \times 10^{10}$  cfu (colony-forming units). Non-motile mutants of *F. johnsoniae* produced a similar LD<sub>50</sub>. *F. columnare* caused increased death rates following both injection and bath infections. There was considerable strain variation in LD<sub>50</sub>, with the most lethal strain tested producing an LD<sub>50</sub> of  $3.2 \times 10^6$  cfu injected and  $1.1 \times 10^6$  cfu ml<sup>-1</sup> in bath experiments, including skin damage. The LD<sub>50</sub> of *F. columnare* in zebra fish without skin damage was  $>1 \times 10^8$ , indicating an important effect of skin damage.

**KEY WORDS:** *Flavobacterium columnare* · *Flavobacterium johnsoniae* · Zebra fish

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## INTRODUCTION

*Flavobacterium columnare* is the causative agent of columnaris disease (CD), a serious problem in US catfish and trout farming industries, producing significant economic loss (Wagner et al. 2002). *F. columnare* infects a large range of fish, including trout, goldfish, carp, eels, white capping, and perch (Austin & Austin 1999 and references therein). Most of the incidence of CD reported is in temperate fish such as salmonids, catfish, and carp, although reports have been made of infection of tropical fish such as black mollies (Decostere et al. 1998). *F. columnare* infections occur primarily in epithelial tissue, including gills and skin. Adult fish are often present with a characteristic 'saddleback lesion', resulting in loss of pigment and scales around the dorsal fin (Austin & Austin 1999). Fish mortality in aquaculture settings can reach 100% (Austin & Austin 1999).

*Flavobacterium johnsoniae* is not normally considered a serious fish pathogen, but has been linked to several disease outbreaks, most notably in farmed barramundi *Lates carcalifer* in Australia and Tasmania

(Carson et al. 1993). *F. johnsoniae* and *F. columnare* exhibit similar phenotypes, and *F. johnsoniae* strains have been mis-identified as *F. columnare* during disease outbreaks (Darwish et al. 2004). *F. johnsoniae* has several advantages over *F. columnare* in the laboratory, including ease of growth and handling, rapid doubling time, and a robust genetic system allowing the generation and complementation of mutants (McBride 2004).

One of the common characteristics of flavobacterial fish pathogens is their extreme strain variability. Different strains of *Flavobacterium columnare* and *F. psychrophilum* have been shown to have differences in virulence in numerous species (Decostere et al. 1998, Madsen & Dalsgaard 2000). There is also strain variation in the ability of *F. psychrophilum* and *F. johnsoniae* to accept and maintain plasmids used in genetic analysis (Alvarez et al. 2004, D. W. Hunnicutt unpubl. data).

Recent reports of *Flavobacterium columnare* infection of tropical fish (Decostere et al. 1998), along with the common reports of infection of other cyprinids

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(Bootsma & Clerx 1976) raise the possibility that infection of zebra fish *Danio rerio* might occur. Zebra fish have been widely used as a model system for the study of vertebrate development. They present many attractive qualities for this work, including small size, ease of care, a rapid life cycle, and transparent early stages that allow direct observation of development. Due in part to these advantages, a powerful genetic system has been developed for zebra fish, allowing the generation and selection of mutant strains; a genome sequencing project is also currently in process (Yoder et al. 2002).

Although zebra fish are a common experimental system, there are, to our knowledge, no reports concerning flavobacterial infection of this species. The online Zebra Fish Information Network, a resource maintained by the Institute for Neurology at the University of Oregon, lists 'Bacterial Gill Disease' as an occasional problem with fish housed in poor conditions and attributes this to 'gliding bacteria' (<http://zfin.org/zirc/disMan/diseaseManual.php#Gliding%20Bacteria>).

The goal of the current study was to determine whether *Flavobacterium johnsoniae* or *F. columnare* infect zebra fish and to examine some of the parameters of such an infection. The development of zebra fish as a model system for flavobacterial pathogenesis could be of use in vaccine development and other areas of fish disease research.

## MATERIALS AND METHODS

### Bacterial strains, media, and growth conditions.

All bacterial strains were stored at  $-80^{\circ}\text{C}$  before being revived on CYE agar plates (10 g casitone, 5 g yeast extract, 1 g  $\text{MgSO}_4$ , 10 ml 1 M Tris, pH 7.0). *Flavobacterium columnare* strains and *F. johnsoniae* ATCC 43622 were initially grown on plates for 48 h at  $25^{\circ}\text{C}$ , before being transferred to 5 ml of CYE broth and grown on a shaker at  $23^{\circ}\text{C}$ . Additional *F. johnsoniae* strains (ATCC 17601, CJ588) were grown in 5 ml of CYE broth in a  $30^{\circ}\text{C}$  shaker. CJ588 required the addition of erythromycin ( $100\ \mu\text{g ml}^{-1}$ ) to CYE media in order to maintain a transposon (Hunnicuttt & McBride 2000). Turbidity measurements were made on a Scienceware Model 800-3 Klett meter, with a K66 Red (640 to 700 nm) filter, and were compared to plate counts to establish the concentration of viable cells in the cultures used for infection studies. Final colony-forming units (cfu) per milliliter were confirmed by plate counts taken at the time the culture was used. *F. johnsoniae* plates were incubated at  $30^{\circ}\text{C}$  for 1 d prior to colony counting. *F. columnare* plates were incubated at  $25^{\circ}\text{C}$  for 2 d prior to colony counting.

**Zebra fish care.** Wild type zebra fish *Danio rerio* were obtained from Segrest farms (USA) through a local pet store, fed once per day, and were allowed to acclimate in 10 gallon aquaria (37.8 l) at  $27^{\circ}\text{C}$  for at least 3 d prior to infection. Zebra fish were anesthetized with Tris-buffered tricaine (3-aminobenzoic acid ethylester, pH 7.0) at a concentration of  $192\ \mu\text{g ml}^{-1}$ . After infection, fish were housed in mouse cages (VWR) containing deionized water and kept at room temperature ( $\sim 23^{\circ}\text{C}$ ). Euthanasia of zebra fish was performed by placing the fish in a solution of Tris-buffered tricaine at a concentration of  $320\ \mu\text{g ml}^{-1}$ .

**Bacterial preparation.** A 1 ml aliquot of an overnight starter culture was transferred to a sterile Klett flask containing 24 ml of CYE media. Cultures were allowed to grow at  $20^{\circ}\text{C}$  to a density between 30 and 70 Klett units ( $1$  to  $2 \times 10^8$  cfu  $\text{ml}^{-1}$ ) to ensure the bacteria were in the logarithmic growth phase. For *Flavobacterium columnare* cultures, this required 12 to 24 h. Cultures were diluted to the desired concentrations in CYE broth. In order to concentrate *F. johnsoniae* when necessary, 20 ml cultures were centrifuged for 10 min at 2940 g. The supernatant was removed, and the pellet was suspended in fresh medium to generate a  $1 \times 10^{11}$  cfu  $\text{ml}^{-1}$  suspension.

### Sequencing of *Flavobacterium columnare* 16S rRNA genes.

A 25 ml overnight culture of *F. columnare* 14-56 was centrifuged (2940 g for 10 min), and the pellet was resuspended in 3.4 ml 50 mM Tris and 1 mM EDTA, pH 8.1. The cell suspension was then treated with 40  $\mu\text{l}$  Proteinase K ( $20\ \text{mg ml}^{-1}$ ), 20  $\mu\text{l}$  RNase ( $1\ \text{mg ml}^{-1}$ ), and 400  $\mu\text{l}$  10% sodium dodecyl sulphate (SDS) for 1 h at  $37^{\circ}\text{C}$ . The mixture was then diluted with 4 ml 50 mM Tris and 1 mM EDTA, pH 8.1, followed by extraction with 5 ml equilibrated phenol and 5 ml chloroform:iso-amyl alcohol (24:1), and centrifuged (17400 g for 10 min). Phenol-chloroform extraction was repeated 3 times. The aqueous layer was then transferred to a cold, 50 ml glass tube, and 5 M NaCl was added to bring the solution to 0.2 M. Ice-cold ethanol was added to 2.5 times the volume. The DNA was then spooled and rinsed in 70% ethanol. The DNA was allowed to air dry for 1 h before being resuspended in 1 ml of TE buffer. The purity of the DNA was assessed by 260/280 absorbance. Sequencing of the 16S rRNA gene was conducted by the USDA Center for Cool and Cold Water Aquaculture, Kearneysville, West Virginia, USA. DNA analysis was conducted using Sequencher (Gene Codes) and BLAST software.

**Infection of zebra fish by abrasion-bath culture.** All infection protocols were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC No. 18922). Zebra fish were anesthetized in 200 ml deionized water containing  $192\ \mu\text{g}$

ml<sup>-1</sup> MS-222 and placed with the dorsal side upward on moistened cheesecloth between the open jaws of a hemostat. The zebra fish were abraded by removing 3 to 4 scales from the left side next to the dorsal fin by scraping the area with a 29 gauge needle (Catalog No. BD-309301; VWR). The fish were then allowed to regain consciousness in water containing specific concentrations of bacteria ranging from 10<sup>5</sup> to 10<sup>7</sup>. After 1 h, the fish were removed from solution and placed into tanks containing deionized water.

**Infection of zebra fish through injection.** Injections were carried out essentially as described by Neely et al. (2002). Zebra fish were anesthetized and placed, either supine for intraperitoneal (IP) injections or prostrate for intramuscular (IM) injections, on moistened cheesecloth between the open jaws of a hemostat. During IP injections, the needle (29 gauge) was positioned parallel to the fish's spine and inserted cephalad into the abdomen just posterior to the pectoral fins. The needle was then inserted to the end of the bevel (1.5 mm), and 10 µl of the *Flavobacterium johnsoniae* suspension was injected into the fish.

For IM-injected fish, the needle was positioned at a 45° angle in relation to the fish's spine and inserted to the end of the bevel. After the needle was inserted, it was rotated 90° to help ensure that all of the 10 µl injection remained within the fish. In addition to rotation, the needle remained in the muscle for an additional 5 s after injection. Both IP and IM experiments were conducted with 6 fish at a single bacterial concentration ranging from 1 × 10<sup>8</sup> to 1 × 10<sup>11</sup> cfu ml<sup>-1</sup>.

**Detection of *Flavobacterium columnare* in infected fish.** To confirm that test individuals were free from *F. columnare* before experimental infection and that death was due to infection, representative fish were screened before infection and 24 h post-infection. The fish were euthanized, and internal organs and muscle tissue were removed. Total DNA was isolated using the DNAeasy kit (Quiagen) and used as template for the PCR-based *F. columnare* detection methods of Toyama et al. (1996) and Bader et al. (2003a) with the following modification: rTaq polymerase was used for PCR (Takara Bio). PCR products were run on a 0.7% agarose gel and stained with Syber Green.

**LD<sub>50</sub> (mean lethal dose) calculation.** The LD<sub>50</sub> for the various strains and infection methods was determined using the method of Reed & Muench (1938). Injections of *Flavobacterium johnsoniae* ATCC1701 and CJ588 were repeated 3 times on groups of 6 fish at each concentration, as were abrasion-bath infections with *F. columnare* 14-56 and ATCC49512. Abrasion-bath infections with *F. johnsoniae* strains were repeated twice on groups of 6 fish at each concentration, as were injection infections with *F. columnare*.

## RESULTS

### Growth characteristics of *Flavobacteria* tested

Numerous media were tested to determine the best growth conditions for the infection experiments. CYE media proved to support robust, planktonic growth of the *Flavobacterium columnare* strains tested. Several other media supported the growth of *F. columnare*, but growth tended to be in mucoid clumps, which prevented us from accurately correlating viable counts with turbidity measurements. Thus, CYE was used throughout the infection experiments. Turbidity readings on the Klett meter of 25 to 30 produced viable colony counts of approximately 1 × 10<sup>8</sup> cfu ml<sup>-1</sup> with all species tested.

### Signs of *Flavobacterium johnsoniae* infection

*Flavobacterium johnsoniae* ATCC 17061-injected zebra fish *Danio rerio* showed symptoms within 12 to 24 h. Inflammation and scale loss around the site of injection were common, as was a red discoloration around the jaw and gills and a yellow discoloration over the whole animal (Fig. 1A). Many fish also developed small lesions between the pelvic and caudal fins. Fish that survived >48 h generally recovered fully. Prior to death, fish exhibited additional signs of distress, including increased inflammation of gills, discoloration, scale loss, lethargy, and loss of appetite.

### *Flavobacterium johnsoniae* injection and bath infection results

*Flavobacterium johnsoniae* ATCC 17061 and ATCC 43622 were both tested for the ability to infect zebra fish using the abrasion-bath method. Neither strain caused increased mortality or visible signs of infection at concentrations of up to 10<sup>7</sup> cfu ml<sup>-1</sup>. Injection of *F. johnsoniae* ATCC 17061 (either IP or IM) resulted in fish mortality (Table 1). The LD<sub>50</sub> of wild type *F. johnsoniae* was 4.2 × 10<sup>10</sup> cfu following IP injection and 3.2 × 10<sup>10</sup> cfu following IM injection.

### *Flavobacterium johnsoniae* pathogenesis in zebra fish does not require motility

In order to determine the importance of gliding motility to virulence, the injection experiments were repeated with CJ588, a non-motile *Flavobacterium johnsoniae* mutant. The LD<sub>50</sub> was 2.29 × 10<sup>10</sup> cfu following IP injection and 1.44 × 10<sup>10</sup> cfu following IM

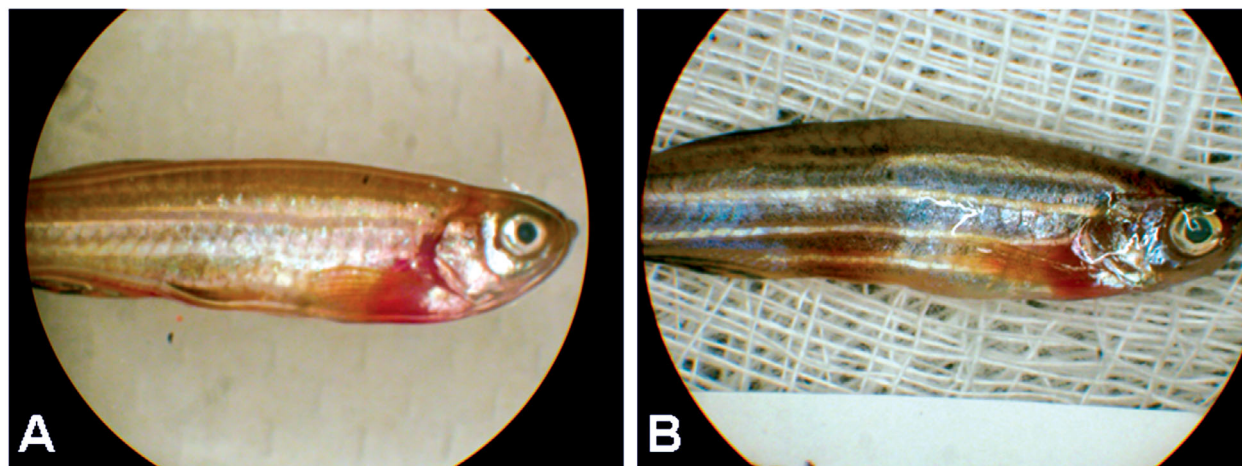


Fig. 1. *Danio rerio* infected with *Flavobacterium johnsoniae* and *F. columnare*. (A) A representative zebra fish 48 h following intramuscular injection with  $10^{10}$  colony-forming units of *F. johnsoniae* ATCC 17601. (B) A representative zebra fish 24 h following abrasion-bath infection with  $1 \times 10^6$  cfu ml<sup>-1</sup> *F. columnare* 14-56. Note classic saddleback lesion

injection. These data suggest that gliding motility is not required for *F. johnsoniae* pathogenesis in zebra fish.

#### 16S RNA gene sequence analysis of *Flavobacterium columnare* 14-56

To confirm the identity of the environmental isolate *Flavobacterium columnare* 14-56, we PCR amplified and sequenced the 16S rRNA gene. BLAST alignment of 1376 bp showed 99.93% identity to *F. columnare* ATCC 49512 (NCBI Accession No. DQ005508) and *F. columnare* IFO 15943 (NCBI Accession No. AB078047).

#### *Flavobacterium columnare* infection of zebra fish

*Flavobacterium columnare* Strain 14-56 caused rapid mortality and visible signs of infection in abraded zebra fish following immersion in bath cultures.

Table 1. *Flavobacterium johnsoniae* in *Danio rerio*. Mean lethal dose (LD<sub>50</sub>) of motile (ATCC 17601) and non-motile (CJ588) *F. johnsoniae* in zebra fish. cfu: colony-forming units

<i>F. johnsoniae</i> strains	LD <sub>50</sub>
<b>ATCC 17061</b>	
Intraperitoneal injection	$4.2 \times 10^{10}$ cfu
Intramuscular injection	$3.2 \times 10^{10}$ cfu
<b>CJ588</b>	
Intraperitoneal injection	$2.3 \times 10^{10}$ cfu
Intramuscular injection	$1.4 \times 10^{10}$ cfu

Fish developed a characteristic saddleback lesion within 24 h, with death occurring between 24 and 48 h post-infection (Fig. 1B). The LD<sub>50</sub> for *F. columnare* 14-56 was  $1.1 \times 10^6$  cfu ml<sup>-1</sup>. *F. columnare* ATCC 23463 also caused rapid mortality in zebra fish following the abrasion-bath inoculation method (Table 2). The LD<sub>50</sub> for *F. columnare* ATCC 23463 was  $1.1 \times 10^7$  cfu ml<sup>-1</sup>. Injection of either strain of *F. columnare* also resulted in fish mortality, with the LD<sub>50</sub> for *F. columnare* 14-56 being  $3.2 \times 10^6$  cfu and the LD<sub>50</sub> for ATCC 23463 being  $7.2 \times 10^7$  cfu. To confirm that fish death was due to *F. columnare* infection, PCR-based detection of *F. columnare* was conducted of tissue samples taken from representative fish using the method of Toyama et al. (1996). Samples were taken from fish 24 h after infection by scar-bath method from both infected fish and uninfected controls. The results can be seen in Fig. 2. Both *F. columnare* 14-56 and ATCC 23463 produced bands of the expected size (1.1 kb), as did the tissue samples taken from infected fish. DNA isolated from uninfected control fish pro-

Table 2. *Flavobacterium columnare* in *Danio rerio*. Mean lethal dose (LD<sub>50</sub>) of *F. columnare* strains in zebra fish. cfu: colony-forming units

<i>F. columnare</i> strains	LD <sub>50</sub>
<b>ATCC 23463</b>	
Abrasion-bath	$1.1 \times 10^7$ cfu ml <sup>-1</sup>
Bath only	$>1 \times 10^8$ cfu ml <sup>-1</sup>
Intraperitoneal injection	$4.2 \times 10^{10}$ cfu
Intramuscular injection	$3.2 \times 10^{10}$ cfu
<b>Fc14-56</b>	
Abrasion-bath	$1.1 \times 10^6$ cfu ml <sup>-1</sup>
Bath only	$>1 \times 10^8$ cfu ml <sup>-1</sup>

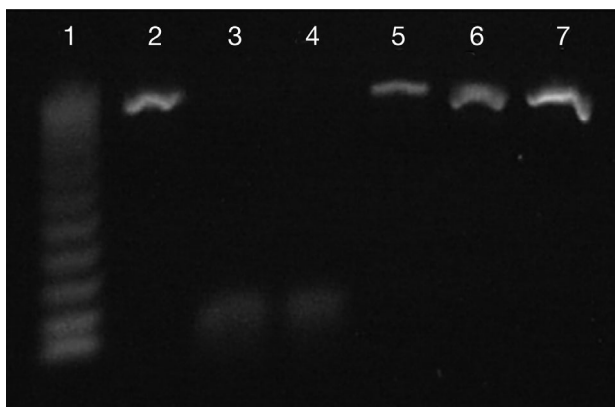


Fig. 2. *Flavobacterium columnare* in *Danio rerio*. Detection of *F. columnare* 14-56 in infected zebra fish by *F. columnare*-specific PCR. Lane 1: 100 bp marker (BioRad); lane 2: *F. columnare* 14-56; Lanes 3 and 4: uninfected control fish; lanes 5 and 6: infected fish; lane 7: *F. columnare* ATCC 23463

duced no bands. Similar results were obtained using the PCR assay of Bader et al. (2003a) (data not shown).

Unabraded controls were run for all abrasion-bath experiments. Zebra fish with intact skins bathed in the cultures showed no increase in mortality over medium-only controls and exhibited no visible signs of infection at the concentrations tested (up to  $10^8$  cfu ml<sup>-1</sup>). This suggests that mechanical injury is required for efficient *Flavobacterium columnare* infection of zebra fish.

## DISCUSSION

In addition to its use as a model for vertebrate development, zebra fish *Danio rerio* have been used as a model system for *Streptococcus iniae* infection (Neely et al. 2002). Injections with this Gram-positive pathogen resulted in increased fish mortality. That study showed the utility of zebra fish as a model system by repeating infection experiments in immunocompromised mutants. This kind of work is only possible in a model host for which a robust genetics system exists. Knowing characteristics of the host that increase or decrease its susceptibility to flavobacterial pathogens would be useful to the breeders of fish stocks.

*Flavobacterium columnare* infection has been demonstrated in a wide variety of fish species. Here, we report on the experimental infection of adult zebra fish *Danio rerio* by *F. columnare* and *F. johnsoniae*. Although both organisms can produce increased mortality in zebra fish, only *F. columnare* acts as an aggressive pathogen in zebra fish under the conditions tested.

*Flavobacterium johnsoniae* was tested for its ability to infect zebra fish for 2 reasons: (1) it has been shown

to infect the barramundi (Carson et al. 1993); and (2) it has been the model system for the study of bacterial gliding motility in the Cytophaga/Bacterooides/Flavobacterium group (McBride 2004). It is also the only Flavobacterium for which a robust genetics system exists (McBride 2004). Gliding motility may be linked to *F. johnsoniae*'s ability to digest large polymers (McBride et al. 2003). Gliding motility is also a common characteristic of 4 flavobacterial fish pathogens, *F. johnsoniae*, *F. columnare*, *F. psychrophilum*, and *F. branchiophilum* (Bader & Starliper 2002). Our study, however, did not reveal any link between gliding motility and virulence in *F. johnsoniae* infections of zebra fish. Non-motile mutants did not show reduced virulence when compared to the wild type.

This may be due to the high *Flavobacterium johnsoniae* loads needed for lethal infection of zebra fish. Such large numbers of bacteria may simply be carried through the blood stream and have no need for motility. Alternatively, motility, and its associated large polymer digestion capability, simply may not be involved in pathogenesis of injected *F. johnsoniae* in zebra fish. Since *F. johnsoniae* did not infect zebra fish in bath trials at the highest bacterial concentrations tested, we do not know whether gliding motility is important to infection of the skin. *F. johnsoniae* ATCC 43622 was originally isolated from a presumed outbreak of CD and identified as *F. columnare* (Darwish et al. 2004). Our results, however, suggest that in zebra fish this strain is no more pathogenic than the *F. johnsoniae* type strain ATCC 17601 (data not shown). The high doses of *F. johnsoniae* required to induce lethal infection in zebra fish indicated that it is not a pathogen in this fish species.

The virulence and behavior of *Flavobacterium columnare*, like *F. psychrophilum*, is very strain dependent (Shamshudin & Plumb 1996, Madsen & Dalsgaard 2000, Thomas-Jinu & Goodwin 2004, Figueiredo et al. 2005). Multiple isolates from disease outbreaks, as well as the ATCC type strain, were tested in preliminary experiments for their suitability of use in challenge trials (data not shown). *F. columnare* 14-56 was chosen as the central strain in this study because of its relative ease of growth and handling, and its virulence. ATCC 23463 consistently grew in mucoid clumps in broth culture, making cell enumeration, injection, and bath cultures difficult.

The level of virulence we measured for *Flavobacterium columnare* 14-56 in zebra fish is comparable to that reported by other researchers in different fish species. Bader et al. (2003b) reported significant mortality of channel catfish after bath inoculation with  $10^8$  cfu ml<sup>-1</sup> of *F. columnare* ARS1, and Soltani et al. (1994) reported LD<sub>50</sub>s of  $1.3 \times 10^3$  cfu ml<sup>-1</sup> for barramundi and  $1.7 \times 10^5$  cfu ml<sup>-1</sup> for goldfish. An important finding of

our study was the requirement of skin damage for efficient infection of zebra fish. The LD<sub>50</sub> for abraded fish was >2 orders of magnitude lower than that seen in unabraded fish. Similar results in catfish were reported recently; mechanical abrasion or heat branding of catfish skin before infection with *F. columnare* ARS1 significantly reduced the 96 h LD<sub>50</sub> to 10<sup>4.3</sup> and 10<sup>5.8</sup> cfu ml<sup>-1</sup>, respectively (Bader et al. 2006).

Zebra fish have been suggested as an immunological model system and as a system for bacterial pathogenesis (Neely et al. 2002, Yoder et al. 2002). The genetic tools available for manipulation of this species make it possible to test specific immune genes for their importance in resisting *Flavobacterium columnare* infection. Future studies using the infection model described here with additional strains of *F. columnare* and immune-deficient zebra fish may provide insight into the mechanisms of disease production.

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