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Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*

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ABSTRACT: The present study identifies an emerging disease associated with an aquatic *Francisella*-like bacterium that can cause mortality in hybrid striped bass *Morone chrysops* × *M. saxatilis* reared intensively in freshwater. Clinically affected fish were lethargic, had scattered haemorrhagic cutaneous lesions and diffuse gill pallor. The head kidney and spleen were markedly swollen and contained numerous interstitial granulomas; histological examination revealed small, pleomorphic Gram-negative coccobacilli within vacuolated cells. The bacterium could not be cultured from head kidney homogenates either with standard or enriched microbiological media or following inoculation of a Chinook salmon embryo (CHSE)-214 cell line. No amplification product was obtained from head kidney DNA by polymerase chain reaction (PCR) assay using *Piscirickettsia salmonis*-specific primers. PCR analysis of infected head kidney homogenate with primers designed for the eubacterial 16S rRNA produced a single amplicon. Phylogenetic analysis of this DNA sequence demonstrated that the sequence aligned most closely with members of the genus *Francisella*, identified from tilapia *Oreochromis* spp. in Taiwan and an aquatic *Francisella* species that was recently isolated from the three-line grunt *Parapristipoma trilineatum* in Japan. This *Francisella*-like disease was transmitted to naïve hybrid striped bass fingerlings by intraperitoneal injection of tissue homogenates prepared from a natural outbreak. All fish developed gross and histological lesions identical to those from natural outbreaks. Intracellular Gram-negative bacteria were observed within the cytoplasm of cells (presumably macrophages) within the granulomas, but bacteria were not recovered. The 16S DNA sequence of the bacterium obtained from tissues of experimentally infected fish was identical to that obtained from the fish used as infected donor tissue.

KEY WORDS: *Francisella*-like bacterium · Intracellular bacterial disease · Hybrid striped bass · Emergent disease in aquaculture

INTRODUCTION

Infectious diseases of cultured finfish, including those due to bacterial pathogens, are a major risk factor limiting the success and viability of aquaculture (Meyer 1991). Bacteria can cause significant mortality and economic loss in finfish reared in both freshwater and marine environments, and several broad-host-range bacterial disease complexes have arisen with the continued intensification of aquaculture production practices (Yii et al. 1997, Low et al. 1999, Heckert et al. 2001, Mauel & Miller 2002, Decostere et al. 2004). Conditions conducive to the manifestation of disease during intensive aquaculture will undoubtedly continue to be a source of the emergence of novel bacterial pathogens in finfish.

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Intracellular bacterial pathogens can cause some of the most recalcitrant aquaculture diseases. Examples include *Renibacterium salmoninarum*, *Mycobacterium* spp., *Photobacterium damselae* subsp. *piscicida* and *Piscirickettsia salmonis* (Grayson et al. 1999, van der Sar et al. 2004, McCarthy et al. 2005). Diseases associated with rickettsia or rickettsia-like organisms (RLO) have been reported in a broad group of freshwater and marine fish hosts throughout a diverse geographic range (Fryer & Mauel 1997). The first finfish RLO to be isolated, characterized, and shown to cause disease was *P. salmonis*, a Gram-negative obligate intracellular pathogen recovered from an epizootic affecting coho salmon in southern Chile (Fryer et al. 1990, 1992). Since then *Piscirickettsia*-like organisms (PLOs), including *P. salmonis*, have been recognized as the cause of piscirickettsiosis or salmonid rickettsial septicemia (SRS) that affects cultured salmonids and non-salmonids from both freshwater and marine environments (Garces et al. 1991, Lannan & Fryer 1993, Comps et al. 1996, Almendras & Fuentealba 1997, Chen M.F. et al. 2000, Chen S.C. et al. 2000, Fryer & Hedrick 2003, Athanassopoulou et al. 2004, Arkush et al. 2005, McCarthy et al. 2005).

Recently, a PLO syndrome has been described in cultured tilapia from Taiwan, Jamaica, and Indonesia, as well as several US states including Hawaii, Florida, South Carolina and southern California (Chen et al. 1994, Chern & Chao 1994, Mauel & Miller 2002, Mauel et al. 2003, 2005). These studies have concluded that while this disease seems to be caused by a PLO, it does not appear to be related to *Piscirickettsia salmonis* because *P. salmonis*-specific PCR primers and standard tissue culture techniques fail to detect the presence of this pathogen (Mauel et al. 2003, 2005). Indeed, some infections that seem to be caused by a PLO may in fact be caused by a novel intracellular bacterial pathogen.

A newly emergent finfish pathogen that appears to belong to the genus *Francisella* has been shown to cause mortality in cultured Norwegian cod *Gadus morhua* and three-line grunt *Parapristipoma trilineatum* from Japan (Fukuda et al. 2002, Nylund et al. 2006). Naïve fish that received an intraperitoneal (i.p.) injection of a homogenate from diseased tissues or an axenic culture of the bacterium, were found on death to have granulomas containing the intracellular bacterium (Fukuda et al. 2002, Kamaishi et al. 2005, Nylund et al. 2006). Phylogenetic analysis of the 16S rRNA from bacteria isolated from diseased cod and three-line grunt demonstrated this agent to be a new species of *Francisella* (Kamaishi et al. 2005, Nylund et al. 2006). This represents the first report of an aquatic *Francisella* pathogen isolated from marine fish and establishes both the genus *Francisella* and a potentially new species of this genus as an emerging intracellular bacterial pathogen of teleost fish.

During routine diagnostic evaluations of moribund hybrid striped bass, we identified an intracellular bacterial infection that closely resembled the PLO disease described in Hawaiian tilapia (Mauel et al. 2003, 2005) and also shared many similarities with the *Francisella* species pathogen recently isolated from three-line grunt and cod (Kamaishi et al. 2005, Nylund et al. 2006). Here, we describe the clinical, bacteriological and pathological features of this disease in hybrid striped bass reared in a high density, freshwater recirculation production facility in southern California, USA. While we were able to routinely reproduce the disease and associated lesions in naïve fish by injection of infected tissue homogenates, our efforts to culture the bacterium from these infections failed. A molecular diagnostics analysis has led us to conclude that this condition was not associated with *Piscirickettsia salmonis* but rather with an aquatic *Francisella*-like pathogen similar to that described from three-line grunt.

MATERIALS AND METHODS

Background. Over the past several years, a distinct disease syndrome has been recognized during routine diagnostic evaluation of moribund juvenile hybrid striped bass (sunshine bass, *Morone chrysops* × *M. saxatilis*) reared in a high-density, recirculation, freshwater facility. To determine its cause, representative moribund fish underwent necropsy, including bacteriological, histopathological and ultrastructural examination of infected tissues.

Necropsy. Moribund fish were sacrificed with an overdose of MS-222 (Finquel, Argent Laboratories), examined for the presence of gross external lesions and a blood sample obtained by caudal venipuncture. For each fish, 2 heparinized capillary tubes of whole blood were collected and centrifuged for 5 min at room temperature (Readacrit Centrifuge). The packed cell volume (haematocrit) was measured for each fish and then averaged for all animals collected from within the same production tank. The external cranial surface of each animal was swabbed with 100% isopropanol, the brain exposed via cranial puncture, and tissue was aseptically streaked onto routine and various selective bacteriological media. The abdominal wall was flooded with alcohol, the abdomen opened, and the head and trunk kidney, liver and spleen were streaked onto various culture media for routine bacterial isolation (see 'Bacteriology' below).

Light and electron microscopy. Peripheral blood smears and head kidney and spleen impression smears

were air-dried, fixed in absolute methanol for 10 min, and stained by the Wright-Giemsa and Gram methods (Hardy Diagnostics). Representative tissues for histological examination were collected and preserved in Bouin's solution overnight, then transferred to 70% ethanol. Tissues were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). Giemsa, Ziehl-Neelson, and Gram staining of serial sections was used to enhance detection of other bacteria. For electron microscopy, approximately 0.2 g of affected head kidney was placed into roughly 20 volumes of an ice-cold 2.0% glutaraldehyde/1.5% formaldehyde fixative solution (IMEB) and held at 4°C until processed for ultrastructural examination. Trimmed samples were post-fixed for 1 h in 1% osmium tetroxide and then routinely processed into Spurr's resin. Sections were mounted on uncoated grids and stained with uranyl acetate and lead citrate.

Bacteriology. Tryptic soy agar (TSA) plates containing 5% sheep blood (blood agar [BA]; Hardy Diagnostics) were employed as the primary isolation medium for recovery of aerobic bacteria from affected tissues. Initial culture efforts were inconclusive; thus, additional media and incubation conditions were used in an attempt to provide a more complete nutritional spectrum for bacterial isolation. For all subsequent culture attempts, head kidney tissue from infected fish was also streaked onto the following commercially available media: brain heart infusion agar (BHIA), BHIA plus 5% sheep blood, BHIA plus 1.0% NaCl, MacConkey's agar, Mueller-Hinton agar plus 5% sheep blood, Middlebrook 7H10 agar, Lowenstein-Jensen (LJ) and LJ-Gruft modification slants, chocolate agar and SP4 agar (for isolation of *Mycoplasma* spp.). All media were purchased from a commercial supplier (Hardy Diagnostics) and were incubated from 22 to 28°C under aerobic, microaerophilic and anaerobic environments as described by the manufacturer (BBL GasPak Systems; Becton-Dickinson).

Tissue culture. Attempts to culture the bacterium from hybrid striped bass head kidney tissues in established cell lines followed the procedures described for *Piscirickettsia salmonis* (Fryer et al. 1990). Briefly, tissues from freshly dead fish were removed aseptically and homogenized in minimal essential medium (MEM) without sodium bicarbonate supplemented with 2% fetal bovine serum, 20 mM L-glutamine and buffered with 25 mM HEPES (MEM-2+HEPES) and without antibiotics. The homogenate was centrifuged at 208 × *g* at 4°C for 1 min and the supernatant was serially diluted in MEM-2+HEPES. Serial 10-fold dilutions of the supernatant were then inoculated on to monolayers of Chinook salmon embryo (CHSE)-214 cells in 24-well plates using antibiotic-free Eagle's minimal essential medium supplemented with 10% fetal bovine serum. The cells were incubated at 15°C and examined for evidence of cytopathic effects for a period of 21 d.

Experimental reproduction of disease. To confirm the infectious nature of the intracellular bacteria observed in the infected tissues of moribund fish, the head kidney and spleen from infected fish were aseptically dissected and placed in a sterile Petri dish. Each organ was stabbed and streaked onto the various microbiological media described above. Following this, a small piece of each tissue was removed and placed into a pre-weighed vial containing 2 ml of phosphate buffered saline (PBS, pH 7.2–7.4). The vials were weighed to the nearest 0.1 mg and homogenized with a hand-held tissue grinder (Tissue Tearor; Biospec Products). Following homogenization, roughly half of the head kidney homogenate was filtered through a 0.22 µm membrane (Millipore) and both the head kidney and spleen preparations were kept cool until required for challenge. For molecular identification and tissue culture, paired head kidney tissue samples (approximately 0.2 g each) were aseptically placed in either 5 ml of absolute ethanol or in a sterile 15 ml centrifuge tube, respectively, and held on ice until processed.

Experimental infection of naïve hybrid striped bass fingerlings (average weight 4 to 5 g) was tested using i.p., per os (p.o.) and gill surface (GS) routes of exposure (Almendras et al. 1997). Prior to IP injection, each tissue preparation was also diluted 1 in 10 with PBS and loaded into a tuberculin syringe equipped with a 26 gauge × 3/8 inch needle (Becton Dickinson). Groups of 8 naïve juvenile hybrid striped bass (10 to 12 cm total length, 7 to 11 g) from a healthy laboratory population, were maintained in 100 l circular tanks that received aerated geothermal well water at 26 ± 1°C under flow through conditions (inflow rate of 3 l min^{-1}). Water chemistry (dissolved oxygen, alkalinity, hardness, pH, ammonia-nitrogen) were monitored at the start, middle, and end of the experiment. Prior to challenge all fish were anaesthetized with MS-222 (150 mg l^{-1}) after which one group received a 0.1 ml i.p. injection of the various head kidney or splenic homogenates. Control fish were treated in an identical manner and received an i.p. injection of 0.1 ml of each filtered tissue homogenate or PBS.

Attempts to transmit the disease by the p.o. or GS routes of exposure were made using the undiluted homogenized head kidney inoculum (2 groups of 3 fish for each route). For the p.o. exposure route, a 3 inch (7.62 cm) long sleeve of sterile polyethylene tubing (0.0729 inch inner diameter, 0.1409 inch outer diameter; Cole-Parmer Instrument Company) was placed over an 18 gauge × 1 inch (2.5 cm) needle and connected to a 1 ml tuberculin syringe. Following anaesthesia, the tubing was gently inserted down the esoph-

agus and into the stomach where 0.1 ml of the tissue homogenate was delivered. For the GS route of exposure, fish were placed on their right side, the left operculum lifted and 0.1 ml of the homogenate was dropped onto the gill surface and the fish were held out of the water for 30 s. Following each experimental exposure route, all fish were placed into their respective experimental tanks for recovery. Fish were fed daily ad libitum and monitored for morbidity and mortality for 12 d.

Molecular identification. Head kidney tissues obtained from a total of 3 individual hybrid striped bass with clinical signs of infection were examined for the presence of *Piscirickettsia salmonis* DNA by the procedures described by Mauel et al. (1996) with minor modifications. The primer sequences and the conditions described for the PCR were identical to those described by Mauel et al. (1996) but the DNA isolation from tissues followed standard procedures for the Qiagen DNeasy™ kit, rodent tail protocol.

Amplification of eubacterial 16S rDNA was also attempted with universal primers from DNA extracted from head kidney tissue (50 to 100 mg) collected from 2 hybrid striped bass. Kidney tissues were collected aseptically as described and then homogenized in 0.5 ml of sterile PBS (Sigma). This sample, KST-1, represented the head kidney tissue obtained from a fish during a naturally occurring outbreak (i.e. the donor fish used to experimentally reproduce the disease in naïve hybrid striped bass fingerlings). This sample was verified to contain large numbers of the pleomorphic Gram-negative coccobacilli via histological examination of a portion of this tissue after the disease transmission experiment was started. The KST-2 sample was head kidney tissue removed from a moribund fingerling 4 d after receiving an i.p. injection of a 1:10 dilution of KST-1. A portion (~0.1 ml) of each homogenate was resuspended in 0.5 ml DNAzol® (MRC), supplemented with Proteinase K (100 µg), and total genomic DNA (gDNA) was extracted following the manufacturer's protocol. A region of the 16S rRNA gene was amplified from gDNA preparations using 2 universal bacterial primers: Primer 6, 5′-GCYTAACACATGCAAGTCGA and Primer 52, 5′-CCAGCAGCCGCGGTAATACG (Brunk et al. 1996). Briefly, 100 ng of gDNA was added to 25 µl reactions containing 0.5 µM of each primer, 0.2 mM dNTPs and 1 U AmpliTaq Gold® DNA Polymerase, LD (Applied Biosystems). Cycling conditions were as follows: denaturation at 94°C for 2 min; amplification for 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 45 s and a final extension at 72°C for 4 min. PCR products were verified on 1% agarose gels (expected size ~550 bp), and purified using a QiaQuick PCR Cleanup Kit (Qiagen) following the manufacturer's instructions. Sequence data were generated in both directions using PCR primers (Primer 6 and Primer 52) and ABI Dye-Terminator chemistry that was visualized on an automated sequencer (Model 373; Applied Biosystems).

Sequences attained from the 2 hybrid striped bass (HSB) head kidney samples (KST-1 and KST-2) were edited manually (SeqEd v1.02, Applied BioSystems) and compared with16S rRNA sequences in GenBank using BLAST search algorithms (Altschul et al. 1990). A selection of known bacterial species with significant BLAST homology, as well as several known fish pathogens (Table 1), were obtained from the database and aligned with the unknown sequences using ClustalW options in Vector NTI (Informax). The resulting dataset of orthologous 16S rRNA sequences (500 bp) was used to generate similarity estimates and a distance-based (neighbor-joining; distances generated using the methods of Jukes & Cantor 1969) consensus tree from 1000 bootstrap replicates in PAUP* (vers. 4.0b8; Sinauer Associates).

RESULTS

Clinical and gross presentation of disease

Affected production tanks had increased morbidity and a daily, low-level increase in mortality after onset of an infection. Affected fish usually ranged from 10 to 350 g in weight, showed loss of appetite, were dis-

Table 1. Reference bacteria used for the present study and their respective GenBank sequence accession numbers

Bacterial species	Accession No.
Francisella tularensis subsp. *tularensis* FSC 199	AY968225
Francisella tularensis subsp. *holarctica* FSC 257	AY968231
Francisella tularensis subsp. *mediaasiatica* FSC 149	AY968236
Francisella tularensis subsp. *novicida* FSC 040	AY 968237
Francisella philomiragia FSC 153	Z21933
Francisella sp. Ehime-1	AB194068
cf. *Francisella* sp. CYH-2002	AF385857
Tilapia parasite TPT-541	AF206675
Wolbachia persica ATCC VR 331	M21292
Aeromonas salmonicida	X60405
Photobacterium damselae	AB026844
Piscirickettsia salmonis (type culture, LF-89)	U36941
Piscirickettsia salmonis (sea bass PLO, SBPLO)	AY542956
Agrobacterium tumefaciens	AY972446

tinctly darker in pigmentation and were lethargic. The highest prevalence of infection occurred from January to June when the water temperatures ranged from 20 to 28°C. As the summer progressed and water temperatures increased above 29 to 30°C, there was an obvious facility-wide decline in the prevalence of disease. All other production parameters were reported to fall within normal limits for intensive hybrid striped bass culture. Blood samples were obtained from 18 moribund and 18 healthy fish collected from 5 different production tanks that displayed the typical signs of the disease. Haematocrit values for moribund fish ranged from 13.6 to 22.9% (mean 17.8%) while those obtained from healthy fish from the same production unit ranged from 42.4 to 49.7% (mean 46%).

All moribund fish had a mild to moderate degree of bilateral exophthalmia and displayed a marked to severe diffuse branchial pallor. Another common finding was the presence of numerous focal to multifocal, haemorrhagic skin lesions scattered over the abdomen (Fig. 1). Gill wholemounts indicated the presence of low numbers of *Trichodina* and *Ambiphyra* protozoa as well as varying numbers of bacteria closely associated with the lamellar epithelial surface. In many instances moribund fish had evidence of opercular and facial haemorrhage and/or congestion, presumably as a result of the labored gill ventilation commonly observed in moribund fish. Internally, the most obvious gross change involved extensive swelling of the head kidney and spleen (Fig. 2). The extent of kidney inflammation was anatomically restricted to the cranial region; rarely did the swelling extend to the medial aspect of the trunk kidney. Preliminary necropsies indicated that the head kidney and spleen were 7.7- to 9.9-fold heavier than those from healthy fish (data not shown). Diffuse hepatic and atrial cardiac pallor in the absence of any gross lesions, an absence of overt peritoneal inflammation but low quantities of a clear, watery fluid, and a gastrointestinal tract that was devoid of feed, were consistently found in all infected fish.

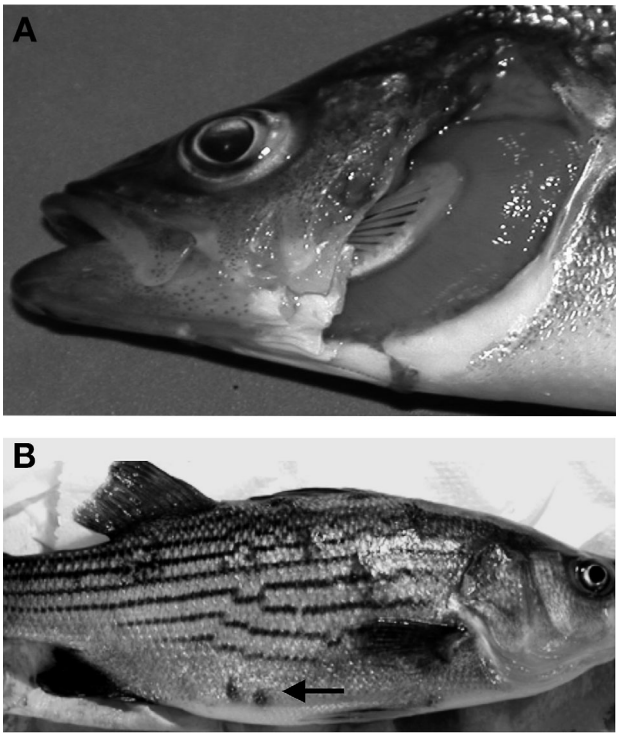

Fig. 1. *Morone chrysops* × *M. saxatilis.* (A) Gill and (B) skin lesions in hybrid striped bass naturally infected with a *Francisella*-like bacterium. Note in (A) the diffuse gill pallor and (B) several raised haemorrhagic foci (arrow) on the ventrolateral body wall

Histopathological and ultrastructural observations

From the naturally occurring outbreaks examined during this study, the most dramatic histopathological changes involved the head kidney and spleen (Fig. 3). The interstitium of both these organs had multifocal areas of brightly eosinophilic inflammation and necrosis associated with a pyogranulomatous inflammatory infiltrate or granulomas that were surrounded by degenerate, vacuolated cells (presumably macrophages) that contained low to moderate numbers of small, pleomorphic Gram-negative, non-acid fast coccobacilli (Fig. 4). In the head kidney, degeneration and necrosis of the interrenal and chromaffin tissues was not common, although degeneration of these tissues was apparent in more advanced cases, perhaps as the result of pressure necrosis from the granulomas. Furthermore, granulomatous foci were present in the posterior renal interstitium, sometimes associated with tubular degeneration and necrosis. In these instances, morphologically similar intracellular bacteria were seen within vacuolated cells at the periphery of the foci.

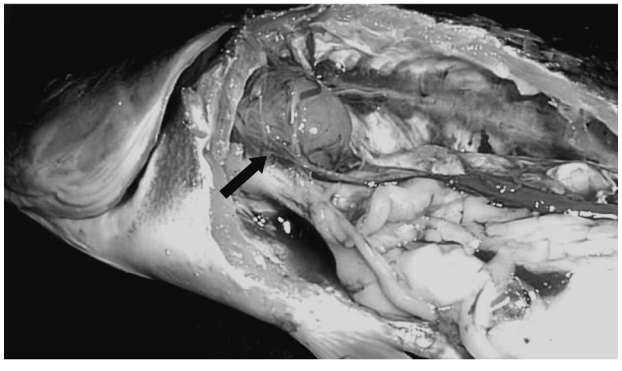

Fig. 2. *Morone chrysops* × *M. saxatilis.* Dramatic swelling of head kidney (arrow) in hybrid striped bass infected with *Francisella*-like bacterium

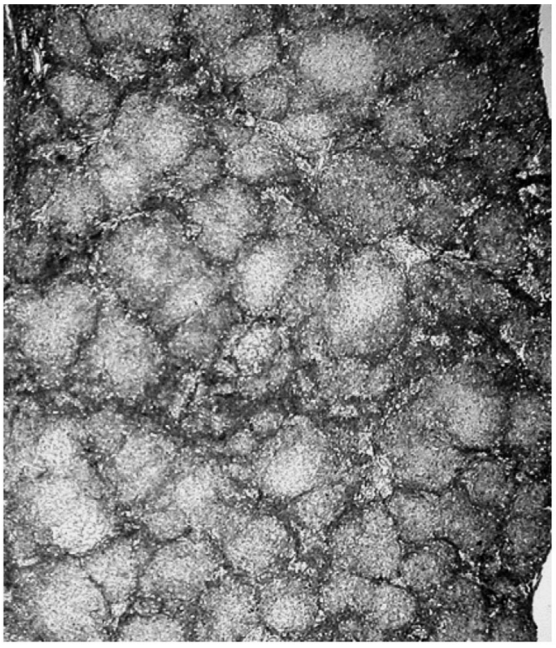

Fig. 3. *Morone chrysops* × *M. saxatilis.* Numerous granulomas throughout the spleen of hybrid striped bass naturally infected with *Francisella*-like bacterium using hematoxylin and eosin, H&E (×4)

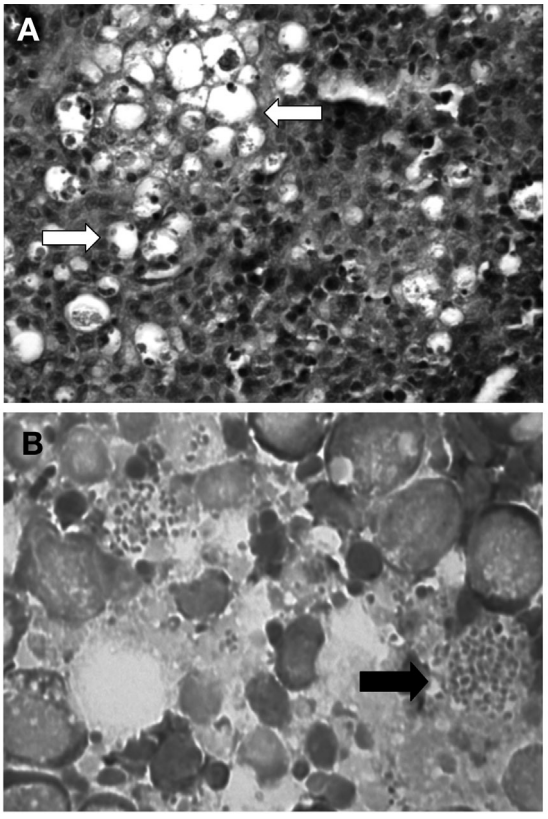

Fig. 4. *Morone chrysops* × *M. saxatilis.* Histopathological changes in head kidney of hybrid striped bass naturally infected with *Francisella*-like bacterium. (A) Granulomatous foci containing cells undergoing degeneration and pyknosis (white arrows) using H&E (×20). (B) Head kidney imprint demonstrating large numbers of small, coccoid-shaped bacteria within cells and free in the interstitium (black arrow) using the Wright-Giemsa staining method (×40)

Splenic changes associated with the marked pyogranulomatous presentation of this condition were almost entirely restricted to the ellipsoids. Again, much of the interstitium contained mononuclear inflammatory cells that appeared to be in the process of organizing into more discrete granulomatous foci and granulomas. At the periphery of these regions were numerous degenerating cells with pyknotic nuclei; they appeared to be devoid of cytoplasm, but each contained numerous small, coccobacilli-shaped bacteria. Fibrinoid necrosis and haemorrhage and varying degrees of erythrophagocytosis were occasionally associated with the extensive degeneration and necrosis of ellipsoids. Despite these dramatic changes, the splenic capsule appeared intact, although small focal areas of inflammation were sometimes seen on the serosal surface.

Additional histopathological changes common among all outbreaks were observed in the heart and gills. There was extensive endothelial hypertrophy and hyperplasia in the atrium, although intracellular coccobacilli were not common. A mononuclear inflammatory infiltrate was present in the interstitium of the gill filaments, often with intracellular coccobacilli seen within some of the inflammatory cells. Of note was the absence of overt liver and brain pathology. Although small granulomatous foci were occasionally observed in the liver of freshly dead fish, intracellular bacteria were not common. An incidental finding of protozoan parasites (mainly *Trichodina* spp. and *Ambiphyra* spp.) and various bacteria associated with the gill epithelium, was considered normal for hybrid striped bass reared under intensive, recirculation culture conditions.

Ultrastructurally, the bacteria were easily seen within macrophages (Fig. 5). They were situated in closely apposed clusters within the cytoplasm of infected cells, and apparently not within a cytoplasmic vacuole. Preliminary measurements of the intracellular bacteria indicated that they averaged 1.2 × 0.8 µm in size.

Bacteriology

In the outbreaks examined in this study, no single bacterial species was routinely recovered on standard or enriched culture media. The most common obser-

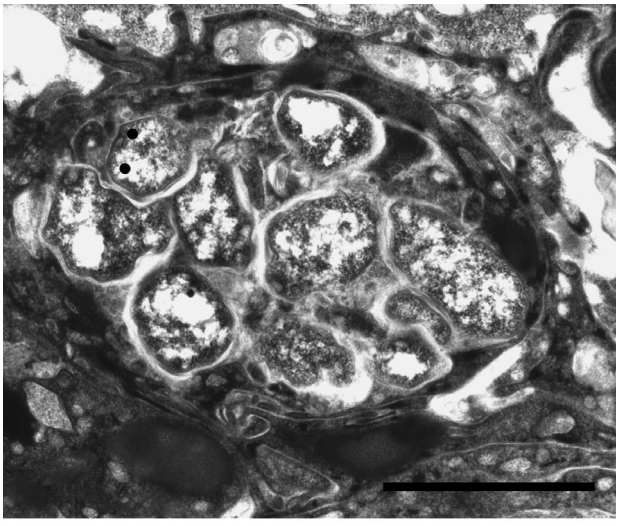

Fig. 5. *Morone chrysops* × *M. saxatilis.* Multiple coccoid bacteria (black dots) within the cytoplasm of a head kidney macrophage in experimentally infected hybrid striped bass. Intracellular bacteria do not appear to reside within cytoplasmic vacuoles (scale bar = 1.5 µm)

vation consistent among all outbreaks was partial haemolysis that developed on the BA plates after 1 to 3 d post inoculation. This change was seen only in the area that was directly streaked with the infected head kidney or spleen tissue but bacterial colonies were never observed growing in this region. Bacteria were not isolated from tissues that were incubated under microaerophilic or anaerobic conditions. Attempts to isolate the bacterium in tissue culture were unsuccessful as examinations of CHSE-214 cells inoculated with extracts from infected hybrid striped bass tissues revealed no evidence of cytopathic effects upon initial inoculation (14 d) or following subculture and observation for an additional 14 d.

Experimental reproduction of disease

Both the head kidney and splenic tissue homogenates (approx. 0.1 g ml^{-1} PBS) caused mortality in the hybrid striped bass fingerlings (Table 2). Injection of the undiluted head kidney and splenic homogenate resulted in mortality in 7 of 8 test fish (87.5%), while there was 100% mortality in fish that received the diluted tissue homogenate. In contrast, mortality was not observed in fingerlings that received PBS or the head kidney homogenate previously filtered through the 0.22 µm membrane. Similarly, neither p.o. nor branchial exposure to the head kidney homogenate caused mortality under the experimental conditions employed. Water quality parameters throughout the experiment were as follows; dissolved oxygen ranged from 11–15 mg l^{-1}, alkalinity and hardness ranged from 68–74 and 6–10 mg l^{-1} (both as $CaCO_3$), respectively, and total ammonia-nitrogen and pH from 0.01–0.03 and 7.6–7.8 mg l^{-1}, respectively.

In fish that received an i.p. injection of diluted head kidney and spleen homogenates, mortality began at Day 5 post-injection and continued until Day 12, when the experiment was terminated due to the complete mortality in these groups (data not shown). At necropsy, moribund fish displayed diffuse gill pallor, and a markedly swollen head kidney and spleen, both of which contained numerous granulomas. The histopathological features of the experimental infection closely paralleled those seen in the natural outbreaks. One notable difference was the dramatically greater number of intracellular bacteria present within the degenerating cells at the periphery of the granulomas. At the completion of the experiment, all surviving control animals appeared clinically healthy, displayed an active interest in feed, and had no histological or bacteriological indication of infectious disease.

Molecular identification

The PCR designed to amplify a portion of the 16S rRNA gene of *Piscirickettsia salmonis* failed to yield a product from the 3 separate hybrid striped bass samples examined that were known to contain the intracellular bacterium based on Wright-Giemsa-stained

Table 2. *Morone chrysops* × *M. saxatilis*. Experimental reproduction of aquatic *Francisella*-like disease in naïve hybrid striped bass fingerlings using tissue homogenates prepared from a naturally infected hybrid striped bass. Mortality was monitored for 12 d post injection. Values for head kidney and spleen: g tissue in phosphate-buffered saline (PBS) prior to homogenization, and tissue preparation diluted with PBS

Group (treatment type)	n	Mortality	% Mortality
Head kidney (0.1 g ml^{-1})	8	7	87.5
Head kidney (1:10)	8	8	100.0
Spleen (0.1 g ml^{-1})	8	7	87.5
Spleen (1:10)	8	8	100.0
Head kidney (0.22 µm filtered)	8	0	0.0
Control (PBS)	8	0	0.0
Per os (gastric gavage)	3	0	0.0
Branchial exposure	3	0	0.0

imprints (data not shown). Positive controls consisting of DNA extracts from known *P. salmonis* infected salmonid tissues analyzed in parallel with the hybrid striped bass head kidney extracts were strongly positive. In contrast, a portion of the 16S rRNA gene was PCR amplified from total genomic DNA preparations of HSB head kidney tissues with universal bacterial primers. Single PCR amplicons (~550 bp) were obtained from both samples (KST-1 and KST-2), and resulting sequence data were found to be 100% identical, yielding 428 bp of consensus sequence data after alignments and editing (GenBank Accession No. DQ377177). Results from BLAST queries revealed that KST-1 and KST-2 had 100% identity to partial 16S rRNA gene sequences of an undescribed intracellular bacterium (*Francisella* sp.) isolated from tilapia in Taiwan (GenBank Accession Nos. AF206675 and AF385857) and a recently isolated *Francisella* sp. from the three-line grunt (GenBank Accession No. AB194068, Kamaishi et al. 2005). Known species of bacteria in the database with the highest levels of sequence homology were members of the genus *Francisella* and included *F. philomiragia* (98.2% similarity) and *F. tularensis* subsp. *novicida* (95.4% similarity). The KST-1 and KST-2 sequences shared 95.2% similarity with *F. tularensis* subsp. *holarctica*, while *F. tularensis* subsp. *tularensis*, and *F. tularensis* subsp. *mediasiatica* also shared 95.2% similarity. Comparisons to the intracellular Gram-negative fish pathogen, *Piscirickettsia salmonis*, revealed only moderate similarities (<86% similarity).

A consensus tree, based on a phylogenetic analysis (neighbor-joining) of evolutionary distances between KST-1 and KST-2, members of the genus *Francisella*, *Wolbachia persica* (a close relative) and a selection of known fish pathogens, is presented in Fig. 6. Results strongly support the clustering of KST-1 and KST-2 (100% similarity) with the other *Francisella* spp. associated with or isolated from fish species, and this sequence type is most closely related to *Francisella philomiragia* (100% bootstrap support). The separation of this clade from other described subspecies of *F. tularensis* (*novicida*, *holarctica, tularensis, mediasiatica*) is also strongly supported (97% bootstrap support). The levels of divergence between the sequences presented here (KST-1, KST-2) and the other known species of *Francisella* (1.9 to 5.2%), suggest that this may represent a new species.

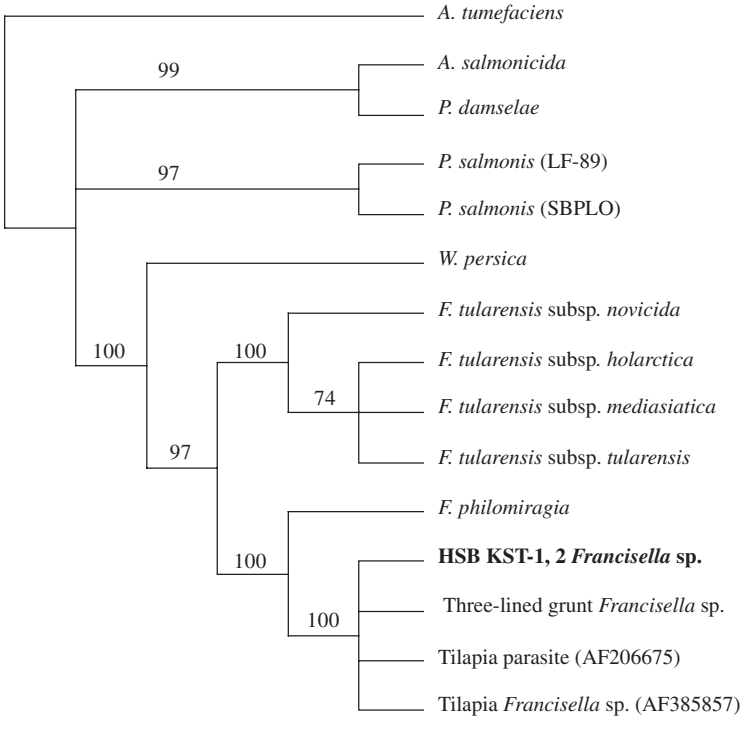

Fig. 6. Distance-based tree (neighbor-joining) of partial 16s rRNA sequences of KST-1 and KST-2 hybrid striped bass head kidney sequences, members of the genera *Francisella*, *Wolbachia persica* (a close relative) and a selection of known fish pathogens. *Agrobacterium tumefaciens* was used as an outgroup. GenBank Accession Nos. are given for tilapia. *P. damselae*: *Photobacterium damselae*

DISCUSSION

Our examinations of the causes of a disease in hybrid striped bass reared in a high-density freshwater production facility have demonstrated an association with a novel intracellular Gram-negative *Francisella*-like bacterial pathogen. 16S rRNA sequence analyses of head kidney tissues from both a natural and an experimental infection were identical and consistent with a *Francisella*-like bacterium. The bacterium we routinely observed in hybrid striped bass tissues appears to be similar to the *Francisella* sp. from diseased cod and the three-line grunt because i.p. injection of naïve fish with infected tissue homogenates reproduced the disease and mortality (Fukuda et al. 2002, Kamaishi et al. 2005, Nylund et al. 2006). Using similar procedures (Abd et al. 2003, Petersen et al. 2004, Kamaishi et al. 2005), we have recently isolated a morphologically similar organism from hybrid striped bass and have established its pathogenicity in hybrid striped bass fingerlings (V. E. Ostland unpubl. obs.). Phenotypic and molecular characterization studies and virulence studies of this aquatic *Francisella*-like isolate are currently in progress.

Based on 428 base pairs of 16S rRNA sequence, our phylogenetic analysis suggests that the *Francisella*-like bacterium associated with mortality in hybrid striped bass is most closely related to similar agents described from tilapia in Taiwan and three-line grunt from Japan (Kamaishi et al. 2005). Following the aquatic *Francisella*-like agents, the next nearest relative to the hybrid striped bass bacterium is *Francisella philomiragia*; it is less closely related to the multiple subspecies of *F. tularensis*, the causative agent of tularemia in humans and animals. There is increasing evidence that members of the genus *Francisella* are more widespread throughout various soil and water environments than was previously realized (Barns et al. 2005). The isolation and identification of *Francisella*-like bacteria associated with diseases both in fresh and marine aquatic environments in our own studies and in those of others supports this hypothesis. Our studies also indicate that this group of newly recognized agents should be considered as yet another example of emerging intracellular bacterial pathogens among cultured freshwater and marine finfish.

These preliminary findings demonstrate that genetically identical *Francisella*-like pathogens can cause mortality in hybrid striped bass, several species of tilapia in Taiwan, and in three-line grunt and, more recently, cod. The successful isolation of the etiological agent has, however, only been reported from the marine species. Moreover, considering the bacteriological, molecular, microscopic, and ultrastructural similarities between the hybrid striped bass disease and the piscirickettsiosis-like organism in Hawaiian tilapia (HTPLO), we hypothesize that both diseases may, in fact, be caused by the same or a very closely related pathogen because molecular and histopathological studies have detected an identical agent in feral Mossambique tilapia *Oreochromis mossambicus* in southern California, USA (V. E. Ostland unpubl. obs.). Further efforts to isolate and characterize the intracellular bacteria associated with this disease syndrome in tilapia in southern California are also underway.

The temperature preferences of 2 aquatic *Francisella* sp. isolates studied so far range from 6 to 25°C but neither can grow at 37°C (Kamaishi et al. 2005, Nylund et al. 2006). Similarly, we were unable to propogate the *Francisella*-like bacterium recovered from hybrid striped bass at 37°C, but this isolate consistently grew at 28°C (V. E. Ostland unpubl. obs.). Furthermore, clinical disease associated with the *Francisella*-like bacterium in intensively reared hybrid striped bass seems to be restricted to water temperatures ranging from 20 to 28°C. The prevalence of natural outbreaks of this disease in hybrid striped bass declines significantly when water temperature reaches 29 to 30°C and clinical disease essentially disappears when the water temperature exceeds 31°C (V. E. Ostland unpubl. obs.). Many new isolates of *Francisella*-like bacteria can be common soil and water inhabitants but they can be difficult to culture from these environmental habitats (Petersen et al. 2004, Barns et al. 2005). Recent evidence suggests that ubiquitous aquatic protozoa, such as the free-living amoeba *Acanthamoeba castellanii*, might serve as an important environmental reservoir (Abd et al. 2003). Collectively, these observations strongly suggest that aquatic *Francisella* sp. isolated from fish are likely nonpathogenic to mammals and thus should pose no threat to humans or other terrestrial animals.

In our experience, mortality associated with *Francisella*-like bacteria can be controlled with oxytetracycline (OTC)-medicated feed at 3.8 g OTC per 0.45 kg of food when fed at 3% body weight per day for 10 d (as prescribed under veterinary supervision). This is consistent with previous reports that feeding OTC to tilapia decreased PLO-associated mortality (Chern & Chao 1994, Mauel & Miller 2002, Mauel et al. 2003). Antibiotics have been used extensively to attempt to control SRS infections but they are costly, difficult to deliver to the intracellular environment where these pathogens localize, and perhaps most importantly, none has proved to be consistently effective against these infections (Smith et al. 1997). To effectively prevent and control piscirickettsial and other intracellular bacterial fish diseases, vaccine development is perhaps the most logical alternative (Kuzyk et al. 1996). Currently, there are no antibiotics approved in the USA to control aquatic *Francisella* infections of teleosts.

The disease caused by this novel *Francisella*-like bacterium appears to affect juvenile hybrid striped bass in their first year of production, since diagnostic monitoring of different age classes throughout a typical production cycle suggest that fish greater than 0.6 kg (re)develop clinical disease less frequently (V. E. Ostland unpubl. obs.). It is not known whether this apparent resistance is due to survival from a previous episode of disease, thus conferring protection due to acquired immunity, or is merely the result of an age-related phenomenon linked to immune competency. Histopathological examination of hybrid striped bass experimentally infected with this *Francisella*-like pathogen indicates that the pathogen quickly enters the intracellular environment and, over the course of several days, causes a dose-dependent increase in the degeneration and necrosis of cells of the presumably mononuclear leucocytic lineage in the head kidney and spleen, leading to intense, localized inflammation and granuloma formation (V. E. Ostland unpubl. obs.). This observation seems to parallel the cytopathogenicity and apoptosis described during *Francisella tularen-*

sis infections, where this pathogen can localize in the murine phagosome but also has the capacity to alter the phagosomal maturation process and enter the cytoplasm of the macrophage (Fortier et al. 1995, Lai et al. 2001, Clemens et al. 2004). This may also help explain why we were unable to observe the aquatic *Francisella*-like bacterium within a distinct cytoplasmic vacuole, a potentially relevant diagnostic observation to help differentiate between infections caused by intracellular Gram-negative pathogens such as *Piscirickettsia salmonis* and *Francisella* sp.

Vaccination is considered the most prudent measure for the prevention of tularemia in humans and animals, especially in light of the possible threat of *Francisella tularensis* as a bioweapon (Greenfield & Bronze 2003, Titball & Oyston 2003). While antibiotics are considered one of the most important tools for a rapid first line of response for *Francisella* infections in mammals, including humans, wide-spread use of antibiotics to control these infections in the face of continued emergence of antibiotic-resistant human and animal pathogens, is unlikely to be sanctioned by regulators (Navas 2002). Thus, a thorough understanding of the biology, virulence mechanisms and pathogenesis of this *Francisella*-like bacterium in hybrid striped bass that will facilitate the development of a safe, potent, bacterin, live attenuated, or subunit vaccine will help ensure the long-term success of aquaculture in the face of emerging aquatic *Francisella* spp. infections.

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