Reference: *Biol. Bull.* **193:** 269–270. (October, 1997)

Identification of Toadfish-Pathogenic Bacteria Based on a Comparative Molecular Approach

Supaya Wenuganen[1], Robert Bullis, Roxanna Smolowitz (Marine Biological Laboratory, Woods Hole, Massachusetts 02543), and Elena Barbieri[2]

Opsanus tau (oyster toadfish) is an important laboratory animal at the Marine Biological Laboratory (MBL); they are used as a model for studying the mechanisms of hearing and balance.

A new disease, bacterial pericarditis, which is caused by a gram negative bacterium, has caused mortalities in the Marine Resources Center (MRC). Determining the identity of this pathogen, which has not been previously seen in toadfish (1), is important to the health management program for this species. A rapid and accurate method, based on the 16S rRNA gene, for the identification of bacterial pathogens was used to address this problem (2, 3).

Bacterial strains (n = 15) were isolated from individual infected toadfishes (n = 12) during the outbreak of bacterial pericarditis that occurred from July 1996 to April 1997 at the MRC. Brain heart infusion agar (BHIA; Difco, Detroit, MI) with 1.2% NaCl, was used to isolate and purify bacterial cultures. All isolates were first tested for antibiotic resistance. BHIA antibiotic plates, including ampicillin (50 μg/ml), kanamycin (25 μg/ml), tetracycline (10 μg/ml), and vancomycin (100 μg/ml), were used. The plates were incubated for 24 h at room temperature.

Bacterial DNA was isolated from each isolate using the bead beater procedure and standard phenol extraction. Universal prokaryote primers specific to the ends of prokaryotic 16S rRNA were used to amplify this gene. DNA hybridization (checker board) was applied to the 16S rRNA of all isolates using 16S rRNA complementary probes: universal prokaryotic, enteric, betas, deltas, sulfate reducing bacteria, flavobacteria, gram positive low GC, and spirochete.

To screen the PCR products and compare the 16S rDNA in all isolates, restriction fragment length polymorphism analysis (RFLP) was performed using four tetrameric restriction enzymes, *Hae*III, *Hin*PI, *Msp*I, and *Rsa*I (4). To confirm the results obtained from the RFLP analysis, denaturing gradient gel electrophoresis (DGGE) was applied. DGGE primers were used to amplify the 16S rRNA gene, and the PCR products were run in a 40%–60% urea formamide gradient acrylamide gel. After the RFLP and DGGE comparisons, representative samples with a common RFLP DNA pattern and DGGE band positions were chosen for sequencing with an ABI automatic apparatus. Sequences obtained were compared to the non-redundant nucleotide database at the National Center for Biotechnology Information using the BLAST (Basic Local Alignment Tool) algorithm. Phylogenetic analyses were done according to Paster (5).

All isolates (#1–#15) tested for antibiotic resistance were susceptible to ampicillin, kanamycin, and tetracycline, and were resistant to vancomycin. If antibiotic treatment is needed, ampicillin and tetracycline can be used on the infected fish. Kanamycin, however, is not recommended because spontaneous resistance develops. The resistance of the bacteria to vancomycin is not surprising because this antibiotic cannot penetrate the gram negative outer membrane (6).

All of the fifteen 16S rRNA genes from the bacterial isolates gave positive signals with enteric and universal probes, but no signal was detected with betas, deltas, SRBs, flavobacteria, gram positive low GC, or spirochete probes. This result indicates that all of the isolates tested by checkerboard hybridization can be classified as enteric bacteria. The comparative RFLP analysis of the 16S rRNA gene confirmed that all 15 isolates can be classified into two distinctive patterns: Group I includes isolates #1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14; and group II includes isolates #3, 7, 15. *Hin*PI enzyme cut these 16S rRNA fragments more frequently than any other tested enzyme. DGGE analysis revealed that the group I isolates are identical. Sequence analysis of 16S rRNA genes of the group I isolates (#2, 4, 6, 14) indicates that they can be classified into the gamma purple cluster and are phylogenetically closely related to *Escherichia coli.* Current analyses suggest that the isolates could be a new species not yet described. Sequence analysis of 16S rRNA group II isolates (#3 and #15) indicates that they are phylogenetically close to the *Photobacterium* cluster. Photobacteria, commonly found in healthy marine fish (7, 8), are presumed to be contaminants.

The molecular analysis of group I bacteria (12 of 15) confirms that the predominant isolates are identical, and suggests that this coliform-like bacteria is the causative agent of the disease. Work is being conducted to fulfill Koch's postulates, and pulsed field gel electrophoretic (PFGE) analysis should provide additional information. Moreover, from the sequence data obtained, we will design a 16S rDNA primer that is specific for the etiological agent and can be used for the rapid detection of this pathogen.

We thank the MBL Microbial Diversity Course staff and faculty, particularly Abigail Salyers and E. Leadbetter, for valuable discussions, and Bruce Paster for assistance with the molecular analyses. This research was supported by the NASA Life Sciences Program.

Literature Cited

1. **Smolowitz, R., and R. Bullis. 1997.** *Biol. Bull.* **193:** 270–271.
2. **Avanis-Aghajani, E., K. Jones, D. Chapman, and C. Brank. 1994.** *Biol. Techniques* **17:** 144–147.
3. **Wiik, R., E. Stackebrandt, O. Valle, F. L. Daae, O. M. Rodseth, and K. Andersen. 1995.** *Int. J. Syst. Bacteriol.* **43:** 421–428.
4. **Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. Karl. 1996.** *Appl. Environ. Microbiol.* **67:** 2501–2507.

[1] IUC for Biotechnology, Bogor Agricultural University, Bogor, 16680, Indonesia.

[2] Istituto di Scienze Tossicologiche Igienistiche e Ambientali, University of Urbino, Urbino, 61029, Italy.

5. **Paster, B., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996.** *Appl. Env. Microbiol.* **62:** 346–352.
6. **Salyers, A. A., and D. D. Whitt. 1994.** ASM Press. Washington, DC.
7. **Austin, B. 1983.** *J. Mar. Biol. Ass. UK.* **63:** 585–592.
8. **Austin, B. 1982.** *J. Appl. Bacteriol.* **52:** 253–268.

Reference: *Biol. Bull.* **193:** 270–271. (October, 1997)

Bacterial Pericarditis: A New Disease of Toadfish

R. Smolowitz (Laboratory for Aquatic Animal Medicine and Pathology, University of Pennsylvania, Marine Biological Laboratory, Woods Hole, Massachusetts 02543), and R. Bullis[1]

The toadfish, *Opsanus tau,* is an important research animal used in the study of diabetes, muscle physiology, sound reception, and equilibrium. Toadfish are sediment dwellers that live inshore on rocky bottom and reefs. During the winter they migrate to deeper waters, bury themselves, and remain torpid till the spring (1). In the spring and summer, personnel of the Marine Biological Laboratory's Marine Resource Center (MRC) collect toadfish from various areas along the Massachusetts coast. These animals are kept over the winter to supply specimens for research.

Toadfish captured during the spring and early summer of 1996 were held in ambient water untill September, then were placed in treated water held at about 10°–15°C through the winter. Beginning at the end of July 1996 and continuing until April 1997, mortalities due to a new disease, termed here bacterial pericarditis, were identified in the adult populations of toadfish held at the MRC. Over the 8-month period, an epidemic level of this disease, 51% (24/47), was identified in the dead or moribund toadfish submitted for necropsy. At necropsy, 33% (8/24) of the fish with bacterial pericarditis exhibited swollen "coconut belly" abdomens that contained up to 140 ml of serosanguinous fluid that clotted when exposed to air (Fig. 1A). Internally, 79% (19/24) of the affected fish showed an accumulation of up to 5 ml of white/tan to pink, thick, flocculent pericardial exudate (Fig. 1B). Occasionally, the exudate appeared more diphtheritic and encased the heart. Multifocal discrete white/tan nodules were common in the myocardium of the ventricle. Eighty-three percent (20/24) of the fish showed nodules in the liver that ranged from 6 cm to <1 mm (Fig. 1C). In 4% (1/24) of the cases, a large nodule at the hilus of the liver, without apparent pericardial component, was associated with ascites. Nodules (2 cm to <1 mm diameter) were also identified in the kidney (17%, 4/24), in both male and female gonads (42%, 10/24) and in the male accessory sex glands (17%, 3/18) of several fish. In 8% (2/24) of the cases, the air bladder was filled with thick, flocculent, white/tan exudate.

Tissues were removed at necropsy, processed in paraffin, and stained with hematoxylin and eosin using standard methods (2). Histologically, the pericardial inflammation consisted of necrotic cells, macrophages and lesser numbers of heterophils and lymphocytes. Rod-shaped bacteria were abundant both within inflammatory cells and free in the debris (Fig. 1D, 1E). Multifocally fibroblastic proliferation forming granulation tissue was noted at the junction of the pericardial exudate and the epicardium. Multifocal discrete nodules seen in many organs were characterized by central liquefactive necrosis intermixed with variable numbers of bacteria. Many macrophages and a lesser number of heterophils surrounded and isolated the necrotic debris from the surrounding organ parenchyma, but only rarely were encapsulating fibroblastic-like macrophages seen (3) (Fig. 1F). In addition, the livers showed severe edema accompanied by dilation of the space of Disse, bile stasis within canaliculi and hepatocytes, and atrophy of the hepatic cords.

The most characteristic gross lesions identified in this disease were pericarditis and hepatic nodules. Pericarditis and myocarditis caused poor myocardial function resulting in secondary congestion of the liver. In animals showing a more chronic and severe pericarditis, ascites resulted in the gross signs of "coconut belly." Rarely, large nodules at the hepatic hilus resulted in hepatic blood stasis and ascites. Bacterial pericarditis devastated the adult toadfish population held at the MRC over the winter of 1996–1997. This disease, characterized grossly by severe pericarditis, has not been previously reported in toadfish or other fish and has not been previously identified in MRC toadfish. The origin of the bacterium causing this disease is not known but has been classified as *E. coli*-like. Work is being conducted to further classify the bacterium.

We thank the Elizabeth Wadman and the staff of the MRC for their support and help.

Literature Cited

1. **Guder, E. W. 1908.** *Bull. U.S. Bur. Fish.* **28:** 1071–1099.
2. **Howard, D. W., and C. S. Smith. 1983.** *Histological Techniques for Marine Bivalve Mollusks.* NOAA Technical Memorandum NMFS-F/NEC-25, United States Dept. of Commerce, National Technical Information Service, Springfield, VA.
3. **Noga, E., M. J. Dykstra, and J. F. Wright. 1989.** *Vet. Path.* **26:** 429–437.

[1] LAAMP, U. of Penn., MBL, Woods Hole, MA.