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Molecular and Phenotypic Characterization of Strains of *Photobacterium damselae* subsp. *piscicida* Isolated from Hybrid Striped Bass Cultured in Louisiana, USA

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Abstract.—Photobacteriosis, which is caused by the bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) was found to be a primary cause of mortality in hybrid striped bass (striped bass *Morone saxatilis* × white bass *M. chrysops*) cultured in brackish water in southern Louisiana. The disease was diagnosed on 50 occasions from 1990 through 2000 by the Louisiana Aquatic Diagnostic Laboratory, and mortality ranged from 5% to 90% in individual culture units. Strains of the bacterium from the Louisiana coast of the Gulf of Mexico were identified by means of biochemical tests, guanine–cytosine ratio, and Aquarapid Pp kit. Strains were characterized according to their biochemical phenotype, enzyme activities, plasmid profile, genotypic profile as determined by randomly amplified polymorphic DNA (RAPD) analysis, and antimicrobial susceptibility. When compared with representative isolates from the Chesapeake Bay, Greece, Japan, and Israel, Louisiana Gulf Coast isolates of *P. damselae* subsp. *piscicida* were found to be almost identical in biochemical phenotype and enzyme activity. The isolates differed in their plasmid profiles and antimicrobic susceptibilities. Louisiana isolates were found to possess a unique plasmid banding profile relative to strains from other geographic locations. The Louisiana isolates typically produced two large plasmid bands greater than 30 kilo–base pairs (kb) and two smaller bands 8.0 kb and 5.0 kb in size. Isolates from Israel and Greece exhibited similar banding patterns but were different from Louisiana and Japanese isolates. Resistance to Romet and Terramycin by some Louisiana strains was the result of acquisition of an R plasmid. When analyzed by RAPD, the Louisiana Gulf Coast strains were found to belong to clonal lineage group 2, which displays a fingerprint similar to that of the Japanese strains.

The culture of hybrid striped bass (striped bass *Morone saxatilis* × white bass *M. chrysops*) is a growing industry in the United States, production increasing from 220 metric tons in 1987 to approximately 5,400 metric tons in 2000 (National Marine Fisheries Service 2001). Hybrid striped bass mariculture began in the late 1980s in the marshes of the Louisiana coast of the Gulf of Mex-

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ico using pond, net-pen, raceway, and cage culture techniques. Four farms were in operation by 1990, shipping whole-fish product primarily to markets in the Atlantic Coast states. The largest operation had the potential to produce approximately 150,000 kg of marketable fish per year. However, since the fall of 1990, yearly outbreaks of photobacteriosis have had a devastating impact on this burgeoning industry, entailing losses in the millions of dollars.

The causative agent of the disease previously known as fish pasteurellosis was originally described after a massive fish kill in Chesapeake Bay, which destroyed approximately 50% of the natural populations of white perch *M. americanus* and striped bass (Snieszko et al. 1964). An isolate from this outbreak was deposited in the American Type Culture Collection, Manassas, Virginia (ATCC 17911). Based on a variety of physical and biochemical characteristics, the bacterium was tentatively placed in the genus *Pasteurella*. The pathogen was studied morphologically, physiologically, and serologically by Janssen and Surgalla (1968), who concluded that the bacterium was a new species and proposed the name *Pasteurella piscicida*. The name was never validated by bacterial taxonomists due to physiological inconsistencies between this bacterium and those in the genus *Pasteurella*, such as lack of nitrate reductase, tolerance of pH values outside the normal range, halophilia, lower optimum growth temperature, and unusual host range. The bacterium was not included in Bergey's *Manual of Systematic Bacteriology* (Mannheim 1984) or the *Approved Lists of Bacterial Names* (Skerman et al. 1989). Nevertheless, the name was used in the literature until 1995, when the organism was formally renamed *Photobacterium damsela* subsp. *piscicida* based on 16S ribosomal RNA sequences (Gauthier et al. 1995). The name was later corrected to *P. damselae* subsp. *piscicida* (Truper and DeClari 1997).

After the original outbreak, the bacterium was found to be responsible for smaller fish kills involving natural populations of striped bass in Chesapeake Bay (Paperna and Zwerner 1976) and western Long Island Sound (Robohm 1983). Among cultured fish in the United States, photobacteriosis was first documented in striped bass reared in earthen, brackish water ponds on the Alabama Gulf Coast at the Claude Peteet Mariculture Center, Alabama Marine Resources Division (Hawke et al. 1987). In this instance, approximately 49,000 fingerlings (weight, 30–40 g) that were being raised for stock enhancement were lost to the disease in a 20-d period. Although *P. damselae* subsp. *piscicida* has remained an important pathogen of cultured yellowtail *Seriola quinqueradiata* in Japan since the late 1960s (Kubota et al. 1970; Egusa 1980), it has recently emerged as a serious pathogen of cultured fish in the Mediterranean. The disease has been documented from cultured seabream (also known as the gilthead bream) *Sparus auratus* in Spain (Toranzo et al. 1991), Portugal (Baptista et al. 1996), Malta (Bakopoulos et al. 1997), and Italy (Magariños et al. 1992) and from cultured sea bass *Dicentrarchus labrax* (also known as European bass *M. labrax*) in France (Magariños et al. 1992), Turkey (Candan et al. 1996), and Greece (Bakopoulos et al. 1995). Photobacteriosis has also caused serious economic losses in hybrid striped bass reared in ponds in Israel (Nitzan et al. 2001). The importance of *P. damselae* subsp. *piscicida* as a pathogen of wild and farmed fish in Japan and Europe has been established in several reviews (Austin and Austin 1993; Magariños 1996; Plumb 1999).

The only U.S. strain that has been subjected to phenotypic, antigenic, and molecular characterization is ATCC 17911, which was isolated from the Chesapeake Bay. This study is the first to characterize strains affecting commercial aquaculture from the U.S. Gulf Coast.

Methods

Culture Facilities

Hybrid striped bass fingerlings (40–50 g) were stocked at the following rates into four culture facilities: 60 fish/m^3 in 8.1-m^3 floating cages at farm A, 35–44 fish/m^3 in 340-m^3 fixed net-pens at farm B, 60 fish/m^3 in 10.6-m^3 floating cages at farm C, and in 4.8-ha ponds (stocking density unknown) at farm D. Fish were fed a commercial hybrid striped bass feed (Burris Mill and Feed, Franklinton, Louisiana). Hybrid striped bass were harvested when they reached a mean weight of approximately 1 kg. Supplemental and emergency aeration devices were available at farm B but not at the other farms. Oxygenation of the water, waste removal, and maintenance of water quality depended primarily on the tidal movement of water through the floating cages and fixed net-pens.

Isolation and Maintenance of Bacterial Strains

Diseased hybrid striped bass were voluntarily submitted to the Louisiana Aquatic Diagnostic Laboratory (LADL), School of Veterinary Medicine, Louisiana State University, Baton Rouge,

TABLE 1.—Strains of *Photobacterium damselae* subsp. *piscicida* used in molecular, biochemical, and physical comparisons.

Strain	Donor[a]	Host	Geographic origin
1079A	D. Maragoudaki	Sea bass	Greece
EP 94-001	T. Aoki	Yellowtail	Japan
93-320	R. Ariav	Hybrid striped bass	Israel
29687	ATCC	Yellowtail	Japan
17911	ATCC	White perch	Maryland
90-744	LADL	Hybrid striped bass	Louisiana
91-197	LADL	Hybrid striped bass	Louisiana
93-173	LADL	Hybrid striped bass	Louisiana
94-069	LADL	Hybrid striped bass	Louisiana
95-390	LADL	Hybrid striped bass	Louisiana

[a] Investigators or labs providing strains for use in the study are as follows: Takashi Aoki, Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan; ATCC, American Type Culture Collection, Manassas, Virginia; Raanan Ariav, Kibbutz Ma'agan Michael, Israel; LADL, Louisiana Aquatic Diagnostic Laboratory, Department of Pathobiological Sciences, Louisiana State University, Baton Rouge; Dimitri Maragoudaki, Zoological Institute, University of Leuven, Leuven, Belgium.

from Louisiana mariculture farms. Bacterial cultures were isolated by streaking samples of liver, kidney, and spleen on brain-heart infusion agar (BHIA; Difco Laboratories, Detroit, Michigan) supplemented with 2% NaCl or tryptic soy agar (TSA) II blood agar (5% sheep blood; Baltimore Biological Laboratories, Cockeysville, Maryland) and incubating them at 28°C for 48 h. Bacterial strains from Louisiana, strains donated from other laboratories, and those purchased from the American Type Culture Collection were archived in an ultralow freezer at −70°C in 500-μL aliquots of brain-heart infusion broth (BHIB) supplemented with 2% NaCl and 20% glycerol. The hosts, geographic origins, and laboratory sources of 10 representative strains used for comparison in this study are listed in Table 1.

Phenotypic Analysis

Morphological, physiological, and biochemical tests were conducted according to methods outlined for halophilic organisms in the *Manual of Clinical Microbiology* (Murray et al. 1995). Additional biochemical testing was done at 28°C and 4 d incubation with the API 20E and 50CH systems (BioMérieux-Vitek, Inc., Hazelwood, Missouri). The enzyme activity of various strains was determined with the API-ZYM system (BioMérieux-Vitek) incubated for 5 h at 28°C. Inoculation, addition of reagents, and reading of results were done according to the manufacturer's protocol.

Identification by Aquarapid Pp Kit

Identification of Louisiana strains was also accomplished using the Aquarapid Pp test kit (Bionor A/C, Skein, Norway). Tests were conducted on spleen, liver, and kidney tissues from naturally infected fish, negative control tissues, and negative control tissues seeded with strain LA 91-197. The Aquarapid Pp consists of a plastic substrate (spoons) coated with specific polyclonal antibodies against *P. damselae* subsp. *piscicida* produced in sheep. These antibodies bind target bacteria present in homogenized fish tissue placed in the plastic spoons. The spoons are rinsed and an alkaline phosphatase antisheep conjugate added, followed by substrate. A pink color reaction is indicative of a positive result. A description of the use of Bionor kits for diagnosis of *P. damselae* subsp. *piscicida* has been given previously (Romalde et al. 1995a, 1995b). Tests were performed according to the manufacturer's instructions.

Antimicrobic Susceptibility

Antimicrobic susceptibility was determined by means of the Kirby−Bauer disk diffusion method (Barry and Thornsberry 1980) with commercially available impregnated paper discs (BBL Microbiology Systems, Cockeysville, Maryland). Methods for the standardized testing of aquatic pathogens (NCCLS 2003) were not yet been published at the time this work was done; however, an effort was made to adhere as closely as possible to the methods proposed by Alderman and Smith (2001). Vibriostatic agent 0/129 was dissolved in chloroform and added to sterile paper disc blanks (BBL Microbiology Systems) at 150 μg per disk. Lawn cultures of the strains to be tested were made from a 0.5 McFarland standardized inoculum on Mueller–Hinton agar (BBL Microbiology Systems)

plates with 1% NaCl and no more than five evenly spaced discs per plate. The following chemotherapeutic agents (μg/disc) were employed: ampicillin (10), chloramphenicol (30), kanamycin (30), erythromycin (15), oxytetracycline (30), nitrofurantoin (300), sulfadimethoxine–ormetoprim (25), amoxicillin–clavulinic acid (30), and the vibriostatic agent 0/129 (150). Plates were incubated for 48 h at 28°C in a normal atmosphere.

Molecular Analysis

Plasmid content.—Cultures of the representative isolates were started in 5 mL BHIB with 1% NaCl. The cultures were grown for 24 h at 28°C in 17-mm × 100-mm glass test tubes placed in a roller apparatus (CEL-GRO; Laboratory Line, Inc., Melrose Park, Illinois) and harvested by centrifugation. Plasmid DNA was isolated by a modification of the alkaline lysis method of Birnboim and Doly (1979) as outlined in Sambrook et al. (1989). DNA samples (10–20 μL) containing approximately 0.1 μg DNA/μL were mixed with 5 μL tracking dye and electrophoresed through 0.7% agarose gels in tris acetate–EDTA (TAE) buffer at 10 V for 18 h in a horizontal apparatus (Biorad Laboratories, Richmond, California). Gels were stained with 0.5 μg ethidium bromide per milliliter for 30 min and destained with distilled water for 2 h. A supercoiled ladder (2.067–16.21 kilo–base pairs [kb]; Gibco BRL, Gaithersburg, Maryland) was included as a molecular weight standard in all electrophoresis runs.

Detection of R plasmids.—Evidence of R plasmids was determined by conjugation and plasmid transfer between LADL 91-278, a resistant Gulf Coast strain of *P. damselae* subsp. *piscicida* and *Escherichia coli* nm 554, a strain that is Terramycin and Romet sensitive, contains no plasmid DNA, and lacks the gene for plasmid mobilization. Strains LADL 91-278 and *E. coli* nm 554 were cross-streaked on BHIA with 2% NaCl and cultured for 12 h at 25°C. Due to its slower growth rate, strain LADL 91-278 was streaked first and allowed to grow for 8 h at 25°C prior to cross-streaking with *E. coli*. The co-cultured cells were then restreaked on BHIA with 10 μg tetracycline per milliliter and incubated for 24 h at 37°C, a temperature that selects against the growth of *P. damselae* subsp. *piscicida*. Plasmid minipreps were made from the transconjugates and R plasmids visualized by agarose gel electrophoresis of plasmid DNA by means of methods outlined previously.

Chromosomal DNA extraction.—Starter cultures of *P. damselae* subsp. *piscicida* strains LADL 91-197 and ATCC 29687 were grown to log phase in 100 mL BHIB with 2% NaCl. Two-liter broth cultures were inoculated with 20 mL of the starter culture. A sample of the starter broth culture was streaked on a TSA II blood agar plate to check for purity. The cultures were grown for 16 h at 25°C and 200 revolutions per minute on a rotary shaker (New Brunswick Scientific, New Brunswick, Connecticut) and the cells harvested by centrifugation at 4,000 × gravity (*g*) for 30 min. DNA was extracted and purified by the chloroform–isoamyl alcohol method of Marmur and Doty (1961). The purity of each preparation was determined spectrophotometrically. A value of 1.8 for the ratio of optical density at 260 nm to optical density at 280 nm was used to demonstrate a lack of protein and RNA contamination.

Denaturation temperature and guanine–cytosine (G+C) ratio.—The midpoint denaturation temperature (T_m) of each DNA preparation was determined in a computerized Gilford Response II UV-VIS spectrophotometer with a thermocuvette, heating block, cooling water assembly, and printer (Gilford Systems, Inc., Oberlin, Ohio). Diluted DNA solutions were loaded into removable quartz microcuvettes and the instrument programmed for temperature increases of 1°C per minute. Printouts of the thermal denaturation profiles were produced and the molar percentage of guanine plus cytosine calculated by the equation of Norgard and Bartel (1978), namely,

$$\%G+C = 100 \times (T_m/50.2) - 0.990.$$

Randomly amplified polymorphic DNA (RAPD) analysis.—DNA was isolated from representative strains by means of a modified protocol of Ausubel (1994) or the InstaGene Matrix (BioRad). The modified method of Ausubel is as follows: Cultures of the various isolates were started in 4 mL BHIB with 1% NaCl. These cultures were grown for 24 h at 28°C in 17-mm × 100-mm glass test tubes placed in a roller apparatus (CEL-GRO) and harvested by centrifugation. The pellets were resuspended in 500 μL of tris–EDTA buffer (pH 8.0). Twenty-five μL of a 10% solution of sodium dodecyl sulfate and 0.6 μL of RNaseA (100 mg/mL) were added and incubated at 37°C for 2 h. Three μL of proteinase K (20 mg/mL) was added and incubated at 37°C for 2 h. The DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol, transferred to a phase lock gel tube (Eppendorf, Hamburg, Germany), and centrifuged at 10,000 × *g* for 10 min. The top

aqueous DNA layer was removed, precipitated with 1/10 volume of 3M NaAc and 2.5 volumes of absolute ethanol, and stored overnight at −20°C. After centrifugation at 10,000 × *g* for 15 min, the DNA pellet was resuspended in an appropriate amount of double-distilled water. The DNA solutions were stored at 4°C until they were used for polymerase chain reaction (PCR) reactions. Two μL (20 ng) of each DNA solution was used in the respective amplification reactions. The DNA analysis was carried out with Ready-To-Go RAPD analysis beads (Amersham Biosciences, Piscataway, New Jersey) according to the manufacturer's instructions. Briefly, a randomly designed 10-mer oligonucleotide set comprised of six different primers was available for use in different PCR reactions with DNA from the various reference strains. The oligonucleotide primer sequences have been described elsewhere (Romalde et al. 1999). The PCRs were carried out in a final volume of 25 μL using 1.5 units of *Taq* polymerase, 10 mM tris–HCl (pH 9.0), 50 mM KCl, 15 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 25 pmol of each primer and, as stated before, 2 μL (20 ng) of DNA solution.

When the InstaGene Matrix was used for DNA extraction, 1 μL of DNA solution was added to the reaction mixture. The PCR s were carried out in a GeneAmp 9700 (PE Applied Biosystems, Foster City, California). The GeneAmp 9700 was programmed for the following amplification cycle: start at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 35°C for 1 min, and elongation at 72°C for 2 min; this was carried out over 30 cycles. The reaction was held at 72°C for 5 min, followed by a reduction to 4°C indefinitely. Only the results from reactions with primer P4 (AAGAGCCCGT) are presented since this primer had previously demonstrated reproducible bands that can differentiate strains of *P. damselae* subsp. *piscicida* into two distinct groups (Magariños et al. 2000). One μL of DNA products containing approximately 0.7 μg DNA/μL resulting from the PCR amplification was mixed with 2 μL tracking dye and 10 μL double-distilled water and electrophoresed through 1.5% agarose gels in TAE buffer with 0.5 μg of ethidium bromide per milliliter at 50 V for 3 h in a horizontal apparatus (Biorad). A molecular weight standard, 100-bp ladder (New England Biolabs, Beverly, Massachusetts) was included in all the electrophoresis runs as a reference marker. Gels were photographed and digitally captured on a FluorChem 8800 (Alpha Innotech 1993) using Alpha Ease FC software.

Results

Isolation and Maintenance of the Etiologic Agent

From December 1990 through December 2001, photobacteriosis was diagnosed from 50 cases of diseased hybrid striped bass submitted to the Louisiana Aquatic Diagnostic Laboratory (LADL; Table 2). The bacterium was readily isolated from the spleen, liver, and kidney of moribund specimens on TSA II agar plates with 5% sheep blood. Isolated cultures of the bacteria were archived in the LADL collection at −70°C following confirmatory identification.

Disease Progression and Therapy

The mortality rates during the different outbreaks were variable, depending on the use of antibiotic feeds, water temperature, and water quality conditions. In some instances mortality was less than 5%, but in many individual culture units (cages or net-pens) it exceeded 90% over a 30-d period (R. Fernandez, Lafourche Mariculture, personal communication, 1994). The control of epizootics on fish farms was problematic because of the rapid onset of disease. The acute nature of the infection facilitated its spread through much of the fish population before a diagnosis could be made and medicated feeds administered. Medicated feeds were not particularly effective because infected fish became anorexic. Control of the disease was further complicated by the fact that no antibiotics have been approved for use with hybrid striped bass by the U.S. Food and Drug Administration. In cases where Terramycin- or Romet-medicated feeds were used, an emergency compassionate Investigational New Animal Drug (INAD) permit was granted by the U.S. Food and Drug Administration, Center for Veterinary Medicine. Amoxicillin-medicated feeds were administered in 1997 under an INAD issued to Gurvey and Berry Co., Inc., Toronto, Ontario. The feeding rates of the floating pellets were intended to achieve therapeutic doses of 80 mg/kg fish/d of Terramycin-medicated feed for 12 d, 50 mg/kg fish/d of Romet-medicated feed for 5 d, and 110 mg/kg fish/d of amoxicillin-trihydrate-medicated feed for 12 d. Effective therapy was hindered by the emergence of Romet-, Terramycin-, or amoxicillin-resistant strains of the pathogen in 1991, 1994, and 1997 following the use of the medicated feeds (Table 2).

Moribund fish collected during disease episodes typically swam listlessly near the surface and ex-

TABLE 2.—Cases submitted to the Louisiana Aquatic Diagnostic Laboratory from which *Photobacterium damselae* subsp. *piscicida* was isolated, 1990–2000.

Case	Farm[a]	Date	Water temperature (°C)	Salinity (‰)	Size (cm)	Culture type	Antibiotic susceptibility[b]
90-744	A	Dec 20, 1990	20	15	30	Cage	S
91-197	B	Mar 28, 1991	24	08	22	Net-pen	S
91-198	B	Mar 28, 1991	24	08	22	Net-pen	S
91-216	B*	Apr 12, 1991	24	10	18	Net-pen	S
91-217	B	Apr 12, 1991	24	10	18	Net-pen	S
91-218	B	Apr 12, 1991	24	10	18	Net-pen	S
91-245	A*	Apr 19, 1991	25	13	30	Cage	S
91-276	B	Apr 23, 1991	22	06	22	Net-pen	R
91-278	B	Apr 23, 1991	22	06	25	Net-pen	R
91-289	A	Apr 30, 1991	23	06	25	Cage	R
91-317	A	May 8, 1991	25	08	25	Cage	R
91-318	A	May 8, 1991	25	08	25	Cage	R
91-368	A	May 31, 1991	26	03	28	Cage	R
92-196	C	Jun 5, 1992	28	[c]	25	Cage	S
92-207	D	Jun 9, 1992	28	[c]	25	Pond	S
93-173	C	Jun 4, 1993	28	06	25	Cage	S
94-056	B	Mar 31, 1994	18	07	25	Net-pen	S
94-069	B	Apr 8, 1994	20	07	35	Net-pen	S
94-070	B	Apr 8, 1994	20	07	15	Net-pen	S
94-084	B*	Apr 13, 1994	25	10	30	Net-pen	S
94-085	B	Apr 13, 1994	25	10	30	Net-pen	S
94-086	B	Apr 13, 1994	25	10	20	Net-pen	S
94-088	B	Apr 13, 1994	24	10	30	Net-pen	S
94-108	B	May 4, 1994	26	10	10	Raceway	S
94-109	B	May 4, 1994	26	10	10	Net-pen	R
94-110	B	May 4, 1994	26	10	10	Net-pen	R
94-118	B	May 5, 1994	26	10	10	Raceway	S
94-130	B	May 5, 1994	25	10	15	Net-pen	[c]
94-165	B	Jun 2, 1994	27	05	15	Net-pen	R
94-425	B	Dec 2, 1994	18	15	20	Net-pen	S
95-390	B	Nov 8, 1995	17	15	20	Net-pen	S
95-393	B	Nov 10, 1995	17	17	25	Net-pen	S
96-330	B	Dec 3, 1996	14	17	13	Net-pen	S
96-331	B	Dec 3, 1996	14	17	15	Net-pen	S
97-099	B	Mar 4, 1997	23	10	13	Net-pen	S
97-132	B*	Mar 21, 1997	20	10	14	Net-pen	S
97-135	B	Mar 25, 1997	22	10	14	Net-pen	S
97-146	B	Apr 3, 1997	19	10	15	Net-pen	S
97-147	B	Apr 3, 1997	19	10	15	Net-pen	S
97-163	B	Apr 18, 1997	20	10	12	Net-pen	S
97-164	B	Apr 18, 1997	20	10	15	Net-pen	S
97-202	B	May 6, 1997	26	10	16	Net-pen	S
97-235	B	Jun 4, 1997	29	10	20	Net-pen	R
97-236	B	Jun 4, 1997	29	10	20	Net-pen	R
98-251	B	Nov 20, 1998	22	13	30	Net-pen	S
98-262	B	Dec 3, 1998	20	13	30	Net-pen	S
99-053	B	Feb 23, 1999	17	18	15	Net-pen	S
99-103	B	Mar 22, 1999	20	18	15	Net-pen	S
99-523	B	Nov 19, 1999	19	18	20	Net-pen	S
00-074	B	Mar 4, 2000	24	21	13	Net-pen	S

[a] Asterisks indicate farms on which oxytetracycline- and Romet-medicated feeds were administered in 1991 and 1994 and amoxicillin-medicated feeds in 1997.

[b] Antimicrobial susceptibility to Terramycin and Romet as determined by disc diffusion on Mueller–Hinton medium with 2% NaCl; S = susceptible, R = resistant.

[c] No data reported with case.

hibited no obvious external clinical signs. A few individuals exhibited diffuse hemorrhages in the operculum. Internally, diseased fish had enlarged, friable spleens that occasionally exhibited small white spots in the parenchyma. No other obvious internal clinical signs were noted. Histopathological lesions in the internal organs were consistent with those described previously (Hawke et al.

1987). The white spots in the spleen, typically seen in cases of ''pseudotuberculosis'' in yellowtail, were only observed in advanced cases of the disease when antibiotic therapy had slowed the course of infection.

The yearly outbreaks of photobacteriosis typically occurred in the spring, although fall outbreaks were seen in 1990, 1995, 1998, and 1999. The affected fish ranged from 10 to 35 cm in length, and high mortality rates were noted in all size-classes. Mortalities were observed at water temperatures between 14°C and 29°C and salinities between 3‰ and 21‰ (Table 2). Cumulative mortality was greater than 50% of cultured stocks in 1991, 1994, 1995, and 1997 on affected farms and included many valuable, food-size fish (Fernandez, personal communication, 2002).

Phenotypic and Biochemical Characteristics of Louisiana Isolates

Gram stains of representative isolates of *P. damselae* subsp. *piscicida* from kidney, spleen, and blood revealed gram-negative rods that exhibited bipolar staining and pleomorphism. Streaks of samples of kidney, liver, and spleen on 5% sheep blood TSA II agar produced pure cultures of a single bacterial colony type after 48 h of incubation. The colonies were 2–3 mm in diameter, smooth, raised, entire, translucent, slightly viscid, and nonhemolytic.

Preliminary tests showed the bacterial isolates to be nonmotile, gram-negative rods 0.5–0.8 μm in width and 0.8–2.5 μm in length that were catalase positive, oxidase positive, fermentative with no gas produced in glucose motility deeps, and having an alkaline/acid reaction with negative H_2S production in triple sugar iron (TSI) slants. Bacterial cells were plump and rod shaped in early log phase growth and tended to become smaller and more coccoid in older cultures. The bacteria were sensitive to the vibriostatic agent 0/129 and required 0.5% salt in the medium for growth. All isolates produced a code number of 2005004 in the API 20E system. The results of the phenotypic analysis of representative strains are included in Table 3. No variation in phenotype among strains from different geographic locations was detected, although some strains were delayed or negative for maltose fermentation.

Comparison of Enzyme Activities

The results of enzyme activities (as determined in the API-ZYM system) are reported in Table 4. All strains tested gave similar results, with the exception of the test for alkaline phosphatase. The Louisiana and Japanese strains were uniformly negative in this test, although a slight color intensity score of 2 was recorded. The strains from Greece, Israel, and Maryland were strongly positive in the alkaline phosphatase reaction (with an intensity score of 4) and the acid phosphatase reaction (with an intensity score of 5). The reaction intensity scores were uniformly equal to 3 for all strains tested for esterase, esterase–lipase, and leucine arylamidase. All strains gave a result of 0 in tests for lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase.

Plasmid Content

Native plasmids.—The approximate size of plasmids (kb) was determined from multiple electrophoresis runs and comparison with the supercoiled DNA ladder. The ladder could accurately measure the size of smaller plasmids (<30 kb), though the size of larger plasmids could only be estimated. The Louisiana strains of *P. damselae* subsp. *piscicida* were found to possess four identical plasmid bands, with the exception of strain 90-744, which was lacking all of the plasmids except one (Figure 1). The Louisiana strains typically had two large plasmid bands that were above the 30-kb level and two smaller bands that were determined to be 8.0 and 5.0 kb in size. Strain 90-744 lacked all but the 5.0-kb plasmid. No strains from the other geographic locations examined in this study or reported in the literature possess the same plasmid profile. The strains from Japan, EP 94-001 and ATCC 29687, possessed bands sized at 3.7 and 5.0 kb. This is similar to those reported for other pathogenic Japanese strains (3.5 and 5.1 kb; Zhao and Aoki 1992). Strain ATCC 29687 possessed one high-molecular-weight plasmid, and strain EP 94-001 possessed two high-molecular-weight plasmids above the 30-kb level. Strain EP 94-001 possessed two faint plasmid bands at 8.5 and 7.5 kb in addition to the smaller plasmids (Figure 2). Strain ATCC 29687 exhibited very faint bands that were sized at 9.5 and 6.0 kb. The strains from Israel and Greece were almost identical, exhibiting bands of 8.5 and 9.5 kb. Strain ATCC 17911 did not exhibit any plasmid DNA, which is consistent with the findings of Zhao and Aoki (1992). Thus, plasmid profile is a characteristic that can be used to differentiate Louisiana isolates from those from other locations.

TABLE 3.—Phenotypic characteristics of representative strains of *Photobacterium damselae* subsp. *piscicida*. Symbols are as follows: +, positive reaction; −, negative reaction; (+), weak positive reaction; V, variable reaction; O, oxidative reaction; and F, fermentative reaction.

Test	Strain			
	Louisiana	(Japan) EP 001	(Greece) 1079A	(Israel) 93-320
Gram stain	−	−	−	−
Motility	−	−	−	−
Oxidase	+	+	+	+
Catalase	+	+	+	+
Voges-Proskauer	+	+	+	+
Indole production	−	−	−	−
Nitrate reduction	−	−	−	−
Citrate utilization	−	−	−	−
H_2S production	−	−	−	−
O/F glucose	O/F	O/F	O/F	O/F
Gas from glucose	−	−	−	−
Growth at temperature				
5°C	−	−	−	−
15°C	+	+	+	+
25°C	+	+	+	+
30°C	+	+	+	+
37°C	−	−	−	−
Growth at salinity				
0% NaCl	−	−	−	−
3% NaCl	+	+	+	+
5% NaCl	−	−	−	−
Arginine dihydrolase	+	+	+	+
Lysine decarboxylase	−	−	−	−
Ornithine decarboxylase	−	−	−	−
Tryptophan deaminase	−	−	−	−
β-galactosidase (ONPG)[a]	−	−	−	−
Urease	−	−	−	−
Gelatinase	−	−	−	−
Hemolysis (5% sheep blood)	−	−	−	−
Acid production from				
Glucose	+	+	+	+
Mannose	+	+	+	+
Galactose	+	+	+	+
Fructose	+	+	+	+
Ribose	+	+	+	+
N-Acetyl glucosamine	+	+	+	+
Maltose	V	(+)	(+)	(+)
Glycerol	(+)	(+)	(+)	(+)
Erythritol	−	−	−	−
Arabinose	−	−	−	−
Xylose	−	−	−	−
Adonitol	−	−	−	−
β-Methyl-xyloside	−	−	−	−
Sorbose	−	−	−	−
Rhamnose	−	−	−	−
Dulcitol	−	−	−	−
Inositol	−	−	−	−
Mannitol	−	−	−	−
Sorbitol	−	−	−	−
α-Methyl-D-mannoside	−	−	−	−
α-Methyl-D-glucoside	−	−	−	−
Amygdalin	−	−	−	−
Arbutin	−	−	−	−
Esculin	−	−	−	−
Salicin	−	−	−	−
Cellobiose	−	−	−	−
Lactose	−	−	−	−
Melibiose	−	−	−	−
Saccharose	−	−	−	−
Trehalose	−	−	−	−
Inulin	−	−	−	−

TABLE 3.—Continued.

Test	Strain			
	Louisiana	(Japan) EP 001	(Greece) 1079A	(Israel) 93-320
Melitzitose	–	–	–	–
Raffinose	–	–	–	–
Amidon	–	–	–	–
Glycogen	–	–	–	–
Xylitol	–	–	–	–
β-Gentiobiose	–	–	–	–
Turanose	–	–	–	–
Lyxose	–	–	–	–
Tagatose	–	–	–	–
Fucose	–	–	–	–
Arabitol	–	–	–	–
Gluconate	–	–	–	–
2 or 5 ceto-gluconate	–	–	–	–

[a] [Author: Please spell out ONPG.]

R-plasmids.—Transconjugates of *P. damselae* subsp. *piscicida* LADL 91-278 and *E. coli* nm 554 were selected after cross-streaking on BHIA plates. Only tetracycline-resistant colonies of *E. coli* were recovered on the medium following incubation. Plasmid minipreps of the resulting tetracycline-resistant colonies of *E. coli* revealed the acquisition of a high-molecular-weight plasmid (data not shown). Since the strain of *E. coli* used in this study did not possess the gene for plasmid mobilization, the process was presumed to be initiated by a gene present on the R plasmid in *P. damselae* subsp. *piscicida*.

RAPD Analysis

Of the six primers included in the RAPD analysis kit, only primer 4 has been shown to separate *P. damselae* subsp. *piscicida* into two distinct clonal groups. Fingerprint patterns of PCR products from the Louisiana strains place them in clonal group 2, which has a pattern similar to that of strains from Japan (Figure 3). The DNA fingerprints of the strains from Greece, Israel, and the Chesapeake Bay were placed in clonal group 1, which is consistent with the previous report of Magariños et al. (2000).

Antimicrobic Susceptibility

The results of the antimicrobic susceptibility evaluations are shown in Table 5. Three of the strains tested, 91-197 (Louisiana), 93-320 (Israel), and 1079A (Greece), showed uniform susceptibility to the antimicrobial agents used. The other

TABLE 4.—Enzyme activities displayed by live cell suspensions of representative strains of *Photobacterium damselae* subsp. *piscicida*.

Strain[a]	Enzyme activity[b]						
	2	3	4	6	11	12	18
1079A (Greece)	4	3	3	3	5	1	3
93-320 (Israel)	4	3	3	3	5	1	3
ATCC 17911	4	3	3	3	5	1	4
ATCC 29687	2	3	3	3	4	1	4
EP 94-001 (Japan)	2	3	3	3	4	1	4
90-744 (LADL)	2	3	3	3	4	1	4
91-197 (LADL)	2	3	3	3	4	1	4
93-173 (LADL)	2	3	3	3	4	1	4
94-069 (LADL)	2	3	3	3	4	1	4
95-390 (LADL)	2	3	3	3	4	1	4

[a] Abbreviations are as follows: ATCC, American Type Culture Collection; LADL, Louisiana Aquatic Diagnostic Laboratory.

[b] Numbers match the API code, as follows: 2, alkaline phosphatase; 3, esterase; 4, esterase–lipase; 6, leucine arylamidase; 11, acid phosphatase; 12, napthol-AS-BI-phosphohydrolase; and 18, *N*-acetyl-β-glucosaminidase. Reactions are scored from 0 to 5 based on color intensity following exposure to bright fluorescent light for 30 min after the addition of the reagents. Reaction intensities of 3, 4, and 5 are considered positive; reaction intensities of 0, 1, and 2 are considered negative.

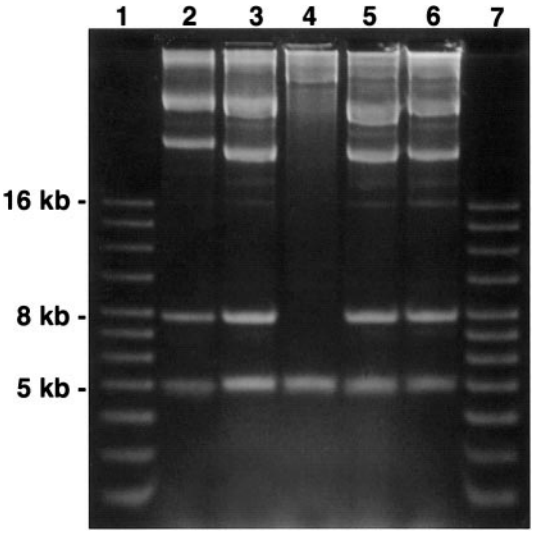

FIGURE 1.—Agarose gel electrophoresis of plasmid DNA minipreps showing the profiles of Louisiana strains. Lanes are as follows: (1) the supercoiled DNA ladder, (2) LADL 94-069, (3) LADL 95-390, (4) LADL 90-744, (5) LADL 91-197, (6) LADL 93-173, and (7) the supercoiled DNA ladder.

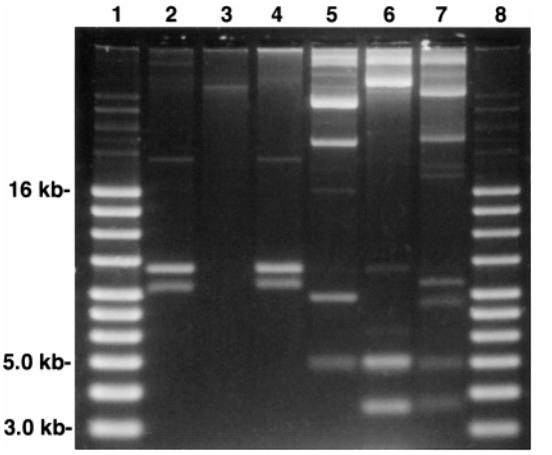

FIGURE 2.—Agarose gel electrophoresis of plasmid DNA minipreps showing the profiles of representative strains. Lanes are as follows: (1) the supercoiled DNA ladder, (2) LADL 93-320 (Israel), (3) ATCC 17911 (Maryland), (4) 1079A (Greece), (5) LADL 91-197 (Louisiana), (6) ATCC 29687 (Japan), (7) EP 94-001 (Japan), and (8) the supercoiled DNA ladder.

two strains, EP 94-001 (Japan) and 91-278 (Louisiana), exhibited varying resistance patterns. Strain 91-278 was resistant to Romet (SOR25) and oxytetracycline, and strain EP 94-001 was resistant to oxytetracycline, erythromycin, kanamycin, and chloramphenicol.

Identification by Aquarapid Pp

Louisiana strains were detected in fish tissue producing a positive reaction with the Aquarapid Pp test kit. The kit was able to detect the pathogen in both naturally infected fish from Louisiana fish farms and spleen tissue from specific-pathogen-free hybrid striped bass seeded with strain LADL 91-197. This indicates that the Louisiana strains share some surface antigens with other strains that the kit was designed to detect, so that use of the kit is acceptable for diagnosis of photobacteriosis in the United States.

G+C Ratio

Melting curves were plotted for a representative Louisiana strain (91-197) and ATCC 29687. The melting temperature or midpoint of the thermal denaturation (T_m) was determined graphically on the Gilford Response II UV-VIS spectrophotometer to be 72°C for each strain. The DNA base composition, expressed as %(G + C), was calculated according to the formula of Norgard and

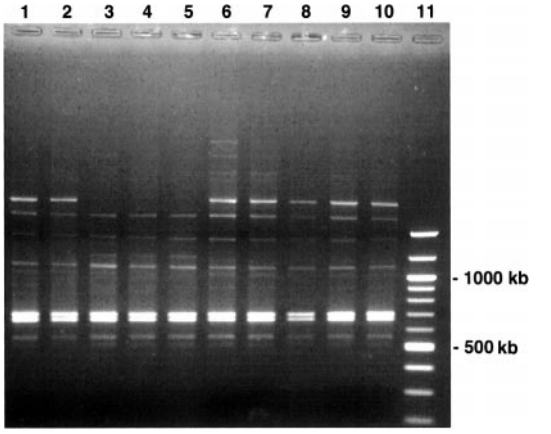

FIGURE 3.—Randomly amplified polymorphic DNA (RAPD) profiles of representative strains of *Photobacterium damselae* subsp. *piscicida* from Louisiana, Israel, Greece, and Japan as well as strains ATCC 17911 from Maryland and ATCC 29687 from Japan that were generated from RAPD primer 4. Lanes are as follows: (1) LADL 90-744 (Louisiana), (2) LADL 91-197 (Louisiana), (3) ATCC 17911, (4) 1079A (Greece), (5) LADL 93-320 (Israel), (6) EP 94-001 (Japan), (7) ATCC 29687, (8) LADL 93-173 (Louisiana), (9) LADL 94-069 (Louisiana), (10) LADL 95-390 (Louisiana), and (11) the 100-bp ladder.

TABLE 5.—Antimicrobic susceptibility of representative strains of *Photobacterium damselae* subsp. *piscicida*. Values are zone diameters (mm); asterisks denote resistant strains.

Strain[a]	Antimicrobial agent[b]							
	AM10	AMC30	C30	SOR25	T30	E15	K30	0/129
91-197	40	40	34	30	42	30	20	26
91-278	36	30	27	10*	10*	20	15	24
93-320	34	34	30	30	36	20	15	30
EP 94-001	36	38	12*	26	10*	0*	0*	25
1079A	30	30	29	30	32	20	17	30

[a] Strains 91-197 and 91-278 were from Louisiana, strain EP 94-001 from Japan, strain 93-320 from Israel, and strain 1079A from Greece.

[b] Abbreviations are as follows: AM10, ampicillin (10 μg); AMC30, amoxicillin–clavulinic acid (30 μg); C30, chloramphenicol (30 μg); SOR25, sulfadimethoxine–ormetoprim (25 μg); T30, oxytetracycline (30 μg); E15, erythromycin (15 μg), K30, kanamycin (30 μg); and 0/129, vibriostatic agent (150 μg).

Bartel (1978) to be 44.40. This is consistent with the published range for the genus *Photobacterium*.

Discussion

This report is the first to document the occurrence of photobacteriosis in a commercially cultured fish species in the United States and the first to characterize strains from the U.S. Gulf Coast using molecular methods. The negative impact of photobacteriosis on the Louisiana hybrid striped bass mariculture industry is significant and has contributed to the failure of this potentially lucrative industry. Four farms on the Louisiana Gulf Coast were involved in hybrid striped bass mariculture in 1990, and only one remains in operation today. Hybrid striped bass are currently not being produced at this farm due to the occurrence of photobacteriosis; the red drum *Sciaenops ocellatus*, which is apparently resistant to the disease, is being grown in its place. The farm's owners would prefer to produce the hybrid striped bass due to its higher market value but are hesitant to return to culturing this species until improved drugs or vaccines are available for the control of photobacteriosis.

Strains of *P. damselae* subsp. *piscicida* from various geographic locations have been shown to be almost identical by means of conventional phenotypic and serologic techniques. Several different investigators have come to the conclusion that this is a highly homogeneous bacterial species (Magariños et al. 1996; Austin and Austin 1993), although more sophisticated molecular methods have allowed the discrimination of isolates from different locations. The results of this study comparing Louisiana isolates with strains from Greece, Japan, Israel, and the Chesapeake Bay corroborate these findings in the areas of biochemical phenotype and enzyme activities. Louisiana strains were detected by means of the Aquarapid Pp, which indicates some antigenic homogeneity. The overall homogeneity within the species makes *P. damselae* subsp. *piscicida* an excellent candidate for vaccine development.

By means of RAPD analysis, the Louisiana strains were assigned to clonal group 2, which includes the Japanese isolates. Of interest is the fact that the Chesapeake Bay isolate, ATCC 17911, belongs to clonal group 1, which includes the European isolates. There was good correlation between the banding patterns obtained in this study and those of Magariños et al. (2000). Slight variability in the intensity of bands was noted on mutiple PCR runs, but generally the results were very consistent regardless of the method used for DNA extraction. With DNA obtained by the method of Ausubel (1994) the exact amount of DNA in the reaction mixture was known, whereas with the InstaGene Matrix the same volume was used in each reaction but the exact amount of DNA was not quantified.

Variability among strains from different geographic locations was detected in the native plasmid profile and antimicrobic susceptibility. The location of two large bands (>30 kb) and two smaller bands (corresponding to 8 and 5 kb) are unique to Louisiana strains. The variability in plasmids seen in strain 90-744 is most likely the result of multiple passages on agar media before it was archived and the curing of the plasmids. This was the first strain isolated from Louisiana, and it was subjected to numerous passages and testing prior to being archived. This may also explain the lack of plasmids in ATCC 17911. The difference in the size of one of the large plasmids in strain LADL 94-069 was a consistent finding and may indicate

some variation within the Louisiana isolates. The resistance to Romet and Terramycin by certain Louisiana strains was the result of feeding medicated feeds and the subsequent acquisition of an R plasmid. Antibiotic resistance typically occurred on fish farms 2–3 weeks after the administration of medicated feeds; the exception was 1997, when resistance appeared approximately 6 weeks after the administration of such feeds. Interestingly, resistance was a transient event and did not persist from one year to the next.

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