Vol. 57: 51–58, 2003 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published December 3

Construction of a safe, stable, efficacious vaccine against *Photobacterium damselae* ssp. *piscicida*

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ABSTRACT: Vaccination with bacterial auxotrophs, particularly those with an interruption in the common pathway of aromatic amino-acid biosynthesis, known as the shikimate pathway, has been shown to be effective in the prevention of a variety of bacterial diseases. In order to evaluate this approach to vaccine development in the important marine pathogen *Photobacterium damselae* subsp. *piscicida*, the *aroA* gene of the shikimate pathway was identified from a *P. damselae* subsp. *piscicida* genomic library by complementation in an *aroA* mutant of *Escherichia coli*. The complementing plasmid was isolated and the nucleotide sequence of the *P. damselae* subsp. *piscicida* genomic insert was determined. Subsequent analysis of the DNA-sequence data demonstrated that the identified plasmid contained 3464 bp of *P. damselae* subsp. *piscicida* DNA, including the complete *aroA* gene. The sequence data was used to delete a 144 bp *MscI* fragment, and the kanamycin resistance gene (*kan*) from transposon Tn*903* was ligated into the *MscI* site. This Δ*aroA*::*kan* construct was sub-cloned into a suicide plasmid and transferred to a wild-type *P. damselae* subsp. *piscicida* by conjugation and allelic exchange. One selected mutant, LSU-P2, was confirmed phenotypically to require supplementation with aromatic metabolites for growth in minimal media, and was confirmed genotypically by PCR and DNA sequencing. Further, LSU-P2 was demonstrated to be avirulent in hybrid striped bass and to provide significant protection against disease following challenge with the wild-type strain.

KEY WORDS: *Photobacterium damselae* subsp. *piscicida* · Vaccination · Fish · Immunization · Hybrid striped bass · Live attenuated vaccine

INTRODUCTION

Coastal marine aquaculture in warm water has grown substantially in the past 10 to 15 yr. Concomitant with growth of the industry in coastal areas, however, has been the emergence of the bacterial disease agent *Photobacterium damselae* ssp. *piscicida*, previously known as *Pasteurella piscicida*, which has seriously impacted and restricted the expansion of commercial aquaculture in warm-water coastal areas. Although relatively unknown outside of Japan prior to 1990, where photobacteriosis caused dollar losses in excess of $20 million annually in cultured yellowtail (Sano & Fukuda 1986), the recent evolution of coastal aquaculture in the USA and the Mediterranean region has created ideal conditions for this highly pathogenic, halophilic organism to proliferate. In Louisiana from 1990 to 1995, 32 cases with heavy mortalities were reported in coastal hybrid striped-bass farms (Louisiana Aquatic Animal Diagnostic Lab [LAADL] case records), with 4 farms closing as a direct result of *P. damselae* ssp. *piscicida* losses. In Israel, 4 of the 5 farms on the Mediterranean failed because of losses to *P. damselae* ssp. *piscicida*, and losses of hybrid striped bass at the only surviving site exceeded $1 million (Nitzan 2001). It is also important to note that gilthead seabream *Sparus aurata* and seabass *Dicentrarchus labrax*, species that are being cultured in Israel, Europe and the Mediterranean, are also highly susceptible to *P. damselae* ssp. *piscicida* infection. Production of hybrid striped bass, seabream

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and seabass throughout the Mediterranean region is estimated to be in the in the region of tens of thousands of tons, and *P. damselae* ssp. *piscicida* has become a serious problem region-wide for all of these species (Baudin-Laurencin et al. 1991, Ceschia et al. 1991, Toranzo et al. 1991).

Previous attempts at vaccination against *Photobacterium damselae* ssp. *piscicida* yielded equivocal results (Romalde & Magarinos 1997), with the most promising reports involving the use of a live attenuated strain (Kusuda & Hamaguchi 1988) and a toxoid-enriched formalin-killed bacterin (Morinigo et al. 2002). Evidence in our laboratory demonstrating that *P. damselae* ssp. *piscicida* is capable of invasion and intracellular growth in phagocytic cells and fish cell lines (Elkamel & Thune 2003, Elkamel et al. 2003) indicates that a strong cell-mediated immune (CMI) response will be important in establishing protection (Kaufmann 1999). Because virulent strains of *P. damselae* ssp. *piscicida* are invasive by both oral and immersion routes of exposure, a live attenuated strain of *P. damselae* ssp. *piscicida* that retains its ability to invade the host and temporarily persist in the tissues should stimulate an effective CMI response, and provide protection against subsequent challenge with the wild-type strain. Other bacterial fish pathogens with an interruption in the common pathway of aromatic amino-acid biosynthesis, known as the shikimate pathway, are attenuated and confer varying degrees of protection against subsequent challenge by virulent pathogens (Vaughan et al. 1993, Thune et al. 1999). Bacterial pathogens with stable defects in the *aro* genes of the shikimate pathway require supplementation with aromatic metabolites for growth; because the metabolites required are essentially unavailable in vertebrate tissues, they are incapable of sustained growth in those tissues and are, thus, nutritionally attenuated. Consequently, the objective of this study was to produce and evaluate an *aroA* mutant of *P. damselae* ssp. *piscicida* as a live attenuated vaccine.

MATERIALS AND METHODS

Experimental fish. Hybrid striped-bass fingerlings used in this study were obtained from freshwater earthen ponds to ensure no prior exposure to *Photobacterium damselae* ssp. *piscicida*, and were reared in a specific pathogen-free (SPF) laboratory at the Louisiana State University School of Veterinary Medicine (LSU-SVM). The SPF fish were fed a maintenance diet and held at a salinity of 1 to 3 ppt and a temperature of 28°C.

Bacteria, vectors, and media. Bacterial strains, plasmids, and their sources are listed in Table 1. *Escherichia coli* were grown at 37°C with Luria-

Table 1. Bacterial strains, plasmids and their sources used in the study

Bacterial strain or plasmid	Description	Source
Bacteria		
Escherichia coli RT2829	*E. coli* K-12 *aroA354 recA supE44 glnV42*	Thune et al. (1999)
Photobacterium damselae ssp. *piscicida* 91-197	Wild-type *P. damselae* ssp. *piscicida* isolated from a moribund hybrid striped bass	Louisiana State University Aquatic Diagnostic Lab
LSU-P2	As 91-197 except Δ*aroA::kan* (*kan*: kanamycin resistance gene)	This study
Plasmid		
pBK-CMV	Plasmid cloning vector (Km^r)	Stratagene
pPD23	pBK-CMV derivative with 3464 bp fragment of the *Photobacterium damselae* ssp. *piscicida* chromosome containing the *aroA* gene	This study
pBluescript	Cloning vector (Ap^r)	Stratagene
pPD24	pBluescript derivative with 3464 bp fragment of the *Photobacterium damselae* ssp. *piscicida* chromosome containing the *aroA* gene	This study
pPD25	Ap^r, Km^r, pPD24 derivative with 144 bp MscI deletion in the *aroA* gene and 1.7 kb *Bam*HI fragment of transpososn Tn*903* Km^r cassette inserted in the MscI deletion site	This study
pGP704	Ap^r, with R6K *ori*, *mob* RP4, polylinker from M13 tg131	Miller & Mekalanos (1988)
pPD26	Ap^r, Km^r, pGP704 derivative containing the Δ*aroA::kan Photobacterium damselae* ssp. *piscicida* gene from pPD25 inserted in *Eco*RI site	This study

Bertani (LB) broth on agar plates (Sambrook et al. 1989). *Photobacterium damselae* ssp. *piscicida* were grown at 25 to 28°C with brain-heart infusion (BHI) broth or agar plates, with 1 to 2% added NaCl. Lambda Zap Express bacteriophage (Stratagene) were grown in *E. coli* XL1-Blue MRF′ (Stratagene) with LB agar plates and NZYM top agarose (Sambrook et al. 1989). *E. coli* minimal media (Atlas 1993), with and without aromatic supplementation, was used to grow and evaluate complementation of *E. coli* Strain RT2829. Eagle's minimal essential media, made according to the recipe provided by Sigma Chemicals but without glutamine, aromatic amino acids, or indicators, was used to evaluate auxotrophy of the *P. damselae* ssp. *piscicida* mutants. Aromatic supplementation of minimal media consisted of 220 µM tyrosine, 200 µM tryptophan, 240 µM phenylalanine, 65 µM 2,3-dihydroxybenzoate, 100 µM hydroxybenzoate, and 70 µM *p*-aminobenzoic acid. Bacterial cells resulting from conjugations between *P. damselae* and *E. coli* were grown at 28°C on LB plates with 1% added NaCl.

The F′ episome in *Escherichia coli* XL1-Blue MRF′ was maintained with tetracycline selection at 12.5 µg ml^{-1}. Ampicillin (Ap) at 200 µg ml^{-1} was used to maintain pBluescript SK– (Stratagene), pGP704, and their derivatives. Tetracycline (Tc) at 20 µg ml^{-1} was used to maintain and select for Tn10 insertions. Kanamycin (Km) at 50 µg ml^{-1} was used to maintain plasmids derived from the pBK-CMV phagemid, plasmids carrying the kanamycin resistance cassette from Tn*903* (*kan*), and the *Photobacterium damselae* ssp. *piscicida* insertion/deletion mutant, LSU-P2. Colistin (Col) at 10 µg ml^{-1} was used for counterselection against *E. coli* SM10 *pir* following conjugations. For blue-white screening of DNA cloned into pBluescript, *E. coli* XL1-Blue MRF′ was spread on LB plates that had been previously spread with 100 µl of 100 mM isopropylthio-β-D-galactoside (IPTG) and 40 µl of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and allowed to dry, similar to the procedure of Sambrook et al. (1989).

Isolation of the *aroA* gene. Genomic DNA was isolated from *Photobacterium damselae* ssp. *piscicida* as described by Ausubel et al. (1994). The DNA was partially digested with *Sau3*AI restriction endonuclease (New England Biolabs) for 20 min to optimize the yield of fragments averaging 6 kb in length. Fragments were ligated to *Bam*HI-digested (New England Biolabs) Lambda ZAP™ Express (Stratagene) with T4 DNA Ligase (New England Biolabs), packaged using Gigapack Gold Packaging Extract (Stratagene), and amplifed once prior to storage at –70°C. Following excision of the library in XL1-Blue MRF′, the recovered plasmids were used to transform *Escherichia coli* RT2829, and then plated on minimal media with Km to select for plasmids carrying the *aroA* gene by complementation. Plasmid pPD23, containing a 3.6 kb insert of *P. damselae* ssp. *piscicida* DNA, was selected for sequence analysis.

Sequencing and sequence analysis. Both strands of the *Photobacterium damselae* ssp. *piscicida* DNA carried in the plasmid pPD23 were sequenced. Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen) and PCR DNA was purified with a Qiaquick PCR purification kit (Qiagen). Sequencing reactions were performed as per the manufacturer's recommendation using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems) and samples were analyzed by capillary electrophoresis using the ABI 310 automated sequencer (PE Applied Biosystems). Generated sequences were assembled, analyzed, and aligned with other sequences using the Wisconsin Sequence Analysis Package Version 8.0 (Genetics Computer Group) and PC/Gene (Intelligenetics).

Vaccine strain construction. Using the generated sequence data, a deletion/insertion mutation of the *Photobacterium damselae* ssp. *piscicida aroA* gene carried on pPD23 was constructed as depicted in Fig. 1. Briefly, the inserted DNA carrying the *aroA* gene was removed from pPD23 by digestion with *Spe* I and *Not* I and ligated into the *Spe* I and *Not* I sites of pBluescript, creating pPD24. This step was necessary because pBK-CMV, the plasmid obtained from the Lambda ZAP excision, contains a gene encoding Km resistance, the same antibiotic resistance encoded on the marker used to insertionally mutate the *aroA* gene. Plasmid pPD24 was then digested with *Msc*I, which deleted a 144 bp fragment of the *aroA* gene. The kanamycin resistance gene from the transposon Tn*903* (*kan*) (GenBank #V00359) was ligated into the *MscI* site. This construct was sub-cloned into the π-protein based suicide plasmid pGP704, transferred to a wild-type *P. damselae* ssp. *piscicida* by conjugation, and incorporated into the chromosome by allelic exchange. Putative mutants were selected on media containing Km and Col, and vector loss was confirmed by establishing sensitivity to Ap. One mutant, LSU-P2, was selected for confirmation of aromatic auxotrophy and its Δ*aroA*::*kan* construction.

Phenotypic and genotypic confirmation of LSU-P2. The *Photobacterium damselae* ssp. *piscicida* Δ*aroA*::*kan* construction of LSU-P2 was confirmed phenotypically by culturing both *P. damselae* ssp. *piscicida* 91-197 and LSU-P2 in minimal media with 2% NaCl, with and without supplementation of aromatic metabolites. LSU-P2 failed to grow without supplementation, while *P. damselae* ssp. *piscicida* 91-197 was able to grow in either medium, confirming an auxotrophic phenotype for LSU-P2.

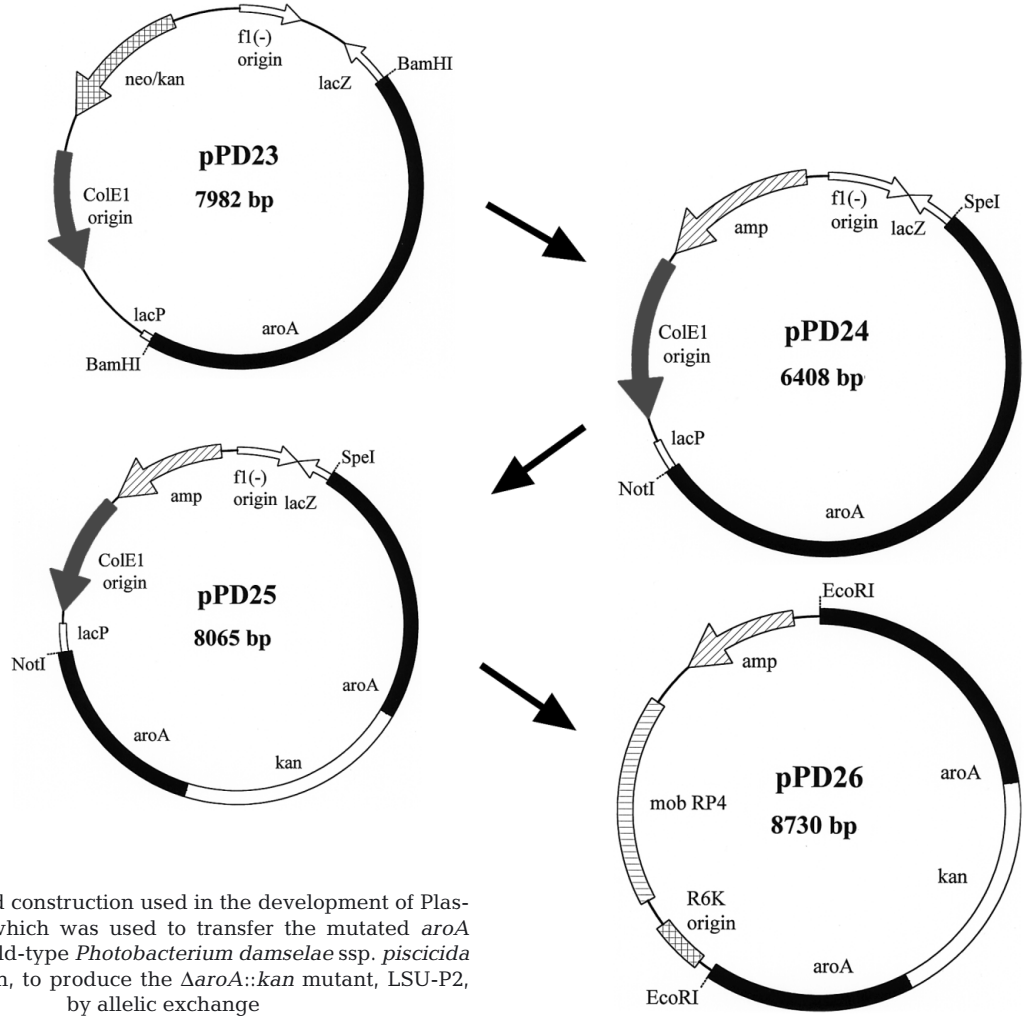

Fig. 1. Plasmid construction used in the development of Plasmid pPD26, which was used to transfer the mutated *aroA* gene to the wild-type *Photobacterium damselae* ssp. *piscicida* by conjugation, to produce the Δ*aroA*::*kan* mutant, LSU-P2, by allelic exchange

The *Photobacterium damselae* ssp. *piscicida* Δ*aroA*::*kan* genetic construction was confirmed in a series of PCR reactions using genomic DNA from LSU-P2 and *P. damselae* ssp. *piscicida* 91-197 as templates. Primer pairs and expected amplification products included: kan+ (GenBank #V00359, bp 1400 to 1417) and kan– (GenBank #V00359, bp 2007 to 2024), which should amplify a 625 bp fragment of the Tn*903 kan* sequence; aro-4 (GenBank #AY066024, bp 940 to 957), specific for the *P. damselae* ssp. *piscicida aroA* gene sequence and kan–, which should amplify a 1439 bp fragment from the Δ*aroA*::*kan* construct; and aro+6 (GenBank #AY066024, bp 940 to 957), specific for the *P. damselae* ssp. *piscicida aroA* gene sequence and kan+, which should amplify a 1144 bp fragment from the Δ*aroA*::*kan* construct. Primer locations and amplification results are depicted in Fig. 2. The anticipated 1439 bp product of the aro–4 and kan– primers, and the anticipated 1144 bp product of aro+6 and kan+ primers were both sequenced, and the sequence data confirmed proper alignment to the predicted Δ*aroA*::*kan* sequence.

Stability of the mutant construct. In order to assess the stability of the insertion/deletion mutation, LSU-P2 was passed 30 times in BHI-broth without Km selection. As a brief explanation, after 24 h triplicate cultures were 'passed' by transferring 50 µl to fresh BHI-broth tubes. After 30 passes, 500 µl was removed from each replicate, and the cells were pelleted and resuspended in sterile, distilled/deionized water. The suspended cells were boiled for 10 min and clarified by centrifugation at 2500 RCF. Using the aro+6 and aro–4 primers, which should amplify a 391 bp fragment from the wild-type *aroA* gene, and the kan+/kan– primers, which should amplify a 625 bp fragment of the Tn*903 kan* gene, supernatants were evaluated for the presence of the wild-type genotype by PCR.

Attenuation studies. For the safety studies, fish were moved from the SPF laboratory into the challenge sys-

tems at a density of 5 fish per system, and were acclimated for 1 wk prior to challenge. Challenge systems consisted of 2 interconnected 30 l tanks, one housing the fish, and a second equipped with a HydroSponge filter (Aquatic Eco-Systems) for biological filtration. Water quality parameters were monitored daily and adjusted as needed. Water temperatures were maintained at 22°C and salinity at the time of challenge was 10 ppt. Ammonia levels were undetectable and nitrite levels were minimally detectable, but were not of concern due to the elevated salinity.

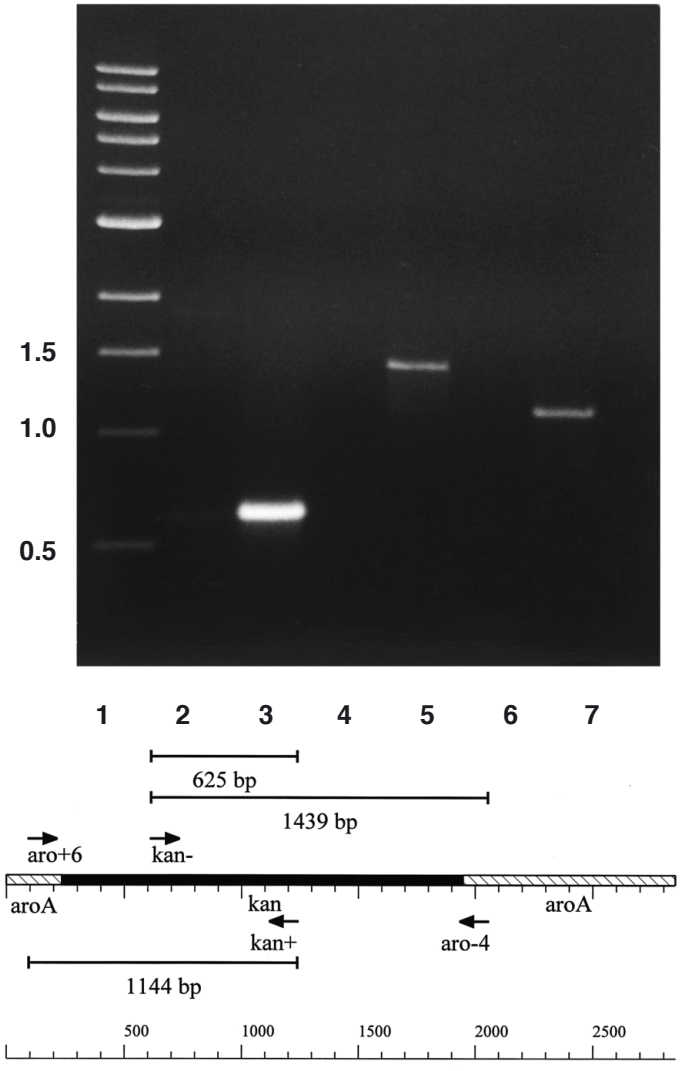

Fig. 2. Genotypic confirmation of the mutant LSU-P2 Δ*aroA*::*kan* construction by PCR. Primers used are identified by arrows on the graphic representation of the Δ*aroA*::*kan* region. Predicted amplification products are also depicted on the graphic representation as lines above and below the Δ*aroA*::*kan* fragment. Actual products of the amplification are presented in the agarose gel, with Lanes 2, 4, and 6 using wild-type *Photobacterium damselae* ssp. *piscicida* 91-197 DNA. Lanes 3, 5, and 7 contain the predicted 525, 1439, and 1144 bp fragments amplified from the appropriate primer pairs with LSU-P2 DNA as the template. kan: kanamycin resistance gene

For the LSU-P2 injection challenge, each dose was conducted in triplicate with 5 fish per system, with fish weighing an average of 55 g. Doses of 100 µl containing 1×10^6, 1×10^5, 1×10^4, and 1×10^3 colony forming units (CFU) g fish^{-1} were injected intraperitoneally, and a fifth group was injected with 100 µl of sterile 0.9% saline. For injection, fish were anesthetized with 100 mg l^{-1} of tricaine methanesulfonate (MS-222) in a separate aerated tank at a salinity of 10 ppt. In the wild-type challenge, fish weighed an average of 65 g and were injected with doses of 1×10^3, 2×10^2, 1×10^2, 2×10^1, 1×10^1, and 1×10^0 CFU g fish^{-1}.

To evaluate the safety of LSU-P2 by immersion, fish were removed from their tanks and placed in an 18 l tank with heavy aeration at a salinity of 10 ppt. Three groups of 5 fish were immersed in 10^8 CFU ml^{-1}, and 3 groups were immersed in 10^7 CFU ml^{-1} for 15 min, after which they were moved to a 10 ppt rinse tank for 10 min and then returned to the experimental systems. As a negative control, 15 fish were immersed in a 2:3 dilution of BHI broth, with 2% NaCl and 10 ppt tank water for 15 min.

In all challenge experiments, mortalities were recorded twice daily for 10 d post-challenge. Spleen samples were aseptically removed from all dead fish, transferred to trypticase soy agar (TSA) with 5% sheep blood, and confirmed to be *Photobacterium damselae* ssp. *piscicida* using API20E strips (bioMérieux). Finally, at the conclusion of the study, representative spleen samples from convalescent fish were cultured to evaluate possible *P. damselae* ssp. *piscicida* carrier status.

Vaccine efficacy. LSU-P2 was subsequently evaluated for attenuation and for its ability to induce protective immunity in hybrid striped bass. A total of 360 fish weighing an average 74 g were stocked into 3 systems of 4 tanks each (30 fish per tank, 120 per system) and acclimated for 3 wk. Treatments included fish vaccinated with LSU-P2, fish sham-vaccinated in dilution water only, and fish that were non-vaccinated and non-challenged. Fish were removed from the tanks for vaccination and transferred to a plastic tank containing 100 l of either vaccine diluted 1:1000 (4.0×10^6 CFU ml LSU-P2^{-1}), or diluent water for 15 min. Fish were challenged 6 wk post-vaccination by immersion exposure to 9000 CFU ml^{-1} of the wild type parent, *P. damselae* ssp. *piscicida* 91-197. Dead fish were removed from the tanks twice daily and spleen samples were aseptically removed to transfer to blood-agar plates to confirm *P. damselae* ssp. *piscicida* as the cause of death.

RESULTS

Photobacterium damselae ssp. *piscicida aroA* gene

Results of sequencing pPD23 revealed that the *Photobacterium damselae* ssp. *piscicida* DNA insert was 3464 bp in length and verified the presence of the *P. damselae* ssp. *piscicida aroA* gene. Subsequent double-strand sequencing of 1910 bp of the insert revealed that the *P. damselae* ssp. *piscicida aroA* gene is 1281 bp long, encoding 427 amino acids, with 66.2% identity at the nucleotide level and 73.4% codon identity to the *aroA* gene of *Escherichia coli*. The nucleotide sequence for the 1910 bp fragment containing the 1281 bp *P. damselae aroA* gene appears in the EMBL-GenBank-DDBJ nucleotide sequence databases (www.ncbi.nlm.nih.gov) under accession number AY066024.

Stability of the mutant construct

Amplification by PCR using the aro+6 and aro–4 primers resulted in detection of the predicted 391 bp fragment in the wild-type *Photobacterium damselae* ssp. *piscicida* 91-197, but failed to detect the presence of the fragment associated with the wild-type genome in the LSU-P2 30 d cultures. The kan+ and kan– primers amplified the predicted 625 bp fragment of the kanamycin resistance cassette from the sub-passed LSU-P2, but yielded no products from the wild-type. These results indicate that the genotype of LSU-P2 remained stable after 30 consecutive passes in BHI broth without Km selection.

Attenuation studies

Mortality rates for the attenuation studies are presented in Table 2. For LSU-P2 by injection, rates ranged from 0 to 100% at doses ranging from 1.0×10^2 CFU g fish^{-1} to 1.0×10^6 CFU g fish^{-1}, with an LD_{50} dose calculated at 8.7×10^4 CFU g fish^{-1}. This contrasts to the wild-type *Photobacterium damselae* ssp. *piscicida*, which had a mortality rate of 73% when injected with a single CFU g fish^{-1}, making an LD_{50} calculation impossible. Despite the high level of virulence of the wild type by injection, no fish died in either the 10^8 or 10^7 CFU ml^{-1} dose when delivered by immersion. The lack of mortality in hybrid striped bass following immersion exposure to 10^8 CFU ml LSU-P2^{-1} contrasts to an immersion LD_{50} of 5.4×10^2 CFU ml^{-1} reported by Hawke (1996) for the wild type *P. damselae* ssp. *piscicida* 91-197 in the same species.

Vaccine efficacy

No apparent stress resulted from the vaccination procedure and no mortalities were recorded for 6 wk post-exposure. Results following immersion challenge with the wild-type *Photobacterium damselae* ssp. *piscicida* 91-197 indicated that a significant level of protection was evident in the vaccinated fish, with an 85% relative percent survival (RPS) (Table 3).

DISCUSSION

These results indicate that an *aroA* mutant of *Photobacterium damselae* ssp. *piscicida* is strongly attenuated, but retains its ability to invade hybrid striped bass following immersion exposure. In addition, significant protection against subsequent exposure to the wild-type *P. damselae* ssp. *piscicida* was conferred to hybrid striped bass following a brief immersion exposure, suggesting the potential utility of LSU-P2 as a live attenuated vaccine. LSU-P2 is also very stable, with no reversion to the wild type after 30 subpasses. Because

Table 2. Percent mortality for hybrid striped bass after intraperitoneal injection or immersion exposure to the *aroA* mutant of *Photobacterium damselae* ssp. *piscicida*, LSU-P2, or wild-type *P. damselae* ssp. *piscicida*, Strain LA91-197. CFU: colony-forming units; ND: not done

Treatment (dose)	LA91-197	LSU-P2
Injection (10^0 CFU g fish^{-1})	73	ND
Injection (10^1 CFU g fish^{-1})	87	ND
Injection (10^2 CFU g fish^{-1})	87	0
Injection (10^3 CFU g fish^{-1})	100	0
Injection (10^4 CFU g fish^{-1})	ND	0
Injection (10^5 CFU g fish^{-1})	ND	53
Injection (10^6 CFU g fish^{-1})	ND	100
Immersion (10^7 CFU ml^{-1})	ND	0
Immersion (10^8 CFU ml^{-1})	ND	0

Table 3. Results of hybrid striped bass immersion challenge with virulent *Photobacterium damselae* ssp. *piscicida* 6 wk following immersion vaccination with LSU-P2. Mortality data are mean values of 4 replicate tanks with 30 fish per tank. RPS: Relative % survival = (1 – [% mortality vaccinates/ % mortality controls]) × 100; **: significantly different from the non-vaccinated group by ANOVA ($p < 0.01$)

Treatment	Mean % mortality ± SD	RPS
Non-vaccinated, non-challenged	0.00	
Non-vaccinated	91.65 ± 6.9	
LSU-P2	13.3 ± 11.0**	85.5

the Km resistance cassette of Tn*903* had the insertion sequences and the transposase encoding regions removed prior to ligation into the *P. damselae* ssp. *piscicida aroA* gene, it is probable that the Δ*aroA*::*kan* construction of LSU-P2 is incapable of reversion.

Compared to the *aroA* strains of *Edwardsiella ictaluri* previously evaluated for immersion vaccination of fish, the level of protection seems to correlate to the relative virulence of the wild type. In a previous study, Thune et al. (1999) determined that the injection LD_{50} for wild type *E. ictaluri* was ca. 40 CFU g fish^{-1}, and that immersion in 10^7 CFU ml H_2O^{-1} resulted in 73.3% mortality. Subsequently, a single immersion vaccination in an *aroA* mutant strain reduced mortality from 86.2% in non-vaccinated controls to 31.2% in vaccinates, following immersion challenge in 4×10^7 of the wild-type strain, a relative percent survival of 63.8. In contrast, wild-type *Photobacterium damselae* ssp. *piscicida* is more virulent in hybrid striped bass, with an injection LD_{50} of less than 1 CFU g fish^{-1} and mortality of 91.65% following immersion in only 9×10^3 CFU ml^{-1} (Hawke 1996). Because of this increased virulence, immersion vaccination in only 4.0×10^6 CFU ml^{-1} resulted in a reduction of mortality from 91.65% in non-vaccinated fish to 13.3% in vaccinated fish, an RPS of 85.5%.

Previous attempts to vaccinate against photobacteriosis by immersion provided equivocal results. Fukuda & Kusuda (1981) reported protection using a formalin-killed bacterin to vaccinate yellowtail, *Seriola quinqueradiata*, by a variety of methods. The same authors reported variable success with formalin-killed cells and purified *Photobacterium damselae* ssp. *piscicida* lipopolysaccharide LPS (Fukuda & Kusuda 1985), and later reported variable success with multiple doses and routes of administration, also with a formalin-killed bacterin (Kusuda & Hamaguchi 1987). Finally, Kusuda & Hamaguchi (1988) suggested that formalin-killed cells were not effective in the control of *P. damselae* ssp. *piscicida* in yellowtail, and reported 25.3% challenge mortality in yellowtail vaccinated with a live-attenuated strain compared to 57.3% mortality in fish vaccinated with a formalin-killed bacterin, 78.7% mortality in fish vaccinated with a heat-killed bacterin, and 81.3% mortality in non-vaccinated fish. Magariños et al. (1994) used a formalin-killed whole-cell *P. damselae* ssp. *piscicida* bacterin (WCB) and a formalin-killed whole-cell *P. damselae* ssp. *piscicida* bacterin enriched with heat-inactivated extracellular products (WCEB) to vaccinate gilthead seabream *Sparus aurata* by immersion. Their results indicated that challenged WCB-vaccinated fish demonstrated 35% mortality compared to 40% in the non-vaccinated controls, while challenged WCEB-vaccinated fish demonstrated 25% mortality compared to 60% in the non-vaccinated controls. Magariños et al. (1999) later reported 60% mortality in controls, versus 6 to 14% in vaccinates using a similar toxin-enriched whole-cell bacterin in 50 mg gilthead seabream, but insufficient replication of the treatments precluded statistical analysis of the data. Finally, Morinigo et al. (2002) used a divalent *Vibrio alginolyticus* and *P. damselae* ssp. *piscicida* toxoid-enriched whole-cell bacterin to vaccinate gilthead seabream, and demonstrated statistically significant protection from disease, with RPS values ranging from 70 to 86%.

Data presented here compares favorably to that for the killed, toxoid-enriched vaccine, which had an overall RPS of 75.5% for gilthead seabream (Morinigo et al. 2002) and 85.5% in the present study. A direct comparison of the 2 vaccines is necessary, however, for an accurate analysis of their relative efficacy. In addition, practical concerns regarding costs of production, storage and delivery procedures, and regulatory questions need to be addressed. The strong efficacy of the *aroA* strain with a single immersion dose following a 1:1000 dilution, however, indicates that use in the field would be practical, especially if greater dilution and lower doses are equally effective. Further studies to optimize dose and time of immersion, as well as to evaluate duration of immunity and methods for field transport and delivery, will determine the ultimate practicality of vaccination in the field.

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Editorial responsibility: Carey Cunningham, Aberdeen, UK

Submitted: January 3, 2002; Accepted: August 8, 2003
Proofs received from author(s): November 7, 2003