Short communication

First isolation of *Photobacterium damselae* ssp. *damselae* from cultured redbanded seabream, *Pagrus auriga* Valenciennes, in Spain

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Redbanded seabream, *Pagrus auriga* Valenciennes (*Sparidae*), has a wide geographical distribution and a very important commercial value in European countries. Recently, this species has been successfully cultured in the C.I.F.P.A. 'El Toruño' (El Puerto de Santa Maria, Cadiz, Spain) as a result of extensive studies on its reproductive cycle, nutrition and growth (Prieto, Cañavate & Cardenas 2003). However, as a consequence of this intensive culture, two disease outbreaks with moderate mortality rates have been recorded.

Photobacterium damselae ssp. *damselae* is an autochthonous inhabitant of both estuarine and marine waters (Ghinsberg, Drasinover, Sheinberg & Nitzan 1995). This bacterial species comprises strains that produce skin ulcers and haemorrhagic septicaemia in a wide range of fish species, and, in addition, it may be a primary pathogen for mammals, including humans (Fouz, Larsen, Nielsen, Barja & Toranzo 1992; Shin, Shin, Suh, Ryang, Rew & Nolte 1996).

Although the pathogenicity to mammals constitutes one of the main characteristics to differentiate this subspecies from *P. damselae* ssp. *piscicida* (Fouz, Toranzo, Marco-Noales & Amaro 1998), they also differ in important biochemical and physiological traits, as well as in host specificity (Magariños, Toranzo & Romalde 1996). In contrast to *P. damselae* ssp. *piscicida*, which is serologically homogeneous, at least four serotypes of *P. damselae* ssp. *damselae* have been recognized (Fouz *et al.* 1992).

In the present study, *P. damselae* ssp. *damselae* has been isolated from two epizootic outbreaks affecting cultured redbanded seabream. The characterization of the bacterial isolates was performed on the basis of phenotypic characteristics compared with reference strains of both the subspecies of *P. damselae*. In addition, enzymatic properties of the extracellular products and the susceptibility pattern to several antimicrobials were established.

Diseased fish were anaesthetized with MS-222 in sea water (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 65 mg mL^{-1} prior to sampling for microbial isolation. Samples were collected from eyes, spleen, liver and kidney, and seeded on tryptic soy agar and broth (Difco Lab., Detroit, MI, USA) supplemented with 1.5% NaCl (TSAS, TSBS), marine agar (Difco), thiosulphate-citrate-bile salt-sucrose agar (TCBS; Difco), and TCBS supplemented with 1.5% NaCl (TCBS-1). The inoculated media were incubated at 22 °C for 2–5 days.

All the isolates were subjected to taxonomical analysis according to *Bergey's Manual of Determin-*

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ative Bacteriology (Holt, Krieg, Sneath, Staley & Williams 1994). The API-20E system (BioMerieux, Madrid, Spain) was used following the manufacturer's instructions. For comparative purposes, *P. damselae* ssp. *damselae* ATCC 33539 and *P. damselae* ssp. *piscicida* ATCC 17911 were used.

An antibiogram test was performed following the disc diffusion technique described by Barry & Thornsberry (1991). The antimicrobial agents (BioMerieux) and concentrations used are shown in Table 1. Bacterial isolates were adjusted to 0.5 McFarland's scale (NCCLS 2001), streaked onto Mueller-Hinton agar plates (Oxoid, Madrid, Spain) with 1% NaCl, and incubated at 22 °C for 24 h. NCCLS standards (2001) were used for evaluation of the results.

Bacterial extracellular products (ECPs), obtained according to Liu (1957), were used for the determination of enzymatic activity. Briefly, a volume of a bacterial culture in TSBS was spread on a cellophane overlaid TSAS plate and incubated at 22 °C for 48 h. Bacterial cells were harvested in saline solution (pH 7) and subsequently removed by centrifugation. Supernatants were filtered through 0.45-μm pore-size membrane filters and used as crude ECPs. Enzymatic activities were evaluated with the API-ZYM system (BioMerieux).

The 50% lethal dose (LD_{50}) was determined using redbanded seabream with weights ranging from 5 to 10 g. These animals were injected intraperitoneally in groups of five fish with bacterial doses ranging from 10^2 to 10^8 CFU and kept at 22 °C for 2 weeks. Bacteriological analyses were carried out and death was considered as caused by the inoculated bacteria only if the strain used for inoculation was isolated in pure culture.

In order to amplify a fragment of the 16S rDNA gene, sequences from *Vibrio*, *Aeromonas* and *Photobacterium* spp. were aligned (Megalign v5.53; DNASTAR, Madison, WI). Forward (5′-GGC-TCA-CCA-AGG-CGA-CGA-TCC-CTA-3′) and reverse (5′-GGA-CTT-AAC-CCA-ACA-TTT-CAC-AAC-ACG-AG-3′) primers were located in conserved regions yielding an amplicon of 833 bp. Polymerase chain reaction (PCR) amplification was carried out in a 25 μL reaction mixture containing 5 pmol of each primer, 200 μM of each dNTP, 1x PCR buffer, 2 mM $MgCl_2$, 1.5 U Bio*Taq* polymerase (BioLine, London, UK) and 1 μL of a boiled colony suspension. PCR cycles were: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. Cycle sequencing was performed with a Bigdye® Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) on a 377 DNA sequencer (Applied Biosystems). The sequences were analysed using Sequencing Analysis v3.4.1 (Applied Biosystems) and Seqman v5.53 (DNASTAR). The 16S rDNA gene sequences were used in a BLAST search to retrieve the most closely related sequences. Selection of sequences for the final analysis was performed according to the results of the BLAST query.

A multiplex-PCR approach, using two primer pairs directed to internal regions of the 16S rRNA and *ureC* genes, was employed to differentiate between the subspecies of *P. damselae* (Osorio, Toranzo, Romalde & Barja 2000). Isolates of the *damselae* ssp. yield two amplification fragments

Table 1 Antimicrobial susceptibility patterns of isolates of *Photobacterium damselae* ssp. *damselae* from redbanded seabream and *P. damselae* ssp. *piscicida* ATCC 17911

Antimicrobials	Concentration (μg per disc)	*P. damselae* ssp. *damselae*	*P. damselae* ssp. *piscicida*
Chloramphenicol	30	S	S
Amoxycillin	25	R	S
Erythromycin	15	R	R
Flumequine	30	S	S
Trimethoprim–Sulfametoxazole	25	S	S
Oxolinic acid	10	S	S
Nitrofurantoin	300	S	S
Kanamycin	30	R	R
Nalidixic acid	30	S	S
Ampicillin	10	R	S
Tetracycline	30	R	S
Oxytetracycline	30	R	S
Enrofloxacin	5	S	S
Streptomycin	10	R	R
Novobiocin	30	R	S
Clindamycin	2	R	NT

S, sensitive; R, resistant; NT, not tested.

corresponding to 16S rRNA (267 bp) and *ureC* (448 bp) genes, whereas isolates of the *piscicida* ssp. show only the 267 bp product.

Losses of 10% of redbanded seabream occurred in two outbreaks (December 2003 and May 2004) over a period of 2–33 weeks, affecting fish ranging from 80 to 300 g. The external signs were exophthalmia and dark skin pigmentation. Internally, fish showed ascitic fluid in the abdominal cavity, and an enlarged and pale liver with congested blood vessels and dark areas. The gall bladder was filled with bile and the spleen was also enlarged. These gross clinical signs were similar to those previously described for vibriosis and pasteurellosis in other cultured sparid fish (Balebona, Andreu, Bordas, Zorrilla, Moriñigo & Borrego 1998; Zorrilla, Balebona, Moriñigo, Sarasquete & Borrego 1999), but the typical white tubercles on the spleen provoked by *P. damselae* ssp. *piscicida* were never observed.

Isolated bacteria were Gram-negative rod-cocci, cytochrome-oxidase positive and motile organisms, and were identified as *Photobacterium damselae* ssp. *damselae* according to the criteria established by Holt *et al.* (1994), Zorrilla *et al.* (1999) and Rajan, Lin, Ho & Yang (2003). This result was confirmed using the API-20E system where the numerical code obtained was 6015004, which is similar to that of the *P. damselae* ssp. *damselae* reference strain. The phenotypic pattern of *P. damselae* ssp. *damselae* isolates matched that obtained for the reference strain ATCC 33539. This study shows that there are several traits which could be considered keys for phenotypic discrimination between both the subspecies of *P. damselae*, such as the lysine decarboxylase test, susceptibility to vibriostatic agent O/129 (150 μg) and polymyxin B, and utilization of D-galactose and D-mannose as carbon and energy sources. In addition, both the *P. damselae* ssp. were clearly differentiated by culture on TCBS-1, as previously reported by Rajan *et al.* (2003).

Although the biochemical and physiological characteristics tested can be useful in discrimination of both subspecies of *P. damselae*, a genetic characterization has been performed to confirm the phenotypic results. The sequencing of a fragment of the 16S rDNA gene clearly showed that the isolates from redbanded seabream belong to the *P. damselae* complex (bootstrap value of 100%), which agrees with results reported by Gauthier, Lafay, Ruimy, Breittmeyer, Nicolas, Gauthier & Christen (1995) and Osorio, Collins, Toranzo, Barja & Romalde (1999). The observation of two amplification bands of 267 and 448 bp in the multiplex-PCR assay (Fig. 1) demonstrated that the isolates from redbanded seabream are members of the *damselae* ssp.

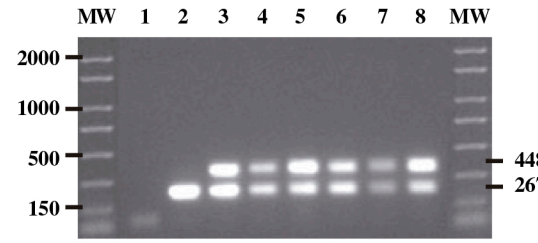

Figure 1 Multiplex-polymerase chain reaction assay of redbanded seabream isolates. Lanes: MW, molecular weight marker (Sigma); 1, negative control; 2, *Photobacterium damselae* ssp. *piscicida* ATCC 17911; 3, *P. damselae* ssp. *damselae* ATCC 33539; 4–8, redbanded seabream isolates. Numbers on the left indicate the size (bp) of the molecular weight marker. Size of amplification fragments (bp) is shown on the right.

Only one resistotype was recorded, showing susceptibility to several chemotherapeutic agents used for the treatment of bacterial outbreaks in aquaculture (Table 1). On the other hand, *P. damselae* ssp. *piscicida* strain ATCC 17911 was only resistant to kanamycin, streptomycin and erythromycin (Table 1). The susceptibility or

Table 2 Exoenzymatic activity patterns of *Photobacterium damselae* ssp. *damselae* isolates from redbanded seabream and *P. damselae* ssp. *piscicida* ATCC 17911

Activity	*P. damselae* ssp. *damselae*	*P. damselae* ssp. *piscicida*
Alkaline phosphatase	30–40[a]	20–30
Esterase	10–20	10–20
Esterase–lipase	20–30	20–30
Lipase	0	0
Leucine arylamidase	5–10	5–10
Valine arylamidase	0	0
Cystine arylamidase	0	0
Trypsin	0	0
α-chemotrypsin	5–10	0
Acid phosphatase	>40	20–30
Phosphohydrolase	5–10	10–20
α-galactosidase	0	0
β-galactosidase	0	0
β-glucuronidase	0	0
α-glucosidase	0	0
β-glucosidase	0	0
N-acetyl-β-glucosaminidase	20–30	20–30
α-mannosidase	0	0
α-fucosidase	0	0

[a] Concentration expressed as nmol.

resistance to tetracyclines and beta-lactams is the most important difference between the subspecies (Bakopoulos, Adams & Richards 1995; Zorrilla *et al.* 1999).

Extracellular products of *P. damselae* ssp. *damselae* isolates presented only one exoenzymatic activity pattern determined by the API-ZYM gallery. This pattern included low levels (< 10 nmol) of leucine arylamidase, alpha-chemotrypsin and phosphohydrolase; moderate levels (10–30 nmol) of esterase, esterase–lipase and *N*-acetyl-β-glucosaminidase; and high levels (> 30 nmol) of acid and alkaline phosphatase. Negative results were obtained for the other exoenzymatic activities tested (Table 2).

The LD_{50}, determined by redbanded seabream intraperitoneal inoculation, was 3.9×10^5 CFU g^{-1} of fish weight. Thus, the isolates can be considered moderately virulent for *P. auriga* (Santos, Toranzo, Barja, Nieto & Villa 1988).

This is the first report of the isolation of *P. damselae* ssp. *damselae* from redbanded seabream, a sparid fish newly cultured in southern Spain. A low variability in the biochemical profile among the *P. damselae* ssp. *damselae* isolates tested was observed (acetoine production), although these profiles were clearly different from that of *P. damselae* ssp. *piscicida*. The analysis of *ureC* and 16S rRNA genes confirmed the phenotypic characterization of the isolates as *P. damselae* ssp. *damselae*.

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References

Bakopoulos V., Adams A. & Richards R.H. (1995) Some biochemical properties and antibiotic sensitivities of *Pasteurella piscicida* isolated from Greece and comparison with strains from Japan, France and Italy. *Journal of Fish Diseases* **18,** 1–8.

Balebona M.C., Andreu M.J., Bordas M.A., Zorrilla I., Moriñigo M.A. & Borrego J.J. (1998) Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). *Applied and Environmental Microbiology* **64,** 4269–4275.

Barry A.L. & Thornsberry C. (1991) Susceptibility tests: diffusion test procedures. In: *Manual of Clinical Microbiology* (ed. by A. Balows, W.J. Hausler Jr, K.L. Hermann, H.D. Isenberg & H.J. Shadomy), pp. 1117–1125. American Society for Microbiology, Washington, DC.

Fouz B., Larsen J.L., Nielsen B., Barja J.L. & Toranzo A.E. (1992) Characterization of *Vibrio damsela* strain isolated from turbot *Scophthalmus maximus* in Spain. *Diseases of Aquatic Organisms* **12,** 155–166.

Fouz B., Toranzo A.E., Marco-Noales E. & Amaro C. (1998) Survival of fish virulent strains of *Photobacterium damselae* subsp. *damselae* in seawater under starvation conditions. *FEMS Microbiology Letters* **168,** 181–186.

Gauthier G., Lafay B., Ruimy R., Breittmeyer V., Nicolas J.L., Gauthier M. & Christen R. (1995) Small-subunit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Sniezko *et al.*) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damselae* subsp. *piscicida* comb. nov. *International Journal of Systematic Bacteriology* **45,** 139–144.

Ghinsberg R.C., Drasinover V., Sheinberg Y. & Nitzan Y. (1995) Seasonal distribution of *Aeromonas hydrophila* and *Vibrio* species in Mediterranean coastal water and beaches: a possible health hazard. *Biomedical Letters* **51,** 151–159.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. & Williams S.T. (1994) *Bergey's Manual of Determinative Bacteriology.* Williams and Wilkins Co., Baltimore.

Liu P.V. (1957) Survey of hemolysin production among species of pseudomonads. *Journal of Bacteriology* **74,** 718–727.

Magariños B., Toranzo A.E. & Romalde J.L. (1996) Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Annual Review of Fish Diseases* **6,** 41–64.

NCCLS (2001) *Performance Standards for Antimicrobial Susceptibility Testing*, 11th Supplement. National Committee for Clinical Laboratory Standards of Antimicrobial Susceptibility Testing, Wayne.

Osorio C.R., Collins M.D., Toranzo A.E., Barja J.L. & Romalde J.L. (1999) 16S RNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. *Applied and Environmental Microbiology* **65,** 2942–2946.

Osorio C.R., Toranzo A.E., Romalde J.L. & Barja J.L. (2000) Multiplex PCR assay for *ureC* and 16S rRNA genes clearly discriminates between both subspecies of *Photobacterium damselae*. *Diseases of Aquatic Organisms* **40,** 177–183.

Prieto A., Cañavate J. & Cardenas S. (2003) Crecimiento de larvas de hurta (*Pagrus auriga*) en cultivo. *IX Congreso Nacional de Acuicultura*, Cadiz, Spain, p. 386.

Rajan P.R., Lin J.H.-Y., Ho M.-S. & Yang H.-L. (2003) Simple and rapid detection of *Photobacterium damselae* ssp. *piscicida* by a PCR technique and plating method. *Journal of Applied Microbiology* **95,** 1375–1380.

Santos Y., Toranzo A.E., Barja J.L., Nieto T.P. & Villa T.G. (1988) Virulence properties and enterotoxin production of *Aeromonas* strains isolated from fish. *Infection and Immunity* **56,** 3285–3293.

Shin J.D., Shin M.G., Suh S.P., Ryang D.W., Rew J.S. & Nolte F.S. (1996) Primary *Vibrio damsela* septicemia. *Clinical and Infectious Diseases* **22,** 856–857.

Zorrilla I., Balebona M.C., Moriñigo M.A., Sarasquete C. & Borrego J.J. (1999) Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damselae* ssp. *piscicida*, from sole, *Solea senegalensis* (Kaup). *Journal of Fish Diseases* **22,** 167–172.

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