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Phenotypic characterization of the marine pathogen *Photobacterium damselae* subsp. *piscicida*

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The taxonomic position of *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish pasteurellosis, is controversial as this organism has also been described as '*Pasteurella piscicida*'. To clarify the taxonomic position of the pathogen, a total of 113 *P. damselae* subsp. *piscicida* strains and 20 *P. damselae* subsp. *damselae* strains, isolated from different geographical areas and from the main affected fish species, were analysed using 129 morphological and biochemical tests, including the commercial API 20E and API CH50 test systems. For comparison, the type strains of other *Photobacterium* species (i.e. *Photobacterium leiognathi* and *Photobacterium angustum*) were included in the analyses. The results were statistically analysed by unweighted pair group average clustering and the distance between the different clusters was expressed as the percentage disagreement. The analyses showed that, based on morphological and biochemical identification tests, *P. damselae* subsp. *piscicida* is related to other *Photobacterium* species. However, it is clearly distinguishable from *P. damselae* subsp. *damselae* and no phenotypic evidence was found to include *P. damselae* subsp. *piscicida* as a subspecies in the species *P. damselae*.

Keywords: phenotypic characterization, numerical taxonomy, *Photobacterium*, *Photobacterium damselae* subsp. *piscicida*

INTRODUCTION

Photobacterium damselae subsp. *piscicida* is the causative agent of the fish disease pasteurellosis. This disease was first observed in natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963 in Chesapeake Bay, USA (Snieszko *et al.*, 1964). In 1969, the pathogen became economically significant with detrimental fish losses in the Japanese fish culture industry (Kusuda & Yamaoka, 1972). Until recently, Europe was considered to be free of fish pasteurellosis. However, in 1990 several outbreaks of fish pasteurellosis occurred in cultured populations of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in different European countries including France (Baudin Laurencin *et al.*, 1991), Italy (Ceschia *et al.*, 1991), Spain (Toranzo *et al.*, 1991), Greece (Bakopoulos *et al.*, 1995), Portugal (Baptista *et al.*, 1996), Turkey (Candan *et al.*, 1996), and Malta (Bakopoulos *et al.*, 1997). Pasteurellosis continues to be a serious problem in the intensive culture of different fish species in the Mediterranean Sea and Japan.

The taxonomic position of *P. damselae* subsp. *piscicida* is controversial. Based on morphological and biochemical characterization, the organism was first placed in the genus *Pasteurella* and given the name '*Pasteurella piscicida*' or 'fish killer' (Janssen & Surgalla, 1968). Although this species is clearly distinguishable from the other species within the genus *Pasteurella* (i.e. by positive reactions for arginine dihydrolase and the methyl red test, and negative reactions for nitrate reduction and growth at 37 and 42 °C), the organism remained in this genus. However, De Ley *et al.* (1990) reported that, based on analysis of rRNA cistrons, '*Pasteurella piscicida*' should be classified in the family *Vibrionaceae*. This argument was supported by fatty acid methyl ester profiling (Romaldo *et al.*, 1995). More recently, Gauthier *et al.* (1995) found that, based on rRNA sequence and

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Abbreviations: TCBS agar, thiosulfate/citrate/bile/sucrose agar; UPGMA, unweighted pair group method with averages.

DNA–DNA hybridization data, the pathogen was closely related to *P. damselae* and proposed that it be placed in the genus *Photobacterium* as a subspecies of *P. damselae*.

In this report, the taxonomic position of the pathogen *P. damselae* subsp. *piscicida* was clarified by standard and commercial (API 20E and API CH50) morphological and biochemical identification tests.

METHODS

Bacterial strains and culture conditions. A total of 137 strains was used in this study. Diagnoses were obtained and verified by comparing the reaction profiles of the strains with the description of the species. One-hundred-and-thirteen strains were identified as *P. damselae* subsp. *piscicida*, 20 as *P. damselae* subsp. *damselae*, one as *Photobacterium angustum*, two as *Photobacterium leiognathi* and one as *Photobacterium fischeri*. The *P. damselae* subsp. *piscicida* collection contained isolates from Europe (58), Japan (43) and the USA (two), 36 of which were isolated from sea bass, 22 from sea bream and 34 from yellowtail. These strains were all isolated between 1963 and 1997. The *P. damselae* subsp. *damselae* collection consisted of one strain isolated from human, one from brown shark, one from oyster, one from seawater, seven from shrimp and six from different fish species. Information concerning the source of the remaining *P. damselae* subsp. *damselae* strains was not available. The reference and type strains included in this study were: *P. damselae* subsp. *piscicida* ATCC 17911 and NCIMB 25918^T; *P. damselae* subsp. *damselae* ATCC 35083, ATCC 33539^T and NCIMB 2184^T (= ATCC 33539^T); *P. leiognathi* LMG 4228^T; *P. angustum* NCIMB 1895^T; and *P. fischeri* ATCC 25918 (a complete list of strains may be obtained from the author).

Bacterial cultures were maintained on long-term preservation medium (West & Colwell, 1984) and reactivated on brain/heart infusion agar (Difco) to which 1 % (w/v) NaCl was added (BN medium) and incubated for 48 h at 26 °C.

Morphological and biochemical testing of the isolates. All media contained a final NaCl concentration of 1·5 % (w/v) and the isolates were incubated for 72 h at 26 °C unless noted otherwise. The Gram reaction, nitrate reduction, luminescence, the indole reaction, salt tolerance for growth (0, 0·5, 3, 6, 8 and 10 %, w/v, NaCl), and production of alginase, amylase, deoxyribonuclease, gelatinase and lipase (Tween 80) were tested according to the methods described by West & Colwell (1984). Motility was determined by the hanging drop method. The oxidase reaction was tested on oxidase test disks (bioMérieux) and catalase production was assessed with 3 % (v/v) H_2O_2. β-Haemolysis and antibiotic resistance to vibriostatic agent O/129 (10 and 150 μg; Oxoid) and novobiocin (5 μg; Diagnostics Pasteur) were recorded on tryptic soy agar supplemented with 5 % (v/v) sheep blood containing 0·5 % (w/v) NaCl (bioMérieux). The bacteria were scored as sensitive for these antibiotics if an inhibition zone could be detected. Temperature tolerance for growth was examined on BN plates incubated at 4, 37 and 42 °C. The methyl red and Voges–Proskauer tests were conducted in MR–VP medium (Difco). Fermentative versus oxidative metabolism of glucose, gas production from glucose (inverted Durham tube), and lysine and ornithine decarboxylase production (Moeller) were analysed as described by Twedt (1978). Arginine dihydrolase was assessed by the method of Thornley (1960). Reaction on Kligler iron agar (Difco), growth and reaction on thiosulfate/citrate/bile/sucrose (TCBS) agar (Difco) and MacConkey agar (No. 3; Oxoid) were examined. Tartrate utilization was tested on phenol red tartrate agar (Difco). Growth on citrate as the sole source of energy was tested on Simmons citrate agar (Difco). Utilization of malonate, production of phenylalanine deaminase, aesculin hydrolysis, casein hydrolysis and the phosphatase reaction were examined according to the methods described by Smibert & Krieg (1984). Elastinase production was tested on elastinase agar as described by Hsu *et al.* (1981). The Gram reaction, catalase and oxidase activity, and motility were determined after 24 h. Phenylalanine deaminase activity, aesculin hydrolysis, growth on Simmons citrate agar, use of malonate and tartrate, and growth temperature tolerance were examined after 7 d. TCBS and MacConkey media and BN plates, used to test growth temperature tolerance, were inoculated from a McFarland standard No. 3 bacterial suspension in saline (NaCl 1·5 %, w/v). Antibiotic resistance was determined by the disk diffusion method on Mueller–Hinton agar (Difco). The following antimicrobial agents were tested: amoxicillin (25 μg), ampicillin (10 μg), chloramphenicol (30 μg), flumequine (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), nitrofurantoin (300 μg), oxacillin (1 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg), and trimethoprim–sulfamethoxazole (1·25 + 23·75 μg) (bioMérieux). Antibiotic resistance was determined according to the bioMérieux manual.

API 20E and API CH50 (bioMérieux) reactions were carried out as described in the bioMérieux manual and incubated for 72 h. The carbohydrate test tubes were sealed with sterile mineral oil.

Numerical taxonomy. The results of the 137 strains (including reference and type strains) examined by the 129 different tests were scored as 1 (positive) and 0 (negative) and incorporated in a larger data matrix (137 strains/129 characters). Unweighted pair group method with averages (UPGMA) clustering (Sneath & Sokal, 1973) was performed using the cluster analysis program of Statistica for Windows. The distance between the different clusters was expressed in percentage disagreement.

Test reproducibility was evaluated by duplicating tests with two strains; the corresponding error was estimated according to Sneath & Johnson (1972).

RESULTS

Numerical taxonomy

The results of the 137 strains tested against 129 biochemical tests were analysed by UPGMA clustering based on the percentage disagreement. The dendrogram is presented in Fig. 1. Table 1 gives the reaction profiles obtained for each of the clustered and unclustered strains included in this analysis. The probability of test error was 3·6 %.

At the level of 13·5 % disagreement, three clusters (1, 2 and 3) and one unclustered strain could be distinguished. The clusters differed in terms of arginine dihydrolase and lysine decarboxylase activity, motility, β-haemolysis, growth in 0·5 % NaCl, Voges–Proskauer test, growth at 37 °C, nitrate reduction, Kligler test, growth on TCBS and MacConkey media, production of amylase and phosphatase, antibiotic resistance

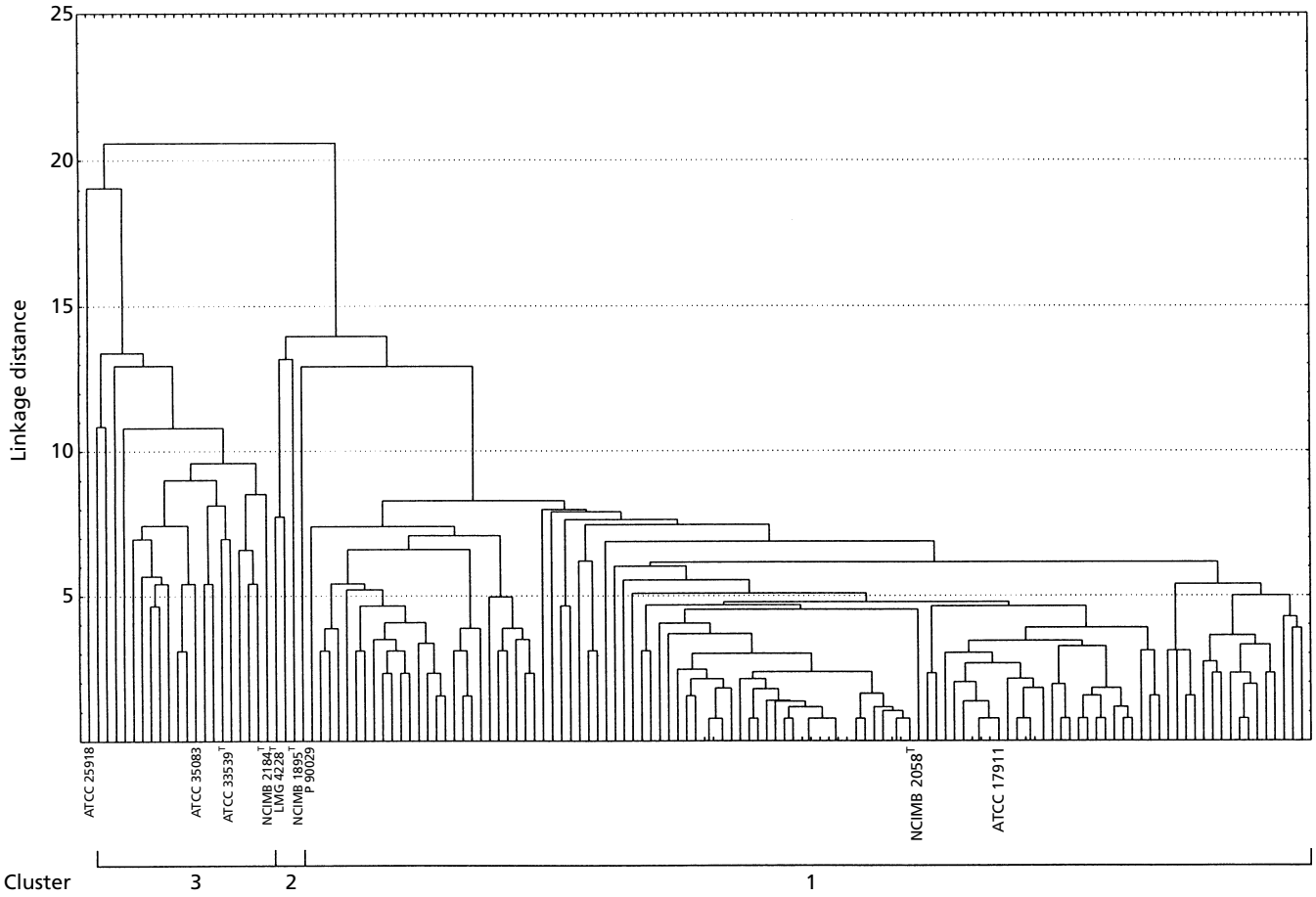

Fig. 1. Dendrogram based on UPGMA clustering using the percentage disagreement.

(penicillin G, ampicillin, kanamycin and amoxicillin), utilization of tartrate, and fermentation of glycerol, salicin, cellobiose, maltose, trehalose and amidon.

Cluster 1 formed at 13% disagreement and contained the majority of all strains tested (113 strains or 82·5%). The reference and type strains of *P. damselae* subsp. *piscicida* (ATCC 17911 and NCIMB 2058^T, respectively) were located in this cluster. All but one of the strains clustered at a much lower degree of dissimilarity (7%). The aberrant strain, P 90029, which joined the other bacteria from cluster 1 at 13% disagreement, was isolated in 1990 in Japan from a diseased yellowtail. This bacterium could be clearly distinguished from the other strains by a positive reaction for urease and by the ability to ferment maltose and lactose in the API CH50 gallery. The bacteria from cluster 1 in general were characterized by a negative reaction for nitrate reduction and the inability to produce phosphatase. All bacteria were Simmons citrate-negative, although 18% of them scored positive for Simmons citrate in the commercial API 20E test system.

Cluster 2 formed at 13·5% disagreement and consisted of three strains: the type strains of *P. leiognathi* (LMG 4228^T) and *P. angustum* (NCIMB 1895^T) and one additional *P. leiognathi* strain which was donated by John Lee (Department of Biochemistry, University of Georgia, Athens, GA, USA). Although the two type strains clustered together, they could still be distinguished from one another. *P. angustum* differed from *P. leiognathi* by positive reactions for oxidase, catalase, gelatinase, caseinase, DNase, methyl red, aesculin hydrolysis, and fermentation of saccharose and maltose, and negative reactions for nitrate reduction, growth at 37 °C, luminescence, fermentation of glycerol and gluconate, and sensitivity to kanamycin and novobiocin. Cluster 2 joined cluster 1 at 14% disagreement.

Cluster 3 formed at 13·5% disagreement and contained 20 strains. The bacteria from cluster 3 produced gas and amylase, were resistant to amoxicillin and fermented glycerol and maltose. Although the two different isolates (ATCC 33539^T and NCIMB 2184^T) of the type strain of *P. damselae* subsp. *damselae* are both located in this cluster they could be distinguished from one another. In contrast to ATCC 33539^T, NCIMB 2184^T was characterized by positive reactions for methyl red, amylase production, citrate utilization,

Table 1. Reaction profiles obtained for the different clusters and *P. fischeri*

The numbers in the table refer to the number of strains that give a positive reaction for a given character (percentages are given in parentheses); +, all strains positive; −, all strains negative. All strains showed negative reactions for Gram-staining, ornithine decarboxylase, H_2S production, TDA, indole production (standard test, not commercial test), growth in 0, 8 and 10% NaCl, growth at 4 °C, malonate utilization, and fermentation of mannose, inositol, sorbitol, rhamnose, melibiose, D-arabinose, L-arabinose, erythritol, D-xylose, L-xylose, adonitol, methyl β-xyloside, L-sorbose, dulcitol, mannitol, methyl α-D-mannoside, amygdalin, inulin, melezitose, D-raffinose, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, β-gentiobiose, 2-ketogluconate and 5-ketogluconate. All strains were positive for oxidative and fermentative reactions, growth in 3% NaCl and fermentation of D-glucose, D-fructose and D-mannose.

	Cluster 1	**Cluster 2**	**Cluster 3**	***P. fischeri***
No. of strains in each cluster	113	3	20	1
API 20E				
β-Galactosidase	−	−	1 (5)	−
Arginine dihydrolase	108 (96)	+	19 (95)	−
Lysine decarboxylase	−	−	11 (55)	+
Citrate utilization	20 (18)	−	3 (15)	−
Urease	1 (< 1)	−	17 (85)	−
Indole production	−	−	1 (5)	−
Voges–Proskauer test	109 (96)	+	+	+
Gelatinase	−	1 (33)	4 (20)	−
Fermentation of:				
Glucose	110 (97)	+	+	+
Saccharose	−	1 (33)	1 (5)	−
Amygdalin	−	−	3 (15)	+
Oxidase activity	103 (91)	2 (67)	15 (75)	+
Catalase activity	105 (93)	2 (67)	19 (95)	+
Motility	−	−	19 (95)	+
β-Haemolysis	−	−	17 (85)	−
Sensitivity to:				
O/129 (10 μg)	75 (66)	+	14 (70)	−
O/129 (150 μg)	93 (82)	+	+	+
Novobiocin (5 μg)	93 (82)	2 (67)	18 (90)	+
Growth at:				
0·5% NaCl	97 (86)	+	19 (95)	−
6% NaCl	3 (3)	+	18 (90)	+
37 °C	61 (54)	1 (33)	19 (95)	−
42 °C	1 (< 1)	−	11 (55)	−
Voges–Proskauer test	110 (97)	+	15 (75)	−
Methyl red reaction	110 (97)	2 (67)	19 (95)	−
Arginine dihydrolase	108 (96)	+	19 (95)	−
Lysine decarboxylase	−	1 (33)	5 (25)	+
Nitrate reduction	−	1 (33)	17 (85)	+
Simmons citrate reaction	−	−	4 (20)	−
Tartrate	2 (2)	1 (33)	4 (20)	+
TCBS growth	−	1 (33)	19 (95)	−
TCBS sucrose	−	−	1 (5)	−
Kligler test:				
Glucose	81 (72)	+	18 (90)	−
Lactose	85 (75)	+	16 (80)	−
Gas	−	−	9 (45)	−
MacConkey growth	52 (46)	2 (67)	19 (95)	−
Lipase production	108 (96)	2 (67)	15 (75)	+
Amylase production	60 (53)	−	+	−
Gelatinase production	−	1 (33)	3 (15)	−
DNase production	83 (73)	1 (33)	+	+

Table 1. *(cont.)*

	Cluster 1	Cluster 2	Cluster 3	*P. fischeri*
Phenylalanine deaminase	–	–	1 (5)	–
Caseinase production	1 (< 1)	1 (33)	–	–
Phosphatase production	1 (< 1)	+	+	+
Aesculin hydrolysis	–	1 (33)	6 (30)	–
Luminescence	–	2 (67)	–	–
Gas production	–	–	13 (65)	–
Sensitivity to:				
Flumequine	110 (97)	+	18 (90)	+
Tetracycline	88 (78)	+	17 (85)	+
Gentamicin	111 (98)	+	17 (85)	+
Neomycin	83 (73)	+	11 (55)	+
Chloramphenicol	84 (74)	+	19 (95)	+
Penicillin G	103 (91)	–	–	–
Flumiquine	112 (99)	+	+	+
Oxacillin	2 (2)	–	–	–
Ampicillin	107 (95)	+	1 (5)	–
Streptomycin	64 (57)	–	1 (5)	–
Trimethoprim–sulfamethoxazole	112 (99)	+	18 (90)	+
Kanamycin	81 (72)	2 (67)	9 (45)	–
Amoxicillin	108 (96)	–	–	–
API CH50				
Fermentation of:				
Glycerol	1 (< 1)	2 (67)	+	–
Ribose	96 (85)	+	+	+
Galactose	105 (93)	+	+	+
Methyl α-D-glucoside	–	–	2 (10)	–
N-Acetylglucosamine	111 (98)	+	+	+
Arbutin	–	–	2 (10)	–
Aesculin	–	–	2 (10)	–
Salicin	1 (< 1)	–	3 (15)	+
Cellobiose	–	–	17 (85)	+
Maltose	2 (2)	1 (67)	+	+
Lactose	1 (< 1)	–	–	–
Saccharose	2 (2)	–	2 (10)	–
Trehalose	–	–	16 (80)	–
Amidon	–	–	16 (80)	+
Glycogen	–	–	6 (30)	–
D-Turanose	1 (< 1)	–	3 (15)	–
Gluconate	–	2 (67)	–	–

sensitivity to trimethoprim–sulfamethoxazole, and negative reactions for oxidase, urease, Voges–Proskauer test, aesculin hydrolysis, gas production, and sensitivity to gentamicin, neomycin and kanamycin. Cluster 3 joined clusters 1 and 2 at 20·5% disagreement.

The unclustered strain, *P. fischeri* ATCC 25918, joined cluster 3 at 19% disagreement and was characterized by a negative reaction on the Kligler test, sensitivity to O/129 (10 μg), production of lysine decarboxylase and growth in 6% NaCl. It should be noted that the bacterium scored positive for the Voges–Proskauer test in commercial medium, but negative in MR–VP medium.

DISCUSSION

The probability of test error was calculated as 3·6%, which is an acceptable value according to the criteria of Sneath & Johnson (1972). This means that the different reaction profiles for the two isolates of the type strain *P. damselae* subsp. *damselae*, ATCC 33539^T and NCIMB 2184^T, are probably not due to a lack of test reproducibility. It is known, however, that maintenance of a strain within a laboratory by repeated subculture can result in a decrease in its vigour and the loss or change of some characteristics (Bryant *et al.*, 1986).

The reaction profiles of some of the bacteria were not

completely in agreement with the general profile of the species to which they belonged for a number of tests, e.g. oxidase and catalase activity, production of lipase, growth in 0·5% NaCl, and methyl red and Voges–Proskauer tests. A number of strains had a weak positive reaction for these tests. In the analysis of results, these reactions were scored as negative.

The reaction profiles of the 113 strains of cluster 1 are basically in agreement with the amended description of *P. damselae* subsp. *piscicida* made by Gauthier *et al.* (1995). The bacteria only differed by negative reactions for the fermentation of mannose, saccharose and raffinose. These aberrant reactions can be explained by the different reaction conditions used for the API CH50 gallery. In contrast to Gauthier *et al.* (1995), a 1·5% saline suspension was used as inoculum in this study. Moreover, the incubation temperature and time were increased to 26 °C and 72 h, respectively. Phenotypic characterization showed that *P. damselae* subsp. *piscicida* is a Gram-negative, non-motile coccobacillus with bipolar staining. The bacterium is characterized by positive reactions for arginine dihydrolase, methyl red and Voges–Proskauer tests, production of oxidase and fermentation of ribose, and negative reactions for nitrate reduction, indole production, production of alginase and gelatinase, growth at 42 °C and fermentation of saccharose. A full description of *P. damselae* subsp. *piscicida* is given in Table 1. Strain P 90029 differed from the other cluster 1 bacteria by aberrant reactions on the API 20E and API CH50 galleries (see Results). Due to the negative reactions for arginine dihydrolase and fermentation of glucose and the positive reaction for urease production, P 90029 exhibited an API 20E index profile of 0011004. Based on positive reactions for arginine dihydrolase, Voges–Proskauer test and fermentation of glucose, *P. damselae* subsp. *piscicida* generally displays a unique code of 2005004 (Toranzo *et al.*, 1991). In this analysis, only 76% (86 out of 113) strains exhibited this index profile. Aberrant reactions for arginine dihydrolase, citrate utilization and Voges–Proskauer test have already been observed by Kent *et al.* (1982), Hawke *et al.* (1987) and Candan *et al.* (1996). Based on these findings, it is concluded that the API 20E test system is not always a useful tool for the identification of *P. damselae* subsp. *piscicida*. It is suggested that in addition to API 20E, 'classical' tests such as salt tolerance for growth (0, 0·5, 3, 6, 8 and 10% NaCl), growth temperature tolerance (4, 37 and 42 °C), arginine dihydrolase according to Thornley (1960), classical methyl red and Voges–Proskauer tests, and nitrate reduction, gas production from glucose, and plate tests for the hydrolysis of alginate, casein, elastin, gelatin, starch and DNA should be performed. Based on results of the classical tests, the reaction profile of P 90029 is in agreement with that of *P. damselae* subsp. *piscicida*.

Cluster 3 represents the subspecies *P. damselae* subsp. *damselae*. The reaction profile of this subspecies is in agreement with the description of Gauthier *et al.* (1995) with the exception of aesculin hydrolysis.

Based on phenotypic characterization, cluster analysis grouped *P. damselae* subsp. *piscicida* (formerly '*Pasteurella piscicida*') together with the *Photobacterium* species at a level of 19% disagreement or 81% agreement. Furthermore, analysis showed that the bacterium is more related to *P. angustum* and *P. leiognathi* than to *P. damselae* subsp. *damselae*. This result is not in agreement with the genotypic analyses of Gauthier *et al.* (1995), which showed that '*Pasteurella piscicida*' and *P. damselae* were closely related. Both bacteria shared the same 16S rRNA sequence and exhibited 80% DNA–DNA hybridization. Based on these findings, '*Pasteurella piscicida*' was classified as a subspecies of *P. damselae* and renamed *P. damselae* subsp. *piscicida*. The present study, on the other hand, found no morphological and/or biochemical evidence to include '*Pasteurella piscicida*' as a subspecies of *P. damselae*. As shown in Table 1, the species could be clearly distinguished from *P. damselae* subsp. *damselae* by negative reactions for motility, nitrate reduction, growth at 37 and 42 °C, growth on TCBS and MacConkey media, β-haemolysis, production of urease and amylase, and the inability to ferment amidon, cellobiose, maltose and trehalose.

In conclusion, based on the present phenotypic analysis, the species '*Pasteurella piscicida*' should be placed along with the other *Photobacterium* strains in the genus *Photobacterium* according to positive reactions for arginine dihydrolase and methyl red, and fermentation of ribose, and negative reactions for nitrate reduction, production of alginase and fermentation of sucrose. Because results obtained by phenotypic analyses were not in accordance with those of the genotypic analyses performed by Gauthier *et al.* (1995), further studies involving ribotyping, random amplification of polymorphic DNA and pulsed-field gel electrophoresis are underway to unravel the taxonomic position of this marine pathogen.

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