Small-Subunit rRNA Sequences and Whole DNA Relatedness Concur for the Reassignment of *Pasteurella piscicida* (Snieszko et al.) Janssen and Surgalla to the Genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov.

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The taxonomic status of *Pasteurella piscicida* (strain NCIMB 2058T [T = type strain] and a strain isolated from the environment) was investigated by performing phylogenetic analyses of small-subunit rRNA sequences, DNA-DNA hybridization analyses, and biochemical characterization analyses. The results of the phylogenetic analyses and the levels of DNA-DNA complementarity demonstrated conclusively that *Pasteurella piscicida* is extremely closely related to *Photobacterium damsela* ATCC 33539T. Since the two taxa exhibited a level of DNA-DNA relatedness of 86%, they are members of the same species. The high level of DNA relatedness and the presence of specific morphological and biochemical characteristics support the hypothesis that two subspecies should be recognized. On the basis of its phylogenetic position, we concluded that *Pasteurella piscicida* should be renamed *Photobacterium damsela* subsp. *piscicida* comb. nov.

In 1963, an epizootic destroyed 50% of the white perch (*Roccus americanus*) and striped bass (*Roccus saxatilis*) in the upper Chesapeake Bay. The bacteria responsible for this fish disease were quickly isolated (24) and were later shown to cause considerable losses in marine aquaculture environments (reviewed in reference 15). These bacteria, which are not present in healthy fish, cause infection and death when they are inoculated into fish (1). Virulence seems to be caused by a thermolabile phospholipase (16), which has also been found in other fish pathogens, including *Aeromonas salmonicida* (14) and *Photobacterium damsela* (13).

On the basis of its general physiology (gram-negative cells that are non-motile, oxidase positive, and rod shaped and exhibit bipolar staining), the bacterium that was responsible for the epizootic was first placed in the genus *Pasteurella*, without a specific designation (24). The name *Pasteurella piscicida* was later proposed (11) despite the inability of the organism to reduce nitrate and its tolerance of pH values and temperatures that were unusual for members of the genus *Pasteurella* and despite the fact that it was the only marine representative of the genus. A later general analysis led to the conclusion that *Pasteurella piscicida* might be closely related to the genus *Vibrio*, but characteristics such as the lack of motility, variable Gram staining reactions, and changing from rods to coccoids were thought to be incompatible with this genus and led to an alternative suggestion that the genus *Arthrobacter* would be a more suitable genus for this organism (21). Finally, on the basis of rRNA cistrons similarity data, *Pasteurella piscicida* appeared to be a member of the family *Vibrionaceae* (7). Probably because of the uncertainty concerning the correct position of the organism, the name *Pasteurella piscicida* did not appear on the Approved Lists of Bacterial Names (22).

Interest in this bacterium increased in 1990 when it affected many different marine fish in Europe. A comparison of strains isolated from the coasts of Europe with strains obtained from Japan and North America showed that these organisms formed a phenotypically, serologically, and genetically homogeneous taxon (15). In the meantime, molecular probes were characterized in order to rapidly identify *Pasteurella piscicida* and to try to prevent spread of the disease (29, 30). In these studies the workers examined probe specificity only with what were thought to be related species (i.e., mostly other *Pasteurella* species) (29, 30). Therefore, it is important that the taxonomic status of *Pasteurella piscicida* be determined with precision, particularly if this bacterium in fact does not belong to the genus *Pasteurella*.

While we were working on a field study of fish pathogens, a bacterium isolated from the environment was identified as *Pasteurella piscicida* by classical methods, but as a member of the family *Vibrionaceae* by small-subunit rRNA sequence analysis. To resolve this inconsistency, we also obtained and analyzed a *Pasteurella piscicida* reference strain. In our study we performed a phylogenetic analysis of the small-subunit rRNA sequences of both strains, a DNA-DNA hybridization analysis, and a biochemical characterization analysis. We found that bacteria formerly known as *Pasteurella piscicida* are indeed members of the family *Vibrionaceae* (19) and that their levels of genetic relatedness to *Photobacterium damsela* should lead to a change in the taxonomic status of these organisms.

MATERIALS AND METHODS

Bacterial strains and bacteriological media. The wild strain of *Pasteurella piscicida* used in this study was isolated from sea bass (*Dicentrarchus labrax*) obtained from the Mediterranean Sea by workers at the Centre National d’Études Vétérinaire et Alimentaires, Laboratoire de Pathologie des Animaux Aquatiques, Biot, France. Reference strain *Pasteurella piscicida* NCIMB 2058T (T = type strain) was obtained directly from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. These two strains were grown at 22°C on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.3 M NaCl or were stored frozen at −70°C in brain heart infusion broth containing 20% (vol/vol) glycerol. *Photobacterium damsela* ATCC 33539T was obtained directly from the American Type Culture Collection, Rockville, Md. This strain was maintained at 22°C.
on marine agar 2216 (Difco) or was stored frozen at -70°C in marine broth containing 20% (vol/vol) glycerol.

**Phenotypic characterization.** Most of the methods used to characterize *Pasteurella piscicida* have been previously described (3–5). The exceptions were the tests performed with API 20E, API 20NE, and API 20CH galleries (API-System; Biomerieux, Marcy l’Etoile, France); in these tests the preparations were supplemented with 0.3 M NaCl and incubated at 22°C. The following tests were assessed by using an electronic microscope, using cells grown in 0.25× and 1× marine broth (Difco) and collected at the mid-exponential and late stationary phases of growth. The cells were negatively stained with phosphotungstic acid by using the method of Jahn (10) and were observed with a Philips model CM2 transmission electron microscope operated at 100 kV.

Assimilation of carbohydrates was verified as described by Jansen and Sargalla (11), and susceptibility to antibiotics was determined as described by Gauthier and Breittmayer (9). The ability to accumulate poly-β-hydroxybutyrate (PHB) as an extracellular reserve product was determined by using the media described by Reichelt and Baumann (18); cells were examined for the presence of PHB granules by phase-contrast microscopy. Whether an organism accumulated PHB was also determined by spectrophotometry (26) by using cells grown under nitrogen-limiting conditions with glucose as the sole source of carbon and energy.

**DNA amplification.** The method used to prepare bacterial DNA for PCR was derived from the method of Sritahan and Barker (25). Bacteria were grown on marine agar and then were suspended in 200 μl of a lysis solution (10 mM Tris [pH 8.0], 1 mM EDTA, 1% Triton X-100) and boiled for 5 min. Following a single chloroform extraction, 5 μl of supernatant was used to amplify small-subunit rRNA genes. To do this, we used two primers, which corresponded to positions 1498 to 1509 of the *Escherichia coli* small-subunit rRNA sequence. An initial denaturation step was performed at 95°C for 180 s; this was followed by annealing at 52°C for 60 s and extension at 72°C for 90 s. The preparation was then subjected to 25 cycles consisting of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s. A final extension step was performed at 72°C for 5 min. This amplification procedure produced 1.5-kb DNA molecules.

**Direct sequencing of PCR products.** After purification on a 1% low-melting-point agarose gel, PCR products were sequenced directly as described previously (19).

**Phylogenetic analysis.** Small-subunit rRNA sequences were aligned and examined by using a set of programs developed in our laboratory (available from R. Christen). The sequences used in the phylogenetic analyses (Fig. 1) were the following conserved regions of the small-subunit rRNA sequences: positions 42 to 74, 93 to 453, 473 to 836, 849 to 1004, 1023 to 1130, and 1137 to 1426 (*Escherichia coli* small-subunit rRNA sequence numbering).

Phylogenetic analyses were performed by using three different methods. A neighbor-joining method (20) was used in a preliminary analysis. The resulting topologies were then further investigated by performing maximum-likelihood and maximum-parsimony analyses. For the maximum-likelihood analyses we used the F12m program rewritten by G. J. Olsen (University of Illinois, Urbana) and compiled on a Hewlett-Packard model 700 workstation, while the maximum-parsimony analyses were performed with the PAUP program for Macintosh computers (27). In the latter case, the analyses were performed by using the branch-and-bound option or the heuristic option when the branch-and-bound option was too time-consuming. The robustness of each topology was evaluated by performing a maximum-parsimony analysis through 100 bootstrap replications (heuristic search). Trees were drawn by using the nplot program for Macintosh computers developed by M. Gouy (Université de la Rive Sud, Lausanne, Switzerland) which allows transformation of a formal tree representation (Newick’s format) into “MacDraw” drawings.

**DNA-DNA hybridization.** DNA was prepared by a procedure adapted from the procedure of Marmar (17). Bacteria were incubated overnight at 25°C with shaking. Cells were collected by centrifugation at 13,000 × g for 20 min and then resuspended in saline-EDTA buffer (0.1 M EDTA, 0.15 M NaCl [pH 8] containing 1.4% sodium dodecyl sulfate. After 5 min of incubation at 60°C, sodium perchlorate (final concentration, 0.8 M) and an equal volume of a chloroform-isooctanol alcohol solution (24:1, vol/vol) were added. The mixture was shaken for 10 min and then centrifuged at 4,300 × g for 20 min. Then 2 volumes of cold (−20°C) ethanol was slowly added to the upper aqueous phase, and the resulting precipitate was collected, resuspended, and digested with RNase (final concentration, 0.6 U/ml) at 37°C for 30 min. Following two chloroform-isooctanol alcohol extractions, the DNA was precipitated with ethanol and resuspended in 10 μl of 1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0); then 0.5 volume of 2-propanol was added drop by drop with slow shaking. The resulting precipitate was washed with 70% ethanol, dried, and dissolved in 0.1× SSC. Absorbance ratios (A260/280 and A260/230) were used to assess the purity of each DNA preparation. After repeated washing by passage through a 1.0-ml column of DNA cleanup resin, the DNA was eluted from the resin by using 5 μl of ultra-pure water. The resulting DNA was used for the radioactive DNA labeling reaction. After denaturation, the DNA was allowed to hybridize with DNA from each isolate. 

**RESULTS**

Small-subunit rRNA sequences and phylogenetic analysis. The small-subunit rRNA sequences of strain NCIMB 2058 and the wild strain of *Pasteurella piscicida* were identical. Because of this, all phylogenetic analyses were performed with a single *Pasteurella piscicida* small-subunit rRNA sequence. Most small-subunit rRNA sequences for *Vibrio* and *Photobacterium* strains have been determined in our laboratory (19). All sequences were aligned by examining the alignment of the sequences in a database containing more than 1,800 aligned eubacterial small-subunit rRNA sequences.

The results of phylogenetic analyses in which we used representatives of all eubacterial phyla and all subdivisions of the *Proteobacteria* confirmed that *Pasteurella piscicida* belonged to a well-defined taxon which is known as the gamma-3 subgroup of the phylum *Proteobacteria*. This taxon includes the families *Enterobacteriaceae*, *Aeromonadaceae*, *Pasteurellaceae*, and *Vibrionaceae* (19, 28). The phylogenetic position of *Pasteurella piscicida* was then investigated in more detail by using only representatives of these four families. The analyses were performed by using three phylogenetic methods: the neighbor-joining method, the maximum-parsimony method, and the maximum-likelihood method. The phylogenetic positions of the bacteria which we investigated were always the same, regardless of which method was used or order of these methods were chosen (results are summarized in Fig. 1). *Pasteurella piscicida* and *Photobacterium damselae* always formed a monophyletic group (tax as determined by all methods; 100% of bootstrap replications). A detailed analysis of Fig. 1 revealed that when we used the maximum-parsimony method and the heuristic search option, we obtained two equally most-parsimonious trees (length, 847; consistency index, 0.466; retention index, 0.761) that differed only in the positions of *Haemophilus aphrophilus* and *Haemophilus ducreyi*. Because the branch-and-bound approach was too time-consuming when the whole set of data was used, this analysis was performed with a reduced set of data (data for 15 taxa representing the four families which we studied). In this analysis we obtained eight equally most-parsimonious trees (length, 492; consistency index, 0.644; retention index, 0.772), all of which confirmed the placement of *Pasteurella piscicida* in the *Vibrionaceae* and the association of this organism with *Photobacterium damselae*; these results completely agreed with the results obtained when we used the neighbor-joining and maximum-likelihood methods. The robustness of the data was also demonstrated by the support of 100% of the bootstrap replications. A direct analysis of DNA
sequences revealed that the rRNA sequences of Pasteurella piscicida and Photobacterium damselfish differed by only one nucleotide among the 1,434 positions sequenced (the nucleotides at one position for Pasteurella piscicida and two positions for Photobacterium damselfish remained undetermined).

Morphological and biochemical description of Pasteurella piscicida. (i) Morphology. The two strains of Pasteurella piscicida which we studied were facultatively anaerobic, gram-negative, straight rods which exhibited bipolar staining, and neither of the strains was bioluminescent. Neither strain produced flagella under the cultural conditions used in this study.

(ii) Growth, halotolerance, and Na+ requirement. The wild Pasteurella piscicida isolate grew in ordinary media supplemented with 0.1 to 0.5 M NaCl, and strain NCIMB 2058T grew in ordinary media containing 0.2 to 0.5 M NaCl; optimal growth occurred in the presence of 0.3 M NaCl. Both strains exhibited an absolute requirement for Na+ since they did not grow in the presence of Na+ concentrations lower than 2 × 10^{-2} M. Growth was optimal in brain heart infusion medium containing 0.3 M NaCl, and the osmotic requirements ranged from 0.5 to 3% NaCl. Pasteurella piscicida grew in appropriate media at 25 and 35°C but not at 4 and 37°C, and at initial pH values of 6 to 9. The two strains accumulated the storage product PHB when they were grown on medium containing glucose, but they were not able to utilize the exogenous monomer dl-3-hydroxybutyrate.

(iii) Biochemical characterization. The two Pasteurella pisc-
Piscicida strains used glucose, mannose, glutamate, adipate, L-proline, and N-acetylgulcosamine as sole carbon sources but did not use xylose, cellobiose, gluconate, acetate, pyruvate, and β-hydroxybutyrate. Glucose, fructose, and mannose were fermented anaerobically by these organisms; galactose, sucrose, and raffinose were weakly fermented; and L-arabinose, D-mannitol, L-rhamnose, D-glucitol, trehalose, D-xylose, maltose, melibiose, lactose, cellobiose, glycerol, and galactitol were not fermented. Oxidase, catalase, phospholipase, arginine decarboxylase, lecithinase, tweenase 80, tweenase 20, and DNAse were not produced. The strains differed only in their ability to hydrolyze starch (the amylase test was negative for strain NCIMB 2058T).

(iv) Susceptibility to antibiotics. Both strains of Pasteurella piscicida were susceptible to chloramphenicol and tetracycline on marine agar and to the vibriostatic agent pteridin O/129 and novobiocin on Mueller-Hinton medium. They were moderately susceptible to neomycin and gentamicin and resistant to streptomycin, kanamycin, and erythromycin on marine agar.

Description of Photobacterium damselae. We also examined the characteristics described above for Photobacterium damselae. Photobacterium damselae differed from Pasteurella piscicida in the following ways: Photobacterium damselae was flagellated and grew at 37°C and at pH values of 5 to 10; the urease and nitrate reductase tests were positive; and xylose, nitrate reductase, agarase, and alginate tests were negative. Gelatin was not liquified, and none of the strains used xylose, cellobiose, gluconate, acetate, pyruvate, and streptomycin, kanamycin, and erythromycin on marine agar.

Accumulation of PHB. The results of the two methods used to study accumulation of PHB (microscopy and spectrophotometry) demonstrated that PHB was accumulated as an intracellular reserve product by Photobacterium damselae, Pasteurella piscicida, Photobacterium leiognathi, and V. hollosae ATCC 33564T. Only the spectrophotometric method proved that V. hollosae ATCC 33508T accumulated PHB (detection of PHB granules by phase-contrast microscopy was not feasible). V. logei and Vibrio fisheri did not accumulate PHB.

DNA-DNA hybridization. Our DNA-DNA hybridization analysis revealed that there was a high level of DNA-DNA relatedness (95%) between the two strains of Pasteurella piscicida tested, which confirmed that they belong to the same species. A level of relatedness of 80% confirmed that Pasteurella piscicida and Photobacterium damselae belong to the same bacterial species.

DISCUSSION

Our small-subunit rRNA sequence analyses revealed unambiguously that Pasteurella piscicida should be affiliated with the family Vibrionaceae, which is consistent with data from previous rRNA cistron similarity studies (7). More precisely, all of the phylogenetic methods used (neighbor joining, maximum likelihood, maximum parsimony) revealed that the relationship between Pasteurella piscicida and Photobacterium damselae is monophyletic. However, two bacteria can be considered members of the same species only when they exhibit a level of genomic DNA relatedness of more than 70% (12). Since the level of complementarity of the DNAs of Pasteurella piscicida NCIMB 2058T and Photobacterium damselae ATCC 33539T was 80%, it is appropriate to consider these organisms members of a single species.

The criteria used to discriminate one subspecies from another differ according to the genus (12). Morphological and biochemical analyses revealed a number of important traits such as the presence of a flagellum and urease and nitrate reductase activities, which could be used to differentiate the organisms which we studied. Thus, strains ATCC 33539T and NCIMB 2058T are different enough to be considered members of different subspecies. Because these strains belong to the family Vibrionaceae, the generic name Pasteurella cannot be retained. Thus, it is appropriate to reject the name Pasteurella piscicida and to rename strain NCIMB 2058T Photobacterium damsela subsp. piscicida comb. nov.; Photobacterium damsela should be renamed Photobacterium damsela subsp. damsela comb. nov. (type strain, ATCC 33539T).

Table 1 summarizes the main phenotypic traits that differentiate Photobacterium species and subspecies, including the two Photobacterium damsela subspecies. All Photobacterium spp. are able to accumulate PHB as an extracellular reserve product, a property that was of considerable taxonomic importance in the definition of the genus Photobacterium (2). On the basis of their ability to grow at either a low temperature (4°C) or a high temperature (35°C), these bacteria could be divided into a psychrotolerant group (Photobacterium phosphoreum, Photobacterium angustum) and a thermotolerant group (Photobacterium leiognathi, Photobacterium damsela subsp. damsela, Photobacterium damsela subsp. piscicida). Photobacterium damsela subsp. piscicida is the only nonflagellated member of the genus Photobacterium.

In a previous phylogenetic analysis based on nearly complete small-subunit rRNA sequences, Ruimy et al. (19) showed that the family Vibrionaceae can be divided into the following three monophyletic groups: (i) V. logei and V. fisheri; (ii) all Photobacterium species plus V. hollosae and V. costicola; and (iii) all remaining Vibrio species. On the basis of the phylogenetic analysis results and results of PHB accumulation analyses, reassignment of V. hollosae and V. costicola to the genus of Photobacterium should now be considered.

In terms of morphology, it is interesting that the presence of a flagellar structure is an important characteristic that can be used to distinguish the two subspecies of Photobacterium damsela. The variability observed in this species weakens the meaning of this structural trait for taxonomic purposes. It also suggests that the presence of flagella could be a rather versatile characteristic and that loss of flagella may result from divergent evolution even in very closely related bacteria. In terms of genetics, we observed one difference between the rRNA sequences of the two subspecies which we investigated. The correlation among species identification, rRNA sequences, and levels of DNA-DNA hybridization has been discussed previously (8), and it is now clear that (i) phylogenetic analysis of rRNA sequences is now the fastest method to determine to which bacterial species a new strain is most closely related, (ii) there is at the present time no reason to believe that a strict relationship should be found for all genera regarding the number of differences observed in the rRNA sequences of two bacteria and their genetic relatedness when the entire genome is considered, and (iii) there is probably no unique criterion for defining a bacterial species, since the definition of a bacterial species is related to its ecophysiology as much as it is to its genetic composition.

Amended description of Photobacterium damsela subsp. piscicida (Snieszko et al. 1964) Gauthier, Lafay, Breittmayer, Ruimy, Nicolas, Gauthier, and Christen comb. nov. Photobacterium damsela subsp. piscicida (L. n. piscis, a fish; L. suff. -cida, from L. v. caedo, to cut or to kill; M.L. n. piscicida, fish killer). Cells are gram-negative, nonflagellated, straight rods which exhibit bipolar staining. Facultatively anaerobic. Accumulates the storage product PHB when cells are grown on medium
containing glucose, but does not utilize the exogenous monomer D,L-β-hydroxybutyrate. Oxidase, catalase, phospholipase, and lecithinase positive. Urease and nitrate reductase negative. No growth occurs in the absence of sodium ions. Not bioluminescent. Does not produce extracellular gelatinase, agarase, phospholipase A, or phospholipase C.

### Table 1. Characteristics that differentiate *Photobacterium damselae* sp. *piscicida* from other *Photobacterium* species and subspecies

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<th>Characteristic</th>
<th><em>Photobacterium damselae</em> subsp. <em>piscicida</em></th>
<th><em>Photobacterium damselae</em></th>
<th><em>Photobacterium leognathi</em></th>
<th><em>Photobacterium angustum</em></th>
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* Data from reference 23 and this study.
* Data from reference 3.
* - negative; +, positive; d, variable; ND, not determined.

### References


