Pseudomonas baetica sp. nov., a fish pathogen isolated from wedge sole, *Dicologlossa cuneata* (Moreau)

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Five Gram-negative bacterial isolates, recovered from an outbreak that occurred in March 2006 in Huelva, Spain, affecting adult diseased cultured wedge sole [*Dicologlossa cuneata* (Moreau)], were characterized phenotypically and genotypically in order to clarify their taxonomic position. On the basis of 16S rRNA gene sequence analysis, the isolates were included in the genus *Pseudomonas*, within the *Pseudomonas fluorescens*-related species group, their closest relatives being the *Pseudomonas jessenii* and *Pseudomonas koreensis* subgroups. The highest sequence similarities were recorded with the type strains of *Pseudomonas reinekei*, *P. moorei*, *P. umsongensis*, *P. jessenii* and *P. mohnii* (99.4–99.3% similarity). Sequence analysis of the housekeeping genes *gyrB* and *rpoD* clearly differentiated the isolates from currently described *Pseudomonas* species, the highest sequence similarities recorded to type strains being below 95% for both genes. Phylogenetic analysis using concatenated sequences of the three genes showed *Pseudomonas moraviensis* DSM 16007^T and *P. koreensis* DSM 16610^T as the closest reference strains. DNA–DNA hybridization assays with related strains confirmed that these isolates belong to a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas baetica* sp. nov. is proposed. The type strain is strain a390^T (=CECT 7720^T =LMG 25716^T). The novel species could be easily distinguished from phylogenetically related species by several phenotypic characteristics, including gelatin hydrolysis, acid production from glucose and growth at 6% NaCl. Virulence assays revealed that the novel species is pathogenic for wedge sole.

Pseudomonas Migula 1894 is a genus of gammaproteobacteria that belongs to the family *Pseudomonadaceae*. These Gram-negative rods display great metabolic diversity that enables them to utilize a wide range of organic compounds and they occupy an important ecological position in the

carbon cycle. Pseudomonads are ubiquitous, being found in a diverse range of ecosystems, and are also important as pathogens of animals and plants (Tryfinopoulou *et al.*, 2002; Bossis *et al.*, 2000; Yamamoto *et al.*, 2000). The genus has undergone repeated taxonomic revision. DNA–rRNA hybridization experiments carried out by Palleroni *et al.* (1973) showed that the genus *Pseudomonas*, as classically defined, in fact consisted of a conglomeration of genera that could be separated clearly into five rRNA similarity groups. Nowadays, only rRNA group I, which contains the type species *Pseudomonas aeruginosa*, is considered the ‘true’ genus *Pseudomonas* or *Pseudomonas sensu stricto*, while the members of the other groups have been reclassified into newly created genera. The genus *Pseudomonas sensu stricto*, composed of several groups, includes both fluorescent and non-fluorescent species (Kerstens *et al.*, 1996; Anzai *et al.*, 1997; Verhille *et al.*, 1999b; Scarpellini *et al.*, 2004).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB* and *rpoD* gene sequences of strains a390^T, a391, a393, a398 and a399 are respectively FM201274, FM201278 and FN678357 (a390^T); FM201275, FM201279 and FN678358 (a391); FN678353, FN678354 and FN678359 (a393); FM201276, FN678355 and FN678360 (a398); and FM201277, FN678356 and FN678361 (a399). Those for the *rpoD* gene sequences of *P. jessenii* DSM 17150^T, *P. reinekei* DSM 18361^T and *P. moorei* DSM 12647^T are respectively FN678364, FN678362 and FN678363.

Four supplementary figures and a supplementary table are available with the online version of this paper.

According to this classification, some *Pseudomonas* species have been reported to be causative agents of fish disease. Among these, *Pseudomonas anguilliseptica* is considered the most significant for cultured fish, having been described from many species, including turbot (Toranzo *et al.*, 2005). However, other species of the genus may also be serious pathogens, such as *Pseudomonas plecoglossicida* in ayu, *Pseudomonas chlororaphis* in amago trout, *P. aeruginosa* in South-American catfish, *Pseudomonas putida* in ayu, rainbow trout and yellowtail and *Pseudomonas fluorescens* in carp, sea bream and tilapia (Austin & Austin, 2007). The present paper reports the description of *Pseudomonas baetica* sp. nov., a novel fish pathogen isolated from wedge sole [*Dicologlossa cuneata* (Moreau)], an economically valuable flatfish species that is being cultured in south-west Spain in order to diversify marine aquaculture.

Samples for bacterial isolation were taken from livers of moribund wedge sole and cultured on *Flexibacter maritimus* medium (FMM) (Pazos *et al.*, 1996) at 20 °C for 24–96 h. Isolates were subcultured onto tryptic soy agar (TSA; Difco). Strains were stored in sterile seawater containing 20 % glycerol at –80 °C. The Gram reaction was determined according to the KOH method proposed by Buck (1982) and by the Gram-staining method. To investigate physiological and biochemical characteristics, we used the methods proposed by Smibert & Krieg (1981) for the following tests: motility, cytochrome oxidase, catalase, oxidation/fermentation (OF), arginine dihydrolase, decarboxylation of lysine and ornithine (Moeller's method), reduction of nitrate, Simmons' citrate, indole production, acid production from lactose, mannose and salicin and the extracellular enzyme activities urease, caseinase, gelatinase, lipase (Tween 80) and amylase and hydrolysis of aesculin. Gas production from glucose was tested according to MacFaddin (1980). Temperature tolerance was tested by checking growth on TSA at 4, 30, 35, 40 and 44 °C and tolerance of salinity was tested with growth on nutrient agar supplemented with 0, 3, 6, 8, 10 and 12 % (w/v) NaCl. Growth on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) was also tested. Fluorescein and pyocyanin production was tested on King media B and A (King *et al.*, 1954), respectively. Haemolysis activity was assayed on Columbia agar with 5 % sheep blood (BD-BBL). All tests were incubated aerobically at 20 °C. Commercial miniaturized API 20E, API 20NE and API ZYM galleries (bioMérieux) were also utilized according to the manufacturer's instructions, with incubation at 20 °C. Carbon utilization tests were carried out using Biolog GN2 MicroPlates as described in the manufacturer's manual. The reference strains *P. aeruginosa* ATCC 27853, *P. fluorescens* CECT 378^T and *P. putida* CECT 385 were included as controls to determine the specificity of the identification system. Drug sensitivity of the isolates was determined on Mueller–Hinton agar by the disc diffusion method using the following chemotherapeutic agents (µg per disc; Oxoid): ampicillin (10), novobiocin (30) and the vibriostatic agent O/129 (10 and 150). Antibiofilm readings were performed

after 48 h incubation at 20 °C. Strains with inhibition diameters greater than 1.5 cm were considered as susceptible.

Five strains (a390^T, a391, a393, a398 and a399) were isolated from the livers of several cultivated adult wedge sole during an outbreak with a moderate mortality rate that occurred in an experimental marine farm located in Huelva, in south-west Spain, in March 2006. Although they were dying, the fish showed no external or internal signs of disease. On the basis of morphological, physiological and biochemical characteristics, pure cultures of the isolates were tentatively included in the genus *Pseudomonas*. All the strains were Gram-negative, oxidase-positive, motile, strictly aerobic, large irregular rods, able to grow in 0–6 % NaCl at 4–30 °C. They were also positive for arginine dihydrolase, gelatinase, fluorescein production and denitrification. Other tests carried out are summarized in the species description. Altogether, the five isolates examined were phenotypically homogeneous, showing only minor discrepancies. The API 20E, API ZYM and Biolog profiles were identical for all five strains except for the results of one (acid from melibiose), three (alkaline phosphatase, esterase C4 and leucine arylamidase) and five (utilization of monomethyl succinate, L-histidine, L-threonine, putrescine and 2-aminoethanol) tests, respectively. According to the Biolog MicroLog3 software, all five isolates were identified as *P. fluorescens* biotype G. However, no amplification fragment was obtained when primers 16SPSEfluF and 16SPSER were used, designed to recognize all *P. fluorescens* biotypes (Scarpellini *et al.*, 2004). On the other hand, several differentiating phenotypic characteristics were found between these strains and closely related *Pseudomonas* species (Table 1). The most discriminatory tests were hydrolysis of gelatin, acid production from glucose, growth at 6 % NaCl, absence/presence of fluorescein and utilization as a sole carbon source of *N*-acetyl-D-glucosamine, formic acid, D-galacturonic acid, D-glucosaminic acid, hydroxyl-L-proline, inosine and uridine.

Since it has been reported recently by Mulet *et al.* (2010) that analysis of three concatenated gene sequences (16S rRNA, *gyrB* and *rpoD*) is sufficient for reliable phylogenetic analysis of the genus *Pseudomonas*, this approach was employed here. For 16S rRNA gene and housekeeping gene sequence analysis, DNA was extracted from a single bacterial colony by boiling for 10 min in distilled water and quantified by spectrophotometry (A_{260}). Partial 16S rRNA, *gyrB* and *rpoD* gene sequences were obtained using universal primers 20F and 1500R, described by Kawasaki *et al.* (1993), UP1 and UP2r, described by Yamamoto & Harayama (1995), and 70F and 70R, described by Yamamoto & Harayama (1998), respectively. The PCR products were purified with the GenElute PCR Clean-up kit (Sigma) and sequenced directly using an ABI Prism 373A DNA Sequencer (Applied Biosystems). PCRs and sequencing were performed generally as indicated in the literature in each case. Sequencing of the *rpoD* gene was also performed for the type strains of *Pseudomonas jessenii*, *Pseudomonas reinekei* and *Pseudomonas moorei*, since these sequences were absent from the GenBank/EMBL databases. Sequence corrections and analysis were

Table 1. Characteristics that differentiate *Pseudomonas baetica* sp. nov. from closely related *Pseudomonas* species

Species: 1, *P. baetica* sp. nov.; 2, *P. moorei*; 3, *P. umsongensis*; 4, *P. mohnii*; 5, *P. reinekei*; 6, *P. jessenii*; 7, *P. fluorescens*; 8, *P. koreensis*; 9, *P. moraviensis*. Data were taken from Verhille *et al.* (1999a, b), Kwon *et al.* (2003), Buller (2004), Tvřzová *et al.* (2006), Cámara *et al.* (2007) and this work. +, 90% or more strains positive; v+, 75–89% strains positive; v, 26–74% strains positive; v–, 11–25% strains positive; –, 0–10% strains positive; (+), weak positive reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Arginine dihydrolase	+	+	+	+	–	–	+	+	–
Nitrate reduction	–	–	+	–	–	+	v	–	–
Hydrolysis of:									
Tween 80	–	–	–	–	–	–	ND	+	ND
Gelatin	+	–	–	–	–	–	+	v+	+
Casein	+	ND	ND	ND	ND	+	ND	–	–
Acid from glucose	+	–	–	–	–	ND	+	–	–
Growth at 6% NaCl	+	–	–	–	–	–	ND	ND	ND
Fluorescein production	+	–	+	–	–	+	+	+	+
Utilization of:*									
Tween 40	+	–	+	–	–	+	+	+	+
Tween 80	+	–	+	–	–	+	+	+	+
N-Acetyl-D-glucosamine	+	–	–	–	–	(+)	+	+	ND
L-Arabinose	+	+	+	+	+	+	+	+	+
D-Arabitol	+	–	v	–	+	+	+	+	+
D-Fructose	+	–	+	–	–	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	v	+	–	+	+	+	+
D-Mannose	+	–	+	(+)	–	+	+	+	+
D-Psicose	–	–	v	–	–	–	–	–	+
Trehalose	–	–	–	–	–	–	+	–	+
Acetic acid	+	–	+	–	+	+	+	+	+
Formic acid	–	–	+	(+)	+	+	–	–	+
D-Galactonic acid lactone	+	+	+	+	+	+	+	+	+
D-Galacturonic acid	–	+	+	+	+	–	+	v	–
D-Glucosaminic acid	–	+	+	(+)(+)	–	+	+	+	+
D-Glucuronic acid	–	+	v	+	–	–	+	v	–
α -Hydroxybutyric acid	–	–	+	–	(+)	–	+	+	+
p-Hydroxyphenylacetic acid	–	–	–	–	–	+	–	–	–
α -Ketobutyric acid	–	–	+	–	–	–	+	v	ND
α -Ketoglutaric acid	+	+	+	+	+	+	+	–	+
α -Ketovaleric acid	–	–	+	–	–	(+)	–	+	+
Malonic acid	+	–	+	(+)(+)	+	+	+	+	+
Propionic acid	+	–	+	(+)(+)	+	+	+	+	+
Quinic acid	+	+	+	+	+	+	+	+	+
D-Saccharic acid	+	+	+	+	+	+	+	+	+
Succinamic acid	–	–	–	–	–	+	+	–	+
Glucuronamide	–	–	–	–	–	–	+	+	ND
L-Alaninamide	–	–	+	–	(+)	+	–	+	+
D-Alanine	+	(+)	+	+	–	+	+	+	+
L-Alanyl glycine	+	–	+	–	–	+	+	+	+
Glycyl L-glutamic acid	–	–	+	–	–	–	+	+	ND

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
L-Histidine	v+†	+	+	–	–	+	+	+	+
Hydroxy-L-proline	–	+	v	+	–	+	–	+	+
L-Leucine	+	–	+	–	–	+	+	+	+
L-Ornithine	–	–	+	–	–	+	+	+	–
L-Pyrogutamic acid	+	+	+	–	+	+	+	v	+
L-Threonine	v+†	–	+	–	–	+	+	+	+
γ -Aminobutyric acid	+	+	+	+	–	+	+	+	+
Inosine	+	–	–	–	–	–	+	+	+
Uridine	+	–	–	–	–	–	+	+	+
Phenylethylamine	–	–	+	(+)	+	+	–	–	–
Putrescine	v+†	(+)	+	(+)	–	+	+	+	ND
2-Aminoethanol	v+†	(+)	+	–	(+)	+	+	+	+
DL- α -Glycerol phosphate	–	–	–	–	–	+	+	–	+

*Data for utilization of substrates as carbon sources for *P. jessenii* and *P. fluorescens* were obtained in this work using the type strains of these species.

†Type strain negative.

performed with the DNASTAR Seqman program (Lasergene). Sequences of phylogenetically related species were obtained after BLAST (<http://blast.ncbi.nlm.nih.gov/>) and EzTaxon (<http://www.eztaxon.org/>; Chun *et al.*, 2007) searches. Phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood algorithms (Tamura *et al.*, 2007; Posada, 2008; FigTree version 1.1.2, <http://tree.bio.ed.ac.uk/>). Distance matrices were calculated using Kimura's two-parameter correction. Stability of groupings was estimated by bootstrap analysis (1000 replicates) using the program MEGA version 4.0 (Tamura *et al.*, 2007).

Phylogenetic analysis based on 16S rRNA gene sequences placed the isolates in rRNA group I (*Pseudomonas sensu stricto*), specifically within the *P. fluorescens*-related species group, their closest relatives being the *Pseudomonas jessenii* and *Pseudomonas koreensis* subgroups. However, all the strains were clearly differentiated from other *Pseudomonas* species, grouping in a separate cluster (Figs 1 and S1, available in IJSEM Online). According to the EzTaxon database, the closest relatives were *P. jessenii* CIP 105274^T (99.5% similarity), *P. moorei* DSM 12647^T (99.4%), *P. reinekei* DSM 18361^T (99.3%), *P. umsongensis* KACC 10847^T (99.3%), *P. mohnii* DSM 18327^T (99.3%), *P. moraviensis* DSM 16007^T (99.2%), and *P. koreensis* DSM 16610^T (99.2%). When using BLAST, the type strains *Pseudomonas Vancouverensis* DSM 17555^T and *P. migulae* CIP 105470^T and reference strains *P. fluorescens* ATCC 17573, *P. fluorescens* Pf0-1 and *P. putida* ATCC 17522 also showed similarity above the limit of intraspecific variability (98.7%) proposed by Stackebrandt & Ebers (2006). Nevertheless, in previous studies of the genus *Pseudomonas*, it has been shown that strains with 16S rRNA gene sequence similarities greater than even 99% may belong to different species (Uchino *et al.*, 2001; Behrendt *et al.*, 2003; Hauser *et al.*,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains of *Pseudomonas baetica* sp. nov. and related members of the genus *Pseudomonas*. *P. anguilliseptica* NCIMB 1949^T and *P. aeruginosa* ATCC 10145^T were used as an outgroup. The stability of groupings was estimated by bootstrap analysis of 1000 replicates; numbers at nodes show percentage bootstrap values. Similar tree topology was obtained with the maximum-likelihood algorithm (Fig. S1). Bar, 0.005 substitutions per nucleotide position.

2004). Sequence similarities between the five wedge-sole isolates under study were 99.9–100 %.

Phylogenetic analyses based on housekeeping genes were more discriminative. The highest similarities between *gyrB*

gene sequences were to *Pseudomonas marginalis* ATCC 10844^T (94.6 %), *P. fluorescens* ATCC 17467 (94.5 %), *P. fluorescens* Pf0-1 (93.8 %) and *P. koreensis* DSM 16610^T (93.4 %), other strains showing values below 93 %. The phylogenetic tree derived from these sequences illustrates

the position of the five isolates under study, clearly separated from other *Pseudomonas* species (Fig. S2). *gyrB* gene sequence analysis is considered a highly reliable tool for bacterial species determination (Harayama & Yamamoto, 1996; Yamamoto & Harayama, 1996, 1998; Yamamoto *et al.*, 1999, 2000; Soler *et al.*, 2004; Zeigler, 2003). In fact, there is a high correlation between phylogenetic distance based on *gyrB* sequences and total genome relatedness as analysed by DNA–DNA hybridization, the limit for intraspecific variability being 0.041 (Yamamoto *et al.*, 1999). The genetic distance of the five isolates under study was 0.000–0.004, showing values with other related strains above 0.060. According to *rpoD* gene sequences, the closest relatives were *P. fluorescens* ATCC 17573 (95.1 % similarity) and Pf0-1 (91.3 %), *P. moraviensis* LMG 24280^T (92.5 %) and *P. koreensis* LMG 21318^T (91.4 %), other strains displaying similarity of 90 % or lower. The phylogenetic tree derived from *rpoD* sequences is shown in Fig. S3.

Phylogenetic trees based on concatenated sequences of the 16S rRNA gene and the two housekeeping genes (*gyrB* and *rpoD*) confirmed the clustering of the five isolates with high bootstrap values and their distinction from currently known *Pseudomonas* species (Figs 2 and S4). Concatenated sequences of the five isolates shared 99.6–100 % similarity. The closest type strains were *P. moraviensis* DSM 16007^T and *P. koreensis* DSM 16610^T (95.7 %). The reference strain *P. fluorescens* ATCC 17573 also showed high similarity (96.5 %), but the *P. fluorescens* type strain did not (93.7 %). Nevertheless, all these strains showed similarity with 107 *Pseudomonas* species below the limit of intraspecific variability (97 %) observed by Mulet *et al.* (2010) using the same three genes. In addition, our results suggest that some reference strains of *P. fluorescens* (Pf0-1 and ATCC 17573) are misidentified, supporting previous findings (Goris *et al.*, 2007; Mulet *et al.*, 2010).

For DNA–DNA hybridization assays, DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen) and the concentration and purity of each sample were determined by measuring the A_{260} and A_{260}/A_{280} ratio, respectively. DNA–DNA hybridization assays were performed by the plate method proposed by Ziemke *et al.* (1998), combining the hydroxyapatite method with non-radioactive detection of released DNA. The hybridization temperature (T_m) was 65 °C. Levels of DNA–DNA relatedness were determined between strain a390^T and the other four isolates and the type strains *P. moraviensis* LMG 24280^T, *P. koreensis* LMG 21318^T, *P. jessenii* DSM 17150^T, *P. reinekei* DSM 18361^T, *P. moorei* DSM 12647^T, *P. migulae* DSM 17966^T and *P. fluorescens* ATCC 13525^T. In addition, reciprocal hybridizations were performed with the most closely related type strains.

DNA–DNA hybridization experiments with other *Pseudomonas* type strains, selected by consideration of phenotypic and gene sequence analysis, were done in duplicate and confirmed the results obtained previously. Levels of DNA reassociation between strain a390^T and the reference

Pseudomonas strains were all less than the threshold value for species delineation of 70 % (Wayne *et al.*, 1987) and ranged from 39.4 ± 4.5 to 60.4 ± 1.4 % (Table 2). Values obtained in reciprocal hybridizations were 44.7, 45.3, 45.3 and 47.6 % for the type strains of *P. reinekei*, *P. migulae*, *P. jessenii* and *P. moorei*, respectively. The DNA–DNA hybridization values with the other four wedge-sole isolates were above this value and ranged from 74 to 96 %, proving that these isolates belong to a unique species (Table 2).

Preparation of fatty acid methyl esters (FAMES) from strain a390^T and the type strains of its closest relatives grown at 25 °C on TSA-1 plates was performed according to the instructions of the Microbial Identification System (MIDI) as described by Sasser (1990). FAMES were analysed by gas chromatography in an Agilent 6850 GC system equipped with a split injector, flame-ionization detector and a fused silica column (30 m $\times$ 0.2 mm $\times$ 0.33 mm). The operating system was Chemstation (Agilent) and Sherlock MIS 6.0 (MIDI) and the aerobic bacteria library TSBA6/RTSBA6.10 was utilized as the MIDI database. The temperature program ramped from 170 to 288 °C at 28 °C min⁻¹ with a split ratio of 40 : 1 and head pressure of 144.8 kPa. FAME analysis was conducted on three samples of each strain, cultured and treated under identical conditions. FAME composition was consistent with previously published results for members of the genus *Pseudomonas* (*Pseudomonas* rRNA group I; Cámara *et al.*, 2007), with saturated and unsaturated C₁₆ and C₁₈ fatty acids being the predominant compounds (Table S1). Discrimination fell at the level of 2-OH, 3-OH and 17 : 0 cyclo ω 7c fatty acids, as demonstrated previously for species within this group (Cámara *et al.*, 2007). The considerable amount of 12 : 0 fatty acids and the detection of only trace amounts of 14 : 0 fatty acids supported the inclusion of strain a390^T, together with its closest neighbours, within intragenic cluster II defined by Yamamoto *et al.* (2000).

The virulence of strain a390^T, selected for experimental infections, was tested by two routes of infection: intraperitoneal injection and immersion. Two groups of 10 healthy wedge sole, weighing approximately 2 and 15 g, were challenged by intraperitoneal injection with 10⁶ cells per fish, while two other groups of 10 fish weighing approximately 4 g were challenged by bath immersion for 18 h, as described previously by Avendaño-Herrera *et al.* (2006), with a bacterial suspension of 10⁷ cells ml⁻¹. A control group of 10 fish (challenged with PBS) was included in each virulence assay. After bacterial challenge, experimental and control fish were kept in 18 l tanks at 20 °C in continually flowing seawater and mortalities were recorded daily for 18 days. Dead fish were removed and subjected to standard bacteriological examination. The Ethics Committee for Animal Experiments of the University of Santiago de Compostela approved the protocols of this study.

Strain a390^T caused significant mortality to wedge sole by intraperitoneal injection (100 % mortality 3 days after exposure) but not by bath immersion (10 % mortality within 18 days). Experimentally infected fish did not show

Fig. 2. Neighbour-joining phylogenetic tree based on concatenated sequences of the housekeeping genes *gyrB* and *rpoD* and the 16S rRNA gene, showing the position of strains of *P. baetica* sp. nov. *P. anguilliseptica* NCIMB 1949^T and *P. aeruginosa* ATCC 10145^T were used as an outgroup. The stability of groupings was estimated by bootstrap analysis of 1000 replicates; numbers at nodes show percentage bootstrap values. Similar tree topology was obtained with the maximum-likelihood algorithm (Fig. S4). Bar, 0.01 substitutions per nucleotide position.

clinical signs of disease, but the inoculated strain was recovered from all dead fish. None of the control fish died during the various assays.

In summary, results from phenotypic characterization, 16S rRNA and housekeeping gene sequence analysis and DNA–DNA hybridization confirm that the five wedge-sole isolates represent a novel *Pseudomonas* species that is pathogenic for fish, for which the name *Pseudomonas baetica* sp. nov. is proposed, with a390^T as the type strain.

Description of *Pseudomonas baetica* sp. nov.

Pseudomonas baetica (ba.e'ti.ca. L. fem. adj. *baetica* of or belonging to Hispania Baetica, the name of the former province of the Roman Empire covering the south-west region of Spain).

Cells are Gram-negative, motile, large irregular rods. Colonies on TSA are small (up to approx. 0.5 mm in diameter after 48 h of incubation), round and whitish. Metabolism is oxidative. Cytochrome oxidase and catalase

Table 2. DNA–DNA hybridization between *Pseudomonas baetica* sp. nov. a390^T and isolates a391, a393, a398 and a399 and type strains of closely related *Pseudomonas* species

DNA–DNA hybridization experiments performed with type strains of other *Pseudomonas* species were done in duplicate. Reciprocal values with reference type strains are given in parentheses.

Strain	Hybridization (%)
<i>P. baetica</i> sp. nov. a390 ^T	(100)
a391	96
a393	84
a398	74
a399	91
<i>P. moraviensis</i> LMG 24280 ^T	49.9 ± 1.0
<i>P. koreensis</i> LMG 21318 ^T	46.7 ± 2.2
<i>P. moorei</i> DSM 12647 ^T	60.4 ± 0.4 (47.6)
<i>P. reinekei</i> DSM 18361 ^T	60.4 ± 1.4 (44.7)
<i>P. jessenii</i> DSM 17150 ^T	57.9 ± 6.9 (45.3)
<i>P. migulae</i> DSM 17966 ^T	50.9 ± 4.1 (45.3)
<i>P. fluorescens</i> ATCC 13525 ^T	39.4 ± 4.5

are produced. Growth occurs at 4–30 °C in the presence of 0–6 % NaCl. Fluorescein pigment is produced, but pyocyanin is not. Arginine dihydrolase is produced, but lysine and ornithine are not decarboxylated. Nitrate to nitrite reduction test is negative, but denitrification to N₂ gas is positive. Does not grow on TCBS agar. Voges–Proskauer, indole and H₂S production and ONPG (β -galactosidase) tests are negative. Simmons' citrate test is positive. Haemolytic on blood agar. Known strains hydrolyse gelatin and casein, but not starch, Tween 80, aesculin or urea. Acid is produced from glucose and mannose, but not from mannitol, inositol, sorbitol, rhamnose, sucrose, amygdalin, arabinose, lactose or salicin. Acid production from melibiose is variable (type strain negative). Gas is not produced from glucose. The following substrates are utilized as sole carbon sources for growth (Biolog GN2): Tweens 40 and 80, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-fructose, D-galactose, α -D-glucose, D-mannitol, D-mannose, methyl pyruvate, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-gluconic acid, β -hydroxybutyric acid, α -ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-leucine, L-proline, L-pyroglyutamic acid, L-serine, γ -aminobutyric acid, urocanic acid, inosine, uridine and glycerol. The following substrates are not used: α -cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, adonitol, cellobiose, i-erythritol, L-fucose, gentiobiose, *myo*-inositol, lactose, lactulose, maltose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, formic acid, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid,

α -ketovaleric acid, sebacic acid, succinamic acid, glucuronamide, L-alaninamide, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-ornithine, L-phenylalanine, D-serine, DL-carnitine, thymidine, phenylethylamine, 2,3-butanediol, DL- α -glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Variable results for utilization of L-histidine, L-threonine, putrescine, 2-aminoethanol and monomethyl succinate (type strain negative). The following enzymes are produced (API ZYM): esterase lipase (C8) and acid phosphatase; most strains, including the type strain, produce alkaline phosphatase, esterase (C4) and leucine arylamidase but not α -fucosidase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase or α -mannosidase. The cellular fatty acid composition is dominated by saturated and unsaturated C₁₆ and C₁₈ fatty acids (type strain profile available in Table S1). Sensitive to (μ g per disc) novobiocin (30) and resistant to the vibriostatic agent O/129 (10 and 150) and ampicillin (10).

The type strain is a390^T (=CECT 7720^T =LMG 25716^T), isolated in 2006 from the liver of a diseased wedge sole on the south-west coast of Spain and pathogenic for this flatfish species. A reference strain is a391 (=CECT 7721 =LMG 25717), isolated during the same outbreak.

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