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Pseudomonas plecoglossicida sp. nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*

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A new *Pseudomonas* species, for which the name *Pseudomonas plecoglossicida* is proposed, was isolated from cultured ayu (*Plecoglossus altivelis*) with bacterial haemorrhagic ascites. The causative agent was similar to *Pseudomonas putida* biovar A in its phenotypic characteristics and on the basis of 16S rRNA gene sequence analysis, but it reduced nitrate to nitrite. Furthermore, it was distinguished phenotypically from *Pseudomonas putida* biovar A by utilization of D-malate, L-(+)-tartrate, *m*-tartrate and nicotinate. The levels of DNA–DNA hybridization between the isolate strain FPC 951^T and other reference strains of *Pseudomonas* species, including *Pseudomonas putida*, were less than 50%. The G+C content of the DNA of FPC 951^T was 62·8 mol%. Strain FPC 951^T (= ATCC 700383^T) is designated the type strain of the new species.

Keywords: *Pseudomonas plecoglossicida*, taxonomy, fish pathogen, ayu, *Plecoglossus altivelis*

INTRODUCTION

Since the early 1990s, a new bacterial disease has caused mass mortalities among pond-cultured ayu (*Plecoglossus altivelis*) in Japan. Wakabayashi *et al.* (1996) named it bacterial haemorrhagic ascites (BHA) of ayu, because bloody ascites commonly occurred. Recently, pejerrey (*Odonthestes bonariensis*) farmed in Japan has also been affected with BHA. A pseudomonad has always been isolated from the internal organs of the diseased fish. The isolates, when inoculated artificially, had high mortality to ayu and could be reisolated from the inoculated fish.

The organisms are Gram-negative, aerobic, rod-shaped bacteria, motile by several polar flagella. They are cytochrome-oxidase-positive and oxidize but do not ferment glucose. Most of the isolates have the API 20 NE profile 1-140-457, which is not found in the API profile database (API 20NE analytical profile index, 5th edition; BioMérieux) (Wakabayashi *et al.*, 1996).

Some *Pseudomonas* species have been reported to be causative agents of fish disease: *Pseudomonas anguilliseptica* was described as a pathogen in eel (*Anguilla japonica*) (Wakabayashi & Egusa, 1972) and ayu (Nakai *et al.*, 1985), *Pseudomonas fluorescens* in carp (Shiose *et al.*, 1974), sea bream (*Evynnis japonica*) (Kusuda *et al.*, 1974) and tilapia (*Sarotherodon niloticus*) (Miyashita, 1984), *Pseudomonas chlororaphis* in amago trout (*Onchorhynchus rhodurus*) (Hatai *et al.*, 1975) and *Pseudomonas putida* in yellowtail (*Seriola quinqueradiata*) (Kusuda & Toyoshima, 1976).

The genus *Pseudomonas* is recognized as a large and widely diverse bacterial group (Palleroni *et al.*, 1973). Up to the present, many arguments about the taxonomy of this genus have been put forward (Segers *et al.*, 1994; Tamaoka *et al.*, 1987; Willems *et al.*, 1989, 1990; Yabuuchi *et al.*, 1990, 1992, 1995). Its classification has been basically done with phenotypic characterization (Stanier *et al.*, 1966) and nucleic acid homology (Palleroni *et al.*, 1973). Palleroni *et al.* (1973) subdivided *Pseudomonas* species into five main groups on the basis of rRNA–DNA hybridization. According to this classification, all fish-pathogenic *Pseudomonas* belong to rRNA group I, which has been recently regarded as *Pseudomonas sensu stricto* (Kersters *et al.*, 1996).

In this paper, we present evidence that the isolates from ayu with BHA constitute a new species on the basis of phenotypic characteristics and molecular

The DDBJ accession number for the 16S rRNA gene sequence of *Pseudomonas plecoglossicida* FPC 951^T is AB009457.

genetic methods, 16S rRNA gene (rDNA) sequence analysis and DNA–DNA hybridization. We propose the name *Pseudomonas plecoglossicida* sp. nov. for the bacterium.

METHODS

Bacterial strains. The following six bacterial strains studied were isolated from ayu with BHA: strains FPC 951^T (= ATCC 700383^T) and FPC 952 isolated in March 1994 in Tokushima Prefecture, strains FPC 941 (= ATCC 700384) and FPC 940 in January 1994 in Shiga Prefecture, strain FPC 975 in August 1995 in Nagano Prefecture, and strain FPC 976 in June 1991 in Wakayama Prefecture, Japan. The strains were isolated by streaking portions of kidney tissues on tryptic soy agar (TSA). The bacteria were stored in tryptic soy broth (TSB) containing 10 % glycerol at −80 °C. All of the results were based on cultures incubated at 25 °C unless otherwise noted.

Phenotypic characterization. Cell morphology was recorded from light microscope observations of a Gram-stained smear preparation and also from electron microscope observations of a negatively stained preparation.

Biochemical characteristics, firstly, were determined with commercially produced kit systems (API 20 NE and API ZYM; BioMérieux). To investigate further physiological and biochemical characteristics, we used methods described by Stanier *et al.* (1966) and Palleroni & Doudoroff (1972) for the following tests: the oxidase reaction, the accumulation of poly-β-hydroxybutyrate, levan formation from sucrose, the egg-yolk reaction, the production of extracellular lipase, hydrolysis of gelatin and starch, and denitrification. The temperature range for growth was examined in TSA using a temperature gradient incubator (TN-3; Toyo kagaku sangyo) for 7 d. Fluorescent pigment was observed on King medium A and King medium B (Eiken Chemical) (King *et al.*, 1954). Arginine dihydrolase was determined in Møller's decarboxylase base (Difco Laboratories) (MacFaddin, 1980). Carbon source utilization tests were performed in the basal medium of Stanier *et al.* (1966) and Véron (1975) by using 96-well microplates described by Ishimaru *et al.* (1995). Growth was determined at 25 °C for 7 d and growth greater than the control without carbon source was regarded as positive.

DNA extraction. Chromosomal DNA was extracted by a slightly modified procedure of Wilson (1987). Five hundred milligrammes wet weight bacterial cells was resuspended in 11·3 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0). Cells were lysed by 10 % SDS and proteinase K (20 mg ml^{-1}; Sigma). After cetyltrimethylammonium bromide/NaCl treatment, the DNA was further purified by chloroform/isoamyl alcohol (24:1, v/v), phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and RNase A (10 mg ml^{-1}; Sigma). The DNA concentration and purity of each DNA sample were determined by measuring the A_{260} and A_{260}/A_{280} ratio, respectively.

16S rDNA sequence determination. The 16S rRNA genes were amplified using PCR (Saiki *et al.*, 1988) with *Taq* polymerase (Toyobo) and the universal primer pair of 20F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1500R (5′-GGT TAC CTT GTT ACG ACT T-3′) described by Weisburg *et al.* (1991). PCR amplifications were performed in 50 µl reaction mixture containing buffer, 1·5 mM $MgCl_2$, 0·5 nmol each deoxynucleoside triphosphate, 10 pmol each primer, 2·5 U *Taq* polymerase and the template DNA, by using the DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer) with the following temperature profile: an initial denaturation at 94 °C for 2 min; 30 cycles of denaturation (2 min at 94 °C), annealing (1·5 min at 50 °C) and extension (2 min at 72 °C); and a final extension at 72 °C for 2 min. The PCR products purified with a Microcon-100 microconcentrator (Amicon) were sequenced directly using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequence reaction mixtures were electrophoresed and analysed with an Applied Biosystems model 377A DNA sequencer.

Phylogenetic analysis. The 16S rDNA sequences determined were then aligned with reference sequences obtained from databases using the CLUSTAL W program (Thompson *et al.*, 1994). Pairwise evolutionary distances of them were calculated using Kimura's two-parameter model (Kimura, 1980). A phylogenetic tree from distance matrices was constructed by the neighbour-joining method (Saitou & Nei, 1987), and the topology of it was evaluated using a CLUSTAL W bootstrap analysis with 1000 replicates.

DNA–DNA hybridization. DNA homology determination followed the quantitative dot blot hybridization method described by Ezaki *et al.* (1988) using photobiotin (Vector laboratories) for labelling DNA probes and streptavidin–alkaline phosphatase conjugate (Gibco-BRL) and the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (Kirkegaard & Perry Lab.) for detecting hybridized spots, according to the protocols supplied by the manufacturers. Quantification of hybridized spots was determined essentially as described by Hiraishi *et al.* (1991) using a densitograph imaging analyser (type AE-6920-MFS; Atto) for measuring the spots. Each hybridization experiment was done twice.

DNA base composition. The G+C content of DNA was determined by the thermal denaturation method (Marmur & Doty, 1962). DNA from *Escherichia coli* strain K-12 was used as a standard.

Nucleotide sequence accession numbers. The 16S rRNA gene sequence of *Pseudomonas plecoglossicida* FPC 951^T has been deposited in DDBJ under accession number AB009457. The strain designations and accession numbers of 16S rRNA gene sequences of other species that were compared with the FPC 951^T sequence in this study are as follows: *Pseudomonas aeruginosa* LMG 1242^T (Z76651), *Pseudomonas asplenii* LMG 2137^T (Z76655), *Pseudomonas aureofaciens* DSM 6698^T (Z76656), *Pseudomonas chlororaphis* LMG 5004^T (Z76657), *Pseudomonas flavescens* B62^T (U01916), *Pseudomonas fluorescens* IAM 12022^T (D84013), *Pseudomonas mendocina* LMG 1223^T (Z76664), *Pseudomonas putida* biovar A DMS 291^T (Z76667), *Pseudomonas putida* biovar B MnB1 (U70977), *Pseudomonas stutzeri* ATCC 17591 (U26261), *Escherichia coli* MRE 600 (J01859), *Burkholderia cepacia* ATCC 25416 (M22518), *Comamonas testosteroni* ATCC 11996 (M11224), *Brevundimonas diminuta* ATCC 11568 (M59064) and *Stenotrophomonas maltophilia* ATCC 13637 (M59158).

RESULTS

Phenotypic characteristics

Six isolates had the same phenotypic properties. Cells were Gram-negative rods measuring 0·5–1 × 2·5–4·5 µm. They were motile by polar multitrichous

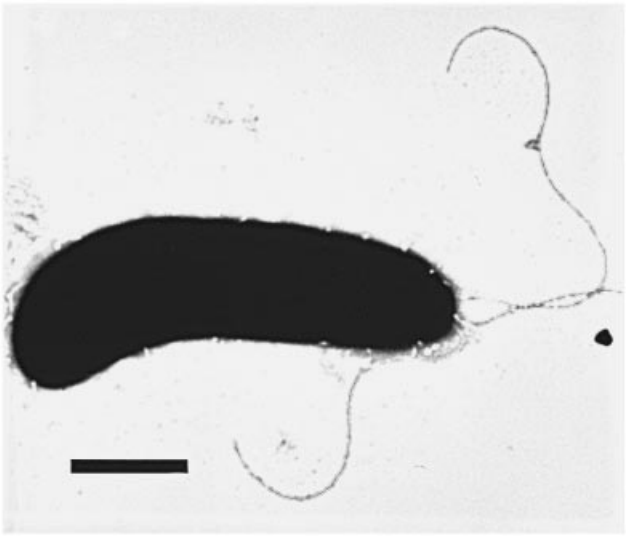

Fig. 1. Electron micrograph of negatively stained cells of *Pseudomonas plecoglossicida* FPC 951^T. Bar, 1 μm.

flagella (Fig. 1), and sometimes single or no flagellated cells were observed. On tryptic soy medium, growth occurred at 10–30 °C in the presence of 0–5% NaCl, and at pH 5–9. Metabolism was respiratory, not fermentative. The cytochrome oxidase test was positive. Nitrate reduction was positive, but denitrification was negative. Fluorescent pigment was produced weakly on King medium B. A summary of phenotypic properties is shown in Table 1.

16S rRNA gene sequence analysis

Two strains of the isolate, FPC 951^T and FPC 941, were selected to use for 16S rRNA gene sequence analysis. *Pseudomonas plecoglossicida* FPC 951^T had the same 16S rDNA sequence as strain FPC 941. A phylogenetic tree derived from 16S rRNA gene sequences was used to illustrate the position of *Pseudomonas plecoglossicida* FPC 951^T and other *Pseudomonas* species belonging to rRNA groups I–V in Fig. 2. It indicated that *Pseudomonas plecoglossicida* FPC 951^T was placed in rRNA group I. The similarities (%) in 16S rDNA sequences of *Pseudomonas plecoglossicida* FPC 951^T with other closely related *Pseudomonas* species belonging to rRNA group I were: *Pseudomonas aeruginosa* LMG 1242^T, 95·9%; *Pseudomonas asplenii* LMG 2137^T, 98·4%; *Pseudomonas aureofaciens* DSM 6698^T, 97·1%; *Pseudomonas chlororaphis* LMG 5004^T, 97·1%; *Pseudomonas flavescens* B62^T, 98·2%; *Pseudomonas fluorescens* IAM 12022^T, 96·2%; *Pseudomonas mendocina* LMG 1223^T, 97·4%; *Pseudomonas putida* biovar A DMS 291^T,

Table 1. Characteristics for differentiating *Pseudomonas plecoglossicida* from other *Pseudomonas* species

Data for all *Pseudomonas* species other than *Pseudomonas plecoglossicida* are from Palleroni (1984). +, 90% or more strains are positive; −, 90% or more strains are negative; d, 11–89% of strains are positive.

Characteristic	*P. plecoglossicida*	*P. aeruginosa*	*P. fluorescens*	*P. chlororaphis*	*P. aureofaciens*	*P. putida* bv. A	*P. putida* bv. B	*P. stutzeri*	*P. mendocina*
No. of flagella	>1	1	>1	>1	>1	>1	>1	1	1
Fluorescent pigments	+	d	d	+	+	+	d	−	−
Growth at 4 °C	−	−	d	+	+	d	+	−	−
Growth at 41 °C	−	+	−	−	−	−	−	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	−	+
Denitrification	−	+	d	+	−	−	−	+	+
Levan formation from sucrose	−	−	d	+	+	−	−	−	−
Gelatin liquefaction	−	+	+	+	+	−	−	−	−
Lecithinase (egg yolk)	−	−	d	+	d	−	−	−	−
Lipase (Tween 80 hydrolysis)	−	±	d	+	d	d	d	+	+
Utilization of:									
Mannitol	−	+	d	+	+	d	d	d	−
D-Xylose	−	−	d	−	−	d	d	−	−
L-Arabinose	−	−	d	−	+	d	+	−	−
D-Mannose	−	−	d	+	+	d	d	−	−
Sucrose	−	−	d	+	d	−	d	−	−
Trehalose	−	−	d	+	d	−	−	−	−
2-Ketogluconate	+	+	d	+	d	d	+	−	−
D-Malate	+	d	d	d	−	d	d	d	d
D(−)-Tartrate	−	−	d	−	−	d	d	−	−
L(+)-Tartrate	−	−	d	d	−	d	d	−	d
m-Tartrate	−	−	d	−	−	d	−	−	−
m-Inositol	−	−	d	+	+	−	−	−	−
Propylene glycol	+	+	d	−	−	d	+	+	+
Phenyl acetate	+	+	d	d	+	d	+	−	−
Testosterone	−	−	−	−	−	−	+	−	−
L-Lysine	+	+	d	d	d	+	d	−	−
L-Citrulline	+	d	d	d	d	d	d	−	−
L-Tryptophan	−	d	d	+	+	−	+	−	−
L-Kynurenine	−	+	d	+	+	−	+	−	−
Anthranilate	−	+	d	+	+	−	+	−	−
Nicotinate	−	−	d	−	−	d	+	−	−
DNA mol% G+C	62·8	67·2	59·4–61·3	63·5	63·6	62·5	60·7	60·6–66·3	62·8–64·3

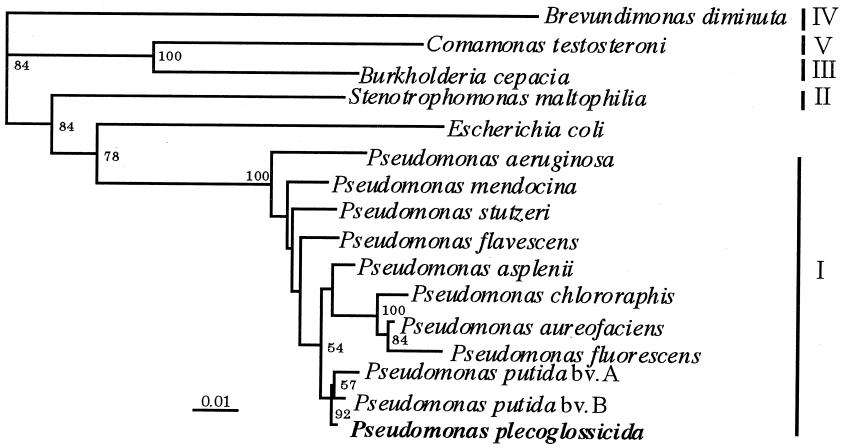

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of *Pseudomonas plecoglossicida*, *E. coli*, and other species belonging to *Pseudomonas* rRNA groups I–V. Roman numerals on the right indicate the number of the *Pseudomonas* rRNA group described by Palleroni *et al.* (1973). The tree was generated by the neighbour-joining method. Numbers at nodes show the levels of bootstrap support based on data for 1000 replicates; only values that are more than 50% are shown. Bar, 0·01 substitution per nucleotide position.

Table 2. Percentage DNA–DNA hybridization between *Pseudomonas plecoglossicida* sp. nov. strains FPC 951^T and FPC 941 and closely related *Pseudomonas* species

Species	Strain*	DNA–DNA hybridization (%)	
		FPC 951^T	FPC 941
Pseudomonas plecoglossicida	FPC 951^T	100	
	FPC 941	96	100
	FPC 975	94	108
	FPC 976	96	104
Pseudomonas putida bv. A	ATCC 12633^T	47	47
	IAM 1218	24	26
	IAM 1235	32	33
Pseudomonas putida bv. B	ATCC 17472	33	35
	ATCC 23483	40	40
Pseudomonas asplenii	ATCC 23835^T	21	20
Pseudomonas aureofaciens	IAM 12353	22	24
Pseudomonas chlororaphis	IAM 12354^T	41	37
Pseudomonas flavescens	ATCC 51555^T	22	21
Pseudomonas fluorescens	IAM 12022^T	37	39
Pseudomonas mendocina	JCM 5966^T	23	23
Pseudomonas stutzeri	IAM 12668^T	21	19

*FPC, Fish Pathogen Collection, University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Manassas, VA, USA; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; JCM, Japan Collection of Microorganisms, The Institute for Physical and Chemical Research (RIKEN), Saitama, Japan.

98·9%; *Pseudomonas putida* biovar B MnB1, 99·6%; *Pseudomonas stutzeri* ATCC 17591, 97·8%.

DNA–DNA hybridization

DNA–DNA hybridization experiments were performed with other *Pseudomonas* species selected by consideration of phenotypic characteristics and 16S rRNA gene sequence analysis. The reference *Pseudomonas* species and the results in this experiment are given in Table 2. Levels of DNA similarity between the two isolated strains and the reference *Pseudomonas* species were all less than 50%, and ranged from 19 to 47% (Table 2). Four isolates obtained from different places showed more than 90% DNA homology.

DNA base composition

The G+C content of *Pseudomonas plecoglossicida* FPC 951^T was 62·8 mol% as determined by the thermal denaturation procedure.

DISCUSSION

The six isolates examined in this study were found to represent one homogeneous group of bacteria and are thought to belong to a single species. They were placed in the genus *Pseudomonas* (Palleroni, 1984) on the basis of their phenotypic characteristics.

According to *Bergey's Manual of Systematic Bacteriology* (Palleroni, 1984), the new species is classified

phenotypically by the following key characteristics: poly-β-hydroxybutyrate is not accumulated as a carbon reserve material, fluorescent pigment is produced, and arginine dihydrolase is present. Although the bacterium is phenotypically similar to *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens* and *Pseudomonas putida*, it can be clearly distinguished from them all, except *Pseudomonas putida*, by gelatin liquefaction (Table 1).

The results of comparative analysis of the 16S rRNA gene sequence showed that *Pseudomonas plecoglossicida* FPC 951^T belonged to rRNA group I, which is the genus *Pseudomonas sensu stricto* (Fig. 2) (Kersters *et al*., 1996). Furthermore, the species which had the highest similarity with *Pseudomonas plecoglossicida* FPC 951^T was *Pseudomonas putida* biovar B, strain MnB1 (= ATCC 23483) (99·6%), and the next was *Pseudomonas putida* biovar A, strain IAM 1236^T (= ATCC 12633^T) (98·6%). From the phylogenetic tree (Fig. 2) and the levels of 16S rDNA similarity, there is no doubt that *Pseudomonas plecoglossicida* FPC 951^T is assigned to rRNA group I and is most closely related to *Pseudomonas putida*.

Concerning the 16S rRNA gene sequence, around 20 different *Pseudomonas putida* sequences have been registered in the DDBJ database. Most of them are rather short, with lengths about 1330 bp. Moore *et al*. (1996) reported that 1344 nucleotide positions were conserved in all *Pseudomonas* species, so we selected ~1450 bp sequences, not those shorter ones, for 16S rDNA sequence analysis in this study. The level of 16S rDNA similarity between *Pseudomonas plecoglossicida* FPC 951^T and *Pseudomonas putida* biovar B MnB1 was more than 99%, but they were clearly different in phenotypic characteristics (Table 1) and DNA–DNA homology (Table 2). Some papers report that 16S rDNA sequences are so conservative that they are appropriate for determining inter- and intrageneric relationships but not useful in defining species (Fox *et al*., 1992; Stackebrandt & Goebel, 1992).

DNA–DNA hybridization was performed with *Pseudomonas plecoglossicida* FPC 951^T and FPC 941 and reference strains selected on the basis of phenotypic characterization and 16S rDNA similarity. To investigate highly related organisms at the species level, DNA–DNA hybridization experiments are indispensable. At present, the phylogenetic definition of a species generally includes strains with approximately 70% or greater DNA–DNA relatedness (Wayne *et al*., 1987). DNA–DNA hybridization revealed that *Pseudomonas putida* biovar A ATCC 12633^T had the highest homology with *Pseudomonas plecoglossicida* FPC 951^T and FPC 941, but its value was 47% (Table 2). From this result, we conclude that *Pseudomonas plecoglossicida* FPC 951^T and FPC 941 belong to a new *Pseudomonas* species.

The species *Pseudomonas putida* shows wide diversity phenotypically and genetically (Palleroni & Doudoroff, 1972; Elomari *et al*., 1994). In particular, *Pseudomonas putida* biovar A is fairly heterogeneous with respect to DNA homology and other genetic properties, but relatively homogeneous with respect to phenotypic properties (Palleroni & Doudoroff, 1972). The species, for example, was classified into one or two groups by commercialized systems phenotypically (Grimont *et al*., 1996), while it was classified into more than four groups by ribotyping (Brosch *et al*., 1996), and had 3% diversity in 16S rRNA gene sequence genetically (Moore *et al*., 1996). There has been room for argument on the grouping of *Pseudomonas putida* (Barrett *et al*., 1986) as stated above. Our new isolate is sure to be close to *Pseudomonas putida*, but it can be distinguished by the following characteristics: a positive nitrate reduction (Elomari *et al*., 1994) and no utilization of D-malate, L-(+)-tartrate, *m*-tartrate or nicotinate as sole carbon source divide it from *Pseudomonas putida* biovar A; while no growth at 4 °C and no use of L-arabinose, testosterone, L-tryptophan, L-kynurenine, anthranilate or nicotinate as sole carbon source divide it from *Pseudomonas putida* biovar B (Table 1).

In addition to the differences above, results from the 16S rDNA sequence analysis and DNA–DNA hybridization confirm that the organism is different from all *Pseudomonas* species previously published and should be recognized as a new species, for which we propose the name *Pseudomonas plecoglossicida* sp. nov.

Description of *Pseudomonas plecoglossicida* sp. nov.

Pseudomonas plecoglossicida (ple.co.glos.si.ci′da. M.L. masc. n. *Plecoglossus* systematic name of a fish genus; L. suffix masc. n. *-cida* killer; M.L. masc. n. *plecoglossicida* the killer of *Plecoglossus*, the genus to which ayu belongs).

Cells are Gram-negative rods (0·5–1 × 2·5–4·5 μm), and are motile by means of polar multitrichous flagella, and in some cases exhibit single or no flagellum. Intracellular granules of poly-β-hydroxybutyrate are not accumulated. Metabolism is oxidative. Growth occurs at temperatures between 10 and 30 °C in the presence of 0–5% (w/v) NaCl. Catalase and cytochrome oxidase are produced. Fluorescent pigment is produced weakly on King medium B. Hydrolysis of gelatin, starch and Tween 80 is negative. Lecithinase is not produced. Arginine dihydrolase is produced. Lysine and ornithine are not decarboxylated. These organisms are not able to form levan from sucrose. They reduce nitrate to nitrite but do not denitrificate it to N_2 gas. They are haemolytic on blood agar. They utilize the following substrates as sole carbon sources for growth: caprate, succinate, citrate, L-alanine, β-alanine, L-aspartate, L-glutamate, L-arginine, L-tyrosine, glucose, D-fructose, gluconate, 2-ketogluconate, D-malate, propylene glycol, ethanol, n-butanol, phenyl acetate, glycine, L-leucine, L-valine, L-lysine, L-ornithine, L-citrulline, L-histidine and

L-phenylalanine. They do not use the following compounds as sole carbon sources: maltose, cellobiose, lactose, starch, inulin, L-threonine, mannitol, D-xylose, L-arabinose, L-rhamnose, D-mannose, D-galactose, sucrose, trehalose, adipate, D-(−)-tartrate, L-(+)-tartrate, *m*-tartrate, erythritol, sorbitol, *m*-inositol, adonitol, geraniol, phenol, testosterone, L-tryptophan, L-kynurenine, anthranilate or nicotinate. The following enzymes are produced (determined by using API ZYM): alkaline phosphatase, caprylate esterase (C_8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and phosphoamidase. The following enzymes are not produced (determined by using API ZYM): butyrate esterase (C_4), myristate lipase (C_{14}), cystine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. The G+C content of the DNA is 62·8 mol%. The type strain is FPC 951^T; it has been deposited in the American Type Culture Collection, Manassas, VA, USA, as strain ATCC 700383^T. This strain was isolated in 1994 from bacterial haemorrhagic ascites of ayu (*Plecoglossus altivelis*) in Tokushima Prefecture, Japan. One additional strain, FPC 941 (= ATCC 700384), which was isolated in 1994 from the diseased ayu in Shiga Prefecture in Japan, is a reference strain of this species.

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REFERENCES

Barrett, E. L., Solanes, R. E., Tang, J. S. & Palleroni, N. J. (1986). *Pseudomonas fluorescens* biovar V: its resolution into distinct component groups and the relationship of these groups to other *P. fluorescens* biovars, *P. putida*, and to psychrophilic pseudomonads associated with food spoilage. *J Gen Microbiol* **132**, 2709–2721.

Brosch, R., Lefèvre, M., Grimont, F. & Grimont, P. A. D. (1996). Taxonomic diversity of pseudomonads revealed by computer-interpretation of ribotyping data. *Syst Appl Microbiol* **19**, 541–555.

Elomari, M., Izard, D., Vincent, P., Coroler, L. & Leclerc, H. (1994). Comparison of ribotyping analysis and numerical taxonomy studies of *Pseudomonas putida* biovar A. *Syst Appl Microbiol* **17**, 361–369.

Ezaki, T., Dejsirilert, S., Yamamoto, H., Takeuchi, N., Liu, S. & Yabuuchi, E. (1988). Simple and rapid genetic identification of *Legionella* species with photobiotin-labeled DNA. *J Gen Appl Microbiol* **34**, 191–199.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Grimont, P. A. D., Vancanneyt, M., Lefèvre, M., Vandemeulebroecke, K., Vauterin, L., Brosch, R., Kersters, K. & Grimont, F. (1996). Ability of Biolog and Biotype-100 systems to reveal the taxonomic diversity of the pseudomonads. *Syst Appl Microbiol* **19**, 510–527.

Hatai, K., Egusa, S., Nakajima, M. & Chikahata, H. (1975). *Pseudomonas chlororaphis* as a fish pathogen. *Bull Jpn Soc Sci Fish* **41**, 1203.

Hiraishi, A., Hoshino, Y. & Satoh, T. (1991). *Rhodoferax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the '*Rhodocyclus gelatinosus*-like' group. *Arch Microbiol* **155**, 330–336.

Ishimaru, K., Akagawa-Matsushita, M. & Muroga, K. (1995). *Vibrio penaeicida* sp. nov., a pathogen of kuruma prawns (*Penaeus japonicus*). *Int J Syst Bacteriol* **45**, 134–138.

Kersters, K., Ludwig, W., Vancanneyt, M., Vos, P. D., Gillis, M. & Schleifer, K. (1996). Recent changes in the classification of the pseudomonads: an overview. *Syst Appl Microbiol* **19**, 465–477.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

King, E. O., Ward, M. K. & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**, 474–477.

Kusuda, R. & Toyoshima, T. (1976). Characteristics of a pathogenic *Pseudomonas* isolated from cultured yellowtail. *Fish Pathol* **11**, 133–139.

Kusuda, R., Toyoshima, T. & Nishioka, J. (1974). Characteristics of a pathogenic *Pseudomonas* isolated from cultured crimson sea breams. *Fish Pathol* **9**, 71–78.

MacFaddin, J. F. (1980). *Biochemical Tests for Identification of Medical Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Miyashita, T. (1984). *Pseudomonas fluorescens* and *Edwardsiella tarda* isolated from diseased tilapia. *Fish Pathol* **19**, 45–50.

Moore, E. R. B., Mau, M., Arnscheidt, A., Böttger, E. C., Hutson, R. A., Collins, M. D., Peer, Y. V. D., Wachter, R. D. & Timmis, K. N. (1996). The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intrageneric relationships. *Syst Appl Microbiol* **19**, 478–492.

Nakai, T., Hanada, H. & Muroga, K. (1985). First records of *Pseudomonas anguilliseptica* infection in cultured ayu, *Plecoglossus altivelis*. *Fish Pathol* **20**, 481–484.

Palleroni, N. J. (1984). Genus I. *Pseudomonas* Migula 1894, 237^{AL} (nom. cons. opin. 5, jud. comm. 1952, 237). In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 141–199. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Palleroni, N. J. & Doudoroff, M. (1972). Some properties and taxonomic subdivisions of the genus *Pseudomonas*. *Annu Rev Phytopathol* **10**, 73–100.

Palleroni, N. J., Kunisawa, R., Contopoulou, R. & Doudoroff, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *Int J Syst Bacteriol* **23**, 333–339.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Segers, P., Vancanneyt, M., Pot, B., Torck, U., Hoste, B.,

Dewettinck, D., Falsen, E., Kersters, K. & Vos, P. D. (1994). Classification of *Pseudomonas diminuta* Leifson and Hugh 1954 and *Pseudomonas vesicularis* Büsing, Döll, and Freytag 1953 in *Brevundimonas* gen. nov. as *Brevundimonas diminuta* comb. nov. and *Brevundimonas vesicularis* comb. nov., respectively. *Int J Syst Bacteriol* **44**, 499–510.

Shiose, J., Wakabayashi, H., Tominaga, M. & Egusa, S. (1974). A report on a disease of cultured carp due to a capsulated *Pseudomonas*. *Fish Pathol* **9**, 79–83.

Stackebrandt, E. & Goebel, B. M. (1992). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966). The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol* **43**, 159–271.

Tamaoka, J., Ha, D. M. & Komagata, K. (1987). Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. *Int J Syst Bacteriol* **37**, 52–59.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Véron, M. (1975). Nutrition et taxonomie des Enterobacteriaceae et bacteries voisines. I. Methods d'étude des auxanogrammes. *Ann Microbiol Inst Pasteur* **126A**, 267–274.

Wakabayashi, H. & Egusa, S. (1972). Characteristics of a *Pseudomonas* sp. from an epizootic of pond-cultured eels (*Anguilla japonica*). *Bull Jpn Soc Sci Fish* **38**, 577–587.

Wakabayashi, H., Sawada, K., Ninomiya, K. & Nishimori, E. (1996). Bacterial hemorrhagic ascites of ayu caused by *Pseudomonas* sp. *Fish Pathol* **31**, 239–240.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Weisburg, W. G., Barns, M. S., Pelletier, A. D. & Lane, J. D. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Willems, A., Busse, J., Goor, M. & 8 other authors (1989). *Hydrogenophaga*, a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and '*Pseudomonas carboxyflava*'), and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). *Int J Syst Bacteriol* **39**, 319–333.

Willems, A., Falsen, E., Pot, B., Jantzen, E., Hoste, B., Vandamme, P., Gillis, M., Kersters, K. & Ley, J. D. (1990). *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *Int J Syst Bacteriol* **40**, 384–398.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1.–2.4.2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.

Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T. & Yamamoto, H. (1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* **34**, 99–119.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251–1275.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H. & Nishiuchi, Y. (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol Immunol* **39**, 897–904.