***Pseudoalteromonas peptidolytica* sp. nov., a novel marine mussel-thread-degrading bacterium isolated from the Sea of Japan**

Kasthuri Venkateswaran† and Nobuhiko Dohmoto

Author for correspondence: Kasthuri Venkateswaran. Tel: +1 818 393 1481. Fax: +1 818 393 4176.
e-mail: kjenkat@jpl.nasa.gov

Nippon Suisan Kaisha Ltd,
Central Research
Laboratory, Hachioji City,
Tokyo 192, Japan

A new bacterial species belonging to the genus *Pseudoalteromonas* is described on the basis of phenotypic characterization, and sequence analysis of its 16S rRNA-coding and gyrase B (*gyrB*) genes. Ten strains, isolated from sea water of Yamato Island, Sea of Japan, were Gram-negative, yellow, motile, polarly flagellated, aerobic, rod-shaped eubacteria and had a G+C content of 42 mol%. Analysis of the 16S rDNA sequence revealed a clear affiliation between these strains and members of the γ -Proteobacteria. High similarity values were found with members of the genus *Pseudoalteromonas* and this was supported by fatty acid profiles. The 16S rDNA sequence similarity between strain F12-50-A1^T and *Pseudoalteromonas piscicida* was very high (99.1%). However, molecular characterizations employing small subunit 16S rDNA sequences were at the limits of resolution for the differentiation of species in this genus. As a result, DNA–DNA hybridization and sequence analyses of a more rapidly evolving *gyrB* gene were performed. Our assertion that this strain represents a distinct bacterial species within the genus *Pseudoalteromonas* was supported by both of these molecular analyses. Species-specific PCR probes were designed for the *gyrB* gene and used for the rapid screening of F12-50-A1^T-like strains, thereby confirming the species. As these strains cleave complex protein compounds of the *Mytilus edulis* foot by secreting proteases, the name *Pseudoalteromonas peptidolytica* sp. nov. is proposed, with strain F12-50-A1^T (= MBICC F1250A1^T) as the type strain.

Keywords: *Pseudoalteromonas peptidolytica*, phylogeny, marine mussel thread, *gyrB*, 16S rDNA

INTRODUCTION

Marine biofouling due to sedentary animals poses serious problems by increasing fluid frictional resistance of ship hulls and in pipelines, clogging of fishing nets and corrosion (Costerton *et al.*, 1987). Previous work has shown that the permanent adhesive of *Mytilus edulis* is a protein containing large amounts of hydroxyproline (13%) and 3,4-dihydroxyphenyl-alanine (Dopa, 11%). Laboratory experiments have shown that treatment of the protein with clostridial

collagenase reduces the molecular mass by 10%. Although trypsin treatment extensively degraded this polyphenolic protein, the major tryptic peptide contains 10-amino-acid (Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys) repeats. This non-degradable peptide sequence was reported to be repeated as often as 75 times in the polyphenolic protein of *M. edulis* (Waite, 1983). Isolation, purification and characterization of extracellular products of a novel *Pseudoalteromonas* strain F12-50-A1^T that are responsible for the degradation of polyphenolic protein are reported elsewhere (N. Dohmoto & K. Venkateswaran, data not shown).

Despite the applied microbiological relevance and diversity of function embodied by the *Pseudoalteromonas* group, the phylogenetic breadth and organization of this group remains incompletely addressed (Baumann *et al.*, 1984; Gauthier *et al.*,

† **Present address:** Jet Propulsion Laboratory, NASA M/S 89-5, Planetary Protection Technologies, 4800 Oak Grove Dr., Pasadena, CA 91109, USA.

Abbreviation: Dopa, 3,4-dihydroxyphenyl-alanine.

The GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper are given in Table 3.

1995). Introduced as *Pseudomonas* (Buck *et al.*, 1963), the genus has undergone several name changes. In 1972, four species were assigned to the genus *Alteromonas* and since then, the genus *Alteromonas* has been used as a refuge for Gram-negative, heterotrophic, aerobic bacteria with a single polar flagellum which is typical of *Pseudomonas* members except for the low G+C content (38–50 mol %). However, with recent improvements in the applicability of molecular approaches for microbial phylogeny, efforts are underway to clarify the phylogenetic organization of this genus. One result has been the reassessment of the distinctions between the genera *Alteromonas*, *Pseudoalteromonas*, *Shewanella* and *Moritella* (Gauthier *et al.*, 1995). Gauthier *et al.* (1995) described 13 species in the genus *Pseudoalteromonas*. To this day, the *Pseudoalteromonas* group remains, almost by default, the phenotypic resting place for a growing conglomerate of non-fermentative aerobes associated with marine habitats. The phenotypic boundaries of and within this group remain ill-defined for lack of a diagnostically informative pattern of phenotypic characteristics. Furthermore, a number of novel species and *Candidatus* have been recently introduced including an anti-fouling-compound-producing *Pseudoalteromonas tunicata* (Holmstrom *et al.*, 1998), a saprophytic *Pseudoalteromonas bacteriolytica* (Sawabe *et al.*, 1998) and two psychrophilic species, *Pseudoalteromonas antarctica* (Bozal *et al.*, 1997) and *Pseudoalteromonas prydzensis* (Bowman, 1998).

While the gene sequence of the small subunit of the 16S rRNA molecule is accepted for the definition of phylogenetic relationships between organisms (Woese, 1987), this molecule at times lacks the specificity required for the differentiation of close relatives (Fox *et al.*, 1992; Venkateswaran *et al.*, 1998, 1999; Yamada *et al.*, 1999). Nine of 17 well-described species of the genus *Pseudoalteromonas* showed 16S rDNA sequence similarities of >98 %. To circumvent this limitation, the more rapidly evolving *gyrB* gene (Edgell & Doolittle, 1997) has been used as a high-resolution molecular identification marker for distinguishing strains of *Vibrio* (Venkateswaran *et al.*, 1998), the *Bacillus cereus* group (Yamada *et al.*, 1999), *Acinetobacter* (Yamamoto *et al.*, 1999), *Pseudomonas* (Yamamoto & Harayama, 1995, 1996), *Shewanella* (Venkateswaran *et al.*, 1999) and now *Pseudoalteromonas*.

METHODS

Bacterial strains and growth conditions. Micro-organisms included in this study were isolated from the waters of the Sea of Japan using the *R/V Sohegen Maru*. Other strains were purchased from various culture collections. The isolation of marine mussel-thread-degrading bacteria was carried out as described by Dohmoto *et al.* (1993). For routine cultivation, cells were grown aerobically in marine broth (Difco) at 25 °C.

Preparation and degradation of mussel thread. Mussel thread was prepared by following the procedures of

Dohmoto *et al.* (1993). Briefly, mussel threads were removed from the bivalve and, after repeated washing and drying, threads were treated with ethyl acetate and ethanol before rinsing with distilled water. Filter-dried mussel thread was used as sole carbon substrate (0.5 %, w/v) throughout the period of the study. The degradation potential was calculated after appropriate incubation conditions (Dohmoto *et al.*, 1993) in terms of the dry weight reduction from the initial weight of the added mussel thread. Specific activity was measured by the ninhydrin method (Sarath *et al.*, 1989).

Phenotypic characterization. Routine biochemical tests were carried out according to established procedures (Baumann *et al.*, 1984). The ability to grow at an NaCl concentration of 1–10 % was determined in 1 % (w/v) sterile tryptone water supplemented with the appropriate NaCl concentration, and the ability to grow without NaCl was determined in 1 % tryptone water. Sugars and amino acids were tested on a minimal medium at a concentration of 1 % as described elsewhere (Baumann *et al.*, 1984). Additional phenotypic characteristics were determined by the BIOLOG microbial identification system. For the determination of cell shape and size and the detection of flagella, cells were negatively stained with osmium chloride according to the methods of Cole & Popkin (1981) and observed with an H-600 Hitachi transmission electron microscope.

Fatty acid analysis. Cells were cultivated overnight in marine broth (Difco) at 30 °C with vigorous shaking. Cellular fatty acids were extracted from lyophilized cells, methylated and analysed by GC (Ringelberg *et al.*, 1994). GC conditions and verification of peaks were as described elsewhere (Ringelberg *et al.*, 1994).

Molecular characterization

(i) DNA extraction and DNA–DNA hybridization. Purified genomic DNA (Johnson, 1981) from liquid-grown cultures was used as the template for PCR amplification unless otherwise specified. PCR assays were performed in a DNA Thermal Cycler (Perkin Elmer). The G+C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization was carried out as described by Huß *et al.* (1983) and Escara & Hutton (1980).

(ii) 16S rDNA. Universal eubacterial primers (Bact 11 and 1492; *Escherichia coli* numbering) were used to amplify the 1.5 kb PCR fragment according to the protocols established by Ruimy *et al.* (1994). Amplicons thus generated were purified and sequenced as described below.

(iii) *gyrB* gene. Primers (UP-1 and UP-2r) within the known DNA sequence (Yamamoto & Harayama, 1995) were added to the PCR mixture at a concentration of 1 µM and the solution was subjected to 30 cycles of PCR (Venkateswaran *et al.*, 1998). PCR-amplified 1.2 kb *gyrB* products were sequenced directly following purification on Qiagen columns. The PCR-amplified products were checked by agarose gel electrophoresis (Venkateswaran *et al.*, 1998). Suitable molecular size markers were included in each gel.

(iv) Sequencing. The identity of a given PCR product was verified by sequencing using the dideoxy chain termination method with a Sequenase DNA sequencing kit (United States Biochemical) and with an ABI 373A automatic sequencer as recommended by the manufacturer (Perkin Elmer).

(v) Phylogenetic analysis and sequence alignment. The phylogenetic relationships of organisms covered in this study were

determined by comparison of individual 16S rDNA sequences with the sequences deposited in the ribosomal database. *gyrB* sequences were compared with ca. 500 other *gyrB* sequences in the database maintained by our laboratory and S. Harayama (Marine Biotechnology Institute, Kamaishi, Iwate, Japan). Evolutionary trees were constructed with the PAUP (Swofford, 1990), PHYLIP (Felsenstein, 1990) and ARB program packages (Strunk & Ludwig, 1995). Nucleotide sequence accession numbers are given in Table 3.

(vi) *Pseudoalteromonas peptidolytica*-specific primers. Oligonucleotide primers (PP-1, forward primer, 5' ACG ACG TCA AGT CAT CAT GG; PP-2r, reverse primer, 5' GTA AGC GAA TCA CTT AAA GT) based on the *gyrB* gene sequence of the strain F12-50-A1^T were synthesized (Beckman) according to manufacturer's instructions. These primers were able to amplify a 205 bp fragment. When whole bacterial cells were used as templates for PCR in the absence of a DNA extraction step, freshly grown cells from agar plates or cells from liquid culture that had been centrifuged and washed in PBS [0.1 M, pH 7.5, containing 2% (w/v) NaCl] were used. In some experiments, DNA was purified and used as a template. PCR assays were performed in a DNA Thermal Cycler (Perkin Elmer). PCR amplification of a *P. peptidolytica*-specific 205 bp fragment was performed by using 30 cycles, each consisting of 1 min at 94 °C, 1.5 min at 58 °C, 2.5 min at 72 °C and a final extension step at 72 °C for 7 min. Following DNA amplification, the 205 bp amplicon was analysed by submarine gel electrophoresis, stained and visualized under UV illumination. Suitable molecular size markers were included in each gel.

RESULTS

Degradation of marine mussel thread and synthetic decapeptide

Of 1000 strains screened, only ten strains showed 35% degradation of mussel thread (Dohmoto *et al.*, 1993). Strain F12-50-A1^T exhibited a specific activity 2295 units mg⁻¹ against the marine mussel thread and this accounted for a degradation of 65% in terms of dry weight reduction. Similarly, the crude extracellular products of strain F12-50-A1^T showed high marine mussel thread degradation (50–70%).

Further characterization of ten strains that showed marine mussel thread degrading ability was carried out. Phenotypic characterization (ten strains), fatty acid methyl ester analysis (three strains) and molecular analysis including 16S rDNA, *gyrB* sequences and DNA–DNA hybridization (one strain) were performed using various sets of strains. A species-specific primer based on the *gyrB* gene of strain F12-50-A1^T was designed and the identity of the species was checked for all ten strains.

Morphological and physiological characteristics

Strain F12-50-A1^T and the other nine strains were Gram-negative, rod-shaped, non-spore-forming organisms. They grew well at 35 °C in marine broth. Cells are 2–3 µm in length and 0.7–0.8 µm in diameter,

Fig. 1. Transmission electron micrograph of *Pseudoalteromonas peptidolytica* showing its morphology and flagellar position. Bar, 1 µm.

and are motile by means of a single unsheathed polar flagellum (Fig. 1). On marine agar incubated at 35 °C, young colonies are yellow and circular, with a diameter of 1–2 mm, smooth and convex with regular edges. Biochemical characterization of ten strains of these marine mussel-thread-degrading bacteria is presented in Table 1. Strains were positive for cytochrome oxidase, catalase, gelatinase, lipase, amylase and alginase and negative for the production of arginine dihydrolase and decarboxylases of lysine and ornithine. They did not show denitrification. They were unable to ferment glucose and hydrogen sulfide was not produced from thiosulfate. Indole and ketone were not produced. The carbon substrate profile of F12-50-A1^T and the nine other strains, as measured by the BIOLOG system, showed an identification match for *Pseudoalteromonas*. Phenotypically, these strains resembled *Pseudoalteromonas luteoviolacea*.

Optimum growth conditions

These strains grew at 15–37 °C (with optimum growth at 30–35 °C) and at pH 6–10 (optimum 6–7). No growth was seen in the absence of sodium ions. As these strains were isolated from the pelagic waters of the Sea of Japan, they are halophilic and grew at NaCl concentrations of 1–10%, with a good yield at 6%.

Table 1. Biochemical characteristics of pigmented species of *Pseudoalteromonas*

Strains: 1, *P. peptidolytica* ($n = 10$); 2, *P. aurantia* ATCC 33046^T; 3, *P. citrea* NCIMB 1889^T; 4, *P. luteoviolacea* ATCC 33492^T; 5, *P. piscicida* ATCC 15057^T; 6, *P. rubra* ATCC 29570^T; 7, *P. tunicata* CCUG 26757^T. All strains are straight rods; grow at 25 °C and 1–3 % NaCl; produce oxidase and gelatinase; utilize D-glucose and N-acetylglucosamine; do not denitrify; are non-luminescent; do not produce β -galactosidase, arginine dihydrolase or lysine and ornithine decarboxylases; do not utilize D-galactose, lactose, sucrose, salicin, D-mannitol, glycerol, citrate, DL-lactate, γ -aminobutyrate, sarcosine, putrescine, α -ketoglutarate, D-sorbitol, DL-malate, acetate, *m*-hydroxybenzoate or methanol as sole carbon source.

Characteristic	1*	2	3	4	5	6	7
Growth at temperature:							
4 °C	0	+	–	–	–	–	+
35 °C	90	–	+	+	–	+	–
40 °C	90	–	+	–	–	–	–
Growth at 6–10 % NaCl	100			–	–		
Production of:							
Amylase	100	+	+	+	–	+	
Chitinase	0	–	+	–	–	–	
Lipase	100	+	+	+	–	+	
Alginase	90	–	–	–	–	–	
Utilization of:							
D-Mannose	0	+	+	–	+	+	+
D-Fructose	0	+	+	–	–	+	–
Maltose	100	–	–	+	–	+	+
Succinate	100	–	–	–	–	–	
Fumarate	100	–	–	–	–	–	
Pigmentation	Yellow	Yellow	Yellow	Purple	Yellow	Red	Green
G + C content (mol %)	41–42	38–43	41–45	42	42	46–48	42–43

* The percentages of positive strains (out of 10) are shown.

Cellular fatty acid composition

Strain F12-50-A1^T and two other strains tested contained straight-chain, terminally branched and mono-unsaturated fatty acids with a composition of $24 \pm 1\%$, $55 \pm 3\%$ and $15 \pm 3\%$, respectively (Table 2). Among those acids measured, palmitoleic (16:1 ω 7c) and palmitic acid (16:0) were the major fatty acids in F12-50-A1^T. Fatty acid methyl ester analysis of one of the closely related species, *Pseudoalteromonas piscicida*, showed similar profiles. Our results are in agreement with fatty acid methyl ester profiles for *Pseudoalteromonas antarctica* (Bozal *et al.*, 1997), *P. tunicata* (Holmstrom *et al.*, 1998) and seven other type strains of *Pseudoalteromonas* species (Svetashev *et al.*, 1995). Because of the similar fatty acid composition, fatty acid methyl ester profiles could not differentiate between the *Pseudoalteromonas* species.

Molecular phylogenetic analysis

The marine mussel-thread-degrading strains isolated during this study closely resembled *P. luteoviolacea* by conventional phenotypic characterization and fatty acid methyl ester profiles (Svetashev *et al.*, 1995). However, these strains were the only pseudoalteromonads showing degradation of marine mussel

thread. To confirm the species identity, the molecular phylogeny of this strain was analysed.

(i) 16S rDNA sequence analysis. The 16S rDNA sequences of all known γ -*Proteobacteria* were compared with that of F12-50-A1^T. All phylogenetic analyses of its 16S rDNA sequence unambiguously demonstrated that F12-50-A1^T belonged to the phylum γ -*Proteobacteria*. Their phylogenetic relationships were then analysed, and this study was repeated with several different subdomains of the 16S rDNA sequence and bootstrapping analysis was performed to avoid sampling artifacts. The resulting analysis indicated that F12-50-A1^T shared a close phylogenetic relationship with the species of *Pseudoalteromonas*, *Alteromonas*, *Shewanella* and *Vibrio*. Neighbour-joining, parsimony and maximum-likelihood analyses were then undertaken on this subset of bacteria, using several subdomains of the 16S rDNA. In all analyses, F12-50-A1^T was most closely associated with members of the genus *Pseudoalteromonas*.

The similarities in the 16S rDNA nucleotide sequences between F12-50-A1^T and 17 *Pseudoalteromonas* species were between 89 and 99 % (Table 3). A variation of only 1 % was found between F12-50-A1^T and *P. piscicida*, and 3 % between F12-50-A1^T and

Table 2. Fatty acid composition of *P. peptidolytica* and related species

Fatty acid	Fatty acid methyl esters (%) produced in strains:			
	<i>P. peptidolytica</i>			<i>P. piscicida</i>
	F12-50-A1 ^T	F5-100-B2	F5-100-C1	ATCC 15057 ^T
10:0	0.93	0.71	1.02	0.68
10:0 3OH	2.16	1.91	1.94	1.62
Unknown 11.798	1.64	1.72	1.64	1.28
Unknown 12.486	0.09			0.12
11:0		0.10		
11:0 3OH		0.61	0.77	
11:0 iso 3OH	0.12			
12:0	1.85	2.12	1.91	1.86
12:0 iso 3OH	0.22	0.44	0.18	0.16
12:1 3OH	0.11	0.16	0.20	0.09
12:0 3OH	6.14	5.96	5.01	6.35
13:1	0.14			
14:0	3.11	2.91	2.01	4.35
15:1 ω 8c	0.35	0.69	0.93	
15:0 iso				
15:0		0.87	0.90	
16:0 iso	0.12	0.21		0.15
16:1 ω 9c	0.87	1.21	1.34	1.53
16:0	17.46	16.68	17.21	20.65
17:1 ω 8c	0.19	2.11	1.92	
17:0		0.67	0.61	
18:1 ω 7t	3.03			5.81
18:1 ω 9c	0.39	0.63	0.55	0.50
18:0	0.93	1.18	1.21	1.18

Pseudoalteromonas rubra. A very high variation (11 %) was noticed between F12-50-A1^T and *P. bacteriolytica*. Such a high degree of variation within a well-described genus is uncommon. This value is somewhat close to the variation seen between F12-50-A1^T and *Alteromonas macleodii* (12.5 %).

A phylogenetic tree based on 16S rDNA sequences is shown in Fig. 2. The branching order of this tree shows two distinct clusters in which *P. bacteriolytica* evolved earlier than the rest with a distinct stock adjacent to *A. macleodii*. Non-pigmented strains exhibited five sub-clusters and pigmented strains showed four sub-clusters: two individual clades for *P. prydzensis* and *P. tunicata*; one clade composed of *Pseudoalteromonas citrea* and *Pseudoalteromonas aurantia*; and the other clade composed of *P. luteoviolacea*, *P. piscicida*, *P. rubra* and strain F12-50-A1^T.

(ii) **gyrB sequence analysis.** The 1.2 kb nucleotide sequences of the *gyrB* gene were determined for *A. macleodii*, 11 available well-described *Pseudoalteromonas* species and F12-50-A1^T. Although 16S rDNA sequences had 99 % similarity between phenotypically distinct F12-50-A1^T and *P. piscicida*, the 12 % variation seen in *gyrB* nucleotide sequences between them supports their species differentiation.

The similarities in *gyrB* nucleotide sequences with F12-50-A1^T and other species were between 75 and 88 % (Table 3). It is obvious that F12-50-A1^T had greater similarity with pigmented *Pseudoalteromonas* (82–88 %) than with non-pigmented species (78–80 %). A very high variation (21 %) was observed between F12-50-A1^T and *Pseudoalteromonas haloplanktis* subsp. *haloplanktis*. This value is somewhat close to the variation seen between F12-50-A1^T and *A. macleodii* (25 %). Amino acid translation of *gyrB* nucleotide sequences of F12-50-A1^T exhibited similarities of 92–96 % and 88–90 % with pigmented and non-pigmented *Pseudoalteromonas* species, respectively.

The relative phylogenetic positions occupied by each of the *Pseudoalteromonas* type strains, based on *gyrB* nucleotide sequences, are shown in Fig. 3. An unrooted tree was generated by the maximum-likelihood method. The branching order of the *gyrB*-based tree resembles that of a similar tree based on 16S rDNA nucleotide sequences. The seven non-pigmented strains formed a distinct cluster. The other five pigmented strains, *P. aurantia*, *P. citrea*, *P. luteoviolacea*, *P. piscicida* and strain F12-50-A1^T, clustered into a distinct group of perfect integrity with regard to

Table 3. Percentage similarities of F12-50-A1^T strain sequences with other *Pseudoalteromonas* species and accession numbers of various nucleotide sequences

Bacterial strain	Strain no.	Place of isolation	Sequence similarities (%):			Accession no. for the sequences of:	
			16S rDNA	<i>gyrB</i>		16S rDNA*	<i>gyrB</i> †
				Nucleic acid	Amino acid		
<i>A. macleodii</i>	NCIMB 1963 ^T	Sea water	87.7	75.3	82.3	X82145	AF007272
<i>P. antarctica</i>	CECT 4664 ^T	Sea water, Antarctica	93.7	NT	NT	X98336	
<i>P. atlantica</i>	NCIMB 301 ^T	Seaweed, Canada	94.7	79.8	88.6	X82134	AF007274
<i>P. aurantia</i>	ATCC 33046 ^T	Sea water	93.5	87.7	96.2	X82135	AF007275
<i>P. bacteriolytica</i>	IAM 14594 ^T	Red spot disease, Laminaria	89.0	NT	NT	D89929	
<i>P. carrageenovora</i>	NCIMB 302 ^T	Sea water, Canada	94.6	80.0	90.3	X82136	AF007276
<i>P. citrea</i>	NCIMB 1889 ^T	Sea water	93.5	82.1	92.2	X82137	AF007277
<i>P. denitrificans</i>	ATCC 43337 ^T	Sea water, Norway	92.3	NT	NT	X82138	
<i>P. espejiana</i>	NCIMB 2127 ^T	Sea water, California, USA	94.9	79.3	88.4	X82143	AF007278
<i>P. haloplanktis</i>	NCIMB 2084 ^T	Sea water	93.7	79.1	88.9	X67024	AF007279
subsp. <i>haloplanktis</i>							
<i>P. luteoviolacea</i>	ATCC 33492 ^T	Sea water, France	96.4	82.8	94.7	X82144	AF007280
<i>P. nigrifaciens</i>	ATCC 19375 ^T	Butter	94.4	78.2	87.7	X82146	AF007281
<i>P. peptidolytica</i>	F12-50-A1 ^T	Sea water, Japan				AF007286†	AF007285
<i>P. piscicida</i>	ATCC 15057 ^T	Red Tide sea water, Florida, USA	99.1	87.9	97.1	X82215	AF139115
<i>P. prydzensis</i>	ACAM 620 ^T	Sea ice, Prdyz Bay, Antarctica	92.7	NT	NT	U85855	
<i>P. rubra</i>	ATCC 29570 ^T	Sea water, France	97.0	NT	NT	X82147	
<i>P. haloplanktis</i> subsp. <i>tetraodonis</i>	NCIMB 13177 ^T	Skin slime of pufferfish	91.5	NT	NT	X82139	AF007283
<i>P. tunicata</i>	CCUG 26757 ^T	Tunicates, Australia	95.2	79.8	88.4	Z25522	
<i>P. undina</i>	NCIMB 2128 ^T	Sea water, California, USA	94.1	79.5	89.6	X82140	AF007284

* Data retrieved from GenBank database for comparison.

† Generated for this study.

NT, Not tested.

species. The branching order of the tree based on the amino acid sequences of *gyrB* (Fig. 4) was very similar to that based on the *gyrB* nucleotide sequences. However, three distinct subclusters were noted among the non-pigmented *Pseudoalteromonas* species.

(iii) **DNA–DNA hybridization.** DNA–DNA hybridization was performed between F12-50-A1^T (Table 4), three pigmented strains (2.0–3.4%), one non-pigmented *Pseudoalteromonas* species (*P. haloplanktis* subsp. *haloplanktis*, 1.0%) and *E. coli* (0.2%) and *Bacillus subtilis* (0.8%). None of the values were above the 70% similarity that would place the strain within the same species. In particular, the similarity between *P. peptidolytica* and *P. piscicida* was only 2.0%; this pair showed 99.1% similarities in their 16S rDNA sequences. Similarly, *P. aurantia* and *P. citrea* showed only 10% DNA–DNA hybridization values but exhibited 99.7% similarity in their 16S rDNA sequence.

(iv) **Designing *P. peptidolytica*-specific PCR primers.** A species-specific primer set of 20 bp each was designed

to specifically detect and differentiate F12-50-A1^T from other related species. When these primers were used to generate 205 bp PCR products, F12-50-A1^T could be differentiated from *P. piscicida*. A total of 160 strains comprising 72 different species was screened for both the 1.2 kb *gyrB* gene and F12-50-A1^T-specific 205 bp fragments. These species include members of the genera *Pseudoalteromonas*, *Alteromonas*, *Vibrio*, *Aeromonas*, *Plesiomonas*, *Photobacterium* and *Halo-**monas*. A specific band of 205 bp was noticed only for the ten strains that degrade marine mussel thread but no other bacterial species exhibited any amplicon. However, PCR amplification using primer set UP-1 and UP-2r revealed a 1.2 kb *gyrB* fragment in all strains examined, thus ascertaining the presence of the DNA gyrase B subunit. Thus, the primers developed and described here (PP-1 and PP-2r) are specific to F12-50-A1^T and the other nine marine mussel-thread-degraders and could be used to screen this species. This indirect approach saves cost and labour but confirms the specificity of the species.

Fig. 2. Phylogenetic tree of type strains of various *Pseudoalteromonas* species based on 16S rDNA nucleotide sequences.

Fig. 3. Phylogenetic tree of type strains of various species of *Pseudoalteromonas* based on *gyrB* nucleotide sequences.

DISCUSSION

Phenotypic characteristics, such as positive oxidase and gelatinase reactions, the absence of glucose fermentation, a strict respiratory metabolism, an ability to grow only in higher NaCl concentrations, and a G+C content of 42 mol% seem to be sufficient to warrant the inclusion of F12-50-A1^T in the genus *Pseudoalteromonas*. Although some key characteristics could differentiate the strain F12-50-A1^T and nine marine mussel-thread-degraders from other species of *Pseudoalteromonas*, conventional biochemical testing

and fatty acid methyl ester profiles place these strains within the pigmented species of *Pseudoalteromonas*. Molecular methods are tacitly less susceptible to artifactual misinterpretation than culture-based approaches. It has been revealed that organisms with less than 97% similarity over the 16S rRNA gene do not yield DNA reassociation values of more than 60%; hence, rDNA sequence analysis may be used as a surrogate for DNA reassociation (Stackebrandt & Goebel, 1994).

Strain F12-50-A1^T shares >96% similarity in 16S rDNA sequences with three pigmented *Pseudoalteromonas* species. However, phenotypic characters of F12-50-A1^T and nine other strains are different from these species. *P. luteoviolacea* shows a distinct purple pigmentation, and all three species do not grow at high NaCl concentration (6–10%). Furthermore, F12-50-A1^T and other closely related species exhibited very low DNA–DNA similarities which suggests that this strain represents a new species. The species-specific PCR confirmed that nine of the other marine mussel-thread-degraders belong to the same species as F12-50-A1^T. In addition, F12-50-A1^T and the nine other strains of the proposed novel species secrete proteases that are not produced by any of the known *Pseudoalteromonas* species.

In general, *Pseudoalteromonas* species could not be differentiated based on the 97% 16S rDNA threshold level. Among *Pseudoalteromonas* species known, the percentage of nucleotide substitutions in the 16S rDNA sequences varied from 0.3 to 11.0%. It should be noted that some of the established phenotypically distinct species grouped together and had <3.0% dissimilarities in their 16S rDNA nucleotide sequences. For example: *P. antarctica*, *Pseudoalteromonas atlantica*, *Pseudoalteromonas carrageenovora*, *Pseudoalteromonas espejiana*, *P. haloplanktis* subsp. *haloplanktis*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas haloplanktis* subsp. *tetraodonis* and

Fig. 4. Phylogenetic tree of type strains of various species of *Pseudoalteromonas* based on *gyrB* amino acid sequences.

Table 4. DNA–DNA hybridization of various *Pseudoalteromonas* species

Strains: 1, *P. peptidolytica* F12-50-A1^T; 2, *P. aurantia* NCIMB 2033^T; 3, *P. citrea* NCIMB 1889^T; 4, *P. piscicida* ATCC 15057^T.

Bacterium	Strain no.	Similarity (%) to labelled DNA from:			
		1	2	3	4
<i>P. peptidolytica</i>	F12-50-A1 ^T	100.0			
<i>P. aurantia</i>	NCIMB 2033 ^T	3.0	100.0		
<i>P. citrea</i>	NCIMB 1889 ^T	3.4	10.0	100.0	
<i>P. piscicida</i>	ATCC 15057 ^T	2.0	NT	12.0	100.0
<i>P. luteoviolacea</i>	ATCC 33492 ^T	2.2	11.0	12.0	8.0
<i>P. haloplanktis</i> subsp. <i>haloplanktis</i>	NCIMB 2084 ^T	1.0	8.0	14.0	8.0
<i>E. coli</i>	ATCC 25922	0.2	NT	NT	NT
<i>B. subtilis</i>	ATCC 6051	0.8	NT	NT	NT

NT, Not tested.

Pseudoalteromonas undina formed a group that exhibited <2.5% dissimilarity; a second group, *P. citrea* and *P. aurantia*, showed only 5 bp substitution in their 1.5 kb 16S rDNA sequences and a third group, *P. luteoviolacea*, *P. piscicida*, *P. rubra* and F12-50-A1^T strain, showed <3.0% dissimilarity.

The base substitution frequency of *gyrB* is much higher than that of the 16S rRNA gene, making it a more appropriate choice for differentiating close phylogenetic relationships (Ochman & Wilson, 1987). Based on the comparison of *gyrB* sequences of various genera (Yamamoto & Harayama, 1995, 1996; Venkateswaran *et al.*, 1998, 1999; Yamada *et al.*, 1999), a species cut-off value of 90% was proposed (Venkateswaran *et al.*, 1999). Even though the 16S rDNA similarities are inconclusive at 97% in some bacterial genera, when the *gyrB* criteria are applied, low similarities were noted among closely related species. In this study, the *gyrB* sequences of F12-50-A1^T and *P. piscicida* showed

only 88% similarity that would confirm a species status for F12-50-A1^T. This was further verified by their low DNA–DNA reassociation values.

Description of *Pseudoalteromonas peptidolytica* sp. nov.

Pseudoalteromonas peptidolytica (pep.ti.do.ly'ti.ca. Gr. n. *peptos* a class of organic compounds; Gr. adj. *lytica* dissolving; M.L. fem. adj. *peptidolytica* peptide repeats dissolving)

Cells of the type strain are rod-shaped. They are 2–3 µm in length and 0.7–0.8 µm in diameter. Gram-negative, aerobic, polarly flagellated bacteria. Neither endospores nor capsules formed. Colonies on marine agar medium are circular, smooth, convex with regular edges and yellow-pigmented. Sodium ions are essential for growth. Able to grow at 15–40 °C with optimum growth at 30 °C. Produces cytochrome oxidase,

catalase, gelatinase, lipase, amylase and alginase. Aerobically utilizes maltose, succinate, fumarate, glucose and *N*-acetylglucosamine. Does not produce H₂S from thiosulfate or denitrify. Does not produce β -galactosidase, arginine dihydrolase or lysine and ornithine decarboxylases. Based on both 16S rDNA and *gyrB* nucleotide sequences, this bacterium belongs to the γ -*Proteobacteria* and is a member of the genus *Pseudoalteromonas*. The type strain F12-50-A1^T (=MBICC F1250A1^T) was isolated from the surface waters of the Yamato Islands, in the Sea of Japan.

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REFERENCES

- Baumann, P., Gauthier, M. J. & Baumann, L. (1984). Genus *Alteromonas* Baumann, Baumann, Mandel and Allen, 1972, 418. In *Bergey's Manual of Systematic Bacteriology*, pp. 243–352. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Bowman, J. (1998). *Pseudoalteromonas prydzensis* sp. nov., a psychrophilic, halotolerant bacterium from Antarctic sea ice. *Int J Syst Bacteriol* **48**, 1037–1041.
- Bozal, N., Tudela, E., Rossello-Mora, R., Lalucat, J. & Guinea, J. (1997). *Pseudoalteromonas antarctica* sp. nov., isolated from an Antarctic coastal environment. *Int J Syst Bacteriol* **47**, 345–351.
- Buck, J. D., Meyers, S. P. & Leifson, E. (1963). *Pseudomonas* (*Flavobacterium*) *piscicida* Bein comb. nov. *J Bacteriol* **86**, 1125–1126.
- Cole, R. M. & Popkin, T. J. (1981). Electron microscopy. In *Manual of Methods for General Bacteriology*, pp. 34–51. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilaw, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annu Rev Microbiol* **41**, 435–464.
- Dohmoto, N., Venkateswaran, K., Nose, T., Tanaka, H., Miki, W. & Miyachi, S. (1993). Marine mussel-thread-degrading bacteria and partial purification of proteinases responsible for degradation. *J Mar Biotechnol* **1**, 83–87.
- Edgell, D. R. & Doolittle, W. F. (1997). Archaea and the origin(s) of DNA replicon proteins. *Cell* **89**, 995–998.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19**, 1315–1327.
- Felsenstein, J. (1990). PHYLIP manual version 3.3. University Herbarium, University of California, Berkeley, CA, USA.
- Fox, G. E., Wisotzkey, J. D. & Jurtschuk, 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.
- Gauthier, G., Gauthier, M. & Christen, R. (1995). Phylogenetic analysis of genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int J Syst Bacteriol* **45**, 755–761.
- Holmstrom, C., James, S., Neilan, B. A., White, D. C. & Kjelleberg, S. (1998). *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int J Syst Bacteriol* **48**, 1205–1212.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Bacteriology*, pp. 34–51. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilaw, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Ochman, H. & Wilson, A. C. (1987). Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J Mol Evol* **26**, 74–86.
- Ringelberg, D. B., Townsend, G. T., DeWeerd, K. A., Suflita, J. M. & White, D. C. (1994). Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by its signature lipopolysaccharide branched-long-chain hydroxy fatty acids. *FEMS Microbiol Ecol* **14**, 9–18.
- Ruimy, R., Breittmayer, V., ElBaze, P., Lafay, B., Boussemart, O., Gauthier, M. & Christen, R. (1994). Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small subunit rRNA sequences. *Int J Syst Bacteriol* **44**, 416–426.
- Sarath, G., De La Motte, R. S. & Wagner, F. W. (1989). Proteolytic enzymes. In *Proteases Assay Methods*, pp. 25–55. Edited by R. J. Beynon & J. S. Bonds. Oxford: IRL/Oxford University Press.
- Sawabe, T., Makino, H., Tatsumi, M., Nakano, K., Tajima, K., Iqbal, M. M., Yumoto, I., Ezura, Y. & Christen, R. (1998). *Pseudoalteromonas bacteriolytica* sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*. *Int J Syst Bacteriol* **48**, 769–774.
- Stackebrandt, E. & Goebel, B. M. (1994). 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.
- Strunk, O. & Ludwig, W. (1995). ARB – a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany.
- Svetashev, V. I., Vysotskii, M. V., Ivanova, E. P. & Mikhailov, V. V. (1995). Cellular fatty acids of *Alteromonas* species. *Syst Appl Microbiol* **18**, 37–43.
- Swofford, D. (1990). PAUP: phylogenetic analysis using parsimony, version 3.0. Computer program distributed by the Illinois Natural History Survey, Champaign, IL, USA.
- Venkateswaran, K., Dohmoto, N. & Harayama, S. (1998). Cloning and nucleotide sequence of *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of the pathogen in shrimp. *Appl Environ Microbiol* **64**, 681–687.
- Venkateswaran, K., Moser, D. P., Dollhopf, M. E. & 10 other authors (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49**, 705–724.
- Waite, J. H. (1983). Evidence for a repeating 3,4-dihydroxy-

phenylalanine- and hydroxyproline-containing decapeptide in the adhesive protein of the mussel, *Mytilus edulis* L. *J Biol Chem* **258**, 2911–2915.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Yamada, S., Ohashi, E., Agata, N. & Venkateswaran, K. (1999). Cloning and nucleotide sequences of *gyrB* of *Bacillus cereus*, *B. thuringiensis*, *B. anthracis* and *B. mycoides* and its application in the detection *B. cereus* in rice. *Appl Environ Microbiol* **65**, 1483–1490.

Yamamoto, S. & Harayama, S. (1995). PCR amplification and

direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.

Yamamoto, S. & Harayama, S. (1996). Phylogenetic analysis of *Acinetobacter* strains on the nucleotide sequences of *gyrB* genes and on the amino acid sequences of their products. *Appl Environ Microbiol* **46**, 506–511.

Yamamoto, S., Bouvet, P. J. & Harayama, S. (1999). Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA-DNA hybridization. *Int J Syst Bacteriol* **49**, 87–95.