***Pseudoalteromonas ulvae* sp. nov., a bacterium with antifouling activities isolated from the surface of a marine alga**

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A dark-purple marine bacterium that inhibits the germination of marine algal spores and the settlement of invertebrate larvae has been characterized and assessed for taxonomic assignment. Two strains, designated UL12^T and UL13, were isolated from the surface of the common marine alga *Ulva lactuca*. Based on 16S rDNA sequencing, UL12^T and UL13 were found to show the highest similarity (97%) to members of the genus *Pseudoalteromonas*. DNA–DNA hybridization studies demonstrated less than 28% genomic DNA relatedness between these isolates and closely related *Pseudoalteromonas* species and greater than 65% homology between UL12^T and UL13. The two isolates were found to display identical characteristics and are strict aerobes, motile by means of single polar flagella, exhibit non-fermentative metabolism and require sodium ions for growth. The isolates hydrolyse gelatin and can utilize citrate, maltose, mannose and glucose but not trehalose, sucrose, fructose, lactose or glycerol as sole carbon sources. The molecular evidence together with the phenotypic characteristics show that this bacterium constitutes a new species within the genus *Pseudoalteromonas*. The name *Pseudoalteromonas ulvae* sp. nov. is proposed for this bacterium and the type strain is UL12^T (= UNSW 095600^T = NCIMB 13762^T).

Keywords: *Pseudoalteromonas ulvae*, antifouling bacteria, marine epiphytic bacteria, 16S rDNA sequencing

INTRODUCTION

The genus *Pseudoalteromonas* includes both pigmented and non-pigmented, Gram-negative, rod-shaped, heterotrophic marine bacteria that are motile by means of polar flagella. This recently described genus is the result of extensive taxonomic revision on the basis of the phylogenetic relationships among members of the genera *Alteromonas*, *Shewanella* and *Moritella*. Gauthier *et al.* (1995) suggested that the genus *Alteromonas* be divided into two genera, with the majority of species being reclassified within the new genus *Pseudoalteromonas*, leaving *Alteromonas macleodii* as the sole species of *Alteromonas*.

Species of the genus *Pseudoalteromonas*, the majority

of which appear to be associated with eukaryotic hosts (Holmström & Kjelleberg, 1999), are isolated frequently from marine waters around the world. Species have been isolated from various animals such as tunicates (Holmström *et al.*, 1998), mussels (Ivanova *et al.*, 1996, 1998), pufferfish (Simidu *et al.*, 1990) and sponges (Ivanova *et al.*, 1998) and from a range of marine algae (Akagawa-Matsushita *et al.*, 1992; Yoshikawa *et al.*, 1997). The bacterial strains in this study were isolated from the surface of the marine alga *Ulva lactuca*. Both strains have been shown to inhibit the settlement of larvae of the marine invertebrate *Balanus amphitrite* and the germination of spores of the green alga *U. lactuca* and a species of the red alga *Polysiphonia* (Egan *et al.*, 2000). The aim of this study was to describe by phenotypic and genetic characterization the isolates designated UL12^T and UL13 for the purpose of taxonomic assignment. On the basis of high 16S rDNA sequence similarity and low DNA-relatedness values to other *Pseudoalteromonas* species,

The GenBank/EMBL/DBJ accession numbers for the 16S rDNA sequences of isolates UL12^T and UL13 are AF172987 and AF172988, respectively.

together with morphological and biochemical characteristics, it is proposed that isolates UL12^T and UL13 constitute a new species named *Pseudoalteromonas ulvae* sp. nov.

METHODS

Source of inoculum and isolation. Two strains, UL12^T and UL13, were isolated from the surface of the common marine alga *U. lactuca*, which was collected from the rocky intertidal zone near Sydney, on the east coast of Australia. The algal thallus was suspended in sterile nine-salts solution (NSS) [containing (l⁻¹): 17.6 g NaCl, 1.47 g Na₂SO₄, 0.08 g NaHCO₃, 0.25 g KCl, 0.04 g KBr, 1.87 g MgCl₂·6H₂O, 0.41 g CaCl₂·2H₂O, 0.008 g SrCl₂·6H₂O, 0.008 g H₃BO₃; pH 7] and surface bacteria were removed by vortexing. Aliquots of the samples were then spread on the complex marine medium VNSS agar [VNSS agar consists of NSS plus (l⁻¹): 1.0 g peptone, 0.5 g yeast extract, 0.5 g glucose, 0.01 g FeSO₄·7H₂O, 0.01 g Na₂HPO₄ and 15 g agar] and incubated at 23 °C for 48 h. Morphologically distinct bacterial colonies were selected. Bacteria were stored at -70 °C in 30% glycerol.

Phenotypic characterization. Bacterial strains were routinely grown on VNSS agar and incubated at 23 °C to provide inocula for biochemical tests. Oxidative or fermentative utilization of glucose was determined by the method of Hugh & Leifson (1953). Catalase activity was determined by the method of Skerman (1967) and oxidase activity was tested according to Kovacs (1956). The optimal salt concentration for growth of isolates UL12^T and UL13 was tested using the medium VNSS with NaCl concentrations of 0–10% (w/v). Growth on the rich medium Luria broth (LB20), containing 10 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 20 g NaCl l⁻¹, and tryptone soy broth (TSB) (Oxoid) was also assessed. Marine minimal medium (MMM) (Östling *et al.*, 1991) was used during tests for growth of isolates on different substrates as sole carbon and energy sources at concentrations of 4 g l⁻¹. Susceptibility of UL12^T and UL13 to the antibiotics gentamicin, tetracycline, ampicillin, kanamycin, streptomycin, carbenicillin, chloramphenicol, spectinomycin and penicillin G was tested at concentrations of 50 and 100 µg ml⁻¹ in VNSS medium. Sensitivity to the vibriostatic agent O/129 was tested using discs at a concentration of 150 µg ml⁻¹. Activities of arginine dihydrolase, tryptophan deaminase, lysine decarboxylase and ornithine decarboxylase were determined using the API 20E system (bioMérieux). Exponential-phase bacterial cells were washed three times with MMM before being inoculated into the test cupules. Motility was determined by visualization of cells under phase microscopy with a 100× oil-immersion objective.

Negative staining and electron microscopy. Cell morphology, size and flagellum characteristics were determined by transmission electron microscopy. One drop of cell suspension from an overnight bacterial culture was mixed with a drop of sodium phosphotungstate (2% aqueous) for 30 s on a Formvar 300-square copper grid. The grid was blotted using filter paper and dried for 10 min before examination on a Hitachi H7000 electron microscope at 10000× magnification.

16S rDNA amplification and sequencing. Genomic DNA was extracted from late-exponential-phase bacterial cells using the XS buffer method as described by Tillet & Neilan

(2000). The 16S rRNA gene was amplified from genomic DNA using PCR with standard primers corresponding to positions 27 in the forward direction and 1492 in the reverse direction of the *Escherichia coli* 16S rRNA gene sequence. The thermal profile consisted of 25 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 2 min. PCR products were visualized on a 1% agarose gel using a molecular mass standard to estimate size and concentration of product. Single band products were purified by ethanol precipitation. Approximately 100 ng template DNA was then sequenced in a thermocycling reaction with BigDye terminator cycle sequencing mix (Applied Biosystems) and analysed on an ABI 377 DNA sequencing system.

Phylogenetic analysis. DNA sequences were aligned using the multiple sequence alignment tools CLUSTAL W and PILEUP (GCG, 1992). Ambiguous and gap positions were deleted manually and the alignment was confirmed and checked against both primary and secondary structure considerations of the 16S rRNA molecule. The aligned sequences were applied to genetic-distance and maximum-parsimony methods for phylogenetic inference. Genetic distances were calculated using the formulae of Jukes & Cantor (1969), Kimura (1980) and the maximum-likelihood method (Felsenstein, 1981). Phylogenetic inference protocols EDNAML, EDNADIST, NEIGHBOR, EDNAPARS, CONSENSE and SEQBOOT were supplied by the PHYLIP packages (version 3.57c; Felsenstein, 1989). All sequence manipulation and phylogeny programs were made available through the Australian National Genome Information Service (ANGIS, Sydney, Australia).

DNA–DNA hybridization. Levels of genomic relatedness were determined by performing DNA–DNA dot-blot hybridizations with radioactively labelled genomic DNA. Target genomic DNA was denatured by boiling for 10 min and then chilling quickly on ice. Duplicate aliquots containing 50 ng denatured genomic DNA from *Pseudoalteromonas aurantia*, *Pseudoalteromonas citrea*, *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas piscicida*, *Pseudoalteromonas rubra*, *Pseudoalteromonas tunicata* and the isolates UL12^T and UL13 were dotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech). Membranes were allowed to air dry and the DNA was subsequently fixed by UV cross-linking. Pre-hybridization was performed at 42 °C for 1 h in Rapid-hyb buffer (Amersham Pharmacia Biotech). Genomic DNA of strain UL12^T was labelled by nick translation (Rigby *et al.*, 1977) using a Nick translation kit (Roche) and Redivue [α -³²P]dCTP (Amersham Pharmacia Biotech). Hybridizations were performed in the pre-hybridization buffer with 10 ng labelled probe ml⁻¹ at 42 °C for 16 h. After hybridization, the membranes were washed once in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.1% (w/v) SDS at room temperature for 20 min and then washed twice in 0.5× SSC, 0.1% (w/v) SDS at 55 °C for 15 min with a final, high-stringency wash in 0.5× SSC, 0.1% (w/v) SDS at 65 °C for 15 min. The degree of probe binding was determined by exposing the membrane to a phosphorimager screen (Bio-Rad) overnight; thereafter, the images were captured with a Bio-Rad GS425 imager (greater than three log signal response linearity). Image analysis was performed using the Bio-Rad software package MULTI-ANALYST. The signal produced by hybridization of the probe to the homologous target DNA was taken to be 100% and percentage hybridization for each test species was calculated. Hybridization experiments were repeated twice.

RESULTS AND DISCUSSION

The two strains UL12^T and UL13 isolated from the surface of the marine alga *U. lactuca* appeared as small, regular, dark-purple colonies on VNSS agar and were Gram-negative, motile rods as viewed by phase microscopy. The cells examined by electron microscopy were 1.7–2.5 µm long and 1–1.5 µm wide and possessed single polar flagella (Fig. 1).

Isolates UL12^T and UL13 were found to be identical with respect to specific physiological and biochemical features. Both isolates are strict aerobes and are capable of growth at 4 °C but not at 35 °C. The optimum temperature for growth was found to be 23 °C and both isolates could grow within a pH range of 5.5–12 (optimum at pH 8). The isolates required sodium ions at a concentration of at least 0.1 % (w/v) NaCl, with the optimum concentration for growth being 1–2.5 % (w/v) NaCl.

The isolates exhibited gelatinase and tryptophan deaminase activity, while β-galactosidase, arginine dihydrolase, lysine/ornithine decarboxylase and urease activity were not detected. Growth on different carbon and energy sources showed that the bacterium utilizes citrate, maltose and Tween 20 within 2 d of incubation, L-proline after 4 d of incubation and glucose and mannose after 7 d of incubation. Strains UL12^T and UL13 were unable to ferment sugar and displayed little or no oxidative acid production as demonstrated by the Hugh–Leifson test. In addition,

the isolates were positive for both catalase and oxidase activity.

Cells were sensitive to gentamicin, tetracycline, ampicillin, kanamycin, streptomycin, carbenicillin, chloramphenicol and spectinomycin at 50 µg ml⁻¹ but were resistant to penicillin G at concentrations of up to 100 µg ml⁻¹. Both isolates were sensitive to the vibriostatic agent O/129 at 150 µg ml⁻¹.

After 3 d of incubation on LB20 and TSB, isolates UL12^T and UL13 grew as small white colonies. Streaking the white colonies from an LB20 or TSB agar plate onto an agar plate containing VNSS medium resulted in the formation of dark-purple colonies after 24 h of incubation. Variation in the level of pigment expression depending on the growth medium is a characteristic shared by other related species such as *P. tunicata* (Holmström *et al.*, 1998), *Pseudoalteromonas nigrifaciens* (Ivanova *et al.*, 1996), *Pseudoalteromonas denitrificans* (Enger *et al.*, 1987) and *Shewanella hanedai* (Baumann *et al.*, 1984).

The DNA sequencing strategy used in this investigation generated 1355 and 1403 bases of the 16S rRNA gene for UL12^T and UL13, respectively. The resulting sequences were aligned with other closely related 16S rRNA gene sequences within the EMBL, GenBank and RDP databases. The derived multiple sequence alignment (1191 characters) was used to generate pairwise sequence identity and genetic distances between UL12^T, UL13 and related bacteria. Several phylogenetic trees were constructed using different methods, including genetic-distance matrices and maximum-parsimony. Statistical evaluation of the derived genetic divergences was performed by bootstrap resampling (100 replicates) of the sequence data.

The tree topology shown in Fig. 2 was identical to other statistical representations of the sequence data. Strains UL12^T and UL13 were found to belong to the γ-3 subclass of the *Proteobacteria* and in a lineage with members of the genus *Pseudoalteromonas*, as supported by high bootstrap values for the cluster (Fig. 2). Isolates UL12^T and UL13 shared 16S rRNA similarity of 99.8 % while, within the genus *Pseudoalteromonas*, both had a range of sequence similarities of 91–97 % to the other members included in this analysis. The highest sequence similarities outside this genus were 92 % for members of the genera *Vibrio* and *Photobacterium* and 91 % similarity to the genus *Shewanella*. The highest 16S rDNA sequence identity shared with other species was 97 % with *P. piscicida*. However, isolates UL12^T and UL13 shared more phenotypic characteristics with *P. tunicata*. When comparing a number of different taxonomic parameters (Table. 1), the two isolates differed from the *P. tunicata* type strain by only four traits; the inability to grow at 35 °C, differences in pigmentation, the ability to utilize citrate and the inability to use trehalose as a sole carbon source. Comparison of the new isolates with other strains of *Pseudoalteromonas*

Fig. 1. Electron micrograph of a cell of strain UL12^T. Bar, 1 µm.

Fig. 2. Distance matrix tree based on a 1191 bp sequence alignment of the 16S rDNA genes of *Pseudoalteromonas ulvae* UL12^T and UL13 with those of members of the genus *Pseudoalteromonas* and other closely related bacteria. Distances were calculated according to the algorithm of Jukes & Cantor (1969) and trees were constructed by the neighbour-joining method of Saitou & Nei (1987). '*Marinobacter arcticus*' was chosen as the outgroup. Bootstrap values (100 replicates) are indicated at branching points. Bar, 1 substitution per 100 nucleotide positions.

(Table 1) showed that most strains differed by four to eight characteristics.

In view of the high 16S rDNA sequence similarity between UL12^T and UL13 and other *Pseudoalteromonas* species, DNA–DNA hybridization studies were performed between these isolates and close phylogenetic neighbours. Both UL12^T and UL13 were found to have low levels of hybridization (13.6–28.5%) to other *Pseudoalteromonas* species including *P. aurantia* ATCC 33046^T, *P. citrea* NCIMB 1889^T, *P. luteoviolacea* NCIMB 1893^T, *P. piscicida* ATCC 15057^T, *P. rubra* ATCC 29570^T and *P. tunicata* CCUG 26757^T. These values are below the currently accepted limit of DNA relatedness (70%) for the phylogenetic definition of a species (Stackebrandt & Goebel, 1994) and therefore give further evidence that the isolates represent a novel species with the genus *Pseudo-*

alteromonas. In contrast, hybridization levels of 68.5% were found between isolates UL12^T and UL13. This value, while being slightly below the 70% limit for species delineation, when taken together with the high 16S rDNA sequence similarity and the phenotypic similarities between the isolates suggests that UL12^T and UL13 may belong to the same species.

Cells of strain UL12^T are capable of inhibiting the settlement of invertebrate larvae and marine algal spores, including those of the green alga *U. lactuca*, from which the strain was isolated (Egan *et al.*, 2000). Many strains within this genus have been shown to produce extracellular metabolites that are active against other organisms including agarases, toxins and bacteriolytic substances. For example, *P. piscicida* releases a toxin thought to be responsible for fish mortality (Bein, 1954), *Pseudoalteromonas tetradonis*

Table 1. Differential characteristics of *Pseudoalteromonas* species

Taxa are identified as: 1, *P. ulvae*; 2, *P. tunicata*; 3, *P. piscicida*; 4, *Pseudoalteromonas undina*; 5, *P. rubra*; 6, *P. citrea*; 7, *P. aurantia*; 8, *P. luteoviolacea*. Data were taken from this study and from Baumann *et al.* (1984), Gauthier (1982), Hansen *et al.* (1965) and Holmström *et al.* (1998). Characters are scored as: +, positive; –, negative; d, 11–89% of strains are positive; ND, not determined. Pigmentation is indicated as: p, purple; g, green; y, yellow; r, red. All strains are motile, hydrolyse gelatin, display oxidative activity and are negative for utilization of glycerol.

Characteristic	1	2	3	4	5	6	7	8
Growth at:								
4 °C	+	+	–	d	–	–	+	–
35 °C	–	+	+	–	+	d	–	+
Pigmentation	p	g	y	–	r	y	y	p
Utilization of:								
Mannose	+	+	+	–	+	+	+	–
Sucrose	–	–	+	+	–	–	–	–
Maltose	+	+	+	+	–	–	d	+
Sorbitol	–	–	ND	–	–	–	–	–
Fructose	–	–	+	–	–	+	+	–
Citrate	+	–	+	–	–	–	–	–
Lactose	–	–	ND	–	–	–	–	–
Melibiose	–	–	ND	–	ND	–	–	–
Trehalose	–	+	ND	+	+	+	+	+
L-Proline	+	+	ND	d	ND	–	–	+

(formerly *Pseudoalteromonas haloplanktis* subsp. *tetradonidis*) produces the neurotoxin tetrodotoxin, which causes pufferfish poisoning (Simidu *et al.*, 1990), and *Pseudoalteromonas bacteriolytica* strains display strong bacteriolytic activity and are believed to be the causative agent of red-spot disease in the brown alga *Laminaria japonica* (Sawabe *et al.*, 1998). The well-studied species *P. tunicata* also produces several extracellular components that target a range of organisms including bacteria, fungi, invertebrate larvae and algae (Holmström *et al.*, 1998).

The marine isolates UL12^T and UL13 are different from previously characterized *Pseudoalteromonas* species. The 16S rDNA sequence, together with the DNA relatedness values, show clearly that isolates UL12^T and UL13 make up a novel species within the genus *Pseudoalteromonas*. Both isolates display phenotypic and biochemical characteristics typical of *Pseudoalteromonas* species, including a requirement for sodium ions, motility by a single flagellum and the presence of oxidase and catalase activity, gelatinase activity and oxidative metabolism. The main phenotypic features of isolates UL12^T and UL13 closely resemble those of *P. tunicata*. However, in addition to the features listed in Table 1, both isolates can be distinguished from *P. tunicata* by the lack of a sheathed flagellum and a strictly aerobic metabolism in isolates UL12^T and UL13 (Holmström *et al.*, 1998).

Therefore, on the basis of phenotypic and genetic characterization, isolates UL12^T and UL13 can be considered as a new species, for which the name *Pseudoalteromonas ulvae* sp. nov. is proposed.

Description of *Pseudoalteromonas ulvae* sp. nov.

Pseudoalteromonas ulvae (ul'vae. N.L. gen. n. *ulvae* of *Ulva*, the generic name of the host alga, *U. lactuca*).

Strictly aerobic, Gram-negative, rod-shaped cells that are 1.75–2.5 µm in length and 1–1.5 µm in width. Cells are motile by means of single polar flagella. Growth on VNSS medium results in small, dark-purple colonies, while growth on LB20 or TSB with 2% NaCl results in the formation of white colonies. Does not ferment sugar in the Hugh–Leifson test. Sodium ions are required for growth, with the optimum NaCl concentration being 1–2.5%. Can grow within a pH range of 5.5–12; the optimum pH for growth is pH 8. Slow growth occurs at 4 °C and no growth is detectable at 35 °C. Oxidase- and catalase-positive. Utilizes citrate, maltose, L-proline, glucose, mannose and Tween 20 but not trehalose, lactose, sucrose, fructose, glycerol, raffinose, sorbitol, melibiose, xylose, cellobiose, erythritol, L-glutamine, arabinose, rhamnose or DL-serine as sole carbon and energy sources. Positive for hydrolysis of gelatin and displays tryptophan deaminase activity. Strains UL12^T and UL13 are negative for H₂S production and no β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or urease activity is detected. Sensitive to tetracycline, ampicillin, kanamycin, streptomycin, carbenicillin, chloramphenicol and spectinomycin at 50 µg ml^{–1} and sensitive to the vibriostatic agent O/129 at 150 µg ml^{–1}. Resistant to penicillin G at up to 100 µg ml^{–1}. Isolated from the surface of a marine alga, *Ulva lactuca*, collected from the rocky intertidal zone off the eastern coast of Australia.

The type strain, UL12^T, has been deposited in the Culture Collection of the University of New South Wales as UNSW 095600^T and in the National Collection of Industrial and Marine Bacteria, Aberdeen, UK, as NCIMB 13762^T. Nucleotide sequences for the 16S rDNA genes of isolates UL12^T and UL13 have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers AF172987 and AF172988, respectively.

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