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Pseudoalteromonas bacteriolytica sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*

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An aerobic, polarly flagellated marine bacterium that produces a prodigiosin-like pigment was isolated from the red-spotted culture beds of *Laminaria japonica*. Five isolates had unique bacteriolytic activity for both Gram-positive and -negative bacteria, which had never been observed among *Alteromonas* or related species. The isolates were identified as the causative agent of red spot disease of *L. japonica* seeds. The phenotypic features of the isolates were similar to these of *Pseudoalteromonas rubra* ATCC 29570^T, but they could be differentiated using 10 traits (growth at 37 °C, requirement for organic growth factors, bacteriolytic activity, utilization of sucrose, *N*-acetylglucosamine, fumarate, succinate, D-galactose, L-proline and acetate). The G C content of DNAs from the isolates was 44–46 mol%. The isolates constitute a new species, distinct from the other *Alteromonas* and *Pseudoalteromonas* species, as shown by DNA–DNA hybridization experiments and phylogenetic clustering of 16S rRNA gene sequences, for which the name *Pseudoalteromonas bacteriolytica* sp. nov. (type strain = IAM 14595^T) is proposed. A set of phenotypic features which differentiate this new species from closely related *Pseudoalteromonas* and *Alteromonas* species is provided.

Keywords: *Pseudoalteromonas bacteriolytica* sp. nov., bacteriolytic activity, red spot disease, *Laminaria*

INTRODUCTION

In 1984, a bacterium producing a prodigiosin-like red pigment was isolated from red-spotted culture beds of *Laminaria japonica* (7). The bacterium was an aerobic, polarly flagellated marine bacterium, and it was suggested that the bacteria could be assigned to the genus *Alteromonas* (7). In addition, the bacterium showed a broad spectrum of bacteriolytic activity. Presently, at least six bacteriolytic substances, including bacteriolytic enzymes, have been detected in culture supernatants and cell-bound fractions (19, 20). An ecological function of this bacteriolytic activity has been proposed, which would allow maintenance of the bacterial population in oligotrophic aquatic environments (21).

The DDBJ/GenBank/EMBL accession number for the sequence of *Pseudoalteromonas bacteriolytica* (IAM 14595^T) is D89929.

Originally consisting of four species, the genus *Alteromonas* included Gram-negative, aerobic, non-pigmented, polarly flagellated species of marine bacteria, which differed from the genus *Pseudomonas* by a lower G+C content (1, 2). Recently, following phylogenetic analyses of 16S rDNA sequences, the genus was divided into two new genera, the emended genus *Alteromonas* and the new genus *Pseudoalteromonas*. Now, at least 14 species, most of which were previously *Alteromonas* species, are included in the genus *Pseudoalteromonas*, and the emended genus *Alteromonas* is restricted to a single species, *Alteromonas macleodii*, with two subspecies (8, 15). Bacteriolytic *Pseudoalteromonas* or *Alteromonas* strains have never been described previously. The precise taxonomic position of the aforementioned bacteriolytic bacterium therefore remains uncertain. In this study, DNA–DNA hybridizations, phenotypic characterization and phylogenetic analyses were performed to clarify the taxonomic assignment of the causative agent of red

spot disease of *L. japonica* seeds. All of the data suggest that this bacterium is a new species of *Pseudoalteromonas*, for which we propose the name *Pseudoalteromonas bacteriolytica*.

METHODS

Bacterial strains. Strains used in this study are listed in Table 1. Five strains (No. 8R, E-1, E-2 and A3), including the type strain IAM 14595^T had been isolated from red-spotted culture beds of *L. japonica* (7). These isolates of *Pseudoalteromonas bacteriolytica* were maintained on CSY-3 agar medium containing casitone (Difco) 1·0 g, Difco bactosoytone 1·0 g, Difco yeast extract 1·0 g, ferric ammonium citrate 0·1 g, and 1000 ml natural seawater, pH 7·5 (19). The stock cultures were maintained in CSY-3 broth containing 20 % glycerol (v/v). All reference strains were maintained on ZoBell 2216E agar medium (13).

Morphological, biochemical and physiological characterization. Conventional phenotypic characteristics of *P. bacteriolytica*, *P. rubra*, *P. haloplanktis* subsp. *haloplanktis* and *A. macleodii* were determined by the methods described by Baumann *et al.* (2), Hidaka & Sakai (9), Holt *et al.* (10), Leifson (11), Oppenheimer & ZoBell (13), Ostle & Holt (14) and West *et al.* (22). Bacteriolytic activity was determined by the formation of a clear zone on the plate using the CSY-3 agar medium including freeze-dried cells of *Micrococcus luteus* (Seikagaku Kogyo). This medium was prepared according to the methods of Yumoto *et al.* (20). Briefly, 0·5 ml viable cell suspension of *M. luteus* (approx. 10^{10} ml^{-1} in natural seawater) was added to 2·5 ml melted agar (0·8 % in natural seawater) at 45 °C. It was then poured onto a base of CSY-3 agar medium. After 5 d incubation, the formation of a clear zone around the spotted culture was determined.

Determination of G + C content and DNA–DNA hybridization. DNA from bacterial strains was prepared by the procedures of Marmur (12), with minor modification. G + C contents of DNA were determined according to the melting temperature (T_m) of the DNA. DNA–DNA hybridization experiments were performed in microdilution wells using a fluorometric direct binding method (5) under conditions previously described by Sawabe *et al.* (17). DNAs from *Pseudoalteromonas bacteriolytica* IAM 14595^T and *Pseudoalteromonas rubra* ATCC 29570^T were labelled with photobiotin (Vector Laboratories). The hybridization of the biotinylated DNA to immobilized DNAs was performed under optimal conditions following pre-hybridization, and then biotinylated DNA that hybridized to immobilized DNA was detected by a fluorimetric method after binding streptavidin-β-galactosidase to labelled DNA. 4-Methylumbelliferyl-β-D-galactopyranoside (6×10^{-4} M; Wako) was added to each well as fluorogenic substrate for β-galactosidase prior to incubation at 30 °C. Fluorescence intensity of the well was then measured using the MicroFluoro reader (MTP-22; Corona Electric) at wavelengths of 360 nm for excitation and 450 nm for emission. DNA–DNA homology was calculated according to the method of Ezaki *et al.* (6).

DNA amplification and sequencing. Bacterial DNAs for PCR were prepared according to the methods of Enright *et al.* (4). One hundred nanograms of DNA templates were

Table 1. DNA relatedness among *Pseudoalteromonas, Alteromonas* and *Marinomonas* strains

Strain		G + C content (mol %)	Reassociation (%) with biotinylated DNA from:	
			***P. bacteriolytica* IAM 14595^T**	***P. rubra* ATCC 29570^T**
P. bacteriolytica	IAM 14595^T	46	100·0	3·4
P. bacteriolytica	No. 8R	44	92·4	NT
P. bacteriolytica	E-1	45	99·3	NT
P. bacteriolytica	E-2	44	98·3	NT
P. bacteriolytica	A-3	46	93·5	NT
P. rubra	ATCC 29570^T	46	5·6	100·0
A. macleodii	IAM 12920^T	46·2*	2·6	3·9
P. espejiana	IAM 12640^T	41·4*	3·3	4·1
P. atlantica	NCIMB 301^T	41·2*	4·5	3·2
P. carrageenovora	NCIMB 302^T	39·5*	3·9	2·2
P. marinovulgaris	ATCC 14394	NT	3·1	3·4
P. nigrifaciens	IAM 13010^T	40·6*	4·1	4·5
P. haloplanktis	IAM 12915^T	41·6*	3·7	5·8
P. haloplanktis	ATCC 19648	40·5*	4·1	6·0
P. undina	IAM 12922^T	40·1*	3·9	4·3
P. piscicida	NCIMB 645^T	43–46*	3·7	7·5
M. communis	IAM 12914^T	47·0*	3·3	2·2
M. vaga	IAM 12923^T	48·4*	3·1	2·8

NT, Not tested.

* Data from reference 2.

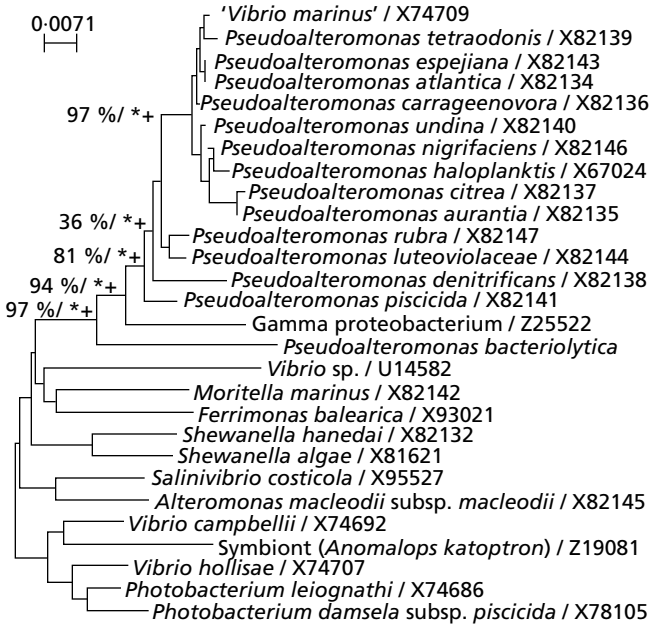

Fig. 1. Unrooted phylogenetic tree showing phylogenetic relationships for a selection of bacteria belonging to the gamma 3 subclass, *Pseudoalteromonas* and *Alteromonas*. The figure combines the results of three analyses, neighbour-joining, maximum-parsimony and maximum-likelihood. The topology shown was obtained by neighbour-joining, and the percentages are the result of a bootstrap analysis using 100 replications. Branches also obtained in the maximum-likelihood analysis are indicated by * ($P < 0{\cdot}01$). Monophyletic units also obtained in the most parsimonious tree are indicated by +. Scale bar, 0·0071 accumulated changes per nucleotide.

used in a PCR to amplify the small-subunit rRNA genes as previously described by Sawabe *et al*. (17). PCR conditions were as follows; the initial denaturation step at 94 ° C for 180 s, an annealing step at 55 °C for 60 s and an extension step at 72 °C for 90 s. The thermal profile then consisted of 30 cycles. The amplification primers used in this study gave a 1·5 kbp PCR product and corresponded to positions 25–1521 in the *Escherichia coli* sequence. The PCR products were purified by PEG 6000 and directly sequenced by using a *Taq* FS dye terminator sequencing kit (ABI) and the protocol recommended by the manufacturer. DNA sequencing was performed with an Applied Biosystems model 373A automated sequencer (17). Nine sequencing primers were used for sequencing (17).

Phylogenetic analysis. The sequences were aligned and studied using a set of programs developed by R. Christen. In all phylogenetic analyses, we used the sequences determined in this study and small-subunit rDNA sequences obtained from the EMBL database. For Fig. 1, the following sequences were used: *Pseudoalteromonas denitirificans* ATCC 43337^T, X82138; *Pseudoalteromonas citrea* NCIMB 1889^T, X82137; *Pseudoalteromonas aurantia* ATCC 33046^T, X82135; *Pseudoalteromonas atlantica* IAM 12927^T, X82134; *Pseudoalteromonas espejiana* NCIMB 2127^T, X82143; *Pseudoalteromonas carrageenovora* ATCC 12662^T, X82136; *Pseudoalteromonas undina* NCIMB 2128^T, X82140; *Pseudoalteromonas haloplanktis* ATCC 14393, X67024; *Pseudoalteromonas nigrifaciens* NCIMB 8614^T, X82146; *Pseudoalteromonas tetraodonis* IAM 14160^T, X82139; *Pseudoalteromonas piscicida* C201 CERBOM, X82141; *Pseudoalteromonas rubra* ATCC 29570^T, X82147; *Pseudoalteromonas luteoviolacea* NCIMB 1893^T, X82144; *Alteromonas macleodii* subsp. *macleodii* IAM 12920^T, X82145; '*Vibrio marinus*' ATCC 15831^T, X74709; *Vibrio* sp., U145842; *Moritella marinas* NCIMB 1144^T, X82142; *Ferrimonas balearica*, X93021; *Shewanella hanedai* CIP 103207^T, X82132; *Shewanella algae*, X81621; *Salinivibrio costicola* NCIMB 701^T, X95527; *Vibrio campbellii* ATCC 25920^T, X74692; symbiont *Anomolops katoptron*, Z19081; *Vibrio hollisae* ATCC 33564^T, X74707; *Photobacterium leiognathi* ATCC 25521^T, X74686; and *Photobacterium damsela* subsp. *piscicida* NCIMB 2058, X78105.

Domains used to construct the dendrogram shown in Fig. 1 were regions of the small-subunit rDNA sequences available for all sequences and excluding positions likely to show homoplasy: positions 251–434, 493–590, 653–835, 855–997, 1045–1114, 1156–1354 (*E. coli* small-subunit rDNA sequence J01695 numbering). Phylogenetic analyses were performed by using three different methods, neighbour-joining (16), maximum-likelihood (options QFYG, fastDNAml program of G. J. Olsen, University of Illinois, Urbana, USA) and maximum-parsimony [PAUP 3.0s for the Macintosh, heuristic search (18)]. The robustness of each topology was checked by using the neighbour-joining method and 100 bootstrap replications. Trees were drawn by using the njplot program for the Macintosh (M. Gouy, CNRS URA 243, Université Claude Bernard, Lyon, France).

RESULTS AND DISCUSSION

The five strains isolated from red-spotted culture beds appeared as polarly flagellated, Gram-negative, non-fermentative rods (Table 2). The bacterium required salt for its growth, did not accumulate poly-β-hydroxybutyrate and did not reduce nitrate (Table 2). No peritrichous flagella were observed when the bacterium was cultivated on solid media. The G+C contents of the strains were 44–46 mol% (Table 1), suggesting that this bacterium should be assigned either to the genus *Alteromonas* or to the genus *Pseudoalteromonas* (2, 10).

Specific biochemical and physiological features of strain IAM 14595^T, No. 8R, E-1, E-2 and A-3 are shown in Table 2. Morphological, biochemical and physiological characteristics of these strains including IAM 14595^T were most closely related to that of *Pseudoalteromonas rubra* ATCC 29570^T among *Alteromonas*, *Pseudoalteromonas*, *Marinomonas* and related species. Phenotypic characteristics of *Pseudoalteromonas haloplanktis* subsp. *haloplanktis* IAM 12915^T, type species of *Pseudoalteromonas* (8), or *Alteromonas macleodii* IAM 12920^T, type species of *Alteromonas* (8), are also quite different from our strains (Table 2). Ten traits were different between these species: bacteriolytic activity, growth at 37 °C, requirement for organic growth factors, utilization of sucrose, *N*-acetylglucosamine, fumarate, succinate, D-galactose, L-proline and acetate (Table 2). *Pseudoalteromonas denitrificans* also produced prodigiosin-like pigment (3), but a significant number of bio-

Table 2. Phenotypic characteristics for distinguishing *Pseudoalteromonas bacteriolytica* from *P. rubra*, *P. haloplanktis* subsp. *haloplanktis* and *Alteromonas macleodii*

O, Oxidative; pol, polar flagellum; WP, weakly positive; d, different reactions; ( ), result of *P. bacteriolytica* IAM 14595^T.

Characteristic	*P. bacteriolytica* IAM 14595^T, No. 8R, E-1, E-2 and A-3	*P. rubra* ATCC 29570^T	*P. haloplanktis* subsp. *haloplanktis* IAM 12915^T	*A. macleodii* IAM 12920^T
Pigmentation	+	+	−	−
Water-insoluble	Red	Red		
Salt requirement	+	+	+	+
OF test	O	O	O	O
Flagellar arrangement	pol	pol	pol	pol
Growth at:				
4 °C	−	−	+	−
37 °C	−	+	−	+
40 °C	−	−	−	+
Oxidase	+	+	+	+
Catalase	WP	+	+	+
Production of:				
Amylase	+	+	−	+
Alginase	−	−	−	+
Agarase	−	−	−	−
Lipase	+	+	+	+
Chitinase	−	−	−	−
Gelatinase	+	+	+	+
Caseinase	+	+	+	+
κ-Carrageenase	−	−	−	−
Bacteriolytic activity	+	−	−	−
NO_3^- reduced to NO_3^-	−	−	−	−
Requirement for organic growth factors	+	−	−	−
Utilization of:				
D-Mannose	+	+	−	−
D-Fructose	+	+	+	+
Sucrose	+	−	−	+
Maltose	d (−)	+	+	+
D-Gluconate	−	−	+	+
D-Glucosamine	+	+	−	−
N-Acetylglucosamine	−	+	−	+
D-Mannitol	d (−)	−	+	+
Fumarate	+	−	+	−
Succinate	+	−	+	+
D-Sorbitol/citrate	−	−	−	−
meso-Erythritol/glycerol/ m-hydroxybenzoate/DL-malate/ α-ketoglutarate	−	−	−	−
D-Galactose	+	−	−	+
Cellobiose	d (−)	−	+	+
Melibiose	−	−	−	+
Lactose	−	−	−	+
Xylose	−	−	−	+
Trehalose	d (+)	+	+	−
γ-Aminobutyrate	−	−	−	+
L-Proline	−	+	+	+
Acetate	+	−	+	+
Pyruvate	d (+)	+	+	+

Table 2 *(cont.)*

Characteristic	*P. bacteriolytica* IAM 14595^T, No. 8R, E-1, E-2 and A-3	*P. rubra* ATCC 29570^T	*P. haloplanktis* subsp. *haloplanktis* IAM 12915^T	*A. macleodii* IAM 12920^T
L-Tyrosine	d (−)	−	−	+
Aconitate	−	−	−	−
L-Glutamate	d (+)	−	+	+
D-Glucose/propinonate	+	+	+	+
D-Glucronate/putrescine/δ-aminovarate	−	−	−	−
Growth on KCN	−	−	−	−
PHB accumulation	−	−	−	−

chemical characteristics including G+C content of the species were different from our strains.

DNA–DNA hybridization results (Table 1) showed that the four other isolates were more than 90% similar to IAM 14595^T. DNA–DNA homology values between IAM 14595^T and representatives of the genera *Pseudoalteromonas*, *Marinomonas* and *Alteromonas*, used in this study were distinctively low (Table 1). There was no significant homology when *Pseudoalteromonas rubra* ATCC 29570^T was photobiotinylated and hybridization was performed between *Pseudoalteromonas rubra* ATCC 29570^T and IAM 14595^T (Table 1).

The 16S rDNA sequence of strain IAM 14595^T was aligned by comparison to a database containing about 5000 already aligned eubacterial small-subunit rDNA sequences. The results of broad phylogenetic analyses clearly showed that the sequence which we studied belonged to the gamma subclass of the *Proteobacteria* (data not shown), and more precisely, to the gamma 3 subclass. More detailed analyses showed that it could be included in the *Pseudoalteromonas* genus, with which it formed a robust clade (and in agreement with phenotypic data) although it was the deepest branch of the genus *Pseudoalteromonas* (Fig. 1). Homology levels for nucleotides of 16S rDNAs from *Pseudoalteromonas bacteriolytica* and closely related species were very low, ranging from 87·7% (against gamma proteobacterium Z25522) to 90·3% (against *Pseudoalteromonas carrageenovora* and *Pseudoalteromonas espejiana*). This finding was also revealed by the DNA–DNA homology results, in which IAM 14595^T showed a very low level of relatedness with the other species of *Pseudoalteromonas* (Table 1). It was also separated from *Alteromonas macleodii* subsp. *macleodii* (Fig. 1). From the results of the DNA–DNA hybridization (Table 1) and 16S rRNA (Fig. 1) experiments, it was revealed that the strain IAM 14595^T and the other four strains, which are the causative agents of red spot disease of *L. japonica*, should be recognized as a new species. The name proposed for this bacterium with a unique bacteriolytic activity and that induces damages in *Laminaria* seed supply is *Pseudoalteromonas bacteriolytica*.

Description of *Pseudoalteromonas bacteriolytica* sp. nov.

Pseudoalteromonas bacteriolytica (bac.te.rio.ly′ti.ca. Gr. n. *baktron* rod or staff; Gr. adj. *lytica* dissolving; M.L. fem. adj. *bacteriolytica* bacteria-dissolving).

Gram-negative, strictly aerobic, polarly flagellated bacterium isolated from red-spotted culture beds of *Laminaria japonica*. Cells are rod-shaped, with rounded ends, and are 0·6–0·9 μm in diameter and 1·9–2·5 μm long when the organism is grown on CSY-3 agar medium; the cells occur singly or in pairs. No endospores or capsules are formed. Peritrichous flagellation is not observed when the organism is cultivated on solid media. Colonies on CSY-3 agar medium are red (although non-pigmented colonies frequently occurred), circular, and smooth and convex with entire edges. Mesophilic and neutrophilic chemo-organotroph which grows at temperatures ranging from 15 to 35 °C. No growth occurs at 37 °C. Positive for acid production from glucose, hydrolysis of starch, Tween 80 and casein; weakly positive for catalase; positive for oxidase; assimilates D-mannose, D-fructose, sucrose, D-glucosamine, fumarate, succinate, D-glucose, D-galactose, acetate and propionate. Negative for luminescence, production of fluorescein and pyocyanin; hydrolysis of agar, chitin and κ-carrageenan; nitrate reduction; growth on KCN; accumulation of poly-β-hydroxybutyrate; assimilation of D-gluconate, *N*-acetylglucosamine, D-sorbitol, citrate, *meso*-erythritol, glycerol, DL-malate, α-ketoglutarate, m-hydroxybenzoate, melibiose, lactose, D-glucronate, xylose, putrescine, δ-aminovarate, γ-aminobutyrate, L-proline, aconitate (Table 2). The G+C content of the DNA is 44–46 mol%. The type strain is IAM 14595^T (Table 1).

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