Pseudoalteromonas sagamiensis sp. nov., a marine bacterium that produces protease inhibitors

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A marine bacterium producing protease inhibitors was isolated from neritic sea water and was studied phenotypically, genotypically and phylogenetically. This bacterium (strain B-10-31T) produced three types of protease inhibitor, namely, marinostatin, monastatin and leupeptin, which were considerably different in terms of their chemical structure and properties. Strain B-10-31T was a rod-shaped, non-spore-forming, Gram-negative, strictly aerobic bacterium that was motile by means of one polar flagellum. The strain required Na+ for growth and exhibited optimal growth at 27 ± 6°C, pH 8 ± 0 and 2% (w/v) NaCl. It utilized various substrates, such as D-glucose, maltose, maltotriose, N-acetylglucosamine, L-threonine, L-serine, L-arginine, L-proline, L-α-alanine and L-glutamate, as the sole energy source. Ubiquinone-8 was the major respiratory quinone. The major fatty acids were C16:0, C16:1ω7c, C16:1ω9c and C18:1ω7c. The G+C content of the DNA of strain B-10-31T was 42 ± 0 mol%. Phylogenetic analysis, based on 16S rDNA sequences, showed that the strain clustered in the γ-Proteobacteria. The aerobic marine bacterium Pseudoalteromonas bacteriolytica was the species most closely related to the new isolate (90 ± 4% 16S rDNA sequence similarity); other described species in the γ-Proteobacteria cluster showed low levels of sequence similarity with strain B-10-31T (< 90%). Based on the above results, it is proposed that the novel marine bacterium should be classified as a new species, for which the name Pseudoalteromonas sagamiensis (type strain B-10-31T = JCM 11461T = DSM 14643T) is proposed.

Previously, we isolated a marine bacterium (strain B-10-31T) that produces protease inhibitors (Imada et al., 1985a) and tentatively identified it as Alteromonas sp. Three different types of protease inhibitor produced by this isolate were characterized. The first one was a serine protease inhibitor called marinostatin, and a related compound, which had an inhibitory activity against serine proteases such as subtilisin (Imada et al., 1986a). The second one was a thiol protease inhibitor called monastatin, which had an inhibitory activity against the protease produced by a bacterium pathogenic to fish (Imada et al., 1985b). The third one was leupeptin, which had an inhibitory activity against both thiol and serine proteases (Hamato et al., 1992).

Marinostatins were the first protease inhibitors isolated from marine bacteria. These compounds have been characterized based on their amino acid sequences (Imada et al., 1986b), their reactive site (Takano et al., 1991), their nucleotide sequences (Miyamoto et al., 1998) and the conditions of their production in strain B-10-31T (Imada et al., 1985c). However, the exact taxonomic position of strain B-10-31T remained to be determined.

In this study, we have characterized strain B-10-31T more thoroughly from a taxonomic viewpoint. On the basis of the results of this study, we conclude that our isolate should be classified as a new species of Pseudoalteromonas, for which we propose the name Pseudoalteromonas sagamiensis.

Strain B-10-31T was originally isolated from coastal sea water at the Aburatsubo Inlet of Sagami Bay in Kanagawa Prefecture, Japan, at a depth of 5 m. The culture was maintained on modified PESS II (MP) agar medium [per
litrage sea water: 1·0 g Bacto-soytone, 1·0 g Proteose peptone no. 3, 0·1 g Bacto-yeast extract, 2·0 g Polypepton, 1·5 g Bacto-agar (pH 7·5) [Imada et al., 1985a] at 20 °C and subcultured every 5 months. Gram staining, catalase test, oxidase test, nitrate reduction test, tests for the production of protease, gelatinase, amylase, alginase, chitinase and lecithinase, and tests for the hydrolysis of Tween 80 and tributyryl were performed as described previously (Imada et al., 1985a). Accumulation of poly-β-hydroxybutyrate and organic growth factor requirement were determined according to the method of Baumann et al. (1971). The oxidation–fermentation (O/F) test was performed using MOF medium for marine bacteria (Leifson, 1963). Unless stated otherwise, test media were prepared with aged sea water and the cultivation temperature was 27 °C. The ranges of temperatures and pH values for growth of the strain were determined in the same medium containing NaCl at concentrations ranging from 0 to 7 % (w/v) instead of natural sea water. For carbon assimilation tests, cells of strain B-10-31T were Gram-negative rods that were motile by means of one polar flagellum, as described previously (Imada et al., 1985a). Colonies on the agar medium had light-yellow pigmentation, and were flat, circular and wrinkled. A light-brown pigment was produced around old colonies (after 2 days incubation) on PYG-S plate medium. Spores were not observed microscopically. The strain was weakly catalase- and oxidase-positive. Accumulation of poly-β-hydroxybutyrate as an intracellular reserve product was not observed. Organic growth factors were required. The strain produced protease, gelatinase and amylase, but not alginase, chitinase or lecithinase. It hydrolysed Tween 80, but not tributyryl. Nitrate reduction was not observed. It required Na⁺ for growth and was able to grow in the presence of 0·5–5 % (w/v) NaCl, with the optimum being 2 %. The temperature range for growth was 15–35 °C, with the optimum being 27 °C. It grew well at pH 6·0–8·5; the optimum pH for growth was 8·0. Oxidative acid formation from D-glucose was observed in MOF medium. Spores were not observed microscopically. The inhibitory zone of growth was observed after 2 days incubation. The strain was susceptible to fradiomycin, gentamicin, lividomycin, ribostamycin, streptomycin, erythromycin, oleandomycin, rifampicin, chloramphenicol and tetracycline, but not to dibekacin, kanamycin, lincomycin, ampicillin, carbencillin, oxacillin, penicillin G, the vibriostatic agent O-129 or vancomycin.

Quinones were extracted, fractionated and analysed by spectrochromatography, as described previously (Hiraiishi et al., 1996). HPLC analysis indicated that ubiquinone-8 accounted for 95 % of the total quinone content of the strain. The other quinones detected were ubiquinone-6 and ubiquinone-7. Menaquinones were not detected.

Cellular fatty acid composition was analysed by using the GC system described previously (Nakamura et al., 1995). Unsaturated fatty acids C₁₆:₁₀9°C, C₁₆:₁₁₀°C and C₁₈:₁₁₀7°C were the major fatty acids detected with compositions of 22.15 % and 27 % (total fatty acids), respectively. The saturated fatty acid C₁₆:₀ was also detected (11 % total fatty acids). The hydroxy fatty acid C₁₂:₀₃-OH was detected in an appreciable amount (7 % total fatty acids). Iso-branched fatty acids were not detected.

Genomic DNA was prepared by the procedure of Marmur (1961). The DNA base content was determined by the HPLC method, as described previously (Katayama-Fujimura et al., 1984). The DNA G + C content of strain B-10-31T was found to be 42·0 mol%.

The 16S rDNA of strain B-10-31T was amplified by PCR, sequenced using an Applied Biosystems Dye Terminator Cycle Sequencing kit and analysed using an Applied Biosystems 373A DNA sequencer as described previously (Kobayashi et al., 2000). Sequence data were compiled from overlapping sequence data using the GENETYX computer program. Nucleotide substitution rates (Kmut values) (Kimura, 1980) were determined and a distance matrix tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL W program (Thompson et al., 1994). The sequence at positions 49–1321, based on Escherichia coli numbering (Weisburg et al., 1991), was aligned in this study. The reference sequences of organisms related to strain B-10-31T were obtained from the DDBJ/EMBL/GenBank databases. 16S rDNA sequence analysis revealed that strain B-10-31T belongs to the γ-Proteobacteria and is related to members of the genera Pseudoalteromonas, Alteromonas, Idiomarina, Thalassomonas and Colwellia. Phylogenetic analysis showed that the strain does not belong to any of the previously described genera [Fig. 1 and complete tree available in IJSEM Online (http://ijsem.sgmjournals.org)]. The 16S rDNA gene sequence of strain B-10-31T exhibited similarities to those of Pseudoalteromonas, Alteromonas, Idiomarina, Thalassomonas, Colwellia and Glaciecola species as follows: 90·4 % (Pseudoalteromonas bacteriolytica) to 86·6 % (Pseudoalteromonas antarctica), 87·7 % (Alteromonas alvinellae and Alteromonas macleodii), 90·3 % (Idiomarina abyssalis) to 89·3 % (Idiomarina zobellii), 89·7 % (Colwellia maris) to 86·2 % (Colwellia hornerae), 89·0 % (Thalassomonas
B-10-31T. In this study, phylogenetic trees were also constructed by different methods, such as the maximum-likelihood method contained within the PHYLIP package (Felsenstein, 1993), and similar phylogenetic trees to that available from IJSEM Online were obtained.

Phenotypic studies also revealed that strain B-10-31T could not be assigned to any of the four previously described genera of aerobic marine bacteria. Table 1 shows a comparison of the characteristics of strain B-10-31T with those of related genera, namely, *Idiomarina*, *Colwellia*, *Thalassomonas* and *Glaciecola*, which accommodate Gram-negative, rod-shaped bacteria common in marine habitats. Species of aerobic marine bacteria that belong to the genus *Idiomarina*, namely, *I. zobellii* and *I. abyssalis*, have been isolated from sea water at a depth of 4000–5000 m (Ivanova et al., 2000). Strain B-10-31T showed the second highest (only 0·1 % lower than *P. bacteriolytica*) 16S rDNA sequence similarity (90·3 %) to *I. abyssalis*. Phenotypic comparison showed significant differences between strain B-10-31T and *Idiomarina* species, including cellular fatty acid composition (presence or absence of iso-branched fatty acids), G+C content of genomic DNA, ability to grow at low temperatures and utilization of various substrates such as D-glucose and maltose. Various phenotypic properties distinguished strain B-10-31T from species of the genera *Colwellia*, *Thalassomonas* and *Glaciecola*. Growth at 4 °C, chitinase production and oxygen sensitivity distinguished strain B-10-31T from *Colwellia* species. The genomic DNA G+C content clearly distinguished strain B-10-31T from the *Thalassomonas* species: *T. viridans* has a higher G+C content (48·4 mol%) than strain B-10-31T. The production of green pigment and utilization of substrates such as D-fructose and sucrose also distinguished strain B-10-31T from *T. viridans*. Phenotypic properties distinguishing strain B-10-31T from *Glaciecola* species included colony profile, growth at 4 °C and utilization of various substrates such as D-glucose and maltose.

According to the genotypic and phylogenetic studies described here, we conclude that strain B-10-31T should be classified in a new genus as a new species. However, we decided that the new isolate should be classified as a *Pseudoalteromonas* species at this time, because the phenotypic differences between strain B-10-31T and related genera found so far seemed to be too small to warrant generic separation. Therefore, we propose the name *Pseudoalteromonas sagamiensis* to accommodate our new isolate. Strain B-10-31T is the type and only strain of this species.

Fig. 1. Phylogenetic tree showing the relationship of *Pseudoalteromonas sagamiensis* B-10-31T with strains of related genera, based on a comparison of 16S rDNA sequences. The tree, constructed using the neighbour-joining method (Saitou & Nei, 1978), was based on a comparison of positions 49–1321 (E. coli numbering; Weisburg et al., 1991). Bootstrap values, obtained with 1000 bootstrap resamplings, are given at branch points of interest. Nucleotide sequence accession numbers are given in parentheses. The tree was rooted with the 16S rDNA sequence of *Escherichia coli* (X91217).
Table 1. Phenotypic characteristics useful for differentiating strain B-10-31T from four related genera that occur in marine habitats

Data were obtained from Bowman et al. (1998), Ivanova et al. (2000) and Macían et al. (2001). +, Positive; −, negative; V, varies between strains; ND, not determined.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>B-10-31T</th>
<th>Idiomarina</th>
<th>Colwellia</th>
<th>Thalassomonas</th>
<th>Glaciecola</th>
</tr>
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<tbody>
<tr>
<td>Flagellar arrangement</td>
<td>Polar</td>
<td>Polar</td>
<td>Polar</td>
<td>Polar</td>
<td>ND</td>
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<tr>
<td>Pigmentation</td>
<td>Yellow</td>
<td>None</td>
<td>None</td>
<td>Green</td>
<td>Pink-red, pale-pink</td>
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<tr>
<td>Oxygen sensitivity</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Facultatively anaerobic</td>
<td>Aerobic</td>
<td>ND*</td>
</tr>
<tr>
<td>Growth with NaCl (%)</td>
<td>1-5-5</td>
<td>0-6-15</td>
<td>&gt;2-5</td>
<td>2-4</td>
<td>ND</td>
</tr>
<tr>
<td>Psychrophilic growth (at 4 °C)</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Accumulation of poly-β-hydroxybutyrate</td>
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<td>−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>Gelatinase production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
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<td>Chitinase production</td>
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<td>V</td>
<td>ND</td>
<td>−</td>
<td>−</td>
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<td>Utilization of:</td>
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<td></td>
<td></td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>D-Fructose</td>
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<td>−</td>
<td>ND</td>
<td>+</td>
<td>−</td>
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<tr>
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<td>−</td>
<td>ND</td>
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<td>−</td>
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<tr>
<td>Sucrose</td>
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<td>−</td>
<td>ND</td>
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<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Lactose</td>
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<tr>
<td>Major fatty acid type</td>
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<td>Straight</td>
<td>Straight</td>
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<tr>
<td>Major isoprenoid quinone</td>
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<td>Q-8</td>
<td>ND</td>
<td>ND</td>
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<td>DNA G + C content (mol%)</td>
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<td>48-50</td>
<td>40-46</td>
<td>48-4</td>
<td>40-46</td>
</tr>
</tbody>
</table>

*Growth in the presence of between 0-5 × and 1-5 × sea water.

Description of Pseudoalteromonas sagamiensis sp. nov.

Pseudoalteromonas sagamiensis (sa.ga.mi.en’sis. N.L. adj. sagamiensis referring to Sagami Bay, the place of isolation).

Cells are Gram-negative rods that are 0.7–1.4 μm wide and 2.5–3.7 μm long. Motile by means of one polar flagellum. Spore formation is not observed. Strict aerobe. Colonies on PYG-D plate medium supplemented with 2% (w/v) NaCl are flat, circular and wrinkled and have a light-yellow pigmentation. A light-brown pigment is produced around 2-day-old colonies. Marine bacterium that grows in the presence of 1-5-5% (w/v) NaCl, with the optimum being 2% NaCl. Grows well at 15 and 35 °C, but not at 10 or 40 °C; optimum growth at 27 °C. Grows well at pH 6-0 and 8-5; optimum growth at pH 8-0. Weakly catalase- and oxidase-positive. Oxidative acid formation from D-glucose is observed. Produces some protease inhibitors, namely, marinostatin, monastatin, lysozyme, and leupeptin. Produces protease, gelatinase and amylase, but not alginate, chitinase or lecinthase. Able to hydrolyse Tween 80, but not tributyrin. Nitrate reduction and production of hydrogen sulfide are not observed. Poly-β-hydroxybutyrate is not accumulated as an intracellular reserve product. Organic growth factors are required. D-Glucose, maltose, maltotriose, N-acetylglucosamine, L-threonine, L-serine, L-arginine, L-proline, L-α-alanine and L-glutamate are utilized as sole carbon and energy sources. D-Mannose, D-galactose, D-fructose, sucrose, cellobiose, melibiose, lactose, salicin, D-glucaronate, fumarate, DL-lactate, DL-glycerate, citrate, D-mannitol, glycerol, sarcosine, putrescine, D-sorbitol, DL-malate, 2-oxoglutarate, D-ribose, D-xyllose, L-arabinose, L-rhamnose, trehalose, gluconurate, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, heptanoate, caprylate, L-tartrate, DL-β-hydroxybutyrate, pyruvate, meso-inositol, propylene glycol, ethanol, n-propanol, n-butanol, glycerine, D-α-alanine, β-alanine, L-isoleucine, L-lysine, L-ornithine, L-citrulline, L-histidine, betaine and trigonelline are not utilized. Susceptible to fradiomycin, gentamicin, lividomycin, lincomycin, streptomycin, erythromycin, oleandomycin, rifampicin, chloramphenicol and tetracycline, but not to dibekacin, kanamycin, lincomycin, ampicillin, carbencillin, oxacillin, penicillin G, the vibrio-static agent O-129 or vancomycin. Ubiquinone-8 is the major respiratory quinone. Menaquinone is absent. The major fatty acids are C16:0, C15:0iso7c, C16:1ω9c and C18:1ω7c. 16S rDNA sequence analyses places the species among the γ-Proteobacteria.

The type strain is strain B-10-31T (=JCM 11461T=DSM 14643T). The G+C content of its DNA is 42.0 mol%. Isolated from neritic sea water at the Aburatsubo Inlet of Sagami Bay in Kanagawa Prefecture, Japan, at a depth of 5 m.
Note added in proof
Since this article was submitted for publication, three more species of Pseudoalteromonas have been described, Pseudoalteromonas agarivorans (Romanenko et al., 2003b), Pseudoalteromonas phenolica (Isanesteyo & Kamei, 2003) and Pseudoalteromonas maringlutinosa (Romanenko et al., 2003a).

References