\textit{Pseudoalteromonas phenolica} sp. nov., a novel marine bacterium that produces phenolic anti-methicillin-resistant \textit{Staphylococcus aureus} substances

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Four strains of aerobic, Gram-negative rods, motile by means of a single polar flagellum, that produced phenolic anti-methicillin-resistant \textit{Staphylococcus aureus} (MRSA) substances and brown-pigmented colonies, were isolated from sea water. The G + C content of the DNA ranged from 39.9 to 40.6 mol\%. The isolates grew at 18–37°C and pH 6.5–9.5 (optimal pH 7.5–9) and in medium containing 1–5 \% (w/v) NaCl (optimal NaCl concentration 2–3.5 \%). The isolates grew optimally in medium dissolved in 40–100 \% artificial sea water. Based on 16S rDNA similarities, the novel strains were closely related to \textit{Pseudoalteromonas luteoviolacea} and \textit{Pseudoalteromonas piscicida}, with 96.3 and 95.7 \% sequence similarity, respectively. However, the strains could be differentiated from \textit{P. luteoviolacea} by seven traits and from \textit{P. piscicida} by 10 traits. Analysis of DNA–DNA relatedness to these related species revealed low levels of DNA hybridization (19.6 \% to \textit{P. luteoviolacea} and 22.4 \% to \textit{P. piscicida}). However, the type strain, O-BC30\(^T\), and the other three bacterial isolates showed high DNA relatedness to each other, ranging from 84.8 to 93.7 \%. Based on the results of phenotypic characterization, phylogenetic analysis based on 16S rDNA sequences and DNA–DNA hybridization, it is concluded that these isolates represent a novel species in the genus \textit{Pseudoalteromonas}. Because the type strain, O-BC30\(^T\) (= IAM 14989\(^T\) = KCTC 12086\(^T\)), produces phenolic anti-MRSA substances, the name proposed for this novel species is \textit{Pseudoalteromonas phenolica} sp. nov.

\textbf{INTRODUCTION}

The genus \textit{Pseudoalteromonas} includes both pigmented and non-pigmented, heterotrophic bacteria, cells of which are Gram-negative rods, motile by single polar flagella. The genus was proposed by Gauthier et al. (1995), who divided \textit{Alteromonas} into two genera, \textit{Alteromonas} and \textit{Pseudoalteromonas}, based on 16S rDNA sequences. Species in the genus \textit{Pseudoalteromonas}, typically associated with eukaryotic hosts (Holmström & Kjelleberg, 1999), are frequently isolated from marine animals such as sponges (Ivanova et al., 2002b), tunicates (Holmström et al., 1998) and mussels (Ivanova et al., 1996, 1998) and also from marine algae such as \textit{Ulva lactuca} (Egan et al., 2001) and \textit{Laminaria japonica} (Sawabe et al., 1998). Several species in the genus, such as \textit{Pseudoalteromonas luteoviolacea} (Gauthier, 1982; Novick & Tyler, 1985), \textit{Pseudoalteromonas rubra} (Gauthier, 1976), \textit{Pseudoalteromonas citrea} (Gauthier, 1977), \textit{Pseudoalteromonas aurantia} (Gauthier & Brittymayer, 1979) and \textit{Pseudoalteromonas peptidolytica} (Venkateswaran & Dohmoto, 2000), were isolated from sea water. Members of \textit{Pseudoalteromonas} are also known to be producers of bioactive substances (Holmström & Kjelleberg, 1999). \textit{P. luteoviolacea} (Gauthier, 1982; Hanefeld et al., 1994), \textit{P. rubra} (Gauthier, 1976), \textit{P. aurantia} (Gauthier & Brittymayer, 1979), \textit{Pseudoalteromonas rutherica} (Ivanova et al., 2002a) and \textit{Pseudoalteromonas maricaloris} (Ivanova et al., 2002b) have been determined as antibiotic-producing bacteria. Recently, we isolated a novel species in the genus \textit{Pseudoalteromonas} that produces anti-methicillin-resistant \textit{Staphylococcus aureus} (MRSA) substances.

MRSA causes a wide range of human diseases, ranging from minor skin infections to life-threatening deep infections such as pneumonia, endocarditis, meningitis, post-operative infection, septicemia and toxic shock syndrome. The high prevalence of MRSA around the world makes it a serious public health problem, since this Gram-positive pathogen has become multi-drug-resistant...
(Archer & Bosilevac, 2001; Hiramoto et al., 2001; Isnansetyo et al., 2001; Kaatz et al., 2000; Witte, 1999). In an effort to discover alternative antibiotics against MRSA, we screened bacteria that originated from the marine environment for anti-MRSA activity. Four strains of brown-pigmented marine bacteria with potent anti-MRSA activity were isolated independently from sea water collected from the same location and at the same sampling time. These isolates were then identified by pheno-typic and genotypic characterization to represent a novel species in the genus Pseudoalteromonas. This paper describes this novel bacterial species and proposes the name *Pseudoalteromonas phenolica* sp. nov.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains were isolated on 30 July 1999. The temperature and salinity of the sea water at the sampling site were 27 °C and 38.3%o. The samples were obtained with sterile polyprene bottles and transported to our laboratory in a cool-box. The sample was smeared on ZoBell 2216E agar medium containing (l) 5 g polypepton (Nihon Seiyaku) and 1 g yeast extract (Nihon Seiyaku) dissolved in 75% Jamarine S artificial sea water (ASW) (Jamarine Laboratory) (Sako et al., 1996a, b). The plates were incubated at 25 °C for 24 h. Bacterial colonies on the plate were then overlaid with MRSA suspended at 10^6 cells ml^-1 in tryptic soy medium (Difco) with 0.8% agar; the plates were then incubated at 25 °C for a further 24 h. When antagonistic activity of colonies was observed against MRSA, these active colonies were isolated on ZoBell 2216E agar medium. Active isolates designated O-BC20, O-BC30T, O-BC40(B) and O-BCX3 were isolated and are described in this paper. The reference strains *P. luteoviolacea* IAM 14710^T*, P. pseudoalteromonas piscicida* IAM 12932^T*, *P. pseudoalteromonas haloplanktis* subsp. *haloplanktis* IAM 12915^T* and *Alteromonas macleodii* IAM 12920^T* were provided by the IAM Culture Collection (University of Tokyo, Japan). All of these strains were routinely cultivated aerobically at 25 °C on ZoBell 2216E agar medium.

**Physiological and biochemical characterization.** Morphological observation and phenotypic tests including catalase, cytochrome oxidase, citrinase, aagarase, arginine dihydrolase, cellulase, gelatinase, lecithinase, DNase and lipase activities were carried out based on the methods described by Smibert & Krieg (1994). Oxidative or fermentative metabolic activity of glucose was determined by the method of Hugh & Leifson (1953). The requirement for NaCl was tested in a medium containing (l) 5 g MgCl_2, 2 g MgSO_4, 0.5 g CaCl_2, 1 g KCl, 5 g peptone (Sigma-Aldrich) and various concentrations of NaCl at pH 7.5, adjusted with KOH (Smibert & Krieg, 1994). Growth in various concentrations of ASW was examined in ASW formula 2 (Smibert & Krieg, 1994) supplemented with 5 g peptone l^-1, pH 7.5. Determination of substrate utilization as single carbon and energy sources was performed in marine minimal medium (Ostling et al., 1991; Holmström et al., 1998) containing 1% (w/v) substrate of interest. Growth under anaerobic conditions was tested in an anaerobic jar system (GasPak system; BBL).

**Electron microscopy.** For observation of cell morphology and flagellation, bacterial cells grown overnight on ZoBell 2216E agar were suspended in physiological saline solution. One drop of the suspension was negatively stained with one drop of 1% phosphotungstic acid after the suspension was placed on a carbon-coated copper grid. Observation was carried out under a transmission electron microscope (model H-800; Hitachi).

**Molecular characterization.**

**DNA base composition.** DNA preparation was carried out based on the procedure of Marmur (1961), with minor modifications. To extract and deproteinize the DNA, phenol/chloroform/isoamyl alcohol (25:24:1) was used instead of chloroform/isoamyl alcohol (24:1) used in the standard procedure. The G + C content of DNA was determined by the HPLC method, as described previously (Tamaoka & Komagata, 1984). The G + C content of DNA from *Escherichia coli* was used as a reference.

**Amplification of 16S rDNA and sequencing.** Bacterial DNA was extracted from cells by using the EZDNA Bacterial DNA kit (Omega Biotek) as recommended by the manufacturer. PCR was carried out to amplify the 16S rDNA with a Perkin-Elmer model GeneAmp PCR System 2400 thermal cycler (Applied Biosystems) with universal primers 27f and 1492r corresponding to positions 27 in the forward direction and 1492 in the reverse direction of the *E. coli* 16S rDNA sequence (Brosius et al., 1978). The amplification was performed using the PCR conditions described by Sawabe et al. (1998). The thermal profile consisted of 30 cycles of denaturation at 94 °C for 180 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s. The PCR product was purified by polyethylene glycol (PEG 6000) precipitation and used as the template for sequencing.

The ABI PRISM Dye Terminator cycle sequencing kit (Applied Biosystems) was used following the protocols described by the manufacturer. The PCR cycle-sequencing product was purified by using CENTRI-SEP columns (Princeton Separations) according to the procedure described by the manufacturer. 16S rDNA sequences were determined with a Perkin-Elmer model ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

**Phylogenetic analysis and alignment.** A similarity search with the 16S rDNA sequence was performed with 16S rDNA sequences available in the GenBank/EMBL/DDBJ databases using the BLAST algorithm (Altschul et al., 1990). Multiple sequence alignment of representative sequences was carried out by using the CLUSTAL W program, version 1.8 (Thomson et al., 1994). A neighbour-joining analysis (Saitou & Nei, 1987) and bootstrap analysis of 1000 data resamplings were performed to determine the robustness of each topology. A phylogenetic tree was made by using the program TREEVIEW (Page, 1996). 16S rDNA sequence similarity values to representative 16S rDNA sequences retrieved from GenBank were calculated by the computer program GENETYX (Software Development).

**DNA–DNA hybridization.** DNA–DNA hybridization was carried out in microplate wells (Black Maxisorp; Nunc) using a fluorimetric method (Ezaki et al., 1989; Willems et al., 2001). The fluorescence intensity was detected by a fluorescence multi-well plate reader (Cytolfluor II; PerSeptive Biosystems). The two most closely related strains, *P. luteoviolacea* IAM 14710^T* and *P. piscicida* IAM 12932^T*, a non-pigmented *Pseudoalteromonas* strain, *P. haloplanktis* subsp. *haloplanktis* IAM 12915^T*, *A. macleodii* IAM 12920^T* and *E. coli* XL-1 Blue (Stratagene) were used as reference strains.

**RESULTS AND DISCUSSION**

This paper proposes an anti-MRSA substance-producing bacterium isolated from the marine environment as a novel species in the genus *Pseudoalteromonas*. Strain O-BC30^T* and the other three strains were Gram-negative rods, motile by a single polar flagellum. These bacteria were aerobic and non-fermentative, utilized glucose oxidatively, hydrolysed starch and did not produce H_2S from thiosulphate. DNase,
The 16S rDNA sequence (1450 bases) of strain O-BC30T was closest to *P. luteoviolacea*, *P. piscicida* and *P. rubra* (formerly *Alteromonas luteoviolacea*, *Pseudomonas piscicida* and *Alteromonas rubra*; Gauthier et al., 1995) with 96-3, 95-7 and 95-7 % sequence similarity, respectively. These sequence similarity data are consistent with the phylogenetic analysis (Fig. 1), which grouped these species in the same clade. Strain O-BC30T exhibited 95-1, 95-0 and 94-2 % 16S rDNA sequence similarity, respectively, to the other three members of this clade, *Pseudoalteromonas flavipulchra*, *P. maricaloris* and *P. peptidolytica*.

The 16S rDNA sequence similarity between *P. luteoviolacea* and *P. piscicida* and strain O-BC30T is less than 97-0 %, implying that these strains will not reassociate with more than 60 % DNA–DNA relatedness (Rosselló-Mora & Amann, 2001; Stackebrandt & Goebel, 1994). However, the 97 % threshold level of 16S rDNA similarity can not differentiate the genus *Pseudoalteromonas* to the species level (Venkateswaran & Dohmoto, 2000).

The DNA relatedness between O-BC30T and the type strains of the genus *Pseudoalteromonas* is less than 60 % at the DNA–DNA relatedness level (Venkateswaran & Dohmoto, 2000). However, the 97 % threshold level of 16S rDNA similarity can not differentiate the genus *Pseudoalteromonas* to the species level (Venkateswaran & Dohmoto, 2000).

### Table 1. Differential characteristics between *Pseudoalteromonas phenolica* sp. nov. and closely related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
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<tr>
<td>Pigmentation</td>
<td>Brown</td>
<td>Purple</td>
<td>–</td>
<td>–</td>
<td>Red</td>
<td>Yellow</td>
<td>Orange</td>
<td>Yellow</td>
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<tr>
<td>Growth at 37 °C</td>
<td>+</td>
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<td>+</td>
<td>–</td>
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<td>Catalase</td>
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<td>–</td>
<td>+</td>
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<td>Utilization of:</td>
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<tr>
<td>Maltose</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>D-Mannose</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Sucrose</td>
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<td>+</td>
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<tr>
<td>Laevulose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>ND</td>
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<td>D-Arabinose</td>
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<td>–</td>
<td>ND</td>
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<td>D-Mannitol</td>
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<td>N-Acetylg glucosamine</td>
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<td>Citrate</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>+</td>
<td>ND</td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glutamate</td>
<td>d</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>DL-Malate</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>Fumarate</td>
<td>+</td>
<td>–</td>
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<td>Acetate</td>
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<td>Pyruvate</td>
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<td>L-Tyrosine</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Tween 20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>G+C content of DNA (mol%)</td>
<td>39-9</td>
<td>40-6</td>
<td>40-43</td>
<td>43-46</td>
<td>41-45</td>
<td>46-48</td>
<td>41-42</td>
<td>41-7 ± 0-4</td>
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</tbody>
</table>

All of the strains grew well at 25–37 °C and slowly at 18 °C, but did not grow at 5 or 45 °C. The strains grew at pHs ranging from 6-5 to 9-5, with optimum growth at pH 7:5–9. Growth was not seen on TCBS agar medium (Holt & Krieg, 1994), but the bacteria grew well on Marine agar 2216 (Difco) and ZoBell 2216E agar. The strains grew optimally in ZoBell 2216E broth dissolved in 40–100 % ASW and slowly in 20–30 % ASW, but did not grow in medium containing 12-5 % ASW. Growth occurred in medium containing NaCl at concentrations of 1 to 5 % (optimal concentration 2-3-5 %).

The 16S rDNA sequence (1450 bases) of strain O-BC30T was aligned by comparison with available sequences from GenBank/EMBL/DDBJ. The result revealed that strain O-BC30T was closest to *P. luteoviolacea*, *P. piscicida* and *P. rubra* (formerly *Alteromonas luteoviolacea*, *Pseudomonas piscicida* and *Alteromonas rubra*; Gauthier et al., 1995) with 96-3, 95-7 and 95-7 % sequence similarity, respectively. These sequence similarity data are consistent with the phylogenetic analysis (Fig. 1), which grouped these species in the same clade. Strain O-BC30T exhibited 95-1, 95-0 and 94-2 % 16S rDNA sequence similarity, respectively, to the other three members of this clade, *Pseudoalteromonas flavipulchra*, *P. maricaloris* and *P. peptidolytica*.
of the most closely related species, \textit{P. luteoviolacea} IAM 14710\textsuperscript{T} and \textit{P. piscicida} IAM 12932\textsuperscript{T}, was respectively 19-9 and 22-4\%. DNA–DNA hybridization was also performed between O-BC30\textsuperscript{T} and a non-pigmented \textit{Pseudoalteromonas} strain, \textit{P. haloplanktis} subsp. \textit{haloplanktis} IAM 12915\textsuperscript{T}, \textit{A. macleodii} IAM 12920\textsuperscript{T} and \textit{E. coli} XL-1 Blue, resulting in DNA–DNA relatedness of 14-1, 11-9 and 14-1\%. In addition, the DNA relatedness values between the other three bacterial isolates [O-BC20, O-BC40(B) and O-BCX3] and the reference strains were also very low, ranging from 3-7 to 27-3\%. All of the DNA relatedness values were less than 70\%, suggesting that all strains studied and reference strains could not be placed within the same species. In contrast, the DNA relatedness between strain O-BC30\textsuperscript{T} and the other three novel strains ranged from 84-8 to 93-7\%, suggesting that these four strains should be included in the same species. Phylogenetic analysis based on 16S rDNA sequences and DNA–DNA hybridization therefore reveals that these four strains of anti-MRSA substance-producing bacteria should be included in the genus \textit{Pseudoalteromonas} as a novel species.

The phenotypic characteristics and substrate utilization profiles also revealed that these anti-MRSA substance-producing marine bacteria can be differentiated from \textit{P. luteoviolacea} by seven traits (growth at 37 °C, pigmentation and utilization of sucrose, laevulose, fumarate, glutamate and acetate), from \textit{P. piscicida} by 10 traits (pigmentation, growth at 35 and 37 °C and utilization of sucrose, laevulose, \textit{N}-acetylglucosamine, trehalose, citrate, \textit{D}-malate, fumarate and acetate), from \textit{P. rubra} by eight traits (catalase, pigmentation and utilization of maltose, sucrose, laevulose, fumarate, acetate and Tween 20) and from \textit{P. haloplanktis} subsp. \textit{haloplanktis} by seven traits (catalase, pigmentation, growth at 35 and 37 °C and utilization of \textit{D}-mannose, \textit{N}-acetylglucosamine, trehalose and fumarate) (Table 1). In conclusion, the phenotypic characteristics also strongly suggest that the four strains of the anti-MRSA substance-producing marine bacteria represent a novel species in the genus \textit{Pseudoalteromonas}.

The most closely related species, \textit{P. luteoviolacea}, is reported to be an antibiotic-producing bacterium (Baumann \textit{et al.}, 1984; McCarthy \textit{et al.}, 1985; Hanefeld \textit{et al.}, 1994). However, the nature and antibacterial spectra of the antibiotics produced by strain O-BC30\textsuperscript{T} are different from those produced by \textit{P. luteoviolacea}. Strain O-BC30\textsuperscript{T} produces phenolic antibiotics, which are only active against Gram-positive bacteria. However, \textit{P. luteoviolacea} produces antibiotics against both Gram-positive and -negative bacteria. Because strain O-BC30\textsuperscript{T} produces phenolic anti-MRSA substances, the name proposed for this novel species is \textit{Pseudoalteromonas phenolica} sp. nov.

\textbf{Description of \textit{Pseudoalteromonas phenolica} sp. nov.}

\textit{Pseudoalteromonas phenolica} (phe.no’li.ca. N.L. n. phenolum phenol; N.L. fem. adj. \textit{phenolica} related to phenol).


The type strain, strain O-BC30\textsuperscript{T} (=IAM 14989\textsuperscript{T} =KCTC
12086T), was isolated from sea water collected off Ogasawara Island, Tokyo, Japan.

REFERENCES


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