Pseudoxanthomonas taiwanensis sp. nov., a novel thermophilic, N₂O-producing species isolated from hot springs

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Two novel thermophilic bacterial strains, with an optimum growth temperature of between 50 and 60 °C, were isolated from the Chi-ban Hot Springs in eastern Taiwan. Strains CB-225 and CB-226T were aerobic, thermophilic, non-sporulating, yellow-pigmented heterotrophic organisms. These strains exhibited an unusual denitrification reaction, reducing nitrite, but not nitrate, with the production of N₂O only. On the basis of a phylogenetic analysis of 16S rDNA sequences, DNA–DNA similarity data, morphological, physiological and biochemical characteristics, and fatty acid compositions, it was found that the novel strains belonged to the genus Pseudoxanthomonas and represented a novel species within this genus, for which the name Pseudoxanthomonas taiwanensis is proposed; the type strain is CB-226T (= ATCC BAA-404T = CCRC 17172T). P. taiwanensis differs from the only member of the genus Pseudoxanthomonas, the mesophilic species Pseudoxanthomonas broegbernensis, in that it exhibits a higher growth temperature and different morphological characteristics, such as the absence of polar flagella.

Keywords: thermophilic, Pseudoxanthomonas, nitrous oxide

INTRODUCTION

Micro-organisms associated with hot springs of geothermal areas have received considerable interest in recent years. Many thermophilic micro-organisms that inhabit extreme environments have been discovered and studied (Brock, 1978; Kristjansson & Stetter, 1992; Vieille & Zeikus, 2001). Thermophilic micro-organisms can obtain energy from extreme environments via autotrophic or heterotrophic bioenergetic pathways. Denitrification is an ability of bacterial cells that enables them to obtain energy from the dissimilatory reduction of nitrate (NO₃⁻) or nitrite (NO₂⁻), generating final products such as nitrous oxide (N₂O) or nitrogen (N₂). The denitrification reaction usually occurs in a few strains of fungi and many bacterial taxa, but is rarely seen in thermophilic bacteria (Knowles, 1982; Payne, 1973).

The distribution of denitrifying organisms among the Bacteria follows a distinct pattern, with a diverse range of denitrifying species belonging to the various subclasses of the Proteobacteria. Denitrifying microbes, with evolutionary significance, are also present among the halophilic and hyperthermophilic branches of the Archaea (Zumft, 1997). Denitrifying bacteria have frequently been isolated from biofilters used in waste-gas treatment, and have been shown to belong to the genera Stenotrophomonas and Xanthomonas (Lipski et al., 1992; Lipski & Altendorf, 1997). Finkmann et al. (2000) also reported the isolation of strains belonging to several novel genera and species from biofilters; these strains had similar denitrification characteristics and belonged to the genera Stenotrophomonas, Luteimonas and Pseudoxanthomonas. An investigation of the physiological properties of these strains revealed that they all possessed denitrifying activities. All of the strains reduced NO₂⁻, but not NO₃⁻, with the only product of this reduction being N₂O.

In this study, two novel thermophilic strains with similar truncated denitrification characteristics were...
isolated from the Chi-ban Hot Springs in eastern Taiwan. On the basis of DNA G+C content determinations, morphological and phenotypic characteristics, DNA–DNA similarity data and phylogenetic data, the strains were found to represent a new species within the genus *Pseudoxanthomonas*, for which the name *Pseudoxanthomonas taiwanensis* is proposed.

**METHODS**

**Isolation of bacterial strains.** Samples of hot-spring water, solfatara soil and mud were collected from hot springs in the Chi-ban area, Taitung, Taiwan. Water samples were transported without temperature control and analysed within 24 h of collection. Aliquots (100 µl) of untreated water samples were spread directly onto *Thermus* agar plates (Williams & da Costa, 1992), which were subsequently sealed in plastic bags and incubated at 50 °C for 7 days. Yellow-pigmented colonies were picked from the plates and subcultured to purity. Strains CB-226<sup>ã</sup>, CB-225 and other isolates were preserved in *Thermus* medium containing 15% (v/v) glycerol at −70 °C.

*Pseudoxanthomonas broegbernensis* DSM 12573<sup>T</sup> (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used for comparison with the two novel strains. Strains CB-225 and CB-226<sup>ã</sup> were deposited in the American Type Culture Collection (Manassas, VA 20110–2209, USA) and the Culture Collection and Research Center (Bioresource Collection and Research Center, PO Box 246, Hsinchu, Taiwan, 30099) as strains ATCC BAA-451 (= CCRC 17197) and ATCC BAA-404<sup>T</sup> (= CCRC 17172<sup>T</sup>), respectively.

**Morphological and ultrastructural characteristics.** Light microscopy and transmission electron microscopy were used to observe the morphology of cells. For light microscopy, a Zeiss AxioScope microscope equipped with a Nikon Coolpix 990 digital camera was used to obtain photomicrographs.

For transmission electron microscopy, bacterial strains were grown for 48 h and then washed by centrifugation. For negative-staining, 5 µl of liquid culture was dropped onto Formvar/carbon-coated grids (300 mesh) and stained with 1% phosphotungstic acid (pH 7/0). Electron micrographs were generated with a Hitachi model H7100 electron microscope.

**Phenotypic characteristics.** Biochemical and tolerance tests on strains CB-226<sup>ã</sup> and CB-225 and *P. broegbernensis* DSM 12573<sup>T</sup> were performed as described previously (Santos et al., 1989; Manaia & da Costa, 1991; Tenreiro et al., 1995; Van den Moover & Swings, 1990) in *Thermus* broth or on *Thermus* agar incubated at the appropriate temperatures for 3 days (CB-225 and CB-226<sup>ã</sup>, 50 and 30 °C; DSM 12573<sup>T</sup>, 30 °C). The pH range for growth was determined by measuring the turbidity (at 660 nm) of liquid cultures grown using appropriate biological buffers (Chung et al., 1997).

Filter-sterilized carbon sources (2.0 g l<sup>−1</sup>), ammonium sulfate (0.5 g l<sup>−1</sup>) and yeast extract (0.2 g l<sup>−1</sup>) were added to *Thermus* basal salts for single carbon source assimilation tests. Growth was determined by measuring the turbidity (at 600 nm) of liquid cultures in 125 ml flasks at the optimal growth temperature for 4 days. Positive and negative control cultures were grown in *Thermus* and minimal media, respectively. All growth experiments were performed in triplicate.

**Denitrification reactions.** Denitrification reactions were performed in *Thermus* broth in tubes with serum-bottle stoppers (Neyra & Dobereiner, 1977; Neyra & Van Berkum, 1977). The medium was supplemented with either 20 mM KNO<sub>3</sub> or 20 mM KNO<sub>2</sub>, to provide nitrous ions. After the bottles containing the medium had been inoculated and sealed, aerobic and microaerobic conditions were obtained as described previously (Balderston et al., 1976; Bazylnski et al., 1986; Payne, 1973). The tubes were incubated for 5 days at 30 °C (*P. broegbernensis* DSM 12573<sup>T</sup>, and strains CB-225 and CB-226<sup>ã</sup>) or at 50 °C (strains CB-225 and CB-226<sup>ã</sup>). Gas samples were collected and then analysed by using a Shimadzu GC-14A GC apparatus equipped with a thermal conductivity detector (TCD) and fitted with a Porapak Q (80–100 mesh) column (200 cm x 3 mm). The temperatures of the injection port and the TCD were set to 70 and 100 °C, respectively. The carrier gas was high-purity helium, and the column head pressure was set to 196 kPa, giving a linear velocity of 40 ml min<sup>−1</sup>.

**Fatty acid composition.** The cultures used for analyses of fatty acid composition were grown on *Thermus* agar plates at 30 or 50 °C for 48 h, as described above. Fatty acid methyl esters (FAMEs) were obtained from fresh, wet biomass by saponification, methylation and extraction as described by Kuykendall et al. (1988). The FAMEs were separated and analysed by GC/MS, using a HP 6890 gas chromatograph fitted with a 5% phenylmethyl siloxane capillary column (30 m x 0.25 mm; Hewlett Packard 5MS) and equipped with a HP 5973 mass-selective detector. The carrier gas was high-purity helium, the column head pressure was 140 kPa, the septum purge was 1 ml min<sup>−1</sup> and the injection port temperature was 25 °C. The oven was programmed to operate at 60 °C for 1 min, 60–140 °C at a rate of 25 °C min<sup>−1</sup>, 140–200 °C at a rate of 5 °C min<sup>−1</sup> and finally at 200 °C for 10 min<sup>−1</sup>. Identification and quantification of the FAMEs, as well as the numerical analysis of the fatty acid profiles, were performed by using the standard MIS Library Generation Software (Microbial ID).

**Determination of DNA G+C content and of DNA–DNA similarity.** The DNA G+C content of the strains was obtained by using HPLC, as specified by Mesbah et al. (1989): φ phage DNA was used as the control. DNA–DNA similarity data were obtained by using dot-blot hybridization and radioisotope detection. The probes were prepared by random-prime labelling (redprime II; Amersham Pharmacia Biotech) with [32P]dCTP. The hybridization procedures, optimal hybridization temperatures and buffers used were as detailed by Johnson (1984) and Kristjansson et al. (1994). The radioactive signals were detected by radioautography with an X-ray film (Kodak X-OMAT); the images were exposed for the minimum time required, to prevent saturation of the film. The signal produced by self-hybridization of the probe with its homologous target DNA was defined as 100% hybridization; the percentage similarity values were calculated for duplicate dots. Signals were analysed densitometrically using the GEL-PRO program (version 3.0).

**16S rDNA sequence determination and phylogenetic analysis.** Extraction of genomic DNA from strains CB-225 and CB-226<sup>ã</sup>, PCR-mediated amplification of their 16S rDNA genes and sequencing of the purified PCR products were performed according to Rainey et al. (1996). Sequences were analysed by electrophoresis, using a model 373A automated DNA sequencer (Applied Biosystem). The 16S rDNA sequences of the two novel strains were compared with those contained within the EMBL database (Maidak et al., 1994)
RESULTS AND DISCUSSION

Isolation of strains and morphological characteristics

Samples were taken from hot-spring water (n = 1), solfataric soil (n = 1) and mud (n = 1) from the Chibian Hot Springs, Taitung, Taiwan. The in situ temperature was 50–75 °C, and the pH values of the samples, as determined at the ambient temperature, were between pH 6·0 and 8·0. Red- and yellow-pigmented colonies were observed on the surface of Thermus agar plates following incubation of the samples at 50 °C for 72 h. After serial transfer and purification of the colonies, two novel light-yellow pigmented isolates (CB-225 and CB-226) with various morphological and physiological properties were selected for further study.

Cells of strains CB-225 and CB-226T were Gram-negative and formed single rods. The cells were non-spore-forming and non-motile. When grown on Thermus agar plates, colonies of the novel strains had a light-yellow pigmentation. Transmission electron microscopy of cells of the novel strains revealed that strains CB-225 and CB-226T appeared as single rods that were approximately 0·5–0·8 µm in diameter and 0·9–1·4 µm in length (Fig. 1a). No polar flagella were observed for strains CB-225 and CB-226T, unlike typical cells of P. broegbernensis DSM 12573T, which have a long polar flagellum (Fig. 1b).

Phenotypic and biochemical characteristics

Strains CB-225 and CB-226T grew between 30 and 60 °C; the optimum growth temperature was 50 °C when the strains were grown in Thermus medium (Fig. 2a). The pH range for growth of the strains was pH 6–11; the optimum pH was around 8·0 at the optimum growth temperature (Fig. 2b). These characteristics differ from those of the mesophilic species P. broegbernensis DSM 12573T, which grows poorly at 37 °C.

Several biochemical characteristics, such as the presence of cytochrome oxidase and catalase and the ability to hydrolyse Tween 80 and DNA, were shared by strains CB-225 and CB-226T and P. broegbernensis DSM 12573T. However, strains CB-225 and CB-226T were β-glucosidase-positive (P. broegbernensis DSM 12573T is β-glucosidase-negative) and utilized fewer carbon sources than P. broegbernensis DSM 12573T.
et al., 1996; Yabuuchi et al., 1983), but the end products of denitrification for these bacteria have not been analysed comprehensively. Only some of the strains reported by Finkmann et al. (2000), and strains CB-225 and CB-226T (this study), are known to follow denitrification sequences that reduce NO$_3^-$ to N$_2$O.

With varying oxygen concentrations and a higher growth temperature (50 °C), strains CB-225 and CB-226T exhibited higher denitrification activities. A similar result has been reported for *Nitrosomonas europaea*, which reduces NO$_3^-$ to N$_2$O under limited oxygen concentrations (Poth & Focht, 1985). The results obtained for our novel strains may follow from the low in situ concentration of oxygen and the high temperature of the geothermal environments from which these strains were isolated.

The final product of NO$_3^-$ reduction by the novel strains was N$_2$O, a potent greenhouse gas (like CO$_2$ or CH$_4$). The efficiency of the greenhouse effect of N$_2$O exceeds that of CO$_2$, and the atmospheric ozone chemistry of N$_2$O is of great concern (Crutzen, 1981; Dickinson & Ciccone, 1986). The main sources of N$_2$O are biomass burning, nylon manufacturing and bacterial activity due to the gaseous flux between soil and the atmosphere (Conrad, 1996; Thiemens & Trogler, 1991). Other sources, such as waste-water treatment plants, may also emit N$_2$O. In this study, the denitrifying activities of thermophilic bacteria in geo-

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**Table 1. Biochemical features that distinguish strains CB-225 and CB-226 from *P. broegbernensis***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Fructose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Melibiose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Raffinose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ribitol</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>L-Serine</td>
<td>+*</td>
<td>-</td>
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</tbody>
</table>

*P. taiwanensis* CB-225 produced a weakly positive reaction for utilization of L-serine when it was grown at 30 °C.

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The fatty acid compositions

Table 2 details the compositions of the fatty acids of the three strains studied here, which were obtained following extraction, methylation and saponification of the lipids and their subsequent GC/MS analysis. The fatty acids of *P. broegbernensis* DSM 12573$^T$ and the novel strains CB-225 and CB-226$^T$ were analysed after growth of the strains at different temperatures. *P. broegbernensis* DSM 12573$^T$ was tested at 30 °C and strains CB-225 and CB-226$^T$ were tested at 30 and 50 °C. In the fatty acid profiles of strains CB-225 and CB-226$^T$, C$_{15:0}$iso, C$_{16:0}$iso and C$_{17:0}$iso were present at high levels; the relative proportion of C$_{15:0}$iso, was...
The total fatty acid content, were detected in strains the higher ratio of C

16S rRNA genes of strains CB-225 and CB-226 T

Following PCR amplification and sequencing of the 16S rDNA sequence analysis, DNA G

"&

higher than that of C

C

and 23

*Unknown fatty acids with equivalent chain lengths are 21

Strains: 1, P. broegbernensis DSM 12573 T and strains CB-225 and CB-226 T when grown

given in any of the strains grown at 30 or 50 °C.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13:0 iso</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C13:0 iso 2-OH</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C13:0 iso 3-OH</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>12.0</td>
<td>6.2</td>
<td>6.5</td>
<td>6.4</td>
<td>6.0</td>
</tr>
<tr>
<td>C13:0 iso</td>
<td>32.4</td>
<td>28.4</td>
<td>29.6</td>
<td>27.3</td>
<td>29.0</td>
</tr>
<tr>
<td>C15:0 anteiso</td>
<td>31.8</td>
<td>10.3</td>
<td>5.5</td>
<td>11.3</td>
<td>4.8</td>
</tr>
<tr>
<td>C16:0</td>
<td>0.7</td>
<td>0.6</td>
<td>0.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>C16:0 iso</td>
<td>6.9</td>
<td>36.0</td>
<td>38.9</td>
<td>35.9</td>
<td>41.5</td>
</tr>
<tr>
<td>C16:1 cis9</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>1.3</td>
<td>3.7</td>
<td>2.5</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td>C17:1 iso cis9</td>
<td>5.5</td>
<td>4.6</td>
<td>2.7</td>
<td>4.3</td>
<td>2.2</td>
</tr>
<tr>
<td>C17:0 iso</td>
<td>3.6</td>
<td>5.1</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17:0 iso</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17:0</td>
<td>1.5</td>
<td>3.0</td>
<td>1.2</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>C16:0 2-OH</td>
<td>0.9</td>
<td>18</td>
<td>1.0</td>
<td>14</td>
<td>0.9</td>
</tr>
<tr>
<td>C18:1 iso cis 9</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown 1*</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown 2</td>
<td>1.8</td>
<td></td>
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</tr>
</tbody>
</table>

* Unknown fatty acids with equivalent chain lengths are 21:174 and 23:174.

higher than that of C15:0 anteiso. Strains CB-225 and CB-226 T could be distinguished from P. broegbernensis DSM 12573 T by the high level of C16:0 iso present and the higher ratio of C15:0 iso to C13:0 anteiso. Alterations in the fatty acid compositions of strains CB-225 and CB-226 T were observed upon temperature shift. Several unknown fatty acids, which represented about 3% of the total fatty acid content, were detected in strains CB-225 and CB-226 T when they were grown at 50 °C.

16S rDNA sequence analysis, DNA G+C content and DNA–DNA similarity

Following PCR amplification and sequencing of the 16S rRNA genes of strains CB-225 and CB-226 T, a sequence of 1539 nt in length was determined. Comparing the 16S rDNA sequences of strains CB-225 and CB-226 T to those available in the EMBL database (http://www.ebi.ac.uk) indicated that the two novel strains belonged to the Xanthomonas group of the γ-Proteobacteria and, more precisely, to the genus Pseudoxanthomonas. A more detailed sequence analysis, based on a dataset that consisted of at least 1400 unambiguous nucleotides between positions 28 and 1526 (Escherichia coli numbering; Brosius et al., 1978) of the 16S rDNA sequences, revealed that strains CB-225 and CB-226 T were most closely related to P. broegbernensis. The similarity between the 16S rDNA sequences of the CB-225/CB-226 T cluster and P. broegbernensis DSM 12573 T was 96.3%. A pairwise comparison of the 16S rDNA sequences of strains CB-225 and CB-226 T demonstrated that the level of similarity between these novel isolates was 99.9%. Fig. 3 shows a 16S-rDNA-based phylogenetic dendrogram, generated by the neighbour-joining method, depicting the phylogenetic position of the two novel strains.

The G+C content of the DNA of strain CB-225 was 69.9 mol% and that of strain CB-226 T was 70.1 mol%, as determined by the HPLC method. The DNA–DNA similarity between the novel isolates and P. broegbernensis DSM 12573 T was also determined. Strains CB-225 and CB-226 T showed 96.5% DNA–DNA similarity with each other. The high degree of DNA–DNA similarity and the high levels of 16S rDNA similarity between the two novel isolates indicated that they both belonged to the same species. The separate species status of strains CB-225 and CB-226 T was demonstrated by the hybridization values obtained when they were hybridized with P. broegbernensis DSM 12573 T; strains CB-225 and CB-226 T showed 41.2 and 37.4% DNA–DNA similarity, respectively, with P. broegbernensis DSM 12573 T.

Our 16S rDNA sequence analysis clearly demonstrated that strains CB-225 and CB-226 T are members of the genus Pseudoxanthomonas and that they have a unique taxonomic position within the γ-Proteobacteria. 16S rDNA sequence data also showed that the strains belong to a single species that is distinct from P. broegbernensis DSM 12573 T; this distinctness was confirmed by DNA–DNA similarity studies. Based on their morphological characteristics, 16S rDNA sequences, distinctive fatty acid compositions and
unusual denitrification reaction, we propose that strains CB-225 and CB-226 are classified as a novel species within the genus *Pseudoxanthomonas*, as *Pseudoxanthomonas taiwanensis*.

**Description of *Pseudoxanthomonas taiwanensis* sp. nov.**

*Pseudoxanthomonas taiwanensis* (tai.wan.en’sis. N.L. fem. adj. taiwanensis of Taiwan, referring to where the organism was first isolated).

Aerobic. Colonies are light yellow. Cells are Gram-negative, predominantly single straight rods, usually 0.5–0.8 μm in diameter and 0.9–1.4 μm in length. Flagella are absent. Thermophilic, with a growth temperature range of 30–60 °C; the optimum temperature is 50 °C. pH range for growth is 6–11; the optimum pH is about 8.0. Cytochrome oxidase-positive. Other characteristics of the species can be found in Tables 1 and 2 and within the text. DNA G+C content is between 69.9 and 70.1 mol%. Predominant fatty acids are C₁₅:0 iso and C₁₆:0 iso; C₁₄:0 3-OH and C₁₇:0 3-OH are also present. Denitrification occurs. Reduces NO₃⁻, but not NO₂⁻, yielding only N₂O as the end product. Isolated from the Chi-ban Hot Springs, Taitung, Taiwan. The type strain of *Pseudoxanthomonas taiwanensis* is CB-226T (= ATCC BAA-404T = CCRC 17172T).

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Pseudoxanthomonas taiwanensis sp. nov.


