Pseudomonas atagosis sp. nov., and Pseudomonas akappagea sp. nov.,
New Soil Bacteria Isolated from Samples on the Volcanic Island Izu
Oshima, Tokyo

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Abstract
During the exploration of microbial natural resources, two strains of Pseudomonas, PS14T and PS24T, were isolated from samples taken from Izu Oshima, a volcanic island located 120 km southwest of central Tokyo. Phylogenetic analysis based on 16S rRNA gene sequences showed that PS14T was most similar to Pseudomonas baetica a390T (99.6%) and Pseudomonas helmanticensis OHA11T (99.5%), and that PS24T was most similar to Pseudomonas qingdaonensis JJ3T (98.8%) and Pseudomonas lutea OK2T (98.7%). The major fatty acids of these two strains were C16:0 and C17:0 cyclo, summed feature 3 (C16:1 ω6c and/or C16:1 ω7c), and summed feature 8 (C18:1 ω7c and/or 18:1 ω6c). The phylogenetic analyses, DNA-DNA hybridization results and phenotypic traits indicated that PS14T and PS24T constitute two novel species, Pseudomonas atagosis sp. nov. (type strain PS14T = CECT 9940T, = LMG 31496T) and Pseudomonas akappagea sp. nov. (type strain PS24T = CECT 9941T, = LMG 31497T), respectively. The sequence data of the draft genomes of PS14T and PS24T were deposited in the GenBank database under accession numbers VXCA00000000 and VXCP00000000, respectively, and the sequence data of their 16S rRNA genes were deposited in the GenBank database under accession numbers MN396717 and MN382268, respectively.

Introduction
The genus Pseudomonas was first described at the end of the nineteenth century [1]. Pseudomonas strains are Gram-negative, rod-shaped, motile, catalase-positive and oxidase-positive bacterial cells. These bacteria have been isolated from various environments worldwide, including soil, animals, plants, and water [2]. To date, the List of Prokaryotic Names with Standing in Nomenclature (https://www.bacterio.net) includes 255 species of Pseudomonas, including 18 subspecies.

During the exploration of microbial natural resources, we collected soil samples from Izu Oshima, in January 2017. PS14T was isolated from soil collected at Mt. Atago, which is located in the northwest part of the island. Mt. Atago, where Castanopsis sieboldii trees grow, is the transitional final stage, known as a climax community. PS24T was isolated from a red scoria cone located on the west coast of the island, which the local inhabitants call Akappage. The present study describes the phenotypic and phylogenetic characteristics of these two strains. These characteristics indicate that these two strains represent novel species of the genus Pseudomonas.

Materials and Methods

Strains and Growth Conditions
Approximately 5 g of the ground surface was collected at eight locations on Izu Oshima, including Mt. Atago (34° 77’ 06” N, 139° 35’ 98” E) and Akappage (34° 77’ 50” N, 139° 34’ 99” E). Approximately 0.5 g of soil samples were
suspended in 5 ml of 0.9% NaCl solution, and 0.1 ml of the suspension of each sample was spread onto *Pseudomonas* spp. selective medium (Pseudomonas CFC/CN agar, Merck). The plates were incubated for 48 h at room temperature. 20 and 76 colonies appeared from the Mt. Atago and Akapage samples, and several colonies with different colony morphologies were selected and purified with a single colony isolation. PS14T and PS24T were two of the selected isolates. The reference strains *Pseudomonas helmanticensis* LMG 28168T, *Pseudomonas lutea* LMG 21974T, *Pseudomonas rhizophoerae* LMG 21640T and *Pseudomonas bohemia* LMG 30182T were obtained from Belgian Coordinated Collections Microbiology (BCCM), *Pseudomonas koreensis* JCM 14769T and *Pseudomonas bohemia* LMG 30182T were imported under the permit of the Minister of Agriculture, Forestry and Fisheries, Japan, in accordance with the Plant Protection Law. All these strains were cultured in tryptic soy broth (TSB, Becton Dickinson).

**Morphological, Physiological and Biochemical Studies**

Cell morphology was examined by scanning electron microscopy (Hitachi S-4800). Colony morphology was assessed on tryptic soy agar (TSA, Becton Dickinson) plates after culturing for 24 h at 28 °C. Growth at various temperatures was tested by culturing in Luria–Bertani broth (LB, Becton Dickinson) for 24 h at 28 °C. Growth at different temperatures was tested on tryptic soy agar (TSA, Becton Dickinson) plates after culture. Cell morphology was examined by scanning electron microscopy (Hitachi S-4800). Colony morphology was assessed on tryptic soy agar (TSA, Becton Dickinson) plates after culturing for 24 h at 28 °C. Growth at various temperatures was tested by culturing in Luria–Bertani broth (LB, Becton Dickinson) for 24 h at 28 °C. Growth at different temperatures was tested on tryptic soy agar (TSA, Becton Dickinson) plates after culture. Oxidase activity was assessed using Cytochrome Oxidase Test Strips (Nissui, Tokyo, Japan). Catalase activity was analyzed by dropping 3% hydrogen peroxide solution onto the cells and monitoring the production of bubbles. Growth at different NaCl concentrations was assessed in nutrient broth (Becton Dickinson) [3] containing 0, 1, 2, 3, 4, 5, 6, and 7% NaCl. Growth at different pH levels (5, 6, 7, 8, 9, and 10) was investigated by adding hydrochloric acid or sodium hydroxide to 7.5 ml of twofold-higher TSB and 3 ml of buffer agent (MOPS for pH 5 to pH 7, HEPES for pH 8 to pH 9, and CAPS for pH 9 to pH 10). The broth was diluted with sterile water to adjust the TSB concentration to onefold. API 20 NE strips (bioMérieux) and Biolog GN3 MicroPlates were used according to the manufacturers’ instructions. API 20 NE and GN3 tests for *Pseudomonas granadensis* DSM 28040T was performed by German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

**Chemotaxonomic Characterization**

Fatty acid methyl ester analysis was performed at Techno Suruga Laboratory Co., Ltd (Shizuoka, Japan). Fatty acids were prepared as described by MIDI Microbial Identification System [5] and analyzed using the Sherlock Microbial Identification (MIDI) system (version 6.0).

**Genomic DNA Preparation, Sequencing, and Assembly**

Genomic DNA was extracted from PS14T and PS24T using QIAamp DNA Mini Kits (Qiagen), and genomic libraries of both strains were prepared using Nextera XT DNA Library Preparation Kits (Illumina). Paired-end sequencing was performed using MiSeq Reagent Kits v3 (600-cycles) through the Illumina MiSeq platform. De novo assembly was performed using CLC Genomics Workbench v7 (Qiagen). The DNA sequences of the 16S rRNA genes were analyzed using BigDye® Terminator v3.1 Cycle Sequencing Kits and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Life Technolo-gies, Carlsbad, CA), along with the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTATCCGTCGCA-3′) [6].

**Phylogenetic Analysis**

Sequences were aligned using CLUSTAL W software and phylogenetic trees were constructed using MEGA 7.0 software [7]. Evolutionary distances were calculated using Tamura’s 3-parameter model [8]. To account for heterogeneity of substitution rate among nucleotide sites, the discrete gamma model with 5 categories was used. Phylogenetic trees were reconstructed using maximum-likelihood (ML) methods [9]. The sequences of all *Pseudomonas* type strains used for the analysis except *Pseudomonas helmanticensis* LMG 28168T were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database and EzBioCloud (https://www.ezbiocloud.net/). *Pseudomonas helmanticensis* LMG 28168T (GOLD ID Gp0112928) was retrieved from Department of Energy Joint Genome Institute (https://www.jgi.doe.gov) under Genomes Online Database IMG.
Genome Analysis

The similarity of the sequenced genomes to genomes of other type strains was determined based on the Average Nucleotide Identity with OrthoANIu algorithm [10] and Genome-to-Genome-Distance (GGDC) version 2.1 software [11]. The GGDC results were based on formula 2, which is independent of genome length and is therefore recommended to use for incomplete draft genomes.

Results and Discussion

Phylogenetic trees were constructed based on the 16S rRNA sequences (1459 bp) of PS14T and PS24T and of representative Pseudomonas strains (Fig. 1). GenBank accession numbers are listed in Table S1. The highest interspecific sequence similarities that were found between strain PS14T and its phylogenetic neighbors were Pseudomonas baetica a390T (99.6%) and P. helmanticensis OHA11T (99.5%), and that of PS24T were P. qingdaonensis JJ3T (98.8%) and P. lutea OK2T (98.7%).

Figure 2 is a phylogenetic tree constructed based on concatenated sequences of 16S rRNA and three housekeeping genes linked in the order 16S rRNA (1459 bp)–gyrB (801 bp)–rpoD (718 bp)–rpoB (915 bp) (Fig. 2). These sequences were retrieved from the genome sequences, and GenBank accession numbers of these genes are listed in Tables S1 and S2. Strain PS14T clusters in a separate branch that is related to a group including P. baetica, P. helmanticensis and P. koreensis. PS24T was placed near P. qingdaonensis and P. rhizosphaerae. These results indicate that both of these Izu Oshima strains belong to the P. fluorescens lineage, but they are distinct from other species in that lineage.

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P. atagosis PS14T (MN396717)

- P. helmanticensis OHA11T (HG940537)
- P. baetica a390T (PKLC01000001)
- P. koreensis Ps_9-14T (LT629687)
- P. jessenii CIP105274T (NIWT01000013)
- P. granadensis F-278770T (LT629778)
- P. vancouverensis Dha-51T (AJ011507)
- P. laurysulfativorans AP3_22T (MF554631)
- P. lini CFBP5737T (AY035996)
- P. syringae KCTC 12500T (AYTM02000002)
- P. fluorescens NCTC 10038T (LS483372)
- P. rhizosphaerae IH5T (AY152673)
- P. lutea OK2T (AY364537)
- P. bohemica IA19T (MG190030)
- P. akappagea PS24T (MN382268)
- P. qingdaonensis JJ3T (PHTD01000020)
- P. japonica NBRC 103040T (BBIR01000146)
- P. putida NBRC 14164T (AP013070)
- P. straminea IAM1598T (D84023)
- P. oleovorans DSM1045T (UGUV01000002)
- P. aeruginosa DSM50071T (CP012001)

Acinetobacter baumanii ATCC19606T (HE651907)

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Fig. 1 Maximum-likelihood (ML) tree based on 16S rRNA gene sequences (1459 bp) showing the relationships of the strains, P. atagosis sp. nov. PS14T and P. akappagea sp. nov. PS24T, with related type strains of the genus Pseudomonas. The ML tree was reconstructed using Tamura’s 3-parameter model +G. The discrete gamma model with 5 categories were used. Bootstrap values, expressed as percentages of 1000 replications, are shown at the branching points. GenBank accession numbers are given in parentheses and in Table S1.
Genomic Analysis

The DNA G+C contents of PS14<sup>T</sup> and PS24<sup>T</sup> were found to be 59.6% and 60.2%, respectively. Assessments of ANI scores and dDDH values of PS14<sup>T</sup>, PS24<sup>T</sup> and closely related strains are listed in Table S3. The highest correlations were between PS14<sup>T</sup> and *P. helmanticensis*, with an ANI score of 88.3% and a dDDH score of 35.7%, and between PS24<sup>T</sup> and *P. qingdaonensis*, with an ANI score of 80.8% and a dDDH score of 24.5%. These ANI and dDDH scores were lower than the cutoff values for species delineation (> 95% for ANI and > 70% for dDDH) [12], indicating that PS14<sup>T</sup> and PS24<sup>T</sup> are likely novel species of the genus *Pseudomonas*.

Chemotaxonomic Characterization

The major fatty acids detected in the Izu Oshima strains were found to be C<sub>16:0</sub>, C<sub>17:0 cyclo</sub>, summed feature 3 (C<sub>16:1ω6c</sub> and/or C<sub>16:1ω7c</sub>), and summed feature 8 (C<sub>18:1ω7c</sub> and/or C<sub>18:1ω6c</sub>) (Table 1). This profile is similar to that of related strains. Both PS14<sup>T</sup> and PS24<sup>T</sup> possess three fatty acids generally detected in the genus *Pseudomonas*, namely C<sub>10:0 3-OH</sub>, C<sub>12:0 3-OH</sub> and C<sub>12:0 3-OH</sub> [11].
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Table 1  Cellular fatty acid content of PS14T, PS24T and closely related strains

|        | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| C12:0 2OH | 5.3    | 2.8    | 4.9    | 6.4    | 4.7    | 6.2    | 2.9    | 2.8    | 4.0    | 9.0    |
| C12:0 3OH | 4.5    | 3.5    | 2.9    | 4.0    | 2.5    | 5.1    | 4.2    | 3.5    | 3.9    | 3.4    |
| C10:0 3OH | 3.2    | 3.3    | 2.4    | 3.6    | 3.2    | 4.2    | 2.2    | 1.5    | 4.3    | 8.4    |
| C12:0    | 1.6    | 4.3    | 2.0    | 2.1    | 1.5    | 5.0    | 5.7    | 4.9    | 6.2    | 3.0    |
| C14:0    | 32.8   | 29.1   | 31.9   | 30.0   | 31.9   | 20.0   | 19.3   | 25.6   | 30.0   | 27.5   |
| C17:0 cyclo | 11.5   | 7.0    | 5.1    | 2.0    | 6.9    | 1.5    | 1.9    | 35.4   | 37.2   | 33.4   |
| C18:0    | TR     | TR     | TR     | TR     | TR     | 1.7    | ND     | TR     | TR     |       |
| Summed feature 3a | 27.2   | 32.6   | 32.9   | 42.4   | 35.6   | 41.9   | 35.4   | 37.2   | 33.4   | 25.8   |
| Summed feature 8b | 10.7   | 15.3   | 15.6   | 8.5    | 12.4   | 13.4   | 27.8   | 21.2   | 7.4    | 11.4   |

Growth Conditions, Physiology, Morphology, and Biochemical Characteristics

The phenotypic features of PS14T and PS24T are presented in Table 2. The phenotypic features of PS14T were similar to those of P. koreensis, although they differed in utilization of gelatin hydrolysis, d-fucose, d-arabitol, l-histidine, glucuronamide, and α-keto-glutaric acid. PS24T was found to be more restricted than PS14T, with n-glucose being the only sugar source found to be utilized by PS24T.

Description of Pseudomonas atagosis sp. nov.

This strain, which has been named for Atago Mountain, the source of the original sample, was found to be Gram-negative, motile, rod-shaped, oxidase-positive, and catalase-positive. Cells were observed to be 1.0–1.7 μm long and 0.4–0.6 μm wide. Colonies grown on TSA agar for 24 h at 28 °C were moist and creamy-white in color due to extracellular substances. Concentrated cell pellet was beige-colored. Growth was observed at temperatures of 5–36 °C, with optimum growth at 24–28 °C. The strain could grow in the presence of 0–4% (w/v) NaCl and at pH between 5 and 8 but did not produce a fluorescent pigment when grown on King B agar. Major fatty acids were C16:0, C17:0 cyclo, summed feature 3 (C16:1 ω6c and/or C16:1 ω7c), and summed feature 8 (C18:1 ω7c and/or 18:1 ω6c). On API 20NE tests, this strain was positive for L-arginine, gelatin, d-glucose, l-asparagine, l-arginine, l-aspartic acid, l-glutamic acid, l-pyroglutamatic acid, l-serine, d-gluconic acid, glucuronamide, mucic acid, quinic acid, d-saccharic acid, l-lactic acid, citric acid, α-keto-glutaric acid, l-malic acid, γ-amino-butyric acid, β-hydroxy-D,L-butyric acid, propionic acid, and acetic acid.

This strain, which has been named for the source of the original sample, was found to be Gram-negative, motile, rod-shaped, oxidase-positive, and catalase-positive. Cells were observed to be 1.0–1.7 μm long and 0.4–0.6 μm wide. Colonies grown on TSA agar for 24 h at 28 °C were beige in color. Growth was observed at temperatures of 5–36 °C, with optimum growth at 24–28 °C. These bacteria could grow in the presence of 0–3% (w/v) NaCl and at pH between 5 and 8 but did not produce a fluorescent pigment when grown on King B agar. Major fatty acids were C16:0, C17:0 cyclo, summed feature 3 (C16:1 ω6c and/or C16:1 ω7c), and summed feature 8 (C18:1 ω7c and/or 18:1 ω6c). On API 20NE tests, this strain was positive for potassium nitrate, d-glucose, potassium tellurite, and aztreonam. The G+C content of the type strain is 59.5%. The type strain, PS14T (= CECT 1913), was isolated from soil collected at Mt. Atago, which is located in the northwest part of Izu Oshima, Tokyo, Japan.

Description of Pseudomonas akappagea sp. nov.

This strain, which has been named for the source of the original sample, the coastal area of Izu Oshima island, called “Akappage” by the local inhabitants, was found to be Gram-negative, motile, rod-shaped, oxidase-positive, and catalase-positive. Cells were observed to be 1.0–1.7 μm long and 0.4–0.6 μm wide. Colonies grown on TSA agar for 24 h at 28 °C were beige in color. Growth was observed at temperatures of 5–36 °C, with optimum growth at 24–28 °C. These bacteria could grow in the presence of 0–3% (w/v) NaCl and at pH between 5 and 8 but did not produce a fluorescent pigment when grown on King B agar. Major fatty acids were C16:0, C17:0 cyclo, summed feature 3 (C16:1 ω6c and/or C16:1 ω7c), and summed feature 8 (C18:1 ω7c and/or 18:1 ω6c). On API 20NE tests, this strain was positive for potassium nitrate, d-glucose, potassium tellurite, and aztreonam. The G+C content of the type strain is 59.5%. The type strain, PS14T (= CECT 1913), was isolated from soil collected at Mt. Atago, which is located in the northwest part of Izu Oshima, Tokyo, Japan.
assays showed that these bacteria can utilize α-D-glucose, glycerol, α-serine, l-alanine, l-arginine, l-aspartic acid, l-glutamic acid, l-histidine, l-pyroglutamic acid, l-serine, l-gluconic acid, mucic acid, quinic acid, methyl pyruvate, l-lactic acid, citric acid, α-keto-glutaric acid, l-malic acid, bromo-succinic acid, γ-amino-butryric acid, β-hydroxy-DL-butyric acid, propionic acid, acetic acid, and formic acid and was able to grow in the presence of 1% sodium lactate, α-serine, trehalomycycin, rifamycin SV, lincomycin, guanidine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, potassium tellurite, and aztreonam. The G+C content of the type strain is 60.2%. The type strain, PS24T (= CECT 9941T, = LMG 31497T), was isolated from located on the west coast of Izu Oshima, Tokyo, Japan.

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| Table 2 Phenotypic characteristics that differentiate the strains PS14T and PS24T from the closely related type strains |
|---------------------------------------------------------------|
|                                                                 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| GC content (%) | 59.6 | 60.2 | 59.2 | 58.8 | 60.2 | 60.5 | 62.0 | 60.2 | 59.5 | 64.2 |
| Fluorescence | + | - | + | + | + | + | + | - | - | + |
| Activity of enzymes (API 20 NE test) |
| Potassium nitrate | - | + | - | - | - | - | + | + | - | - |
| L-arginine | + | - | - | + | + | - | - | - | - | + |
| Gelatine (bovine origin) | + | - | + | + | + | + | + | - | - | - |
| Growth on (API 20 NE test) |
| D-mannose | w | - | + | + | + | + | + | - | - | - |
| D-mannitol | + | - | + | + | + | + | + | - | - | - |
| N-acetyl-glucosamine | + | - | + | + | + | + | + | - | - | - |
| Phenylacetic acid | - | + | - | - | - | - | - | - | + | + |
| Carbon sources (Biolag GN3) |
| D-galactose | + | - | + | + | w | + | + | w | + | - |
| D-fucose | w | - | + | + | w | - | - | - | w | w |
| Inosine | w | - | w | + | - | - | - | w | w | w |
| D-arabitol | - | - | + | + | - | - | - | - | + | - |
| D-fructose-6-PO4 | - | - | w | + | w | - | - | - | w | w |
| D-aspartic acid | - | - | - | - | - | - | - | - | - | - |
| D-serine | - | + | - | + | w | - | - | - | - | + |
| Glycyl-L-proline | - | - | - | - | - | - | - | - | - | - |
| Pectin | - | - | - | - | + | + | + | - | - | - |
| D-galacturonic acid | - | - | - | - | w | - | - | + | + | - |
| L-galactonic acid lactone | - | - | - | - | - | - | - | + | + | - |
| D-glucuronic acid | - | - | + | - | w | - | - | + | + | - |
| Glucuronamide | + | - | + | + | w | - | - | w | w | w |
| Mucic acid | + | + | + | - | w | + | - | w | w | - |
| Quinic acid | + | + | + | + | + | + | + | - | - | - |
| D-saccharic acid | + | - | + | + | w | w | - | - | + | - |
| P-hydroxy-phenylacetic acid | - | - | - | - | - | - | - | - | - | - |
| D-malic acid | - | - | w | - | - | - | - | + | + | - |
| Bromo-succinic acid | - | + | w | - | - | - | - | - | - | - |
| Tween 40 | - | - | w | + | w | - | - | - | - | - |
| α-keto-butyric acid | + | + | + | + | + | + | + | - | - | - |
| Acetoacetic acid | - | - | - | - | - | - | - | - | + | + |

1, PS14T; 2, PS24T; 3, P. helmanticensis OHA11T; 4, P. baetica a390T; 5, P. granadensis DSM 28040T; 6, P. koreensis 9-14T; 7, P. rhizosphaerae IH5T; 8, P. lutea OK2T; 9, P. bohemica IAT; 10, P. qingdaonensis JJ3T. All data were obtained in this study, except taxon 4, which were from reference [15] and for fluorescent data of taxon 5, which were from reference [13].

+ positive, − negative, w weakly positive (GN3, extremely faint color, or with small purple flecks or clumps)
and interpretation, or the decision to submit the work for publication. The genome sequence data of *P. helmentics* OHA11 was produced by the US Department of Energy Joint Genome Institute (https://www.jgi.doe.gov/) in collaboration with the user community. We thank Dr. Tadao Kunihio of Techno Suruga Laboratory Co., Ltd for providing technical assistance and Ms. Kana Nishitani of Global Nature Club for acting as a professional field guide and sharing her geographical knowledge of Izu Oshima.

**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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