# *Cupriavidus pinatubonensis* sp. nov. and *Cupriavidus laharis* sp. nov., novel hydrogen-oxidizing, facultatively chemolithotrophic bacteria isolated from volcanic mudflow deposits from Mt. Pinatubo in the Philippines

Yoshinori Sato,[1] Hirofumi Nishihara,[1] Masao Yoshida,[1] Makiko Watanabe,[2] Jose D. Rondal,[3] Rogelio N. Concepcion[3] and Hiroyuki Ohta[1]

Correspondence
Hiroyuki Ohta
hohta@mx.ibaraki.ac.jp

[1]Department of Bioresource Science, Ibaraki University College of Agriculture, Ami-machi, Ibaraki 300-0393, Japan

[2]Department of Environmental Science and Technology, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama 226-8503, Japan

[3]Soils Research and Development Centre, Bureau of Soils and Water Management, Diliman, Quezon City, Philippines

Taxonomic studies were performed on ten hydrogen-oxidizing, facultatively chemolithotrophic bacteria that were isolated from volcanic mudflow deposits derived from the eruption of Mt. Pinatubo in the Philippines in 1991. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these isolates belonged to the genus *Cupriavidus* of the *Betaproteobacteria*; sequence similarity values with their nearest phylogenetic neighbour, *Cupriavidus basilensis*, were 97·1–98·3 %. In addition to phylogenetic analysis, results of whole-cell protein profiles and biochemical tests revealed that these strains were members of two distinct species. DNA–DNA hybridizations and whole-cell protein profiles enabled these isolates to be differentiated from related *Cupriavidus* species with validly published names. The isolates were aerobic, Gram-negative, non-sporulating, peritrichously flagellated rods. Their G+C contents ranged from 65·2 to 65·9 mol% and their major isoprenoid quinone was ubiquinone Q-8. On the basis of these results, two novel species are proposed, *Cupriavidus pinatubonensis* sp. nov. [nine strains, with 1245$^T$ (=CIP 108725$^T$=PNCM 10346$^T$) as the type strain] and *Cupriavidus laharis* sp. nov. [one strain, the type strain 1263a$^T$ (=CIP 108726$^T$=PNCM 10347$^T$)]. It is also suggested that *Ralstonia* sp. LMG 1197 (=JMP 134) should be included in the species *C. pinatubonensis*.

One of the major subjects in terrestrial microbiology is to elucidate the role of microbes in the process of soil genesis and soil ecosystem development. New substrates derived from volcanic eruptions, e.g. lava, tephra and volcanic ash, contain negligible amounts of organic carbon and fixed nitrogen when first deposited (Chadwick *et al.*, 1999). Consequently, rates of vegetation growth and other ecosystem processes are often constrained by the supply of nitrogen and carbon in early developing ecosystems on volcanic deposits. Under these conditions, carbon and energy sources for microbial community development derive from the colonization of phototrophs or lichens (Jackson & Keller, 1970; Kurina & Vitousek, 1999; Crews *et al.*, 2001), dry and wet depositions of organic matter (King, 2003) and atmospheric inputs of trace gases (e.g. CO, $H_2$ and $CH_4$) (Conrad, 1996). Recently, King (2003) showed that atmospheric CO and hydrogen utilization contribute 2–4 % and 15–20 % of total respiratory reducing equivalent flow, respectively, in intact cores from recent Hawaiian volcanic deposits.

Previously, bacterial diversity of very recent volcanic deposits sampled from volcanic mudflow-affected areas around Mt. Pinatubo, the Philippines, has been analysed by quinone profiling (Ohta *et al.*, 2003). Subsequently, pure bacterial cultures were isolated from such volcanic deposit

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 1243, 1245$^T$, 1246a, 1247, 1250, 1266, 1278a, 1244a, 1249 and 1263a$^T$ are AB121220–AB121224, AB121227, AB121228, AB054960, AB055427 and AB054961, respectively.

A figure showing whole-cell protein electrophoretic profiling of the group P strains is available as supplementary material in IJSEM Online.

samples and then subjected to comparative 16S rRNA gene sequence analysis. Analysis revealed that 25 % of total culturable bacteria were phylogenetically related to a hydrogen-oxidizing bacterium, *Ralstonia eutropha* [recently transferred to the novel genus *Wautersia* (Vaneechoutte *et al.*, 2004) and further reclassified as a synonym of *Cupriavidus necator* (Vandamme & Coenye, 2004)], with similarities of 97·3–98·2 % (Sato *et al.*, 2004). Furthermore, most of the *C. necator*-related bacterial strains from the mudflow deposits grew chemolithoautotrophically with $H_2$ as electron donor and $CO_2$ as carbon source and also possessed hydrogenase activity (Sato *et al.*, 2004). In the present study, the *C. necator*-related bacteria were further characterized and described using physiological characterization, DNA–DNA hybridization, determination of DNA base composition and whole-cell protein profiles. On the basis of these data, the two representative strains (1245$^T$ and 1263a$^T$) should be classified as the type strains of two novel species.

The ten strains under study (1243, 1244a, 1245$^T$, 1246a, 1247, 1249, 1250, 1263a$^T$, 1266 and 1278a) were isolated in 1998 from the 0·00–0·25 m depth layer of the volcanic deposits at two sites [site N (15° 15′ 17″ N 120° 34′ 59″ E) and site S1 (2 km east of site N)] that had been hit repeatedly by mudflows during successive rainy seasons after the violent eruption of 1991 on the east side of Mt. Pinatubo (Ohta *et al.*, 2003; Sato *et al.*, 2004). *C. necator* JCM 11282 (the type strain of *W. eutropha*), *Cupriavidus gilardii* JCM 11283$^T$, *Cupriavidus oxalaticus* JCM 11285$^T$ and *Cupriavidus pauculus* JCM 11286$^T$ were obtained from the Japan Collection of Microorganisms, Wako-shi, Japan. *Cupriavidus basilensis* DSM 11853$^T$ was obtained from the DSMZ, Braunschweig, Germany, and *Ralstonia* sp. LMG 1197 (Pemberton *et al.*, 1979), *C. necator* LMG 8453$^T$, *Cupriavidus campinensis* LMG 19282$^T$, *Cupriavidus metallidurans* LMG 1195$^T$, *Cupriavidus respiraculi* LMG 21510$^T$ and *Cupriavidus taiwanensis* LMG 19424$^T$ were from BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium. All strains were grown aerobically at 27 °C on nutrient broth (NB) [1 % (w/v) Ehrlich meat extract (Kyokuto Seiyaku), 1 % (w/v) Tryptticase peptone (Becton Dickinson) and 0·5 % (w/v) NaCl, pH 7·0 with 1 M NaOH] or 100-fold diluted NB ($10^{-2}$ NB), unless otherwise stated.

Classical phenotypic tests and analysis of isoprenoid quinones were performed as described by Ohta & Hattori (1983). Growth of the strains at 41 °C on NB was monitored for 7 days. The API 20NE kit system (bioMérieux) was used according to the recommendations of the manufacturer. The substrate utilization profile was tested in a $10^{-2}$ NB liquid medium supplemented with each of the following compounds: sucrose, D-fructose, acetate, formate, glutamate, lactate, benzoate, caffeate, catechol, ferulate, guaiacol, phenol, vanillate, 2-hydroxybenzoate, 3,4-dihydroxybenzoate and 4-hydroxybenzoate. All compounds were sterilized by filtration and added to autoclaved $10^{-2}$ NB medium. Sugars were added at a concentration of 0·2 % (w/v); organic acids and aromatic compounds were added at 0·02 % (w/v). Growth was measured after 2 days incubation and utilization was assessed by comparing growth in the presence and absence of an added compound. To compare cellular protein profiles, cells were grown for 48 h on NB at 27 °C. Whole-cell lysates were prepared essentially as described by Ohta *et al.* (1993) and one-dimensional analytical SDS-PAGE was performed by the method of Laemmli (1970) with a 12·5 % separating gel and 4·5 % stacking gel. Proteins were visualized by silver staining with a commercial kit (Daiichi Pure Chemicals). The similarity between all pairs of electrophoresis patterns was calculated by the simple matching coefficient, expressed as a percentage. Cluster analysis was performed by the unweighted pair group method using arithmetic averages (UPGMA) clustering technique. The complete 16S rRNA gene sequences determined previously (Sato *et al.*, 2004) were compared with those of the type strains of *Cupriavidus* species. The CLUSTAL W algorithm (Thompson *et al.*, 1994) was used to align sequences and to construct a neighbour-joining tree with 1000 bootstrap iterations. For determination of DNA base composition, DNA was extracted, purified by phenol treatment (Saito & Miura, 1963) and enzymically degraded into nucleosides. The nucleoside mixture was separated by reversed-phase HPLC as described by Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). For enzymic development, alkaline phosphatase–streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as substrate and chemiluminescence was determined with a Wallac 1420 ARVOsx multilabel counter as described previously (Ushiba *et al.*, 2003).

The ten bacteria isolated from the volcanic mudflow deposits were strictly aerobic, Gram-negative, non-sporulating, catalase-positive rods (0·3–0·6 × 0·8–1·6 μm). Their phenotypic characteristics determined by the API 20NE kit test were very similar. All of them reduced nitrate to nitrite but not further to $N_2$, possessed urease and oxidase activities and assimilated gluconate, caprate, adipate, malate, citrate and phenylacetate. None of the strains produced indole, acids from glucose, β-glucosidase, protease or β-galactosidase. None of the strains assimilated arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine or maltose. Strain 1247 utilized glucose, but the other strains did not. Six strains (1243, 1244a, 1245$^T$, 1247, 1250 and 1278a) produced arginine dihydrolase.

When the almost-complete 16S rRNA gene sequences of the ten strains comprising 1523–1525 nt were compared to each other, nine strains (1243, 1244a, 1245$^T$, 1246a, 1247, 1249, 1250, 1266 and 1278a) formed a homogeneous group with very high similarities (99·7–100 %): the group was named group P. The similarities of the group P strains to the other strain (1263a$^T$) were 98·6–98·7 %. Phylogenetic analysis of the sequences of the ten strains under study, five *Ralstonia* species, *Ralstonia* sp. LMG 1197 and nine *Cupriavidus*

species confirmed that the ten strains belonged to the genus *Cupriavidus* and formed a separate phylogenetic cluster in the tree (Fig. 1). Within the cluster, strain 1263a$^T$ formed a distinct branch with group P (a bootstrap resampling value of 94 %). The closest relatives of the group P strains and 1263a$^T$ among the type strains of established *Cupriavidus* species were *C. basilensis*, with sequence similarities of 98·1–98·3 % and 97·1 %, respectively, and *C. taiwanensis*, with sequence similarities of 98·0–98·2 % and 97·8 %, respectively. *Ralstonia* sp. LMG 1197 clustered together with the group P strains and was clearly separate from the clusters of *Ralstonia* species. The 16S rRNA gene sequence similarities between *Ralstonia* sp. LMG 1197 and strains of group P and 1263a$^T$ were 99·5–99·7 % and 98·6 %, respectively. The homogeneity of group P was further confirmed by the same overall protein-banding pattern in whole-cell protein electrophoretic profiling (see Supplementary Fig. S1 in IJSEM Online). In the following taxonomic analyses, strain 1245$^T$ was used as the representative of group P and tested together with strain 1263a$^T$.

Because *Ralstonia* sp. LMG 1197 clustered with the group P strains on the phylogenetic tree, the genomic relatedness between strain 1245$^T$ and *Ralstonia* sp. LMG 1197 was determined by DNA–DNA hybridization. Strain 1245$^T$ exhibited a high level (91 %) of DNA–DNA relatedness to *Ralstonia* sp. LMG 1197, indicating that these strains are related each other at the species level. DNA–DNA hybridization values were also determined with the following pairs of strains: 1245$^T$/1263a$^T$ (44 %); 1245$^T$/*C. basilensis* DSM 11853$^T$ (46 %); 1263a$^T$/*C. basilensis* DSM 11853$^T$ (50 %); and 1263a$^T$/*Ralstonia* sp. LMG 1197 (53 %). These values did not reveal relatedness at the species level for any of these pairs. Strains 1245$^T$ and 1263a$^T$ and the type strains of established *Cupriavidus* species were further compared by SDS-PAGE of whole-cell proteins, a method that has often been proven to differentiate at the species level (Pot *et al.*, 1994; Kersters & De Ley, 1975): indeed, in the genera *Ralstonia* and *Cupriavidus*, high protein electrophoretic similarity correlates with a high level of DNA–DNA hybridization (Coenye *et al.*, 1999, 2003a; Vandamme *et al.*, 1999; Goris *et al.*, 2001; Vandamme & Coenye, 2004). UPGMA cluster analysis of SDS-PAGE protein profiles showed that strain 1245$^T$ and *Ralstonia* sp. LMG 1197 formed a cluster at the similarity level of 87 % and were distinct from strain 1263a$^T$ (67 % similarity) and the other type strains of *Cupriavidus* species (less than 78 % similarity) (Fig. 2).

The predominant respiratory lipoquinone detected in strains 1245$^T$ and 1263a$^T$ was ubiquinone-8 (Q-8) and the DNA G+C contents of the strains were 65·9 and 65·2 mol%, respectively. Utilization of aromatic compounds was included in the phenotypic characterization because *Ralstonia* sp. LMG 1197, which is closely related to strain 1245$^T$, is known to be able to degrade several aromatic and chlorinated aromatic compounds (Clement

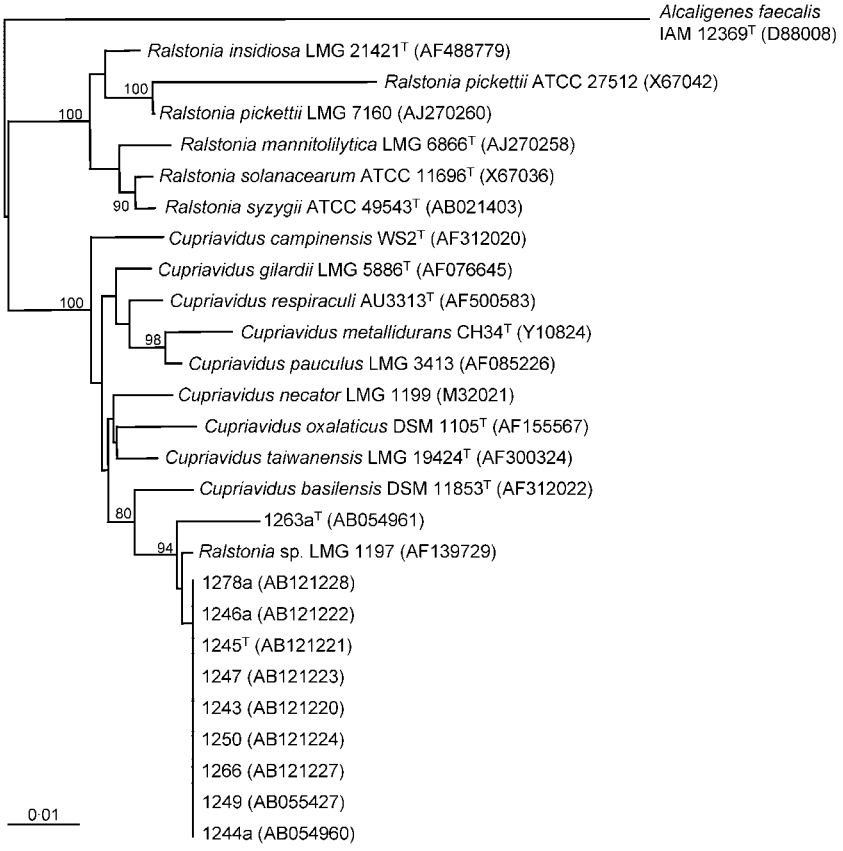


**Fig. 1.** Neighbour-joining tree (Saitou & Nei, 1987) based on 16S rRNA gene sequences (continuous stretch, 1409–1420 nt) showing the positions of strains 1245$^T$ and 1263a$^T$ among their phylogenetic neighbours. Sequences of reference species were obtained from DDBJ and GenBank and the 16S rRNA coding sequence of *Alcaligenes faecalis* IAM 12369$^T$ was selected as an outgroup. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). The tree was visualized using TREEVIEW (Page, 1996). Numbers on branch nodes are bootstrap values (only those above 50 % are shown) expressed as a percentage (1000 resamplings). Bar, 0·01 substitutions per nucleotide position.

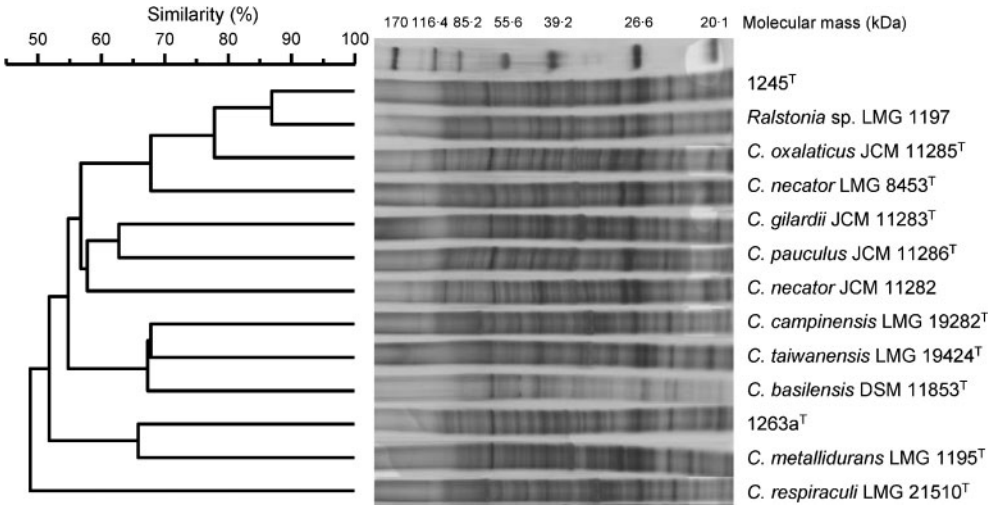


**Fig. 2.** Whole-cell protein profiles of strains 1245$^T$ and 1263a$^T$ and the type strains of established *Cupriavidus* species and the corresponding dendrogram derived from UPGMA cluster analysis of simple matching coefficient.

*et al.*, 1995). Utilization of ten aromatic compounds and six organic acids by strain 1245$^T$, strain 1263a$^T$, *Ralstonia* sp. LMG 1197 and *C. basilensis* DSM 11853$^T$ was tested and substrate utilization profiles were compared. Seven out of ten aromatic compounds were used by strain 1245$^T$, *Ralstonia* sp. LMG 1197 and *C. basilensis* DSM 11853$^T$. The utilization profile of strain 1245$^T$ was the same as that of *Ralstonia* sp. LMG 1197: benzoate, caffeate, catechol, phenol, 2-hydroxybenzoate, 3,4-dihydroxybenzoate and 4-hydroxybenzoate were used, but ferulate, guaiacol and vanillate were not used. *C. basilensis* DSM 11853$^T$ also used a wide range of aromatic compounds: benzoate, caffeate, catechol, phenol, 2-hydroxybenzoate, 4-hydroxybenzoate and vanillate. In contrast, strain 1263a$^T$ utilized a narrower range of aromatic compounds (caffeate, catechol, phenol and vanillate). Other phenotypic characteristics of strains 1245$^T$ and 1263a$^T$ are shown in Table 1 or are given in the species description.

**Table 1.** Phenotypic characteristics of strain 1245$^T$, strain 1263a$^T$, *Ralstonia* sp. LMG 1197, *C. basilensis* DSM 11853$^T$, *C. necator* JCM 11282 and other members of the genus *Cupriavidus*

Strains/species: 1, strain 1245$^T$; 2, *Ralstonia* sp. LMG 1197; 3, strain 1263a$^T$; 4, *C. oxalaticus*; 5, *C. basilensis* DSM 11853$^T$; 6, *C. necator* JCM 11282; 7, *C. taiwanensis*; 8, *C. campinensis*; 9, *C. metallidurans*; 10, *C. pauculus*; 11, *C. gilardii*; 12, *C. respiraculi*. Data are consolidated from our experiments and Goris *et al.* (2001), Chen *et al.* (2001), Vandamme *et al.* (1999), Coenye *et al.* (1999, 2003b), Jenni *et al.* (1988) and Sahin *et al.* (2000). +, Positive; −, negative; d, variable result within the species; NA, no data available.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nitrate to nitrite reduction | + | + | + | + | − | + | + | + | + | − | − | d |
| Nitrate to $N_2$ reduction | − | − | − | + | − | + | NA | − | d | − | − | NA |
| Growth at 41 °C | − | − | + | − | − | + | − | + | − | + | + | + |
| Arginine dihydrolase | + | + | + | + | − | + | − | − | − | − | + | − |
| Urease | + | + | + | + | − | + | − | + | − | + | − | − |
| Assimilation of: | | | | | | | | | | | | |
| *N*-Acetyl-D-glucosamine | − | − | − | − | − | + | − | − | − | − | − | − |
| Adipate | + | + | + | + | + | + | − | + | + | + | d | + |
| Citrate | + | + | + | + | + | + | d | − | + | + | d | − |
| Phenylacetate | + | + | + | + | + | + | + | + | + | + | d | − |
| Acetate | + | + | − | − | − | + | − | − | − | − | − | − |
| Lactate | + | + | − | + | − | + | + | + | + | + | + | + |
| Glutamate | + | + | − | + | − | + | + | + | + | + | + | + |
| Vanillate | − | − | + | − | + | − | − | − | − | − | − | − |
| 3,4-Dihydroxybenzoate | + | + | − | − | − | + | − | − | − | + | − | − |

On the basis of the data presented, two novel species within the genus *Cupriavidus* are proposed, with the names *Cupriavidus pinatubonensis* sp. nov. (type strain 1245$^T$) and *Cupriavidus laharis* sp. nov. (type strain 1263a$^T$). In addition, our results indicate that *Ralstonia* sp. LMG 1197 should be placed in the novel species *C. pinatubonensis*. The phenotypic characteristics that differentiate the two novel species from other *Cupriavidus* species are summarized in Table 1.

### Description of *Cupriavidus pinatubonensis* sp. nov.

*Cupriavidus pinatubonensis* (pin.a.tu.bo.nen′sis. N.L. masc. adj. *pinatubonensis* pertaining to Mt. Pinatubo, the volcano on Luzon Island, the Philippines, from which first strains were isolated).

Cells are aerobic, Gram-negative, non-sporulating, peritrichously flagellated rods (0·3–0·6 × 0·8–1·6 μm). Colonies on 100-fold diluted NB agar are circular, entire, convex, opaque and white. Growth is observed in NB and 10-fold and 100-fold diluted NB at 27, 30 and 41 °C. Oxidase, catalase and urease are present; protease, β-galactosidase and β-glucosidase are not present. No indole production or production of acid from glucose occurs. Nitrate is reduced to nitrite, but not further to $N_2$. Assimilates gluconate, caprate, adipate, malate, citrate, phenylacetate, D-fructose, acetate, glutamate, lactate, benzoate, caffeate, catechol, phenol, 2-hydroxybenzoate, 3,4-dihydroxybenzoate and 4-hydroxybenzoate, but not glucose, arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine, maltose, sucrose, formate, ferulate, guaiacol or vanillate. Chemolithoautotrophic growth occurs in the presence of hydrogen, oxygen and carbon dioxide. The major isoprenoid quinone is ubiquinone Q-8.

The type strain is 1245$^T$ ( = CIP 108725$^T$ = PNCM 10346$^T$), isolated from volcanic mudflow deposits sampled around Mt. Pinatubo, the Philippines. The DNA G+C content of strain 1245$^T$ is 65·9 mol%.

### Description of *Cupriavidus laharis* sp. nov.

*Cupriavidus laharis* (la.har′is. N.L. gen. n. *laharis* of a lahar, a volcanic mudflow).

Cells are Gram-negative, aerobic, non-sporulating, peritrichously flagellated rods (0·3–0·6 × 0·8–1·6 μm). Colonies on 100-fold diluted NB agar are circular, entire, convex, opaque and white. Growth is observed in NB and 10-fold and 100-fold diluted NB at 27 and 30 °C, but not at 41 °C. Oxidase, catalase and urease are present; protease, β-galactosidase and β-glucosidase are not present. No indole production or production of acid from glucose occurs. Nitrate is reduced to nitrite, but not further to $N_2$. Assimilates gluconate, caprate, adipate, malate, citrate, phenylacetate, D-fructose, acetate, caffeate, catechol, phenol and vanillate, but not sucrose, formate, glutamate, lactate, benzoate, ferulate, guaiacol, 2-hydroxybenzoate, 3,4-dihydroxybenzoate or 4-hydroxybenzoate. Chemolithoautotrophic growth occurs in the presence of hydrogen, oxygen and carbon dioxide. The major isoprenoid quinone is ubiquinone Q-8.

The type strain is 1263a$^T$ ( = CIP 108726$^T$ = PNCM 10347$^T$), isolated from volcanic mudflow deposits sampled around Mt. Pinatubo, the Philippines. The DNA G+C content of strain 1263a$^T$ is 65·2 mol%.

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