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Isolation, characterization and identification of thiosulfate-oxidizing strain MU2A-22 isolated from volcanic deposits in Miyake-jima island, Japan

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Abstract: [Objective] A bacterium MU2A-22^T, which was isolated from 131-year-old volcanic deposits of Miyake-jima island (Japan), has been characterized for its capability of thiosulfate oxidizing. **[Methods]** The culture-based method was used to isolate and identify strain MU2A-22^T for its phenotypic characteristics and taxonomic position. **[Results]** The strain MU2A-22^T was Gram-negative, short rod- to coccus-shaped. This bacterium could utilize D-glucose, L-arabinose, gluconate, adipate and dL-malate as sole carbon source. Strain MU2A-22^T was able to use thiosulfate as an energy source with the optimum concentration of 2.5 mmol/L. The optimum growth condition was 25 °C−30 °C and pH was 6.0−8.0 respectively. 16S rRNA gene sequence analysis indicated that this strain was closely related to *Paracoccus solventivorans* 6637^T within *Alphaprotebacteria* (97% 16S rRNA gene sequence similarity). Its possess of the large-subunit gene of ribulose 1,5-bisphosphate carboxylase/oxygenase (*rbcL*) has also been identified. The cellular fatty acid profiles was characterized of the genus *Paracoccus.* The major fatty acids (>10%) were $C_{18:1}$(74.7%) and $C_{18:0}$(12.1%). DNA-DNA relatedness between strain MU2A-22^T and *P. solventivorans* 6637^T was 49.3%. G+C contents was 66.5%−66.7%. **[Conclusion]** As for the above results, strain MU2A-22^T seemed to be a novel species in the genus *Paracoccus* with its accession number of GQ452286 and the name of *Paracoccus scorialis* sp. nov. was proposed.

Keywords: Volcanic deposits, Bacterium isolation, Thiosulfate-oxidizing, *rbcL*, Novel species

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日本三宅岛火山土壤中硫代硫酸盐氧化菌的分离、生物学特性及鉴定

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摘 要: 【目的】利用培养法从日本三宅岛火山土壤(堆积年限 131 年)中分离到一株能氧化分解硫代硫酸盐的细菌 MU2A-22^T。【方法】用培养法对该菌株 MU2A-22^T 进行了生理生化性质以及分类学位置上的确定。【结果】菌株 MU2A-22^T 为革兰氏阴性, 短杆状或球状。理化性质表明该菌株能利用葡萄糖、L-阿拉伯糖、葡萄糖酸盐、己二酸酯、dL-苹果酸钠、硫代硫酸钠(最适浓度为 2.5 mmol/L)为唯一碳源进行自养生长。最适生长温度为 25 ℃−30 ℃, 最适 pH 为 6.0−8.0。菌株 MU2A-22^T 的 16S rRNA 序列与菌株 *Paracoccus solventivorans* 6637^T 亲缘关系最近, 序列相似性为 97%, 编码核酮糖-1,5-二磷酸羧化酶/加氧酶(Rubisco)的基因也被确定。对 *Paracoccus* 属内几种近缘菌的脂肪酸分析, 证明菌株 MU2A-22^T 中含有 *Paracoccus* 属的特征氨基酸, 其中含量大于 10%的分别为 $C_{18:1}$(74.7%) 和 $C_{18:0}$(12.1%)。DNA-DNA 杂交实验表明, 菌株 MU2A-22^T 与 *Paracoccus solventivorans* 6637^{T}DNA 的相似度为 49.3%。MU2A-22^T 菌株 G+C 含量为 66.5%−66.7%。【结论】菌株 MU2A-22^T 为 *Paracoccus* 属内的一新种菌(登录号 GQ452286), 命名为 *Paracoccus scorialis* sp. nov.。

关键词: 火山土壤, 细菌分离, 硫代硫酸盐氧化, *rbcL*, 新菌种

New terrestrial ecosystem process can be influenced by many factors, such as photosynthesis and biological N_2 fixation, or atmospheric deposition (For example, dissolved in precipitation or by dry deposition of particles and gases) or rock-derived elements such as calcium, magnesium, potassium and phosphorous entering. Volcanic deposits have been regarded as an ideal model ecosystem to investigate its development. The analysis of microbial community in the volcanic deposits of Hawaiian and other ecosystems led to the isolation of various function bacterial groups. Hudosn *et al.*[1] isolated a resembled *Bacillus schlegelii*, capable of heterotrophic growth and autotrophic growth in the presence of hydrogen and carbon dioxide. From geothermal volcanic soil of Mount Erebus, Ross Island, Antarctica, Myrica-nodulating *Frankia* has also been identified[2] in five volcanic deposits, a series of deposits ages ranging from 20-year-old to 162-year-old Hawaiian lava. *Cupriavidus pinatubonensis* and *Cupriavidus laharis* with the capability of hydrogen-oxidizing have been isolated from the young lahar (mud flow) (age, 2−3yr) collected from Mt. Pinatubo, Luzon Island, the Philippines[3].

Miyake-jima island (55.14 km^2, 814.5 m in height), is an active volcano island, situated in the western rim of the Pacific Ocean (34° 05’ N, 139° 31’E), about 180 km south of Tokyo. For this island, the eruptions of 1874-y, 1940-y, 1962-y,

1983-y, and 2000-y were recorded in the recent history. Land covered with lava from the series of eruptions was suited to a chronosequence study of the bacteria community structures. Despite ecological research on vegetation on this island has been done much, very little is known about microbial species and functional diversity in the volcanic deposits. In addition, the information about the influence of the microorganisms on sustainable ecosystems is also very limited.

In the present research work, we selected the northeast site of this island for the sample gathering, which was covered by scoria deposited in 1867-y eruption. The bacterial community in those volcanic deposits was analyzed by culture-based method and the representative isolate was tested on its physiological and biochemical characteristics.

1 Materials and Methods

1.1 Site description and sampling

The sampling site (MU2A) are located at the northeast side of Miyake-jima island. Site MU2A was colonized by *Alnus sieboldiana*, *Prunus lannesiana* and *Persea thunbergii* Kosterm[4]. Collected samples were stored in plastic bags and kept at 4 °C until the bacteriological analysis.

1.2 Chemical analysis of samples

Total organic carbon was analyzed using a Shimadu TOC-V total organic carbon analyzer according to the manufacture's instructions. Total carbon and nitrogen were measured on a Yanaco CHN Corder type MT-6 (Yanaco Analytical Instruments Corp., Kyoto, Japan). Slurries (1: 2.5 mass ratios of samples and deionized water) were used to determine pH. The sample water content was estimated by drying material at 105 °C overnight.

1.3 Isolation of bacteria

The enumeration and isolation of culturable bacteria were carried out with 100-fold diluted nutrient broth (DNB) agar medium[5]. In brief, 5 g of fresh sample was suspended with 45 mL of sterile distilled by ultrasonicating for 5 min at 100 W. Consequently, the suspensions were diluted appropriately with sterile water, and then inoculated into sterile DNB agar medium. Four replicates were done and incubated at 30 °C for 4 weeks. The isolation procedure was the same as previously described and bacteria were stored at room temperature in semisolid DNB stab cultures.

1.4 Phenotypic tests of *Paracoccus scorialis* sp. nov.

Cell morphology and Gram stain for the pure cultures of isolates were examined as previously described[5]. Growth temperature range was tested by incubating the isolates in 10-fold diluted nutrient broth (10^{-1} NB) liquid medium at 4 °C, 30 °C, 37 °C and 40 °C for a two-week period. The pH range (pH 4−10, using increments of 2 pH unit) for growth was determined by assessing changes in OD_{550} over the incubation time (up to 7 days) in 10^{-1} NB medium[6]. The final pH was adjusted using NaOH and HCl solutions. Other phenotypic characteristics of strain MU2A-22^T were examined by the methods of Ohta & Hattori[5]and API 20NE kit system (bioMérieux) according to the recommendation of the manufacture.

1.5 Thiosulfate-oxidizing activity

The activity of oxidizing thiosulfate was determined in succinate/mineral medium that containing (mg/L): NH_4Cl 400, K_2HPO_4 800, KH_2PO_4 300, sodium succinate 2 700, yeast extract 100, $ZnSO_4{\cdot}7H_2O$ 0.02, MoO_3 0.003, H_3BO_3 0.003, $MnSO_4{\cdot}5H_2O$ 0.003, $CoCl_2{\cdot}6H_2O$ 0.003, and $CuSO_4{\cdot}5H_2O$ 0.0015. A filter-sterile solution of thiosulfate was added to the medium at final concentrations of 10 to 30mmol/L. Cultures were grown at 30 °C with shaking and the growth was followed by measuring OD_{550}.

1.6 Phylogenetic analysis of *Paracoccus scorialis* sp. nov.

Genomic DNA of the bacterial cells cultured on DNB agar plates was extracted according with

the protocol of Wang and Wang[7]. Polymerase chain reaction (PCR) amplification of 16S rRNA gene of each isolate was carried out using the following primers 10F (*Escherichia coli* positions 10−27) and 1541R (*E. coli* positions 1541−1521)[4]. The PCR conditions and the purification of PCR products were essentially the same as described previously[4]. The presence of the large-subunit gene of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) (*rbcL*) in the representative isolates has also been examined using the primers K2F and V2R[8]: K2F, 5′-ACCA[C/T]CAAGCC[G/C]AAG CT[C/G]GG-3′;V2R, 5′-GCCTTC[C/G]AGCTTG CC[C/G]ACC[G/A]C-3′. The PCR cycles consist of an initial denaturation step of 3 min at 94 °C and a hot start at 80 °C, followed by 30 cycles of 45 s at 94 °C, 60 s at 62 °C, and 90 s at 72 °C, with a final extension of 20 min at 72 °C.

Amplified nucleotide sequences were determined by ABI PRISM™ Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster, USA) and read on an Applied Biosystems 3100 DNA sequencer. The primers used for sequencing were primers 10F and K2F for the genes of 16S rRNA and *rbcL* respectively. The gene sequences were compared with similar DNA sequences retrieved from the DDBJ/EMBL/GenBank databases using the BLAST program[9]. For phylogenetic analysis of the sequence datasets, the CLUSTAL W program[10] was utilized and a tree was constructed by the neighbor-joining method[11]. Phylogenetic analysis of *rbcL* of representative isolates was performed using the deduced amino acid sequences.

1.7 Fatty acids components

Cellular fatty acids were extracted with 5% HCl-methanol, as described by Ikemoto *et al*.[12]., Fatty acids profiles were analyzed by a model GC-14B gas chromatography (Shimadzu Corp., Kyoto, Japan) equipped with a capillary column ULBON HR-SS-10 (0.23 mm×50 m: Shimadzu Corp., Kyoto, Japan) and a hydrogen flame ionization detector.

1.8 DNA G+C contents and DNA-DNA hybridization

To determine the DNA G+C contents, genomic DNA was prepared according to the method of Saitou & Miura[13] and digested with P1 nuclease using the DNA GC kit (Yamasa Shoyu). The G+C contents were determined by reversed-phase HPLC as described by Tamaoka & Komagata[14].

DNA-DNA hybridization was carried out with photobiotin-labelled probes in microplates wells, as described by Ezaki *et al*.[15]. Alkaline phosphatase-streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as substrate and Wallac 1420 ARVOsx multilabel counter was used for the determination of chemiluminescence as described by Ushiba *et al*.[16].

1.9 Nucleotide sequence accession numbers

Accession numbers of the partial 16S rRNA sequences for isolates were obtained by depositing partial sequences with GenBank of National Center for Biotechnology Information (www. ncbi. nih. gov). The accession numbers (in parenthesis) for the isolates were shown in the phylogenetic trees.

2 Results

2.1 Chemical analysis of volcanic deposit samples

The pH of the samples was very low, about 4. Water contents, total carbon and total nitrogen were 21. 5, 37.9 and 2.3 respectively.

2.2 Phenotypic characteristics of *Paracoccus scorialis* sp. nov.

To obtain a comprehensive understanding, several phenotypic characteristics were examined. The representative of strain MU2A-22^T (GQ452286) was a Gram-negative, short rod- to coccus-shaped bacterium. This optimal growth temperature and pH ranged from 25 °C−30 °C and 6.0−8.0 respectively. Comparing with its closest strain *P. solventivorans* 6637^T, the same characteris-

tics were found for their activity of arginine dihydrolase, urease, β-glucosidase, protease, β-galactosidase, oxidase, catalase. However, there were differences between strain MU2A-22^T (GQ452286) and *P. solventivorans* 6637^T, with the former utilizing D-glucose, L-arabinose, gluconate, adipate and dL-malate. In this study, figure 1 showed strain MU2A-22^T (GQ452286) could obtain energy for its growth via the oxidation of thiosulfate, and the optimum growth yield was at about 2.5 mmol/L thiosulfate (Fig. 1). Recently, Lu *et al.* [17] isolated *Limnobacter litoralis* KP1-19^T(AB366174) from a 22-year-old volcanic deposits in Miyake-jima island and also identified its capability of using thiosulfate for its growth as the energy source. The above results provided a possible explanation that some similar properties in volcanic soil existed although their deposit ages were different.

2.3 Phylogenetic position of *Paracoccus scorialis* sp. nov. in genus of *Paracoccus*

A total of 47 bacterial strains were isolated from sample MU2A. The phylogenetic analysis illustrated the relationships of the isolates with their closest affiliates in the database based on their 16S rRNA gene sequences. From the phylognetic analysis, the predominant strains (25.9%) were allocated in *Alphaprotebacteria*. Strain MU2A-22^T (GQ452286), as a represent strain, was randomly selected from those predominant isolates and its much more exact position has been determined in Fig. 2. 16S rRNA gene sequence similiarity between *Paracoccus scorialis* sp. nov and *P. solventivorans* (Y07705) was 97%.

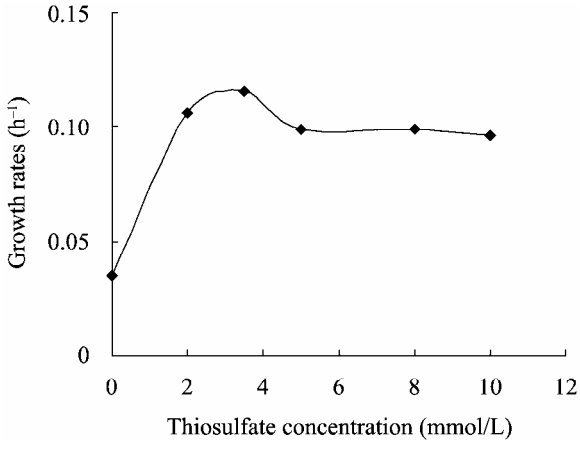

Fig. 1 Effect of thiosulfate on the growth of strain MU2A-22^T (GQ452286) which was cultured at 30 °C with shaking in the succinate medium

图 1 菌株 MU2A-22^T (GQ452286)对硫代硫酸盐的利用结果

Note: The growth was determined by measuring the optical density at 550 nm (OD_{550}).

2.4 Phylogenetic analysis of amplified *rbcL* gene from *Paracoccus scorialis* sp. nov.

Many of the obligate lithotrophs, which include sulfide-, sulfur-, metal-, ammonium-and nitrite-oxidizing bacteria, have been studied thoroughly. In contrast, few of the facultative lithotrophs have been described in detail. In this study, the *rbcL* gene of strain MU2A-22^T (GQ452286) was successfully amplified and the phylogenetic analysis revealed that it belonged to the FormIC subgroup (Fig. 3). This result was consistent with the previously report of FormIC dominated by facultative lithotrophs among the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*[18−20].

2.5 Test of fatty acids profiles, DNA-DNA hybridization and G+C contents

The fatty acids profiles of MU2A-22^T (GQ452286) and the other several *Paracoccus* species were very similar (Table 1), and the main fatty acids are $C_{18:1}$ (74.7%, 77.3%, 69.6%, 84.6% and 89.9% respectively) and $C_{18:0}$ (12.1%, 7.5%, 17.6%, 5.3%) respectively.

Table 1 Fatty acids analysis for strain MU2A-22^T (GQ452286) and its several related species of genus *Paracoccus*.

The values for the hybridization of strain MU2A-22^T (GQ452286) and *P. solventivorans* DSM 6637^T (Y07705) and *P. alkenifer* DSM11593^T (Y13827) and *P. koreensis* NBRC102292^T (AB187584) were 49.3%, 50.9% and 44.9% respectively. Such relatedness are low enough for

strain MU2A-22^T (GQ452286) to be classed into a novel *Paracoccus* species [21]. G+C contents of the bacterium were 66.5%−66.7%, ranging in genus *Paracoccus*.

3 Discussion

In the present study, culturable approach coupled with a molecular approach, in which case PCR amplification of 16S rRNA gene sequence based on bacterial identification was attempted to analyze bacterial communities in the 131-year-old volcanic deposits in Miyake-jima island in Japan. Moreover, the characterization of the predominant *Paracoccus*-related isolates was performed.

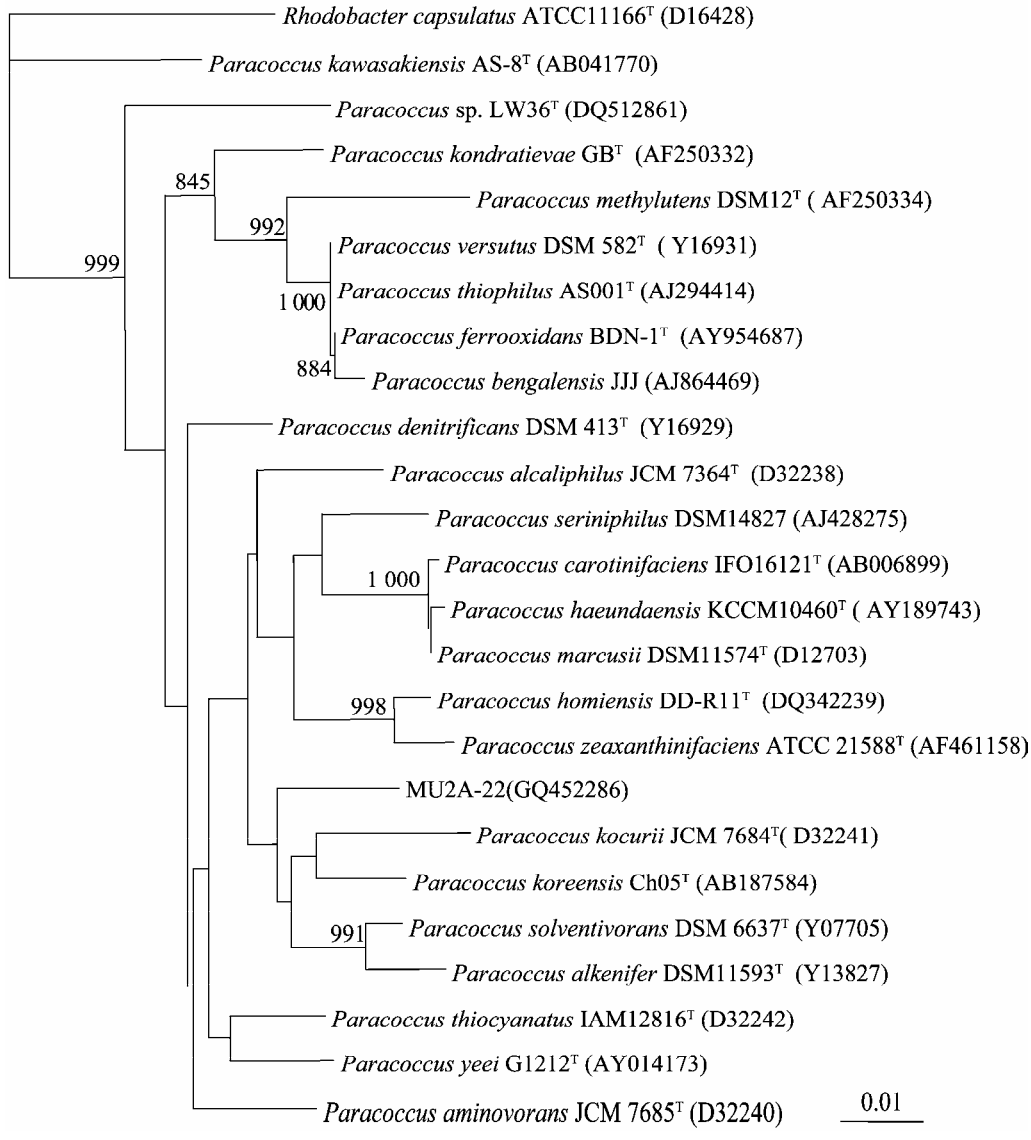

Fig. 2 Neighbor-Joining tree showing the phylogenetic affiliation of strain MU2A-22^T(GQ452286) with almost *Paracoccus* species based on 16S rRNA gene sequences

图 2 利用邻接法构建的基于菌株 MU2A-22^T (GQ452286)及相关菌株 16S rRNA 序列的系统发育树

Note: The sequence from *Rhodobacter capsulatus* ATCC11166^T(D16428) was used as an outgroup. Scale 0.1 represented the distance of evolution.

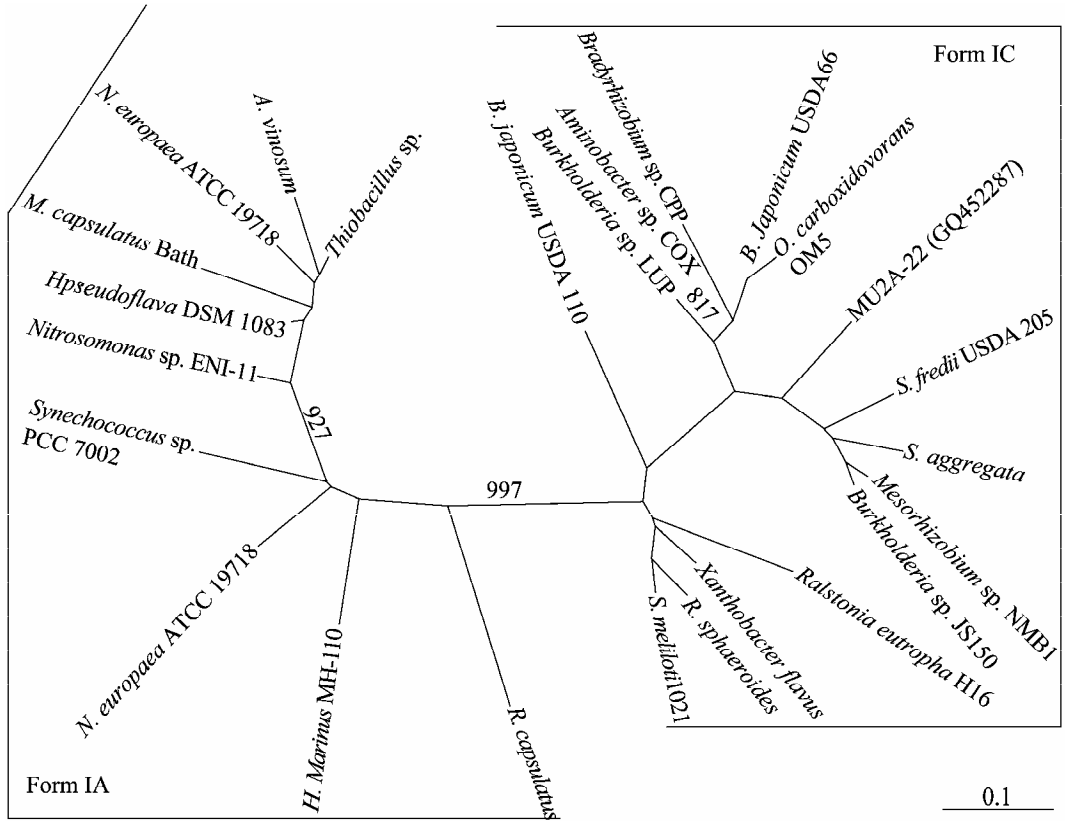

Fig. 3 Neighbor-Joining phylogenetic tree based on deduced amino acid sequences showing the affiliation of MU2A-22^T(GQ452286) *rbcL* sequence with FormIC *rbcL* sequences

图 3 利用邻接法构建的基于菌株 MU2A-22^T (GQ452286)及相关菌株氨基酸序列的系统发育树

Note: Values along branches indicate bootstrap percentages of >80%, based on 1 000 replicates. The accession numbers: Strain MU2A-22^{T}GQ452286; *Allochromatium vinosum*, P22849; *Aminobacter* sp. COX, AY422046; *Bradyrhizobium japonicum* USDA110, AF04180; *Bradyrhizobium* sp. CPP, AY422047; *Bradyrhizobium japonicum* USDA 6, AY422048; *Burkholderia* sp. LUP, AY422050; *Burkholderia* sp. JS150, AY422049; *Hydrogenovibrio marinus* MH-110, AB122070; *Hydrogenophaga pseudoflava* DSM1083, U55037; *Mesorhizobium* sp. NMB1, AY422051; *Methylococcus capsulatus* Bath, AF447860; *Nitrosomonas europaea* ATCC19718, NP_841943; *Nitrosomonas* sp. ENI-11, AB061373; *Oligotropha carboxidovorans* OM5, AY422052; *Prochlorococcus marinus* SS120, AE017126; *Ralstonia eutropha* H16, NP_943062; *Rhodobacter sphaeroides*, M64624; *Rhodobacter capsulatus*, L82000; *Sinorhizobium fredii* USDA 205, AY422053; *Sinorhizobium meliloti* 1021, AL591985; *Stappia aggregata*, AY422055; *Synechococcus* sp. PCC7002, D13971; *Thiobacillus* sp., M34536; *Xanthobacter flavus* H4-14, X17252. The scale bar shows 0.10 substitutions per site.

Table 1 Fatty acids analysis for strain MU2A-22^T(GQ452286) and its several related species of genus *Paracoccus*
表 1 MU2A-22^T(GQ452286)及其边缘 *Paracoccus* 菌株脂肪酸测定结果

Strains	$C_{10:0}$ 3-OH	$C_{16:0}$	$C_{17:0}$ iso	$C_{17:0}$	$C_{12:0}$ 3-OH	$C_{18:0}$	$C_{14:0}$ 2-OH	$C_{19:0}$	$C_{18:1}$	$C_{19:0}$ cyclo	$C_{20:1}$ ω9t
1	3.6	2.0	–	2.9	0.9	12.1	2.6	1.2	74.7	–	–
2	1.1	1.4	–	0.6	0.4	7.5	0.5	–	77.3	10.9	0.1
3	3.6	4.0	0.8	1.4	0.5	17.6	1.7	–	69.6	–	–
4	1.2	–	–	2.4	–	7.6	1.2	0.6	84.6	–	2.4
5	1.7	–	–	0.5	–	5.3	1.0	–	89.9	–	1.6

Note: 1: Strain MU2A-22^T(GQ452286); 2: *P. kocurii* 16713^T; 3: *P. koreensis* 102292^T; 4: *P. solventivorans* 6637^T ; 5: *P. alkenifer* 11593^T.

The results presented here proved the possible novel species of the genus *Paracoccus* were the predominant members colonizing in the 131-years-old volcanic deposits. For Miyake-jima island, the research on the bacterial diversity analysis in the young volcanic deposits (23-year-old), another site KP1, has ever been carried out by Lu *et al*.[17]. In that study, *Herbaspirillum*-and *Limnobacter*-related strains have been detected to be the predominant isolates. The possible reason for their colonizing in that very limited organic matter environment was they could utilize H_2 as the energy source and CO_2 as the carbon source for *Herbaspirillum*-related strains, and for *Limnobacter*-related strains, their predominance was due to their living sites very near the sea shore, the sulfur compounds could be input and assimilated by them as the energy source. The ability of oxidizing thiosulfate for isolated *Paracoccus*-related bacteria seems to be consistent with *Limnobacter*-related strians.

Besides strain MU2A-22^T(GQ452286), the other known *Paracoccus* species up to date were all isolated from the polluted environment and showed their versatile metabolisms, such as degrading toxic compound reported by Neef *et al*.,[22] N, N-dimethylformamide by Urakami *et al*.,[23], thiocyanate by Katayama *et al*., [24], tetramethylammonium by Ohara *et al*.,[25], acetone by Siller *et al*.,[26]. This was some how similar to strain MU2A-22^T (GQ452286), which was isolated from the destroyed volcanic environment and also showed a wide variety of assimilating substrates. In addition, in the study of Lu *et al*. [17], *Paracoccus*-related strains were also detected in KP1 site, another point in Miyake-jima island. However, the amounts of those isolates were very small in KP1 site. It is highly likely that such groups might have migrated to these volcanic environment from the separated areas from Miyake-jima island, and accumulated many more in the old volcanic site MU2A (131-year-old) than in the young site KP1 with the age of 23 years.

Our data proved strain MU2A-22^T(GQ452286) could earn the energy by oxidizing thiosulfate for its growth. This property was very similar to several reported *Paracoccus* species, such as *P. sulfuroxidans*, *P. bengalensis*, which were identified by Liu *et al*.[27] and Ghosh *et al*.[28] respectively, also can utilize sulfur-compounds for their growth. This identical metabolic property could be used as a weak support for strain MU2A-22^T(GQ452286) belonging to the family of the genus *Paracoccus*.

Recently, *Limnobacter*-related strain KP1-19^T (AB366174), as a representive of the predominance colonizing in another KP1 site in Miyake-jima island, has been tested for its thiosulfate utilization. That result showed it could utilize thiosulfate as the energy source for its growth [9]. This seems to provide the support on the appearance of the predominant *Paracoccus*-related strains in the similar volcanic environment and the influence on the development of microbes from their living environments.

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征 稿 简 则

1 刊物简介与栏目设置

《微生物学通报》是由中国科学院微生物研究所和中国微生物学会主办的, 以微生物学应用基础研究及技术创新与应用为主的综合性学术期刊。刊登内容包括: 工业微生物学、海洋微生物学、环境微生物学、基础微生物学、农业微生物学、食品微生物学、兽医微生物学、药物微生物学、医学微生物学、病毒学、酶工程、发酵工程、代谢工程等领域的最新研究成果, 产业化新技术和新进展, 以及微生物学教学研究和改革等。设置的栏目有: 研究报告、专论与综述、生物实验室、高校教改纵横、名课讲堂、教学与科研成果展示、显微世界、专题专栏、专家论坛、书讯、会讯等。

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3 写作要求

来稿要求论点明确, 数据可靠, 简明通顺, 重点突出。

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