Characterization of *Variovorax* Strain C6d Isolated from the Algae-bacteria Consortia

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Abstract

The rhizosphere microorganisms can form beneficial, pathogenic, or neutral relationships. These relationships can promote plant growth and productivity. Among them, a number of *Variovorax* isolates from the rhizosphere were isolated. Bacteria *Variovorax* strain C6d (AB552893) was isolated from the non axenic culture of *Chlorella* spp., C6. The cell was Gram-negative, motile, non-spore-forming, short and rod-shaped (0.5-1.0x1.5-2.0µm). Colonies were in white colour after 7 days on 10-fold diluted Nutrient Broth. The strain was able to tolerate NaCL to 1.0% but not to 4.0% of NaCl. It grew quite well at temperatures ranging from 10°C to 37°C, yet did not show any growth at 4°C and 42°C. The dominant isoprenoid quinone was ubiquinone 8 (Q8). The major fatty acid composition of this strain was summed feature 3, 16:0 and 18:1:w7. The DNA G+C content of strain C6d was 70.4 mol%.

Keywords

Variovorax, Characterization, algae-bacteria consortia

Introduction

The phycosphere is the zone surrounding an algal cell within which microorganisms are influenced by algal products. Not a small number of microalgae are living in soil, and the phycosphere in soil may represent a good microhabitat for soil bacteria. Studies on the associations between green microalgae (phylum *Chlorophyta*, kingdom *Plantae*) and bacteria are considered to be of great value, because the form is not only a microorganism-microorganism association but, at the same time, may also be a primitive plant-microorganism association.

Rhizosphere microbes that can form beneficial, pathogenic, or neutral relationships. Understanding these associations can inform strategies for manipulating the plant's microbiome to promote plant growth and productivity. Among the microorganisms isolated from the rhizosphere are a number of *Variovorax* isolates (Jennifer, 2019). *Variovorax* are known for degrading a variety of toxins and organic compounds (Futamata *et al.,* 2005). *Variovorax* also plays an

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important role in the natural cycling of biogenic chemicals including degrading a variety of contaminants such as pesticides (Han *et al.*, 2011).

The genus *Variovorax* belongs to the family *Comamonadaceae* in the class Betaproteobacteria, phylum *Proteobacteria*. The species *Variovorax paradoxus* was originally described as *Alcaligenes paradoxus* by Davis in 1969 and re-classified by Willems *et al.* in 1991 as *V. paradoxus*. Four other members have so far been described, *Variovorax dokdonensis* Yoon *et al.* (2006a), *Variovorax soli* (Kim *et al.,* 2006), *Variovorax boronicumulans* (Miwa *et al.,* 2008), and *Variovorax ginsengisoli* (Im *et al.* 2010; Tuan *et al.,* 2018) reported three more novel species of soil bacteria, *Variovorax ureilyticus*, *Variovorax rhizosphaerae*, and *Variovorax robiniae*.

Variovorax are microorganisms of interested due to their diverse metabolic capabilities. Many species of *Variovorax* have been isolated from contaminated soils and groundwater around the world and of those, some are able to biodegrade of both biogenic compounds and anthropogenic contaminants. For example, Mahan *et al.* (2017) isolated *Variovorax* sp. strain JS1663 that was able to degraded N-nitroglycine (NNG). This is the key enzyme involved in its catabolism. The interaction between *Variovorax* with other microbe and/or plants makes *V. paradoxus* ideal for studies investigating its potential for different applications.

Variovorax strain *C6d* was isolated from the culture of *Chlorella* spp., and C6 was initially isolated in November 2005 from the soil collected in the experimental field of the University of Tokyo, Japan (Otsuka *et al.*, 2008a). The algal culture has been maintained without purification in C liquid medium (Ichimura, 1971) for long term. The cultivation conditions were a 16:8 light-dark cycle with a light intensity of about 10-20 µmol photons m^{-2} s^{-1} at 25°C in the light and 20°C in the dark. The algal culture was serial subcultivated once every four weeks. C medium contains no carbon sources for general heterotrophic bacteria (Vu *et al.,* 2010).

Currently, few studies the characterization of *Variovorax* isolated from algal cultures have been reported. In this study, the taxonomic position of the bacteria *Variovorax* strain C6d is discussed based on phenotypic, genotypic and chemotaxonomic properties. This study gave us a good understanding of the bacteria associated with *Chlorella*, and opened a chance for conducting further studies on the effects of the consortia on the soil and environment.

Materials and Methods

Identification of bacteria

DNA of the isolate was extracted according to Ashida *et al.* (2010). The almost full length of the 16S ribosomal RNA (rRNA) gene of the isolate was amplified by polymerase chain reaction (PCR) with the forward primer 27F and the reverse primer 1492R that corresponds to the positions 8-27 (AGAGTTTGATCCTGGCTCAG) and 1510-1492 (GGTTACCTTGTTACGACTT) in the *Escherichia coli* 16S rRNA gene numbering, respectively and a thermal cycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplification was carried out with the initial denaturation step at 94°C for 10min, then 27 cycles including denaturation at 94°C for 30 sec, annealing at 55°C for 30sec, and extension at 72°C for 1min. The final extension was done at 72°C for 7min, and then the mixtures were held at 4°C. Amplification of the PCR products of the proper size was confirmed by electrophoresis through a 1.5% agarose gel, followed by staining with ethidium bromide. MicroSpin S-400 HR columns (GE Healthcare Biosciences, Oslo, Norway) were used to purify the amplified products then directly sequenced as described by Saito *et al.* (2008). The DNA sequence chromatograms were edited and analyzed by using Finch TV ver. 1.4 (Geospiza, Seattle, WA, USA; http://www.geospiza.com/finchtv/) software and bidirectional sequences were aligned by Clustal W ver. 2.0 (Larkin *et al.*, 2007) to obtain the consensus sequence for each region of the 16S rRNA gene of a strain. The type strains of species phylogenetically closely related to the

isolated strains were searched for using the SeqMatch program (Cole *et al.*, 2007) based on the similarity of the almost full-length 16S rRNA gene sequences. Clustal W version 2.0 program was used to analyse the phylogenetic relationships of the isolated strains and the closely related species, and identify the strains at the genus level.

Gram staining and morphological characteristics

Gram staining was conducted based on the described methods by Gerhardt *et al.* (1994).The morphological characteristics of the cells were observed by photos taken by JEOL Datum (Tokyo, Japan) using transmission electron microscopy with negative staining.

Physiological tests

The physiological profile of the strains was determined using API 20NE microtest galleries (bioMérieux® S.A., Marcy-l'Etoile, France) according to the manufacture's instructions. In addition, Biolog Gen III MicroPlate (Biolog, Inc) that allows testing 71carbon source and 23 chemical sensitivity was used to determine the assimilation and the oxidation patterns of these strains, respectively.

The growth of the bacteria at 4, 10, 15, 20, 26, 30, 37, and 42°C was determined by adding 100µL of the subculture into a glass tube containing pure 10mL of pure 10^{-1} NB medium and evaluated after two weeks of the inoculation started. The growth of the bacteria was determined by the measurement of the OD at 600 nm compared to a blanked control of the culture media without bacteria.

Isoprenoid quinones

The isoprenoid quinones were extracted and analyzed according to the method described by Collins & Jones (1981) with some modifications. The bacterial cells were vacuum freeze-dried in Freevac (Tozai Tsusho Co., Ltd, Japan) to remove the water content. About 100mg of dried cells was transferred to a 100-mL glass flask and covered with by aluminium foil. Twenty milliliters (20mL) of extraction solvent

(chloroform – methanol 2:1) was added and stirred for 2h at room temperature. The sample solution was filtered by a 5A Advantec filter paper (Advantec, Toyo Roshi Kaisha, Ltd., Japan). The procedure was repeated. The filtrates were combined and evaporated in vacuo using a rotary evaporator (R-3000 Rotavapor; Büchi Labortechnik AG, Germany) at 40rpm at 30°C. The quinones were extracted again by using 5mL of acetone and evaporated in the vacuo. Two-hundred microliters (200µL) of 99.5% methyl ethanol was added to dissolve the quinones. The quinone fractions and the reference quinones were separated by thin-layer chromatography (TLC) using silica gel 60F (Merck, Ltd., Japan) and developed in the chromatographic chamber containing 100mL hexane-diethyl ether (85:15). After drying the quinone spots were detected under UV light at 275nm. The quinone spots were scraped off and collected in a tube and 500µL of acetone was added to extract the quinones. The quinone solution was concentrated by a stream of N_2 gas and dissolved in 200µL of ethanol. The extracted quinones and the reference quinones were analyzed by a high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with a 5C18R Cosmosil column (Nacalai Tesc) and using methanol-isopropanol (2:1) as the solvent. The oven temperature was set at room temperature with a pump rate of 1 mL min^{-1}, and chromatograms were read at 270nm.

Whole cellular fatty acids

Whole cellular fatty acids of the bacteria were extracted and analyzed according to the Sherlock Microbial Identification (MIDI) systems. The cell sample was saponified with 1mL of Sherlock Reagent 1, shook for 10sec and heated at 100°C for 5min, then shook again for 10sec and re-heated for 25min. After a total of 30 min of saponification in the water bath at 100°C, the sample was cooled in a cold tap water to room temperature. The cell suspension was methylated with 2mL of Sherlock Reagent 2, shook for five to ten seconds, heated at 80°C ± 1°C for 10min, and cooled rapidly to room temperature. The fatty acid methyl esters were extracted by adding 1.25mL of Sherlock Reagent 3 and gently mixed

for 10min. The lower phase was removed and discarded by Pasteur pipette. The remaining upper phase was washed with 3mL of Sherlock Reagent 4 and mixed for 5min to remove free fatty acids and residual reagents from the organic extract. The suspension was centrifuged at $2,000 \times g$ for 5min to separate to two phases completely. About 2/3 of the upper phase extract solution was transferred to a clean GC sample vial using clean Pasteur pipette and analyzed by the 6890 Series GC system equipped with 7860 Series Injector (Agilent). Helium was used as the carrier gas. A 2µL injection volume of the samples was used. The injector temperature was 250°C, the column temperature was increased from 120 to 240°C at a rate of 5°C min^{-1} and the gas chromatography-mass spectrometry transfer line temperature was 280°C. Fatty acid methyl esters (FAMEs) were identified on the basis of their retention times and their mass spectra.

G + C content of the genomic DNA

The G+C content of the genomic DNA was determined by HPLC according to the method described by Mesbah *et al.* (1989). Briefly, 25µL of DNA solution containing from 2 to 25µg of DNA in a 1.5 microtube was incubated at 60ºC for 1h, then heated at 100ºC for 2min, and rapidly cooled in an ice bath and flash centrifuged. An equimolar mixture of nucleotides for analysis of DNA base composition (Yamasa Shoyu, Choshi, Japan) was used as the quantitative standard. The standard solution, made up of 10µL of the above nucleotides and 15µL of sterile distilled water was prepared under the same condition. Then, 25µL acetate buffer (40mM, pH 5.3) containing 2mM ZnSO$_4$, 20µL sterile distilled water, and 5µL P1 nuclease solution (20 U mL^{-1}) were added to the DNA solution and incubated at 37ºC for 2h. Then 2.5µL of glycine buffer (0.1 M, pH 10.4) and 2µL of 1/10 alkaline phosphate were added to the DNA solution and incubated overnight at 37ºC. After the incubation period, 10µL of the sample solution was injected into the HPLC equipped with a 5C18R Cosmosil column (Nacalai Tesc), and 0.2M ammonium phosphate-acetonitrile (40:1) as solvent. The column temperature was maintained at 40ºC with 1.0mL per min speed and read at 270nm. The G+C content was analyzed by Chromatopac using method 42 to obtain the factors C, G, T, and A.

Results and Discussion

Identification of bacteria

Strain C6d-NBRC 108627^{T} was isolated from the non-axenic culture of *Chlorella* sp and formed colonies with a smooth surface, translucent white color, moist texture, raised elevation, and circular margin on tenfold Nutrient Broth agar. This strain was identified as a *Variovorax* bacterium based on the neighbour-joining tree (**Figure 1**). The nearly full length of the 16S rRNA gene sequence of strain C6d, positions 28 to 1491 (*Escherichia coli* 16S rRNA gene numbering) was compared with those of the type strains of *Variovorax* bacteria. Sequence similarities of the 16S rRNA gene between strain C6d and the type strains of *V. bronicumulans, V. soli*, *V. paradoxus*, *V. ginsengisoli*, and *V. dokdonensis* were 96.9%, 96.7%, 96.6%, 96.6%, and 88.9%, respectively (**Figure 1**).

Morphological and phenotypic characteristics

The cell of the strain C6d was Gram-negative, motile using flagella and occurred as single cells, non-spore-forming, short and rod-shaped (**Figure 2**). The cellular size was about 0.5-1.0×1.5-2.0µm. According to Satola *et al.* (2013), cells of Variovorax are Gram-negative, motile, and oval or rod-shaped, motile with flagella and live in pairs or single cells, straight to slightly curved rods and the size are amounts to 0.3-0.6×0.7-3.0µm, yellow colonies, slimy, and shiny. The morphological characteristics of strain C6d were nearly the same of other members of the genus *Variovorax*.

Chemotaxonomic analysis

The dominant isoprenoid quinone of strain C6d was ubiquinone 8 (Q8) as the same as the other type strains of the genus. The major fatty acid composition of this strain was summed feature 3, 16:0, and 18:1:w7, comprising more than 80% in total (**Table 1**). Summed feature 7 and summed feature 4 were detected in all members of this genus but not detected in strain C6d. These two fatty acids were probably

Table 1. Cellular fatty acid profiles of strain C6d

Fatty acid	C6d
C10:0 3OH	-
C12 : 0	1.1
C14 : 0	1.4
C14:0 2-OH	-
C15 : 0	1.5
C16 : 0	25.8
C17 : 0	0.94
C17 : 0 cyclo	5.0
18:1w7c	17.4
Summed feature 3[a]	37.9

Note: [a] Summed features are groups of two or three fatty acids which cannot be separated by GLC with the MIDI system. Summed feature 3 consisted of the following fatty acid: 16:1 w7c/15 iso 2OH.
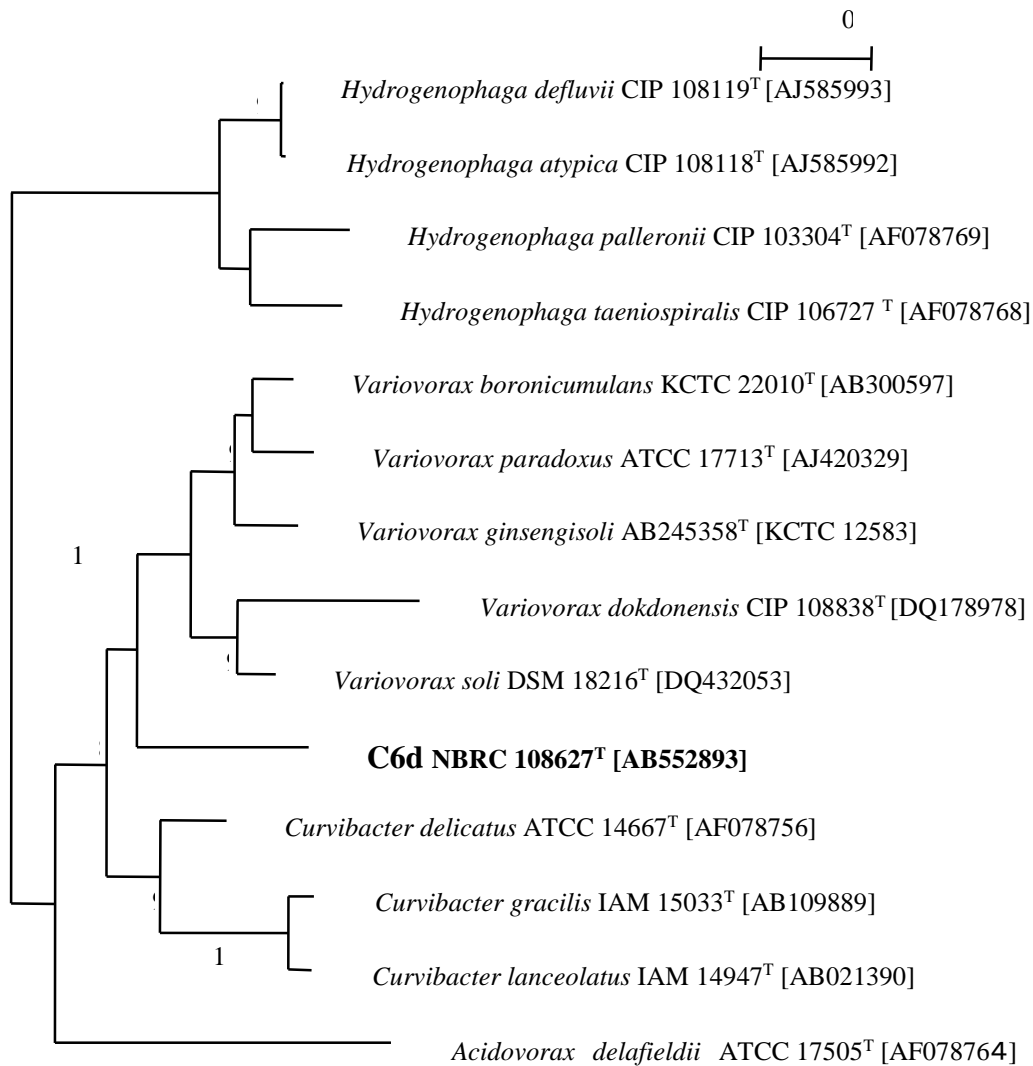
0

Hydrogenophaga defluvii CIP 108119^T [AJ585993]

Hydrogenophaga atypica CIP 108118^T [AJ585992]

Hydrogenophaga palleronii CIP 103304^T [AF078769]

Hydrogenophaga taeniospiralis CIP 106727 ^{T} [AF078768]

Variovorax boronicumulans KCTC 22010^T [AB300597]

Variovorax paradoxus ATCC 17713^T [AJ420329]

Variovorax ginsengisoli AB245358^T [KCTC 12583]

Variovorax dokdonensis CIP 108838^T [DQ178978]

Variovorax soli DSM 18216^T [DQ432053]

C6d NBRC 108627^T [AB552893]

Curvibacter delicatus ATCC 14667^T [AF078756]

Curvibacter gracilis IAM 15033^T [AB109889]

Curvibacter lanceolatus IAM 14947^T [AB021390]

Acidovorax delafieldii ATCC 17505^T [AF078764]

Figure 1. Neighbour-joining tree constructed based on the nearly full length of the 16S rRNA gene sequences of the bacterial strain C6d and their phylogenetically close species. The number of nodes indicate the bootstrap value of occurrence in 1000 trials (only values greater than 700 (70%) are shown).
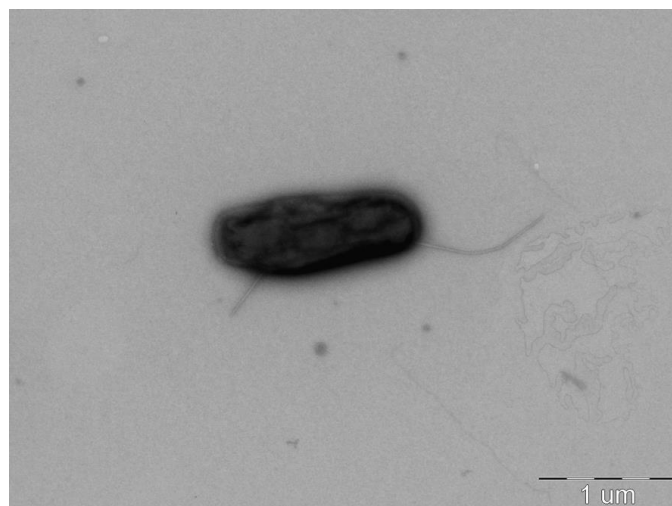
Figure 2. Transmission electron microscopy picture of *Variovorax* sp. C6d

identified as 18:1w7c and summed feature 3 in the present study. The differences in minor fatty acid composition between strain C6d and the type strains of *Variovorax* can also be attributed to the differences in culture conditions such as media used for cultivating the strain for fatty acid analysis. In this study, 1/10 NB was used but in the other studies, R2A was used as the medium to grow the strains. The DNA G+C content of strain C6d was 70.4mol%, which was within the range of 66.0 to 71.2mol% of the genus *Variovorax* (Im *et al.* 2010; Tuan *et al.*, 2018).

Characterization and description of *Variovorax* C6d

The cell was Gram-negative, motile, non-spore-forming, short and rod-shaped (0.5-1.0x 1.5-2µm) (**Figure 2**). Colonies were white in colour after 7 days on 10-fold diluted Nutrient Broth. The strain was able to tolerate NaCl to 1.0% but not to 4.0%, and grew well at 10 to 30°C but did not grow at 4 and 37°C. Strain C6d was positive for nitrate reduction and also for oxidation of D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-galactose, 3-methyl glucose, L-lactate, L-histidine, malate, D-fucose, L-fucose, L-rhamnose, inosine, myo-Inositol, glycerol, D-glucose-6-PO$_4$, D-fructose-6-PO$_4$, β-methyl-D-glucoside, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-neuraminic acid, N-acetyl-D-galactosamin, D-aspartic acid, L-arginine, L-glutamic acid, L-serine, L-histidine, Lpyroglutamic acid, D-gluconic acid, D-glucoronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, D-malic acid, L-malic acid, L-lactic acid, and α-keto-glutaric acid, as sole carbon sources, but negative for dextrin, D-maltose, D-raffinose, α-D-lactose, D-melibiose, α-D-glucose, D-manose, D-fructose, D-sorbitol, D-manitol, D-arabitol, gelatin, glycyl-L-proline, L-alanine, D-serine, D-galacturonic acid, L-galatonic acid lactone, propionic acid, acetic acid, citric acid, acetoacetic acid, methyl pyruvate, tween 40, D-lactic acid methyl ester, bromo-succinic acid, gamma aminobutyric acid, p-hydroxyl-Phenylacetic acid, α-hydroxyl-butyric acid, and β- hydroxyl-D,L-butyric acid (**Table 2**). The major cellular fatty acids are C16: 0, summed feature 3, and C18:1w7c. Q-8 was the predominant isoprenoid quinone. The G+C content of the genomic DNA was 70.4mol%. The strain was C6d^T (= NBRC 108627^T).

Conclusion

The results of this study showed that *Variovorax* not only occurs in contaminated soils and groundwater, but also associates with algae. The strain C6d -NBRC 108627^T isolated from the nonaxenic algal culture had all of the characteristics of the members of *Variovorax*. These results provide to a chance for continued research on the potential of using C6d in the field

Table 2. Physiological characteristics of strain C6d

Characteristics	C6d
Cell shape	Rods
Cell width (µm)	0.5-1.0
Cell length (µm)	1.5-2.0
Nitrate reduction	+
Urease	-
a-Glucosidase	-
D-Glucose	-
L-Arabinose	-
D-Mannose	-
D-Mannitol	-
Gluconate	+
Adipate	-
Citrate	w
Phenylacetate	-
D-Sorbitol	-
DNA G+C content (mol%)	70.4

Note: +, positive; -, negative; w, weak reactions.

of reclamation of polluted soil and the environment.

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