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Characterization of carbazole degrading marine bacterium strain OC6S isolated from seawater of Kobe, Japan, a probable novel species and genus from'*Alphaproteobacteria*'class

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Abstract

The carbazole-degrading marine bacterium OC6S^T was isolated from seawater collected off the coast of Kobe, Japan. Phylogenetic analysis of 16S rRNA revealed that this strain is distinct from other known orders of class '*Alphaproteobacteria*'. The closest related bacterium was *Kordiimonas gwangyangensis* GW14-5^T, although 16S rRNA similarity was only 90.1%. Strain OC6S^T was Gram-negative, motile, rod-shaped, catalase-positive, oxidase-positive, nitrate-reducing, and mesophilic. Respiratory quinone was Q-10 for both aerobic and anaerobic growth, and the DNA G+C content was 58.3 mol%. Optimal cell growth was observed at 16-37 ℃, pH 5.5-10, and at NaCl concentrations up to 5%. The dominant fatty acids were C18 : 1 ω7*c* (41.54%), C14 : 0 2-OH (14.21%), and C17 : 1 *ω*6*c* (10.10%). The results suggest that this marine bacterium may be representing a novel genus and species within '*Alphaproteobacteria*'.

INTRODUCTION

Phylum *Proteobacteria* is subdivided into five classes: '*Alphaproteobacteria*','*Betaproteobacteria*','*Gammaproteobacteria*', '*Deltaproteobacteria*', and '*Epsilonproteobacteria*'. Currently, orders that are recognized as members of '*Alphaproteobacteria*': *Caulobacterales*, '*Kopriimonadale'*, *Kordiimonadales*, *Magnetococcales* ,'*Parvularculales*', *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Rickettsiales*, *Sneathiellales*, *Sphingomonadales* and *Kiloniellales* (NCBI taxonomy website, 2013). Notably, recently identified orders, '*Kopriimonadales'*, *Kordiimonadales* [1], *Sneathiellales* [2] and *Kiloniellales* [3] were isolated from marine environments. During the course of isolating carbazole degrading bacteria from seawater samples of sea surrounding Japan, we isolated a novel marine bacterium belonging to the `*Alphaproteobacteria*`. In this study we report the identification and characterization of strain OC6S^T, which may represents a probable novel genus and species in the class '*Alphaproteobacteria*'.

METHODS

Strain OC6S^T was isolated from samples collected off the coast of Kobe, Japan. Each 10 L sample was filtered using a vacuum driven filter (Steritop; Millipore, Billerica, MA, USA), and the sediments were re-suspended in 30 mL of basal medium (100 mg NH_4NO_3, 10 mg KH_2PO_4, 2.5 mg Fe-EDTA, and 5 mg yeast extract in 1 L of sterile-filtered seawater, pH 8.0). Next, 1 mL of this concentrated sample was inoculated into 200 mL of basal medium containing 0.1% (w/v) carbazole. This sample was enriched several times for approximately 6 months at 30℃. Strain OC6S^T was then isolated from colonies that formed clear zones when grown on 1.5% (w/v) agar plates (Nacalai Tesque, Kyoto, Japan) containing basal medium and 0.1% (w/v) carbazole.

For growth test of temperature, Growth temperature was tested at 12 ℃, 16 ℃, 20 ℃, 25 ℃, 30 ℃, 37 ℃ and 40 ℃.

For growth NaCl concentration test, NaCl concentration for growth was tested at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0% NaCl. For growth pH test, strain OC6S^T was grown in the media whose pH was adjusted to be 5.0, 5.5, 6.0, 7.0, 8.0, 9.0 and 10.0. For growth test under anaerobic condition, strain OC6S^T was grown in Marine broth 2216 medium at 30 ℃ under anaerobic condition.

For growth substrate test, strain OC6S^T was grown in 2 ml of an artificial seawater mineral salts medium [4] containing vitamin solution (2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg riboflavine, 5 mg thiamine-HCl, 5 mg nicotinamide, 5 mg calcium pantothenate, 0.1 mg vitamin B_{12} and 5 mg p-aminobenzoic acid in 1 L of sterile-filtered water) and each 0.1% (w/v) D (+) glucose, D (-) mannose, L (+) arabinose, D (-) sorbitol, D (-) fructose, D (-) mannitol, Maltose, D (+) galactose, Sucrose, D-xylose, Glycerin, D (+) cellobiose, L (+) rhamnose, Glycine, L-glutamine, Potassium hydrogen phthalate, D (+) raffinose, Lactose monohydrate, L-ascorbic acid, Benzoic acid, Salicylic acid and Pyruvic acid for a week at 30 ℃. After cultivation for a week, absorbance at 600 nm of each medium was measured by spectrophotometer.

For biochemical and phenotypic analyses, strain OC6S^T was grown in Marine broth 2216 (Becton Dickinson, Sparks, MD, USA) for 72 h at 30 ℃, under both aerobic and anaerobic conditions. The strain was characterized according to the methods

described by Cowan and Steel [5]. Gram-staining was carried out using Favor-G Nissui (Nissui Pharmaceutical, Tokyo, Japan). Microscopy was performed using a BX50F4 optical microscope (Olympus, Tokyo, Japan) and a JEM-2000EX transmission electron microscope (JEOL, Tokyo, Japan). Quinone was extracted using the method described by Nishijima *et al.*, 1997 [6] and examined using the Waters 600 high-performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA, USA).

RESULTS AND DISCUSSION

Strain OC6S^T was Gram-negative and formed colonies that were light yellow, uniformly circular (1.0-2.0 mm in diameter), smoothly convex, and semitransparent. Catalase and oxidase activity were detected, but glucose oxidation was not observed. Microscopy revealed that OC6S^T is a motile, rod-shaped bacterium with a polar monotrichous flagellum (Fig. 1). Optimal growth was observed at 16-37 °C, pH 5.5-10.0, and NaCl concentrations of up to 5% (w/v). No growth was observed under at 12 °C and 40 °C, pH 5.0, NaCl-free conditions, while growth occurred under anaerobic conditions. Strain OC6S^T was also examined using the API 20NE system (version 7.0; BioMérieux, Lyon, France), but no reaction was observed other than nitrate reduction. As a result of growth substrate, strain OC6S^T utilized L-glutamine as a sole carbon source, while did not utilized D (+) glucose, D (-) mannose, L (+) arabinose, D (-) sorbitol, D (-) fructose, D(-) mannitol, Maltose, D (+) galactose, Sucrose, D-xylose, Glycerin, D (+) cellobiose, L (+) rhamnose, Glycine, Potassium hydrogen phthalate, D (+) raffinose, Lactose monohydrate, L-ascorbic acid, Benzoic acid, Salicylic acid and Pyruvic acid. Ubiquinone Q-10 was detected under both aerobic and anaerobic growth conditions. Phenotypic characteristics of strain OC6S^T were concluded in table 1.

The cellular fatty acid composition of strain OC6S^T was profiled using the Sherlock Microbial Identification system (version 5.0; MIDI, Newark, DE, USA), and fatty acid extraction and analyses were conducted according to the manufacturer's protocol. The bacterium was composed of three primary fatty acids: C18:1 ω7*c* (41.54%), C14:0 2-OH (14.21%), and C17:1ω6*c* (10.10%). Detailed fatty acid compositions of strain OC6S^T and members of '*Alphaproteobacteria*' are shown in table 2.

The DNA G+C content was determined via HPLC (LC-10; Shimadzu, Kyoto, Japan) using the method described by Katayama *et al*., 1984 [7]. The DNA G+C content of strain OC6S^T was 58.3 mol%. Next, 16S rRNA gene fragments were isolated from strain OC6S^T, amplified using primers described by Maeda *et al.* 2009 [8], and sequenced on an ABI PRISM 310 genetic analyzer using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. The sequence was then compared to a variety of 16S rRNA gene sequences deposited in GenBank, DDBJ, and EMBL. BLAST analysis showed that strain OC6S^T grouped under class '*Alphaproteobacteria*', among the genera *Kordiimonas*, *Mesorhizobium*, and *Ensifer*. However, the closest related bacterium, *Kordiimonas gwangyangensis* GW14-5^T, showed only 90.1% similarity, suggesting that OC6S^T is distinct at the family or order level. A phylogenetic tree was constructed using 16S rRNA sequences from type strains that represent every order with '*Alphaproteobacteria*' (Fig. 2) [9]. Gene sequences were aligned using CLUSTAL W and the tree was constructed using the neighbor-joining method and MEGA software version 4.0 [10, 11]. Our phylogenetic analysis indicated that strain OC6S^T is distinct from other type strains within '*Alphaproteobacteria*' (Fig. 2).

Furthermore, strain OC6S^T was clearly distinct from even its closest relative, *K. gwangyangensis* GW14-5^T. The DNA G+C content of strain OC6S^T (58.3 mol%) was markedly higher than that of strain GW14-5^T (39.3 mol%) (Table 1). The dominant fatty acid in strain OC6S^T was C18:1ω7*c* (41.54%), whereas this fatty acid was not detected in strain GW14-5^T (Table 2).

Table 1. Phenotypic characteristics of strain OC6S^T in comparison to *Kordiimonas gwangyangensis* GW14-5^T [1].

Characteristic	strain OC6S^T	*K. gwangyangensis* GW14-5^T
Cell size (μm)	0.3-0.4×1.0-2.0	0.25×1.3-1.4
Colony size (mm)	1.0-2.0	2.0-3.0
Growth temperature (°C)	16-37	17-44
Growth pH	5.5-10.0	6.0-8.5
NaCl growth range (%)	0.5-5.0	0.5-4.0
DNA G+C content (mol%)	58.3	39.3
O2 requirement	-	+
Oxidase/catalase activity	+/+	+/w
Nitrate reduction	+	-

Fig. 1. Transmission electron micrograph of cells of strain OC6S^T with a polar monotrichous flagellum. Bar, 500 nm.

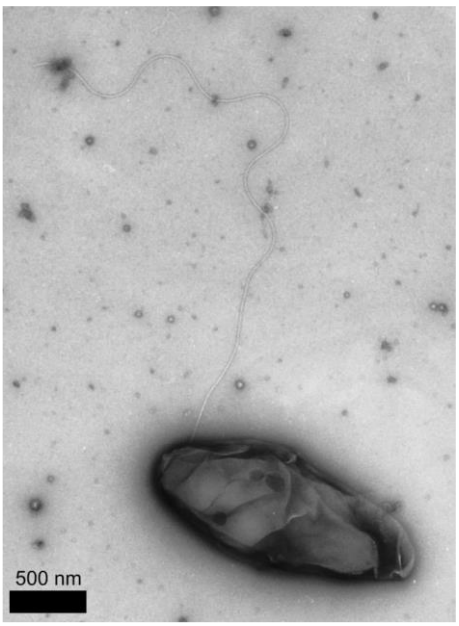
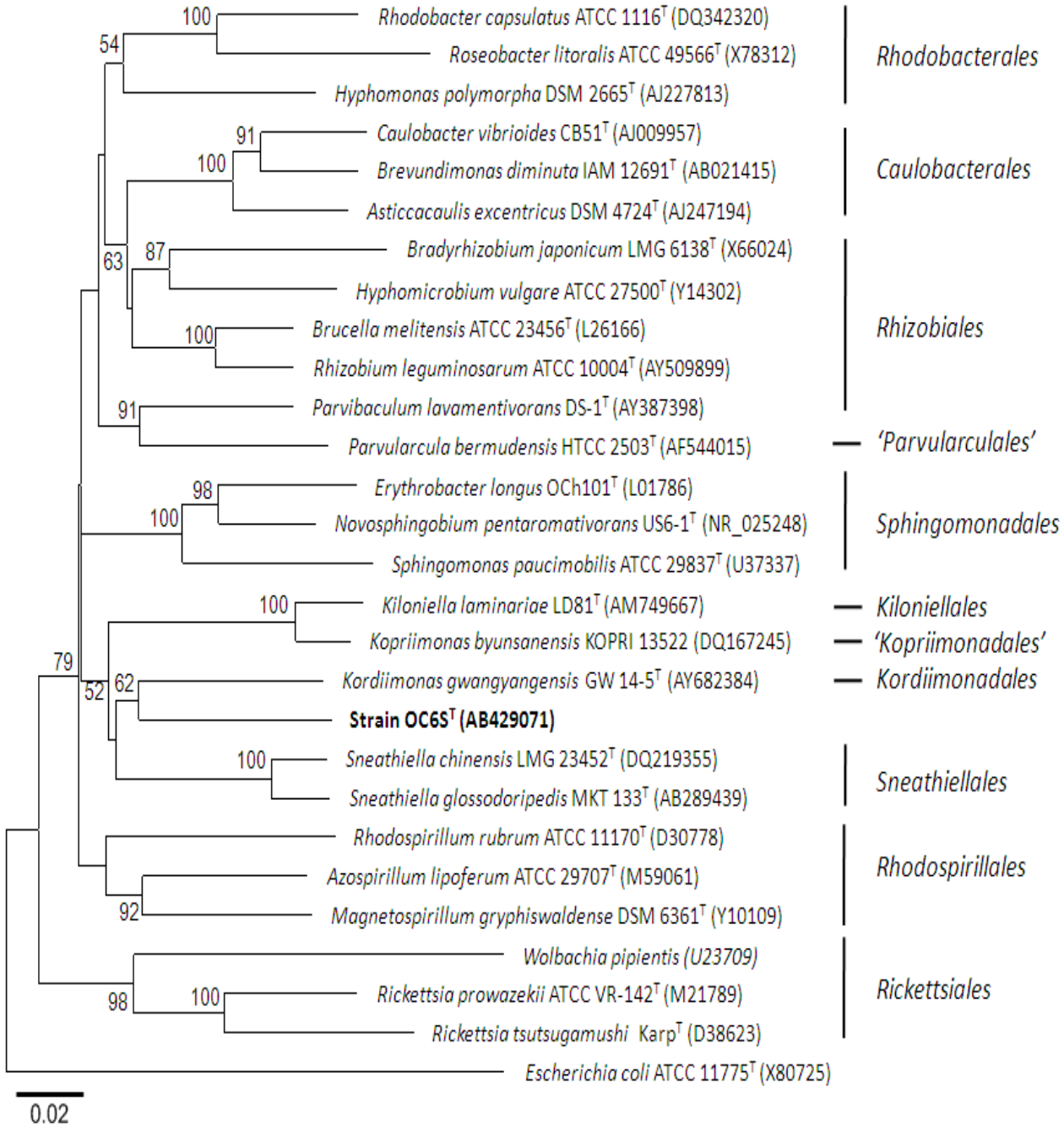


Table 2. Total fatty acids of contents (%) of strain OC6S^T and other members of 'Alphaproteobacteria'. Strains: 1, strain OC6S^T; 2, *Kordiimonas gwangyangensis* GW14-5^T [1]; 3, *Parvularcula bermudensis* HTCC 2503^T [12]; 4, *Parvibaculum lavamentivorans* DSM 13023^T [13]; 5, *Novosphingobium pentaromativorans* US6-1^T [14]; 6, *Caulobacter vibrioides* DSM 9893^T [15]; 7, *Paracoccus haeundensis* KCCM 10460^T [16]; 8, *Kiloniella laminariae* LD81^T [3]; 9, *Sneathiella chinensis* LMG 23452^T [2]. -,fatty acids not detected or less than 1%.

Fatty acid	1	2	3	4	5	6	7	8	9
Saturated fatty acids									
$C_{10:0}$ 3-OH	-	-	-	-	-	-	2.1	-	-
$C_{12:0}$	2.2	-	5.2	-	-	-	-	-	-
$C_{12:0}$ alde	-	-	-	-	-	-	-	1.7	-
$C_{13:0}$	1.2	-	-	-	-	-	-	-	-
$C_{13:0}$ 2-OH	1.3	-	-	-	-	-	-	-	-
$C_{14:0}$	7.6	-	2.9	-	-	-	-	-	-
$C_{14:0}$ 2-OH and/or 3-OH	14.2	-	-	6.3	19.7	3.9	1.5	1.2•	2.7
$C_{15:0}$	3.7	1.9	-	-	-	-	-	-	2.1
$C_{15:0}$ 2-OH and/or 3-OH	1.4	-	-	-	-	-	-	-	-
$C_{15:0}$ iso	-	15.1	-	-	-	-	-	-	-
$C_{16:0}$	2.6	2.7	8.6	6.5	3.5§	1.1	-	8.5	17.2
$C_{16:0}$ 3-OH	1.6	-	-	1.2	-	-	-	-	-
$C_{17:0}$	-	2	-	-	-	-	-	1.1ø	-
$C_{17:0}$ iso	-	12.6	-	-	-	-	-	-	-
$C_{17:0}$ anteiso	-	3.5	-	-	-	-	-	-	-
$C_{18:0}$	-	-	4	9.7	-	1.3	7.8	3.5#	-
Unsaturated fatty acids									
$C_{12:1}$ 5c	-	-	-	-	-	-	2.0∫	-	-
$C_{15:1}$ ω6c and ω8c	1.1	-	-	-	-	-	-	-	-
$C_{15:1}$ iso	-	1.4	-	-	-	-	-	-	-
$C_{16:1}$*	-	1	-	1.4	8.8	-	-	30.7	10
$C_{17:1}$ ω6c	10.1	-	-	-	2	-	-	-	6.4
$C_{17:1}$ ω8c	1.2	-	-	-	-	-	-	-	-
$C_{17:1}$ iso		46.2	-	-	-	-	-	-	-
$C_{18:1}$†	41.5‡	4.2	79.3	53.2	64	88.8	84.3	48.6	47.1
Remainder									
$C_{17:0}$ cyclo	-	2.6	-	-	-	-	-	-	-
$C_{19:0}$ cyclo¶	-	-	-	21.7	-	-	-	1.4	9.8
Unidentified, etc.	8	4.5	-	-	-	3.3	-	2.3	2.3

∗Includes the one or more of the following: C16 : 1 ω5*c*, C16 : 1 ω7*c* and/or C16 : 1 ω9*c*.
†Includes the one or more of the following: C18 : 1 ω5*c*, C18 : 1 ω7*c*, C18 : 1 ω9*c*, C18 : 1 ω9*t* and/or C18 : 1 ω12*t*.
‡Content for C18 : 1 ω7*c* only.
¶Includes C19 : 0 cyclo 2-OH and/or C19 : 0 cyclo ω8*c*.
§Includes C16 : 0 2-OH.
∫Includes C17 : 1 ω8*c*.
•Includes C16 : 1 iso.
øIncludes C17 : 0 3-OH.
#Includes C18 : 0 3-OH.

Fig. 2. Neighbour-joining tree of nearly complete 16S rRNA gene sequences showing relationships between strain OC6S^T and other members of the '*Alphaproteobacteria*'. Bootstrap values



CONCLUSION

These differences in phylogeny and base and fatty acid composition suggest that strain OC6S^T represents a novel species and genus within a new family in the class '*Alphaproteobacteria*'. Strain OC6S^T was deposited to culture collection centers with accession numbers of NBRC 105636^T =JCM 15931T =KCTC 22627^T.

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