***Paracoccus carotinifaciens* sp. nov., a new aerobic Gram-negative astaxanthin-producing bacterium**

Akira Tsubokura, Hisashi Yoneda and Haruyoshi Mizuta

Author for correspondence: Akira Tsubokura. Tel: +81 45 625 7171. Fax: +81 45 625 7298.
e-mail: tsubokura.akira@nisseki.co.jp

Central Technical Research
Laboratory, Nippon Oil
Company Ltd, 8 Chidori-cho,
Naka-ku, Yokohama
231-0815, Japan

The strain E-396^T, isolated from soil, was Gram-negative, aerobic, orange-pigmented, rod-shaped, motile by peritrichous flagella and astaxanthin-producing. This organism produced carotenoids, mainly astaxanthin, and did not produce bacteriochlorophyll. The ubiquinone system was Q-10. Analysis of the 16S rRNA sequence of strain E-396^T showed it to be a member of the α -3 subclass of the *Proteobacteria*, forming a cluster with the species of the genus *Paracoccus*. On the basis of the production of orange pigments and motility by peritrichous flagella, together with DNA–DNA reassociation data, it is concluded that the new isolate should be classified into a new species of the genus *Paracoccus*, *Paracoccus carotinifaciens* sp. nov. The type strain is E-396^T (= IFO 16121^T).

Keywords: *Paracoccus*, *Paracoccus carotinifaciens*, *Proteobacteria*, carotenoid, astaxanthin

INTRODUCTION

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is a carotenoid widely distributed in nature and is present in marine animal tissues such as those of red sea bream, salmon and lobsters (Fujita *et al.*, 1983; Johnson, 1991; Nelis & De Leenheer, 1991). The following organisms have been reported to produce astaxanthin: the basidiomycetous yeast *Phaffia rhodozyma* (Miller *et al.*, 1976), the green alga *Haematococcus pluvialis* (Bubrick, 1991), the Gram-positive bacterium *Brevibacterium* 103 (Iizuka & Nishimura, 1969) and the Gram-negative marine bacterium *Agrobacterium aurantiacum* (Yokoyama *et al.*, 1994). We screened many isolates which produced orange or red pigments from soil samples. As a result, we were able to isolate a strain capable of producing astaxanthin.

In this paper, we describe the results of a taxonomic and 16S rDNA sequence analysis of the astaxanthin-producing strain. We concluded that this strain should be classified as a new species of the genus *Paracoccus*, and *Paracoccus carotinifaciens* sp. nov. is proposed for the strain, with type strain E-396^T (= IFO 16121^T).

METHODS

Bacterial strains. The orange-pigmented and astaxanthin-producing bacterial strain, E-396^T (= IFO 16121^T), was isolated from soils collected in the Kanagawa Prefecture, Japan. This strain was isolated on nutrient agar medium (Eiken), and was maintained on the same medium by serial inoculation. The type strains of known species of the genus *Paracoccus* were maintained on BY agar medium (Katayama-Fujimura & Kuraishi, 1980).

Phenotypic characteristics. A strain was grown on nutrient agar and was submitted to morphological and physiological characterization. Morphological observation and principal biochemical characterization by use of API 20E strips (bioMérieux) were carried out on the isolated strain. The temperature ranges for growth were determined by incubating cells for 10 d on plates of nutrient agar at the following temperatures: 4, 10, 25, 28, 33, 37 and 40 °C. The pH ranges for growth were determined by incubating cells in nutrient broth medium at 28 °C for 10 d at the following pH: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. Acid and gas production from carbohydrates was determined using a modification of the Hugh and Leifson medium (Hugh & Leifson, 1953) containing (l⁻¹) 2 g Bacto-tryptone (Difco), 5 g NaCl, 1 g yeast extract (Difco), 0.3 g K₂HPO₄, 80 mg bromothymol blue, 2 g agar and 1% (w/v) carbohydrate. The following compounds were tested: L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol, inositol and glycerol. The ability to use inorganic nitrogen sources was examined using a medium containing (l⁻¹) 10 g

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glucose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.01 g yeast extract (Difco) and 1–2 g inorganic nitrogen source. Growth was monitored by measuring turbidity after 7 d incubation at 28 °C with shaking. Denitrification was determined by the growth and the formation of gas in a stab culture of nutrient agar containing 0.1% (w/v) agar. Formation of 3-ketolactose was examined by the method of Bernaerts & De Ley (1963). Catalase activity was determined by the presence of bubbles in a 3% hydrogen peroxide solution (Takeuchi *et al.*, 1996). Oxidase activity was determined by using oxidase testing paper (Nissui Seiyaku). Slime production was examined both on a medium containing glucose as a carbon source and a medium containing sucrose as described by Jordan (1984).

Chemotaxonomic characteristics. Quinones and fatty acids were analysed by the method described by Komagata & Suzuki (1987). Bacteriochlorophyll formation was determined by examining the absorption spectra of extracted pigments as described by Trüper & Pfennig (1981). Cells were prepared by cultivation in anaerobic conditions in light or under aerobic conditions. Sphingolipid formation was determined by the method of Kawahara *et al.* (1991). Production of carotenoids was examined by the following method. Carotenoids were extracted with 0.5 ml dichloromethane/methanol (4:1), to which was added 0.5 ml n-hexane. The pigment was loaded into HPLC columns (silica column: Wako-sil 5SIL-120, Wako Pure Chemical). The mobile phase was a mixture of n-hexane/dichloromethane/methanol (10:8:1). Pigments were monitored by measuring the A_{470} . Astaxanthin was identified by using the standard solution of authentic astaxanthin.

Electron microscopy. Cultured cells were suspended in 0.1 M phosphate buffer (pH 7.2). The cells were fixed with 2% glutaraldehyde, washed with 0.05 M cacodylate buffer and postfixed with 1% osmium tetroxide. The fixed cells were dehydrated in ethanol, and the ethanol was replaced with propylene oxide before embedding in Epon resin and sectioning with an ultramicrotome. Sections were examined with a Philips EM420 transmission electron microscope.

DNA base composition and DNA–DNA hybridization. The G + C content of the genomic DNA was determined by the method of Tamaoka & Komagata (1984). DNA–DNA hybridizations were carried out by the photobiotin method using micro plates described by Ezaki *et al.* (1990).

Determination of the 16S rDNA sequence and phylogenetic analysis. The 16S rDNA sequence for strain E-396^T was determined by the cloning method according to Anzai *et al.* (1997). Phylogenetic analysis was carried out as described by Anzai *et al.* (1997). The determined sequence was compared with those of the α -2, α -3 *Proteobacteria*.

RESULTS

Cell shape, Gram stain and motility

Strain E-396^T was Gram-negative, motile by means of peritrichous flagella, rod-shaped and non-spore-forming. The cells were 0.3–0.75 μm in diameter and 1.0–5.0 μm in length (Fig. 1). The cells were elongated or swollen at the early stage of culture when the organisms were grown in a liquid medium containing yeast extract (1%).

Fig. 1. Electron micrograph of a thin-sectioned cell of strain E-396^T grown on nutrient agar for 24 h at 25 °C. Bar, 0.2 μm .

Physiological characteristics

On nutrient agar, this strain formed round, smooth, orange colonies. The physiological and chemotaxonomic characteristics of strain E-396^T are summarized in Table 1. Strain E-396^T was able to grow between 10 and 33 °C, with optimum growth at 28 °C, and the pH range for growth was 6.0–9.0, with an optimum pH of 6.5–7.5. Strain E-396^T utilized ammonium as a nitrogen source but did not utilize nitrate. Denitrification was not performed. 3-Ketolactose was not produced. This strain was catalase-positive, oxidase-positive and urease-negative. Gelatin and starch were not hydrolysed. This strain did not form slime on either glucose agar or sucrose agar.

Chemotaxonomic characteristics

The major isoprenoid quinone was ubiquinone-10. The major non-hydroxyl fatty acid was $\text{C}_{18:1}$, which constituted 82% of the total non-hydroxyl acids. 3-Hydroxyl fatty acids were present, with 3-OH $\text{C}_{10:1}$ predominating. Sphingolipids were not produced. Because the strain was not able to grow anaerobically in light, the formation of bacteriochlorophyll was examined using cells cultured aerobically. This strain did not produce bacteriochlorophyll under aerobic conditions. The major carotenoid was astaxanthin;

Table 1. Characteristics of strain E-396^T

Character	Strain E-396 ^T
Morphological characters	
Cell width (µm)	0.3–0.75
Cell length (µm)	1.0–5.0
Polymorphism	+
Motility	+
Spores	–
Optimum temp. for growth (°C)	28
Optimum pH for growth	7
Utilization of:	
D-Glucose	+
L-Arabinose	–
D-Mannose	+
D-Mannitol	+
N-Acetyl-D-glucosamine	–
Maltose	+
Potassium gluconate	+
n-Capric acid	–
Adipic acid	–
DL-Malic acid	+
Sodium citrate	–
Phenyl acetate	–
Acid or gas production from:	Acid/gas
L-Arabinose	+/-
D-Xylose	+/-
D-Glucose	+/-
D-Mannose	+/-
D-Fructose	-/-
D-Galactose	+/-
Maltose	+/-
Sucrose	+/-
Lactose	+/-
Trehalose	+/-
D-Sorbitol	+/-
D-Mannitol	+/-
Inositol	-/-
Glycerol	+/-
Nitrate reduction	–
Denitrification	–
Production of:	
Urease	–
Oxidase	+
Catalase	+
β-Galactosidase	+
Arginine dihydrolase	–
Pigment	+(orange to red)
Indole	–
3-Ketolactose	–
Slime	–
Hydrolysis of:	
Aesculin	+
Starch	–
Gelatin	–
Chemotaxonomic characteristics	
Quinone system	Q-10
G + C content (mol %)	67
Bacteriochlorophyll	–
Sphingolipid	–
Major fatty acids	
Non-hydroxyl acids	C _{18:1}
3-Hydroxyl fatty acids	3-OH C _{10:0}
Major carotenoids	Astaxanthin

Table 2. Reassociation values (%) between DNAs from strain E-396^T and *Paracoccus* species

Organism	Strain E-396 ^T
Strain E-396 ^T	100
<i>Paracoccus aminophilus</i> IAM 14245 ^T	23
<i>Paracoccus aminovorans</i> IAM 14244 ^T	22
<i>Paracoccus denitrificans</i> IAM 12479 ^T	21
<i>Paracoccus kocurii</i> IAM 14243 ^T	26
<i>Paracoccus versutus</i> IAM 12814 ^T	23

other carotenoids were also detected. The G + C content of the DNA of strain E-396^T was 67 mol %.

DNA–DNA hybridizations

The DNA–DNA reassociation among strain E-396^T and the genus *Paracoccus* species is shown in Table 2. The DNA–DNA binding values between strain E-396^T and five species of the genus *Paracoccus* are between 21 and 26 %.

16S rRNA sequence and phylogenetic tree analysis

Nearly full-length fragments of the 16S rDNA from strain E-396^T were amplified by PCR, and one of these fragments was sequenced. The phylogenetic positions of the new isolate within the α-3 subclass of the *Proteobacteria* are shown in Fig. 2. Strain E-396^T was closely related to species belonging to the genus *Paracoccus* and formed a cluster with those species. The bootstrap value to the *Paracoccus* cluster including strain E-396^T from the other α-3 *Proteobacteria* was 57%. The sequence of strain E-396^T exhibited the highest levels of similarity to the sequences of *Paracoccus alcaliphilus*. The levels of similarity between the sequence of strain E-396^T and that of *P. alcaliphilus*, *Paracoccus thiocyanatus* and *Paracoccus solventivorans*, which were not subjected to DNA–DNA hybridization assay, were 95.9, 94.9 and 94.9 %, respectively.

DISCUSSION

In the present study, a strain of astaxanthin-producing bacteria was isolated. Phylogenetic analysis was carried out based on 16S rRNA gene sequence information for this strain, E-396^T, and the analysis showed that the newly isolated astaxanthin-producing bacterium falls into the α-3 subclass of the *Proteobacteria* and forms a cluster with the species of the genus *Paracoccus*. This implies that the new isolate should be classified as a member of the genus *Paracoccus*. At the time of writing the genus *Paracoccus* consisted of the following eight species: *P. alcaliphilus* (Urakami *et al.*, 1989), *Paracoccus aminophilus* (Urakami *et al.*, 1990), *Paracoccus aminovorans* (Urakami *et al.*, 1990), *Para-*

Fig. 2. Phylogenetic relationships among the species of the α-3 Proteobacteria and representative species of the other α Proteobacteria. The tree was drawn by the neighbour-joining method, and the results of bootstrap analysis (1000 replications) are shown on each branch. *Escherichia coli* was used as the root organism. Accession numbers are in parentheses. Bar, 0.012 K_{nuc} .

coccus denitrificans, *Paracoccus kocurii* (Ohara *et al.*, 1990), *P. solventivorans* (Siller *et al.*, 1996), *P. thiocyanatus* (Katayama *et al.*, 1995) and *Paracoccus versutus* (Katayama *et al.*, 1995) [two new species have been validated recently: *Paracoccus alkenifer* (Lipski *et al.*, 1998) and *Paracoccus marcusii* (Harker *et al.*, 1998)]. The DNA–DNA reassociation levels between strain E-396^T and five known *Paracoccus* species are sufficiently low to assure classification of the isolate as a new species of the genus *Paracoccus*. With regard to *P. alcaliphilus*, *P. thiocyanatus* and *P. solventivorans*, which were not subjected to DNA–DNA hybridization assay, the levels of similarity of their 16S rDNA sequences imply that strain E-396^T can be differentiated from these species genotypically. Our isolate can

be distinguished from the known species of the genus *Paracoccus* by its phenotypic characteristics, including motility, pigment production, denitrification and urease activity (Table 3).

Photosynthetic bacterial genera *Rhodobacter* (Imhoff *et al.*, 1984), *Rhodovulum* (Hiraishi & Ueda, 1994) and *Roseobacter* (Shiba, 1991) contain carotenoids of the spheroidene series as photosynthetic pigments. In contrast, the newly isolated non-photosynthetic bacteria produce the polar carotenoid astaxanthin as a major pigment. An elucidation of the function of carotenoids produced by this strain has become a subject of considerable interest.

On the basis of its phenotypic and chemotaxonomic

Table 3. Characteristics differentiating *P. carotinifaciens* from other species

Strain	Motility	Colour of colonies*	Denitrification	Urease
<i>P. carotinifaciens</i>	+	Orange to red	—	—
<i>P. alcaliphilus</i> †	—	Creamy white	—	+
<i>P. aminophilus</i> ‡	—	Creamy white	—	—
<i>P. aminovorans</i> ‡	—	Creamy white	—	—
<i>P. denitrificans</i> §	—	White	+	+
<i>P. kocurii</i> §	—	Creamy white	+	—
<i>P. solventivorans</i>	—	NA	+	NA
<i>P. thiocyanatus</i> ¶	—	Creamy white	+	—
<i>P. versutus</i> ¶	+ or —**	Creamy white	+	—

NA, No data available.

* Data from this study. Colonies were grown on BY agar medium.

† Data from Urakami *et al.* (1989).

‡ Data from Urakami *et al.* (1990).

§ Data from Ohara *et al.* (1990).

|| Data from Siller *et al.* (1996).

¶ Data from Katayama *et al.* (1995).

** There are positive and negative reports for motility (Katayama *et al.*, 1995).

characteristics, 16S rDNA sequence analysis and DNA–DNA hybridization studies, we propose that strain E-396^T should be placed in a new species, *Paracoccus carotinifaciens*. A description of the new species is given below.

Description of *Paracoccus carotinifaciens* sp. nov.

Paracoccus carotinifaciens (ca.ro.ti.ni.fa'ci.ens. M.L. neut. n. *carotinum* carotene; L. part. adj. *faciens* making/producing; M.L. part. adj. *carotinifaciens* carotene/carotenoid-producing).

Gram-negative motile rods by means of peritrichous flagella. Not spore forming. Cells range from 0.3 to 1.0 µm in diameter and 1.0 to 5.0 µm in length. Cells are elongated or swollen at the early stage of culture when the organisms are grown in a liquid medium containing yeast extract (1%). Colonies are circular and orange to red. The optimal growth temperature is between 25 and 30 °C. No growth occurs at 37 °C. Oxidase and catalase are positive. Urease is negative. Aerobic. Nitrate is not reduced. Denitrification is not performed. Non-phototrophic. Slime is not produced. Ubiquinone-10 is the major quinone. Bacteriochlorophyll is not produced. Capable of producing astaxanthin. The major cellular non-hydroxyl fatty acid is unsaturated C_{18:1}. The major hydroxyl fatty acid is 3-OH C_{10:1}. Utilizes D-glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate and DL-malic acid. Acid production from L-arabinose, D-xylose, D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol and glycerol. DNA G + C composition is 67 mol%. The type strain is IFO 16121^T.

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