Paracoccus haeundaensis sp. nov., a Gram-negative, halophilic, astaxanthin-producing bacterium

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An aerobic, non-motile, Gram-negative, orange-pigmented, rod-shaped, astaxanthin-producing marine bacterium was isolated from the Haeundae Coast, Korea. This strain, BC74171^T, produced carotenoids, mainly astaxanthin. All the type strains of the genus *Paracoccus* were compared with strain BC74171^T using 16S rRNA gene sequence analysis, fatty acid patterns and physiological reaction profiles. Based on the results of these analyses, it is proposed that strain BC74171^T represents a novel species, *Paracoccus haeundaensis* sp. nov. The type strain is BC74171^T (=KCCM 10460^T =LMG P-21903^T).

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is a carotenoid that is widely distributed in nature and is present in marine animal tissues, including those of red sea bream, salmon and lobster (Fujita *et al.*, 1983; Johnson & An, 1991; Nelis & De Leenheer, 1991). Organisms that produce astaxanthin include the basidiomycetous yeast *Phaffia rhodozyma* (Miller *et al.*, 1976), the green alga *Haematococcus pluvialis* (Bubrick, 1991), and the Gram-negative bacteria '*Agrobacterium aurantiacum*' (Yokoyama *et al.*, 1994), *Paracoccus marcusii* (Harker *et al.*, 1998), *Paracoccus carotinifaciens* (Tsubokura *et al.*, 1999) and *Paracoccus* sp. strain MBIC 01143 (Misawa *et al.*, 1995).

The genus *Paracoccus* consists of Gram-negative, oxidase- and catalase-positive bacteria that show substantial metabolic versatility. At present, the genus *Paracoccus* includes 17 recognized species. Phylogenetically, the genus belongs to the α -3 subclass of the *Proteobacteria*. In this study, results of a taxonomic study and a phylogenetic analysis based on 16S rRNA gene sequence comparisons are described. Data show that a newly isolated marine strain, BC74171^T, should be classified as a representative of a novel species within the genus *Paracoccus*. The name *Paracoccus haeundaensis* sp. nov. is proposed; the type strain is BC74171^T (=KCCM 10460^T =LMG P-21903^T) and its characteristics are described.

Published online ahead of print on 19 March 2004 as DOI 10.1099/ijs.0.63146-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BC74171^T is AY189743.

A transmission electron micrograph of strain BC74171^T and a table showing selected characteristics of *Paracoccus haeundaensis* sp. nov. and related *Paracoccus* species are available as supplementary material in IJSEM Online.

An orange-pigmented, astaxanthin-producing bacterial strain, BC74171^T, was isolated from sea water collected on the Haeundae Coast, Korea. This strain was isolated on nutrient agar medium (Difco) and maintained on PPES-II medium (0.2 % polypeptone, 0.1 % Bacto-yeast extract, 0.1 % Bacto-soytone, 0.1 % Bacto-tryptone, 10 p.p.m. ferric citrate and 3 % NaCl) by serial inoculation. Strain BC74171^T was Gram-negative and rod-shaped. The cells ranged from 0.3 to 0.7 μ m in diameter and 0.8 to 2.5 μ m in length. Cells were non-motile and non-spore-forming. Colonies on agar were smooth, flat and bright orange in colour.

Cultured cells were suspended in 0.1 M phosphate buffer (pH 7.2). Cells were fixed with 2 % glutaraldehyde, washed with 0.05 M cacodylate buffer and post-fixed with 1 % osmium tetroxide. 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 JEM 1200EX-II transmission electron microscope (Fig. 1; see also the transmission electron micrograph available as supplementary material in IJSEM Online).

Strain BC74171^T was cultivated for 2 days at 25 °C on modified trypticase soy agar (TSA, 2 % NaCl added) adjusted to pH 8.0. Harvesting, saponification, methylation and extraction of the fatty acid methyl esters were performed according to Sasser (1990). Fatty acid methyl esters were identified by GC-MS, as described previously (Lipski & Altendorf, 1997).

The fatty acid profile ($C_{18:1}$, 84.32 %; $C_{18:0}$, 7.79 %; $C_{10:0}$ 3-OH, 2.06 %; $C_{12:1}$ *cis*5, 2.0 %; $C_{14:0}$ 3-OH, 1.47 %; $C_{17:0}$, 0.80 %; $C_{16:0}$, 0.78 %; and unknown peaks, 0.78 %) is characteristic of the α -*Proteobacteria*. The major cellular non-hydroxyl fatty acid is unsaturated $C_{18:1}$. The major

Fig. 1. Transmission electron micrograph of cells of *Paracoccus haeundaensis* strain BC74171^T during the exponential stage of growth. Bar, 200 nm.

hydroxyl fatty acid is C_{10:0} 3-OH. The bright orange colouration of the bacterium was shown to result from biosynthesis of carotenoids within the cells. Pigments were extracted with acetone, analysed by HPLC and monitored by measuring A₄₇₀. One of the major pigments that accumulated in the cell wall was astaxanthin, which was identified by spectroscopic analysis using a standard solution of authentic astaxanthin.

Strain BC74171^T, *P. marcusii* DSM 11574^T and *Paracoccus* sp. strain MBIC 01143 were grown on PPES-II medium and subjected to physiological characterization. The temperature range for growth was determined by incubating cells for 10 days on plates of nutrient agar at the following temperatures: 4, 10, 20, 25, 28, 30, 37, 40 and 50 °C. The pH range for growth was determined by incubating cells in PPES-II broth medium at 25 °C for 10 days at the following pH: 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, 10.5 and 11.0. NaCl tolerance was measured in trypticase soy broth (TSB) at concentrations of 0–10 % (w/v). Motility was determined with an optical microscope using the hanging-drop technique (Skerman, 1967). To investigate its morphological and physiological characteristics, strain BC74171^T was, in most cases, cultivated on TSA or in broth with 2 % NaCl added, at 25 °C. Acid production from some carbohydrates was detected using the method of Hugh &

Leifson (1953). Growth was monitored by measuring turbidity after 10 days incubation at 25 °C with shaking. Starch hydrolysis was determined as described by Cowan & Steel (1965). Denitrification was determined by growth and gas formation in a stab culture of nutrient agar containing 0.1 % (w/v) agar. Catalase activity was determined by the presence of bubbles in a 3 % H₂O₂ solution. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Urease production was determined as described by Lanyi (1987).

Strain BC74171^T grew at 20–37 °C, but not at 4, 10, 40 or 50 °C; the optimal growth temperature was 25–30 °C. Slow growth was observed in TSB in the absence of NaCl. The optimum NaCl concentration for growth was 1–6 %. When the NaCl concentration in the medium was increased to 7 %, growth was slow. No growth occurred in the presence of more than 8 % (w/v) NaCl. Growth was very slow or inhibited below pH 6.0 and above pH 10.5. Glucose was not fermented. Metabolism was aerobic.

Extraction of genomic DNA and amplification of the 16S rRNA gene were carried out as described by Rainey *et al.* (1992). The PCR product was ligated into a pGEM-T vector (Promega), sequenced using a Termination Sequencing Ready Reaction kit (Perkin Elmer) and electrophoresed using an ABI 377 genetic analyser (Perkin Elmer). The resulting sequence data were analysed with programs BLASTN and BLASTX of GenBank. Sequences were aligned using the program CLUSTAL W. Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. To indicate confidence in the branching order, bootstrap analysis (1000 replications) was completed for both the distance and parsimony methods. A phylogenetic dendrogram was obtained using the program TREEVIEW (Fig. 2). The full-length 16S rRNA gene from strain BC74171^T was sequenced. The sequence of strain BC74171^T shared the greatest similarity with the sequences of *P. marcusii* and *P. carotinifaciens* (99.8 and 99.6 % similarity, respectively).

The G + C content of the genomic DNA was determined by the method of Tamaoka & Komagata (1984). The DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values in each sample were excluded; the DNA–DNA relatedness values are expressed as the mean of the remaining three values.

The G + C content of the DNA of strain BC74171^T was 66.9 mol% (by HPLC analysis). DNA–DNA hybridization was performed to determine the genomic relatedness between strain BC74171^T and some *Paracoccus* species. Strain BC74171^T exhibited mean levels of DNA–DNA relatedness to the type strains of *Paracoccus denitrificans*, *Paracoccus thiocyanatus*, *Paracoccus versutus* and *P. marcusii*,

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence analysis of members of the genus *Paracoccus*. Bar, Jukes & Cantor (1969) distance and maximum-parsimony distance (1 nt substitution per 100 nt). Numbers on branch nodes are bootstrap values (1000 replications).

and *Paracoccus* sp. strain MBIC 01143 of 8, 7, 6, 47 and 11 %, respectively, when each of their DNAs was used separately as a labelled probe.

The genus *Paracoccus* comprises a group of aerobic, Gram-negative bacteria that are catalase- and oxidase-positive and reduce nitrate. Seventeen recognized species of this genus have been identified. Details of physiological characteristics of *P. haeundaensis* and several type strains belonging to the genus *Paracoccus* are available as supplementary material in IJSEM Online.

The 16S rRNA gene sequence of *P. haeundaensis* shared high similarity with the sequences of *P. marcusii* and *P. carotinifaciens* and all three are astaxanthin-producing bacteria. The type strain of *P. carotinifaciens* is deposited only in IFO (= IFO 16121^T) as a patented strain and is not publicly available. In a case like this, *P. carotinifaciens* contravenes the revised Rule 27(3) and Rule 30 of the Bacteriological Code (Euzéby & Tindall, 2004). Therefore, direct biochemical comparison and DNA–DNA hybridization studies between *P. haeundaensis* and *P. carotinifaciens* could not be performed. The biochemical characteristics of *P. carotinifaciens* were taken from a published report (Tsubokura *et al.*, 1999). *P. marcusii* occurs as a coccus or short rod and forms short chains, whereas both *P. haeundaensis* and *P. carotinifaciens* occur as rods, but do not form chains. *P. carotinifaciens* has flagella, whereas *P. haeundaensis* does not. Moreover, the species have different phenotypic characteristics. *P. haeundaensis* does not utilize

mannitol, maltose or mannose, whereas both *P. marcusii* and *P. carotinifaciens* utilize these compounds. In addition, *P. haeundaensis* hydrolyses starch, whereas neither *P. marcusii* nor *P. carotinifaciens* does.

Description of *Paracoccus haeundaensis* sp. nov.

Paracoccus haeundaensis (hae.un'dae.n.sis. N.L. adj. *haeundaensis* referring to Haeundae beach in Busan, Korea, where the type strain was isolated).

Gram-negative, non-motile, rod-shaped and non-spore-forming. Cells are 0.3–0.7 µm in diameter and 0.8–2.5 µm in length. Colonies are orange to red in colour. Optimal growth temperature is 25 °C; no growth occurs below 10 °C or above 40 °C. The optimum NaCl concentration for growth is 1–6 % (w/v). No growth occurs in the presence of more than 8 % (w/v) NaCl. Optimal pH for growth is 8. Capable of producing astaxanthin. The major cellular non-hydroxyl fatty acid is unsaturated C_{18:1}. The major hydroxyl fatty acid is C_{10:0} 3-OH. The following carbon and energy sources can be used for growth: D-arabinose and galactose. No growth occurs on D-glucose, lactose, maltose, sucrose, trehalose, D-mannitol, D-sorbitol, inositol, D-raffinose, D-fructose, D-mannose, dimethylformamide, L-glutamic acid, acetone, L-leucine, L-asparagine, L-rhamnose, salicin, D-cellobiose, adonitol, dulcitol, D-xylose or glycerol. L-Ornithine and L-lysine decarboxylases, and arginine dihydrolase are not detected. Citrate utilization test is

positive. Starch is hydrolysed. No indole is produced from tryptophan. Cytochrome oxidase and catalase reactions are positive. Urease-negative. Nitrate is reduced. Denitrification does not occur. Glucose is not fermented. Metabolism is aerobic. DNA G+C content is 66.9 mol%.

The type strain is BC74171^T (=KCCM 10460^T=LMG P-21903^T).

Acknowledgements

We would like to thank Professor J. Hirschberg (The Hebrew University of Jerusalem, Israel) for providing us with the type strain of *Paracoccus marcusii*. Also, we thank Dr N. Misawa (Marine Biotechnology Institute, Japan) for allowing us to use *Paracoccus* sp. strain MBIC 01143. This work was supported by the Special Research Fund (2000-3) of Algenetech through Pukyong National University, Korea.

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