Paracoccus marinus sp. nov., an adonixanthin diglucoside-producing bacterium isolated from coastal seawater in Tokyo Bay

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Two novel marine, Gram-negative, non-motile, catalase- and oxidase-positive, aerobic bacteria were isolated from coastal seawater in Tokyo Bay. Analysis of almost-complete 16S rRNA gene sequences showed that the two isolates are members of the genus *Paracoccus*, sharing highest 16S rRNA gene sequence similarity (96.5 %) with *Paracoccus aminophilus* NBRC 16710^T. The DNA–DNA reassociation values between *P. aminophilus* NBRC 16710^T and these isolates were only 10–20 %, in contrast to the high DNA relatedness between the two isolates (89 %). At least 1 % (w/v) NaCl was required for growth. Cellular fatty acid profiles revealed C_{18:1ω7c} as the major component and C_{10:0} 3-OH as the major hydroxy fatty acid. Ubiquinone-10 was detected as the major respiratory quinone. The G + C content of the genomic DNA of both strains was 69 mol%. On the basis of DNA–DNA hybridization data and physiological and chemotaxonomic characteristics, it is proposed that these strains should be placed in a novel species, *Paracoccus marinus* sp. nov. The type strain is KKL-A5^T (=NBRC 100637^T =CIP 108500^T); KKL-B9 (=NBRC 100640) is a reference strain.

Many marine bacteria are difficult to cultivate because of their oligotrophic nature. To cultivate such bacteria, which may not be adapted for growth in nutrient-rich media, Button *et al.* (1993) used natural seawater (containing a very low concentration of organic carbon) as the growth medium. This technique was further improved to meet high-throughput culturing (Connon & Giovannoni, 2002) and many novel microbial strains, including those that had previously been believed to be ‘unculturable’, have been isolated. The high-throughput culturing approach was used to study the microbial ecology of seawater in Tokyo Bay and two novel strains of the genus *Paracoccus* were isolated.

Seawater samples were collected from Kisarazu port on the east side of Tokyo Bay and total cell counts were analysed using 4',6-diamidino-2-phenylindole (DAPI) staining. The samples were diluted in sterile seawater to give 1–5 cells ml⁻¹. Each 1 ml diluted sample was dispensed into 2 ml 96-deep-well plates, which were sealed with the 96-well-plate cap and incubated at 20 °C. After 1 week of incubation, microbial growth in each well was examined by

DAPI staining using 100 µl sample. Wells showing multiplication of microbes were used to inoculate quarter-strength marine agar 2216 (Difco) plates to isolate colony-forming bacteria. Colonies grown on the plates were subsequently subcultured and purified on the same medium. Two strains, KKL-A5^T and KKL-B9, were thus isolated and subjected to polyphasic characterization.

For routine cultivation, half-strength marine agar 2216 (HSMa) diluted with artificial seawater (Naigai Chemicals) at 25 °C was used. The KKL strains were stored at –80 °C in artificial seawater with 20 % (v/v) glycerol for long-term preservation.

To determine the phylogenetic position of these strains, genomic DNA was extracted by using the Qiagen DNeasy Tissue kit according to the manufacturer's protocol and 16S rRNA gene fragments were PCR-amplified using 20 ng genomic DNA and a set of universal primers (27f and 1492r; Brosius *et al.*, 1978). Amplified products were purified using the Qiagen PCR purification kit before sequencing, which was carried out using an ABI Big Dye Terminator Cycle Sequence kit (version 3.1) and an Applied Biosystems 3730 DNA Analyzer. A comparison of almost-complete 16S rRNA gene sequences of the two KKL strains using the dynamic programming algorithm (Needleman & Wunsch, 1970; <http://www.ebi.ac.uk/emboss/align/>) showed that the sequences were 99.9 %

Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KKL-A5^T and KKL-B9 are AB185957 and AB185959, respectively.

similar to each other. A BLAST search showed that the novel strains are members of the genus *Paracoccus*. The 16S rRNA gene sequences of type strains of species of the genus *Paracoccus* with validly published names were retrieved from the DDBJ/EMBL/GenBank databases and pairwise comparisons were made between 16S rRNA gene sequences of the KKL strains and those of the type strains. It was shown that, of all the sequences studied, that of *Paracoccus aminophilus* NBRC 16710^T shared the highest 16S rRNA gene sequence similarity (96.5 %) with the two strains. The 16S rRNA gene sequences of the type strains and the KKL strains were multiply aligned using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Adachi & Hasegawa, 1996) algorithms. The robustness of the phyletic lines was evaluated by bootstrap resampling analysis (Felsenstein, 1985) of 1000 replicates for neighbour-joining and 100 replicates for maximum-likelihood. As shown in Fig. 1, the KKL strains grouped with *P. aminophilus* NBRC 16710^T. Although the bootstrap value obtained for the node was low, the results of the phylogenetic analysis were congruent with those of pairwise alignments of 16S rRNA gene sequences, identifying *P. aminophilus* NBRC 16710^T as the closest relative. DNA–DNA hybridization was performed between the KKL strains and *P. aminophilus* NBRC 16710^T. Genomic DNA was extracted using the protocol of Minamisawa (1990) and the fluorometric method of Ezaki *et al.* (1989) was used for DNA–DNA hybridization in 50 % formamide at 50 °C. The DNA–DNA reassociation value between strains KKL-A5^T and KKL-B9 was 89 %; however, values between the KKL strains and *P. aminophilus* were considerably lower (10–23 %).

The G + C content of chromosomal DNA was determined by the HPLC method of Mesbah *et al.* (1989). The G + C contents of both KKL strains were 69 mol%.

These strains were further tested for a number of key characters using standard procedures as described below. Colony morphology was observed on HSMA plates at 25 °C. Four-day-old colonies were round, uniform, small (1–2 mm) and dull orange in colour. Cell morphology from 3-day-old colonies on HSMA plates was observed under a transmission electron microscope (H7600; Hitachi) after negative staining with 1 % (w/v) phosphotungstic acid. Gram staining was performed as described by Cowan & Steel (1993). The method described by Buck (1982) for the differentiation of Gram-positive and Gram-negative bacteria was also used. The KKL strains were Gram-negative, non-motile, short rods, measuring 0.5–0.8 µm wide and 0.8–1.2 µm long. Catalase activity was tested by mixing cells from colonies grown on HSMA plates with 3 % (v/v) hydrogen peroxide on a glass slide; oxidase activity was tested by spotting the cells on a cytochrome oxidase strip (Nissui Pharmaceuticals). Both strains tested positive for catalase and oxidase. The presence of carotenoid pigments was examined as described by Takaichi *et al.* (2006). The major carotenoid (nearly 80 % of the total) was adonixanthin diglucoside (Takaichi *et al.*, 2006). Growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C) was examined on HSMA plates and in marine broth 2216. The KKL strains grew optimally at 25–35 °C and very weakly at 10 and 37 °C. No growth was observed on plates incubated at 4, 40 or 45 °C. The ability to grow at different pH values was examined in marine broth 2216 (Difco) adjusted to a final pH with either HCl (pH 4.0, 5.0 and 6.0) or NaOH (pH 8.0, 9.0 and 10.0). Both strains grew at pH 6.0–9.0; optimal growth was at pH 7.0–8.0. However, no growth was observed at pH 4.0, 5.0 or 10.0. The ability of the KKL strains to grow in different salinities was assessed in one-fifth-strength LB medium (2 g Bacto tryptone and 1 g yeast extract per litre MilliQ water) with 0, 1, 2, 3, 4, 5, 6, 7, 10,

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing the phylogenetic relationship between strains KKL-A5^T and KKL-B9 (*Paracoccus marinus* sp. nov.) and related species. Bootstrap values of ≥ 500 are shown at the nodes. Closed circles show the nodes that were also recovered in maximum-likelihood analysis. Bar, 0.005 K_{nuc} .

12 and 15 % (w/v) NaCl. Strains grew in 1–4 % (w/v) NaCl (optimally in 2–3 % NaCl); very weak or no growth was observed in one-fifth-strength LB without NaCl or supplemented with 5–15 % (w/v) NaCl. Utilization of sodium nitrate (7.8 g l⁻¹), ammonium sulfate (7.8 g l⁻¹), sodium glutamate (20 g l⁻¹), Casamino acids (10 g l⁻¹; Difco) and peptone (10 g l⁻¹; Difco) as nitrogen sources was tested in medium N (artificial seawater containing 1.0 g glucose l⁻¹ and 0.2 g NaHCO₃ l⁻¹, pH 7.0). Only peptone was utilized by the KKL strains. Utilization of different carbon sources was tested by using artificial seawater supplemented with casein hydrolysate (10 mg l⁻¹; Fluka) and yeast extract (0.1 g l⁻¹) and the following substrates, each at a concentration of 0.2 % (w/v): D-arabinose, D-fructose, D-galactose, D-glucose, α -D-lactose, maltose, D-mannose, sucrose, trehalose, acetate, formate, lactate, pyruvate, propionate, succinate, arginine, asparagine, ethanol, *myo*-inositol, D-mannitol and D-sorbitol (Ruiz-Ponte *et al.*, 1998). The KKL strains assimilated relatively few carbon sources, as listed in the species description. Degradation of starch, casein, chitin, DNA, gelatin and Tweens 20, 40 and 80 and the reduction of nitrate and nitrite were tested according to the protocols of Cowan & Steel (1993). Both strains degraded Tween 20, but were negative for the degradation of starch, casein, chitin, DNA, gelatin and Tweens 40 and 80 and for the reduction of nitrate and nitrite. API 20NE strips (bioMérieux) and GN2 microplates (Biolog) were used to characterize the strains according to the manufacturers' instructions except that inocula were prepared according to Rüger & Krambeck (1994). Biolog microplates and API strips were analysed after 3 and 2 days incubation at 25 °C, respectively. The strains were not able to utilize any substrate provided within the Biolog GN2 microplates. The strains also tested negative for all tests included in the API 20NE strips (reduction of nitrate and nitrite, production of indole from tryptophan, production of acid from glucose and the presence of urease, β -galactosidase and β -glucosidase).

For chemotaxonomic characterization, fatty acid analysis and quinone analysis was performed. Strains were cultivated for 3 days on marine agar 2216 at 25 °C before cells were harvested for fatty acid analysis using the Sherlock Microbial Identification System according to the MIDI instructions (Sasser, 1990). The cellular fatty acid profile of the KKL strains was characterized by a large proportion of C_{18:1} ω 7c (85–87 %) with C_{10:0} 3-OH (2 %) as the major hydroxy fatty acid. Other fatty acids detected were C_{18:0} (4–5 %), C_{17:0} (1–2 %), C_{19:0} 10-methyl (2 %) and an unknown fatty acid of equivalent chain-length 11.79 (2 %). The following fatty acids were detected in trace amounts: C_{16:0}, C_{19:0}, C_{17:1} ω 8c, C_{18:0} 3-OH and C_{20:1} ω 7c. This fatty acid profile is characteristic of the *Alphaproteobacteria*, including members of the genus *Paracoccus* (Kelly *et al.*, 2006).

The protocol of Nakagawa & Yamasato (1993) was used for analysis of isoprenoid quinones. Ubiquinone-10 was found as the major quinone in both strains.

Four members of the genus *Paracoccus* that shared the highest pairwise 16S rRNA gene sequence similarities with the two isolates, namely *P. aminophilus* (96.5 %), *Paracoccus seriniphilus* (96.2 %), *Paracoccus zeaxanthinifaciens* (96.1 %) and *Paracoccus koreensis* (96 %), were selected for comparison of biochemical, physiological and chemotaxonomic characters with those of the KKL strains (Table 1). Taking into account the physiological and molecular properties of the KKL strains, it is proposed that these two strains should be classified as representatives of a novel species of the genus *Paracoccus*. The name *Paracoccus marinus* sp. nov. is proposed.

Description of *Paracoccus marinus* sp. nov.

Paracoccus marinus (ma.ri' nus, L. masc. adj. *marinus* of or belonging to the sea, marine).

Gram-negative, aerobic, non-motile, short rod-shaped cells, 0.5–0.8 μ m wide and 0.8–1.2 μ m long. Colonies on

Table 1. Differential characteristics of strains KKL-A5^T and KKL-B9 (*Paracoccus marinus* sp. nov.) and related species

Taxa: 1, strains KKL-A5^T and KKL-B9 (*P. marinus* sp. nov.; data from this study); 2, *P. aminophilus* (Urakami *et al.*, 1990); 3, *P. seriniphilus* (Pukall *et al.*, 2003); 4, *P. zeaxanthinifaciens* (Berry *et al.*, 2003); 5, *P. koreensis* (La *et al.*, 2005). –, Negative; +, positive; w+, weakly positive; NA, no data available.

Characteristic	1	2	3	4	5
Colony colour*	DO	W-LY	None	YO	W
Major pigment type†	AD	None	None	ZA	None
Nitrate reduction	–	+	+	–	–
Nitrite reduction	–	–	–	–	+
Growth at/in:					
40 °C	–	–	–	+	NA
37 °C	w+	–	+	+	+
0 % NaCl	–	+	–	NA	NA
3 % NaCl	+	w+	+	+	NA
6 % NaCl	–	NA	+	+	NA
Growth on:					
Methanol	NA	–	NA	NA	NA
Mannitol	–	–	+	+	–
Inositol	–	–	+	+	–
Fructose	+	–	+	NA	NA
Urease	–	–	–	+	–
β -Galactosidase	–	NA	+	+	–
β -Glucosidase	–	NA	–	+	–
Major 3-OH fatty acids					
C _{10:0} 3-OH	+	+	+	+	+
C _{14:0} 3-OH	–	+	–	–	–
C _{18:0} 3-OH	+	–	–	+	–
DNA G + C content (mol%)	69	63	63	67–68	69

*DO, Dull orange; LY, light yellow; w, white; YO, yellow–orange.

†AD, Adonixanthin diglucoside; ZA, zeaxanthin.

marine agar 2216 are circular, convex, smooth and dull orange in colour. Major carotenoid is adonixanthin diglucoside. Catalase- and oxidase-positive, but negative for β -glucosidase and β -galactosidase. Growth occurs between 10 and 35 °C (optimum 25–35 °C) and at pH 6–9 (optimum pH 7.0–8.0). NaCl (1–4 %, w/v) is required for growth (optimum 2–3 %). Growth does not occur at NaCl concentrations of 5 % (w/v) and higher. Utilizes peptone, but not ammonium sulfate, sodium glutamate, sodium nitrate or Casamino acids as nitrogen sources. D-Fructose, D-galactose, D-glucose, lactate, pyruvate and asparagine are utilized as carbon sources, but D-arabinose, α -D-lactose, maltose, D-mannose, sucrose, trehalose, acetate, formate, propionate, succinate, arginine, ethanol, *myo*-inositol, D-mannitol and D-sorbitol are not utilized. Starch, gelatin, casein, chitin, DNA, aesculin, urea, arginine and Tweens 40 and 80 are not hydrolysed, but Tween 20 is hydrolysed. Indole is not produced from tryptophan and acid is not produced from glucose. Nitrate and nitrite are not reduced. The major fatty acid is C_{18:1} ω 7 ζ ; C_{10:0} 3-OH is present as the major hydroxy fatty acid component. Ubiquinone-10 is the major respiratory quinone.

The type strain is KKL-A5^T (=NBRC 100637^T =CIP 108500^T), isolated from the seawater of Kisarazu port, Tokyo Bay; a reference strain, KKL-B9 (=NBRC 100640), was isolated from the same source. The DNA G+C content of the type strain is 69 mol%.

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