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Note

Paracoccus zeaxanthinifaciens sp. nov., a zeaxanthin-producing bacterium

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A comprehensive taxonomic re-evaluation was performed on the marine, zeaxanthin-producing bacterium formerly classified as [*Flavobacterium*] sp. strain R-1512 (ATCC 21588). This strain, together with two other previously described marine isolates, [*Flavobacterium*] strain R-1506 and *Paracoccus* sp. strain MBIC 3966, were found to comprise a new species of the genus *Paracoccus*. The name *Paracoccus zeaxanthinifaciens* sp. nov. is proposed, with ATCC 21588^T (=R-1512^T =LMG 21293^T) designated as the type strain.

Carotenoids are C_{40} isoprenoid compounds, some of which are used commercially as nutritional supplements, pharmaceuticals and food colourants for humans and as pigments for animal feed. The currently industrially important carotenoids are produced mainly by chemical synthesis (β-carotene, canthaxanthin and astaxanthin) or by extraction from natural sources (lutein/zeaxanthin from marigold, capsanthin/capsorubin from paprika). Commercial production of carotenoids using micro-organisms has been achieved in some cases. For instance, β-carotene is produced by fermentation with the fungus *Blakeslea trispora* (Sibeyn & de Pater, 1998) or by pond culture using the halotolerant alga *Dunaliella salina* (Borowitzka, 1999). Lycopene production has also been reported in *B. trispora* (Marcos *et al.*, 2000). Astaxanthin is produced by fermentation using the red yeast *Xanthophyllomyces dendorous* (formerly named *Phaffia rhodozyma*) (Jacobson *et al.*, 2000) or in photobioreactors or open ponds using the alga *Haematococcus pluvialis* (Lorenz & Cysewski, 2000; Olaizola, 2000).

Zeaxanthin (3,3′-dihydroxy-β-carotene) is a yellow carotenoid that has application in poultry pigmentation and in the prevention of age-related macular degeneration in humans. In the mid-1960s, scientists at Hoffman-La Roche isolated several marine bacteria that produced zeaxanthin (Schocher & Wiss, 1975). One bacterium, given the strain designation R-1512, was deposited at the American Type Culture Collection as strain ATCC 21588. Using the accepted taxonomic standards of that time, the zeaxanthin-producing bacterium was classified as a member of the genus *Flavobacterium*, but no species designation was assigned. An extensive mutagenesis and screening programme was conducted in the late 1960s/early 1970s to isolate mutants of strain R-1512 with higher zeaxanthin productivities. With respect to the present work, two such mutants are significant. These mutants, listed in ascending order of their zeaxanthin productivities, are R1534 and R114. A variety of other mutants have been used over the years for biochemical studies of carotenoid biosynthesis (Britton *et al.*, 1977; Goodwin, 1972; McDermott *et al.*, 1974; Mohanty *et al.*, 2000).

In this report, we present the results of an extensive taxonomic analysis of strain ATCC 21588 (=R-1512) that shows that this bacterium and two other independent isolates comprise a new species in the genus *Paracoccus*. The

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The GenBank accession numbers for the 16S rRNA gene sequences of ATCC 21588^T and R-1506 are AF461158 and AF461159, respectively.

Results of DNA fingerprinting (AFLP) analysis and the carotenoid production profile for all pigmented strains used in this work are available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

Table 1. Bacteria used in this work

Taxon	Strain designation used*	Other strain designations*	Source of isolation	Reference
P. zeaxanthinifaciens	ATCC 21588^T	R-1512^T LMG 21293^T	Seaweed collected off the coast of the African Red Sea	Schocher & Wiss (1975)
	R114	PTA-3335^{PP}	Mutant derived from R-1512^T	This study
	R1534	PTA-3336^{PP}	Mutant derived from R-1512^T	This study
	R-1506	PTA-3431^{PP}	Seaweed collected off the coast of the African Red Sea	Schocher & Wiss (1975)
	MBIC 3966	94-209	The Jellyfish Lake, Palau	T. Hamada, Marine Biotechnology Institute, Kamaishi Laboratories, Kamaishi, Iwate, Japan
Paracoccus sp.	MBIC 3024	[*Alcaligenes*] sp. strain PC-1	Marine sample, Okinawa Islands, Japan	Misawa *et al.* (1995), Yokoyama *et al.* (1994)
	MBIC 4017	HI3	Himekayu spa, Japan	T. Hamada
	MBIC 4020	HI9	Himekayu spa, Japan	T. Hamada
P. marcusii	DSM 11574^T	MH1^T	Nutrient agar plate contaminant, Israel	Harker *et al.* (1998)
P. carotinifaciens	E-396^T	NBRC (IFO) 16121^T	Soil, Japan	Tsubokura *et al.* (1999a)
P. solventivorans	DSM 6637^T	L1^T LMG 19740^T	Soil (1 m depth) near defunct natural gas company	Siller *et al.* (1996)

*ATCC, American Type Culture Collection, Manassas, VA, USA; PTA, ATCC patent strain designation; ^{PP}, ATCC patent pending designation; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; LMG, Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, Ghent University, Belgium; MBIC, Marine Biotechnology Institute Collection, Kamaishi Institute, Kamaishi Iwate, Japan; NBRC, Biological Resource Center, Chiba, Japan (NBRC holds strains formerly held by IFO, Institute for Fermentation, Osaka, Japan).

name *Paracoccus zeaxanthinifaciens* is proposed, with ATCC 21588^T (=R-1512^T =LMG 21293^T) being designated as the type strain.

The bacterial strains used are listed in Table 1. Strain R-1512^T (=ATCC 21588^T) and strain R-1506 are two independent isolates from the same initial screening of marine bacteria for zeaxanthin-producing strains. Strains R114 and R1534 are mutants derived from strain R-1512^T by classical mutagenesis and screening for improved zeaxanthin production. Strains R114, R1534, and R-1506 were recently deposited (according to the terms of the Budapest Treaty) at the patent depository at the ATCC and were given the strain designations PTA-3335^{PP}, PTA-3336^{PP} and PTA-3431^{PP}, respectively. Strains MBIC 3024, MBIC 3966, MBIC 4017 and MBIC 4020 were identified as members of the genus *Paracoccus* by their 16S rDNA gene sequences [deposited in EMBL by T. Hamada, Marine Biotechnology Institute (MBI), Kamaishi Laboratories, Kamaishi, Iwate, Japan], and were obtained from H. Kasai, MBI. It should be noted that MBIC 3024 is another designation for [*Alcaligenes*] sp. strain PC-1, a previously described ketocarotenoid-producing bacterium (Misawa *et al.*, 1995; Yokoyama *et al.*, 1994). *Paracoccus marcusii* DSM 11574^T and *Paracoccus carotinifaciens* E-396^T are type strains of carotenoid-producing species (Harker *et al.*, 1998; Tsubokura *et al.*, 1999a) and were obtained from DSMZ, the German Collection of Microorganisms and Cell Cultures and The National Institute of Advanced Industrial Science and Technology (Japan), respectively. *Paracoccus solventivorans* DSM 6637^T (Siller *et al.*, 1996) was also obtained from DSMZ, and was included in some comparisons as a representative non-carotenogenic species of *Paracoccus*.

Depending on the experiment, strains were grown in either BBL Trypticase Soy Broth or Difco Marine Broth. Media were supplemented where necessary with 1·5 % (w/v) Difco Bacto agar. The cultivation temperatures used are specified in the relevant sections below, except in the case of cultures used for preparation of DNA, which were all grown at 28 °C. All cultures were grown aerobically unless indicated otherwise.

Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). Genes encoding 16S rRNA were amplified from genomic DNA from strains ATCC 21588^T, R114, R1534 and R-1506 by PCR. Forward primer 16F27 [5′-AGA GTT TGA TCC TGG CTC AG-3′] was used for strains R1534 and R-1506, while forward primer 16F38 [5′-CTG GCT CAG GAC/T GAA CGC TG-3′] was used for strains ATCC 21588^T and R114. The reverse primer 16R1522 [5′-AAG GAG GTG ATC CAG CCG CA-3′] was

used for all strains. Purification of PCR products, DNA sequencing using five forward and three reverse primers, and sequence assembly were performed as described by Coeyne *et al.* (1999), but using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit. Phylogenetic analysis was performed using the software package GeneCompar version 2.0 (Applied Maths) after including the consensus sequences from strains ATCC 21588^T, R114, R1534 and R-1506 in an alignment of small-subunit ribosomal sequences collected from the EMBL database. A similarity matrix was created by homology calculation with a gap penalty of 0 % and after discarding unknown bases. A tree was constructed using the neighbour-joining method.

Genomic DNA was prepared according to the protocol of Wilson (1987). The G+C content of the DNAs was determined by HPLC according to the method of Mesbah *et al.* (1989) as modified by Logan *et al.* (2000). Reported values are the mean of three measurements on the same DNA sample. DNA–DNA hybridizations were performed using the initial renaturation rate method (De Ley *et al.*, 1970). The hybridization temperature was 81·5 °C. Values are the mean of at least two determinations.

The same purified DNA as was used for the DNA–DNA hybridization experiments was used for AFLP analysis (Vos *et al.*, 1995). The experimental procedures, the adaptors ligated to the sticky ends generated by cleavage with the restriction enzymes *Apa*I and *Taq*I and the primers A01 and T01 were as described by Huys *et al.* (1996). The other primers used were: A02, 5′-GACTGCGTACAGGCCCC-3′; A03, 5′GACTGCGTACAGGCCCG3′; T02, 5′-CGATGAGTCCTGACCGAC-3′; T03, 5′-CGATGAGTCCTGACCGAG-3′. Primers A01, A02 and A03 were labelled at their 5′ end with [^{32}P]ATP (Amersham International) using T4 kinase (Pharmacia Biosciences). The six primer combinations (PCs) used for AFLP were: PC A, A01 + T01; PC B, A01 + T02; PC D, A02 + T01; PC I, A03 + T03; PC G, A03 + T01; PC H, A03 + T02. The electrophoretic patterns were scanned, numerically analysed with GelCompar version 4.2 software (Applied Maths), compared using the Pearson product–moment correlation coefficient and clustered using the unweighted pair group method with averages (UPGMA) linking.

Extraction of cellular fatty acids and determination of the fatty acid compositions by gas chromatography was carried out using the Microbial Identification System (MIDI) according to the instructions of the manufacturer. For this test, bacteria were grown for 24 h at 28 °C on Trypticase Soy Agar.

For testing the aerobic utilization of carbon sources, Biolog-SF-N MicroPlate microtitre plates containing 95 substrates were used (Harker *et al.*, 1998), with the exception that the substrate in well E6 was DL-lactic acid methyl ester instead of the usual sodium salt of DL-lactic acid. Cells were grown for 24 h at 28 °C on Marine Agar. A cell suspension with a density equivalent to 0·5 McFarland units was prepared in sterile distilled water. From this suspension, 18 drops were transferred into 21 ml AUX medium (API 20NE; bioMérieux) and mixed gently. One hundred microlitres of the suspension was transferred to each well of the Biolog MicroPlates, and the plates were incubated at 30 °C. Wells were visually checked for growth after 48 h and after 6 days. Also, at 6 days the visual scoring was confirmed by reading the microtitre plates using the Biolog plate reader.

Nine selected tests [reduction of nitrate to nitrite, denitrification, production of indole from tryptophan, acid production from glucose (fermentation), arginine dihydrolase, urease, aesculin hydrolysis (β-glucosidase), gelatin hydrolysis and β-galactosidase] were performed using API 20NE strips (bioMérieux). For these tests, cells were grown for 24 h at 28 °C on Marine Agar. Cell suspensions were prepared and strips inoculated according to the instructions of the manufacturer. Strips were incubated at 28 °C and results determined after 24 and 48 h. For testing the temperature, pH and salt concentration ranges for growth, cells were first grown for 24 h at 28 °C on Marine Agar. Cell suspensions having a density of between 1 and 2 McFarland units were then prepared in sterile distilled water. These suspensions were used as inocula for each test. For determining the temperature and salt concentration ranges for growth, three drops of cell suspension were transferred onto the surface of each Marine Agar plate. One drop was diluted by streaking, the other two drops were left undisturbed. In the case of the temperature test, plates were incubated at 10, 25, 30, 33, 37 and 40 °C. In the case of the salt tolerance test, the Marine Agar plates had been supplemented with NaCl to reach final concentrations of (w/v) 3, 6 and 8 % and all were incubated at 28 °C. For both tests, plates were checked for growth after 24 h, 48 h and 5 days. Growth was determined as visual growth (confluent in the drops and as colonies in the streaks with diluted inoculum) and scored relative to the growth under the control condition (30 °C for the temperature test and no added NaCl for the salt tolerance test). To test the pH range for growth, three drops of cell suspension were transferred into tubes containing 10 ml Marine Broth having final pH values after autoclaving of pH 6·1, 6·3, 7·0, 7·7, 8·1 and 9·1. The cultures were incubated with shaking at 28 °C. Growth was checked at 24 h, 48 h, 3 days and 6 days. Growth was determined as increased turbidity (measured as % transmission using the Biolog turbidimeter) and scored relative to growth at pH 7·0 (control). Anaerobic growth was tested by streaking a loopful of freshly grown cells on Marine Agar plates, with or without addition of 0·1 % (w/v) KNO_3, and incubating at 30 °C in an atmosphere of 10 % CO_2 and 90 % N_2. To test starch hydrolysis, a loopful of freshly grown cells was applied to Marine agar plates supplemented with 0·2 % (w/v) soluble starch and plates were checked for clear zones after 48 h incubation at 30 °C by flooding of the plates with lugol. Production of poly-β-hydroxybutyrate (PHB) was tested using the crotonic acid method (Daniels *et al.*, 1994). *Escherichia coli* LMG 2092^T and *Ralstonia eutrophus* LMG

1201 served as negative and positive controls, respectively, for PHB production.

Colony pigmentation was observed visually and recorded after 5 days growth at 28 °C on Marine Agar. Cell morphology and motility were observed using an Olympus light microscope equipped with phase-contrast optics (magnification ×1000). Cells were grown for 24 h at 28 °C on Marine Agar, and cell suspensions were made in sterile saline for microscopic examination.

For analysis of carotenoids by HPLC, 50 ml Marine Broth was inoculated with approximately 0·5 ml of an overnight liquid culture (grown in Marine Broth), and incubated for 24 h at 28 °C with shaking. Cells (approx. 0·35 g wet wt) were collected by centrifugation, washed with saline, and again collected by centrifugation. Carotenoids were extracted from the cells by suspending the pellet in 4 ml tetrahydrofuran (Fluka 87370). Solids were removed from the extracts by centrifugation. Samples were analysed by a reversed phase HPLC method that allowed simultaneous determination of astaxanthin, adonixanthin, zeaxanthin, canthaxanthin, β-carotene and lycopene. The method is also able to separate the main *cis*-isomers of zeaxanthin. Chromatography was performed using an Agilent 1100 HPLC system equipped with a temperature-controlled autosampler and a diode array detector. The column was a YMC Carotenoid C30 column (5 micron, steel, 250 mm long × 4·6 mm i.d.; Waters). The guard column was a Pelliguard LC-18 cartridge (20 mm; SUPELCO). The mobile phase was a methanol/methyl tert-butyl ether (MeOH/TBME) gradient having the following parameters (all percentages expressed as v/v): start, 80 % MeOH/20 % TBME; 10 min, 65 % MeOH/35 % TBME; 20 min, 10 % MeOH/90 % TBME. The flow rate was 1·0 ml min^{-1}. The injection volume and column temperature were 10 µl and 15 °C, respectively. Carotenoids were detected by absorbance at 450 nm. Quantification of carotenoids was performed with a two level calibration using external standards. Calculations were based on peak areas. The selectivity of the method was checked by injecting standard solutions of the relevant carotenoid reference compounds. The target compounds (all-*trans*-carotenoids) were completely separated and showed no interference. Some minor *cis* isomers may co-elute, although these potentially interfering isomers are rare and may be neglected in routine analysis. The retention times (given in min) for the different carotenoids using this HPLC method were as follows: astaxanthin, 6·99; adonixanthin, 7·50; 15-*cis*-zeaxanthin, 7·80; 13-*cis*-zeaxanthin, 8·23; all-*trans*-zeaxanthin, 9·11; canthaxanthin, 9·95; cryptoxanthin, 13·45; β-carotene, 17·40 and lycopene, 21·75. The linearity, sensitivity and reproducibility of the method with respect to detection of zeaxanthin were tested. A linear range was found from 0·1 µg ml^{-1} to 250 µg ml^{-1} zeaxanthin (correlation coefficient 0·9998). The lower limit of detection for zeaxanthin was determined to be 60 µg l^{-1}. Using a higher injection volume and optimization of the integration parameters, it is possible to lower the detection limit to approximately 5 µg l^{-1}. The retention time for all-*trans*-zeaxanthin was very stable [relative standard deviation (RSD), 0·2 %]. The peak area reproducibility, based on ten repetitive analyses of the same culture sample, was determined to be 0·17 % RSD for all-*trans*-zeaxanthin.

The complete sequence of the 16S rRNA genes from the zeaxanthin-producing isolate, strain ATCC 21588^T (=R-1512^T), its two mutant derivatives, R114 and R1534, and the independent zeaxanthin-producing isolate, strain R-1506, were determined (1404, 1404, 1415 and 1415 nucleotides, respectively). The sequences from ATCC 21588^T, R114 and R1534 were identical. The sequences from R-1506 differed in only one nucleotide (position 1350) from the sequence from ATCC 21588^T. This demonstrated that the two independent zeaxanthin-producing isolates are phylogenetically highly related and are likely to belong to the same species. Comparison of the ATCC 21588^T and R-1506 sequences with those publicly available at the EMBL library showed that these bacteria, formerly classified as unidentified species of *Flavobacterium*, should be reclassified as members of the genus *Paracoccus* (for a review of the taxonomy of the genus *Paracoccus*, see Baj, 2000). However, the sequence similarities observed with all 14 currently validly described *Paracoccus* species was <97 %, the limit for a possible relatedness at the species level (Stackebrandt & Goebel, 1994). This indicated that strains ATCC 21588^T and R-1506 belonged to one or two new species of *Paracoccus*. Sequence similarities of >97 % were observed between the 16S rDNA sequences of strains ATCC 21588^T and R-1506 and several unnamed *Paracoccus* strains, suggesting that one or more of the unnamed (MBIC) strains could be related at the species level to strains ATCC 21588^T and/or R-1506. Based on cluster analysis (Fig. 1), strains ATCC 21588^T, R114, R1534, R-1506 and MBIC 3966 were selected for DNA–DNA hybridization experiments to analyse species relatedness. The sequence similarity of the selected MBIC strains and any of the 14 validly described *Paracoccus* species was <97 % (data not shown).

The mol% G+C of the DNA from the investigated strains and the results of DNA hybridization experiments are summarized in Table 2. Strains ATCC 21588^T, R114, R1534, R-1506 and MBIC 3966 showed a DNA reassociation value of ⩾88 %, clearly above the generally accepted limit (70 %) for species delineation (Wayne *et al.*, 1987), and therefore belong to a new species within the genus *Paracoccus*, for which the name *P. zeaxanthinifaciens* is proposed. The G+C content of these five strains ranged from 66·9 to 67·7 mol%, thus remaining within 1 mol%, characteristic for a well defined species (Vandamme *et al.*, 1996). On the other hand, the low DNA relatedness between strains MBIC 3024, MBIC 4017 and MBIC 4020 themselves, and between these strains and strains ATCC 21588^T, R114, R1534, R-1506 and MBIC 3966 showed that MBIC 3024, MBIC 4017 and MBIC 4020 each belong to a different additional new species within the genus *Paracoccus*.

P. zeaxanthinifaciens strains ATCC 21588^T, R114, R1534,

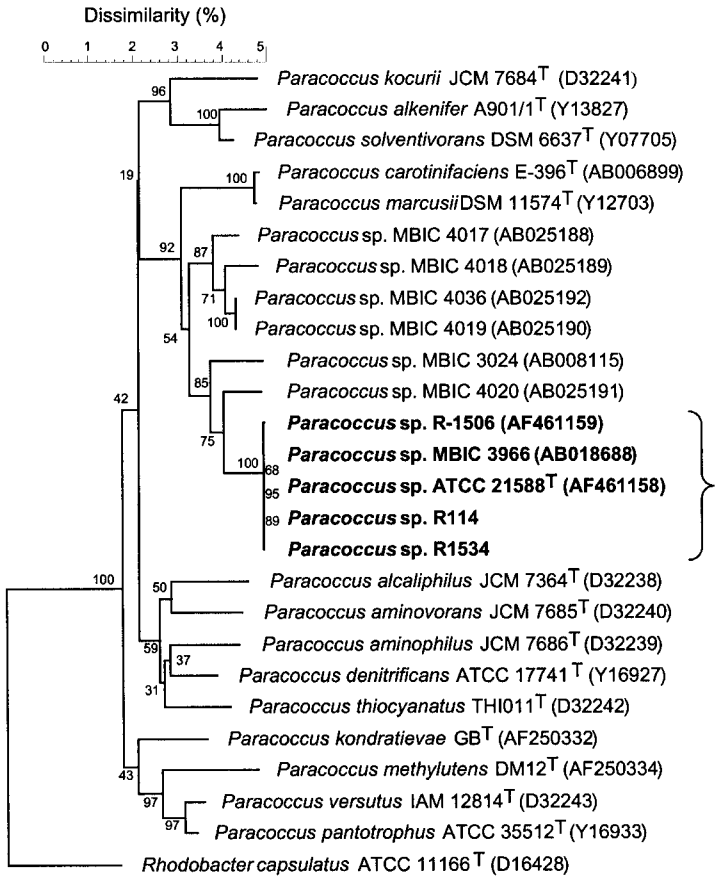

Fig. 1. Distance matrix tree showing the phylogenetic relatedness of *P. zeaxanthinifaciens* strains (in bold) and other species of *Paracoccus* based on sequence comparison of 16S rRNA genes. *Rhodobacter capsulatus* was used as the outgroup and bootstrap probability values are indicated at the branch points (100 trees resampled). Sequence accession numbers are given in parentheses after the strain designations.

R-1506 and MBIC 3966 were compared by AFLP to evaluate their infraspecies relatedness. *P. marcusii* DSM 11574^T was included as an outlier. In all six primer combinations used, the DNA fingerprints of strains ATCC 21588^T, R114 and R1534 were highly similar if not identical. In cases where minor differences were observed, reproducibility was not evaluated. With all primer combinations, strains ATCC 21588^T, R114 and R1534 could be clearly discriminated from strains R-1506 and MBIC 3966. The fingerprints of strains R-1506 and MBIC 3966, although similar to each other, showed distinct differences. Under the conditions used, the strains of *P. zeaxanthinifaciens* clustered at a mean level of 58 % similarity, and the cluster can clearly be discriminated from the profile of *P. marcusii* DSM 11574^T. The results obtained with two of the primer combinations used are shown as representative examples in the supplementary data system in IJSEM Online (http://ijs.sgmjournals.org). These results indicate, therefore, that at present the new species of *Paracoccus* described here contains three different wild-type strains.

Table 2. G+C content of DNA from the *Paracoccus* spp. strains selected and DNA reassociation between the strains

Strain	G+C (mol%)	DNA reassociation (%)
P. zeaxanthinifaciens		
ATCC 21588^T	67·6	100
R114	67·5	100 100
R1534	67·7	96 97 100
R-1506	67·5	94 88 90 100
MBIC 3966	66·9	93 ND ND 88 100
***Paracoccus* sp.**		
MBIC 3024	65·4	31 ND ND 31 32 100
MBIC 4017	67·2	32 ND ND 31 24 24 100
MBIC 4020	68·4	27 ND ND 25 23 25 34 100

ND, Not determined.

Table 3 summarizes the mean cellular fatty acid composition of the 3 wild-type strains constituting *P. zeaxanthinifaciens* and the fatty acid composition of *P. marcusii* DSM 11574^T, *P. carotinifaciens* E-396^T and *P. solventivorans* DSM 6637^T. All four species showed a comparable cellular fatty acid profile, with 18 : 1ω7*c* as the major compound. This is consistent with fatty acid compositions reported previously for the three type strains (Harker *et al.*, 1998; Tsubokura *et al.*, 1999a; Siller *et al.*, 1996), and indeed with the fatty acid composition characteristic of the entire genus *Paracoccus* (Baj, 2000). No attempt was made to determine the significance of the minor differences in fatty acid composition between *P. zeaxanthinifaciens* and the three type strains, as variability of these minor compounds within the latter *Paracoccus* species could not be assessed since these species currently contain only the type strain analysed.

Table 3. Cellular fatty acid composition (mean percentage of total) of *P. zeaxanthinifaciens* and of related *Paracoccus* species

Strains: 1, *P. zeaxanthinifaciens* (ATCC 21588^T, R-1506 and MBIC 3966); 2, *P. marcusii* DSM 11574^T; 3, *P. carotinifaciens* E-396^T; 4, *P. solventivorans* DSM 6637^T. ND, Not detected.

Fatty acid	1	2	3	4
10:0 3-OH	4·9 (1·0)	6·2	3·4	3·6
Unidentified 11·799*	3·6 (0·4)	4·9	2·8	3·0
Unidentified 15·275*	1·6 (0·3)	2·9	1·1	ND
16:0	0·3 (0·3)	ND	0·3	0·7
17:1ω8*c*	ND	ND	0·6	0·8
17:0	0·2 (0·1)	ND	0·3	1·3
18:1ω7*c*	80·2 (1·5)	80·3	84·0	79·0
18:0	3·6 (0·6)	2·6	5·2	6·6
18:0 3-OH	0·7 (0·4)	ND	ND	ND
19:0	ND	ND	ND	0·7
20:1ω7*c*	0·8 (0·2)	ND	0·2	2·0
Summed feature 2†	2·9 (0·5)	3·0	2·1	2·6
Summed feature 3†	0·7 (0·6)	ND	0·2	ND

*Unknown fatty acid; the number indicates the equivalent chain-length.

†Summed feature consisting of one or more of the following fatty acids which cannot be separated by the Microbial Identification System: 2, 14:0 3-OH and 16:1 iso I; 3, 15:0 iso 2-OH, 16:1ω7*c* and 16:1ω7*t*.

Aside from the genetic properties described above that show that strains ATCC 21588^T, R-1506 and MBIC 3966 comprise a new species of *Paracoccus*, these strains were easily differentiated biochemically and physiologically from the currently accepted species of *Paracoccus*. All species of *Paracoccus* except for *P. marcusii* DSM 11574^{T}and *P. carotinifaciens* E-396^T are capable of reducing nitrate to nitrite, and in seven of those species, denitrification is performed (Baj, 2000). Strains ATCC 21588^T, R-1506 and MBIC 3966 were incapable of nitrate reduction or denitrification. Colony pigmentation further distinguishes the latter strains from all other species of *Paracoccus*. Among the established species of *Paracoccus*, only *P. marcusii* DSM 11574^T, *P. carotinifaciens* E-396^T and *P. thiocyanatus* THIO11^T exhibit pigmented colonies (Baj, 2000). In the case of *P. thiocyanatus* THIO11^T, the reddish pigment was determined to not be a carotenoid(s) (Harker *et al.*, 1998). Although *P. marcusii* DSM 11574^{T}and *P. carotinifaciens* E-396^T do produce carotenoids, they produce mainly ketocarotenoids (Hirschberg & Harker, 1999; Tsubokura *et al.*, 1999b), hence the orange-pink colony pigmentation. Strains ATCC 21588^T (R114, R1534), R-1506 and MBIC 3966 produce exclusively zeaxanthin (see below), giving rise to the yellow-orange colony pigmentation.

Strains ATCC 21588^T, R-1506 and MBIC 3966 were subjected to a battery of other phenotypic tests and compared more specifically to the two known carotenoid-producing species *P. marcusii* DSM 11574^T and *P. carotinifaciens* E-396^T. Of the 95 carbon sources tested as growth substrates using the Biolog SF-N system, 12 could be used, and 53 could not be used by all strains of *P. zeaxanthinifaciens* (these carbon sources are listed below in the description of the new species). The strains gave variable growth responses to the remaining 25 substrates. Based on this test, *P. zeaxanthinifaciens* could be distinguished from *P. marcusii* DSM 11574^T and *P. carotinifaciens* E-396^T by the inability of *P. zeaxanthinifaciens* to use seven carbon sources (adonitol, erythritol, gentiobiose, methyl β-glucoside, D-sorbitol, xylitol and quinic acid). Two additional carbon sources that were utilized by *P. zeaxanthinifaciens* (L-asparagine and L-aspartic acid) were not used for growth by *P. marcusii* DSM 11574^T. The growth characteristics of *P. marcusii* DSM 11574^T on the nine carbon sources mentioned above are the same as those reported by Harker *et al.* (1998), with the exception of methyl β-glucoside, which in their hands did not support growth of the organism. Tsubokura *et al.* (1999a), in the original description of *P. carotinifaciens* E-396^T, did not report whether these nine carbon sources can be used by this strain, so no comparison with the present results can be made.

P. zeaxanthinifaciens grew weakly or not at all at 10 °C, but grew well from 25 to 40 °C. Neither *P. marcusii* DSM 11574^T nor *P. carotinifaciens* E-396^T could grow at 40 °C. *P. zeaxanthinifaciens* grew on Marine Agar supplemented with 3, 6 or 8 % NaCl, and in Marine Broth having a final pH ranging from 6·1 to 9·1. In contrast, *P. marcusii* DSM 11574^T and *P. carotinifaciens* E-396^T grew very poorly in the presence of 8 % NaCl, and in addition *P. marcusii* DSM 11574^T was not able to grow at pH 9·1. Urease was positive for *P. zeaxanthinifaciens* (although weak for the two mutants) and negative for *P. marcusii* DSM 11574^T and *P. carotinifaciens* E-396^T.

Cells of *P. zeaxanthinifaciens* strains were coccoid to short rods with dimensions of 0·8–0·9 × 1·1–1·5 μm. *P. marcusii* DSM 11574^T, *P. carotinifaciens* E-396^T and *P. solventivorans* DSM 6637^T all appeared as short rods. The shape and size of these cells was similar to the original reports (Harker *et al.*, 1998; Tsubokura *et al.*, 1999a; Siller *et al.*, 1996).

Table 4 summarizes the results of the physiological tests that differentiated between *P. zeaxanthinifaciens*, *P. marcusii* and *P. carotinifaciens*. Other characteristics that are shared by all members of *P. zeaxanthinifaciens* but not discriminative towards *P. marcusii* or *P. carotinifaciens*, are given in the species description.

The carotenoid production profile for all pigmented strains used in this work is available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org). Zeaxanthin was the only carotenoid produced by strains ATCC 21588^T, R114, R1534, R-1506 and MBIC 3966. All other strains produced a mixture of the ketocarotenoids canthaxanthin

Table 4. Tests that discriminate *P. zeaxanthinifaciens* from *P. marcusii* and *P. carotinifaciens*

Strains: 1, *P. zeaxanthinifaciens* (ATCC 21588^T, R-1506 and MBIC 3966); 2, *P. marcusii* DSM 11574^T; 3, *P. carotinifaciens* E-396^T. Growth was scored relative to each control condition (specified in Methods). The data shown are results measured after 5 days (temperature and salt ranges for growth, colony pigmentation, urease activity) or 6 days (pH range for growth and carbon sources utilized). +, Good growth (equivalent to the control condition); +/−, very poor growth; −, no growth.

Test	1	2	3
Growth at 40 °C	+	−	−
Growth with 8 % NaCl	+	+/−	+/−
Growth at pH 9·1	+	−	+
Growth on:			
Adonitol	−	+	+
Erythritol	−	+	+
Gentobiose	−	+	+
Methyl β-glucoside	−	+	+
D-Sorbitol	−	+	+
Xylitol	−	+	+
Quinic acid	−	+	+
L-Asparagine	+	−	+
L-Aspartate	+	−	+
Urease	+	−	−
Colony pigmentation	Yellow-orange	Orange-pink	Orange-pink

and astaxanthin (a small amount of the ketocarotenoid adonixanthin was also detected in MBIC 4017). These results are consistent with the previous reports that strain ATCC 21588^T produces only zeaxanthin (Schocher & Wiss, 1975) and that *P. marcusii* DSM 11574^T and *P. carotinifaciens* E-396^T produce primarily ketocarotenoids (Hirschberg & Harker, 1999; Tsubokura *et al.*, 1999b).

Based on the work reported here, we propose that strains ATCC 21588^T (=R-1512^T), R-1506 and MBIC 3966 be classified as a new species of the genus *Paracoccus*, having the name *Paracoccus zeaxanthinifaciens* sp. nov., and further propose that ATCC 21588^T (=R-1512^T =LMG 21293^T) be designated as the type strain.

Description of *Paracoccus zeaxanthinifaciens* sp. nov.

Paracoccus zeaxanthinifaciens (ze.a.xan.thi.ni.fa′ci.ens. N.L. neut. n. *zeaxanthinum* zeaxanthin; L. part. pres. *faciens* making/producing; N.L. adj. *zeaxanthinifaciens* zeaxanthin-producing).

Short rods to cocci, 0·8–0·9 × 1·1–1·5 μm, growing singly, in pairs or in short chains. Non-motile and Gram-negative. Colonies on Marine Agar are circular, convex, smooth and deep yellow to orange due to accumulation of the carotenoid zeaxanthin. Astaxanthin and other ketocarotenoids are not produced. Growth is strictly aerobic. Catalase, oxidase and β-galactosidase are positive. β-Glucosidase is positive, but weak for the type strain. Arginine dihydrolase is negative. Indole is not produced from tryptophan and glucose is not fermented. Starch and gelatin are not hydrolysed. Nitrate is not reduced to nitrite, and denitrification is not performed. PHB is produced. Grows weakly or not at all at 10 °C, but grows well from 25 to 40 °C. Grows on Marine Agar containing 3–8 % NaCl, and grows in Marine Broth at pH 6·1–9·1. Utilizes D-arabitol, D-galactose, α-D-glucose, *myo*-inositol, α-lactose, D-mannitol, D-melibiose, D-trehalose, L-asparagine, L-aspartic acid, L-glutamic acid and L-pyroglutamic acid for growth, but does not utilize α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, i-erythritol, L-fucose, gentobiose, methyl β-glucoside, D-raffinose, L-rhamnose, D-sorbitol, xylitol, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-glucosaminic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid methyl ester, malonic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, D-alanine, L-histidine, hydroxy-L-proline, L-phenylalanine, D-serine, L-threonine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glucose 1-phosphate or glucose 6-phosphate. The major non-hydroxyl fatty acid is $C_{18:1(\omega7c)}$ and the major hydroxyl fatty acid is 3-OH $C_{10:0}$. The G+C content of the DNA from the strains currently within the species ranges from 66·9 to 67·7 mol%. The type strain is ATCC 21588^T =R-1512^T =LMG 21293^T, and it was isolated from seaweed collected from the coast of the African Red Sea. Reference strains are R-1506 and MBIC 3966.

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