Erythrobacter flavus sp. nov., a slight halophile from the East Sea in Korea

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Two Gram-negative, motile, non-spore-forming, yellow-pigmented and slightly halophilic strains (SW-46^T and SW-52) were isolated from sea water of the East Sea, Korea, and subjected to a polyphasic taxonomic study. Strains SW-46^T and SW-52 were characterized chemotaxonomically by having ubiquinone-10 (Q-10) as the predominant respiratory lipoquinone and C_{18:1} ω 7c as the major fatty acid. Their DNA G + C content was 64.0–64.1 mol%. Strains SW-46^T and SW-52 showed 1 bp difference in their 16S rDNA sequences and a mean DNA–DNA relatedness level of 94.4 %. Phylogenetic analysis based on 16S rDNA sequences showed that strains SW-46^T and SW-52 fall within the α -subclass of the *Proteobacteria* and form a coherent cluster with *Erythrobacter longus*, *Erythrobacter litoralis* and *Erythrobacter citreus*. Levels of 16S rDNA similarity between strains SW-46^T and SW-52 and the type strains of these three *Erythrobacter* species were 96.5–97.9 %. Levels of DNA–DNA relatedness between strains SW-46^T and SW-52 and the type strains of *E. longus*, *E. litoralis* and *E. citreus* were 3.6–14.7 %. Therefore, on the basis of phenotypic properties, phylogeny and genomic data, strains SW-46^T and SW-52 should be placed in the genus *Erythrobacter* as a novel species, for which the name *Erythrobacter flavus* sp. nov. is proposed. The type strain is SW-46^T (=KCCM 41642^T =JCM 11808^T).

The genus *Erythrobacter* was proposed by Shiba & Simidu (1982) to accommodate Gram-negative, ovoid to rod-shaped and aerobic chemo-organotrophs. Species of this genus are red or orange in colour and contain bacteriochlorophyll *a* and carotenoids (Shiba & Simidu, 1982; Yurkov *et al.*, 1994). However, a novel *Erythrobacter* species that produces a yellow pigment and lacks bacteriochlorophyll *a*, *Erythrobacter citreus*, has recently been described (Denner *et al.*, 2002). Phylogenetic analyses based on 16S rDNA sequences have shown that the genus *Erythrobacter* falls within the α -subclass of the *Proteobacteria* and is closely related to the genera *Erythromicrobium* and *Porphyrobacter* (Yurkov *et al.*, 1994; Anzai *et al.*, 2000; Denner *et al.*, 2002).

The classification of *Erythrobacter* as a genus separate from *Erythromicrobium* and *Porphyrobacter* is warranted by only small phenotypic differences (Shiba, 1991; Fuerst *et al.*, 1993; Yurkov *et al.*, 1994). However, the separation of the cluster that comprises *Erythrobacter* from the cluster that comprises the genera *Erythromicrobium* and *Porphyrobacter* has been supported by a high bootstrap resampling value (Denner *et al.*, 2002). Nevertheless, the three genera mentioned above may have to be taxonomically re-evaluated by using additional phenotypic, particularly chemotaxonomic, data or detailed phylogenetic analysis.

There are three *Erythrobacter* species with validly published names: *Erythrobacter longus* (Shiba & Simidu, 1982), *Erythrobacter litoralis* (Yurkov *et al.*, 1994) and *E. citreus* (Denner *et al.*, 2002). The genus *Erythrobacter* is characterized chemotaxonomically by having C_{18:1} as the major fatty acid and by a DNA G + C content of 60–67 mol% (Shiba & Simidu, 1982; Fuerst *et al.*, 1993; Yurkov *et al.*, 1994). However, Shiba (1991) reported that the type strain of *E. longus* has a DNA G + C content of 57.4 mol%.

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Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains SW-46^T and SW-52 are AF500004 and AF500005, respectively.

All three *Erythrobacter* species have been isolated from marine environments (Shiba & Simidu, 1982; Yurkov *et al.*, 1994; Denner *et al.*, 2002). Recently, two slightly halophilic bacterial strains, SW-46^T and SW-52, were isolated from sea water of Hwajinpo Beach, East Sea, Korea. The two isolates were phylogenetically most closely related to the genus *Erythrobacter*, based on the result of 16S rDNA sequence comparison. Colonies of the two strains were observed to be yellow on marine agar, unlike the first two *Erythrobacter* species that were described. Accordingly, the aim of the present study was to establish the exact taxonomic status of the two isolates by a polyphasic taxonomic approach. In this work, we describe the morphological, phenotypic, phylogenetic and genomic characteristics of strains SW-46^T and SW-52. On this basis, we propose a novel species of the genus *Erythrobacter*, *Erythrobacter flavus* sp. nov., for strains SW-46^T and SW-52.

Strains SW-46^T and SW-52 (= KCCM 41643 = JCM 11809) were isolated by using the dilution-plating technique on marine agar 2216 (MA; Difco). *E. longus* DSM 6997^T, *E. litoralis* DSM 8509^T and *E. citreus* DSM 14432^T, which were obtained from DSMZ, Germany, were used as reference strains. Cell biomass of strains SW-46^T and SW-52 and reference strains was obtained from marine

broth 2216 (MB; Difco) cultures grown at 30 °C, for respiratory lipoquinone analysis and DNA extraction. All strains were cultivated on a gyratory shaker at 150 r.p.m. For fatty acid methyl ester (FAME) analysis, cell mass of strains SW-46^T and SW-52, *E. longus* DSM 6997^T and *E. litoralis* DSM 8509^T was obtained from agar plates after 5 days cultivation at 30 °C on MA. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM); presence or absence of flagella was examined by TEM using cells from exponentially growing cultures. The cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). Growth at various NaCl concentrations was investigated in MB. Growth at 4–55 °C was measured on MA. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with MA that had been prepared anaerobically. Presence or absence of bacteriochlorophyll *a* was examined by registration of the *in vitro* absorption spectrum of a methanol extract of cells at 768–769 nm. Susceptibility to antibiotics was detected on agar plates by using antibiotic discs (concentrations shown in Table 1). Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by

Table 1. Differential phenotypic characteristics of *Erythrobacter flavus* sp. nov., *E. longus*, *E. litoralis* and *E. citreus*

+, Positive; –, negative; w+, weakly positive; ND, not determined; v, variable. Data in parentheses are for the type strain. All species are rod-shaped and positive for catalase and oxidase activities, susceptibility to chloramphenicol (100 µg per disc; 30 µg per disc for *E. citreus*) and utilization of acetate. All species are negative for Gram-staining, spore formation, motility, susceptibility to polymyxin B (100 U per disc; 300 U per disc for *E. citreus*) and utilization of malate.

Characteristic	<i>E. flavus</i>	<i>E. longus</i> *	<i>E. litoralis</i> *	<i>E. citreus</i> †
Colour of colonies	Yellow	Orange	Red or orange	Yellow
Nitrate reduction	–	v (+)	–	+
H ₂ S production	–	–	ND	ND
Hydrolysis of:				
Gelatin	–	+	–	ND
Starch	+	–	–	(–)
Utilization of:				
Glucose	–	+	+	v (w+)
Fructose	–	ND	+	–
Citrate	–	–	–	v (w+)
Formate	–	–	–	ND
Pyruvate	+	+	+	–
Glutamate	–	+	+	ND
Succinate	–	–	+	ND
Lactate	v	–	+	–
Susceptibility to:				
Penicillin (20 U; 10 U for <i>E. citreus</i>)	–	–	–	v
Streptomycin (50 µg; 10 µg for <i>E. citreus</i>)	–	–	–	v
Optimum growth temperature (°C)	30–37	25–30	25–30	ND
DNA G+C content (mol%)	64.0–64.1	60–64 (60.7)	67	62.0–62.4

*Data from Shiba & Simidu (1982) and Yurkov *et al.* (1994).

†Data from Vybiral *et al.* (1999) and Denner *et al.* (2002).

oxidation of 1 % *p*-aminodimethylaniline oxalate. Hydrolysis of aesculin and nitrate reduction were determined as described by Lányi (1987). Hydrolysis of casein, starch and Tween 80 and urease activity were determined as described by Cowan & Steel (1965). Hydrolysis of gelatin was studied as described by Cowan & Steel (1965), with the modification that artificial sea water was used. The artificial sea water contained [(1 distilled water)⁻¹]: 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Levring, 1946). Hydrolysis of hypoxanthine, tyrosine and xanthine was examined on MA plates with substrate concentrations as described by Cowan & Steel (1965). H₂S production was tested as described by Bruns *et al.* (2001). Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994).

Strains SW-46^T and SW-52^T had identical morphological characteristics for their cells and colonies. Cells of both strains were rods, approximately 0.7–0.9 µm wide and 1.5–2.5 µm long after 3 days cultivation at 30 °C on MA. Gram-staining reaction was negative. Cells of strains SW-46^T and SW-52 had a single polar flagellum and no spores were observed. Colonies on MA were yellow, smooth, glistening, circular, convex with entire margins and 1.0–1.5 mm in diameter after 3 days incubation at 30 °C. Cell morphologies of strains SW-46^T and SW-52^T were similar to those of *Erythrobacter* species, whereas colonies of the two strains were similar to those of *E. citreus* but different in colour from those of *E. longus* and *E. litoralis* (Table 1) (Shiba & Simidu, 1982; Yurkov *et al.*, 1994; Denner *et al.*, 2002). Strains SW-46^T and SW-52 were similar in most of their cultural and physiological characteristics: they grew optimally at 30–37 °C and grew at 10 and 42 °C, but not at 4 °C or above 43 °C. The optimal pH for growth was 6.5–7.5; no growth was observed at pH 4.5. Strains SW-46^T and SW-52 grew optimally in the presence of 2–5 % (w/v) NaCl, but did not grow without NaCl. Strain SW-46^T did not grow in the presence of > 14 % NaCl and strain SW-52 did not grow in the presence of > 13 % NaCl. Neither strain grew on MA under anaerobic conditions. Both strains showed catalase, oxidase and urease activities. Starch, Tween 80 and tyrosine were hydrolysed. No hydrolysis of aesculin, casein, gelatin, hypoxanthine or xanthine was observed. H₂S was not produced. Nitrate was not reduced to nitrite. Acid was produced from D-cellobiose and maltose. Acid production from D-trehalose was found only in strain SW-52. Acetate, butyrate and pyruvate were utilized for growth. Bacteriochlorophyll *a* was not detected *in vitro* in either strain. Phenotypic characteristics of strains SW-46^T and SW-52 were compared with those of *Erythrobacter* species (Table 1); strains SW-46^T and SW-52 were found to have physiological properties that were distinguishable from those of other *Erythrobacter* species.

Respiratory lipoquinones were analysed by using reversed-phase HPLC (Komagata & Suzuki, 1987). For quantitative

analysis of cellular fatty acid composition, a loop of cell mass was harvested and FAMES were prepared and identified by following the instructions of the Microbial Identification system (MIDI). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984); DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

The predominant respiratory lipoquinone of strains SW-46^T and SW-52, *E. longus* DSM 6997^T and *E. litoralis* DSM 8509^T was ubiquinone-10 (Q-10), the same as that of *E. citreus* (Denner *et al.*, 2002). Cellular fatty acid profiles of strains SW-46^T and SW-52 are shown in Table 2, together with those of *E. longus* DSM 6997^T, *E. litoralis* DSM 8509^T and *E. citreus* RE35F/1^T. Strains SW-46^T and SW-52 had cellular fatty acid profiles that contained large amounts of saturated and unsaturated fatty acids (Table 2). The major fatty acid found in strains SW-46^T and SW-52 was C_{18:1}ω7c, at a peak ratio of approximately 45–46 % (Table 2). The fatty acid profiles of the two strains were similar to those of the type

Table 2. Cellular fatty acid profiles of *Erythrobacter* species

Strains: 1, *E. flavus* SW-46^T; 2, *E. flavus* SW-52; 3, *E. longus* DSM 6997^T; 4, *E. litoralis* DSM 8509^T; 5, *E. citreus* RE35F/1^T. Values are percentage of total fatty acids. —, Not detected. Fatty acids that represented <0.5 % in all strains were omitted.

Fatty acid	1	2	3	4	5*
Saturated:					
C _{15:0}	3.0	3.4	2.8	0.5	0.3
C _{16:0}	6.4	7.6	11.4	2.7	8.2
C _{17:0}	7.3	8.3	10.1	—	—
C _{18:0}	0.5	0.5	1.0	—	—
Branched:					
anteiso-C _{15:0}	—	—	—	—	0.6
anteiso-C _{16:0}	—	—	—	—	0.7
anteiso-C _{17:0}	—	—	—	—	1.2
Unsaturated:					
C _{16:1} ω5c	—	—	—	0.7	3.6
C _{17:1} ω6c	6.2	5.1	3.9	28.5	5.6
C _{17:1} ω8c	8.1	6.7	4.8	4.3	0.7
C _{18:1} ω5c	—	—	—	2.2	4.2
C _{18:1} ω7c	46.2	44.9	50.9	46.9	49.3
11-Methyl-C _{18:1} ω7c	5.5	7.1	—	3.7	4.4
Hydroxy:					
C _{14:0} 2-OH	0.9	0.9	5.0	2.9	0.9
C _{15:0} 2-OH	9.5	8.7	8.6	2.6	—
C _{16:0} 2-OH	1.4	1.3	1.5	1.5	—
C _{18:1} 2-OH	0.3	0.4	—	—	2.0
iso-C _{16:0} 3-OH	—	—	—	1.1	1.0
Summed feature 3†	4.6	4.9	—	2.3	14.9

*Data from Denner *et al.* (2002).

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

strains of *E. longus* and *E. citreus*. The fatty acid profile of *E. longus* DSM 6997^T obtained in this study was similar to that reported by Fuerst *et al.* (1993). However, there was a noteworthy difference in the proportion of C_{17:1ω6c} between strains SW-46^T and SW-52 and *E. litoralis* DSM 8509^T. The DNA G+C contents of strains SW-46^T and SW-52 were 64.0 and 64.1 mol%, respectively.

Chromosomal DNA was isolated and purified according to a method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. 16S rDNA was amplified by PCR with two universal primers, as described previously (Yoon *et al.*, 1998). The PCR product was purified by using a QIAquick PCR Purification kit (Qiagen). Sequencing of the purified 16S rDNA was performed by using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically by using an Applied Biosystems model 377 automatic DNA sequencer. Alignment of sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated by using the algorithm of Jukes & Cantor (1969) with the program DNADIST. The stability of relationships was assessed by bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset, by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

The 16S rDNA sequences of two strains determined in this study each comprised 1442 nucleotides, which represents approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. There is only 1 bp difference between the 16S rDNA sequences of strains SW-46^T and SW-52. The strains were found to have highest 16S rDNA similarity to members of the α -Proteobacteria. Strains SW-46^T and SW-52 exhibited 16S rDNA similarity levels of 96.5–97.9% with the type strains of *E. longus*, *E. litoralis* and *E. citreus*, but <96.5% to other species used in the phylogenetic analysis. In the phylogenetic tree based on the neighbour-joining algorithm, strains SW-46^T and SW-52 formed a coherent cluster with *E. citreus*, *E. longus* and *E. litoralis* (Fig. 1). The relationship between this cluster and the clade that comprised the genera *Erythromicrobium* and *Porphyrobacter* was supported by a bootstrap confidence level of 100%. This tree topology was also generated by the maximum-parsimony and maximum-likelihood algorithms (data not shown). When the neighbour-joining and maximum-parsimony algorithms were used, the relationship between the cluster that comprised strains SW-46^T and SW-52 and *Erythrobacter* species and the clade that comprised the genera *Porphyrobacter*

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *Erythrobacter flavus* sp. nov. SW-46^T and SW-52 and representatives of related taxa, based on 16S rDNA sequences. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch-points. Bar, 0.01 substitutions per nucleotide position.

and *Erythromicrobium* was supported by high bootstrap resampling values.

DNA–DNA hybridization was performed fluorometrically by using photobiotin-labelled DNA probes and microdilution wells (Ezaki *et al.*, 1989). Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values for each sample were excluded; DNA relatedness values are means of the remaining three values. Strains SW-46^T and SW-52 exhibited DNA–DNA relatedness levels of 94.0 and 94.7% when their DNA was used individually as a labelled DNA probe. Accordingly, by considering the criterion of DNA–DNA similarity for definition of a species in current bacterial systematics (Wayne *et al.*, 1987), strains SW-46^T and SW-52 should be classified as members of the same species. Levels of DNA–DNA relatedness between strains SW-46^T and SW-52 and the type strains of *E. longus*, *E. litoralis* and *E. citreus* are shown in Table 3; they support the conspicuous genomic distinctiveness of strains SW-46^T and SW-52.

Phenotypically, strains SW-46^T and SW-52 are similar to *E. citreus* in pigmentation and the absence of

Table 3. Levels of DNA–DNA relatedness

Strain	Reassociation with (%)	
	Strain SW-46 ^T	Strain SW-52
Strain SW-46 ^T	100	94.7
Strain SW-52	94.0	100
<i>E. longus</i> DSM 6997 ^T	4.2	3.6
<i>E. litoralis</i> DSM 8509 ^T	6.0	7.4
<i>E. citreus</i> DSM 14432 ^T	13.1	14.7

bacteriochlorophyll *a*, and are different in these respects from *E. longus* and *E. litoralis*. The 16S rDNA sequence analysis provides evidence that both strains belong to the *Erythrobacter* 16S rDNA cluster. In this study, cellular fatty acid and respiratory lipoquinone analyses were performed on the type strains of *E. longus* and *E. litoralis*, as well as on strains SW-46^T and SW-52; the results have been compared with those of *E. citreus*. The predominant respiratory lipoquinone and fatty acid profiles of strains SW-46^T and SW-52 were found to be similar to those of the type strains of the other *Erythrobacter* species, confirming their intra-generic relationship. In view of these combined morphological, chemotaxonomic and phylogenetic analyses, strains SW-46^T and SW-52 warrant classification in the genus *Erythrobacter*. The level of DNA–DNA relatedness and phenotypic characteristics confirmed that the strains constitute a separate species. Therefore, on the basis of the data presented, we propose to include strains SW-46^T and SW-52 in the genus *Erythrobacter* as *Erythrobacter flavus* sp. nov.

Description of *Erythrobacter flavus* sp. nov.

Erythrobacter flavus (fla'vus. L. masc. adj. *flavus* yellow, the colour of colonies or pigment).

Non-spore-forming rods, 0.7–0.9 × 1.5–2.5 µm on MA. Gram-staining reaction is negative. Motile by means of a single polar flagellum. Colonies are yellow, smooth, glistening, circular, convex with entire margins and 1.0–1.5 mm in diameter after 3 days cultivation at 30 °C on MA. Optimal temperature for growth is 30–37 °C. Growth occurs at 10 and 42 °C, but not at 4 °C or above 43 °C. Optimal pH for growth is 6.5–7.5. Growth occurs at pH 5.0, but not at pH 4.5. Optimal growth occurs in the presence of 2–5 % (w/v) NaCl. No growth occurs in the absence of NaCl or in the presence of >14 % NaCl. No growth occurs under anaerobic conditions on MA. Catalase-, oxidase- and urease-positive. Starch, Tween 80 and tyrosine are hydrolysed. Aesculin, casein, gelatin, hypoxanthine and xanthine are not hydrolysed. H₂S is not produced. Nitrate is not reduced to nitrite. Susceptible to chloramphenicol. Resistant to penicillin, streptomycin and polymyxin B. Acid is produced from D-cellobiose and maltose. Acid production from D-trehalose is variable. Acid is not produced from adonitol, L-arabinose, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, D-mannitol, D-mannose, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, stachyose, sucrose or D-xylose. Acetate, butyrate and pyruvate are utilized for growth. Glucose, fructose, glutamate, citrate, malate, succinate, formate, methanol, ethanol and benzoate are not utilized. Utilization of lactate is variable. The predominant respiratory lipoquinone is ubiquinone-10. The major fatty acid is C_{18:1ω7c}. The DNA G + C content is 64.0–64.1 mol% (determined by HPLC).

The type strain (SW-46^T = KCCM 41642^T = JCM 11808^T) and reference strain (SW-52 = KCCM 41643 = JCM 11809)

were isolated from sea water of Hwajinpo Beach, East Sea, Korea.

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