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A salt lake extremophile, *Paracoccus bogoriensis* sp. nov., efficiently produces xanthophyll carotenoids

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A Gram-negative obligate alkaliphilic bacterium (BOG6[T]) that secretes carotenoids was isolated from the outflow of Lake Bogoria hot spring located in the Kenyan Rift Valley. The bacterium is motile by means of a polar flagellum, and forms red colonies due to the production of xanthophyll carotenoid pigments. 16S rRNA gene sequence analysis showed this strain to cluster phylogenetically within the genus *Paracoccus*. Strain BOG6[T] is aerobic, positive for both catalase and oxidase, and non-methylotrophic. The major fatty acid of the isolate is C18: 1ω7c. It accumulated polyhydroxybutyrate granules. Strain BOG6[T] gave astaxanthin yield of 0.4 mg/g of wet cells indicating a potential for application in commercial production of carotenoids. On the basis of its genotypic characteristics, fatty acid composition and physiological reaction profiles, it is proposed that the isolate may be assigned to the genus *Paracoccus* as *Paracoccus bogoriensis* sp. nov. The type strain is BOG6[T] (=DSM16578 =LMG22798). The GenBank 16S rDNA nucleotide sequence accession number is AJ580352.

Key words: *Paracoccus bogoriensis*, *alpha-proteobacteria*, alkaliphile, xanthophylls, astaxanthin, extremophile, carotenoids.

INTRODUCTION

Xanthophylls are C40 oxygenated carotenoid pigments that are synthesised *de novo* by a wide variety of plants, bacteria and archaea but not by animals (Johnson and Schroeder, 1995; Ong and Tee, 1992). Xanthophylls have considerable interest due to their widespread use in the food, nutraceutical and pharmaceutical industries (Borowitzka, 1988; Dufossé, 2006). The hydroxy ketocarotenoid astaxanthin (3, 3'- dihydroxy- , '- carotene-4, 4'-dione) is a potent biological antioxidant with stronger antioxidant activity than –carotene and Vitamin E (Johnson and An, 1991). Astaxanthin is used in fish

used in fish aquaculture to impart pleasant colours to salmon and trout (Jacobson et al., 2003; Lorenz and Cysewski, 2000). It is also the natural pigment for some varieties of tilapia. It is marketed as a nutritional supplement for humans (Guerin et al., 2003). Biological activities of astaxanthin in humans include potentiation of the immune system (Bendich, 1989; Jynouchi et al., 1995) and suppression of certain cancers (Palozza et al., 2009; Miyashita, 2009). Commercially, xanthophllys are produced from microorganisms such as the red yeast *Phaffia rhodozyma* and the green algae *Haematococcus pluvialis* (Nelis and Leenher, 1991) as well as by chemical synthesis. However the current sources are not able to satisfy world demand, hence the need to search for alternative sources. Some bacteria that belong to the genus *Paracoccus sp.* are known to secrete astaxanthin and are

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are under patent protection.

More than 20 species of the genus *Paracoccus* have been described in literature (Kelly et al., 2006a, 2006b). The most highly studied member of the genus is *Paracoccus denitrificans* , first described by Beijeirinck and Minkman (1910) as *Micrococcus denitrificans* and later renamed by Davis et al. (1969). The genome of *P. denitrificans* was sequenced in 2004. It is not known to produce carotenoids*.*

Paracoccus species are Gram negative, catalase-positive, oxidase-positive bacteria that contain C18: 1 7c as a major component of the cellular fatty acids and are metabolically versatile (Kelly et al., 2006b). Members of the species that have been shown to produce carotenoids, and are under intellectual property protection include: *P. carotinifaciens, P. marcusii*, *P. zeaxanthinifaciens* and *P. haeundaensis* (Berry et al., 2003; Hirschberg and Harker, 1999; Tsubokura et al., 1999a, Lee et al., 2004).

The *Paracoccus* have been isolated from diverse environments such as soil, sludge and sea water. Lake Bogoria, the site of isolation of strain BOG6[T], is an alkaline-saline, Athalassic lake located in the Kenyan Rift Valley. It covers an area of 20 km^2 and its pH ranges from 9 - 11 while the lake temperature fluctuates between 30 - 90°C depending on the site. The alkalinity is due to its unique geochemistry. It lies in a closed basin that was created by a tilt faulting. The predominant bedrock is alkaline trachyte lavas of the lower Pleistocene era. Evaporation and leaching causes concentration of salts particularly sodium carbonate (McCall, 1967; Grant et al., 1990). The large temperature variation is due to the hot springs and geysers that dot its shores and emit their outflow into the lake.

Our investigations were conducted in a bioprospecting effort to find new and robust biocatalysts and secondary metabolites from microorganisms of the Kenyan soda lakes. The hot springs of Lake Bogoria were chosen for their high temperature and pH. Thus this environment was deemed to provide a unique habitat for isolation of thermohalotolerant alkaliphiles.

The purpose of this paper is to establish that isolate BOG6[T] produces carotenoids of industrial interest, and further to describe its taxonomic position. A taxonomic approach encompassing morphological and metabolic properties and molecular methods was employed to characterise the isolate BOG6[T]. For construction of phylogenetic trees, the 16S ribosomal RNA (rRNA) gene sequence was used. 16S rRNA is classically used in construction of phylogenetic trees because it is an orthologous gene whose original function of encoding the small sub-unit ribonucleoprotein, is still retained among bacterial species. The 16S rRNA sequence is also one of the most evolutionarily conserved and widespread sequences in nature. These features make the 16S RNA gene ideal in molecular taxonomy (Woese et al., 1990; Pace, 1997).

MATERIALS AND METHODS

Isolation and growth conditions

Water and soil samples were taken from the out-flow of the hot springs of Lake Bogoria (0° 22'S and 36° 05'E), inoculated overnight at 37°C in a modified Horikoshi (MH) medium (Horikoshi and Akiba, 1982) made up of yeast extract 5 gl^{-1}; peptone 5 gl^{-1}; glucose 2 gl^{-1}; KH_2PO_4 1 gl^{-1}; $MgSO_4.7H_2O$ 0.2 gl^{-1}; agar 15 g l^{-1}; Na_2CO_3 (7.5% w/v) 40 ml l^{-1}; Trace elements (SL 10) 1 ml l^{-1}. Colonies were further isolated on the same medium. Strain BOG6[T], which formed bright red colonies was selected for further analysis. Unless otherwise stated, strain BOG6[T] was routinely grown on a modified Horikoshi (MH) medium at 37°C.

Phylogenetic analysis and G+C content of the DNA

Genomic DNA was prepared as described in Golyshina et al. (2000). The 16S rDNA was amplified using the primer pair F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTACGACTT- 3') that amplifies bacterial 16S rDNA from position 27 to 1492 in the *E. coli* numbering system. The primers were supplied by MWG Biotech, Ebersberg, Germany. The PCR amplifications were done under the conditions described previously (Yakimov et al., 1998, Golyshina et al., 2000). The amplification products were purified from agarose gel using the QIAXEX II extraction kit (Qiagen) using the manufacturer's protocol. Purified 16S rDNA was sequenced directly in an Applied Biosystems 373A DNA Sequencer using *Thermus aquaticus* (*Taq*) DNA polymerase with the PCR primers (F27 and R1492) in the forward and reverse directions, and the Perkin-Elmer fluorescent dye-labelled dideoxy-nucleotides (Perkin-Elmer, USA). The reactions were incubated in a Perkin-Elmer thermocycler, and after removal of excess primers the fluorescent labeled fragment sets were resolved by electrophoresis in the ABI 373A automatic sequencer and bases called by the program ABI Sequence Analysis Software v3.3 for Macintosh (Apple Inc, USA).

The sequence was added to an alignment of homologous bacterial 16S rDNA primary structures using the MUSCLE program (Edgar, 2004). Phylogenetic analyses were performed using MEGA software, version 4.0 (Tamura et al., 2007) with distance-based neighbour-joining, maximum-parsimony and maximum-likelihood methods (Felstein, 1985; Saitou and Nei, 1987; Tamura et al., 2004). Confidence in the topology obtained from these analyses was gauged using bootstrap resampling methods in MEGA software, version 4.0 and included 1000 replications. Bayesian analysis was completed using MRBAYES, version 3.1.2. (Ronquist and Huelsenbeck, 2001) using four-chain Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) analysis. The general time-reversible model of nucleotide evolution was used with an assumption of a discrete gamma distribution (GTR+I+C). Trees were sampled every 1000 generations for a total of 1 000 000 trees. The first 1000 trees were deleted as the 'burn-in' of the chain. The log-likelihood scores of sample points against generation time were plotted using TRACER software, version 1.4.1 (available at http://tree.bio.ed.ac.uk/software/tracer/) to ensure stationarity was achieved. Trees and model parameter values were sampled from a target distribution generated when chains converged. Bayesian consensus trees were built using the 'sum t' option in MRBAYES. Branch support was calculated as posterior probability.

The G + C content were determined by HPLC according to the method of Mesbah et al. (1989) as modified by Logan et al. (2000).

Morphological, physiological and biochemical analyses

Mid-logarithmically growing cells of the isolate BOG6[T] were pro-

cessed as described in Yakimov et al. (1998) and Golyshina et al. (2000). Electron micrographs of ultrathin sections negatively stained and shadow-casted bacterial cells were recorded with a 1024 x 1024 CCD-camera in an energy-filtered transmission elec-tron microscope CEM 902 (Zeiss, Oberkochen, Germany) in magnification range from x7000 to x30000 at 80 kV.

The temperature range for growth was determined by growing the strain on modified Horikoshi (MH) medium and incubating at 4, 10, 25, 30, 37, 40, 42, 45, 50 and 55°C for 7 days. Growth was consi-dered positive if the strain formed visible colonies on the solid medium. The pH range for growth was investigated by inoculating the cells in liquid MH medium at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 9.5, 10.0 and 10.5 and incubating, with shaking at 37°C for 7 days. For the determination of halotolerance, the MH solid medium was supplemented with 1 - 20% w/v NaCl, and incubated at 37°C for 7 days.

The metabolic profile of strain BOG6[T] was tested using Biolog GN2 microplates (Biolog, CA, USA). The strain was grown on MH agar medium at 37°C for 18 h. Bacterial biomass was scraped from the agar plates and resuspended in M9 minimal medium (Sambrook et al., 1989) supplemented with sodium carbonate so-lution to pH 9.5. The OD_{595} was adjusted to 0.35 and 150 l of the suspension was added into each well. After incubating for 24 h at 37°C reduction of tetrazolium dye to formazan was determined by Emax precision microplate reader (Molecular Devices, PA, USA). The ability to utilize individual substrates was confirmed by inocu-lating the cells in M9 minimal medium supplemented with trace elements SL 10 ($FeCl_2.4H_2O$ 1.5 gl^{-1}; $ZnCl_2$ 0.07 gl^{-1}; $MnCl_2.4H_2O$ 0.1 gl^{-1}; $CoCl_2.H_2O$ 0.19 gl^{-1}; $CuCl_2.2H_2O$ 0.002 gl^{-1}; $NiCl_2.6H_2O$ 0.024gl^{-1}; $Na_2MoO_4.2H_2O$ 0.036 gl^{-1}) and the substrate.

Tests for utilization of additional substrates were performed using the API 20 NE system (bioMérieux, France) which tests nitrate reduction, indole production from tryptophan, presence of urease, arginine dihydrolase, -galactosidase, -glucosidase, cytochrome oxidase, protease, glucose fermentation and assimilation of several other substrates.

To test the production of other enzymes, 1.5 ml of an overnight liquid culture was centrifuged for 1 min at 10 000 g. The pellet was resuspended in 0.5 ml 100 mM sodium phosphate buffer pH 7.0 and sonicated for 5 min. The lysate was cleared by centrifugation at 10 000 g for 30 s. 3 µl of the supernatant was added to 30 µl of the following substrates: pNP- galactoside, pNP- galactoside, pNP- glucoside, pNP- glucoside and pNP- fucoside. All the sub-strates were prepared in concentrations of 100 mM except for pNP-fucoside (20 mM) in 100 mM sodium phosphate buffer pH 7.0. The reaction mixture was incubated for 1 h at 37°C. The enzyme activity was revealed by development of a yellow colour in the reaction mixture when pNP is released. Hydrolysis of the glycosidic linkage was confirmed by running the reaction products on thin layer chromatography (TLC).

Chemotaxonomic characterisation

Fatty acids were extracted using a modified Bligh-Dyer procedure, as described previously (Bligh and Dyer, 1959; Vancanneyt et al., 1996). Bacterial biomass (1 g) was suspended in 100 ml methanol/dichloromethane/phosphate buffer (52.6:26.3:21.1) and sonicated for 15 min (Labsonic U, Braun, Germany). After overnight incuba-tion at room temperature 153.4 ml methanol/dichloromethane/phosphate buffer (35.4:61:57) was added followed by 5 min sonication. The samples were centrifuged at 5860 g for 15 min to separate the phases. The dichloromethane phase was filtered through dry sodium sulphate and a hydrophobic filter. The metha-nol/phosphate buffer phase was re-extracted by the addition of 25 ml dichloromethane, then centrifuged and filtered. This total lipid fraction was analysed by gas chromatography.

High perfomance liquid chromatography

Carotenoids were extracted from cell pellets in acetone, which was subsequently evaporated under a stream of nitrogen gas. The caro-tenoids extract was loaded into an HPLC silica column (Agilent Zorbax SB- 18 column) with ethyl acetate as the mobile phase. Astaxanthin was detected at 481 nm.

Mass spectrometry

Fast-atom-bombardment mass spectrometry (FAB-MS) was per-formed in the negative mode with a mixture of triethanolamine and tetramethylurea (2:1, v/v) as a matrix, on the first of two mass spec-trometers of a tandem high resolution instrument of EIBIE2B2 configuration (JMS-HX/HX110A;JEOL) at 10 kV accelerating voltage. Resolution was set to 1:2000. The JEOL FAB gun was operated at 6 kV with xenon.

RESULTS

Microscopy and ultrastructural analysis

From shadow-casted (Figure 1A) and negatively stained samples, strain BOG6[T] cells were found to be mono-polarly and monotrichously flagellated (Figure 1A: fl). Cells generally had a short-rod or coccoid shape with cellular dimensions from 1.100 - 1.500 µm in diameter and a mean of 1.113 µm. Ultrathin sectioned bacteria showed a clear-cut architecture of the cell wall, which showed the presence of an outer membrane (Figure 1B; om), which outlined the electron transluscent periplasmic space but did not show a pronounced murein layer. The cell interior showed a dense cytoplasmic matrix. This matrix occasionally contained electron transluscent non-membrane contoured inclusions. The inclusions are presumably polyhydroxybutyrate storage polymers. The bacterial chromosome is shown as the condensed DNA fibres within the cytoplasm (Figure 1C: BChr). Nearly all cells were studded with vesicles immediately beneath the cytoplasmic membrane (Figure 1C: arrows), often con-centrated in the polar region. They appear to be limited by a membrane-like envelope, as outlined by its double-track ultrastructure (Figure 1B: arrowheads). These vesi-cles are in general 18.7 - 68.1 nm in size with a mean of 46.8. Cells were motile and negative to the Gram's stain. Colonies on MH agar were flat and red in colour.

Chemotaxonomic properties

Fatty acid analyses revealed that C18: 1ω7c was the major compound at 78.9 of the total lipid content. Other fatty acids were present at the following amounts: C 18:0 (7.6); C12:0 (4.2); C18: 1 12c (2.8); C 16:0 (1.9) and C 14:0 (1.7).

The presence of astaxanthin was determined by HPLC analysis and mass spectroscopy. The retention time on HPLC was identical to a synthetic astaxanthin standard at 20.7 min. The $_{max}$ of the purified astaxanthin in ethyl acetate was 476.8 nm. Mass spectral analysis for both
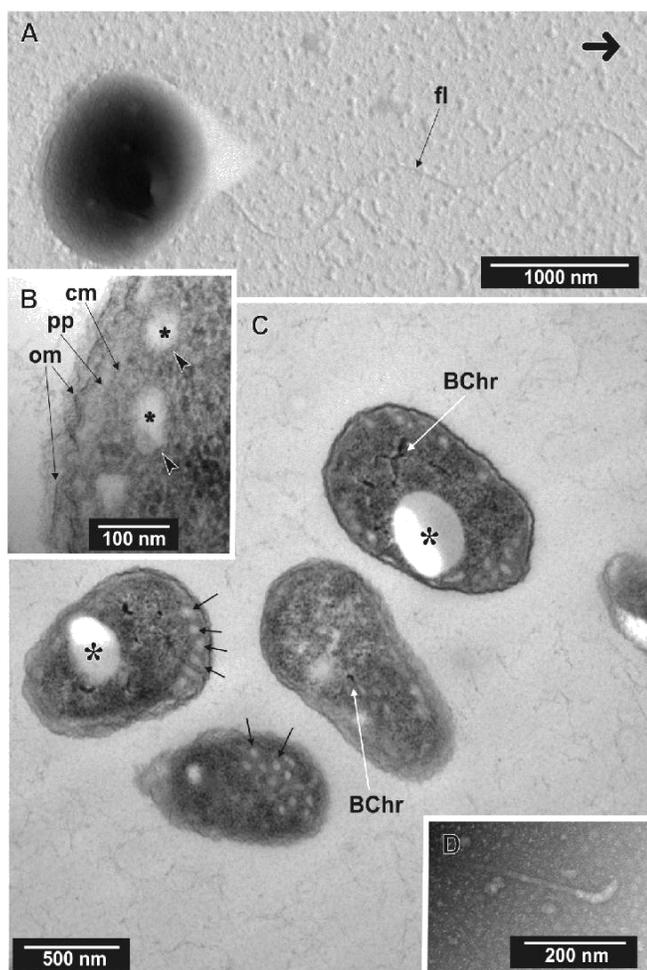
Figure 1. Transmission electron microscopy of mid- exponential phase cells of *P. bogoriensis* . (A) Shadow-casted flagellated coccoid cell. The flagellum (fl) shows the characteristic sinusoidal shape. Arrow indicates the shadowing direction. (B) Detailed view of the cell, close to the cell wall; the outermost layer is outer membrane (om), which limits the periplasm (pp) on one side. The cytoplasmic membrane (cm) is only weakly visible because of the low contrast. Asterisks indicate cytoplasmic vesicles, out lined by a membrane like surface (arrowheads). (C) Survey view of ultrathin sectioned cells, which distinctly show the bacterial chromosome (B.Chr), polyhydroxybutyrate inclusions (asterisks), partially sectioned and cytoplasmic vescles (arrows). (D) Negatively stained flagellum hook.

sample and synthetic astaxanthin standard showed peaks at 597.4 and 619.4 corresponding to [M+H]$^+$ and[M+Na]$^+$ respectively. The other mass spectral peak at 565.4 [M+H]$^+$ corresponded to the structure of canthaxanthin. Total carotenoids and astaxanthin yields were 3.7 and 0.4 mg/g of cells respectively.

Phylogenetic analysis

Tree topologies generated by different methods were

similar with no supported conflicts. Strain BOG6^T 16S rDNA nucleotide sequence was more similar to *P. aestuarii* (98%), *P. zeaxanthinifaciens* (97%), *P. marinus* (97%) and *P. homiensis* (96%). Strain BOG6^T clustered with *P. aestuarii* in the phylogenetic tree (Figure 2), and branch separation between the two microorganisms was supported by a bootstrap percentage of 99% (50% regarded as significant) and posterior probability (PP) of 100% (94% regarded as significant).
G+ C content of DNA was 66.7 mol %

Growth at different temperatures and pH

Strain BOG6^T grew within the temperature range of 30 - 45°C, with optimum temperature for growth at 40°C. The isolate has therefore a higher temperature optimum compared to other astaxanthin producing species of the *Paracoccus* described in literature. For example *P. marcusii* and *P. carotinifaciens* do not grow at temperatures above 33°C (Harker et al., 1998; Tsubokura et al., 1999b).

  Strain BOG6^T could grow in pH range of 7.5 - 10.5 with the optimum pH for growth at 9.5. Little growth was recorded at pH 7 or below. Strain BOG6^T is thus an obligate alkaliphile which is not a common trait among the *Paracoccus* species. All previously described species of *Paracoccus* with the exception of *P. aestuarii* and *P. alcaliphilus,* grow well at or below pH 7. Strain BOG6^T grows well in the presence of up to 6.5% NaCl, thus is halotolerant. *P. aestuarii* does not grow in media containing NaCl concentrations beyond 5%.

Physiological tests

Metabolically, strain BOG6^T could utilize L-arabinose, D-sorbitol, L-rhamnose and raffinose. These substrates are assimilated by neither *P. aestuarii* nor *P. zeaxanthifaciens*, close phylogenetic relatives of strain BOG6^T. Moreover, unlike strain BOG6^T, *P. homiensis* does not utilize L-arabinose, maltose, D-glucose and quinic acid. In contrast to *P. marcusii,* strain BOG6^T does not utilise citrate, inositol and formic acid as carbon sources. Moreover strain BOG6^T could not utilize methanol, methylamine, trimethylamine and dimethylsulfoxide, substrates which are metabolised by some members of the *Paracoccus* genus.

  Tests for enzymatic activities showed that strain BOG6^T constitutively produce and glucosidases, and galactosidases and an alkaline protease. –fucosidase activity was however not observed.

DISCUSSION

Strain BOG6^T produces carotenoids, with astaxanthin as the major carotenoid (0.4 mg/g of cells). Most of the carotenoids are found within intracellular vesicles. Six other
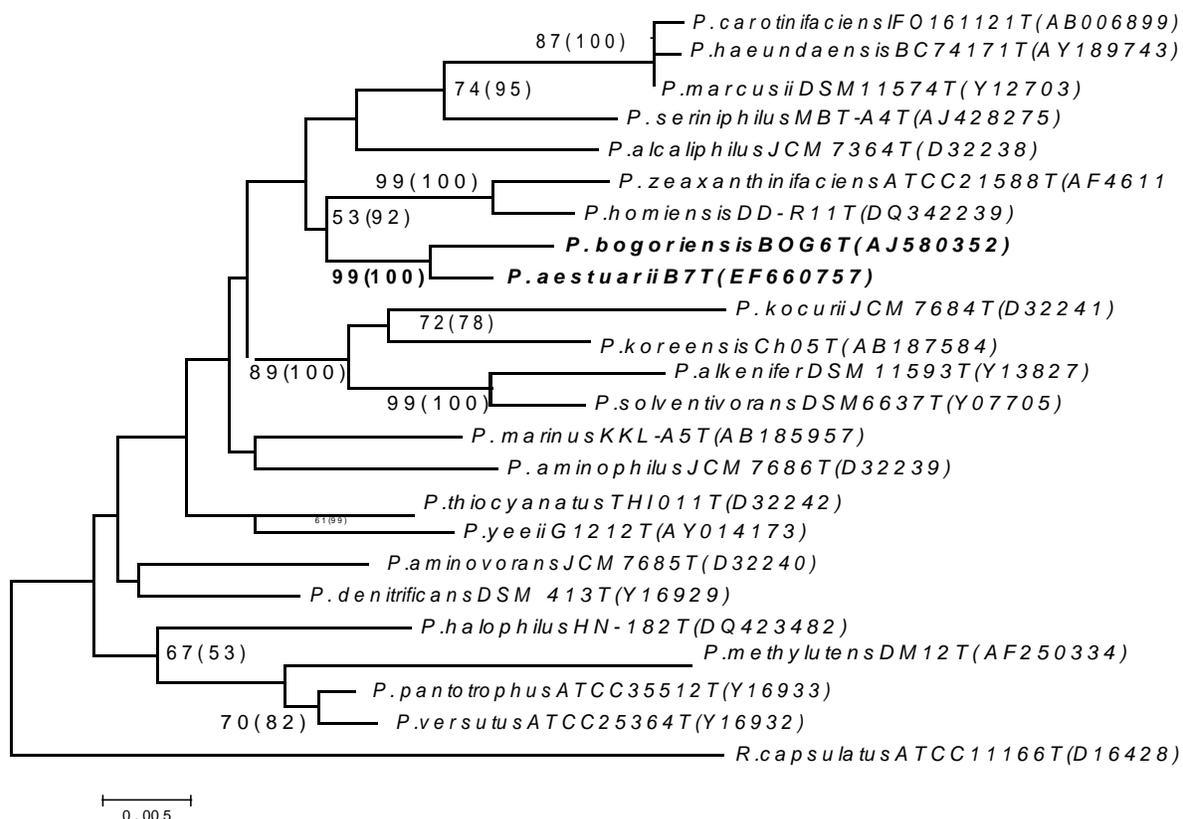
Figure 2. Maximum-likelihood phylogenetic tree of strain BOG6[T] (*Paracoccus bogoriensis*) and the most closely related micro-organisms, created using MEGA software, version 4 (Tamura et al., 2007). Numbers reflect bootstrap support percentages (of 1000 replications) generated in MEGA software, version 4.0; numbers in parentheses are Bayesian posterior probabilities generated using MRBAYES, version 3.1.2 (Huelsenbeck and Ronquist, 2001). Only those bootstrap percentages of at least 50% have been shown as they demonstrate good support. The sequence of *Rhodobacter capsulatus* ATCC 11166[T] was used as the out-group. *Bar* 0.5% sequence divergence.

other species of *Paracoccus: P. marcusii, P. zeaxanthinifaciens, P. carotinifaciens, P. homiensis, P. haeundaensis* and *P. aestuarii* have also been shown to produce carotenoids. The spectrum of carotenoids produced by these bacteria differs. Phylogenetically, strain BOG6[T] is close to *P. aestuarii* and *P. zeaxanthinifaciens* which produce astaxanthin and zeaxanthin as the major carotenoids respectively. *P. zeaxanthinifaciens* was not found to produce astaxanthin. Further study of the three species is of merit to shed insight into the genotypic factors responsible for the differing phenotypes.

Motility is an additional phenotype that varies amongst members of the *Paracoccus* genus. Only three other members of the genus- *P. carotinifaciens, P. homiensis* and *P. versutus* (Katayama et al., 1995) have so far been described as motile. Strain BOG6[T] was motile and possessed a polar flagellum with a characteristic hook clearly visible under electron microscopy. It is curious that its close phylogenetic relatives, *P. aestuarii* and *P. zeaxanthinifaciens* are non-motile. However, phylogenetic clustering based on 16S rRNA gene sequences is due to ancestral relationships (Kelly et al., 2006b, Tsubokura et

al., 1999b), and it is therefore probable that motility is a functional morphotype that evolved independently in each species.

The major fatty acid of Strain BOG6[T] was C18: 1ω7c (78.9%). This falls within the range of 68.9 - 84.3% reported for the other species of *Paracoccus* (Kelly et al., 2000; Roh et al., 2009) and similar to that of the members of the - subclass of *proteobacteria* (Kelly et al., 2000). Furthermore strain BOG6[T] accumulated poly-hydroxybutyrate that is a common trait among the *Paracoccus* when growing under carbon sufficient conditions (Kelly et al., 2000).

Strain BOG6[T] showed strong alkaliphilic characteristic (pH range 7.5 -10.5). This is not a dominant trait among the *Paracoccus. P. aestuarii* and *P. alcaliphilus* (pH range 7 - 9.5) (Urakami et al., 1989; Roh et al., 2009) are also an alkaliphiles. Most of the alkaliphiles have been isolated from marine sources. Strain BOG6[T] is the first member of the genus to be isolated from a salt lake environment. The saline environment from which strain BOG6[T] was isolated does nonetheless have some similarities with the sea environments, notably both are highly

saline, suggesting similar evolutionary trajectories for these organisms. It could be possible that the bacterium was introduced into the lake by the migratory birds that travel from the marine habitats to the lake.

Like other members of the *Paracoccus* genus strain BOG6[T] was found to be a versatile heterotroph capable of assimilating a variety of substrates. Other distinguishing characteristics of strain BOG6[T] have been summarized in Table 1.

The G + C content of strain BOG6[T] was 66.7 mol %. The range for the *Paracoccus sp* described to date in literature is 61.3 - 71.0 mol % (Roh et al., 2009). Phylogenetically strain BOG6T clustered closest to *P. aestuarii* (Figure 2). A publication reporting *P. aestuarii* appeared in April 2009 (Roh et al., 2009) when this manuscript was under preparation. DNA-DNA hybridization could therefore not be performed between the microorganism and strain BOG6[T]. The results reported here nonetheless unequivocally show critical genotypic and phenotypic differences between the two strains, for example, the differences in motility, C18: 1 ω7c fatty acid content (78.9% for strain BOG6[T] compared to 68.9% for *P. aestuarii*), G + C content (see above) and temperature of growth (Table 1). The metabolic variability between strain BOG6[T] and *P. aestuarii* is additionally striking: Of the substrates under comparison- *P. aestuarii* could not assimilate any of the L-saccharides such as L-fucose, L-rhamnose, L-arabinose, L-xylose or L-sorbose while strain BOG6[T] utilized all of them. In contrast strain BOG6[T] does not uti-lize the structurally peculiar sugar *myo*-inositol and citrate which are carbon sources for *P. aestuarii*.

As already noted above, while the species of genus *Paracoccus* share features such as having Gram negative coccoid or coccobacilloid cells, accumulation of poly-hydroxybutyrate, possession of C18: 1 ω7c as the major fatty acids and a high mol% G + C content (ranging between 61.3 -71% for most); certain physiological and molecular characteristics can be used to differentiate the genus *Paracoccus* at the species level.

Table 1 shows some of the characteristics that distinguish strain BOG6[T] as well as other members of the *Paracoccus* genus from one another. The *Paracoccus* species can be differentiated on the basis of motility, variant temperature and pH requirements, pigmentation and metabolic diversity. The optimal temperature for the majority of the *Paracoccus* has been stated by Kelly et al. (2006) to range between 25 - 37°C. Some species are capable of autotrophic growth such as *P. dentrificans*, *P. pantotrophus* and *P. thiocyanatus* while others are capable of chemolithotrophy or autotrophy, for example *P. marcusii, P. zeaxanthinifaciens* and *P. carotinifaciens*. Strain BOG6[T] could not grow in media containing me-thanol as the only carbon source, indicating it was unable to grow methylotrophically- which biochemically is a form of autotrophy. Further, members of the *Paracoccus* genus diverge in the optimal temperature required for growth at the species level. For instance *P. aminovorans,*

P. alcaliphilus , *P. solventirans* and *P. marcusii* cannot grow beyond temperatures above 37°C, while others such as *P. homiensis*, *P. zeaxanthinifaciens* or strain BOG6[T] are optimally cultivated above 37°C. Some species grow cannot grow at all in acidic media (for example *P. alcaliphilus* and strain BOG6[T]). In addition some *Paracoccus* species (e.g. *P. seriniphilus*) cannot produce carotenoid pigments, hence this property serves to distinguish various *Paracoccus* species.

At the molecular level, comparison of 16S rRNA gene sequences of the *Paracoccus* have shown that clustering depicts ancestral relationships with the - subclass of the proteobacteria with the closest relatives being the genus *Rhodobacter* (Tsubokura et al., 1999b). 16S rRNA gene sequence similarity within the genus is in the range of 93.5 - 99.8% (Kelly et al, 2006). Strain BOG6[T] has a sequence similarity closest to *P. aestuarii* (98%). This is not unusual as high sequence similarity is common among members of the *Paracoccus* genus. For example *P. carotinifaciens* and *P. marcusii* share 99.8% sequence similarity (Kelly et al., 2006b). High sequence similarity does not therefore indicate species identity among the *Paracoccus.* The phylogenetic distinction among the *Paracoccus* species is supported by the long branches in the dendrogram (Figure 2) that is demonstrative of a lack of close relationship with the nearest neighbor. Moreover bootstrap values from maximum likelihood trees and posterior values from the Bayesian analysis (Figure 2) support high degree of divergence among the *Paracoccus* species including strain BOG6[T], showing these are true species. Kelly et al. (2006b) have in fact predicted that the high degree of genetic divergence shows that many *Paracoccus* species still remain uncovered.

Taken together these genotypic and phenotypic characteristics of strain BOG6[T] show that it belongs to a new species in the genus *Paracoccus*. Its production of many enzymes active at alkaline pH, make the bacterium a potential microorganism for biotechnological exploitation. Its production of commercially important xanthophylls such as astaxanthin underscores this potential. Since it is tolerant for high pH, this suggests it would be less susceptible to culture contamination in a production process.

The study has added to the body of described bacteria that make up Lake Bogoria microbial community- a subject of numerous ecological investigations over the past three decades. Further studies would be required to determine the precise role strain BOG6[T] plays in this complex ecosystem. The name *Paracoccus bogoriensis* sp. nov. is proposed for strain BOG6[T] .

Description of *Paracoccus bogoriensis*

P. bogoriensis (bo.go.ri.en .sis. N.L. masc. adj. *bogoriensis* pertaining to Lake Bogoria, the location from which the organism was first isolated).

Cells are Gram-negative, motile, aerobic, non-spore forming cocci to short rods 0.99 - 1.11 × 1.1 - 1.5 μm in

Table 1. Differential characteristics of strain BOG6[T] from other *Paracoccus* species. Data from Berry et al. (2003), Harker et al. (1998), Kelly et al. (2006b), Kim et al. (2006, Lee et al. (2004), Roh et al. (2009) and Tsubokura et al. (1999b). +, present; -, absent; NR, not reported.

Characteristic	Strain BOG6[T]	*P. aestuarii*	*P. zeaxanthinifaciens*	*P. homiensis*	*P. marcusii*	*P. carotinifaciens*	*P. denitrificans*	*P. alcaliphilus*	*P. aminophilus*	*P. aminovorans*	*P. solventivorans*	*P. versutus*	*P. kocurii*	*P. thiocyanatus*	*P. seriniphilus*
Motility	+	-	-	+	-	+	-	-	-	-	-	+	-	-	-
Growth at 40 ºC	+	-	+	+	-	-	NR	-	-	-	NR	NR	NR	NR	-
Growth on:															
D-Sorbitol	+	-	-	+	+	+	+	+	-	+	-	+	-	+	+
myo-Inositol	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+
Raffinose	+	-	-	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	-
Quinic acid	+	NR	-	-	+	+	NR	NR	NR	NR	NR	NR	NR	NR	-
L-Asparagine	+	NR	+	+	-	+	+	+	+	+	+	+	+	+	+
L-Aspartic acid	+	NR	+	-	-	+	NR	NR	NR	NR	NR	NR	NR	NR	+
Acetic acid	+	NR	-	+	-	NR	+	+	+	+	+	+	+	+	+
Citric acid	-	+	-	+	+	NR	+	+	-	-	-	-	-	-	+
Formic acid	-	NR	-	+	+	NR	+	-	-	-	+	+	+	-	+
Methanol	-	NR	NR	NR	-	NR	+	+	-	-	-	+	-	-	NR
Rhamnose	+	-	-	+	-	NR	-	NR	NR	NR	NR	NR	NR	NR	-
Pigmentation	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-

size. Colonies on MH agar are circular, smooth, convex and orange to red in colour due to the accumulation of astaxanthin. Growth occurs between 30 and 45°C with an optimum at 40°C and at pH 7.5 - 10.5 with an optimum at pH 9.5. Catalase- and oxidase-positive. Does not reduce nitrate to nitrite, and does not perform denitrification. Ferments glucose and hydrolyses starch. Utilizes L-arabinose, dextrin, I-erythritol, D-fructose, D-galactose, α-D-glucose, glycerol, α-D lactose, maltose, D-mannose, D-melibiose, raffinose, L-rhamnose, saccharose, sorbitol, L-sorbose, sucrose, trehalose, ribose, acetic acid, formic acid, D-gluconic acid, D-glucuronic acid, β−hydroxybutyric acid, palmitic acid, succinic acid, quinic acid, L-alanine, L- asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine and Tween 80. Does not utilize α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, m-inositol, gelatin, DL-α-glycerol phosphate, glycogen, citric acid, lactic acid, methanol, methylamine, L- glycine, L- lysine, L-methionine and L-threonine. The G+C content is 66.7 mol %. The type strain is BOG6[T] (=DSM16578 =LMG22798). The GenBank 16S rDNA nucleotide sequence accession number is AJ580352.

REFERENCES

Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants, and degenerative diseases of aging Proc. Natl. Acad. Sci. USA. 90: 7915-22.

Beijeirinck MW, Minkman DCJ (1910). Bildung und Verbrauch von Stick-oxydul durch Bakterien. Zentralbl. Bakteriol. (in German) 25: 30–63.

Bendich A (1989). Carotenoids and the immune response. J. Nutr. 119: 112-115.

Berry A, Janssens D, Hümbelin M (2003). *Paracoccus zeaxanthinifaciens* sp nov, a zeaxanthin-producing bacterium. Int. J. Syst. Evol. Micro. 53: 231-238.

Bligh EG, Dyer WJ (1959). A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Borowitzk MA (1988). Vitamins and fine chemicals from microalgae, In Borowitzka, M. A. and L. J. Borowitzka (eds.), Micro-algal Biotechnology. Cambridge University Press, Cambridge, U.K. pp. 153-196.

Britton G (1995). Structure and properties of carotenoids in relation to function. FASEB J. 9: 1551-1558.

Davis DH, Doudoroff M, Stanier RY (1969). Proposal to reject the genus Hydrogenomonas: taxonomic implications. Int. J. Syst. Bacteriol. 19: 375–390.

Dufossé L (2006). Microbial Production of Food Grade Pigments. Food Technol. Biotechnol. 44: 313–321

Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32: 1792-1797.

Felstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Golyshina VO, Pivovarova TA, Karavaiko GI, Kondrat'eva TF, Moore ERB, Abraham W-R, Lünsdorf H, Timmis KN, Yakimov MM and Golyshin PN (2000). *Ferroplasma acidiphilum* gen nov, sp nov, an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam nov, comprising a distinct lineage of the Archaea. Int. J. Syst. Evol. Micro. 50: 997-1006.

Grant WD, Mwatha WE, Jones BE (1990). Alkaliphiles: ecology, diversity and applications. FEMS Microbiol Rev 75: 255-270.

Guerin M, Huntley ME, Olaizola M (2003). Haematococcus astaxanthin: Applications for human health and nutrition. Trends Biotechnol. 21:210 –216.

Harker M, Hirschberg J, Oren A (1998). *Paracoccus marcusii* sp nov, an orange Gram-negative coccus. Int. J. Syst. Bacteriol. 48: 543-548.

Hirschberg J, Harker M (1999). Carotenoid-producing bacterial species and process for production of carotenoids using same. US Patent 5: 935-808.

Horikoshi K, Akiba T (1982). Alkalophilic Microorganisms, Springer, Berlin, Heidelberg and New York.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754 - 755.

Jones BE, Grant WD, Collins NC, Mwatha WE (1994). Alkaliphiles: diversity and identification. In: Priest FG, Ramos-Comerzana A, Tindall BJ (eds) Bacterial diversity and systematics Plenum, New York, pp. 195-229.

Jacobson GK, Jolly SO, Sedmak JJ, Skatrud TJ, Wasileski JM (2003). Astaxanthin over-producing strains of *Phaffia rhodozyma*, methods for their cultivation and their use in animal feeds. US patent 200(300): 49241.

Johnson EA, An GH (1991). Astaxanthin from Microbial Sources. Crit. Rev. Biotechnol. 11: 297- 326.

Johnson E, Schroeder W (1995). Microbial carotenoids. Adv. Biochem. Eng. Biotechnol. 53: 119 - 178.

Jyonouchi H, Sun S, Gross M (1995). Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without Vitamin A activity, enhances *in vitro* immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr. Cancer 23: 171-83.

Katayama Y, Hiraishi A, Kuraishi H (1995). *Paracoccus thiocyanatus* sp nov, a new species of thyocyanate-utilizing facultative chemolithotroph, and transfer of Thiobacillus versutus to the genus *Paracoccus* as *Paracoccus versutus* comb nov with emendation of the genus. Microbiol. 141: 1469-1477.

Kelly DP, Rainey FA, Wood AP (2000). The genus *Paracoccus:* In The Prokaryotes: an evolving electronic resource for the microbiological community, release 3.4. Edited by M Dworkin, N Falkow, H Rosenberg, KH Schleifer, E Stackenbrandt New York: Springer http://141.150.157.117:8080/prokPUB/index.htm

Kelly DP, Euzéby JP, Goodhew CF, Wood AP (2006). Redefining *Paracoccus denitrificans* and *Paracoccus pantotrophus* and the case for a reassessment of the strains held by international culture collections. Int. J. Syst. Bacteriol. 56: 2495 - 2500.

Kelly DP, Rainey FA, Wood AP (2006). The genus *Paracoccus:* In The Prokaryotes, Edited by Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt. Springer, New York. 5: 232–249.

Kim BY, Weon HY, Yoo SH, Kwon SW, Cho YH, Stackebrandt E and Go SJ (2006). *Paracoccus homiensis* sp nov, isolated from a sea-sand sample. Int. J. Syst. Evol Microbiol. 56: 2387-90

Lee JH, Kim YS, Choi TJ, Lee WJ, Kim YT (2004). *Paracoccus haeundaensis* sp. nov., a Gram-negative, halophilic, astaxanthin-producing bacterium. Int. J. Syst. Evol. Microbiol. 54: 1699-702.

Logan JM, Burnens A, Linton D, Lawson AJ, Stanley J (2000). *Campylobacter lanienae* sp. nov., a new species isolated from workers in an abattoir. Int. J. Syst. Evol. Microbiol. 50: 865-72.

Lorenz RT, Cysewski GR (2000). Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. Trends Biotechnol. 18(4): 160-7.

Mesbah M, Premachandran U, Whitman WB (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. 39: 159-167.

McCall GH (1967). Geology of Nakuru-Thompson's Falls – Lake Hannington area Geological survey of Kenya Rep. No 1978

Miki W (1991).Biological functions and activities of animal carotenoids. Appl Chem. 63: 141-6.

Miyashita K (2009). Function of Marine Carotenoids. Forum Nutr. 61: 136-146.

Nelis HT, de Leenher AP (1991). Microbial sources of carotenoid pigments used in food and feed. J. Appl. Bacteriol. 70: 181-191.

Ong ASH, Tee ES (1992). Natural sources of carotenoids from plants and oils. Meth. Enzymol. 213: 142-167.

Pace NR (1997). A molecular view of microbial diversity and the biosphere. Science 276: 734–740.

Palozza P, Torelli C, Boninsegna A, Simone R, Catalano A, Mele MC, Picci N (2009). Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells. Cancer Lett. In Press. E-Pub doi:10.1016/j.canlet.2009.03.031

Pfander H (1992). Carotenoids: an overview. Meth. Enzymol. 213: 3-13.

Roh SW, Nam Y-D, Chang H-W, Kim K-H, Kim M-S et al. (2009). Paracoccus aestuarii sp. nov., isolated from tidal flat Sediment. Int. J. Syst. Evol. Microbiol. 59: 790–794.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd ed Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Snodderly DM (1995). Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am. J. Clin. Nutr. 62: 1448-1461.

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101: 11030-11035.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Tsubokura A, Yoneda H, Takaki M, Kiyota T (1999a). Bacteria for production of carotenoids. US Patent 5:858-761.

Tsubokura A, Yoneda H, Mizuta H (1999b). *Paracoccus carotinifaciens* sp nov, a new aerobic Gram-negative astaxanthin-producing bacterium. Int. J. Syst. Bacteriol. 49: 277-282.

Urakami T, Tamaoka J, Suzuki K, Komagata K (1989). *Paracoccus alcaliphilus* sp nov, an alkaliphilic and facultatively methylotrophic bacterium. Int. J. Syst. Bacteriol. 39: 116-121.

Vancanneyt M, Witt S, Abraham W-R, Kersters K, Fredrickson HL (1996). Fatty acid content in whole-cell hydrolysates and phospholipids of pseudomonads: a taxonomic evaluation. Syst. Appl. Microbiol. 19: 528-540.

Woese CR, Kandler O, Wheelis ML (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. U. S. A. 87: 4576–4579.

Yakimov MM, Golyshin PN, Lang S, Moore ERB, Abraham W -R, Lünsdorf H, Timmis K N (1998). *Alcanivorax borkumensis* gen nov sp nov, a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol. 48: 339-348.