Chelatococcus sambhunathii sp. nov., a moderately thermophilic alphaproteobacterium isolated from hot spring sediment

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A moderately thermophilic bacterial strain (HT4^T) isolated from a hot spring sediment was characterized phenotypically and phylogenetically. Cells were Gram-negative, aerobic, non-sporulating, rod-shaped and motile by means of a single polar flagellum. Both oxidase and catalase activities were positive. Heterotrophic growth was observed at pH 6.0–8.5 and at 20–50 °C; optimum growth occurred at pH 7.5–8.0 and 37–42 °C. The major cellular fatty acids were C_{14:0} 3-OH, C_{18:0} 3-OH, C_{18:1} 2-OH, C_{18:1} ω7c and C_{19:0} cyclo ω8c. The DNA G+C content of strain HT4^T was 67.8 mol%. 16S rRNA gene sequence analysis indicated that strain HT4^T clustered within the radiation of the genus *Chelatococcus* and showed 99.0% similarity with *Chelatococcus daeguensis* CCUG 54519^T and 96% similarity with *Chelatococcus asaccharovorans* DSM 6462^T. However, levels of DNA–DNA relatedness between strain HT4^T and *Chelatococcus daeguensis* CCUG 54519^T and *Chelatococcus asaccharovorans* DSM 6462^T were 52 and 20%, respectively. On the basis of the phenotypic, physiological and chemotaxonomic data, 16S rRNA gene sequence analysis and DNA–DNA hybridization results, strain HT4^T is considered to represent a novel species of the genus *Chelatococcus*, for which the name *Chelatococcus sambhunathii* sp. nov. is proposed. The type strain is HT4^T (=DSM 18167^T=JCM 14988^T).

Exploration of bacterial species in hot springs is of importance owing to the intriguing biogeochemistry and extreme conditions of these environments. The vast majority of bacterial species belonging to the most recent lines of descent, such as the *Proteobacteria*, are mesophilic, although slightly thermophilic, moderately thermophilic and even extremely thermophilic species have been described from these bacterial lineages. The *Alphaproteobacteria* includes many mesophilic species, but also a few slightly thermophilic or moderately thermophilic species, namely *Rubritepida flocculans* (Alarico *et al.*, 2002), *Porphyrobacter cryptus* (Rainey *et al.*, 2003), *Phenylobacterium lituiforme* (Kanso & Patel, 2004), *Acidicaldus organivorus* (Johnson *et al.*, 2006), *Nesiotobacter exalbescens* (Donachie *et al.*, 2006) and *Rubellimicrobium thermophilum* (Denner *et al.*, 2006). Several hot springs exist on the Indian subcontinent (Gupta *et al.*, 1975), but the micro-organisms present in these environments have been little studied (but

see Ghosh *et al.*, 2003). This paper describes a bacterial strain (HT4^T) isolated from a hot spring located at Athamallik, Orissa, India. Based on the results of the present polyphasic taxonomic study, strain HT4^T is considered to represent a novel species of the genus *Chelatococcus*.

The hot spring at Athamallik is situated at 80 m above sea level. It has a series of outlets within a radius of 70 m. The water temperature at the main outlet was 56 °C and in the surrounding areas ranged from 43 to 50 °C. The pH of sediment samples taken for analysis was 7.4. Sediment samples were transported to the laboratory without temperature control and subsequently transferred into 250-ml conical flasks containing 50 ml nutrient broth (Difco) and incubated on a shaker (ISF-1-V; Adolf Kuhner AG) at 200 r.p.m. and 45 °C. After 2 days incubation, the contents of the flasks were serially diluted and plated onto nutrient agar medium (Difco) and incubated at 37 °C for 4 days. Several colonies that developed at 37 °C were picked and purified by repeated streaking on the same medium. A colony with a mucoid cream colour was selected for further analysis; this was designated strain HT4^T.

Colonies of strain HT4^T grown on nutrient agar were circular, 1.5–2.5 mm in diameter, mucoid and smooth.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HT4^T is DQ322070.

A supplementary table detailing the fatty acid composition of strain HT4^T and other members of the genus *Chelatococcus* is available with the online version of this paper.

Single colonies appeared within 2–3 days at 37 °C. Cell morphology was examined by using transmission electron microscopy (model FEI MORGAGNI 268D). The presence of flagella was determined by using cells that were placed onto a carbon-coated grid and negatively stained with 2.0% phosphotungstic acid (Sharma *et al.*, 1989). Cells were rod-shaped, 0.8–1.0 µm wide and 2.3–2.7 µm long, motile by means of a single polar flagellum and generally occurred singly (Fig. 1). Cells stained Gram-negative and did not form spores. Oxidase activity was assayed with discs impregnated with dimethyl *p*-phenylenediamine (Himedia). Catalase activity was assayed by mixing a pellet of a freshly centrifuged culture with a drop of hydrogen peroxide (10%, v/v). Anaerobic growth was determined on tripticase soy agar (TSA; MP Biomedical LLC) and on TSA supplemented with potassium nitrate (0.1%, w/v) in an anaerobic jar (BD GasPak EZ system; Becton Dickinson), prepared according to the manufacturer's instructions. The jar was incubated at 37 °C and examined after 7 days. All other routine biochemical tests and procedures were performed as described by Panda *et al.* (2009). Phenotypic properties of strain HT4^T are given in the species description and characteristics that differentiate strain HT4^T from *Chelatococcus daeguensis* CCUG 54519^T and *Chelatococcus asaccharovorans* DSM 6462^T are given in Table 1.

Growth occurred at 20–50 °C, with optimum growth at 37–42 °C. Heterotrophic growth was observed at pH 6.0–8.5, with optimum growth at pH 7.5–8.0. Utilization of organic compounds other than amino acids was examined

Fig. 1. Electron micrograph of a cell of strain HT4^T, showing a single polar flagellum. Bar, 1 µm.

in mineral salts medium containing potential organic carbon sources at a concentration of 0.5% (w/v). Utilization of amino acids as sole carbon sources was tested by using single amino acids at a concentration of 0.2% (w/v). The mineral salts medium contained (per litre distilled water): 4.0 g Na₂HPO₄, 1.5 g KH₂PO₄, 0.01 g CaCl₂·5H₂O, 1.0 g NH₄Cl and 0.5 g MgSO₄·7H₂O. The medium was adjusted to pH 7.5 with 10 M NaOH. Utilization of nitrilotriacetate was tested by using the synthetic medium defined by Egli *et al.* (1988).

As strain HT4^T was isolated from a hot spring, aerobic growth on reduced sulfur compounds was tested in mineral salts medium supplemented with different inorganic sulfur sources (thiosulfate, tetrathionate or elemental sulfur) at a concentration of 0.1–1.0 g l⁻¹ in addition to yeast extract powder (2.0 g l⁻¹). The concentration of sulfur compounds in the culture filtrates was determined as described by Kelly *et al.* (1969). Sulfate content was determined according to Berglund & Sorbo (1960) and Gleen & Quastel (1952). Strain HT4^T was unable to utilize these sulfur sources; it therefore appears to be a chemo-organotroph. Yeast extract-mineral salts medium containing 0–5.0% (w/v) NaCl was inoculated and incubated at 37 °C for 4 days to test for salt tolerance. On this medium strain HT4^T was able to grow in the presence of 0–3.0% (w/v) NaCl but not in the presence of 4% NaCl.

Antibiotic resistance of strain HT4^T was checked on nutrient agar containing different concentrations of antibiotics. Strain HT4^T was resistant to kanamycin (50 µg ml⁻¹) and neomycin (30 µg ml⁻¹), but was susceptible to streptomycin (25 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), tetracycline (15 µg ml⁻¹), nalidixic acid (20 µg ml⁻¹), rifampicin (20 µg ml⁻¹) and ampicillin (40 µg ml⁻¹).

For estimation of the G + C content of the genomic DNA of strain HT4^T, DNA was enzymically degraded into nucleotides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC (Shimadzu Corp.) by using a VYDAC201 SP54 analytical column (C18, 5 µm, 250 × 4.6 mm) equipped with a guard column (201 GD54H; Vydac). Operating conditions were as described by Tamaoka & Komagata (1984): temperature 45 °C; 10 µl sample; solvent 0.3 M (NH₄)H₂PO₄/acetonitrile, 40:1 (v/v); pH 4.4; 1.3 ml min⁻¹. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The G + C content of the genomic DNA of strain HT4^T was 67.8 mol%, a value consistent with membership of the genus *Chelatococcus* (Egli & Auling, 2005).

Cellular fatty acids were analysed from cells grown on TSA plates for 3 days at 30 °C. Cells were saponified and transmethylated as described by Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were separated by using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID), which consisted of an Agilent model 6890N gas chromatograph. The fatty acid profile of strain HT4^T comprised C_{16:0} (1.2% of the total), C_{17:0} (1.5%), C_{18:0} (1.7%), C_{14:0} 3-OH (3.0%), C_{18:0} 3-OH (3.4%), C_{18:1}

Table 1. Differential characteristics between strain HT4^T (*C. sambhunathii* sp. nov.) and the type strains of recognized *Chelatococcus* species

+, Positive; –, negative. All tests were performed concurrently with the same methods and under the same conditions.

Characteristic	Strain HT4 ^T	<i>Chelatococcus daeguensis</i> CCUG 54519 ^T	<i>Chelatococcus asaccharovorans</i> DSM 6462 ^T
Isolation source	Hot spring	Textile waste matter	Wastewater, soil
Cell morphology	Rods	Rods	Diplococci
Motility	+	+	–
Hydrolysis of gelatin	+	–	+
NaCl tolerance range (w/v)	0.0–3.0	0.0–5.0	0.0–2.5
Utilization of:			
Mannose, xylose, gluconate, D-arabinose, cysteine	–	+	+
Glycerol, salicin, inositol, adonitol, cellobiose, xylitol, aesculin, rhamnose, galactose, methionine, threonine, tyrosine, lysine	–	+	–
Glycine	+	–	+
Nitrilotriacetate	+	–	+
Acid production from:			
Glucose, inositol, arabinose, rhamnose	–	+	–
pH for growth			
Range	6.0–8.5	5.5–10	5.5–9.5
Optimum	7.5–8.0	7.0–7.5	7.0–8.0

2-OH (7.1 %), C_{18:1}ω7c (73.1 %), C_{19:0} cyclo ω8c (7.8 %) and C_{20:1}ω7c (0.8 %). Although strain HT4^T showed a fatty acid profile typical of the genus *Chelatococcus* (Yoon *et al.*, 2008), it could be distinguished from the type strain of its closest phylogenetic neighbour (see Supplementary Table S1 in IJSEM online), *Chelatococcus daeguensis* CCUG 54519^T, based on the absence of C_{16:1}ω7c, iso-C_{15:0} 2-OH and C_{17:0} cyclo.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were carried out as described by Das *et al.* (1996). The gel-purified 16S rRNA gene was sequenced by using a CEQ Dye terminator cycle sequencing kit in a model CEQ 8000 automated DNA sequencer (Panda *et al.*, 2009). The 16S rRNA gene sequence of strain HT4^T was aligned with representative reference sequences of members of the *Alphaproteobacteria* by using MEGA version 4.0 (Tamura *et al.*, 2007). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Tree topologies and statistical significance of branch points were tested based on 100 bootstrap resamplings of the neighbour-joining data (Felsenstein, 1985).

Comparative analysis of 1378-nt positions of the 16S rRNA gene sequence of strain HT4^T with those of other members of the *Alphaproteobacteria* showed that strain HT4^T was related closely to members of this lineage (Fig. 2). 16S rRNA gene sequence analysis indicated that strain HT4^T was related most closely to *Chelatococcus daeguensis* CCUG 54519^T (99 % similarity) (Yoon *et al.*, 2008), followed by *Chelatococcus asaccharovorans* DSM 6462^T (96 %), *Beijerinckia mobilis*

DSM 2326^T and *Corbulabacter subterraneus* DSM 14364^T (94 %), and *Bosea thiooxidans* DSM 9653^T (93 %).

DNA–DNA hybridization experiments were carried out between strain HT4^T and *Chelatococcus daeguensis* CCUG 54519^T and *Chelatococcus asaccharovorans* DSM 6462^T following the method of Ezaki *et al.* (1989) by using a ³²P-labelled probe. Experiments were performed in triplicate with either HT4^T or *Chelatococcus daeguensis* DNA as probe. Mean levels of DNA–DNA relatedness between strain HT4^T and *Chelatococcus daeguensis* CCUG 54519^T and *Chelatococcus asaccharovorans* DSM 6462^T were 52 and 20 %, respectively. Therefore, considering the 70 % DNA–DNA relatedness cut-off point recommended for bacterial species delineation (Wayne *et al.*, 1991; Stackebrandt & Goebel, 1994), strain HT4^T should be regarded as representing a novel species of the genus *Chelatococcus*.

Based on phenotypic properties, cellular fatty acid analysis, phylogenetic analysis and DNA–DNA relatedness data, strain HT4^T is considered to represent a novel species of the genus *Chelatococcus*, for which the name *Chelatococcus sambhunathii* sp. nov. is proposed.

Description of *Chelatococcus sambhunathii* sp. nov.

Chelatococcus sambhunathii (sam.bhu.na.thi'i. N.L. masc. gen. n. *sambhunathii* of Sambhunath, named after Dr Sambhunath De, an eminent microbiologist in India).

Fig. 2. Neighbour-joining phylogenetic tree showing the position of strain HT4^T among related taxa based on 16S rRNA gene sequences. Bootstrap values as percentages of 100 resamplings are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

Cells are rods that are 0.8–1.0 µm wide and 2.3–2.7 µm long. Cells usually occur singly, and are motile by means of a single polar flagellum. Gram-negative. Catalase- and oxidase-positive. Able to grow at 20–50 °C and pH 6.0–8.5. Colonies on nutrient agar medium are round, mucoid and smooth. Positive for arginine dihydrolase, gelatin liquefaction, growth on MacConkey agar, King's A and King's B media, and nitrate reductase. Negative for the methyl red and Voges–Proskauer tests, indole production, urease, starch hydrolysis and H₂S production. Anaerobic growth occurs in the presence of nitrate. Positive for assimilation of proline, asparagine, valine, glutamine, glutamic acid, histidine, alanine, aspartic acid, leucine, isoleucine, serine and citrate, but negative for assimilation of sucrose, mannitol, sorbitol, oxalate, raffinose, sorbose, maltose, lactose, phenylalanine, arginine and tryptophan. The fatty acid profile comprises C_{16:0}, C_{17:0}, C_{18:0}, C_{14:0} 3-OH, C_{18:0} 3-OH, C_{18:1} 2-OH, C_{18:1} ω7c, C_{19:0} cyclo ω8c and C_{20:1} ω7c. The DNA G+C content of the type strain is 67.8 mol%.

The type strain, HT4^T (=DSM 18167^T=JCM 14988^T), was isolated from a hot spring in India.

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