Caenispirillum bisanense gen. nov., sp. nov., isolated from sludge of a dye works

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Two Gram-negative, non-spore-forming, motile and helical-shaped bacterial strains, K92^T and K93, were isolated from sludge from a dye works in Korea, and their taxonomic positions were investigated by means of a polyphasic approach. Strains K92^T and K93 grew optimally at 37 °C and pH 7.0–8.0 in the presence of 0.5 % (w/v) NaCl. They contained Q-10 as the predominant ubiquinone and C_{18:1}ω7c as the major fatty acid. The major polar lipids were phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified amino-group-containing lipids that were ninhydrin-positive. Their DNA G + C contents were 70.0 mol%. The 16S rRNA gene sequences of K92^T and K93 showed no differences, and the two strains had a mean DNA–DNA relatedness of 93 %. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains K92^T and K93 formed a distinct evolutionary lineage within the *Alphaproteobacteria*. The 16S rRNA gene sequences of strains K92^T and K93 exhibited similarity values of less than 91.5 % with respect to the 16S rRNA gene sequences of other members of the *Alphaproteobacteria*. The two strains were distinguishable from phylogenetically related genera through differences in several phenotypic properties. On the basis of the phenotypic, phylogenetic and genetic data, strains K92^T and K93 represent a novel genus and species, for which the name *Caenispirillum bisanense* gen. nov., sp. nov. is proposed. The type strain of *Caenispirillum bisanense* is K92^T (=KCTC 12839^T=JCM 14346^T).

In an attempt to investigate the microbial community in sludge from the wastewater treatment plant of a dye works in Korea, many bacterial strains were isolated and characterized taxonomically. This study focuses on two of these isolates, designated strains K92^T and K93. Comparative 16S rRNA gene sequence analysis indicated that strains K92^T and K93 form a deep branch within the *Alphaproteobacteria*. Accordingly, the aim of the present work was to determine the exact taxonomic positions of strains K92^T and K93 by using a polyphasic characterization that included the determination of phenotypic properties, a detailed phylogenetic analysis based on 16S rRNA gene sequences and analysis of genetic relatedness data.

A sludge sample collected from the wastewater treatment plant of a dye works at Daegu, Korea, was used as the source for the isolation of bacterial strains. Strains K92^T and K93 were isolated on nutrient agar (Difco) and trypticase soy

agar (TSA; Difco) at 30 °C, using the standard dilution plating technique. *Aquaspirillum itersonii* subsp. *itersonii* LMG 4337^T, *Aquaspirillum itersonii* subsp. *nipponicum* LMG 7370^T, *Aquaspirillum peregrinum* subsp. *peregrinum* LMG 4340^T and *Aquaspirillum peregrinum* subsp. *integrum* LMG 5407^T were obtained from the Laboratorium voor Microbiologie Universiteit Gent (LMG), Ghent, Belgium. The morphological, physiological and biochemical characteristics of strains K92^T and K93 were investigated using routine cultivation on TSA at 37 °C. Cell morphology was examined by light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–50 °C) was measured on TSA. Growth in the absence of NaCl and at various NaCl concentrations (0.5 and 1.0–10.0 %, w/v, in increments of 1.0 %) was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. The pH range for growth was determined in nutrient broth (Difco) that had been adjusted to various pH values (pH 4.5–10.5 in

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains K92^T and K93 are respectively EF100694 and EF100695.

A transmission electron micrograph of a cell of strain K92^T and cellular fatty acid profiles of strains K92^T and K93 and related taxa are available as supplementary material with the online version of this paper.

increments of 0.5 pH units) prior to sterilization by the addition of HCl or Na₂CO₃. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). DNase activity was examined by using DNase test agar with methyl green (Difco). Aesculin hydrolysis and nitrate reduction were studied as described previously (Lanyi, 1987). For *in vivo* pigment-absorption spectrum analysis, the two novel strains were cultivated aerobically in the dark at 37 °C in liquid *Erythromicribium/Roseococcus* medium (Yurkov *et al.*, 1994; DSMZ medium no. 767) with the modification that trisodium citrate was used instead of acetate. The cultures were washed twice, by centrifugation, using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson 450 sonicator. After removal of the cell debris by centrifugation, the absorption spectrum of the supernatant was examined in a Beckman Coulter DU800 spectrophotometer. Antibiotic susceptibility was tested on TSA plates by using antibiotic discs containing the following concentrations: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; and carbenicillin, 100 µg. The utilization of various substrates, activities of various enzymes and other physiological and biochemical properties were tested by using the API 20E, API 20NE, API 50 CH and API ZYM systems (bioMérieux); the cells were suspended in AUX medium, according to the manufacturer's instructions, to inoculate the API 50 CH system.

Cell biomass for DNA extraction and for isoprenoid quinone and polar lipid analyses was obtained from cultivation in trypticase soy broth at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by a PCR using two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For fatty acid methyl ester analysis, cell mass of strains K92^T and K93 was harvested from TSA plates after incubation for 2 days at 37 °C, and cell mass of the four *Aquaspirillum* reference strains was harvested after incubation for 3 days at 28 °C on solid medium (LMG medium no. 8) that contained (l⁻¹ distilled water) 1 g succinic acid, 10 g peptone, 1 g (NH₄)₂SO₄, 1 g MgSO₄·7H₂O, 2 mg FeCl₃·6H₂O, 2 mg MnSO₄·H₂O and

15 g agar (pH 7.0). The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The presence of phosphatidylcholine was identified by spraying with Dragendorff reagent (Sigma). The DNA G + C content was determined using the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed and the resulting nucleotides analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strains K92^T and K93 are given in the genus and species descriptions (see below), in Table 1 and in Supplementary Fig. S1 (available in IJSEM Online). The two strains were found to be identical in all of the morphological, cultural, physiological and biochemical characteristics tested in this study, except with regard to susceptibility to polymyxin B. Strains K92^T and K93 did not produce bacteriochlorophyll *a* aerobically in the dark. Sonicated *in vivo* cell extracts of the two strains showed an absorption maximum only at 409 nm, which indicated the absence of bacteriochlorophyll *a* and only the presence of a carotenoid. Extracts obtained using acetone/methanol did not show *in vitro* maximal absorption peaks. The almost-complete 16S rRNA gene sequences of strains K92^T and K93 (comprising 1441 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA sequence) were found to be identical. A comparative 16S rRNA gene sequence analysis showed that strains K92^T and K93 form a distinct phylogenetic lineage within the *Alphaproteobacteria* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strains K92^T and K93 joined the phylogenetic clade comprising *A. itersonii* and *A. peregrinum*, with which they exhibited the highest 16S rRNA gene sequence similarity values (91.3–91.5 %). The sequence similarity values with respect to the other species used in the phylogenetic analysis were in the range 81.4–91.2 % (Fig. 1). Strains K92^T and K93 possessed a mean level of DNA–DNA relatedness of 93 % when their DNAs were used individually as labelled DNA probes for cross-hybridization, indicating that the two strains represent the same genomic species (Wayne *et al.*, 1987). In view of the combined phenotypic, phylogenetic and genetic similarities, strains K92^T and K93 could be considered as members of the same species.

The predominant isoprenoid quinone detected in strains K92^T and K93 was Q-10 (peak area ratio, approx. 94–95 %).

Table 1. Differential phenotypic characteristics of strains K92^T and K93, *A. itersonii* and *A. peregrinum*

Taxa: 1, strains K92^T and K93; 2, *A. itersonii*; 3, *A. peregrinum*. Unless indicated, data for reference taxa were taken from Krieg (1984). +, Positive; –, negative; v, variable. Cells of all taxa are helical in shape, all of the taxa are positive for catalase, oxidase and aesculin hydrolysis and all are negative for Gram-staining, indole production and hydrolysis of casein and gelatin.

Characteristic	1	2	3
Growth at 3% (w/v) NaCl	+	–	–
Growth on MacConkey agar	+	+*	–†
Anaerobic growth with KNO ₃	+	+	–
Nitrate reduction to nitrite	+	–	–
H ₂ S production	–	+	+
Hydrolysis of:			
DNA	+	+*	–†
Starch	+	–	–
Urea	–	–	+
Temperature range for growth (°C)	15–47	12–42	11–40
pH range for growth	6.5–10.0	5.5–9.0	5.5–9.0
Utilization of:‡			
Adipate	+	–	–
Citrate	+	–	–
Malate	+	+	–
Glycerol	+	v§	–
Aesculin	–	–	+
D-Fructose	–	+	+
Ribose	+	–	–
Gluconate	+	–	–
DNA G+C content (mol%)	70.0	60–66 (<i>T_m</i>)	60–64 (<i>T_m</i>)

*Not determined for *A. itersonii* subsp. *nipponicum*.

†Not determined for *A. peregrinum* subsp. *integrum*.

‡Data from this study.

§Positive for *A. itersonii* subsp. *nipponicum* but negative for *A. itersonii* subsp. *itersonii*.

Strains K92^T and K93 had cellular fatty acid profiles that contained large amounts of unsaturated, straight-chain and hydroxy fatty acids; the major component was C_{18:1}ω7c (see Supplementary Table S1 in IJSEM Online). The major polar lipids detected in strains K92^T and K93 were phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified amino-group-containing lipids that were ninhydrin-positive. Strain K92^T and strain K93 had DNA G+C contents of 70.0 mol%.

Strains K92^T and K93 were shown to be most closely related phylogenetically to *A. itersonii* and *A. peregrinum* (Fig. 1). However, the 16S rRNA gene sequence similarity values were too low (<91.5%) to allow assignment of the two isolates to any recognized genus within the *Alphaproteobacteria*. In the phylogenetic tree based on 16S rRNA gene sequences, it was found that *A. itersonii* and *A.*

peregrinum are distantly related to the clade comprising *Aquaspirillum serpens*, the type species of the genus *Aquaspirillum* (Fig. 1). *A. serpens* is not only phylogenetically distantly related to strains K92^T and K93, but is also affiliated to the *Betaproteobacteria*. *A. itersonii* and *A. peregrinum* also showed low 16S rRNA gene sequence similarity values (91.0–91.2%) with respect to each other. Accordingly, *A. itersonii* and *A. peregrinum* may have to be reclassified as members of two different novel genera rather than the genus *Aquaspirillum*. Strains K92^T and K93 are distinguishable from *A. peregrinum*, which has Q-9 as the predominant ubiquinone (Sakane & Yokota, 1994). The fatty acid profiles of strains K92^T and K93 are similar to those of *A. itersonii* and *A. peregrinum* strains (see Supplementary Table S1 in IJSEM Online). However, strains K92^T and K93 show minor differences, relative to the two *Aquaspirillum* species, in the proportions of several unsaturated fatty acids, although these differences may result from differences in cultivation conditions (see Supplementary Table S1 in IJSEM Online). Strains K92^T and K93 are also distinguishable from *A. itersonii* and *A. peregrinum* through differences in several phenotypic properties, as shown in Table 1. The two strains are distinguishable from some phylogenetically related phototrophic purple non-sulfur bacteria, including the members of the genera *Rhodospira*, *Roseospira* and *Rhodospirillum*, by the absence of bacteriochlorophyll *a* and by differences in the predominant isoprenoid quinone types (Pfennig *et al.*, 1997; Imhoff *et al.*, 1998; Guyoneaud *et al.*, 2002). The low levels of 16S rRNA gene sequence similarity between strains K92^T and K93 and all other members of the family *Alphaproteobacteria*, together with the differential phenotypic properties, suggest that strains K92^T and K93 represent a novel genus and species within the *Alphaproteobacteria*, for which the name *Caenispirillum bisanense* gen. nov., sp. nov. is proposed.

Description of *Caenispirillum* gen. nov.

Caenispirillum (Cae.ni.spi.ril'lum. L. n. *caenum* sludge, mud; Gr. n. *spira* a spiral; N.L. dim. neut. n. *spirillum* a small spiral; N.L. neut. n. *Caenispirillum* a small spiral isolated from sludge).

Cells are Gram-negative, non-spore-forming and helical in shape. The predominant ubiquinone is Q-10. The major fatty acid is C_{18:1}ω7c. The major polar lipids are phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified amino-group-containing lipids that are ninhydrin-positive. The DNA G+C content is 70.0 mol%. The type species is *Caenispirillum bisanense*.

Description of *Caenispirillum bisanense* sp. nov.

Caenispirillum bisanense (bi.san.en'se. N.L. neut. adj. *bisanense* of Bisan, Daegu, Korea, from where the type strain was isolated).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain K92^T and representatives of some related taxa. Bootstrap percentages (based on 1000 replications) greater than 50% are shown at branch points. *E. coli* ATCC 11775^T was used as an out-group (not shown). Bar, 0.05 substitutions per nucleotide position.

Exhibits the following properties in addition to those given in the genus description. Cells are 0.5–0.7 × 0.7–7.0 µm. Motile by means of a single polar flagellum. Colonies on TSA are circular, raised, smooth, glistening, greyish-yellow in colour and 1.5–2.5 mm in diameter after incubation for 2 days at 37 °C. Growth occurs at 15 and 47 °C, the optimum temperature being 37 °C; growth does not occur at 10 or 48 °C. Growth is observed at pH 6.5 and 10.0, but not at pH 6.0 or 10.5; optimal growth occurs at pH 7.0–8.0. Optimal growth occurs in the presence of 0.5% (w/v) NaCl; growth occurs in the absence of NaCl and in the presence of 5% (w/v) NaCl, but not in the presence of greater than 6% (w/v) NaCl. Tweens 20, 40, 60 and 80 are hydrolysed, but hypoxanthine, tyrosine and xanthine are not. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. The following substrates are not utilized: caprate, phenylacetate, erythritol, D-arabinose, L-arabinose, D- and L-xylose, adonitol, methyl β-D-xyloside, galactose, glucose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, 2-ketogluconate and 5-ketogluconate. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, penicillin G, streptomycin and tetracycline, but not to lincomycin or oleandomycin. Susceptibility to polymyxin B is variable (positive for type strain). In assays with the API ZYM system, alkaline phosphatase, esterase (C4) and esterase lipase (C8) are present and naphthol-AS-BI-phosphohydrolase is weakly present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent.

The predominant ubiquinone is Q-10. The major fatty acid is C_{18:1}ω7c. The major polar lipids are phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified amino-group-containing lipids that are ninhydrin-positive. The DNA G+C content is 70.0 mol%. Other phenotypic characteristics are shown in Table 1.

The type strain, K92^T (= KCTC 12839^T = JCM 14346^T), was isolated from sludge from the wastewater treatment plant of a dye works at Daegu, Korea.

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- to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira medisalina* comb. nov. *Int J Syst Bacteriol* **48**, 793–798.
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