Asticcacaulis benevestitus sp. nov., a Novel Psychrotolerant Dimorphic Prosthecate Bacterium from Tundra Wetland Soil

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ABSTRACT

An isolate of Gram-negative, aerobic, heterotrophic, non-pigmented, dimorphic prosthecate bacteria was obtained from tundra wetland soil and designated as strain Z-0023^T. The cells of this strain undergo a dimorphic life cycle and develop a non-adhesive stalk at a site, which is not coincidental with the center of the cell pole, a characteristic typical of representatives of the genus *Asticcacaulis*. A highly distinctive feature of the cells of strain Z-0023^T is presence of a conical, bell-shaped sheath that appears on cells grown at low temperatures. This prosthecate bacterium is psychrotolerant, moderately acidophilic organism capable of growth between 4 and 28ºC (optimum 15-20ºC) and between pH 4.5 and 8.0 (optimum 5.6-6.0). The major phospholipid fatty acid is 18:1*ω*7*c* and the major phospholipids are phosphatidylglycerols. The G+C content of the DNA is 60.4 mol%. On the basis of 16S rRNA gene sequence similarity, strain Z-0023^T is most closely related to *Asticcacaulis biprosthecium* (98% similarity), *Asticcacaulis taihuensis* (98%), and *Asticcacaulis excentricus* (95%). However, low DNA-DNA relatedness to these organisms and a number of distinctive features of the tundra wetland isolate indicated that it represented a novel species of the genus *Asticcacaulis*, for which the name *Asticcacaulis benevestitus* sp. nov. is proposed (type strain Z-0023^T = DSM 16100^T = ATCC BAA-896^T).

Keywords: *Asticcacaulis benevestitus* sp. nov, dimorphic prosthecate bacteria, psychrotolerant microorganisms, microbial communities in tundra.

Abbreviations: DPB – dimorphic prosthecate bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Asticcacaulis benevestitus* strain Z-0023^T is AM087199.

Dimorphic prosthecate bacteria (DPB) are present in almost any sample of freshwater or seawater and in many types of soils (Henrici, Johnson, 1935; Poindexter, 1964, 1981a, 1999; Nikitin et al., 1966; Belyaev, 1968; Staley, 1968; Staley *et al*., 1987; Lapteva, 1987). An important characteristic of DPB is their ability to metabolize organic materials available in extremely low quantities and to tolerate a prolonged nutrient scarcity (Larson, Pate, 1975; Poindexter, 1981b; Vasilyeva, Zavarzin, 1995).

DPB are characterized by asymmetric cell reproduction. Each reproductive event in these bacteria produces two siblings: a sessile cell with a cellular stalk (a prostheca) and a motile cell with a polar flagellum. On the basis of the morphology of the reproductive stage (fission or budding), two fundamentally different types of DPB are distinguished: caulobacteria (*Caulobacter, Brevundimonas, Maricaulis* and *Asticcacaulis*) and hyphomicrobia (*Hyphomicrobium*, *Pedomicrobium*, and *Hyphomonas*) (Poindexter, 1999; Abraham *et al.*, 1999). Caulobacteria are especially widespread in the environments were nutrient concentrations and ambient temperatures are low (Poindexter, 1981a, 1999; Staley *et al*., 1987). The taxonomy of caulobacteria has relied primarily upon morphological criteria for a long time. Recently, a polyphasic approach, comprising 16S rDNA sequencing, lipid analysis and NaCl tolerance characterizations, was used to analyze a large set of strains of these bacteria (Abraham *et al*., 1999; 2001). It has been shown that caulobacteria form two different phylogenetic lineages within the *Alphaproteobacteria*, one comprising the freshwater and brackish water representatives of the group *Caulobacter – Brevundimonas – Asticcacaulis*, and the other comprising the marine species of the genus *Maricaulis*.

All known representatives of the genus *Asticcacaulis* have been isolated from freshwater samples. Sessile cells of these bacteria develop subpolar or lateral prosthecae, which do not possess adhesive material at their tips. Instead, the cells bear holdfast material directly on their surfaces. To date, this genus comprised three validly described species, *Asticcacaulis excentricus* (Poindexter, 1964), *Asticcacaulis biprosthecium* (Pate *et al*., 1973)

and *Asticcacaulis taihuensis* (Liu *et al*., 2005). Representatives of the genus *Asticcacaulis* are rarely observed and even more rarely isolated. In this report, we describe a novel species of this genus that has been isolated from tundra wetland peat.

The peat sample was collected from a depth of 3 to 6 cm of a shrub tundra wetland, northeast of Vorkuta in the Polar Ural, Russia (68ºN, 52ºE). 1g of peat was homogenized in 10 ml of sterile water and 1 ml of this suspension was used to inoculate 100 ml of a liquid dilute peptone-yeast extract (PY) medium containing 0.005% peptone and 0.005% yeast extract, pH 6.8, supplemented with 1% (v/v) vitamin stock solution and 2% (v/v) modified Hutner's basal salts as described by Staley (1968). For enrichment of psychrophilic bacteria, the incubation was carried out for 2 months at 6ºC. The resultant enriched cell suspension was spread-plated onto the surface of the PY medium supplemented with sodium acetate (0.1%, w/v) and solidified with Difco agar (2%, w/v), and the plates were further incubated at 6ºC. As colonies become visible, they were successively re-streaked onto fresh plates with the same agar medium until a culture with uniform colonies was obtained. The isolate, designated Z-0023, was maintained both on agar slants and in liquid cultures using above described PY medium with acetate (PY-A) and PYG medium (0.1% peptone, 0.1% yeast extract, 0.1% glucose, pH 6.7). Cell morphology and cell life cycle, as well as culture purity, were examined using phase-contrast and electron microscopy. In the latter case, the samples were stained with 1% (w/v) uranyl acetate. For preparation of thin sections, cells were collected from agar plates and pre-fixed with 1.5% (w/v) glutaraldehyde in 0.05M cacodylate buffer (pH 6.5) for 1 h at 4ºC and then fixed in 1% (w/v) OsO_4 in the same buffer for 4 h at 20ºC. After dehydration in an ethanol series, the samples were embedded in a Spurr epoxy resin. Thin sections were cut on an LKB-4800 microtome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol. The specimen samples were examined with a JEM-100C transmission electron microscope. Growth of the isolate was monitored by nephelometry at 600 nm for 2-4 weeks in PY-A and PYG liquid media under a variety of

conditions, including temperatures of 4-37ºC, pH 4.5-8.3 and NaCl concentrations of 0.1-5.0% (w/v). Variations in the acidity level were achieved by mixing 0.05M solutions of Na_2HPO_4 and KH_2PO_4 to create media with the same ionic strength. To determine the range of potential growth substrates of strain Z-0023, the following carbon sources were tested using liquid PY medium with addition of the respective compounds at a concentration of 0.1% (w/v): D-glucose, L-arabinose, D-ribose,D- xylose, D-galactose, D-fructose, lactose, D-maltose, sucrose, D-cellobiose, D-mannose, D-melibiose, raffinose, L-rhamnose, D-trehalose, D-mannitol, D-sorbitol, starch, acetate, propionate, butyrate, pyruvate, malate, fumarate, succinate, citrate, methanol, ethanol, propanol, butanol, L-arginine, DL-alanine, L-phenylalanine, L-glutatate, L-lysine, L-proline, L-hydroxyproline, L-serine, L-tryptophan. Growth was examined after 8 days of incubation at 20ºC. Sensitivity to antibiotics was tested by spreading 2-day-old cell suspension onto PYG agar medium and applying filter discs containing the following antibiotics: polymixin M (300 U), benzylpenicillin (10 U), ristomycin (30 μg), canomycin (30 μg), monomycin (30 μg), tetracyclin (30 μg), erythromycin (15 μg), streptomycin (30 μg), levomycetin (30 μg). Growth was assessed after 2 days of incubation at 20ºC.

For fatty acid analyses, cells of strain Z-0023 were grown on PYG agar plates at 10 and 20°C. Cells were saponified (15 % (w/v) NaOH, 30 min, 100°C), methylated to fatty acid methyl esters (FAMEs) (methanolic HCl, 10 min, 80°C) and extracted (hexane/methyl-tert-butyl ether (1:1, v/v)) as described in detail by Osterhout *et al.* (1991). Fatty acid methyl esters were analyzed on a gas chromatograph equipped with a flame ionization detector and an autosampler. Separation of fatty acid methyl esters was achieved using fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5 % phenyl methyl silicone (film thickness 0.33 μm; HP Ultra 2). Injection temperature was 250°C and detector temperature was 300°C. The oven program was 150°C for 2 min, then increased from 150° to 289°C at 4°C min^{-1}, followed by an isothermal period of 11 min. The instrument was equipped with a

flame ionization detector and an autosampler; H_2 served as the carrier gas. Fatty acid methyl esters were identified by comparison with standards or by gas chromatography – mass spectrometry (Abraham *et al*., 1998). For polar lipid fatty acid analysis, lipids were extracted, using a modified Bligh-Dyer procedure (Bligh & Dyer, 1959) as described previously (Abraham *et al.,* 1997). The extract of the total lipids was analysed by electrospray ionization (ESI) using a quadrupol-time-of-flight mass spectrometer (Yakimov *et al*., 2004).

Genomic DNA from strain Z-0023 was extracted using a sodium dodecyl sulphate-based assay as described previously (Dedysh *et al*., 1998). The DNA base composition of strain Z-0023^T was determined by thermal denaturation using a Unicam SP1800 spectrophotometer (UK) at a heating rate of 0.5°C min^{-1}. The mol % G+C value was calculated with the equation of Owen et al. (1969): GC % = T_m·2.08 - 106.4. The DNA of *Escherichia coli* K-12 was used as the standard. DNA-DNA hybridization of strain Z-0023^T and two species of the genus *Asticcacaulis*, i.e. *Asticcacaulis excentricus* (DSM-4724^T) and *Asticcacaulis biprosthecium* (DSM-4723^T), was done on nitrocellulose membrane filters (Hybond-N, Amersham International, UK) according to Lysenko *et al*. (1988). Genome size of strain Z-0023 ^{T} was calculated with the equation of De Ley *et al*. (1970): M = (98.37 – 0.91 × GC %)/k, where k is DNA re-association rate. PCR-mediated amplification of the 16S rRNA gene from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA) was performed using primers Eub9f and Eub1492r and reaction conditions described by Lane (1991). The 16S rRNA gene amplicons were purified using QIAquick spin columns (Qiagen) and sequenced on an ABI Prism 377 DNA sequencer using BigDye terminator chemistry, as specified by the manufacturer (PE Applied Biosystems). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al*., 2004).

Small (1-2 mm in diameter), smooth, circular, convex with an entire edge, opaque white colonies were observed on PY-A agar after 2 weeks of incubation at 6ºC. Single colonies were successively selected from plates for re-streaking until a pure culture, designated Z-0023^T, was obtained. Microscopic examination revealed that strain Z-0023^T is represented by Gram-negative, motile, rod-shaped cells, 0.5-0.7 μm in width and 1.4-2.0 μm in length (Fig. 1a). These cells reproduced by binary fission and underwent a dimorphic life cycle during which a sessile cell with a cellular stalk (a prostheca) divides to give a rise to a motile cell with a polar flagellum (Fig. 1b). A single, non-adhesive prostheca of these bacteria was 0.10-0.15 μm in diameter and had an excentral, sub-polar location on the cell (Fig. 1d,c), which is characteristic of representatives of the genus *Asticcacaulis* (Poindexter, 1964; Pate *et al*., 1973). The prostheca length ranged from 0.5 to 5.0 μm depending on cultivation conditions and attaining maximal length in defined minimal media. In cells grown on rich complex media (for example, on PYG medium), prosthecae were short or unobservable, while the cells themselves appeared significantly (up to ten fold) elongated and misshapen.

The major distinctive feature of the cells of strain Z-0023^T was presence of conical, bell-shaped sheath, which was attached to the sessile cell at the point of juncture of prostheca and cell (Fig. 1d, e, f). Cell division occurs within this sheath (Fig. 1d). Interestingly, these clothes-like sheathes appeared on cells grown at low temperatures (below 10ºC), while the population grown at moderate temperatures (15-25ºC) consisted of sheath-free cells.

In stationary liquid cultures, most of the growth of strain Z-0023^T occurred in the form of a pellicle that developed at the surface of the medium. In liquid cultures incubated on a rotary shaker, the cells were evenly dispersed throughout the culture.

Similar to other known representatives of the genus *Asticcacaulis*, strain Z-0023^T was not capable of growth in a mineral medium with glucose or some other compound as the only

growth substrate. It had an absolute requirement for the presence of growth factors in cultivation media. Thus, utilisation of a given carbon compound was assumed to have occurred when growth was distinctly heavier in its presence than on the basal PY medium alone. The compounds tested and the results are presented in Table S1 (Supplementary material). Most sugars, ethanol, and some organic acids (acetate, malate, fumarate, and succinate) were the preferable growth substrates. Strain Z-0023^T differed from *A. excentricus* and *A. biprosthecium* by the inability to utilise pyruvate, and it could also be differentiated from *A. taihuensis* by the inability to utilise D-cellobiose, D-mannose and D-melibiose.

Strain Z-0023^T grew in the pH range 4.5-8.3 with the optimum at pH 5.6-6.0. The temperature range for growth was 4-28ºC with the optimum at 15-20ºC. The culture generation time under optimal temperature conditions, calculated from increases in OD_{600} in the exponential phase of growth, was in the range 30-35 h. Both the specific growth rate and the growth yield of the culture at 25-28ºC were significantly lower than those attained at 15-20ºC. No growth occurred at 37ºC.

Compared with *A. excentricus* DSM 4724^T and *A. biprosthecium* DSM 4723^T (Abraham *et al*., 2001), NaCl was not required for growth of strain Z-0023 and this isolate has less tolerance of dissolved salts. Growth inhibition of 50% was observed in the presence of NaCl in the medium at concentrations of 1-1.5% (w/v), whereas NaCl at concentrations above 2.0% completely inhibited growth. Strain Z-0023 was susceptible to antibiotics that inhibit prokaryotic protein synthesis, i. e. streptomycin, tetracycline, erythromycin, levomycetin, monomycin.

Whole-cell fatty acid compositions and the comparison of glyco- and phospholipid fatty acid profiles of strains Z-0023^T, *A. biprosthecium* DSM 4723^T, *A. excentricus* DSM 4724^T and *A. taihuensis* T3-B7^T are shown in Table 1 and Table S2 (Supplementary material), respectively. Similar to other known members of the genus *Asticcacaulis*, 18:1ω7*c* was the major fatty acid in strain Z-0023^T. The distinguishing feature of the fatty

acid profile of strain Z-0023^T was the absence of the 12:1 3-OH, which up to now was considered a common feature of the FAME profiles in *Asticcacaulis* (Abraham *et al.*, 2001). Glycolipids present in all *Asticcacaulis* spp. are α-D-glucopyranosyl- and α-D-glucopyranuronosyl-diacylglycerols, also common in *Caulobacter*, *Brevundimonas* and some other *Alphaproteobacteria*. In strain Z-0023^T four different glycolipids could be detected, among them a glycolipid of 796 Da previously only detected in *A. biprosthecium* DSM 4723^T but lacking in *A. excentricus* DSM 4724^T. The (-)-ESI mass spectra of the phospholipids of the strain Z-0023^T showed clear differences to those of the other type strains (Table S3, Supplementary material). Phospholipids of this bacterium were of the phosphatidyl-glycerol (PG) type and six different phospholipids could be identified analogous to *A. biprosthecium* DSM 4723^T and *A. excentricus* DSM 4724^T. Interestingly, most of the phospholipids detected in strain Z-0023^T have been also found in *A. excentricus* DSM 4724^T but not in *A. biprosthecium* DSM 4723^T.

Comparative sequence analysis of the 16S rRNA gene revealed that strain Z-0023^T belongs to the *Alphaproteobacteria* and, more precisely, that it is included in the phylogenetic lineage comprising the freshwater and brackish water representatives of the group *Caulobacter – Brevundimonas – Asticcacaulis* (Fig. 2). The new isolate from tundra wetland peat was most closely related to the three known representatives of the genus *Asticcacaulis*, i.e. *A. excentricus* DSM 4724^T (95% sequence similarity), *A. taihuensis* T3-B7^T (98%), and *A. biprosthecium* DSM 4723 ^{T} (98%). The DNA G+C content of strain Z-0023 was 60.4 mol% and the genome size was 2.5×10^9 Da. DNA-DNA hybridization values of strain Z-0023 with *A. biprosthecium* DSM 4723^T and *Asticcacaulis excentricus* DSM 4724^T were 40 and 35%, respectively.

The combined morphological and genotypic characteristics reported above indicate that strain Z-0023^T belongs to the genus *Asticcacaulis*. However, compared with *A. biprosthecium* and *A. excentricus*, the novel strain has greater tolerance to cold temperatures and low pH, but

has higher sensitivity to dissolved salts. We were not able to compare temperature and pH growth ranges of strain Z-0023^T with those of *A. taihuensis*, since these characteristics have not been reported for the latter bacterium. The unique morphologic feature of novel strain from tundra wetland was presence of bell-shaped sheathes that appeared on cells grown at low temperatures. Strain Z-0023^T also differed from all three known species of the genus *Asticcacaulis* with regard to the assimilation of some substrates (Table 2) and absence of the 12:1 3-OH fatty acid in FAME composition. Thus, we propose a novel species, *Asticcacaulis benevestitus* sp. nov., for this prosthecate bacterium from tundra wetland peat. The major characteristics differentiating this novel species from the other species of the genus *Asticcacaulis* are summarized in Table 2.

Description of *Asticcacaulis benevestitus* sp. nov.

Asticcacaulis benevestitus (be.ne.vesti'tus. L. adv. *bene* well; L. part. adj. *vestitus* clothed/clad; N.L. masc. part. adj. *benevestitus* well clad).

Rod-shaped, Gram-negative cells, 0.5-0.7 by 1.4-2.0 μm. Reproduces by binary fission. Undergoes a dimorphic life cycle during which a sessile cell with one excentral, sub-polar prostheca divides to give rise to a motile cell with a single, polar flagellum. A single, non-adhesive prostheca of 0.10-0.15 μm in diameter arises from a sub-polar site. At temperatures below 10ºC most prosthecate cells are embedded in conical bell-shaped sheath, which is attached to the cell at the point of juncture of prostheca and cell. Colonies are opaque white, smooth, circular, convex with an entire edge, and 1-2.5 mm in diameter after 7 days of growth at 20ºC on PYG medium. Chemo-organotrophic aerobe. Carbon sources include glucose, sucrose, xylose, maltose, galactose, arabinose, lactose, fructose, ethanol, acetate, malate, fumarate, succinate, raffinose, rhamnose, trehalose. Yeast extract is required for growth. Temperature range for growth is 4-28ºC (optimum at 15-20ºC). Growth occurs between pH 4.5 and 8.3, with an optimum at pH 5.6-6.0. NaCl is not required for growth and tolerated up to a concentration of 2.0% (w/v). Susceptible to antibiotics that inhibit

prokaryotic protein synthesis. The major PLFA is 18:1ω7c and the major phospholipids are phosphatidylglycerols. The G+C content is 60.4 mol%. Genome size is 2.5×10^9 Da. The type strain, Z-0023^T (= DSMZ 16100^T = ATCC BAA-896^T) was isolated from a tundra wetland in the Vorkuta region, northern Russia.

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Table 1. Whole-cell fatty acid compositions of the novel prosthecate isolate Z-0023 and other representatives of the genus *Asticcacaulis*

FAME	**12:0**	**12:0 3-OH**	**12:1 3-OH**	**14:0**	**15:0a**	**15:0**	**16:1 ω7/ω9**	**16:0**	**16:0 iso**	**16:0 2-OH**	**17:0 cyclo7,8**	**17:0**	**17:1 ω6c**	**17:1 ω8c**	**18:1ω7**	**18:0**	**19:0 cyclo 8,9**
Strain Z-0023	-	-	-	-	-	3,4	14,8	10,6	-	-	2,2	1,7	-	-	57,5	0,9	-
A. biprosthecium	2,5	-	7,5	1,1	-	2,9	13,8	20,8	-	-	-	-	1,0	-	40,7	-	-
A. excentricus	-	-	4,2	-	-	2,3	7,4	17,3	1,8	-	-	1,8	1,5	1,1	57,7	-	-
A. taihuensis	-	2,3	2,6	1,1	2,6	1,5	7,4	28,7	-	10,4	-	-	-	-	41,9	-	1,5

Table 2. Major characteristics that distinguish *Asticcacaulis benevestitus* sp. nov. and other species of the genus *Asticcacaulis*

Characteristic	***A. excentricus*** [a]	***A. biprosthecium*** [b]	***A. taihuensis*** [c]	***A. benevestitus***
Number of prosthecae per cell	1	1-2	1	1
Presence of a sheath	-	-	-	+
Utilization of:				
Piruvate	+	+	nd	-
L-Arabinose	v	-	+	+
Sucrose	+	-	+	+
D-Mannose	+	-	+	-
D-Cellobiose	nd	nd	+	-
D-Melibiose	nd	nd	+	-
Temperature optimum, ºC	30	30	nd	15-20
Optimum pH	6.5	7.2	nd	5.6-6.0
NaCl is required for growth	+	+	nd	-
Growth at NaCl > 2% (w/v)	+	+	-	-
Presence of 12:1 3-OH fatty acid	+	+	+	-
G + C composition (mol%)	55	61	59	60.4

[a]- Data are from Poindexter (1964); [b] – data are from Pate *et al*. (1973); [c] – data are from Liu *et al*. (2005); v – variable, nd – not determined.

Figure captions

Fig. 1. (A) Phase-contrast micrograph of cells of strain Z-0023^T grown on PY-A medium for 3 weeks at 6ºC; bar, 10 μm; (B-E) electron micrographs of cells of strain Z-0023^T; (F) electron micrograph of a section taken through the area of juncture of cell and prostheca of strain Z-0023^T. (B-F) bars, 0.5 μm. p – prostheca, f – flagellum, sh – sheath, sb – stalk bands.

Fig. 2. 16S rDNA-based neighbour-joining tree showing the phylogenetic position of strain Z-0023^T in relation to *Asticcacaulis biprosthecium*, *Asticcacaulis excentricus*, *Asticcacaulis taihuensis,* prosthecate bacteria of the genera *Caulobacter*, *Brevundimonas*, *Maricaulis* and some other representative members of the *Alphaproteobacteria*. Bootstrap values (1000 data resamplings) >50% are shown. 16S rDNA sequence of gammaproteobacterial *Pseudomonas stutzeri* (AF143245) was used as an outgroup. The scale bar represents 0.1 substitutions per nucleotide position.