Andersenella baltica gen. nov., sp. nov., a novel marine bacterium of the *Alphaproteobacteria* isolated from sediment in the central Baltic Sea

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A bacterial isolate from the Baltic Sea, designated strain BA141^T, was characterized for its physiological and biochemical features, fatty acid profile, pigment spectrum, DNA G + C content and phylogenetic position based on comparative 16S rRNA gene sequence analysis. The strain was isolated from the surface of sediment in a deep basin of the central Baltic Sea. Phylogenetic analysis of the 16S rRNA gene sequence revealed a clear affiliation with the *Alphaproteobacteria*, and showed that the closest phylogenetic relationship was with the genus *Rhodobium*. The G + C content of the DNA was 61.2 mol%. Cells of strain BA141^T were red-pigmented, Gram-negative, pleomorphic to rod-shaped, non-motile and catalase- and oxidase-positive. Growth was observed at salinities of 0.8–6 ‰, with optimum growth at 1.5–3 ‰. The temperature range for growth was 10–37 °C, with optimum growth at 25–30 °C. The fatty acids were dominated by unsaturated fatty acids (>86 ‰); the number of fatty acids detected was very low, with 18 : 1 ω 7c (73 ‰) as the predominant fatty acid; other major fatty acids were 19 : 0 cyclo 8c (10 ‰) and 16 : 0 (8 ‰). The pigment spectrum indicated the presence of carotenoids and unknown pigment(s) with an absorption maximum at 430 nm, but not bacteriochlorophyll *a*. According to phylogenetic analysis based on the 16S rRNA gene sequence and the phenotypic features, strain BA141^T represents a new genus and species. The name *Andersenella baltica* gen. nov., sp. nov. is proposed, with strain BA141^T (=CIP 109499^T=LMG 24028^T) as the type strain.

A novel bacterium was obtained from the surface layer of anoxic sediment in the central Baltic Sea (Brettar *et al.*, 2006). Members of the *Alphaproteobacteria* are considered to represent a major fraction of marine bacteria (Giovannoni & Rappé, 2000). Most of this knowledge is based on environmental sequence data rather than on cultured bacteria, because members of the *Alphaproteobacteria* tend to be more difficult to culture than members of the *Gammaproteobacteria*. Giovannoni & Rappé (2000) concluded that this was due to their high degree of adaptation to specific environmental conditions, which

renders them less amenable to cultivation on the one hand, but very successful in marine ecosystems on the other.

The novel isolate studied here was most closely related to *Rhodobium orientis*. Garrity *et al.* (2004) proposed the family 'Rhodobiaceae' comprising the genera *Rhodobium* and *Roseospirillum*. Phylogenetic studies by Lee *et al.* (2005) showed that there were major inconsistencies in the formation of this family and suggested that additional taxonomic studies were needed before the classification of the genus *Rhodobium* could be confirmed.

Strain BA141^T was isolated during a cruise onboard RV *Aranda* in September 1998 from the black anoxic surface of sediment (sediment top layer 0–1 cm depth, 5 °C, 11 ‰ salinity) collected at a sampling site in the central Baltic Sea, station TEILI1 (59° 26' 07" N 21° 30' 02" E; at a depth of 165 m). The sediment sample was subsampled under sterile conditions from a large sample volume. Details of the environmental conditions, sampling and isolation procedures are given elsewhere (Brettar *et al.*, 2002;

Abbreviation: PHA, polyhydroxyalkanoate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain BA141^T are AM712634 and AM712635, respectively.

A table detailing results obtained using API and Biolog GN2 and tests for hydrolysis of and growth on various substrates for strain BA141^T is available with the online version of this paper.

Brettar & Rheinheimer, 1992; Höfle & Brettar, 1995). The sediment sample was serially diluted with sterile seawater, spread on agar plates and incubated in the dark for 3 weeks. The medium used for isolation was half-strength ZoBell agar (Oppenheimer & ZoBell, 1952). The strain grew well on half-strength ZoBell agar, in half-strength marine 2216 broth (Difco) and on marine agar (Difco).

The isolate was tested for a number of key characteristics using standard procedures (Gerhardt *et al.*, 1994), such as Gram behaviour (KOH string test), cell size, motility and morphology (via phase-contrast microscopy, and electron microscopy after Pt/C shadow casting or thin sectioning) and cytochrome oxidase and catalase (3 % H₂O₂). Growth on 1 % yeast extract, 1 % peptone, 0.2 % β -hydroxybutyrate and MacConkey agar was tested. In addition, the production of indole, nitrate reduction and hydrolysis of aesculin, casein, tyrosine, starch, gelatin and DNA were tested. Chitinase, cellulase and pectinase activities were tested as described by Atlas (1993).

For electron microscopy, samples from a liquid culture of strain BA141^T were adsorbed onto carbon-Formvar foil, washed with water, blotted and air-dried. They were shadow-casted at 15 deg elevation with platinum-carbon and analysed using an energy-filter transmission electron microscope (CEM902; Zeiss), as described by Golyshina *et al.* (2000). Embedding and thin-sectioning were performed as described by Yakimov *et al.* (1998).

In addition, strain BA141^T was characterized by using the whole test spectra of the identification systems API 50 CH, API 20NE, API ZYM (bioMérieux) and Biolog GN2 at 28 °C. Growth at different temperatures was assessed at 4, 10, 20, 25, 30, 37 and 42 °C. Growth at different salinities was tested at 0, 0.8, 1.0, 1.5, 3, 6 and 10 %, with salt being added as NaCl or Biomaris sea salt. Growth at different pH was tested at pH 5.7, 7 and 9.7 (pH adjusted using bicarbonate buffer or HCl, with growth assessed based on the occurrence of visible colonies on agar). For these tests we used half-strength marine broth or agar (Difco 2216), except for the salinity test for which half-strength salt-free ZoBell medium was supplemented with the respective amount of NaCl or Biomaris sea salt.

Pigment analysis was performed according to Hiraishi *et al.* (1995, 2000), i.e. the same procedure as used for analysis of the genus *Rhodobium* (Hiraishi *et al.*, 1995). Briefly, after growth in half-strength marine broth, cells were washed three times in PBS and either (i) resuspended in 50 mM phosphate buffer (pH 7.0), sonicated for 3 min and centrifuged to remove cell debris or (ii) extracted in ice-cold acetone/methanol solution (7:2, v/v) in the dark for 12 h. Absorption spectra of the resultant cell extracts were recorded spectrophotometrically from 300–900 nm.

Genomic DNA was prepared from individual colonies as described by Moore *et al.* (1996). 16S rRNA genes were amplified by PCR according to Mullis & Faloona (1987). The gyrase B (*gyrB*) gene sequence was amplified by PCR as

described by Yamamoto & Harayama (1995). The PCR-products were sequenced directly as described by Moore *et al.* (1999).

For phylogenetic analysis based on the 16S rRNA gene sequence, the most similar sequences were identified by running BLAST queries at NCBI (nr database), with the filter option set to false. These sequences were included and aligned within a local database of 289 000 previously aligned and analysed bacterial 16S rRNA gene sequences. In a first analysis, new sequences were carefully aligned (manual adjustments) and analysed by phylogeny (BIONJ, as detailed below). This was done to demonstrate that the novel strain was not related to any previously described genus. Among the 117 related sequences, 22 sequences of known type strains were selected and analysed further using three phylogenetic methods (BIONJ, maximum-likelihood and maximum-parsimony). For the neighbour-joining analysis, distance matrices were calculated using the Kimura two-parameter correction, BIONJ was used according to Gascuel (1997), and maximum-likelihood and maximum-parsimony (using the Global option) were from PHYLIP (Felsenstein, 1993). Bootstrap analyses were performed using BIONJ based on 1000 replications. The phylogenetic trees were drawn using TreeDyn (Chevenet *et al.*, 2006). The results of the final phylogenetic analysis are shown in Fig. 1. Phylogenetic analysis based on *gyrB* gene sequences was not successful due to a lack of available *gyrB* gene sequences in the phylogenetic neighbourhood of strain BA141^T. Therefore, only the GenBank/EMBL/DDBJ number for the *gyrB* gene sequence of strain BA141^T is provided.

The DNA G+C content (mol%) of strain BA141^T was determined using HPLC analysis of hydrolysed DNA according to Tamaoka & Komagata (1984) and Mesbah *et al.* (1989).

For analysis of the cellular fatty acid profile, strain BA141^T was grown on half-strength marine agar 2216 for 24 h at 28 °C. Fatty acid methyl esters were obtained from washed cells by saponification, methylation and extraction. Gas chromatography analysis was controlled by MIS software (Microbial ID) and the peaks were automatically integrated and identified using the microbial identification software package (Sasser, 1990).

Cells of strain BA141^T were Gram-negative, non-motile, pleomorphic rods, 1–5 µm in length and 0.5–1 µm in width (Fig. 2a–c). On marine agar, strain BA141^T formed small dark-red-pigmented opaque colonies. When grown in half-strength marine broth, the culture had a light to intense orange-red colour. Cells harvested from liquid culture were bright-red in colour. Strain BA141^T was slow-growing, with irregular lag phases after inoculation. To obtain visible growth on agar or in liquid culture, an incubation time of about 1 week at room temperature was required, with 2–4 weeks incubation for a densely grown culture.

Electron microscopy of shadow-casted cells showed that the cells had irregular shapes, often with branching (Fig. 2a

Fig. 1. Unrooted phylogenetic tree resulting from the analysis of nearly complete 16S rRNA gene sequences of the most related type strains. The topology shown was obtained using neighbour-joining based on 1000 bootstrap replications. Bootstrap percentages are indicated as well as branches that were also found by using maximum-parsimony (indicated by a plus sign) and maximum-likelihood ($P < 0.01$) (indicated by an asterisk) and therefore that define robust clusters. Bar, 0.01 substitutions per nucleotide position.

and c–e). In liquid culture, cells formed star-like cell aggregates. Ultrastructural analysis using electron microscopy revealed the presence of polyhydroxyalkanoate (PHA) deposits. Enlargement of the cells was often observed in the vicinity of the PHA deposits. Neither budding cells, flagella nor intracytoplasmic membranes were observed, as was the case for *Rhodobium orientis* (Hiraishi *et al.*, 1995).

All details on the physiological and biochemical traits are summarized in the species description and in Supplementary Table S1 (available in IJSEM Online). In general, strain BA141^T showed no substrate use in the API 50 CH and API 20NE test systems and a rather limited and mostly weak substrate use was recorded using the API ZYM test system. Substrates used in the Biolog GN2 test systems were mostly organic acids plus L-arabinose, alaninamide and glycerol. As a general rule, phenotypic features were rated as positive when a weak or more pronounced signal was obtained (for details see Supplementary Table S1).

The pigment spectrum of the sonicated cell extract showed a major peak at 430 nm, with shoulder peaks around 470, 500 and 530 nm (Fig. 3). In addition, a minor peak occurred at 365 nm. No peaks above 600 nm were

observed, indicating that strain BA141^T did not contain bacteriochlorophyll *a*. The large peak at 430 nm was not identified; the shoulder peaks on the large peak between 470 and 530 nm were considered to indicate the presence of carotenoids. The pigment spectrum was very different from that of *Rhodobium orientis*, which showed pronounced peaks for bacteriochlorophyll *a* at 800 and 870 nm, but no peak at 430 nm (Hiraishi *et al.*, 1995). In the acetone/methanol extract of strain BA141^T a broad peak from 450 to 550 nm was observed, supporting the presence of carotenoids.

In terms of phenotypic features, strain BA141^T differed with respect to many of those of the phylogenetically closest related species *Rhodobium orientis* (Table 1). Differences were observed in the presence of flagella, formation of budding cells, presence of bacteriochlorophyll *a* and intracytoplasmic membranes, and loss of pigmentation in oxic cultures. In terms of physiological features, they differed regarding denitrification, and the use of sugars and alcohols (Hiraishi *et al.*, 1995). The two species were able to use a broad spectrum of organic acids.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BA141^T did not form a robust clade

Fig. 2. Ultrastructural analysis of cells of strain BA141^T grown in half-strength marine broth (21 °C, daylight). (a) Survey view of ultrathin-sectioned bacteria showing the overall features and often irregular contours of the cells. These cytoplasmic dilatations (indicated by asterisks) are often related to the presence of PHA deposits. (b) Detail of a bacterial cell, revealing the presence of an outer membrane (OM), indicative of a Gram-negative type of cell wall. (CM, cytoplasmic membrane; CP, cytoplasm). (c, d, e) Overall morphologies of Pt/C shadow-casted cells, which especially reveal the pleomorphic feature of individual cells (c, e) and star-like aggregates (d). Arrows in (c) and (d) indicate the shadowing direction. Bars, 1 µm (a, d and e), 50 nm (b) and 500 nm (c).

with any other previously described genus; a grouping with the genus *Rhodobium* was revealed by using BIONJ and maximum-parsimony, but not maximum-likelihood (Fig. 1). In addition, the bootstrap support of the neighbour-joining analysis was low (52%; Fig. 1). This is a strong indication that strain BA141^T represents a new genus. *Rhodobium orientis* (GenBank no. D30792) had a 16S rRNA gene sequence similarity of 92.5% with strain BA141^T. Also, with most species of the genus

Mesorhizobium, a 16S rRNA gene sequence similarity of 92% or below was observed. In comparison to 16S rRNA gene sequences derived from cultured bacteria, 16S rRNA gene sequence similarities of above 93% were not observed.

The DNA G+C content of strain BA141^T was 61.2 mol% (Table 1). Values for strains of the related species *Rhodobium orientis* ranged from 61.5 to 65.7% and are therefore within the same range as that for strain BA141^T.

Fig. 3. Absorption spectra of cell extracts of strain BA141^T obtained from sonicated (solid line) and from acetone/methanol (dashed line) extracted cells.

The fatty acid profile was dominated by unsaturated fatty acids (>86 %) and showed a low number of detected fatty acids. The fatty acid profile was composed of 14:0 (0.5 %), 16:1 ω 11 c (1.0 %), 16:0 (8.2 %), 16:0 2-OH (1.0 %), 18:1 ω 9 c (1.8 %), 18:1 ω 7 c (73.3 %), 19:0 cyclo ω 8 c (9.9 %) and an unknown component (ECL 11.799, 4.3 %). Thus the fatty acid spectrum was strongly dominated by 18:1 ω 7 c . The fatty acid profile of strain BA141^T was similar to those of members of the genus

Mesorhizobium, with the closest similarity with *Mesorhizobium plurifarum* (Tighe *et al.*, 2000).

Based on 16S rRNA gene sequence analysis, strain BA141^T belongs to the *Alphaproteobacteria*, but is clearly identified as representing a new genus. Also, from a phenotypic perspective, the most closely related species and the only described representative of the genus *Rhodobium* at the time of writing, *Rhodobium orientis*, showed many features that were different from those of strain BA141^T, i.e. presence of flagella, formation of budding cells, presence of bacteriochlorophyll *a* and intracytoplasmic membranes, loss of pigmentation in oxic cultures, and many physiological features, such as denitrification, and broader use of sugars and alcohols (Hiraishi *et al.*, 1995).

Based on the polyphasic approach used, we propose a new genus and species, *Anderseniella baltica* gen. nov., sp. nov., to accommodate the novel Baltic Sea isolate, with strain BA141^T as the type strain.

Description of *Anderseniella* gen. nov.

Anderseniella (An.der.sen.i.el'la. N.L. dim. n. *Anderseniella* named in honour of the late marine scientist Valérie Andersen, Observatoire Océanologique de Villefranche sur Mer, France, in recognition of her valuable work on marine ecosystems).

Cells are Gram-negative, rod-shaped to pleomorphic, and oxidase- and catalase-positive. Non-flagellated and red-pigmented. Growth is heterotrophic and aerobic. Pre-

Table 1. Differential phenotypic features between strain BA141^T (*Anderseniella baltica* sp. nov.) and *Rhodobium orientis*

+, Positive; –, negative. Data for *Rhodobium orientis* are from Hiraishi *et al.* (1995).

Feature	Strain BA141 ^T	<i>Rhodobium orientis</i>
Site of isolation	Central Baltic Sea, basin (165 m); sediment surface	Japan, coast, tidal pools; seawater and mud
Size (μm)	0.5–1.0 × 1.0–5.0	0.7–0.9 × 1.0–3.2
Motility	–	+
Culture colour at aerobic growth	Orange–red	(several flagella)
Bacteriochlorophyll <i>a</i>	–	Colourless
Intracytoplasmic membranes	–	+
Budding cells	–	+
DNA G + C content (mol%)	61.2	61.5–65.7
Denitrification	–	+
Utilization of:		
Formate	+	–
Valerate	–	+
Succinate	–	+
L-Arabinose	+	–
Fructose	–	+
Glucose	–	+
Mannitol	–	+
Sorbitol	–	+
Glycerol	+	–

dominant fatty acid is 18:1 ω 7c. Other major fatty acids are 16:0 and 19:0 cyclo ω 8c. Cells contain carotenoids but not bacteriochlorophyll *a*. Cells contain PHA. Member of the *Alphaproteobacteria*. The type species is *Anderseniella baltica*.

Description of *Anderseniella baltica* sp. nov.

Anderseniella baltica (bal'ti.ca. N.L. fem. adj. *baltica* from the Baltic Sea, referring to the source of the type strain).

Exhibits the following properties in addition to those given in the species description. Colonies are small, opaque, circular, smooth, convex and entire. Forms dark-red colonies and light to intense orange-red liquid cultures (>1 week, 25 °C, half-strength marine broth). Cells are partially branching (width 0.5–1.0 μ m, length 1.0–5.0 μ m). Cells form star-like aggregates in liquid culture. Grows at oxic to low oxic conditions. Does not reduce nitrate to nitrite. Temperature for growth ranges from 10 to 37 °C, with optimum growth at 25–30 °C. Salinity range for growth is from 0.8 to 6 ‰ of final concentration of artificial sea salt; addition of NaCl alone is not sufficient for growth. Grows at pH 7–7.5; no growth occurs at pH 5.7 or 9.7. Grows on 1% yeast extract and 1% peptone, but not on aesculin, casein or β -hydroxybutyrate. Does not hydrolyse starch, gelatin, tyrosine, DNA, cellulose, chitin or pectin. Does not produce indole. Does not grow on MacConkey agar. Acid production is not observed using the API 50 CH test system. All API 20NE tests are negative. In the API ZYM test system, alkaline phosphatase, esterases (C4 and C8) and leucine arylamidase are positive; all other tests are negative. In the Biolog GN2 system, positive results are obtained for L-arabinose, glycerol, alaninamide and the organic acids succinic acid monomethyl ester, acetic acid, formic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, α -ketobutyric acid, DL-lactic acid, succinamic acid and urocanic acid; all other tests are negative. In general, the use of organic acids is better than for other substrates. All unmentioned tests of the API and Biolog GN2 test systems are negative. A detailed overview of all positive and negative test results for the biochemical tests is given in Supplementary Table S1 (in IJSEM Online). The DNA G+C content of the type strain is 61.2 mol%.

The type strain, BA141^T (=CIP 109499^T=LMG 24028^T), was isolated from the surface layer of anoxic sediment in the central Baltic Sea.

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