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Laurent Urios, H el ene Agogu e, Fran oise Lesongeur, Erko Stackebrandt, Philippe Lebaron. *Balneola vulgaris* gen. nov., sp. nov., a member of the phylum Bacteroidetes from the north-western Mediterranean Sea. *International Journal of Systematic and Evolutionary Microbiology*, 2006, 56 (8), pp.1883-1887. 10.1099/ijs.0.64285-0 . hal-01355090

HAL Id: hal-01355090

<https://hal.science/hal-01355090v1>

Submitted on 22 Aug 2016

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Balneola vulgaris gen. nov., sp. nov., a member of the phylum Bacteroidetes from the north-western Mediterranean Sea

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A novel aerobic, Gram-negative bacterium, named 13IX/A01/164^T, was isolated from surface waters in the coastal north-western Mediterranean Sea. Cells were motile, straight rods, 2.5 µm long and 0.2 µm wide, and formed orange colonies on marine agar medium. The G+C content of the genomic DNA of strain 13IX/A01/164^T was 42 mol%. Phylogenetic analysis of the 16S rRNA gene sequence placed the strain in the phylum *Bacteroidetes* within the family *Crenotrichaceae*. On the basis of 16S rRNA gene sequence comparison and physiological and biochemical characteristics, this isolate represents a novel species of a new genus, for which the name *Balneola vulgaris* gen. nov., sp. nov. is proposed. The type strain of *Balneola vulgaris* is 13IX/A01/164^T (=DSM 17893^T=CIP 109092^T=OOB 256^T).

Micro-organisms were the first form of life on Earth and they are found everywhere in nature, including extreme environments like hot springs and salt marshes. Samples collected in these hostile environments have revealed a wide range of micro-organisms that have adapted not only to survive, but also to reproduce under extreme conditions. Many of these organisms belong to the *Archaea* and they sometimes exhibit very interesting properties that have potential in biotechnological applications. For this reason, there is an increasing interest in exploring microbial diversity in these ecosystems (Demirjian et al., 2001). However, although the majority of thermophilic and extremely halophilic micro-organisms belong to the *Archaea*, members of the Bacteria with similar characteristics and properties have been isolated. For example, the orders *Thermales* (Rainey & da Costa, 2001) and *Thermotogales* (Huber & Stetter, 1992) encompass 13 genera of thermophilic strains and the genera *Halorhodospira* (Imhoff & Süling, 1996) and *Salinibacter* (Anton et al., 2002) are composed of extremely halophilic species. Some recently described genera, such as *Rhodothermus* and *Thermonema*, belong to the family *Crenotrichaceae* within the *Bacteroidetes*. This family is remarkable as all its members described to date exhibit characteristics that are regarded as extremophilic. Species of the genera *Rhodothermus* (Alfredsson et al., 1988; Sako et al., 1996) and *Thermonema* (Hudson et al., 1989; Tenreiro et al., 1997) are thermophilic and have optimal growth temperatures between 60 and 80°C. Isolates of the genus *Salinibacter* are extremely halophilic and require at least 150 g salt l⁻¹ for growth. In this paper, a novel strain of the family *Crenotrichaceae*, isolated from the north-western Mediterranean Sea, is described; this strain represents the first member of this family that does not exhibit the extreme characteristics that are found in other members of the family.

Samples were collected in September 2001 in the bay of Banyuls-sur-Mer (42°29' N 3° 08' E) by submerging a sterile bottle and opening it at a depth of 0,5 m (Agogu e et al., 2004). Subsamples were spread on marine agar plates (MA 2216; Difco) and incubated at 25°C for 2 weeks. Colonies were picked and purified after at least three subcultures. Among these colonies, an isolate that formed orange coloured colonies was obtained and referenced as strain 13IX/A01/164^T (Agogu e et al., 2005).

Microscopic observations (Olympus AX70) indicated that cells of strain 13IX/A01/164T were motile rods, approximately $2,5\pm 0,4$ μm long and $0,2\pm 0,06$ μm wide. No gliding motility was observed. Cells were negatively stained for transmission electron microscopy (Raguénès et al., 1997). A polar flagellum was observed (Fig. 1). The Ryu KOH reaction (Powers, 1995) led to immediate cell lysis that was confirmed by phase-contrast microscopy (Olympus AX70). This positive reaction indicated that cells were Gram-negative.

The strain was grown in marine broth medium (MB 2216; Difco). Marine broth medium with a range of salinities was prepared according to the composition provided by the manufacturer with the appropriate NaCl concentration. To study growth at a range of pH values, MES, PIPES, AMPSO or MOPS (Sigma) was added to marine broth medium to reach the required pH. Cultures were incubated at 30°C under aerobic conditions. Methods for the determination of growth parameters were as reported by Wery et al. (2001b). Growth was observed at 10–40°C, with optimum growth at 30°C (see Supplementary Fig. S1a available in IJSEM Online). The strain grew at NaCl concentrations of 0–70 g l^{-1} ; an optimum concentration for growth could be defined at 10 g l^{-1} , but growth rates were quite similar at 5 and 20–40 g l^{-1} (Supplementary Fig. S1b). Growth occurred at pH 5.0–10.0, with a clear optimum at pH 8.0. A linear increase in the growth rate was observed from pH 5.0 to 8.0; at pH 9.0, growth decreased dramatically to 60% of the value obtained at pH 8.0 (Supplementary Fig. S1c). The shortest generation time observed was 23 min. The ability to use different substrates was investigated with Biolog GN2 MicroPlates (De Groote et al., 1999) according to the manufacturer's instructions except for the incubation time (measurements were performed every hour for 24 h). Positive reactions are indicated in Table 1. Weak positive reactions were observed for cellobiose, lactose and chydroxybutyrate.

Enzyme activities were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, chymotrypsin and acid phosphatase exhibited positive reactions. Colour reactions for esterase (C8) and cystine arylamidase were weak. Catalase and oxidase tests were performed; the strain was found to be catalase-positive and oxidase-negative. The presence of flexirubin

pigments was investigated according to McCammon & Bowman (2000); a negative reaction was observed.

Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID) using cells grown in MB. As cells did not grow on the medium recommended by the MIDI system (trypticase soy broth), the fatty acids obtained could not be compared directly to those of the MIDI database. Extracts were analysed using a Hewlett Packard model HP6890A GC equipped with an FID as described previously (Kämpfer & Kroppenstedt, 1996). The fatty acid composition of strain 13IX/A01/164T was as follows: 15 : 0 iso (23.5 %), 15 : 0 iso 2-OH (11.3 %), 17 : 1v8c (11 %), 15 : 0 (10.7 %), 15 : 1v6c (10.6 %), 13 : 0 iso (6.5 %), 17 : 1v9c iso (6.5 %), 15 : 0 anteiso (4.1 %), 17 : 1v6c (3.2 %), 14 : 0 iso (2.3 %), 16 : 0 iso (2.3 %), 17 : 0 iso (1.5 %), 16 : 0 (1.4 %), 17 : 0 (1 %), 15 : 1v8c (1 %), and 16 : 1v5c (1 %). Although fatty acid data are missing for species of *Salinibacter*, those of *Rhodothermus* (Silva et al., 2000) have significantly different compositions, with major amounts (>20% each) of 17 : 0 anteiso, 15 : 0 anteiso and 17 : 0 iso.

Genomic DNA was extracted as described by Wery et al. (2001a). The G+C content was determined by thermal denaturation using the method of Marmur & Doty (1962) and conditions reported by Raguénès et al. (1997). The G+C content of the genomic DNA of strain 13IX/A01/164^T was 41.8±1.1 mol%. The 16S rRNA gene was amplified and sequenced as described by Agogué et al. (2005). The sequence was compared to those available in GenBank using BLAST (Altschul et al., 1997). Alignments and similarities were obtained by the CLUSTAL X method. The phylogenetic reconstruction was produced using PHYLO_WIN (Galtier et al., 1996) with the Jukes–Cantor correction for determination of the distance matrix, followed by neighbor joining (Saitou & Nei, 1987) for determination of the best phylogenetic tree. Maximum-likelihood methods (Felsenstein, 1981) were also employed for phylogenetic analysis. Bootstrap values were determined according to Felsenstein (1985). Strain 13IX/A01/164^T was phylogenetically affiliated to the family *Crenotrichaceae* within the phylum *Bacteroidetes* (Fig. 2) and was most closely related to *Rhodothermus marinus* NR-32^T (16S rRNA gene sequence similarity of 87

%). Strain 13IX/A01/164^T was also closely related to strains of *Salinibacter ruber*, *Thermonema rossianum* and *Thermonema lapsum*.

Some characteristics of strain 13IX/A01/164^T were quite similar to those of its nearest relatives, including colony pigmentation, salinity growth range (with the exception *Salinibacter ruber*, which is extremely halophilic) and pH growth range (with a slightly higher optimum) (Table 1). Nevertheless, strain 13IX/A01/164^T exhibited no extreme characteristics, it is neither thermophilic nor halophilic, and the environment from which it was isolated clearly differed from those where the most closely related species were found. No filamentous cells, such as those observed with *Thermonema* strains, were observed. The DNA G+C content of strain 13IX/A01/164^T was significantly lower (by 5–24 mol%) than those of related species.

Based on phenotypic and genotypic differences between strain 13IX/A01/164^T and its nearest relatives, it is proposed that strain 13IX/A01/164^T should be assigned as a representative of a novel species in a new genus belonging to the *Crenotrichaceae*. Because of the geographical origin of strain 13IX/A01/164^T and its lack of original characteristics compared with the extremophilic behaviour of the most closely related species, the name *Balneola vulgaris* gen. nov., sp. nov. is proposed.

Description of Balneola gen. nov.

Balneola (Bal.ne'o.la. M.L. fem. n. *Balneola* the ancient name of Banyuls, referring to the area of isolation of the first characterized strain).

Aerobic, motile, Gram-negative rod growing optimally at 30°C and pH 8.0. Catalase-positive and oxidase-negative. The major fatty acids are 15 : 0 iso (23.5 %), 15 : 0 iso 2-OH (11.3 %), 17 : 1v8c (11 %), 15 : 0 (10.7 %), 15 : 1v6c (10.6 %), 13 : 0 iso (6.5%) and 17 : 1v9c iso (6.5 %). Phylogenetically affiliated with the phylum Bacteroidetes within the family *Crenotrichaceae*. The type species is *Balneola vulgaris*.

Description of Balneola vulgaris sp. nov.

Balneola vulgaris (vul.ga'ris. L. fem. adj. vulgaris common, referring to the lack of specific characteristics).

Forms orange colonies on MA medium. Grows at 10– 40°C (optimum 30°C), pH 5.0– 10.0 (optimum pH 8.0) and a salinity range of 0–50 g l⁻¹ (optimum 20 g l⁻¹). Positive reactions with Biolog GN2 plates are obtained for N-acetylgalactosamine, adonitol, arabinose, arabitol, erythritol, fructose, fucose, glucose, lactulose, maltose, methyl glucoside, sorbitol, acetate, hydroxyphenylacetate and propionate. Positive reactions with the API ZYM system are obtained for alkaline phosphatase, leucine arylaminidase, valine arylaminidase, trypsin, chymotrypsin and acid phosphatase. Other characteristics are given in Table 1.

The type strain is 13IX/A01/164^T (=DSM 17893^T=CIP 109092^T=OOB 256^T), isolated from a water column in the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E). The DNA G+C content of strain 13IX/A01/164^T is 42 mol%.

Acknowledgements

We thank Laurent Intertaglia from the OOB and Magali Cros for their technical assistance. This work was partly funded by the AIRWIN project 'Structure and role of biological communities involved in the transport and transformation of persistent pollutants at the marine AIR–Water INterface' (contract EVK3-CT2000-00030). The project was also carried out in the frame of the MarBEF Network of Excellence 'Marine Biodiversity and Ecosystem Functioning' which is funded in the Community's Sixth Framework Programme (contract no. GOCE-CT- 2003-505446). This publication is contribution number MPS- 06027 of MarBEF. It was also partly funded by the French program 'Bio-diversité et Changement Global – project: development of a coastal microbial observatory' from the Institut Français de la Biodiversité.

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Table 1. Comparison of some characteristics of strain 13IX/A01/164^T and closely related species
 + : Positive; - negative; (+), weakly positive; ND, no data available.

| Characteristic | Strain 13IX/A01/164 ^T | <i>Thermonema rossianum</i> | <i>Thermonema lapsum</i> | <i>Salinibacter ruber</i> | <i>Rhodothermus marinus</i> |
|--|----------------------------------|-----------------------------|------------------------------------|---------------------------------|--|
| Isolation source | | | | | |
| Location | Bay of Banyuls, France | Bay of Naples, Italy | Rotorua area, New Zealand | Mallorca and Santa Paula, Spain | Reykjanes, Iceland, and Tachibana Bay, Japan |
| Environment | Water column | Saline hot springs | Terrestrial non-saline hot springs | Crystallizer ponds of salterns | Shallow submarine hot springs |
| Sample type | Water | Water | Water | Brine | Water and sediment |
| DNA G+C content (mol%) | 41.8±1.1 | 50.9 | 47 | 66.3–67.7 | 64.4–66.6 |
| Colony colour | Orange | Yellow | Yellow | Red | Reddish |
| Shape | Rods | Filamentous cells | Filamentous cells | Rods or slightly curved | Rods |
| Cell dimensions (mm) | 2.0–3.0x0.2 | ND | ≥60 (length) | 2.0–6.0x0.4 | 2–10 |
| Motility | + | 2 | + | + | 2 |
| Temperature for growth (°C) | | | | | |
| Range | 10–40 | 35–65 | 35–65 | 27–52 | 50–85 |
| Optimum | 30 | 60 | 60 | 37–47 | 65–80 |
| Salinity for growth (g l ⁻¹) | | | | | |
| Range | 0–50 | 5–50 | 0–30 | 150–300 | 10–60 |
| Optimum | 20 | 10–30 | 0 | 200 | 20–30 |
| pH for growth | | | | | |
| Range | 5.0–10.0 | >5.0 and <10.0 ^a | <8.0 ^b | 6.0–8.5 | 5.5–9.0 |
| Optimum | 8.0 | 7.0–7.5 | 6.5 | 6.5–8.0 | 7.0 |
| Substrates utilized* | | | | | |
| Tween 80 | 2 | ND | ND | 2 | ND |
| Fructose | + | ND | ND | 2 | ND |
| Galactose | 2 | ND | 2 | 2 | + |
| Glucose | + | ND | 2 | 2 | + |
| Inositol | 2 | ND | 2 | ND | ND |
| Lactose | (+) | ND | 2 | 2 | + |
| Maltose | + | ND | ND | 2 | + |
| Mannitol | (+) | ND | ND | 2 | ND |
| Rhamnose | 2 | ND | 2 | ND | ND |
| Sorbitol | + | ND | 2 | 2 | 2 |
| Sucrose | 2 | ND | 2 | 2 | + |
| Acetate | + | ND | 2 | 2 | + |
| Citrate | 2 | ND | ND | ND | 2 |
| Gluconate | 2 | ND | 2 | ND | 2 |
| Succinate | 2 | ND | 2 | 2 | 2 |
| Alanine | 2 | ND | 2 | ND | ND |
| Asparagine | 2 | ND | ND | ND | 2 |
| Aspartate | 2 | ND | ND | ND | + |
| Glutamate | 2 | ND | 2 | ND | + |
| Leucine | 2 | ND | ND | ND | 2 |
| Phenylalanine | 2 | ND | ND | ND | 2 |
| Proline | 2 | ND | 2 | ND | 2 |
| Serine | 2 | ND | ND | ND | 2 |
| Threonine | 2 | ND | ND | ND | 2 |

*Results of substrate utilization tests were not given in enough detail in the description of *T. rossianum*.

^aNo growth was observed at pH 5.0 or pH 10.0.

^bNo growth was observed at pH 8.0; the lower pH limit for growth is unknown.

Figure 1. Electron micrograph of a negatively stained cell of strain 13IX/A01/164^T harvested during the exponential phase. A single polar flagellum is shown. Bar, 1 μ m.

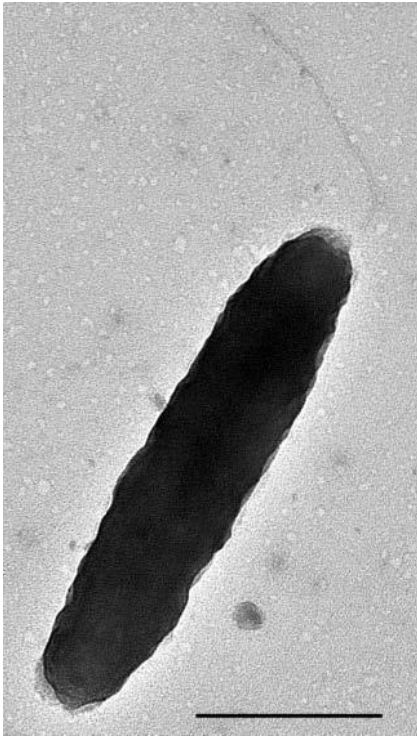


Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain 13IX/A01/164^T. *Bacteroides massiliensis* B8463^T was used as an outgroup. Accession numbers are indicated in parentheses. The tree corresponds to an unrooted tree obtained by the neighbour-joining algorithm (Kimura corrections). Bootstrap percentages from 500 replicates are displayed on their relative branches.

