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Halorhabdus rudnickae sp. nov., a halophilic archaeon isolated from a salt mine borehole in Poland

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ABSTRACT

Two halophilic archaea, designated strains WSM-64^T and WSM-66, were isolated from a sample taken from a borehole in the currently unexploited Barycz mining area belonging to the "Wieliczka" Salt Mine Company, in Poland. Strains are red pigmented and form non-motile cocci that stain Gram-negative. Strains WSM-64^T and WSM-66 showed optimum growth at 40 °C, in 20% NaCl and at pH 6.5–7.5. The strains were facultative anaerobes. The major polar lipids of the two strains were phosphatidylglycerol (PG2), phosphatidylglycerol phosphate methyl ester (PGP-Me) and sulfated diglycosyl diether (S-DGD). Menaquinone MK-8 was the major respiratory quinone. The DNA G+C content of strain WSM-64^T was 61.2 mol% by HPLC method; 61.0 mol% by genome sequencing. Analysis of the almost complete 16S rRNA gene sequence indicated that the strains WSM-64^T and WSM-66 (99.7% identity) represented a member of the genus *Halorhabdus* in the family *Halobacteriaceae*. Both strains formed a distinct cluster and were most closely related to *Halorhabdus tiamatea* SARL4B^T and *Halorhabdus utahensis* AX-2^T (DSM 12940^T) (95.4% and 95.6%, respectively). ANI values of WSM-64^T with the closest relative type strains were <78.5%. Based on 16S rRNA gene sequence and whole genome analyses, physiological and biochemical characteristics we describe a new species represented by strain WSM-64^T (=DSM 29498^T =CECT 8673^T) for which we propose the name *Halorhabdus rudnickae* sp. nov.

The genus *Halorhabdus* comprise two species, *Halorhabdus utahensis* isolated from sediment of Great Salt Lake, USA and *Halorhabdus tiamatea* isolated from the brine sediment interface of the Shaban Deep brine in the Red Sea [2,20]. The species *Hrd. utahensis* grows under fully oxic and anoxic conditions, while *Hrd. tiamatea* grows under anoxic to micro-oxic conditions of up to about 5% O_2. Growth of *Hrd. tiamatea* in fully oxic conditions is extremely poor or does not occur at all [21]. Several haloarchaea have been isolated from the interior of salt mines, some of which belong to new genera [19,25]. However, the organism we describe here was isolated from the hypersaline environment of borehole number E 632 in the currently unexploited Barycz mining area belonging to the "Wieliczka" Salt Mine Company, in Poland.

Strains WSM-64^T and WSM-66 were isolated from a water sample of borehole number E 632 in the former Barycz mining area, in Poland (49°59′05″ N 20°00′52″ E), with a pH of 6.5 and a salinity of 25.5%. The detailed geological and hydrological characteristics of the sampling area has been described by d'Obyrn et al. [5].

Water samples were maintained without temperature control for 6 days, and then 1 to 5 ml were filtered through membrane filters (Gelman type GN-6; pore size 0.45 μm; diameter

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47 mm). The filters were placed on the surface of solidified medium 372 (*Halobacteria* medium) in the list of recommended media for microorganisms at the DSMZ (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium372.pdf) except that the NaCl concentration was 25% (w/v) instead of 20% (w/v). The plates were wrapped in plastic to prevent evaporation and incubated at 45 °C for up to 20 days. Single isolated colonies where selected and sub-cultured onto agar plates with the same media to obtain a pure culture. The selection of organisms was carried out trying to cover all possible colony morphologies to cover the widest range of diversity as possible. Cultures were purified by subculturing and the isolates stored at −70 °C in *Halobacteria* medium with 15% (w/v) glycerol.

Total genomic DNA was extracted following the method of Nielsen et al. [11], and used for the different analyses performed. Random amplified polymorphic DNA (RAPD) analysis was carried out as described by Wiedmann-al-Ahmad et al. [22] using the primer OPA3 (5′-AGTCAGCCAC-3′). The G+C content of DNA was determined by high-performance liquid chromatography as described by Mesbah et al. [10] and by genome sequencing. 16S rRNA gene sequences were reviewed, corrected and assembled using Sequencher v4.9 software (Gene Codes Corp., USA). Alignments and tree reconstructions were performed using the ARB software package version 5.5 [9]. The new sequences were added to the reference LTP123 [23] database, and aligned using the SINA tool (SILVA Incremental Aligner) [14], implemented in the ARB software package. Final alignments were manually improved following the reference alignment in ARB-editor. Complete sequences were used to reconstruct de novo trees using the neighbor-joining algorithm implemented in the ARB software package; and the RAxML algorithm [18] using 100 bootstrap replicates. For the reconstructions we used different datasets of LTP type strains, as well as different filters implemented in the LTP123 database (i.e. no filter, 30% and 50% conservational filters) to evaluate the branching orders. Whole genome sequencing was performed at the FISABIO Sequencing and Bioinformatics services (Valencia, Spain) using Illumina Miseq technology; DNA libraries were generated following the Nextera XT Illumina protocol (Nextera XT Library Prep kit [FC-131-1024]). We used 0.2 ng μl^{-1} purified gDNA to initiate the protocol. The libraries were sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3 [MS-102-3001]) on a MiSeq Sequencer according to manufacturer's instructions. After sequencing and quality check, the final dataset resulted in 3,116,141 joined and unjoined reads with a mean read length of 291,18 bp (sd: 84,19). The assembly was performed using MIRA program [3]. 2,999,074 reads passed assembly quality check resulted in 24 assembled contigs (the larger: 569,420 bp). After manual revision, the final assembly was of 6 contigs with a total length of 3,367,133 bp. Genome to genome comparisons to calculate the average nucleotide identity (ANI) and tetranucleotide regression values as taxonomic thresholds were performed using the program JSpecies using the default parameters [15].

Unless otherwise stated, phenotypic characterization was performed under aerobic conditions in *Halobacteria* medium at 40 °C as previously described [1,2,12,13,17]. Characterization of the type strains of *Hrd. tiamatea* (SARL4B^T) and *Hrd. utahensis* (DSM 12940^T) was performed in this lab, using the same methods and were not repeated [2].

Cell morphology, motility and presence or absence of poly-β-hydroxybutyrate were examined by phase contrast microscopy during the exponential phase of growth. The growth temperature range of the strains was examined with 5 °C increments between 15 and 60 °C by measuring the turbidity (610 nm) of cultures incubated in 300 ml metal-capped Erlenmeyer flasks, containing 100 ml of medium. The pH range for growth was examined at 40 °C in the same medium by using 50 mM citrate-phosphate buffer, MES, HEPES, TAPS, and CAPSO over a pH range from 5.0 to 9.5 with intervals of 0.5 pH units. Growth with added salt was determined in liquid medium with NaCl ranging between 0 and 30% (w/v) with intervals of 5%. The requirement for Mg^{2+} was tested under optimal growth conditions, by supplementing the liquid medium with 0%, 0.5%, 1%, 2%, 5% (w/v) of $MgSO_4 \cdot 7H_2O$ and with 5% increments between 5 and 60%. Susceptibility to antibiotics was determined in liquid medium by the addition of filter-sterilized antibiotics (25 $\mu g\,ml^{-1}$) [12,13]. Catalase and oxidase activities were examined as described previously [17]. Other enzymatic characterization tests were performed using the API ZYM system (bioMérieux) as recommended by the manufacturer but with salinity adjusted to 20% NaCl (w/v) and incubated at 40 °C. The production of hydrogen sulphide and indole, the methyl red test reaction and the Voges-Proskauer reaction were performed as described previously [17] in DSMZ medium 372. Anaerobic growth was assessed in cultures in the same medium supplemented with nitrate, L-arginine, DMSO and TMAO (each at 3.0 g l^{-1}) and incubated in anaerobic chambers (GENbox anaer, bioMérieux). Single-carbon source assimilation tests were performed in minimal *Halobacteria* medium DSMZ 372 with 0.01% of yeast extract (without casamino acids, Na-glutamate and Na_3-citrate) to which filter-sterilized ammonium sulfate (0.5 g l^{-1}) and the carbon source (2.0 g l^{-1}) were added. Single carbon source assimilation was examined by measuring the turbidity of cultures incubated at 40 °C in 20 ml screw capped tubes containing 10 ml of medium for up to 7 days. Acid production from carbohydrates was determined by using API 50 CH system (bioMérieux) according to the manufacturer's instructions, using API 50 CHB/E medium containing 20% NaCl and 2% $MgSO_4.7H_2O$ (microtubes were covered with mineral oil). Results were recorded after 3, 7 and 15 days of incubation at 40 °C.

Polar lipids were extracted from freeze-dried cells; individual polar lipids were separated by one-dimensional and two-dimensional thin-layer chromatography and visualized as described previously [6]. To visualize phospholipids, aminolipids, glycolipids and total lipids the following reagents were used respectively, molybdenum blue, ninhydrin, α-naphthol-sulfuric acid and molybdophosphoric acid [6]. Lipoquinones were extracted, purified by thin-layer chromatography and separated by high performance liquid chromatography [7].

From the incubated filters, 35 isolates could be brought to pure culture. Among them, ten with identical RAPD patterns were recovered being the most abundant morphotype. Two of which were designated WSM-64^T and WSM-66, and chosen for further characterization (Supplementary Fig. S1). The 16S rRNA gene phylogenetic reconstruction based on both maximum likelihood and neighbor joining analyses affiliated both newly isolated strains with *Hrd. tiamatea* (SARL4B^T) and *Hrd. utahensis* AX-2^T (DSM 12940^T) type strains (Fig. 1). WSM-64^T, WSM-66 shared identities of >99.7%; whereas the identity with the *Hrd. tiamatea* and *Hrd. utahensis* type strains was always <95.6%. Both *Hrd. tiamatea* and *Hrd. utahensis* type strains shared an identity of 99.2%. The sequence divergence between *Halorhabdus* spp. type strains and the new isolates appears well in the range where a putative new genus could be drawn [24]. However, the monophyletic nature of the branch including all such sequences (Fig. 1) indicated that the best option is to classify the new isolates within the existing *Halorhabdus* genus.

As the genome sequences of the *Hrd. tiamatea* (SARL4B^T) and *Hrd. utahensis* (DSM 12940^T) type strains (RefSeq NC_021921 and NC_013158 respectively) were available in the public repositories, the almost complete genome sequence of strain WSM-64^T was obtained for comparative studies. The sequencing approach rendered a total of 2.9 × 10^6 reads assembled in a total of 6 contigs, and the assembled stretches rendered a genome draft with a size of 3.36 Mb with an average coverage of 240.48 fold. The automatic annotation by means of Prodigal program identified a total