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Novel isolates double the number of chemotrophic species and allow the first description of higher taxa in *Acidobacteria* subdivision 4☆

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ABSTRACT

Despite their high phylogenetic diversity and abundance in soils worldwide, *Acidobacteria* represent an enigmatic bacterial phylum. Four novel *Acidobacteria* strains were isolated from Namibian semiarid savannah soils using low-nutrient cultivation media and extended incubation periods. 16S rRNA gene sequence analyses placed the isolates within *Acidobacteria* subdivision 4. Sequence identities with their closest relatives *Aridibacter famidurans* and *Blastocatella fastidiosa* were ≤94.9%. The Gram-negative, non-motile, rod-shaped, aerobic, and chemoorganotrophic bacteria grew at minimum doubling times of 5–14 h and formed tiny white to pinkish colonies. Major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylglycerol. The major isoprenoid quinone was MK-8. The major fatty acid methyl esters comprised *iso*-$C_{15:0}$, *iso*-$C_{15:1}$H/$C_{13:0}$ 3-OH, and $C_{16:1}\omega7c/C_{16:1}\omega6c$. Based on a polyphasic taxonomic characterization, strain Ac_18_E7^T (=DSM 26557^T = LMG 28656^T) represented a novel species and genus, *Tellurimicrobium multivorans* gen. nov., sp. nov. The other strains constituted three independent species of the novel genus *Stenotrophobacter* gen. nov., *Stenotrophobacter terrae* sp. nov. (Ac_28_D10^T = DSM 26560^T = LMG 28657^T), *S. roseus* sp. nov. (Ac_15_C4^T = DSM 29891^T = LMG 28889^T), and *S. namibiensis* sp. nov. (Ac_17_F2^T = DSM 29893^T = LMG 28890^T). These isolates doubled the number of established species and permitted the description of higher taxa of *Acidobacteria* subdivision 4. The family *Blastocatellaceae* fam. nov. is proposed in order to summarize the currently known oligotrophic, slightly acidophilic to neutrophilic mesophiles from arid soils. The superordinated order *Blastocatellales* ord. nov. and *Blastocatellia* classis nov. also include the terrestrial species *Pyrinomonas methylaliphatogenes* and the anoxygenic photoheterotrophic species *Chloracidobacterium thermophilum* from microbial mats.

Approximately 60 of the 90 recognized bacterial phyla are comprised of only a few or even no cultivated representatives [2,37]. Members of the phylum *Acidobacteria* represent a major fraction of soil bacterial communities where they constitute up to 79% of all bacterial cells [22]. Whereas >70,000 different sequence types of *Acidobacteria* have been detected by culture-independent molecular methods [3,4,20,23,26,32,34,62], only 31 species have been cultivated and validly described to date [41]. After the isolation and characterization of the first representative *Acidobacterium capsulatum* [25] in 1991, the rate of description of novel species has substantially increased over the past few years [9,16,19,26,50,58,60] due to better insights into the specific adaptations of these bacteria [13,15,18,26,29,36,38,40] and subsequently improved cultivation methods [13,16,40]. However, the successful isolation of novel *Acidobacteria* to date has been strongly biased toward a single subdivision.

Abbreviations: DPG, diphosphatidylglycerol; DDH, DNA-DNA hybridization; FAMEs, fatty acid methyl esters; MK, menaquinone; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGL, phosphoglycolipid; SD, subdivision; SE, soil extract; SSE, soil solution equivalent; TLC, thin-layer chromatography.

☆ The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Ac_15_C4^T, Ac_17_F2^T, Ac_18_E7^T, and Ac_28_D10^T are KP638489, KP638491, KP334257, and KF840371, respectively.

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The phylum *Acidobacteria* is divided into 26 subdivisions [3]. The majority of the presently described species are affiliated with *Acidobacteria* subdivision 1 (21 species, including one species "Acidipila rosea" with a not yet validated name). Additional species are known for subdivisions 3 (two species), 4 (five species), 8 (three species) and 23 (one species). "*Thermotomaculum hydrothermale*" has been described as a species of subdivision 10 [21], but its name has also not yet been validated. Subdivision 4 (SD 4) includes five validly described species that are classified as four genera, namely *Blastocatella fastidiosa* [16], *Aridibacter famidurans* and *A. kavangonensis* [19], *Pyrinomonas methylaliphatogenes* [9], and *Chloracidobacterium thermophilum* [50]. Except for *C. thermophilum*, which has an anoxygenic photoheterotrophic and microaerophilic metabolism, the other representatives of SD 4 are characterized as rather slow-growing aerobic chemoorganoheterotrophs [9,14,16,19] that use a narrow spectrum of carbon and energy sources, and are able to grow in low-nutrient media [9,16,19].

In the present study, a high throughput cultivation approach was used to enrich and isolate additional *Acidobacteria* of underrepresented subdivisions. Soil samples from Namibian semiarid savannah soils located in Mashare (Kavango region; Supplementary Fig. S1) yielded four novel strains designated Ac_15_C4^T, Ac_17_F2^T, Ac_18_E7^T, and Ac_28_D10^T that represented four additional novel *Acidobacteria* SD 4 species. Strain Ac_28_D10^T was isolated from an old agricultural flood plain soil (17°53′32.4″ S, 20°11′15.4″ E) with a slightly acid pH (pH 6.6 and 5.7 measured in distilled water and in 2 mM $CaCl_2$, respectively). Strains Ac_18_E7^T and Ac_15_C4^T were isolated from two different fallow soils (17°55′0.02″ S, 20°06′18.7″ E and 17°53′37.9″ S, 20°14′50.7″ E, respectively); the former being a Kalahari sandy soil with a slightly acidic pH (pH 6.2 in distilled water and 5.2 in 2 mM $CaCl_2$) and the latter constituting an old flood plain soil with a slightly basic pH (pH 8.2 in distilled water and 7.3 in 2 mM $CaCl_2$). All three soil samples were collected in spring 2011. The fourth strain, Ac_17_F2^T, originated from a riparian woodland soil (17°53′35.5″ S, 20°14′57.5″ E) with a near-neutral pH (7.6 in distilled water and 7.2 in 10 mM $CaCl_2$) sampled in spring 2012.

According to their original pH, soil samples were dispersed in 10 mM HEPPS buffered at pH 8.0 (Ac_15_C4^T), in 10 mM HEPES buffered at pH 7.0 (Ac_17_F2^T) or in 10 mM MES buffered at pH 6.0 (Ac_18_E7^T and Ac_28_D10^T) and total bacterial cell numbers were determined after staining with SYBR Green I (Life Technologies). Cultivation experiments were set up in sterile 96-well microtiter plates containing 180 μL of growth medium per well. Each well was inoculated with 20 μL soil suspension containing 10 or 100 bacterial cells. Strains Ac_28_D10^T and Ac_15_C4^T grew in soil solution equivalent (SSE)/Cmix medium buffered at pH 6.0 with MES and at pH 8.0 with HEPPS, respectively (Supplementary Materials and Methods). Strain Ac_18_E7^T was cultivated in SSE/HD 1:10 medium (=DSMZ medium 1426) buffered at pH 6.0 (Supplementary Materials and Methods). For cultivation of strain Ac_17_F2^T, medium with soil extract (SE)/HD 1:10 buffered at pH 7.0 was employed (Supplementary Materials and Methods). After six weeks incubation at 20 °C in the dark, bacterial growth was detected by turbidity [19]. All positive enrichments were screened for growth of *Acidobacteria* by group-specific diagnostic PCR using the primer pair Acido31f [4] and 907r [28]. Cultures containing *Acidobacteria* were streaked on the same media used for initial enrichment but solidified with 1.5% (w/v) agar, and strains were isolated by subsequent streaking. Unless otherwise stated, all strains were routinely grown aerobically at 25 °C in SSE/HD 1:10 buffered at pH 5.5 (strains Ac_18_E7^T and Ac_28_D10^T) or at pH 7.0 (strains Ac_15_C4^T and Ac_17_F2^T). Data for reference strains were taken from the literature, since the cultivation conditions in previous studies were comparable (*Blastocatella fastidiosa* A2-16^T, *Aridibacter famidurans* A22_HD_4H^T, and *A. kavangonensis* Ac_23_E3^T). The two strains *Pyrinomonas methylaliphatogenes* K22^T and *Chloracidobacterium thermophilum* B^T required specific growth conditions due to their different physiology [9,16,19,50] and hence could not be tested in the media used for the former strains.

Phenotypic characterization was performed as described previously [16,19]. Cells of all four strains stained Gram-negative and had a similar morphology. They were short and rod-shaped, with lengths of up to 2.5 μm and widths of up to 0.7 μm (Table 1). Spores or capsules were not observed under any growth condition. All strains divided by binary fission. Whereas strain Ac_18_E7^T formed small aggregates of up to 20 cells (Supplementary Fig. S2a), the other three strains occurred as single cells or as pairs (Supplementary Fig. S2b–d). Unlike *Blastocatella fastidiosa* A2-16^T, all strains isolated in the present study were non-motile and formed only very small colonies that were usually less than 1 mm in diameter. After 1 week of growth on solid SSE/HD 1:10 medium, strain Ac_18_E7^T produced white, circular, convex colonies with regular edges that exhibited a bright pinkish color. Colonies of strains Ac_15_C4^T, Ac_17_F2^T, and Ac_28_D10^T were circular, convex with regular edges and pink in color.

The temperature and pH ranges and their optima for growth were determined in duplicate or triplicate under oxic conditions using liquid SSE/HD 1:10 medium, as described previously [19]. All strains were mesophiles with broad temperature optima and a broad temperature growth range. None of them grew below 14 °C or above 43 °C (Table 1). The optima for growth were found at neutral or slightly acidic pH (5.5–7.9). However, the pH growth range was alkaline (Ac_18_E7^T) or even highly alkaline (other strains) (Table 1). With respect to both parameters, the four strains resembled *Blastocatella* and *Aridibacter* species, although they showed a narrower range of optimum values. In contrast, *Pyrinomonas methylaliphatogenes* K22^T and *Chloracidobacterium thermophilum* B^T have been shown to be moderate thermophiles [9,50]. Doubling times under optimal conditions for strains Ac_15_C4^T, Ac_17_F2^T, Ac_18_E7^T, and Ac_28_D10^T were 5.1 h, 13.8 h, 12.0 h and 11.9 h, respectively (Table 1). With respect to growth kinetics, only strain Ac_15_C4^T resembled the existing *Aridibacter* strains (doubling times 5.5–6.1 h), whereas all other new isolates grew significantly slower than even *B. fastidiosa* A2-16^T (doubling time 9.8 h).

The four novel strains exhibited an aerobic chemoorganotrophic metabolism. This type of metabolism is shared by all members of SD 4 *Acidobacteria* except for the anoxygenic photoheterotrophic strain *C. thermophilum* B^T [9,50]. All strains except Ac_28_D10^T showed catalase activity. Strains Ac_15_C4^T, Ac_17_F2^T, and Ac_28_D10^T were also cytochrome-*c* oxidase negative in contrast to Ac_18_E7^T and *P. methylaliphatogenes* K22^T. API 20NE and API ZYM galleries (BioMérieux) were used following the manufacturer's instructions and were examined after 48 h (API 20NE) or 4 h (API ZYM) incubation. Like *B. fastidiosa* A2-16^T, *A. famidurans* A22_HD_4H^T and *A. kavangonensis* Ac_23_E3^T, the four strains were unable to reduce nitrate to nitrate, ferment glucose, hydrolyze urea, or anaerobically metabolize the amino acid arginine with the release of ammonium. However, they showed a strong hydrolysis of aesculin, except strain Ac_18_E7^T that only exhibited a weak response. Unlike *B. fastidiosa* A2-16^T, none of the strains produced indole from the degradation of the amino acid tryptophan and, unlike both species of the genus *Aridibacter*, they did not hydrolyze ortho-nitrophenyl-β-galactoside (ONPG) by the enzyme *β*-galactosidase. Strains Ac_17_F2^T and Ac_18_E7^T did not hydrolyze gelatin, which was similar to *A. kavangonensis* Ac_23_E3^T (Table 1). Strains Ac_15_C4^T, Ac_17_F2^T, Ac_18_E7^T, and Ac_28_D10^T were characterized by exoenzymatic activity towards phosphate-containing compounds (alkaline and acid phosphatases and naphtol-AS-BI-phosphohydrolase), proteins (leucine arylamidase and trypsin), lipids (esterase lipase C8), glycosides, and amino sugars (N-acetyl-β-glucosaminidase) (Table 1). This is a property shared with the genera *Blastocatella, Aridibacter*, and *Pyrinomonas*