Aeromonas rivuli sp. nov., isolated from the upstream region of a karst water rivulet

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Two freshwater isolates (WB4.1-19^T and WB4.4-101), sharing 99.9 % 16S rRNA gene sequence similarity, were highly related to *Aeromonas sobria* (99.7 % similarity; 6 bp differences). A phylogenetic tree derived from a multi-locus phylogenetic analysis (MLPA) of the concatenated sequences of five housekeeping genes (*gyrB*, *rpoD*, *recA*, *dnaJ* and *gyrA*; 3684 bp) revealed that both strains clustered as an independent phylogenetic line next to members of *Aeromonas molluscorum* and *Aeromonas bivalvium*. The DNA–DNA reassociation value between the two new isolates was 89.3 %. Strain WB4.1-19^T had a DNA–DNA relatedness value of <70 % with the type strains of the other species tested. Phenotypic characterization differentiated the two novel strains from all other type strains of species of the genus *Aeromonas*. It is concluded that the two new strains represent a novel species of the genus *Aeromonas*, for which the name *Aeromonas rivuli* sp. nov. is proposed, with the type strain WB4.1-19^T (=CECT 7518^T=DSM 22539^T=MDC 2511^T).

The genus *Aeromonas* (family *Aeromonadaceae*, class *Gammaproteobacteria*), includes bacteria that are considered autochthonous of aquatic environments and are often associated with fish and human diseases (Martin-Carnahan & Joseph, 2005; Figueras, 2005; Janda & Abbott, 2010). At the time of writing, the genus includes more than 20 recognized species (Martin-Carnahan & Joseph, 2005; Saavedra *et al.*, 2006; Miñana-Galbis *et al.*, 2007; Demarta *et al.*, 2008; Martínez-Murcia *et al.*, 2008; Beaz-Hidalgo *et al.*, 2009; Alperi *et al.*, 2010b). *Aeromonas* group 501 has been recently described and named as *Aeromonas diversa* (Miñana-Galbis *et al.*, 2010) and two further species, *Aeromonas taiwanensis* and *Aeromonas sanarellii*, have also been described recently (Alperi *et al.*, 2010a). The taxonomy of the genus *Aeromonas* is considered complex when using either classical identification tools, such as phenotypic characteristics, or the 16S rRNA gene (Martínez-Murcia *et al.*, 2005; Ørmen *et al.*, 2005). 16S rRNA gene sequence analysis is considered to be a robust tool that is widely used in bacterial taxonomy. A threshold value for sequence similarity of about 97 % was proposed for the latter gene, below which strains exhibit sufficiently low DNA–DNA reassociation values (i.e. <70 %) to be considered as representing separate species (Stackebrandt & Ebers, 2006, and references therein). Recently, based on a broader dataset, the threshold value was increased to 98.7–99.0 % in order to facilitate taxonomic studies without sacrificing the quality and precision of a 'species' description (Stackebrandt & Ebers, 2006). In the genus *Aeromonas*, only one species (*Aeromonas simiae*) shows 16S rRNA gene sequence similarities below 97 % (96.8 %), while many of the others show values greater than 99.0 %. Strains of *Aeromonas bestiarum*, *Aeromonas salmonicida* and *Aeromonas piscicola* possess an identical sequence for this gene (Martínez-Murcia *et al.*, 2005; Beaz-Hidalgo *et al.*, 2009; Alperi *et al.*, 2010a, b). These high 16S rRNA gene sequence similarities and the microheterogeneities found in this gene hamper its utility for analysis of this genus (Alperi *et al.*, 2008, 2010a, b).

The use of housekeeping genes has been recommended as part of a polyphasic approach for the genomic circumscription of species and as a means of differentiating taxa from neighbouring species detected by, for example, 16S rRNA gene sequences (Stackebrandt *et al.*, 2002). We have

Abbreviations: MLPA, multi-locus phylogenetic analysis; VP, Voges–Proskauer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *rpoD*, *recA*, *dnaJ* and *gyrA* gene sequences of strains WB4.1-19^T and WB4.4-101 are FJ976900, FJ969434, FJ969433, FJ969435, FJ969432 and FJ969436, and FJ976899, FJ969439, FJ969437, FJ969440, FJ969441 and FJ969438, respectively.

Supplementary figures and tables are available with the online version of this paper.

introduced sequence analyses of housekeeping genes (*gyrB* and *rpoD*) for studies on the phylogenetic relationships between members of the genus *Aeromonas* (Yáñez *et al.*, 2003; Soler *et al.*, 2004). This approach turned out to constitute a turning point in the taxonomy of aeromonads as these genes, in common with other genes investigated recently (*rpoB*, *dnaJ*, *recA* and *cpn60*) by other authors (Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009), show a much higher resolution than 16S rRNA gene sequences. The analysis of housekeeping genes has not only led to some proposals for the reclassification of existing taxa (Martínez-Murcia *et al.*, 2009), but has also enabled the recognition of novel species of the genus *Aeromonas*, e.g. *Aeromonas aquariorum* and *Aeromonas tecta* (Martínez-Murcia *et al.*, 2008; Demarta *et al.*, 2008) and, more recently, *A. piscicola*, *A. fluvialis*, *A. taiwanensis* and *A. sanarellii* (Beaz-Hidalgo *et al.*, 2009; Alperi *et al.*, 2010a, b).

In a recent environmental study, the taxonomic diversity of aerobic bacteria (n=681) in a karst water rivulet in northern Germany (Westerhöfer Bach) was evaluated and 40 different genera and about 60 novel phylospecies were identified (Cousin *et al.*, 2008). Fifteen of the recovered isolates, belonging to the genus *Aeromonas* on the basis of partial 16S rRNA gene sequences (432 bp), were sent to our laboratory for further molecular characterization. The present communication describes the polyphasic approach adopted for the classification of a novel species of the genus *Aeromonas*.

All 15 isolates were cultured on sheep blood agar at 30 °C and DNA was extracted from single colonies using InstaGene Matrix (Bio-Rad Laboratories). The conditions for *rpoD* (820 bp) and 16S rRNA gene (1503 bp) sequence analysis, including primers, amplification conditions and sequencing, were as previously described (Martínez-Murcia *et al.*, 1992; Soler *et al.*, 2004). The sequences obtained were aligned independently with the sequences of the type and reference strains of all the members of the genus *Aeromonas* taken from our in-house database (constructed with our own sequences) and those that are available in GenBank, using the CLUSTAL_X program version 1.8 (Thompson *et al.*, 1997). Genetic distances were obtained using Kimura's two-parameter model (Kimura, 1980). Evolutionary trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the MEGA4 program (Tamura *et al.*, 2007) and maximum-parsimony method (for the 16S rRNA gene) using PAUP* version 4.0b10 (Swofford, 2002). The stability of the relationships was assessed by bootstrapping (1000 replicates).

The phylogenetic analysis based on the *rpoD* gene revealed that 13 of the 15 strains belonged to recognized species of the genus *Aeromonas*, while two strains (WB4.1-19^T and WB4.4-101) grouped as an individual lineage within the *A. molluscorum*/*A. bivalvium* cluster (data not shown). The two novel isolates shared 98.6 % *rpoD* gene sequence similarity (9 bp differences between their sequences). Sequence similarity with the closest species, *A. molluscorum*, was 94.3 % for strain WB4.1-19^T and 94.5 % for strain WB4.1-101. These values are below the minimum intra-species similarity of 97.4 % previously established for the *rpoD* gene in the genus *Aeromonas* (Soler *et al.*, 2004).

The almost complete 16S rRNA gene (1503 bp) was sequenced from both novel strains and revealed that the strains were highly related to each other (99.9 % similarity; 2 bp differences) sharing a very high gene sequence similarity (99.7 %) with *Aeromonas sobria* NCIMB 12065^T. These results were in agreement with those obtained at the DSMZ laboratory (Braunschweig, Germany) using partial sequences (432 bp) of the 16S rRNA gene. The similarities found for the two novel strains were in line with those shown by other species and indicated that the 16S rRNA gene threshold value for delineation of species of the genus *Aeromonas* (without considering those that show 100 % similarity) should be 99.5–99.7 %, which is higher than the new values of 98.7–99.0 % recently proposed by Stackebrandt & Ebers (2006).

In the phylogenetic trees constructed using the 16S rRNA gene sequences, the two new isolates clustered next to *A. sobria* NCIMB 12065^T. This association was only supported by low bootstrap values (<50 %) no matter whether the neighbour-joining (Fig. 1) or maximum-parsimony algorithms (see Supplementary Fig. S1 in IJSEM Online) were used to determine phylogeny. Chromatogram analysis of the 16S rRNA gene sequences of both strains showed microheterogeneities in two positions (1011 and 1018) for strain WB4.1-19^T and in four positions (258, 469, 1355, 1357) for strain WB4.4-101 (see Supplementary Table S1). Microheterogeneities have been described for other species of the genus *Aeromonas* (Alperi *et al.*, 2008, 2010a, b).

The multi-locus phylogenetic analysis (MLPA) involved sequencing the *gyrB* (923 bp), *rpoD* (652 bp), *recA* (600 bp), *dnaJ* (800 bp) and *gyrA* (709 bp) genes. A phylogenetic tree was constructed with the concatenated sequences of these five genes (3684 bp). This analysis was performed at the Molecular Diagnostic Center (MDC), Orihuela, Spain, as described by Alperi *et al.* (2010a).

The MLPA tree revealed, in agreement with all five single-gene phylogenies and in contrast to that of the 16S rRNA gene, that strains WB4.1-19^T and WB4.4-101 were not phylogenetically related to *A. sobria*, but appeared as an independent branch in a cluster that included *A. molluscorum* and *A. bivalvium* (Fig. 2). Species delineation based on the analysis of five housekeeping genes was recommended by the ad-hoc committee (Stackebrandt *et al.*, 2002), but only the papers that described the species *A. fluvialis* (Alperi *et al.*, 2010b) and *A. taiwanensis* and *A. sanarellii* from clinical isolates (Alperi *et al.*, 2010a), have complied with this recommendation.

DNA–DNA reassociation experiments were performed between the two novel isolates and between strain

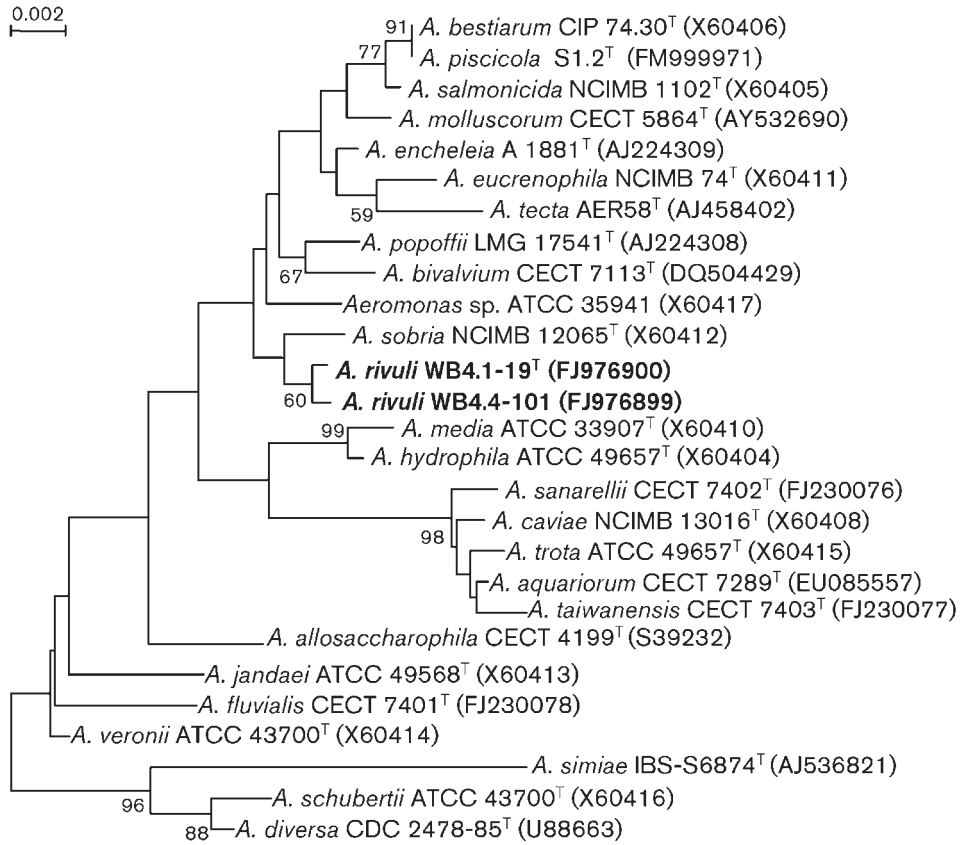

Fig. 1. Unrooted neighbour-joining phylogenetic tree derived from the 16S rRNA gene sequences showing the relationships of strains WB4.1-19^T and WB4.4-101 with all other species of the genus *Aeromonas*. Numbers at nodes indicate bootstrap values >50 % (percentage of 1000 replicates). Bar, 0.002 estimated nucleotide substitutions per site.

WB4.1-19^T and the type strains of the closest species, *A. sobria*, *A. molluscorum* and *A. bivalvium*, as well as with *A. bestiarum*, *A. encheleia*, *A. eucrenophila*, *A. piscicola*, *A. popoffii*, *A. salmonicida* and *A. tecta*. DNA extraction and DNA–DNA reassociation experiments were conducted as previously described (Alperi *et al.*, 2010b). The DNA–DNA reassociation value between strains WB4.1-19^T and WB4.4-101 was 89.5 % (±6.5 %), while the type strains of the other species of the genus *Aeromonas* showed values below the 70 % threshold established for species delineation (Stackebrandt *et al.*, 2002; see Supplementary Table S2).

Optimal growth temperature and pH were determined in tryptic soy broth (TSB, Difco) after 24 h by optical density. Cell sizes, morphologies and the presence of flagella were determined by electron microscopy using previously described methods (Collado *et al.*, 2009). Both strains were straight, non-spore-forming, non-encapsulated rods that were motile by means of polar flagella (see Supplementary Fig. S2).

The cultural characteristics of strains WB4.1-19^T and WB4.4-101, i.e. the size and colour of colonies and production of a brown diffusible pigment, were determined on tryptic soy agar (TSA, Difco) at 30 °C for 24 h. Sheep blood agar (Biomedics) was used to evaluate haemolysis under the same conditions. Twenty eight phenotypic tests selected from Abbott *et al.* (2003) and also performed in a previous study (Alperi *et al.*, 2010b) were used for the characterization of strains WB4.1-19^T and WB4.4-101. In addition, tests were performed to determine the utilization of L-arabinose, L-histidine, L-proline, DL-lactate and salicin as carbon sources and for the hydrolysis of starch. All tests were conducted in triplicate at 30 °C. Some tests were further confirmed using commercial identification kits (API 20NE and API 20E; bioMérieux). Additional tests included in the latter kits, together with the assimilation/oxidation reactions of 49 carbohydrates using the API 50CH kit (bioMérieux) were also considered. Phenotypic characteristics that differentiated both new isolates from other species of the genus *Aeromonas* are presented in Table 1. All type strains of recognized species of the genus *Aeromonas*, including the recently described members, were tested (Table 1) under identical conditions to those used for strains WB4.1-19^T and WB4.4-101.

Interestingly the two novel isolates showed phenotypic similarity with *A. molluscorum* as they were the only taxa

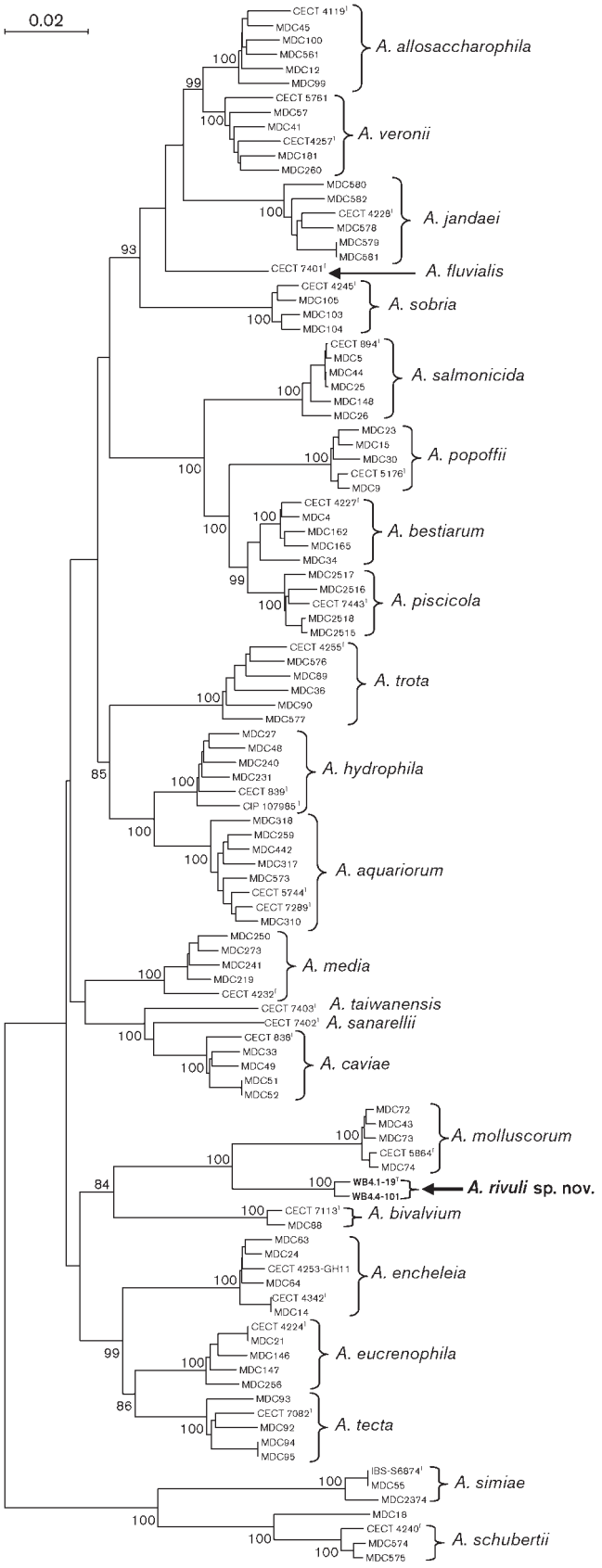

Fig. 2. Unrooted neighbour-joining phylogenetic tree derived from the MLPA of concatenated sequences of five housekeeping genes (*gyrB*, *rpoD*, *recA*, *dnaJ* and *gyrA*; 3684 bp) showing the relationships of strains WB4.1-19^T and WB4.4-101 with all other species of the genus *Aeromonas*. Numbers at nodes indicate bootstrap values >70 % (percentage of 1000 replicates). Bar, 0.02 estimated nucleotide substitutions per site.

that all gave a negative result in tests for indole, Voges–Proskauer (VP) reaction, gas from glucose and for ornithine- and lysine decarboxylase. The characteristics that differentiated the novel isolates from *A. molluscorum* were their ability to hydrolyse starch, but not to use or produce acid from L-arabinose. Other useful tests to differentiate the two novel strains from other species of the genus *Aeromonas* include their inability to produce indole, gas from D-glucose, VP, lysine decarboxylase and β-haemolysis from sheep blood agar (Table 1).

Based on molecular and phenotypic evidence, it is concluded that strains WB4.1-19^T and WB4.4-101 represent a novel species of the genus *Aeromonas*, for which the name *Aeromonas rivuli* sp. nov. is proposed.

Description of *Aeromonas rivuli* sp. nov.

Aeromonas rivuli (ri′vu.li. L. gen. masc. n. *rivuli* of/from a rivulet, a small creek).

Cells are Gram-negative, non-spore-forming motile rods with a polar flagellum and are 2.0–2.5 μm long and 0.5–0.7 μm wide. Oxidase and catalase-positive, reduces nitrates to nitrites and is resistant to the vibriostatic agent O/129 (150 μg). Colonies on TSA are opaque, beige in colour and 2.0–2.5 mm in diameter after 48 h incubation at 30 °C and 1.0–2.0 mm at 37 °C. No brown diffusible pigment is produced on TSA at 25 °C or 30 °C. No haemolysis is observed on sheep blood agar at 30 °C. Growth occurs at 7–37 °C and at 0–3 % NaCl (w/v). Optimal growth is at 30 °C and at pH 8.7–9.0 after 24 h on TSB. Positive for the β-galactosidase test, ADH, hydrolysis of aesculin, gelatin, starch, arbutin, Tween 80 and DNA. Negative result in tests for ornithine- and lysine decarboxylase, VP, production of indole from tryptophan, gas from glucose, hydrogen sulphide from cysteine and hydrolysis of elastin. Utilization of citrate is variable. Able to utilize glycerol, D-galactose, maltose, starch, glycogen, sucrose, L-histidine, L-proline, salicin and D-mannitol, but not DL-lactate, L-arabinose, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Acid is produced from glycerol (only with API 50CH), D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, salicin, maltose, sucrose and trehalose. Does not produce acid from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, lactose, melibiose, inulin, melezitose,

Table 1. Key phenotypic characteristics that differentiate strains WB4.1-19^T and WB4.4-101 from other species of the genus *Aeromonas*

Taxa: 1, *A. rivuli*; 2, *A. hydrophila*; 3, *A. bestiarum*; 4, *A. salmonicida*; 5, *A. caviae*; 6, *A. media*; 7, *A. eucrenophila*; 8, *A. sobria*; 9, *A. veronii* biovar *sobria*; 10, *A. jandaei*; 11, *A. veronii* biovar *veronii*; 12, *A. schubertii*; 13, *A. trota*; 14, *A. encheleia*; 15, *A. allosaccharophila*; 16, *A. popoffii*; 17, *A. simiae* (data from Harf-Monteil *et al.*, 2004); 18, *A. molluscorum* (Miñana-Galbis *et al.*, 2004); 19, *A. bivalvium* (Miñana-Galbis *et al.*, 2007); 20, *A. aquariorum* (Martínez Murcia *et al.*, 2008); 21, *A. tecta* (Demarta *et al.*, 2008); 22, *A. fluvialis* (Alperi *et al.*, 2010b); 23, *A. piscicola* (Beaz-Hidalgo *et al.*, 2009); 24, *A. taiwanensis* (Alperi *et al.*, 2010a); 25, *A. sanarellii* (Alperi *et al.*, 2010a). +, 85–100 % of strains positive; −, 0–15 % of strains positive; V, 16–84 % of strains positive. All tests were performed for type strains and the results are expressed in parentheses as (+) or (−); ND, no data available; ODC, ornithine decarboxylase; LDC, lysine decarboxylase. Data for species 1–16 were obtained from Abbott *et al.* (2003) with the exception of tests indicated as ND, these authors performed tests at 35 °C, with the exceptions of *A. popoffii* and *A. sobria* which were at 25 °C. Tests for taxa 17, 21, 22, 24 and 25 were performed at 30 °C (as in the present study). Tests for taxa 18, 20 and 23 were performed at 25 °C. Tests for taxon 19 were performed at 25–30 °C.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
β-Haemolysis	−	+(+)	+(+)	V(+)	V(−)	V(−)	+(+)	−(+)	+(+)	+(+)	+(+)	V(−)	V(+)	V(+)	V(−)	−(−)	−(−)	V*(−)	−*(−)	ND(+)	+(+)	−(−)	+(+)	−(−)	−(−)
Indole	−	+(+)	+(+)	+(−)	V(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	−(−)	+(+)	+(+)	+(+)	V(−)	−(−)	−(−)	+(+)	+(+)	−(−)	+(+)	+(+)	+(+)	+(+)
VP	−	+(+)	V(+)	V(−)	−(−)	−(−)	−(−)	−(−)	+(+)	+(+)	V(−)	V(−)	−(−)	−(−)	−(−)	+(+)	−(−)	−(−)	−(−)	−†(−)	V(+)	−(−)	+(+)	−(−)	−(−)
ODC	−	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	+(+)	−(−)	−(−)	−(−)	V(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)
LDC	−	+(+)	V(+)	V(−)	−(−)	−(−)	−(−)	+(+)	+(+)	+(+)	+(+)	V(−)	+(+)	−(−)	+(+)	−(−)	+(+)	−(−)	+(+)	+(+)	V(+)	−(−)	+(+)	−(−)	−(−)
Glucose (gas)	−	+(+)	V(+)	V(−)	−(−)	−(−)	V(+)	V(+)	+(+)	+(+)	+(+)	−(−)	V(+)	V(+)	+(+)	+(+)	−(−)	−(−)	−(−)	+(+)	+(+)	+(+)	+(+)	−(−)	−(−)
Hydrolysis of:																									
Aesculin	+	+(+)	V(+)	+(+)	V(+)	V(+)	V(+)	−(−)	−(−)	−(−)	+(+)	−(−)	−(−)	V(+)	V(+)	−(−)	V(−)	+(+)	+(+)	+(+)	V(+)	−(−)	+(+)	+(+)	+(+)
Starch	+	+‡(+)	ND(+)	ND(+)	+‡(+)	+§(+)	ND(+)	+‡(+)	ND(+)	ND(+)	ND(+)	ND(+)	ND(+)	V‖(+)	ND(+)	+¶(+)	ND(+)	−(−)	+(+)	+(+)	ND(+)	ND(−)	+(+)	+(+)	+(+)
Acid from:																									
Glycerol	−#	+(+)	+(+)	+(+)	V(−)	V(+)	−(−)	+(+)	+(+)	+(+)	+(+)	−(−)	V(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)
Sucrose	+	+(+)	+(+)	+(+)	+(+)	+(+)	V(+)	+(+)	+(+)	−(−)	+(+)	−(−)	V(−)	V(+)	+(+)	−(−)	+(+)	+(+)	+(+)	+(+)	−(−)	+(+)	+(+)	+(+)	+(+)
L-Arabinose	−	V(+)	+(+)	+(+)	+(+)	+(+)	V(+)	−(−)	−(+)	−(−)	−(−)	−(−)	−(−)	−(−)	V(+)	V(+)	−(−)	+(+)	+(+)	−(−)	−(−)	−(−)	−(−)	+(+)	+(+)
Salicin	+	V(−)	V(+)	V(−)	V(+)	V(+)	V(−)	−(−)	−(−)	−(−)	+(+)	−(−)	−(−)	−(+)	−(−)	−(−)	−(−)	ND(+)	+(+)	+(+)	V(−)	+(+)	+(+)	+(+)	+(+)
Lactose	−	V(−)	−(−)	+(−)	V(−)	V(+)	−(+)	−(−)	−(−)	−(−)	V(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	−(−)	+(+)	−(−)	−(−)	−(−)
Utilization of:																									
DL-Lactate	−	V(+)	−(−)	−(−)	+(−)	V(+)	−(−)	−(−)	−(−)	−(−)	−(−)	V(−)	+(+)	−(−)	−(−)	V(+)	ND(−)	V(−)	+(+)	−(−)	−(−)	ND(−)	−(−)	ND(+)	ND(+)
L-Arabinose	−	V**(+)	ND(+)	−**(−)	+**(+)	+**(+)	+**(+)	−**(−)	−**(+)	−**(−)	−**(+)	−**(−)	−**(−)	−**(−)	+**(+)	ND(+)	ND(+)	+(+)	+(+)	ND(−)	−(−)	−(−)	ND(−)	+(+)	+(+)

*Result from Demarta *et al.* (2008), performed at 30 °C.

†VP negative result was taken from the species description of Martínez-Murcia *et al.* (2008) and not from the table where there was an later erratum, (+).

‡Result from Miñana Galbis *et al.* (2002), performed at 25 °C.

§Result from Allen *et al.* (1983), performed at 22 °C.

‖Result from Esteve *et al.* (1995), test performed at 28 °C.

¶Result from Huys *et al.* (1997), test performed at 28 °C.

#Acid production from glycerol was negative when determined in tube but positive when tested by API 50CH.

**Result from Valera & Esteve (2002), tests performed at 28 °C.

raffinose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol or L-arabitol. Production of acid from cellobiose is variable. The API 20NE and API 20E profiles for strains WB4.1-19^T and WB4.1-101 were 5576354 and 3006167, respectively.

The type strain, WB4.1-19^T (=CECT 7518^T=DSM 22539^T=MDC 2511^T), was isolated from a karst hard water creek, Westerhöfer Bach, located at the north-western slope of the Harz Mountain, Lower Saxony, Germany. The isolation site was 350 m downstream from the discharge site.

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