Acetobacter senegalensis sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (*Mangifera indica* L.)

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A thermotolerant acetic acid bacterium, designated strain CWBI-B418^T, isolated in Senegal from mango fruit (*Mangifera indica*), was characterized in detail by means of genotypic and phenotypic methods. The novel strain was strictly aerobic and exhibited optimal growth on YGM medium at 35 °C. Cells were Gram-negative, motile and coccoid. The strain was assigned to the genus *Acetobacter* on the basis of 16S rRNA gene sequence analysis. DNA–DNA hybridization experiments with its phylogenetically closest relatives showed that strain CWBI-B418^T represented a novel *Acetobacter* genospecies. The DNA G + C content of strain CWBI-B418^T was 56.0 mol%. Phenotypic characteristics enabling the differentiation of strain CWBI-B418^T from phylogenetically related *Acetobacter* species were: production of 2-keto-D-gluconic acid from D-glucose, but not 5-keto-D-gluconic acid, production of catalase but not oxidase, growth on yeast extract with 30 % D-glucose, growth with ammonium as sole nitrogen source with ethanol as carbon source, utilization of glycerol and ethanol but not maltose or methanol as carbon sources, and growth in the presence of 10 % ethanol. Based on the genotypic and phenotypic data presented, strain CWBI-B418^T clearly represents a novel *Acetobacter* species, for which the name *Acetobacter senegalensis* sp. nov. is proposed. The type strain is CWBI-B418^T (=LMG 23690^T = DSM 18889^T).

The taxonomy of acetic acid bacteria (AAB) has substantially changed in recent years. According to De Ley *et al.* (1984), AAB were classified in the family *Acetobacteraceae* in two genera, *Acetobacter* and *Gluconobacter*. At the time

of writing, AAB are classified in nine genera, namely *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconoacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathanella* and *Neosassa* in the family *Acetobacteraceae*, class *Alphaproteobacteria* (Cleenwerck *et al.*, 2002; Loganathan & Nair, 2004; Jojima *et al.*, 2004; Yukphan *et al.*, 2005). AAB are widespread in nature and are generally capable of oxidizing ethanol to acetic acid. This enables them to grow in wine, cider, sake and kombucha tea, resulting in their use for the production of different kinds of vinegar and beverages (Trček & Teuber, 2002). In tropical areas such as sub-Saharan Africa, where the ambient temperature is regularly above 30 °C, industrial

Abbreviation: AAB, acetic acid bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of CWBI-B418^T is AY883036.

A table comparing the oxidation of carbon sources by strain CWBI-B418^T and *Acetobacter tropicalis* is available with the online version of this paper.

vinegar production is almost exclusively performed by using thermotolerant AAB, as they considerably reduce the costs associated with the use of cooling water, which otherwise rapidly become prohibitive (Sow *et al.*, 2005; Ndoye *et al.*, 2006).

Two *Acetobacter* strains, *Acetobacter* sp. CWBI-B418^T and *Acetobacter pasteurianus* CWBI-B419, were recently isolated from mango fruit (*Mangifera indica*) in Senegal and 'Dolo' (local beer obtained by fermenting cereal products) in Burkina Faso, respectively. These strains were selected as potential strains for vinegar production in sub-Saharan Africa on the basis of their ability to grow and produce acetic acid at higher temperatures (Ndoye *et al.*, 2006). The two strains were used in a study of artisanal production of spirit vinegar of 6 % via the Orleans method (unpublished data) and were evaluated as freeze-dried starters in an acetification process (Ndoye *et al.*, 2007). The present study deals with the determination of the taxonomic position of strain CWBI-B418^T in the genus *Acetobacter*. The polyphasic characterization of the novel strain by means of genotypic and phenotypic methods falls within the framework of a project on sustainable and durable natural resource management and valorization in Senegal and Burkina Faso. Based on the results obtained, we conclude that strain CWBI-B418^T represents a novel species of the genus *Acetobacter*.

Acetobacter reference strains used in this study were obtained from the BCCM/LMG Bacteria Collection (<http://bccm.belspo.be>). Reference strains were grown according to the supplier's specifications, unless indicated otherwise. Strain CWBI-B418^T was cultivated on medium 13 agar from the BCCM/LMG *Catalogue of Cultures* (<http://bccm.belspo.be/db/>; 2.5 % D-mannitol, 0.5 % yeast extract, 0.3 % peptone, 1.5 % agar) by incubation at 28 °C under aerobic conditions for 1–3 days and then checked for purity. The strain grew well on this medium at 28 °C and appeared as visible, opaque, beige colonies within 1 day of incubation. Small colony variations were noted, with some colonies being smooth and others slimy, irregular and spreading. Previous studies showed that the strain exhibited good growth between 25 and 40 °C, with optimal growth at 35 °C on YGM medium (1 % yeast extract, 2 % glucose, 2 % D-mannitol, with 2.5 % ethanol and 0.5 % acetic acid added aseptically after sterilization), but slow growth at 42 °C (Ndoye *et al.*, 2006).

Cell morphological characteristics, including shape and size, were determined from cells grown at 28 °C for 1 day on medium 13 under aerobic conditions. Gram staining was carried out according to the method of Hucker & Conn (1923). Cells of strain CWBI-B418^T were Gram-negative, non-spore-forming coccoids, approximately 0.8 µm in width and 1.2–2 µm in length. Cells occurred singly, in pairs or in short chains and occasionally in long chains.

Conventional biochemical tests were performed according to standard methods (De Ley *et al.*, 1984). Oxidase activity was tested by using 1 % *N,N,N',N'*-tetramethyl

p-phenylenediamine. Catalase activity was tested by adding young cells to a drop of a 10 % H₂O₂ solution and observing production of O₂. Strain CWBI-B418^T was oxidase-negative and catalase-positive. It was able to oxidize ethanol to acetic acid and acetate and lactate to CO₂ and H₂O, two biochemical properties typical of AAB.

The phylogenetic affiliation of strain CWBI-B418^T was previously determined by Ndoye *et al.* (2006) based on 16S rRNA gene sequence analysis, where it was shown that it belonged to the genus *Acetobacter*. Previous studies (Lisdiyanti *et al.*, 2001; Cleenwerck *et al.*, 2002) showed the existence of two stable sublineages in the genus *Acetobacter*. In the present study a new phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987) (Fig. 1) using the BioNumerics 4.5 software package (Applied Maths). Unknown bases were discarded from the calculations. Bootstrapping analysis (Felsenstein, 1985) was undertaken to test the statistical reliability of the topology of the neighbour-joining tree by using 1000 bootstrap resamplings of the data. Fig. 1 shows that strain CWBI-B418^T belonged to the stable subcluster containing *Acetobacter oeni*, *A. estunensis*, *A. nitrogenifigens*, *A. aceti*, *A. tropicalis*, *A. cerevisiae*, *A. orleanensis*, *A. malorum*, *A. orientalis*, *A. cibinongensis* and *A. indonesiensis*. 16S rRNA gene sequence similarities obtained by pairwise alignment with the BioNumerics 4.5 software package between strain CWBI-B418^T and the type strains of recognized *Acetobacter* species were 99.3 % for *A. tropicalis*, 98.7 % for *A. indonesiensis*, 98.5 % for *A. cibinongensis*, 98.4 % for *A. cerevisiae*, 98.4 % for *A. orientalis*, 98.3 % for *A. malorum*, 98.1 % for *A. oeni*, 98.1 % for *A. orleanensis*, 98.0 % for *A. syzygii*, 98.0 % for *A. estunensis*, 98.0 % for *A. pomorum*, 97.9 % for *A. aceti*, 97.8 % for *A. lovaniensis*, 97.7 % for *A. pasteurianus*, 97.5 % for *A. nitrogenifigens* and 97.5 % for *A. peroxydans*.

Strain CWBI-B418^T was hybridized to all phylogenetically related recognized *Acetobacter* species to determine whether it belonged to one of these species. DNA for DNA–DNA hybridizations and DNA base composition analysis was prepared according to the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). DNA–DNA hybridizations were performed by using a modification of the microplate method described by Ezaki *et al.* (1989) (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. A × B and B × A) were performed and the variation between them was within the limits of the method, of which the mean standard deviation is ± 7 % (Goris *et al.*, 1998). The DNA relatedness percentages presented are the mean of a minimum of four hybridization experiments, including the reciprocal reactions. Strain CWBI-B418^T showed an intermediate level of DNA–DNA relatedness with *A. tropicalis* LMG 19825^T (52 %), and low levels with *A. estunensis* LMG 1626^T (9 %), *A. oeni* LMG 21952^T (6 %), *A. aceti* LMG 1504^T (7 %), *A. cerevisiae* LMG 1625^T (18 %), *A. orleanensis* LMG 1583^T (22 %), *A. malorum* LMG 1746^T (18 %), *A. orientalis*

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain CWBI-B418^T and related species of the family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value calculated based on 1000 subsets. Bar, 1 nt substitution per 100 nt.

LMG 21417^T (15 %), *A. cibinongensis* LMG 21418^T (17 %), *A. indonesiensis* LMG 19824^T (21 %) and *A. nitrogenifigens* LMG 23498^T (3 %). As these values are clearly below 70 %, the generally accepted limit for species delineation (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002), the results indicate that strain CWBI-B418^T represents a novel genospecies within the genus *Acetobacter*. The DNA base composition of strain CWBI-B418^T was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage λ DNA (Sigma) was used as a calibration reference. The DNA G + C content of strain CWBI-B418^T was 56.0 mol%. This value is consistent with those of recognized members of the genus *Acetobacter* (Lisdiyanti *et al.*, 2000, 2001; Cleenwerck *et al.*, 2002; Silva *et al.*, 2006).

To confirm finally whether strain CWBI-B418^T represented a novel *Acetobacter* species, phenotypic characteristics that enable it to be differentiated from recognized *Acetobacter* species were examined as described by Cleenwerck *et al.* (2002). Strain CWBI-B418^T could be differentiated from most species of the genus *Acetobacter* by its ability to grow on yeast extract with 30 % D-glucose, the ability to grow with

ammonium as sole nitrogen source with ethanol as carbon source and the ability to grow in the presence of 10 % ethanol (Table 1). Strain CWBI-B418^T differed from *A. tropicalis*, its phylogenetically closest neighbour, by the ability to grow in ammonium with ethanol, the ability to grow in 10 % ethanol, the ability to grow on yeast extract with 30 % D-glucose and the inability to grow on maltose.

Additional characteristics differentiating strain CWBI-B418^T from *A. tropicalis* were determined by using API 50CH strips (bioMérieux), following the manufacturer's guidelines for oxidation. The bacterial suspensions were made up in API 50CHL medium, except that bromocresol purple was substituted by bromocresol green (0.17 g l⁻¹). The colour change in the cup was read after 1, 2 and 3 days incubation at 30 °C. The results obtained indicated that strain CWBI-B418^T differed from *A. tropicalis* by the ability to produce acid from D-glucose and D-xylose aerobically (see Supplementary Table S1 in IJSEM Online).

The results presented above allow the genotypic and phenotypic differentiation of strain CWBI-B418^T from all

Table 1. Differential characteristics between strain CWBI-B418^T and related *Acetobacter* species

Taxa: 1, *A. cerevisiae* (four strains); 2, *A. malorum* LMG 1746^T; 3, *A. pasteurianus* (seven strains); 4, *A. pomorum* LMG 18848^T; 5, *A. peroxydans* (two strains); 6, *A. lovaniensis* LMG 1617^T; 7, *A. orleanensis* (four strains); 8, *A. indonesiensis* (two strains); 9, *A. tropicalis* (two strains); 10, *A. estunensis* (three strains); 11, *A. aceti* (four strains); 12, *A. syzygii* LMG 21419^T; 13, *A. cibinongensis* LMG 21418^T; 14, *A. orientalis* LMG 21417^T; 15, *A. oeni* B13^T; 16, *A. nitrogenifigens* RG1^T; 17, strain CWBI-B418^T. +, Positive; –, negative; w, weakly positive; v, variable; ND, not determined. Data for taxa 1–11 were taken from Cleenwerck *et al.* (2002), data for taxa 12–15 were taken from Silva *et al.* (2006) and data for taxon 16 were taken from Dutta & Gachhui (2006), except where indicated otherwise.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Formation from D-glucose:																	
5-Keto-D-gluconic acid	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	ND	–
2-Keto-D-gluconic acid	+	+	v	–	–	+	+	+	+	+	+	–	+	+	–	ND	+
Growth in ammonium with ethanol	–	–	–	–	+	+	–	–	–	+	+	–	w	–	–	+	+
Growth on carbon sources:																	
Glycerol	+	+	v	+	–	+	+	+	+	v	+	+	+	+	+	+	+
Maltose	–	–	v	–	+	–	v	+	+	–	v	+	–	w	–	+	–
Methanol	–	+	–	–	–	+	–	–	–	–	–	–	–	w	–	–	–
Growth in 10% ethanol	ND	+	+	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Growth on yeast extract + 30% D-glucose	–	+	v	–	–	–	–	–	–	–	–	–	+	–	–	+	+
Catalase	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Motility	–	–	v†	–‡	+	v (mostly +)†	v†	v†	v†	+	v†	+	–	v (mostly +)†	+	+	+
DNA G+C content (mol%)	56.0–57.6	57.2	53.2–54.3	52.1	59.7–60.7	57.1–58.9	55.7–58.1	54.0–54.2	55.6–56.2	59.2–60.2	56.9–58.3	54.3–55.4†	53.8–54.5†	52.0–52.8†	58.1	64.1	56.0

*Data obtained in this study.

†Data taken from Lisdiyanti *et al.* (2000, 2001).

‡Data taken from Sokollek *et al.* (1998).

recognized *Acetobacter* species. Strain CWBI-B418^T should therefore be classified as representing a novel species of the genus *Acetobacter*, for which the name *Acetobacter senegalensis* sp. nov. is proposed.

Description of *Acetobacter senegalensis* sp. nov.

Acetobacter senegalensis (se.ne.gal.en'sis. N.L. m. adj. *senegalensis* referring to the country from where the type strain of this species was isolated).

Cells are Gram-negative, non-spore-forming, coccoid, approximately 0.8 µm wide and 1.2–2 µm long. Cells occur singly, in pairs or in short chains and occasionally in long chains. Oxidase-negative and catalase-positive. Strictly aerobic. Optimal growth temperature on YGM medium is 35 °C, but good growth is observed between 28 and 40 °C. On medium 13 agar, colonies appear within 1 day of incubation at 28 °C and are circular, convex, opaque, beige and approximately 0.6 mm in diameter. Characterized by the combination of the following phenotypic features: able to grow on yeast extract with 30 % D-glucose, able to grow with ammonium as sole nitrogen source with ethanol as carbon source, able to grow in the presence of 10 % ethanol, able to grow on glycerol as carbon source but not on maltose or methanol as carbon source, and produces 2-keto-D-gluconic acid from D-glucose, but not 5-keto-D-gluconic acid. The G + C content of the DNA is 56.0 mol%.

The type strain, CWBI-B418^T (= LMG 23690^T = DSM 18889^T), was isolated from mango fruit in Senegal (sub-Saharan Africa).

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