Acetobacter ghanensis sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans

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Twenty-three acetic acid bacteria, isolated from traditional heap fermentations of Ghanaian cocoa beans, were subjected to a polyphasic taxonomic study. The isolates were catalase-positive, oxidase-negative, Gram-negative rods. They oxidized ethanol to acetic acid and were unable to produce 2-ketogluconic acid, 5-ketogluconic acid and 2,5-diketogluconic acid from glucose; therefore, they were tentatively identified as *Acetobacter* species. 16S rRNA gene sequencing and phylogenetic analysis confirmed their position in the genus *Acetobacter*, with *Acetobacter syzygii* and *Acetobacter lovaniensis* as their closest phylogenetic neighbours. (GTG)₅-PCR fingerprinting grouped the strains in a cluster that did not contain any type strains of members of the genus *Acetobacter*. DNA–DNA hybridization with the type strains of all recognized *Acetobacter* species revealed DNA–DNA relatedness values below the species level. The DNA G + C contents of three selected strains were 56.9–57.3 mol%. The novel strains had phenotypic characteristics that enabled them to be differentiated from phylogenetically related *Acetobacter* species, i.e. they were motile, did not produce 2-ketogluconic acid or 5-ketogluconic acid from glucose, were catalase-positive and oxidase-negative, grew on yeast extract with 30 % glucose, grew on glycerol (although weakly) but not on maltose or methanol as carbon sources, and did not grow with ammonium as sole nitrogen source and ethanol as carbon source. Based on the genotypic and phenotypic data, the isolates represent a novel species of the genus *Acetobacter* for which the name *Acetobacter ghanensis* sp. nov. is proposed. The type strain is R-29337^T (= 430A^T = LMG 23848^T = DSM 18895^T).

Acetic acid bacteria (AAB) are Gram-negative, obligate aerobic bacteria that are widespread in nature. They are united in the family *Acetobacteraceae*, which currently includes the genera *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathania*, *Neosasaia* and *Granulibacter* (Cleenwerck *et al.*, 2002; Loganathan & Nair, 2004; Jojima *et al.*, 2004; Yukphan *et al.*,

2005; Greenberg *et al.*, 2006). A common feature of AAB (with the exception of the genus *Asaia*) is the ability to oxidize ethanol to acetic acid under neutral and acidic (pH 4.5) conditions. Because of this characteristic, AAB are often involved in the production of fermented foods, either in a beneficial (chocolate products, coffee, vinegar, nata de coco and speciality beers) or detrimental (spoilage of beers, wines and ciders) manner (Kerstens *et al.*, 2006).

AAB contribute to the development of high-quality cocoa since they play a critical role in the spontaneous fermentation of cocoa beans, regarded as the first stage in chocolate production (Schwan & Wheals, 2004). They oxidize ethanol, initially produced by yeasts, to acetic acid, which then diffuses into the beans, and this – in combination with heat produced by this exothermic bioconversion – causes the

Abbreviation: AAB, acetic acid bacteria.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains R-29337^T, 444B and 384 are EF030713, DQ887337 and DQ887338, respectively.

A maximum-parsimony tree based on 16S rRNA gene sequences of *Acetobacter ghanensis* sp. nov. and related species of the family *Acetobacteraceae* is available with the online version of this paper.

death of the seed embryo as well as the end of fermentation. In turn, biochemical changes in the beans are initiated, leading to the formation of precursor molecules that are necessary for the development of the characteristic aroma, flavour and colour of the beans. These properties are further developed during drying, roasting and final processing of well-fermented cocoa beans to produce cocoa powder and chocolate (Hansen *et al.*, 1998; Thompson *et al.*, 2001).

Today, little is known about AAB associated with cocoa bean fermentation, although strains of the genera *Acetobacter* and *Gluconobacter* are most commonly found (Schwan & Wheals, 2004). During a biodiversity study on micro-organisms involved in the spontaneous fermentation of Ghanaian cocoa beans, 132 AAB strains were isolated. These strains, together with 64 AAB reference strains, including the type strains of most recognized AAB species, were investigated by rep-PCR fingerprinting using the (GTG)₅-primer, a genotypic technique that has proven to be useful for rapid and reliable species recognition and typing of lactobacilli (Gevers *et al.*, 2001) and enterococci (Švec *et al.*, 2005). (GTG)₅-PCR fingerprinting revealed a cluster of 23 AAB isolates that did not contain any AAB type strain, suggesting that they could represent a novel AAB species (L. De Vuyst, N. Camu, T. De Winter, K. Vandemeulebroecke, V. Van de Perre, M. Vancanneyt, P. De Vos and I. Cleenwerck, unpublished results). Genotypic and phenotypic data demonstrate that these 23 AAB isolates indeed represent a novel species of the genus *Acetobacter*, for which the name *Acetobacter ghanensis* sp. nov. is proposed.

The 23 AAB strains (430A^T, 131, 134A, 141B, 153A, 384, 429, 415, 421, 130B, 422, 118A, 440, 112A, 444B, 110, 140A, 118B, 444A, 428A, 435, 430B1 and 387A) were isolated from seven traditional heap fermentations of Ghanaian cocoa beans, performed during the main- and mid-crop of 2004. Deoxycholate-mannitol-sorbitol (DMS) medium was used and incubation was carried out at 42 °C for 1–4 days. The isolates were preserved at –80 °C in MYP medium [2.5 % (w/v) D-mannitol, 0.5 % (w/v) yeast extract and 0.3 % (w/v) bacteriological peptone (Oxoid)], supplemented with 25 % (v/v) glycerol as cryoprotectant. Isolates were recovered by incubation at 30 °C in MYP broth under aerobic conditions for 1–4 days. Isolates 444B, 430A^T and 415 were deposited in the Research Collection of the Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium, as strains R-29336, R-29337^T and R-29338, respectively. On solidified MYP medium [1.5 % (w/v) agar], the novel strains appeared as beige, round, convex, smooth, shiny colonies with a diameter of approximately 0.8 mm after 4 days incubation. The type strains of the *Acetobacter* species used in this study were obtained from the BCCM/LMG Bacteria Collection (<http://www.belspo.be/bccm/>). They were grown according to the provider's specifications, unless indicated otherwise.

Cells of the 23 isolates were tested for their Gram reaction, cell shape and cell size. Catalase activity was tested by adding young cells from MYP agar to a drop of 20 % (v/v) hydrogen

peroxide solution. Oxidase activity was tested using the Oxidase DrySlide testkit (Becton Dickinson). *Lactobacillus plantarum* LMG 6907^T (a catalase-negative, oxidase-negative lactic acid bacterium) and *Acetobacter aceti* LMG 1504^T (a catalase-positive, oxidase-negative AAB) were used as controls. Cells of all isolates were Gram-negative rods, approximately 0.8 µm wide and 1.5–2.5 µm long. Cells occurred singly or in pairs and were oxidase-negative and catalase-positive.

Production of acetic acid from ethanol and gluconic acid from glucose were tested following growth of the strains at 30 °C for 48 h in basal medium [0.05 % (w/v) yeast extract and 0.3 % (w/v) vitamin-free Casamino acids (Difco)] plus ethanol (0.3 %, w/v) and GY medium [5 % (w/v) D-glucose and 0.5 % (w/v) yeast extract], respectively, using high pressure anion-exchange chromatography-conductivity following the protocol of Van der Meulen *et al.* (2006). The production of 2- and 5-ketogluconic acids and 2,5-diketogluconic acid from glucose were determined by the methods described by Gossele *et al.* (1980). All isolates oxidized ethanol to acetic acid and produced gluconic acid from glucose. They did not produce 2-ketogluconic acid, 5-ketogluconic acid or 2,5-diketogluconic acid from glucose. These characteristics indicated that the strains probably belong to the genus *Acetobacter* that, at the time of writing, comprises 16 species with validly published names (Lisdiyanti *et al.*, 2000, 2001; Cleenwerck *et al.*, 2002; Silva *et al.*, 2006; Dutta & Gachhui, 2006).

Fatty acids were extracted and analysed according to the recommendations of the commercial identification system MIDI (Microbial Identification System) from reference strains (*A. aceti* LMG 1261^T, *Acetobacter lovaniensis* LMG 1579^T, *Acetobacter orleanensis* LMG 1583^T and *Acetobacter pasteurianus* LMG 1262^T) and six of the isolates (430A^T, 444B, 415, 131, 153A and 110) from cells grown on MYP agar for 24 h at 28 °C under aerobic conditions. *Stenotrophomonas maltophilia* LMG 958^T was used as the control. The predominant fatty acid was the straight-chain, unsaturated C_{18:1}ω7c fatty acid, which accounted for approximately 50 % total fatty acid content. This is in agreement with results obtained previously for AAB (Urakami *et al.*, 1989; Franke *et al.*, 1999; Dellaglio *et al.*, 2005; Kersters *et al.*, 2006). Other fatty acids found in the isolates in minor but still significant amounts were C_{16:0} (6–16 %), C_{16:0} 2-OH (9–12 %), C_{14:0} 2-OH (5–7 %), C_{14:0} (3–5 %) and C_{19:0} cyclo ω8c (5–15 %). Compared with reported data for species of the genus *Acetobacter* (Urakami *et al.*, 1989; Franke *et al.*, 1999), the novel isolates contained high levels of C_{19:0} cyclo ω8c.

16S rRNA gene sequences of strains R-29337^T, 444B and 384 were determined as described by Franz *et al.* (2006) with the following modifications. DNA for 16S rRNA gene sequencing was extracted from strain R-29337^T by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002) and from strains 444B and 384 by the method of Gevers *et al.* (2001), except that mutanolysin was substituted

by proteinase K [0.0025 g proteinase K ml⁻¹ Tris/HCl (10 mM) + EDTA (1 mM) buffer (pH 8.0)]. Nearly complete sequences were determined using the eight sequencing primers listed by Coenye *et al.* (1999), except for strain R-29337^T where primer BKL1 (position 536–516 according to the *Escherichia coli* 16S rRNA gene sequence numbering; 5'-GTATTACCGCGGCTGCTGGCA-3') was used instead of primer PD. Pairwise similarities between the obtained consensus sequences and 16S rRNA gene sequences from the EMBL database were calculated with the BioNumerics 4.5 software package (Applied Maths) using an open gap penalty of 100 % and a unit gap penalty of 0 %. Phylogenetic trees were constructed with the BioNumerics 4.5 software package using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Felsenstein, 1985) methods. Bootstrapping analysis (Felsenstein, 1985) was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data.

The neighbour-joining tree (Fig. 1) was slightly different to the maximum-parsimony tree (see Supplementary Fig. S1 in IJSEM Online); however, both trees clearly showed that strains R-29337^T, 444B and 384 belonged to the genus *Acetobacter* and, more precisely, to the subcluster containing *A. lovaniensis*, *Acetobacter syzygii*, *Acetobacter peroxydans*, *A. pasteurianus* and *Acetobacter pomorum* (Lisdiyanti *et al.*, 2001; Cleenwerck *et al.*, 2002; Dutta & Gachhui, 2006). The 16S rRNA gene sequence similarities obtained by pairwise alignment showed that isolates R-29337^T, 444B and 384 were most closely related to each other (100 %) and to *A. syzygii* (99.7 %) and *A. lovaniensis* (99.5 %); lower similarities were observed with *A. pomorum* (98.1 %), *A. peroxydans* (97.9 %), *Acetobacter orientalis* (97.8 %), *A. pasteurianus* (97.7 %), *Acetobacter cibinongensis* (97.7 %), *Acetobacter tropicalis* (97.7 %), *Acetobacter indonesiensis* (97.7 %), *A. aceti* (97.6 %), *Acetobacter estunensis* (97.4 %), *Acetobacter oeni* (97.3 %), *Acetobacter cerevisiae* (97.2 %), *Acetobacter malorum* (97.1 %), *A. orleanensis* (96.8 %) and *Acetobacter nitrogenifigens* (96.4 %).

Hybridizations between DNA from isolate R-29336 (= 444B) and DNA from isolates R-29337^T (= 430A^T), R-29338 (= 415) and the type strains of all recognized species of the genus *Acetobacter* were carried out. DNA for DNA–DNA hybridizations and DNA base composition analysis was prepared by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). Only high-molecular-mass DNA with A₂₆₀/A₂₈₀ and A₂₃₄/A₂₆₀ absorption ratios of 1.8–2.0 and 0.40–0.60, respectively, was used. DNA–DNA hybridizations were performed according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the microplate method described by Ezaki *et al.* (1989). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. A × B and B × A) were performed and their variation was within the limits of this method (Goris *et al.*, 1998). The DNA binding values reported are mean values of at least four hybridization experiments, including the reciprocal

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter ghanensis* sp. nov. and related species of the family Acetobacteraceae. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

reactions. Strain R-29336 showed high DNA–DNA hybridization values with strains R-29337^T and R-29338 (99 and 88 %, respectively) and intermediate DNA binding values with the type strains of *A. syzygii* LMG 21419^T (46 %) and *A. lovaniensis* LMG 1617^T (47 %), phylogenetically the most closely related *Acetobacter* species. Low DNA–DNA hybridization values were found with *A. pomorum* LMG 18848^T (18 %), *A. peroxydans* LMG 1635^T (18 %), *A. orientalis* LMG 21417^T (11 %), *A. pasteurianus* LMG 1262^T (20 %), *A. cibinongensis* LMG 21418^T (11 %), *A. tropicalis* LMG 19825^T (11 %), *A. indonesiensis* LMG 19824^T (14 %), *A. aceti* LMG 1504^T (5 %), *A. estunensis* LMG 1626^T (10 %), *A. oeni* LMG 21952^T (6 %), *A. cerevisiae* LMG 1625^T (15 %), *A. malorum* LMG 1746^T (9 %), *A. orleanensis* LMG 1583^T (13 %) and *A. nitrogenifigens* LMG 23498^T (4 %). These DNA–DNA hybridization data prove that the cocoa isolates represent a novel genospecies within the genus *Acetobacter* (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002).

The DNA base composition of strains R-29336, R-29337^T and R-29338 was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage λ DNA (Sigma) was used as the calibration reference. The G+C content ranged from 56.9 to 57.3 mol%, which is consistent with DNA G+C contents of members of the genus *Acetobacter* (Lisdiyanti *et al.*, 2000, 2001; Cleenwerck *et al.*, 2002; Silva *et al.*, 2006).

Isolates R-29336, R-29337^T and R-29338 were tested for their ability to grow on the carbon sources glycerol (0.3 %), maltose (0.3 %), methanol (0.3 %) and ethanol (10 %), and on yeast extract with 30 % glucose as described previously (Cleenwerck *et al.*, 2002). The utilization of ammonium as the sole nitrogen source with ethanol as carbon source was also tested as described previously (Cleenwerck *et al.*, 2002). The three novel isolates were able to grow on glycerol (weakly), but not on maltose or methanol as carbon source. Growth on ethanol was variable (strain R-29337^T was negative). All isolates grew on yeast extract with 30 % glucose, but were unable to grow with ammonium as sole nitrogen source and ethanol as carbon source. Phenotypic differentiation from phylogenetically related *Acetobacter* species is summarized in Table 1. The isolates differed from *A. syzygii* mainly by their growth on yeast extract with 30 % glucose, but not on maltose. They differed from *A. lovaniensis* by their inability to produce 2-ketogluconic acid from glucose, and their growth on yeast extract with

30 % glucose, but not on methanol or on ethanol with ammonium as nitrogen source.

The results presented above allow the 23 *Acetobacter* isolates from traditional heap fermentations of Ghanaian cocoa beans to be differentiated genotypically and phenotypically from the 16 currently recognized species of the genus *Acetobacter*. These isolates should therefore be classified as representatives of a novel species for which the name *Acetobacter ghanensis* sp. nov. is proposed.

Description of *Acetobacter ghanensis* sp. nov.

Acetobacter ghanensis (gha'nen.sis. N.L. masc. adj. *ghanensis* pertaining to Ghana, referring to the country where the first isolates of this species were obtained).

Cells are Gram-negative rods, approximately 0.8 μ m wide and 1.5–2.5 μ m long. Cells occur singly or in pairs. Oxidase-negative. Catalase-positive. Strictly aerobic. On MYP agar, colonies are beige, round, convex, smooth and shiny and approximately 0.8 mm in diameter after incubation at 28 °C for 4 days. Ethanol is oxidized to acetic acid. Gluconic acid is produced from glucose. Unable to produce 2-ketogluconic acid, 5-ketogluconic acid or 2,5-diketogluconic acid from glucose. C_{18:1} ω 7c is the predominant fatty acid (approx. 50 %); other fatty acids in significant amounts are C_{16:0} (10–16 %), C_{16:0} 2-OH (9–11 %), C_{14:0} 2-OH (5–7 %), C_{14:0} (4–5 %) and C_{19:0} cyclo ω 8c (5–10 %).

Table 1. Differential characteristics of *Acetobacter ghanensis* sp. nov. and related species of the genus *Acetobacter*

Taxa: 1, *A. ghanensis* sp. nov. (3 strains); 2, *A. syzygii* LMG 21419^T; 3, *A. lovaniensis* LMG 1617^T; 4, *A. cerevisiae* (4 strains); 5, *A. malorum* LMG 1746^T; 6, *A. pasteurianus* (7 strains); 7, *A. pomorum* LMG 18848^T; 8, *A. peroxydans* (2 strains); 9, *A. orleanensis* (4 strains); 10, *A. indonesiensis* (2 strains); 11, *A. tropicalis* (2 strains); 12, *A. estunensis* (3 strains); 13, *A. aceti* (4 strains); 14, *A. cibinongensis* LMG 21418^T; 15, *A. orientalis* LMG 21417^T; 16, *A. oeni* B13^T; 17, *A. nitrogenifigens* RG1^T. +, Positive; –, negative; W, weakly positive; V, variable; ND, not determined. Unless indicated otherwise, data for taxon 1 were obtained in this study; data for taxa 2 and 14–16 were taken from Silva *et al.* (2006); data for taxa 3–13 were taken from Cleenwerck *et al.* (2002); and data for taxon 17 were taken from Dutta & Gachhui (2006).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Formation from glucose:																	
5-Ketogluconic acid	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	ND
2-Ketogluconic acid	–	–	+	+	+	V	–	–	+	+	+	+	+	+	+	–	ND
Growth in ammonium with ethanol	–	–	+	–	–	–	–	+	–	–	–	+	+	W	–	–	+
Growth on carbon sources:																	
Glycerol	W	+	+	+	+	V	+	–	+	+	+	V	+	+	+	+	+
Maltose	–	+	–	–	–	V	–	+	V	+	+	–	V	–	W	–	+
Methanol	–	–	+	–	+	–	–	–	–	–	–	–	–	–	W	–	–
Growth in 10 % ethanol	V	–*	–*	ND	ND	+	–*	–*	–*	–*	–*	–*	–*	–*	–*	+	+
Growth on YE+30 % glucose	+	–	–	–	+	V	–	–	–	–	–	–	–	+	–	–	+
Catalase	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
DNA G+C content (mol%)	56.9–57.3	54.3–55.4*	57.1–58.9	56.0–57.6	57.2–54.3	53.2–54.3	52.1–60.7	59.7–58.1	55.7–58.1	54.0–54.2	55.6–56.2	59.2–60.2	56.9–58.3	53.8–54.5*	52.0–52.8*	58.1	64.1

*Data taken from Lisdiyanti *et al.* (2001).

Able to grow on yeast extract with 30 % glucose, on glycerol (although weakly), but not on maltose or methanol. Unable to grow with ammonium as sole nitrogen source and ethanol as carbon source. The G + C content of the DNA varies from 56.9 to 57.3 mol%. The DNA G + C content of the type strain is 57.3 mol%. Can be differentiated genotypically from other species of the genus *Acetobacter* by DNA–DNA hybridization and (GTG₅)-PCR fingerprinting.

The type strain, strain R-29337^T (=430A^T=LMG 23848^T=DSM 18895^T), was isolated from a traditional heap fermentation of Ghanaian cocoa beans.

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