

Acetobacter fabarum sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation

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Six acetic acid bacterial isolates, obtained during a study of the microbial diversity of spontaneous fermentations of Ghanaian cocoa beans, were subjected to a polyphasic taxonomic study. (GTG)₅-PCR fingerprinting grouped the isolates together, but they could not be identified using this method. Phylogenetic analysis based on 16S rRNA gene sequences allocated the isolates to the genus *Acetobacter* and revealed *Acetobacter lovaniensis*, *Acetobacter ghanensis* and *Acetobacter syzygii* to be nearest neighbours. DNA–DNA hybridizations demonstrated that the isolates belonged to a single novel genospecies that could be differentiated from its phylogenetically nearest neighbours by the following phenotypic characteristics: no production of 2-keto-D-gluconic acid from D-glucose; growth on methanol and D-xylose, but not on maltose, as sole carbon sources; no growth on yeast extract with 30% D-glucose; and weak growth at 37 °C. The DNA G+C contents of four selected strains were 56.8–58.0 mol%. The results obtained prove that the isolates should be classified as representatives of a novel *Acetobacter* species, for which the name *Acetobacter fabarum* sp. nov. is proposed. The type strain is strain 985^T (=R-36330^T =LMG 24244^T =DSM 19596^T).

Acetic acid bacteria (AAB) play an important role in cocoa bean fermentation (Schwan & Wheals, 2004; Thompson *et al.*, 2007). They oxidize ethanol, produced by yeasts, to acetic acid. This volatile acid, combined with the heat produced by the exothermic bioconversion, causes the death of the seed embryo and the end of the fermentation. The cocoa bean fermentation process leads to the formation of precursor molecules that are very important for development of the aroma, flavour and colour of the beans (Hansen *et al.*, 1998; Thompson *et al.*, 2007). *Acetobacter* and *Gluconobacter* species are most commonly found in cocoa bean fermentations (Ardhana & Fleet, 2003;

Schwan & Wheals, 2004; Lagunes Gálvez *et al.*, 2007; Nielsen *et al.*, 2007; Camu *et al.*, 2007; De Vuyst *et al.*, 2007). However, isolations of AAB from such fermentations have so far been performed on a very limited number of media, e.g. Nielsen *et al.* (2007) isolated AAB from glucose-yeast extract-calcium carbonate (GYC) agar (5% D-glucose, 1% yeast extract, 3% calcium carbonate, 2% agar) (concentrations of all medium components are listed as w/v) and Camu *et al.* (2007) isolated them from deoxycholate-mannitol-sorbitol (DMS) agar (1% peptone, 0.3% yeast extract, 1.5% calcium lactate, 0.1% D-glucose, 0.1% sorbitol, 0.1% D-mannitol, 0.1% potassium phosphate, 0.01% sodium deoxycholate, 0.002% magnesium sulphate, 0.003% bromocresol, 0.01% cycloheximide, 1.8% agar, pH 4.5; Guiraud, 1998). Several studies have pointed out that even minor modifications in the composition of the isolation medium can result in the isolation of representatives of so far unknown AAB taxa (Yamada *et al.*, 2000; Lisdiyanti *et al.*, 2001, 2003). For this reason, it has been suggested that a combination of media be used to avoid selective isolation of AAB (Lisdiyanti *et al.*, 2003).

Abbreviation: AAB, acetic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 985^T and 1145 are AM905849 and AM905850, respectively.

A maximum-parsimony tree based on nearly complete 16S rRNA gene sequences and additional differential characteristics of *A. fabarum* and phylogenetically related species are available as supplementary material with the online version of this paper.

During a study of the microbial biodiversity of spontaneous fermentations of Ghanaian cocoa beans, 209 AAB isolates were collected from four different culture media: DMS agar (Guiraud, 1998); acetic acid medium (AAM) agar (1 % D-glucose, 0.5 % ethanol, 0.3 % acetic acid, 1.5 % peptone, 0.8 % yeast extract; Lisdiyanti *et al.*, 2001); basal medium with ethanol (BME) agar [0.05 % yeast extract, 0.3 % vitamin-free Casamino acids (Difco), 0.3 % ethanol]; and glucose-yeast extract (GY) agar (5 % D-glucose, 0.5 % yeast extract). The agar concentration in all media was 1.5 % (w/v). The isolates were investigated by (GTG)₅-PCR fingerprinting, a technique that has proven useful for rapid and reliable species identification and classification of lactobacilli (Gevers *et al.*, 2001), enterococci (Švec *et al.*, 2005) and AAB (De Vuyst *et al.*, 2007; Cleenwerck *et al.*, 2007). A group of six AAB isolates that could not be identified was revealed (Fig. 1). The present study deals with the further characterization of these isolates and shows that they represent a novel species of the genus *Acetobacter*.

The six AAB isolates were collected from three of the four different culture media mentioned above: four isolates from AAM agar (isolates 900, 1145, 950 and 1104), one from BME agar (isolate 985^T) and one from GY agar (isolate 1039). No isolates were obtained from DMS medium, the medium that was used by Camu *et al.* (2007) to investigate AAB involved in cocoa fermentations. This observation confirms the importance of the use of a combination of media (Lisdiyanti *et al.*, 2003) to avoid selective isolation of AAB. The isolates were preserved as described previously (Cleenwerck *et al.*, 2007) and recovered on MYP agar [2.5 % D-mannitol, 0.5 % yeast extract, 0.3 % bacteriological peptone, 1.5 % agar (Oxoid); w/v] and Z1 agar (2 % yeast extract, 2 % calcium lactate, 1.5 % agar; w/v) by incubation at 28 °C under aerobic conditions for 1–4 days. Isolates 985^T, 1145, 950 and 1039 were deposited in the Research Collection of the Laboratory of Microbiology as R-36330^T, R-36331, R-36458 and R-36459, respectively. 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.

For microscopy, the isolates were grown on Z1 agar. Cells were tested for their Gram reaction, cell shape and size and catalase and oxidase activities according to methods described previously (Cleenwerck *et al.*, 2002). After incubation at 28 °C for 3 days, the isolates appeared as rough, shiny, beige, round colonies that were raised and wavy, with a diameter of approximately 0.8 mm. Cells of all isolates were motile, Gram-negative, coccoid rods (0.8 × 1.2–3.0 µm) that occurred singly or in pairs and were oxidase-negative and catalase-positive.

Sequences of the 16S rRNA genes of isolates 985^T and 1145 were determined following the protocol described by Franz *et al.* (2006) with the modification that DNA for sequencing was extracted by the method of Wilson (1987) as applied by Cleenwerck *et al.* (2002). Nearly complete 16S rRNA gene sequences were obtained using the primers *Gamma, *PD, *O, *3, *R, Gamma, 3 and BKL1 (Coenye *et al.*, 1999; Cleenwerck *et al.*, 2007). Pairwise similarities between the 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. The statistical reliability of the topology of the trees was evaluated by bootstrap analysis (Felsenstein, 1985). 16S rRNA gene sequence analysis revealed that the isolates were affiliated to the genus *Acetobacter* and, more precisely, in the sublineage (Lisdiyanti *et al.*, 2001; Cleenwerck *et al.*, 2002, 2007; Dutta & Gachhui, 2006) containing *Acetobacter lovaniensis*, *A. ghanensis*, *A. syzygii*, *A. peroxydans*, *A. pasteurianus* and *A. pomorum* (Fig. 2 and Supplementary Fig. S1, available in IJSEM Online). Furthermore, the 16S rRNA gene sequence similarities obtained by pairwise alignment showed that

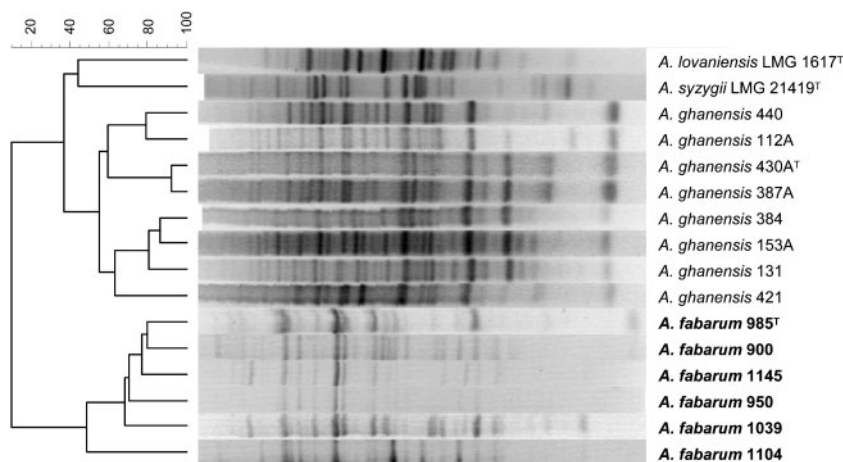


Fig. 1. (GTG)₅-PCR fingerprints of the six isolates of *Acetobacter fabarum* sp. nov. and their closest phylogenetic relatives. The dendrogram was derived from unweighted pair-group cluster analysis (UPGMA) of the fingerprints. Levels of linkage are expressed as Pearson similarity coefficients.

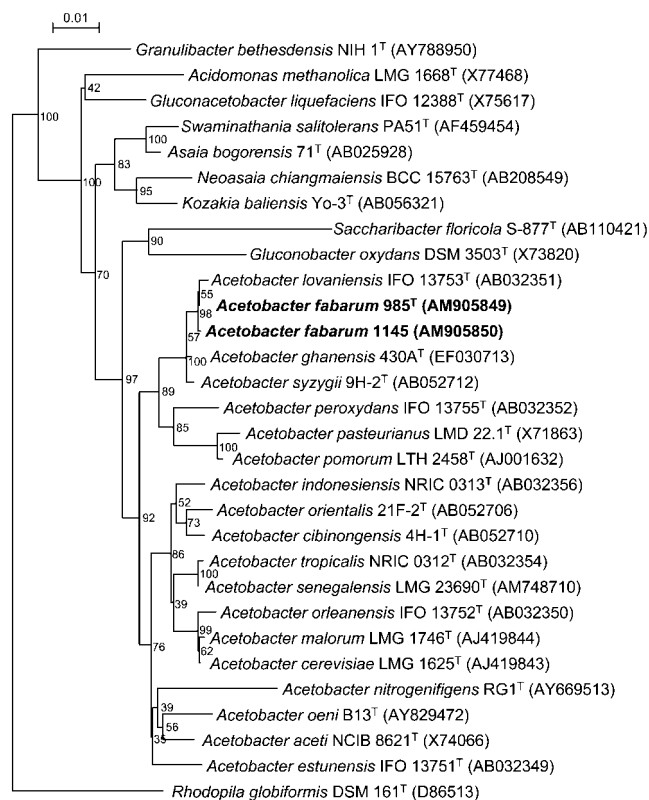


Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strains of *A. fabarum* sp. nov. and related species of the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap percentages calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions.

isolates 985^T and 1145 were closely related to each other (99.9% similarity) and to the type strains of *A. lovaniensis* (99.9%), *A. ghanensis* (99.7%) and *A. syzygii* (99.5%). The isolates were clearly less related to the type strains of other recognized *Acetobacter* species: i.e. *A. peroxydans* and *A. pomorum* (97.9%), *A. senegalensis* (97.6%), *A. orientalis*, *A. cibirongensis*, *A. tropicalis* and *A. indonesiensis* (97.5%), *A. pasteurianus* and *A. aceti* (97.4%), *A. estunensis* and *A. oeni* (97.1%), *A. cerevisiae* and *A. malorum* (97.0%), *A. orleanensis* (96.9%) and *A. nitrogenifigens* (96.6%).

DNA–DNA hybridizations were performed between isolate 985^T and the type strains of the phylogenetically related species *A. lovaniensis*, *A. ghanensis*, *A. syzygii*, *A. peroxydans*, *A. pomorum* and *A. pasteurianus*, and also between isolates 985^T, 1145, 950 and 1039. 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 A_{260}/A_{280} and A_{234}/A_{260} 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 in the presence of 50% formamide. Reciprocal reactions (e.g. A × B and B × A) were performed for every DNA pair and their variation was within the limits of this method (Goris *et al.*, 1998). The level of DNA–DNA binding found between isolate 985^T and the type strains of phylogenetically related species was intermediate (52% with *A. lovaniensis* LMG 1617^T, 35% with *A. ghanensis* R-29337^T and 32% with *A. syzygii* LMG 21419^T) or low (18% with *A. peroxydans* LMG 1635^T, 18% with *A. pomorum* LMG 18848^T and 20% with *A. pasteurianus* LMG 1262^T). The level of DNA–DNA binding between isolates 985^T, 1145, 950 and 1039 varied between 82 and 97%. The DNA–DNA hybridization data demonstrate that the cocoa isolates represent a novel genospecies within the genus *Acetobacter* (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). The DNA G + C contents of isolates 985^T, 1145, 950 and 1039 were determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as calibration reference. The DNA G + C contents of isolates 985^T, 1145, 950 and 1039 were 56.8–58.0 mol%, which is consistent with those of members of the genus *Acetobacter* (Lisdiyanti *et al.*, 2000, 2001; Silva *et al.*, 2006; Ndoye *et al.*, 2007; Cleenwerck *et al.*, 2007).

The production of gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was determined for isolates 985^T, 1145, 950 and 1039 by the method described by Gosselé *et al.* (1980) using high-pressure anion-exchange chromatography with conductivity detection (Van der Meulen *et al.*, 2006) instead of TLC. Physiological characteristics of isolates 985^T, 1145, 950 and 1039 that enable differentiation from recognized *Acetobacter* species were examined by methods reported previously (Cleenwerck *et al.*, 2002, 2007). Additionally, the isolates and strains of *A. lovaniensis*, *A. ghanensis* and *A. syzygii*, their phylogenetically nearest relatives, were examined for other physiological characteristics such as growth at different temperatures (28, 34, 37 and 42 °C) on GY agar (5% D-glucose, 1% yeast extract, 1.5% agar) and growth on the carbon sources D-xylose, D-fructose, D-galactose and D-glucose. The isolates could be differentiated from *A. lovaniensis*, *A. ghanensis* and *A. syzygii* by their inability to produce 2-keto-D-gluconic acid from D-glucose (differentiation from *A. lovaniensis*), their ability to grow on methanol (differentiation from *A. ghanensis* and *A. syzygii*) and D-xylose (differentiation from *A. lovaniensis*) but not on maltose as carbon sources (differentiation from *A. syzygii*), their inability to grow on yeast extract with 30% D-glucose (differentiation from *A. ghanensis*) and their weak growth at 37 °C (differentiation from *A. lovaniensis*, *A. ghanensis* and *A. syzygii*) (Table 1 and Supplementary Table S1).

In conclusion, the six isolates originating from Ghanaian cocoa bean fermentations constitute a taxon that can be differentiated genotypically and phenotypically from the 18 currently recognized *Acetobacter* species and should therefore be classified as representatives of a novel species,

Table 1. Differential characteristics of *Acetobacter fabarum* sp. nov. and recognized *Acetobacter* species

Strains: 1, *A. fabarum* sp. nov. (4 strains); 2, *A. lovaniensis* LMG 1617^T; 3, *A. ghanensis* (3 strains); 4, *A. syzygii* LMG 21419^T; 5, *A. pasteurianus* (7 strains); 6, *A. pomorum* LMG 18848^T; 7, *A. peroxydans* (2 strains); 8, *A. indonesiensis* (2 strains); 9, *A. orientalis* LMG 21417^T; 10, *A. cibinongensis* LMG 21418^T; 11, *A. tropicalis* (2 strains); 12, *A. senegalensis* (3 strains); 13, *A. orleanensis* (4 strains); 14, *A. malorum* LMG 1746^T; 15, *A. cerevisiae* (4 strains); 16, *A. nitrogenifigens* RG1^T; 17, *A. oeni* B13^T; 18, *A. aceti* (4 strains); 19, *A. estumensis* (3 strains). +, Positive; -, negative; w, weakly positive; v, variable (the result of the type strain of *A. fabarum* is given in parentheses). Except where indicated, data were taken from the following sources: column 1, this study; 2, 5–8, 11, 13–15 and 18–19, Cleenwerck *et al.* (2002); 3, Cleenwerck *et al.* (2007); 4, 9, 10 and 17, Silva *et al.* (2006); 12, Ndoye *et al.* (2007) (*A. senegalensis* CWBI-B418^T) and Camu *et al.* (2007) (*A. senegalensis* 108B and 420A); 16, Dutta & Gachhui (2006). Phenotypic characteristics taken from Cleenwerck *et al.* (2002, 2007), Silva *et al.* (2006), Ndoye *et al.* (2007) and Camu *et al.* (2007) were examined as described by Cleenwerck *et al.* (2002). YE, Yeast extract.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Formation from D-glucose of:																			
5-Keto-D-gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	+	+	-
2-Keto-D-gluconic acid	-	+	-	-	v	-	-	+	+	+	+	+	+	+	+	ND	-	+	+
Growth in ammonium with ethanol	v (+)	+	-	-	-	-	+	-	-	w	-	+	-	-	-	+	-	+	+
Growth on carbon sources																			
Glycerol	+	+	w	+	v	+	-	+	+	+	+	+	+	+	+	+	+	+	v
Maltose	-	-	-	+	v	-	+	+	w	-	+	-	v	-	-	+	-	v	-
Methanol	+	+	-	-	-	-	-	-	w	-	-	-	-	+	-	-	-	-	-
Growth in 10% ethanol	v (-)	- ^{a*}	v	- ^a	+ ^a	- ^a	- ^a	- ^a	- ^a	- ^a	- ^a	+	- ^a	+ ^b	ND	+	+	- ^a	- ^a
Growth on YE + 30% D-glucose	-	-	+	-	v	-	-	-	-	+	-	+	-	+	-	+	-	-	-
Catalase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
DNA G+C content (mol%)	56.8– 58.0	57.1– 58.9	56.9– 57.3	54.3– 55.4†	53.2– 54.3	52.1	59.7– 60.7	54.0– 54.2	52.0– 52.8†	53.8– 54.5†	55.6– 56.2	55.6– 56.0	55.7– 58.1	57.2	56.0– 57.6	64.1	58.1	56.9– 58.3	59.2– 60.2

*Data taken from: *a*, Lisdiyanti *et al.* (2001); *b*, Ndoye *et al.* (2007).

†Data were taken from Lisdiyanti *et al.* (2001) and are ranges for four (*A. syzygii*), nine (*A. orientalis*) and two (*A. cibinongensis*) strains, including the type strains.

for which the name *Acetobacter fabarum* sp. nov. is proposed.

Description of *Acetobacter fabarum* sp. nov.

Acetobacter fabarum (fa.ba'rum. L. gen. pl. n. *fabarum* of beans, referring to the initial isolation of this species from cocoa beans).

Cells are Gram-negative, motile, coccoid rods, approximately 0.8 µm wide and 1.2–3.0 µm long. Cells occur singly or in pairs. Oxidase-negative. Catalase-positive. On Z1 agar, colonies are beige, round, raised, wavy, rough, shiny and approximately 0.8 mm in diameter after incubation at 28 °C for 3 days. Ethanol is oxidized to acetic acid. Gluconic acid is produced from D-glucose. Unable to produce 2-keto-D-gluconic acid or 5-keto-D-gluconic acid from D-glucose. Able to grow on methanol and D-xylose but not on maltose as sole carbon source. Unable to grow on yeast extract with 30% D-glucose. Growth with ammonium as sole nitrogen source and on glycerol and 10% ethanol as carbon source is variable between strains. The DNA G+C content varies from 56.8 to 58.0 mol%. Can be differentiated genotypically from other *Acetobacter* species by DNA–DNA hybridization and (GTG)₅-PCR fingerprinting.

The type strain is strain 985^T (=R-36330^T =LMG 24244^T =DSM 19596^T), isolated from a Ghanaian cocoa bean heap fermentation. The DNA G+C content of strain 985^T is 57.6 mol%.

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