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IMPROVED CLASSIFICATION AND IDENTIFICATION OF ACETIC ACID BACTERIA BASED ON MOLECULAR TECHNIQUES

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CONTENTS

LIST OF ABBREVIATIONS

2KGADH	2-keto-D-gluconate dehydrogenase
2KGR	2-keto-D-gluconate reductase
5KGR	5-keto-D-gluconate reductase
A.	*Acetobacter*
AAB	acetic acid bacteria
ABC transporter	ATP-binding cassette transporter
Ac.	*Acidomonas*
ADH	membrane-bound alcohol dehydrogenase
AFLP®	amplified fragment length polymorphism
ALDH	membrane-bound acetaldehyde dehydrogenase
As.	*Asaia*
ATCC	American Type Culture Collection, USA
BCCM™/LMG	Bacteria Collection of the Belgian Coordinated Collections of Microorganisms, Ghent University, Belgium
BLAST	basic local alignment search tool
BMR	Bio Molecular Research Center, University of Padua, Italy
BNF	biological nitrogen fixation
CFU	colony forming units
CGD	chronic granulomatous disease
CTAB	hexadecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell cultures, Germany
EDTA	ethylene diamine tetraacetic acid
EMBL	European Molecular Biology Laboratory, Cambridge, UK
ERIC	enterobacterial repetitive intergenic consensus
FDA	Food and Drug Administration, USA
G.	*Gluconacetobacter*
GADH	D-glucononate dehydrogenase
GDH	glucose dehydrogenase
Gl.	*Gluconobacter*
Gr.	*Granulibacter*
HPAEC	high pressure anion exchange chromatography
HPLC	high performance liquid chromatography
HGT	horizontal gene transfer

IFO	Institute for Fermentation, Osaka, Japan
K.	*Kozakia*
LMD	Laboratorium voor Microbiologie, Technische Universiteit Delft, The Netherlands
LM-UGent	Laboratory of Microbiology, Ghent University, Belgium
MLSA	multilocus sequence analysis
MPDH	membrane bound polyoldehydrogenase
N.	*Neoasaia*
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)
NCIB	National Collection of Industrial and Marine Biology, U.K.
NCIMB	National Collection of Industrial, Marine and Food Bacteria, U.K.
NBRC	NITE Biological Resource Centre, Japan
NRIC	NODAI Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PQQ	pyrroloquinoline quinone
Q-9	ubiquinone-9
Q-10	ubiquinone-10
RAPD	randomly amplified polymorphic DNA
REP	repetitive extragenic palindromic
rep-PCR	interspersed repetitive sequence-based polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
S.	*Saccharibacter*
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sw.	*Swaminathania*
TCA	tricarboxylic acid cycle
UPGMA	unweighted pair group method using arithmetic averages

PART I

BACKGROUND AND OBJECTIVES, LITERATURE STUDY, SHORT OVERVIEW OF THE EXPERIMENTAL WORK

BACKGROUND AND OBJECTIVES

In may 1998, I joined the BCCM^{TM}/LMG Bacteria Collection, which is one of four complementary research-based service culture collections of the Belgian Co-ordinated Collections of Micro-organisms (BCCM™) (http://bccm.belspo.be/index.php). The BCCM^{TM}/LMG Bacteria Collection provides, as stated above, services and regularly requested services are identification or classification, characterization and molecular typing of microorganisms.

Acetic acid bacteria (AAB) comprise a group of microorganisms that are of interest to the industry. They play a beneficial role in the production of several fermented foods and beverages, such as vinegars, cocoa-based products, kombucha and nata de coco; may cause spoilage to others such as beer, wine and cider; and find applications in the production of fine chemicals (e.g. vitamine C and miglitol) and of bacterial cellulose, a biopolymer with particular properties to be used as food thickener, paper additive, diaphragm for headphones, artificial skin, etc. Additionally, some AAB have been described as plant growth promotors; whereas others have recently been described as human pathogens.

A lot of research has been done on AAB, partly by the hostlab to which the BCCM^{TM}/LMG Bacteria Collection is connected. However, when I joined the Collection, several weaknesses could be noticed in their classification, especially in the genera *Acetobacter* and *Gluconacetobacter*.

The goal of this thesis was to improve the classification of AAB on the basis of molecular techniques and to provide methods that enable fast and accurate species level identification and classification of these microorganisms.

CHAPTER 1

LITERATURE STUDY

1.1. GENERAL CHARACTERISTICS OF AAB

AAB are generally defined as Gram-negative, coccoid or rod-shaped, obligate aerobic bacteria that have the ability to incompletely oxidize a wide range of sugars, alcohols and sugar alcohols. At present, AAB are classified into 10 genera and 49 species (Bacterial Nomenclature Up-to-date, DSMZ, May 2008; Table 1.1). A common feature to most AAB is the ability to oxidize ethanol to acetic acid, except *Asaia* (*As*.) that cannot produce acetic acid from ethanol and *Saccharibacter* (*S*.) and *Granulibacter* (*Gr*.) that form negligible or very little acetic acid from ethanol.

Table 1.1. Classification of the *Acetobacteraceae* (10 genera, 49 species)

Species[a]	Reference	Species[a]	Reference
Acetobacter aceti	Lisdiyanti *et al.*(2000)	*Gluconacetobacter europaeus* [d]	Yamada *et al.* (1997)
Acetobacter cerevisiae	Cleenwerck *et al.* (2002)	*Gluconacetobacter hansenii* [d]	Yamada *et al.* (1997)
Acetobacter cibinongensis [b]	Lisdiyanti *et al.* (2001)	*Gluconacetobacter intermedius*	Yamada (2000)
Acetobacter estunensis [c]	Lisdiyanti *et al.*(2000)	*Gluconacetobacter johannae*	Fuentes-Ramírez *et al.* (2001)
Acetobacter ghanensis	Cleenwerck *et al.* (2007)	*Gluconacetobacter kombuchae*	Dutta & Gachhui (2007)
Acetobacter indonesiensis [c]	Lisdiyanti *et al.*(2000)	***Gluconacetobacter liquefaciens*** [d]	Yamada *et al.* (1997)
Acetobacter lovaniensis [c]	Lisdiyanti *et al.*(2000)	*Gluconacetobacter nataicola*	Lisdiyanti *et al.* (2006)
Acetobacter malorum	Cleenwerck *et al.* (2002)	*Gluconacetobacter oboediens*	Yamada (2000)
Acetobacter nitrogenifigens	Dutta & Gachhui (2006)	*Gluconacetobacter rhaeticus*	Dellaglio *et al.* (2005)
Acetobacter oeni	Silva *et al.* (2006)	*Gluconacetobacter sacchari*	Franke *et al.* (1999)
Acetobacter orientalis [b]	Lisdiyanti *et al.* (2001)	*Gluconacetobacter saccharivorans*	Lisdiyanti *et al.* (2006)
Acetobacter orleanensis [c]	Lisdiyanti *et al.*(2000)	*Gluconacetobacter swingsii*	Dellaglio *et al.* (2005)
Acetobacter pasteurianus	Lisdiyanti *et al.*(2000)	*Gluconacetobacter xylinus* [d]	Yamada *et al.* (1997)
Acetobacter peroxydans	Lisdiyanti *et al.*(2000)	*Gluconobacter albidus* [e]	Yukphan *et al.* (2004c)
Acetobacter pomorum	Sokollek *et al.* (1998b)	*Gluconobacter cerinus*	Katsura *et al.* (2002)
Acetobacter senegalensis	Ndoye *et al.* (2007a)	*Gluconobacter frateurii*	Mason & Claus (1989)
Acetobacter syzygii [b]	Lisdiyanti *et al.* (2001)	*Gluconobacter kondonii*[g]	Malimas *et al.* (2007)
Acetobacter tropicalis [c]	Lisdiyanti *et al.*(2000)	***Gluconobacter oxydans***	Mason & Claus (1989)
Acidomonas methanolica	Urakami *et al.* (1989)	*Gluconobacter thailandicus* [e]	Tanasupawat *et al.* (2004)
Asaia bogorensis	Yamada *et al.* (2000)	***Granulibacter bethesdensis***	Greenberg *et al.* (2006b)
Asaia krungthepensis	Yukphan *et al.*(2004b)	***Kozakia baliensis***	Lisdiyanti *et al.* (2002)
Asaia siamensis	Katsura *et al.* (2001)	***Neoasaia chiangmaiensis*** [f]	Yukphan *et al.* (2005)
Gluconacetobacter azotocaptans	Fuentes-Ramírez *et al.* (2001)	***Saccharibacter floricola***	Jojima *et al.* (2004)
Gluconobacter diazotrophicus [d]	Yamada *et al.* (1997)	***Swaminathania salitolerans***	Loganathan & Nair (2004)
Gluconacetobacter entanii	Schüller *et al.* (2000)		

[a]The type species of each genus is indicated in bold

[b]Validated in 2002; [c]Validated in 2001

[d]The original spelling of the genus name *Gluconoacetobacter* (Yamada *et al.*, 1997) was corrected to *Gluconacetobacter* at the occasion of its validation (1998, Validation List no. 64, International Journal of Systematic Bacteriology 48, 327-328);

[e]Validated in 2005; [f]Validated in 2006; [g]Validated in 2008

Acetic acid can be oxidized further to CO_2 and H_2O (overoxidation of ethanol) by strains of *Acetobacter* (*A.*), *Gluconacetobacter* (*G.*) and *Acidomonas* (*Ac.*). Oxidation of acetic acid is weak in *Asaia*, *Kozakia* (*K.*), *Swaminathania* (*Sw.*) and *Granulibacter*, and absent in *Gluconobacter* (*Gl.*), *Saccharibacter* and *Neoasaia* (*N.*). Gluconic acid is usually produced from glucose. Most AAB have a relatively high tolerance to acidic conditions, which is essential for organisms producing large amounts of acid. Most strains are able to grow at pH values lower than 5. AAB are oxidase negative and generally catalase positive. Enlarged, irregular cell forms (involution forms) may occur and their appearance or disappearance seems to be correlated with changes of pH, temperature or medium composition (Cleenwerck & De Vos, 2008; De Ley *et al.*, 1984a; Kersters *et al.*, 2006; Sievers & Swings, 2005).

1.2. HABITATS, METABOLISM, APPLICATIONS AND CONCERNS OF AAB

1.2.1. Habitats

AAB are ubiquitous in the environment. They occur in sugary and alcoholic, slightly acid niches and have been isolated from plant material (e.g. fruits like apples, apricots, bananas, coconuts, figs, guavas, grapes, mandarins, mangoes, oranges, papayas, passion fruits, pears, peaches, pineapples, persimmons; flowers; sugarcane and coffee plants) and insects (e.g. honey bees and the pink sugar cane mealy bug *Saccharicoccus sacchari*), and also from wine, cider, beer, sake (Japanese rice wine), palm wine, tequila, cocoa wine, souring fruit juices, soft drinks, fruit-flavoured water, vinegar, honey, brewer's yeast, flour, kefir, nata, kombucha, natural cocoa bean fermentations, fermented meat, sugarcane juice, garden soil, sewage, etc. (overview: see Kersters *et al.*, 2006). Recently, isolates from clinical origin have been reported (Greenberg *et al.*, 2006a, b; Snyder *et al.*, 2004; Tuuminen *et al.*, 2006, 2007). The isolation sources per genus are briefly given below.

Gluconobacter strains are mainly isolated from sugar-rich habitats, such as fruits and flowers. They are generally tolerant to high sugar concentrations, but can also be found in alcoholic niches (Lisdiyanti *et al.*, 2003; Sievers & Swings, 2005).

Acetobacter and *Gluconacetobacter* strains are often isolated from alcohol-containing niches. From Pasteur's time on their occurrence in wines, beer and vinegar has been reported by many authors (see 1.2.3.1, 1.2.4.1), and they have been isolated from fermented foods as well. *Acetobacter* strains are also frequently recovered from fruits (De Ley *et al.*, 1984b; Lisdiyanti *et al.*, 2003; Seearunruangchai *et al.*, 2004;

Sievers & Swings, 2005).

Most strains of *Ac. methanolica*, the only species of the genus *Acidomonas* thus far, have been isolated from unique niches, namely sludge from sewage-treatment plants and a yeast fermentation process where methanol was the sole source of carbon and energy. One strain was isolated from sugarcane (Lisdiyanti *et al.*, 2003; Urakami *et al.*, 1989; Yamashita *et al.*, 2004).

Most strains of the genus *Asaia* have been isolated from tropical flowers collected in Thailand, Indonesia and the Philippines (Huong *et al*., 2007; Kersters *et al.*, 2006; Lisdiyanti *et al.*, 2003; Yukphan *et al*., 2006a, b). *As. bogorensis* strains are most frequently isolated (Huong *et al.*, 2007; Yukphan *et al*., 2006a, b). Recently, an *Asaia* strain was isolated from fruit-flavoured bottled water (Moore *et al.,* 2002a) and strains, assigned to *As. bogorensis* on the basis of partial 16S rRNA gene sequence analysis, were reported as opportunistic human pathogens (Snyder *et al*., 2004; Tuuminen *et al*., 2006, 2007).

The genera *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia* and *Granulibacter* consist for the moment of only one species. *K. baliensis* strains have been isolated from ragi (starter for fermented foods) and palm brown sugar (Lisdiyanti *et al.*, 2003), *Sw. salitolerans* strains from wild rice (Loganathan & Nair, 2004), *S. floricola* strains from the pollen of Japanese flowers (Jojima *et al*., 2004) and *N. chiangmaiensis* strains from tropical flowers (Yukphan *et al.,* 2005). *Gr. bethesdensis* is the second species in the family of *Acetobacteraceae* that is associated with human disease. Strains of this species were isolated from patients with chronic granulomatous disease and appear to be genuine pathogens. The niche from which the strains infected the patients is not (yet) known (Fredricks & Ramakrishnan, 2006; Greenberg *et al*., 2006a, b).

1.2.2. Metabolism

AAB are of interest to the industry, mainly for their capacity to incompletely oxidize different kinds of alcohols, sugars, sugar alcohols and organic acids, but also for production of bacterial cellulose and as growth promotors of plants. A lot of research has been performed to unravel the metabolism for the oxidations and cellulose production and the mechanisms of plant growth promotion, with the aim of trying to control or improve the related bioprocesses.

AAB oxidize alcohols, sugars and sugar alcohols via two different pathways. The substrates can either be transported through the cytoplasmic membrane and oxidized by $NAD(P)^+$ dependent cytosolic dehydrogenases or they can be oxidized by $NAD(P)^+$

independent dehydrogenases that are located in the cytoplasmic membrane. The membrane-bound dehydrogenases are linked to the respiratory chain, which contains ubiquinone Q-10 as the major quinone component in all tested genera, except in *Acetobacter*, where ubiquinone Q-9 is the major component. The terminal acceptor for the electrons derived from the oxidative processes is oxygen. The process of incomplete oxidation is an exothermic reaction which is accompanied by heat development. AAB that are thermotolerant have an advantageous trait for industrial oxidation processes, because the costs of cooling during fermentation can be reduced (Adachi *et al.*, 2003; De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002; Keliang & Dongzhi, 2006; Moonmangmee *et al*., 2000).

The oxidative capacity of strains of the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* has been investigated thoroughly, but is not yet fully elucidated. The oxidative capacity towards sugars is more pronounced in *Gluconobacter* strains, while the production of acetic acid from ethanol is more efficient in *Gluconacetobacter* and *Acetobacter*. It has been shown that accumulation of acetic acid, gluconic acid and other oxidation products in the medium by members of these genera is mainly due to the activity of the membrane-bound dehydrogenases. The reactive centres of these enzymes are oriented towards the periplasmatic space, and therefore substrates only have to cross the outer membrane, where they are transformed to oxidation products, which are released nearly quantitatively into the medium via porines of the outer membrane (De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002; Kersters *et al*., 2006).

AAB oxidize sugar alcohols according to the Bertrand-Hudson rule in a stereospecific manner. This feature makes AAB very useful to the industry for the synthesis of sugar derivates such as L-sorbose that can otherwise only be obtained by complex protection group chemistry (Deppenmeier *et al.*, 2002).

Acetobacter, *Gluconacetobacter* and *Gluconobacter* catabolize sugars by means of the hexose monophospate shunt. Glycolysis is present in *G. diazotrophicus*, but absent or very weak in other studied species of these genera, due to lack of phosphofructokinase. The tricarboxylic acid cycle (TCA) is complete in *Acetobacter*, *Acidomonas* and *Gluconacetobacter*, but incomplete in *Gluconobacter* since succinate dehydrogenase has not been found. The enzymes of the TCA cycle are used for the oxidation of organic acids and this explains why *Gluconobacter* is incapable to oxidize acetic acid and many other organic acids further to CO_2 and H_2O. Strains which are able to grow on Hoyer's medium with ethanol as sole source of carbon and $(NH_4)_2SO_4$ as the only source of nitrogen, utilize the enzymes of the glyoxylate bypass. (De Ley *et al.*, 1984a; De Muynck *et al.*, 2007; Prust *et al.*, 2005; Saravanan *et al.*, 2008 ; Sievers &

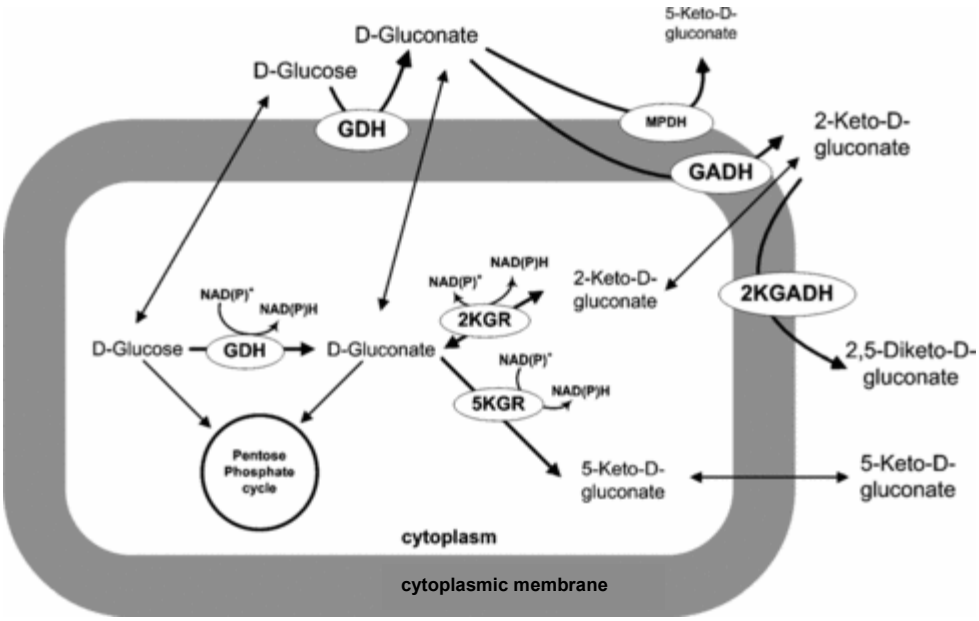


Fig. 1.1. Glucose pathways in *Gl. oxydans* (De Muynck *et al*., 2007). Glucose can be directly oxidized by membrane-bound glucose dehydrogenase (GDH) to D-gluconate, and this can be further oxidized to 2-keto-D-gluconate and 2,5-diketo-D-gluconate by membrane-bound D-gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGADH), respectively (Matsushita *et al*., 1994). *Gl. oxydans* is also able to accumulate 5-keto-D-gluconate in the culture medium using membrane-bound major polyoldehydrogenase (MPDH). Glucose can also be taken up in the cytoplasm where it can be converted to D-gluconate by a cytosolic glucose dehydrogenase. This D-gluconate can be oxidized by 2-keto-D-gluconate reductase (2KGR) and 5-keto-D-gluconate reductase (5KGR). The cytosolic 2KGR can also reduce 2-keto-D-gluconate back to D-gluconate. D-gluconate can also be dissimilated via the hexose monophospate shunt. D-gluconate production by *Gl. oxydans* has been shown to be mainly due to the activity of the membrane bound GDH, as its activity is 30-fold higher than that of the cytosolic GDH (Pronk *et al*., 1989).

Swings, 2005).

Recently, the complete genome sequence of *Gl. oxydans* 621H was determined with the aim to further understand its overall metabolism and to obtain insight into the oxidative potential of this strain (Prust *et al.*, 2005). *Gl. oxydans* 621H contains a circular chromosome of 2702 kb and five plasmids of 163, 27, 15, 13 and 3 kb, respectively. There are 2664 protein-encoding open reading frames (ORFs) (2432 on the chromosome and 232 on the plasmids), four copies of rRNA operons and 55 genes encoding tRNAs. Biological roles have been assigned to 1877 ORFs. 75 ORFs,

encoding putative dehydrogenases of unknown function, have been identified. The organism contains many membrane-bound dehydrogenases that enable it to grow and to survive in nutrient-rich environments. The enzymes rapidly oxidize sugars to acids that are difficult to assimilate by most microorganisms, whereas *Gl. oxydans* can easily take advantage of these substrates. Furthermore, the formation of acids leads to a decrease in the pH value and prevents propagation of many other microorganisms (Gullo & Giudici, 2008). The information of the genome sequence analysis results in a better understanding of the metabolism of *Gl. oxydans* and may lead to new strategies for the employment of this microorganism in a greater variety of incomplete oxidations than today is the case (De Muynck *et al*., 2007; Prust *et al*., 2005). Recently an easy cloning and expression vector system for *Gl. oxydans* was constructed with the major aim to facilitate the characterization of the biological role of several gene products (Schleyer *et al*., 2008).

The metabolic pathways of ethanol, D-glucose (Fig. 1.1), D-sorbitol, ribitol, glycerol and quinate in *Gl. oxydans* are briefly discussed by De Muynck *et al.* (2007). Pathways of carbon metabolism in *G. xylinus and G. diazotrophicus* are discussed by Ross *et al.* (1991) and by Saravanan *et al*. (2008), respectively.

1.2.3. Applications

AAB have many applications that are based on their capacity to incompletely oxidize different kinds of alcohols, sugars, sugar alcohols and organic acids. The main applications in which this property is used concern the production of several foods and beverages (e.g. vinegar, kombucha, chocolate; see 1.2.3.1 and 1.2.3.2) and chemical products (e.g. L-sorbose involved in the synthesis of vitamin C; see 1.2.3.3). For the latter, *Gl. oxydans* is most frequently used by the industry (De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002; Keliang & Dongzhi, 2006).

Besides these applications, AAB are also used for the production of polysaccharides (mainly bacterial cellulose; see 1.2.3.4) and as growth promotors of plants (see 1.2.3.5).

1.2.3.1. Vinegar

Vinegar is an aqueous solution of acetic acid that is produced by AAB from a dilute ethanol solution. Several natural sugar-containing juices or mashes that are first converted by fermentation to ethanol solutions can serve as raw materials for vinegar production. In the past, vinegar production was always linked with wine making and the

word vinegar derives from the French "vin" and "aigre" ("vinaigre"), meaning "sour wine" (Kersters *et al.*, 2006). Today, many types of vinegar are available on the market such as wine vinegar, cider vinegar, sherry vinegar, balsamic vinegar, traditional balsamic vinegar, vinegar based on citrus wine, malt vinegar, rice vinegar (e.g. komesu and kurosu) and kasuzu (vinegar from sake lees). Vinegar is used as a flavouring ingredient and for conservation of meat and vegetables (Deppenmeier *et al.*, 2002; Kersters *et al.*, 2006).

The metabolic process of acetic acid formation involves the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase and the oxidation of acetaldehyde to acetic acid by aldehyde dehydrogenase. The production of acetic acid is mainly due to the activity of membrane-bound alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) (De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002). These enzymes are tightly linked to the respiratory chain and have been studied well in multiple AAB. Both enzymes are composed of 2 to 3 subunits, but have different cofactors (pyrroloquinoline quinone for ADH, pterin molybdenum for ALDH). The genes encoding the two largest subunits of the ADH enzyme are clustered in the same transcription polarity and are generally cotranscribed. The genes encoding ALDH are organized as an operon. Each gene of the ADH and ALDH subunits contains a signal sequence encoding a leader peptide, responsible for translocation of the proteins through the cytoplasmic membrane (Sievers & Swings, 2005). ADH plays a role in the resistance of AAB to acetic acid. Trček *et al.* (2006) compared ADH of *G. europaeus*, *G. intermedius* and *A. pasteurianus* strains, isolated from industrial vinegar reactors and found a correlation between acetic acid resistance, the activity of ADH and its stability in the presence of acetic acid. Other mechanisms conferring to acetic acid resistance are: the assimilation of acetic acid by enzymes, such as aconitase and citrate synthase, and the export of acetic acid by an ATP-binding cassette (ABC) transporter. Both mechanisms have been described for *A. aceti.* They are induced by acetic acid and serve to reduce the intracellular acetic acid concentration. Overexpression of the genes corresponding to aconitase and the ABC transporter resulted in an improvement of growth in the presence of a high concentration of acetic acid and in an increase in the final yield of acetic acid, probably due to maintenance of a low level of intracellular acetic acid concentration (Nakano & Fukaya, 2008; Nakano *et al*., 2006). ALDH is sensitive to the concentration of oxygen. ALDH activity decreases when the oxygen concentration is too low, which results in an accumulation of acetaldehyde in the fermentation medium (Muraoka *et al.*, 1983). ADH and ALDH have a broad substrate specificity and oxidize branched alcohols and aldehydes into the corresponding carboxylic acids. This feature is useful to

the food industry. Aldehydes produced during food processing can react with amino groups to form coloured materials. Addition of AAB to foods such as mayonnaise, prevents food coloration and reduces off-flavours, since carboxylic acids react little with amino acids (Nomura *et al*., 1995; Sievers & Swings, 2005).

The production of vinegar is documented as early as about 4000 B.C. by the Babylonians (Kersters *et al.*, 2006). Today, three different methods are used for the production of vinegar. In the open-vat method (Orleans method or French method), the ethanol solution is placed in vats with considerable exposure to air. The vinegar is produced by AAB which develop as a surface film on top of the liquid where the oxygen concentration is higher. This method is also referred to as "surface culture fermentation". The second system is the trickling generator process ("quick process" or German method) in which alcohol is trickled from the top through a tank filled with beech-wood shavings on which the bacteria grow. Air is introduced through holes in the bottom and passes upward so that the contact between bacteria and oxygen is improved. The third technology is based on submerged fermentation in a "Frings acetator", a fermentor with an aeration system fixed at the bottom. This method is widely used for commercial vinegar production. The efficiency of this process is very high (Deppenmeier *et al.*, 2002; Kersters *et al.*, 2006).

Vinegar is generally produced by inoculation of "seed vinegar", which is a microbiologically undefined starter culture, generally obtained from a previous fermentation. The lack of defined pure starter cultures is mainly due to problems with isolation, cultivation and preservation of vinegar AAB. Several authors have mentioned that even after successful isolation and cultivation it is extremely difficult to handle the isolates and to preserve their high acetic acid resistance under laboratory conditions (Gullo & Guidici, 2008; Schüller *et al.*, 2000; Sokollek *et al.*, 1998a; Trček *et al.*, 2006). Many studies have been performed to characterize AAB involved in vinegar production. Strains isolated from vinegar seem to be distributed mainly in the genera *Acetobacter* and *Gluconacetobacter* (Deppenmeier *et al.*, 2002; Kersters *et al.*, 2006; Yamada, 2003), of which the taxonomy has recently undergone many changes (see 1.4). Therefore caution needs to be taken when interpreting old data. Several studies have recently been performed to characterize AAB from vinegar using molecular techniques. Five novel species involved in industrial submerged vinegar production have been described since Entani *et al*. (1985) developed a double layer agar technique (using an acetic acid/ethanol medium) that allowed their cultivation: *G. europaeus*, *G. oboediens, A. pomorum*, *G. intermedius* and *G. entanii* (Boesch *et al*., 1998; Schüller *et al*., 2000; Sievers *et al*., 1992; Sokollek *et al*., 1998b). Other species reported to be involved in

vinegar production are: *A. aceti*, *A. pasteurianus*, *A. malorum*, *A. senegalensis* and *G. hansenii* (De Vero *et al*. 2006; Gullo & Giudici, 2008; Nanda *et al*., 2001; Ndoye *et al.*, 2007a; Trček, 2005; Trček & Teuber, 2002).

1.2.3.2. Other fermented beverages and food products

Palm wine is a typical tropical beverage derived from the fermentation of sugary palm sap. Palm wines are very popular in Africa, South America and the Far East. A rather complex microbiota is present in palm wines (Swings & De Ley, 1977). AAB appear in palm sap fermentation after 2-3 days, after the sugar of the sap is fermented to ethanol by yeasts and *Zymomonas*. *A. pasteurianus, A. lovaniensis, A. indonesiensis and A. tropicalis* strains have been isolated from palm wines, as well as *Gluconobacter* strains (Amoa-Awua *et al.*, 2007; Kersters *et al.*, 2006; Lisdiyanti *et al.*, 2003).

Nata is a white to creamy-yellow to pinkish, firm, gelatine-like substance formed through bacterial action on sugared fruit juices. Nata-de-coco and nata-de-pina are traditional dessert delicacies in the Philippines and other Southeast Asian countries. Nata is a form of cellulose produced by AAB belonging to the *G. xylinus*/*G. hansenii* cluster (Bernardo *et al.*, 1998; Iguchi *et al.*, 2000). *A. pasteurianus*, *A. orleanensis*, *A. lovaniensis*, *Gl. oxydans* and *Gl. frateurii* were also found in the process of nata production (Kersters *et al*., 2006; Lisdiyanti *et al.*, 2003, 2006).

Kombucha is a slightly acidulous and sparkling beverage obtained by the fermentation of black tea to which sugar and "tea fungus", a symbiotic culture of yeasts and AAB, has been added. More than thousand years ago tea fungus was already used in Japan, China and India. Nowadays, it is used in Russia, Poland, the Balkan Germany, Eastern Europe, Spain, Italy, France and Switzerland (Kersters *et al.*, 2006). *A. aceti*, *A. pasteurianus*, *A. nitrogenifigens*, *G. xylinus*, *G. intermedius, G. kombuchae* and *Gl. oxydans* have been isolated from kombucha (Boesch *et al.*, 1998; Dutta & Gachhui, 2006, 2007; Kersters *et al*., 2006).

Cocoa (powder) and chocolate are products that are processed from fermented cocoa beans. Raw cocoa beans have an astringent, unpleasant taste and flavour, and fermentation, drying and roasting is necessary to obtain the desired characteristic cocoa and chocolate flavour, aroma and taste (Camu *et al*., 2007). The actual fermentation takes place in the pulp in which the beans are embedded inside a pod. The fermentation starts spontaneously after the beans and viscous pulp are removed from the pods, and it generally lasts for 3 up to 6 days. The pulp is rich in sugars (mainly glucose and fructose) and the initial pH is relatively low, primarily due to a high concentration of

citric acid. Yeasts, lactic acid bacteria and AAB are involved in the natural fermentation of cocoa beans. AAB start to dominate the process when oxygen concentration increases due to the disappearance of the pulp by pectinolytic activity of yeasts. AAB oxidize ethanol produced by yeasts from sugars present in the pulp (10-15 %) to acetic acid. Part of this acid diffuses into the beans and this, together with heat produced during this exothermic reaction (the temperature can go up to 50 °C) leads to the death of the seed embryo and the end of the fermentation. Ethanol, acids and water, that diffused into the cotyledons initiate an array of biochemical and enzymatic reactions that lead to the formation of the precursors of cocoa flavour, aroma and colour. It has been shown that hydrolytic enzymes inside the beans are activated by microbial metabolites such as acetic acid (reviews: Ardhana & Fleet, 2003; Camu *et al*., 2007; Schwan & Wheals, 2004). The following AAB species have been reported in recent studies to be associated with cocoa bean fermentations: *A. pasteurianus*, *A. peroxydans*, *A. lovaniensis*, *A. syzygii*, *A. ghanensis*, *A. tropicalis*, *A. senegalensis*, *A. aceti*, *G. liquefaciens*, *G. xylinus* and *Gl. oxydans* (Ardhana & Fleet, 2003; Camu *et al*., 2007, 2008; Cleenwerck *et al*., 2007; Lagunes Gálvez *et al.*, 2007; Nielsen *et al*., 2007; Schwan & Wheals, 2004).

1.2.3.3. Organic chemicals

AAB can be used for the production of organic chemicals. For this, *Gl. oxydans* is often used by the industry, because it can rapidly incompletely oxidize a great variety of carboxyhydrates, alcohols and related compounds, and oxidation products are excreted nearly quantitatively into the medium. It is used in the production of vitamin C and other products such as D-gluconic acid, 2,5-diketo-D-gluconic acid, 5-keto-D-gluconic acid, dihydroxyaceton, L-ribulose, shikimate, xylonic acid, 6-amino-6-deoxy-L-sorbose and D-tagatose (Chun *et al*., 2006; reviews: De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002; Keliang & Dongzhi, 2006).

Vitamin C is a water soluble vitamin that is indispensable for several physiological functions. It is an essential nutrient for humans, non-human primates and a few other mammals as they cannot synthesize this vitamin. In addition, vitamin C has antioxidant properties and might have a role in cancer treatment. Vitamin C has been industrially produced for about 70 years. Today, vitamin C production is economically one of the most important industrial processes. A considerable part of vitamin C is manufactured from D-glucose via the seven-step Reichstein process, which is largely a chemical process, except for the second step that is the biotransformation of D-sorbitol to L-

sorbose by *Gl. oxydans*. This step is highly important. Vitamin C has several optical isomers due the asymmetric carbon-atoms C4 and C5, however only the L-isomer is biologically active. The stereospecific biotransformation from D-sorbitol to L-sorbose by *Gl. oxydans* makes that protection group chemistry can be avoided (Bremus *et al*., 2006; De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002). The key enzyme for this biotransformation is D-sorbitol dehydrogenase (De Muynck *et al.*, 2007; Deppenmeier *et al.*, 2002). In the past 20 years, several innovative systems have been proposed in order to simplify the production of vitamin C. In most of these approaches microorganisms have been used to produce 2-keto-L-gulonic acid as intermediate that can be converted to vitamin C by conventional catalysis. Strains belonging to the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*, sometimes genetically engineered, have been used (Deppenmeier *et al.*, 2002; reviewed by Bremus *et al*., 2006).

D-gluconic acid (bioproduction from D-glucose) and its salts are used in the formulation of food, pharmaceutical and hygienic products. The main derivate of gluconic acid is sodium gluconate which has the property to chelate calcium and other di- and trivalent metal ions. Its applications are in the food (detergent in bottle washing), textile and concrete industry, as well as in metallurgy. D-gluconic acid is one of the top-ten organic chemicals produced from sugar (De Muynck *et al.*, 2007). For its production, microbial fermentation utilising the fungus *Aspergillus niger* is most widely used. However, the process using *Gl. oxydans* has gained significant importance (Ramachandran *et al*., 2006). In future, *Ac. methanolica*, which produces only gluconic acid from glucose, might supersede *Gl. oxydans* for this production (Kersters *et al.*, 2006).

5-keto-D-gluconic acid (bioproduction from D-glucose) is a precursor of L-(+) tartaric acid, which has many applications, for example, as an antioxidant in the food industry, a chiral reagent in organic synthesis and an acidic reducing agent in the textile industry. Xylaric acid and a number of savoury flavour compounds can also be produced from 5-keto-D-gluconic acid (Keliang & Dongzhi, 2006). The bioproduction of 5-keto-D-gluconic acid by *Gl. oxydans* DSM 2343 was recently optimized by genetic engineering (Merfort *et al*., 2006).

Dihydroxyacetone (bioproduction from glycerol) is a widespread chemical product that is used as a sun tanning agent and as precursor for the synthesis of various fine chemicals and pharmaceuticals (Wei *et al*., 2007). The bioproduction of dihydroxyaceton by *Gl. oxydans* DSM 2343 was also recently optimized by genetic engineering (Gätgens *et al.*, 2007).

L-ribulose (bioproduction from ribitol) is an important chiral lead molecule that is

used for the synthesis of, among other, L-ribose, a high value rare sugar. L-ribose is used in the preparation of antiviral drugs (nucleoside-analogues), that gain importance in the treatment of severe viral diseases, like those caused by the HIV or hepatitis virus (De Muynck *et al*., 2006, 2007).

Shikimate (bioproduction from quinate) is a key intermediate for aromatic amino acids as well as for large numbers of antibiotics, alkaloids, and herbicides. It is also a precursor for oseltamivir, a product mentioned to protect people from a pandemic flu infection. A strategy for high shikimate production from quinate using two enzymatic systems of *Gl. oxydans* IFO 3244 has been proposed (Adachi *et al*., 2006).

Xylonic acid (bioproduction from xylose) has recently been proposed as a water reducer for concrete (Chun *et al*., 2006)

6-amino-6-deoxy-L-sorbose (bioproduction from 1-amino-1-deoxy-D-sorbitol) is a key intermediate in the production of miglitol (N-hydroxyethyl-1-deoxynojirimycin), a drug that was approved by the FDA in 1996 to treat type II diabetes. Nowadays it is the first choice for this treatment (Deppenmeier *et al.*, 2002).

Gl. oxydans dehydrogenases find, because of their unique properties, also application in biofuel cell (Kamitaka *et al.*, 2007; Ramanavicius *et al.*, 2005) and biosensor technology (reviewed by Švitel *et al.*, 2006), and co-enzyme regeneration (De Muynck *et al.*, 2007).

1.2.3.4. Bacterial cellulose

Cellulose is the main component of plant cell walls, but also a representative of microbial extracellular polymers. Strains belonging to the species *G. xylinus* are regarded as the most efficient producers of bacterial cellulose and because of this they have been used intensively as model organisms for studies on cellulose. Investigations have focused on the mechanisms of polymer synthesis as well as on cellulose structure and properties, which determine mainly its use (reviews: Bielecki *et al.*, 2002; Iguchi *et al.*, 2000; Ross *et al.*, 1991; Saxena & Brown, 2005). Synthesis of bacterial cellulose is a multistep process, involving a large number of enzymes and proteins. In cellulose synthesis, a large number of β-1,4-glucan chains are simultaneously polymerized by cellulose synthase, a membrane bound enzyme, which utilizes UDP-glucose as substrate. The β-1,4-glucan chains are secreted in the extracellular space through linearly ordered pores in the membrane where they aggregate to subfibrils which are crystallized into microfibrils, these into bundles and the latter into ribbons (Bielecki *et al.*, 2002; Kersters *et al.*, 2006). In *G. xylinus*, the genes for cellulose biosynthesis are

localized in an operon.

Bacterial cellulose is chemically the same in its primary composition as cellulose produced by other organisms (higher plants, algae), but the degree of polymerization and crystallinity as well as other physical properties are unique to each organism. Bacterial cellulose is characterized by its chemical purity, a very fine nanostructure and some unique physical properties such as high hydrophilicity, great adsorbtivity and high mechanical strength. The macroscopic morphology of bacterial cellulose is influenced by the culture conditions during polymer synthesis. A static culture will synthesize a cellulose pellicle on the surface of the broth, whereas granules are formed in shaken cultures. Cellulose pellicles and granules show differences in their 3D-structure, which contribute to differences in their properties (Bielecki *et al.*, 2002; Iguchi *et al.*, 2000; Saxena & Brown, 2005; Saxena *et al.*, 1994).

Cellulose pellicles have been proposed to have multiple functions in the growth and survival of the organisms in nature. Cells of cellulose producing strains are entrapped in the polymer matrix that keeps part of the bacteria into contact with oxygen, facilitates nutrient supply, since the concentration of nutrients in the polymer is enhanced due to its adsorptive properties, and increases resistance against unfavourable changes in the environment, like variations in pH (Bielecki *et al.*, 2002; De Muynck *et al.*, 2007; Ross *et al.*, 1991; Saxena & Brown, 2005; Saxena *et al.*, 1994). Bacterial cellulose composites with special features can be created by adding compounds during or after polymer synthesis (Brown & Laborie, 2007; Czaja *et al.*, 2007; Shah & Brown, 2005).

Because of the special features of bacterial cellulose, a variety of product applications is possible. An overview of these features and potential applications can be found at http://www.botany.utexas.edu/facstaff/facpages/mbrown/position1.htm. The prevalent potential application of bacterial cellulose is in the biomedical field (e.g. artificial skin and vessels; controlled-drug release carriers; reviews: Czaja *et al.*, 2007 and Hoenich, 2006). At present, bacterial cellulose is mainly applied in food products (e.g. nata, kombucha), paper products, acoustics (e.g. acoustic diaphragm for headphones and loudspeakers), electronics and medical devices (e.g. membranes, temporary wound coverage). Extensive commercial usage of bacterial cellulose requires efficient fermentation for bulk production, which is at present not available. A lot of recent research focuses therefore on strain improvement (by screening for suitable strains and genetic engineering) and production process development (Bae & Shoda, 2005; Chien *et al.*, 2006; De Muynck *et al.*, 2007; Hutchens *et al.*, 2007). One of the problems faced during cellulose synthesis is the tendency for formation of non-cellulose producing mutants which become dominating in the population in cultures aerated by

shaking or stirring (Krystynowicz *et al.*, 2005; Ross *et al.*, 1991).

Several AAB species, besides *G. xylinus*, contain cellulose-producing strains, such as *G. oboediens*, *G. intermedius*, *G. hansenii*, *G. swingsii*, *G. rhaeticus*, *G. kombuchae* and *Gl. oxydans* (Boesch *et al.*, 1998; Dellaglio *et al.*, 2005; Dutta & Gachhui, 2007; Lisdiyanti *et al.*, 2006). Many cellulose-producing strains display significant differences in cellulose synthesis and in the structure of the synthesized polymer. From a bioengineering point of view, structural differences are of great importance since they can be used to create hybrid materials with desired properties (Czaja *et al.*, 2007). Most of the cellulose-producing AAB produce besides cellulose a water soluble polysaccharide called acetan that leads to an increase in the viscosity of the medium, which seems to play an important role in cellulose biosynthesis (Bae *et al.*, 2004; De Muynck *et al.*, 2007; Ishida *et al.*, 2003).

1.2.3.5. Plant growth promotion

Biological nitrogen fixation (BNF), is the enzymatic reduction of atmospheric dinitrogen (N_2) to ammonia, catalyzed by nitrogenase. Bacteria capable of BNF may, under certain conditions, improve growth and yield of crops, and may be used to avoid or reduce the use of N-fertilizers, which are widely applied to improve yield of agriculturally important crops. Several studies have revealed that endophytic diazotrophs have a higher BNF contribution potential than rhizosphere diazotrophs (review: Baldani & Baldani, 2005).

Nitrogen-fixing AAB have been found in association with various plants and they have been classified in several novel species. The first nitrogen-fixing AAB were isolated from inside tissues of sugarcane and described as *A. diazotrophicus* (Cavalcante & Döbereiner, 1988; Gillis *et al.*, 1989), later renamed *G. diazotrophicus* (Yamada *et al.*, 1997). *G. azotocaptans* and *G. johannae,* were the names given to nitrogen-fixing AAB isolated from the rhizosphere and rhizoplane of coffee plants together with *G. diazotrophicus*, but different in several characteristics (Fuentes-Ramírez *et al.*, 2001; Jiménez-Salgado *et al.*,1997). A salt-tolerant nitrogen-fixing AAB, isolated from rhizosphere, roots and stems of mangrove-associated wild rice, was named *Sw. salitolerans* (Loganathan & Nair., 2004). *A. nitrogenifigens* and *G. kombuchae* were described for nitrogen-fixing AAB isolated from kombucha tea. The latter species is capable to produce cellulose and clusters phylogenetically with the "cellulose-producing" *Gluconacetobacter* species (see 1.5) instead of with the "nitrogen-fixing" species of this genus (Dutta & Gachhui, 2006, 2007). The first report on the presence of

nitrogen-fixing property in the genus *Acetobacter* was made by Muthumarasamy *et al*. (2005), who isolated *A. peroxydans* capable of fixing nitrogen, from roots and stems of cultivated wetland rice.

Since the first report of *G. diazotrophicus* as an endophyte of sugarcane (Cavalcante & Döbereiner, 1988), it has been detected as endophyte and in the rhizosphere of other types of plants such as sweet potato, cameroon grass, finger millet, coffee plants, tea plants, ragi, rice, pineapple, mango, banana, carrot, radish and beetroot, as well as in the rhizosphere from sugarcane. *G. diazotrophicus* was apart from this also isolated from mycorriza spore fungi and the sugarcane mealy bug, *Saccharococcus sacchari*. *G. sacchari*, another *Gluconacetobacter* species, isolated from the sugarcane mealy bug and endophytically associated with sugarcane, is unable to fix nitrogen. *G. azotocaptans* was recently isolated from the rhizosphere of corn (reviews: Baldani & Baldani, 2005; Pedraza, 2008; Saravanan *et al*., 2008).

G. diazotrophicus has been studied thoroughly, because its interaction with sugarcane is regarded as a model for the association between an endophytic nitrogen-fixing prokaryote and a monocot (Baldani & Baldani, 2005; Lee *et al.*, 2000, Madhaiyan *et al.*, 2004; Pedraza, 2008). Although their interaction is not fully understood, different reports indicate that this diazotroph is able to promote plant growth through several mechanisms. Besides nitrogen-fixation, phytohormone production, solubilization of mineral nutrients like P and Zn, and biocontrol, have been reported (Madhaiyan *et al.*, 2004; reviews: Pedraza, 2008; Saravanan *et al.*, 2008).

The genes involved in nitrogen-fixation by *G. diazotrophicus* are located on the chromosome in a cluster called the *nif-fix* cluster. Lee *et al*. (2000) sequenced and analyzed such cluster and observed that the overall arrangement of the genes resembled that of the *nif-fix* cluster of *Azospirillum brasilense*, whereas the individual gene products were more similar to those in species of the *Rhizobiacaea* or in *Rhodobacter capsulatus* (Kersters *et al.*, 2006; Lee *et al.*, 2000; Pedraza, 2008). The cluster was 30.5 kb in size, encoded for 32 proteins and represented the largest assembly of contiguous *nif-fix* and associated genes so far characterized in any diazotrophic prokaryote (Lee *et al.*, 2000). Studies on the genetic diversity of *G. diazotrophicus* revealed a rather limited diversity (Baldani & Baldani, 2005; Caballero-Mellado & Martínez-Romero, 1994; Caballero-Mellado *et al.*, 1995; Jiménez-Salgado *et al.*, 1997; Pedraza, 2008). Recently, a genome sequencing project has been initiated for the type strain of *G. diazotrophicus* Pal 5^T, as well as a program for investigation of sugarcane gene expression during the association with the diazotroph. The latter study aims to verify if the plant plays an active role in the association. It is expected that both programs will contribute

significantly to a better understanding of the bacteria/plant association (Baldani & Baldani, 2005; Pedraza, 2008).

Although studies showed that *G. diazotrophicus* is a promising biofertilizer for sugarcane and perhaps other agriculturally important crops, recent data suggest that it is premature to use it for this purpose. Several studies have revealed that nitrogen-fixation by these endophytes depends on the bacterial strain used as well as the plant (species, cultivar, age, etc.), environmental conditions and nutritional status of the soil. Additionally, different reports indicate that this diazotroph is able to promote plant growth through several mechanisms besides nitrogen-fixation (see above). More research is therefore recommended (reviews: Baldani & Baldani, 2005; Pedraza, 2008).

1.2.4. Concerns

Besides their beneficial use, AAB also account for less desirable activities. AAB have been reported to spoil beverages, such as beer and wine (see 1.2.4.1), to cause bacterial rot in apples and pears and pink disease in pineapples (see 1.2.4.2). Recently, *As. bogorensis* and *Gr. bethesdensis* have been reported as human pathogens (see 1.2.4.3).

1.2.4.1. Spoilage of beverages

AAB have been mentioned to cause spoilage to many kinds of beverages such as wine, cider, beer, tequila, fruit juices, soft drinks and fruit-flavoured water. The infection of these beverages by AAB may lead to symptoms such as acidification, formation of a film, formation of clouds or precipitates, off-flavours, colour changes and ropiness (Kersters *et al.*, 2006).

AAB are part of the natural microbiota of grapes and wine, but their presence is not appreciated by wine makers as they can increase the concentrations of acetic acid and other metabolites in wine to levels that are considered undesirable. To avoid wine spoilage by AAB, their population has to be kept to a minimum across the whole process. Studies on population dynamics of AAB during wine making revealed that in grape must *Gl.* oxydans is mainly found, whereas during wine fermentations *A. aceti*, *A. pasteurianus*, *G. liquefaciens* and *G. hansenii* are most frequently isolated (Bartowsky & Henschke, 2008; Bartowsky *et al*, 2003; Du Toit & Lambrechts, 2002; González *et al.*, 2004, 2005; Joyeux *et al*., 1984; Ruiz *et al*., 2000). Other AAB species that have been isolated from grapes or wine are *A. cerevisiae*, *A. oeni* and *A. tropicalis* (Prieto *et*

al., 2007; Silhavy & Mandl, 2006; Silva *et al.*, 2006). Spoilage of wine by AAB after packaging has been reported in bottles sealed with natural cork closures and stored in the vertical upright position, enabling oxygen to ingress into the bottle (Bartwosky *et al.*, 2003).

AAB have been known as beer spoilers for many years. Nowadays infections of beer by AAB are reduced by modern sanitation practice, by the increased used of stainless steel and by the controlled use of anaerobic conditions (Kersters *et al.*, 2006). AAB can still cause problems in cask beer production. They are picked up at the end of the production process and will develop in the cask after air has entered to replace the beer drawn from it. The major beer spoiling AAB are *G. xylinus*, *A. pasteurianus* and *Gl. oxydans*. *Acetobacte*r is part of the normal microbiota of lambic beer (a Belgian beer obtained by spontaneous fermentation) (Kersters *et al.*, 2006).

Fruit juices and related beverages are very selective media, allowing only certain acid-tolerant microorganisms to develop. *Gluconobacter* is considered to be a typical spoiler of soft drinks (Kersters *et al.*, 2006; Sievers & Swings, 2005). An *Asaia* isolate and *Gl. sacchari* were recently reported as spoilers of fruit-flavoured bottled water. It may be mentioned here that the *Asaia* isolate from fruit-flavoured bottled water could not be identified using phenotypic methods generally used in food laboratories and had a natural resistance to several medically important antibiotics, particularly those employed against the gram-negative microorganisms. The source of the contamination with *Asaia* was suggested to be the natural fruit juices that were used for the flavouring of the water, whereas the contamination with *Gl. sacchari* probably originated from the sugar syrup used in the manufacture of the drink (Moore *et al.*, 2002a, b).

1.2.4.2. Bacterial rot in apples and pears and pink disease of pineapples

AAB have been reported to cause bacterial rot of apples and peers, which is characterized by different shades of browning and by tissue degradation. AAB that have been isolated from diseased apples and pears have been classified in the genera *Acetobacter* and *Gluconobacter* (De Ley *et al.*, 1984a; Gillis & De Ley, 1980; Kersters *et al.*, 2006; Sievers & Swings, 2005; Van Keer *et al.*, 1981). Bacterial brown rot could be induced by artificial inoculation, using an inoculum size as low as 100 cells (Van Keer *et al.*, 1981). Recently, *Gl. oxydans* was described as being able to degrade patulin, a mycotoxin that is considered of great concern in apples and apple products, to a less-toxic compound, ascladiol (Ricelli *et al.*, 2007).

AAB have also been reported as causative agent of pink disease in pineapples

(Gosselé & Swings, 1985). However, recent studies indicate that the real causative organism belongs to a novel species that is phylogenetically related to *Pantoea citrea* (Cha *et al.*, 1997; Pujol & Kado, 2000; recent results from a collaborative project with the University of Pretoria, SA on *Pantoea*). The disease itself is recognizable by the appearance of a pink to brown colour after heating the fruit (e.g. during canning), although the fruit has an almost normal appearance before heating.

1.2.4.3. AAB as (opportunistic) human pathogens

Several recent reports mention the involvement of AAB in clinical infections. Isolates assigned to *As. bogorensis* on the basis of partial 16S rRNA gene sequence analysis, were reported to cause bacteremia in 2 patients with a history of intravenous drug abuse (Tuuminen *et al*., 2006, 2007), and peritonitis in a patient with end-stage renal disease, who was on peritoneal dialysis for 5 years (Snyder *et al*., 2004). The isolates could not be identified with routine identification methods used in clinical laboratories. The sources of the infection remained unknown, although Tuuminen *et al.* (2006, 2007) speculated that in the case of the drug abusers some narcotic substances might had been contaminated with the organisms. In 2 patients infections occurred despite prophylactic antibiotic coverage (Snyder *et al.*, 2004; Tuuminen *et al.*, 2006). The isolate from the drug abuser with the long-term antibiotic treatment appeared extremely resistant to almost all antibiotics that are routinely used for gram-negative rods. The isolates from the two drug abusers were compared and appeared to be distinct strains (Tuuminen *et al.*, 2007). As mentioned above (1.2.1), strains of *As. bogorensis* are the most frequently isolated strains of this genus and *Asaia* has been detected as spoilage organism in fruit-flavoured bottled water (see 1.2.4.1; Moore *et al.,* 2002a). The above mentioned data indicate that *Asaia* strains might present for some people a health risk.

Gr. bethesdensis is a recently described species of a new genus of the family *Acetobacteraceae* that is reported as human pathogen. Indeed, strains of this species were recently isolated from patients with chronic granulomatous disease (CGD), a rare inherited disease characterized by an increased susceptibility to life-threatening infections with a narrow spectrum of catalase-producing bacteria (e.g. *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia complex*, *Nocardia* species) and fungi (*Aspergillus* species). Patients with CGD often present clinical symptoms such as pneumonia or lymphadenitis. The isolates could also in this case not be identified with the routine methods used in the clinical lab, and were tentatively allocated to the family

Acetobacteraceae on the basis of 16S rRNA gene sequence analysis. Specific immune response by high titer antibody, ability to cause similar disease in CGD but not in wild type mice and recovery of the introduced isolates from pathogenic lesions in these mice proofed them to be genuine CGD pathogens. *Gl. oxydans* 621H, on the other hand, caused little pathology in the CGD mouse model, suggesting that the virulence factors in *Gr. bethesdensis* are not likely to be widespread in the *Acetobacteraceae* family. The niche from which the strains infected the patients is also unknown. Further, it was noted that the isolates showed a high level of resistance to almost all classes of antibiotics and treatment failed with the two antibiotics to which the bacteria were transiently susceptible upon isolation (Fredricks & Ramakrishnan, 2006; Greenberg *et al*., 2006a, b). Recently, the complete genome sequence of *Gr. bethesdensis* CGD NIH1^T was determined in order to obtain insight into the mechanisms that confer its pathogenicity (Greenberg *et al.*, 2007). Strain CGD NIH1^T contains a circular chromosome of 2708 kb and no plasmids. There are 2437 putative ORFs (of which 1470 share sequence similarity with ORFs of the genome of *Gl. oxydans* 621H), 3 copies of rRNA operons and 52 genes encoding tRNA. *Gr. bethesdensis* contains ORFs potentially important for virulence, adherence, DNA uptake, and methanol utilization that are not present in the genome of *Gl. oxydans*. % GC values and best BLAST analysis suggested that some of these unique ORFs were recently acquired. Comparison of the genome of *Gr. bethesdensis* to published genomes of other CGD pathogens demonstrated conservation of some putative virulence factors, suggesting possible common mechanisms involved in pathogenesis in CGD. The data further suggested that virulence related-ORFs appeared in some cases to be acquired through horizontal transfer. Genotyping of *Gr. bethesdensis* isolates from different patients using a custom micro-array revealed that this taxon is genetically diverse. Further study of *Granulibacter* genomics will shed light on what makes an acetic acid bacterium pathogenic and on which characteristics are important for pathogenicity in CGD (Greenberg *et al.*, 2007).

Acetobacter, *Gluconacetobacter* and *Gluconobacter* are not known to have any pathogenic effect towards man or animals (De Ley *et al.*, 1984a; Sievers & Swings, 2005).

1.3. THE BASIC CONCEPTS OF BACTERIAL TAXONOMY

Taxonomy is an essential discipline in biology as it provides a reference system for biological knowledge. For prokaryotes, it essentially comprises: (i) classification, i.e. the ordering of microorganisms into groups or taxa using a polyphasic approach that

combines phylogenetic information, genotypic data and phenotypic data (biochemical, physiological and morphological characteristics) (ii) nomenclature, i.e. the labelling of individual groups in the framework with a binomial name according to strict rules, and (iii) identification, i.e. the act of determining whether an unknown organism belongs to a particular group in a previously made classification (Gevers *et al.*, 2006; Goodfellow, 2000; Ludwig, 2007; Vandamme *et al*., 1996). In 1987, it was agreed that the complete DNA sequence should be the reference standard to determine phylogeny and that phylogeny should determine taxonomy (Wayne *et al.*, 1987).

In modern bacterial taxonomy, a range of techniques to determine both phenotypic and genotypic characteristics are available, but whole genome DNA-DNA hybridization and 16S rRNA gene sequencing play a central role (reviews: Gevers *et al.*, 2006; Ludwig, 2007; Sohier *et al*., 2008; Vandamme *et al*., 1996).

Whole genome DNA-DNA hybridization is the reference technique for species delineation; with a bacterial species (the basic taxonomic unit) being defined as a group of strains with approximately 70 % or more DNA-DNA relatedness, with 5 °C or less difference in their melting temperature (ΔT_m) and a certain degree of phenotypic consistency (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987). When the bacterial species was defined, this technique was concluded to be the best approach for measuring overall sequence similarity between two entire genomes. Recently, it was shown that DNA-DNA hybridization data are indeed consistent with data obtained from whole genome sequences (Coenye & Vandamme, 2004; Goris *et al*., 2007). However, DNA-DNA hybridization has certain important drawbacks e.g. it is time-consuming and variability in the results has been noticed between different laboratories and methodologies (Saddler, 2005; Stackebrandt *et al*., 2002). Moreover, overall DNA-DNA hybridization values of 50 % or less have limited informative value and therefore DNA-DNA hybridizations are not suitable for the estimation of genetic distances between distantly related species (Martens *et al*., 2008). For these reasons, bacterial taxonomists are encouraged to look for alternative, equivalent or superior methods (Gevers *et al*., 2006; Stackebrandt *et al*., 2002).

Comparitive analysis of 16S rRNA gene sequences is also extensively used. It has been shown valuable for measuring the degree of relatedness between prokaryotes, and phylogenetic trees derived from their sequences have become the backbone of modern bacterial systematics (Vandamme *et al*., 1996; Woese, 1987). In contrast to DNA-DNA hybridization, this technique is suitable to estimate the degree of relatedness between prokaryotes above the species level (Stackebrandt & Goebel, 1994). At and below the species level, the technique is generally not appropriate because of its too low

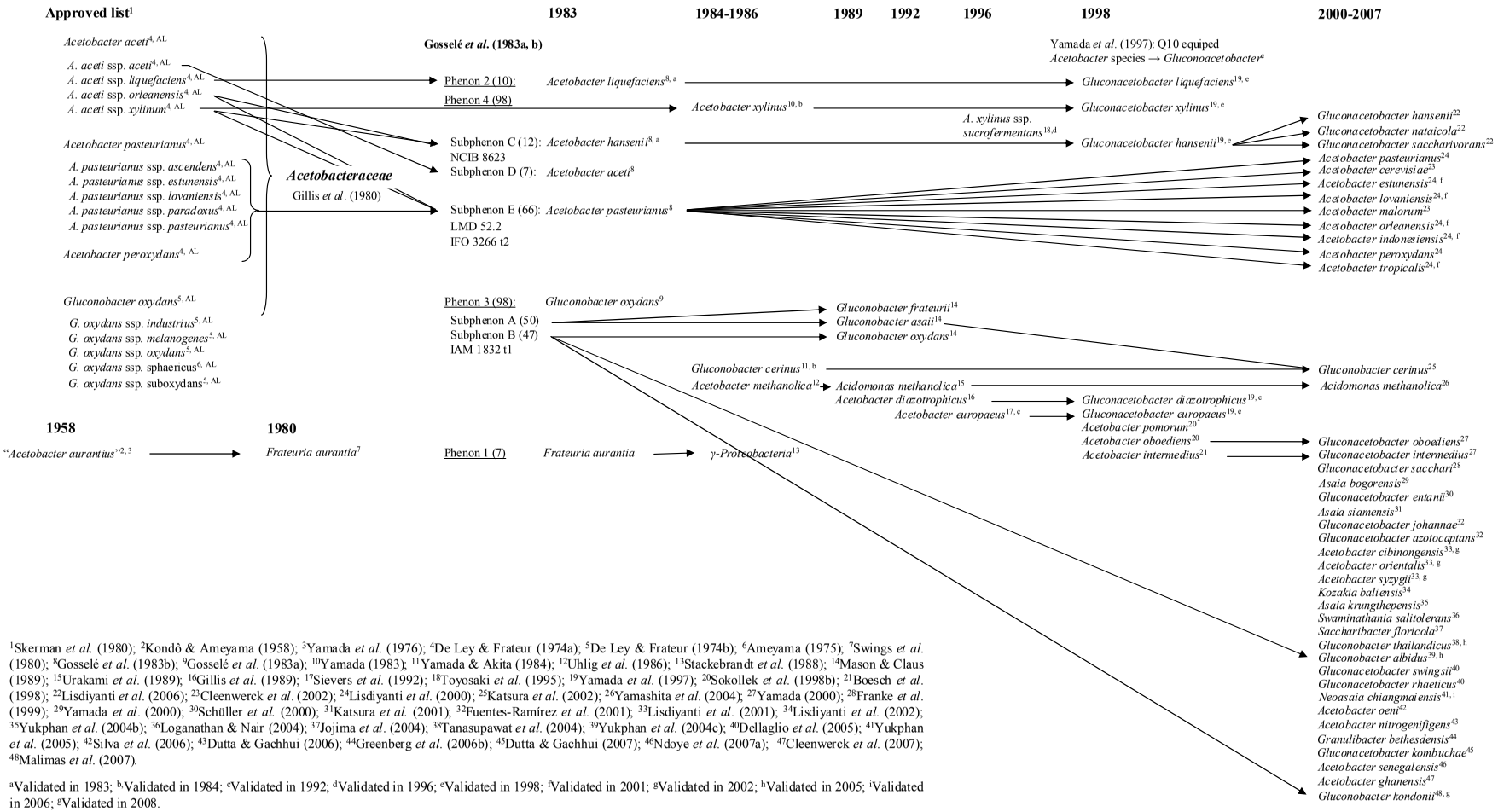
taxonomic resolution. It has been shown that strains with (nearly) identical 16S rRNA gene sequences may show DNA-DNA hybridization values significantly lower than 70 %; however, strains that show less than 97 % 16S r RNA gene sequence similarity (or 98.7-99 % according to more recent data; Stackebrandt & Ebers, 2006) are not likely to have more than 60 to 70 % DNA-DNA relatedness. Therefore, 16S rRNA gene sequence analysis can be used to decide whether additional genotypic tests are mandatory for classification at the species level (Stackebrandt & Goebel, 1994). A concern that has been raised about 16S rRNA trees is that they are single-gene trees which may not adequately reflect organismal phylogenetic relationships, for various reasons, such as possible horizontal gene transfer (HGT), variable mutation rates and variable rates of recombination.

A novel approach that has recently been put forward to investigate phylogenetic relationships is multilocus sequence analysis (MLSA) of housekeeping genes. MLSA has been reported to be suitable for species delineation and for investigation of relationships at the intraspecies level. In addition, it has been mentioned to surpass DNA-DNA hybridization by its ability to give information on interspecies relationships and by providing clear species/genomovar bounderies (Martens *et al*., 2008). However, development of a universal MLSA approach (as for 16S rRNA gene sequencing) is most probably not possible, and it is expected that taxon-specific approaches will be necessary (Coenye *et al*., 2005).

1.4. TAXONOMY OF AAB

The history of the taxonomy of AAB is quite long and dates back to 1898 when *Acetobacter* was introduced by Beijerinck, while *Gluconobacter* was proposed by Asai in 1935. The early classifications of these bacteria were based mainly on morphological, physiological and biochemical characteristics. For example, in the eight edition of Bergey's Manual of Determinative Bacteriology, the AAB consisted of the genera *Acetobacter* and *Gluconobacter* (De Ley & Frateur, 1974a, b), according to the capacity to oxidize acetate and lactate to CO_2 and H_2O (*Acetobacter*, +; *Gluconobacter*, -) and the flagellation pattern (*Acetobacter*, peritrichous; *Gluconobacter*, polar). The genus *Acetobacter* comprised 3 species and 9 subspecies, *A. aceti* (with 4 subspecies: *A. aceti* subsp. *aceti, A. aceti* subsp. *liquefaciens*, *A. aceti* subsp. *xylinum*, *A. aceti* subsp. *orleanensis*), *A. pasteurianus* (with 5 subspecies: *A. pasteurianus* subsp. *pasteurianus*, *A. pasteurianus* subsp. *estunensis*, *A. pasteurianus* subsp. *paradoxus*, *A. pasteurianus* subsp. *ascendens*, *A. pasteurianus* subsp. *lovaniensis*) and *A. peroxydans*. These species

Fig 1.2. Overview of the nomenclatural changes of the acetic acid bacteria since the publication of the Approved Lists of Bacterial Names (Skerman et al., 1980).



[1]Skerman *et al.* (1980); [2]Kondô & Ameyama (1958); [3]Yamada *et al.* (1976); [4]De Ley & Frateur (1974a); [5]De Ley & Frateur (1974b); [6]Ameyama (1975); [7]Swings *et al.* (1980); [8]Gosselé *et al.* (1983b); [9]Gosselé *et al.* (1983a); [10]Yamada (1983); [11]Yamada & Akita (1984); [12]Uhlig *et al.* (1986); [13]Stackebrandt *et al.* (1988); [14]Mason & Claus (1989); [15]Urakami *et al.* (1989); [16]Gillis *et al.* (1989); [17]Sievers *et al.* (1992); [18]Toyosaki *et al.* (1995); [19]Yamada *et al.* (1997); [20]Sokollek *et al.* (1998b); [21]Boesch *et al.* (1998); [22]Lisdiyanti *et al.* (2006); [23]Cleenwerck *et al.* (2002); [24]Lisdiyanti *et al.* (2000); [25]Katsura *et al.* (2002); [26]Yamashita *et al.* (2004); [27]Yamada (2000); [28]Franke *et al.* (1999); [29]Yamada *et al.* (2000); [30]Schüller *et al.* (2000); [31]Katsura *et al.* (2001); [32]Fuentes-Ramírez *et al.* (2001); [33]Lisdiyanti *et al.* (2001); [34]Lisdiyanti *et al.* (2002); [35]Yukphan *et al.* (2004b); [36]Loganathan & Nair (2004); [37]Jojima *et al.* (2004); [38]Tanasupawat *et al.* (2004); [39]Yukphan *et al.* (2004c); [40]Dellaglio *et al.* (2005); [41]Yukphan *et al.* (2005); [42]Silva *et al.* (2006); [43]Dutta & Gachhui (2006); [44]Greenberg *et al.* (2006b); [45]Dutta & Gachhui (2007); [46]Ndoye *et al.* (2007a); [47]Cleenwerck *et al.* (2007); [48]Malimas *et al.* (2007).

[a]Validated in 1983; [b]Validated in 1984; [c]Validated in 1992; [d]Validated in 1996; [e]Validated in 1998; [f]Validated in 2001; [g]Validated in 2002; [h]Validated in 2005; [i]Validated in 2006; [g]Validated in 2008.

and subspecies could be differentiated on the basis of nine phenotypic features namely catalase, ketogenesis, formation of D-gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose, growth on Hoyer-Frateur medium containing 3 % ethanol and formation of cellulose, γ-pyrones and coffee-brown water soluble pigment (De Ley & Frateur, 1974a, b; Gosselé *et al.*, 1983b). The genus *Gluconobacter* contained one single species, *Gl. oxydans*, with 4 subspecies (*Gl. oxydans* subsp. *oxydans*, *Gl. oxydans* subsp. *industrius*, *Gl. oxydans* subsp. *suboxydans*, *Gl. oxydans* subsp. *melanogenes*). All names of *Acetobacter* and *Gluconobacter* species and subspecies mentioned in the Bergey's Manual (De Ley & Frateur, 1974a, b) appeared on the Approved Lists of Bacterial Names (Skerman *et al*., 1980), together with the name of a fifth subspecies of *Gluconobacter* (*Gl. oxydans* subsp. *sphaericus*) described by Ameyama (1975) (Fig. 1.2).

The last 30 years, the taxonomy of AAB has undergone many changes in accordance with the development and application of new technologies. Gillis & De Ley (1980) proposed to unite the genera *Acetobacter* and *Gluconobacter* in the family *Acetobacteraceae* on the basis of rRNA-DNA hybridization data supported by phenotypic data. The two genera showed high rRNA cistron similarity and formed a separate branch in a rRNA superfamily which was later shown to belong to the α-subdivision of the *Proteobacteria* (Stackebrandt *et al.*, 1988). With the introduction of computer technology, objective comparison of large sets of characteristics for large numbers of strains became possible and researchers expected that numerical analyses of phenotypic characteristics would yield superior classifications. Gosselé *et al.* (1983a, b) examined a wide variety of *Acetobacter* and *Gluconobacter* strains (> 200) representing all at that time validly described species and subspecies by numerical analyses of 177 phenotypic features and by polyacrylamide gel electrophoresis of their soluble proteins. Their conclusion was that the above-mentioned subspecies should be ruled out and several classificatory changes were proposed. *Gl. oxydans* (phenon 3) remained the only species of the genus *Gluconobacter*, but without the subspecies recognition (Gosselé *et al.*, 1983a). Numerical analysis revealed although two distinct groups, but the authors decided that too few differences were in favour of a subspecies classification. Within *Acetobacter*, *A. aceti* (subphenon D) and *A. pasteurianus* (subphenon E) were given an emended description. Further, two new species were proposed: *A. liquefaciens* sp. nov. (phenon 2), that contained all *A. aceti* subsp. *liquefaciens* strains and *A. hansenii* sp. nov. (subphenon C), that contained several *A. aceti* subsp. *xylinum* strains, three *A. aceti* subsp. *orleanensis* and two *A. pasteurianus* subsp. *pasteurianus* strains (Gosselé *et al.*, 1983b). In the same year, Yamada (1983) elevated *A. aceti* subsp. *xylinum* to the species

level on the basis of its ubiquinone system comprising Q-10.

Mid eighties, the first DNA-DNA relatedness studies with AAB strains were published by Yamada *et al.* (1984) and Micales *et al.* (1985). Both authors separately re-investigated the genus *Gluconobacter* by DNA-DNA hybridizations. Their results revealed the existence of at least 3 genospecies within *Gluconobacter* and led to the description of three novel species (*Gl. cerinus*, *Gl. frateurii* and *Gl. asaii*) and an emended description of *Gl. oxydans* (Mason & Claus, 1989; Yamada & Akita, 1984). At the end of the eighties, Gillis *et al.* (1989) were the first to describe a novel species of the *Acetobacteraceae* based on a polyphasic approach, combining phenotypic and genotypic data, namely *A. diazotrophicus*. Shortly later, the first phylogenetic studies of AAB based on 16S rRNA gene sequences were published and showed that these organisms formed a cluster within the *Alphaproteobacteria* (Sievers *et al.*, 1994a, b, 1995) confirming hereby the findings of Gillis & De Ley (1980). A major change in the classification of the AAB was introduced when Yamada *et al.* (1997, 1998) proposed the genus *Gluconacetobacter* on the basis of partial 16S rRNA sequences and chemotaxonomic comparisons of ubiquinone systems. As a result, the Q-10 containing *Acetobacter* species, *A. xylinus* (Yamada, 1983), *A. liquefaciens* (Gosselé *et al.*, 1983b), *A. hansenii* (Gosselé *et al.*, 1983b), *A. diazotrophicus* (Gillis *et al.*, 1989) and *A. europaeus* (Sievers *et al.*, 1992) were transferred to the genus *Gluconacetobacter*.

Since 1998, polyphasic taxonomic studies of AAB from novel niches and from culture collections, led to the description of several novel genera, novel species and new combinations. At present, AAB are classified into 10 genera and 49 species (Bacterial Nomenclature Up-to-date, DSMZ, May 2008; Table 1.1). Fig. 1.2 gives an overview of the nomenclatural changes of AAB since the publication of the Approved Lists of Bacterial Names (Skerman *et al*., 1980).

1.5. PHYLOGENY OF AAB BASED ON 16S RRNA GENE SEQUENCES

AAB belong phylogenetically to the *Alphaproteobacteria*, where they form a distinct line of descent together with the related genera *Acidicaldus*, *Acidiphilium*, *Acidisphaera*, *Acidocella*, *Belnapia*, *Craurococcus*, *Muricoccus*, *Paracraurococcus*, *Rhodopila*, *Rhodovarius*, *Roseococcus*, *Roseomonas*, *Rubritepida* and *Teichococcus* (Fritz *et al*., 2004; Johnson *et al*., 2006; Kämpfer *et al.*, 2004; Kersters *et al.*, 2006; Lee *et al*., 2005; Meyer, 2005; Reddy *et al*., 2006; Sievers & Swings, 2005). The *Acetobacteraceae* constitute on this branch a subcluster that is supported by high bootstrap values (Lee *et al.*, 2005). Phylogenetic trees based on almost

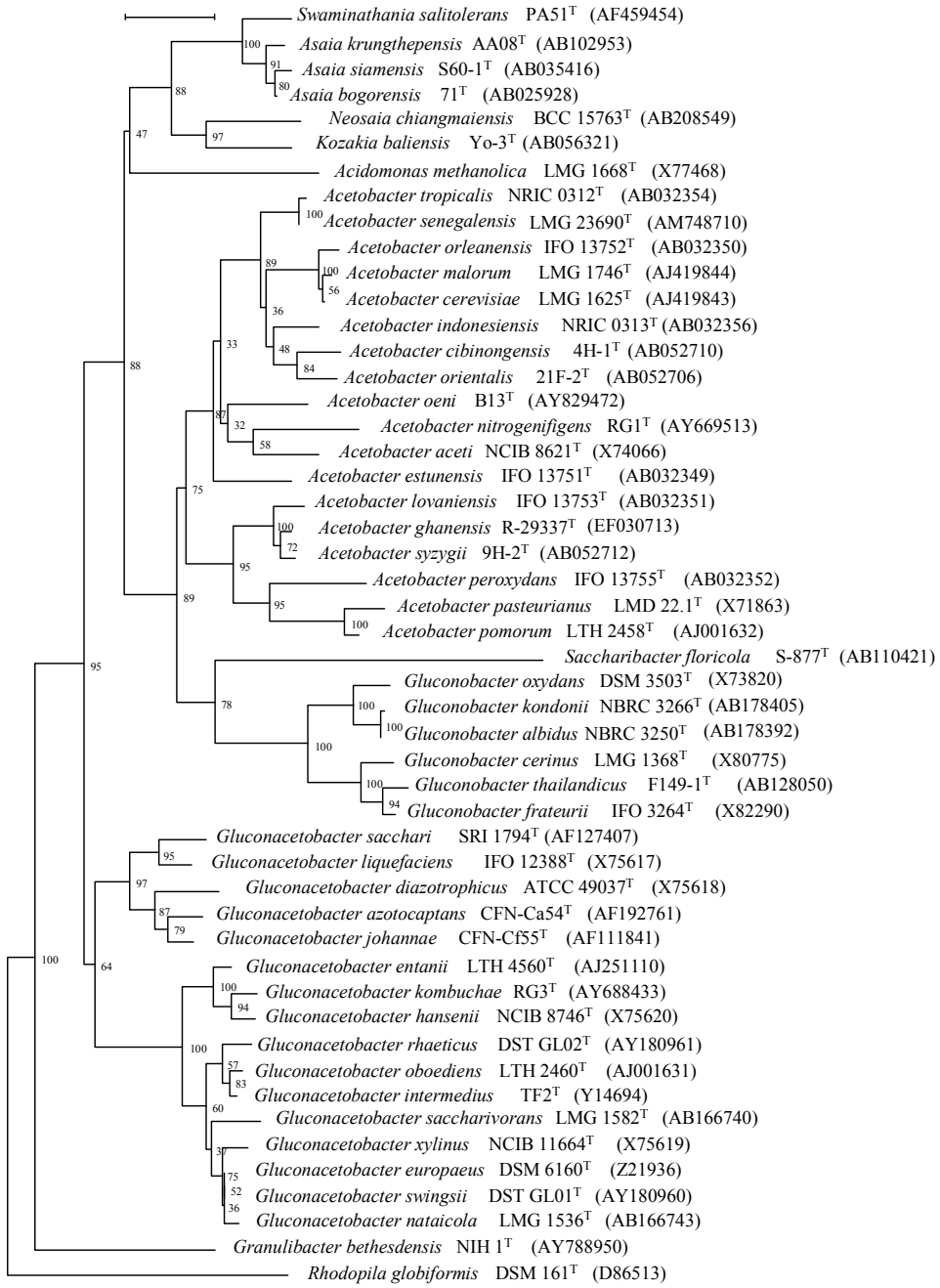


Fig. 1.3. Phylogenetic relationships of the *Acetobacteraceae* based on nearly complete 16S rRNA gene sequences of the type strains of all recognized species of this family. *Rhodopila globiformis* DSM 161^T was used as outgroup. The phylogenetic tree was constructed with the BioNumerics 4.61 software package (Applied Maths, Belgium) using the neighbour-joining method (Saitou & Nei, 1987). The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. Bar indicates 1% estimated sequence divergence.

complete 16S rRNA gene sequences reflecting the distant relationships of the AAB among members of the *Alphaproteobacteria* were recently constructed by Kersters *et al.* (2006), Lee *et al*. (2005) Greenberg *et al.* (2006b) and Sievers & Swings (2005),. A phylogenetic tree based on almost complete 16S rRNA gene sequences reflecting the distant relationships of all validly described species of the family is shown in Fig. 1.3.

The overall 16S rRNA gene sequence similarity between the type strains of the type species of the 10 genera of AAB ranges from 92.1 to 99.0 %, the latter found between *Asaia* and *Swaminathania* (Greenberg *et al.*, 2006b; Loganathan & Nair, 2004). Within the genera consisting of multiple species it ranges as follows: for *Acetobacter* from 95.4 to 99.9 %, for *Asaia* from 99.6 to 99.8 %, for *Gluconacetobacter* from 96.4 to 100 % and for *Gluconobacter* from 98.3 to 99.6 % (Cleenwerck *et al.*, 2002; Dellaglio *et al.*, 2005; Dutta & Gachhui, 2006; Katsura *et al.*, 2001; Lisdiyanti *et al.*, 2001, 2006; Silva *et al.*, 2006; Tanasupawat *et al.*, 2004; Yukphan *et al.*, 2004b, c). Within the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* two subclusters are noticed (Cleenwerck *et al.*, 2002; Dellaglio *et al*., 2005; Dutta & Gachhui, 2006; Lisdiyanti *et al.*, 2001, 2006; Silva *et al.*, 2006; Yamada & Yukphan, 2008). Several species are phylogenetically very closely related, sharing ≥ 99.5 % 16S rRNA gene sequence similarity i.e. *A. cerevisiae*, *A. malorum* and *A. orleanensis*; *A. tropicalis* and *A. senegalensis*; *A. ghanensis*, *A. syzygii* and *A. lovaniensis*; *As. bogorensis*, *As. krungthepensis* and *As. siamensis*; *G. intermedius*, *G. oboediens*, *G. rhaeticus*; *G. europaeus*, *G. swingsii*, *G. nataicola* and *G. xylinus*; *Gl. albidus* and *Gl. kondonii*; *Gl. frateurii* and *Gl. thailandicus*.

1.6. IDENTIFICATION OF AAB

Multiple studies have shown that identification of AAB is often difficult (Cleenwerck & De Vos, 2008; Greenberg *et al.*, 2006b; Kersters *et al.*, 2006; Moore *et al.*, 2002; Tuuminen *et al.*, 2006). While identification to the genus level can generally be achieved by comparitive 16S rRNA gene sequence analysis and/or some phenotypic tests (Cleenwerck & De Vos, 2008; Kersters *et al*., 2006; Yamada & Yukphan, 2008; Table 1.2), identification at the species level can be problematic (Cleenwerck & De Vos, 2008; Kersters *et al*., 2006). Species identifications based on phenotypic data alone are not recommended for several reasons. First, for some species, such as *A. tropicalis*, *A. indonesiensis* and *A. orleanensis*, phenotypic differential characteristics have not been found. Second, some strains (e.g. strains involved in industrial vinegar fermentations and belonging to *G. europaeus* or *G. entanii*) are difficult to cultivate,

which makes it very hard or even impossible to subject them to classical phenotypic tests (Bartowsky *et al*., 2003; Boesch *et al*., 1998; Schüller *et al*., 2000; Sievers *et al*., 1992; Sokollek *et al*., 1998a, b). Also, results of phenotypic tests are sometimes difficult to interprete (Cleenwerck & De Vos, 2008; Kersters *et al.,* 2006; Lisdiyanti *et al*., 2006; Yamashita *et al*., 2004). Contradictory data have been reported for example for the production of acids from sugars and sugar alcohols and it was mentioned that this might be due to slight differences in the procedures used, such as differences in employed media and detection methods (Yamashita *et al.*, 2004). Inclusion of positive and negative controls is often lacking, but must be regarded as a *conditio sine qua non* to enhance the reliability of the phenotypic analysis. Furthermore, spontaneous mutations often occur in AAB. Loss of acetic acid resistance and ethanol oxidizing ability, loss of ability to produce cellulose, as well loss of various other physiological properties have been reported for *Acetobacter* spp., *Gluconacetobacter* spp. and *Gluconobacter* spp. (De Muynck *et al.*, 2007; Iversen *et al.*, 1994; Kersters *et al.*, 2006; Kondô & Horinouchi, 1997; Navarro *et al*., 1999; Ohmori *et al.*, 1982; Sokollek *et al.*, 1998a, Yamada, 1983). These spontaneous mutations appeared in some cases to be due to the presence of insertion sequences (Coucheron, 1991; Iversen *et al.*, 1994). The genome of *Gl. oxydans* 621H contains 82 insertion sequences and 103 transposase genes, i.e. repeated DNA elements that are known to be involved in genomic rearrangements (Prust *et al*., 2005). For all these reasons, several authors have advized to use genotypic data as basis for species identification of AAB.

In the last decade, researchers have therefore increasingly supplemented or replaced phenotypic tests by molecular DNA-based methods. DNA-DNA hybridizations and sequence analysis of 16S rRNA genes, 16S-23S rDNA spacer regions, and genes as *adhA* (encoding subunit I of PQQ dependent alcohol dehydrogenase) and *recA* (encoding the DNA repair protein RecA) have been applied, as well as DNA-based fingerprinting techniques like restriction fragment length polymorphism (RFLP) analysis of PCR amplified rDNA (mainly 16S rRNA genes and 16S-23S rDNA spacer regions), randomly amplified polymorphic DNA (RAPD) fingerprinting, repetitive sequence based PCR (rep-PCR) using REP (Repetitive Extragenic Palindromic sequences) and ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) primers, pulsed-field gel electrophoresis (PFGE) and plasmid profiling. Several species-specific and group-specific probes and PCR's have also been developed (reviewed by Cleenwerck & De Vos, 2008).

However, for many methods the taxonomic resolution is not unambiguously clear, because its evaluation has often been based on a set of strains containing insufficient

Table 1.2. Differential characteristics of the genera of the family *Acetobacteraceae*

Taxa are listed as: 1, *Gluconobacter*; 2, *Acetobacter*; 3, *Gluconacetobacter*; 4, *Acidomonas*; 5, *Asaia*; 6, *Kozakia*; 7, *Swaminathania*; 8, *Saccharibacter*; 9, *Neoasaia*; 10, *Granulibacter*. Unless indicated otherwise, data for taxa 1-3 and 5-6 were taken from Lisdiyanti *et al.* (2002), data for taxon 4 were taken from Yamashita *et al.* (2004), data for taxon 7 were taken from Loganathan & Nair (2004), data for taxa 8 was taken from Jojima *et al.* (2004), data for taxon 9 were taken from Yukphan *et al.* (2005), data for taxon 10 were taken from Greenberg *et al.* (2006b). Symbols: +: positive, -: negative, w: weak positive, v: variable, nd: not determined; nm: non-motile, pl: polar, pr: peritrichous

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Production of acetic acid	+	+	+	+	-	+	+	v (w/-)	+	v (w/-)
Oxidation of:										
acetate to CO_2 and H_2O	-	+	+	+	w	w	w	-	-	w
lactacte to CO_2 and H_2O	-	+	+	v (-/w)	w	w	w	w	-	+
Growth in the presence of 0.35% acetic acid (pH 3.5)	+	+	+	+	-	+	+	-	+	nd
Growth in the presence of 1% KNO_3[a]	-	-	-	+	-	-	+	nd	-	nd
Production of keto-D-gluconic acid from D-glucose:										
2,5-diketo-D-gluconic acid	v	-	v	-	-	-	nd	nd	nd	nd
5-keto-D-gluconic acid	+	v	v	-	+	+	nd	+	+	nd
2-keto-D-gluconic acid	+	v	v[b]	-	+	+	nd	+	+	nd
Production of hydoxyacetone from glycerol	+	v	v	-	v	+	+	-	w	-
Growth on methanol as carbon source	-	v[c]	-	+	-	-	-	-	-	+
Production of water soluble brown-pigment(s)	v	-	v	-[a]	-	-	+	-	-	nd
Production of γ-pyrones from:										
D-glucose	v	-	v	nd	-	-	nd	nd	nd	nd
D-fructose	+	-	-	nd	v	v	nd	nd	nd	nd
Acid production from:										
L-arabinose	+	v	v	+	+	+	+	+	+	nd
D-arabinose	+	-	-	v	+	v	nd	-	w	nd
D-xylose	+	v	v	+	+	+	v	+	+	-
L-rhamnose	-	-	-	-	v	-	-	-	w	nd
D-glucose	+	v	+	+	+	+	+	+	+	w
D-galactose	+	v	+	+	+	+	+	+	+	nd
D-mannose	+	v	v	+	+	+	+	+	+	nd
D-fructose	+	-	+	-	+	-	v	v	+	nd
L-sorbose	+	-	v	nd	+	-	nd	-	-	nd
Melibiose	+	-	-	v	+	+	nd	+	+	nd
sucrose	+	-	-	-	+	v	nd	+	+	-
raffinose	-	-	-	-	-	+	nd	-	+	nd
D-mannitol	+	-	v	-	v	-	-	+	w	-
D-sorbitol	+	-	-	-	v	-	+	-	+	-
dulcitol	-	-	-	-	v[g]	-	v	-	w	-
glycerol	+	-	+	+	+	+	+	-	+	v (w/-)
ethanol	+	+	+	+	-	+	+	-	+	+
Production of cellulose	-	-	v	-	-	-	nd	-	nd	nd
Production of levan-like mucous substance(s) from sucrose	-	v	-	-	-	+	nd	-	-	nd
Growth in the presence of 30 % D-glucose	-	v[d]	v	+	+	-	nd	+	+	nd
Motility and flagellation	nm or pl[e]	nm or pr	nm or pr	nm or pl	nm or pr	nm	pr	nm	nm	nm
Major ubiquinone	Q-10	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	nd
G+C content (mol%)[f]	54-64	52-64	56-67	62-63	59-61	56-57	57-60	52-53	63.1	59

[a]Data taken from Loganathan & Nair (2004), [b]Data taken from Dellaglio *et al*. (2005); [c]Contradictory reports were published for the growth on methanol (Cleenwerck *et al*., 2002; Gosselé *et al*., 1983b; Sokollek *et al*., 1998b; Uhlig *et al*., 1986; Urakami *et al*., 1989); [d]Data taken from Cleenwerck *et al*. (2002); [e]Data taken from Gosselé *et al*. (1983a); [f]Data taken Cleenwerck & De Vos (2008); [g]Data taken from Katsura *et al*. (2001).

representatives per taxon and lacking phylogenetic relatives. Nevertheless, it is likely that the resolution power of techniques based on 16S rRNA genes will not reach the species level, especially because high 16S rRNA gene sequence similarities are noticed between several species of the family (see 1.5). Accurate identification of novel AAB isolates is therefore often not easy and may require sequencing of the 16S rRNA gene as well as DNA-DNA hybridizations (Cleenwerck & De Vos, 2008; Kersters *et al.*, 2006).

SHORT OVERVIEW OF THE EXPERIMENTAL WORK

This work aimed at improving the classification of AAB on the basis of molecular techniques and at providing methods that enable fast and accurate species level identification and classification of these microorganisms.

The first experimental work of this thesis started in May 1998, when AAB were classified into four genera, and was performed **to improve the classification of** two of these genera, namely ***Acetobacter* and *Gluconacetobacter***. These genera contained at that time 7 species (10 by the end of 1998), of which 5 had been delineated mainly on the basis of phenotypic features, namely *A. aceti*, *A. pasteurianus*, *G. hansenii*, *G. liquefaciens* and *G. xylinus* (Gosselé *et al*., 1983b; Yamada, 1983), and it was not clear if they would fit the bacterial species as defined by Wayne *et al*. (1987). It was decided to perform DNA-DNA hybridizations, the technique recommended for species delineation, with 49 strains representing all *Acetobacter* and *Gluconacetobacter* species described at that time (Table 1.3 and 1.4). Of these 49 strains, 37 were classified within the phenotypically delineated species and 36 were enclosed in the study of Gosselé *et al*. (1983b), who investigated 98 strains of these genera by numerical analysis of 177 phenotypic features. The DNA-DNA hybridizations revealed 17 misclassified strains within the species delineated mainly on the basis of phenotypic tests, of which most within *A. pasteurianus*. Chapter 2 deals with the investigation of the phenotypically misclassified *Acetobacter* strains using a polyphasic taxonomic approach. The results of the DNA-DNA hybridizations with the 49 strains are spread over chapters 2, 3 (3.2) and 4 (4.1, 4.2).

In chapter 3, isolates from different sources were classified at the species level also using a polyphasic taxonomic approach. The classification of most of these isolates was time-consuming, generally because of the number of DNA-DNA hybridizations that needed to be performed.

At the end of 2004, when AAB were classified into 8 genera and 35 recognized species with several species being phylogenetically closely related (see 1.4 and 1.5), a new study was initiated with the aim **to find DNA-based molecular methods that enable fast and accurate species level identification and classification of a broad range of AAB**, in order to replace or at least reduce the amount of laborious DNA-DNA hybridizations. A literature study revealed that several genomic methods had been applied on AAB, but many studies were based on a limited number of strains and lacking phylogenetic relatives, and therefore the taxonomic value of these methods was

Table 1.3. List of 26 *Acetobacter* strains selected for the DNA-DNA hybridization study: 4 *A. aceti* strains, 21 *A. pasteurianus* strains and the type strain and only strain of *A. pomorum* according to their species designation at the time of selection

Strains are listed under their current species designations. LMG, BCCM/LMG Bacteria Collection, Ghent University, Belgium; NCIB, the National Collections of Industrial and Marine Biology, Aberdeen, U.K.; LMD, Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan.

Strain	Synonymous strain designation	Species designation at the time of selection	Isolator of the strain; year of isolation; geographical origin of the isolate; source from which the strain was isolated (if known)
A. aceti			
LMG 1496	LMG 24WR[a]	*A. aceti*	
LMG 1504[T]	NCIB 8621[Ta]	*A. aceti*	M. Arends; 1923; beech-wood shavings of vinegar plant
LMG 1535	LMG Ch31[a]	*A. aceti*	J. Frateur; Belgium; vinegar plant
***Acetobacter* sp.**			
LMG 1531	NCIB 8941[a]	*A. aceti*	the Netherlands; quick vinegar
A. cerevisiae			
LMG 1599	NCIB 6425[a]	*A. pasteurianus*	J. Tosic; United Kingdom; brewers' yeast and bee
LMG 1625[T]	NCIB 8894[Ta]	*A. pasteurianus*	Toronto, Canada; beer (ale) in storage
LMG 1682	C101[a]	*A. pasteurianus*	R. Gilliland; Ireland; beer
LMG 1699	MARTIN 2[a]	*A. pasteurianus*	P. Martin; 1977; United Kingdom; brewery
A. estunensis			
LMG 1572	LMG E[a]	*A. pasteurianus*	Bristol, United Kingdom; cider
LMG 1626[T]	NCIB 8935[Ta]; IFO 13751[Tc]	*A. pasteurianus*	Bristol, United Kingdom; cider
A. indonesiensis			
LMG 1588[c]	LMD 39.6[a,]	*A. pasteurianus*	
A. lovaniensis			
LMG 1617[T]	NCIB 8620[Ta]; IFO 13753[Tc]	*A. pasteurianus*	J. Frateur; 1929; Becquevoort, Belgium; sewage on soil
A. malorum			
LMG 1746[T]	LMG 76.10[a]	*A. pasteurianus*	J. Swings; 1976; Ghent, Belgium; rotting apple
A. orleanensis			
LMG 1583[T]	NCIB 8622[Ta]; IFO 13752[Tc]	*A. pasteurianus*	J. Frateur; 1927; Belgium; beer
A. pasteurianus			
LMG 1262[T]t1[b]	LMD 22.1[T] t1[a, b]	*A. pasteurianus*	M. Beijerinck; the Netherlands; beer
LMG 1555	NCIB 8163[a]	*A. pasteurianus*	
LMG 1629	A[a]	*A. pasteurianus*	J. Falcão de Morais; Recife, Brazil; fermented *Agave sisalina* juice
LMG 1630	EQ[a]	*A. pasteurianus*	J. Falcão de Morais; Recife, Brazil; sugar cane bagasse
LMG 1658	MM 80[a]	*A. pasteurianus*	M. Maung; Myanmar; toddy palm
LMG 1659	MM 73[a]	*A. pasteurianus*	M. Maung; Myanmar; toddy palm
LMG 1686	LMD 31.4[a]	*A. pasteurianus*	A. van Rossen; 1928; the Netherlands; vinegar from dry raisins
A. peroxydans			
LMG 1633	LMD 53.9[a]	*A. pasteurianus*	N. Wieringa-Wijsman; 1950; the Netherlands; ditch water
LMG 1635[T]	NCIB 8618[Ta] IFO 13755[Tc]	*A. pasteurianus*	A. Struyck; Delft, the Netherlands; ditch water
A. pomorum			
LMG 18848[T]	LTH 2458[T]	*A. pomorum*	S. Sokollek; Esslingen, Germany; submerged cider vinegar fermentation
A. tropicalis			
LMG 1663	592[a]	*A. pasteurianus*	United Kingdom; fermenting putrified meat
LMG 1754	LMG 79.18[a]	*A. pasteurianus*	R. Viane; 1979; Ivory Coast; fruit of *Ficus capensis*

[a]Strain number used by Gosselé *et al*. (1983b); [b]The type strain of *A. pasteurianus* shows two stable colony types, t1 and t2, that give identical protein profiles by SDS-PAGE; [c]Strain reclassified by Lisdiyanti *et al*. (2000)

Table 1.4. List of 23 *Gluconacetobacter* strains selected for the DNA-DNA hybridization study: 5 *G. diazotrophicus* strains, 2 *G. europaeus* strains, 3 *G. hansenii* strains, 3 *A. intermedius* strains, 4 *G. liquefaciens* strains, the type strain and only strain of *A. oboediens* and 5 *G. xylinus* strains according to their species designation at the time of selection

Strains are listed under their current or proposed (see 4.2) species designations. LMG, BCCM/LMG Bacteria Collection, Ghent University, Belgium; NCIB, the National Collections of Industrial and Marine Biology, Aberdeen, U.K.; LMD, Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands.

Strain	Synonymous strain designation	Species designation at the time of selection	Isolator of the strain; year of isolation; geographical origin of the isolate; source from which the strain was isolated (if known)
G. diazotrophicus			
LMG 7602	Pal 2	*G. diazotrophicus*	J. Döbereiner; Alagoas, Brazil; *Saccharum officinarum*, root
LMG 7603^{T}	Pal 5^{T}	*G. diazotrophicus*	J. Döbereiner; Alagoas, Brazil; *Saccharum officinarum*, root
LMG 7971	PP 4	*G. diazotrophicus*	J. Döbereiner; Pernambuco, Brazil; *Saccharum officinarum*, stem
LMG 8065	PR 4	*G. diazotrophicus*	J. Döbereiner; Rio de Janeiro, Brazil; *Saccharum officinarum*, stem.
LMG 8067	PR 20	*G. diazotrophicus*	J. Döbereiner; Rio de Janeiro Brazil, *Saccharum officinarum*, root.
G. europaeus			
LMG 1510$^{(4.2.)}$	NCIB 613[a]	*G. xylinus*	A. Jørgensen; Copenhagen, Denmark; vinegar
LMG 1521$^{(4.2.)}$	NCIB 7029[a]	*G. xylinus*	
LMG 18494	Trcek V3	*G. europaeus*	J. Trček; 1995; Ljubljana, Slovenia; red wine vinegar produced in submerged bioreactor
LMG 18890^{T}	DES 11^{T}	*G. europaeus*	Esslingen, Germany; submerged culture vinegar generator
G. hansenii			
LMG 1524	NCIB 8246[a]	*G. hansenii*	M. Aschner; Jerusalem, Israel; vinegar.
LMG 1527^{T}	NCIB 8746Ta	*G. hansenii*	
LMG 1528	NCIB 8747[a]	*G. hansenii*	
G. intermedius			
LMG 18909^{T}	TF2^{T}	*A. intermedius*[c]	Switzerland; commercially available tea fungus beverage (Kombucha)
G. liquefaciens			
LMG 1348	NCIB 9417[a]	*G. liquefaciens*	1935; Japan; fruit
LMG 1381^{T}	NCIB 9136Ta	*G. liquefaciens*	T. Asai; 1935; Japan; *Diospyros* sp., dried fruit
LMG 1509	LMD 53.1[a]	*G. liquefaciens*	N. Wieringa-Wijsman; 1950; canal water
LMG 1728	Cho 222[a]	*G. liquefaciens*	Hawaii, United States; *Ananas comosus*, pink diseased fruit
G. oboediens			
LMG 18849^{T}	LTH 2460^{T}	*A. oboediens*[c]	S. Sokollek; Esslingen, Germany; red wine vinegar fermentation
LMG 18907[b]	JK3	*A. intermedius*[c]	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor
LMG 18908[b]	JKD	*A. intermedius*[c]	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor
G. xylinus			
LMG 1515^{T}	NCIB 11664Ta	*G. xylinus*	G. Bertrand; Mountains ash berries
LMG 1518$^{(4.2.)}$	NCIB 5346[a]	*G. xylinus*	
LMG 18788	BPR 2001	*G. xylinus*	Tokyo, Japan; black cherry

[a]Strain number used by Gosselé *et al*. (1983b); [b]Strain for which DNA of good quality for DNA-DNA hybridizations could not be extracted; [c]The species *A. oboediens* and *A. intermedius* were phylogenetically belonging to the genus *Gluconacetobacter* and were later transferred to this genus by Yamada (2000).

not unambiguously clear (see 1.6, reviewed by Cleenwerck & De Vos, 2008). It was decided to evaluate two fingerprinting techniques, **rep-PCR and amplified fragment length polymorphism (AFLP®)** that had previously been proven useful for the above mentioned goal (Duim *et al*., 2001; Gevers *et al*., 2001; Huys *et al.*, 1996; Janssen *et al*., 1997; Rademaker *et al*., 2000; Švec *et al*., 2005) on a broad collection of AAB. For the rep-PCR technique, 64 reference strains including 31 type strains, and 132 isolates from Ghanaian, fermented cocoa beans were used for evaluation. For the AFLP® technique, 135 reference strains and 15 additional strains, representing all currently recognized species, were investigated. The results of these evaluations are presented in chapter 4 (4.1, 4.2). In chapter 5 $(GTG)_5$-PCR DNA fingerprinting data are used in the description of two novel species of the genus *Acetobacter*: *A. ghanensis* (5.1) and *A. fabarum* (5.2).

An overview of the chapters with experimental work is given below:

Chapter 2 deals with the re-examination of the genus *Acetobacter* with descriptions of two novel *Acetobacter* species, *A. cerevisiae* and *A. malorum*.

Chapter 3 deals with the identification and classification of AAB from different sources (rice, apple fruit juice, wine and mango fruit) using a polyphasic approach. In this chapter, four novel species are described, namely *G. swingsii* and *G. rhaeticus* (3.2), *A. oeni* (3.3) and *A. senegalensis* (3.4).

Chapter 4 describes the evaluation of the DNA fingerprinting techniques rep-PCR (4.1) and AFLP® (4.2) for fast and accurate species level identification and classification of AAB.

Chapter 5 shows the integration of $(GTG)_5$-PCR DNA fingerprinting data in the description of two novel *Acetobacter* species, *A. ghanensis* (5.1) and *A. fabarum* (5.2), both isolated from traditional heap fermentations of Ghanain cocoa beans.

PART II

EXPERIMENTAL WORK

CHAPTER 2

RE-EXAMINATION OF THE GENUS *ACETOBACTER*, WITH DESCRIPTIONS OF *ACETOBACTER CEREVISIAE* SP. NOV. AND *ACETOBACTER MALORUM* SP. NOV.

Redrafted from:
Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. & Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int J Syst Evol Microbiol* 52, 1551-1558.

SUMMARY

Thirty-four *Acetobacter* strains, representing *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Acetobacter peroxydans*, *Acetobacter lovaniensis*, *Acetobacter estunensis*, *Acetobacter orleanensis*, *Acetobacter indonesiensis* and *Acetobacter tropicalis*, were subjected to a polyphasic study that included DNA-DNA hybridizations, DNA base ratio determinations, 16S rRNA gene sequence analysis and phenotypic characterization. Two new species are proposed, *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. The type strains of these species are respectively LMG 1625^T (= DSM 14362^T = NCIB 8894^T = ATCC 23765^T) and LMG 1746^T (= DSM 14337^T).

INTRODUCTION

AAB are classified into the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas* and the recently described genus *Asaia* (Yamada *et al.*, 2000). These genera join phylogenetically into a broad rRNA cluster within the α-subclass of the *Proteobacteria*, the AAB lineage.

The genus *Acetobacter* is differentiated from the other genera by its Q-9 ubiquinone system and by the oxidation of acetate and lactate to CO_2 and H_2O (De Ley & Frateur,

1974a; Yamada *et al.*, 1997, 2000). At the time of writing, the genus consisted of nine species: *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Acetobacter peroxydans*, *Acetobacter lovaniensis*, *Acetobacter estunensis*, *Acetobacter orleanensis*, *Acetobacter indonesiensis* and *Acetobacter tropicalis*. These species were delineated mainly on the basis of DNA-DNA relatedness and phylogenetic relationships (Lisdiyanti *et al.*, 2000; Sokollek *et al.*, 1998b).

In the course of a larger study set up to improve the identification of *Acetobacteraceae*, 16 strains from the heterogeneous species *A. pasteurianus* as well as 17 reference strains representing all recognized *Acetobacter* species, were genotypically and phenotypically analysed. DNA homology data demonstrate that five strains, currently allocated to *A. pasteurianus*, could not be assigned to any known *Acetobacter* species. Four of these strains constituted a separate but homogeneous taxon for which the name *Acetobacter cerevisiae* sp. nov. is proposed. The name *Acetobacter malorum* sp. nov. is proposed for strain LMG 1746^{T}.

MATERIAL AND METHODS

Bacterial strains. Strains used in this study are listed in Table 2.1. They were checked for purity by plating on medium 13 from the *Catalogue of Cultures* of the BCCM^{TM}/LMG Bacteria Collection (Janssens *et al.*, 1998) containing 2.5 % D-mannitol, 0.5 % yeast extract, 0.3 % peptone and 1.5 % agar.

DNA isolation. Total DNA for the determination of the DNA base composition and for DNA-DNA hybridizations was prepared by the method of Wilson (1987), with minor modifications. Cells were washed with RS buffer (0.15 M NaCl, 10 mM EDTA, pH 8.0) and then suspended and lysed in a Tris/EDTA buffer (10 mM Tris with up to 200 mM EDTA, pH 8.0) containing RNaseA (Sigma), SDS (Serva) and proteinase K (Merck) to final concentrations of 400 µg ml^{-1}, 2 % (w/v) and 200 µg ml^{-1} respectively. NaCl (5 M stock solution) and CTAB/NaCl solution [10 % (w/v) hexadecyltrimethylammonium bromide in 0.7 M NaCl] were added to final concentrations of 1 M and 13.3 % (v/v) respectively. To obtain DNA solutions free of RNA, a second RNase treatment was performed. RNase A was added to the DNA solutions to a final concentration of 100 µg ml^{-1} and incubated at 37 °C for 1 h. Finally the degraded RNA was removed by chloroform extraction. The DNA was dissolved in 0.1 x SSC (0.15 M NaCl, 0.015 M citric acid, 0.4 M NaOH, pH 7.0) to obtain a

Table 2.1. List of strains studied

Strains are listed under their proposed species designations. LMG, BCCM/LMG Bacteria Collection, Ghent University, Belgium; NCIB, the National Collections of Industrial and Marine Biology, Aberdeen, U.K.; LMD, Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan.

Strain	Synonymous strain designation	Current species designation	Isolator of the strain; year of isolation; geographical origin of the isolate; source from which the strain was isolated (if known)
A. pasteurianus			
LMG 1555	NCIB 8163[a]	*A. pasteurianus*	
LMG 1686	LMD 31.4[a]	*A. pasteurianus*	A. van Rossen; 1928; the Netherlands; vinegar from dry raisins
LMG 1262[T]t1[b]	LMD 22.1[T] t1[a, b]	*A. pasteurianus*	M. Beijerinck; the Netherlands; beer
LMG 1630	EQ[a]	*A. pasteurianus*	J. Falcão de Morais; Recife, Brazil; sugar cane bagasse
LMG 1629	A[a]	*A. pasteurianus*	J. Falcão de Morais; Recife, Brazil; fermented *Agave sisalina* juice
LMG 1658	MM 80[a]	*A. pasteurianus*	M. Maung; Myanmar; toddy palm
LMG 1659	MM 73[a]	*A. pasteurianus*	M. Maung; Myanmar; toddy palm
A. pomorum			
LMG 18848[T]	LTH 2458[T]	*A. pomorum*	S. Sokollek; Esslingen, Germany; submerged cider vinegar fermentation
A. peroxydans			
LMG 1633	LMD 53.9[a]	*A. pasteurianus*	N. Wieringa-Wijsman; 1950; the Netherlands;, ditch water
LMG 1635[T]	NCIB 8618[T a]	*A. peroxydans*	A. Struyck; Delft, the Netherlands; ditch water
A. lovaniensis			
LMG 1617[T]	NCIB 8620[T a]	*A. lovaniensis*	J. Frateur; 1929; Becquevoort, Belgium; sewage on soil
A. estunensis			
LMG 1626[T]	NCIB 8935[T a]	*A. estunensis*	Bristol, United Kingdom; cider
LMG 1572	LMG E[a]	*A. pasteurianus*	Bristol, United Kingdom; cider
LMG 1580	LMD 50.6[a]	*A. estunensis*	1950; Leiden, the Netherlands; beer
A. aceti			
LMG 1531	NCIB 8941[a]	*A. aceti*	the Netherlands; quick vinegar
LMG 1535	LMG Ch31[a]	*A. aceti*	J. Frateur; Belgium; vinegar plant
LMG 1504[T]	NCIB 8621[T a]	*A. aceti*	M. Arends; 1923; beech-wood shavings of vinegar plant
LMG 1496	LMG 24WR[a]	*A. aceti*	
A. cerevisiae			
LMG 1625[T]	NCIB 8894[a]	*A. pasteurianus*	Toronto, Canada; beer (ale) in storage
LMG 1599	NCIB 6425[a]	*A. pasteurianus*	J. Tosic; United Kingdom; brewers' yeast and bee
LMG 1699	MARTIN 2[a]	*A. pasteurianus*	P. Martin; 1977; United Kingdom; brewery
LMG 1682	C101[a]	*A. pasteurianus*	R. Gilliland; Ireland; beer
A. malorum			
LMG 1746[T]	LMG 76.10[a]	*A. pasteurianus*	J. Swings; 1976; Ghent, Belgium; rotting apple
A. orleanensis			
LMG 1583[T]	NCIB 8622[T a]	*A. orleanensis*	J. Frateur; 1927; Belgium; beer
LMG 1592	NCIB 2224[a]	*A. orleanensis*	Manufacture of vinegar
LMG 1608	NCIB 8088[a]	*A. orleanensis*	M. de Monchy; 1932; Netherlands; beer
LMG 1545	IFO 3296[a]	*A. orleanensis*	K. Kondô; 1941; Aichi, Japan; film in fermentor of rice vinegar
A. indonesiensis			
LMG 19824[T]	NRIC 0313[T]	*A. indonesiensis*	Indonesia; fruit of zirzak (*Annona muricata*)
LMG 1588	LMD 39.6[a]	*A. indonesiensis*	
LMG 1571	LMD 39.2[a]	*A. indonesiensis*	
A. tropicalis			
LMG 19825[T]	NRIC 0312[T]	*A. tropicalis*	Indonesia; coconut juice
LMG 19826	NRIC 0321	*A. tropicalis*	Indonesia; lime
LMG 1754	LMG 79.18[a]	*A. pasteurianus*	R. Viane; 1979; Ivory Coast; fruit of *Ficus capensis*
LMG 1663	592[a]	*A. pasteurianus*	United Kingdom; fermenting putrified meat

[a] Strain number used by Gosselé *et al*. (1983b).

[b] The type strain of *A. pasteurianus* shows two stable colonial variations, t1 and t2, that give identical proteinprofiles by SDS-PAGE.

concentration of 0.3-0.8 mg ml^{-1}. DNA quantity and quality were determined by measuring the absorptions at 260, 280 and 234 nm. Only high-quality DNA with A_{260}/A_{280} and A_{234}/A_{260} ratios of 1.8-2.0 and 0.40-0.60 was selected for further use. The size of the DNA was checked by agarose gelelectrophoresis. Only high-molecular-mass DNA was used.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) and Goris *et al.* (1998). Briefly, biotinylated probe DNA was sheared by ultrasonication, denaturated and then hybridized with single stranded unlabeled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 50 °C in a hybridization solution containing 50 % formamide (2 x SSC, 5 x Denhardt's solution, 50 % formamide, 2.5 % dextran sulfate, low-molecular-mass denaturated salmon sperm DNA to a final concentration of 100 µg ml^{-1}, 1.25 µg biotinylated probe DNA ml^{-1}). After 3 h, the hybridization solution was removed and streptavidin / β-D-galactosidase (Sigma) was added to the wells. After 10 min incubation at 37 °C, the wells were washed and 4-methylumbelliferyl-β-D-galactoside (Sigma) was added. The fluorescence intensity was measured with a HTS7000 Bio Assay Reader (Applied Biosystems). Salmon sperm DNA (Sigma) was used as negative control in all experiments. The DNA percentages are means, based on at least two inderpendent hybridization experiments. Reciprocal reactions (e.g. A x B and B x A) were performed and also considered as independent hybridization experiments.

Determination of the DNA G+C content. The G+C content of DNA was determined by HPLC according to the method of Mesbah *et al.* (1989) using a Waters Symmetry Shield RP_8 column thermostated at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ with 1.5 % acetonitrile (pH 4.0). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

Sequencing of 16S rRNA genes. 16S rRNA genes were amplified using oligonucleotide primers complementary to highly conserved regions of these genes. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' (hybridizing at positions 8-27, according to the *Escherichia coli* numbering system), the reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3' (hybridizing at positions 1541-1522). PCR products were purified using a QIA quick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Purified PCR products were sequenced by using the

ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an Applied Biosystems 377 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The eight sequencing primers used are listed in Coenye *et al.* (1999a). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems).

Phylogenetic analysis. The 16S rRNA gene sequences determined and sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics 1.01 software package (Applied Maths). Unknown bases were discarded from the calculations. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data. The strain numbers, species names and accession numbers of 16S rRNA gene sequences retrieved from EMBL for use in the phylogenetic analysis are presented in Fig. 2.1.

Phenotypic characterization. Cell shape and cell size were determined of cells grown aerobically at 28 °C for 2-4 days on medium 13. For the type strain of *A. pomorum*, cell shape and cell size was also determined for cells grown aerobically at 28 °C for 2 days on RAE agar (Sokollek & Hammes, 1997). Gram staining was carried out by the method of Hucker & Conn (1923). Oxidase activity was tested using 1 % N,N,N',N'-tetramethyl *p*-phenylenediamine (Kovacs, 1956). Catalase activity was tested by adding young cells to a drop of a 10 % H_2O_2 solution and observing production of O_2. The production of 2- and 5-keto-D-gluconic acid was determined by the method described by Gosselé *et al.* (1980). The utilization of ammonium as the sole nitrogen source in the presence of ethanol as carbon source was tested using Frateur's modified Hoyer ethanol/vitamins medium (De Ley *et al.*, 1984b) containing 2.5 % agar. Growth was checked after 7 days incubation at 28 °C. Growth on the carbon sources glycerol, maltose and methanol was tested in basal medium (0.05 % yeast extract, 0.3 % vitamin-free Casamino acids, 2.5 % agar) with the carbon source to be tested added at a final concentration of 0.3 %. Medium without the carbon source was used as a control. Growth was evaluated after 7 days incubation at 28 °C. Growth in 30 % D-glucose was tested in a medium containing 0.5 % yeast extract and 30 % D-glucose. Growth was checked after 7 days incubation at 28 °C under stationary conditions. Growth with n-propanol as carbon source was examined as described by Sokollek *et al.* (1998b).

RESULTS AND DISCUSSION

DNA-DNA relatedness. The results of DNA-DNA hybridizations of all strains examined are shown in Table 2.2. DNA-DNA hybridization data revealed that four strains LMG 1625^T, LMG 1599, LMG 1699 and LMG 1682 displayed a high level of DNA-DNA relatedness (66-85 %) and low levels of relatedness to the recognized *Acetobacter* species. The species name *Acetobacter cerevisiae* sp. nov. is proposed for this taxon. Strain LMG 1746^T showed DNA-DNA relatedness at an intermediate level (50-53 %) to the *A. cerevisiae* strains and at low levels to the recognized *Acetobacter* species. The name *Acetobacter malorum* sp. nov. is proposed for this strain. The DNA-DNA hybridization data also revealed that the type strain of *A. pomorum*, LMG 18848^T, is related to strains of *A. pasteurianus* at an intermediate level (51-58 %). The latter result clearly differs from the result obtained by Sokollek *et al.* (1998b), who found only 17 % DNA-DNA relatedness between the type strain of *A. pomorum*, LTH 2458^T, and the type strain of *A. pasteurianus*, DSM 3509^T. The discrepancy between these data could be explained by the fact that Sokollek *et al*. (1998b), who used the membrane method, did not perform reciprocal reactions, which are important to obtain unequivocal results. The hybridization data also showed that four strains, LMG 1633, LMG 1572, LMG 1754 and LMG 1663, currently classified as *A. pasteurianus*, have DNA-DNA binding values of less than 22 % to the type strain of *A. pasteurianus* and should be allocated to *A. peroxydans* (LMG 1633), *A. estunensis* (LMG 1572) and *A. tropicalis* (LMG 1754, LMG 1663).

G+C content. The G+C content of the *Acetobacter* strains studied are shown in Table 2.2 and ranged from 52.1 to 60.7 mol%, whereas the G+C span of a species was limited to 2.4 mol%. The latter results show that the G+C content of *Acetobacter* species is no longer too broad for a species, as was previously the case especially for *A. pasteurianus*, which had a G+C span of 9.7 mol% (Gosselé *et al.*, 1983b, Swings *et al.*, 1992). Analogous results were obtained by Lisdiyanti *et al.* (2000). The G+C content of the strains belonging to *A. cerevisiae* varied from 56.0 to 57.6 mol%. The *A. malorum* strain LMG 1746^T had a G+C content of 57.2 mol%.

Phylogenetic analysis based on 16S rRNA gene sequences. The nearly complete 16S rRNA gene sequences (1436-1444 nucleotides) of *A. cerevisiae* LMG 1625^T, *A. malorum* LMG 1746^T and ten strains representing the recognized *Acetobacter* species

Table 2. 2. DNA-DNA binding values and DNA G+C content of *Acetobacter* strains studied

Mean standard deviation of DNA-DNA hybridization is ± 7% (Goris *et al*., 1998).

Strain	G+C content (mol %)	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	26	27	28	29	30	31	32	33	34
A. pasteurianus																																			
1. LMG 1555	54.3	100																																	
2. LMG 1686	53.5		100																																
3. LMG 1262 ^{T}t1	53.4	78	82	100																															
4. LMG 1630	53.7		79	71	100																														
5. LMG 1629	53.6		90	70		100																													
6. LMG 1658	53.2		66	62	59		100																												
7. LMG 1659	53.7			69			66	100																											
A. pomorum																																			
8. LMG 18848 ^{T}	52.1	51	58	53	51	55		55	100																										
A. peroxydans																																			
9. LMG 1633	60.7			6					9	100																									
10. LMG 1635 ^{T}	59.7			13					23	73	100																								
A. lovaniensis																																			
11. LMG 1617 ^{T}	57.4		21	14		18			20	17	16	100																							
A. estunensis																																			
12. LMG 1626 ^{T}	59.2			20					13	9		10	100																						
13. LMG 1572	60.2	19	21	21	19	3			12	7		9	98	100																					
14. LMG 1580	59.5												92	92	100																				
A. aceti																																			
15. LMG 1531	58.3		16	18		6			12			16				100																			
16. LMG 1535	57.0		14	10		8			8			14				59	100																	
17. LMG 1504 ^{T}	56.9	7	8	7	9	6	5	6	6	10		13	17	17		53	100	100																	
18. LMG 1496	56.9		6	9		7			7			12				55	104	95	100															
A. cerevisiae																																			
19. LMG 1625 ^{T}	57.6		37	32		19			27	10		11	15	17		18	16	12	15	100															
20. LMG 1599	56.8			30					23				25					11		77	100													
21. LMG 1699	56.0			35					24				27					8		74	76	100												
22. LMG 1682	57.4		22	32		13			19			18	15			18	10	9	9	85	80	66	100											
A. malorum																																			
23. LMG 1746 ^{T}	57.2	10	11	7	8	6			9	7		9	5	5				3		53	50			100										
A. orleanensis																																			
24. LMG 1583 ^{T}	55.7			12					12	11		10	6	7				4		33	37	37	40	28	100									
25. LMG 1592	58.0*																								90*	100								
26. LMG 1608	58.1*																								98*		100							
27. LMG 1545	57.3*																								77*	89*	85*	100						
A. indonesiensis																																			
28. L MG 19824 ^{T}	54.0																			18									100						
29. LMG 1588	54.2			17					16	7		15	13	18				9		20	26	32	20	10	12				88*	100				
30. LMG 1571	54.1																												85*	83	100			
A. tropicalis																																			
31. LMG 19825 ^{T}	56.0																			34									18			100		
32. LMG 19826	55.6																												19			81	100		
33. LMG 1754	56.2			18			11	12										7															100	
34. LMG 1663	55.9		22	20		14	6	14	10	6		14	13	13		10	10	9	8	27			24	12	14				20	17		86	93	76	100

*Data taken from Lisdiyanti *et al.* (2000).

were determined and compared with deposited 16S rRNA gene sequences of strains of the recognized *Acetobacter* species and of the type species of the other genera in the family *Acetobacteraceae*. A phylogenetic tree, reflecting the positions of these strains within the AAB lineage, was generated using the neighbour-joining method and is shown in Fig. 2.1. Bootstrap values supported the topology of the tree. The tree showed

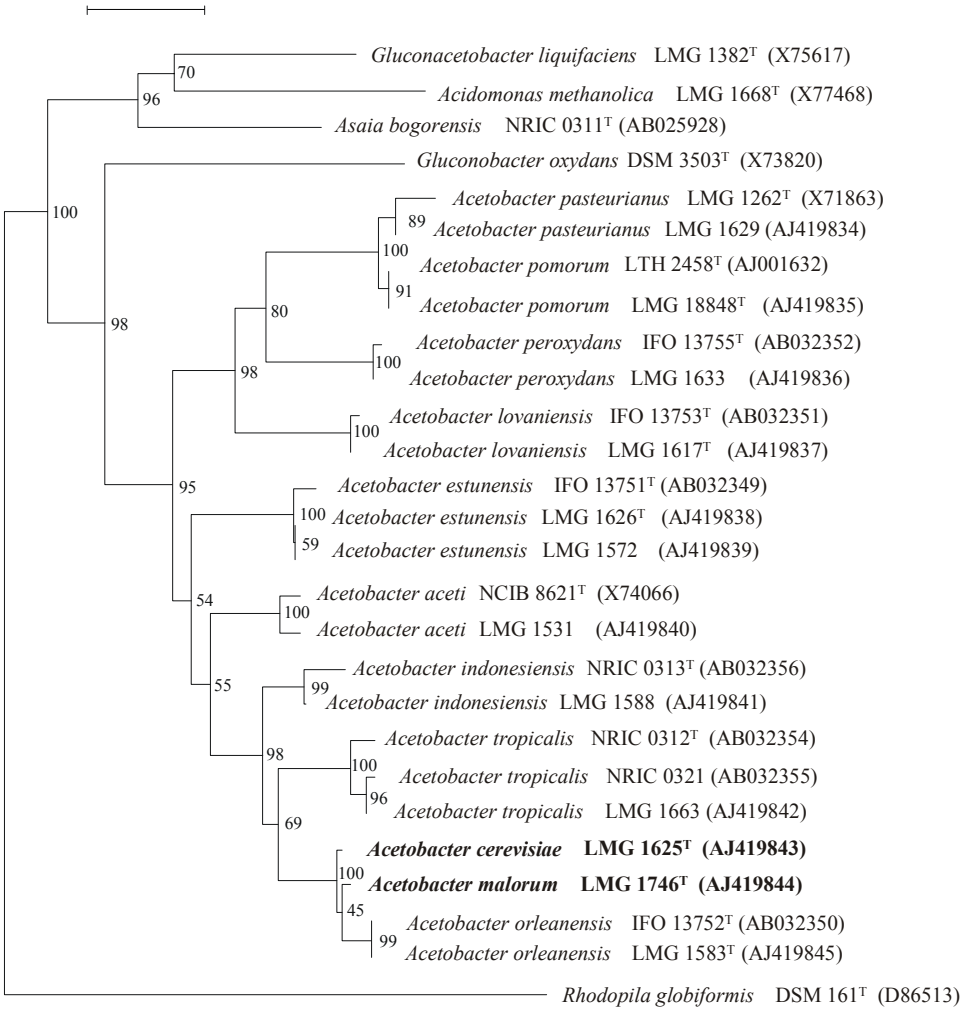


Fig. 2.1. Neighbour-joining tree reflecting the phylogenetic position of *A. cerevisiae* sp. nov. LMG 1625^T and *A. malorum* sp. nov. LMG 1746^T within the acetic acid bacteria based on almost complete 16S rRNA gene sequences. *Rhodopila globiformis* DSM 161^T was used as an outgroup in this analysis. Bar, 1 % sequence dissimilarity. Numbers at branching points indicate bootstrap percentages derived from 500 samples.

that the genus *Acetobacter* formed two major rRNA groups, one containing the species *A. pasteurianus*, *A. pomorum*, *A. peroxydans* and *A. lovaniensis* and the second containing *A. cerevisiae* LMG 1625^T, *A. malorum* LMG 1746^T and the species *A. estunensis*, *A. aceti*, *A. indonesiensis*, *A. tropicalis* and *A. orleanensis*. It is noteworthy that the *Acetobacter* species within each rRNA group showed more than 97.2 % 16S rRNA gene sequence similarity and that strains belonging to the same *Acetobacter* species showed more than 99.6 % 16S rRNA gene sequence similarity. *A. cerevisiae* LMG 1625^T and *A. malorum* LMG 1746^T showed 99.9 % 16S rRNA gene sequence similarity, indicating that they are phylogenetically very closely related. Both strains shared 99.6 % 16S rRNA gene sequence similarity to *A. orleanensis*. Somewhat lower values were observed to members of *A. estunensis*, *A. aceti*, *A. indonesiensis* and *A. tropicalis* (97.6-98.9 %). The 16S rRNA gene sequence of *A. pomorum* LMG 18848^T was compared to the sequence deposited for *A. pomorum* LTH 2458^T (EMBL accession no. AJ001632). The two sequences were 100 % identical over 1440 bases reinforcing the assumption that we used the same strain as Sokollek *et al.* (1998b).

Phenotypic characteristics. Table 2.3 gives characteristics useful in the differentiation of the species of the genus *Acetobacter*. *A. cerevisiae* and *A. malorum* could not be differentiated from the recognized *Acetobacter* species by an exclusive phenotypic characteristic. However, the combination of growth on methanol as carbon source, the inability to grow on ammonium as nitrogen source with ethanol as the carbon source and the ability to grow on 30 % D-glucose, allowed the differentiation of *A. malorum* from the other *Acetobacter* species. *A. cerevisiae* could be differentiated from the species *A. pomorum*, *A. peroxydans*, *A. lovaniensis*, *A. indonesiensis*, *A. tropicalis*, *A. estunensis* and *A. aceti* by the combination of the following phenotypic characteristics: production of 2-keto-D-gluconic acid (but no 5-keto-D-gluconic acid) from D-glucose, the inability to grow on ammonium as nitrogen source with ethanol as the carbon source and the inability to grow on maltose as a carbon source. Phenotypic characteristics for its differentiation from *A. pasteurianus* and *A. orleanensis* were not found. It is important to mention that some strains of *A. pasteurianus*, *A. pomorum*, *A. orleanensis*, *A. indonesiensis* and *A. tropicalis*, even without the addition of *A. cerevisiae*, have phenotypic characteristics that are similar to one another. For identification of these strains at the species level, genotypic characterization (as determining DNA similarity) is required.

The type strains of *A. pomorum* and *A. pasteurianus* were evaluated for growth in the presence of 30 % D-glucose, growth on methanol as carbon source and growth on *n*-

propanol with ammonium as the sole nitrogen source, three features useful in the differentiation of the two species according to Sokollek *et al.* (1998b). In our hands however, LMG 18848^T could not be distinguished from the type strain of *A. pasteurianus* by any of these features.

Table 2.3. Characteristics that differentiate the species of the genus *Acetobacter*

Taxa are listed as: 1, *A. cerevisiae* sp. nov. (4 strains); 2, *A. malorum* sp. nov. LMG 1746^T; 3, *A. pasteurianus* (7 strains); 4, *A. pomorum* LMG 18848^T; 5, *A. peroxydans* (2 strains); 6, *A. lovaniensis* LMG 1617^T; 7, *A. orleanensis* (4 strains); 8, *A. indonesiensis* (2 strains i.e. LMG 1588 and LMG 1571); 9, *A. tropicalis* (2 strains i.e. LMG 1754 and LMG 1663); 10, *A. estunensis* (3 strains); 11, *A. aceti* (4 strains). Characters are scored as: +, positive; -, negative; v, variable. For variable characters, the number of strains testing positive and the result for the type strain are given in parentheses. Abbreviation: YE, yeast extract.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Formation from D-glucose:											
5-keto-D-gluconic acid	-	-	-	-[1]	-	-	-	-	-	-	+
2-keto-D-gluconic acid	+	+	v (2 +, -)	-[1]	-	+	+	+	+	+	+
Growth in ammonium with ethanol	-	-	-	-	+	+	-	-	-	+	+
Growth on carbon sources:											
Glycerol	+	+	v (2 +, -)	+	-	+	+	+	+	v (1 +, +)	+
Maltose	-	-	v (2 +, -)	-	+	-	v (1 +, -)	+	+	-	v (1 +, -)
Methanol	-	+	-	-	-	+	-	-	-	-	-
Growth on YE + 30 % D-glucose	-	+	v (1 +, -)	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	-	+	+	+	+	+	+
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[2]	55.7-58.1	54.0-54.2	55.6-56.2	59.2-60.2	56.9-58.3

[1]Data taken from Sokollek *et al*. (1998b).

[2]Data taken from Lisdiyanti *et al*. (2000).

Some controversial reports exist on the growth of *Acetobacter* strains on mannitol agar (Boesch *et al.*, 1998; Franke *et al.*, 1999; Gosselé *et al.*, 1983b; Lisdiyanti *et al*., 2000; Yamada, 2000; Yamada *et al.*,1997). In our hands, all *Acetobacter* strains grew on medium 13 (Janssens *et al*, 1998), also known as MYP agar (2.5 % mannitol, 0.5 % yeast extract, 0.3 % peptone and 1.5 % agar), although in most cases this growth was not abundant. Medium 13 is however very useful to maintain *Acetobacter* strains viable on plates for a more extended period.

In conclusion, on the basis of DNA-DNA reassociation values, DNA base compositions, phylogenetic relationships and phenotypic characteristics, this study revealed the existence of two novel *Acetobacter* species, *A. cerevisiae* (type strain LMG 1625^T) and *A. malorum* (type strain LMG 1746^T). It also showed that four *A. pasteurianus* strains should be renamed: LMG 1633 should be allocated to *A. peroxydans*, LMG 1572 should be allocated to *A. estunensis*, whereas LMG 1754 and LMG 1663 should be assigned to *A. tropicalis*.

Furthermore this study confirms that *Acetobacter* strains should be classified and identified mainly on the basis of genotypic characteristics, as identification on the basis of phenotypic tests does not always lead to clear-cut results. The results above show that DNA-DNA hybridization is the recommended technique for accurate identification of *Acetobacter* strains.

Description of *Acetobacter cerevisiae* sp. nov.

Acetobacter cerevisiae (ce.re.vi'si.a.e. L. fem. gen. n. cerevisiae of beer, referring to the source from which most strains have been isolated).

Cells are Gram-negative, ellipsoidal to rod-shaped, approximately 1.0 x 2.0-4.0 µm in size, occurring singly, in pairs, occasionally in short chains. Involution forms like swollen cells occur in some strains. Cells are non-motile. Endospores are not detected. Colonies are beige to brown, round, regular to wavy, raised, smooth with a diameter of 0.3-0.5 mm on MYP agar. Obligately aerobic. Oxidase negative. Catalase positive. Characterized by the combination of the following phenotypic features: 2-keto-D-gluconic acid is produced from D-glucose, 5-keto-D-gluconic acid is not produced from D-glucose, no growth with ammonium as the sole nitrogen source on ethanol as carbon source, no growth on maltose or methanol as carbon source (Table 2.3). The range of the G+C content of the DNA is 56.0 to 57.6 mol%. The type strain is LMG 1625^T (= DSM 14362^T = NCIB 8894^T = ATCC 23765^T), which has a G+C content of 57.6 mol% and has been isolated from beer (ale) in storage at Toronto, Canada (Kozulis & Parsons, 1958).

Description of *Acetobacter malorum* sp. nov.

Acetobacter malorum (ma.lo'rum. L. neut. gen. pl. n. *malorum* of apples, referring to the isolation of the type strain of a rotting apple).

Cells are Gram-negative, ellipsoidal, approximately 0.9 x 1.1-1.3 µm in size, occurring singly or in pairs. Cells are non-motile. Endospores are not detected. Colonies are beige, round, regular to wavy, convex, smooth with a diameter of 0.5 mm on MYP agar. Obligately aerobic. Oxidase negative. Catalase positive. Characterized by the combination of the following phenotypic features: 2-keto-D-gluconic acid is produced from D-glucose, 5-keto-D-gluconic acid is not produced from D-glucose, no growth with ammonium as the sole nitrogen source on ethanol as carbon source, no growth on maltose as carbon source, growth on methanol as carbon source (Table 2.3). The type strain is LMG 1746^T (= DSM 14337^T), which has a G+C content of 57.2 mol% and was isolated from a rotting apple in Ghent, Belgium (Gosselé *et al.*, 1983a, b).

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CHAPTER 3

IDENTIFICATION AND CLASSIFICATION OF AAB FROM DIFFERENT SOURCES (RICE, APPLE JUICE, WINE AND MANGO FRUIT) USING A POLYPHASIC APPROACH

3.1. NATURAL ASSOCIATION OF *GLUCONACETOBACTER DIAZOTROPHICUS* AND DIAZOTROPHIC *ACETOBACTER PEROXYDANS* WITH WETLAND RICE

Redrafted from:
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SUMMARY

The family *Acetobacteraceae* currently includes three known nitrogen-fixing species, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae* and *Gluconacetobacter azotocaptans*. In the present study, acetic acid-producing nitrogen-fixing bacteria were isolated from four different wetland rice varieties cultivated in the state of Tamilnadu, India. Most of these isolates were identified as *Gluconacetobacter diazotrophicus* on the basis of their phenotypic characteristics and PCR assays using specific primers for that species. Based on 16S rRNA gene sequence analysis and DNA-DNA reassociation experiments the remaining isolates were identified as *Acetobacter peroxydans*, another species of the *Acetobacteraceae* family, thus far never reported as diazotrophic. The presence of *nifH* genes in *A. peroxydans* was confirmed by PCR amplification with *nifH* specific primers.

INTRODUCTION

Nitrogen is known as an important element in plant growth since it is often the primary nutrient limiting the plant growth in many ecosystems. In agriculture, crop productivity is mainly supported by the use of inorganic N fertilizers, which are expensive and damaging for the environment. From ecological and economical perspectives plant-associated biological nitrogen fixation is desirable especially for economically important crops such as rice, which is the staple diet for more than two fifths of the world's population. There are several reports on the association of nitrogen-fixing bacteria to various crops as *Azospirillum* sp. with wheat (Baldani *et a*l., 1983), *Herbaspirillum seropedicae* with maize (Baldani *et al*., 1986), *Gluconacetobacter diazotrophicus* with sugarcane (Cavalcante & Döbereiner, 1988; Gillis *et al*., 1989) and *Gluconacetobacter johannae* and *Gluconacetobacter azotocaptans* with coffee plants (Fuentes-Ramírez *et al*., 2001). *H. seropedicae* (Baldani *et al*., 1986), *Burkholderia vietnamiensis* (Gillis *et al*., 1995), *Rhizobium leguminosarum* bv *trifolii* (Yanni *et al*., 1997), *Azoarcus* (Engelhard *et al*., 2000), *Serratia marcescens* (Gyaneshwar *et al*., 2001) and innumerable species of *Pseudomonas* (Vermeiren *et al*., 1999; Watanabe *et al*., 1987) have been found in association with rice plants. In the family *Acetobacteraceae*, three nitrogen-fixing species have been described: *G. diazotrophicus*, *G. johannae* and *G. azotocaptans* (Fuentes-Ramírez *et al*., 2001; Gillis *et al*., 1989; Jimenez-Salgado *et al*., 1997). *G. diazotrophicus*, originally described as *Acetobacter diazotrophicus* (Gillis *et al*., 1989) and later transferred to the genus *Gluconoacetobacter* (Yamada *et al*., 1997), which was subsequently corrected to *Gluconacetobacter* (Yamada *et al*., 1998), was the first nitrogen-fixing *Acetobacteraceae* species described and it has been investigated thoroughly with regard to its nitrogen-fixing ability (James *et al*., 1994; Muñoz-Rojas *et al*., 2003; Sevilla *et al*., 1998). Yet it is only since recently that there is evidence that it is a nitrogen contributor for crops (Sevilla *et al*., 2001).

Many reports on AAB mention that these strains are mainly associated with sugar or ethanol-rich environments (Swings, 1992), but recent studies show that their distribution is wider (Jiménez-Salgado *et al*., 1997). *G. diazotrophicus* was originally isolated from sugarcane (Gillis *et al*., 1989), but recent studies show that this species is also associated with other sugar-rich plants as sweet sorghum, sweet potato and pineapple plants (Paula *et al*., 1991; Tapia-Hernandez *et al*., 2000) as well as with sugar-poor plants as coffee and ragi (Jiménez-Salgado *et al*., 1997; Loganathan *et al*., 1999). *G. johannae* and *G. azotocaptans* isolates were recovered from coffee (Fuentes-

Ramírez *et al*., 2001).

The present study describes the identification and characterization of nitrogen-fixing AAB *G. diazotrophicus* and *A. peroxydans* recovered from wetland rice cultivated in India.

MATERIAL AND METHODS

Isolation of diazotrophic AAB from rice. Samples were collected in triplicate from rhizosphere soil, roots and stems from four different rice varieties (*Oryza sativa* cv Co 36, cv Co 39, cv Ponni and cv IR 50) cultivated in flooded soils of Tamilnadu, a Southern state in India, during either flowering stage or just before flowering stage. The rhizosphere soil samples were serially diluted and 0.1 ml aliquots were inoculated into vials containing 5 ml of N-free semisolid LGI medium with an initial pH of 6.0 with 1, 10 or 30 % cane sugar (w/v) and 0.005 % yeast extract (w/v) (Reis *et al*., 1994) and incubated at 32 °C for 5 days. The roots were surface sterilized with 70 % ethanol for 5 min and 0.2 % mercuric chloride for 30 s and then washed several times with sterile water. Basal stem portions were cut into 5 cm pieces, surface sterilized by dipping in 95 % ethanol and then washed 6 times with sterile water. From each end of the stem pieces 1 cm portions were removed by cutting with a sterile blade (Gyaneshwar *et al*., 2001). The outer sterility of the root and stem samples was verified by rolling them on Tryptic Soy agar (Sigma). Afterwards the samples were homogenized with a mortar and pestle in sterile phosphate buffered saline (PBS) and serially diluted. 0.1 ml aliquots were inoculated into vials containing the semisolid LGI medium described for the rhizosphere soil.

Vials with a white to yellow surface pellicle were assayed for acetylene reduction activity as described by Hardy *et al.* (1973). Pellicles of the nitrogenase positive vials were sub-cultured in fresh medium before they were streaked on LGI agar plates with 10 % cane sugar (w/v) and incubated at 32 °C for 10 days. Acid-producing deep yellow and yellowish orange colonies with dark center were picked up for further analysis. The isolates and their sources are presented in Table 3.1. Strains TNCSF 42, TNCSF 47, TNCSF 36 and TNCSF 49 are deposited in the BCCM/LMG Bacteria Collection as LMG 22174, LMG 22175, LMG 21769 and LMG 21770, respectively.

Reference strains. The reference strains used in this study were *G. diazotrophicus* LMG 7603^T, *G. johannae* ATCC 700987^T, *G. azotocaptans* ATCC 700988^T and *A.*

peroxydans LMG 1635^T.

Table 3.1. Source of diazotrophic acetic acid bacteria isolates from wetland rice (*Oryza sativa*)

Strain	Rice variety	Soil pH	Source	Region
G. diazotrophicus				
TNCSF 1	Co 39	7.4	Stem tissue	Madhuranthagam, Kanchipuram
TNCSF 2	Ponni	7.2	Root tissue	Nugumbal, Kanchipuram
TNCSF 21	Ponni	6.5	Root tissue	Chidambaram, Cuddalore
TNCSF 41	Ponni	7.0	Rhizosphere soil	Villupuram, Villupuram
TNCSF 42	IR 50	7.3	Root tissue	Thirumangalam, Madurai
TNCSF 44	Co 36	6.2	Root tissue	Thirukazhuakundram, Kanchipuram
TNCSF 45	Co 39	7.0	Stem tissue	Thanjore, Thanjore
TNCSF 46	Ponni	7.6	Root tissue	Chengalpattu, Kanchipuram
TNCSF 47	Ponni	7.2	Stem tissue	Valajabad, Kanchipuram
A. peroxydans				
TNCSF 20	Co 36	7.7	Root tissue	Nugumbal, Kanchipuram
TNCSF 36	IR 50	7.3	Root tissue	Thirumangalam, Madurai
TNCSF 37	Ponni	6.5	Root tissue	Virudunagar, Tirunelveli
TNCSF 38	Co36	6.3	Stem tissue	Chidambaram, Cuddalore
TNCSF 49	Ponni	7.0	Root tissue	Villupuram, Villupuram
TNCSF 50	Co36	6.2	Stem tissue	Thozhudhur, Trichy

Strains TNCSF 42, TNCSF 47, TNCSF 36 and TNCSF 49 were deposited in the BCCM/LMG Bacteria Collection as LMG 22174, LMG 22175, LMG 21769 and LMG 21770, respectively.

Phenotypic characterization. Colony morphology and pigmentation were observed on LGI agar with 10 % (w/v) cane sugar and potato agar with 10 % (w/v) cane sugar (Cavalcante & Döbereiner, 1988). Pigmentation was also checked on GYC agar, pH 4.5 (Micales *et al*., 1985). Oxidase and catalase tests were determined using commercially available discs (Hi media). The ability to utilize various carbon substrates was assayed in LGI medium with NH_4Cl (0.1 %) and supplemented with 0.5 % (w/v) of the appropriate filter sterilized carbon compound instead of cane sugar. The ability to utilize various amino acids was tested in LGI medium with sorbitol (0.5 %) instead of cane sugar, and supplemented with 0.1 % of an appropriate filter sterilized L-amino acid. LGI medium with both NH_4Cl (0.1 %) and sorbitol (0.5 %), and LGI medium lacking both, were used as positive and negative controls respectively.

Species-specific PCRs for *G. diazotrophicus*, *G. azotocaptans* and *G. johannae*. The isolates and the type strains of the nitrogen-fixing AAB *G. diazotrophicus* LMG 7603^T, *G. johannae* ATCC 700987^T and *G. azotocaptans* ATCC 700988^T were grown in SYP (Caballero-Mellado & Martinez-Romero, 1994) for 48 h at 32 °C. PCR amplifications were performed on supernatant of a single colony that was suspended in 50 µl sterile water and boiled for 5 min at 95 °C (Kirchhof *et al*., 1998) using primers

that were generated on the basis of 16S rRNA gene sequences. The PCR specific for *G. diazotrophicus* was performed using two species-specific primers: AC (5'-CTGTTTCCCGCAAGGGAC-3') and DI (5'-GCGCCCCATTGCTGGGTT-3') described by Sievers *et al.* (1998). The PCRs specific for *G. johannae* and *G. azotocaptans* were performed following the protocol described by Fuentes-Ramírez *et al*. (2001) using a species-specific primer, L927Gj (5'-GAAATGAACATCTCTGCT-3') for *G. johannae* and L923Ga (5'- AATGCTCAATCTGAACA–3') for *G. azotocaptans*, in combination with the universal primer U475 (5'-AATGACTGGGCGTAAAG-3').

16S rRNA gene sequencing and phylogenetic analysis. DNA for 16S rRNA gene sequencing was prepared by alcalic extraction from cells grown on medium 13 from the Catalogue of cultures of the BCCM/LMG Bacteria Collection (Janssens *et al.*, 1998) at 28 °C. A fragment of the 16S rRNA gene of strains TNCSF 36 and TNCSF 49 was amplified using oligonucleotide primers complementary to highly conserved regions of the 16S bacterial rRNA genes. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' (positions 8-27, according to the *Escherichia coli* numbering system), the reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3' (positions 1541-1522). PCR products were purified using a QIA quick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Purified PCR products were partially sequenced by using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an Applied Biosystems 377 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The sequencing primers used were *Gamma, Gamma and PD (Coenye *et al.*, 1999a). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). The partial 16S rRNA gene sequences determined and sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL library, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics 1.0 software package (Applied Maths). The comparison of the sequence data was based on 900 bases of the 16S rRNA genes. Unknown bases were discarded from the calculations. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the tree using 1000 bootstrap resamlings of the data. The strain numbers, species names and accession numbers of the 16S rRNA gene sequences retrieved from EMBL for use in the phylogenetic analysis are presented in Fig. 3.1.

DNA preparation for G+C content determination and for DNA-DNA hybridizations. High-molecular-mass DNA for determination of the G+C content and

for DNA-DNA hybridizations was prepared from cells grown aerobically on Z1 medium (2.0 % yeast extract, 2.0 % calcium lactate and 1.5 % agar) at 28 °C by the method of Wilson (1987) with minor modifications (Cleenwerck *et al*., 2002).

Determination of the DNA G+C content. The G+C content of the DNA was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) and Goris *et al.* (1998). Hybridizations were performed under stringent conditions at 47 °C in a hybridization solution containing 50 % formamide (2 x SSC, 5 x Denhardt's solution, 50 % formamide, 2.5 % dextran sulfate, low-molecular-mass denatured salmon sperm DNA to a final concentration of 100 µg ml^{-1}, 1.25 µg biotinylated probe DNA ml^{-1}). The DNA-DNA relatedness percentages presented are means based on at least two hybridization experiments. Reciprocal reactions (e.g. A x B and B x A) were performed and the variation between them was within the limit of this method (Goris *et al*., 1998).

***nifH* PCR.** The *nifH* gene was amplified by PCR using the primer set PolF/PolR and the conditions described previously (Poly *et al*., 2001).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences of strains TNCSF 36 and TNCSF 49 determined in this study were deposited in EMBL under the accession numbers AJ517177 and AJ517178, respectively.

RESULTS

Isolation of diazotrophic AAB from rice. Fifteen gram-negative, acid producing, nitrogen-fixing isolates were recovered from rhizosphere soil, roots and stems of rice cultivated in India (Table 3.1). The phenotypic characteristics of the isolates were determined and compared with those of the known nitrogen-fixing AAB *G. diazotrophicus*, *G. azotocaptans* and *G. johannae* (Table 3.2). One group of 9 strains showed phenotypic characteristics typical of *G. diazotrophicus*. They formed after 10 days of incubation yellow colonies (1.5 - 3.0 mm) on LGI agar with 10 % cane sugar (w/v) and chocolate brown colonies on potato agar with 10 % cane sugar (w/v). On

GYC solid medium they produced water-soluble brown pigments. They also utilized the same carbon and nitrogen substrates as *G. diazotrophicus* (Table 3.3). The

Table 3.2. Characteristic features of acetobacteria isolates from rice

Strains: 1, *Gluconacetobacter diazotrophicus* LMG 7603^T; 2, *Gluconacetobacter johannae* ATCC 700987^T; 3, *Gluconacetobacter azotocaptans* ATCC 700988^T; 4, *G. diazotrophicus* rice isolates; 5, *A. peroxydans* rice isolates.

Determinative characters*	**1**	**2**	**3**	**4**	**5**
Size and shape of the colony on LGI (10 % sugar) after 10 days	1.5-3 mm, convex, round, smooth	1.5-3 mm, convex, round, smooth	1.5-3 mm, convex, round, smooth	1.5-3 mm, convex, round, smooth	0.8-1.5 mm, flat, dry
Pigmentation on LGI (10 % sugar) after 10 days	yellow	yellow	yellow	yellow	yellowish orange, dark centered
Pigmentation on potato agar (10% sugar) after 10 days	chocolate brown	pale brown	brown (water soluble), agar diffusing	chocolate brown	pale brown, dark centered
Gas production during isolation process	-	-	-	-	+
Successive sub-culturing is essential (~ 60 times)	-	-	-	-	+
Growth on methanol	-	-	-	-	+
Growth on malate	-	+	-	-	+
Growh in the presence of 150 mM of nitrate	-	-	-	-	+

* Cells of the type strains and the rice isolates were Gram-negative, oxidase-positive and catalase-negative. All oxidized ethanol and D-glucose, grew in the presence of 30 % sucrose and possessed acetylene reduction activity.

other group with the remaining 6 strains differed from the known nitrogen-fixing AAB. They formed after 10 days of incubation small (0.8 to 1.5 mm) yellowish-orange colonies with a dark center on LGI agar with 10 % cane sugar (w/v) and pale brown colonies with a dark center on potato agar with 10 % cane sugar (w/v). On GYC solid medium they did not produce the water-soluble brown pigments. They also differed in their ability to utilize some carbon and nitrogen substrates (Table 3.3). The isolation of these strains required successive sub-culturing of the pellicles up to 50-60 times in fresh medium. Gas production was noticed during the isolation process. These strains were only isolated from roots and stems but not from rhizosphere soil.

Table 3.3. Growth of *Gluconacetobacter* species and *Acetobacter peroxydans* on different substrates

Strains: 1, *Gluconacetobacter diazotrophicus* LMG 7603^T; 2, *Gluconacetobacter johannae* ATCC 700987^T; 3, *Gluconacetobacter azotocaptans* ATCC 700988^T; 4, *G. diazotrophicus* rice isolates; 5, *A. peroxydans* rice isolates. ++, Good growth; +, medium growth; -, no growth.

Substrates*	**1**	**2**	**3**	**4**	**5**
Sugars (0.5 %)					
Sorbitol	++	++	+	++	++
Inositol	+	+	-	+	++
Sodium pyruvate	+	+	-	+	++
Glycerol	++	+	-	++	++
L-rhamnose	+	+	-	+	+
Ribose	+	+	+	+	++
Lactose	-	-	-	-	++
Maltose	+	+	+	+	++
Fructose	+	++	++	+	+
Arabinose	++	++	-	++	++
Trehalose	+	+	-	+	++
Raffinose	++	+	-	++	++
Erythritol	++	++	+	++	++
Galactose	++	++	+	++	++
Cellobiose	+	+	-	+	+
Sodium acetate	+	+	-	+	++
Xylose	++	++	-	++	++
Starch	-	-	-	-	+
Sugar alcohols (0.5 %)					
Ethanol	+	+	-	+	++
Methanol	-	-	-	-	++
n-Butanol	-	+	-	-	++
Organic acids(0.5 %)					
Succinic	-	-	-	-	++
Adipic	-	-	-	-	++
Malonic	+	+	-	+	++
Valeric	-	-	-	-	++
Fumaric	-	-	-	-	++
Acetic	-	-	-	-	++
Hippuric	-	-	-	-	++
Malic	-	+	-	-	++
Citric	-	-	-	-	++
L-aminoacids					
L-Cysteine	++	-	++	++	-
L-Glutamine	++	-	++	++	++
L-Proline	++	-	-	++	++
L-Tryptophan	++	++	-	++	-

* The type strains and the rice isolates showed good growth on D-glucose, D-mannose and L-aspartic acid. No growth was observed on oxalic acid or L-glycine.

PCRs specific for *G. diazotrophicus*, *G. johannae* and *G. azotocaptans*. Recently, clear and fast PCR methods were developed for the identification of *G. diazotrophicus* (Sievers *et al*., 1998), *G. johannae* and *G. azotocaptans* (Fuentes-Ramírez *et al*., 2001). To examine whether the rice isolates could be classified as any of the known nitrogen-fixing AAB, PCR reactions with the described species-specific primers were performed. The 9 strains forming the yellow colonies on LGI medium reacted positive in the PCR assay specific for *G. diazotrophicus* (based on the amplification of a specific 16S rRNA gene fragment) giving a PCR product of 445 bp, the size expected for this species. The results showed that these isolates can be assigned to the species *G. diazotrophicus*. The 6 strains forming the yellowish-orange colonies on LGI medium did not yield any PCR product in any of the species-specific PCR tests. These results together with the phenotypic properties of these strains indicate that these isolates belong within the *Acetobacteraceae* family but not to any of the known nitrogen-fixing AAB species thus far described in this family. To identify these isolates further tests were performed.

Phylogenetic positions of TNCSF 36 and TNCSF 49. The 16S rRNA gene of two representative strains of the group forming the yellowish orange colonies on LGI medium, TNCSF 36 and TNCSF 49, was partially sequenced (729 and 948 nucleotides respectively were determined) in order to determine the phylogenetic positions of the strains. Comparison of the partial 16S rRNA gene sequences of both strains revealed 99.7 % sequence similarity and comparison of the partial 16S rRNA gene sequences determined with sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL library, indicated that both strains belonged to the genus *Acetobacter* (Fig. 3.1). The 16S rRNA gene sequence of TNCSF 49 (948 nucleotides) showed significant sequence similarity for possible relatedness at the species level (> 97 %) with *A. peroxydans* IFO 13755^T (100 %), *A. pomorum* LTH 2458^T (97.9 %), *A. pasteurianus* LMG 1262^T (97.5 %), *A. lovaniensis* IFO 13753^T (97.4 %) and *A. syzygii* NRIC 0483^T (97.4 %). To the other species of *Acetobacter* the similarity was below 97 %.

DNA-DNA relatedness and DNA base composition. TNCSF 36 and TNCSF 49 were hybridized against each other and against the type strain of *A. peroxydans* LMG 1635^T their closest relative (100 % 16S rRNA gene sequence similarity). Both strains had a high value of DNA binding to one another (99 %) and to LMG 1635^T (87-89 %). The DNA G+C content of TNCSF 36 and TNCSF 49 for both strains was 60.5 mol% which is comparable to the G+C content of *A. peroxydans* LMG 1635^T (59.7 mol%).

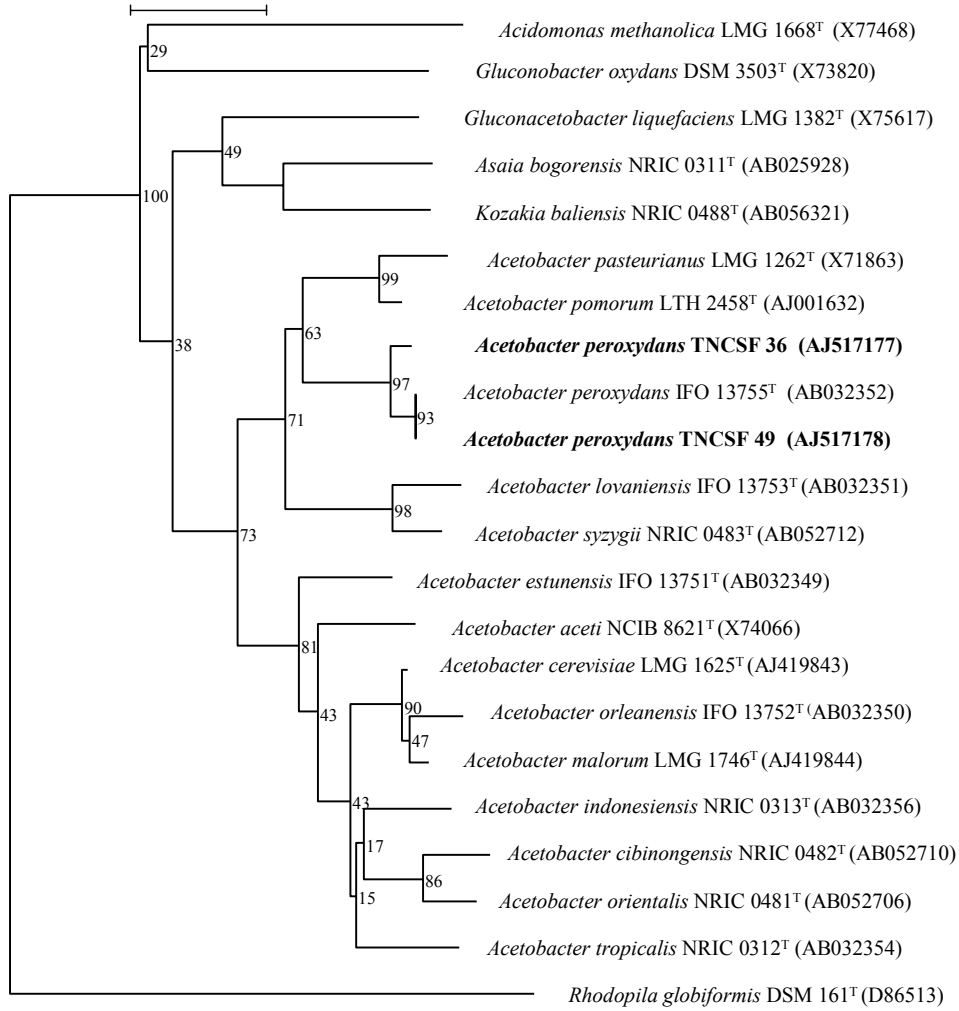

Fig. 3.1. Neighbour-joining tree (based on 900 bases of the 16S rRNA gene sequences) reflecting the phylogenetic positions of TNCSF 36 and TNCSF 49 within the acetic acid bacteria. *Rhodopila globiformis* DSM 161^T was used as an outgroup in this analysis. The bar indicates 1 % sequence dissimilarity. Numbers at branching points indicate bootstrap percentages derived from 1000 samples.

These data show that both strains belong to the species *A. peroxydans.*

Presence of *nif* genes. *A. peroxydans* belongs to the *Acetobacteraceae* family as *G. diazotrophicus*. Hitherto, *A. peroxydans* has never been described as a diazotrophic species. Because *nif* genes are necessary for nitrogen fixation, a PCR with *nifH* specific primers was performed with the *A. peroxydans* isolates TNCSF 36 and TNCSF 49 and with the type strain LMG 1635^T of this species. The strains yielded a PCR product of

about 360 bp confirming the presence of *nif* genes (Fig. 3.2).

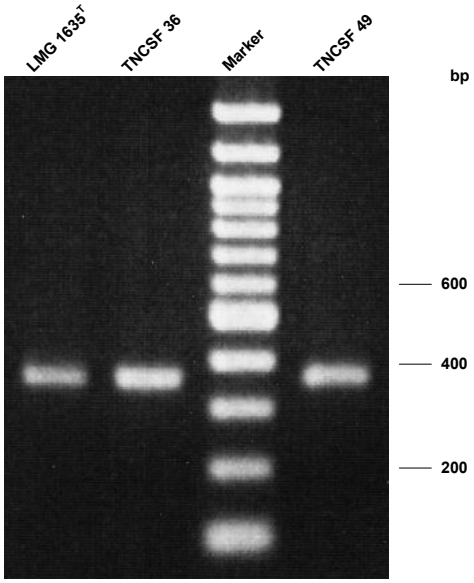

Fig. 3.2. *nifH* PCR amplification products of *A. peroxydans* LMG 1635^T (lane 1), TNCSF 36 (lane 2) and TNCSF 49 (lane 4) separated by agarose gel electrophoresis. Lane 3: molecular weight marker (100 bp ladder).

Acetylene reduction of *A. peroxydans* LMG 1635^T. The acetylene reduction (nitrogenase) activity of LMG 1635^T was examined by the acetylene reduction assay and compared to that of the novel *A. peroxydans* isolates. While LMG 1635^T showed an acetylene reduction activity lower than 10 nmol h^{-1} per culture (5 ml), the *A. peroxydans* isolates TNCSF 36 and TNCSF 49 produced 30 and 35 nmol of ethylene h^{-1} per culture, respectively. It is noteworthy that the type strain of *A. peroxydans* as well as the novel isolates of this species showed an inconsistent nitrogenase activity even among replicates from the same assay.

DISCUSSION

Several studies have focused on nitrogen-fixing bacteria associated with rice (Engelhard *et al.*, 2000; Gillis *et al.*, 1995; Gyaneshwar *et al.*, 2001; Ueda *et al.*, 1995;

Watanabe *et al.*, 1987; Yanni *et al.*, 1997) but the association of AAB with rice cultivated under field conditions has never been reported. The present study shows that nitrogen-fixing AAB are also found in natural association with rice plants.

The AAB isolates recovered from rice and capable of fixing nitrogen were assigned to the species *G. diazotrophicus* and *A. peroxydans*. The identification of the latter as a nitrogen-fixing species is surprising as this species was described already in 1925 (Swings, 1992) and was thus far never reported as diazotrophic. The type strain and novel isolates of *A. peroydans* showed low and inconsistent acetylene reduction activities, compared to *G. diazotrophicus*, but their diazotrophic ability was supported by the presence of *nifH* genes. The present study reveals that nitrogen-fixing AAB are not restricted to the genus *Gluconacetobacter*.

The isolation of *G. diazotrophicus* from wetland rice is not surprising. It is conceivable that the occurrence of this species, reported as a frequent colonizer of sugar-rich plants (Cavalcante & Döbereiner, 1988; Paula *et al*., 1991; Tapia-Hernandez *et al*., 2000), in the rhizosphere as well as inside rice plants, originates from sugarcane residues left over during prior cropping. Some rice varieties sampled in Madhuranthagam and Kanchipuram (Table 3.1) were cultivated as a rotation crop in sugarcane fields. However, *G. diazotrophicus* was also recovered from the inside of rice plants cultivated in some other fields where there was no report of earlier sugarcane cultivation (e.g. strain TNCSF 42, isolated from the root tissue of variety IR 50 cultivated at Thirumangalam, Madurai; Table 3.1). The mechanism of dispersion of *G. diazotrophicus* among non-vegetatively propagated plants such as rice remains to be revealed. The presence of VAM fungi spores, which can carry *G. diazotrophicus* (Paula *et al*., 1991) in soils, the role of mealy bugs, known as host of *G. diazotrophicus* (Ashbolt & Inkerman, 1990; Caballero-Mellado *et al.*, 1995) and commonly occurring pests in sugarcane as well as in rice crops, could play a role.

In the present study the number of colonies of both *G. diazotrophicus* and *A. peroxydans*, isolated from the surface sterilized roots and stems of rice varieties cultivated in flooded fields in South India, was in the range of 10^2-10^3 CFU g^{-1} fresh weight. Previously, it has been reported that the number of *G. diazotrophicus* found in adult sugarcane plants were in the range of 10^5-10^7 CFU g^{-1} fresh tissues (Cavalcante & Döbereiner, 1988; Reis *et al*., 1994), but in mature sugarcane cultivated in Brazil, the number of *G. diazotrophicus* found, was only 10 to 10^2 CFU g^{-1} fresh weight (Reis *et al*., 1994). It has been suggested that nitrogen fertilization in high levels may promote the growth of non-nitrogen-fixing bacteria and simultaneously inhibits the multiplication rate of diazotrophic bacteria (Fuentes-Ramírez *et al*., 1999; Kirchhof *et*

al., 1998; Muthukumarasamy *et al*., 1999). Although, no exact data are available about the levels of nitrogen fertilization applied in the fields where the samples were collected, generally 100–125 kg nitrogen ha^{-1} is recommended in those areas. The low prevalence of AAB in association with rice can also be due to the low amount of their preferential sugar substrates present in the environment of this plant. Recently it has been demonstrated that prevalence and persistence of *G. diazotrophicus* populations also differ according to the sugarcane varieties they harbour (Muñoz-Rojas & Caballero-Mellado, 2003). Because this study is the first report about the natural association of two nitrogen-fixing AAB species with rice plants, it would be of great interest to determine the occurrence and the persistence of these species in other rice varieties cultivated in different regions of India and worldwide. A similar study in South Korea (manuscript in preparation[1]) indicates that *G. diazotrophicus* is naturally associated with South Korean rice variety Hwasambyeo in low numbers (10^4 g^{-1} fresh tissue).

The association of nitrogen-fixing *G. diazotrophicus* and *A. peroxydans* with rice may be important for agriculture as both species may be supplying a part of the nitrogen that rice requires. In the case of sugarcane it has already been proven that *G. diazotrophicus* is a nitrogen contributor (Sevilla *et al*., 2001) and also could even be more beneficial for sugarcane plant growth by mechanisms other than nitrogen fixation (Muñoz-Rojas & Caballero-Mellado, 2003). But in the case of rice plants, though there was a report on the biological nitrogen fixation (BNF) activity (James *et al*., 2000), there is no evidence that any of the associated bacteria or a group of bacteria, reported so far, be responsible for this observed BNF.

ACKNOWLEDGEMENTS

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[1]Muthukumarasamy *et al*. (2007)

3.2. DESCRIPTION OF *GLUCONACETOBACTER SWINGSII* SP. NOV. AND *GLUCONACETOBACTER RHAETICUS* SP. NOV., ISOLATED FROM ITALIAN APPLE FRUIT

Redrafted from:
Dellaglio, F., Cleenwerck, I., Felis, G. E., Engelbeen, K., Janssens, D. & Marzotto, M. (2005). Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit. *Int J Syst Evol Microbiol* 55, 2365-2370.

SUMMARY

Two Gram-negative, rod-shaped, non-spore-forming bacteria (DST GL01^T and DST GL02^T) were isolated from apple fruit juice in the region of the Italian Alpes. On the basis of 16S rRNA gene sequence similarities, strains DST GL01^T and DST GL02^T were shown to belong to the α-subclass of the *Proteobacteria*, and, in particular, to the genus *Gluconacetobacter*, in the *Gluconacetobacter xylinus* branch (98.5-100 %). Chemotaxonomic data (major ubiquinone, Q-10; predominant fatty acid, $C_{18:1}\omega 7c$, accounting for approximately 50 % of the fatty acid content) support the affiliation of both strains to the genus *Gluconacetobacter*. The results of DNA-DNA hybridizations, together with physiological and biochemical data, allowed genotypic and phenotypic differentiation between the strains DST GL01^T and DST GL02^T and from the 11 validly published *Gluconacetobacter* species. They therefore represent two new species, for which the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov. are proposed, with the type strains DST GL01^T (LMG 22125^T = DSM 16373^T) and DST GL02^T (LMG 22126^T = DSM 16663^T), respectively.

INTRODUCTION

Species of the genus *Gluconacetobacter* can be phylogenetically subgrouped in two clusters: 'N_2-fixing' (*Gluconacetobacter diazotrophicus*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter sacchari*, *Gluconacetobacter azotocaptans* and

Gluconacetobacter johannae) and 'cellulose-producing' (*Gluconacetobacter xylinus*, *Gluconacetobacter europeus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter hansenii* and *Gluconacetobacter entanii*). Characterization of a new isolate on the basis of these phenotypic traits might, however, lead to an erroneous identification, as each cluster contains strains lacking these properties (Bernardo *et al.*, 1998; Franke *et al.*, 1999; Fuentes-Ramírez *et al.*, 2001). For instance, the ability to synthesize cellulose is easily lost in many strains and cannot be used as a determinative feature (Swings, 1992).

The species belonging to this genus, most of which have been proposed since 1998 (Boesch *et al.*, 1998; Franke *et al.*, 1999; Fuentes-Ramírez *et al.*, 2001; Schüller *et al.*, 2000; Sokollek *et al.*, 1998b; Yamada, 2000), are strongly correlated at the phylogenetic level.

In this study we present a comprehensive taxonomic analysis of two cellulose-producing strains of AAB (DST GL01^T and DST GL02^T), isolated from apple fruit juice in the South Tyrol region of Italy (Val Venosta), by means of the study of phenotypic and chemotaxonomic properties, the analysis of the phylogenetic marker 16S rRNA gene, the DNA base composition and DNA-DNA relatedness. We provide evidence that the two new isolates represent two different new cellulose-producing species within the genus *Gluconacetobacter*, for which we propose the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov.

MATERIAL AND METHODS

Two cellulose-producing strains DST GL01^T and DST GL02^T were isolated from organic apple juice prepared with fruits from the Val Venosta region in Italy. Both strains were propagated in the synthetic medium ACE (50 g l^{-1} glucose, 5 g l^{-1} yeast extract, pH 6.5) after aerobic incubation at 28 °C for 6 days without shaking. Oxidation of ethanol was verified on Medium 2 (30 g l^{-1} yeast extract, 0.022 g l^{-1} bromocresol green, 2 % (v/v) ethanol, 15 g l^{-1} agar, pH 6.5) described by Swings (1992).

Cell shape and cell size were determined from cells grown aerobically at 28 °C for 3 days on ACE agar medium. Gram staining, oxidase and catalase activity were carried out as described previously (Cleenwerck *et al.*, 2002).

DNA for 16S rRNA gene sequencing was isolated from cellulose-producing cultures (10 ml) preliminarily homogenized by an Ultra Turrax T25 (IKA, Italy) at 8500 r.p.m. for 15 s and filtered through sterile gauzes to eliminate the polysaccharide matrix. Cells

were collected by centrifugation, washed three times, suspended in 0.5 ml of TE buffer (pH 8.0), supplemented with 10 g lysozym l^{-1}, and incubated at 37 °C for 2 h. The DNA was isolated by the CTAB method (Cleenwerck *et al.*, 2002). PCR amplification of the 16S rRNA genes was conducted as described by Boesch *et al.* (1998). Amplification products were purified from a 1 % (w/v) agarose gel by the QIAEX II Gel Extraction System (Qiagen). Sequencing was carried out on purified PCR amplicons at the Bio Molecular Research Center (BMR), University of Padua, Italy. The 16S rRNA gene sequences determined and sequences of strains belonging to the same phylogenetic group, retrieved from the EMBL library, were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics 3.50 software package (Applied Maths, Belgium). Unknown bases were discarded from the calculations. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data. A maximum parsimony analysis was also performed with the program DNAPARS of the PHYLIP package (version 3.5c), using the default options.

Analyses of the respiratory quinones were carried out by the Identification Service of the DSMZ and Dr. Brian Tindall, Braunschweig, Germany. Respiratory lipoquinones were determined from cells grown on Sabouraud-glucose (2 %) liquid medium, pH 5.7, at 29 °C. The respiratory lipoquinones were extracted from 100 mg of freeze-dried cell material based on the two stage method described by Tindall (1990a, b) using methanol:hexane. They were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel (Art. NO. 805 023; Macherey-Nagel), using hexane:tert-butylmethylether (9:1, v/v) as solvent. UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analyzed on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (2 x 125 mm, 3 µm particle size, RP18; Macherey-Nagel) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

Fatty acid profiles were determined from cells grown for 48 hours at 28 °C under aerobic conditions on M129 from the Catalogue of Cultures of the BCCM/LMG Bacteria Collection (Janssens *et al.*, 1998), containing 5.0 % glucose, 0.5 % yeast extract and 1.5 % agar (final pH 6.5). Fatty acids were prepared and identified following the instructions of the MIDI (Microbial Identification) system.

High-molecular-mass DNA for DNA-DNA hybridizations was prepared by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). The type strains were cultured as recommended by the BCCM/LMG Bacteria Collection, except for *G. entanii* for which a pellet of cells was kindly supplied to us by Dr. Hertel

(University of Hohenheim, Germany). Strains LMG 18909^T, LMG 1515^T and DST GL02^T were subjected to a mild alkaline hydrolysis step before cell lysis, as described by Willems *et al.* (2001b). DNA quantity and quality were determined by measuring absorption at 260, 280 and 234 nm. Only high quality DNA with A_{260}/A_{280} and A_{234}/A_{260} ratios of 1.8-2.0 and 0.40-0.60, respectively, was selected for further use. The size of the DNA was estimated by agarose gel electrophoresis. Only high-molecular-mass DNA was used. DNA-DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.*, (1989) (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998). The hybridization temperature was 48 °C. Reciprocal reactions (e.g. A x B and B x A) were performed. The DNA binding values reported are the mean values of a minimum of four hybridization experiments, the reciprocal reactions included.

The G+C content of DST GL01^T and DST GL02^T was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

DST GL01^T and DST GL02^T were phenotypically characterized. The production of 2- and 5-ketogluconic acid was determined by the method described by Gosselé *et al.* (1980). The tolerance to 3 % (v/v) ethanol was tested in acid medium (5 g l^{-1} yeast extract, pH 3.0). The ability to grow on different carbon sources was tested using a standardized and miniaturized assimilation test, ID 32C (Biomérieux), following the manufacturer's guidelines, using bacterial suspensions with an A_{600} of 0.7 to inoculate the galleries. The composition of the minimal medium and the carbon substrates tested are reported in the protocol from the kit. The ability to grow on different carbon sources was determined after 15 days of incubation at 28 °C. Cellulose production was tested in the presence of 1 % (w/v) glucose, 1 % (w/v) fructose, 1 % (w/v) sucrose, 2 % (w/v) ethanol and 2 % (v/v) glycerol in a liquid medium containing 5 g yeast extract l^{-1}.

RESULTS AND DISCUSSION

The two cellulose-producing isolates DST GL01^T and DST GL02^T obtained from organic apple juice were capable to oxidize ethanol to acetic acid and further to CO_2 and H_2O, indicating that they are AAB belonging to *Acetobacter*/*Gluconacetobacter* and not to *Gluconobacter* (Swings, 1992). They consisted of Gram-negative, non-motile coccoid rods, that were catalase-positive and oxidase-negative.

Nearly complete 16S rRNA gene sequences of DST GL01^T and DST GL02^T (1446 bp and 1336 bp, respectively) were determined and compared with 16S rRNA gene

sequences from AAB, retrieved from the EMBL library. The comparison revealed that DST GL01^T and DST GL02^T belonged to the genus *Gluconacetobacter*, and in

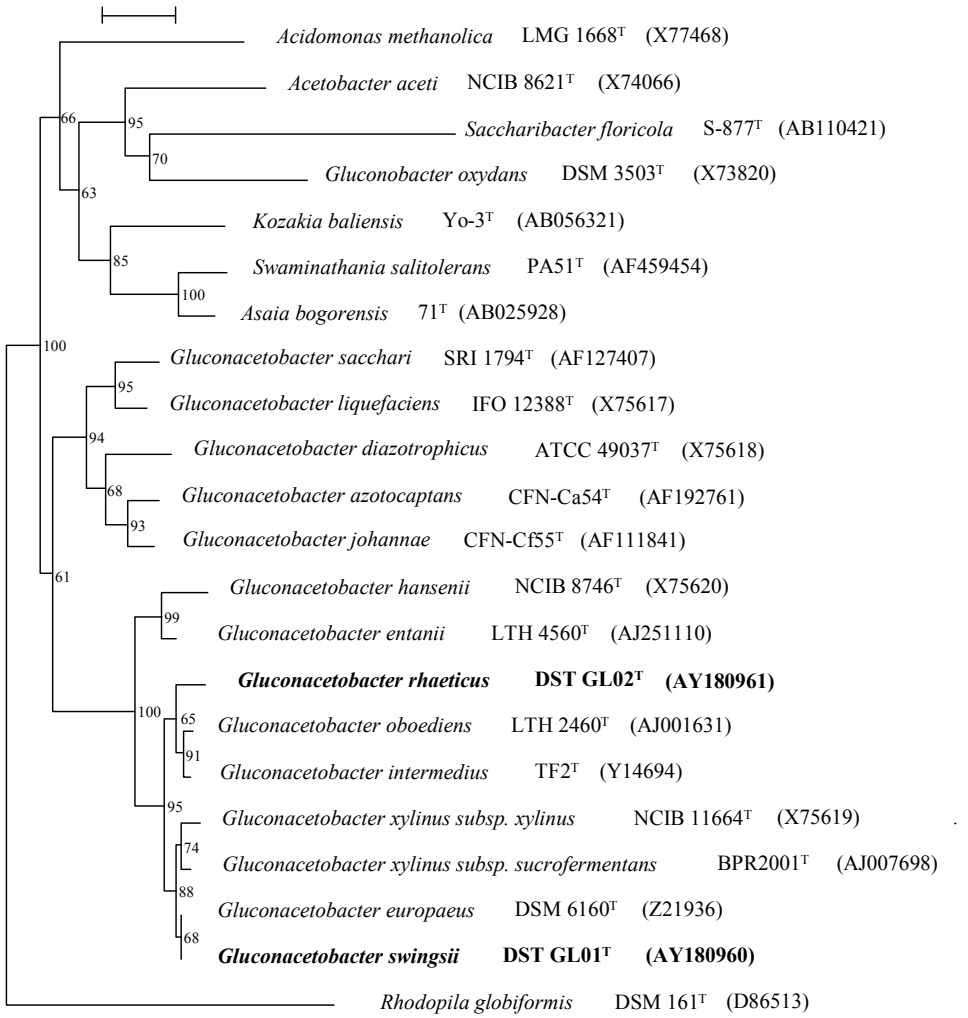

Fig. 3.3. Phylogenetic tree, obtained with the neighbour-joining method and based on almost complete 16S rRNA gene sequences, showing the positions of *Gluconacetobacter swingsii* DST GL01^T and *Gluconacetobacter rhaeticus* DST GL02^T within the acetic acid bacteria. *Rhodopila globiformis* DSM 161^T was used as an outgroup in the analysis. Bar, 1 % sequence dissimilarity. Numbers at branching points indicate bootstrap percentages derived from 1000 samples. Bar, 1 nt substitution per 100 nt.

particular to the *G. xylinus* branch. The 16S rRNA gene sequences of DST GL01^T and DST GL02^T showed 98.5-100 % similarity to sequences of reference strains of the *G. xylinus* branch. The levels of similarity to the 16S rRNA gene sequences of other

validly described species of the *Acetobacteraceae* family were below 97.2 %. A neighbour-joining tree, reflecting the positions of these strains within the AAB lineage, is shown in Fig. 3.3. Parsimony analysis confirmed the phylogenetic placement of the strains in the 'cellulose-producing' *Gluconacetobacter* species cluster.

DST GL01^T and DST GL02^T contained Q-10 as major ubiquinone, a common feature of strains belonging to the genus *Gluconacetobacter* (Yamada *et al.*, 2000), and large amounts of the fatty acid $C_{18:1}\omega 7c$ (approximately 50 % of the fatty acid content). The other fatty acids found in smaller but still significant amounts were $C_{16:0}$ (13.1 %), $C_{17:0}$ (9.8 %) and $C_{16:0}$ 2-OH (6.6 %) for DST GL01^T and $C_{14:0}$ 2-OH (19.5 %), $C_{16:0}$ 2-OH (16.5 %) and $C_{16:0}$ (6.9 %) for DST GL02^T. The fatty acid profiles of DST GL01^T and DST GL02^T are comparable to those obtained for other *Gluconacetobacter* species (Franke *et al.*, 1999; Urakami *et al.*, 1989).

DST GL01^T and DST GL02^T were hybridized with the type strains of the 'cellulose-producing' *Gluconacetobacter* species: *G. oboediens* LMG 18849^T, *G. intermedius* LMG 18909^T, *G. europaeus* LMG 18890^T, *G. xylinus* subsp. *xylinus* LMG 1515^T, *G. xylinus* subsp. *sucrofermentans* LMG 18788^T, *G. hansenii* LMG 1527^T and *G. entanii* LTH 4560^T. The level of DNA-DNA binding between DST GL01^T and DST GL02^T (45 %) and between these strains and the type strains of the *G. xylinus* branch (≤ 60 %) demonstrates a relatedness below the species level.

In this study the DNA-DNA binding values between the type strains of the *G. xylinus* branch were also determined (except for *G. entanii* for which it is very difficult to obtain enough DNA for hybridizations) to obtain a clear view on the DNA-DNA relatedness between these strains (see Table 3.4). From the current literature data, this was not really possible due to multiple factors. The reported DNA binding values are difficult to compare with each other as in many cases different DNA-DNA hybridization methods were used. DNA binding values between some of these species are only vaguely reported, such as the DNA binding values between *G. intermedius* and *G. xylinus* and between *G. intermedius* and *G. hansenii* (DNA binding values are below 60 %). But most of all, DNA binding values between some species have not been reported, such as those between *G. oboediens* and *G. intermedius*, *G. oboediens* and *G. hansenii*, *G. entanii* and *G. intermedius*. The DNA binding value obtained between *G. intermedius* and *G. europaeus* (57 %) is comparable to the 60 % reported by Boesch *et al.* (1998). The values between *G. oboediens* and *G. intermedius* (63 %), and between *G. oboediens* and *G. hansenii* (24 %), demonstrate that they represent different species. The DNA binding values between *G. oboediens* and *G. xylinus* (68 %), and between *G. oboediens* and *G. europaeus* (51 %) are higher than the values reported by Sokollek *et*

al. (1998b) (34 and 25 %, respectively). The discrepancy between these data is probably due to the fact that Sokollek *et al.* (1998b) did not perform reciprocal reactions, which are very important to obtain unequivocal results. The DNA homology value between *G. xylinus* subsp. *xylinus* and *G. xylinus* subsp. *sucrofermentans* (56 %) is comparable to the 58.2 % reported by Toyosaki *et al.* (1995). This value is lower than DNA binding values found between some other species in the *G. xylinus* branch and therefore the question could be raised whether *G. xylinus* subsp. *sucrofermentans* should be elevated at the species level.

Table 3.4. DNA-DNA binding values (%) between DST GL01^T and DST GL02^T and the type strains of related *Gluconacetobacter* species

The variation between reciprocal hybridizations and repeated experiments is ± 7 % (Goris *et al*., 1998). The DNA relatedness percentages reported are the mean of minimum 4 hybridization experiments, the reciprocal reactions included. The value between brackets is the difference that was found between the reciprocal values. Abbreviation: ND, not determined.

Strain	1	2	3	4	5	6	7	8	9
1. *G. oboediens* LMG 18849^T	100								
2. *G. intermedius* LMG 18909^T	63 (18)	100							
3. *G. europaeus* LMG 18890^T	68 (15)	57 (7)	100						
4. *G. xylinus* subsp. *xylinus* LMG 1515^T	51 (6)	50 (4)	50 (5)	100					
5. *G. xylinus* subsp. *sucrofermentans* LMG 18788^T	39 (6)	38 (30)	25 (2)	56 (25)	100				
6. *G. hansenii* LMG 1527^T	24 (5)	19 (3)	24 (9)	16 (2)	13 (17)	100			
7. *G. entanii* LTH 4560^T	ND	ND	ND	ND	ND	ND	100		
8. *G. swingsii* DST GL01^T	52 (26)	57 (12)	60 (4)	49 (19)	33 (10)	20 (11)	16 (0)	100	
9. *G. rhaeticus* DST GL02^T	34 (10)	35 (12)	33 (10)	37 (6)	38 (18)	21 (2)	16 (0)	45 (6)	100

The G+C content of the DNA's from DST GL01^T and DST GL02^T was determined and was 61.7 and 63.4 mol%, respectively.

The phenotypic characteristics of DST GL01^T and DST GL02^T are given in the species descriptions below. The characteristics that differentiate the two strains from each other and from their phylogenetically closest neighbours are given in Table 3.5 (additional characteristics differentiating DST GL01^T from DST GL02^T are available in Table 3.6). DST GL01^T can be differentiated from *G. xylinus* by the ability to grow on 3 % ethanol in the presence of 4-8 % acetic acid, the ability to grow on ethanol as carbon sources and the ability to grow in the presence of 30 % D-glucose. It can be differentiated from *G. europaeus* by the ability to grow in the absence of acetic acid, the ability to grow on ethanol, sucrose, sorbitol, D-mannitol and D-gluconate as carbon

sources and the inability to grow on D-ribose as carbon source. DST GL02^T can be differentiated from *G. oboediens* by the ability to produce 5-keto-D-gluconic acid from D-glucose, the ability to grow on ethanol, D-xylose, sorbitol and D-mannitol as carbon sources and the inability to grow on D-gluconate as carbon source. It can be differentiated from *G. intermedius* by the ability to produce 2- and 5-keto-D-gluconic acid from D-glucose. DST GL01^T and DST GL02^T did not grow in liquid medium shaken on a rotary shaker. Their cell proliferation seems to be strictly correlated to cellulose production (Kamide *et al.*, 1990).

Table 3.5. Characteristics differentiating *G. swingsii* sp. nov. and *G. rhaeticus* sp. nov. from each other and from the phylogenetically closely related *Gluconacetobacter* species

Species: 1, *G. swingsii* sp. nov.; 2, *G. rhaeticus* sp. nov.; 3, *G. oboediens* (data from Sokollek *et al.*, 1998b); 4, *G. intermedius* (data from Boesch *et al.*, 1998); 5, *G. xylinus* (data from Sokollek *et al.*, 1998b); 6, *G. europaeus* (data from Sokollek *et al.*, 1998b); 7, *G. hansenii* [data from Gosselé *et al.* (1998) and Navarro *et al.* (1999)]; 8, *G. entanii* (data from Schüller *et al.*, 2000). Symbols: +, positive; -, negative; v, variable; w, weak ; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8
Production of keto-D-gluconates from D-glucose:								
2-Keto-D-gluconate	+	+	+	-	+	NR	+	-
5-Keto-D-gluconate	+	+	-	-	+	NR	+	-
Growth without acetic acid	+	+	+	+	+	-	+	-
Growth on 3 % ethanol in the presence of 4-8 % acetic acid	+	+	+	NR	-	+	-	+
Growth on carbon sources:								
Ethanol	+	+	-	NR	-	-	-	NR
D-Fructose	+	+	+	NR	+	+	v	+
Maltose	+	+	+	NR	+	+	v	+
D-Ribose	-	+	+	NR	-	+	-	NR
D-Xylose	+	+	-	NR	+	+	-	NR
Sucrose	+	+	+	NR	+	-	v	+
Sorbitol	+	+	-	NR	+	-	-	W
D-Mannitol	+	+	-	NR	+	-	v	-
D-Gluconate	+	-	+	NR	+	-	v	-
Growth in the presence of 30 % D-glucose	+	+	+	NR	-	-	-	NR

Table 3.6. Additional characteristics that distinguish *G. swingsii* sp. nov. from *G. rhaeticus* sp. nov.

Characteristic	1	2
Growth on carbon sources:		
Erythritol, D-Galactose, L-Arabinose, Melibiose, Palatinose, Rhamnose	-	+
Glucuronate, Glucosamine	+	-
Cellulose production from:		
Sucrose 1 % (w/v), Fructose 1 % (w/v)	-	+

Overall, the results obtained indicate that DST GL01^T and DST GL02^T represent two novel species of the genus *Gluconacetobacter*, for which we propose the names *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., respectively.

Description of *Gluconacetobacter swingsii* sp. nov.

Gluconacetobacter swingsii (swing´.si.i. N.L. gen. n. *swingsii* of Swings, in honour of Jean Swings who studied and reviewed AAB).

Cells are Gram-negative, coccoid, approximately 0.9 x 1.5-2.5 μm in size, occurring singly or in pairs. Cells are non-motile. Endospores are not detected. Colonies are beige, regular, convex and smooth with a diameter of 0.8 mm on M129. Oxidase-negative. Catalase-positive. D-glucose is oxidized to 2- and 5- keto-D-gluconic acid. Acetic acid is not required for growth. Growth is observed in 3 % (v/v) ethanol in acid medium, pH 3.0. Growth occurs on cellobiose, D-xylose, maltose, D-mannitol, melezitose, raffinose, sorbitol, sorbose, trehalose, D-gluconate, glucuronate and glucosamine as carbon source, but not on erythritol, galactose, L-arabinose, melibiose, palatinose, rhamnose and D-ribose as carbon source. Cellulose is produced from D-glucose, ethanol and glycerol, and not from D-fructose and sucrose. The ubiquinone system consists of Q-10 as major and Q-9 as minor components. $C_{18:1}\omega 7c$ is the major fatty acid (50 %), other fatty acids in smaller but still significant amounts are $C_{16:0}$ (13.1 %), $C_{17:0}$ (9.8 %) and $C_{16:0}$ 2-OH (6.6 %). The DNA G+C content of the type strain is 61.7 %. Isolated from apple juice in South Tyrol region, Italy. The type strain is DST GL01^T (= LMG 22125^T = DSM 16373^T).

Description of *Gluconacetobacter rhaeticus* sp. nov.

Gluconacetobacter rhaeticus (rhae´.ti.cus L. masc. adj. *rhaeticus* pertaining to Rhaetia, Latin denomination of South Tyrol region in Italy, where the type strain was isolated).

Cells are Gram-negative, coccoid, approximately 0.9 x 1.5-2.5 µm in size, occurring singly, in pairs or in short chains. Cells are non-motile. Endospores are not detected. Colonies are beige, regular, convex and weakly rough with a diameter of 1.0 mm on M129. Oxidase-negative. Catalase-positive. D-glucose is oxidized to 2- and 5- keto-D-gluconic acid. Acetic acid is not required for growth. Growth is observed in 3 % (v/v) ethanol in acid medium, pH 3.0. Growth occurs on cellobiose, D-xylose, maltose, D-mannitol, melezitose, raffinose, sorbitol, sorbose, trehalose, erythritol, galactose, L-arabinose, melibiose, palatinose, rhamnose and D-ribose as carbon source, but not on D-gluconate, glucuronate and glucosamine as carbon source. Cellulose is produced from D-glucose, ethanol, glycerol, fructose and sucrose. The ubiquinone system consists of Q-10 components. $C_{18:1}\omega 7c$ is the major fatty acid (50 %), other fatty acids in smaller but still significant amounts are $C_{14:0}$ 2-OH (19.5 %), $C_{16:0}$ 2-OH (16.5 %) and $C_{16:0}$ (6.9 %). The DNA G+C content of the type strain is 63.4 %. Isolated from apple juice in South Tyrol region, Italy. The type strain is DST GL02^T (= LMG 22126^T = DSM 16663^T).

ACKNOWLEDGEMENTS

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3.3. *ACETOBACTER OENI* SP. NOV., ISOLATED FROM SPOILED RED WINE

Redrafted from:
Silva, L. R., Cleenwerck, I, Rivas, R., Swings, J., Trujillo, M. E., Willems, A. & Velázquez, E. (2006). *Acetobacter oeni* sp. nov., isolated from spoiled red wine. *Int J Syst Evol Microbiol* 56, 21-24.

SUMMARY

A bacterial strain, designated B13^T, was isolated from spoiled red wine in the Dão region, Portugal. The strain was Gram-negative, strictly aerobic, rod-shaped and motile. Phylogenetic analysis on the basis of 16S rRNA gene sequences indicated that B13^T belonged to the genus *Acetobacter* within the *Alphaproteobacteria*. The closest related species was *Acetobacter aceti* with 98.4 % 16S rRNA gene sequence similarity. DNA-DNA hybridizations showed that B13^T constituted a taxon separate from the *Acetobacter* species with validly published names. The DNA G+C content of B13^T was 58.1 mol%. Phenotypic characteristics of B13^T allowed its differentiation from the recognized *Acetobacter* species. B13^T produced 5-ketogluconic acid from glucose, but no 2-ketogluconic acid. It produced catalase but no oxidase. It utilized glycerol, but not maltose, ethanol or methanol as carbon sources. On the basis of the results obtained, B13^T represents a novel species for which the name *Acetobacter oeni* sp. nov. is proposed. The type strain is B13^T (= LMG 21952^T, CECT 5830^T).

INTRODUCTION

The genus *Acetobacter* currently comprises 23 species with validly published names, nine of which have been subsequently transferred to other genera. The *Acetobacter* species were delineated mainly on the basis of DNA-DNA relatedness and phylogenetic relationships (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2000, 2001; Sokollek *et al.*, 1998b). In the present study we describe a novel *Acetobacter* species, isolated from spoiled red wine.

MATERIAL AND METHODS

During a study of red wine from the Dão region, that has been spoiled due to the presence of volatile phenols produced by *Dekkera bruxellensis* (Silva *et al.*, 2004), a bacterial strain, B13^T, was recovered. The strain was isolated by spreading 1 ml spoiled red wine samples aseptically on YEDC plates (glucose 7 g l^{-1}, yeast extract 3 g l^{-1}, calcium carbonate 20 g l^{-1} and agar 17 g l^{-1}).

Strain B13^T was grown on nutrient agar during 48 h at 22 °C to check for motility by phase-contrast microscopy. Gram staining of cells was carried out according to the classical Gram procedure described by Doetsch (1981).

DNA for 16S rRNA gene sequencing, was extracted as described by Rivas *et al.* (2001). Amplification and sequencing of the nearly complete 16S rRNA gene were performed as described previously (Rivas *et al.*, 2003). The sequence determined was compared with sequences from GenBank using the BLAST program (Altschul *et al.*, 1990). Phylogenetic analysis was performed using the BioNumerics 4.0 software package (Applied Maths, Belgium). The sequence determined was aligned with similar sequences retrieved from the EMBL database. Nucleotide substitution rates were calculated using the two-parameter method of Kimura (1980). Bootstrap analysis based on 1000 replications was undertaken to test the robustness of the phylogenetic tree (Felsenstein, 1985).

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). DNA-DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. A x B and B x A) were performed and the variation between them was within the limits of this method (Goris *et al.*, 1998). The DNA-DNA relatedness percentages presented are the mean of minimum 4 hybridization experiments, including the reciprocal reactions. The DNA G+C content was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference.

Phenotypic characteristics were examined as described by Cleenwerck *et al.* (2002).

RESULTS AND DISCUSSION

Strain B13^T grew well on YEDC plates and produced large haloes due to the solubilization of the calcium carbonate by the large amounts of acid secreted by the strain. On this agar, the colonies from the new isolate were mucoid and opaque cream.

Strain B13^T was a Gram negative, rod-shaped and motile organism with peritrichous flagella (0.8-0.9 x 3.6-5.1 μm).

The nearly complete 16S rRNA gene sequence of B13^T (1484 nucleotides) was obtained. B13^T was located in the *Alphaproteobacteria*, within the genus *Acetobacter*.

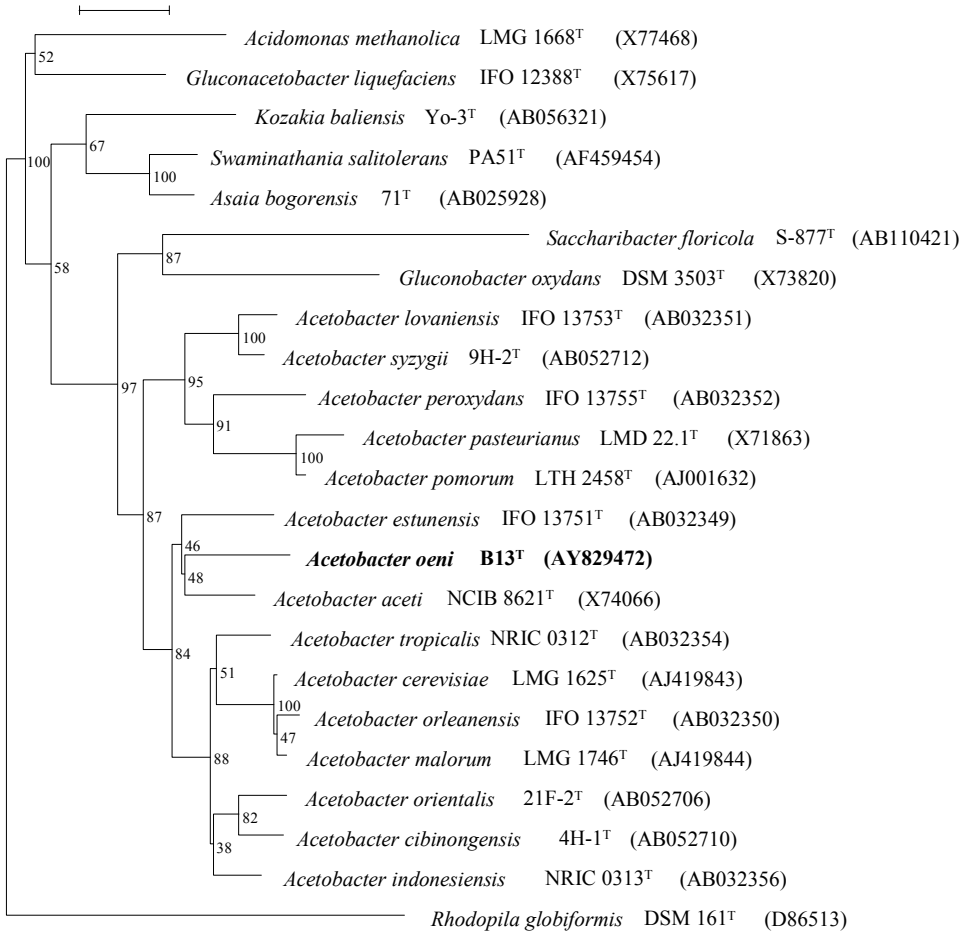

Fig. 3.4. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter oeni* B13^T and other related species of the family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

The sequence similarities of B13^T to the type strains of *Acetobacter aceti*, *Acetobacter tropicalis*, *Acetobacter estunensis*, *Acetobacter cerevisiae*, *Acetobacter indonesiensis*, *Acetobacter malorum*, *Acetobacter orleanensis*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Acetobacter pomorum*, *Acetobacter pasteurianus* and *Acetobacter peroxydans* were 98.3, 98.1, 98.0, 97.9, 97.9, 97.8, 97.6, 97.6, 97.5, 97.4, 97.1, 97.0, 96.7, and 96.4 % respectively. Fig. 3.4 shows the phylogenetic position of strain B13^T within the genus *Acetobacter*.

Strain B13^T showed low DNA-DNA relatedness (≤ 20 %) with the type strains of *A. aceti* (13 %), *A. tropicalis* (14 %), *A. estunensis* (20 %), *A. cerevisiae* (13 %), *A. indonesiensis* (7 %), *A. malorum* (5 %), *A. orleanensis* (6 %), *A. orientalis* (6 %), *A. cibinongensis* (7 %), *A. syzygii* (10 %), *A. lovaniensis* (15 %), *A. pomorum* (12 %), *A. pasteurianus* (16 %) and *A. peroxydans* (13 %). The DNA G+C content of B13^T was 58.1 mol%.

The phenotypic characteristics of B13^T are described in the species description. Phenotypic characteristics differentiating B13^T from the validly described *Acetobacter* species are given in Table 3.7. Strain B13^T can be differentiated from the other species of the genus *Acetobacter* on the basis of 2- and /or 5- keto-D-gluconic acid production from D-glucose, which are the main diagnostic characters for this genus. The ability of the strain to grow in the presence of 10 % ethanol also distinguishes strain B13^T from most *Acetobacter* species. Moreover, strain B13^T differs from the closest phylogenetically related species, *A. aceti* and *A. estunensis*, as it is unable to grow with ammonium as the sole nitrogen source with ethanol as the carbon source.

The results presented above allow the genotypic and phenotypic differentiation of B13^T from the 14 *Acetobacter* species with validly published names. Strain B13^T should therefore be classified as representing a novel species for which we propose the name *Acetobacter oeni* sp. nov.

Description of *Acetobacter oeni* sp. nov.

Acetobacter oeni (oe'ni. N.L. gen. n. *oeni* of wine from Gr. gen. n. *oinou* of wine).

Cells are motile non-spore forming rods, 3.6-5.1 μm in length and 0.8-0.9 μm in diameter. Gram negative, strictly aerobic. The optimal growth temperature on YEDC is 28 °C. Colonies on YEDC are circular convex, cream, opaque and usually 1 to 2 mm in

Table 3.7. Differential characteristics of *Acetobacter oeni* B13^T compared with closely related species

Taxa: 1, *A. oeni* B13^T; 2, *A. cerevisiae* (4 stra ins); 3, *A. malorum* LMG 1746 ^{T}; 4, *A. pasteurianus* (7 strains); 5 , *A. pomorum* LMG 18848 ^{T}; 6, *A. peroxydans* (2 strains); 7, *A. lovaniensis* LMG 1617^T; 8, *A. orleanensis* (4 strains); 9, *A. indonesiensis* (2 strains); 10, *A. tropicalis* (2 strains); 11 , *A. estunensis* (3 strains); 12, *A. aceti* (4 strains); 13, *A. syzygii* LMG 21419^T; 14, *A. cibinongensis* LMG 21418 ^{T}; 15, *A. orientalis* LMG 21417 ^{T}. +, Positive; -, negative; w, weak positive; v, variable; ND, not determined. Unless indicated otherwise, data for taxa 1 and 13-15 were obtained in this study; data for taxa 2-12 were taken from Cleenwerck *et al.* (2002).

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Formation from D-glucose:															
5-keto-D-gluconic acid	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-
2-keto-D-gluconic acid	-	+	+	v	-	-	+	+	+	+	+	+	-	+	+
Growth in ammonium with ethanol	-	-	-	-	-	+	+	-	-	-	+	+	-	w	-
Growth on carbon sources:															
Glycerol	+	+	+	v	+	-	+	+	+	+	v	+	+	+	+
Maltose	-	-	-	v	-	+	-	v	+	+	-	v	+	-	w
Methanol	-	-	+	-	-	-	+	-	-	-	-	-	-	-	w
Growth in presence of 10 % ethanol	+	ND	ND	+*	-*	-*	-*	-*	-*	-*	-*	-*	-*	-*	-*
Growth on YE + 30 % D-glucose	-	-	+	v	-	-	-	-	-	-	-	-	-	+	-
Catalase	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
DNA G+C content (mol%)	58.1	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*

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

diameter within 4 days growth at 28 °C. Produces 5-keto-D-gluconic acid from D-glucose but no 2-keto-D-gluconic acid. Oxidizes D-glucose in media containing ammonium nitrate as nitrogen source, but is unable to ferment D-glucose in the same media. Produces catalase, but no oxidase. Unable to grow with ammonium as the nitrogen source with ethanol as carbon source. Growth in the presence of 10 % ethanol. Utilizes glycerol as a carbon source, but not maltose or methanol. No growth in the presence of 30 % D-glucose. The DNA G+C content of the type strain is 58.1 mol%. The type strain B13^T (= LMG 21952^T = CECT 5830^T), was isolated from spoiled red wine of the Dão region, Portugal.

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3.4. *ACETOBACTER SENEGALENSIS* SP. NOV., A THERMOTOLERANT ACETIC ACID BACTERIUM ISOLATED IN SENEGAL (SUB-SAHARAN AFRICA) FROM MANGO FRUIT (*MANGIFERA INDICA* L)

Redrafted from:

Ndoye, B., Cleenwerck, I., Engelbeen, K., Dubois-Dauphin, R., Guiro, A. T., Van Trappen, S., Willems, A. & Thonart, P. (2007a). *Acetobacter senegalensis* sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (*Mangifera indica* L.). *Int J Syst Evol Microbiol* 57, 1576-1581.

SUMMARY

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 hybridizations 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).

INTRODUCTION

The taxonomy of AAB has substantially changed in the last years. According to De Ley *et al.* (1984a), AAB were classified in the family *Acetobacteraceae* in two genera, *Acetobacter* and *Gluconobacter*. At the time of writing, AAB are classified in 9 genera namely *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconoacetobacter*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathania* and *Neoasaia* in the family *Acetobacteraceae*, class *Alphaproteobacteria* (Cleenwerck *et al.*, 2002; Jojima *et al.*, 2004; Loganathan & Nair, 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 *et al.*, 2002). In tropical areas such as sub-Saharan Africa, where the ambient temperature is regularly above 30 °C, industrial 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 (Ndoye *et al.*, 2006; Sow *et al.*, 2005).

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 (submitted for publication[1]) and were evaluated as freeze-dried starters in an acidification process (Ndoye *et al.*, 2007c). The present study deals with the determination of the taxonomic position of strain CWBI-B418^T in the genus *Acetobacter*. The polyphasic characterisation 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 valorisation 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*.

[1]Ndoye *et al.* (2007b)

MATERIAL AND METHODS

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; w/v) by incubation at 28 °C under aerobic conditions for 1-3 days and then checked for purity.

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). Conventional biochemical tests were performed according to standard methods previously described in the ninth edition of Bergey's Manual of Systematic Bacteriology (De Ley *et al.*, 1984a). Oxidase activity was tested using 1 % N, N, N', N'-tetramethyl *p*-phenylenediamine. Catalase activity was tested by adding young cells to a drop of a 10 % H_2O_2 solution and observing production of O_2.

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 (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2001) showed the existence of two stable sublineages in *Acetobacter*. In the present study a new phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987) using the BioNumerics 4.5 software package (Applied Maths, Belgium) to refine its phylogenetic position. Unknown bases were discarded from the calculations. Bootstrapping analysis (Felsenstein, 1985) was undertaken to test statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data.

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). DNA-DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. A x B and B x A) were performed and the variation between them was within the limits of this method, of which the mean standard deviation is ± 7 % (Goris *et al.*, 1998). The DNA-DNA relatedness percentages presented are the mean of minimum 4 hybridization experiments, including the reciprocal reactions. 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 lambda DNA (Sigma) was used as calibration reference.

Phenotypic characteristics for differentiation of CWBI-B418^T from recognized *Acetobacter* species were examined as described by Cleenwerck *et al.* (2002). Additional phenotypic characteristics of strain CWBI-B418^T were determined using API 50CH strips (Biomérieux), following the manufacturer's guidelines for oxidation. The bacterial suspensions were made 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 of incubation at 30 °C.

RESULTS AND DISCUSSION

Strain CWBI-B418^T grew well on medium 13 agar from the BCCM/LMG *Catalogue of Cultures* medium at 28 °C and appeared as visible, opaque, beige colonies within 1 day of incubation. Small colonial 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 an optimal growth at 35 °C on YGM medium (1 % yeast extract, 2 % D-glucose, 2 % D-mannitol; w/v; with 2.5 % ethanol and 0.5 % acetic acid added aseptically after sterilization; v/v), but slow growth at 42 °C (Ndoye *et al.*, 2006).

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. Strain CWBI-B418^T was oxidase-negative and catalase-positive. It was able to oxidize ethanol to acetic acid and acetate and lactate to CO_2 and H_2O, two biochemical properties typical of certain AAB.

Fig. 3.5 shows that strain CWBI-B418^T belonged to the genus *Acetobacter* and more precisely to the stable subcluster containing *Acetobacter oeni*, *Acetobacter estunensis*, *Acetobacter nitrogenifigens*, *Acetobacter aceti*, *Acetobacter tropicalis*, *Acetobacter cerevisiae*, *Acetobacter orleanensis*, *Acetobacter malorum*, *Acetobacter orientalis*, *Acetobacter cibinongensis* and *Acetobacter 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. estunensis*, 98.0 % for *Acetobacter*

syzygii, 98.0 % for *Acetobacter pomorum*, 97.9 % for *A. aceti*, 97.8 % for *Acetobacter lovaniensis*, 97.7 % for *Acetobacter pasteurianus*, 97.5 % for *Acetobacter nitrogenifigens* and 97.5 % for *Acetobacter peroxydans*.

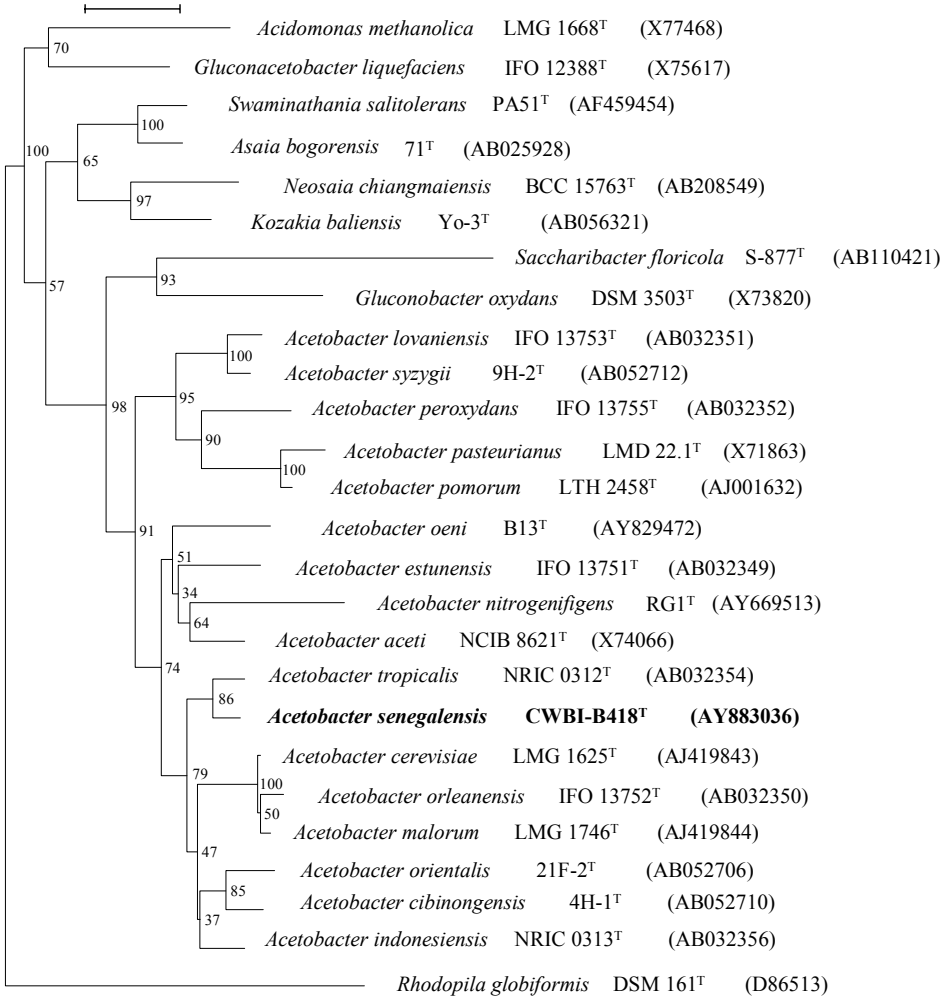

Fig. 3.5. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter senegalensis* CWBI-B418^T and related species of the family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

Strain CWBI-B418^T was hybridized to all phylogenetically related recognized *Acetobacter* species to determine whether it belonged to one of these species. Strain CWBI-B418^T showed an intermediate level of DNA-DNA relatedness with *A. tropicalis*

Table 3.8. Differential characteristics between strain CWBI-B418^T and related *Acetobacter* species

Taxa are listed as: 1, *A. cerevisiae* (4 strains); 2, *A. malorum* LMG 1746^T; 3, *A. pasteurianus* (7 strains); 4, *A. pomorum* LMG 18848^T; 5, *A. peroxydans* (2 strains); 6, *A. lovaniensis* LMG 1617^T; 7, *A. orleanensis* (4 strains); 8, *A. indonesiensis* (2 strains); 9, *A. tropicalis* (2 strains); 10, *A. estunensis* (3 strains); 11, *A. aceti* (4 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* RG 1^T; 17, *A. senegalensis* 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); data for taxon 16 were taken from Dutta & Gachhui (2006); data for taxon 17 were obtained in this study; except 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	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	ND	-
2-Keto-D-gluconic	+	+	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	+[1]	+[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	-[2]	+[2]	+	+
Growth on YE + 30 % D-Glucose	-	+	v	-	-	-	-	-	-	-	-	-	+	-	-	+	+
Catalase	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+		+
Motility	-	-	v[2]	-[3]	+[2]	v (mostly +)[2]	v[2]	v[2]	v[2]	+[2]	v[2]	+[2]	-[2]	v (mostly +)[2]	+	+	+
G+C content of DNA (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[2]	53.8-54.5[2]	52.0-52.8[2]	58.1	64.1	56.0

[1]Data obtained in this study.

[2]Data taken from Lisdiyanti *et al*. (2000, 2001).

[3]Data taken from Sokollek *et al*. (1998).

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* 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 (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987), the results prove that strain CWBI-B418^T represents a novel genospecies within the genus *Acetobacter*. 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* (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2000, 2001; Silva *et al.*, 2006).

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 3.8). 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 (Table 3.8), but also by the ability to produce acid from D-glucose and D-xylose aerobically (Table 3.9).

Table 3.9. Oxidation of carbon sources by the type strains of *A. senegalensis* and *A. tropicalis*

+++, Positive after 1 day; ++, positive after 2 days; +, positive after 3 days; -, negative after 3 days.

Carbon sources	***A. tropicalis* LMG 19825^T**	***A. senegalensis* CWBI-B418^T**
N-Acetyl-glucosamine	+	+
Aesculine	+++	+++
Fructose	+	+
D-Galactose	+	+
D-Glucose	-	+
Glycerol	+	+
Maltose	-	-
Mannitol	+	+
Ribose	+	+
Sorbitol	-	-
D-Xylose	-	+

The results presented above allow the genotypic and phenotypic differentiation of strain CWBI-B418^T from the all 15 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. 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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CHAPTER 4

EVALUATION OF THE DNA FINGERPRINTING TECHNIQUES REP-PCR AND AFLP® FOR IDENTIFICATION AND CLASSIFICATION OF AAB

4.1. VALIDATION OF THE $(GTG)_5$-REP-PCR FINGERPRINTING TECHNIQUE FOR RAPID CLASSIFICATION AND IDENTIFICATION OF AAB, WITH A FOCUS ON ISOLATES FROM GHANAIAN FERMENTED COCOA BEANS

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SUMMARY

Amplification of repetitive bacterial DNA elements through the polymerase chain reaction (rep-PCR fingerprinting) using the $(GTG)_5$ primer, referred to as $(GTG)_5$-PCR fingerprinting, was found a promising genotypic tool for rapid and reliable species identification of AAB. The method was evaluated with 64 AAB reference strains, including 31 type strains, and 132 isolates from Ghanaian, fermented cocoa beans, and was validated with DNA-DNA hybridization data. Most reference strains, except for example all *Acetobacter indonesiensis* strains and *Gluconacetobacter liquefaciens* LMG 1509, grouped according to their species designation, indicating the usefulness of this technique for identification at the species level. Moreover, exclusive patterns were obtained for most strains, suggesting that the technique can also be used for characterization below species level or typing of AAB strains. $(GTG)_5$-PCR fingerprinting allowed us to differentiate four major clusters among the fermented cocoa bean isolates, namely *A. pasteurianus* (cluster I, 100 isolates), *A. syzygii*- or *A. lovaniensis*-like (cluster II, 23 isolates) and *A. tropicalis*-like (clusters III and IV containing 4 and 5 isolates, respectively). *A. syzygii*-like and *A. tropicalis*-like strains from cocoa bean fermentations were reported for the first time. Validation of the method and indications for reclassifications of some AAB and existence of new

Acetobacter species were obtained through 16S rRNA gene sequencing analyses and DNA-DNA hybridizations. Reclassifications refer to *A. aceti* LMG 1531, *Ga. xylinus* LMG 1518 and *Ga. xylinus* subsp. *sucrofermentans* LMG 18788^T.

INTRODUCTION

AAB are Gram-negative, ellipsoidal to rod-shaped, obligate aerobic bacteria that oxidize alcohols or sugars incompletely, leading to the accumulation of organic acids as end-products (De Ley *et al.*, 1984a; Sievers & Swings, 2005; Swings *et al.*, 1992). They are widespread in nature and most of them are capable of oxidizing ethanol as substrate to acetic acid in neutral and acidic (pH 4.5) media. These characteristics make that they are involved in the production of fermented foods, either in a beneficial (chocolate products, coffee, vinegar, nata de coco, and specialty beers) or detrimental (spoilage of beers, wines, and ciders) way, and in the production of commercially important fine chemicals (Kersters *et al.*, 2006).

Today, little is known about AAB associated with cocoa bean fermentation, the first step in cocoa and chocolate production (Ardhana & Fleet, 2003; Carr *et al.*, 1979*;* Ostovar & Keeney, 1973*;* Passos & Passos, 1985*;* Schwan & Wheals, 2004; Thompson *et al.*, 1997). During this spontaneous fermentation, AAB oxidize ethanol, initially produced by yeasts, to acetic acid. This volatile acid diffuses into the beans and this, in combination with heat produced by this exothermic bioconversion, causes the 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 a characteristic aroma, flavour, and colour of the beans (Hansen *et al.*, 1998). These properties are further developed during drying, roasting, and final processing of well-fermented cocoa beans (Thompson *et al.*, 2001b). The activity of AAB is thus essential for the production of high-quality cocoa. Strains of *Acetobacter* and *Gluconobacter* are mostly found (Schwan & Wheals, 2004).

The taxonomy of AAB has undergone many changes in recent years. Several genera and species of AAB have been newly described. AAB are currently classified into ten genera and 45 species, namely *Acetobacter* (16 species), *Gluconobacter* (5 species), *Acidomonas* (1 species), *Gluconacetobacter* (15 species), *Asaia* (3 species), *Kozakia* (1 species), *Saccharibacter* (1 species), *Swaminathania* (1 species), *Neoasaia* (1 species), and *Granulibacter* (1 species), in the family *Acetobacteraceae* as a branch of the

acidophilic bacteria in the α-subdivision of the *Proteobacteria* (Dellaglio *et al.*, 2005*;* Dutta & Gachhui, 2006*;* Greenberg *et al.*, 2006b*;* Jojima *et al.*, 2004*;* Lisdiyanti *et al.*, 2006*;* Loganathan & Nair, 2004*;* Sievers & Swings, 2005*;* Silva *et al.*, 2006*;* Tanasupawat *et al.*, 2004*;* Yukphan *et al.*, 2004b, c, 2005).

As AAB are involved in the production of food, their identification is important information for technologists, who try to control the bioprocesses involved. The identification of AAB to species level has traditionally been performed by studying physiological and chemotaxonomic properties such as growth at low pH and growth in the presence of 0.35 % (v/v) acetic acid, production of acetic acid and gluconic acid from ethanol and D-glucose, respectively, the presence of unique ubiquinones and cellular fatty acids, and protein electrophoretic patterns (De Ley *et al.*, 1984a; Swings *et al.*, 1992). However, the identification methods based on phenotypic characteristics of AAB are not reliable and very time-consuming. Therefore, they have been complemented or replaced by different molecular techniques, in particular DNA-DNA hybridizations (Cleenwerck *et al.*, 2002*;* Dellaglio *et al.*, 2005*;* Lisdiyanti *et al.*, 2000, 2001) and PCR-based genomic fingerprinting techniques such as Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified 16S rRNA (González *et al.*, 2004, 2006a, b; Poblet *et al.*, 2000; Ruiz *et al.*, 2000) or 16S-23S rDNA spacer regions (González *et al.*, 2006a; Ruiz *et al.*, 2000; Trček, 2005; Trček & Teuber, 2002; Yukphan *et al.*, 2004a), Randomly Amplified Polymorphic DNA (RAPD) fingerprinting (Bartowsky *et al.*, 2003; Trček *et al.*, 1997), and PCR amplification of repetitive bacterial DNA elements (rep-PCR) using the REP (Repetitive Extragenic Palindromic sequences) or ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) primers (González *et al.*, 2004; Nanda *et al.*, 2001). Many of these methods were tested on a limited number of strains and failed to discriminate between closely related AAB species or were discriminating below species level.

The aim of this study was to obtain a method for fast and possibly high-throughput classification and identification of a broad range of AAB. Rep-PCR fingerprinting using the (GTG)$_5$ primer, referred to as (GTG)$_5$-PCR fingerprinting, has been found a promising genotypic tool for rapid and reliable species identification and typing of lactobacilli (Gevers *et al.*, 2001) and enterococci (Švec *et al.*, 2005). In this study, (GTG)$_5$-PCR fingerprinting was evaluated for AAB, using reference strains of AAB as well as AAB isolates from Ghanaian, fermented cocoa beans. In addition, validation of the method and indications for reclassifications of some AAB strains from culture collections and existence of new *Acetobacter* species from fermented cocoa beans were obtained through 16S rRNA gene sequencing analyses and DNA-DNA hybridizations.

MATERIALS AND METHODS

Strains and growth conditions. Two sets of AAB were included in this study. The first group consisted of AAB reference strains (64 in total, including 31 type strains) and is listed in Table 4.1. These AAB were obtained, together with the type strain of *Lactobacillus plantarum* LMG 6907^T, from the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium). All reference strains were grown according to the provider's specifications (http://www.belspo.be/bccm/), unless indicated otherwise. The second group consisted of 132 AAB isolates from Ghanaian, fermented cocoa beans. They were maintained frozen at -80 °C in MYP medium (2.5 % D-mannitol, 0.5 % yeast extract, 0.3 % bacteriological peptone Oxoid, Basingstoke, UK; w/v), supplemented with 25 % (v/v) glycerol as cryoprotectant, and recovered by incubation at 30 °C in MYP medium under aerobic conditions for 1-4 days.

Phenotypic tests. All isolates were tested for their Gram reaction, cell shape, cell size and mobility, from cells grown at 30 °C in MYP medium under aerobic conditions for 1-4 days. Catalase activity was detected by the appearance of oxygen gas bubbles from 20 % (v/v) hydrogen peroxide solution by colonies on MYP agar (MYP medium supplemented with 1.5 % agar; w/v). *L. plantarum* LMG 6907^T (catalase-negative lactic acid bacterium) and *Acetobacter aceti* LMG 1504^T (catalase-positive acetic acid bacterium) were used as controls.

The production of acetic acid from ethanol and gluconic acid from D-glucose was tested following growth of the strains at 30 °C for 4 days in basal medium (0.05 % yeast extract, 0.3 % vitamin-free casamino acids (Difco, Detroit, MI); w/v; plus ethanol 0.3 %, v/v) and GY medium (5 % D-glucose, 0.5 % yeast extract; w/v), respectively. The production of 2- and 5-keto-D-gluconic acid from ethanol and D-glucose was tested following growth in basal medium plus ethanol and GY medium, respectively, at 30 °C for 4 days. Residual substrates and metabolites were quantified by high pressure liquid chromatography (HPLC) with refractive index detection for D-glucose and ethanol, and high pressure anion exchange chromatography (HPAEC) with ion suppression and conductivity detection for acetic acid, gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, basically following the protocols of Van der Meulen *et al.* (2006). All tests were done in duplicate.

DNA preparation. Total DNA was extracted from the reference strains by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). For the

isolates it was extracted either as described by Gevers *et al.* (2001), except that mutanolysin was substituted by proteinase K (VWR International, Darmstadt, Germany) in an amount of 2.5 mg ml^{-1} of Tris-HCl (10 mM)-EDTA (1 mM) buffer (pH 8.0), or by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). All isolates were grown in MYP medium for 1-4 days, except for some strains that grew better in GY medium. *Asaia siamensis* and *Asaia bogorensis* were grown in M17 medium (5 % glucose, 3 % calcium carbonate, 1 % yeast extract; w/v).

Rep-PCR fingerprinting. A set of 64 reference strains and 132 isolates was subjected to rep-PCR fingerprinting with the oligonucleotide primer pair REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-IIICGNCGNCATCNGGC-3′) and with the single oligonucleotide primer (GTG)$_5$ (5′-GTGGTGGTGGTGGTG-3′) (Gevers *et al.*, 2001; Versalovic *et al.*, 1994). The reproducibility of (GTG)$_5$-PCR was tested by amplifying DNA from twelve randomly chosen strains several times. In addition, each PCR reaction was controlled for reproducibility by inclusion of the type strain of *L. plantarum* LMG 6907^T. Optimal PCR conditions for each of the primer sets used were as described by Versalovic *et al.* (1994). PCR amplifications were performed with a DNA T3 thermal cycler (Biometra, Westburg, The Netherlands) using Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The PCR products were electrophoresed in a 1.5 % (w/v) agarose gel (15 × 20 cm) for 16 h at a constant voltage of 55 V in 1 × TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) at 4 °C. The rep-PCR profiles were visualized under UV light after staining of the gel with ethidium bromide, and digital image capturing was done using the Gel Doc EQ system (Biorad, Hercules, CA). The resulting fingerprints were analyzed using the BioNumerics version 4.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation coefficient, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles.

DNA-DNA hybridizations. Only high-molecular-mass DNA with A_{260}/A_{280} and A_{234}/A_{260} absorption (A) ratios of 1.8-2.0 and 0.40-0.60, respectively, was used for DNA-DNA hybridizations. DNA-DNA hybridizations were performed according to a modification (Cleenwerck *et al.*, 2002 and Goris *et al.*, 1998) of the microplate method described by Ezaki *et al.* (1989). The hybridization temperature was 46 ± 1 °C (*Acetobacter*) or 49 ± 1 °C (*Gluconacetobacter*). 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 the means of a minimum of four hybridization experiments, including the reciprocal reactions.

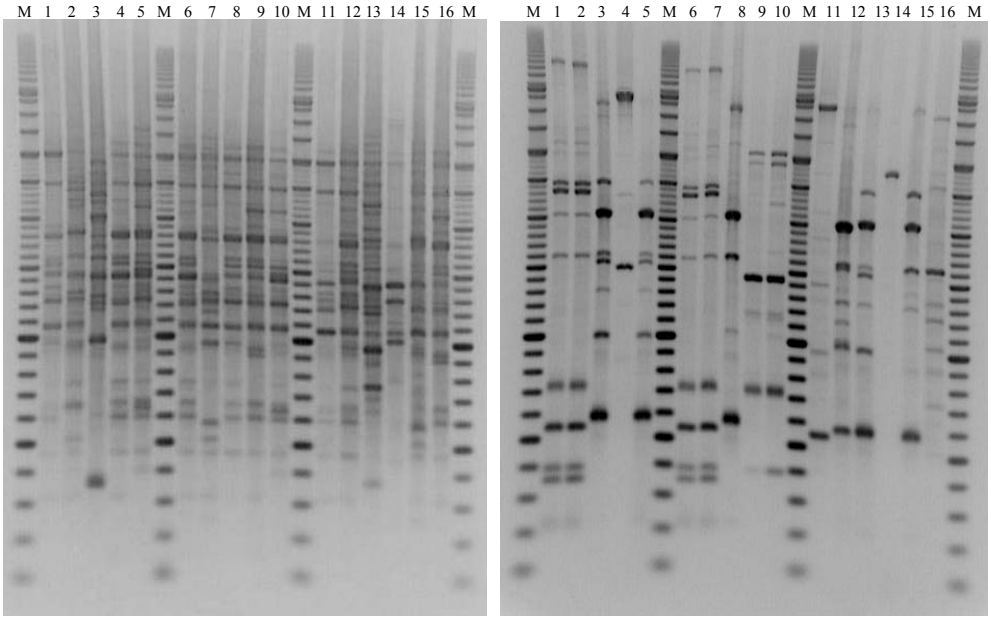
16S rRNA gene sequencing. A fragment of the 16S rRNA gene (corresponding with the positions 8-1541 in the *Escherichia coli* numbering system) was amplified by PCR using the conserved primers pA (5′ AGA GTT TGA TCC TGG CTC AG 3′) and pH (5′ AAG GAG GTG ATC CAG CCG CA 3′). PCR-amplified 16S rRNA genes were purified using the NucleoFast® 96 PCR Cleanup Kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and purified using the Montage™ SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA). Sequencing was performed using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). Nearly complete sequences were determined using the eight sequencing primers listed in Coenye *et al.* (1999a), except for R-29337 where the primer BKL1 (position according to the *E. coli* 16S rRNA gene sequence numbering: 536–516; sequence: 5′ GTA TTA CCG CGG CTG CTG GCA 3′) was used instead of the primer PD. Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). Sequence similarities between the consensus sequences and small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL (EMBL-EBI, Hinxton, Cambridge, UK) were calculated pairwise using an open gap penalty of 100 % and a unit gap penalty of 0 % with the software package BioNumerics version 4.5 (Applied Maths).

RESULTS

Phenotypic characterization of isolates from Ghanaian, fermented cocoa beans

Cells of all isolates were motile or non-motile rods, Gram-negative, and catalase-positive. All isolates grew in basal medium plus ethanol and in GY medium. They all oxidized ethanol to acetic acid and produced gluconic acid from D-glucose in the respective media. Most of the isolates produced 2-keto-D-gluconic acid from D-glucose in very small amounts and did not produce 5-keto-D-gluconic acid.

A



B

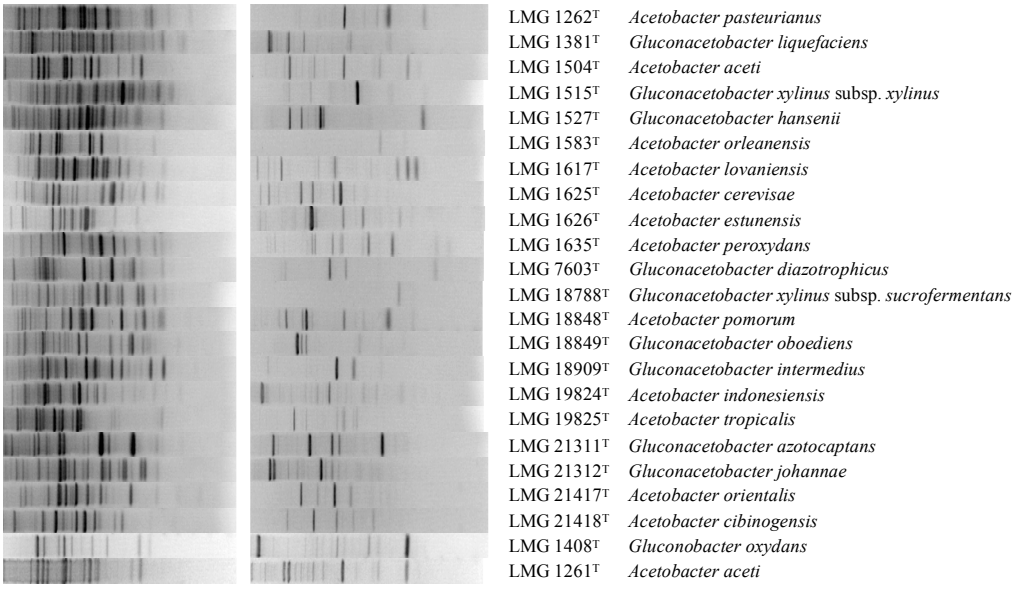


Fig. 4.1. Rep-PCR banding patterns of AAB reference strains and isolates. (A) Picture of a rep-PCR gel with (GTG)$_5$-PCR (left part) and REP-PCR (right part) fingerprints of AAB isolates; lane assignments 1 to 16 correspond with isolates 103, 109, 110, 117, 119, 121, 122, 123, 126, 127, 128, 129, 131, 133, 138 and 139, respectively, while the lanes marked with M are molecular size markers (mixture of a 100-bp and 5000-bp ladder; Biorad). (B) Digitized (GTG)$_5$-PCR (left part) and REP-PCR (right part) fingerprints of AAB reference strains.

Rep-PCR fingerprinting: primer evaluation

The $(GTG)_5$ primer as well as the REP1R-I and REP2-I primer set generated DNA fragments of 300 to 4000 bp for AAB reference strains and isolates (Fig. 4.1). The $(GTG)_5$ primer resulted in banding patterns containing generally between 10 and 30 visualized bands, while the REP1R-I and REP2-I primer set generated banding patterns containing generally less than 10 bands (Fig. 4.1). The $(GTG)_5$ primer was preferred for further evaluation for rapid classification, identification and typing of AAB.

The similarity between the $(GTG)_5$-PCR patterns, obtained through amplification of DNA from twelve strains several times, ranged between 89 and 96 % (results not shown). The inclusion of the type strain of *L. plantarum* LMG 6907^T in each PCR reaction and verification of its banding pattern maintained a high reproducibility (data not shown).

Evaluation of $(GTG)_5$-PCR fingerprinting for classification and identification of AAB

Reference strains

The results of the numerical analysis of the generated $(GTG)_5$-PCR banding patterns of a total of 64 reference AAB strains are shown in Fig. 4.2. All reference strains grouped in separate clusters according to their respective taxonomic designations, except for *A. peroxydans* (LMG 1633 and LMG 1635^T) and *A. indonesienis* (LMG 1571, LMG 1588, and LMG 19824^T) strains that were dispersed, and *A. aceti* LMG 1531, *G. liquefaciens* LMG 1509 and LMG 1728, *G. xylinus* LMG 1518 and *G. xylinus* subsp. *sucrofermentans* LMG 18788^T, which did not cluster with the other strains from their respective taxa.

To evaluate the $(GTG)_5$-PCR technique for species identification, it is obligatory to know the exact species identity of the reference AAB used. All *Acetobacter* (35 strains) and some *Gluconacetobacter* (9 strains) reference strains were previously subjected to DNA-DNA hybridizations (Cleenwerck *et al.*, 2002; Dellaglio *et al.*, 2005; Silva *et al.*, 2006) using the method of Ezaki *et al.* (1989). In the frame of this study additional hybridizations were performed between *A. peroxydans* LMG 1635^T and LMG 1633 and on the remaining *Gluconacetobacter* strains (Table 4.1).

Comparing the results obtained by $(GTG)_5$-PCR fingerprinting (Fig. 4.2) with the DNA-DNA hybridization results (Table 4.1), revealed that strains that did not cluster

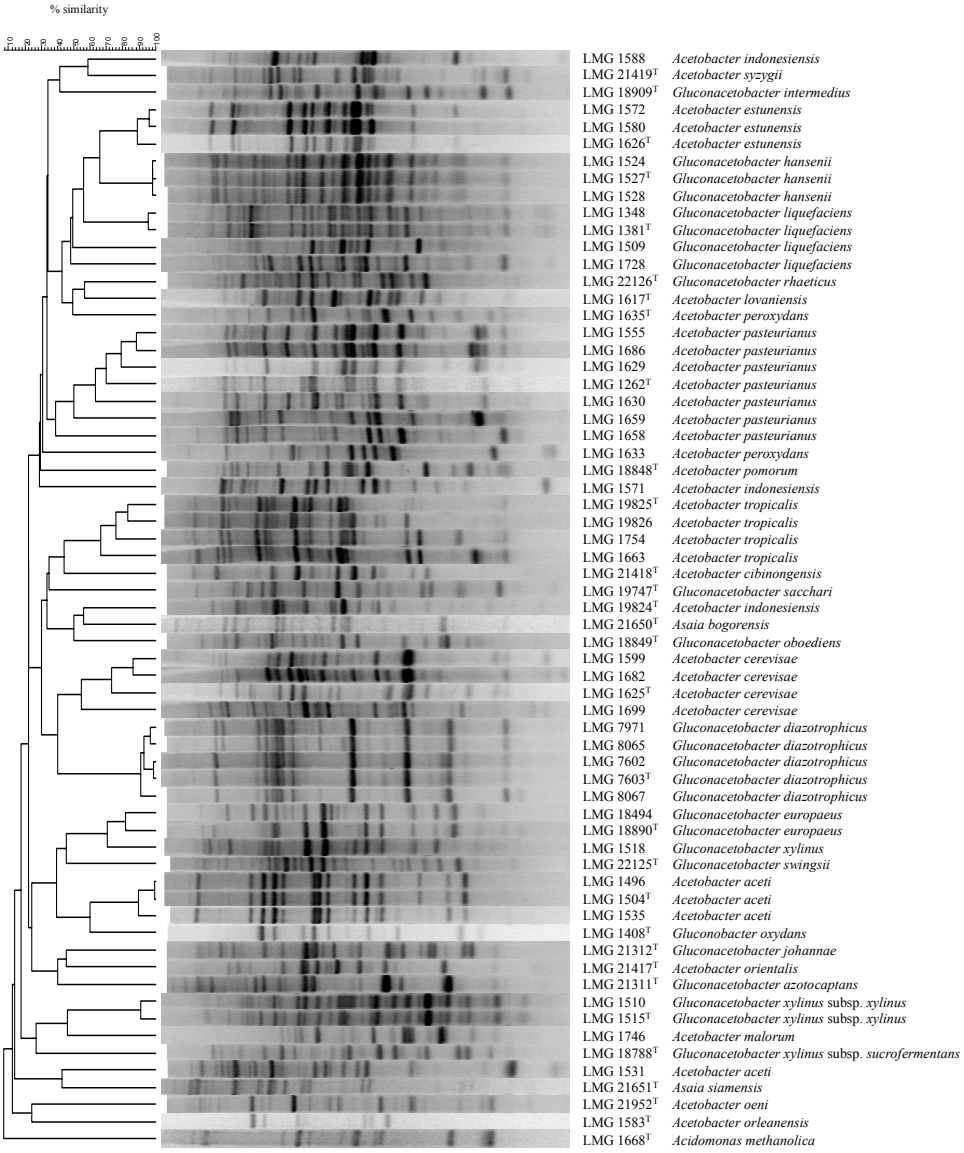


Fig. 4.2. (GTG)$_5$-PCR banding patterns of a total of 64 reference AAB strains, representing all validly described *Acetobacter species* (35 strains), 12 *Gluconacetobacter* species (25 strains) and other AAB, namely *Asaia bogorensis* LMG 21650^T, *Asaia siamensis* LMG 21651^T, *Acidomonas methanolica* LMG 1668^T and *Gluconobacter oxydans* LMG 1408^T. The dendrogram was generated after cluster analysis of the digitized fingerprints and was derived from UPGMA linkage of Pearson correlation coefficients.

with their respective taxa generally showed less or approximately 70 % DNA-DNA relatedness: this is the generally accepted limit for species delineation (Stackebrandt *et al.*, 2002). For example, *G. liquefaciens* LMG 1728, *A. peroxydans* LMG 1633, *G. xylinus* LMG 1518, and *A. aceti* LMG 1531 showed a DNA-DNA relatedness of 68–69

Table 4.1. Acetic acid bacteria reference strains and DNA-DNA relatedness of *Acetobacter peroxydans* LMG 1635^T and LMG 1633 and of *Gluconacetobacter* strains, determined in the frame of this study.

≥ 70 % DNA-DNA binding; 30-69 % DNA-DNA binding.

		28	29	36	37	38	39	40	41	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1. *A. aceti*	LMG 1496																										
2. *A. aceti*	LMG 1504^T																										
3. *A. aceti*	LMG 1531																										
4. *A. aceti*	LMG 1535																										
5. *A. cerevisiae*	LMG 1599																										
6. *A. cerevisiae*	LMG 1625^T																										
7. *A. cerevisiae*	LMG 1682																										
8. *A. cerevisiae*	LMG 1699																										
9. *A. cibinongensis*	LMG 21418^T																										
10. *A. estunensis*	LMG 1572																										
11. *A. estunensis*	LMG 1580																										
12. *A. estunensis*	LMG 1626^T																										
13. *A. indonesiensis*	LMG 1571																										
14. *A. indonesiensis*	LMG 1588																										
15. *A. indonesiensis*	LMG 19824^T																										
16. *A. lovaniensis*	LMG 1617^T																										
17. *A. malorum*	LMG 1746^T																										
18. *A. oeni*	LMG 21952^T																										
19. *A. orientalis*	LMG 21417^T																										
20. *A. orleanensis*	LMG 1583^T																										
21. *A. pasteurianus*	LMG 1262^T																										
22. *A. pasteurianus*	LMG 1555																										
23. *A. pasteurianus*	LMG 1629																										
24. *A. pasteurianus*	LMG 1630																										
25. *A. pasteurianus*	LMG 1658																										
26. *A. pasteurianus*	LMG 1659																										
27. *A. pasteurianus*	LMG 1686																										
28. *A. peroxydans*	LMG 1633	100																									
29. *A. peroxydans*	LMG 1635^T	63	100																								
30. *A. pomorum*	LMG 18848^T																										
31. *A. syzygii*	LMG 21419^T																										
32. *A. tropicalis*	LMG 1663																										
33. *A. tropicalis*	LMG 1754																										
34. *A. tropicalis*	LMG 19825^T																										
35. *A. tropicalis*	LMG 19826																										
36. *G. azotocaptans*	LMG 21311^T			100																							
37. *G. diazotrophicus*	LMG 7602				100																						
38. *G. diazotrophicus*	LMG 7603^T				96	100																					
39. *G. diazotrophicus*	LMG 7971				93	74	100																				
40. *G. diazotrophicus*	LMG 8065				95	75	97	100																			
41. *G. diazotrophicus*	LMG 8067				86	94	89	92	100																		
42. *G. europaeus*	LMG 18494																										
43. *G. europaeus*	LMG 18890^T									100																	
44. *G. hansenii*	LMG 1524										100																
45. *G. hansenii*	LMG 1527^T										97	100															
46. *G. hansenii*	LMG 1528										100	100	100														
47. *G. intermedius*	LMG 18909^T													100													
48. *G. johannae*	LMG 21312^T														100												
49. *G. liquefaciens*	LMG 1348															100											
50. *G. liquefaciens*	LMG 1381^T															100	100										
51. *G. liquefaciens*	LMG 1509															91	82	100									
52. *G. liquefaciens*	LMG 1728															69	68	69	100								
53. *G. oboediens*	LMG 18849^T																			100							
54. *G. rhaeticus*	LMG 22126^T																				100						
55. *G. sacchari*	LMG 19747^T																					100					
56. *G. swingsii*	LMG 22125^T																						100				
57. *G. xylinus subsp. xylinus*	LMG 1510																							100			
58. *G. xylinus subsp. xylinus*	LMG 1515^T									50*														77	100		
59. *G. xylinus subsp. xylinus*	LMG 1518									79														61	42	100	
60. *G. xyl. subsp. sucrofermentans*	LMG 18788^T									25*														57	56*	20	100
61. *Acidomonas methanolica*	LMG 1668^T																										
62. *Asaia bogorensis*	LMG 21650^T																										
63. *Asaia siamensis*	LMG 21651^T																										
64. *Gluconobacter oxydans*	LMG 1408^T																										

*Data taken from Dellaglio *et al*. (2005).

%, 63 %, 42–61 % and 53–59 %, respectively, with other strains from *G. liquefaciens*, *A. peroxydans*, *G. xylinus* and *A. aceti*, respectively. The DNA-DNA relatedness between *A. peroxydans* LMG 1635^T and LMG 1633 determined in this study was slightly lower than the one reported previously (Cleenwerck *et al.*, 2002). For *G. xylinus* LMG 1518, the (GTG)$_5$-PCR fingerprint was visually very similar to these of *G. europaeus* strains LMG 18890^T and LMG 18894. Hybridizations showed a DNA-DNA relatedness of 77 % with *G. europaeus* LMG 18890^T. *G. xylinus* subsp. *sucrofermentans* LMG 18788^T showed a DNA homology of 56 % with *G. xylinus* subsp. *xylinus* LMG 1515^T. For a few strains, such as for all *A. indonesiensis* strains (sharing > 80 % DNA homology with each other) and *G. liquefaciens* LMG 1509, (GTG)$_5$-PCR fingerprints generated none or not enough group-specific bands, in comparison with the profiles of the other AAB strains.

Fermented cocoa bean isolates

To evaluate the applicability of (GTG)$_5$-PCR for identification of unknown isolates, the 132 fermented cocoa bean isolates were subjected to (GTG)$_5$-PCR fingerprinting (Fig. 4.3). The (GTG)$_5$-PCR banding patterns of the isolates were clustered with those of the AAB reference strains and were found to be dispersed over four major clusters, with patterns sharing more than 36 % similarity and visually quite similar (Fig. 4.3). The biggest group of isolates (cluster I, containing 100 isolates) clustered with *A. pasteurianus* reference strains and was therefore assigned to that species. The 32 remaining isolates were dispersed over three clusters, with 23 (cluster II), 4 (cluster III), and 5 (cluster IV) isolates, respectively. These clusters did not contain any of the reference strains and therefore these strains could not be identified to species level.

Isolates 150, 165D and 406 of cluster I were subjected to 16S rRNA gene sequencing analysis and DNA-DNA hybridizations to test the validity of their identification as *A. pasteurianus*. They showed 99.5-99.8 % 16S rRNA gene sequence similarity with the type strains of *A. pomorum* and *A. pasteurianus* and less than 98.1 % with the type strains of other *Acetobacter* species (the EMBL accession numbers for the 16S rRNA gene sequences of isolates 150, 165D and 406 are DQ887334, DQ887336 and DQ887335, respectively) and a DNA-DNA relatedness at species level amongst each other and with the type strain of *A. pasteurianus* (68–80 %) and below species level with the type strain of *A. pomorum* (42–47 %). These results prove that cluster I consisted of *A. pasteurianus* strains.

To verify if clusters II, III, and IV represented possible new AAB species,

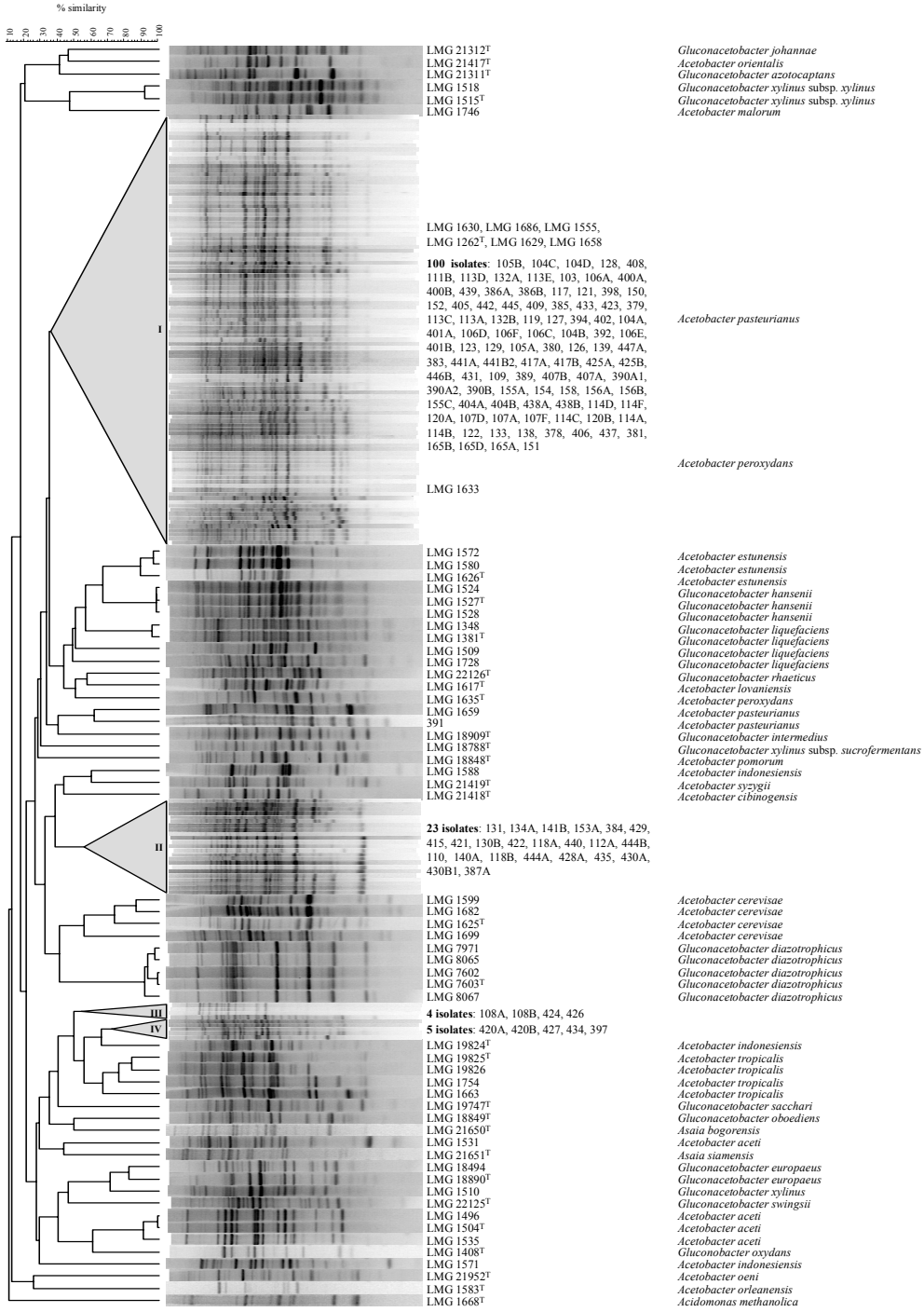


Fig. 4.3. $(GTG)_5$-PCR banding patterns of the AAB reference strains and 132 fermented cocoa bean isolates. The dendrogram, derived from UPGMA linkage of Pearson correlation coefficients, shows four different clusters of isolates, represented as clusters I (100 isolates), II (23 isolates), III (4 isolates) and IV (5 isolates).

representative strains of each of them were subjected to 16S rRNA gene sequence analysis and the phylogenetic closest neighbours were determined. The 16S rRNA gene sequence similarities obtained showed that 384 and 444B (cluster II) were most closely related to *A. syzygii* (99.7 %) and *A. lovaniensis* (99.5 %), while 108B and 420A (cluster III) and 426 and 434 (cluster IV) were most closely related to *A. tropicalis* (99.9 %). The EMBL accession numbers for the 16S rRNA gene sequences of 108B, 384, 420A, 426, 434 and 444B are DQ887339, DQ887338, DQ887340, DQ887342, DQ887341 and DQ887337, respectively.

DNA-DNA hybridizations between 444B and *A. syzygii* LMG 21419^T and *A. lovaniensis* LMG 1617^T revealed a DNA homology value of 46 % and 47 %, respectively. DNA-DNA hybridizations between 108B, 420A and *A. tropicalis* LMG 19825^T revealed a high DNA homology value between both isolates (75 %) and intermediate values between these isolates and *A. tropicalis* LMG 19825^T (54–58 %).

DISCUSSION

Highly conserved repetitive DNA elements, such as Repetitive Extragenic Palindromic (REP) elements, Enterobacterial Repetitive Intragenic Consensus (ERIC) elements and BOX elements, seem to be widely distributed in the genomes of various bacterial groups. The amplification of the sequences between each of these repetitive elements has been used to generate DNA fingerprints of several Gram-negative and Gram-positive bacterial species (Beyer *et al.*, 1998; De Urraza *et al.*, 2000; González *et al.*, 2004; Guinebretier *et al.*, 2001; Lanoot *et al.*, 2004; Pooler *et al.*, 1996; Sander *et al.*, 1998; Wieser & Busse, 2000). Also, (GTG)$_5$ sequences seem to be widely distributed in the genomes of various bacterial groups, as the amplification of the sequences between these repetitive elements has generated typical DNA fingerprints of several lactobacilli (Gevers *et al.*, 2001) and enterococci (Švec *et al.*, 2005), allowing their rapid and reliable species identification and typing. We tested the usefulness of this technique with reference strains of most of the species of AAB and found that it generated complex banding patterns in comparison with the REP primer pair. The reproducibility of the (GTG)$_5$-PCR fingerprints from AAB was similar to that obtained by Gevers *et al.* (2001) with (GTG)$_5$-PCR fingerprints from lactobacilli. Moreover, most reference strains grouped according to their species designation and exclusive patterns were obtained for most strains, indicating the usefulness of this technique for identification at the species level and characterization below species level or typing of

AAB strains.

To evaluate the $(GTG)_5$-PCR technique for species identification, $(GTG)_5$-PCR fingerprinting data were compared with DNA-DNA hybridization data. The latter data are recognized as the data needed for species delineation (Stackebrandt *et al.*, 2002) and for accurate identification of *Acetobacter* and *Gluconacetobacter* strains (Cleenwerck *et al.*, 2002; Dellaglio *et al.*, 2005; Lisdiyanti *et al.*, 2000, 2001). A good correlation was noticed between both data, the clustering of $(GTG)_5$-PCR fingerprints being in line with the 70 % DNA-DNA relatedness limit, which is generally accepted for species delineation (Stackebrandt *et al.*, 2002). The results presented in this paper thus show that $(GTG)_5$-PCR DNA fingerprinting is useful for identification and classification of AAB at the species level.

The data presented in this paper also underline some taxonomic problems. For *A. aceti* LMG 1531 the current classification in the species *A. aceti* can be discussed. Strain LMG 1531 has been assigned to *A. aceti* subsp. *aceti* and cannot be differentiated from other *A. aceti* strains phenotypically (Gosselé *et al.*, 1983b). For this reason strain LMG 1531 has never been removed from *A. aceti*, despite its low DNA-DNA relatedness with other *A. aceti* strains. Also for *G. xylinus* LMG 1518 several arguments exist that the strain has been misclassified. Based on its $(GTG)_5$-PCR fingerprint and its DNA-DNA relatedness to *G. europaeus* (79 %), strain LMG 1518 should be reclassified as *G. europaeus*. For *G. xylinus* subsp. *sucrofermentans* LMG 18788^T the DNA-DNA relatedness (56 %) to *G. xylinus* subsp. *xylinus* is comparable to the 58.2 % reported by Toyosaki *et al.* (1995), but the $(GTG)_5$-PCR fingerprints of both subspecies are different. Therefore, the question can be raised whether *G. xylinus* subsp. *sucrofermentans* should be elevated at the species level. Also, for *A. peroxydans* LMG 1633 the current classification can be questioned on the basis of the results presented in this paper.

For a few strains, such as all *A. indonesiensis* strains (sharing > 80 % DNA homology with each other) and *G. liquefaciens* LMG 1509, $(GTG)_5$-PCR fingerprinting does not seem to be adequate for species identification. It has been shown before that rep-PCR may yield whether or not species-specific banding patterns when analyzing different species of a genus (Pooler *et al.*, 1996; Wieser & Busse, 2000). In many cases, the $(GTG)_5$-PCR fingerprints generated for AAB were strain-specific and $(GTG)_5$-PCR fingerprinting can therefore be used for typing of AAB as well. Alternatively, rep-PCR with the REP1R-I and REP2-I primer pair can also be used for typing.

$(GTG)_5$-PCR fingerprinting allowed us to differentiate four major clusters among the 132 isolates from fermented cocoa beans tested, with decreasing number of isolates

encompassing *A. pasteurianus* (cluster I), *A. syzygii*- or *A. lovaniensis*-like (cluster II), and *A. tropicalis*-like (clusters III and IV). The presence of *A. pasteurianus* in cocoa bean fermentations has been reported before (Ardhana & Fleet, 2003; Passos & Passos, 1985). Moreover, previous studies have shown that members of the genus *Acetobacter* are found more frequently than members of the genus *Gluconobacter* (Schwan & Wheals, 2004), probably reflecting their preference for ethanol and sugars, respectively. In general, *Acetobacter* and *Gluconacetobacter* strains are mainly isolated from fermented foods, while *Gluconobacter* strains are mainly isolated from fruits and flowers (Lisdiyanti *et al.*, 2003). The major species found in wine fermentation are *A. aceti*, *A. pasteurianus* and *G. oxydans* (González *et al.*, 2005). *A. pasteurianus* is also involved in rice vinegar production (Nanda *et al.*, 2001). *A. syzygii*(-like) and *A. tropicalis*(-like) have never been associated with cocoa bean fermentation before. Moreover, *A. syzygii* mainly occurs in flowers and fruits and is seldom isolated from fermented foods, in contrast with *A. tropicalis* that is found in fruits and fermented foods (Lisdiyanti *et al.*, 2001, 2003). *A. tropicalis* and *A. syzygii* were not discovered at the time of the microbiological analyses of fermented cocoa beans performed before (Ardhana & Fleet, 2003; Carr *et al.*, 1979, Ostovar & Keeney, 1973; Passos & Passos, 1985; Schwan & Wheals, 2004; Thompson *et al.*, 1997). Interestingly, the results of the present paper indicate that clusters II, III, and IV represent two novel *Acetobacter* species[1].

To summarize, as far as we know this is the first study in which a fast molecular method for AAB was validated by DNA-DNA hybridization data. The (GTG)$_5$-PCR fingerprinting technique presented in this paper offers significant advantages over identification methods based on phenotypic characteristics of AAB, but also over many currently used molecular techniques, hence enabling its implementation as high-throughput methodology. Manual (GTG)$_5$-PCR fingerprinting allows identification of AAB in one working day and can be used for classification and identification of AAB at the species level, as well as for characterization of AAB below species level (typing). (GTG)$_5$-PCR fingerprinting can quickly increase our knowledge of the ecology and biodiversity of AAB and help us to more accurately determine their growth behaviour and beneficial or undesirable role during various stages of food fermentation such as vinegar production, vinification, and cocoa bean fermentation.

[1]Cluster II: *Acetobacter ghanensis* sp. nov. (chapter 5.1); clusters III and IV: *A. senegalensis* (Camu *et al*., 2007)

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4.2. DIFFERENTIATION OF SPECIES OF THE FAMILY *ACETOBACTERACEAE* BY AFLP DNA FINGERPRINTING AND RECLASSIFICATION OF *GLUCONACETOBACTER KOMBUCHAE* AS *GLUCONACETOBACTER HANSENII*

Redrafted from:
Cleenwerck, I., De Wachter, M., De Vuyst, L. & De Vos, P. (2008). Differentiation of species of the family *Acetobacteraceae* by AFLP® DNA fingerprinting and reclassification of *Gluconacetobacter komuchae* as *Gluconacetobacter hansenii*. *Int J Syst Evol Microbiol* (submitted).

SUMMARY

Amplified fragment length polymorphism (AFLP®) DNA fingerprinting was investigated as a tool for fast and accurate identification of AAB at the species level. One hundred and thirty five reference strains and 15 additional strains, representing the 50 recognized species of the family *Acetobacteraceae*, were subjected to AFLP® analysis using the restriction enzyme combination *Apa*I/*Taq*I and the primer combination A03/T03. The reference strains were previously subjected to either DNA-DNA hybridizations or 16S-23S rDNA spacer region sequence analysis and were regarded as being accurately classified at the species level. The present study revealed that six of these strains have to be reclassified, namely *Gluconacetobacter europaeus* LMG 1518 and *Gluconacetobacter xylinus* LMG 1510 as *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, respectively; *Gluconacetobacter kombuchae* LMG 23726^T as *Gluconacetobacter hansenii*; and *Acetobacter orleanensis* LMG 1545, LMG 1592 and LMG 1608 as *Acetobacter cerevisiae*. Cluster analysis of the AFLP® DNA fingerprints of the reference strains revealed one cluster for each species, showing a linkage level below 50 % with other clusters, except for *Acetobacter pasteurianus*, *Acetobacter indonesiensis*, and *Acetobacter cerevisiae*. The latter species were separated into two, two, and three clusters, respectively. The AFLP® data further supported to classify *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius*, for which at present confusion exists concerning their taxonomic status, as different taxa. The 15 additional strains could all be identified at the species level. AFLP® analysis further revealed that some species harbour genetically diverse strains, whereas other species consist of strains showing similar banding patterns, indicating a more

limited genetic diversity. It can be concluded that AFLP® DNA fingerprinting is suitable for accurate identification and classification of a broad range of AAB, as well as for determination of intraspecific genetic diversity.

INTRODUCTION

AAB are Gram-negative, coccoid or rod-shaped, obligate aerobic bacteria that have the ability to incompletely oxidize a wide range of carbohydrates, alcohols, and sugar alcohols. They are at present classified into ten genera comprising 50 recognized species in the family *Acetobacteraceae* of the *Alphaproteobacteria*, i.e., *Acetobacter* (19 species), *Acidomonas* (1 species), *Asaia* (3 species), *Gluconacetobacter* (16 species), *Gluconobacter* (6 species), *Granulibacter* (1 species), *Kozakia* (1 species), *Neoasaia* (1 species), *Saccharibacter* (1 species), and *Swaminathania* (1 species) (Cleenwerck & De Vos, 2008; Cleenwerck *et al.*, 2007; Dutta & Gachhui, 2007; Malimas *et al.*, 2007; Ndoye *et al*., 2007a; Yamada & Yukphan, 2008).

AAB are of interest to the industry. They play a beneficial role in the production of several fermented foods and beverages, such as vinegars, cocoa-based products, kombucha, and nata de coco, but may also cause spoilage to others such as beer, wine and cider. Additionally, they find applications in the production of fine chemicals (e.g. vitamine C and miglitol) and of bacterial cellulose. Some AAB have been described as plant growth promotors; whereas others have recently been described as human pathogens (Greenberg *et al.*, 2006b; Kersters *et al.*, 2006; Tuuminen *et al.*, 2007).

Multiple studies have shown that accurate identification of AAB isolates is difficult (Cleenwerck & De Vos, 2008; Greenberg *et al.*, 2006b; Kersters *et al.*, 2006; Moore *et al.*, 2002a; Tuuminen *et al.*, 2006). While identification to the genus level can generally be achieved by 16S rRNA gene sequence analysis and/or some phenotypic tests (Cleenwerck & De Vos, 2008; Kersters *et al.*, 2006; Yamada & Yukphan, 2008), identification at the species level can be problematic (Cleenwerck & De Vos, 2008; Kersters *et al.*, 2006). Species identifications based on phenotypic data solely are not recommended for several reasons. First, for some species, such as *Acetobacter tropicalis*, *Acetobacter indonesiensis*, and *Acetobacter orleanensis*, phenotypic differential characteristics have not been found. Second, some strains, such as strains involved in industrial vinegar fermentations and belonging to *Gluconacetobacter europaeus* and *Gluconacetobacter entanii*, are difficult to cultivate, which makes it very

hard or even impossible to subject them to phenotypic tests (Boesch *et al.*, 1998; Bartowsky *et al.*, 2003; Schüller *et al.*, 2000; Sievers *et al.*, 1992; Sokollek *et al.*, 1998a, b). Furthermore, results of phenotypic tests are sometimes difficult to interprete (Kersters *et al.*, 2006; Lisdiyanti *et al.*, 2006; Yamashita *et al.*, 2004) and spontaneous mutations often occur in AAB (Kersters *et al.*, 2006; Prust *et al.*, 2005). Loss of acetic acid tolerance and ethanol-oxidizing ability, or of the ability to produce cellulose as well as loss of various other physiological properties have been reported for *Acetobacter* spp., *Gluconacetobacter* spp. and *Gluconobacter* spp. (De Muynck *et al.*, 2007; Kersters *et al.*, 2006; Navarro *et al.*, 1999; Ohmori *et al.*, 1982; Sokollek *et al.*, 1998a; Yamada, 1983). These spontaneous mutations appeared in some cases to be due to the presence of insertion sequences (Coucheron, 1991; Iversen *et al.*, 1994; Kondô & Horinouchi, 1997). For instance, the genome of *Gluconobacter oxydans* 621H contains 82 insertion sequences and 103 transposons, i.e., repeated DNA elements that are known to be involved in genomic rearrangements (Prust *et al.*, 2005). For all these reasons, several authors have advized to use genotypic data as basis for species identification of AAB.

In the last decade, researchers have therefore increasingly abandoned phenotypic tests or complemented them by molecular DNA-based methods. DNA-DNA hybridizations and sequence analysis of 16S rRNA genes, 16S-23S rDNA spacer regions, and genes as *adhA* (encoding subunit I of pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase) and *recA* (encoding the DNA repair protein RecA) have been applied, as well as DNA fingerprinting techniques such as restriction fragment length polymorphism (RFLP) analysis of PCR-amplified rDNA (mainly 16S rRNA genes and 16S-23S rDNA spacer regions), randomly amplified polymorphic DNA (RAPD) fingerprinting, rep-PCR using the $(GTG)_5$, REP (Repetitive Extragenic Palindromic sequences), and ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) primers, pulsed-field gel electrophoresis (PFGE), and plasmid profiling (reviewed by Cleenwerck & De Vos, 2008). However, for many methods the taxonomic resolution is not unambiguously clear, because its evaluation has often been based on a set of strains with insufficient representatives per taxon and lacking phylogenetic relatives. Nevertheless, it is likely that the resolution power of techniques based on 16S rRNA genes will not reach the species level, especially because high 16S rRNA gene sequence similarities are noticed between several species of the family (Fig. 4.4). For rapid identification and classification of a broad range of AAB, the most appropriate technique at the moment appears to be rep-PCR using the $(GTG)_5$-primer (De Vuyst *et al.*, 2008).

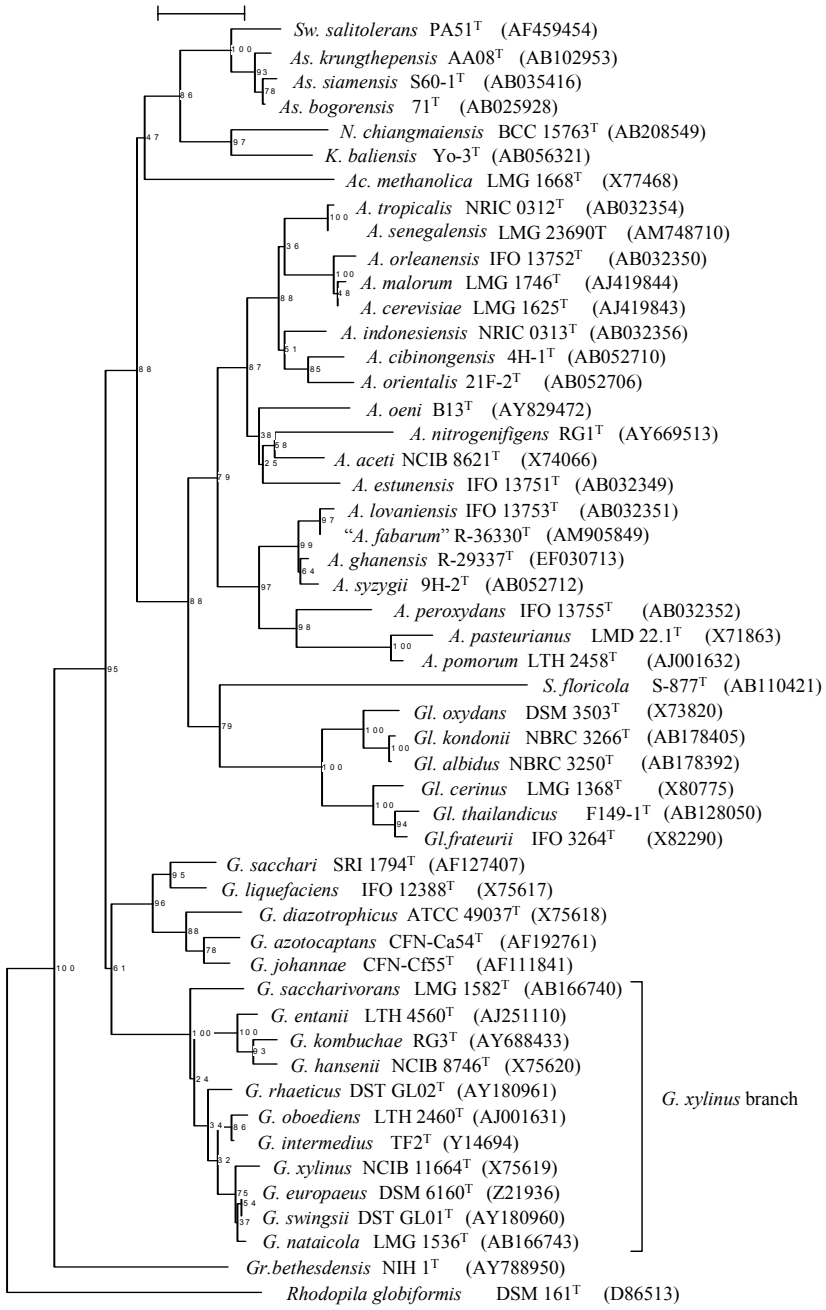


Fig. 4.4. Phylogenetic relationships of the 50 recognized species of the family *Acetobacteraceae* based on nearly complete 16S rRNA gene sequences of the type strains. *Rhodopila globiformis* DSM 161^T was used as outgroup. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. Bar indicates 1 % estimated sequence divergence. *A*., *Acetobacter*; *Ac*., *Acidomonas*; *As*., *Asaia*; *G*., *Gluconacetobacter*; *Gl*., *Gluconobacter*; *Gr*., *Granulibacter*; *K*., *Kozakia*; *N*., *Neoasaia*; *S*., *Saccharibacter*; *Sw*., *Swaminathania*

Amplified fragment length polymorphism (AFLP®) DNA fingerprinting, a universal applicable technique with a high discriminatory power and good reproducibility (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999), has proven to be useful for discrimination at the species level and below, in various taxa such as *Aeromonas* (Huys & Swings, 1999; Huys *et al.*, 1996), *Acinetobacter* (Janssen *et al.*, 1997), *Burkholderia* (Coenye *et al.*, 1999b), *Campylobacter* (Duim *et al.*, 2001; Hänninen *et al.*, 2001), *Stenotrophomonas* (Hauben *et al.*, 1999), lactic acid bacteria including bifidobacteria (Gancheva *et al.*, 1999; Franz *et al.*, 2006; Laursen *et al.*, 2005; Vancanneyt *et al.*, 2002, 2005, 2006), *Xanthomonas* (Rademaker *et al.*, 2000), *Vibrionaceae* (Thompson *et al.*, 2001a), *Bradyrhizobium* (Willems *et al.*, 2001a), *Arcobacter* (On *et al.*, 2003), and *Pantoea* (Brady *et al.*, 2007). In the present study, 150 AAB, representing the 50 recognized species of the family *Acetobacteraceae*, were analyzed by AFLP® DNA fingerprinting, with the aim to investigate if this technique enables fast and accurate identification and classification of AAB at the species level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The 150 strains used in this study are listed in Table 4.2. They were obtained from the BCCM/LMG Bacteria Collection and the Research Collection of the Laboratory of Microbiology (LM-UGent), or kindly provided by the German Collection of Microorganisms and Cell Cultures (DSMZ). Strains were cultivated on GY (5 % glucose, 1 % yeast extract, 1.5 % agar; w/v) or GYAE (5 % glucose, 1 % yeast extract, 1.5 % agar; w/v; 2 % ethanol, 1 % acetic acid; v/v; the latter two components were sterilized separately by filtration and added aseptically to the autoclaved medium) and in some cases on the medium suggested by the provider, by incubation at 28 °C under aerobic conditions.

DNA isolation. Genomic DNA was extracted following the method of Wilson (1987) with minor modifications. Briefly, DNA was extracted from 0.20-1.00 g (wet weight) of cells as previously described (Cleenwerck *et al.*, 2002) or from a loopful of cells using the following protocol. Cells were washed with RS buffer (0.15 M NaCl, 10 mM EDTA, pH 8.0) and then suspended in 360 µl mix containing 340 µl 10 mM Tris/HCl - 25 mM EDTA, pH 8.0, 16 µl RNaseA solution (10 mg ml^{-1}; Sigma), and 4.0 µl proteinase K solution (Merck). Forty µl of preheated (37 °C) SDS/TE solution (20 %

Table 4.2. List of AAB used in this study

Strains are listed under their proposed species designations. LMG, BCCM/LMG Bacteria Collection, Ghent Univerisity, Belgium; R, Research Collection of the Laboratory of Microbiology LM-UGent, Ghent University, Belgium; DSM, German Collection of Microorganisms and Cell Cultures, Germany; NCIMB, National Collection of Industrial, Marine and Food Bacteria, U.K.; LMD, Laboratorium voor Microbiologie, Technische Universiteit Delft, The Netherlands; NBRC, NITE Biological Resource Centre, Japan; NRIC, NODAI Culture Collection Center, Tokyo University of Agriculture, Japan. *A.*, *Acetobacter*; *Ac.*, *Acidomonas*; *As.*, *Asaia*; *G.*, *Gluconacetobacter*; *Gl.*, *Gluconobacter*; *Gr.*, *Granulibacter*; *K.*, *Kozakia*; *N.*, *Neoasaia*; *S.*, *Saccharibacter*; *Sw.*, *Swaminathania;* HG, hybridization group

Strain number	Synonymous strain designation	Species designation at the time of selection	Year of isolation; geographical origin of the isolate; source from which the strain was isolated (if known); note	HG	AFLP group	Proposed species designation
LMG 1496	LMG 24WR	*A. aceti*		*A. aceti*	A12	*A. aceti*
LMG 1504^T	NCIMB 8621^T, NBRC 14818^T	*A. aceti*	1923; beech-wood shavings of vinegar plant	*A. aceti*	A12	*A. aceti*
LMG 1535	LMG Ch31	*A. aceti*	Belgium; vinegar plant	*A. aceti*	A12	*A. aceti*
LMG 1531	NCIMB 8941	*Acetobacter* sp.	cider	related to *A. aceti*	S6	*Acetobacter* sp.
LMG 1545	NBRC 3296, NBRC 3296	*A. orleanensis*	1941; Aichi, Japan; film in fermentor of rice vinegar	*A. cerevisiae*	A29	*A. cerevisiae*
LMG 1592	NCIMB 2224, NBRC 3170	*A. orleanensis*	manufacture of vinegar		A29	*A. cerevisiae*
LMG 1599	NCIMB 6425	*A. cerevisiae*	United Kingdom; brewers' yeast and beer	*A. cerevisiae*	A29	*A. cerevisiae*
LMG 1608	NCIMB 8088, NBRC 3223	*A. orleanensis*	1932; the Netherlands; beer	*A. cerevisiae*	A25	*A. cerevisiae*
LMG 1625^T	NCIMB 8894^T	*A. cerevisiae*	Toronto, Canada; beer (ale) in storage	*A. cerevisiae*	A25	*A. cerevisiae*
LMG 1682	C101	*A. cerevisiae*	Ireland; beer	*A. cerevisiae*	A29	*A. cerevisiae*
LMG 1699	MARTIN 2	*A. cerevisiae*	1977; United Kingdom; brewery	*A. cerevisiae*	S20	*A. cerevisiae*
LMG 21418^T	4H-1^T	*A. cibinongensis*	Indonesia; mountain soursop (*Annona montanae*)	*A. cibinongensis*	S21	*A. cibinongensis*
LMG 1572	LMG E	*A. estunensis*	Bristol, United Kingdom; cider	*A. estunensis*	A15	*A. estunensis*
LMG 1580	LMD 50.6	*A. estunensis*	1950; Leiden, the Netherlands; beer	*A. estunensis*	A15	*A. estunensis*
LMG 1626^T	NCIMB 8935^T, NBRC 13751^T	*A. estunensis*	Bristol, United Kingdom; cider	*A. estunensis*	A15	*A. estunensis*
R-36330^T	LMG 24244^T, 985^T	"*A. fabarum*"	2006; Ghana; fermented cocoa beans	*A. fabarum*	A28	"*A. fabarum*"
R-36331	1145	"*A. fabarum*"	2006; Ghana; fermented cocoa beans	*A. fabarum*	A28	"*A. fabarum*"
R-36458	950	"*A. fabarum*"	2006; Ghana; fermented cocoa beans	*A. fabarum*	A28	"*A. fabarum*"
R-36459	1039	"*A. fabarum*"	2006; Ghana; fermented cocoa beans	*A. fabarum*	A28	"*A. fabarum*"
R-29336	444B	*A. ghanensis*	2004; Ghana; fermented cocoa beans	*A. ghanensis*	A31	*A. ghanensis*
R-29337^T	LMG 23848^T, 430A^T	*A. ghanensis*	2004; Ghana; fermented cocoa beans	*A. ghanensis*	A31	*A. ghanensis*
R-29338	415	*A. ghanensis*	2004; Ghana; fermented cocoa beans	*A. ghanensis*	A31	*A. ghanensis*
LMG 1571	LMD 39.2	*A. indonesiensis*		*A. indonesiensis*	A32	*A. indonesiensis*
LMG 1588	LMD 39.6	*A. indonesiensis*		*A. indonesiensis*	S12	*A. indonesiensis*
LMG 19824^T	NRIC 0313^T, 5H-1^T	*A. indonesiensis*	Indonesia; fruit of zirzak (*Annona muricata*)	*A. indonesiensis*	A32	*A. indonesiensis*
LMG 1617^T	NCIMB 8620^T, NBRC 13753^T	*A. lovaniensis*	1929; Becquevoort, Belgium; sewage on soil	*A. lovaniensis*	S19	*A. lovaniensis*
LMG 1746^T	LMG 76.10^T	*A. malorum*	1976; Ghent, Belgium; rotting apple	*A. malorum*	S15	*A. malorum*
LMG 23498^T	RG1^T	*A. nitronifigens*	2004; India; kombucha tea	*A. nitronifigens*	S5	*A. nitronifigens*
LMG 21952^T	B13^T	*A. oeni*	2002; Viseu Dão region, Portugal; spoiled red wine	*A. oeni*	S8	*A. oeni*
LMG 21417^T	21F-2^T	*A. orientalis*	Indonesia; canna flower (*Canna hybrida*)	*A. orientalis*	A30	*A. orientalis*
LMG 1547*	LMD 39.1	*A. pasteurianus*		*A. orientalis*	A30	*A. orientalis*
LMG 1583^T	NCIMB 8622^T, NBRC 13752^T	*A. orleanensis*	1927; Belgium; beer	*A. orleanensis*	S13	*A. orleanensis*
LMG 1262^T	LMD 22.1 t1^T	*A. pasteurianus*	the Netherlands; beer	*A. pasteurianus*	A13	*A. pasteurianus*
LMG 1555	NCIMB 8163	*A. pasteurianus*		*A. pasteurianus*	A13	*A. pasteurianus*
LMG 1590	LMD 51.1	*A. pasteurianus*	type strain of *A. pasteurianus* subsp. *ascendens*		A13	*A. pasteurianus*
LMG 1591*	LMD 53.6	*A. pasteurianus*	quick vinegar; type strain of *A. pasteurianus* subsp. *paradoxus*		A13	*A. pasteurianus*
LMG 1618*	NCIMB 8757	*A. pasteurianus*	United Kingdom; malt vinegar acetifier		A13	*A. pasteurianus*
LMG 1621*	NCIMB 8760	*A. pasteurianus*	United Kingdom; malt vinegar acetifier		A13	*A. pasteurianus*
LMG 1628	NCIMB 9015	*A. pasteurianus*			A13	*A. pasteurianus*
LMG 1629	A	*A. pasteurianus*	Recife, Brazil; fermented *Agave sisalina* juice	*A. pasteurianus*	A33	*A. pasteurianus*
LMG 1630	EQ	*A. pasteurianus*	Recife, Brazil; sugar cane bagasse	*A. pasteurianus*	A33	*A. pasteurianus*
LMG 1658	MM 80	*A. pasteurianus*	Myanmar; toddy palm	border of *A. pasteurianus*	A33	*A. pasteurianus*
LMG 1659	MM 73	*A. pasteurianus*	Myanmar; toddy palm	border of *A. pasteurianus*	A13	*A. pasteurianus*
LMG 1686	LMD 31.4	*A. pasteurianus*	1928; the Netherlands; vinegar from dry raisins	*A. pasteurianus*	A13	*A. pasteurianus*
LMG 23182*	AWRI B253	*Acetobacter* sp.	1997; Australia; oxidized Shiraz wine showing spoilage		A13	*A. pasteurianus*
R-29340	165D	*A. pasteurianus*	2004; Ghana; fermented cocoa beans	*A. pasteurianus*	A13	*A. pasteurianus*
R-29341	150	*A. pasteurianus*	2004; Ghana; fermented cocoa beans	*A. pasteurianus*	A33	*A. pasteurianus*
R-29342	406	*A. pasteurianus*	2004; Ghana; fermented cocoa beans	*A. pasteurianus*	A13	*A. pasteurianus*
LMG 1633		*A. peroxydans*	1950; the Netherlands; ditch water	border of *A. peroxydans*	A24	*A. peroxydans*
LMG 1635^T	NCIMB 8618^T, NBRC 13755^T	*A. peroxydans*	Delft, the Netherlands; ditch water	*A. peroxydans*	A24	*A. peroxydans*
LMG 21769	TNCSF 36	*A. peroxydans*	1999-2000; Thirumangalam Madurai,Tamilnadu, India; *Oryza sativa*, root tissue	*A. peroxydans*	A24	*A. peroxydans*
LMG 21770	TNCSF 49	*A. peroxydans*	1999-2000; Villupuram Villupuram, Tamilnadu, India; *Oryza sativa*, root tissue	*A. peroxydans*	A24	*A. peroxydans*
LMG 18848^T	LTH 2458^T	*A. pomorum*	Esslingen, Germany; submerged cider vinegar fermentation	*A. pomorum*	S18	*A. pomorum*
LMG 23690^T	CWBI-B418^T	*A. senegalensis*	1995; Casamance, Senegal; mango fruit (*Mangifera indica*)	*A. senegalensis*	A27	*A. senegalensis*
R-29339	108B	*A. senegalensis*	2004; Ghana; fermented cocoa beans	*A. senegalensis*	A27	*A. senegalensis*
R-29343	420A	*A. senegalensis*	2004; Ghana; fermented cocoa beans	*A. senegalensis*	A27	*A. senegalensis*
LMG 21419^T	9H-2^T	*A. syzygii*	Indonesia; fruit of Malay rose apple (*Syzygium malaccense*)	*A. syzygii*	S17	*A. syzygii*
LMG 1663	592	*A. tropicalis*	United Kingdom; fermenting putrified meat	*A. tropicalis*	A26	*A. tropicalis*
LMG 1754	LMG 79.18	*A. tropicalis*	1979; Ivory Coast; fruit of *Ficus capensis*	*A. tropicalis*	A26	*A. tropicalis*
LMG 19825^T	NRIC 0312^T, Ni-6b^T	*A. tropicalis*	Indonesia; coconut juice	*A. tropicalis*	A26	*A. tropicalis*
LMG 19826	NRIC 0321	*A. tropicalis*	Indonesia; lime	*A. tropicalis*	A26	*A. tropicalis*
LMG 1667*	MB 57	*Ac. methanolica*	sludge		A1	*Ac. methanolica*
LMG 1668^T	MB 58^T, IMET 10945^T	*Ac. methanolica*	Leipzig, Germany; fermentation of methanol with *Candida* sp. (not sterile)		A1	*Ac. methanolica*
LMG 1669*	MB 60	*Ac. methanolica*	sludge		A1	*Ac. methanolica*
LMG 1735*	SRI 1214	*Ac. methanolica*	Australia; *Saccharum officinarum*, surface microflora		A1	*Ac. methanolica*
LMG 21650T	71T	*As. bogorensis*	Bogor, Indonesia; flower of the orchid tree (Bauhinia purpurea)		A4	*As. bogorensis*
LMG 23142	87	*As. bogorensis*	Bogor, Indonesia; flower of plumbago (Plumbago auriculata)		A4	*As. bogorensis*
LMG 23143	168	*As. bogorensis*	Yogyakarta, Indonesia; tape ketan (fermented glutinous rice)		A4	*As. bogorensis*

LMG 23083^T	AA08^T	*As. krungthepensis*	Bangkok, Thailand; *Heliconia* sp., flower	A7	*As. krungthepensis*
LMG 23139	AA06	*As. krungthepensis*	Bangkok, Thailand; *Heliconia* sp., flower	A7	*As. krungthepensis*
LMG 23140	AA09	*As. krungthepensis*	Bangkok, Thailand; *Heliconia* sp., flower	A7	*As. krungthepensis*
LMG 21651^T	S60-1^T	*As. siamensis*	Bangkok, Thailand; crown flower (Calotropis gigantea)	A6	*As. siamensis*
LMG 23136	Y85	*As. siamensis*	Bogor, Indonesia; flower of spider lily (Crinum asiaticum)	A6	*As. siamensis*
LMG 23144	D4-1	*As. siamensis*	Bangkok, Thailand; flower of spider lily (Crinum asiaticum)	A6	*As. siamensis*
LMG 23145	I36	*As. siamensis*	Bogor, Indonesia; flower of spider lily (Crinum asiaticum)	A6	*As. siamensis*
LMG 23146	B28S-3	*As. siamensis*	Bogor, Indonesia; flower of ixora (Ixora chinensis)	A6	*As. siamensis*
LMG 21311^T	CFN-Ca54^T	*G. azotocaptans*	1996; Tapachula Chiapas, Mexico; *Coffea arabica* L., rhizosphere	A9	*G. azotocaptans*
LMG 23156	UAP-Ca97	*G. azotocaptans*	1997; Motozintla Chiapas, Mexico; *Coffea arabica* L., rhizosphere	A9	*G. azotocaptans*
LMG 23157	UAP-Ca99	*G. azotocaptans*	1997; Motozintla Chiapas, Mexico; *Coffea arabica* L., rhizosphere	A9	*G. azotocaptans*
LMG 1732	SRI 1205	*G. diazotrophicus*	Australia, *Saccharum officinarum*, surface microflora	A3	*G. diazotrophicus*
LMG 7603^T	Pal 5^T	*G. diazotrophicus*	Alagoas, Brazil; *Saccharum officinarum*, root	A3	*G. diazotrophicus*
LMG 7971	PP 4	*G. diazotrophicus*	Pernambuco, Brazil; *Saccharum officinarum*, stem	A3	*G. diazotrophicus*
LMG 8067	PR 20	*G. diazotrophicus*	Rio de Janeiro Brazil, *Saccharum officinarum*, root	A3	*G. diazotrophicus*
LMG 22174*	TNCSF 42	*G. diazotrophicus*	Thirumangalam Madurai,Tamilnadu, India; *Oryza sativa*, root tissue	A3	*G. diazotrophicus*
LMG 22175*	TNCSF 47	*G. diazotrophicus*	Valajabad Kanchipuram,Tamilnadu, India, *Oryza sativa*, stem tissue	A3	*G. diazotrophicus*
LTH 4560^T		*G. entanii*	Germany; submerged high-acid spirit vinegar fermentation	S10	*G. entanii*
LMG 1380	NBRC 3261	*G. europeaus*	1941; Nishinomiya, Japan; *Myrica rubra*, fruit	A16	*G. europeaus*
LMG 1510	NCIMB 613	*G. xylinus*	Copenhagen, Denmark; vinegar	A16	*G. europeaus*
LMG 1521*	NCIMB 7029	*G. xylinus*	Nairobi, Kenya; from mixed culture NCTC 6716 isolated from vinegar brew	A16	*G. europeaus*
LMG 18494	Trcek V3	*G. europaeus*	1995; Ljubljana, Slovenia; red wine vinegar produced in submerged bioreactor	A16	*G. europeaus*
LMG 18890^T	DES 11^T, DSM 6160^T	*G. europaeus*	Esslingen, Germany; submerged culture vinegar generator	A16	*G. europeaus*
LMG 20956	JK2	*G. europaeus*	1995; Ljubljana, Slovenia; cider vinegar produced in industrial submerged bioreactor	A16	*G. europeaus*
LMG 1524	NCIMB 8246, NBRC 14916	*G. hansenii*	Jerusalem, Israel; vinegar.	A20	*G. hansenii*
LMG 1527^T	NCIMB 8746^T, NBRC 14820^T	*G. hansenii*	Celluloseless mutant 1 derived from NCIB 8745 isolated by M. Aschner, Jerusalem, vinegar	A20	*G. hansenii*
LMG 1528	NCIMB 8747, NBRC 14817	*G. hansenii*	Celluloseless mutant 2 derived from NCIB 8745 isolated by M. Aschner, Jerusalem, vinegar	A20	*G. hansenii*
LMG 23726	RG3	*G. kombuchae*	2004; India; kombucha tea ferment	A20	*G. hansenii*
LMG 18909^T	TF2^T	*G. intermedius*†	Switzerland; commercially available tea fungus beverage (Kombucha)	A22	*G. intermedius*
DSM 11804^T	TF2^T	*G. intermedius*†	Switzerland; commercially available tea fungus beverage (Kombucha)	A22	*G. intermedius*
LMG 21312^T	CFN-Cf55^T	*G. johannae*	1996; Tapachula Chiapas, Mexico; *Coffea arabica* L., rhizosphere	A11	*G. johannae*
LMG 23153	CFN-Cf75	*G. johannae*	1997; Motozintla Chiapas, Mexico; *Coffea arabica* L., rhizosphere	A11	*G. johannae*
LMG 23154	UAP-Cf76	*G. johannae*	1997; Motozintla Chiapas, Mexico, *Coffea arabica* L., rhizoplane	A11	*G. johannae*
LMG 1348	NCIMB 9417, IAM 1836	*G. liquefaciens*	1935; Japan; fruit	A2	*G. liquefaciens*
LMG 1381^T	NCIMB 9136^T, NBRC 12388^T	*G. liquefaciens*	1935; Japan; *Diospyros* sp., dried fruit	A2	*G. liquefaciens*
LMG 1509	LMD 53.1	*G. liquefaciens*	1950; canal water	A2	*G. liquefaciens*
LMG 1536^I	NATA-strain	*G. nataicola*	Manila, Philippines; nata de coco; nata producing organism	S7	*G. nataicola*
LMG 1517	NCIMB 4940, NBRC 14822	*G. oboediens*†		A21	*G. oboediens*
LMG 1688	LMD 22.3	*G. oboediens*†		A21	*G. oboediens*
LMG 1689	LMD 29.8	*G. oboediens*†		A21	*G. oboediens*
LMG 18849^T	LTH 2460^T	*G. oboediens*	Esslingen, Germany; red wine vinegar fermentation	A21	*G. oboediens*
DSM 11826^T	LTH 2460^T	*G. oboediens*†	Esslingen, Germany; red wine vinegar fermentation	A21	*G. oboediens*
LMG 18907*	JK3	*G. intermedius*†	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor	A21	*G. oboediens*
LMG 18908*	JKD	*G. intermedius*†	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor	A21	*G. oboediens*
LMG 22126^I	DST GL02^I	*G. rhaeticus*	2003; Val Venosta, South Tyrol, Italy; organic apple juice	S16	*G. rhaeticus*
LMG 19747^T	SRI 1794^I	*G. sacchari*	Australia; *Saccharum officinarum*, surface microflora	A5	*G. sacchari*
LMG 19748	SRI 1853	*G. sacchari*	Queensland Australia; mealy bug	A5	*G. sacchari*
LMG 1582^T	LMD 29.3	*G. saccharivorans*	1927; Germany; beet juice	A23	*G. saccharivorans*
LMG 1584	LMD 39.5	*G. saccharivorans*		A23	*G. saccharivorans*
LMG 22125^I	DST GL01^I	*G. swingsii*	2003; Val Venosta, South Tyrol, Italy; organic apple juice	S4	*G. swingsii*
LMG 1515^T	NCIMB 11664^T, JCM 7644^T	*G. xylinus*	Mountains ash berries	A14	*G. xylinus*
LMG 1518	NCIMB 5346	*G. europeaus*	O. Verona	A14	*G. xylinus*
LMG 1356^I	NBRC 3250^I	*Gl. albidus*	1941; Japan; *Dahlia coccinea*; *Acetobacter albidus*	A8	*Gl. albidus*
LMG 1375 t1	NBRC 3273	*Gl. albidus*	1941; Nishinomiya, Japan; *Myrica rubra*, fruit	A8	*Gl. albidus*
LMG 1368^T	NBRC 3267^T	*Gl. cerinus*	1941; Osaka, Japan; *Prunus* sp. (cherry)	A19	*Gl. cerinus*
LMG 1376 t1	NBRC 3274	*Gl. cerinus*	1941; Nishinomiya, Japan; *Myrica rubra*, fruit.	A19	*Gl. cerinus*
LMG 1389	NBRC 3275	*Gl. cerinus*	1941; herbal garden, Kyoto, Japan; *Rheum rhabarbarum*, flower	A19	*Gl. cerinus*
LMG 1390	NBRC 3276	*Gl. cerinus*	1941; herbal garden, Kyoto, Japan; *Rheum rhabarbarum*, flower; type strain of *Gluconobacter asaii*	A19	*Gl. cerinus*
LMG 1357 t1	NBRC 3251	*Gl. frateuri*	1941; botanical garden, Kyoto, Japan; *Dahlia* sp., flower	A18	*Gl. frateuri*
LMG 1358	NBRC 3253	*Gl. frateuri*	1941; botanical garden, Kyoto, Japan; *Dahlia* sp., flower	A18	*Gl. frateuri*
LMG 1365^T	NBRC 2364^T	*Gl. frateuri*	1941; Osaka, Japan; *Fragaria ananassa*	A18	*Gl. frateuri*
LMG 1369 t1	NBRC 3268	*Gl. frateuri*	1941; Osaka, Japan; *Prunus* sp. (cherry)	A18	*Gl. frateuri*
LMG 1371	NBRC 3270	*Gl. frateuri*	1941; Osaka, Japan; *Prunus* sp. (cherry)	A18	*Gl. frateuri*
LMG 1379	NBRC 3286	*Gl. frateuri*	1941; Osaka, Japan; *Eriobotrya japonica*, fruit	A18	*Gl. frateuri*
LMG 1367^T t1	NBRC 2366	*Gl. kondonii*	1941; Osaka, Japan; *Fragaria ananassa*	S3	*Gl. kondonii*
LMG 1359	NBRC 3462	*Gl. oxydans*		A10	*Gl. oxydans*
LMG 1385	NBRC 3293	*Gl. oxydans*	1941; Osaka, Japan; Diospyros sp., dried fruit	A10	*Gl. oxydans*
LMG 1393	NBRC 12528	*Gl. oxydans*	Delft, the Netherlands; Amstel beer; assay of nicotinic acid (= niacin), p-aminobenzoic acid, pantothenic acid; type strain of Gluconobacter oxydans subsp. suboxydans	A10	*Gl. oxydans*
LMG 1395	NBRC 3287	*Gl. oxydans*	1941; botanical garden, Kyoto, Japan; Liatris scariosa; flower	A10	*Gl. oxydans*
LMG 1396*		*Gl. oxydans*	beer	A10	*Gl. oxydans*
LMG 1408^T	NBRC 14819^T	*Gl. oxydans*	beer	A10	*Gl. oxydans*
LMG 1424	NBRC 3244	*Gl. oxydans*		A10	*Gl. oxydans*
LMG 1674*		*Gl. oxydans*	1924; Delft, the Netherlands; beer	A10	*Gl. oxydans*

LMG 1486	NBRC 3254	*Gl. thailandicus*	1941; Osaka, Japan; *Fragaria ananassa*	A17	*Gl. thailandicus*
LMG 1487	NBRC 3255	*Gl. thailandicus*	1941; Osaka, Japan; *Fragaria ananassa*	A17	*Gl. thailandicus*
LMG 23137^T	F149-1^T	*Gl. thailandicus*	Bangkok, Thailand; Indian cork tree (*Millingtonia hortensis*), flower	A17	*Gl. thailandicus*
LMG 23138	F142-1	*Gl. thailandicus*	Bangkok, Thailand; glossy ixora (*Ixora lobbii*), flower	A17	*Gl. thailandicus*
DSM 17861^T	CDGNIH1^T	*Gr. bethesdensis*	2003; Bethesda, United States; patient with chronic granulomatous disease, lymph node	S9	*Gr. bethesdensis*
LMG 21812^T	Yo-3^T	*K. baliensis*	1996; Bali, Indonesia; palm brown sugar	S11	*K. baliensis*
LMG 24037^T	AC28^T	*N. chiangmaiensis*	2002; Chiang-Mai, Thailand; red ginger (*Alpinia purpurata*), flower	S2	*N. chiangmaiensis*
LMG 23170^T	S-877^T	*S. floricola*	Kanagawa Prefecture, Japan; pollen	S14	*S. floricola*
LMG 21291^T	PA51^T	*Sw. salitolerans*	1998; Pichavaram mangrove forest Chidambaram, Tamil Nadu, India; wild rice *Porteresia coarctata*	S1	*Sw. salitolerans*

*Strain of which no DNA-DNA hybridization data or 16S-23S rDNA ITS sequence analysis data have (previously) been reported; †The type strains of *G. oboediens* and *G. intermedius* show 63 % DNA relatedness according to Dellaglio *et al.* (2005) and > 70 % according to Sievers & Swings (2005) and Lisdiyanti *et al.* (2006)

SDS in 10 mM Tris/HCl - 1 mM EDTA, pH 8.0; w/v) was added to the mixture, and the whole was incubated at 37 °C for 60 min. After visual verification of cell lysis, 120 µl 5 M NaCl was added and mixed by inversion, before adding 80 µl CTAB/NaCl solution (10 % hexadecyltrimethylammonium bromide in 0.7 M NaCl; w/v). After 20 min of incubation at 65 °C, the lysate was subjected to a phenol/chloroform/isoamylalcohol (49:49:1) extraction using Phase Lock Gel^{TM} tubes (Eppendorf). Ten µl of RNase A solution (10 mg ml^{-1}; Sigma) was added to the carefully transferred aqueous phase and after 15-30 min of incubation at 37 °C, the solution was again subjected to a phenol/chloroform/isoamylalcohol (49:49:1) extraction using Phase Lock Gel^{TM} tubes. The aqueous phase was carefully transferred and mixed with 500 µl ice-cold 100 % isopropanol, and kept on ice for 15 min. Pellets were collected by centrifugation, washed in ice-cold 70 % ethanol, dried, and resuspended in 50-200 µl 0.1 x TE solution, depending on the size of the pellet. The first method was used when the aim was to obtain large amounts (more than 200 µg) of genomic DNA. DNA concentration and purity were estimated by measuring the absorptions at 260 nm, 280 nm and 234 nm. DNA concentration and size were additionally verified by agarose gel electrophoresis using a 1 % (w/v) agarose gel. For *G. entanii* LTH 4560^T, DNA prepared in the frame of the descriptions of *G. swingsii* and *G. rhaeticus* (Dellaglio *et al.*, 2005) was used.

Evaluation of restriction enzymes. Genomic DNA was digested with *Apa*I, *Hin*dIII, *Eco*RI, *Taq*I, *Apa*I/*Taq*I, *Hin*dIII/*Taq*I or *Eco*RI/*Taq*I (Amersham Pharmacia Biotech) in a volume of 20 µl containing 10 units (of each) restriction enzyme, 1 µg DNA, 4 µl buffer [2 µl OPA (10 x; Applied Biosystems), 0.2 µl BSA (10 mg/ml), 0.1 µl DTT (1.0 M), 1.7 µl MilliQ water] and MilliQ water. Digested DNAs were subjected to agarose gel electrophoresis (1 % agarose gel; w/v).

Template preparation, selective PCR, and polyacrylamide gel electrophoresis. Template DNA for selective PCR was prepared essentially as previously described and

so were the primers used (Janssen *et al.*, 1996). One µg of high-molecular-mass DNA was digested with *Taq*I/*Apa*I, *Taq*I/*Hin*dIII or *Taq*I/*Eco*RI, followed by ligation of corresponding double-stranded restriction half-site adaptors (Janssen *et al.*, 1996) using T4 ligase (Amersham Pharmacia Biotech). Template DNA was precipated in a solution containing 100 µl T0.1E (10 mM Tris/HCl - 0.1 mM EDTA, pH 8.0) and used for selective PCR amplification. The following primer combinations were tested: A01/T03, A03/T03, H01/T03, H03/T03, E01/T03 and E03/T03. Each primer consisted of a core region (A-, H-, E- and T-primer core sequences, see Janssen *et al.*, 1996) and a 3'-extension of one nucleotide (01 = adenine as 3'-extension, 03 = guanine as 3'-extension). The hexaprimers were labeled with the fluorescent label 6-FAM at their 5'-end. The amplification mix contained 1.5 µl template DNA, 0.5 µl 4 µM hexaprimer, 0.5 µl 1 µM tetraprimer, and 7.5 µl Amplification Core Mix (Applied Biosystems). The amplification reactions were performed in a GeneAmp PCR system 9700 thermocycler using the 9600 mode (Applied Biosystems) and the temperature program previously described (Thompson *et al.*, 2001a). Selective PCR products were separated using polyacrylamide gel electrophoresis as previously described (Thompson *et al.*, 2001a).

Data analysis. Tracking and normalization of the AFLP® DNA fingerprints, as well as assigning and sizing bands were performed using the GeneScan 3.1 software (Applied Biosystems). Tables with information on the fragments of 50 to 536 base pairs were transferred into the BioNumerics 4.61 software (Applied Maths) for numerical analysis. Fingerprint similarity values were calculated using the DICE coefficient with a position tolerance of 0.2 % to compensate for technical imperfections (optimization tolerance: 0.15 %). A dendrogram was constructed using the unweighted pair group method using arithmetic averages (UPGMA). The zone between 60 and 500 bp was used for comparison.

DNA-DNA hybridizations. Most DNA-DNA hybridizations shown in Tables 4.3 and 4.4 were performed at the BCCM/LMG Bacteria Collection in the frame of previous studies, all according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) of the microplate method described by Ezaki *et al.* (1989). In the frame of this study, some additional hybridizations using the same method were performed. Briefly, genomic DNA for these DNA-DNA hybridizations 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} absorption (A) ratios of 1.8-2.0 and 0.40-0.60, respectively, was used. The hybridization temperature was 48 °C and

hybridizations were carried out in the presence of 50 % formamide. Reciprocal reactions (e.g. A x B and B x A) were performed for every pair of DNA and their variation was within the limits of this method (Goris *et al.*, 1998).

16S rRNA gene sequencing. A nearly complete 16S rRNA gene sequence of G. *kombuchae* LMG 23726^T was determined following the protocol described by Franz *et al.* (2006), using the primers *Gamma, *PD, *O, *3, *R, pA, Gamma, 3 and BKL1 (Cleenwerck *et al.*, 2007; Coenye *et al.*, 1999a). The pairwise similarity between the consensus sequence and the 16S rRNA gene sequence of G. *kombuchae* RG3^T from the EMBL database (AY688433) was calculated with the BioNumerics 4.61 software package (Applied Maths) using an open gap penalty of 100 % and a unit gap penalty of 0 %. A phylogenetic tree based on 16S rRNA gene sequences retrieved from the EMBL database was constructed with the BioNumerics 4.61 software package using the neighbour-joining method (Saitou & Nei, 1987) with an open gap penalty of 100 % and a unit gap penalty of 0 %.

Phenotypic characterization, DNA G+C content determination and *nifH* PCR. The production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was determined by the method described by Gosselé *et al.* (1980) using high pressure anion exchange chromatography with conductivity detection under ion suppression (Van der Meulen *et al.*, 2006) instead of thin layer chromotography. Growth on the carbon sources ethanol, sucrose, sorbitol, and D-mannitol; growth in the presence of 30 % D-glucose; production of acid from galactitol; and production of cellulose were examined by methods previously reported (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2006). The G+C content of DNA prepared for DNA-DNA hybridization was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as calibration reference. *NifH* PCR was performed using the degenerate primers 19F and 407R (Franke *et al.*, 1998).

RESULTS AND DISCUSSION

Choice of restriction enzymes and primers

Genomic DNA of 5 strains covering a DNA G+C content range from 53-67 mol%, namely *A. pasteurianus* LMG 1262^T, *A. tropicalis* LMG 19825^T, *G. hansenii* LMG

1527^T, *G. liquefaciens* LMG 1381^T and *G. azotocaptans* LMG 21311^T, prepared following the protocol previously described (Cleenwerck *et al.*, 2002) and digested with the restriction enzymes *Apa*I, *Hin*dIII, *Eco*RI, *Taq*I, *Apa*I/*Taq*I, *Hin*dIII/*Taq*I and *Eco*RI/*Taq*I was verified for restriction on a 1 % agarose gel, to detect the most suited restriction enzyme combination for preparing template DNA. However, none of the hexacutters appeared to restrict well all genomic DNA and selective PCR using the primer combinations A01/T03 and A03/T03 for *Apa*I/*Taq*I-tDNA, H01/T03 and H03/T03 for *Hin*dIII/*Taq*I-tDNA, and E01/T03 and E03/T03 for *Eco*RI/*Taq*I-tDNA yielded AFLP® patterns with no or only very few bands (data not shown). It was assumed that polysaccharides present in the DNA solutions were inhibiting both restriction endonuclease treatments and PCR (Chan & Goodwin, 1995) and therefore genomic DNA was isolated using a protocol further optimized for removal of polysaccharides from DNA solutions (see Material and Methods). When the tests mentioned above were repeated with this newly prepared DNA, AFLP® patterns yielded between 9 and 125 bands (data not shown). Finally, the restriction enzyme combination *Apa*I/*Taq*I and primer combination A03/T03 were selected for further AFLP® analysis of AAB. This combination generated AFLP® DNA fingerprints exhibiting the most suited characteristics as can be deduced from previous studies (Huys *et al.*, 1996; Thompson *et al.*, 2001a). Each of these fingerprints contained 40 to 106 bands evenly distributed in the range of 50 to 536 bp, whereas 60 % of the DNA fingerprints generated using the other combinations contained less than 40 bands.

Reproducibility

It has been reported previously that DNA fingerprinting can be considered as a highly reproducible technique, making it very suitable to generate databases for identification purposes (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999). In this study, AFLP® DNA fingerprints of 35 strains were generated twice, starting from a new DNA isolation, and genomic DNA of *G. azotocaptans* LMG 21311^T was included in each set of DNA that was subjected to AFLP® analyses. The % similarity between fingerprints obtained for the same strain, after assignment of bands, ranged between 80 and 95 %, except for strains producing a lot of cellulose, such as *G. swingsii* LMG 22125^T and *G. intermedius* LMG 18909^T (= DSM 11804^T). In those cases the % similarity was sometimes lower (70 %). Nevertheless, AFLP® profiles of the same strain still clustered together when all generated AFLP® DNA fingerprints were considered, except if other strains generated very similar profiles (with band pattern similarity above the

reproducibility level), suggesting that the strains could be clonally related.

Identification of AAB at the species level

In the present study, 150 AAB representing the 50 recognized species of the family *Acetobacteraceae*, were analyzed by AFLP® DNA fingerprinting. For 135 of them, DNA-DNA hybridization data or 16S-23S rDNA spacer region sequence analysis data were previously reported (Boesch *et al.*, 1998; Camu *et al.*, 2007; Cleenwerck *et al.*, 2002, 2007, 2008; De Vuyst *et al.*, 2008; Dellaglio *et al.*, 2005; Dutta & Gachhui, 2006, 2007; Franke *et al.*, 1999; Fuentes-Ramírez *et al.*, 2001; Greenberg *et al.*, 2006b; Jojima *et al.*, 2004; Katsura *et al.*, 2001; Lisdiyanti *et al.*, 2000, 2001, 2002, 2006; Loganathan & Nair, 2004; Malimas *et al.*, 2007; Muthukumarasamy *et al.*, 2005; Navarro *et al.*, 1999; Ndoye *et al.*, 2007a; Schüller *et al.*, 2000; Sievers & Swings, 2005; Sievers *et al.*, 1992; Silva *et al.*, 2006; Sokollek *et al.*, 1998b; Takahashi *et al.*, 2006; Trček & Teuber, 2002; Urakami *et al.*, 1989; Yamada *et al.*, 2000; Yukphan *et al.*, 2004b, 2005). Therefore, these strains were assumed to be accurately classified at the species level and were regarded here as reference strains.

In Fig. 4.5, the AFLP® DNA fingerprints of the 150 strains, as well as a UPGMA dendrogram obtained after cluster analysis of these patterns, are shown. Most strains (> 80 %) generated patterns consisting of more than 40 bands, evenly spread in the range of 50-536 bp. Strains generating fewer bands generally belong to the genus *Acetobacter*. For *A. pasteurianus*, the current restriction enzyme combination and primer combination appear not very suitable, as several strains generated profiles containing mainly larger bands (> 200 bp; e.g. A13), while others generated less than 15 bands (e.g. A33).

Nevertheless, the reference strains of a particular species generated profiles that grouped in clusters according to their respective taxonomic designations, with a few exceptions only. First, the species *A. pasteurianus* and *A. indonesiensi*s, were each divided over two AFLP® groups (Table 4.2). Both species were previously shown to contain genetically diverse strains (De Vuyst *et al.*, 2008; Trček & Teuber, 2002), which is in the case of *A. pasteurianus* also reflected in the DNA-DNA relatedness values ranging from 62-90 % (Table 4.3). Second, strains of the species *A. cerevisiae* (LMG 1599, LMG 1625^T, LMG 1682, LMG 1699) and *A. orleanensis* (LMG 1545, LMG 1583^T, LMG 1592, LMG 1608), two phenotypically similar and phylogenetically

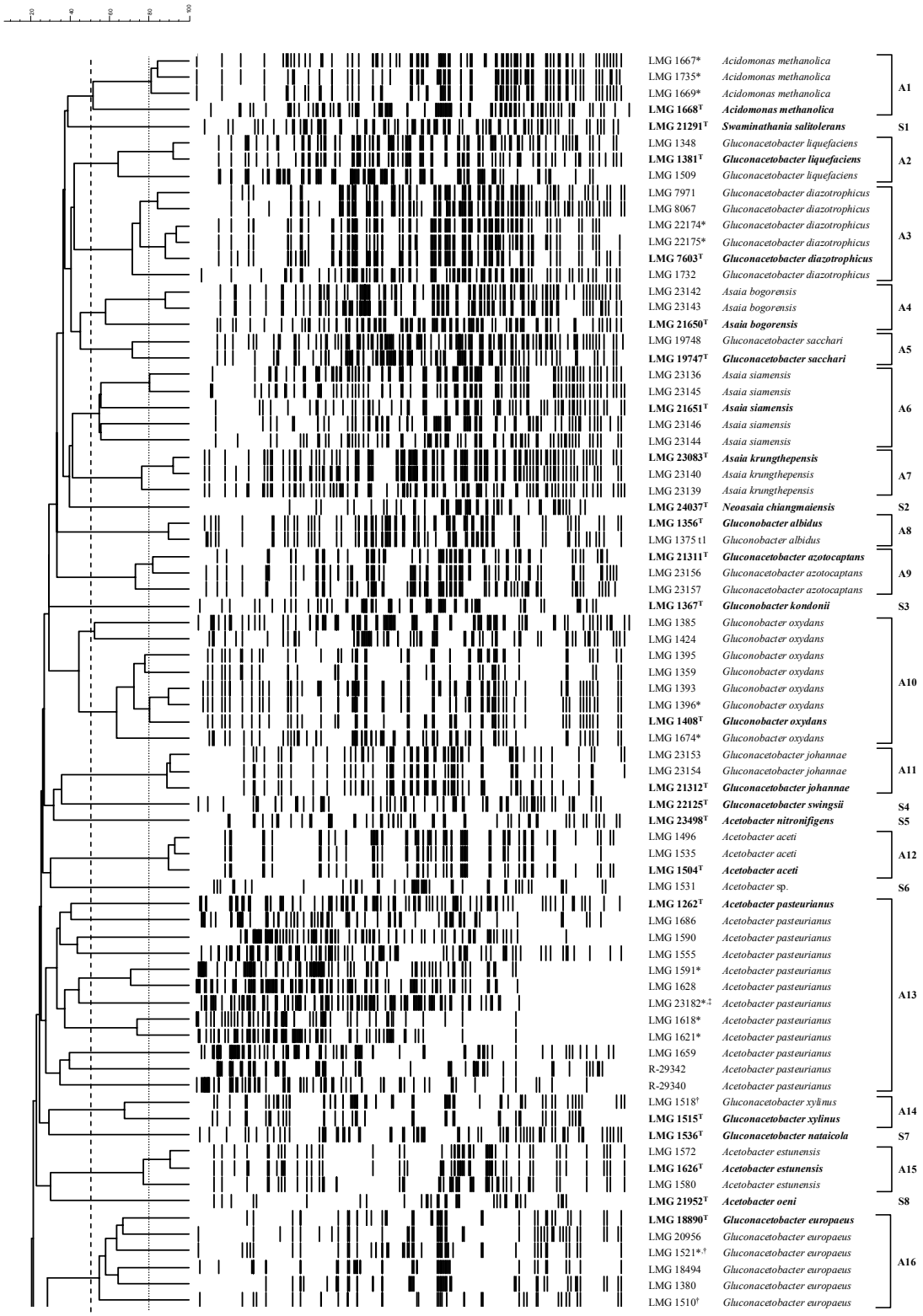
closely related species, as well as *G. europaeus* LMG 1518, *G. xylinus* LMG 1510 and *G. kombuchae* LMG 23726^T (Table 4.2), did not cluster as expected from their species designation and were, therefore, investigated into more detail (see below). It appeared that six of these strains have to be reclassified, namely LMG 1545, LMG 1592, and LMG 1608 as *A. cerevisiae*; LMG 1518 and LMG 1510 as *G. xylinus* and *G. europaeus*, respectively; and LMG 23726^T as *G. hansenii*. After re-assignment of these reference strains *A. cerevisiae* splits up over three AFLP® groups (Table 4.2). It has been mentioned before that species can be divided over different AFLP® clusters (Duim *et al.*, 2001; Huys *et al.*, 1996; Thompson *et al.*, 2001a; Willems *et al.*, 2001a).

In total, 54 AFLP® groups (A1 to A33, containing more than one strain; S1 to S21, containing each one strain) were delineated; each containing strains of the same species and showing a linkage level below 50 % with other groups. Each species is represented by one AFLP® group (except for *A. pasteurianus*, *A. indonesienis*, and *A. cerevisiae*; see above). Strain LMG 1531 (S6), previously assigned to *A. aceti* but removed from this species based on $(GTG)_5$-PCR fingerprint data and its low DNA-DNA relatedness (< 60 %) with true *A. aceti* strains (Table 4.3), showed a separate position, hereby confirming its status as a novel genospecies of *Acetobacter* (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987). Overall, the results with the reference strains revealed the potential of AFLP® for identification and classification of AAB at the species level.

Analysis of the AFLP® data of the 15 additional strains proved the taxonomic value of this AFLP® approach, as all 15 strains could be identified at the species level. Ten strains clustered according to their original taxonomic assignment; while four strains (*A. pasteurianus* LMG 1547, *G. intermedius* LMG 18907 and LMG 18908, and *G. xylinus* LMG 1521) did not (Table 4.2); these appeared all to be misclassified (see below). Finally, LMG 23182, deposited in the BCCM/LMG Bacteria Collection as *Acetobacter* sp. (Table 4.2), could be identified as *A. pasteurianus*, a result that was expected from other research (E. J. Bartowsky, personal communication).

Strains that have to be reclassified

LMG 1545 (A29), LMG 1592 (A29), and LMG 1608 (A25) were classified as *A. orleanensis*, mainly on the basis of DNA-DNA hybridization data (Lisdiyanti *et al.*, 2000). AFLP® DNA fingerprint data of strains of *A. orleanensis* and the closely related species *A. cerevisiae* indicated that LMG 1545, LMG 1592, and LMG 1608 were most probably misclassified, since their AFLP® DNA fingerprints clustered with those of *A.*


20
40
60
80
100
LMG 1667* Acidomonas methanolica
LMG 1735* Acidomonas methanolica
LMG 1669* Acidomonas methanolica
LMG 1668T Acidomonas methanolica
A1
LMG 21291T Swaminathania salitolerans
S1
LMG 1348 Gluconacetobacter liquefaciens
LMG 1381T Gluconacetobacter liquefaciens
LMG 1509 Gluconacetobacter liquefaciens
A2
LMG 7971 Gluconacetobacter diazotrophicus
LMG 8067 Gluconacetobacter diazotrophicus
LMG 22174* Gluconacetobacter diazotrophicus
LMG 22175* Gluconacetobacter diazotrophicus
LMG 7603T Gluconacetobacter diazotrophicus
LMG 1732 Gluconacetobacter diazotrophicus
A3
LMG 23142 Asaia bogorensis
LMG 23143 Asaia bogorensis
LMG 21650T Asaia bogorensis
A4
LMG 19748 Gluconacetobacter sacchari
LMG 19747T Gluconacetobacter sacchari
A5
LMG 23136 Asaia siamensis
LMG 23145 Asaia siamensis
LMG 21651T Asaia siamensis
LMG 23146 Asaia siamensis
LMG 23144 Asaia siamensis
A6
LMG 23083T Asaia krungthepensis
LMG 23140 Asaia krungthepensis
LMG 23139 Asaia krungthepensis
A7
LMG 24037T Neoasaia chiangmaiensis
S2
LMG 1356T Gluconobacter albidus
LMG 1375 t1 Gluconobacter albidus
A8
LMG 21311T Gluconacetobacter azotocaptans
LMG 23156 Gluconacetobacter azotocaptans
LMG 23157 Gluconacetobacter azotocaptans
A9
LMG 1367T Gluconobacter kondonii
S3
LMG 1385 Gluconobacter oxydans
LMG 1424 Gluconobacter oxydans
LMG 1395 Gluconobacter oxydans
LMG 1359 Gluconobacter oxydans
LMG 1393 Gluconobacter oxydans
LMG 1396* Gluconobacter oxydans
LMG 1408T Gluconobacter oxydans
LMG 1674* Gluconobacter oxydans
A10
LMG 23153 Gluconacetobacter johannae
LMG 23154 Gluconacetobacter johannae
LMG 21312T Gluconacetobacter johannae
A11
LMG 22125T Gluconacetobacter swingsii
S4
LMG 23498T Acetobacter nitronifigens
S5
LMG 1496 Acetobacter aceti
LMG 1535 Acetobacter aceti
LMG 1504T Acetobacter aceti
A12
LMG 1531 Acetobacter sp.
S6
LMG 1262T Acetobacter pasteurianus
LMG 1686 Acetobacter pasteurianus
LMG 1590 Acetobacter pasteurianus
LMG 1555 Acetobacter pasteurianus
LMG 1591* Acetobacter pasteurianus
LMG 1628 Acetobacter pasteurianus
LMG 23182*,‡ Acetobacter pasteurianus
LMG 1618* Acetobacter pasteurianus
LMG 1621* Acetobacter pasteurianus
LMG 1659 Acetobacter pasteurianus
R-29342 Acetobacter pasteurianus
R-29340 Acetobacter pasteurianus
A13
LMG 1518† Gluconacetobacter xylinus
LMG 1515T Gluconacetobacter xylinus
A14
LMG 1536T Gluconacetobacter nataicola
S7
LMG 1572 Acetobacter estunensis
LMG 1626T Acetobacter estunensis
LMG 1580 Acetobacter estunensis
A15
LMG 21952T Acetobacter oeni
S8
LMG 18890T Gluconacetobacter europaeus
LMG 20956 Gluconacetobacter europaeus
LMG 1521*,† Gluconacetobacter europaeus
LMG 18494 Gluconacetobacter europaeus
LMG 1380 Gluconacetobacter europaeus
LMG 1510† Gluconacetobacter europaeus
A16

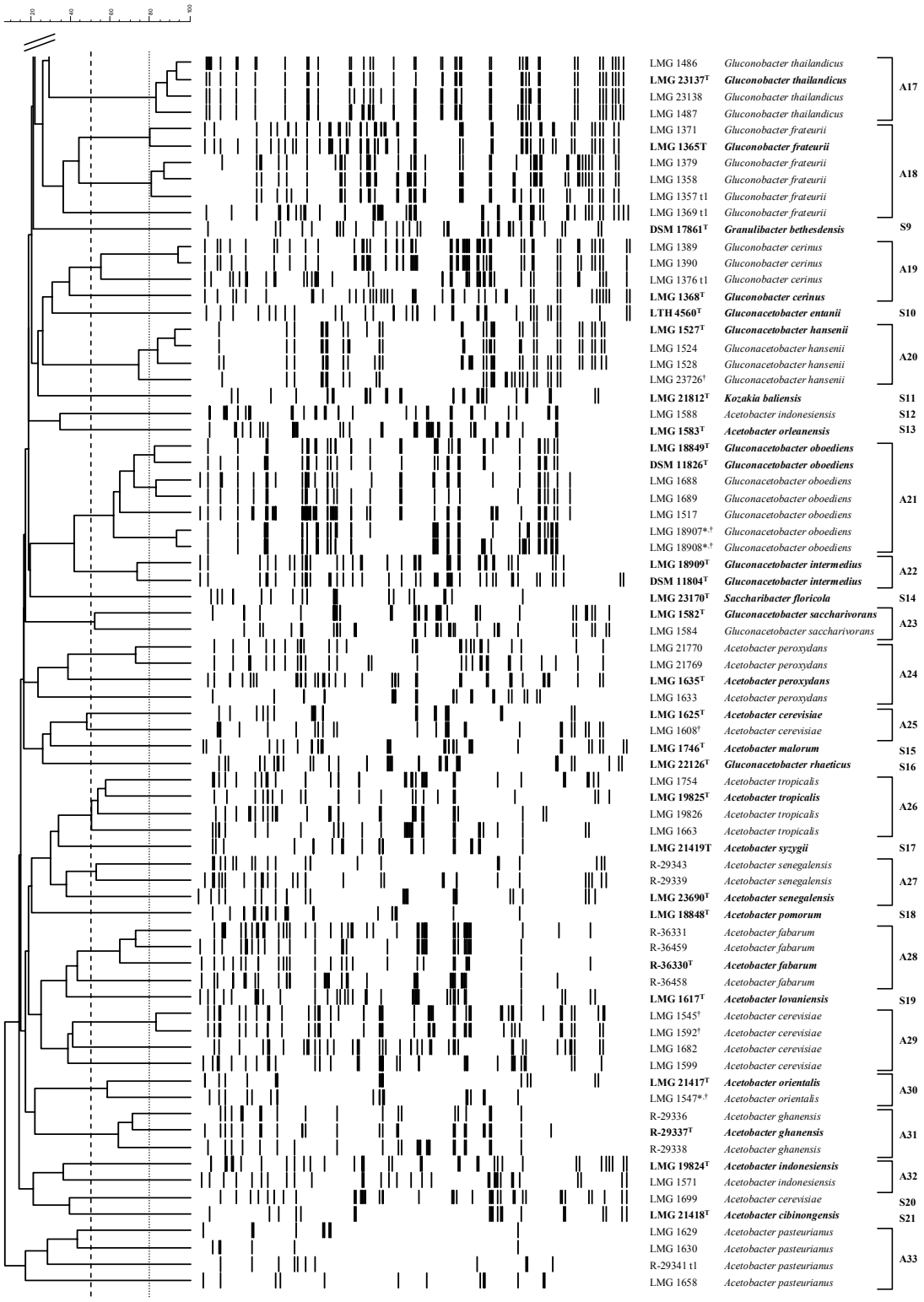

20
40
60
80
100
LMG 1486 Gluconobacter thailandicus
LMG 23137T Gluconobacter thailandicus A17
LMG 23138 Gluconobacter thailandicus
LMG 1487 Gluconobacter thailandicus
LMG 1371 Gluconobacter frateurii
LMG 1365T Gluconobacter frateurii
LMG 1379 Gluconobacter frateurii
LMG 1358 Gluconobacter frateurii A18
LMG 1357 t1 Gluconobacter frateurii
LMG 1369 t1 Gluconobacter frateurii
DSM 17861T Granulibacter bethesdensis S9
LMG 1389 Gluconobacter cerinus
LMG 1390 Gluconobacter cerinus A19
LMG 1376 t1 Gluconobacter cerinus
LMG 1368T Gluconobacter cerinus
LTH 4560T Gluconacetobacter entanii S10
LMG 1527T Gluconacetobacter hansenii
LMG 1524 Gluconacetobacter hansenii A20
LMG 1528 Gluconacetobacter hansenii
LMG 23726† Gluconacetobacter hansenii
LMG 21812T Kozakia baliensis S11
LMG 1588 Acetobacter indonesiensis S12
LMG 1583T Acetobacter orleanensis S13
LMG 18849T Gluconacetobacter oboediens
DSM 11826T Gluconacetobacter oboediens
LMG 1688 Gluconacetobacter oboediens
LMG 1689 Gluconacetobacter oboediens A21
LMG 1517 Gluconacetobacter oboediens
LMG 18907*,† Gluconacetobacter oboediens
LMG 18908*,† Gluconacetobacter oboediens
LMG 18909T Gluconacetobacter intermedius
DSM 11804T Gluconacetobacter intermedius A22
LMG 23170T Saccharibacter floricola S14
LMG 1582T Gluconacetobacter saccharivorans
LMG 1584 Gluconacetobacter saccharivorans A23
LMG 21770 Acetobacter peroxydans
LMG 21769 Acetobacter peroxydans
LMG 1635T Acetobacter peroxydans A24
LMG 1633 Acetobacter peroxydans
LMG 1625T Acetobacter cerevisiae
LMG 1608† Acetobacter cerevisiae A25
LMG 1746T Acetobacter malorum S15
LMG 22126T Gluconacetobacter rhaeticus S16
LMG 1754 Acetobacter tropicalis
LMG 19825T Acetobacter tropicalis
LMG 19826 Acetobacter tropicalis A26
LMG 1663 Acetobacter tropicalis
LMG 21419T Acetobacter syzygii S17
R-29343 Acetobacter senegalensis
R-29339 Acetobacter senegalensis A27
LMG 23690T Acetobacter senegalensis
LMG 18848T Acetobacter pomorum S18
R-36331 Acetobacter fabarum
R-36459 Acetobacter fabarum
R-36330T Acetobacter fabarum A28
R-36458 Acetobacter fabarum
LMG 1617T Acetobacter lovaniensis S19
LMG 1545† Acetobacter cerevisiae
LMG 1592† Acetobacter cerevisiae
LMG 1682 Acetobacter cerevisiae A29
LMG 1599 Acetobacter cerevisiae
LMG 21417T Acetobacter orientalis
LMG 1547*,† Acetobacter orientalis A30
R-29336 Acetobacter ghanensis
R-29337T Acetobacter ghanensis
R-29338 Acetobacter ghanensis A31
LMG 19824T Acetobacter indonesiensis
LMG 1571 Acetobacter indonesiensis A32
LMG 1699 Acetobacter cerevisiae S20
LMG 21418T Acetobacter cibinongensis S21
LMG 1629 Acetobacter pasteurianus
LMG 1630 Acetobacter pasteurianus
R-29341 t1 Acetobacter pasteurianus A33
LMG 1658 Acetobacter pasteurianus

Fig. 4.5. AFLP DNA fingerprints of strains representing the recognized species of the family *Acetobacteracea*. The dendrogram was constructed with the UPGMA method after calculation of the band pattern similarity (%) using the DICE coefficient. *, strain of which no DNA-DNA hybridization data or 16S-23S rDNA ITS sequence analysis data have (previously) been reported; †, strain reclassified on the basis of data presented in this study; ‡, strain identified on the basis of AFLP DNA fingerprint data obtained in this study.

cerevisiae strains, instead of with that of the type strain of *A. orleanensis* LMG 1583^T (Fig. 4.5). Newly performed DNA-DNA hybridizations with LMG 1625^T, LMG 1583^T, LMG 1682, LMG 1545, and LMG 1608 confirmed the classification of LMG 1682 as *A. cerevisiae* (DNA-DNA relatedness of 88 % with *A. cerevisiae* LMG 1625^T versus 34 % with *A. orleanensis* LMG 1583^T), and revealed that LMG 1545 and LMG 1608 should be reclassified in *A. cerevisiae* (Table 4.3). LMG 1592 should also be reclassified as *A. cerevisiae*, as its AFLP® DNA fingerprint showed > 80 % band pattern similarity with that of LMG 1545.

LMG 1518 (A14) and LMG 1510 (A16) were classified as *G. europaeus* and *G. xylinus*, respectively, on the basis of DNA-DNA hybridization data and $(GTG)_5$-PCR DNA fingerprint data (De Vuyst *et al.*, 2008). In the present study, AFLP® analyses were performed for both strains starting from two newly opened LMG ampoules, and on the DNA used in the study of De Vuyst *et al.* (2008). The DNA fingerprint data revealed that the former taxonomic conclusions must have been based on a switch during DNA isolation or strain cultivation; and LMG 1518 and LMG 1510 have to be reassigned to *G. xylinus* and *G. europaeus*, respectively.

LMG 23726^T (A20) is the type strain and only strain of *G. kombuchae* (Dutta & Gachhui, 2007). Its AFLP® DNA fingerprint clustered with AFLP® patterns of strains belonging to *G. hansenii*, its phylogenetically nearest neighbour (99.4 % 16S rRNA gene sequence similarity), indicating that both species could be synonymous. Additional tests confirmed this synonymy. First, the almost complete 16S rRNA gene sequence of *G. kombuchae* LMG 23726^T was determined (1441 nucleotides, AM999342) and revealed *G. kombuchae* RG3^T as neirest neighbour (AY688433, 99.6 % 16S rRNA gene sequence similarity), indicating that LMG 23726^T is a subculture of RG3^T. Second, DNA-DNA hybridizations between *G. kombuchae* LMG 23726^T and *G. hansenii* LMG 1527^T revealed a DNA-DNA relatedness of 81 % during the present study, which is clearly above 70 % (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987) and much higher than the 25 % DNA-DNA relatedness reported by Dutta & Gachhui (2007). *G.*

kombuchae and *G. hansenii* belong phylogenetically to the *G. xylinus* branch (Fig. 4.4; Dutta & Gachhui, 2007; Yamada & Yukphan, 2008), which contains several species showing ≥ 99.4 % 16S rRNA gene sequence similarity. DNA-DNA relatedness values between closely related species of this cluster are generally intermediate (Table 4.4). Third, the DNA G+C content was determined for both strains and was 59.4 mol% for LMG 1527^T and 59.5 mol% for LMG 23726^T, the latter being definitely higher than the 55.8 mol% reported by Dutta & Gacchui (2007). Fourth, recently, Lisdiyanti *et al.* (2006) emended the species description of *G. hansenii*; among ten strains, previously assigned to this species, only three strains were shown to be members (Lisdiyanti *et al.*, 2006). These three strains (LMG 1524, LMG 1527^T, and LMG 1528) as well as *G. kombuchae* LMG 23726^T were investigated for phenotypic characteristics that were reported to enable differentiation of both species from the other species of the *G. xylinus* branch. All four strains produced 2-keto- and 5-keto-D-gluconic acid from D-glucose, acid from galactitol, and were able to grow on D-sorbitol and D-mannitol as energy sources. Growth on the energy sources ethanol and sucrose was variable (LMG 1527^T grew weakly on ethanol, but not on sucrose), as well as growth in the presence of 30 % D-glucose (LMG 1527^T grew weakly in 30 % D-glucose). Cellulose production was clearly noticed for LMG 23726^T, but could not be confirmed for LMG 1524. In general, most previous phenotypic findings were confirmed. Fifth, according to Dutta & Gachhui (2007) the major difference between the type strain of *G. kombuchae* and the related gluconacetobacters was its ability to fix nitrogen. The presence of the *nifH* gene in *G. kombuchae* was confirmed by PCR with the degenerate primers 19F and 407R (Franke *et al.*, 1998) and sequencing of the amplified PCR product (Dutta & Gachhui, 2007). In this study, the four strains all generated a PCR product of the same size (~ 600 bp) using the degenerate primers mentioned above (data not shown), indicating the presence of the *nifH* gene in all these strains. The data presented clearly show that *G. kombuchae* should be reclassified as *G. hansenii*, since both species are synonymous, with the name *G. hansenii* having priority over the name *G. kombuchae* according to rules 38 and 42 of the Bacterial Code (Lapage *et al.*, 1992).

LMG 1547 (A30) was classified as *A. pasteurianus* by Gosselé *et al.* (1983b) based on its position in subphenon E, established on the basis of numerical analysis of 177 phenotypic features. Its AFLP® DNA fingerprint showed much resemblance (~ 55 % band pattern similarity and clear visual similarity) to that of *A. orientalis* LMG 21417^T. DNA-DNA hybridizations between both strains revealed 100 % DNA-DNA relatedness, proving that LMG 1547 is in fact a member of *A. orientalis*.

LMG 18907 and LMG 18908 (A21) were classified as *G. intermedius* on the basis of SDS-PAGE data of their soluble proteins (Boesch *et al.*, 1998). However, their AFLP® DNA fingerprints showed more similarity with AFLP® patterns of *G. oboediens* strains. At present, confusion exists concerning the taxonomic status of *G. oboediens* and *G. intermedius*, due to discrepancies in the DNA-DNA relatedness values reported for their type strains (Cleenwerck & De Vos, 2008). *G. oboediens* and *G. intermedius* were described around the same time in the same year (Boesch *et al.*, 1998; Sokollek *et al.*, 1998b) and were hence not compared with each other upon description. Later studies revealed that both were phylogenetically closely related species of the *G. xylinus* branch (Fig 4.4; Dellaglio *et al.*, 2005; Lisdiyanti *et al.*, 2006; Yamada & Yukphan, 2008).

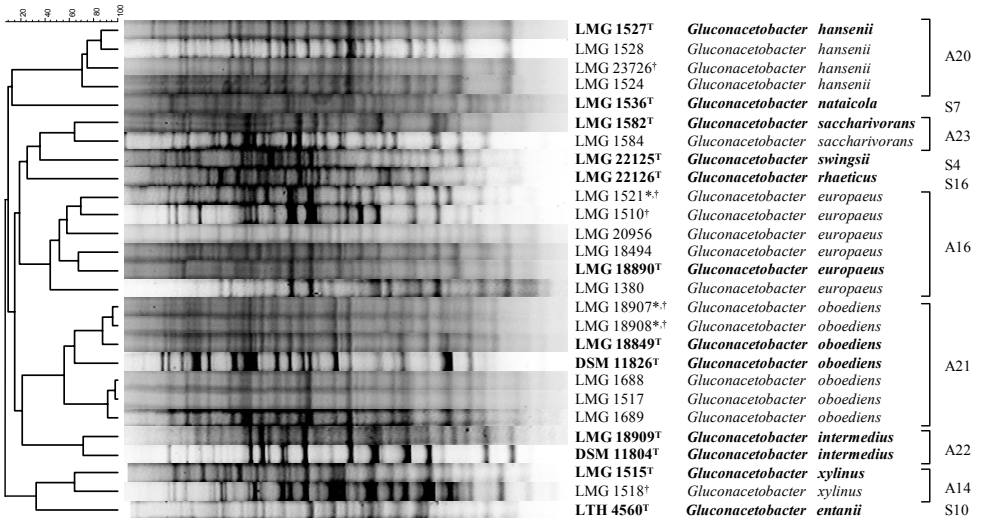


Fig. 4.6. $(GTG)_5$-PCR fingerprints of strains belonging to the species of the *Gluconacetobacter* xylinus branch. The dendrogram was constructed with the UPGMA method after calculation of the band pattern similarity (%) using the Pearson correlation coefficient. *, strain of which no DNA-DNA hybridization data or 16S-23S rDNA ITS sequence analysis data have (previously) been reported; †, strain reclassified on the basis of data presented in this study.

According to Lisdiyanti *et al.* (2006) and Sievers & Swings (2005) both species should be regarded synonymous, mainly because a high DNA-DNA relatedness value was found between their type strains (76 % and > 77 %, respectively). However, according to Dellaglio *et al.* (2005), the type strains shared 63 % DNA-DNA relatedness, which was somewhat lower than the 68 % DNA-DNA relatedness observed between G. *oboediens* LMG 18849^T and G. *europaeus* LMG 18890^T (Table 4.4), suggesting that

both species should better remain separate. The AFLP® DNA fingerprint data supported the conclusion based on the DNA-DNA relatedness of 63 % between the type strains. In this study, additional tests were therefore performed to clarify the taxonomic status of both species. First, *G. intermedius* DSM 11804^T and *G. oboediens* DSM 11826^T, used in the hybridization study of Lisdiyanti *et al.* (2006), were analyzed by AFLP® and their patterns clustered with those of *G. intermedius* LMG 18909^T and *G. oboediens* LMG 18849^T, respectively, with a similarity higher than the reproducibility level. Second, the strains of the species of the *G. xylinus* branch (Fig. 4.4) were subjected to $(GTG)_5$-PCR DNA fingerprinting following the protocol previously described (De Vuyst *et al.*, 2008). Clustering of the $(GTG)_5$ DNA fingerprints (Fig. 4.6) revealed the same groupings as AFLP® DNA fingerprinting. Third, additional DNA-DNA hybridizations with LMG 18849^T, LMG 1517, LMG 1688, and LMG 1619 (A21), and LMG 18909^T (A22) revealed DNA-DNA relatedness values ranging from 53-63 % between *G. intermedius* LMG 18909^T and strains of AFLP® group A21, containing the type strain of *G. oboediens*, whereas values ranging from 68 to 100 % were obtained amongst strains of AFLP® group A21 (Table 4.4). All data together support that *G. intermedius* and *G. oboediens* should better not be regarded synonymous. Consequently, LMG 18907 and LMG 18908 have to be reclassified as *G. oboediens*. Interesting is that the type strain of *G. intermedius* has been isolated from kombucha, whereas strains of AFLP® cluster A21 with a known origin, have been isolated from vinegar.

LMG 1521 (A16) was classified as *A. xylinus* (= G. *xylinus*) on the basis of phenotypic data (Gosselé *et al.*, 1983b). Boesch *et al.* (1998) however noticed that this strain reacted with oligonucleotide probe 23seu, developed for *G. europeaus*. The AFLP® data confirm its classification as *G. europaeus*.

Determination of the genetic diversity amongst strains belonging to the same species

In this study, AFLP® patterns of strains belonging to different genera and generated using the same restriction enzyme combination and primer combination were compared. In Fig. 4.5 it can be noticed that AFLP® patterns of strains belonging to a particular species and containing more than 40 bands generally group at a linkage level of 50 % or more; whereas those with less bands generally cluster at a lower linkage level, as can be noticed for several *Acetobacter* species, with three of them being split up in several AFLP® groups. Also, Janssen *et al.* (1996) noticed that linkage levels of some clusters dropped significantly if the number of bands in the AFLP® patterns decreased.

Table 4.3. DNA-DNA relatedness of *Acetobacter* strains, determined in the frame of this study[1] and previous studies (Camu *et al.*, 2007[7]; Cleenwerck *et al.*, 2002[2], 2007[8], 2008[9]; De Vuyst *et al.*, 2007[6]; Muthukumarasamy *et al.*, 2005[3]; Ndoye *et al.*, 2007a[5]; Silva *et al.*, 2006[4]) at the BCCM/LMG Bacteria Collection

≥ 70 % DNA-DNA binding; 50-69 % DNA-DNA binding

Proposed species designation		Strain number	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	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
A. aceti	1.	LMG 1496	100																																																					
	2.	LMG 1504^{T}	95[2]	100																																																				
	3.	LMG 1531	55[2]	53[2]	100																																																		
	4.	LMG 1535	104[2]	100[2]	59[2]	100																																																		
A. cerevisiae	5.	LMG 1545					100																																																
	6.	LMG 1599		11[2]				100																																															
	7.	LMG 1608							100																																														
	8.	LMG 1625^{T}	15[2]	12[2]	18[2]	16[2]	83[1]	77[2]	75[1]	100																																													
	9.	LMG 1682	9[2]	9[2]	18[2]	10[2]		80[2]		85[2]	100																																												
	10.	LMG 1699		8[2]				76[2]		74[2]	66[2]	100																																												
A. cibinongensis	11.	LMG 21418^{T}											100																																											
A. estunensis	12.	LMG 1572								17[2]				100																																									
	13.	LMG 1580												92[2]	100																																								
	14.	LMG 1626^{T}		17[2]				25[2]		15[2]	15[2]	27[2]		98[2]	92[2]	100																																								
A. fabarum	15.	R-36330^{T}															100																																						
	16.	R-36331															82[9]	100																																						
	17.	R-36458															97[9]	88[9]	100																																				
	18.	R-36459															88[9]	99[9]	89[9]	100																																				
A. ghanensis	19.	R-29336		5[8]						15[8]			11[8]			10[8]					100																																			
	20.	R-29337^{T}															35[9]				99[8]	100																																		
	21.	R-29338																			88[8]	98[8]	100																																		
A. indonesiensis	22.	LMG 1571																						100																																
	23.	LMG 1588		9[2]				26[2]		20[2]	20[2]	32[2]		18[2]		13[2]								83[2]	100																																
	24.	LMG 19824^{T}								18[2]											14[8]			85*	88*	100																															
A. lovaniensis	25.	LMG 1617^{T}	12[2]	13[2]	16[2]	14[2]				11[2]	18[2]			9[2]		10[2]	52[9]				47[6]			15[2]			100																																
A. malorum	26.	LMG 1746^{T}		3[2]				50[2]		53[2]				5[2]		5[2]					9[8]			10[2]			9[2]	100																														
A. nitronifigens	27.	LMG 23498^{T}																			4[8]								100																												
A. oeni	28.	LMG 21952^{T}		13[4]						13[4]			7[4]			20[4]					6[8]			7[4]		15[4]	5[4]		100																													
A. orientalis	29.	LMG 21417^{T}																			11[8]							6[4]	100																												
	30.	LMG 1547																										10[2]		100[1]	100																										
A. orleanensis	31.	LMG 1583^{T}		4[2]				37[2]		33[2]	40[2]	37[2]		7[2]		6[2]					13[8]			12[2]			10[2]	28[2]		6[4]		100																												
A. pasteurianus	32.	LMG 1262^{T}	9[2]	7[2]	18[2]	10[2]		30[2]		32[2]	32[2]	35[2]		21[2]		20[2]	20[9]				20[8]			17[2]			14[2]	7[2]		16[4]			12[2]	100																									
	33.	LMG 1555		7[2]										19[2]																				78[2]	100																								
	34.	LMG 1629	7[2]	6[2]	6[2]	8[2]				19[2]	13[2]			3[2]													18[2]	6[2]						70[2]		100																						
	35.	LMG 1630		9[2]										19[2]														8[2]						71[2]			100																			
	36.	LMG 1658		5[2]																														62[2]				59[2]	100															
	37.	LMG 1659		6[2]																														69[2]					66[2]	100														
	38.	LMG 1686	6[2]	8[2]	16[2]	14[2]				37[2]	22[2]			21[2]													21[2]	11[2]						82[2]		90[2]	79[2]	66[2]		100															
	39.	R-29340																																80[6]							100													
	40.	R-29341																																77[6]								100											
	41.	R-29342																																							68[6]	76[6]	100											
A. peroxydans	42.	LMG 1633																																									100											
	43.	LMG 1635^{T}															18[9]				18[8]						16[2]			13[4]				13[2]									63[6]	100									
	44.	LMG 21769																																										87[3]	100								
	45.	LMG 21770																																										89[3]	99[3]	100							
A. pomorum	46.	LMG 18848^{T}	7[2]	6[2]	12[2]	8[2]		23[2]		27[2]	19[2]	24[2]		12[2]		13[2]	18[9]				18[8]			16[2]			20[2]	9[2]		12[4]			12[2]	53[2]	51[2]	55[2]	51[2]	55[2]		58[2]					23[2]			100							
A. senegalensis	47.	LMG 23690^{T}		7[5]						18[5]			17[5]			9[5]										21[5]		18[5]	3[5]	6[5]	15[5]		22[5]																		100					
	48.	R-29339																																																	79[7]	100			
	49.	R-29343																																																	81[7]	75[6]	100		
A. syzygii	50.	LMG 21419^{T}															32[9]				46[6]									10[4]																										100		
A. tropicalis	51.	LMG 1663	8[2]	9[2]	10[2]	10[2]				27[2]	24[2]			13[2]		13[2]								17[2]	20[2]		14[2]	12[2]						20[2]		14[2]		6[2]	14[2]	22[2]								10[2]					100		
	52.	LMG 1754		7[2]																														18[2]				11[2]	12[2]														76[2]	100	
	53.	LMG 19825^{T}								34[2]											11[8]			18[2]						14[4]																						52[5]	58[6]	54[6]		86[2]		100	
	54.	LMG 19826																						19[2]																														93[2]		81[2]	100

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

Table 4.4. DNA-DNA relatedness of *Gluconacetobacter* strains, determined in the frame of this study[1] and previous studies (De Vuyst *et al.*, 2007[3]; Dellaglio *et al*, 2005[2]) at the BCCM/LMG Bacteria Collection

≥ 70 % DNA-DNA binding; 50-69 % DNA-DNA binding.

Proposed species designation		Strain number	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	26	27	28	29	30	31	32
G. azotocaptans	1.	LMG 21311^T	100																															
G. diazotrophicus	2.	LMG 7603[1]	22[1]	100																														
	3.	LMG 7971		74[3]	100																													
	4.	LMG 8067		94[3]	89[3]	100																												
G. entanii	5.	LTH 4560[1]					100																											
G. europaeus	6.	LMG 1380						100																										
	7.	LMG 1510						69[1]	100																									
	8.	LMG 1521								100																								
	9.	LMG 18494									100																							
	10.	LMG 18890^T						70[1]	73[3]			70[1]	100																					
	11.	LMG 20956							86[1]				76[1]	100																				
G. hansenii	12.	LMG 1524							17[1]				25[1]		100																			
	13.	LMG 1527^T		12[1]		12[1]			13[1]				24[2]		97[3]	100																		
	14.	LMG 1528													100[3]	100[3]	100																	
	15.	LMG 23726														81[1]		100																
G. intermedius	16.	LMG 18909^T							61[1]		49[1]	57[2]			23[1]	19[2]			100															
G. johannae	17.	LMG 21312[1]	57[1]	28[1]															100															
G. liquefaciens	18.	LMG 1348																		100														
	19.	LMG 1381^T	17[1]	20[1]		21[1]									14[1]					100[3]	100													
	20.	LMG 1509																		91[3]	82[3]	100												
G. nataicola	21.	LMG 1536^T																					100											
G. oboediens	22.	LMG 1517						55[1]										53[1]						100										
	23.	LMG 1688																						101[1]	100									
	24.	LMG 1689																56[1]							90[1]	100								
	25.	LMG 18849^T							56[1]			68[2]			24[2]			63[2]						68[1]	69[1]	75[1]	100							
G. rhaeticus	26.	LMG 22126^T					16[2]					33[2]			21[2]			35[2]									34[2]	100						
G. saccharivorans	27.	LMG 19747^T	19[1]	28[1]																	32[1]								100				
G. saccharivorans	28.	LMG 1582[1]																												100				
	29.	LMG 1584																												85*	100			
G. swingsii	30.	LMG 22125^T					16[2]					60[2]			20[2]			57[2]									52[2]	45[2]				100		
G. xylinus	31.	LMG 1518							61[3]		47[1]	63[1]			15[1]			45[1]									54[1]						100	
	32.	LMG 1515^T		13[1]		11[1]			42[3]	43[1]		50[2]		20[1]	16[2]			50[2]			13[1]						51[2]	37[2]			49[2]	77[3]	100	

*Data taken from Lisdiyanti *et al.* (2006).

These data indicate that AFLP® DNA fingerprints with less than 40 bands are more discriminatory at the intraspecies level and less suitable for species level identification.

Nevertheless, some conclusions regarding the intraspecific genetic diversity can be taken from the reported data. For instance, several species, such as *G. oxydans* (A10), *A. pasteurianus* (A13, A33), *G. frateurii* (A18), *G. cerinus* (A19), *A. indonesienis* (S12, A32), and *A. cerevisiae* (A25, A29, S20) harbour genetically diverse strains, whereas other species, such as *G. diazotrophicus* (A3), *A. krungthepensis* (A7), *G. albidus* (A8), *G. azotocaptans* (A9), *G. johannae* (A11), *A. aceti* (A12), *A. estunensis* (A15), *G. thailandicus* (A17), and *G. hansenii* (A20) harbour strains generating quite homogenous patterns. For the *Gluconobacter* strains, the AFLP® data correlate very well with the results of Takahashi *et al.* (2006) based on 16S-23S spacer region sequence analysis data. For the species appearing to have a limited genetic diversity, AFLP® analyses of additional strains (if available) and with other primer combinations (such as A01/T03) are advized to gain a deeper understanding of their genetic diversity. A previous study showed that a better insight into the genetic diversity of *G. diazotrophicus* was obtained when more isolates from sugarcane, as well as from other host plants such as sweet potato and from the mealybug *Saccharococcus sacchari*, were investigated (Caballero-Mellado *et al.*, 1995). Other studies stated that highly related strains may be distinguished by AFLP® when the appropriate set of selective primers is used (e.g. Janssen *et al.*, 1996).

This study further revealed several strains with very similar AFLP® DNA fingerprints, grouping together at a linkage level of 80 % or more, such as *G. liquefaciens* LMG 1381^T and LMG 1348 (A2), and *G. albidus* LMG 1356^T and LMG 1375 t1 (A8). These strains may be clonally related, especially if they have been isolated in the same year from the same source at the same site. In this perspective, it is interesting to note that the four *G. thailandicus* strains (A17) that show very similar AFLP® patterns have different geografical origins, as two strains were recently isolated from flowers collected in Thailand (LMG 23137^T and LMG 23138), whereas the other two strains were isolated in 1941 from *Fragaria ananassa* in Japan (LMG 1486 and LMG 1487). For all these strains, additional analysis, such as AFLP® using other primer combinations or PFGE, could be performed to determine whether these strains are indeed clonally related.

Conclusion

The data in this study demonstrate that AFLP® DNA fingerprinting will permit

accurate identification and classification of a broad range of AAB. Additionally, AFLP® DNA fingerprinting appears to be useful to determine the intraspecific genetic diversity. AFLP® DNA fingerprinting can be regarded as a valuable technique for the taxonomy of AAB.

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CHAPTER 5

DESCRIPTIONS OF TWO NOVEL *ACETOBACTER SPECIES*, BOTH ISOLATED FROM TRADITIONAL HEAP FERMENTATIONS OF GHANAIAN COCOA BEANS

5.1. *ACETOBACTER GHANENSIS* SP. NOV., A NOVEL ACETIC ACID BACTERIUM ISOLATED FROM TRADITIONAL HEAP FERMENTATIONS OF GHANAIAN COCOA BEANS

Redrafted from:
Cleenwerck, I., Camu, N., Engelbeen, K., De Winter, T., Vandemeulebroecke, K., De Vos, P. & De Vuyst, L. (2007). *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. *Int J Syst Evol Microbiol* 57, 1647-1652.

SUMMARY

Twenty-three AAB, 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-keto-D-gluconic acid, 5-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid from D-glucose and were therefore tentatively identified as *Acetobacter* sp. 16S rRNA gene sequencing and phylogenetic analysis confirmed their position in the genus *Acetobacter* with *A. syzygii* and *A. lovaniensis* as their closest phylogenetic neighbours. $(GTG)_5$-PCR fingerprinting grouped the strains in a cluster that did not contain any type strain 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 content 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 of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose, were catalase-positive and oxidase-negative, grew on

yeast extract with 30 % D-glucose, grew on glycerol (although weak) 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).

INTRODUCTION

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*, *Neoasaia* and *Granulibacter* (Cleenwerck *et al.*, 2002; Greenberg *et al.*, 2006b; Jojima *et al.*, 2004; Loganathan & Nair, 2004; Yukphan *et al.*, 2005). A common feature of AAB (with the exception of *Asaia*) is the ability to oxidize ethanol to acetic acid under neutral and acidic (pH 4.5) conditions. This characteristic makes that 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 (Kersters *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 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 a 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.*, 2001b).

Today, little is known about AAB associated with cocoa bean fermentation. *Acetobacter* and *Gluconobacter* strains are mostly commonly found (Schwan & Wheals, 2004). During a biodiversity study on microorganisms involved in the spontaneous fermentation of Ghanaian cocoa beans, 132 AAB strains were isolated.

These strains, together with 64 AAB reference strains, 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 without any AAB type strain, suggesting that they could represent a novel AAB species (De Vuyst *et al.*, 2008). 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.

MATERIALS AND METHODS

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 for their isolation and incubation took place at 42 °C for 1 to 4 days. The isolates were preserved at -80 °C in MYP medium [2.5 % D-mannitol (w/v), 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 as R-29336, R-29337^T and R-29338, 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, unless indicated otherwise.

Cells 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 a 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 acetic acid bacterium) were used as controls.

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-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid from D-glucose was determined by the methods described by Gosselé *et al.* (1980).

Fatty acids were extracted and analyzed 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 6 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.

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} of Tris/HCl (10 mM) + EDTA (1 mM) buffer (pH 8.0)]. Nearly complete sequences were determined using the eight sequencing primers listed in Coenye *et al.* (1999a), except for strain R-29337^T where the 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 the test statistical reliability of the topology of the neighbour-joining tree using 1000 bootstrap resamplings of the data.

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} absorption ratios of 1.8–2.0 and 0.40-0.60, respectively, was used. DNA-DNA hybridizations were performed according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) of the microplate method described by Ezaki *et al.* (1989). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. AxB and BxA) 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 reactions.

The DNA base composition of R-29336, R-29337^T and R-29338 was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as calibration reference.

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 % D-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).

RESULTS AND DISCUSSION

The 23 novel AAB isolates appeared on solidified MYP medium (1.5 % (w/v) agar) as beige, round, convex, smooth, shiny colonies with a diameter of approximately 0.8 mm after 4 days of incubation. Cells of all isolates were Gram-negative rods, approximately 0.8 µm in wide and 1.5-2.5 µm long. Cells occurred singly or in pairs and were oxidase-negative and catalase-positive.

All isolates oxidized ethanol to acetic acid and produced gluconic acid from D-glucose. They did not produce 2-keto-D-gluconic acid, 5-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid from D-glucose. These characteristics indicated that the strains probably belonged to the genus *Acetobacter* that, at the time of writing, comprised 16 recognized species (Cleenwerck *et al.*, 2002; Dutta & Gachhui, 2006; Lisdiyanti *et al.*, 2000, 2001; Silva *et al.*, 2006).

The predominant fatty acid found in the isolates 430A^T, 444B, 415, 131, 153A and 110 was the straight-chain, unsaturated $C_{18:1}\omega 7c$ fatty acid, which accounted for approximately 50 % of the total fatty acid content. This is in agreement with results obtained previously for AAB (Dellaglio *et al.*, 2005; Franke *et al.*, 1999; Kersters *et al.*, 2005; Urakami *et al.*, 1989;). 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 $\omega 8c$ (5-15 %). Compared with reported data for species of the genus *Acetobacter* (Franke *et al.*, 1999; Urakami *et al.*, 1989), the novel isolates contained high levels of $C_{19:0}$ cyclo ω8c.

Nearly complete sequences of strains R-29337^T, 444B and 384 were obtained. A neighbour-joining and a maximum-parsimony tree showing the phylogenetic position of strains R-29337^T, 444B and 384 within the family *Acetobacteraceae* are shown in Fig. 5.1 and Fig. 5.2, respectively. The neighbour-joining tree (Fig. 5.1) is slightly different from the maximum-parsimony tree (Fig. 5.2), however both trees clearly show that strains R-29337^T, 444B and 384 belong to the genus *Acetobacter* and, more precisely, to

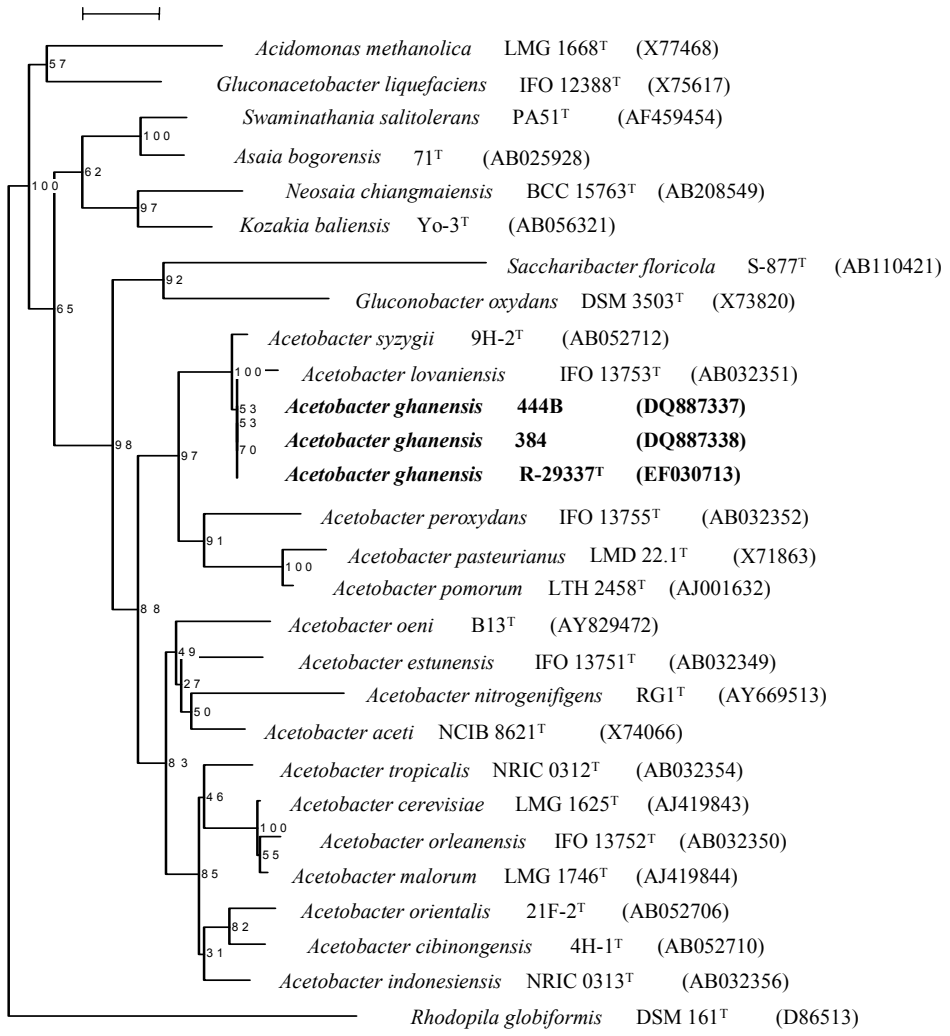

Fig. 5.1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter ghanensis* sp. nov. and related species of the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

the subcluster containing *A. lovaniensis*, *Acetobacter syzygii*, *Acetobacter peroxydans*, *A. pasteurianus* and *Acetobacter pomorum* (Cleenwerck *et al.*, 2002; Dutta & Gachhui, 2006; Lisdiyanti *et al.*, 2001). 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 %),

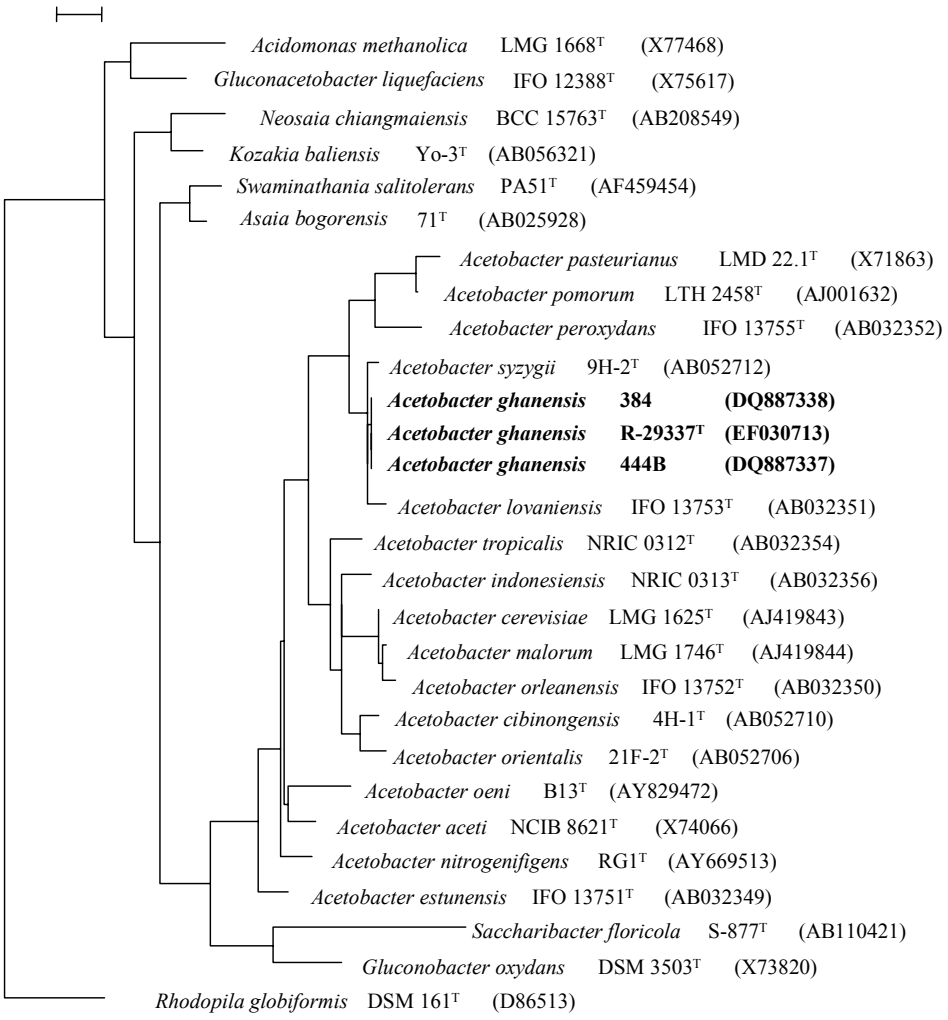

Fig. 5.2. Maximum parsimony tree based on nearly complete 16S rRNA gene sequences of *Acetobacter ghanensis* sp. nov. and related species of the family *Acetobacteraceae*. Bar, 1 nt substitution per 100 nt.

Acetobacter orientalis (97.8 %), *A. pasteurianus* (97.7 %), *Acetobacter cibinongenis* (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) and R-29338 (= 415) and from the type strains of all recognized species of the genus *Acetobacter*, were carried out. Strain R-29336 showed high DNA-DNA binding 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 binding 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. cibinongenis* 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 new genospecies within the genus *Acetobacter* (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987).

The DNA G+C content of isolates R-29336, R-29337^T and R-29338 ranged from 56.9 to 57.3 mol%, which is consistent with DNA G+C contents of members of the genus *Acetobacter* (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2000, 2001; Silva *et al.*, 2006).

The novel isolates R-29336, R-29337^T and R-29338 were able to grow on glycerol (weakly), but not on maltose or methanol as carbon source. Growth on ethanol was variable (R-29337^T was negative). The isolates grew on yeast extract with 30 % D-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 5.1. The isolates differed from *A. syzygii* mainly by their growth on yeast extract with 30 % D-glucose, but not on maltose as the sole carbon source. They differed from *A. lovaniensis* by their inability to produce 2-keto-D-gluconic acid from D-glucose, and their growth on yeast extract with 30 % D-glucose, but not on methanol as the sole carbon source or on ethanol as carbon source with ammonium as nitrogen source.

Table 5.1. Differential characteristics between *Acetobacter ghanensis* sp. nov. and related species of the genus *Acetobacter* species

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* (' 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,; weak 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 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	w	+	+	+	+	v	+	-	+	+	+	v	+	+	+	+	+
Maltose	-	+	-	-	-	v	-	+	v	+	+	-	v	-	w	-	+
Methanol	-	-	+	-	+	-	-	-	-	-	-	-	-	-	w	-	-
Growth in 10 % ethanol	v	-*	-*	ND	ND	+*	-*	-*	-*	-*	-*	-*	-*	-*	-*	+*	+
Growth on YE + 30 % D-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	53.2-54.3	52.1	59.7-60.7	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).

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 µm wide and 1.5-2.5 µm 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 D-glucose. Unable to produce 2-keto-D-gluconic acid, 5-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid from D-glucose. $C_{18:1}\omega 7c$ 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 $\omega 8c$ (5-10 %). Able to grow on yeast extract with 30 % D-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 hybridizations and $(GTG)_5$-PCR fingerprinting. The type strain, strain R-29337^T (= 430^T = LMG 23848^T= DSM 18895^T), was isolated from a traditional heap fermentation of Ghanaian cocoa beans.

ACKNOWLEDGEMENTS

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co-operation with the Belgian Coordinated Collections of Micro-organisms, BCCM), and Barry Callebaut N. V. The cooperation of the Ghanaian Cocoa Producers' Alliance (COCOBOD, Accra, Ghana) and the Cocoa Research Institute of Ghana is highly appreciated. An approval was obtained by the COCOBOD to cooperate with local farmers.

5.2. *ACETOBACTER FABARUM* SP. NOV., A GHANAIAN COCOA BEAN HEAP FERMENTATION ACETIC ACID BACTERIUM

Redrafted from:
Cleenwerck, I., González, Á., Camu, N., Engelbeen, K., De Vos, P. & De Vuyst, L. (2008). *Acetobacter fabarum* sp. nov., a Ghanaian cocoa bean heap fermentation acetic acid bacterium. *Int J Syst Evol Microbiol* (in press).

SUMMARY

Six AABl 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 did not result in their identification. Phylogenetic analysis based on 16S rRNA gene sequences allocated the isolates to the genus *Acetobacter* and revealed *Acetobacter lovaniensis*, *Acetobacter ghanensis* and *Acetobacter syzygii* as nearest neighbours. DNA-DNA hybridizations demonstrated that the isolates belonged to a single new genospecies that could be differentiated from the 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 carbon sources, no growth on yeast extract with 30 % D-glucose, and weak growth at 37 °C. The DNA G+C content of four selected strains ranged from 56.8-58.0 mol%. The results obtained prove that the isolates should be classified as a novel species of the genus *Acetobacter*. The name *Acetobacter fabarum* sp. nov. is proposed. The type strain is R-36330^T (=985^T = LMG 24244^T = DSM 19596^T).

INTRODUCTION

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 the 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; Camu *et al.*, 2007; De Vuyst *et al.*, 2008; Lagunes Gálvez *et al.*, 2007; Nielsen *et al.*, 2007; Schwan & Wheals, 2004). However, isolation of AAB from such fermentations have thus 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; 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 % bromo-cresol, 0.01 % cycloheximide, 1.8 % agar, pH 4.5; w/v; 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 (Lisdiyanti *et al.*, 2001, 2003; Yamada *et al.*, 2000). For this reason it has been suggested to use a combination of media to avoid a selective isolation of AAB (Lisdiyanti *et al.*, 2003).

During a study of the microbial biodiversity of spontaneous fermentations of Ghanaian cocoa beans, 209 AAB isolates were collected from four different culture media: Deoxycholate-Mannitol-Sorbitol (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; w/v; Lisdiyanti *et al.*, 2001), Basal Medium with ethanol (BME) agar (0.05 % yeast extract, 0.3 % vitamin-free casamino acids (Difco), 0.3 % ethanol; w/v) and Glucose-Yeast extract (GY) agar (5 % D-glucose, 0.5 % yeast extract; w/v). The agar concentration was in all media 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 (Cleenwerck *et al.*, 2007; De Vuyst *et al.*, 2008). A group of six AAB isolates that could not be identified was revealed (Fig. 5.3). The six AAB isolates were collected from three of the four different culture media [4 isolates from AAM agar (900, 1145, 950 and 1104), one from BME agar (985^T) and one from GY agar (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*

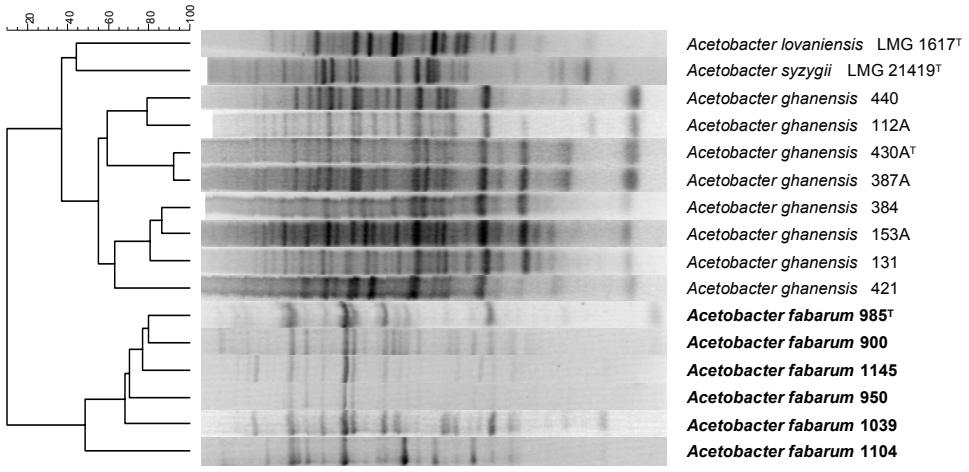

Fig. 5.3. $(GTG)_5$-PCR fingerprints of the six *A. fabarum* isolates and their closest phylogenetic relatives. The dendrogram derived from unweighted pair-group cluster analysis (UPGMA) of the fingerprints. Levels of linkage are expressed as Pearson similarity coefficients.

al., 2003) to avoid selective isolation of AAB. The present study deals with the further characterization of these isolates and shows that they represent a novel species of the genus *Acetobacter*.

MATERIALS AND METHODS

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 % Ca-lactate, 1.5 % agar; w/v) by incubation at 28 °C under aerobic conditions for 1 to 4 days. Isolates 985^T, 1145, 950 and 1039 were deposited in the Research Collection of the Laboratory of Microbiology (LM-UGent) as strains 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 microscopic studies, the isolates were grown on Z1 agar. Cells were tested for their Gram reaction, cell shape and cell size, catalase activity and oxidase activity, following the methods described previously (Cleenwerck *et al.*, 2002).

Sequences of the 16S rRNA genes of isolates R-36330^T and R-36331 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 (Cleenwerck *et al.*, 2007; Coenye *et al.*, 1999a). 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, Belgium) 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).

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} absorption ratios of 1.8–2.0 and 0.40-0.60, respectively, was used. DNA-DNA hybridizations were performed according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) 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 x B and B x A) were performed for every pair of DNA and their variation was within the limits of this method (Goris *et al.*, 1998). The DNA base composition of isolates R-36330^T, R-36331, R-36458 and R-36459 was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as calibration reference.

The production of gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was determined for isolates R-36330^T, R-36331, R-36458 and R-36459 by the method described by Gosselé *et al.* (1980) using high pressure anion exchange chromatography-conductivity (Van der Meulen *et al.*, 2006) instead of thin layer chromatography. Physiological characteristics that enable differentiation of the 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 as growth at different temperatures (28, 34, 37 and 42 °C) on GY agar (5 % D-glucose, 1 % yeast extract, 1.5 % agar, w/v) and growth on the carbon sources D-xylose, D-fructose, D-galactose and D-glucose.

RESULTS AND DISCUSSION

On Z1 medium, the isolates appeared as rough, shiny, beige, round colonies that were raised and wavy, with a diameter of approximately 0.8 mm, after incubation at 28 °C for 3 days. Cells of all isolates were motile, Gram-negative, coccoid rods

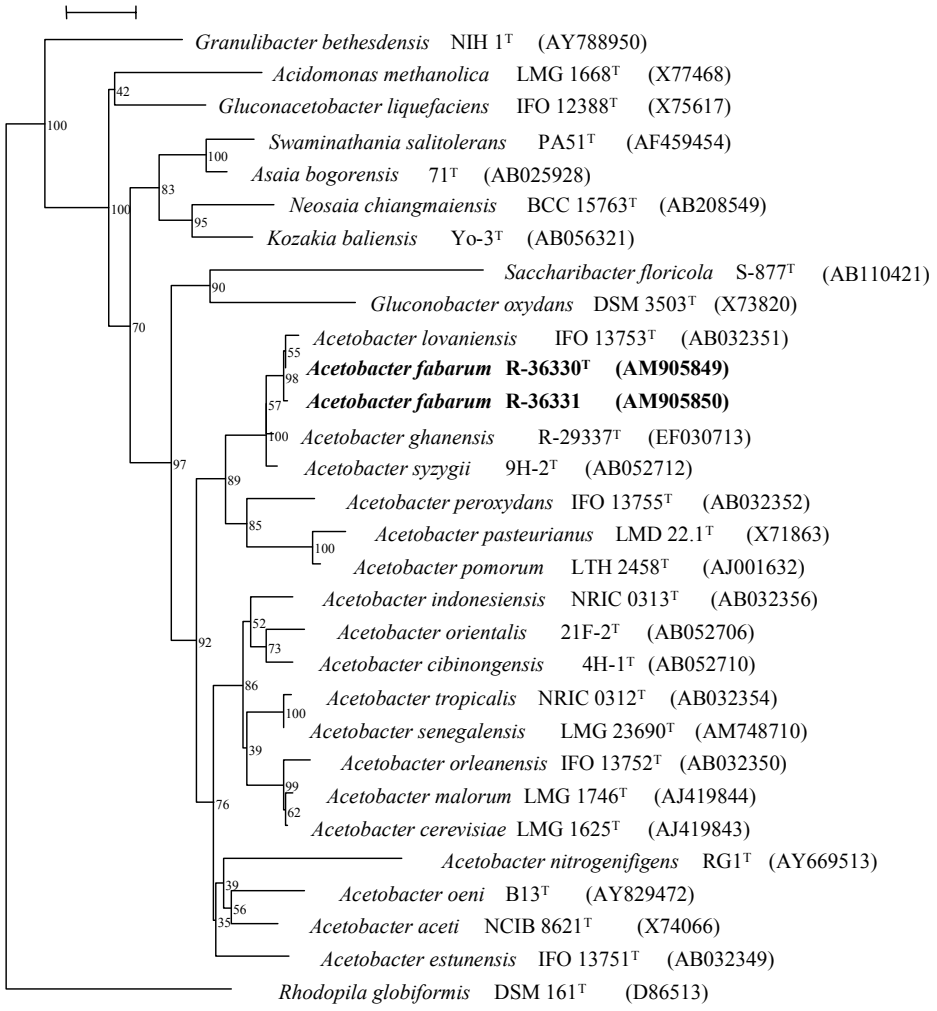

Fig. 5.4. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter fabarum* and related species of the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.

(0.8 x 1.2-3 µm) that occurred singly or in pairs and were oxidase-negative and

catalase-positive.

Nearly complete 16S rRNA gene sequences of isolates R-36330^T and R-36331 were obtained. 16S rRNA gene sequence analysis revealed that the phylogenetic affiliation of the isolates was in the genus *Acetobacter*, and more precisely in the sublineage (Cleenwerck *et al.*, 2002, 2007; Dutta & Gachhui, 2006; Lisdiyanti *et al.*, 2001) containing *A. lovaniensis*, *A. ghanensis*, *A. syzygii*, *A. peroxydans*, *A. pasteurianus* and *A. pomorum* (Fig. 5.4 and Fig. 5.5). Furthermore, the 16S rRNA gene sequence similarities obtained by pairwise alignment showed that isolates R-36330^T and

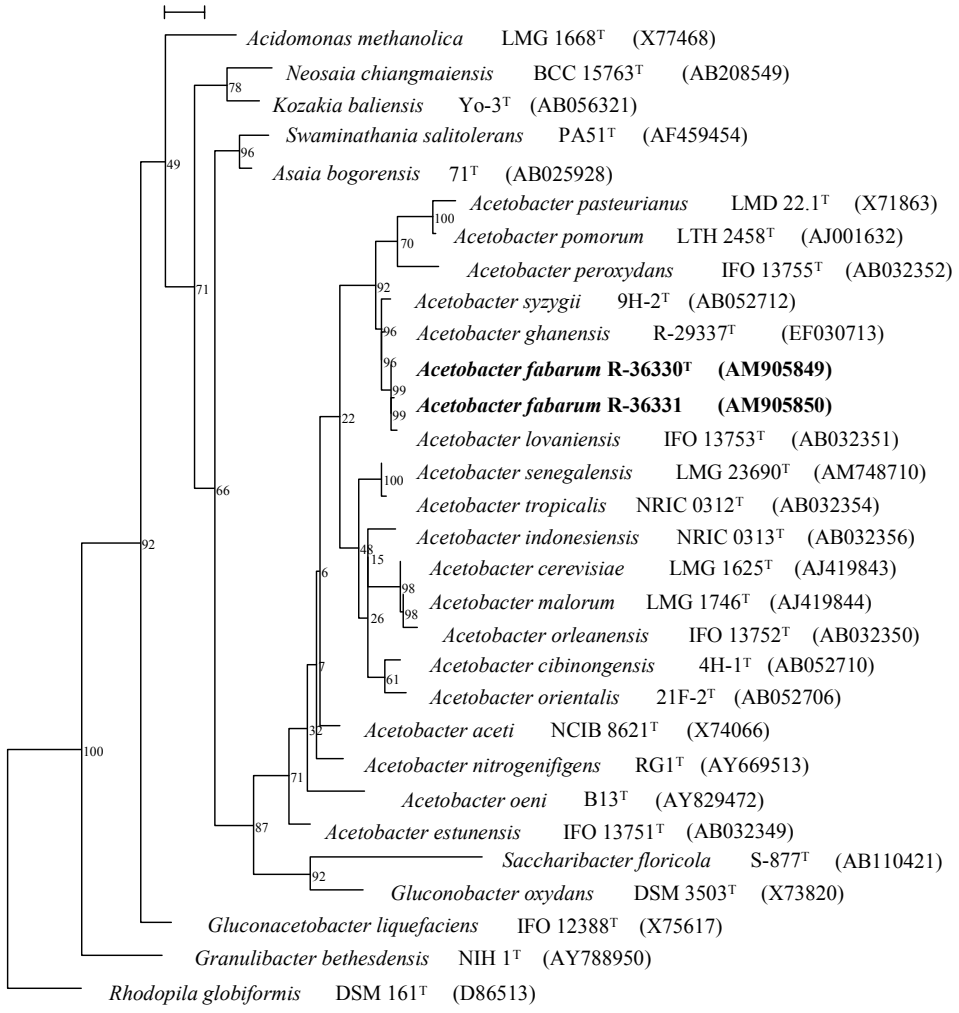

Fig. 5.5. Maximum parsimony tree based on nearly complete 16S rRNA gene sequences of *Acetobacter fabarum* and related species of the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap values calculated for 100 subsets. Bar, 1 nt substitution per 100 nt.

R-36331 were closely related to each other (99.9 %) and to *A. lovaniensis* (99.9 %), *A. ghanensis* (99.7 %) and *A. syzygii* (99.5 %), and definitely less to other recognized *Acetobacter* species: *A. peroxydans* (97.9 %), *A. pomorum* (97.9 %), *A. senegalensis* (97.6 %), *A. orientalis* (97.5 %), *A. cibinongensis* (97.5 %), *A. tropicalis* (97.5 %), *A. indonesiensis* (97.5 %), *A. pasteurianus* (97.4 %), *A. aceti* (97.4 %), *A. estunensis* (97.1 %), *A. oeni* (97.1 %), *A. cerevisiae* (97.0 %), *A. malorum* (97.0 %), *A. orleanensis* (96.9 %) and *A. nitrogenifigens* (96.6 %).

DNA-DNA hybridizations were performed between isolate R-36330^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 the isolates R-36330^T, R-36331, R-36458 and R-36459. The level of DNA-DNA binding found between isolate R-36330^T and the type strains of the 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 R-36330^T, R-36331, R-36458 and R-36459 varied between 82 and 97 %. The DNA-DNA hybridization data demonstrate that the cocoa isolates represent a new genospecies within *Acetobacter* (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987). The DNA G+C content of isolates R-36330^T, R-36331, R-36458 and R-36459 ranged from 56.8 to 58.0 mol% and is consistent with that of members of the genus *Acetobacter* (Cleenwerck *et al.*, 2007; Lisdiyanti *et al.*, 2000, 2001; Ndoye *et al.*, 2007a; Silva *et al.*, 2006).

The isolates could be differentiated from *A. lovaniensis*, *A. ghanensis* and *A. syzygii*, their phylogenetically nearest relatives 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 (Tables 5.2 and 5.3).

In conclusion, the six isolates originating from Ghanaian cocoa bean fermentations, constitute a taxon that can be differentiated, genotypically and phenotypically, from the 18 recognized *Acetobacter* species and should therefore be classified as a new species for which the name *Acetobacter fabarum* sp. nov. is proposed.

Table 5.2. Differential characteristics between *Acetobacter fabarum* and related *Acetobacter* species

Taxa are listed as: 1, *A. fabarum* (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. pomoru*m 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); 1 4, *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. estunensis* (3 strains); +: positive, -: negative, w: weak positive, v: variable. The result of the type strain of *A. fabarum* is given between brackets. Except indicated otherwise: data for taxon 1 were obtained in this study; data for taxa 2, 5-8, 11, 13-15 and 18-19 were taken from Cleenwerck *et al*. (2002); data for taxon 3 were taken from Cleenwerck *et al.* (2007); data for taxa 4, 9, 10 and 17 were taken from Silva *et al*. (2006); data for taxon 12 were taken from Ndoye *et al*. (2007a) (*A. senegalensis* CWBI-B418^{T}) and Camu *et al*. (2007) (*A. senegalensis* 108B and 420A); data for taxon 16 were taken from Dutta & Gachhui (2006) . The phenotypic characteristics taken from Cleenwerck *et al*. (2002, 2007), Silva *et al*. (2006), Ndoye *et al*. (2007a) and Camu *et al*. (2007) were examined as described by Cleenwerck *et al.* (2002).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Formation from D-glucose:																			
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	v (+)	+	w	+	v	+	-	+	+	+	+	+	+	+	+	+	+	+	v
Maltose	-	-	-	+	v	-	+	+	w	-	+	-	v	-	-	+	-	v	-
Methanol	+	+	-	-	-	-	-	-	w	-	-	-	-	+	-	-	-	-	-
Growth in 10 % ethanol	v (-)	-*	v	-*	+*	-*	-*	-*	-*	-*	-*	+	-*	+†	ND	+	+	-*	-*
Growth on YE + 30 % D-glucose	-	-	+	-	v	-	-	-	-	+	-	+	-	+	-	+	-	-	-
Catalase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
DNA G+C content DNA (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 Lisdiyanti et al. (2001).
†Data taken from Ndoye et al. (2007).

Table 5.3. Additional differential characteristics between *Acetobacter fabarum* and the phylogenetically related *Acetobacter* species, *A. lovaniensis*, *A. ghanensis* and *A. syzygii*

++, Strong positive; +, positive; -, negative; w, weak positive; v, variable (the result of the type strain of *A. fabarum* is given between brackets)

Characteristic	*A. fabarum*	*A. lovanienis*	*A. ghanensis*	*A. syzygii*
Growth on carbon sources:				
D-xylose	+	-	+	+
D-galactose	v (-)	-	+	-
Growth on GY at 37°C	w	+	++	++

Description of *Acetobacter fabarum* sp. nov.

Acetobacter fabarum (fa.ba'rum L. gen. pl. n. fabarum of beans, referring to the 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 and 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 and 5-keto-D-gluconic acid from D-glucose. Able to grow on methanol, 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 are variable between strains. The G+C content of the DNA varies from 56.8 to 58.0 mol%. The G+C content of the type strain is 57.6 mol%. Can genotypically be differentiated from other *Acetobacter* species by DNA-DNA hybridizations and $(GTG)_5$-PCR fingerprinting. The type strain is 985^T (= R-36330^T = LMG 24244^T= DSM 19596^T).

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PART III

GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSIONS

CHAPTER 6

GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSIONS

The goals of this work were (i) to improve the classification of AAB on the basis of molecular techniques and (ii) to provide methods that allow fast and accurate identification and classification of these organisms at the species level. This chapter comprises the general outcomes of this work, and perspectives for future studies.

In 1998, when the experimental work for this thesis was started, several weaknesses could be noticed in the classification of AAB, especially in the genera *Acetobacter* and *Gluconacetobacter*. Five species within these genera were delineated mainly on the basis of phenotypic features, namely *A. aceti*, *A. pasteurianus*, *G. hansenii*, *G. liquefaciens* and *G. xylinus* (Gosselé *et al*., 1983b; Yamada, 1983), and not on the basis of a polyphasic approach (1.3.), which is advized to obtain robust classifications. Fourty nine strains representing all species of the genera *Acetobacter* and *Gluconacetobacter* described at that time (Table 1.3 and 1.4), were selected for DNA-DNA hybridization, the reference technique for species delineation. The DNA-DNA hybridization study revealed 17 misclassified strains, all within the species delineated mainly on the basis of phenotypic tests, namely one within *A. aceti*, fourtheen within *A. pasteurianus* and two within *G. xylinus*. The high number of strains misclassified as *A. pasteurianus* was not surprising as this species was suggested to be heterogeneous and to contain more than one species, based on the range of its G+C content (53-63 mol%) which was too broad for a single species (Swings *et al*., 1992). The DNA-DNA hybridization study further revealed that it was difficult to extract DNA of good quality for DNA-DNA hybridization from strains producing much exopolysaccharides. Two strains (LMG 18907 and LMG 18908) even repeatedly failed to give good quality DNA (Table 1.4).

The 15 phenotypically misclassified *Acetobacter* strains were further investigated using a polyphasic taxonomic approach that included DNA-DNA hybridizations, comparitive 16S rRNA gene sequence analysis (of representative strains), DNA G+C content determination, and phenotypic tests (chapter 2) in order to identify them or to classify them as novel bacterial taxa. The 14 strains misclassified as *A. pasteurianus* by phenotypic tests were shown to belong to 2 new *Acetobacter* species, for which the names *A. cerevisiae* (type strain LMG 1625^T, 4 strains) and *A. malorum* (type strain LMG 1746^T, 1 strain) were proposed, and to *A. estunensis* (2 strains), *A. indonesiensis*

(1 strain), *A. lovaniensis* (1 strain), *A. orleanensis* (1 strain), *A. peroxydans* (2 strains) and *A. tropicalis* (2 strains). The latter species were proposed by Lisdiyanti *et al*. (2000) on the basis of a polyphasic taxonomic study including also DNA-DNA hybridizations, 16S rRNA gene sequence analysis, and phenotypic tests on approximately the same set of strains with in addition novel isolates from Indonesian sources. The strain misclassified as *A. aceti* by phenotypic tests showed intermediate DNA-DNA relatedness values with other *A. aceti* strains, but could phenotypically not be differentiated from them. For this reason, its classification as *A. aceti* was maintained.

Comparitive 16S rRNA gene sequence analysis was demonstrated to be useful to classify strains within the genus, but was shown to be of limited value to explore relationships of *Acetobacter* strains at higher taxonomic level. Indeed, within *Acetobacter* two major rRNA groups were found, each containing species showing more than 97 % 16S rRNA gene sequence similarity.

The polyphasic study of *Acetobacter* strains confirmed the existence of the species proposed by Lisdiyanti *et al*. (2000), and also confirmed that diagnostic phenotypic characteristics are difficult to find. Lisdiyanti *et al*. (2000) detected only a few phenotypic characteristics that allowed differentiation of most DNA-DNA hybridization groups, but could not find differentiating phenotypic features for 3 groups, which they named *A. orleanensis*, *A. indonesiensis* and *A. tropicalis*, not following the recommendations of Wayne *et al*. (1987). In this study, a few additional diagnostic phenotypic features could be detected after comparison of the DNA-DNA hybridization data with the large set of phenotypic data obtained by Gosselé *et al.* (1983b), however differentiation of all *Acetobacter* strains remained impossible. It was therefore decided to verify if miniaturized phenotypic systems (i.e. API carbon substrate utilization tests and BIOLOG metabolic profiling) would provide additional discriminatory data. However no useful results were obtained with these systems, since almost no positive reactions were recorded. It is assumed that the slow and sometimes scanty growth of *Acetobacter* strains hampers the use of these techniques.

Based on the polyphasic taxonomic study of *Acetobacter* strains it was recommended not to identify *Acetobacter* strains at the species level on the basis of phenotypic data only, as it could lead to misidentifications, mainly because of the paucity of differential phenotypic features and the difficulties to interprete some phenotypic test results (Cleenwerck & De Vos, 2008; Kersters *et al.*, 2006; Yamashita *et al.*, 2004). In addition, the reliability of the differential phenotypic features for identification was limited because of the limited number of strains tested for some species. In conclusion,

it was recommended to classify and identify *Acetobacter* strains to the species level mainly on the basis of DNA-DNA hybridization data.

In the period following the polyphasic taxonomic study of *Acetobacter*, 19 AAB isolates from different sources (15 from rice, 2 from apple juice, 1 from spoiled red wine and 1 from mango) were classified at the species level using a polyphasic taxonomic approach, combining genotypic as well as phenotypic data (chapter 3). Generally 16S rRNA gene sequence data were used to determine the phylogenetic position of the isolates, and mainly DNA-DNA hybridization data were used to delineate at the species level.

The fifteen isolates from rhizosphere soil, roots and stems from four different **rice** varieties were capable to fix nitrogen and were identified as *G. diazotrophicus* (9 isolates) on the basis of their phenotypic characteristics and a positive reaction in a *G. diazotrophicus*-specific PCR assay, and as *A. peroxydans* (6 isolates) by means of 16S rRNA gene sequence analysis, DNA-DNA hybridizations, G+C content determination and phenotypic tests (chapter 3.1). The identification of several rice isolates as *G. diazotrophicus* was more or less expected as this species was known to be able to fix nitrogen and its occurance in various other crops had been reported (Madhaiyan *et al.*, 2004). On the contrary, the identification of the remaining rice isolates as *A. peroxydans* was surprising as this species was already described in 1925 (Swings, 1992) and was before this study not reported as diazotrophic. However, the type strain and novel isolates of *A. peroxydans* showed low and inconsistent acetylene reduction activities compared to *G. diazotrophicus*, which could explain why its diazotrophic nature was not detected previously. This study further revealed that nitrogen-fixing AAB are not restricted to the genus *Gluconacetobacter*, a finding that was later confirmed by other taxonomists (Dutta & Gacchui, 2006, 2007).

The two isolates from apple juice were capable to produce cellulose and to oxidize ethanol to acetic acid and further to CO_2. These isolates were classified by means of 16S rRNA gene sequence analysis, DNA-DNA hybridizations, fatty acid methyl ester analysis, determination of their ubiquinone composition, as well as classical phenotypic tests, into two novel species of the genus *Gluconacetobacter*, for which the names *G. swingsii* (type strain LMG 22125^T, 1 strain) and *G. rhaeticus* (type strain LMG 22126^T, 1 strain) were proposed (chapter 3.2). Although *Gluconobacter* is considered to be a typical spoiler of fruit juices (Kersters *et al.*, 2006), the classification of the two apple

juice isolates as members of the genus *Gluconacetobacter* was not surprising, as '*Acetobacter*' strains (*Acetobacter* was subdivided in *Acetobacter* and *Gluconacetobacter*, see 1.4) had been found on apples and had been reported to be involved in spoilage of cider, also known as apple wine (Kersters *et al*., 2006). It must be mentioned that the classification of the two apple juice isolates was very-time consuming, because the isolates were shown to belong to the *G. hansenii/G. xylinus* branch, which consists of phylogenetically closely related species (showing > 98.5 % 16S rRNA gene sequence similarity), with many of them containing cellulose-producing strains. As previously mentioned, it was difficult to extract from strains producing much exopolysaccharides DNA of good quality for DNA-DNA hybridization experiments; and in the frame of this investigation the DNA extraction proceduce of Wilson (1987) as modified by Cleenwerck *et al*. (2002) was further optimized by introducing additional steps for removal of exopolysaccharides, because the latter had been reported to be able to interact with denatured DNA and cause erratic DNA-DNA hybridization results (De Ley *et al*., 1970). From all strains DNA could be extracted, and generally technically reliable DNA-DNA relatedness values could be obtained, recognizable by minor differences between values from reciprocal reactions. Finally, DNA-DNA hybridizations were performed between the isolates and the type strains of the species of the *G. xylinus*/*G. hansenii* branch to reveal their DNA-DNA relatedness. The results showed that several species were closely related (intermediate DNA-DNA relatedness values up to 68 %, Table 3.4), and therefore species delineation within this group was found to be difficult.

Finally, the isolate from spoiled red wine and the one from mango were shown to belong to *A. oeni* (type strain B13^T, 1 strain) and *A. senegalensis* (type strain LMG 23690^T, 1 strain) - two novel species in the genus *Acetobacter* - on the basis of 16S rRNA gene sequence analysis, DNA-DNA hybridizations, G+C content determination and phenotypic tests (chapter 3.3 and 3.4). For these species the names were proposed.

As shown above, the classification of *Acetobacter* and *Gluconacetobacter* strains at the species level was often time-consuming, generally due to the amount of DNA-DNA hybridizations that needed to be performed, because of the close phylogenetic relatedness of many *Acetobacter* and *Gluconacetobacter* species.

In the second phase of this study DNA-based molecular methods for fast and accurate identification and classification of AAB at the species-level were explored, in

order to replace or at least reduce the amount of laborious DNA-DNA hybridizations. A study of the literature revealed that several genomic methods had been applied on AAB, but many studies were based on a limited number of strains and lacking phylogenetic relatives, and therefore the taxonomic value of these methods was not unambiguously clear (see 1.6, reviewed by Cleenwerck & De Vos, 2008). It was therefore decided to evaluate rep-PCR and AFLP®, two DNA-based fingerprinting techniques that had been validated as an alternative to DNA-DNA hybridization in other taxa (Duim *et al*., 2001; Gevers *et al*., 2001; Huys *et al.*, 1996; Janssen *et al*., 1997; Rademaker *et al*., 2000; Švec *et al*., 2005) on a broad collection of AAB strains. Both techniques target randomly positioned sites around the genome (repetitive elements in the case of rep-PCR fingerprinting and restriction enzyme sites in the case of AFLP®); were reported to be suitable for building identification databases because of their good reproducibility (Huys *et al.*, 1996; Janssen *et al.*, 1996); are universally applicable; have similar taxonomic information levels, though AFLP® may be regarded to be superior over rep-PCR, because its resolution power is flexible; and are acknowledged as relatively fast and easy to perform, with rep-PCR being technically less complex, which results in less amount of time and work required, making it very suitable for fast screening of a large set of strains.

In this study, rep-PCR DNA fingerprinting using the $(GTG)_5$ primer, referred to as $(GTG)_5$-PCR fingerprinting, was evaluated using 64 AAB reference strains, including 31 type strains and 132 acetic acid producing isolates from heap fermentations of Ghanaian cocoa beans (chapter 4.1); while AFLP® using the restriction enzyme combination *Apa*I/*Taq*I and the primercombination A03/T03, referred to as AFLP®, was evaluated using 135 AAB reference strains and 15 additional AAB strains, representing all species of the family *Acetobacteraceae* at present recognized (chapter 4.2) also including 4 rice isolates from 3.1 and the 2 isolates from Table 1.4 that failed to give good DNA for DNA-DNA hybridization.

Both techniques generated clusters that were largely in agreement with groupings obtained by DNA-DNA hybridizations; and for this reason it was concluded that both allowed fast and accurate identification and classification of a broad range of AAB at the species level, although, minor differences between both techniques could be noticed. Indeed, AFLP® was shown to have a slightly better discriminatory power at the species level (as can be seen from the obtained results for *A. senegalensis*, *A. indonesiensis*, *A. peroxydans* and *G. liquefaciens*); though $(GTG)_5$-PCR fingerprinting appeared to be

more suitable for identification of *A. pasteurianus* strains. Both techniques were also shown to be promising to study the genetic diversity at the intraspecies level, with AFLP® appearing to be more powerful than $(GTG)_5$-PCR fingerprinting, because of its flexible discriminatory power. Indeed, the present AFLP® data suggest that the discriminatory power at the intraspecies level will likely improve for some species by using another primercombination, such as A01/T03.

The techniques $(GTG)_5$-PCR fingerprinting and AFLP® are valuable, because they can increase our understanding of bioprocesses in which AAB are involved. For $(GTG)_5$-PCR fingerprinting, its value was already demonstrated in practice, as this technique has been used to study the diversity and population dynamics of AAB involved in the Ghanaian cocoa bean heap fermentations process (Camu *et al*., 2007, 2008). $(GTG)_5$-PCR fingerprinting revealed a rather limited biodiversity of AAB during this fermentation process. The 132 AAB isolates obtained in 2004 from these fermentation using DMS medium (chapter 4.1) were grouped by $(GTG)_5$-PCR fingerprinting into only 4 distinct clusters (I-IV). Strains of cluster I (100 isolates) grouped with reference strains of *A. pasteurianus* and were assigned to this species; whereas strains of cluster II (23 isolates), III (4 isolates) and IV (5 isolates) could not be identified, because clusters II-IV did not contain any of the reference strains. On the basis of a polyphasic taxonomic study including 16S rRNA gene sequence analyses, DNA-DNA hybridizations and phenotypic tests, strains of clusters III and IV were shown to belong to *A. senegalensis* (Camu *et al*., 2007), while strains of cluster II were shown to belong to a novel species, for which the name *Acetobacter ghanensis* (type strain LMG 23848^T, 23 isolates) was proposed (chapter 5.1). Later, $(GTG)_5$-PCR fingerprinting of 209 AAB isolates obtained in 2005 from these fermentations using four different culture media (DMS, AAM, BME and GY) revealed a group of six AAB isolates that could not be assigned to any of the species present in the database. The isolates were collected from three of the four different culture media, but not from DMS medium, which was previously used. This observation shows the importance of the use of different media to avoid selective isolation of AAB. Using a polyphasic taxonomic approach, these 6 isolates were shown to belong to another novel species, for which the name *Acetobacter fabarum* (type strain LMG 24244^T) was proposed (chapter 5.2). $(GTG)_5$-PCR fingerprinting further revealed that *A. pasteurianus* was present throughout the fermentation, whereas *A. ghanensis*, *A. senegalensis* and *A. fabarum* were not always isolated. In addition, $(GTG)_5$-PCR fingerprint data indicated that only a limited number of AAB strains play a role in the Ghanaian cocoa bean heap

fermentation process (Camu *et al*., 2007, 2008).

Although this study provides methods that allow fast and accurate species level identification of AAB, it remains interesting to look further for alternative methods, because $(GTG)_5$-PCR fingerprinting and AFLP generate data that are not portable (in contrast to sequence data e.g.), which is considered a major disadvantage in modern bacterial taxonomy. In addition, genetic events such as chromosomal rearrangements (including large insertions and deletions) and loss and/or gain of restriction sites and/or primer-binding sites can result in altered patterns (Coenye *et al*., 2005; Gürtler & Mayall, 2001), which may lead to results that are difficult to interpret.

A novel approach that has recently been put forward for investigation of taxonomic relationships of microorganisms is multilocus sequence analysis (MLSA) of protein-coding housekeeping genes (Stackebrandt *et al*., 2002). This method has been reported to be suitable for species delineation and for assessing relationships at the intraspecies level in various taxa (Baldwin *et al*., 2005; De Bruyne *et al*., 2007; Naser *et al*., 2005, 2007; Martens *et al*., 2008; Vitorino *et al*., 2007). In addition, it has been mentioned to surpass DNA-DNA hybridization by its ability to give information on interspecies relationships and by providing clear species/genomovar bounderies (Martens *et al*., 2008). However, development of a universal MLSA approach (as for 16S rRNA gene sequencing) is most probably not possible, and it is expected that taxon-specific approaches will be necessary (Coenye *et al*., 2005).

In order to validate the MLSA approach, the ad hoc committee for re-evalutation of the species definition advized to use a set of strains for which DNA-DNA hybridization data are available and for which intraspecies diversity has been evaluated by DNA fingerprinting methods (Stackebrandt *et al*., 2002). This study provided a set of strains, most of them publicly available, suitable for this purpose; and to further improve the taxonomy of AAB, an MLSA study has been initiated. The results of this study were not included in this thesis, because the number of taxa and/or house-keeping gene sequences available was too limited. However, the preliminairy results indicate that the obtained clusters are in agreement with the current classification, showing the potential of this approach. Yet, weaknesses are revealed as well, as for some strains the selected housekeeping genes cannot be sequenced at present, probably because the currently designed primers are not suitable for them. Additional tests will have to be performed to evaluate if MLSA can be used as an alternative to DNA-DNA hybridizations for this

group of organisms. Nevertheless, MLSA is expected to contribute further to our understanding of AAB, as it will likely reveal information on the phylogenetic relationships of the closely related AAB species, for which 16S rRNA gene sequence analysis is not adequate.

Also culture-independent techniques are needed in AAB research. They can be applied in addition to culture-dependent techniques, to obtain a more detailed view of the targeted AAB population and are useful to study AAB populations in vinegar, because many vinegar strains are difficult to isolate and cultivate. Obviously, isolation and cultivation procedures need to be further optimized.

Further exploration of the diversity of AAB in the environment is also an important topic for future research. It is clear from this study, as well as from other studies, that the diversity of AAB is not yet covered. In the past years, novel species have been detected by using new isolation media and/or by studying novel biotopes. These novel species may possess new features that may lead to new or improved applications.

Finally, implementation of starter cultures in the production of food products and beverages, such as vinegar, will increase process efficiency and reduce the risk of fermentation failure. The characteristics of the raw material, the desired metabolic activities of AAB starter cultures, the applied technology and the desired characteristics of the final product all have to be taken into account and will probably form the basis for strain selection. Studies that combine microbial analyses and metabolic target analyses are essential for the selection of strains to be used in starter cultures. In addition, procedures for cultivation and preservation will also have to be taken into account.

CONCLUSIONS

This study contributed to an improved and more robust classification of AAB and provided two DNA-based fingerprinting techniques that permit fast and reliable identification and classification of a broad range of AAB at the species level. These techniques therefore offer a good alternative to DNA-DNA hybridization. Because $(GTG)_5$-PCR fingerprinting is less complex than AFLP®, it is very suitable to screen a large number of strains in a relatively short time; AFLP® on the other hand appeared to

have a better discriminatory power at the species level, and is therefore to be preferred over $(GTG)_5$-PCR DNA fingerprinting when a few new isolates have to be quickly and accurately identified at the species level. For studies on intraspecies diversity, AFLP® is also to be preferred over $(GTG)_5$-PCR DNA fingerprinting, because of its flexible discriminatory power. Integration of both DNA-based fingerprinting techniques into polyphasic taxonomic studies of AAB is recommended, because it will contribute to a more robust AAB classification. In addition, both techniques can be used to increase our understanding of bioprocesses in which AAB are involved.

SUMMARY

AAB comprise a group of microorganisms that are of interest to the industry. They play a beneficial role in the production of several fermented foods and beverages, such as vinegars, cocoa-based products, kombucha and nata de coco, may cause spoilage to others such as beer, wine and cider, and find applications in the production of fine chemicals (e.g. vitamine C and miglitol) and of bacterial cellulose, a biopolymer with particular properties to be used as food thickener, paper additive, diaphragm for headphones and artificial skin. Additionally, some AAB have been described as plant growth promotors, whereas others have recently been described as human pathogens. Fast and reliable identification of AAB involved in the above-mentioned bioprocesses at the species and strain level is essential to improve our understanding and control of these processes.

The goal of this work was (i) to improve the classification of AAB on the basis of molecular techniques and (ii) to provide methods that enable fast and accurate identification and classification of these organisms at the species level.

In the first part of this thesis, the existing classification of *Acetobacter* and *Gluconacetobacter* was evaluated. DNA-DNA hybridizations were performed with 49 strains representing all species of *Acetobacter* and *Gluconacetobacter* described at that time. This study revealed 17 misclassified strains based on the bacterial species definition of Wayne *et al*. (1987), all within species that were delineated mainly on the basis of phenotypic tests. Most misclassified strains were revealed within *A. pasteurianus*, a species that was suggested to be heterogeneous and to contain more than one species (Swings *et al*., 1992).

The phenotypically misclassified *Acetobacter* strains were further investigated using a polyphasic taxonomic approach that included DNA-DNA hybridizations, DNA G+C content determinations, 16S rRNA gene sequence analysis and phenotypic tests in order to identify them or to classify them as novel bacterial taxa. The misclassified strains were shown to belong to two novel *Acetobacter* species, namely *A. cerevisiae* and *A. malorum*, and to *A. estunensis*, *A. indonesienis*, *A. lovaniensis*, *A. orleanensis, A. peroxydans* and *A. tropicalis* (chapter 2). The latter species were proposed by Lisdiyanti *et al*. (2000) on the basis of a polyphasic taxonomic study also including DNA-DNA hybridizations and 16S rRNA gene sequence analysis, on approximately the same set of

strains with in addition novel isolates from Indonesian sources.

Our study of *Acetobacter* strains confirmed the existence of the *Acetobacter* species proposed by Lisdiyanti *et al*. (2000), and confirmed that diagnostic phenotypic features for *Acetobacter* species are difficult to find. Indeed, comparison of the DNA-DNA hybridization data with the large set of phenotypic data obtained by Gosselé *et al*. (1983b) revealed only a few diagnostic phenotypic features that allowed differentiation of most but not all *Acetobacter* species. The polyphasic taxonomic study demonstrated that *Acetobacter* strains should preferentially be classified and identified at the species level using DNA-DNA hybridization data, until reliable alternative methods would be available.

In chapter 3, 19 isolates from four different sources (15 from rice, 2 from apple juice, 1 from spoiled red wine and 1 from mango) were classified at the species level using a polyphasic approach, combining genotypic as well as phenotypic data. Generally, 16S rRNA gene sequence data were used to determine the phylogenetic position of the isolates, and mainly DNA-DNA hybridization data were used to delineate at the species level. The 15 isolates from rice were found to belong to *G. diazotrophicus* (9 isolates) and *A. peroxydans* (6 isolates); whereas the 4 other isolates were shown to belong to 4 novel species, namely *Gluconacetobacter swingsii* and *Gluconacetobacter rhaeticus* (isolates from apple fruit juice), *Acetobacter oeni* (isolate from spoiled red wine) and *Acetobacter senegalensis* (isolate from mango). The classification of these isolates was in most cases time-consuming, generally due to the number of DNA-DNA hybridizations that needed to be performed, as many AAB species are phylogenetically closely related, showing more than 97 % 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994).

In the second part of this thesis, two DNA-based fingerprinting techniques, namely rep-PCR using the $(GTG)_5$ primer (referred to as $(GTG)_5$-PCR fingerprinting), and AFLP® using the restriction enzyme combination *Apa*I/*Taq*I and the primercombination A03/T03 (referred to as AFLP®) were evaluated for their ability to fast and accurately identify and classify AAB at the species level. For the rep-PCR technique, 64 AAB reference strains including 31 type strains, and 132 AAB isolates from Ghanaian, cocoa beans heap fermentations were used for evaluation. For the AFLP® technique, 135 AAB reference strains and 15 additional AAB strains, representing all currently recognized species of the family *Acetobacteceae*, were investigated. Both techniques were shown to

allow fast and accurate species level identification and classification of a broad range of AAB (chapters 4 and 5), although minor differences between both methods could be noticed. AFLP® was shown to have a slightly better discriminatory power at the species level (as can be seen from the results obtained for *A. senegalensi*s, *A. indonesiensis*, *A. peroxydans* and *G. liquefaciens*); though $(GTG)_5$-PCR fingerprinting appeared to be more suitable for identification of *A. pasteurianus* strains. Both techniques were also shown to be promising to study the diversity at the intra-species level, with AFLP® appearing to be more powerful than $(GTG)_5$-PCR fingerprinting, because of its flexible discriminatory power.

Both techniques can be used to increase our understanding and control of bioprocesses in which AAB are involved. In this regard, $(GTG)_5$-PCR fingerprinting contributed to a better understanding of the Ghanaian cocoa beans heap fermentation process (Camu *et al*., 2007, 2008). Indeed, $(GTG)_5$-PCR DNA fingerprinting revealed a rather limited biodiversity of AAB during fermentation: AAB isolates from such fermentations were grouped into 5 clusters and were shown to belong to two novel species, namely *Acetobacter ghanensis* and *Acetobacter fabarum*, and to *A. pasteurianus* and *A. senegalensis* (4 .1, 5.1 and 5.2). In addition, *A. pasteurianus* was found to be present throughout the fermentation, whereas *A. ghanensis*, *A. senegalensis* and *A. fabarum* were not always isolated. Moreover, $(GTG)_5$-PCR fingerprint data indicated that only a limited number of AAB strains play a key role in the Ghanaian cocoa bean heap fermentation process (Camu *et al*., 2007, 2008).

In conclusion, this study contributed to an improved classification of AAB and provided two DNA-based fingerprinting techniques, $(GTG)_5$-PCR fingerprinting and AFLP® that allow fast and reliable identification and classification of a broad range of AAB at the species level. Integration of both DNA-based fingerprinting techniques into polyphasic taxonomic studies of AAB is recommended, because it will contribute to a more robust AAB classification.

SAMENVATTING

De azijnzuurbacteriën (AZB) omvatten een groep van micro-organismen die van belang zijn voor de industrie. Ze spelen een nuttige rol in de productie van verschillende gefermenteerde voedingsproducten en dranken zoals azijn, cacaogebaseerde producten, 'kombucha' en 'nata de coco'; kunnen bederf veroorzaken aan andere zoals bier, wijn en cider; en vinden toepassingen bij de productie van diverse farmaceutische producten (bvb. vitamine C en miglitol) en van bacterieel cellulose, een biopolymeer met specifieke eigenschappen en diverse toepassingen zoals voedingsbindmiddel, papier additief, diafragma's voor koptelefoons en artificiële huid. Daarnaast bevorderen bepaalde AZB plantengroei, terwijl andere recent beschreven werden als menspathogeen. Vlugge en betrouwbare identificatie van AZB die betrokken zijn bij de hierboven vermelde bioprocessen, op species- en op stamniveau, is van belang om ons inzicht in die processen te vergroten en ze beter te controleren.

Het doel van dit werk was (i) de classificatie van AZB te verbeteren op basis van moleculaire technieken en (ii) methoden te verschaffen die toelaten deze organismen snel en nauwkeurig te identificeren en te classificeren tot op het speciesniveau.

In het eerste deel van deze thesis werd de bestaande classificatie van *Acetobacter* en *Gluconacetobacter* geëvalueerd. Er werden DNA-DNA hybridisaties uitgevoerd met 49 stammen die alle op dat ogenblik beschreven *Acetobacter* and *Gluconacetobacter* species vertegenwoordigden. Deze studie onthulde 17 foutief geclassificeerde stammen volgens de speciesdefinitie van Wayne *et al*. (1987), allemaal binnen species die hoofdzakelijk werden afgebakend op basis van fenotypische testen. Het grootste aantal fenotypisch foutief geclassificeerde stammen werd gevonden in *A. pasteurianus*, een species waarvan gesuggereerd werd dat het heterogeen was en meerdere species bevatte (Swings *et al*., 1992).

De fenotypisch foutief geclassificeerde *Acetobacter* stammen werden verder onderzocht d.m.v. een polyfasische taxonomische aanpak, waarbij gebruik gemaakt werd van DNA-DNA hybridizaties, DNA G+C gehalte bepalingen, 16S rRNA gensequentieanalyse en fenotypische testen, met het doel ze te identificeren of ze te classificeren als nieuwe bacteriële taxa. Er werd aangetoond dat de misgeclassificeerde stammen toebehoorden aan twee nieuwe *Acetobacter* species, namelijk *A. cerevisiae* en *A. malorum*, en aan *A. estunensis*, *A. indonesienis*, *A. lovaniensis*, *A. orleanensis, A.*

peroxydans en *A. tropicalis* (hoofdstuk 2). Deze laatste species werden voorgesteld door Lisdiyanti *et al*. (2000) op basis van een polyfasische taxonomische studie aan de hand van DNA-DNA hybridisaties en 16S rRNA gensequentieanalyse op ongeveer dezelfde set van stammen met bijkomend enkele nieuwe isolaten uit Indonesische bronnen.

Onze studie van *Acetobacter* stammen bevestigde het bestaan van de *Acetobacter* species voorgesteld door Lisdiyanti *et al*. (2000), en bevestigde dat diagnostische fenotypische karakteristieken voor *Acetobacter* species moeilijk te vinden zijn. Inderdaad, de vergelijking van de DNA-DNA hybridisatiegegevens met de grote set van fenotypische gegevens die verkregen werd door Gosselé *et al*. (1983) onthulde maar een beperkt aantal diagnostische fenotypische kenmerken, die een differentiatie mogelijk maakte van de meeste maar niet alle *Acetobacter* species. De polyfasische taxonomische studie toonde aan dat *Acetobacter* stammen best geclassificeerd en geïdentificeerd konden worden tot op het speciesniveau op basis van DNA-DNA hybridisatiegegevens, totdat betrouwbare alternatieve methoden beschikbaar zouden zijn.

In hoofdstuk 3 werden 19 isolaten uit 4 verschillende bronnen (15 uit rijst, 2 uit appelsap, 1 uit bedorven rode wijn en 1 uit mango) geclassificeerd tot op het speciesniveau aan de hand van een polyfasische aanpak. Hierbij werden genotypische en fenotypische gegevens gecombineerd. Over het algemeen werden 16S rRNA gensequentiegegevens gebruikt om de fylogenetische positie van de isolaten te bepalen, terwijl vooral DNA-DNA hybridisatiegegevens gebruikt werden om het speciesniveau te bepalen. De 15 isolaten uit rijst bleken te behoren tot *G. diazotrophicus* (9 isolaten) en *A. peroxydans* (6 isolaten). De 4 overige isolaten behoorden tot 4 nieuwe species, namelijk *Gluconacetobacter swingsii* en *Gluconacetobacter rhaeticus* (isolaten uit appelsap), *Acetobacter oeni* (isolaat uit bedorven rode wijn) en *Acetobacter senegalensis* (isolaat uit mango). De classificatie van deze isolaten was in de meeste gevallen tijdrovend, over het algemeen omwille van het aantal DNA-DNA hybridisaties die dienden uitgevoerd te worden. Dit komt doordat veel AZB species fylogenetisch verwant zijn. Veel species vertonen meer dan 97 % 16S rRNA gensequentiesimilariteit (Stackebrandt & Goebel, 1994).

In het tweede deel van deze studie werd nagegaan of twee DNA-gebaseerde bandpatroon-genererende technieken, meerbepaald rep-PCR d.m.v. de $(GTG)_5$ primer (afgekort als $(GTG)_5$-PCR) en AFLP® d.m.v. de restrictieenzym combinatie *Apa*I/*Taq*I en de primer combinatie A03/T03 (afgekort als AFLP®), het vermogen hadden om AZB

snel en accuraat te identificeren en te classificeren tot op het speciesniveau. Voor de evaluatie van de rep-PCR techniek werd gebruik gemaakt van 64 AZB referentiestammen waarvan 31 typestammen en 132 AZB isolaten uit Ghanese cacaoboonfermentaties.. Voor de evaluatie van de AFLP® techniek werd gebruik gemaakt van 135 AZB referentiestammen en 15 bijkomende AZB stammen, die alle huidig erkende species van de familie *Acetobacteraceae* vertegenwoordigen. Beide technieken lieten vlugge en accurate identificatie en classificatie toe van een brede waaier van AZB (hoofdstukken 4 en 5) hoewel kleine verschillen tussen beide methoden konden worden opgemerkt. AFLP® leek een ietwat beter discriminerend vermogen te hebben op het speciesniveau (te zien aan de resultaten die verkregen werden voor *A. senegalensis*, *A. indonesiensis*, *A. peroxydans* en *G. liquefaciens*), maar voor identificatie van *A. pasteurianus* stammen leek $(GTG)_5$-PCR meer geschikt. Beide technieken bleken tevens veelbelovend om de diversiteit op het intra-speciesniveau te bestuderen. Hoewel, AFLP® lijkt iets meer geschikt te zijn dan $(GTG)_5$-PCR, doordat het een flexibel discriminerend vermogen heeft.

Beide technieken kunnen gebruikt worden om meer inzicht en controle te krijgen in/over de bioprocessen waarin AZB betrokken zijn. $(GTG)_5$-PCR leidde in dit opzicht al tot een beter inzicht in het Ghanese cacaoboon fermentatieproces (Camu *et al*., 2007, 2008). Inderdaad, $(GTG)_5$-PCR onthulde een eerder beperkte biodiversiteit van AZB die betrokken zijn bij dit fermentatie proces: AZB isolaten van dergelijke fermentaties werden gegroepeerd in vijf clusters en bleken te behoren tot twee nieuwe species, namelijk *Acetobacter ghanensis* en *Acetobacter fabarum*, en tot *A. pasteurianus* en *A. senegalensis* (4.1, 5.1 en 5.2). Daarenboven werd *A. pasteurianus* teruggevonden doorheen het ganse fermentatieproces, terwijl *A. ghanensis*, *A. senegalensis* en *A. fabarum* niet altijd geïsoleerd werden. Meer nog, $(GTG)_5$-PCR data doet vermoeden dat slechts een beperkt aantal AZB stammen een belangrijke rol spelen in het Ghanese cacaoboon fermentatieproces (Camu *et al*., 2007, 2008).

Tot slot, deze studie leidde tot een verbeterde classificatie van AZB en verschafte twee DNA-gebaseerde bandpatroon-genererende technieken, $(GTG)_5$-PCR en AFLP®, die een vlugge en betrouwbare identificatie van een brede waaier van AZB tot op het speciesniveau mogelijk maken. Integratie van beide technieken in polyfasische taxonomische studies van AZB wordt aanbevolen, omdat het zal bijdragen tot een meer robuuste AZB classificatie.

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CURRICULUM VITAE

GENERAL INFORMATION

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Born in Bruges, Belgium on May 20, 1969
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EDUCATIONAL BACKGROUND

1987-1992
Ghent University, Ghent, Belgium
Licentiate in Biotechnology
Dissertation: Structure analysis of the gene encoding the human cell-cell adhesion molecule E-cadherin.

1994-1996
Ghent University, Ghent, Belgium
Aggregation Biology

PROFESSIONAL EXPERIENCE

06/1993-10/1993
Scientific researcher at the department of food safety and food quality, Ghent University, Ghent.

11/1993-03/1994
Scientific researcher at the lab of bacteriology and biotechnology, Veterinary and Agrochemical Research Centre (CODA), Brussels.

12/1994-04/1998
Scientific researcher at the lab of bacteriology and biotechnology, CODA, Brussels. Follow up of the project 'Detection of attaching-effacing *Escherichia coli* of bovine origin by means of immunulogical and genetic tests and development of a specific vaccine' (IRSIA-IWONL, 5631A).

05/1998-present
Scientific researcher at the BCCM^{TM}/LMG Bacteria Collection, Ghent University, Ghent. Basic activities: follow up of (i) identification services performed with the techniques whole genome DNA-DNA reassociation, DNA G+G content determination, AFLP, SDS-PAGE, and sequencing of housekeeping genes of LAB (ii) internal quality controls performed with the techniques AFLP and SDS-PAGE and (iii) scientific projects and collaborations.

PUBLICATIONS

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