# Chapter 3
# Acetic Acid Bacteria Taxonomy from Early Descriptions to Molecular Techniques

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## 3.1 Introduction

The exploitation of acetic acid bacteria (AAB) has a long history in fermentation processes and now represents an emerging field in biotechnological applications, especially with regard to the biosynthesis of useful chemicals with a potentially high economic value and, in food science, through the standardization of microbiological processes for the manufacture of both vinegar and other fermented beverages.

Historically, AAB were recognized as 'vinegar bacteria' because the first studies were done on vinegar, and later on wine and beer spoilage. In fact, vinegar AAB are a subset of a larger AAB group, which includes bacteria that interact with flowers, fruits, the rhizosphere of plants, and even human beings (Table 3.1). It is generally recognized that AAB are fastidious microorganisms, which means that many of them are difficult to grow on laboratory media. Many efforts have been made to isolate and culture colonies of AAB. Several media have been suggested and tried, but none of them appears to satisfy the growth requirements of AAB. This has hindered the application of cultivation-based techniques to the study of AAB and, consequently, their taxonomic classification. However, the recent discovery of new culture-independent methods has opened up new horizons for the systematic study of AAB.

Current taxonomic studies are based on a polyphasic approach which, unlike traditional microbiological methods (morphological, physiological and biochemical), takes into account the information derived from metabolism, ecology, genome characterization and phylogeny.

These methods include 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern(s), biochemical assays and physiological and morphological tests. The new approach has resulted in several bacteria, which had previously been misclassified, being placed into new genera and species.

L. Solieri, P. Giudici (eds), *Vinegars of the World*

**Table 3.1** Currently recognized acetic acid bacteria (AAB) genera and species and their type strain isolation source

| Genus | Species | Source[a] | Reference |
|---|---|---|---|
| *ACETOBACTER* | | | Beijerinck, 1898[b] |
| *Acetobacter* | *aceti* | Vinegar | (Pasteur, 1864)<br>Beijerinck 1898 |
| *Acetobacter* | *cerevisiae* | Beer | Cleenwerck et al., 2002 |
| *Acetobacter* | *cibinongensis* | Fruit | Lisdiyanti et al., 2002 |
| *Acetobacter* | *estunensis* | Cider | (Carr, 1958)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *ghanensis* | Cocoa bean | Cleenwerck et al., 2007 |
| *Acetobacter* | *indonesiensis* | Fruit and flower | Lisdiyanti et al., 2000 |
| *Acetobacter* | *lovaniensis* | Soil | (Frateur, 1950)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *malorum* | Apple | Cleenwerck et al., 2002 |
| *Acetobacter* | *nitrogenifigens* | Kombucha tea | Dutta and Gachhui 2006 |
| *Acetobacter* | *oeni* | Wine | Silva et al., 2006 |
| *Acetobacter* | *orientalis* | Canna flower | Lisdiyanti et al., 2002 |
| *Acetobacter* | *orleanensis* | Beer | (Henneberg, 1906)<br>Lisdiyanti et al., 2000 |
| *Acetobacter* | *pasteurianus* | Beer | (Hansen, 1879)<br>Beijerinck and Folpmers 1916 |
| *Acetobacter* | *peroxydans* | Ditch water | Visser't Hooft, 1925 |
| *Acetobacter* | *pomorum* | Industrial vinegar fermentation | Sokollek et al., 1998 |
| *Acetobacter* | *senegalensis* | Mango fruit | Ndoye et al., 2007 |
| *Acetobacter* | *syzygii* | Organic apple juice | Lisdiyanti et al., 2002 |
| *Acetobacter* | *tropicalis* | Coconut | Lisdiyanti et al., 2000 |
| *ACIDOMONAS* | | | Urakami et al., 1989 emend.<br>Yamashita et al., 2004 |
| *Acidomonas* | *methanolica* | Yeast fermentation process | (Uhlig et al., 1986)<br>Urakami et al., 1989 emend.<br>Yamashita et al., 2004 |
| *ASAIA* | | | Yamada et al., 2000 |
| *Asaia* | *bogorensis* | Flower of orchid tree | Yamada et al., 2000 |
| *Asaia* | *krungthepensis* | Heliconia flower | Yukphan et al., 2004 |
| *Asaia* | *siamensis* | Flower of crown flower | Katsura et al., 2001 |
| *GLUCONACETOBACTER* | | | Yamada et al., 1997 |
| *Gluconacetobacter* | *azotocaptans* | Coffee plant | Fuentes-Ramírez et al., 2001 |
| *Gluconacetobacter* | *diazotrophicus* | Sugarcane | (Gillis et al., 1989)<br>Yamada et al., 1997 |
| *Gluconacetobacter* | *entanii* | High-acid industrial vinegar fermentation | Schüller et al., 2000 |
| *Gluconacetobacter* | *europaeus* | High acid vinegar fermentation | (Sievers et al., 1992)<br>Yamada et al., 1997 |
| *Gluconacetobacter* | *hansenii* | Vinegar | (Gosselé et al., 1983)<br>Yamada et al., 1997 emend.<br>Lisdiyanti et al., 2006 |

(continued)

**Table 3.1** (continued)

| Genus | Species | Source[a] | Reference |
|---|---|---|---|
| *Gluconacetobacter* | *intermedius* | 'Tea fungus' beverage (kombucha) | (Boesch et al., 1998) Yamada 2000 |
| *Gluconacetobacter* | *johannae* | Coffee plant | Fuentes-Ramírez et al., 2001 |
| *Gluconacetobacter* | *kombuchae* | Kombucha tea | Dutta and Gachhui, 2006 |
| *Gluconacetobacter* | *liquefaciens* | Dried fruit | (Asai, 1935) Yamada et al., 1997 |
| *Gluconacetobacter* | *nataicola* | Nata de coco | Lisdiyanti et al., 2006 |
| *Gluconacetobacter* | *oboediens* | Industrial red wine vinegar fermentation | (Sokollek et al., 1998) Yamada 2000 |
| *Gluconacetobacter* | *rhaeticus* | Organic apple juice | Dellaglio et al., 2005 |
| *Gluconacetobacter* | *sacchari* | Sugarcane | Franke et al., 1999 |
| *Gluconacetobacter* | *saccharivorans* | Beet juice | Lisdiyanti et al., 2006 |
| *Gluconacetobacter* | *swingsii* | Organic apple juice | Dellaglio et al., 2005 |
| *Gluconacetobacter* | *xylinus* | Mountain-ash berries | (Brown, 1886) Yamada et al., 1997 |
| *GLUCONOBACTER* | | | Asai, 1935 |
| *Gluconobacter* | *albidus* | Flower of dahlia | (ex Kondo and Ameyama, 1958) Yukphan et al., 2005 |
| *Gluconobacter* | *cerinus* | Cherry | (ex Asai, 1935) Yamada and Akita 1984 emend. Katsura et al., 2001 |
| *Gluconobacter* | *frateurii* | Strawberry | Mason and Claus, 1989 |
| *Gluconobacter* | *oxydans* | Beer | (Henneberg, 1897) De Ley 1961 emend. Gosselé et al., 1983 emend. Mason and Claus, 1989 |
| *Gluconobacter* | *thailandicus* | Flower | Tanasupawat et al., 2004 |
| *GRANULIBACTER* | | | Greenberg et al., 2006 |
| *Granulibacter* | *bethesdensis* | Lymph node culture from a patient with chronic granulomatous disease | Greenberg et al., 2006 |
| *KOZAKIA* | | | Lisdiyanti et al., 2002 |
| *Kozakia* | *baliensis* | Palm brown sugar | Lisdiyanti et al., 2002 |
| *FRATEURIA* | | | Swings et al., 1980 |
| *Frateuria* | *aurantia* | Flower and fruit | (ex Kondo and Ameyama, 1958) Swings et al., 1980 |
| *NEOSAIA* | | | Yukphan et al., 2005 |
| *Neosaia* | *chiangmaiensis* | Flower of red ginger | Yukphan et al., 2005 |
| *SACCHARIBACTER* | | | Jojima et al., 2004 |
| *Saccharibacter* | *floricola* | Flower | Jojima et al., 2004 |
| *SWAMINATHANIA* | | | Loganathan and Nair, 2004 |
| *Swaminathania* | *salitolerans* | Mangrove-associated wild rice | Loganathan and Nair, 2004 |

[a] Refers to type strain isolation source.

[b] Quoted in Buchanan et al. (1966).

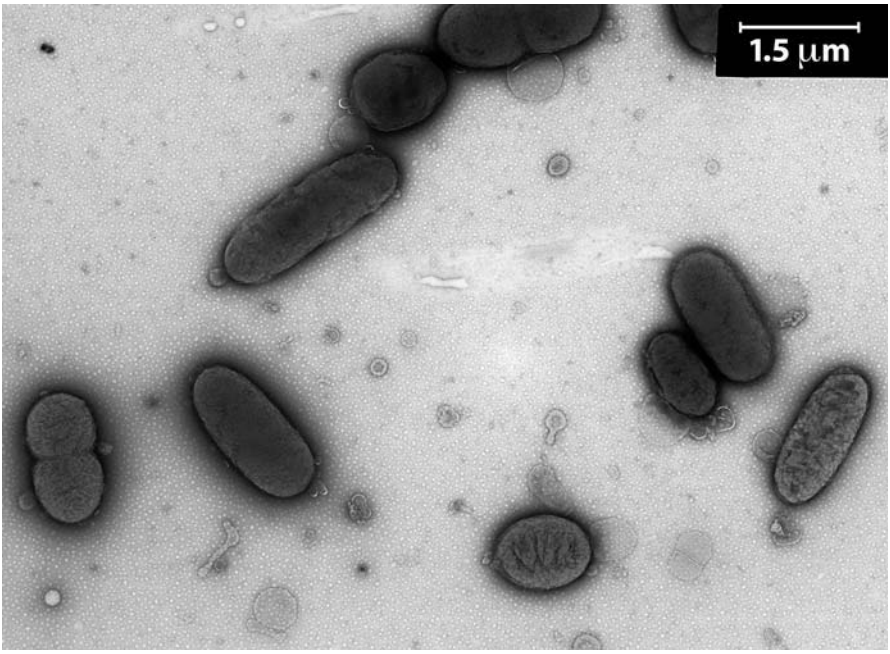


**Figure 3.2** SEM microscope image of acetic acid bacteria (courtesy of Giulio Petroni, Department of Biology, University of Pisa)

## 3.2 Acetic Acid Bacteria Taxonomy

Bacterial taxonomy is a scientifically dynamic area, whose main goal is to give a clear phylogenetic picture of microorganisms, and is an important tool for researchers, scientists and the biotechnological industries. Recent rapid developments in molecular biological techniques, the automation of DNA sequencing, advances in bioinformatic tools and access to sequence databases, has now made it possible for the first time to reveal the microbial 'identity' of microorganisms and has regenerated the study of taxonomy in terms of continuous reclassifications.

Since its discovery, the AAB group has been rearranged several times, with division, renaming, restoration and emendation of genera and species. The taxonomic grouping of AAB originated in 1837 when Kützing, who first observed the organisms in vinegar, described them as a kind of alga and named them *Ulvina aceti* (quoted in Asai, 1968).

Nowadays, ten genera of the *Acetobacteraceae* family are grouped under the collective name 'acetic acid bacteria' (class *α-Proteobacteria*): *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*, *Neoasaia*, *Saccharibacter* and *Swaminathania*, and one genus, *Frateuria*, of the family *Xanthomonadaceae* (class *γ-Proteobacteria*). A phylogenetic tree showing relationships of type strains of the family *Acetobacteraceae* is shown in Figure 3.2.


**Figure 3.2**. A phylogenetic tree reflecting the relationships of the type strains of species belonging to the family *Acetobacteraceae* (courtesy of Wolfgang Ludwig; from Ludwig, 2007)

The current classification is the product of the long and continuous development of bacterial systematics. To obtain a chronological overview of this development, Stackebrandt (2006, 2007) proposed a division of the history of bacterial systematics into four chronological phases that summarize the progress of microbiology over the past 100 years. The first phase, 'early descriptions' (1872-1900), originated with the adoption of the Linnaean paradigm (for plants and animals) also for bacteria. According to this chronological division, the first description of AAB is dated 1868, when Louis Pasteur published the first systematic study, in which he described the 'mother of vinegar' as a mass of living microorganisms which caused acetic acid fermentation. The genus name '*Acetobacter*' appears before 1900, and is usually attributed to Beijerinck (see footnote to Table 3.2).

In the second phase (from 1900 to 1955), physiology began to have an impact on taxonomy, and bacteria were classified first according to morphology, and physiology was then used to discriminate between the most closely related organisms. At this time (1916), the AAB (as quoted in Asai, 1968) were classified by Janke on the basis of their ability to utilize inorganic ammonium salts and acetic acid as the nitrogen and carbon sources, respectively. Later, in 1935, Leifson observed the existence of peritrichous flagella in some *Acetobacter* species and proposed dividing it into *Acetobacter* (only peritrichous or non-flagellated strains with similar physiological characteristics) and *Acetomonas* gen. nov. (polarly flagellated or non-motile strains with similar physiological characteristics) (quoted by Asai, 1968).

In the same year, Asai proposed the division of AAB into two genera, *Acetobacter* and *Gluconobacter* gen. nov., on the basis of their capacity to oxidize ethanol and glucose. In the genus *Acetobacter* were included organisms that exhibited only peritrichous flagellation if motile, that oxidized ethanol strongly, oxidized glucose weakly or not at all, and oxidized acetate and lactate completely. The genus was divided into two subgenera: *Euacetobacter*, including strains able to oxidize ethanol and not glucose; and *Acetogluconobacter*, which included strains able to oxidize ethanol strongly and glucose weakly. The genus *Gluconobacter* included organisms that exhibited only polar flagellation if motile, oxidized glucose strongly, oxidized ethanol weakly or not at all, and did not oxidize acetate and lactate to carbonate. The new genus was also divided into two subgenera: *Gluconoacetobacter* (strains able to oxidize glucose strongly and ethanol weakly) and *Eugluconobacter* (strains able to oxidize glucose but not ethanol) (Asai, 1968). *Gluconobacter* was placed in the family *Pseudomonadaceae*. Since *Acetobacter* showed no close relationship with any existing family, it was placed in the family *Acetobacteraceae*, which had appeared previously in the fifth edition of *Bergey's Manual* (1939).

From 1953 to 1965 other proposals for the systematization of AAB were made by several authors, taking into account the increasing weight of physiology in strain discrimination (for a full report on grouping and proposals, see Asai, 1968). Rainbow and Mitson (1953) proposed the separation of AAB into two groups on the basis of nutritional requirements: (i) lactaphilic group (predominant lactate metabolism) and (ii) glycophilic group (predominant glucose metabolism). This systematization was similar to that proposed by Vaughn in which group I (capable

of oxidizing acetic acid) and group II (not capable of oxidizing acetic acid) corresponded to the lactaphilic and glycophilic groups, respectively.

The third phase of bacterial systematics (1955-1980) corresponds to the great revolution in taxonomy in consequence of the impact of the discovery of the DNA structure by Watson and Crick (1953), which made possible the introduction of appropriate techniques for its analysis and manipulation. Moreover, chemotaxonomy, in which the chemical structures of cell constituents are used to differentiate bacteria into relatedness groups, was integrated into species descriptions (Stackebrandt, 2006). In 1963, a new approach to measuring genetic relatedness among organisms was proposed by McCarthy and Bolton which suggested DNA-DNA hybridization as a powerful tool to distinguish between closely related species. During these years numerical phenotypic analysis and protein sequence analysis were applied to taxonomic research. With the improvement in molecular sequencing techniques, the idea of Zuckerkandl and Pauling (1965) to deduce the phylogenetic history of organisms by comparing the primary structures of macromolecules was able to be adopted.

At this point, it became clear that there was a need to integrate these independent approaches to support species descriptions in a more rational manner. The term 'polyphasic' in relation to bacterial taxonomy was coined by R.R. Colwell in 1968 at the International Conference on Culture Collections (Tokyo), when he documented the unreliability of a single feature as a sole diagnostic criterion for species definition. In 1970, two papers were published on the polyphasic approach to study *Vibrio*-related species (Citarella and Colwell, 1970; Colwell, 1970). From this date the polyphasic approach became more widespread among scientists as a basic and necessary tool to deal with bacterial systematics. With the polyphasic approach, both phenotypic and genetic characterization became the *core* of the 'comprehensive taxonomic' concept.

By the late 1970s, a new technique was available for scientists; this was based on small subunit (SSU) rRNA as an universal marker to measure phylogenetic relationships between microorganisms, as proposed by Carl Woese and co-workers (Woese and Fox, 1977). This revolutionary contribution suggested a natural relationship between microorganisms on which a new prokaryotic systematics could be based. Small subunit rRNA almost perfectly meets the basic requirements of a general phylogenetic marker: i.e. ubiquitous distribution, functional constancy, low frequency of lateral gene transfer and recombination, combined with the availability of a comprehensive database of primary structures. Genes encoding ribosomal RNA, comprising conserved and variable domains, were chosen for most phylogenetic studies and to act as the backbone of modern bacterial systematics (Ludwig, 2007). In the current edition of *Bergey's Manual of Systematic Bacteriology*, the taxonomic outline is derived from the phylogenetic information provided by comparative small unit rRNA analysis (Ludwig and Klenk, 2005).

In the years between 1955 and 1980, AAB taxonomy was subjected to considerable revision in the light of information provided by newly available systematic tools. In 1958, Kondo and Ameyama proposed a new system to classify the genus *Acetobacter* based on carbohydrate oxidation: Group 1 – does not oxidize gluconic

acid, sorbitol and mannitol; and Group 2 – oxidizes gluconic acid, sorbitol and mannitol, and does not oxidize acetic acid. To differentiate between them, several other physiological features were also considered as key elements. In 1960, Stouthamer confirmed the presence of the tricarboxylic acid cycle (TCA) in *Acetobacter* and its absence in *Gluconobacter*; in the same year, Shimwell and Carr proposed the inclusion of this characteristic in distinguishing AAB species.

Since then, a great contribution to AAB taxonomy has been made by the research of De Ley and co-workers. In 1961, De Ley published the first comprehensive study of carbohydrate metabolism, studying the oxidative behaviour of AAB on several substrates, and proposed a classification into three groups. De Ley and Schell (1963) then studied the base composition of DNA, suggesting a close relationship and a possible common phylogenetic origin for *Acetobacter* and *Gluconobacter*. One of the first attempts of AAB DNA hybridization was performed by De Ley and Friedman (1964), whose article started with the following assertion: 'An improved bacterial classification appears to be on the verge of emerging'. They stated that in spite of the great morphological, physiological and biochemical similarities among AAB strains, hybrids are only formed between strains of the same genus with DNA of almost the same base composition.

Starting from 1980, (fourth phase) DNA techniques were incorporated into species description. Consequently, the demarcation of bacterial species shifted away from arbitrary and artificial definitions to ecological and genetic entities sharing the same phylogenetic heritage (Stackebrandt, 2007).

This fourth phase of AAB taxonomy started with an extensive reinvestigation of the group, instigated by several Belgian scientists. An exploration of the internal taxonomic structure of *Acetobacter* and *Gluconobacter* genera and their relationship to each other and to other genera was provided by Gillis and De Ley (1980), who applied the DNA-rRNA hybridization method and reclassified a number of misnamed species in the *Acetobacter* and *Gluconobacter* genera. In the same year, Swings and co-workers re-examined the taxonomic position of strains previously identified as members of *Acetobacter aurantius* by Kondo and Ameyama (1958), and later defined as 'intermediate' strains by Asai because of the difficulty of assigning them to either *Acetobacter* or *Gluconobacter*. On the basis of DNA-rRNA hybridization, they removed the cluster formed by *Acetobacter aurantius* strains from *Acetobacter* and *Gluconobacter* and proposed the new genus, *Frateuria*, with *Frateuria aurantia* sp. nov. as the type strain.

In 1984, Yamada and Kondo divided the genus *Acetobacter* into two subgenera on the basis of differences in the ubiquinone system; the type subgenus *Acetobacter* was characterized by Q-9 quinone, and the type subgenus *Gluconacetobacter* was characterized by Q-10 quinone. In this latter subgenus two species were transferred: *Acetobacter* (*Gluconacetobacter*) *liquefaciens* and *Acetobacter* (*Gluconcetobacter*) *xylinus*. However, Swings (1992) criticized the establishment of the subgenus *Gluconacetobacter* because of the acetate-oxidizing AAB containing Q-10. The subgenus *Gluconacetobacter* was elevated to the generic level on the basis of partial 16S rRNA sequence analysis by Yamada and co-workers in 1997.

Urakami et al. (1989) proposed to transfer methylotrophic species of *Acetobacter* into a new genus, *Acidomonas*, incorporating *Acetobacter methanolicus* in *Acidomonas methanolica* comb. nov., on the basis of methylotrophic ability as a characteristic distinguishing it from other genera. In 1992, Bulygina et al. published a 5S rRNA-based taxonomic study of *Acidomonas*, *Acetobacter* and *Gluconobacter*, in which they supported the proposal of Urakami and co-workers, but criticized the use of chemotaxonomic features such as fatty acid and ubiquinone composition (which can be common to microorganisms belonging to closely related but different genera), affirming the limited value of these features for determining taxonomic rank. They suggested as the main distinguishing characteristic to justify the establishment of the genus *Acidomonas* the ability to utilize methanol, and used sequencing of 5S rRNA to provide evidence that methylotrophic strains of *Acetobacter* are correctly classified as *Acidomonas*. However, no DNA-rRNA data were taken into account when creating this new genus.

From the mid-1980s onwards, a great deal of applied research was done on AAB isolated from industrial vinegar, and two aspects of AAB were highlighted: (i) the need for a basic knowledge of vinegar AAB regarding isolation, cultivation of strains, and preservation of the phenotypic traits for which strains were selected; and (ii) the need for taxonomic clarification of vinegar-related species. In particular, Kittelmann et al. (1989) tested several media for cultivating AAB from industrial vinegar and developed a culture method based on the Wiame agar plate system. Meanwhile, Mariette et al. (1991) studied AAB from wine, spirit and cider acetators for industrial vinegar production, differentiating them by plasmid profile analysis performed on cells taken directly from acetators without intermediate cultivation. In 1992, Sievers and co-workers started to study the microflora of high-acid vinegar fermentation in Switzerland and Germany. The authors clarified the genomic relationship of strains by DNA-DNA hybridization using type strains of *Acetobacter* and *Gluconobacter*. They found very low (0-22%) DNA-DNA similarities with type strains and proposed *Acetobacter europaeus* as a new species. The main biochemical differentiation characteristics of this new species are its strong tolerance to acetic acid (4-8%) and the absolute requirement of acetic acid for growth.

Since 2000, many systematic studies have been done: Lisdiyanti et al. (2000) re-examined the taxonomic position of *Acetobacter* species, confirming the need for DNA-DNA relatedness data for the establishment of *Acetobacter* species. These authors identified taxonomic problems in the genus *Acetobacter* as being due several factors, including the broad range (9.7%) of G+C content of DNA in *Acetobacter pasteurianus*; no comprehensive data for DNA-DNA relatedness between strains identified as *Acetobacter aceti* and those identified as *Acetobacter pasteurianus*; and no consideration of the taxonomic significance of the ubiquinone system (Gosselé et al., 1983).

In the past 7 years, six new genera have been proposed: *Asaia* (Yamada et al., 2000), *Neoasaia* (Yukphan et al., 2005), *Saccharibacter* (Jojima et al., 2004), *Swaminathania* (Loganathan and Nair, 2004), *Kozakia* (Lisdiyanti et al., 2002) and *Granulibacter* (Greenberg et al., 2006). In the latest edition of *Bergey's Manual*, Sievers and Swings (2005) describe the following taxonomic allocation: AAB form

**Table 3.2** Main chronological phases of the study of AAB systematics

| Phase | Status | Reference |
|---|---|---|
| *1872-1900* | ***Early descriptions*** | |
| | First systematics study recognizing that 'mother of vinegar' was a mass of living microorganisms causing acetic acid fermentation | Louis Pasteur 1868 |
| | *Acetobacter* genus | Beijerinck 1898[a] |
| *1900-1955* | ***Bacterial physiology and ecology were first explored and described*** | |
| | Classification according to the capacity to utilize inorganic ammonium salts and acetic acid as nitrogen and carbon sources respectively | Janke 1916[b] |
| | Classification according to flagella and physiological traits | Leifson, 1935[b] |
| | Division into two genera *Acetobacter* and *Gluconobacter* gen. nov. | Asai, 1935 |
| *1955-1980* | ***Availability of new powerful techniques for bacterial description*** | |
| | Presence of tricarboxylic acid (TCA) cycle in *Acetobacter* and absence in *Gluconobacter* | Stouthamer, 1960[b] |
| | First comprehensive study on carbohydrate metabolism | De Ley, 1961 |
| | DNA-rRNA hybridization revealed several *Acetobacter* and *Gluconobacter* misnamed strains | Gillis and De Ley, 1980 |
| *1980 onwards* | ***Modern era*** | |
| | Description of a nitrogen-fixing acetic acid bacterium associated with sugarcane, namely *Acetobacter diazotrophicus* | Gillis et al., 1989 |
| | Nine new genera described: | |
| | *Frateuria* | Swings et al., 1980 |
| | *Acidomonas* | Urakami et al., 1989 |
| | *Gluconacetobacter* | Yamada et al., 1997 |
| | *Asaia* | Yamada et al., 2000 |
| | *Neoasaia* | Yukphan et al., 2005 |
| | *Saccharibacter* | Jojima et al., 2004 |
| | *Swaminathania* | Loganathan and Nair, 2004 |
| | *Kozakia* | Lisdiyanti et al., 2002 |
| | *Granulibacter bethesdensis* (first description of human pathogenic AAB) | Greenberg et al., 2006 |
| | Two full genome sequences available: | |
| | *Gluconobacter oxydans* (strain 621 H) | Prust et al., 2005 |
| | *Granulibacter bethesdensis* (strain CGDNIH) | Greenberg et al., 2006 |

[a] The *Index Bergeyana* (Buchanan et al., 1966) includes the following comment: 'Beijerinck used the vernacular name '*azijnbacterien*' for the acetic bacteria. Apparently at some time before 1900 the vernacular name was rendered into neo-Latin as *Acetobacter* and finally used in publication. There is no record of its formal proposal as a genus.' Kluyver (1940, p. 132), states: 'It is surprising that neither Beijerinck nor Hoyer proposed in their publications the creation of a new genus for the acetic bacteria ... There can be no doubt that, in any case morally, but probably also according to the code of Botanical Nomenclature, Beijerinck is to be considered as the author of the genus *Acetobacter* as it occurs today.' On 1 January 1980, the *Approved Lists of Bacterial Names* followed Kluyver's opinion and cited the genus name *Acetobacter* as *Acetobacter* Beijerinck 1898.

[b] Quoted in Asai (1968).

four major clusters, one containing the *Gluconacetobacter* species with a subcluster comprising *Gluconacetobacter europaeus*, *Gluconacetobacter xylinus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter entanii*, *Gluconacetobacter hansenii*, and one subcluster comprising *Gluconacetobacter sacchari*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae*, and a second cluster containing *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Acetobacter peroxydans*, *Acetobacter pomorum*, *Acetobacter pasteurianus*, *Acetobacter estunensis*, *Acetobacter aceti*, *Acetobacter indonesiensis*, *Acetobacter orleanensis*, *Acetobacter tropicalis*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, and a third cluster comprising *Gluconobacter oxydans*, *Gluconobacter asaii*, *Gluconobacter cerinus* and *Gluconobacter frateurii*. *Kozakia baliensis* forms a sublineage separated from *Asaia bogorensis* and *Asaia siamensis*. *Acidomonas methanolica* represents a distinct phylogenetic line and the separate branching of this organism is supported by most of the treeing analyses.

For a chronological overview, some important milestones in AAB taxonomy are summarized in Table 3.2.

## 3.3 General Concepts and Applications of Taxonomic Techniques to Study AAB at Different Taxonomic Levels

The polyphasic approach to taxonomy is fed by phenotypic, genotypic and phylogenetic information, allowing a 'consensus' species description. However the combination of information from different techniques complicates the interpretation of results in comparison with a monophasic approach, where only one technique is used. Fortunately, scientists nowadays have the ability to standardize methods and apply them to specific cases of interest, allowing for an efficient clustering analysis.

Among the large spectrum of techniques available to polyphasic taxonomy (Table 3.3), some are essential, some are applicable but not necessary, some are interchangeable with each other, others are in some cases unsuitable. Techniques are useful if they are applied in a 'work strategy' that is appropriate for the individual case being studied and the taxonomic level being investigated. Below are described different categories of taxonomic techniques that can be used to study bacteria at different taxonomic levels.

### 3.3.1 Classical Phenotypic Analyses

Classical phenotypic analysis is the most common identification tool for bacteria and constitutes the basis for the formal description of taxa from species to family level. Classical phenotypic characteristics include cell morphology (shape, endospore, flagella, inclusion bodies, Gram staining reaction) and colony aspect (form, colour, size); physiology and biochemical features, comprising information

**Table 3.3** Techniques for studying AAB at different taxonomic levels

| Method | Notes |
|---|---|
| PHENOTYPIC | |
| *Morphological, physiological and biochemical features* | Basis for formal description of taxa from family to strain level<br>Provides descriptive information needed to recognize taxa<br>Highly standardized procedures are required to obtain reproducible results |
| Serotyping | Used for species and strain characterization<br>The need for living bacteria limits the power resolution |
| CHEMOTAXONOMIC | |
| *Cell wall composition, cellular fatty acids, isoprenoid quinines, whole-cell protein analysis* | Robust tools to describe phylogenetically closely related species as genera |
| GENOTYPIC | |
| *Genomic G+C content* | Considered part of the standard description of bacterial taxa |
| *DNA-DNA hybridization* | Acknowledged as the reference method for establishing relationships within and between species<br>Drawbacks:<br>(1) differences in genome size and DNA concentration influence results; (2) plasmid, chromosomal, housekeeping gene and DNA acquired by horizontal gene transfer are not discriminated; (3) results depend on the experimental parameters and are not cumulative; (4) different experiments cannot be compared directly; (5) the respective references (type strains) have to be included in each individual experiment |
| *RFLP* | The method is concerned with differentiation rather than identification of closely related strains |
| *RAPD* | Used for species- and strain-level identification<br>Strict standardization of experimental conditions is needed<br>Fragments are amplified with all ranges of efficiency, and the resulting patterns are very complex and difficult to interpret |
| *DGGE* | Short PCR products (<500 bp) are separated, which limits the phylogenetic information obtained by band sequencing.<br>When applied to complex bacterial communities does not provide reasonable estimates of diversity |
| *AFLP* | Irregular amplification of fragments can hamper the ability to reliably assign bands over multiple gels<br>Very laborious inspection of manual and computer-assisted band analyses |
| *ARDRA* | The main drawback is misinterpretation of pattern<br>Overcome by modern rapid sequencing rDNA |
| *rRNA comparative sequence analysis* | Gold standard for comprehensive phylogenetic analysis<br>Large availability of sequences in public databases |
| *rRNA FISH* | Effective identification of individual cells<br>Three-dimensional localization of cells inside samples |
| *DNA microarrays* | Allow profiling of differential gene expression<br>The main current limitation is the high cost |
| *MLST/MLSA* | Allow intraspecific-level identification<br>Database inconsistent with respect to other molecular markers |

on growth at different temperatures, pH values, salt concentrations or atmospheric conditions, growth in the presence of various substances, and data on the presence or activity of a variety of enzymes. Individually, many of these characteristics have been shown to be irrelevant as parameters for genetic relatedness, yet taken as a whole, they provide descriptive information enabling us to recognize taxa (Vandamme et al., 1996). From a technical point of view, highly standardized procedures are required in order to obtain reproducible results within and between laboratories. Nowadays, miniaturized and automated phenotypic fingerprinting systems have been introduced, which allow more reproducible results.

#### 3.3.1.1 Biotyping Analysis

Biotyping analysis is a combination of biochemical assays, phage typing, serotyping and growth in the presence of specific dyes used to characterize strains at an intraspecific level. Because of the need to use living bacteria, the power resolution is low and the results can be ambiguous, so these techniques are often replaced by DNA-based assays.

### 3.3.2 Chemotaxonomic Analysis

Chemotaxonomy includes the study of chemical constituents of cells (peptidoglycan structure, isoprenoid quinones, lipid and fatty acid composition of cells, polyamines, pigments and mycolic acids). The determination of chemical markers clarifies the cellular chemical composition and provides valuable properties that can be used to critically analyse the phylogenetic clustering of bacterial groups at genus level, thus strongly contributing to the application of a polyphasic approach. Of course, some methods are applicable to a great number of bacterial groups, while others are specific to particular taxa (e.g. peptidoglycan type is very informative, at both genus and species level, for Gram-positive bacteria, which have various types of this constituent; and less informative for Gram-negative bacteria, which have a simpler and more uniform peptidoglycan (Schleifer and Kandler, 1972).

### 3.3.3 Genotypic Methods

Genotypic methods include all the applications stemming from two important milestones in microbial systematics: the discovery of nucleic acids and the use of computers for handling biological data.

#### 3.3.3.1 Determination of the DNA Base Ratio (mol% G+C)

Determination of the DNA base composition (mol% guanosine plus cytosine) is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. Generally, the range observed is not more than 3%

within a well-defined species and not more than 10% within a well-defined genus. It varies between 24% and 76% in the bacterial world. The use of the G+C approach does not provide any phylogenetic information and does not allow an organism to be assigned to a particular taxon; instead it shows discriminating capacity. Different G+C contents indicate different organisms, whereas identical values per se do not necessarily characterize closely related taxa (Ludwig, 2007).

#### 3.3.3.2 DNA-DNA Hybridization

DNA hybridization is acknowledged as the reference method for establishing relationships within and between species (Harayama and Kasai, 2006). On the basis of DNA-DNA hybridization, Wayne et al. (1987) defined a species as an entity that includes strains sharing approximately 70% or higher DNA-DNA relatedness and with a difference of less than 5 °C in the DNA melting temperature between homologous and heterologous DNA hybrids. As cited by Ludwig (2007), a variety of alternative techniques and formats have been developed to measure the amount of heterologous hybrids (end-point measurement) and the kinetics of heterologous hybridization. Initially the technique was laborious and required large amounts of purified DNA, but it has now been miniaturized and microplate formats are now mainly used. Despite several drawbacks of the technique (see Table 3.3), DNA hybridization is the only generally applicable method for determining relationships at lower taxonomic levels where conserved phylogenetic markers fail to achieve resolution.

#### 3.3.3.3 DNA-based Differentiation: Fingerprinting Methods

These methods include indirect, rapid and simple techniques currently popular in many laboratories. RFLP (restriction fragment length polymorphisms), RAPD (random amplification of polymorphic DNA), AFLP (amplified fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis) and DGGE (denaturing gradient gel electrophoresis) are the most widespread techniques which allow the subdivision of species into a number of distinct types. All these techniques are based on the generation and visualization of target DNA fragments after amplification and, in some cases, digestion of amplicons with restriction enzymes. Several general and specific biases are associated with these approaches (see Table 3.3) and often they provide only differentiation information; for these reasons, in the modern taxonomy era, most of them have been replaced by rapid sequencing methods providing much more information for identification and phylogeny.

#### 3.3.3.4 rRNA Comparative Sequence Analysis

rRNA is currently the best target molecule for studying phylogenetic relationships and for creating practical identification systems because it is present in all bacteria, is functionally constant, is composed of highly conserved as well as more variable domains, is subject to a low frequency of lateral gene transfer, and because of the

availability of comprehensive databases containing more than 300,000 full and partial sequences (Ludwig et al., 2004).

When rRNA was first used in taxonomy, sequencing of the smaller subunit 5S molecule resulted in an accumulation of data for numerous bacteria, allowing the establishment of bacterial lineage. Later on, 16S and 23S rRNA gene sequence analysis by direct sequencing partial or whole subunits by using the PCR technique and selected primers, became a powerful tool for assessing the phylogenetic position of bacteria. In general, rRNA-based phylogenetic analysis includes the following steps:

1. DNA isolation from the target bacterium
2. amplification of a partial rRNA target gene sequence
3. alignment of the obtained rRNA sequence with other rRNA sequences available in databases
4. estimation of distances using evolution model
5. reconstruction of a phylogenetic tree from these distances
6. bootstrap analysis.

The large amount of data provided by rRNA comparative sequence analysis was the basis for the development of several probe-based techniques specific to different taxa-level studies. Among these are microarray technology, which shows great potential – offering the advantages of both sequencing and fingerprinting methodologies – and FISH (fluorescent in situ hybridization), which is now a widely applied technique in bacterial identification.

#### 3.3.3.5 Multilocus Sequence Typing/Analysis (MLST/MLSA)

Multilocus sequence typing (MLST) refers to a method for the genotypic characterization of prokaryotes at the intraspecific level, using the allelic mismatches of a small number (usually seven) of housekeeping genes; it is used for recognizing distinct strains within named species. In contrast, multilocus sequence analysis (MLSA) is described as a method for the genotypic characterization of a more diverse group of prokaryotes (including entire genera) using the sequences of single-copy and ubiquitous protein-coding genes, which evolve faster than rRNA (Gevers et al., 2005). At present, the major restriction using these approaches concerns database inconsistencies with respect to the taxonomic spectrum represented for other marker types (Ludwig, 2007). In the future, the availability of full genome sequences of bacteria will allow the application of MLST/MLSA to be considered for species description.

## 3.4 Final Remarks

Our knowledge of AAB began with vinegar as the main ecological niche, but in 2007, after about 200 years of studies, they were for the first time directly linked with human health with the description of the pathogenic species *Granulibacter*

*bethesdensis*. As shown in the taxonomic excursus, the amount of information at our disposal has increased with the development of new, robust and powerful tools of investigation, revealing a complex and fascinating scientific picture. To move forward in the field of AAB taxonomy, future research needs to address genomics, classification and characterization more fully. In this respect, the following two points need to be addressed in order to increase and regulate the stability of AAB taxonomy.

The availability of full genome sequences for all type strains would significantly advance the integration of genomic information into the understanding of microbial diversity and would enable researchers to map phenotypes to genomes. For the AAB, only two complete genome sequences are currently available, those for *Gluconobacter oxydans* 621 H (DSMZ 2343) (Prust et al., 2005) and the type strain *Granulibacter bethesdensis* CGDNIH1T (ATCC BAA-1260T=DSM 17861T) (Greenberg et al., 2006). Making available the entire genome sequences of type strains of the other species will be a vital step to a more complete understanding of the AAB.

The phenomenon of strain evolution affects bacterial species designation; although this drawback is currently known, no solution has been forthcoming to deal with microbial change during the time in laboratory conditions. This problem has crucial implications when clustering bacteria according to phenotypic traits. With respect to AAB, some authors have observed the loss of specific physiological activity as a consequence of spontaneous mutations. For instance, Kondo and Horinouchi, (1997) studied spontaneous high-frequency mutations, resulting from ISs family insertions, which were responsible for genetic instability, leading to deficiencies in various physiological properties of AAB, such as ethanol oxidation and cellulose production. This means that some phenotypic characters cannot be used for taxonomic purposes. Efforts are needed to evaluate microbial changes over time, as well as to guarantee appropriate tools for preserving ‘authentic’ strains.

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