

## **Oral Session II**

### **Classification and Molecular Phylogeny, and Ecology of Acetic Acid Bacteria**

## II-1: Systematics and Ecology of Acetic Acid Bacteria

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Since the beginning of microbiology, microorganisms have been studied from a variety of facets, and classified primarily into taxa on their morphological characteristics. However, the introduction of biochemical and physiological characteristics was inevitable for the classification and identification of bacteria because of their simple morphologies. This does not make an exception of systematics of acetic acid bacteria.

The genus *Acetobacter* was established in 1898 for vinegar bacteria on the oxidation of ethanol to acetic acid. In the middle of the 1930s, the genus *Gluconobacter* was created for acetic acid bacteria, which oxidized glucose more actively than ethanol, and separated from the genus *Acetobacter* which oxidized ethanol more strongly than glucose. In the 1960s, chemotaxonomy was introduced into bacterial systematics, and the genus *Acetobacter* was shown to have ubiquinone 9 (Q-9) and the genus *Gluconobacter* ubiquinone 10 (Q-10).

Further, phylogenetic relationships of bacteria were energetically studied on the basis of 16S rRNA gene sequences in the 1980s. According to Bergey's Manual of Systematic Bacteriology, 2nd edition (2001), the domain *Bacteria* is classified into 24 phyla by the 16S rRNA gene sequence, and acetic acid bacteria are included in the family *Acetobacteraceae* in the class *Alphaproteobacteria*, and 11 genera have been described so far. The genus *Frateuria* in the class *Gammaproteobacteria* is regarded as acetic acid bacteria because of its oxidative metabolism.

Acetic acid bacteria were initially isolated from vinegar and spoiled alcoholic beverages, and their sources are since spreading to flowers, fruits, nata in tropical countries, and other materials. Strains of the genus *Acidomonas* were mainly isolated from sewage. Further, some other acetic acid bacteria were isolated from clinical specimens. Strains of the genus *Asaia* had been considered to have niches on materials in tropical countries, but further investigation revealed their distribution in the subarctic zone - Hokkaido in Japan. The distribution of acetic acid bacteria is geographically worldwide.

Strains of the genus *Asaia* were reported to inhabit the female gut and the male reproductive tract of larvae and adults of *Anopheles stephensi*, an Asian malarial mosquito vector. Further, *Asaia bogorensis* strains were isolated from a patient receiving peritoneal dialysis. A newly described genus in the *Acetobacteraceae*, the genus *Granulibacter* was made it clear to cause the chronic granulomatous disease of humans. Therefore, acetic acid bacteria not only play roles in producing useful substances but are closely related to the association with insect and human diseases.

## II-2: Systematics of the Genus *Gluconobacter* Asai 1935

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In the genus *Gluconobacter* Asai 1935, a single species, *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961 was recognized.<sup>1</sup> The second species was described as *Gluconobacter cerinus* (ex Asai 1935) Yamada and Akita 1984. The third and the fourth species were *Gluconobacter frateurii* Mason and Claus 1989 and *Gluconobacter asaii* Mason and Claus 1989. However, the latter was a junior subjective synonym of *G. cerinus*. The number of species were therefore three in total, viz., *G. oxydans*, *G. cerinus* and *G. frateurii*.

Yukphan et al.<sup>2</sup> examined a number of NBRC strains of *Gluconobacter* for 16S-23S rRNA gene ITS sequence and restriction analyses. The test strains were divided into eight restriction groups. Group I corresponded to *G. oxydans*, Group II corresponded to *G. cerinus*, and Groups III and IV corresponded to *G. frateurii*. Of the eight groups, strains of Group VII was classified as *Gluconobacter albidus* (ex Kondo and Ameyama 1958) Yukphan et al. 2005, as the fourth species of the genus *Gluconobacter*. The fifth species, *Gluconobacter thailandicus* Tanasupawat et al. 2005 (Group III-3<sup>3</sup>) was described.

Malimas et al.<sup>3</sup> divided strains of Group III into five, viz., Group III-1, Group III-2, Group III-3, Group III-4 and Group III-5. And Group III-1 and Group III-4 were further divided into two subgroups, viz., Subgroup III-1a, Subgroup III-1b, Subgroup III-4a and Subgroup III-4b.

The sixth, seventh and eighth species, *Gluconobacter kondonii* Malimas et al. 2008, *Gluconobacter roseus* (ex Asai 1935) Malimas et al. 2008, *Gluconobacter japonicus* Malimas et al. 2008 corresponded respectively to Group VIII, Group VI and Groups III-5 and III-4.

Takahashi et al.<sup>4</sup> analyzed 16S rRNA gene and 16S-23S rRNA gene ITS sequences of a large number of NBRC strains of *Gluconobacter*. Group 5 included NBRC 12467, which was classified as *G. sphaericus* (Ameyama 1975) Malimas et al. 2009, the ninth species.

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## II-3: Nitrogen-fixing acetic acid bacteria: from ecology to genomics.

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The first report of the ability to fix atmospheric nitrogen by members of the *Acetobacteriaceae* family was made by Cavalcante and Döbereiner in 1988<sup>1, 2</sup>. *Gluconacetobacter diazotrophicus*, was isolated from sugarcane plants cropped in Brazil, and described included in the group of acetic acid bacteria because of its ability to overoxidize glucose and ethanol during growth. Beyond these special features, this bacterium is also able to realize two important key biological processes, the nitrogen fixation and the production organic acids<sup>3</sup>, which have important biotechnological applications to agriculture and industries. The *Acetobacteriaceae* family comprises 11 genera<sup>4</sup> and other nitrogen-fixing acetic acid bacteria have been classified into 3 of them, which are *Gluconacetobacter*, *Swaminathania* and *Acetobacter*<sup>5</sup>. Representatives of nitrogen fixing *Gluconacetobacter* species, *G. sacchari*, *G. johannae*, *G. azotocaptans* and *G. kombuchae* have in common the endophytic behaviour among other characteristics<sup>5</sup>.

The RioGene Consortium in Brazil sequenced and annotated the first genome of an acetic acid nitrogen fixing bacteria. The genome of *G. diazotrophicus* comprises more than 3,928 proteins coding genes in a 3.9 Mb chromosome and two cryptic plasmids of 16.6 and 38.8 kb. In *G. diazotrophicus* strain PAL5, the main sugar involved in energy generation for nitrogen fixation is glucose and several putative oxidoreductases involved in sugar and alcohol oxidation are widely distributed in its genome. The nitrogen fixation genes are organized in clusters and the structural genes for nitrogenase, *nifHDK*, are arranged in a cluster of 30.5 kb. The depiction of a comparative genomic analysis involving of *G. diazotrophicus* and other acetic acid or nitrogen fixing bacteria will be shown and discussed in detail.

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## II-4: Microbiological analyses of traditional rice vinegar, Kurozu

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One of the most traditional rice vinegars (rice black-vinegar; Kurozu in Japanese) has been produced since 1800s at Fukuyama-cho area, which is located in the eastern part of Kagoshima prefecture in Japan. To clarify the production system in terms of microbial composition, denaturing gradient gel electrophoresis (DGGE) targeting at small subunit rRNA gene was performed to the traditional rice vinegar fermentation process where the conversion of rice starch into acetic acid proceeded in the same pot. The fungal DGGE profile indicated that the transition from *Aspergillus oryzae* to *Saccharomyces* sp. took place at the initial stage when alcohol production started. The early stage was characterized by the coexistence of *Saccharomyces* sp. and lactic acid bacteria. Almost all the bacterial DGGE bands related to lactic acid bacteria were replaced by bands from *Lactobacillus acetotolerans* and *Acetobacter pasteurianus* at the stage where acetic acid started to accumulate. The microbial succession, tested in three different pots, was found essentially identical<sup>1</sup>.

Also, because the rice vinegar fermentation utilizes no starter cultures other than rice koji, the origin of functionally important microorganisms was clarified through culture-dependent and culture-independent methods.

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## II-5: Natural Imposition of the More Acidophilic Strain in Submerged Wine Vinegar Production

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The modern submerged fermentation for vinegar production is actually the most common industrial process. Rapid acetification as well as high final concentration of acetic acid can thus be achieved. Usually, the inoculum or starter preparation, also called “seed vinegar”, consists of a sample of vinegar drawn from a running traditional (surface culture) or industrial fermentation process and is composed of a mixture of different acetic acid bacteria (AAB).

In this study, acetic acid fermentations were carried out with the submerged, semi-continuous method, using two different inocula obtained from the end product of traditional surface culture fermentations. The aim of this study was to assess the performance of acetic acid fermentation process as influenced by the AAB species present in the starter culture preparation, in correlation with physico-chemical factors such as: alcohol and acetic acid concentrations, oxygen supply on the broth, acetification rate and evolution of total and viable cell number. Morphological evolution of AAB during vinegar production was also studied using scanning and transmission electron microscopy.

Using molecular biology techniques, we identified one inoculum composed of a mixture of 40% *Acetobacter pasteurianus* and 60% *Gluconacetobacter europaeus*, while the other was almost 100% *Acetobacter pasteurianus*. Although both inocula presented different AAB species composition, results showed the imposition of *Gluconacetobacter europaeus* as unique observable species at the end of each process, and a high degree of similarity in the general development of both acetic fermentations. This is consistent with previous observations which showed *Ga. europaeus* as one of the more acidophilic AAB species, since it is able to grow in cultures with a higher acetic acid content, while *A. pasteurianus* is able to grow better in a culture with a lower acetic acid, but higher alcoholic content. In the case of submerged vinegar production, *A. pasteurianus* could be responsible for a shorter adaptation phase, previous to the start point of the acetification process. This AAB species would remain in a non-viable or non-cultivable state when acetic acid content rises, since observed only in low acetic acid concentration conditions (< 9.0%).

The electron microscopy results showed a major transformation in bacterial morphology, starting from small ovoid cells (0.6-1.0  $\mu\text{m}$ ) to small rods (1.0-4.0  $\mu\text{m}$ ). An irregular amorphous layer surrounding the bacteria, observed in all samples, was cytochemically identified as polysaccharides. This layer could be involved in the resistance of AAB against the aggressiveness of the high acetic acid content medium.

## II-6: Genomic Comprehension for Evolutional Flexibility of *Acetobacter pasteurianus*

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It is indispensable that microorganisms utilized in academic and industrial fields are maintained to conserve identical characteristics wherever and whenever used. Extraordinary consideration for storage and handling should be paid to hyper-mutable microorganisms isolated from nature. Attempting to clarify the hyper-mutability, *Acetobacter pasteurianus*, which is a traditional acetic acid brewing bacterium and one of the hyper-mutable bacteria<sup>1</sup>, was subjected for genome DNA sequencing after culturing in the two different environments.

*A. pasteurianus* NBRC(IFO)3283 was maintained by a series of slant passages every three months without colony separation due to avoid the loss of useful features since the deposit of the pure culture into Institute for Fermentation, Osaka (IFO) in 1954 until establishment of the frozen preservation in 1975. Within the 20 years, approximately 2,000 generations occurred and a multi-phenotype cell complex was formed. The first genomic DNA sequencing was completed using the *A. pasteurianus* cell complex stored in 1975. The genome consists of 2.9 Mb chromosome and 6 plasmids, containing totally 3117 protein coding genes including 256 transposons. It was revealed by mutation analyses using 32 isolates from the complex that among the isolates exists a total of eleven discrepancies i.e. single nucleotide mutations, transposon insertions, and hypermutable short tandem repeats. It also illustrated that two mutations out of the discrepancies are involved in the phenotypic differences of sugar synthesis observed in the complex. To elevate efficiency of fermentation, breeding of *A. pasteurianus* was carried out to improve the growth at 42°C, which is over the limited growth temperature. As mapping of mutation sites using a series of breeding strains, single nucleotide substitutions and large-scale deletion at the predicted replication terminal were found.

Alpha-proteobacteria, to which acetic acid bacteria belong, contains many intracellular symbionts and parasites such as bacteria in the families, Rhizobiaceae, Rickettsiaceae, and Brucellaceae. Genomes of such endosymbionts generally show increased evolution rates comparing to ones of closely related free-living bacteria because of adaptation to distinctive intracellular environments, less chances of genome rearrangements with similar species, and transition with small population so-called bottleneck<sup>2</sup>. *A. pasteurianus*, however, is assumed a free-living bacterium, it may have evolved as an exosymbiont with other organisms such as yeasts and lactic acid bacteria in natural niches of seasonal fruits and flowers. In this symposium, I will show the hyper-mutability and the mechanism of *A. pasteurianus* genome, as well as genome comparison with other Acetobacteraceae bacteria, i.e. *Gluconacetobacter xylinus* NBRC(IFO)3288, *Gluconobacter oxydans* 621H<sup>3</sup>, *Granulibacter bethesdensis* CGDNIH1<sup>4</sup>, *Gluconacetobacter diazotrophicus* PAI 5<sup>5</sup> and *Acidiphilium cryptum* JF-5.

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