Kazunobu Matsushita · Hirohide Toyama
Naoto Tonouchi · Akiko Okamoto-
Kainuma *Editors*

Acetic Acid Bacteria

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Preface

Research for acetic acid bacteria (AAB) has a long history since the discovery of AAB by Louis Pasteur and its identification by Martinus Beijerinck in the nineteenth century. In the twentieth century, basic research on the taxonomic study of AAB and on biochemical study for the unique oxidative reactions of AAB progressed as did the industrial applications of AAB not only in vinegar fermentation but also in the bioconversion process for useful chemical or pharmaceutical products. Entering the twenty-first century, AAB research has continued to expand and is expected to show further progress in all aspects of AAB: classification and ecology, physiology and biochemistry, genetics, and biotechnology of vinegar fermentation and other oxidative fermentations. The research on AAB has developed significantly in the last decade, which makes these bacteria more valuable for various industrial uses. Readers can obtain useful, comprehensive information which is exciting with regard to basic science and provides suggestions for better application of these bacteria to a variety of practical production processes as well.

In order to view the future targets or directions of AAB research, we would like to summarize the distinctive physiological properties of AAB and the recent progress on AAB study, especially in the following areas.

(1) Molecular phylogeny and genome study of AAB; (2) Ecological features of AAB: interaction with plants, natural fermentation systems, and insects; (3) Physiological features and living strategies of AAB: rapid oxidation ability, acid resistance, biofilm formation, and genetic instability, and others; (4) Molecular mechanisms of several oxidative fermentations: acetate fermentation, sorbose fermentation, ketogluconate fermentation, and others; (5) Recent biotechnological aspects of AAB: biocatalysts, biosensors, biofuel cells, biocellulose, other useful polysaccharides, and so on.

Yamaguchi, Japan — Kazunobu Matsushita

Contents

Chapter 1
Systematics of Acetic Acid Bacteria

Yuzo Yamada

Abstract Acetic acid bacteria are currently accommodated in the acetous group, the family *Acetobacteraceae*, the class *Alphaproteobacteria*, based on phylogeny, physiology, and ecology. The acetic acid bacteria are classified at present in 17 genera, of which many species have been reported in the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia*, and *Komagataeibacter*. Of the remaining 12 genera, *Acidomonas*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia*, *Ameyamaea*, *Endobacter*, *Nguyenibacter*, and *Swingsia* are monotypic; the genus *Neokomagataea* contains two species. In the class *Gammaproteobacteria*, the genus *Frateuria* has been mentioned taxonomically as pseudacetic acid bacteria. In addition, isolation and identification of acetic acid bacteria are described.

Keywords Acetic acid bacteria • *Acetobacteraceae* • *Alphaproteobacteria* • The acetous group • *Acetobacter* • *Acetobacter aceti* • *Gluconobacter* • *Gluconobacter oxydans* • Pseudacetic acid bacteria • *Gammaproteobacteria* • *Frateuria*

1.1 Introduction

The generic name *Acetobacter*, the oldest name for acetic acid bacteria, was introduced by Beijerinck (1898). However, there is no record of the formal proposal of the generic name as a genus (Komagata et al. 2014; Buchanan et al. 1966; Kluyver 1983). Skerman et al. (1980) cited, 'as it occurs today' in the *Approved Lists of Bacterial Names 1980*, the generic name *Acetobacter* as *Acetobacter* Beijerinck 1898, in which the type species was designated as *Acetobacter aceti* (Pasteur 1864) Beijerinck 1898.

Asai (1935) divided the acetic acid bacteria into two genera: one genus included the species that oxidized ethanol more intensely than D-glucose and had the

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capability of oxidizing acetic acid to carbon dioxide and water, and the other contained the species that are especially isolated from fruit, oxidized D-glucose more intensely than ethanol, and had no capability of oxidizing acetic acid. For the latter genus, the name *Gluconobacter* Asai 1935 was proposed.

Almost 20 years later, the genus '*Acetomonas*' Leifson 1954 was introduced for species that had polar flagellation and were non acetate oxidizing (Leifson 1954). In contrast, the strains of the genus *Acetobacter* had peritrichous flagellation and the capability of oxidizing acetic acid to carbon dioxide and water. The proposals of the two generic names were, of course, the result of confusion in the systematics of acetic acid bacteria (Shimwell 1958; Asai and Shoda 1958; Shimwell and Carr 1959).

De Ley (1961) recognized the priority of the generic name *Gluconobacter* over the generic name '*Acetomonas*.' *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961 was designated as the type species of the genus *Gluconobacter*, because Asai (1935) did not designate the type species (De Ley 1961; De Ley and Frateur 1970).

In acetic acid bacteria, Asai et al. (1964) reported two types of intermediate strains in addition to strains of the genera *Acetobacter* and *Gluconobacter*. One type of the strains had peritrichous flagellation, and the other had polar flagellation despite being acetate oxidizing. The genera *Acetobacter* and *Gluconobacter* were distinguished chemotaxonomically from each other by the presence of the major ubiquinone homologues, that is, Q-9 for the former and Q-10 for the latter (Yamada et al. 1969a). The peritrichously flagellated intermediate strains, which were formerly classified as '*Gluconobacter liquefaciens*' (Asai 1935; Asai and Shoda 1958; Asai 1968) and later regarded as pigment-producing strains of *Acetobacter aceti* (Carr and Shimwell 1960; Kimmit and Williams 1963), had Q-10, which was quite different from the type strain of *Acetobacter aceti* (Q-9), the type species of the genus *Acetobacter*, but similar to strains of the genus *Gluconobacter*. On the contrary, the polarly flagellated intermediate strains, which were once classified as '*Acetobacter aurantium*' (sic) (Kondo and Ameyama 1958), had Q-8, which was never found in any other strains of acetic acid bacteria, and these strains were later classified as *Frateuria aurantia* (ex Kondo and Ameyama 1958) Swings et al. 1980 (Swings et al. 1980).

In the *Approved Lists of Bacterial Names 1980*, the Q-10-equipped peritrichously flagellated intermediate strains were listed as *Acetobacter aceti* subsp. *liquefaciens* (Asai 1935) De Ley and Frateur 1974 (Skerman et al. 1980). The Q-10-equipped strains, which were classified as *Acetobacter liquefaciens* (Asai 1935) Gosselé et al. 1983 (= *A. aceti* subsp. *liquefaciens*) and as *Acetobacter xylinus* (Brown 1886) Yamada 1984 [= *A. aceti* subsp. *xylinus* corrig. (Brown 1886) De Ley and Frateur 1974], were distinguished from the Q-9-equipped strains within the genus *Acetobacter* at the subgeneric level, and the subgenus *Gluconacetobacter* corrig. Yamada and Kondo 1984 was proposed (Yamada and Kondo 1984). However, the subgenus was not accepted in the classification of acetic acid bacteria, along with the genus *Acidomonas* Urakami et al. 1989 for the methanol-assimilating acetic acid bacterium, *Acetobacter methanolicus* Uhlig et al. 1986 (Swings 1992; Sievers et al. 1994).

The subgenus *Gluconacetobacter* was phylogenetically discussed on the basis of the partial 16S rRNA sequences, along with the genus *Acidomonas*, and elevated at the generic level as the genus *Gluconacetobacter* Yamada et al. 1998 with a concomitant existence of the genus *Acidomonas* (Yamada et al. 1997). The type species was designated as *Gluconacetobacter liquefaciens* (Asai 1935) Yamada et al. 1998.

In the genus *Gluconacetobacter*, there were two subclusters in the phylogenetic trees based on 16S rRNA gene sequences (Franke et al. 1999; Yamada et al. 2000). Later, the existence of two phylogenetic groups, that is, the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group, was suggested to be distinguished at the generic level on the basis of morphological, physiological, chemotaxonomic, and ecological characteristics (Yamada and Yukphan 2008). For the latter group, the genus *Komagataeibacter* Yamada et al. 2013 was introduced with the type species, *Komagataeibacter xylinus* (Brown 1886) Yamada et al. 2013 (Yamada et al. 2012a, b).

At the present time, 17 genera are recognized in acetic acid bacteria or the acetous group of the family *Acetobacteraceae* Gillis and De Ley 1980, the class *Alphaproteobacteria* Stackebrandt et al. 1988, viz., *Acetobacter* Beijerinck 1898, *Gluconobacter* Asai 1935, *Acidomonas* Urakami et al. 1989 emend. Yamashita et al. 2004, *Gluconacetobacter* Yamada et al. 1998, *Asaia* Yamada et al. 2000, *Kozakia* Lisdiyanti et al. 2002, *Swaminathania* Loganathan and Nair 2004, *Saccharibacter* Jojima et al. 2004, *Neoasaia* Yukphan et al. 2006, *Granulibacter* Greenberg et al. 2006, *Tanticharoenia* Yukphan et al. 2008, *Ameyamaea* Yukphan et al. 2010, *Neokomagataea* Yukphan et al. 2011, *Komagataeibacter* Yamada et al. 2013, *Endobacter* Ramírez-Bahena et al. 2013, *Nguyenibacter* Vu et al. 2013, and *Swingsia* Malimas et al. 2014 (Fig. 1.1). Of the 17 genera, the 5 genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia*, and *Komagataeibacter* each include a large number of species. However, the remaining 12 genera are monotypic, that is, contain only 1 species, except for the genus *Neokomagataea*, which consists of 2 species.

1.2 Isolation of Acetic Acid Bacteria

The isolation of acetic acid bacteria is in general carried out by an enrichment culture approach (Komagata et al. 2014; Sievers and Swings 2005a). A medium for the enrichment procedure and the isolation of acetic acid bacteria, designated as the pH 3.5 medium (Yamada et al. 1999), is composed, for example, of 1.0 % D-glucose (w/v), 0.5 % ethanol (99.8 %) (v/v), 0.3 % peptone (w/v), 0.2 % yeast extract (w/v), and 0.01 % cycloheximide (w/v), and adjusted at pH 3.5 with hydrochloric acid. In the isolation of acetic acid bacteria capable of fixing atmospheric nitrogen, the LGI medium that contains 10.0 % sucrose (w/v), 0.06 % KH_2PO_4 (w/v), 0.02 % K_2HPO_4 (w/v), 0.02 % $MgSO_4$ (w/v), 0.002 % $CaCl_2$ (w/v), 0.001 % $FeCl_3$ (w/v), and 0.0002 % Na_2MoO_4 (w/v) is used at pH 6.0 (Cavalcante and Döbereiner 1988).

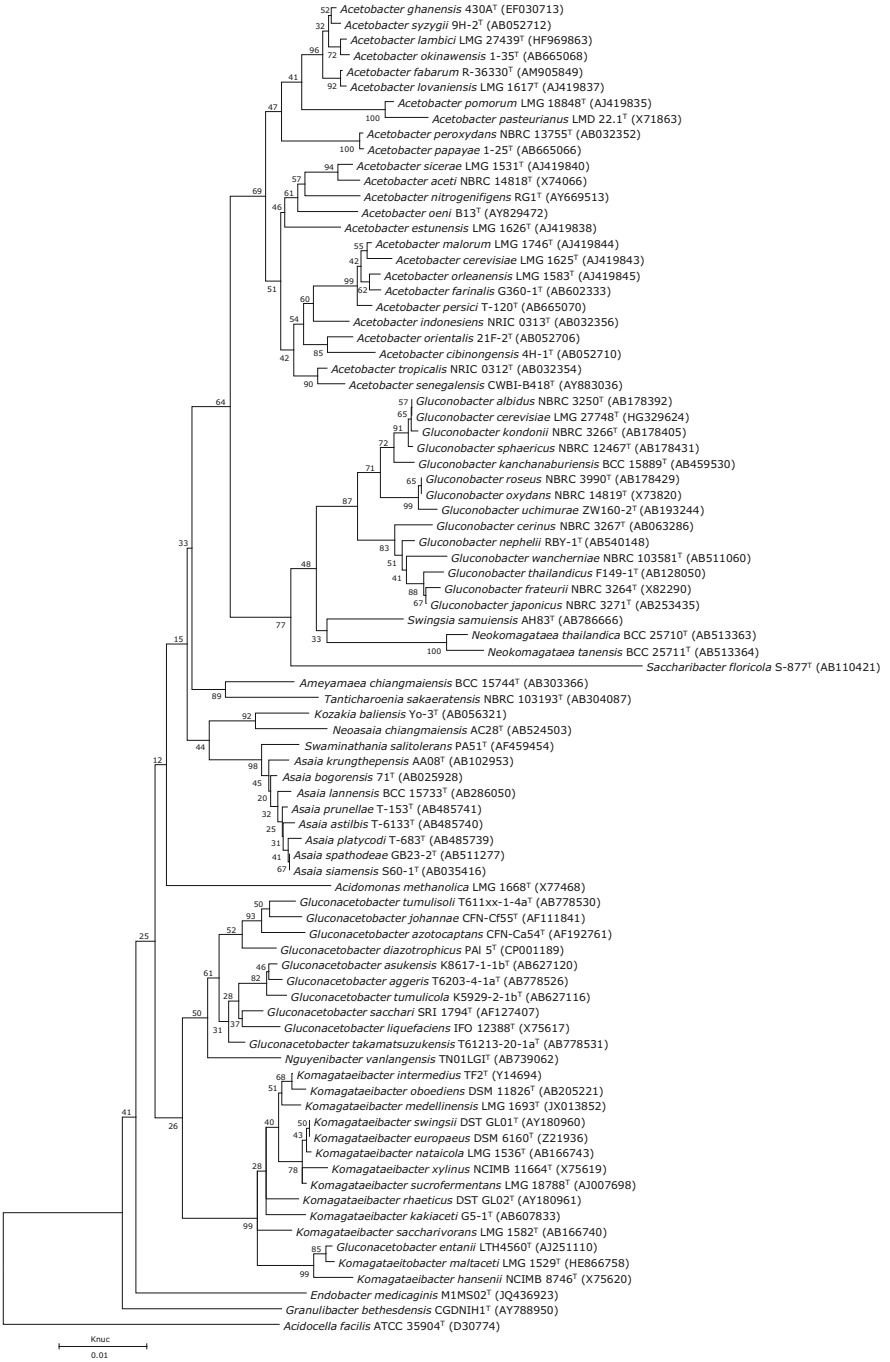

Fig. 1.1 A neighbor-joining phylogenetic tree of acetic acid bacteria. The phylogenetic tree based on 16S rRNA gene sequences of 1213 bases was constructed by using MEGA 5.05 (Tamura et al. 2011). *Numerals* at the nodes of respective branches indicate bootstrap values (%) derived from 1000 replications

When microbial growth is seen in the LGI medium, the culture is transferred to the pH 3.5 medium mentioned previously (Vu et al. 2013). To obtain and purify candidates of acetic acid bacteria, the culture in the pH 3.5 medium is streaked onto agar plates, which are composed of 2.0 % D-glucose (w/v), 0.5 % ethanol (99.8 %) (v/v), 0.3 % peptone (w/v), 0.3 % yeast extract (w/v), 0.7 % calcium carbonate (e.g., precipitated by Japanese Pharmacopoeia) (w/v), and 1.5 % agar (w/v) (Yamada et al. 1999), and the resulting colonies that dissolve calcium carbonate on the agar plates are picked up, inoculated, and incubated on agar slants with the same composition as the agar plates for temporary preservation. The strains isolated were examined again for growth on the pH 3.5 medium.

When the composition, especially the carbon sources, of the medium in the enrichment procedure is changed, the selective isolation of acetic acid bacteria can be expected. In fact, strains of *Asaia bogorensis* and *Asaia siamensis* were first isolated by the use of D-sorbitol or dulcitol instead of D-glucose (Yamada et al. 2000; Katsura et al. 2001). Several kinds of media employed for the enrichment procedure result in the effective isolation of acetic acid bacteria (Lisdiyanti et al. 2003b; Suzuki et al. 2010). Instead of the pH 3.5 medium, the pH 4.5 medium containing 0.03 % acetic acid (v/v) can be used (Yamada et al. 1976).

In the genera that are not monotypic, including more than several species and therefore restricted to *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia*, and *Komagataeibacter* (which are supposed to be taxonomically and ecologically in common but not in rare existence), the generic-level, routine identification for certain strains of acetic acid bacteria can be done by the combination of only two conventional phenotypic tests composed of acetate and lactate oxidation and the production of acetic acid from ethanol (Yamada and Yukphan 2008).

In strains to be assigned to the genus *Acetobacter*, a deep blue color appears quickly and clearly in the acetate and lactate oxidation tests, and acetic acid is produced in the acetic acid production test (Asai et al. 1964; Yamada and Yukphan 2008). In acetate and lactate oxidation, strains to be assigned to the genus *Gluconobacter* show a clear yellow color, and the color change to blue is not so vigorous in strains to be assigned to the genera *Gluconacetobacter* and *Komagataeibacter*, in contrast to the genus *Acetobacter*. The latter two genera, *Gluconacetobacter* and *Komagataeibacter*, are additionally discriminated from each other by water-soluble brown pigment production and cell motility. Strains to be assigned to the former generally produce a water-soluble brown pigment, being motile, but strains to be assigned to the latter do not, being non motile. Strains to be assigned to the genus *Asaia* show no or little acetic acid production from ethanol, differing from the aforementioned four genera, and the color change is very slow in acetate and lactate oxidation. The two conventional tests just described are useful, especially when a large number of isolates are routinely identified or classified at the generic level.

To isolate acetic acid bacteria, sugary and alcoholic materials have widely been utilized as isolation sources. In such cases, the habitats of the acetic acid bacteria are to be the isolation sources (Komagata et al. 2014; Kersters et al. 2006; Sievers and Swings 2005a). Recently, acetic acid bacteria have been found ecologically in a

wide variety of isolation sources, such as activated sludges, rhizosphere soils, soils, pollen, human patients, mosquitoes, a stone chamber of a tumulus, and nodules (Komagata et al. 2014; Kersters et al. 2006; Sievers and Swings 2005a). In addition, acetic acid bacteria that grow on nitrogen-free media have been found (Gillis et al. 1989; Fuentes-Ramírez et al. 2001; Samaddar et al. 2011; Vu et al. 2013).

Most acetic acid bacteria can be maintained at 4 °C for 1 month on agar slants containing an appropriate medium. Long-term preservation of acetic acid bacteria can be achieved by lyophilization or by storage in liquid nitrogen, or by cryoconservation at −80 °C by the use of low-temperature refrigerators and appropriate cryoprotectants (Komagata et al. 2014; Kersters et al. 2006; Sievers and Swings 2005a).

1.3 Identification of Acetic Acid Bacteria

When a certain strain of acetic acid bacteria is isolated, the strain will be assigned to a proper or suitable systematic or taxonomic position. Such a process is called identification. The identification consists of two levels, genus level and species level.

To select acetic acid bacteria from a number of the strains isolated, it is suitable to test the strains for growth on a pH 3.5 medium, which contains, for example, 1.0 % D-glucose (w/v), 0.5 % ethanol (99.8 %) (v/v), 0.3 % peptone (w/v), and 0.2 % yeast extract (w/v); the pH is adjusted to 3.5 with hydrochloric acid (Yamada et al. 1999). A pH 4.0 medium can be used for the growth test. If a certain strain is an acetic acid bacterium, appropriate growth can be seen. If the pH of the medium is adjusted to 4.5, bacteria other than acetic acid bacteria sometimes can grow.

For generic-level identification, the candidates of the acetic acid bacteria obtained are in general subjected to 16S rRNA gene sequence analysis, especially to the construction of phylogenetic trees based on 16S rRNA gene sequences (Komagata et al. 2014). When the phylogenetic trees are constructed by the three methods, viz., the neighbor-joining, maximum parsimony, and maximum likelihood methods, the candidates may be assignable to new taxa, such as new genera (Yamada and Yukphan 2008). On the other hand, some phenotypic feature analyses are applicable to the routine identification of the candidates (Table 1.1).

For specific-level identification, whole-genome DNA–DNA hybridization is necessary and inevitable for the precise identification of the strains that have already been identified or classified at the generic level (Komagata et al. 2014). Of the phenotypic features used for the specific-level identification, acid production from different carbon sources and growth on different carbon sources are generally utilized; however, precise identification would hardly be expected.

Recently, many taxonomic methods have been reported (Komagata et al. 2014; Sievers and Swings 2005a; Cleenwerck and De Vos 2008), for example, isoprenoid quinone analysis and fatty acid composition analysis as chemotaxonomic methods and DNA base composition determination, and 16S–23S rRNA gene internally

Table 1.1 Phenotypic characteristics differentiating the genera of acetic acid bacteria

	Acetobacter	*Gluconobacter*	*Acidomonas*	*Gluconacetobacter*	*Asaia*	*Kozakia*	*Swaminathania*	*Saccharibacter*	*Neoasaia*	*Granulibacter*	*Tanticharoenia*	*Ameyamaea*	*Neokomagataea*	*Komagataeibacter*	*Endobacter*	*Nguyenibacter*	*Swingsia*
Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Flagellation	per[a]	pol[a]	n[c]	per	per	n	per	n	n	n	n	pol	n	n	spol	per	n
Oxidation of																	
Acetate	+	−	+	+	w	w	w	−	−	w	−	+	–	+	−	+	−
Lactate	+	−	−	+	w	w	w	w	−	+	−	w	−	+	−	−	−
Growth on:																	
30% D-Glucose (w/v)	−	–[b]	+	−	+	−	+	+	+	nd	+	−	+	nd	nd	w	+
1% D-Glucose (w/v)	+	+	+	+	+	+	nd	−	nd	nd	nd	+	+	+	+	+	+
Glutamate agar	−	−	nd	+	+	−	+	–[e]	+	+	w	w	+	+	+	+	+
Mannitol agar	vw	+	nd	+	+	+	+	–[e]	+	w	+	+	+	+	+	+	+
Raffinose	−	−	−	−	+	w	nd	nd	+	nd	−	−	−	nd	nd	+	−
Growth in the presence of																	
0.35% acetic acid (w/v)	+	+	+	+	−	+	+	−	+	nd	+	+	−	+	nd	w	−
1% KNO_3 (w/v)	−	−	+	−	−	−	+	nd	−	nd	−	−	−	nd	nd	−	+
Production of acetic acid from ethanol	+	+	+	+	−	+	+	w/−	+	vw	+	+	w	+	+	−	w
Water-soluble brown pigment production	−	–[b]	−	+	−	−	+	−	−	nd	+	−	−	−	nd	+	+
Production of dihydroxyacetone from glycerol	+	+	−	+	w	+	+	−	w	–	+	w	−	+	+	−	+
Production of levan-like polysaccharide	−	−	−	−	−	+	nd	−	−	nd	−	−	−	−	nd	+	−
Assimilation of ammoniac nitrogen on																	
D-Glucose	−	+	w	+	+	−	nd	−	−	+	−	vw	vw	+	+	−	−
D-Mannitol	−	+	w	+	+	−	nd	−	w	nd	−	vw	−	+	nd	w	+
Ethanol	w	−	w	−	−	−	nd	–	−	nd	−	vw	vw	nd	nd	−	−
Production of																	
2-Keto-D-gluconate	+	+	−	+	+	+	nd	+	+	nd	+	+	+	+	nd	+	+

(continued)

Table 1.1 (continued)

Characteristic	*Acetobacter* 1	*Gluconobacter* 2	*Acidomonas* 3	*Gluconacetobacter* 4	*Asaia* 5	*Kozakia* 6	*Swaminathania* 7	*Saccharibacter* 8	*Neoasaia* 9	*Granulibacter* 10	*Tanticharoenia* 11	*Ameyamaea* 12	*Neokomagataea* 13	*Komagataeibacter* 14	*Endobacter* 15	*Nguyenibacter* 16	*Swingsia* 17
5-Keto-D-gluconate	+	+	−	+	+	+	nd	+	+	nd	+	+	+	+	nd	−	+
2,5-Diketo-D-gluconate	−	−[b]	−	+	−	−	nd	−	−	nd	+	−	+	−	nd	+	+
Acid production from																	
D-Mannitol	−	+	w	−	+	−	−	+	w	−	−	−	−	−	−	−	+
D-Sorbitol	−	+	−	−	+(d)	−	+	−	+(d)	−	−	−	−	−	−	−	−
Dulcitol	−	w	−	−	+(d)	−	v	−	w	−	−	−	−	nd	−	−	−
Glycerol	−	+	+	−	+	+	+	−	+	w/-	+	w	−	−	+	−	−
Raffinose	−	−	−	−	+	+	nd	−	+	nd	w	−	−	nd	nd	w	w
Ethanol	+	+	+	+	−	+	+	−	+	+	+	+	−	+	+	−	−
Major quinone	Q-9	Q-10	Q-10	Q-10	Q-10	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
DNA G+C (mol%)	57.2	60.3	62	64.9	60.2	57.2	57.6–59.9[d]	52.3	63.1	59.1	65.6	66.0	56.8	62.5	60.3	69.4	46.9

The characteristics mentioned here are mainly based on those of the type strains of the type species of the respective genera: 1 *Acetobacter aceti* NBRC 14818^T; 2 *Gluconobacter oxydans* NBRC 14819^T; 3 *Acidomonas methanolica* NRIC 0498^T; 4 *Gluconacetobacter liquefaciens* NBRC 12388^T; 5 *Asaia bogorensis* NBRC 16594^T; 6 *Kozakia baliensis* NBRC 16664^T; 7 *Swaminathania salitolerans* PA51^T; 8 *Saccharibacter floricola* S-877^T; 9 *Neoasaia chiangmaiensis* AC28^T; 10 *Granulibacter bethesdensis* CGDNIH1^T; 11 *Tanticharoenia sakaeratensis* AC37^T; 12 *Ameyamaea chiangmaiensis* AC04^T; 13 *Neokomagataea thailandica* AH11^T; 14 *Komagataeibacter xylinus* JCM 7644^T; 15 *Endobacter medicaginis* M1MS02^T; 16 *Nguyenibacter vanlangensis* TN01LGI^T; 17 *Swingsia samuiensis* AH83^T

pol polar, *per* peritrichous, *spol* subpolar, *n* none, + positive, − negative, *w* weakly positive, *vw* very weakly positive, *d* delayed, *v* variable, *nd* not determined

[a]Some strains in the genus are non motile

[b]Some strains in the genus are positive

[c]Some strains of the genus are polarly flagellated

[d]The DNA G+C content of the type strain was not recorded

[e]According to Jojima et al. (2004), growth was shown at 7% glutamate but not at 1% glutamate

transcribed spacer (ITS) sequencing and restriction analysis of ITS as DNA-based molecular methods, in addition to the phenotypic feature analysis, 16S rRNA gene sequence analysis, and the whole-genome DNA–DNA hybridization. The combination of these methods gives more precise information for the identification and the classification of acetic acid bacteria.

1.4 Genera and Species in Acetic Acid Bacteria

The acetic acid bacteria classified in the acetous group constitute the family *Acetobacteraceae* Gillis and De Ley 1980, the class *Alphaproteobacteria* Stackebrandt et al. 1988, together with the acidophilic group (Komagata et al. 2014; Sievers and Swings 2005a; Gillis and De Ley 1980; Stackebrandt et al. 1988). The type genus of the family is *Acetobacter*. Seventeen genera are reported (Table 1.1). The genera and the species listed below are ordered chronologically, because they have their own respective long (or not so long) histories in transitions of generic and specific circumscriptions and in selection of isolation sources.

1.4.1 Acetobacter *Beijerinck 1898*

A.ce.to.bac'ter. L. neut. n. *acetum*, vinegar; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Acetobacter*, vinegar rod.

The genus *Acetobacter* is the oldest in the classification of acetic acid bacteria and the type genus of the family *Acetobacteraceae*. In the *Approved Lists of Bacterial Names 1980*, the three species *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Acetobacter peroxydans* were listed, with their nine subspecies (Skerman et al. 1980). The genus is related phylogenetically to the genera *Gluconobacter*, *Neokomagataea*, *Swingsia*, and *Saccharibacter*. In the genus *Acetobacter*, there are two phylogenetically different groups: the *Acetobacter aceti* group and the *Acetobacter pasteurianus* group.

Cells are gram negative, ellipsoidal to rod shaped, measuring 0.4–1.0 by 1.2–3.0 μm, rarely longer. Cells occur singly or in short chains and occasionally long chains. Peritrichously flagellated when motile; however, *Acetobacter nitrogenifigens* exceptionally has polar flagella (Dutta and Gachhui 2006). Colonies are generally circular, smooth, entire, convex, cream color to beige, opaque, and butyrous on glucose/ethanol/yeast extract/peptone agar.

Strictly aerobic. Catalase positive, but negative in *Acetobacter peroxydans*. Oxidase negative. Acetic acid is produced from ethanol. Acetate and lactate are oxidized to carbon dioxide and water. Does not grow on glutamate agar and very weakly on mannitol agar. Dihydroxyacetone is not usually produced from glycerol, but is produced by a few species. D-Gluconate is produced from D-glucose by all the

species, 2-keto-D-gluconate by a considerable number of species, and 5-keto-D-gluconate by a few species. 2,5-Diketo-D-gluconate is not generally produced. Acid production depends on the kind of sugars, sugar alcohols, and alcohols as well as on the kinds of species and strains. In the type strain of *Acetobacter aceti*, acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, or ethanol (Lisdiyanti et al. 2000). Ammoniac nitrogen is in general hardly utilized.

The optimal growth temperature is around 30 °C. Most species are able to grow at 37 °C but not at 45 °C. Grows at pH 3.5. Most species are not able to grow on 30 % D-glucose (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-9. The DNA G+C content is 53.5–60.7 mol%. For more details of the characteristics, see Komagata et al. (2014).

The type species of the genus is *Acetobacter aceti* (Pasteur 1864) Beijerinck 1898. Twenty-five species are reported.

1.4.1.1 *Acetobacter aceti* (Pasteur 1864) Beijerinck 1898

For the characteristics of the species, refer to Lisdiyanti et al. (2000), Gosselé et al. (1983b), Komagata et al. (2014), and Sievers and Swings (2005b).

The type strain is ATCC 15973^T (= DSM 3508^T = JCM 7641^T = LMG 1261^T = LMG 1504^T = NBRC 14818^T = NCIMB 8621^T), isolated from beechwood shavings of a vinegar plant. The DNA G+C content of the type strain is 57.2 mol%.

1.4.1.2 *Acetobacter pasteurianus* (Hansen 1879) Beijerinck and Folpmers 1916

For the characteristics of the species, refer to Beijerinck and Folpmers (1916), Lisdiyanti et al. (2000), Gosselé et al. (1983b), Komagata et al. (2014), and Sievers and Swings (2005b).

The type strain is LMG 1262^T (=ATCC 33445^T = DSM 3509^T = JCM 7640^T = LMD 22.1^T), isolated from beer, Netherlands. The DNA G+C content of the type strain is 52.7 mol%.

1.4.1.3 *Acetobacter peroxydans* Visser't Hooft 1925

For the characteristics of the species, refer to Visser't Hooft (1925), Lisdiyanti et al. (2000), Gosselé et al. (1983b), Komagata et al. (2014), and Sievers and Swings (2005b).

The type strain is NBRC 13755^T (=ATCC 12874^T = JCM 25077^T = LMG 1635^T), isolated from ditch water, Delft, Netherlands. The DNA G+C content of the type strain is 60.3 mol%.

1.4.1.4 *Acetobacter pomorum* Sokollek, Hertel and Hammes 1998

For the characteristics of the species, refer to Sokollek et al. (1998).

The type strain is LTH 2458^T (= CIP 105762^T = DSM 11825^T = LMG 18848^T), isolated from a submerged cider vinegar fermentation at a factory in the southern part of Germany. The DNA G+C content of the type strain is 50.5 mol%.

1.4.1.5 *Acetobacter estunensis* (Carr 1958) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001

Basonym: *Acetobacter pasteurianus* subsp. *estunensis* (Carr 1958) De Ley and Frateur 1974.

For the characteristics of the species, refer to Lisdiyanti et al. (2000).

The type strain is NBRC 13751^T (= ATCC 23753^T = DSM 4493^T = JCM 21172^T = LMG 1626^T = NCIMB 8935^T), isolated from cider, Bristol. The DNA G+C content of the type strain is 59.7 mol%.

1.4.1.6 *Acetobacter lovaniensis* (Frateur 1950) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001

Basonym: *Acetobacter pasteurianus* subsp. *lovaniensis* (Frateur 1950) De Ley and Frateur 1974.

For the characteristics of the species, refer to Lisdiyanti et al. (2000).

The type strain is NBRC 13753^T (=ATCC 12875^T= DSM 4491^T = JCM 17121^T = LMG 1579^T = LMG 1617^T = NCIMB 8620^T), isolated from sewage on soil by J. Frateur in 1929. The DNA G+C content of the type strain is 58.6 mol%.

1.4.1.7 *Acetobacter orleanensis* (Henneberg 1906) Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001

Basonym: *Acetobacter aceti* subsp. *orleanensis* (Henneberg 1906) De Ley and Frateur 1974.

For the characteristics of the species, refer to Lisdiyanti et al. (2000).

The type strain is NBRC 13752^T (= ATCC 12876^T = DSM 4492^T = JCM 7639^T = LMG 1583^T = NCIMB 8622^T), isolated from beer by J. Frateur in 1929. The DNA G+C content of the type strain is 58.6 mol%.

1.4.1.8 *Acetobacter indonesiensis* Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001

For the characteristics of the species, refer to Lisdiyanti et al. (2000).

The type strain is 5H-1^T (= JCM 10948^T = LMG 19824^T = NBRC 16471^T = NRIC 0313^T), isolated from fruit of zirzak (*Annona muricata*) in Indonesia. The DNA G+C content of the type strain is 53.7 mol%.

1.4.1.9 *Acetobacter tropicalis* Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2001

For the characteristics of the species, refer to Lisdiyanti et al. (2000).

The type strain is Ni-6b^T (= JCM 10947^T = LMG 19825^T = NBRC 16470^T = NRIC 0312^T), isolated from coconut (*Coccos nucifera*) in Indonesia. The DNA G+C content of the type strain is 55.9 mol%.

1.4.1.10 *Acetobacter cerevisiae* Cleenwerck, Vandemeulebroecke, Janssens and Swings 2002

For the characteristics of the species, refer to Cleenwerck et al. (2002).

The type strain is LMG 1625^T (= ATCC 23765^T = DSM 14362^T = JCM 17273^T = NCIMB 8894^T), isolated from beer (ale) in storage at Toronto, Canada. The DNA G+C content of the type strain is 57.6 mol%.

1.4.1.11 *Acetobacter malorum* Cleenwerck, Vandemeulebroecke, Janssens and Swings 2002

For the characteristics of the species, refer to Cleenwerck et al. (2002).

The type strain is LMG 1746^T (= DSM 14337^T = JCM 17274^T), isolated from a rotten apple in Ghent, Belgium. The DNA G+C content of the type strain is 57.2 mol%.

1.4.1.12 *Acetobacter cibinongensis* Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002

For the characteristics of the species, refer to Lisdiyanti et al. (2001).

The type strain is 4H-1^T (= CIP 107380^T = DSM 15549^T = JCM 11196^T = NBRC 16605^T), isolated from mountain soursop (*Annona montana*) in Indonesia. The DNA G+C content of the type strain is 54.5 mol%.

1.4.1.13 *Acetobacter orientalis* Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002

For the characteristics of the species, refer to Lisdiyanti et al. (2001).

The type strain is 21F-2^T (= CIP 107379^T = DSM 15550^T = JCM 11195^T = NBRC 16606^T = NRIC 0481^T), isolated from canna flower (*Canna hybrida*) in Indonesia. The DNA G+C content of the type strain is 52.3 mol%.

1.4.1.14 *Acetobacter syzygii* Lisdiyanti, Kawasaki, Seki, Yamada, Uchimura and Komagata 2002

For the characteristics of the species, refer to Lisdiyanti et al. (2001).

The type strain is 9H-2^T (= CIP 107378^T = DSM 15548^T = JCM 11197^T = NBRC 16604^T = NRIC 0483^T), isolated from fruit of Malay rose apple (*Syzygium malaccense*) in Indonesia. The DNA G+C content of the type strain is 55.3 mol%.

1.4.1.15 *Acetobacter nitrogenifigens* Dutta and Gachhui 2006

For the characteristics of the species, refer to Dutta and Gachhui (2006).

The type strain is RG1^T (= LMG 23498^T = MTCC 6912^T), isolated from Kombucha tea. The DNA G+C content of the type strain is 64.1 mol%.

1.4.1.16 *Acetobacter oeni* Silva, Cleenwerck, Rivas, Swings, Trujillo, Willems and Velázquez 2006

For the characteristics of the species, refer to Silva et al. (2006).

The type strain is B13^T (= CECT 5830^T = LMG 21952^T), isolated from spoiled red wine of the Dão region, Portugal. The DNA G+C content of the type strain is 58.1 mol%.

1.4.1.17 *Acetobacter ghanensis* Cleenwerck, Camu, Engelbeen, De Winter, Vandemeulebroecke, De Vos and De Vuyst 2007

For the characteristics of the species, refer to Cleenwerck et al. (2007).

The type strain is R-29337^T (= 430A^T = DSM 18895^T = LMG 23848^T), isolated from a traditional heap fermentation of Ghanaian cocoa beans. The DNA G+C content of the type strain is 57.3 mol%.

1.4.1.18 *Acetobacter senegalensis* Ndoye, Cleenwerck, Engelbeen, Dubois-Dauphin, Guiro, Van Trappen, Willems and Thonart 2007

For the characteristics of the species, refer to Ndoye et al. (2007).

The type strain is CWBI-B418^T (= DSM 18889^T = LMG 23690^T), isolated from mango fruit in Senegal (Sub-Saharan Africa). The DNA G+C content of the type strain is 56.0 mol%.

1.4.1.19 *Acetobacter fabarum* Cleenwerck, González, Camu, Engelbeen, De Vos and De Vuyst 2008

For the characteristics of the species, refer to Cleenwerck et al. (2008).

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

1.4.1.20 *Acetobacter farinalis* Tanasupawat, Kommanee, Yukphan, Muramatsu, Nakagawa and Yamada 2011

For the characteristics of the species, refer to Tanasupawat et al. (2011a).

The type strain is G360-1^T (= BCC 44845^T = NBRC 107750^T = PCU 319^T), isolated from fermented rice flour. The DNA G+C content of the type strain is 56.3 mol%.

1.4.1.21 *Acetobacter papayae* Iino, Suzuki, Kosako, Ohkuma, Komagata and Uchimura 2013

For the characteristics of the species, refer to Iino et al. (2012a).

The type strain is 1-25^T (= JCM 25143^T = LMG 26456^T = NRIC 0655^T), isolated from a papaya fruit, Okinawa, Japan. The DNA G+C content of the type strain is 60.5 mol%.

1.4.1.22 *Acetobacter okinawensis* Iino, Suzuki, Kosako, Ohkuma, Komagata and Uchimura 2013

For the characteristics of the species, refer to Iino et al. (2012a).

The type strain is 1-35^T (= JCM 25146^T = LMG 26457^T = NRIC 0658^T), isolated from a piece of a stem of sugarcane, Okinawa, Japan. The DNA G+C content of the type strain is 59.3 mol%.

1.4.1.23 *Acetobacter persici* corrig. Iino, Suzuki, Kosako, Ohkuma, Komagata and Uchimura 2013

For the characteristics of the species, refer to Iino et al. (2012a).

The type strain is T-120^T (= JCM 25330^T = LMG 26458^T), isolated from a peach fruit, Okinawa, Japan. The DNA G+C content of the type strain is 58.7 mol%.

1.4.1.24 *Acetobacter lambici* Spitaels, Li, Wieme, Balzarini, Cleenwerck, Van Landschoot, De Vuyst and Vandamme 2014

For the characteristics of the species, refer to Spitaels et al. (2014a).

The type strain is LMG 27439^T (= DSM 27328^T), isolated from fermenting lambic beer. The DNA G+C content of the type strain is 56.2 mol%.

1.4.1.25 *Acetobacter sicerae* Li, Wieme, Spitaels, Balzarini, Nunes, Manaia, Van Landschoot, De Vuyst, Cleenwerck and Vandamme 2014

For the characteristics of the species, refer to Li et al. (2014).

The type strain is LMG 1531^T (= NCIMB 8941^T), isolated from traditionally produced kefir. The DNA G+C content of the type strain is 58.3 mol%.

1.4.2 Gluconobacter *Asai 1935*

Glu.co.no.bac'ter. N. L. neut. n. *acidum gluconicum*, gluconic acid; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Gluconobacter*, gluconate rod.

The genus *Gluconobacter* was proposed by Asai (1935), who selected a variety of fruits for isolation of acetic acid bacteria and found two taxonomic groups in the isolated strains on the oxidation of ethanol and D-glucose. One had intense ethanol oxidizability rather than D-glucose and oxidized acetic acid to carbon dioxide and water, and the other had intense glucose oxidizability rather than ethanol and did not oxidize acetic acid. For the latter group, the generic name *Gluconobacter* was given. In the *Approved Lists of Bacterial Names 1980*, the only species, *Gluconobacter oxydans*, was listed with its five subspecies (Skerman et al. 1980). The DNA G+C content of the species was 54.2–62.8 mol%, with the range of 8.6 mol% (Yamada et al. 1981b).

Cells are gram negative, ellipsoidal to rod shaped, measuring 0.4–1.2 by 1.0–3.0 μm, and polarly flagellated when motile. Colonies are smooth, raised to convex, entire and glistening on ethanol/glucose/yeast extract/calcium carbonate/agar. Some strains produce pink colonies.

Strictly aerobic. Catalase positive and oxidase negative. Acetic acid is produced from ethanol. Acetate and lactate are not oxidized. Grows on mannitol agar, but not on glutamate agar. Dihydroxyacetone is produced from glycerol. D-Gluconate, 2-keto-D-gluconate, and 5-keto-D-gluconate are produced from D-glucose, and a few strains produce 2,5-diketo-D-gluconate. A water-soluble brown pigment is produced in strains of a few species. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, D-fructose, melibiose, D-mannitol, D-sorbitol, glycerol, and ethanol. Grows on D-glucose, D-fructose, D-mannitol, D-sorbitol, and glycerol. Strains of several species require nicotinic acid for growth.

Optimum temperature for growth is 25 °–30 °C. Many species grow at 35 °C, and a few species grow at 37 °C. Optimum pH for growth is around pH 5.5. Most species grow at pH 3.5. acid is $C_{18:1}\omega7c$. The major ubiquinone is Q-10. The DNA G+C content is 54.0–61.5 mol%. Strains of *Gluconobacter* are isolated from fruits, flowers, and other sugar-rich materials. For more details of characteristics, see Komagata et al. (2014).

The type species of the genus is *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961. Fourteen species are reported.

1.4.2.1 *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961

For the characteristics of the species, refer to Asai et al. (1964), Yamada et al. (1981a, b), Gosselé et al. (1983a), Yamada and Akita (1984), Tanaka et al. (1999), Katsura et al. (2002), Komagata et al. (2014), and Sievers and Swings (2005d).

The type strain is ATCC 19357^T (= DSM 3503^T = DSM 7145^T = JCM 7642^T = LMG 1408^T = NBRC 14819^T = NCIMB 9013^T), isolated from beer by J.G. Carr. The DNA G+C content of the type strain is 60.3 mol%.

1.4.2.2 *Gluconobacter cerinus* (ex Asai 1935) Yamada and Akita 1984 emend. Katsura, Yamada, Uchimura and Komagata 2002

Synonym: *Gluconobacter asaii* Mason and Claus 1989.

For the characteristics of the species, refer to Yamada and Akita (1984), Yamada et al. (1984), Mason and Claus (1989), and Katsura et al. (2002).

The type strain is NBRC 3267^T (= ATCC 19441^T = DSM 9533^T = DSM 9534^T = LMG 1368^T = NRRL B-4241^T), isolated from cherry (*Prunus* sp.). The DNA G+C content of the type strain is 55.9 mol%.

1.4.2.3 *Gluconobacter frateurii* Mason and Claus 1989

For the characteristics of the species, refer to Mason and Claus (1989).

The type strain is Kondo 40^T (= NBRC 3264^T = ATCC 49207^T = DSM 7146^T = LMG 1365^T), isolated from strawberry (*Fragaria ananassa*). The DNA G+C content of the type strain is 55.1 mol%.

1.4.2.4 *Gluconobacter albidus* (ex Kondo and Ameyama 1958) Yukphan, Takahashi, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2005

For the characteristics of the species, refer to Yukphan et al. (2004a).

The type strain is NBRC 3250^T (= BCC 14434^T = JCM 20271^T), isolated from a flower of dahlia by Kondo and Ameyama (1958). The DNA G+C content of the type strain is 60.0 mol%.

1.4.2.5 *Gluconobacter thailandicus* Tanasupawat, Thawai, Yukphan, Moonmangmee, Itoh, Adachi and Yamada 2005

For the characteristics of the species, refer to Tanasupawat et al. (2004).

The type strain is F-149-1^T (= BCC 14116^T = JCM 12310^T = NBRC 100600^T = TISTR 1533^T), isolated from a flower of Indian cork tree (*Millingtonia hortensis*) Bangkok, Thailand. The DNA G+C content of the type strain is 55.8 mol%.

1.4.2.6 *Gluconobacter kondonii* Malimas, Yukphan, Takahashi, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2007

For the characteristics of the species, refer to Malimas et al. (2007).

The type strain is Kondo 75^T (= BCC 14441^T = NBRC 3266^T), isolated from strawberry. The DNA G+C content of the type strain is 59.8 mol%.

1.4.2.7 *Gluconobacter roseus* (ex Asai 1935) Malimas, Yukphan, Takahashi, Muramatsu, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2008

For the characteristics of the species, refer to Malimas et al. (2008a).

The type strain is Asai G-2^T (= BCC 14456^T = JCM 20293^T = NBRC 3990^T), isolated from a fruit of kaki (persimmon, *Diasporas kaki*). The DNA G+C content of the type strain is 60.5 mol%.

1.4.2.8 *Gluconobacter sphaericus* (Ameyama 1975) Malimas, Yukphan, Takahashi, Muramatsu, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2008

Basonym: *Gluconobacter oxydans* subsp. *sphaericus* Ameyama 1975.

For the characteristics of the species, refer to Ameyama (1975) and Malimas et al. (2008b).

The type strain is NBRC 12467^T (= BCC 14448^T = LMG 1414^T), isolated from fresh grapes by Ameyama (1975). The DNA G+C content of the type strain is 59.5 mol%.

1.4.2.9 *Gluconobacter kanchanaburiensis* Malimas, Yukphan, Lundaa, Muramatsu, Takahashi, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Suzuki, Tanticharoen and Yamada 2009

For the characteristics of the species, refer to Malimas et al. (2009a).

The type strain is AD92^T (= BCC 15889^T = NBRC 103587^T), isolated from a spoiled fruit of jackfruit (*Artocarpus heterophyllus*). The DNA G+C content of the type strain is 59.5 mol%.

1.4.2.10 *Gluconobacter japonicus* Malimas, Yukphan, Takahashi, Muramatsu, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2009

For the characteristics of the species, refer to Malimas et al. (2009b).

The type strain is Kondo 7^T (= BCC 14458^T = NBRC 3271^T), isolated from a fruit of Chinese bayberry. The DNA G+C content of the type strain is 56.4 mol%.

1.4.2.11 *Gluconobacter wancherniae* Yukphan, Malimas, Lundaa, Muramatsu, Takahashi, Kaneyasu, Tanasupawat, Nakagawa, Suzuki, Tanticharoen and Yamada 2011

For the characteristics of the species, refer to Yukphan et al. (2010).

The type strain is AC42^T (= BCC 15775^T = NBRC 103581^T), isolated from unknown seed. The DNA G+C content of the type strain is 56.6 mol%.

1.4.2.12 *Gluconobacter uchimurae* Tanasupawat, Kommanee, Yukphan, Moonmangmee, Muramatsu, Nakagawa and Yamada 2011

For the characteristics of the species, refer to Tanasupawat et al. (2011b).

The type strain is ZW160-2^T (= BCC 14681^T = NBRC 100627^T), isolated from rakam fruit (*Zalacca wallichiana*). The DNA G+C content of the type strain is 60.5 mol%.

1.4.2.13 *Gluconobacter nephelii* Kommanee, Tanasupawat, Yukphan, Malimas, Muramatsu, Nakagawa and Yamada 2011

For the characteristics of the species, refer to Kommanee et al. (2011).

The type strain is RBY-1^T (= BCC 36733^T = NBRC 10606^T), isolated from rambutan (*Nephelium lappaceum*). The DNA G+C content of the type strain is 57.2 mol%.

1.4.2.14 *Gluconobacter cerevisiae* Spitaels, Wieme, Balzarini, Cleenwerck, Van Landschoot, De Vuyst and Vandamme 2014

For the characteristics of the species, refer to Spitaels et al. (2014b).

The type strain is LMG 27748^T (= DSM 27644^T), isolated from fermenting lambic beer. The DNA G+C content of the type strain is 58.0 mol%.

1.4.3 Acidomonas *Urakami, Tamaoka, Suzuki and Komagata 1989 emend. Yamashita, Uchimura and Komagata 2004*

A.ci.do.mo'nas. L. adj. *acidus*, sour or acid; L. fem. n. *monas*, unit or monad; *Acidomonas*, acidophilic monad.

The genus *Acidomonas* was introduced for the facultatively methylotrophic bacterium, *Acetobacter methanolicus* Uhlig et al. 1986. However, the generic name was not accepted for a long time (Swings 1992; Sievers et al. 1994). The phylogenetic relationship between the genus *Acidomonas* and other genera of acetic acid bacteria was sufficiently remote to establish the new genus (Bulygina et al. 1992; Yamada et al. 1997; Yamashita et al. 2004).

Cells are gram negative, short rods, measuring 0.5–0.8 by 1.5–2.0 μm. Cells occur singly, in pairs, or rarely in short chains, and are either motile with a single polar flagellum or non motile. Colonies are shiny, smooth, circular, convex, entire, beige to pink, and 1–3 mm in diameter on glucose/peptone/yeast extract/malt

extract (PYM) agar (pH 4.5) after 5 days at 30 °C. Pellicles are produced in PYM broth.

Aerobic. Catalase positive and oxidase negative. Acetic acid is produced from ethanol. Acetate is oxidized, but lactate is not or only weakly oxidized. Dihydroxyacetone is not produced from glycerol. D-Gluconate is produced from D-glucose. 2-Keto-D-gluconate, 5-keto-D-gluconate, or 2,5-diketo-D-gluconate is not produced in culture media. Acid is produced from L-arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-mannose, glycerol, ethanol, or methanol. Methanol, ethanol, D-glucose, D-mannose, glycerol, or succinic acid is utilized as a sole source of carbon. Pantothenic acid is essentially required for growth.

Grows on 30 % D-glucose (w/v) and 0.35 % acetic acid (v/v). Grows at pH 3.0. Grows at 30 °C but not at 45 °C. The major cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{18:1}2OH$. The major quinone is Q-10. The DNA G+C content is 62–63 mol%. Strains of *Acidomonas* were abundantly isolated from activated sludges, except for the type strain, but not from vegetables, fruit, decayed wood and leaves, manure, and paddy soil. For more details of characteristics, see Komagata et al. (2014).

1.4.3.1 *Acidomonas methanolica* (Uhlig et al. 1986) Urakami, Tamaoka, Suzuki, and Komagata 1989 emend. Yamashita, Uchimura and Komagata 2004

Basonym: *Acetobacter methanolicus* Uhlig, Karbaum and Steudel 1986.

For the characteristics of the species, refer to Uhlig et al. (1986), Urakami et al. (1989), and Yamashita et al. (2004).

The type strain is MB 58^T (= DSM 5432^T = JCM 6891^T = LMG 1668^T = NRIC 0498^T), isolated from a nonsterile fermentation process for the production of single-cell protein (SCP) from methanol with *Candida* species. The cells of the type strain are non motile, and the DNA G+C content is 62 mol%.

1.4.4 Gluconacetobacter *corrig. Yamada, Hoshino and Ishikawa 1998*

Glu.con.a.ce.to.bac'ter. N. L. neut. n. *acetum gluconicum*, gluconic acid; L. neut. n. *acetum*, vinegar; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Gluconacetobacter*, gluconate-vinegar rod.

The genus *Gluconacetobacter* was introduced by the elevation of the subgenus *Gluconacetobacter* corrig. (ex Asai 1935) Yamada and Kondo 1984 for the Q-10-equipped *Acetobacter* species. Phylogenetically, the genus *Gluconacetobacter* consisted of two groups: the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group. For the latter group, the genus *Komagataeibacter* Yamada et al. 2013 was proposed.

Cells are gram negative rods, measuring 0.6–0.9 by 1.2–2.0 μm, with peritrichous flagella when motile, and occur singly or in pairs. Colonies are generally light brown to brown.

Aerobic. Catalase positive. Oxidase negative. Acid is produced from ethanol. Oxidizes acetate and lactate. Grows on glutamate agar and mannitol agar. A few species produce dihydroxyacetone from glycerol. 2-Keto-D-gluconate is produced from D-glucose. Most of species produce 2,5-diketo-D-gluconate, and a few species produce 5-keto-D-gluconate. Most of the species produce a water-soluble brown pigment. Acid is produced from L-arabinose, D-xylose, D-glucose, D-mannose, or ethanol. Grows on D-glucose, D-fructose, sucrose, D-mannitol, or ethanol. Ammoniac nitrogen is used as a sole nitrogen source. Strains of most species have the activity of nitrogen fixation.

Most of the species grow on 30 % D-glucose (w/v). Grows between 15 ° and 30 °C but not at 37 °C. The optimum growth temperature is around 30 °C. Grows at pH 3.0. The optimum growth pH is about 5.5. The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content is 58–65 mol%. For more details of characteristics, see Komagata et al. (2014).

The type species of the genus is *Gluconacetobacter liquefaciens* (Asai 1935) Yamada et al. 1998. Ten species are reported.

1.4.4.1 *Gluconacetobacter liquefaciens* (Asai 1935) Yamada, Hoshino and Ishikawa 1998

Basonym: *Acetobacter aceti* subsp. *liquefaciens* (Asai 1935) De Ley and Frateur 1974.

Synonyms: *Acetobacter liquefaciens* (Asai 1935) Gosselé, Swings, Kersters, Pauwels and De Ley 1983; '*Gluconobacter liquefaciens*' Asai 1935.

For the characteristics of the species, refer to Asai et al. (1964), Gosselé et al. (1983b), Yamada and Kondo (1984), Navarro and Komagata (1999), Sievers and Swings (2005c), and Komagata et al. (2014).

The type strain is Asai G-1^T (= ATCC 14835^T = DSM 5603^T = JCM 17840^T = LMG 1381^T = LMG 1382^T = NBRC 12388^T), isolated from dried persimmon. The DNA G+C content of the type strain is 64.9 mol%.

1.4.4.2 *Gluconacetobacter diazotrophicus* (Gillis et al. 1989) Yamada, Hoshino and Ishikawa 1998

Basonym: *Acetobacter diazotrophicus* Gillis, Kersters, Hoste, Janssens, Kroppenstedt, Stephan, Teixeira, Döbereiner and De Ley 1989.

For the characteristics of the species, refer to Gillis et al. (1989).

The type strain is Döbereiner PAl 5^T (= ATCC 49037^T = CCUG 37298^T = CIP 103539^T = DSM 5601^T = LMG 7603^T), isolated from roots and stems of sugarcane in Alagoas, Brazil. The DNA G+C content of the type strain is 61 mol%.

1.4.4.3 *Gluconacetobacter sacchari* Franke, Fegan, Hayward, Leonard, Stackebrandt and Sly 1999

For the characteristics of the species, refer to Franke et al. (1999).

The type strain is SRI 1794^T (= CIP 106693^T = DSM 12717^T), isolated from the leaf sheath of sugarcane and from the pink sugarcane mealybug. The DNA G+C content of the type strain is 65 mol%.

1.4.4.4 *Gluconacetobacter johannae* Fuentes-Ramírez, Bustillos-Cristales, Tapia-Hernández, Jiménez-Salgado, Wang, Martínez-Romer and Caballero-Mellado 2001

For the characteristics of the species, refer to Fuentes-Ramírez et al. (2001).

The type strain is CFN-Cf55^T (= ATCC 700987^T = CIP 107160^T = DSM 13595^T), isolated from the rhizosphere of coffee plants. The DNA G+C content of the type strain is 57.96 mol%.

1.4.4.5 *Gluconacetobacter azotocaptans* Fuentes-Ramírez, Bustillos-Cristales, Tapia-Hernández, Jiménez-Salgado, Wang, Martínez-Romero and Caballero-Mellado 2001

For the characteristics of the species, refer to Fuentes-Ramírez et al. (2001).

The type strain is CFN-Ca54^T (= ATCC 700988^T = CIP 107161^T = DSM 13594^T), isolated from the rhizosphere of coffee plants. The DNA G+C content of the type strain is 64.01 mol%.

1.4.4.6 *Gluconacetobacter tumulicola* Tazato, Nishijima, Handa, Kigawa, Sano and Sugiyama 2012

For the characteristics of the species, refer to Tazato et al. (2012).

The type strain is K5929-2-1b^T (= JCM 17774^T = NCIMB 14760^T), isolated from a black viscous substance in a plaster hole at the center of the ceiling in the stone chamber of the Kitora Tumulus in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 64.7 mol%.

1.4.4.7 *Gluconacetobacter asukensis* Tazato, Nishijima, Handa, Kigawa, Sano and Sugiyama 2012

For the characteristics of the species, refer to Tazato et al. (2012).

The type strain is K8617-1-1b^T (= JCM 17772^T = NCIMB 14759^T), isolated from a brown viscous gel on the northeast area of the ceiling in the stone chamber of the Kitora Tumuli in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 65.4 mol%.

1.4.4.8 *Gluconacetobacter tumulisoli* Nishijima, Tazato, Handa, Tomita, Kigawa, Sano and Sugiyama 2013

For the characteristics of the species, refer to Nishijima et al. (2013).

The type strain is T611xx-1-4a^T (= JCM 19097^T = NCIMB 14861^T), isolated from clay soil taken from near a spider web and an ant hole at a plugging stone directly under the plugging stone of the upper north side at the space adjacent to Takamatsuzuka Tumulus in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 66.5 mol%.

1.4.4.9 *Gluconacetobacter takamatsuzukensis* Nishijima, Tazato, Handa, Tomita, Kigawa, Sano and Sugiyama 2013

For the characteristics of the species, refer to Nishijima et al. (2013).

The type strain is T61213-20-1a^T (= JCM 19094^T = NCIMB 14859^T), isolated from soil taken from the left side wall of the west side in the stone chamber exterior during the dismantling work of Takamatsuzuka Tumulus in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 66.6 mol%.

1.4.4.10 *Gluconacetobacter aggeris* Nishijima, Tazato, Handa, Tomita, Kigawa, Sano and Sugiyama 2013

For the characteristics of the species, refer to Nishijima et al. (2013).

The type strain is T6203-4-1a^T (= JCM 19092^T = NCIMB 14860^T), isolated from soil taken from 5 cm below the surface in a bamboo grove of the burial mound of Takamatsuzuka Tumulus in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 65.4 mol%.

1.4.5 Asaia *Yamada, Katsura, Kawasaki, Widyastuti, Saono, Seki, Uchimura and Komagata 2000*

A.sa'i.a. N. L. fem. n. *Asaia*, Asai, named after Professor Toshinobu Asai, a Japanese bacteriologist who contributed to the systematics of acetic acid bacteria.

The strains of the genus *Asaia* were first found and isolated from flowers collected in Indonesia. In the beginning, the distribution of the *Asaia* strains was supposed to be restricted only to the tropical zone, that is, in Thailand, the Philippines, and Indonesia (Yamada and Yukphan 2008). However, the *Asaia* strains were isolated in the temperate zone, in Japan (Suzuki et al. 2010). The strains of the genus *Asaia* produced no or a very small amount of acetic acid from ethanol and did not grow in the presence of 0.35 % acetic acid (v/w).

Cells are gram negative, rod shaped, measuring 0.4–1.0 by 1.0–2.5 μm, and motile with peritrichous flagella. Colonies are smooth, entire, raised, shiny, and light brown, pink, to dark pinkish on glucose/peptone/yeast extract agar.

Aerobic. Catalase positive and oxidase negative. Produces no or a limited amount of acetic acid from ethanol. Oxidizes acetate and lactate to carbon dioxide and water. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is generally produced. Produces 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate. Acid is produced from D-glucose, D-galactose, D-fructose, or other sugars and sugar alcohols. Grows on D-glucose, D-fructose, or D-mannitol. Ammoniac nitrogen is assimilated on D-glucose or D-mannitol.

Grows on 30 % D-glucose (w/v), but not in the presence of 0.35 % acetic acid (v/v). Growth generally occurs between 10 ° and 30 °C, but not at 37 °C. Grows at pH 3.0. The major cellular fatty acid is $C_{18:1}\omega 7c$. The major quinone is Q-10. The DNA G+C content is 58.6–61.0 mol%. For more details of characteristics, see Komagata et al. (2014).

The type species of the genus is *Asaia bogorensis* Yamada et al. 2000. Eight species are reported.

1.4.5.1 *Asaia bogorensis* Yamada, Katsura, Kawasaki, Widyastuti, Saono, Seki, Uchimura and Komagata 2000

For the characteristics of the species, refer to Yamada et al. (2000).

The type strain is 71^T (= JCM 19569^T = NRIC 0311^T), isolated from a flower of orchid tree (*Bauhinia purpurea*) in Bogor, Indonesia. The DNA G+C content of the type strain is 60.2 mol%.

1.4.5.2 *Asaia siamensis* Katsura, Kawasaki, Potacharoen, Saono, Seki, Yamada, Uchimura and Komagata 2001

For the characteristics of the species, refer to Katsura et al. (2001).

The type strain is S60-1^T (= JCM 10715^T = NBRC 16457^T = NRIC 0323^T), isolated from a flower of crown flower (*Calotropis gigantea*), in Bangkok, Thailand. The DNA G+C content of the type strain is 59.3 mol%.

1.4.5.3 *Asaia krungthepensis* Yukphan, Potacharoen, Tanasupapwat, Tanticharoen and Yamada 2004

For the characteristics of the species, refer to Yukphan et al. (2004b).

The type strain is AA08^T (= BCC 12978^T = NBRC 0535^T = TISTER 1524^T), isolated from a heliconia flower (*Heliconia* sp.) in Bangkok, Thailand. The DNA G+C content of the type strain is 60.3 mol%.

1.4.5.4 *Asaia lannensis* corrig. Malimas, Yukphan, Takahashi, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2008

For the characteristics of the species, refer to Malimas et al. (2008c).

The type strain is AB92^T (= BCC 15733^T = NBRC 102526^T), isolated from a flower of spider lily (*Crynum asiaticum*) in Chiang Mai, Thailand. The DNA G+C content of the type strain is 60.8 mol%.

1.4.5.5 *Asaia spathodeae* Kommanee, Tanasupawat, Yukphan, Malimas, Muramatsu, Nakagawa and Yamada 2010

For the characteristics of the species, refer to Kommanee et al. (2010).

The type strain is GB23-2^T (= BCC 36458^T = NBRC 105894^T = PCU 307^T), isolated from a flower of the African tulip (*Sapathodea campanulata*) in Thailand. The DNA G+C content of the type strain is 59.7 mol%.

1.4.5.6 *Asaia astilbis* corrig. Suzuki, Zhang, Iino, Kosako, Komagata and Uchimura 2010

For the characteristics of the species, refer to Suzuki et al. (2010).

The type strain is T-6133^T (= DSM 23030^T = JCM 15831^T), isolated from astilbe (*Astilbe thunbergii* var. *congesta*), Yamanashi, Japan. The DNA G+C content of the type strain is 58.9 mol%.

1.4.5.7 *Asaia platycodi* Suzuki, Zhang, Iino, Kosako, Komagata and Uchimura 2010

For the characteristics of the species, refer to Suzuki et al. (2010).

The type strain is T-683^T (= JCM 25414^T = DSM 23029^T), isolated from balloon flower (*Platycodon grandiflorum*) in Akita, Japan. The DNA G+C content of the type strain is 60.0 mol%.

1.4.5.8 *Asaia prunellae* Suzuki, Zhang, Iino, Kosako, Komagata and Uchimura 2010

For the characteristics of the species, refer to Suzuki et al. (2010).

The type strain is T-153^T (= DSM 23028^T = JCM 25354^T), isolated from self-heal (*Prunella vulgaris*) in Akita, Japan. The DNA G+C content of the type strain is 58.9 mol%.

1.4.6 Kozakia *Lisdiyanti, Kawasaki, Widyastuti, Saono, Seki, Yamada, Uchimura and Komagata 2002*

Ko.za'ki.a. N. L. fem. n. *Kozakia*, Kozaki, named after Professor Michio Kozaki, a Japanese bacteriologist who contributed to the study of microorganisms in tropical regions, especially Southeast Asia.

The genus *Kozakia* is phylogenetically related to the genus *Asaia*. However, the genus *Kozakia* especially differed from the genus *Asaia* in oxidation of ethanol to acetic acid and in production of a large amount of levan-like mucous substances from sucrose.

Cells are gram negative, rod shaped, and non motile, measuring 0.6–0.8 by 2.0–3.0 μm. Colonies are not pigmented.

Strictly aerobic. Catalase positive and oxidase negative. Acetic acid is produced from ethanol. Acetate and lactate are oxidized to carbon dioxide and water, but the activity is weak. Grows on mannitol agar but not on glutamate agar. Dihydroxyacetone is produced from glycerol. D-Gluconate, 2-keto-D-gluconate, and 5-keto-D-gluconate are produced from D-glucose, but 2,5-diketo-D-gluconate is not. A water-soluble brown pigment is not produced from D-glucose. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, raffinose, *meso*-erythritol, glycerol, or ethanol. Methanol is not utilized. Ammoniac nitrogen is not assimilated on glucose, mannitol, or ethanol medium without vitamins. A levan-like mucous substance is produced from sucrose or D-fructose. γ-Pyrone is produced from D-fructose but not from D-glucose.

Growth is not inhibited by 0.35 % acetic acid (v/v) at pH 3.5. Does not grow on 30 % D-glucose (w/v). Grows at pH 3.0 and 30 °C. The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content is 56.8–57.2 mol%. For more details of characteritics, see Komagata et al. (2014).

1.4.6.1 *Kozakia baliensis* Lisdiyanti, Kawasaki, Widyastuti, Saono, Seki, Yamada, Uchimura and Komagata 2002

For the characteristics of the species, refer to Lisdiyanti et al. (2002).

The type strain is Yo-3^T (= DSM 14400^T = JCM 11301^T = NBRC 16664^T= NRIC 0488^T), isolated from palm brown sugar collected in Bali, Indonesia in 1996. The DNA G+C content of the type strain is 57.2 mol%.

1.4.7 Swaminathania *Loganathan and Nair 2004*

Swa.mi.na.tha'ni.a. N. L. fem. n. *Swaminathania*, Swaminathan, named after Swaminathan, an Indian biologist, the father of the Green Revolution in India.

The strains of the genus *Swaminathania*, which were isolated using a nitrogen-free semisolid LGI medium at pH 5.5 from the rhizosphere, roots, and stems of salt-tolerant, mangrove-associated wild rice, were phylogenetically related especially to those of the genus *Asaia*. However, the genus was distinguished phenotypically from the genus *Asaia* by growth on 0.35 % acetic acid (v/v) and 3 % NaCl (w/v) or 1 % KNO_3 (w/v).

Cells are gram negative, straight rods with round ends, measuring approximately 0.7–0.9 by 1.9–3.1 μm, and motile with peritrichous flagella. Colonies are initially yellowish and become dark orange later, smooth and raised, with entire margin on LGI medium.

Aerobic. Catalase positive and oxidase negative. Acetic acid is produced from ethanol under neutral and acidic conditions. Acetate and lactate are oxidized to carbon dioxide and water, but the activity was weak. Grows on mannitol agar and glutamate agar. Acid is produced from L-arabinose, D-glucose, D-galactose, D-mannose, D-sorbitol, glycerol, or ethanol. Methanol is not utilized. A water-soluble brown pigment is produced on glucose/calcium carbonate-containing agar. Strains are able to fix nitrogen. Solubilization of phosphate is shown. Grows intensely in the presence of 0.35 % acetic acid (v/v) at pH 3.5 and 3 % NaCl using 1 % KNO_3 (w/v) as a nitrogen source.

The major cellular fatty acid is $C_{18:1}\omega7c/\omega9t/\omega12t$. The major quinone is Q-10. The DNA G+C content is 57.6–59.9 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.7.1 *Swaminathania salitolerans* Loganathan and Nair 2004

For the characteristics of the species, refer to Loganathan and Nair (2004).

The type strain is PA51^T (= LMG 21291^T = MTCC 3852^T), isolated from mangrove-associated wild rice (*Porteresia coarctata*) in Pichavaram, Tamil Nadu, India. The DNA G+C content of the type strain is not reported.

1.4.8 Saccharibacter *Jojima, Miura, Suzuki, Yokozeki, Yamanaka and Fudo 2004*

Sac.cha.ri.bac'ter. L. neut. n. *sacchrum* or *saccharon*, sugar; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Saccharibacter*, a sugar rod or a rod that grows intensely in a sugar-rich environment.

The strains of the genus *Saccharibacter* that were isolated from the pollen of a Japanese flower were quite remote phylogenetically from the strains of any other genera of acetic acid bacteria. The strains of the genus were osmophilic, showing no growth on 1 % glutamate agar (w/v) but growing on 7 % glutamate agar (w/v). The phylogenetically related genera are *Neokomagataea*, *Swingsia*, and *Gluconobacter*.

Cells are gram negative, straight rods, measuring 0.8–1.0 by 2.5–4.0 μm, and non motile. Colonies are circular, entire, and pale in color on yeast extract/glucose/peptone agar.

Strictly aerobic. Catalase positive and oxidase negative. Produces negligible or very little acetic acid from ethanol. Acetate is not oxidized to carbon dioxide and water, and lactate is weakly oxidized. Grows on mannitol agar and glutamate agar supplemented with 7 % substrates (w/v). Does not grow on common mannitol agar and glutamate agar with 1 % substrates (w/v). Dihydroxyacetone is not produced from glycerol. D-Gluconate, 2-keto-D-gluconate, and 5-keto-D-gluconate are produced from D-glucose. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, sucrose, or D-mannitol. Methanol is not utilized. Ammoniac nitrogen is not assimilated on Hoyer–Frateur medium with D-glucose, D-mannitol, or ethanol. Cellulosic pellicles and water-soluble mucous substances are not produced. Not pigmented.

Grows in the glucose range between 2 % and 40 % (w/v), with an optimum around 10 % (w/v). High glucose concentration, for example, 10 % D-glucose (w/v), is preferable for growth. Osmophilic. No growth occurs in the presence of 0.35 % acetic acid (v/v) at pH 3.5. Temperature for growth ranges from 20 ° to 33 °C; the optimum is around 25–30 °C. The growth pH ranges from pH 4.0 to pH 7.5; the optimum pH is around pH 5.0 to pH 7.0. No growth is observed below pH 4.0. The major cellular fatty acids are $C_{16:0}2OH$ (31.1–41.0 %) and $C_{18:1}\omega7c$ (22.0–29.8 %). The major quinone is Q-10. The DNA G+C content is 52–53 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.8.1 *Saccharibacter floricola* Jojima, Mihara, Suzuki, Yokozeki, Yamanaka and Fudo 2004

For the characteristics of the species, refer to Jojima et al. (2004).

The type strain is S-877^T (= AJ 13480^T = DSM 15669^T = JCM 12116^T), isolated from pollen collected in Kanagawa Prefecture, Japan. The DNA G+C content of the type strain is 52.3 mol%.

1.4.9 Neoasaia *Yukphan, Malimas, Potacharoen, Tanasupawat, Tanticharoen and Yamada 2006*

Ne.o.a.sa'i.a. Gr. adj. *neos*, new; N. L. fem. n. *Asaia*, a bacterial name after Professor Asai, Japan; N. L. fem. n. *Neoasaia*, new *Asaia*.

The strain of the genus *Neoasaia* that was isolated from a flower of red ginger was closely related phylogenetically to those of the genera *Kozakia, Asaia*, and *Swaminathania*. However, the phenotypic characteristic was that of no oxidation of acetate and lactate, differentiating from the foregoing three genera.

Cells are gram negative, rod shaped, measuring 0.8–1.0 by 1.0–2.0 μm, and non motile. Colonies are smooth, raised, entire, shiny, and pink.

Aerobic. Acetic acid is produced from ethanol. Acetate and lactate are not oxidized. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is weakly produced from glycerol. 2-Keto-D-gluconate and 5-keto-D-gluconate are produced from D-glucose. Acid is produced from D-arabinose weakly, L-arabinose, D-xylose, L-rhamnose weakly, D-fructose with delay, D-galactose, D-glucose, D-mannose, melibiose, sucrose, raffinose, D-mannitol weakly, D-sorbitol with delay, dulcitol weakly, *meso*-erythritol, glycerol, or ethanol.

Grows on D-arabinose weakly, L-arabinose, D-xylose, D-fructose, L-sorbose, D-galactose, D-glucose, D-mannose weakly, sucrose, raffinose, D-mannitol, D-sorbitol, dulcitol, *meso*-erythritol, or glycerol. Ammoniac nitrogen is hardly assimilated in the presence of D-glucose or D-mannitol as a carbon source. A water-soluble brown pigment is not produced on a glucose/peptone/yeast extract/calcium carbonate medium, and a levan-like polysaccharide is not produced on a sucrose medium. However, the production of fructan is reported by *Neoasaia chiangmaiensis* NBRC 101099^T (Jacob et al. 2013).

Grows on 30 % D-glucose (w/v) and in the presence of 0.35 % acetic acid (v/v), but not in the presence of 1.0 % KNO_3 (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content is 63.1 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.9.1 *Neoasaia chiangmaiensis* Yukphan, Malimas, Potacharoen, Tanasupawat, Tanticharoen and Yamada 2006

For the characteristics of the species, refer to Yukphan et al. (2005).

The type strain is $AC28^T$ (= BCC 15763^T = NBRC 101099^T), isolated from a flower of red ginger (*Alpinia purpurata*) in Chiang Mai, Thailand, in September 2002. The DNA G+C content of the type strain is 63.1 mol%.

1.4.10 Granulibacter *Greenberg, Porcella, Orcella, Stock, Wong, Conville, Murray, Holland and Zelazny 2006*

Gra.nu.li.bac'ter. L. neut. n. *granulum*, grain; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Granulibacter*, a rod that causes granules or granuloma formation.

The strain of the genus *Granulibacter* isolated first from three patients with chronic granulomatous disease was quite remote phylogenetically from other acetic acid bacteria. The strain grew at optimum temperatures of 35–37 °C and on methanol.

Gram negative, coccobacillus to rod shaped, and non motile. Colonies are convex, entire, smooth, and nondiffusible yellow on a modified glucose/yeast extract/calcium carbonate.

Strictly aerobic. Catalase positive and oxidase negative. Acetic acid is hardly produced from ethanol. Acetate and lactate are oxidized to carbon dioxide and water, but the activity of the former is weak. Grows on glutamate agar but weakly on mannitol agar. Dihydroxyacetone is not produced from glycerol. Acid is produced from D-glucose or ethanol and from glycerol weakly. Methanol can be used as a sole source of carbon. Ammoniac nitrogen is assimilated on glucose. A high concentration of D-glucose, for example, 5 % D-glucose (w/v), is preferable for growth.

Optimum temperature for growth is 35–37 °C. Optimum pH for growth is 5.0–6.5. Grows at pH 3.5. The major cellular fatty acids are $C_{18:1}\omega7c$ and $C_{16:0}$. The major quinone is Q-10 (Yukphan et al. 2009). The DNA G+C content is 59.1 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.10.1 *Granulibacter bethesdensis* Greenberg, Porcella, Stock, Wong, Conville, Murray, Holland and Zelazny 2006

For the characteristics of the species, refer to Greenberg et al. (2006).

The type strain is CGDNIH1^T (= ATCC BAA-1260^T = DSM 17861^T), which was isolated from lymph node culture from a granulomatous disease patient in Bethesda, MD, USA, in 2003. The DNA G+C content of the type strain is 59.1 mol%.

1.4.11 Tanticharoenia *Yukphan, Malimas, Muramatsu, Takahashi, Kaneyasu, Tanasupawat, Nakagawa, Suzuki, Potacharoen and Yamada 2008*

Tan.ti.cha.ro.e'nia. N. L. fem. n. *Tanticharoenia*, named after Dr. Morakot Tanticharoen, Thailand, who contributed to studies of acetic acid bacteria.

The strains of the genus *Tanticharoenia* that were isolated from soil collected in Sakaerat, Nakhon Rachashima, Thailand, constituted an independent cluster

phylogenetically. The strains did not oxidize either acetate or lactate, but grew on 30 % D-glucose (w/v).

Cells are gram negative, rod shaped, measuring 0.6–0.8 by 1.0–1.6 μm, and non motile. Colonies are creamy and smooth with entire margin when grown on glucose/ethanol/peptone/yeast extract/calcium carbonate agar.

Acetic acid is produced from ethanol. Acetate and lactate are not oxidized. Grows on glutamate agar weakly and on mannitol agar. Dihydroxyacetone is produced from glycerol. 2-Keto-D-gluconate, 5-keto-D-gluconate, and 2,5-diketo-D-gluconate are produced from D-glucose. A water-soluble brown pigment is intensely produced on glucose/peptone/yeast extract/calcium carbonate agar. Acid is produced from L-arabinose, D-xylose, D-fructose weakly, D-galactose, D-glucose, D-mannose, melibiose, sucrose weakly, raffinose weakly, *meso*-erythritol, glycerol, or ethanol. Grows on L-arabinose, D-xylose, D-fructose, D-glucose, D-galactose, *meso*-erythritol, D-mannitol, D-sorbitol, glycerol, or sucrose. Ammoniac nitrogen is not assimilated in the presence of D-glucose, D-mannitol, or ethanol as a carbon source.

Grows in the presence of 0.35 % acetic acid (v/v), but not of 1 % KNO_3. Grows on 30 % D-glucose (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content ranges from 64.5 to 65.6 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.11.1 *Tanticharoenia sakaeratensis* Yukphan, Malimas, Muramatsu, Takahashi, Kaneyasu, Tanasupawat, Nakagawa, Suzuki, Potacharoen and Yamada 2008

For the characteristics of the species, refer to Yukphan et al. (2008).

The type strain is AC37^T (= BCC 15772^T = NBRC 103193^T), isolated from soil collected at Sakaerat, Nakhon Ratchasima, Thailand. The DNA G+C content of the type strain is 65.6 mol%.

1.4.12 Ameyamaea *Yukphan, Malimas, Muramatsu, Takahashi, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Hamana, Tahara, Suzuki, Tanticharoen and Yamada 2010*

A.me.ya.ma'e.a. N. L. fem. n. *Ameyamaea*, Ameyama, named after Professor Minoru Ameyama, Japan, who contributed to studies of acetic acid bacteria, especially their biochemical and systematic studies.

The strains of the genus *Ameyamaea* that were isolated from flowers of red ginger collected in Chiang Mai, Thailand, were closely related phylogenetically to strains of the genus *Tanticharoenia*. However, the strains showed oxidation of

acetate and weak oxidation of lactate and no growth on 30 % D-glucose (w/v), differing from those of the genus *Tanticharoenia*.

Cells are gram negative rods, measuring 0.6–0.8 by 1.0–1.8 μm, and motile with polar flagella. Colonies are creamy and smooth with entire margin on glucose/ethanol/peptone/yeast extract/calcium carbonate agar.

Acetic acid is produced from ethanol. Acetate is oxidized to carbon dioxide and water, but lactate is weakly oxidized. Grows on glutamate agar weakly and on mannitol agar. Dihydroxyacetone is weakly produced from glycerol. 2-Keto-D-gluconate and 5-keto-D-gluconate are produced from D-glucose. A water-soluble brown pigment is not produced on glucose/peptone/yeast extract/calcium carbonate agar.

Acid is produced from D-arabinose weakly, L-arabinose, D-xylose, L-rhamnose, D-glucose, D-mannose, D-galactose, *meso*-erythritol, glycerol weakly, melibiose, or ethanol. Grows on D-glucose, D-mannose very weakly, D-galactose, D-xylose, L-arabinose, L-rhamnose, D-fructose, L-sorbose, D-mannitol, D-sorbitol, dulcitol, *meso*-erythritol, glycerol, or melibiose very weakly. Growth is weak on methanol. Ammoniac nitrogen is very weakly assimilated in the presence of D-glucose, D-mannitol, or ethanol as a carbon source.

Grows in the presence of 0.35 % acetic acid (v/v), but not on 30 % D-glucose (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content is 66.0–66.1 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.12.1 *Ameyamaea chiangmaiensis* Yukphan, Malimas, Muramatsu, Takahashi, Kaneyasu, Potacharoen, Tanasupawat, Nakagawa, Hamana, Tahara, Suzuki, Tanticharoen and Yamada 2010

For the characteristics of the species, refer to Yukphan et al. (2009).

The type strain is AC04^T (= BCC 15744^T = NBRC 103196^T), isolated from a flower of red ginger (*Alpinia purpurea*) in Chiang Mai, Thailand. The DNA G+C content of the type strain is 66.0 mol%.

1.4.13 Neokomagataea *Yukphan, Malimas, Muramatsu, Potacharoen, Tanasupawat, Nakagawa, Tanasupawat and Yamada 2011*

Ne.o.ko.ma.ga.ta'ea. N. L. fem. n. *Neokomagataea*, new Komagata, named after Professor Kazuo Komagata, a Japanese microbiologist who contributed to bacterial systematics and phylogeny, especially of acetic acid bacteria.

The strains of the genus *Neokomagataea* that were isolated in Thailand from flowers of lantana and candle bush were related phylogenetically to those of the

genus *Gluconobacter*. The strains of the genus grew on 30 % D-glucose (w/v) but not in the presence of 0.35 % acetic acid (v/v), the latter of which differed from those of the genus *Gluconobacter*.

Cells are gram negative rods, measuring 0.6–0.8 by 1.0–1.6 μm, and non motile. Colonies are smooth, entire, and creamy on glucose/ethanol/peptone/yeast extract/calcium carbonate agar.

Acetic acid is weakly produced from ethanol. Acetate and lactate are not oxidized. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is not produced from glycerol. 2-Keto-D-gluconate, 5-keto-D-gluconate, and 2,5-diketo-D-gluconate are produced from D-glucose. A water-soluble brown pigment is not produced. Acid is produced from L-arabinose weakly, D-xylose, D-glucose, D-galactose weakly, D-fructose, or sucrose. Grows on D-glucose, L-rhamnose weakly, or sucrose. Ammoniac nitrogen is not generally assimilated on D-glucose or ethanol as a source of carbon.

Grows between 1.0 and 30 % D-glucose (w/v). Osmotolerant. Growth does not occur in the presence of 0.35 % acetic acid (v/v) or in the presence of 1.0 % or 2.0 % NaCl (w/v), or 1.0 % KNO_3 (w/v). The major cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, and $C_{18:1}2OH$. The major quinone is Q-10. The DNA G+C content is 51.2–56.8 mol%. For more details of characteristics, see Komagata et al. (2014).

The type species of the genus is *Neokomagataea thailandica* Yukphan et al. 2011. Two species are reported.

1.4.13.1 *Neokomagataea thailandica* Yukphan, Malimas, Muramatsu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2011

For the characteristics of the species, refer to Yukphan et al. (2011).

The type strain is AH11^T (= BCC 25710^T = NBRC 106555^T), isolated from a flower of lantana (*Lantana camera*) at Tan Island, Hat Khanom-Mu Ko Thale Thai National Park, Nakhon-Si-Thammarat, Thailand, in 2007. The DNA G+C content of the type strain is 56.8 mol%.

1.4.13.2 *Neokomagataea tanensis* Yukphan, Malimas, Muramatsu, Potacharoen, Tanasupawat, Nakagawa, Tanticharoen and Yamada 2011

For the characteristics of the species, refer to Yukpohan et al. (2011).

The type strain is AH13^T (= BCC 25711^T = NBRC 106556^T), isolated from a flower of candle bush (*Senna alata*) at Tan Island, Hat Khanom-Mu Ko Thale Thai National Park, Nakhon-Si-Thammarat, Thailand, in 2007. The DNA G+C content of the type strain is 51.2 mol%.

1.4.14 Komagataeibacter *Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013*

Ko.ma.ga.ta.e.i.bac'ter. N. L. fem. n. *Komagataea*, Komagata, the name of a Japanese microbiologist; N. L. masc. n. bacter, rod; N. L. masc. n. *Komagataeibacter*, Komagata rod, which is named after Professor Kazuo Komagata, Japan, who contributed to the bacterial systematics, especially of acetic acid bacteria.

The genus *Komagataeibacter* was introduced for the *Gluconacetobacter xylinus* group of the genus *Gluconacetobacter* based on 16S rRNA gene sequence analysis and morphological, physiological, and ecological characterizations. The 11 species of the genus *Gluconacetobacter* were transferred to the genus *Komagataeibacter* as new combinations. Recently, three new combinations were additionally reported. The phenotypic characteristics of the genus *Komagataeibacter* were generally no motility, no production of 2,5-diketo-D-gluconate from D-glucose, and no water-soluble brown pigment production on glucose/peptone/yeast extract/calcium carbonate medium.

Cells are gram negative rods, measuring 0.5–0.8 by 1.0–3.0 μm, occurring singly, in pairs, or in chains. Non motile. Colonies are circular, smooth, or rough, raised to convex or umbonate, entire, glistening, and white-creamy to beige.

Aerobic. Catalase positive and oxidase negative. Acetic acid is produced from ethanol. Acetate and lactate are oxidized to carbon dioxide and water. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is generally produced from glycerol. D-Gluconate, 2-keto-D-gluconate, and/or 5-keto-D-gluconate are produced from D-glucose, but 2,5-diketo-D-gluconate is not produced.

Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, or ethanol. Grows on D-glucose, D-fructose, maltose, sucrose, or D-mannitol. Ammoniac nitrogen is generally assimilated on D-mannitol. Cellulosic materials are produced by some strains, that is, of *Komagataeibacter xylinus* and *Komagataeibacter nataicola*. A water-soluble brown pigment is not produced on glucose/yeast extract/calcium carbonate medium. γ-Pyrone compounds are not produced.

Grows generally in the presence of 0.35 % acetic acid (v/v). Some species require acetic acid for growth. Grows at pH 3.0. The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content ranges from 58 to 64 mol%. For more details of characteristics, see Komagata et al. (2014).

The type species of the genus is *Komagataeibacter xylinus* (Brown 1886) Yamada et al. 2013. Fourteen species are reported.

1.4.14.1 *Komagataeibacter xylinus* (Brown 1886) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter aceti* subsp. *xylinus* corrig. (Brown 1886) De Ley and Frateur 1974.

Synonyms: *Acetobacter xylinus* (Brown 1886) Yamada 1984; *Gluconacetobacter xylinus* (Brown 1886) Yamada, Hoshino and Ishikawa 1998; '*Bacterium xylinum*' Brown 1886.

For the characteristics of the species, refer to Gosselé et al. (1983b), Lisdiyanti et al. (2006), Navarro and Komagata (1999), Yamada (1983), Sievers and Swings (2005c), and Komagata et al. (2014).

The type strain is NCIMB 11664^T (= DSM 6513^T = JCM 7644^T = LMG 1515^T = NBRC 15237^T = BCC 49175^T), isolated from mountain ash berry by Professor G. Bertrand. The DNA G+C content of the type strain is 62.5 mol%.

1.4.14.2 *Komagataeibacter hansenii* (Gosselé et al. 1983) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter hansenii* Gosselé, Swings, Kersters, Pauwels and De Ley 1983.

Synonyms: *Gluconacetobacter hansenii* (Gosselé et al. 1983) Yamada, Hoshino and Ishikawa 1998; *Gluconacetobacter kombuchae* Dutta and Gachhui 2007.

For the characteristics of the species, refer to Gosselé et al. (1983b), Lisdiyanti et al. (2006), Dutta and Chchhui (2007), Cleenwerck et al. (2009), Komagata et al. (2014), and Sievers and Swings (2005c).

The type strain is NCIMB 8746^T (= DSM 5602^T = JCM 7643^T = LMG 1527^T = NBRC 14820^T = BCC 6318^T), isolated from a local vinegar in Jerusalem, Israel. The DNA G+C content of the type strain is 59.0 mol%.

1.4.14.3 *Komagataeibacter europaeus* (Sievers et al. 1992) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter europaeus* Sievers, Sellmer and Teuber 1992.

Synonym: *Gluconacetobacter europaeus* (Sievers et al. 1992) Yamada, Hoshino and Ishikawa 1998.

For the characteristics of the species, refer to Sievers et al. (1992).

The type strain is DES11^T (= DSM 6160^T = JCM 16935^T = BCC 36446^T), isolated from a submerged culture vinegar generator at a factory in Esslingen in the southern part of Germany. The DNA G+C content of the type strain is not described. The range of DNA G+C content is 56.2–57.3 mol%.

1.4.14.4 *Komagataeibacter oboediens* (Sokollek et al. 1998) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter oboediens* Sokollek, Hertel and Hammes 1998.

Synonym: *Gluconacetobacter oboediens* (Sokollek et al. 1998) Yamada 2000.

For the characteristics of the species, refer to Sokollek et al. (1998) and Yamada (2000).

The type strain is LTH 2460^T (= DSM 11826^T = JCM 16937^T = LMG 18849^T = BCC 36445^T), isolated from a submerged red wine vinegar fermentation at a factory in the southern part of Germany. The DNA G+C content of the type strain is 59.9 mol%.

1.4.14.5 *Komagataeibacter intermedius* (Boesch et al. 1998) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter intermedius* Boesch, Trček, Sievers and Teuber 1998.

Synonym: *Gluconacetobacter intermedius* (Boesch et al. 1998) Yamada 2000.

For the characteristics of the species, refer to Boesch et al. (1998) and Yamada (2000).

The type strain is TF2^T (= DSM 11804^T = JCM 16936^T = BCC 36447^T = LMG 18909^T), isolated from a commercially available tea fungus beverage (Kombucha) in Switzerland. The DNA G+C content of the type strain is 61.55 mol%.

1.4.14.6 *Gluconacetobacter entanii* Schüller, Hertel and Hammes 2000

For the characteristics of the species, refer to Schüller et al. (2000).

The type strain is LTH 4560^T (= BCRC 17196^T = DSM 13536^T = LMG 20950^T = LMG 21788^T), isolated from submerged high-acid industrial vinegar fermentations. The DNA G+C content of the type strain is 58 mol%. The type strain is not available in any culture collections (Yamada et al. 2012b). This species is not listed as a new combination, according to Rule 27 of the Bacteriological Code (Tindall et al. 2006).

1.4.14.7 *Komagataeibacter swingsii* (Dellaglio et al. 2005) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Gluconacetobacter swingsii* Dellaglio, Cleenwerck, Felis, Engelbeen, Janssens and Marzotto 2005.

For the characteristics of the species, refer to Dellaglio et al. (2005).

The type strain is DST GL01^T (= DSM 16373^T = JCM 17123^T = LMG 22125^T = BCC 36451^T), isolated from apple juice in South Tyrol region, Italy. The DNA G+C content of the type strain is 61.7 mol%.

1.4.14.8 *Komagataeibacter rhaeticus* (Dellaglio et al. 2005) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Gluconacetobacter rhaeticus* Dellaglio, Cleenwerck, Felis, Engelbeen, Janssens and Marzotto 2005.

For the characteristics of the species, refer to Dellaglio et al. (2005).

The type strain is DST GL02^T (= DSM 16663^T = JCM 17122^T = LMG 22126^T = BCC 36452^T), isolated from apple juice in South Tyrol region, Italy. The DNA G+C content of the type strain is 63.4 mol%.

1.4.14.9 *Komagataeibacter saccharivorans* (Lisdiyanti et al. 2006) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Gluconacetobacter saccharivorans* Lisdiyanti, Navarro, Uchimura and Komagata 2006.

For the characteristics of the species, refer to Lisdiyanti et al. (2006).

The type strain is LMG 1582^T (= BCC 36444^T = JCM 25121^T = NRIC 0614^T = BCC 36444^T), isolated from beet juice in Germany in 1927. The DNA G+C content of the type strain is 61 mol%.

1.4.14.10 *Komagataeibacter nataicola* (Lisdiyanti et al. 2006) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Gluconacetobacter nataicola* Lisdiyanti, Navarro, Uchimura and Komagata 2006.

For the characteristics of the species, refer to Lisdiyanti et al. (2006).

The type strain is LMG 1536^T (= JCM 25120^T = NRIC0616^T = BCC 36443^T), isolated from nata de coco in the Philippines. The DNA G+C content of the type strain is 62 mol%.

1.4.14.11 *Komagataeibacter sucrofermentans* (Toyosaki et al. 1996) Yamada, Yukphan, Vu, Muramatsu, Ochaikul, Tanasupawat and Nakagawa 2013

Basonym: *Acetobacter xylinus* subsp. *sucrofermentans* Toyosaki, Kojima, Tsuchida, Hoshino, Yamada and Yoshinaga 1996.

Synonym: *Gluconacetobacter sucrofermentans* (Toyosaki et al. 1996) Cleenwerck, De Vos and De Vuyst 2010.

For the characteristics of the species, refer to Toyosaki et al. (1995) and Cleenwerck et al. (2010).

The type strain is LMG 18788^T (= DSM 15973^T = JCM 9730^T = BCC 7227^T), isolated from a cherry. The DNA G+C content of the type strain is 62.7 mol%.

1.4.14.12 *Komagataeibacter kakiaceti* (Iino et al. 2012) Yamada 2014

Basonym: *Gluconacetobacter kakiaceti* Iino, Suzuki, Tanaka, Kosako, Ohkuma, Komagata and Uchimura 2012.

For the characteristics of the species, refer to Iino et al. (2012b) and Yamada (2014).

The type strain is G5-1^T (= JCM 25156^T = NRIC 0798^T = LMG 26206^T), isolated from kaki vinegar collected in Kumamoto Prefecture, Japan in 2005. The DNA G+C content of the type strain is 63.6 mol%.

1.4.14.13 *Komagataeibacter medellinensis* (Castro et al. 2013) Yamada 2014

Basonym: *Gluconacetobacter medellinensis* Castro, Cleenwerck, Trček, Zuluaga, De Vos, Caro, Aguirre, Putaux and Gañán 2013.

For the characteristics of the species, refer to Castro et al. (2013), Yamada et al. (1969b), and Yamada (2014).

The type strain is LMG 1693^T (= NBRC 3288^T = Kondo 51^T), isolated from vinegar by K. Kondo, Japan. The DNA G+C content of the type strain is 60.7 mol%.

1.4.14.14 *Komagataeibacter maltaceti* (Slapšak et al. 2013) Yamada 2014

Basonym: *Gluconacetobacter maltaceti* Slapšak, Cleenwerck, De Vos and Trček 2013.

For the characteristics of the species, refer to Slapšak et al. (2013) and Yamada (2014).

The type strain is LMG 1529^T (= NBRC 14815^T = NCIMB 8752^T), isolated from malt vinegar brewery acetifier by T.K. Walker in 1956. The DNA G+C content of the type strain is 62.5 mol%.

1.4.15 Endobacter *Ramírez-Bahena, Teijedor, Martín, Velázquez and Peix 2013*

En.do.bac'ter. Gr. pref. *endo*, within; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Endobacter*, a rod isolated from the inside of a root nodule of alfalfa.

The strain of the genus *Endobacter* was isolated from a surface-sterilized nodule of alfalfa in Spain; it is quite remote phylogenetically from other acetic acid bacteria and constituted an independent cluster in a phylogenetic tree based on 16S rRNA gene sequences.

Cells are gram negative, coccoid to rod shaped. Motile with subpolar flagella. Colonies are white and mucoid on modified yeast extract mannitol agar.

Aerobic. Catalase positive and oxidase negative. Acetate and lactate are not oxidized. Acetic acid is produced from ethanol. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is produced from glycerol. Acid is produced from D-xylose, D-glucose, glycerol, or ethanol. Grows between 20 ° and 37 °C with an optimum temperature for growth of 28 °C. Ammoniac nitrogen is assimilated on D-glucose. Optimal pH for growth ranges from 5.0 to 7.0, but growth occurs at pH 3.5.

The major cellular fatty acids are $C_{18:1}\omega7c$ (39.94 %), $C_{19:0}$cyclo$\omega8c$ (12.15 %), and $C_{16:0}$ (13.40 %). The major quinone is Q-10. The DNA G+C content is 60.3 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.15.1 *Endobacter medicaginis* Ramírez-Bahena, Teijedor, Martín, Velázquez and Peix 2013

For the characteristics of the species, refer to Ramírez-Bahena (2013).

The type strain is M1MS02^T (= CECT 8088^T = LMG 26838^T), isolated from a surface-sterilized nodule of alfalfa (*Medicago sativa*), Spain. The DNA G+C content of the type strain is 60.3 mol%.

1.4.16 Nguyenibacter *Vu, Yukphan, Chaipitakchonlatarn, Malimas, Muramatsu, Bui, Tanasupawat, Duong, Nakagawa, Pham and Yamada 2013*

Ngu.ye.ni.bac'ter. N. L. masc. n. *Nguyenius*, Nguyen, the name of a famous Vietnamese microbiologist; N. L. masc. n. bacter, rod; N. L. masc. n. *Nguyenibacter*, a rod, which is named after Professor Dung Lan Nguyen, Vietnam, who contributed to the study of microorganisms, especially of strains isolated in Vietnam.

The two strains of the genus *Nguyenibacter* were isolated by the use of nitrogen-free LGI medium. The strains were related phylogenetically to those of the genera *Gluconacetobacter* and *Acidomonas*.

Cells are gram negative rods, measuring 0.6–0.8 by 1.0–1.6 μm. Motile with peritrichous flagella. Colonies are smooth, entire, transparent, and creamy to brownish.

Aerobic. Catalase positive and oxidase negative. Acetic acid is not produced from ethanol. Acetate is oxidized to carbon dioxide and water, but lactate is not oxidized. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is not produced from glycerol. 2-Keto-D-gluconate and 2,5-diketo-D-gluconate are produced from D-glucose. A water-soluble brown pigment is produced. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-fructose weakly, maltose, melibiose, sucrose, or raffinose weakly. Grows on D-glucose, D-galactose, L-arabinose weakly, D-xylose weakly, D-fructose weakly, L-sorbose weakly, maltose, melibiose weakly, sucrose, raffinose, D-mannitol weakly, D-sorbitol weakly, or glycerol. Ammoniac nitrogen is utilized on D-mannitol, but not on D-glucose or ethanol. Growth occurs on N_2-free medium. γ-Pyrone compound is weakly produced. Levan-like polysaccharides are produced from sucrose.

Grows weakly on 30 % D-glucose (w/v), and weakly in the presence of 0.35 % acetic acid (v/v). Growth does not occur on 1.0 % KNO_3 (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content range is 68.1–69.4 mol%. For more details of characteristics, see Komagata et al. (2014).

1.4.16.1 *Nguyenibacter vanlangensis* Vu, Yukphan, Chaipitakchonlatarn, Malimas, Muramatsu, Bui, Tanasupawat, Duong, Nakagawa, Pham and Yamada 2013

For the characteristics of the species, refer to Vu et al. (2013).

The type strain is TN01LGI^T (= BCC 54774^T = NBRC 109046^T = VTCC-B-1198^T), isolated from the rhizosphere of Asian rice collected in Vietnam. The DNA G+C content of the type strain is 69.4 mol%.

1.4.17 Swingsia *Malimas, Chaipitakchonlatarn, Vu, Yukphan, Muramatsu, Tanasupawat, Potacharoen, Nakagawa, Tanticharoen and Yamada 2014*

Swing'si.a. N. L. fem. n. *Swingsia*, Swings, named after Professor Jean Swings, Belgium, who contributed to the systematics of bacteria, especially of acetic acid bacteria.

The two strains of the genus *Swingsia* were isolated from flowers in Thailand and located at an intermediary position phylogenetically between the genera

Gluconobacter and *Neokomagataea*. The strains grew on 30 % D-glucose (w/v) and at 37 °C, but not in the presence of 0.35 % acetic acid (v/v), and acetic acid was sometimes produced weakly from ethanol.

Cells are gram negative rods, measuring 0.6–0.8 by 1.0–1.8 μm. Non motile. Colonies are brownish and smooth with entire margin.

Aerobic. Catalase positive and oxidase negative. Acetic acid is produced weakly from ethanol. Acetate and lactate are not oxidized. Grows on glutamate agar and mannitol agar. Dihydroxyacetone is produced from glycerol. 2-Keto-D-gluconate, 5-keto-D-gluconate, and 2,5-diketo-D-gluconate are produced from D-glucose. A water-soluble pigment is produced. Acid is produced from L-arabinose weakly, D-arabinose weakly, D-xylose weakly, L-rhamnose weakly, D-glucose, D-mannose weakly, D-galactose, D-fructose weakly, D-arabitol weakly, D-mannitol, maltose weakly, lactose weakly, melibiose, sucrose, or raffinose weakly. Grows on D-glucose, D-fructose, sucrose, L-arabitol weakly, D-arabitol weakly, or D-mannitol. Ammoniac nitrogen is utilized on D-mannitol, but not on D-glucose or ethanol. Ammonic nitrogen is assimilated on D-mannitol but not on D-glucose or ethanol. Levan-like polysaccharides are not produced.

Grows on 30 % D-glucose (w/v), but not in the presence of 0.35 % acetic acid (v/v). Growth occurs in the presence of 1.0 % KNO_3 (w/v). The major cellular fatty acid is $C_{18:1}\omega7c$. The major quinone is Q-10. The DNA G+C content range is 46.9–47.3 mol%.

1.4.17.1 *Swingsia samuiensis* Malimas, Chaipitakchonlatarn, Vu, Yukphan, Muramatsu, Tanasupawat, Potacharoen, Nakagawa, Tanticharoen and Yamada 2014

For the characteristics of the species, refer to Malimas et al. (2013).

The type strain is AH83^T (= BCC 25779^T = NBRC 107927^T), isolated from a flower of golden trumpet. The DNA G+C content of the type strain is 46.9 mol%.

1.5 Genus and Species in Pseudacetic Acid Bacteria

Several strains were once isolated and named '*Acetobacter aurantium*' by Kondo and Ameyama (1958). According to the description of the species, the strains were not able to oxidize acetate.

Asai et al. (1964) reinvestigated the strains for phenotypic characteristics and found that they had polar flagellation and oxidized acetate and lactate to carbon dioxide and water, and the strains were named the polarly flagellated intermediate strains. Additional three strains were then newly isolated and confirmed to have polar flagellation and the capability of oxidizing acetate and lactate (Ameyama and Kondo 1967).

In the isoprenoid quinone analysis of the polarly flagellated intermediate strains, Q-8 was detected as the major quinone, indicating that the quinone system obtained was quite different chemotaxonomically from either Q-9 of *Acetobacter* strains or Q-10 of *Gluconobacter* strains (Yamada et al. 1969a, 1976). In the cellular fatty acid composition of the polarly flagellated intermediate strains, *iso*-$C_{15:0}$ acid was found as the major, indicating that the strains were quite different similarly from $C_{18:1}\omega7c$ acid of *Acetobacter* and *Gluconobacter* strains (Yamada et al. 1981a).

For such unique bacterial strains equipped with Q-8 and *iso*-$C_{15:0}$ acid, the name of pseudacetic acid bacteria was given (Yamada 1979; Yamada et al. 1981a, b; Lisdiyanti et al. 2003a). The genus *Frateuria* was later introduced for these strains by Swings et al. (1980). The genus is accommodated in the class *Gammaproteobacteria* Stackebrandt et al. 1988.

1.5.1 Frateuria *Swings, Gillis, Kersters, De Vos, Gosselé and De Ley 1980 emend. Zhang, Liu and Liu 2011*

Fra.teu'ri.a. N. L. fem. n. *Frateuria*, Frateur, named after Professor Joseph Frateur, Belgium, especially in recognition of the study of acetic acid bacteria.

The genus *Frateuria* was long thought to be monotypic. However, a second species was recently reported.

Cells are gram negative, rod shaped, measuring 0.4–0.8 by 0.8–2.0 μm, singly or in pairs, motile with a single polar or subpolar flagellum when motile. Shows luxuriant growth on glucose/yeast extract/calcium carbonate agar. Colonies are flat or circular, and medium turning to brown.

Aerobic. Catalase positive or negative. Oxidase negative or positive. Oxidizes lactate but not acetate (Swings et al. 1980; Lisdiyanti et al. 2003a). Grows on glutamate agar and mannitol agar. Dihydroxyacetone is generally produced from glycerol. Produces D-gluconate, 2-keto-D-gluconate, and 2,5-diketo-D-gluconate from D-glucose but not 5-keto-D-gluconate. Produces a water-soluble brown pigment. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, L-rhamnose, D-galactose, D-glucose, D-mannose, D-fructose, glycerol, or ethanol. Does not grow on methanol. Assimilates ammoniac nitrogen on D-mannitol.

No growth is observed in the presence of 0.35 % acetic acid (v/v). Grows on 20 % D-glucose (w/v) at 34 °C and at pH 3.5. The major cellular fatty acid is *iso*-$C_{15:0}$. The major quinone is Q-8. The DNA G+C content is 62–68 mol%. *Frateuria* strains are isolated from flowers of lily, rose, ladybell, and coconut and fruits of raspberry, mango, rambai, and jackfruit. For more details of characteristics, see Komagata et al. (2014) and Swings and Sievers (2005).

The type species is *Frateuria aurantia* (ex Kondo and Ameyama 1958) Swings et al. 1980. Two species are reported.

1.5.1.1 *Frateuria aurantia* (ex Kondo and Ameyama 1958) Swings, Gillis, Kersters, De Vos, Gosselé and De Ley 1980

Synonym: '*Acetobacter aurantius*' corrig. Kondo and Ameyama 1958.

For the characteristics of the species, refer to Swings et al. (1980), Yamada et al. (1969a, 1976, 1981a, b), and Lisdiyanti et al. (2003a, b).

The type strain is G-6^T (= Kondo 67^T = NBRC 3245^T = ATCC 33424^T = DSM 6220^T = LMG 1558^T), isolated from a flower of lily. The DNA G+C content of the type strain is 65.0 mol%.

1.5.1.2 *Frateuria terrea* Zhang, Liu and Liu 2011

For the characteristics of the species, refer to Zhang et al. (2011).

The type strain is VA24^T (= CGMCC 1.7053^T = NBRC 104236^T), isolated from forest soil of the Changbai Mountains, Heilongjiang Province, China. The DNA G+C content of the type stran is 67.4 mol%.

1.6 Closing Remarks

More than 100 years have already passed since the genus *Acetobacter* Beijerinck 1898 was introduced with the only known species, *Acetobacter aceti* (Pasteur 1864) Beijerinck 1898, for vinegar-producing acetic acid bacteria. Up to 1960, acetic acid bacteria were believed to constitute a quite small taxonomic group, that is, only the one genus. However, their circumstances have entirely changed.

Acetic acid bacteria, including the vinegar-producing bacteria and their relatives, have been found in large numbers by expanding their possible living environments, viz., activated sludge, rhizosphere soils, soils, pollen, human patients, mosquitoes, stone chambers of tumuluses, and nodules of plants, in addition to sugary and alcoholic materials.

Up to the present, the acetic acid bacteria have numbered 17 genera and 84 species. These numbers will be greatly increased in future, pushing back the frontiers of their living environments and their isolation sources, and many new taxa, that is, new genera and new species, will be reported.

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References

Ameyama M (1975) *Gluconobacter oxydans* subsp. *sphaericus*, new subspecies isolated from grapes. Int J Syst Bacteriol 25:365–370

Ameyama M, Kondo K (1967) Carbohydrate metabolism by acetic acid bacteria: Part VI. Characteristics of the intermediate type strains. Agric Biol Chem 31:724–737

Asai T (1935) Taxonomic study of acetic acid bacteria and allied oxidative bacteria in fruits and a new classification of oxidative bacteria. Nippon Nogeikagaku Kaishi 11:674–708 (in Japanese)

Asai T (1968) Acetic acid bacteria: classification and biochemical activities. University of Tokyo Press, Tokyo

Asai T, Shoda K (1958) The taxonomy of *Acetobacter* and allied oxidative bacteria. J Gen Appl Microbiol 4:289–311

Asai T, Iizuka H, Komagata K (1964) The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. J Gen Appl Microbiol 10: 95–126

Beijerinck MW (1898) Über die Arten der Essigbakterien. Zbl Bakteriol Parasitenkd Infektionskr Hyg II 4:209–216

Beijerinck MW, Folpmers T (1916) Formation of pyruvic acid from malic acid by microbes. Verslag van de Gewone Vergaderingen der Wis- en Natuurkundige Afdeeling der Koninklijke Akademie van Wetenschappen te Amsterdam 18: 1198–1200

Boesch C, Trček J, Sievers M, Teuber M (1998) *Acetobacter intermedius* sp. nov. Syst Appl Microbiol 21:220–229

Buchanan RE, Holt JG, Lessel EF Jr (1966) Comment. In: Buchanan RE, Holt JG, Lessel EF Jr (eds) Index Bergeyana. Williams & Wilkins, Baltimore, p 4

Bulygina ES, Gulikova OM, Dikanskaya EM, Netrusov AI, Tourova TP, Chumakov KM (1992) Taxonomic studies of the genera *Acidomonas*, *Acetobacter* and *Gluconobacter* by 5S ribosomal RNA sequencing. J Gen Microbiol 138:2283–2286

Carr JG, Shimwell JL (1960) Pigment-producing strains of *Acetobacter aceti*. Nature (Lond) 186:331–332

Castro C, Cleenwerck I, Trček J, Zuluaga R, De Vos P, Caro G, Aguirre R, Putaux J-L, Gañán P (2013) *Gluconacetobacter medellinensis* sp. nov., cellulose- and non-cellulose-producing acetic acid bacteria isolated from vinegar. Int J Syst Evol Microbiol 63:1119–1125

Cavalcante VA, Döbereiner J (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugar cane. Plant Soil 108:23–31

Cleenwerck I, De Vos P (2008) Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int J Food Microbiol 125:2–14

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

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

Cleenwerck I, González Á, Camu N, Engelbeen K, De Vos P, De Vuyst L (2008) *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. Int J Syst Evol Microbiol 58:2180–2185

Cleenwerck I, De Wachter M, González Á, De Vuyst L, De Vos P (2009) Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*. Int J Syst Evol Microbiol 59: 1771–1786

Cleenwerck I, De Vos P, De Vuyst L (2010) Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter xylinus* subsp. *sucrofermentans* as *Gluconacetobacter sucrofermentans* (Toyosaki et al. 1996) sp. nov., comb. nov. Int J Syst Evol Microbiol 60:2277–2283

De Ley J (1961) Comparative carbohydrate metabolism and a proposal for a phylogenetic relationship of the acetic acid bacteria. J Gen Microbiol 24:31–50

De Ley J, Frateur J (1970) The status of the generic name *Gluconobacter*. Int J Syst Bacteriol 20: 83–95

Dellaglio F, Cleenwerck I, Felis GE, 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

Dutta D, Gachhui R (2006) Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 56:1899–1903

Dutta D, Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 57:353–357

Franke IH, Fegan M, Hayward C, Leonard G, Stackebrandt E, Sly LI (1999) Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. Int J Syst Bacteriol 49: 1681–1693

Fuentes-Ramírez LE, Bustillos-Cristales R, Tapía-Hernández A, Jiménez-Salgado T, Wang ET, Martínez-Romero E, Caballero-Mellado J (2001) Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int J Syst Evol Microbiol 51:1305–1314

Gillis M, De Ley J (1980) Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of *Acetobacter* and *Gluconobacter*. Int J Syst Bacteriol 30:7–27

Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt RM, Stephan MP, Teixeira KRS, Döbereiner J, De Ley J (1989) *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int J Syst Bacteriol 39:361–364

Gosselé F, Swings J, Kersters K, De Ley J (1983a) Numerical analysis of phenotypic features and protein gel electropherograms of *Gluconobacter* Asai 1935 emend. mut. char. Asai, Iizuka, and Komagata 1964. Int J Syst Bacteriol 33:65–81

Gosselé F, Swings J, Kersters K, Pauwels P, De Ley J (1983b) Numerical analysis of phenotypic features and protein gel electrophoregrams of a wide variety of *Acetobacter* strains. Proposal for the improvement of the taxonomy of the genus *Acetobacter* Beijerinck 1898, 215. Syst Appl Microbiol 4:338–368

Greenberg DE, Porcella SF, Stock F, Wong A, Conville PS, Murray PR, Holland SM, Zelazny AM (2006) *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. Int J Syst Evol Microbiol 56:2609–2616

Iino T, Suzuki R, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2012a) *Acetobacter okinawensis* sp. nov., *Acetobacter papayae* sp. nov., and *Acetobacter persicus* sp. nov.: novel acetic acid bacteria isolated from stems of sugarcane, fruits and a flower in Japan. J Gen Appl Microbiol 58:235–243

Iino T, Suzuki R, Tanaka N, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2012b) *Gluconacetobacter kakiaceti* sp. nov., an acetic acid bacterium isolated from a traditional Japanese fruit vinegar. Int J Syst Evol Microbiol 62:1465–1469

Jacob F, Phaff A, Novoa-Carballal R, Rübsam H, Becker T, Vogel RF (2013) Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloidal function. Carbohydr Polym 92:1234–1242

Jojima Y, Mihara Y, Suzuki S, Yokozeki K, Yamanaka S, Fudou R (2004) *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. Int J Syst Evol Microbiol 54:2263–2267

Katsura K, Kawasaki H, Potacharoen W, Saono S, Seki T, Yamada Y, Uchimura T, Komagata K (2001) *Asaia siamensis* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. Int J Syst Evol Microbiol 51:559–563

Katsura K, Yamada Y, Uchimura T, Komagata K (2002) *Gluconobacter asaii* Mason and Claus 1989 is a junior subjective synonym of *Gluconobacter cerinus* Yamada and Akita 1984. Int J Syst Evol Microbiol 52:1635–1640

Kersters K, Lisdiyanti P, Komagata K, Swings J (2006) The family *Acetobacteraceae*: the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes, vol 5, 3rd edn. Springer, New York, pp 163–200

Kimmitt MR, Williams PJLeB (1963) Systematic position of *Gluconobacter liquefaciens*. J Gen Microbiol 31:447–449

Kluyver AJ (1983) Beijerinck: the microbiologist. In: van Iterson G, den Dooren de Jong LE, Kluyver A (eds) Martinus Willem Beijerinck: his life and his work. Science Tech, Madison, pp 132–134

Komagata K, Iino T, Yamada Y (2014) The family *Acetobacteraceae*. In: Rosenberg E, De Long EF, Lory S, Stackebrandt E, Thompson F (eds) The prokaryotes. *Alphaproteobacteria* and *Betaproteobacteria*. Springer, Berlin/Heidelberg, pp 3–78

Kommanee J, Tanasupawat S, Yukphan P, Malimas T, Muramatsu Y, Nakagawa Y, Yamada Y (2010) *Asaia spathodeae* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 56:81–87

Kommanee J, Tanasupawat S, Yukphan P, Malimas T, Muramatsu Y, Nakagawa Y, Yamada Y (2011) *Gluconobacter nephelii* sp. nov., an acetic acid bacterium in the class *Alphaproteobacteria*. Int J Syst Evol Microbiol 61:2117–2122

Kondo K, Ameyama M (1958) Carbohydrate metabolism by *Acetobacter* species: Part 1. Oxidative activity for various carbohydrates. Bull Agric Chem Soc Jpn 22:369–372

Leifson E (1954) The flagellation and taxonomy of species of *Acetobacter*. Antonie Van Leeuwenhoek 20:102–110

Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC, Manaia CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P (2014) *Acetobacter sicerae* sp. nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by *dnaK*, *groEL* and *rpoB* sequence analysis. Int J Syst Evol Microbiol 64:2407–2415

Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2000) Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov. and *Acetobacter estunensis* (Carr 1958) comb. nov. J Gen Appl Microbiol 46:147–165

Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2001) Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov. and *Acetobacter orientalis* sp. nov. J Gen Appl Microbiol 47:119–131

Lisdiyanti P, Kawasaki H, Widyastuti Y, Saono S, Seki T, Yamada Y, Uchimura T, Komagata K (2002) *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the *α-Proteobacteria*. Int J Syst Evol Microbiol 52:813–818

Lisdiyanti P, Yamada Y, Uchimura T, Komagata K (2003a) Identification of *Frateuria aurantia* strains isolated from Indonesian sources. Microbiol Cult Coll 19:81–90

Lisdiyanti P, Katsura K, Potacharoen W, Navarro RR, Yamada Y, Uchimura T, Komagata K (2003b) Diversity of acetic acid bacteria in Indonesia, Thailand and the Philippines. Microbiol Cult Coll 19:91–99

Lisdiyanti P, Navarro RR, Uchimura T, Komagata K (2006) Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov. Int J Syst Evol Microbiol 56:2101–2111

Loganathan P, Nair S (2004) *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). Int J Syst Evol Microbiol 54:1185–1190

Malimas T, Yukphan P, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2007) *Gluconobacter kondonii* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 53:301–307

Malimas T, Yukphan P, Takahashi M, Muramatsu Y, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2008a) *Gluconobacter roseus* (ex Asai 1935) sp. nov., nom. rev., a pink-colored acetic acid bacterium in the *Alphaproteobacteria*. J Gen Appl Microbiol 54:119–125

Malimas T, Yukphan P, Takahashi M, Muramatsu Y, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2008b) *Gluconobacter sphaericus* (Ameyama 1975) comb. nov., a brown pigment-producing acetic acid bacterium in the *Alphaproteobacteria*. J Gen Appl Microbiol 54:211–220

Malimas T, Yukphan P, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2008c) *Asaia lannaensis* sp. nov., a new acetic acid bacterium in the *Alphaproteobacteria*. Biosci Biotechnol Biochem 72:666–671

Malimas T, Yukphan P, Lundaa T, Muramatsu Y, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Suzuki K, Tanticharoen M, Yamada Y (2009a) *Gluconobacter kanchanaburiensis* sp. nov., a brown pigment-producing acetic acid bacterium for Thai isolates in the *Alphaproteobacteria*. J Gen Appl Microbiol 55:247–254

Malimas T, Yukphan P, Takahashi M, Muramatsu Y, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2009b) *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *Alphaproteobacteria*. Int J Syst Evol Microbiol 59: 466–471

Malimas T, Chaipitakchonlatarn W, Vu HTL, Yukphan P, Muramatsu Y, Tanasupawat S, Potacharoen W, Nakagawa Y, Tanticharoen M, Yamada Y (2013) *Swingsia samuiensis* gen. nov., sp. nov., an osmotolerant acetic acid bacterium in the α-*Proteobacteria*. J Gen Appl Microbiol 59:375–384

Mason LM, Claus GW (1989) Phenotypic characteristics correlated with deoxyribonucleic acid sequence similarities for three species of *Gluconobacter*: *G. oxydans* (Henneberg 1897) De Ley 1961, *G. frateurii* sp. nov. and *G. asaii* sp. nov. Int J Syst Bacteriol 39:174–184

Navarro RR, Komagata K (1999) Differentiation of *Gluconacetobacter liquefaciens* and *Gluconacetobacter xylinus* on the basis of DNA base composition, DNA relatedness and oxidation products from glucose. J Gen Appl Microbiol 45:7–15

Ndoye B, Cleenwerck I, Engelbeen K, Dubois-Dauphin R, Guiro AT, van Trappen S, Willems A, Thonart P (2007) *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

Nishijima M, Tazato N, Handa Y, Tomita J, Kigawa R, Sano C, Sugiyama J (2013) *Gluconacetobacter tumulisoli* sp. nov., *Gluconacetobacter takamatsuzukensis* sp. nov. and *Gluconacetobacter aggeris* sp. nov., isolated from Takamatsuzuka Tumulus samples before and during the dismantling work in 2007. Int J Syst Evol Microbiol 63:3981–3988

Ramírez-Bahena MH, Tejedor C, Martín I, Velázquez E, Peix A (2013) *Endobacter medicaginis* gen. nov., sp. nov., isolated from alfalfa nodules in acidic soil. Int J Syst Evol Microbiol 63:1760–1765

Samaddar N, Paul A, Chakravorty S, Chakraborty W, Mukherjee J, Chowdhuri D, Gachhui R (2011) Nitrogen fixation in *Asaia* sp. (family *Acetobacteraceae*). Curr Microbiol 63:226–231

Schüller G, Hertel C, Hammes WP (2000) *Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations. Int J Syst Evol Microbiol 50:2013–2020

Shimwell JL (1958) Flagellation and taxonomy of *Acetobacter* and *Acetomonas*. Antonie Van Leeuwenhoek 24:187–192

Shimwell JL, Carr JG (1959) The genus *Acetomonas*. Antonie Van Leeuwenhoek 25:353–368

Sievers M, Swings J (2005a) Family II. *Acetobacteraceae* Gillis and De Ley 1980. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The *Proteobacteria* Part C: The *Alpha-, Beta-, Delta- and Epsilonproteobacteria*. Springer, New York, pp 41–95

Sievers M, Swings J (2005b) Genus I. *Acetobacter* Beijerinck 1898. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The *Proteobacteria* Part C: The *Alpha-*, *Beta-*, *Delta-* and *Epsilonproteobacteria*. Springer, New York, pp 51–54

Sievers M, Swings J (2005c) Genus VIII. *Gluconacetobacter* Yamada, Hoshino and Ishikawa 1998. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The *Proteobacteria* Part C: The *Alpha-*, *Beta-*, *Delta-* and *Epsilonproteobacteria*. Springer, New York, pp 72–77

Sievers M, Swings J (2005d) Genus IX. *Gluconobacter* Asai 1935. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The *Proteobacteria* Part C: The *Alpha-*, *Beta-*, *Delta-* and *Epsilonproteobacteria*. Springer, New York, pp 77–81

Sievers M, Sellmer S, Teuber M (1992) *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst Appl Microbiol 15:386–392

Sievers M, Ludwig W, Teuber M (1994) Revival of the species *Acetobacter methanolicus* (ex Uhlig et al. 1986) nom. rev. Syst Appl Microbiol 17:352–354

Silva LR, Cleenwerck I, Rivas P, Swings J, Trujillo ME, Willems A, Velázquez E (2006) *Acetobacter oenis* sp. nov., isolated from spoiled red wine. Int J Syst Evol Microbiol 56:21–24

Skerman VBD, McGowan V, Sneath PHA (eds) (1980) Approved lists of bacterial names. Int J Syst Bacteriol 30:225–420

Slapšak N, Cleenwerck I, De Vos P, Trček J (2013) *Gluconacetobacter maltaceti* sp. nov., a novel vinegar producing acetic acid bacterium. Syst Appl Microbiol 36:17–21

Sokollek SJ, Hertel C, Hammes WP (1998) Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. Int J Syst Bacteriol 48:935–940

Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014a) *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. Int J Syst Evol Microbiol 64:1083–1089

Spitaels F, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014b) *Gluconobacter cerevisiae* sp. nov., isolated from brewery environment. Int J Syst Evol Microbiol 64:1134–1141

Stackebrandt E, Murray RGE, Trüper HG (1988) *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the 'purple bacteria and their relatives'. Int J Syst Bacteriol 38:321–325

Suzuki R, Zhang Y, Iino T, Kosako Y, Komagata K, Uchimura T (2010) *Asaia astilbes* sp. nov., *Asaia platycodi* sp. nov. and *Asaia prunellae* sp. nov., novel acetic acid bacteria isolated from flowers in Japan. J Gen Appl Microbiol 56:339–346

Swings J (1992) The genera *Acetobacter* and *Gluconobacter*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes, vol 3, 2nd edn. Springer-Verlag, New York, pp 2268–2286

Swings J, Sievers M (2005) Genus II. *Frateuria* Swings, Gillis, Kersters, De Vos, Gosselé and De Ley 1980. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The *Proteobacteria* Part B: The *Gammaproteobacteria*. Springer, New York, pp 91–93

Swings J, Gillis M, Kersters K, De Vos P, Gosselé F, De Ley J (1980) *Frateuria*, a new genus for "*Acetobacter aurantius*". Int J Syst Bacteriol 30:547–556

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tanaka M, Murakami S, Shinke R, Aoki K (1999) Reclassification of the strains with low G+C contents of DNA belonging to the genus *Gluconobacter* Asai 1935 (*Acetobacteraceae*). Biosci Biotechnol Biochem 63:989–992

Tanasupawat S, Thawai C, Yukphan P, Moonmangmee D, Itoh T, Adachi O, Yamada Y (2004) *Gluconobacter thailandicus* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 50:159–167

Tanasupawat S, Kommanee J, Yukphan P, Muramatsu Y, Nakagawa Y, Yamada Y (2011a) *Acetobacter farinalis* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 57:159–167

Tanasupawat S, Kommanee J, Yukphan P, Moonmangmee D, Muramatsu Y, Nakagawa Y, Yamada Y (2011b) *Gluconobacter uchimurae* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 57:293–301

Tazato N, Nishijima M, Handa Y, Kigawa R, Sano C, Sugiyama J (2012) *Gluconacetobacter tumulicola* sp. nov. and *Gluconacetobacter asukensis* sp. nov., isolated from the stone chamber interior of the Kitora Tumulus. Int J Syst Evol Microbiol 62:2032–2038

Tindall BJ, Kämpfer P, Euzéby JP, Oren A (2006) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 56:2715–2720

Toyosaki H, Kojima Y, Tsuchida T, Hoshino K, Yamada Y (1995) The characterization of an acetic acid bacterium useful for producing bacterial cellulose in agitation cultures: the proposal of *Acetobacter xylinum* subsp. *sucrofermentans* subsp. nov. J Gen Appl Microbiol 41:307–314

Uhlig H, Karbaum K, Steudel A (1986) *Acetobacter methanolicus* sp. nov., an acidophilic facultatively methylotrophic bacterium. Int J Syst Bacteriol 36:317–322

Urakami T, Tamaoka J, Suzuki K, Komagata K (1989) *Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. Int J Syst Bacteriol 39: 50–55

Visser't Hooft F (1925) Biochemische onderzoekingen over het geslacht *Acetobacter*. Dissertation, Technical University Meinema Delft

Vu HTL, Yukphan P, Chaipitakchonlatarn W, Malimas T, Muramatasu Y, Bui UTT, Tanasuapwat S, Duong KC, Nakagawa Y, Pham HT, Yamada Y (2013) *Nguyenibacter vanlangensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 59:153–166

Yamada Y (1979) Classification of microorganisms based on the molecular species of the respiratory quinones. Hakko to Kogyo 37:940–954 (in Japanese)

Yamada Y (1983) *Acetobacter xylinus* sp. nov., nom. rev., for the cellulose-forming and cellulose-less, acetate-oxidizing acetic acid bacteria with the Q-10 system. J Gen Appl Microbiol 29: 417–420

Yamada Y (2000) Transfer of *Acetobacter oboediens* Sokollek et al. 1998 and *Acetobacter intermedius* Boesch et al. 1998 to the genus *Gluconacetobacter* as *Gluconacetobacter oboediens* comb. nov. and *Gluconacetobacter intermedius* comb. nov. Int J Syst Evol Microbiol 50:2225–2227

Yamada Y (2014) Transfer of *Gluconacetobacter kakiaceti*, *Gluconacetobacter medellinensis* and *Gluconacetobacter maltaceti* to the genus *Komagataeibacter* as *Komagataeibacter kakiaceti* comb. nov., *Komagataeibacter medellinensis* comb. nov. and *Komagataeibacter maltaceti* comb. nov. Int J Syst Evol Microbiol 64:1670–1672

Yamada Y, Akita M (1984) An electrophoretic comparison of enzymes in strains of *Gluconobacter* species. J Gen Appl Microbiol 30:115–126

Yamada Y, Kondo K (1984) *Gluconoacetobacter*, a new subgenus comprising the acetate-oxidizing acetic acid bacteria with ubiquinone-10 in the genus *Acetobacter*. J Gen Appl Microbiol 30:297–303

Yamada Y, Yukphan P (2008) Genera and species in acetic acid bacteria. Int J Food Microbiol 125:15–24

Yamada Y, Aida K, Uemura T (1969a) Enzymatic studies on the oxidation of sugar and sugar alcohol: V. Ubiquinone of acetic acid bacteria and its relation to classification of genera *Gluconobacter* and *Acetobacter*, especially of the so-called intermediate strains. J Gen Appl Microbiol 15:181–196

Yamada Y, Nakazawa E, Nozaki A, Kondo K (1969b) Characterization of *Acetobacter xylinum* by ubiquinone system. Agric Biol Chem 33:1659–1661

Yamada Y, Okada Y, Kondo K (1976) Isolation and characterization of "polarly flagellated intermediate strains" in acetic acid bacteria. J Gen Appl Microbiol 22:237–245

Yamada Y, Nunoda M, Ishikawa T, Tahara Y (1981a) The cellular fatty acid composition in acetic acid bacteria. J Gen Appl Microbiol 27:405–417

Yamada Y, Ishikawa T, Yamashita M, Tahara Y, Yamasato K, Kaneko T (1981b) Deoxyribonucleic acid base composition and deoxyribonucleic acid homology in the polarly flagellated intermediate strains. J Gen Appl Microbiol 27:465–475

Yamada Y, Itakura N, Yamashita M, Tahara Y (1984) Deoxyribonucleic acid homologies in strains of *Gluconobacter* species. J Ferment Technol 62:595–600

Yamada Y, Hoshino K, Ishikawa T (1997) The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 61:1244–1251

Yamada Y, Hosono R, Lisdiyanti P, Widyastuti Y, Saono S, Uchimura T, Komagata K (1999) Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. J Gen Appl Microbiol 45:23–28

Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T, Komagata K (2000) *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the *α-Proteobacteria*. Int J Syst Evol Microbiol 50:823–829

Yamada Y, Yukphan P, Vu HTL, Muramatsu Y, Ochaikul D, Nakagawa Y (2012a) Subdivision of the genus *Gluconacetobacter* Yamada, Hoshino and Ishikawa 1998: the proposal of *Komagatabacter* gen. nov., for strains accommodated to the *Gluconacetobacter xylinus* group in the *α-Proteobacteria*. Ann Microbiol 62:849–859

Yamada Y, Yukphan P, Vu HTL, Muramatsu Y, Ochaikul D, Tanasupawat S, Nakagawa Y (2012b) Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). J Gen Appl Microbiol 58:397–404

Yamashita S, Uchimura T, Komagata K (2004) Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989. Int J Syst Evol Microbiol 54:865–870

Yukphan P, Takahashi M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2004a) *Gluconobacter albidus* (ex Kondo and Ameyama 1958) sp. nov., nom. rev., an acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 50:235–242

Yukphan P, Potacharoen W, Tanasupawat S, Tanticharoen M, Yamada Y (2004b) *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. Int J Syst Evol Microbiol 54:313–316

Yukphan P, Malimas T, Potacharoen W, Tanasupawat S, Tanticharoen M, Yamada Y (2005) *Neoasaia chiangmaiensis* gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the *α-Proteobacteria*. J Gen Appl Microbiol 51:301–311

Yukphan P, Malimas T, Muramatsu Y, Takahashi M, Kaneyasu M, Tanasupawat S, Nakagawa Y, Suzuki K, Potacharoen W, Yamada Y (2008) *Tanticharoenia sakaeratensis* gen. nov., sp. nov., a new osmotolerant acetic acid bacterium in the α-*Proteobacteria*. Biosci Biotechnol Biochem 72:672–676

Yukphan P, Malimas T, Muramatsu Y, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Hamana K, Tahara Y, Suzuki K, Tanticharoen M, Yamada Y (2009) *Ameyamaea chiangmaiensis* gen. nov., sp. nov., an acetic acid bacterium in the *α-Proteobacteria*. Biosci Biotechnol Biochem 73:2156–2162

Yukphan P, Malimas T, Lundaa T, Muramatsu Y, Takahashi M, Kaneyasu M, Tanasupawat S, Nakagawa Y, Suzuki K, Tanticharoen M, Yamada Y (2010) *Gluconobacter wancherniae* sp. nov., an acetic acid bacterium from Thai isolates in the *α-Proteobacteria*. J Gen Appl Microbiol 56:67–73

Yukphan P, Malimas T, Muramatsu Y, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2011) *Neokomagataea* gen. nov., with descriptions of *Neokomagataea thailandica* sp. nov. and *Neokomagataea tannensis* sp. nov., osmotolerant acetic acid bacteria of the *α-Proteobacteria*. Biosci Biotechnol Biochem 75:419–426

Zhang J-Y, Liu X-Y, Liu S-J (2011) *Frateuria terrea* sp. nov., isolated from forest soil and emended description of the genus *Frateuria*. Int J Syst Evol Microbiol 61:443–447

Chapter 2
Acetic Acid Bacteria in Production of Vinegars and Traditional Fermented Foods

Yoshikatsu Murooka

Abstract Vinegars produced from various ingredients by acetic acid bacteria are a traditional seasoning condiment used all over the world. Various species of acetic acid bacteria, which are mostly the bacteria that inhabit the ingredients, barrels, or pots, are found in vinegar fermentations. Acetic acid bacteria are also found during fermentations of traditional food production. In these fermentation processes, acetic acid bacteria form a microbial community, a so-called biofilm, with other microbes, and are important in producing acetic acid, which contributes to preserving fermented foods and producing health-related elements. Acetic acid bacteria are also known as spoilers of some delicate foods. The taxonomic characteristics of these acetic acid bacteria have been clarified by recent advanced techniques of genomic analysis.

Keywords Acetic acid bacteria • Vinegar • Fermented food • Spoiler

2.1 Acetic Acid Bacteria in Traditional Vinegar Production

Varieties of vinegars are produced all over the world. Most vinegars are of plant origin, except those from whey or honey. Despite their ingredients, vinegars are produced by two-step processes. The first step is the production of ethanol from a carbohydrate, such as sugars, glucose, sucrose, fructose, or galactose, by yeasts. When we use ingredients containing starch, such as rice, barley, beans, or other cereals, we have to degrade the starch into glucose and maltose using fungi, such as *koji* mold, malt, or purified enzymes, amylases. The second step is the oxidation of ethanol to acetic acid by acetic acid bacteria, which are mostly species of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*, among the 17 members of the genera (see Chap. 1).

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2.1.1 Rice Vinegars

Rice vinegar is made from fermented rice wine (*sake*), which is produced by two processing steps, saccharification by *koji* mold (*Aspergillus oryzae*) from steamed rice and alcohol fermentation by *sake* yeast (*Saccharomyces cerevisiae*) (Murooka et al. 2009). The rice vinegar is produced by a traditional static surface fermentation and a modern continuous or batch submerged fermentation. The final acidity of the fermented vinegar is usually less than 10 %. The dominant acetic acid bacteria in such vinegar productions are *Acetobacter pasteurianus* in surface culture and *Acetobacter xylinum* (*Komagataeibacter xylinus*) in submerged culture (Entani and Masai 1984). However, the acidity produced by these bacteria is not so high. Fourteen strains of acetic acid bacteria isolated from rice vinegar broth with high acidity were classified as a new species, *Acetobacter polyoxogenes* (‘*Gluconacetobacter polyoxogenes*’) (Entani et al. 1985).

2.1.1.1 Polished Rice Vinegar, *Komesu*, and Unpolished Rice Vinegar, *Kurosu*

The *komesu* and *kurosu* are produced from polished and unpolished rice grains, respectively, and *kasuzu* is produced from *sake* lees. The product of *komesu* and *kasusu* is pale amber in color, mild and sweet, and is thus used in *sushi* and seaweed salad, *sunomono* (a vinegar dish), for Japanese cuisine. *Kurosu* is dark black in color and is used mainly as a table condiment and healthy drink in China and Japan (Murooka et al. 2009). The ‘*Gluconacetobacter (Ga.) polyoxogenes’* was chosen to develop a new system for rice vinegar (*komesu*) fermentation using an acetator because the bacterium generates a more preferable flavor and low-level gluconic acid (Aritomi et al. 2008). ‘*Ga. polyoxogenes*’ is quite sensitive to the lack of oxygen.

Identification of the species and characterization of the dominant strains in static surface acetic acid fermentation were required for the stabilization of fermentation procedures and improvement of the strain. Recent advances of genetic analyses including DNA–DNA hybridization, enterobacterial repetitive intergenic consensus (ERIC)-polymerase chain reaction (PCR) and repetitive extragenic palindromic (REP)-PCR, random amplified polymorphic DNA (RAPD), and 16S rRNA sequence analysis with PCR-denaturing gradient gel electrophoresis (DGGE) methods allow identification of species of acetic acid bacteria.

By using ERIC-PCR and RAPD methods and 16S rRNA sequence analysis, Nanda et al. (2001) clarified the acetic acid bacteria involved in the rice vinegars *komesu*, *kurosu*, and *kasuzu*, fermented by the traditional static surface method. In these fermentations, no pure cultured strain had been inoculated since these vinegar productions began one century ago. The acetic acid bacterial layer covering the *moromi* surface is scooped up with a mesh bowl and then gently introduced into a

Fig. 2.1 Transferring inoculation of a crepe pellicle of acetic acid bacteria. The bacteria covering the *moromi* surface are scooped up with a mesh bowl and then gently floated into the pellicle layer of a new *moromi* (Picture provided by Tamanoi Vinegar Co. Ltd. Sakai, Japan)

new batch of *moromi* (Fig. 2.1). The *moromi* is a mash containing alcohol and vinegar with or without the steamed ingredient.

A total of 124 of 126 isolates from early- (1–10 days), middle- (11–20 days), late- (21–32 days), and post- (>32 days) fermentations of *komesu* were the same group of strains that belong to *Acetobacter (A.) pasteurianus*, and only 2 of the 126 strains that appeared at post-fermentation referred to the same species, but were recognized as a group of strains different from the former group of strains on the basis of DNA fingerprinting analyses (Nanda et al. 2001). A total of 50 strains isolated from the early, middle, and late phases of static *kurosu* fermentation were identified to be one group of *A. pasteurianus* (Table 2.1).

2.1.1.2 Sake Lees Vinegar, *Kasuzu*

The production of *sake* lees vinegar is seen in Zhen Jian Xiancu, China. In Japan *kasuzu* has been produced since the Edo period, that is, for about 300 years. The acidity of *kasuzu* is 4.2 to 4.5. A total of 210 acetic acid bacteria isolated from *kasuzu* were classified into four groups (Murooka et al. 2009); some of these strains, which were obtained from the early and middle phases of the fermentation, were classified into *A. pasteurianus*, and others, which were obtained from the late phase of the fermentation, were classified as *Gluconacetobacter intermedius* (*Komagataeibacter intermedius*). Because the physiological characteristics and ERIC-PCR patterns of these strains were different from the type strain of *Ga. intermedius* DSM 11804, these strains were classified into a new subspecies, '*Gluconacetobacter(Ga.) intermedius* subsp. *tamanoi* subsp. nov.' (Murooka et al. 2009).

Gluconobacter oxydans subsp. *sphaericus* (*Gluconobacter sphaericus*) IFO12467 was screened with a culture medium of *kasuzu* as the highest oxidized ability of ethanol to acetic acid (Saeki 1993). The strain showed it was

Table 2.1 Acetic acid bacteria found in production of vinegars and fermented foods

Fermented food	Acetic acid bacteria		Reference
	Genus	Species	
Rice vinegar			
Komesu	*Acetobacter*	*pasteurianus*	Entani and Masai (1984), Ameyama (1990), and Nanda et al. (2001)
	'Gluconacetobacter	*polyoxogenes*[a]	Entani et al. (1985)
Kurosu	*Acetobacter*	*pasteurianus*	Nanda et al. (2001)
Kasuzu	*Acetobacter*	*pasteurianus*	Nanda et al. (2001)
	'Gluconacetobacter	*intermedius* subsp. *tamanoi*'[a]	Murooka et al. (2009)
Fukuyama kurozu	*Acetobacter*	*pasteurianus*	Entani and Masai (1985), Haruta et al. (2006), and Tokunaga et al. (2009)
	Acetobacter	*aceti*	
	Komagataeibacter[b]	*xylinus*	
Wine vinegar			
Wine vinegar	*Komagataeibacter*[b]	*europaeus*	Sievers et al. (1992), Sokllek et al. (1998), and Andres-Barrao et al. (2008)
	Komagataeibacter[b]	*oboediens*	
	Acetobacter	*pomorum*	
	Acetobacter	*pasteurianus*	
Jerez vinegar	*Acetobacter*	ND	Caro et al. (1998)
	Gluconacetobacter	ND	
Palm wine vinegar	*Acetobacter*	ND	Okafor (1975), Uzochukwu et al. (1999), and Amoa-Awua et al. (2007)
Traditional balsamic vinegar	*Komagataeibacter*[b]	*xylinus*	De Vero et al. (2006), Gullo et al. (2006), and Solieri et al. (2006)
	Acetobacter	*pasteurianus*	
	Komagataeibacter[b]	*europaeus*	
	Komagataeibacter[b]	*hansenii*	
	Acetobacter	*aceti*	
	Acetobacter	*malorum*	Cleenwerck et al. (2002)
Cereal vinegar			
Cereal Chinese vinegar	*Acetobacter*	*pasteurianus*	Mao (1998) and Hu and Hao (2004)
	Acetobacter	*aceti*	
	Gluconacetobacter	*liquefaciens*	
	'*Acetobacter*	*rancens*'[a]	
	Komagataeibacter[b]	*hansenii*	
	Komagataeibacter[b]	*xylinus*	
Malt/beer vinegar	*Acetobacter*	*cerevisiae*	Grierson (2009) and Cleenwerck et al. (2002)
	Gluconacetobacter	*sacchari*	Fleet (1998)
	Acetobacter	*lambici*	Spitaels et al. (2014)
Spirit vinegar	*Komagataeibacter*[b]	*intermedius*	Andres-Barrao and Barja (2008) and Andres-Barrao et al. (2011)
	Komagataeibacter[b]	*oboediens*	
Fruit vinegar			
Luck pang vinegar	*Acetobacter*	*aceti*	Uchimura et al. (1991)
Persimmon vinegar	*Acetobacter*	*pasteurianus*	Suenaga et al. (1993)
Cider vinegar	*Komagataeibacter*[b]	*europaeus*	Joshi and Sharma (2009)
	Komagataeibacter[b]	*xylinus*	Li et al. (2014)
	Acetobacter	*sicerae*	

(continued)

Table 2.1 (continued)

Fermented food	Acetic acid bacteria		Reference
	Genus	Species	
Taiwan fruit vinegar	*Acetobacter*	*aceti*	Ou and Chang (2009)
African fruit vinegar	*Acetobacter*	*tropicalis*	Ndoye et al. (2006)
	Acetobacter	*pasteurianus*	
Thai fruit vinegar	*Komagataeibacter*[b]	*maltaceti*	Slapsak et al. (2013)
Kaki vinegar	*Komagataeibacter*[b]	*kakiaceti*	Iino et al. (2012)
Other vinegar			
Whey vinegar	*Komagataeibacter*[b]	*liquefaciens*	Parrondo et al. (2003)
Onion vinegar	*Acetobacter*	*pasteurianus*	Horiuchi et al. (2000) and Gonzalez et al. (2008)
Honey vinegar	*Acetobacter*	ND	Snowdon and Cliver (1996)
	Gluconacetobacter	ND	
Fermented food			
Kombucha tea	*Komagataeibacter*[b]	*intermedius*	Liu et al. (1996), Boesch et al. (1998), and Dutta and Gachhui (2007)
	Komagataeibacter[b]	*kombuchae*	
	Acetobacter	*aceti*	
	Komagataeibacte[b]	*xylinus*	
Nata de coco	*Gluconacetobacter*	*medellinensis*	Iguchi et al. (2004), Supakod and Wongwicham (2012), and Castro et al. (2013)
Cocoa bean	*Acetobacter*	*ghanensis*	Cleenwerck et al. (2007), Camu et al. (2007), Ndoye et al. (2007), and Cleenwerck et al. (2008)
	Acetobacter	*senegalensis*	
	Acetobacter	*fabarum*	
Ragi	*Kozakia*	*baliensis*	Lisdiyanti et al. (2003)
Khao-Khab	*Acetobacter*	*indonesiensis*	Tanasupawat et al. (2011)
	Acetobacter	*orientalis*	
	Acetobacter	*lovaniensis*	
	Acetobacter	*syzygii*	
Apricot	*Acetobacter*	*pomorum*	Fu et al. (2012)
Fermented milk	*Acetobacter*	*malorum*	Fukami et al. (2009) and Kiryu et al. (2012)
	Acetobacter	*orientalis*	
Thai fermented food	*Gluconobacter*	*frateurii*	Monmangmee et al. (2008)
	Acetobacter	*tropicalis*	
	Acetobacter	*pasteurianus*	

Some names of genus and species of bacteria are described as appeared in the references but are not changed to the recent taxonomic nomenclature

ND not determined

[a]Taxonomic nomenclature that is not authorized that is shown as ‘ ’

[b]The original genus name was *Gluconacetobacter*. In the text, the new name is shown in parentheses following the original name

advantageous for the production of submerged vinegar fermentation containing a large amount of gluconate.

2.1.1.3 Vinegar Production in a Ceramic Pot, *Tsubosu*

In China, most black vinegars have been produced from glutinous rice and other cereals such as millets and barley in ceramic pots, and the detailed techniques of 23 kinds of vinegar production were described in China's oldest agricultural treatise book in the Northern Wei period (AD 386–533). In Japan, pot vinegar (*tsubosu*) has been produced from rice, as appeared an old tax record in the Nara period (AD 710–794) (Mazza and Murooka 2009). We can see the *tsubosu* fermentation, which has been produced since 1710, at Fukui, northwest from the historical city of Kyoto, Japan (Fig. 2.2). For more than 200 years the famous black rice vinegar (*kurosu*), *Fukuyama kurozu*, has been produced in Kagoshima Prefecture, which is located in the southern part of Japan.

In Fukuyama *kurozu* brewing, the three reaction steps of saccharification, alcohol, and acetic acid fermentation proceed sequentially and partly in parallel in ceramic pots. No microorganisms except *koji* mold are inoculated in steamed rice and water. Entani and Masai (1985) isolated acetic acid bacteria together with several yeast strains and lactic acid bacteria from the *kurozu*. In acetic acid bacteria, five strains of *Acetobacter pasteurianus*, four strains of *Komagataeibacter xylinus*, and one strain of *A. aceti* were isolated from *moromi* of *kurozu* fermentation (Table 2.1). Haruta et al. (2006) applied the DGGE method targeting small-subunit rRNA genes to analyze the microbial community in *kurozu* fermentation. They found that almost all the bacterial DGGE bands showed *A. pasteurianus* and *Lactobacillus acetotolerans* when acetic acid started to accumulate. The microbial succession was found to be essentially identical when tested in three different pots. At the early stage, some bacterial species differed from those detected by DGGE. By using five PCR primer sets designed to amplify the five specific DNA fragments

Fig. 2.2 Brewing of the ceramic pot vinegar (*tubosu*) (Picture provided by Tobaya-suten Co. Ltd, Fukui, Japan)

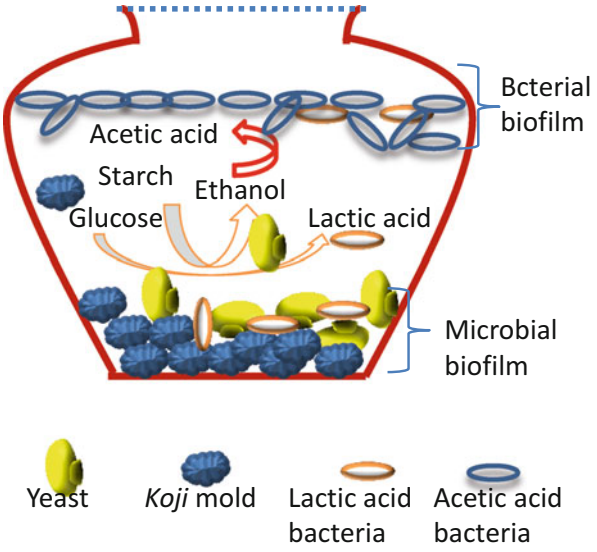

Fig. 2.3 Schematic representation of microbial community (biofilm) formation in Fukuyama *kurozu* fermentation. *Koji* mold, yeasts, and lactic acid bacteria are floating or sunk to form a biofilm at the bottom of the pot, and acetic acid bacteria form a biofilm on the surface of the fermented broth (Figure is revised from the original figure by Furukawa et al. 2013)

within the internal transcribed space region of acetic acid bacteria, *A. pasteurianus* was found in the *kurozu* samples after 60 days of fermentation (Tokunaga et al. 2009). No acetic acid bacterium was detected from 7-day fermented *kurozu*.

As we may expect, an interesting phenomenon was reported by Furukawa et al. (2011, 2013) in that almost all strains of *A. pasteurianus* formed biofilm (pellicles) in mixed culture with various lactic acid bacteria on the surface of the fermentation broth (Fig. 2.3). Yeast, *koji* mold, and lactic acid bacteria formed a biofilm and sank to the bottom of the pot. Okazaki et al. (2010) also found acetic acid bacterial pellicles on the mash surface and identified the dominant bacterial species as *A. pasteurianus*.

Because *tsubosu* including *kurosu* are produced from unpolished rice grains, these black rice vinegars contain many components important for human health, such as amino acids, vitamins, several antioxidants, and a DPPH radical-scavenging compound (Murooka et al. 2009). During 6 months of fermentation and more than 1 year of aging of *kurosu* production, the vinegar contains immunostimulatory components, such as tumor necrosis factor (TNF)-α and Toll-like receptors, which would be responsible for the beneficial health effects of *kurosu* (Hashimoto et al. 2013).

2.1.2 Wine Vinegar

Wine vinegar is the most commonly used vinegar in the households of Mediterranean countries and central Europe. Wine vinegars, red and white, are made from red and white wines, respectively, and usually matured in wood barrels for up to

2 years. During this aging, a mixed flavor and the formation of amino acids and polyphenol compounds are developed (Sellmer-Wilsberg 2009).

A new species of *Acetobacter europaeus* (*Komagataeibacter europaeus*) was isolated from submerged vinegar fermentations in Germany and Switzerland (Sievers et al. 1992). The bacterium had a strong tolerance to acetic acid at a concentration of 4–8 % and the absolute requirement of acetic acid for growth. Sokollek et al. (1998) isolated two new species of *Acetobacter oboediens* (*Komagataeibacter oboediens*) and *Acetobacter pomorum* from red wine and cider vinegar fermentations.

Andres-Barrao et al. (2008) selected two groups of acetic acid bacterial strains as the starter culture for submerged semicontinuous vinegar production. Acetic acid bacteria isolated from a wine vinegar submerged culture were identified as a mixture of 40 % *A. pasteurianus* and 60 % *K. europaeus*, whereas strains in the other culture were almost 100 % *A. pasteurianus*. Although both inocula of the starter presented a different composition of bacterial species, *K. europaeus* was observed at the end of fermentation. This observation showed that *K. europaeus* is an acidophilic species, whereas *A. pasteurianus* grows better in a culture with a low acetic acid but higher alcohol content. The surface layer surrounding the bacteria contains a polysaccharide that must be a biofilm, which could contribute to maintaining the acetic acid bacteria against the high content of acetic acid.

2.1.2.1 Traditional Balsamic Vinegar

Traditional balsamic vinegar is made in Modena and Reggio Emilia, Italy, with cooked grape must, through a three-step process: conversion of sugars to ethanol by yeast, oxidation of ethanol to acetic acid by acetic acid bacteria, and finally at least 12 years of aging by sequential transfer from a larger barrel to a smaller barrel (Fig. 2.4). The product is highly dense and dark-brown in color, having a sweet and sour taste, fruity and complex in flavor (Giudici et al. 2009).

Fig. 2.4 Aging of traditional balsamic vinegar production. Wine and vinegar are refilled from a larger volume of barrel to a smaller barrel per year (Picture taken by author at the Traditional Balsamic Vinegar Museum, Modena, Italy)

A tentative identification of isolated strains from traditional balsamic vinegar was made using the PCR-restriction fragment length polymorphism (RFLP) method with 16S–23S–5S rRNA. The 32 isolates were found to be a *K. xylinus* group, two strains of the *A. pasteurianus* group and one of *A. aceti* (Gullo et al. 2006). By analysis of acetic acid bacteria with the PCR-DGGE method, *K. europaeus*, *Gluconacetobacter (Ga.) hansenii* (*Komagataeibacter hansenii*), *A. pasteurianus*, *A. aceti*, and *A. malorum* were found in the balsamic vinegar fermentation process (De Vero et al. 2006). In general, few acetic acid bacterial species are able to ferment in a sugar concentration higher than 25 % (w/v). De Vero et al. isolated sugar-tolerant acetic acid bacteria as a starter of acetic acid fermentation. The most sugar-tolerant species is *A. malorum*, which was first isolated from spoiled apples and can grow on 30 % of glucose (Cleenwerck et al. 2002). As a starter strain, *A. pasteurianus* is suitable for traditional balsamic vinegar acetification (Gullo and Giudici 2008; Gullo et al. 2009).

2.1.2.2 Jerez Vinegar

Jerez vinegar is produced from sherry wines by following the traditional methods of aging in southwestern Spain. The first written references to Jerez vinegar comes from Columela in the first century AD (Tesfaye et al. 2009). After sherry wine production, the subsequent acetification process encompasses the activity of acetic acid bacteria species of the genera of *Acetobacter* and *Gluconobacter* (Caro et al. 1998).

The aging system is important to determine the quality of Jerez vinegar: the dynamic system, which consists of a series of casks arranged in sequence whose number varies from 3 to 8, and the static aging method are used. In the dynamic system, the wine is introduced at the top cask of the pyramid of casks and the final product is withdrawn from the bottom casks, which are the most aged. The aging period is 6 months to more than 10 years and categorizes three classes of Jerez vinegar, depending on their degree of aging.

2.1.3 Malt/Beer Vinegar

Malt vinegar originated from Britain several hundred years ago. Malt vinegar is produced from malted barley with or without the addition of cereal grains. Malt vinegar is a pale straw-colored liquid, with strong acetous flavor, whereas dark malt vinegar has a dark brown color derived from the addition of barley extract or caramel to the malt vinegar (Grierson 2009). Distilled malt vinegars are obtained by the distillation of malt vinegar. *Acetobacter* sp. is naturally provided from a vessel packed with wood wool. *A. cerevisiae* and *Ga. sacchari* were found in the malt vinegar fermentations (Cleenwerck et al. 2002; Fleet 1998).

2.1.4 Cider Vinegar

Cider vinegar or apple vinegar is made with apple juice or concentrated apple juice through a double fermentation, with alcohol and acetic acid in the same barrel, by naturally occurring yeasts and acetic acid bacteria. Nowadays, cider vinegar is made mainly by submerged culture as is wine vinegar. Cider vinegar is extensively used in several countries including Austria, the UK, the United States (U.S.), and Switzerland (Joshi and Sharma 2009). Cider vinegar is used as a flavoring agent and as food preservation. It is popular in folk medicine and is suggested as a remedy for various diseases, not only for obesity, overweight, and arthritis but also for asthma, coughs, diarrhea, colitis, eczema, and hair loss. *K. europaeus* has been described as the dominant species in industrial submerged-culture vinegar manufacturing in central Europe, whereas *K. xylinus* has been frequently recovered from the traditional process.

Five acetic acid bacteria isolated from a traditionally produced cider and kefir vinegars were grouped on the basis of their MALDI-TOF mass spectrometry (MS) profile. Two strains were classified as members of the genus *Acetobacter*. By DNA sequence analysis of the housekeeping genes *dnaK*, *groEL*, and *rpoB*, two strains were named as a new species, *Acetobacter sicerae* sp. nov. (Li et al. 2014).

Other local fruit vinegars are listed in Table 2.1.

2.1.5 Cereal Vinegar in China

Many kinds of cereal vinegars from starchy raw materials such as corn, wheat, barley, sorghum, and rice are produced in China and Taiwan (Chen et al. 2009). Solid-state fermentation processes are widespread in Asian countries to produce vinegar on a small scale. The traditional and modern methods of cereal vinegar fermentation in China are similar to *tsubosu* production, as described earlier. In the solid-state fermentation of cereal vinegars, several species of acetic acid bacteria, such as *A. pasteurianus*, *A. aceti*, *A. liquefaciens* (*Gluconacetobacter liquefaciens*), '*A. rancens*,' *A. hansenii* (*Komagataeibacter hansenii*), *K. xylinus*, and *Gluconobacter* spp. were found (Mao 1998; Hu and Hao 2004). Other than acetic acid bacteria, *Lactobacillus* and *Bacillus* spp. were found in cereal vinegar fermentations (Wu 2004). Wu et al. (2010) isolated 21 acetic acid bacterial strains from Chinese cereal vinegars produced by solid-state fermentation in different regions of China. Most of the strains belonged to *A. pasteurianus*.

2.1.6 Spirit Vinegar

Spirit vinegar is sometimes reserved for the stronger variety of acetic acid (5–21 %) made from sugar cane or from chemically produced acetic acid. Depending on the bacterial species and the raw material used, the submerged semicontinuous fermentation method allows the production of high-acid vinegar, with final acetic acid concentration up to 15 %. The resistance of acetic acid bacteria to acetic acid and ethanol varies among species (Andres-Barrao and Barja 2008). Fingerprinting techniques with PCR-RFLP and sequencings of 16S rRNA, ITSI region, and partial *adhA* gene showed the presence of four different strains in the culture broth. These strains were classified into two groups, *K. intermedius* and *Ga. oboediens* (*Komagataeibacter oboediens*) (Andrees-Barrao et al. 2011). Because no growth was observed on any culture media when plated with high-acid (>10 %) vinegar samples, acetic acid bacteria from spirit vinegar become nonviable or non-cultivable at high acetic acid concentrations, which reached 10–14 % during the vinegar production process.

2.1.7 Other Local Vinegars

In Turkey, fig (*Ficus carica*) vinegar has been produced in a homemade scale. Acetic acid bacteria were isolated from two different homemade vinegar fermentations together with mold, yeast, and lactic acid bacteria. Acetic acid bacteria were counted ranging between 1.6×10^2 and 8.8×10^2 CFU/ml culture broth (Sengum and Karabiyikli 2008).

Onion vinegar was produced from worthless onions for recycling a vegetable biomass (Gonzalez et al. 2008). *Acetobacter* spp. was used in acetic acid fermentation after onion alcohol fermentation. Horiuchi et al. (2000) produced a new type of onion vinegar rich in amino acids, organic acids, and certain minerals from commercially worthless onions using *A. pasteurianus*.

In Sub-Saharan Africa, 17 acetic acid bacterial strains were isolated from overproducing fruits, such as mangos, and cereals (Ndoye et al. 2006). Two specific acetic acid bacteria were selected for their ability for growth and acetate production at higher temperature. These strains were identified as *Acetobacter tropicalis* and *A. pasteurianus*, respectively, through phylogenetic analysis based on the 16S rRNA gene sequence. Because optimal growth and acetic acid production of the two strains are at 35 ° and 38 °C, respectively, these strains make production of industrial vinegar possible in Sub-Saharan Africa.

In Taiwan, at least 66 fruit vinegars, including 12 mei (apricot), 17 cider, 3 mulberry, 4 lemon, 8 blended, 15 wine, 2 orange, and 5 others (star fruit, blueberry, pineapple, grapefruit, passion fruit) are marketed. Five different strains of *A. aceti* were isolated from pineapple vinegar (Ou and Chang 2009).

Miyazaki et al. (1998) developed new fruit vinegars with marmelo, apple, white asparagus, and pumpkins by inoculation of *A. pasteurianus*, which was isolated from a starter of rice vinegar.

Two novel acetic acid bacterial strains were isolated from traditional *kaki* vinegar, which is produced from a popular fruit, *kaki* (*Diospyros kaki* Thunb.), in Japan. These strains were named *Gluconacetobacter kakiaceti* sp. nov. (*Komagataeibacter kakiaceti*) (Iino et al. 2012).

Slapsak et al. (2013) reported a new species, *Gluconacetobacter maltaceti* sp. nov. (*Komagataeibacter maltaceti*), as a novel vinegar producing acetic acid bacterium isolated from local vinegar in Thailand.

2.1.8 Vinegars Produced by New Technologies

Isolation and characterization of acetic acid bacteria have promoted acetic acid production by new technologies. Continuous operation of a three-phase fluidized-bed bioreactor using immobilized *A. aceti* IAM1802 cells showed that the productivity was little affected by the dilution rate of fermentation, although there was rapid decrease in the productivity in the free cell continuous fermentation (Sun and Furusaki 1990). For innovation of traditional balsamic vinegar production, selected starter cultures are the main technological improvement. Basic traits are efficient oxidation of ethanol, fast rate of acetic acid production, tolerance to a high concentration of acetic acid, no overoxidation, and low pH resistance. Strains of *K. europaeus* and *A. malorum* have the specific traits of tolerance to high concentrations of sugar and ethanol. The wide temperature range in strains of *K. europaeus* and *A. malorum* is also selected for starter cultures because they show suitable characteristics for vinegar production (Gullo and Giudici 2008).

By oxidative fermentation in combination with microbial fuel cell technology, production of vinegar by *A. aceti* was successfully demonstrated through a series of repeated batch fermentations (Tanino et al. 2013). Because the bacterial culture in vinegar production is a mixture of different strains of acetic acid bacteria, vinegar production was easy to optimize by scale-down trial of the fermentation process (Schleputz and Buchs 2014). By inoculation of mixed acetic acid bacteria originated from a spirit vinegar production site in Germany, microtiter plate cultivation with the new custom-made lid provides a platform for high-throughput studies on vinegar production. Results are comparable to those in the 9-l bioreactor.

2.2 Acetic Acid Bacteria in Traditional Fermented Food Production

2.2.1 Nata de Coco

Bacterial cellulose production of *K. xylinus* at the air–liquid interface of coconut water is popularly known as *nata-de-coco*. This unconventional product based on coconut water is native to the Philippines and developed locally first in 1949. *Nata* so formed is more than 90 % water imbibed in cellulose and finds use in desserts, fruit cocktails, and fruit jellies (Jagannath et al. 2008). Pure cultures of *K. xylinus* obtained a consistent product and will benefit the coconut-based farmers and communities to produce good quality *nata* with superior physical properties as the local farmers used batch variations that resulted in overgrowth of contaminated yeast. Cheap media for the preparation of seed culture of *K. xylinus* were a mixture of coconut water and banana juice (1:1) (Supakod and Wongwicham 2012). *Gluconacetobacter medellinensis* sp. nov. (*Komagataeibacter medellinensis*) is a cellulose- and non-cellulose-producing acetic acid bacterium isolated from vinegar (Castro et al. 2013).

Many experiments with production, dissolution, and regeneration of bacterial cellulose extracted from *nata-de-coco* were reported (Valla 2008; Pandey et al. 2014) (see Chap. 3).

2.2.2 Cocoa Bean Heap

The fermentation of the Chanalan cocoa bean heap is seen in Sub-Saharan Africa. The cocoa beans undergo fermentation before processing for chocolate production. After removal of beans and pulp from pods, the pulp-bean mass is piled into heaps, covered with plantain leaves, and spontaneous fermentation starts. Camu et al. (2007, 2008) studied biodiversity of population dynamics and metabolomics in several spontaneous heap fermentations. Culture-dependent and culture-independent molecular methods were used for identification of acetic acid bacteria.

The predominant bacteria were *A. pasteurianus*, and a novel species was found and named *A. ghanensis* (Cleenwerck et al. 2007), *A. senegalensis* (Camu et al. 2007; Ndoye et al. 2007), and *A. fabarum* (Cleenwerck et al. 2008). Acetic acid can penetrate the cocoa beans, kill the embryo, and decompose their internal structure to form flavor and color development. Thus, acetic acid bacteria have an important function during cocoa bean fermentation that contributes to the formation of cocoa flavor precursors and the control of the cocoa bean fermentation process.

More detailed characterizations of acetic acid bacteria concerned with cocoa fermentation and *kombucha* vinegar are described in Chap. 3.

2.2.3 *Are Any Acetic Acid Bacteria Involved in the Formation of Acetic Acid in Japanese Pickles, Soy Sauce, and* Miso *or Korean* Kimchi *?*

In traditional fermented foods in Asia such as soy sauce (*shoyu*), fermented soybean-barley (*miso*) in Japan, and pickles such as *kimchi* in Korea and *tsukemono* in Japan, various organic acids, such as acetic acid, lactic acid, and citric acid, are detected by chemical and liquid chromatographic analyses. The largest amount of lactic acid is contained in Japanese pickles, *tsukemono*, followed by acetic, citric, formic, malic, and glutamic acids (Tomomatsu et al. 1972). More than 13 species of lactic acid bacteria and 10 species of yeast isolated from six kinds of *tsukemono* were identified by 16S rRNA sequence analysis (Asada and Ueno 2009). However, no acetic acid bacteria were detected. By cultivation of isolated yeast strains, acetic acid, citric acid, and succinic acid were detected in the fermentation broth. However, more careful experiments would be required to conclude that the special yeast can form acetic acid.

The volatile and nonvolatile organic acids in *kimchi* during 60 days of fermentation at 10 °C were analyzed by gas chromatography (GC)-MS (Shim and Kim 2012). Acetic acid was observed as the dominant volatile acid until the middle of fermentation, and lactic acid was the major nonvolatile organic acid, which was produced throughout fermentation. Populations of *Leuconostoc* and *Lactobacilli* increased exponentially during 7 days of fermentation, indicating acetic acid and lactic acid were mainly produced by these lactic acid bacteria.

Acetic acid showed inhibitory activity to osmophilic yeasts in brine fermentation of soy sauce (Noda et al. 1982) and a retarding effect on the growth of contaminated bacteria during the *shoyu-koji* making process (Hayashi et al. 1979). However, no existence of acetic acid bacteria has been reported, although many reports demonstrated that groups of lactic acid bacteria appeared during these fermentations. Recently, I received information that acetic acid in *shoyu* is produced by a lactic acid bacterium, *Tetragenococcus* (previously *Pediococcus*) *halophilus* (Kanbe et al. 1978). In *miso* and in several pickles, acetic acid may be formed by this lactic acid bacterium. Because these fermented foods contain high concentrations of salt and are fermented under anaerobic conditions, acetic acid bacteria cannot grow during the fermentations although salt-tolerant lactic acid bacteria and yeasts can. There is a report that some yeast may produce acetic acid in these fermentation processes. However, there is no clear proof that any yeast can form acetic acid (personal communication, Dr. Y. Kaneko, Osaka University).

Nanba and Kato (1983) reported that stimulating effects of inoculation with acetic acid bacteria, including a strain of *Acetobacter* sp., *A. aceti* IFO3281, and the M-1 strain isolated from vinegar mash in acetic acid production, were observed during the production of *miso* and soy sauce.

2.2.4 Other Fermented Foods

Lisdiyanti et al. (2003) isolated a total of 331 strains of acetic acid bacteria from fermented foods, fruits, and flowers in Indonesia, Thailand, and the Philippines. *Acetobacter*, and *Gluconacetobacter* (mostly *Komagataeibacter*) were mainly isolated from fermented foods. *Kozakia baliensis* strains were isolated from *ragi*, a starter for fermented foods. Thermotolerant acetic acid bacteria were isolated from Thai fermented foods (Monmangmee et al. 2008). Some strains can grow at 40 °C. The bacteria were identified as *Gluconobacter frateurii*, *Acetobacter tropicalis*, and *A. pasteurianus*.

Acetic acid bacteria were isolated from traditional fermented rice (*Khao-khab*, nonglutinous rice) and related products in Thailand (Tanasupawat et al. 2011). On the basis of the 16S rRNA and 16S–23S rRNA gene internal transcribed spacer restriction analyses, 25 isolates were divided into six groups and identified at the species level. Group 1, including 5 isolates, were identified as *Acetobacter indonesiensis*; group 2, including 2 isolates, were *A. lovaniensis*; group 3, including 1 isolate, was *A. orientalis*; group 4, including 11 isolates, were *A. pasteurianus*; group 5, including 3 isolates, were *A. syzygii*; and group 6, including 3 isolates, were unidentified and were considered to constitute a new species.

The *Acetobacter pomorum* strain was isolated from the natural fermented broth of apricot dregs in China (Fu et al. 2012).

Acetobacter malorum NCI1683, derived from fermented milk, was tested to determine whether its continuous ingestion could improve cognitive function in healthy middle-aged and elderly persons (Fukami et al. 2009). A 12-week supplementation of *A. malorum* significantly shortened the response times of working memory. Thus, the acetic acid bacterium has a beneficial effect on cognitive function.

In traditional Caucasian fermented milk, so-called Caspian Sea Yogurt in Japan, lactobionic acid is produced from lactose by *A. orientalis*, which is one of the principal bacteria used in fermentation of the yogurt (Kiryu et al. 2012).

2.3 Acetic Acid Bacteria as Spoilers

In wine making, both yeast inoculation and SO_2 keep the population of lactic acid bacteria at a very low level, although acetic acid bacteria were hardly affected by these two practices (Andorra et al. 2008). Wines spoiled by acetic acid bacteria have characteristic volatility, a vinegar-like sourness on the palate, a range of acetic, nutty, or bruised apple aromas, and often a reduction in fruity character (Bartowsky et al. 2003). Bottled red wines, sealed with natural cork closures and stored in a vertical upright position, may develop spoilage by acetic acid bacteria. This spoilage is evident as a distinct deposit of bacterial biofilm in the neck of the bottle at the interface of the wine and the headspace of air. The spoilage is

accompanied with vinegar-, sherry-, bruised apple-, nutty-, and solvent-like off-aromas, depending on the degree of spoilage (Bartowsky and Henschke 2008). The number of *Acetobacter* cells ranged from 4.21 CFU/ml in a Cabernet Sauvignon at 4 °C of storage to 4.52 log CFU/ml in Cabernet Sauvignon at 25 °C of storage. *Acetobacter aceti*, *A. pasteurianus*, and *A. orleanensis* were detected by using real-time PCR. (Kantor et al. 2013)

Acetic acid is generally considered an off-flavor in beer. However, a very few sour beer styles feature it as a desirable flavor characteristic, notably Flanders Red Ale and Oud Bruin. However, most sour mash beers, such as Berliner Weisse, depend on the different souring action of lactic acid bacteria; a vinegar character is generally considered undesirable in these beers. In contrast to conventional beers, which are fermented by carefully cultivated strains of brewer's yeasts, Lambic beer is produced by spontaneous fermentation. An acetic acid bacterium was isolated from fermenting Lambic beer, and the strain was proposed to be a new species, *Acetobacter lambici* sp. nov. (Spitaels et al. 2014).

Although the consumption of soybean protein as a food ingredient has increased, its characteristic beany flavor has hindered its wide utilization. Medium-chain aldehydes such as *n*-hexane appear to be mainly responsible for the objectionable flavor of soybean products. The effective removal of *n*-hexane from soybean protein by acetic acid bacteria was demonstrated (Adachi et al. 1980). These effective acetic acid bacteria were *A. aceti* and *G. suboxydans* (*Gluconobacter oxydans*) (Kobayashi et al. 1992).

An important feature of acetic acid bacteria that is not related to food was reported at the Kitora Tumulus, Nara, located in the central area of Japan (Kagawa et al. 2013). The Kitora Tumulus, which is believed to have been built around the late seventh century, has beautiful mural paintings that were executed directly on a very thin layer of plaster in the stone chamber. In 2005, small holes containing black substances were observed on the ceiling plaster, and an acetic acid bacterium, *Gluconacetobacter* sp., was isolated from the black substances. This is the first example of characterization of acetic acid bacteria isolated from decayed plaster paintings, and it is likely that microbes such as these bacteria have been involved in the deterioration of the plaster. Alcohol in the painting may act as a carbon source for acetic acid bacteria to form acetic acid, which caused the damage to the paintings.

2.4 Advanced Technology in Analysis of Acetic Acid Bacteria

An effective method for grouping acetic acid bacteria genera was defined and evaluated as a tool for preliminary screening of the major bacterial species involved in vinegar production (De Vero and Giudici 2008). Strains of *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Komagataeibacter*, *Asaia*, *Neoasaia*,

Saccharibacter, *Frateuria*, and *Kozakia* were screened on the basis of 16S rRNA sequences using the PCR-DGGE technique.

Whole-genome sequences of the highly acetic acid-resistant bacteria *K. europaeus* as the reference strain and a strain of *K. oboediens* isolated from vinegar fermentation were reported (Andres-Barrao et al. 2011). The genome sequence was also completed for *Gluconacetobacter* sp. strain SXCC-1 isolated from the starter of Chinese Shanxi vinegar (Du et al. 2011).

Several mechanisms have been proposed to explain how acetic acid bacteria resist high concentrations of acetic acid, such as the assimilation of acetate through the tricarboxylic acid (TCA) cycle, the export of acetate by various transporters, and modifications of the outer membrane. Andres-Barrao et al. (2012) compared the proteome of *Acetobacter pasteurianus* for differences between growth in glucose and in ethanol in the presence of acetic acid by using 2D-DIGE. They identified 53 proteins that showed 1.5 fold the minimal level of differential expression. Furthermore, the same group showed that MALDI-TOF MS analysis was a rapid and reliable method for the clustering and identification of acetic acid bacterial species (Andres-Barrao et al. 2013).

References

Adachi O, Tayama K, Shinagawa E, Matsushita K, Ameyama M (1980) Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 44:503–515

Ameyama M (1990) Biochemistry and biotechnology of vinegars. In: Ameyama M, Otsuka S (eds) Sunokagaku. Asakura Shoten, Tokyo, pp 117–118 (in Japanese)

Amoa-Awua WK, Sampson E, Takano-Debrah K (2007) Growth of yeast, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. J Appl Microbiol 102:599–606

Andorra C, Landi S, Mas A, Guillamon JM (2008) Effect of oenological practices on microbial populations using culture-independent techniques. Food Microbiol 25:849–856

Andres-Barrao C, Barja F (2008) Characterization of bacterial population during spirit vinegar production. In: Abstract 2nd international conference on acetic acid bacteria, Nagoya, p 68

Andres-Barrao C, Weber A, Barja F (2008) Natural imposition of the more acidophilic strain in submerged wine vinegar production. In: Abstracts, 2nd international conference on acetic acid bacteria, Nagoya, p 30

Andres-Barrao C, Falquet L, Calderon-Copete SP, Descombes P, Ortega Perez R, Barja F (2011) Genome sequence of the high-acetic acid resistant bacteria *Gluconacetobacter europaeus* LMG18890 and *G. europaeus* LMG18494 (reference strains), *G. europaeus* 5P3, and *Gluconacetobacter oboediens* 174Bp2 (isolated from vinegar). J Bacteriol 193:2670–2671

Andres-Barrao C, Saad MM, Chappuis ML, Boffa M, Perret X, Ortega Perz R, Barjia F (2012) Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. J Proteomics 75:1701–1717

Andres-Barrao C, Benagli C, Chappuis M, Ortega Perez R, Tonolla M, Barja F (2013) Rapid identification of acetic acid bacteria using MALDI-TOF mass spectrometry fingerprinting. Syst Appl Microbiol 36:75–81

Aritomi T, Izawa M, Asada K, Morimoto H (2008) The development of a system for rice vinegar fermentation using acetators. In: Abstracts, 2nd international conference on acetic acid bacteria, Nagoya, p 33

Asada S, Ueno Y (2009) Study on isolation of useful microorganisms. Kyoto Prefecture Chusyo-kigyo Technological Center Report, no. 37, pp 38–42

Bartowsky EJ, Henschke P (2008) Acetic acid bacteria spoilage of bottled red wine: a review. Int J Food Microbiol 125:60–70

Bartowsky EJ, Xia D, Gibson RL, Fleet RL, Henschke PA (2003) Spoilage of bottled wine by acetic acid bacteria. Lett Appl Microbiol 36:307–314

Boesch C, Treck J, Sievers M, Teuber M (1998) *Acetobacter intermedius* sp. nov. Syst Appl Microbiol 21:220–229

Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, Vuyst LD (2007) Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl Environ Microbiol 73:1809–1824

Camu N, Gonzalez A, De Winter T, Schoor AV, De Bruyne K, Vandamme P, Takama JS, Addo SK, De Vuyst LD (2008) Influence of tuning and environmental contamination on the dynamics of populations of acetic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. Appl Environ Microbiol 74:86–98

Caro I, Palacios VM, Perez L (1998) Kinetic models for the acetic acid fermentation. Recent Res Dev Biotechnol Bioeng 1:203–211

Casro C, Cleenwreck I, Trcek J, Zuluaga R, De Vos P, Caro G, Aquirre R, Putauz JL, Ganan P (2013) *Gluconacetobacter medellinensis* sp. nov., cellulose- and non-cellulose-producing acetic acid bacteria isolated from vinegar. Int J Syst Evol Microbiol 63:1119–1125

Chen F, Li L, Qu J, Chen C (2009) Cereal vinegars made by solid-state fermentation in China. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 243–259

Cleenwerck I, Vandemeulebrocecke 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

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

Cleenwerck I, Conzalez A, Camu N, Engelbeen K, De Vos P, De Vuyst L (2008) *Acetobacter fabarum* sp. nov., and acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. Int J Syst Evol Microbiol 58:2180–2185

De Vero L, Giudici P (2008) Genus-specific profile of acetic acid bacteria by 16SrDNA PCR-DGGE. Int J Food Microbiol 125:96–101

De Vero L, Gala E, Guillo M, Solleri L, Landi S, Giudici P (2006) Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiol 23:809–813

Du XJ, Jia SR, Yang Y, Wang S (2011) Genome sequence of *Gluconacetobacter* sp. strain SXCC-1, isolated from Chinese vinegar fermentation starter. J Bacteriol 193:3395–3396

Dutta D, Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 57:353–357

Entani E, Masai H (1984) Acetic acid bacteria from vinegars. J Brew Soc Jpn 79:746 (in Japanese)

Entani E, Masai H (1985) Identification of yeast, lactic acid bacteria and acetic acid bacteria isolated from fermented mash of Fukuyama rice vinegar. J Brew Soc Jpn 80:200–205 (in Japanese)

Entani E, Ohmori S, Masai H (1985) *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J Gen Appl Microbiol 34:475–490

Fleet GH (1998) The microbiology of alcohol beverages. In: Wood BJB (ed) Microbiology of fermented food, vol 1. Blackie, London, pp 217–262

Fu L, Zhang F, Zhang B (2012) Isolation and identification of acetic acid bacteria. http://www.ipcbee.com/vol50/024-ICFEB2013-B10019.pdf

Fukami H, Tachimoto H, Kishi M, Kaga T, Tanaka Y, Koga Y, Shirasawa T (2009) Continuous ingestion of acetic acid bacteria: effect on cognitive function in healthy middle-aged and elderly persons. Anti-Aging Med 6:60–65

Furukawa S, Hirayama S, Fukase S, Ogihara H, Morinaga Y (2011) Mixed biofilm formation among yeast, lactic acid bacteria and acetic acid bacteria and its application. Seibutsu Kogaku Kaishi 89:478–481 (in Japanese)

Furukawa S, Watanabe T, Toyama H, Morinaga Y (2013) Significance of microbial symbiotic coexistence in traditional fermentation. J Biosci Bioeng 116:533–539

Giudici P, Gullo M, Solieri L (2009) Traditional balsamic vinegar. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia , Milan, pp 157–177

Gonzalez-Saiz JM, Pizzarro C, Esteban-Diez I, Rodriguz-Tecedor S (2008) Monitoring of substrate and product concentrations in acetic fermentation process for onion vinegar production by NIR spectroscopy: value addition to worthless onions. Anal Bioanal Chem 391:2937–2947

Grierson B (2009) Malt and distilled malt vinegar. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 135–143

Gullo M, Giudici P (2008) Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection. Int J Food Microbiol 125:46–53

Gullo M, Caggia S, De Vero L, Giudici P (2006) Characterization of acetic acid bacteria in "traditional balsamic vinegar". J Food Microbiol 106:209–212

Gullo M, De Vero L, Giudici P (2009) Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. Appl Environ Microbiol 75:2585–2589

Haruta S, Ueno S, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y (2006) Succession of bacterial and fungal communities during traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int J Food Microbiol 109:79–87

Hashimoto M, Obara K, Ozono M, Furuyashiki M, Ikeda T, Suda Y, Fukase K, Fujimoto Y, Shigehisa H (2013) Separation and characterization of the immunostimulatory components in unpolished rice black vinegar (*kurozu*). J Biosci Bioeng 116:688–696

Hayashi K, Terada M, Mizunuma T, Yokotsuka T (1979) Retarding effect of acetic acid on growth of contaminated bacteria during shoyu-koji making process. J Food Sci 44:359–362

Horiuchi J, Knnno T, Kobayashi M (2000) Effective onion vinegar production by a two-step fermentation system. J Biosci Bioeng 90:289–293

Hu HP, Hao L (2004) Separation and identification of dominant *Acetobacter* spp. from Shanxi aged vinegar. J Shanxi Agric Univ 24:283–285

Iguchi M, Yamanaka S, Budhiono A (2004) Bacterial cellulose, a masterpiece of nature's arts. J Master Sci 35:261–270

Iino T, Suzuki R, Tanaka N, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2012) *Gluconacetobacter kakiaceti* sp. nov., an acetic acid bacterium isolated from a traditional Japanese fruit vinegar. Int J Syst Evol Microbiol 62:1465–1469

Jagannath A, Kalaiselvan A, Manjunatha SS, Raju PS, Bawa AS (2008) The effect of pH, sucrose and ammonium sulphate concentrations on the production of bacterial cellulose (*Nata-de-coco*) by *Acetobacter xylinum*. World Microbiol Biotechnol 24:2593–2599

Joshi VK, Sharma S (2009) Cider vinegar: microbiology, technology and quality. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 198–207

Kagawa R, Sano C, Nishijima M, Tazato N, Kiyuna T, Hayakawa N, Kawanobe W, Udagawa S, Tateishi T, Sugiyama J (2013) Investigation of acetic acid bacteria isolated from the Kitora tumulus in Japan and their involvement in the degradation of the plaster of the mural paintings. Stud Conserv 58:30–40

Kanbe C, Iwasa T, Sakasai T (1978) Production of organic acids by *Pedicoccus halophilis* in soy sauce fermentation. Nogeikagaku-Kaishi 52:329–334 (in Japanese)

Kantor A, Kacaniova M, Pochop J, Petrova J, Hleba L, Felsociova S, Pavelkova A, Rovna K, Bobkova A (2013) Lactic acid and acetic acid bacteria isolated from red wine. J Microbiol Biotechnol Food Sci 2:1704–1715

Kiryu T, Yamauchi K, Masuyama A, Ooe K, Kimura T, Kiso T, Nakano H, Murakami H (2012) Optimization of lactobionic acid production by *Acetobacter orientalis* isolated from Caucasian fermented milk, "Caspian Sea Yogurt". Biosci Biotechnol Biochem 76:361–363

Kobayashi H, Kohya S, Kawashima K, Kim W-S, Tanaka H, Motoki M, Kasamo K, Kusakabe I, Murakami K (1992) Deodorization of soybean protein with microorganisms. Biosci Biotechnol Biochem 56:530–531

Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC, Manala CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P (2014) *Acetobacter sicerae* sp. nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by *dnaK*, *groEL* and *rpoB* sequence analysis. Int J Syst Evol Microbiol 64:2407–2415

Lisdiyanti P, Katsura K, Potacharoen W, Navarro RR, Yamada Y, Uchimura T, Komagata K (2003) Diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines. Microbiol Cult Coll 19:91–99

Liu CH, Hsu WH, Lee FL, Lio CC (1996) The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during haipao fermentation. Food Microbiol 13:407–415

Mao YX (1998) Microorganisms. In: Shanghai Institute of Brewing Science (ed) Technology for fermented conditions. China Light Industry Press, Bejing, pp 14–24

Mazza S, Murooka Y (2009) Vinegars through the ages. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 17–40

Miyazaki S, Otsubo M, Aoki S, Sawatani T (1998) Application of agricultural bio-source to acetic acid fermentation. Kogyo Gijyutsu Kaigi Kenkyu Ronbunsyu 11:14–17 (in Japanese)

Moonmangmee D, Lorliam W, Moonmangmee S, Tanasupawat S (2008) Diversity of thermotolerant acetic acid bacteria from fermented foods isolated in Thailand. In: Abstracts, 2nd International Conference on Acetic Acid Bacteria, Nagoya, p 69

Murooka Y, Nanda K, Yamashita M (2009) Rice vinegars. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 121–133

Nanba T, Kato H (1983) Stimulative effects of miso and soy sauce for acetic acid fermentation. Nippon Shokuhin Kogyo Gakkaishi 30:191–199 (in Japanese)

Nanda K, Taniguchi M, Ujike S, Ishihara N, Mori H, Ono H, Murooka Y (2001) Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (komesu) and unpolished rice vinegar (kurosu) production in Japan. Appl Environ Microbiol 67:986–990

Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro AT, Kere C, Diawara B, Thonart P (2006) Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme Microb Technol 39:916–923

Ndoye B, Cleenwerck I, Engelbeen K, Dubois-Dauphin R, Guiro AT, Van Trappen S, Willems A, Thonart P (2007) *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

Noda F, Hayashi K, Mizunuma T (1982) Influence of pH on inhibitory activity of acetic acid on osmophilic yeasts used in brine fermentation of soy sauce. Appl Environ Microbiol 43:245–246

Okafor N (1975) Microbiology of Nigerian palm wine with particular reference to bacteria. J Appl Bacteriol 38:81–88

Okazaki S, Furukawa S, Ogihara H, Kawarai T, Kitada C, Komenou A, Yamasaki M (2010) Microbiological and biochemical survey on the transition of fermentative processes in Fukuyama pot vinegar brewing. J Gen Appl Microbiol 56:205–211

Ou ASM, Chang RC (2009) Taiwan fruit vinegar. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 223–242

Pandey M, Abeer MM, Amin MGI (2014) Dissolution study of bacterial cellulose (*nata de coco*) from local food industry: solubility behavior & structural changes. Int J Pharm Pharmaceut Sci 6:89–93

Parrondo J, Herrero M, Garcia LA, Diaz M (2003) Production of vinegar from whey. J Inst Brew 109:356–358

Saeki A (1993) Application of *Gluconobacter oxydans* subsp. *sphaericus* IFO12467 to vinegar production. J Ferment Bioeng 75:232–234

Schleputz T, Buchs J (2014) Scale-down of vinegar production into microtiter plates using a custom-made lid. J Biosci Bioeng 117:485–496

Sellmer-Wilsberg S (2009) Wine and grape vinegars. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 145–156

Sengum I Y, Karabiyikli S (2008) Microbiological profile of fig vinegar produced traditionally in Tarkey. In: Abstracts, 2nd international conference on acetic acid bacteria, Nagoya, p 23

Shim SM, Kim JY (2012) Profiling of fermentative metabolites in kimuchi: volatile and non-volatile organic acids. J Korean Soc Appl Biol Chem 55:463–469

Sievers M, Sellmer S, Teuber M (1992) *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst Appl Microbiol 15:386–392

Slapsak N, Cleenwerck I, De Vos P, Treck J (2013) *Gluconacetobacter maltaceti* sp. nov., a novel vinegar producing acetic acid bacterium. Syst Appl Microbiol 36:17–21

Snowdon JA, Cliver DO (1996) Microorganisms in honey. Int J Food Microbiol 31:1–26

Sokllek J, Hertel C, Hammes W (1998) Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. Int J Syst Bacteriol 48:935–940

Solieri L, Landi S, De Vero L, Guidici P (2006) Molecular assessment of industrial vinegar fermentation from traditional balsamic vinegar. J Appl Microbiol 101:63–71

Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014) *Acetobacter lambici* sp. nov., isolated from fermenting Lambic beer. Int J Syst Evol Microbiol 64:1083–1089

Suenaga H, Furuta M, Ohta S, Yamagushi T, Yamashita S (1993) Persimmon vinegar production from brewed persimmon using a horizontal rotary drum reactor. J Jpn Soc Food Sci Technol 40:225–227

Sun Y, Furusaki S (1990) Continuous production of acetic acid using immobilized *Acetobacter aceti* in a three-phase fluidized bed bioreactor. J Ferment Bioeng 69:102–110

Supakod S, Wongwicham A (2012) Cheap media for inoculation of acetic acid bacteria. Adv Mater Res 506:575–678

Tanasupawat S, Kommanee J, Yukphan P, Nakagawa Y, Yamada Y (2011) Identification of *Acetobacter* strains from Thai fermented rice products based on the 16S rRNA gene sequence and 16S-23S rRNA gene internal transcribed spacer restriction analyses. J Sci Food Agric 91:2652–2659

Tanino T, Nara Y, Tsujiguchi T, Ohshima T (2013) Coproduction of acetic acid electricity by application of microbial fuel cell technology to vinegar fermentation. J Biosci Bioeng 116:219–223

Tesfaye W, Morales ML, Garcia-Parrilla C, Troncoso A (2009) Jerez vinegar. In: Giudici P, Solieri L (eds) Vinegars of the world. Springer Italia, Milan, pp 179–195

Tokunaga H, Tanaka H, Hashiguchi K, Nagano M, Arakawa T, Tokunaga M (2009) Rapid detection of acetic acid bacteria in the traditional pot-fermented rice vinegar *Kurozu*. Food Sci Technol Res 15:587–590

Tomomatsu K, Tajiri T, Matsumoto K (1972) Determination of organic acid in Japanese-pickles. Kinki Univ Agric Kiyo 5:169–177 (in Japanese)

Uchimura T, Nishimura Y, Ohara N, Kozaki M (1991) Microorganisms in Luck pang used for vinegar production in Thailand: microorganisms in Chinese starters from Asia. J Brew Soc Jpn 86:62–67

Uzochukwu S, Balogh E, Tucknot OG, Lewis MJ, Ngoddy PO (1999) Role of palm wine yeasts and bacteria in palm wine aroma. J Food Sci Technol 36:301–304

Valla S (2008) Cellulose biosynthesis in *Gluconacetobacter xylinum* – its biochemistry, genetics and application. In: Abstracts, 2nd international conference on acetic acid bacteria, Nagoya, p 38

Wu JH (2004) The ecological distribution and productivity of microbe in Da Qu for Shanxi aged vinegar. Dissertation, Shanxi Agriculture University, PR China

Wu JJ, Gullo M, Chen FS, Giudici P (2010) Diversity of *Acetobacter pasteurianus* stains isolated from solid-state fermentation of cereal vinegars. Curr Microbiol 60:280–286

Chapter 3
Acetic Acid Bacteria in Fermented Food and Beverage Ecosystems

Vasileios Pothakos, Koen Illeghems, David Laureys, Freek Spitaels, Peter Vandamme, and Luc De Vuyst

Abstract Acetic acid bacteria (AAB) are mainly associated with the biotechnological process of vinegar and cellulose production, and although their occurrence in natural fermentation systems has been substantiated, their role remains rather unclear. Members of the *Acetobacteraceae* phylogenetic group have been reported in a range of spontaneous fermentations, such as the last phase of the cocoa bean fermentation process, the manufacturing of acidic beers, and several other slightly acidic beverages (e.g., kombucha, milk kefir, water kefir). In general, AAB are fastidious and obligate aerobes, although they can remain in a viable but nonculturable state when oxygen levels are low, and thus they are regarded as cumbersome to cultivate or isolate using laboratory media. Currently, with the implementation of culture-independent methods, as well as the introduction of metagenomics and high-throughput sequencing technologies, the abundance and functionalities of AAB in food commodities are being determined. Despite their sporadic isolation or incidence in fermentation ecosystems, the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* constitute the most frequently encountered taxa. The complete genome sequencing of strains of representative AAB species has elucidated the gene repertoires related to interesting metabolic features, facilitating the understanding of the key role of AAB in natural fermentation ecosystems.

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3.1 Introduction

Fermented foods and beverages have an important place in human nutrition worldwide, and therefore have been extensively studied throughout the years. Through the bioconversions of nutrients, the organoleptic profile of the food materials is enhanced, the shelf life of susceptible foodstuffs is extended, the availability of indispensable nutrients is improved, and the fermented end products may develop health-promoting properties. Yeasts, filamentous fungi, and lactic acid bacteria (LAB) are predominant in dairy, meat, cereal, alcoholic, and other food and beverage fermentations (Holzapfel and Wood 2014), but little is known about the occurrence and contribution of acetic acid bacteria (AAB) in natural fermentation ecosystems (Raspor and Goranovič 2008). AAB are ubiquitous, aerobic, Gram-negative bacteria belonging to the *Acetobacteraceae*, well adapted to carbohydrate/ethanol-rich commodities, and naturally occur in habitats associated with plants, flowers, and fruits (Bartowsky and Henschke 2008; Yamada and Yukphan 2008). Studies on microbe–insect interactions showed the establishment of symbiotic relationships between AAB and the midgut of numerous taxonomic orders of insects (Crotti et al. 2010; Shin et al. 2011). Apart from their environmental occurrence and the colonization of insect intestines, members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* are primarily found in food and beverage fermentation ecosystems (Raspor and Goranovič 2008). Representative examples are cocoa, milk kefir, water kefir, kombucha, and acidic beers.

3.2 AAB in Fermented Food Ecosystems

3.2.1 Cocoa

After fermentation and drying, cocoa beans, which represent the seeds of the *Theobroma cacao* L. tree, are the raw material for the production of different end products, such as cocoa powder, cocoa butter, and chocolate (Wood and Lass 2001). Manufacturing of these products occurs through a series of processes such as roasting, winnowing, and grinding of fermented dry cocoa beans. However, regardless of the end product, the first post-harvest process consists of a fermentation of the cocoa beans, which is typically carried out in heaps, boxes, baskets, sacks, or trays, or on platforms, in cocoa-producing countries around the Equator, depending on the farming practices applied and the cocoa-producing region involved (Fig. 3.1). The cocoa bean fermentation process is further influenced by

Fig. 3.1 Cocoa bean box fermentation is a commonly used method for fermentation of the cocoa pulp-bean mass. A cascade of three boxes of about 1 m^3 each is an optimal setup for this fermentation process. In a box fermentation process, fermentation of the cocoa pulp-bean mass starts in the upper box; every 24 h, the fermenting cocoa pulp-bean mass is transferred to a lower box. At the end of the fermentation process, the fermented cocoa beans are collected

the variety of the cocoa plant, the quality of the cocoa beans, the implemented farming practices, the harvesting season, and weather conditions (De Vuyst et al. 2010). The fermentation is responsible for the formation of colour and flavour precursors in the cocoa beans, as the result of penetration of acetic acid produced during the fermentation process into the cocoa beans, causing breakdown of the cocoa bean cell walls and membranes. This mechanism enables a variety of biochemical changes, such as sucrose and protein hydrolysis and complexation of polyphenols, the latter being responsible for reducing the astringent and unpleasant flavour present in fresh cocoa beans (Schwan and Wheals 2004; Thompson et al. 2007). Consequently, it is clear that the cocoa bean fermentation process makes a considerable contribution to flavour and colour development of the fermented dry cocoa beans and quality aspects associated with the products derived thereof.

Cocoa pods, the fruits of the cocoa tree, are opened after harvesting, as they contain the cocoa beans surrounded by a white, mucilaginous pulp. It is this carbohydrate-rich pulp that is the substrate for microbial fermentation and not the cocoa beans themselves, albeit that the composition of the latter changes, as already mentioned. The cocoa pulp consists mainly of water, carbohydrates (primarily sucrose, glucose, and fructose), pectin, and organic acids (predominantly citric acid), with smaller amounts of proteins, minerals, and oligoelements (De Vuyst et al. 2010; Schwan et al. 1995). The ratios of the carbohydrates depend on the

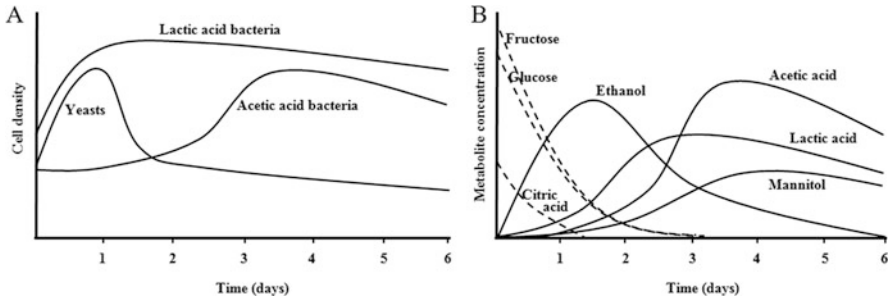

Fig. 3.2 Microbial succession (**a**) and metabolic activities (**b**) of the key factors in the cocoa bean fermentation ecosystem. *Dashed lines* (**b**) represent the substrates present in the cocoa pulp-bean mass; *full lines* represent metabolites produced by the microorganisms present

cocoa variety and the maturity of the cocoa pods. Indeed, sucrose is the main carbohydrate of the cocoa pulp in unripe pods, but it is hydrolysed by cocoa pulp invertase upon maturation.

3.2.1.1 Microbial Ecosystem of the Cocoa Bean Fermentation Process

Although the cocoa bean fermentation process is a spontaneous process, it is characterised by a fairly strict microbial ecosystem (De Vuyst et al. 2010; Schwan and Wheals 2004), which dominates this fermentation process at different stages (Fig. 3.2). In general, a specific succession of yeasts, LAB, and AAB results in well-fermented, high-quality cocoa beans, albeit other bacteria might also be involved (De Vuyst et al. 2010; Papalexandratou et al. 2013; Schwan and Wheals 2004).

3.2.1.2 Yeasts and LAB in Cocoa Bean Fermentation

The carbohydrate-rich cocoa pulp-bean mass has a low pH (pH 3.0–4.0) as a consequence of the presence of citric acid and remains anaerobic upon opening of the cocoa pods because of the tightening of the cocoa pulp-bean mass in the heaps or boxes (Schwan and Wheals 2004; Wood and Lass 2001). These conditions favour the growth of yeasts, possibly originating from the flowers and/or fruits, at the onset of the cocoa pulp-bean mass fermentation process (0–24 h; Fig. 3.2). The yeasts initiate an anaerobic alcoholic fermentation, thereby converting the available carbohydrates (mainly sucrose and glucose) into mainly ethanol and carbon dioxide (De Vuyst et al. 2010; Ho et al. 2013). Furthermore, pectinolytic enzymes produced by the yeasts are involved in the breakdown of the pectin macromolecules, allowing air ingress into the cocoa pulp-bean mass during fermentation as a result of reduction of viscosity and drainage of the cocoa pulp, thereby increasing its porosity (Ardhana and Fleet 2003; Schwan et al. 1997). Also, yeasts might be involved in the consumption of citrate (Schwan and Wheals 2004), although this

functionality has not been consistently found (Daniel et al. 2009; Jespersen et al. 2005). It has been shown that *Hanseniaspora opuntiae*, *H. guilliermondii*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *P. kudriavzevii*, and *Candida tropicalis* are most often the dominant yeast species (Ardhana and Fleet 2003; Ho et al. 2013; Jespersen et al. 2005; Nielsen et al. 2007), although other species might be present as well (Ardhana and Fleet 2003; Daniel et al. 2009; Illeghems et al. 2012; Jespersen et al. 2005; Nielsen et al. 2005, 2007, 2010; Papalexandratou and De Vuyst 2011).

The interplay of microaerobic conditions (from air ingress because of depectinisation), the presence of ethanol that inhibits the growth of the yeasts and the change in temperature and pH upon cocoa pulp-bean mass fermentation allows the growth of other microorganisms, in particular, bacteria such as LAB, which thrive under these altered conditions (24–72 h; Fig. 3.2). Indeed, LAB ferment carbohydrates (such as glucose and fructose) via the homofermentative pathway into lactic acid or via the heterofermentative pathway into lactic acid, acetic acid, or ethanol, and carbon dioxide (Camu et al. 2007; Lefeber et al. 2010). Fructose and citric acid, present in the cocoa pulp, can also be used by heterofermentative LAB as an alternative external electron acceptor, thereby producing mannitol and pyruvic/acetic acid, respectively. Citrate consumption contributes to a pH increase of the cocoa pulp. It has been proposed through simulated cocoa pulp-bean mass fermentations that LAB possessing one or more of these functional properties will be more adapted to and more competitive in the cocoa pulp-bean mass fermentation process and, hence, become the dominant strains (Adler et al. 2013; Lefeber et al. 2010, 2011b). At the onset of the cocoa bean fermentation process, a wide LAB species diversity is often found (Camu et al. 2007, 2008; Garcia-Armisen et al. 2010; Papalexandratou et al. 2011a, b, c, 2013), but the major fermentation process is carried out by a restricted number of LAB species, consisting mainly of heterofermentative members of the genus *Lactobacillus*, such as *Lactobacillus plantarum* and *L. fermentum* (Ardhana and Fleet 2003; Camu et al. 2007, 2008; Crafack et al. 2013; de Melo Pereira et al. 2012, 2013; Garcia-Armisen et al. 2010; Hamdouche et al. 2015; Ho et al. 2013; Kostinek et al. 2008; Lagunes-Gálvez et al. 2007; Lefeber et al. 2011a; Meersman et al. 2013; Nielsen et al. 2007; Papalexandratou et al. 2011a, b, c, 2013).

3.2.1.3 AAB in Cocoa Bean Fermentation

During the later stage of the cocoa bean fermentation process (36–112 h; Fig. 3.2), conditions become favourable for the growth of AAB, namely, a temperature increase above 37 °C, aeration caused by further breakdown of the cocoa pulp, and the availability of substrates such as ethanol (produced by the yeasts) and lactic acid, acetic acid, and mannitol (produced by the LAB) (Camu et al. 2007, 2008). The main activity of AAB is the (incomplete) oxidation of ethanol into acetic acid, an enzymatic two-step reaction carried out in the periplasm by membrane-bound pyrroloquinoline quinone (PQQ)-dependent dehydrogenases, enabling energy

Table 3.1 Documented metabolic activities of acetic acid bacteria (AAB) in different food and beverage fermentation ecosystems

Fermentation ecosystem	Metabolic activities	Effect on the fermentation process
Cocoa beans	Production of acetic acid (via ethanol oxidation)	Temperature increase of the fermentation mass
		Killing of the cocoa bean embryo
		Acidic flavour of the cocoa beans
	Overoxidation of acetic acid into carbon dioxide and water	Temperature increase of the fermentation mass
	Oxidation of lactic acid (conversion into pyruvate)	Formation of additional acetic acid, carbon dioxide, and acetoin
	Oxidation of mannitol	Formation of fructose (used by yeasts and LAB)
	Depletion of residual glucose	Formation of flavour precursors
Kombucha	Formation of a cellulose layer	Harborage of bacterial and fungal microbiota
	Formation of acetic acid, gluconic acid, and glucuronic acid	Characteristic acidic flavour
Lambic beers	Production of acetic acid (via ethanol oxidation)	Characteristic acidic flavour

production and tolerance toward acetic acid (Table 3.1). This acetic acid diffusing into the cocoa beans, together with the elevated temperature of the cocoa pulp-bean mass caused by this oxidation, is responsible for the death of the cocoa bean embryo, which marks the end of the fermentation process (De Vuyst et al. 2010). Next to periplasmic dehydrogenation, ethanol can be converted into acetate intracellularly, mainly to maintain the redox balance of the cell (Adler et al. 2014). *Acetobacter* and *Gluconacetobacter* species are able to overoxidise acetic acid (produced by LAB or AAB) into carbon dioxide via the tricarboxylic acid (TCA) cycle intracellularly, allowing production of extra adenosine triphosphate (ATP). Also, lactic acid can be converted into pyruvate intracellularly, which is then further oxidised into acetic acid, carbon dioxide, and water, or acetoin, depending on the environmental conditions (Adler et al. 2014; Lefeber et al. 2010; Moens et al. 2014). Mannitol can be oxidised into fructose by a membrane-bound dehydrogenase, thereby also allowing production of extra energy (Moens et al. 2014). Typically, glucose is depleted at the end of the cocoa bean fermentation process, as it is metabolised by both yeasts and LAB. However, it has been reported that residual glucose might be available for AAB, for example, in the case of a poor fermentation (De Vuyst et al. 2010; Papalexandratou et al. 2011a). This residual glucose might trigger growth of other AAB, such as glucose-fermenting *Gluconobacter* species, which can convert glucose into gluconates or other fermentation end products (De Vuyst et al. 2010).

The community diversity of AAB associated with the cocoa bean fermentation process is restricted, encompassing species of the genera *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* (Ardhana and Fleet 2003; Camu

et al. 2007, 2008; Garcia-Armisen et al. 2010; Lagunes-Gálvez et al. 2007; Nielsen et al. 2007). However, it is clear that *Acetobacter pasteurianus* is the principal AAB species found during cocoa bean fermentation processes worldwide (Ardhana and Fleet 2003; Camu et al. 2007, 2008; Crafack et al. 2013; de Melo Pereira et al. 2012; Garcia-Armisen et al. 2010; Hamdouche et al. 2015; Ho et al. 2013; Illeghems et al. 2012; Lefeber et al. 2011a; Meersman et al. 2013; Nielsen et al. 2007; Papalexandratou et al. 2011b, c, 2013). This occurrence can be ascribed to the metabolic features of this species related to the utilization of the available substrates in the cocoa pulp-bean mass, its thermotolerance, and its response mechanisms to acidic stress (Illeghems et al. 2013).

3.2.2 Milk Kefir

Milk kefir [Turkish neut. n.< keyif (= joy); aka "kiaphur," "keer," "kepi," "knapon," "kippi," etc.] is a fermented milk beverage, encountered in various geographic locations worldwide, made with milk inoculated with milk kefir grains (Ahmed et al. 2013). The milk kefir grains (harbouring the yeasts and the LAB responsible for the fermentation process) are white, elastic, opaque, and cauliflower-shaped structures consisting of water and water-insoluble kefiran, that is, a polysaccharide produced by the LAB extracellularly. Their real origin is unclear, although they have a history of more than 2000 years; however, they are believed to originate from the Caucasian Mountains (Farnworth 1999; Nielsen et al. 2014). Milk kefir grains serve as a starter culture in a back-slopping practice and are handed over from one person to another, as they increase in mass after each fermentation batch.

Traditionally, the milk kefir grains are brought into a vessel with milk; subsequently, the vessel is closed and left at room temperature for 24–48 h. At the end of the fermentation process, the mixture is sieved and the liquor obtained, that is, the fresh milk kefir, is ready for consumption (Ahmed et al. 2013; Nielsen et al. 2014). The liquor is a viscous, sparkling, and foaming beverage that has an acidic and alcoholic flavour. Apart from being a refreshing beverage, milk kefir is believed to have health-promoting properties (Ahmed et al. 2013; Chen et al. 2012; Maeda et al. 2004; Marsh et al. 2014a; Nielsen et al. 2014).

Milk originating from cattle, goats, and sheep can be used as the main substrate, with or without a preceding heat treatment. The disaccharide lactose, typical for animal milk, can be utilized as an energy source by the microorganisms and for kefiran production. Alternatively, milk substitutes such as walnut, coconut, rice, peanut, soy milk, and a cocoa pulp-based beverage have been used to make milk kefir (Cui et al. 2013; Kesenkas et al. 2011; Liu et al. 2002; Liu and Lin 2000; Nielsen et al. 2014; Puerari et al. 2012). However, these substrates need to be supplemented with glucose, galactose, or sucrose to stimulate the microbiota of the milk kefir grains, because these non-dairy substitutes tend to weaken the inoculum (Nielsen et al. 2014).

3.2.2.1 Yeasts and LAB in Milk Kefir Fermentation

The milk kefir microorganisms, being mainly yeasts and LAB, are predominantly found at the exterior of the grains (Magalhaes et al. 2011; Rea et al. 1996). The LAB counts in milk kefir reach levels of 8–9 log CFU/ml, and the most important members belong to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc*. LAB are responsible for the acidification of the liquor and the replenishing of the milk kefir grains (Nielsen et al. 2014; Pogacic et al. 2013; Ünal and Arslanoglu 2013). In particular, *Lactobacillus kefiri*, *L. parakefiri*, and *L. kefiranofaciens* seem to be fermentation-specific species for the production process of milk kefir (Chen et al. 2008; Dobson et al. 2011; Leite et al. 2012). Alternatively, yeast counts reach levels of 6–7 log CFU/ml, and the most frequently reported species belong to the genera *Saccharomyces*, *Kazachstania*, *Kluyveromyces*, *Torulaspora*, *Pichia*, *Lachancea*, and *Yarrowia* (Ahmed et al. 2013; Marsh et al. 2013; Nielsen et al. 2014). Additionally, bifidobacteria can be found in milk kefir (Dobson et al. 2011; Leite et al. 2012), but their counts are not consistent.

3.2.2.2 AAB in Milk Kefir Fermentation

AAB are occasionally found in milk kefir, although they are absent in several cases (Dobson et al. 2011; Leite et al. 2012; Takizawa et al. 1998; Witthuhn et al. 2004). However, some papers report AAB counts exceeding 8 log CFU/ml (Ahmed et al. 2013; Marsh et al. 2013; Motaghi et al. 1997; Witthuhn et al. 2005), whereby the AAB densities are generally below those of the yeasts and LAB. This great variability in AAB counts cannot be correlated with the organoleptic profiles of the concomitant milk kefirs, suggesting that AAB might not be necessary for a successful fermentation process. In contrast, their occurrence is sporadic and could be attributed to the fact that milk kefir constitutes a commodity that provides substrates (e.g., glucose, ethanol, and lactic acid) which favour AAB proliferation and, depending on the oxygen diffusion in the fermentation vessel, their growth can be triggered or suppressed. Commercial starter cultures for milk kefir have been developed, containing yeasts and LAB species but no AAB (Zeynep et al. 2009), corroborating the hypothesis that the contribution of AAB to milk kefir is minor. The most frequently identified AAB in milk kefir are allocated to the species *Acetobacter aceti* (Motaghi et al. 1997), *A. syzygii* (Kesmen and Kacmaz 2011; Miguel et al. 2010; Ünal and Arslanoglu 2013), *A. lovaniensis* (Ünal and Arslanoglu 2013), other *Acetobacter* species (Bergmann et al. 2010; Garrote et al. 2001; Leite et al. 2012; Marsh et al. 2014a; Puerari et al. 2012), and *Gluconobacter japonicus* (Miguel et al. 2010). Throughout storage of milk kefir, yeast and AAB counts remain constant at around 5–6 log CFU/ml, whereas LAB counts decrease after 7 days (Irigoyen et al. 2005). It has also been found that the acetoin concentration increases during milk kefir fermentation (Güzel-Seydim

et al. 2000), which could be attributed to AAB activity, apart from LAB (Moens et al. 2014). To assess the microbial diversities in the milk kefir ecosystem, a combination of culture-dependent and culture-independent techniques is required (Miguel et al. 2010; Ünal and Arslanoglu 2013).

3.2.3 Water Kefir

Water kefir (aka "ginger beer plants," "aquakefir," "Tibicos," "California bees," "ale nuts," "balm of Gilead," "Bèbées," or "Japanese beer seeds") is also found worldwide (Gulitz et al. 2013; Kebler 1921; Laureys and De Vuyst 2014; Lutz 1899; Pidoux 1989; Pidoux et al. 1988; Ward 1892). It constitutes a fermented beverage made with water, table sugar, (dried) fruits, and water kefir grains (Fig. 3.3) (Laureys and De Vuyst 2014; Gulitz et al. 2013). Similarly to milk kefir, water kefir grains serve as the inoculum for the water kefir fermentation process and are handed on from one individual to another. Water kefir grains are brittle, translucent, cauliflower-shaped structures that consist of water and insoluble dextran, that is, a glucan with contiguous α-(1-6) glycosidic linkages between the residues in the main chain and α-(1-3) branches (Pidoux 1989). The origin of water kefir grains is unclear, but it is speculated that they derive from the leaves of the *Opuntia* cactus (Lutz 1899). Water kefir is also considered beneficial for the health of the individuals who consume it (Marsh et al. 2014a; Moreira et al. 2008; Silva et al. 2009; Rodriguez and Denadra 1995).

Traditionally, water kefir grains are brought into a vessel with water, table sugar, and (dried) fruits, after which the vessel is closed and the mixture ferments at room temperature for 48–96 h. Then, the mixture is sieved, and the water kefir liquor obtained is a sparkling beverage that has a fruity, slightly acidic, and slightly alcoholic flavour. The fruits most commonly used for water kefir fermentation processes are dried figs and lemons. Dried figs provide nutrients (e.g., amino acids, vitamins, and minerals) and enhance the flavour of the beverage. Instead of dried figs, other (dried) fruits such as dates or apricots can be used (Gulitz et al. 2013; Stadie et al. 2013). Lemons are often added to improve the organoleptic properties of the water kefir liquor, but they are not necessary for the fermentation. Sometimes, other ingredients (e.g., tea and herbs) are added, depending on the recipe. Overall, sucrose is the main carbohydrate during the water kefir fermentation process, used as energy source by the microbiota involved and essential for the replenishing of the water kefir grain mass (Laureys and De Vuyst 2014).

3.2.3.1 Yeasts and LAB in Water Kefir Fermentation

The microbiota conducting the water kefir fermentation processes inhabit the external surface of the water kefir grains and are mainly assigned to yeast and LAB taxa (Laureys and De Vuyst 2014; Moinas et al. 1980). The most frequently

Fig. 3.3 Water kefir is a sparkling, fermented beverage made with water, table sugar, (dried) fruits, and water kefir grains that has a fruity, slightly acidic, and slightly alcoholic flavour

identified LAB belong to the species *Lactobacillus hilgardii*, *L. casei/paracasei*, *L. nagelii*, and *Leuconostoc mesenteroides/pseudomesenteroides* (Gulitz et al. 2011, 2013; Laureys and De Vuyst 2014; Stadie et al. 2013). Similarly to milk kefir, LAB are responsible for the production of lactic acid and the generation of additional water kefir grain mass (Laureys and De Vuyst 2014; Pidoux 1989; Pidoux et al. 1988; Waldherr et al. 2010). The most widespread yeast species are *Saccharomyces cerevisiae*, *Dekkera bruxellensis*, and *Zygotorulaspora florentina* (Laureys and De Vuyst 2014). Bifidobacteria can also be found in water kefir (Gulitz et al. 2013; Laureys and De Vuyst 2014).

3.2.3.2 AAB in Water Kefir Fermentation

Acetic acid bacteria (AAB) are either absent from water kefir fermentation processes (Laureys and De Vuyst 2014) or represent a major microbial community (Gulitz et al. 2011; Magalhaes et al. 2010). However, their counts are less than those of yeasts and LAB (Gulitz et al. 2011, 2013; Laureys and De Vuyst 2014; Marsh et al. 2013). Apparently, the presence of AAB is proportional to the oxygen diffused into the fermentation vessel. It has been reported that AAB can remain in a viable but nonculturable (VBNC) state under anaerobic conditions, being metabolically idle, and when oxygen becomes available they start to grow (Bartowsky and

Henschke 2008). In general, *Acetobacter* spp. seem to be better adapted to the water kefir ecosystem compared to *Gluconacetobacter* spp. (Marsh et al. 2013). Still, their role remains undetermined in terms of functionality and interaction with the other microbiota. Overall, *Acetobacter lovaniensis* (Magalhaes et al. 2010; Miguel et al. 2011), *A. fabarum* (Gulitz et al. 2011), *A. cerevisiae*, *A. aceti*, *A. ghanensis* (Stadie et al. 2013), *A. lovaniensis/fabarum* (Laureys and De Vuyst 2014), and *A. sicerae* (Li et al. 2014) have been found in water kefir fermentation ecosystems.

3.2.4 Kombucha

Kombucha (aka "Indischer teepilz," "tea fungus," "fungus japonicus," "Haipao," "theebier," "Cainii grib," "kambuha," "Manchurian mushroom," "Cainii kvass," or "Wolga jellyfish") is a traditional beverage. It occurs worldwide and is prepared with water, table sugar, tea, and a kombucha culture (Dufresne and Farnworth 2000; Hartmann et al. 2000; Jarrell et al. 2000; Mayser et al. 1995). Kombucha supposedly originates from Russia or China (Dufresne and Farnworth 2000; Mayser et al. 1995), although an ancient origin of the name may be from a Doctor "Kombu," who brought a tea ("cha" is the Japanese word for tea) from Korea to Japan as a cure for the digestive troubles of Emperor Ingyo (Dufresne and Farnworth 2000). However, there is some confusion about the name. Although the word kombucha was already used for a fermented tea beverage in a German report from 1928 (Hermann 1928), "kombucha" is the name for an algal tea in Japan. Japanese kombucha is an unfermented beverage made with water and "Dashi Kombu,, an edible brown algae (*Saccharina japonica*) cultivated in China, Japan, and Korea. In Japan, the kombucha discussed in this chapter is the fermented tea beverage called "Koucha-kinoko"; "kou-cha" is the Japanese word for English tea ("kou" stands for red tea and "cha" for green tea) and "kinoko" is the Japanese word for fungi. Nevertheless, to avoid confusion, the beverage that is discussed in this chapter is further referred to as kombucha, in accordance with the majority of the scientific literature about this fermented tea beverage. The floating kombucha layer that serves as the starter culture for the kombucha fermentation process is a tough, opaque, and grayish cellulose layer. The thickness of this layer increases with time and ranges from several millimeters to centimeters, resembling the cellulose layer formed during vinegar fermentations (Jarrell et al. 2000). Kombucha may possess several health-promoting effects (Hartmann et al. 2000; Jarrell et al. 2000; Jayabalan et al. 2014), albeit toxicity caused by contaminating molds has been reported (Currier et al. 1996; Perron et al. 1995).

The kombucha cellulose layer, which harbours the microbiota responsible for the fermentation process, is added to an open vessel containing tea and table sugar and subsequently left to ferment at room temperature for 1–3 weeks (Malbasa et al. 2006). Before consumption, the cellulose layer is removed and the liquor obtained has a very specific flavour, characterized by a sharp acidity. Black or green tea is mainly used for the fermentation process (Hoon et al. 2014), providing

nutrients (e.g., amino acids, vitamins, minerals) to the kombucha culture and contributing to the overall flavour of the beverage. Further, sugar is used as the energy source by the microorganisms and for the formation of the cellulose layer.

3.2.4.1 Yeasts and LAB in Kombucha Fermentation

The cellulose layer harbouring the kombucha culture is a symbiosis of bacteria and yeasts, but the exact microbial composition varies. A broad spectrum of yeast species can be found in kombucha, belonging to the genera *Saccharomyces*, *Zygosaccharomyces*, and *Dekkera* (Jayabalan et al. 2014; Marsh et al. 2014b). LAB can also be present (Marsh et al. 2014b), but a thorough analysis of the LAB species diversity in kombucha is currently lacking.

3.2.4.2 AAB in Kombucha Fermentation

The most characteristic microorganism in kombucha is believed to be *Komagataeibacter* (*K.*) *xylinus*, formerly *A. xylinum* and *Gluconacetobacter* (*Ga.*) *xylinus* (Jayabalan et al. 2014; Mikkelsen et al. 2009; Sievers et al. 1996). This AAB species is considered to be the key microorganism for the formation of the cellulose layer on the surface of the fermenting liquor (Kozaki et al. 1972; Nguyen et al. 2008). However, other AAB species occur in kombucha, such as *Gluconacetobacter kombuchae (Komagataeibacter kombuchae)* (Dutta and Gachhui 2007), *A. pasteurianus* (Liu et al. 1996; Yoshino et al. 1996), and *Komagataeibacter hansenii* (Dutta and Gachhui 2007; Tan et al. 2012), which could potentially produce cellulose. Next to these key microorganisms, a broad diversity of other AAB has been found in kombucha, such as *A. aceti* (Liu et al. 1996), *A. nitrogenifigens* (Dutta and Gachhui 2006), *Ka. intermedius* (Boesch et al. 1998), *Gluconacetobacter saccharivorans* (Wang et al. 2014; Yang et al. 2010), *Ga. sacchari*, *Acetobacter* spp., *Gluconobacter* spp., and *Gluconacetobacter* spp. (Hoon et al. 2014; Marsh et al. 2014b).

AAB are responsible for the main acidification in kombucha, producing acetic acid from ethanol (produced by the yeasts), gluconic acid, and glucuronic acid from glucose (released from sucrose by yeast invertase activity) (Dufresne and Farnworth 2000). Additionally, some *Gluconacetobacter* species originating from kombucha are very efficient in the production of D-saccharic acid 1,4-lactone from glucose (Yang et al. 2010). Lactic acid is not always found in kombucha and thus it is not regarded as a characteristic metabolite (Dufresne and Farnworth 2000). Similarly, citric acid is not a characteristic metabolic product for this beverage but may occur in kombucha (Jayabalan et al. 2008; Malbasa et al. 2011).

An important function of AAB during the kombucha fermentation process is the formation of the floating cellulose layer in which the microbial communities reside (Table 3.1). This floating cellulose layer can be produced by species of *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* from various carbon sources (Nguyen

et al. 2008). Glucose, fructose, and mannitol are the most efficient substrates (Mikkelsen et al. 2009; Nguyen et al. 2008).

Tea leaves are the source of nitrogen during kombucha fermentations. However, certain AAB isolated from kombucha, such as *A. nitrogenifigens* and *K. kombuchae*, are able to fix nitrogen from the atmosphere (Dutta and Gachhui 2006, 2007); this may be another important function of AAB during kombucha fermentation.

Concentrations of vitamins B_1, B_2, B_6, B_{12}, and C increase during kombucha fermentation (Bauer-Petrovska and Petrushevska-Tozi 2000; Jayabalan et al. 2014; Malbasa et al. 2011; Vitas et al. 2013), as well as the antioxidant activity and the phenol content of the tea. This change may be attributed to enzymes produced by the bacteria and/or yeasts present (Chu and Chen 2006; Jayabalan et al. 2014).

3.2.5 Acidic Beers

Beer is among the earliest historically fermented beverages and is a product of a fermented extract that is usually derived from malted barley or other grains (Briggs et al. 2004). Acidic lambic beers, obtained by a long (up to 3 years) spontaneous fermentation and maturation process that is traditionally carried out in wooden casks (Verachtert and Iserentant 1995), are probably the oldest known beers (De Keersmaecker 1996). They are typically brewed around the Senne River valley, an area near Brussels, during the colder months of the year, using 66 % malted barley and 33 % unmalted wheat, according to law regulations (Belgisch Ministerie van Economische Zaken 1993). The mixing of a young lambic beer (typically 1 year old), containing residual fermentable carbohydrates, with an old lambic beer (typically 3 years old) dominated by microbiota able to deplete the carbon sources, results in gueuze beers. These beers are subjected to refermentation in bottles, giving a carbonated end product with a complex flavour profile, constituting a trademark of traditional craftsmanship (Verachtert and Derdelinckx 2005; Spitaels et al. 2015a).

In response to the increasing worldwide interest in Belgian acidic beers, the American craft brewing industry has manufactured American coolship ales (ACA), resembling the lambic beer production method (Bokulich et al. 2012a). However, these beers are a seasonal product from classical breweries, which contrasts to traditional Belgian lambic breweries that exclusively produce lambic beers (Spitaels et al. 2014a).

3.2.5.1 Microbial Succession Patterns During Lambic Beer Fermentation

During the 3 years of the lambic beer fermentation process, a pattern of succession in microbial dominance and activities occurs. The evolution of the microbiota was outlined in the 1970s for the first time by means of culture-dependent approaches,

using biochemical tests for the identification of the bacterial and fungal species involved (Van Oevelen et al. 1977; Verachtert 1983; Verachtert and Dawoud 1984; Verachtert and Derdelinckx 2005). Recent studies with a polyphasic approach, combining culture-based and polymerase chain reaction (PCR) assay-based methods as well as identification techniques with high-throughput and discriminatory capacity [i.e., matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), 16S rRNA gene and housekeeping gene sequencing] delineated the key factors of lambic beer fermentation processes (Spitaels et al. 2014a). Additionally, the microbiota of an ACA fermentation process was primarily studied culture independently (Bokulich et al. 2012a).

In general, four distinct phases are identified in relationship to the microbial profile of acidic beers of spontaneous fermentation. The fermentation starts with members of *Enterobacteriaceae* (e.g., *Enterobacter* spp., *Escherichia/Shigella*, *Hafnia alvei*, *H. paralvei*, *Klebsiella pneumoniae*, *K. oxytoca*, *Citrobacter gillenii*, *Raoultella terrigena*), which are isolated during the first month of the fermentation process, as has been documented for both Belgian lambic beers and ACA beers (Bokulich et al. 2012a; Martens et al. 1991; Spitaels et al. 2014a; Van Oevelen et al. 1977). The consortium of enterobacteria is introduced into the wort while cooling overnight, a practice of traditional breweries, before the wort is transferred into the casks. Hence, these microorganisms originate from the brewery environment, as contamination can be attributed to the resident microbiota of the premise or it can be facilitated through air mediation (Spitaels et al. 2014a). After 1 month, the depletion of glucose, the accumulation of ethanol, and the unfavorable low pH of the wort suppress the growth of the enterobacteria (Martens et al. 1991). Artificial acidification of the wort at the start of the fermentation process, which is generally applied in today's breweries, results in the complete absence of enterobacteria (Spitaels et al. 2015b).

The second phase of the fermentation is referred to as the main or ethanol fermentation phase and extends until the fourth month. During this period, yeasts (e.g., *S. cerevisiae*, *S. pastorianus*, *Hanseniaspora* spp., *Naumovia castelli*, *Candida* spp., and *Dekkara bruxellensis*) are more frequently isolated (Spitaels et al. 2014a; Van Oevelen et al. 1977) along with LAB, such as members of the genera *Pediococcus* and *Lactobacillus* (Bokulich et al. 2012a; Spitaels et al. 2014a). During this phase, carbohydrates are converted into ethanol (yeasts) and lactic acid (LAB). The oxidative yeast species *Hanseniaspora uvarum* can co-occur with the enterobacteria phase (Bokulich et al. 2012a; Spitaels et al. 2014a). During the third phase, characterised by the depletion of carbohydrates, LAB and mainly *Pediococcus damnosus* produce large amounts of lactic acid, defining the acidification stage of the lambic beer fermentation process. Last, the maturation phase for lambic beers starts after 6 or 8 months, with the dominance of *D. bruxellensis* (formerly *Brettanomyces bruxellensis*) (Bokulich et al. 2012a; Spitaels et al. 2014a; Van Oevelen et al. 1977). AAB are recovered from lambic beer fermentation processes throughout the entire period, but their role and contribution are not yet elucidated. The recovery of AAB through plating is usually challenging, however, because of the VBNC state of the cells, during which they remain dormant. The

description of two novel species of AAB (i.e., *A. lambici* and *G. cerevisiae*) underpins as well the limited knowledge concerning the diversity and role of AAB in acidic beers (Spitaels et al. 2014b, c). Apparently, the geographic location and production technology have an impact on the microbial profile and patterns of succession during the long fermentation period (Bokulich et al. 2012a; Spitaels et al. 2014a, 2015a).

3.2.5.2 Sensorial Attributes of Acidic Beers

The unique organoleptic properties of lambic beers are mainly the acidic flavor, the reduced sweetness, and the fruity undertones (Verachtert and Derdelinckx 2005), characteristics of their technological aspects. Members of the *Enterobacteriaceae* family and oxidative yeasts consume carbohydrates in the beginning of the fermentation, favouring the production of 2,3-butanediol and acetic acid and the accumulation of ethanol, respectively (Martens et al. 1992). Subsequently, *Saccharomyces* spp. increase the levels of ethanol, whereas LAB are responsible for the accumulation of lactic acid. The use of unmalted wheat and the turbid mashing step results in a wort that is rich in malto-oligosaccharides or dextrins that are not fermentable by conventional *Saccharomyces* brewing yeasts (Shantha Kumara and Verachtert 1991). Instead, they select for *Dekkera* spp., which harbour α-glucosidase enzymes that facilitate the depletion of dextrins (Shantha Kumara et al. 1993). Further, during the maturation stage, AAB contribute to the production of acetic acid (Table 3.1), while *D. bruxellensis* generates aromatic esters (i.e., ethyl acetate and ethyl lactate), along with long-chain fatty acids, which are also found in 'aging' gueuze beers (Spaepen and Verachtert 1982; Spitaels et al. 2015a). Concomitantly, ethyl hexanoate and ethyl octanoate are produced by *D. bruxellensis*, contributing an overall fruity tone to the beers (Spaepen et al. 1978). After 1 year of fermentation, the density of the wort usually is about 3.5 ^{o}P, but the presence of *D. bruxellensis* and *P. damnosus* can lead to superattenuation up to <1 ^{o}P (Shantha Kumara and Verachtert 1991), which explains the lack of sweetness.

Practically, the core microbiota is responsible for the major characteristics of acidic beer fermentation processes. The application of novel techniques and exhaustive studying of this relatively unknown fermentation process give further insights into the complexity of lambic beer production processes (Spitaels et al. 2014a, 2015b).

3.2.6 (Meta)genomic Approaches on AAB in Fermented Food Ecosystems

Genomics, the study of the entire genome of an individual organism, and metagenomics, the study of the collection of genomes of members of an ecosystem,

have recently achieved great progress through the technological development in next-generation DNA sequencing. These novel applications facilitate massive parallel sequencing of short (environmental) DNA sequences, leading to new research opportunities in the field of genomics-based microbiology. Therefore, these approaches constitute promising tools to assess the microbiota of food fermentation processes, thereby enabling insights into the microbial diversity, the metabolic capacities, and other functional properties of the microorganisms involved (Ercolini 2013).

3.2.6.1 Genomic Studies of Food-Related AAB

Currently, only a few genome sequences of AAB strains are available, of which only nine genome sequences have been completed. The latter encompass *Acetobacter pasteurianus* (Azuma et al. 2009; Illeghems et al. 2013), *Acidiphilium (Ac.) cryptum*, *Ac. multivorum*, *Ga. diazotrophicus* (Bertalan et al. 2009), *G. oxydans* (Ge et al. 2013; Prust et al. 2005), *K. medellinensis* (formerly *Ga. medellinensis/xylinus*) (Ogino et al. 2011), and *K. xylinus* (formerly *Ga. xylinus*) (Ogino et al. 2011). The available genome sequences enabled insights into the metabolism and physiology of AAB (Deppenmeier and Ehrenreich 2009) and revealed their adaptation to different niches, such as micro-aerophilic conditions in the insect gut (Chouaia et al. 2014). Only a few genomic studies associated with AAB involved in food fermentation processes have been carried out: these encompass species of the genera *Acetobacter* (most species), *Gluconacetobacter*, and *Gluconobacter*.

Concerning the genus *Gluconobacter*, the species *G. oxydans*, which is often found in alcoholic beverages (Raspor and Goranovič 2008), is the only species that has been investigated using genomic approaches. Whole-genome sequencing of strains belonging to this species sheds light on their overall metabolism (*G. oxydans* 621H) (Prust et al. 2005) as well as on the mechanisms associated with specific biotechnological features, such as sorbose production (*G. oxydans* H24) (Ge et al. 2013). Information obtained from the genome sequence of *G. oxydans* 621H has been used to investigate specific important enzymes of this strain, such as pyruvate decarboxylase and several membrane-bound dehydrogenases, by the construction of gene-deletion mutants (Peters et al. 2013). Also, analysis of the cofactor metabolism of *G. oxydans* 621H by overproduction of its key enzymes in *Escherichia coli* was feasible using gene sequences derived from the genome sequence (Rauch et al. 2010). Last, the genome annotation of *G. oxydans* 621H facilitated the construction of knockout mutant strains with disrupted genes related to glucose metabolism (Wei et al. 2013).

Within the genus *Acetobacter*, several species originating from food ecosystems have been investigated based on their genome sequence. The finished genome sequence of *A. pasteurianus* 3283, originating from rice vinegar in Japan, was used to investigate its metabolic capacities, genetic stability, and mechanisms towards stress tolerance (Azuma et al. 2009). Later, the draft genome sequences

of *A. pasteurianus* NBRC 101655 and *A. pasteurianus* NBRC 3191 were determined and annotated with *A. pasteurianus* 3283 as a reference (Matsutani et al. 2012). A genome-wide phylogenetic relationship of these three strains was obtained to determine the core genome and the mechanisms of *A. pasteurianus* involved in thermotolerance (Matsutani et al. 2012). Also, the thermotolerant strain *A. pasteurianus* SKU1108 was used in adaptive mutation experiments, involving cultivation of this strain at high temperatures (Matsutani et al. 2013). The genome sequence of two thermo-adapted strains became available, revealing the genes that are essential for an improved thermotolerance (Matsutani et al. 2013). Finally, the complete genome sequence of the cocoa-specific functional starter culture strain *A. pasteurianus* 386B was compared with the aforementioned *Acetobacter pasteurianus* strains (Illeghems et al. 2013). This investigation revealed the metabolic features, niche adaptations, and tolerance towards stress conditions that make this strain an ideal candidate starter culture for controlled cocoa bean fermentation processes. To expand the knowledge of the metabolism of *A. aceti*, the draft genome sequence of *A. aceti* NBRC 14818, a strain originating from ethanol-based vinegar, was determined (Sakurai et al. 2011). Furthermore, the genomic data of *A. aceti* NBRC 14818 were used (i) to reveal the response of this strain to different carbon sources (Sakurai et al. 2011) and ethanol (Sakurai et al. 2012) by construction of a microarray and (ii) to investigate the role of the glyoxylate pathway by constructing knockout mutants (Sakurai et al. 2013). The draft genome sequence of *A. tropicalis* SKU1100, a strain originating from fruit from Thailand, was used to identify the genes involved in its thermotolerance (Soemphol et al. 2011). Additionally, a comparative genome analysis between the latter strain and the genome sequence of *A. pasteurianus* IFO 3283 revealed that the number of arginine-based salt bridges contributes to the thermotolerant character of *A. tropicalis* SKU1100 (Matsutani et al. 2011a).

Several genome sequences of *Gluconacetobacter* species have been determined and analysed. By comparative genome analysis of *Ga. xylinus* NBRC 3288, a strain originating from vinegar and unable to produce cellulose, with different cellulose-producing strains, the genes required for cellulose production were determined (Ogino et al. 2011). Also, whole-genome sequencing of four *Gluconacetobacter* spp. strains originating from red wine and spirit vinegar was performed to further understand the mechanisms contributing to their high acetic acid resistance (Andrés-Barrao et al. 2011).

A genome-wide phylogenetic analysis of the genera *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* has been conducted based on five genome sequences, underlining the phylogenetic relationship within AAB (Matsutani et al. 2011b).

3.2.6.2 16S rRNA Gene-Based Metagenomic Studies Involving AAB

16S rRNA gene-based metagenomics, also referred to as ‘metagenetics’ or ‘metabarcoding’ (Esposito and Kirschberg 2014), is based on the sequencing of (partial) 16S rRNA genes present in a metagenomic sample: this is achieved by

PCR, using primer sets that target the hypervariable regions of the 16S rRNA gene. Sequencing of amplicons of the 16S rRNA gene derived from complex bacterial communities is a widely used method to obtain insight into the community members and their abundance. An increasing number of food-related ecosystems are characterised with this method, such as spoiling meat (Ercolini et al. 2011; Nieminen et al. 2012), and various fermented foods, such as traditional Asian fermented foods (Kim and Park 2014), cheese (Masoud et al. 2011, 2012; Ercolini et al. 2012), and beer (Bokulich et al. 2012a).

Several of the studies mentioned here reported on the presence of AAB. Indeed, *Acetobacteraceae* were found on grapes used for wine production (mainly *Acetobacter*) (Bokulich et al. 2014), in botrytised wine fermentations (mainly *Gluconobacter*) (Bokulich et al. 2012b), as well as on grape marc, the solid parts of grapes used for the production of distilled beverages (Campanaro et al. 2014). In the latter study, several species of the genera *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* are found in the beginning of the fermentation process, after which they decrease rapidly as the anaerobic conditions develop. Other 16S rRNA gene-based metagenomic studies of alcoholic fermented beverages reported on the presence of AAB in pulque (*A. pomorum* and *G. oxydans*), a Mexican fermented agave juice (Escalante et al. 2004), and in makgeolli (*Acetobacter* sp.), a Korean rice beer (Jung et al. 2012). Further, the metagenomic approach revealed the presence of *Acetobacter* species in American coolship ales (Bokulich et al. 2012b).

AAB have also been found in nonalcoholic fermented beverages by means of marker gene-based metagenomic studies. For instance, 16S rRNA gene sequencing has revealed *Gluconacetobacter* as the dominating genus during kombucha fermentation, whereas *Acetobacter* spp. are less abundant (Marsh et al. 2014b). Also, several species within the genera *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* are present as background microbiota in water kefir (Gulitz et al. 2013; Marsh et al. 2013).

Last, state-of-the-art metagenomic sequencing techniques have revealed the presence of AAB in fermented dairy products. Indeed, the presence of *A. lovaniensis* in milk kefir has been found by 454 pyrosequencing (Leite et al. 2012), whereas this species has also been found together with *A. malorum* in kurut (Tibetan fermented yak milk) by construction of a 16S rRNA gene clone library (Liu et al. 2012). Similarly, a 16S rRNA gene clone library of samples from spontaneous cocoa bean fermentation processes carried out in Brazil and Ghana revealed the occurrence of the species *Gluconacetobacter* (Garcia-Armisen et al. 2010).

3.2.6.3 Whole-Genome Metagenomic Studies Involving AAB

In contrast to 16S rRNA gene-based metagenomic studies, whole-genome metagenomic studies of fermented food ecosystems, encompassing shotgun sequencing of all metagenomic DNA, are scarce.

Two whole-genome metagenomic studies report on the presence of *Proteobacteria*, that is, a puer tea fermentation process (Lyu et al. 2013) and a kimchi fermentation process (Jung et al. 2011). However, the presence of AAB was not reported, as the taxonomic resolution within the *Proteobacteria* was insufficient. Further, a metagenomic study of a sample of a Brazilian cocoa bean fermentation process revealed the presence of *A. pasteurianus* and *G. oxydans* at a time point during the fermentation process (30 h) at which they do not proliferate, indicating their survival under microaerobic conditions, the latter resulting from a decrease of the viscosity of the cocoa pulp, allowing ingress of oxygen (Illeghems et al. 2012, 2015). Despite the limited number of studies currently available, application of a metagenomic approach on food fermentation processes, encompassing both marker gene-based and whole-genome metagenomic sequencing, seems to be a robust tool for assessing both the species diversity and the functionality of AAB in various fermented foods and beverages.

References

Adler P, Bolten CJ, Dohnt K, Hansen CE, Wittmann C (2013) Core fluxome and metafluxome of lactic acid bacteria under simulated cocoa pulp fermentation conditions. Appl Environ Microbiol 79:5670–5681

Adler P, Frey LJ, Berger A, Bolten CJ, Hansen CE, Wittmann C (2014) The key to acetate: metabolic fluxes of acetic acid bacteria under cocoa pulp fermentation simulating conditions. Appl Environ Microbiol 80:4702–4716

Ahmed Z, Wang YP, Ahmad A, Khan ST, Nisa M, Ahmad H, Afreen A (2013) Kefir and health: a contemporary perspective. Crit Rev Food Sci Nutr 53:422–434

Andrés-Barrao C, Falquet L, Calderon-Copete SP, Descombes P, Pérez RO, Barja F (2011) Genome sequences of the high-acetic acid-resistant bacteria *Gluconacetobacter europaeus* LMG 18890^T and *G. europaeus* LMG 18494 (reference strains), *G. europaeus* 5P3, and *Gluconacetobacter oboediens* 174Bp2 (isolated from vinegar). J Bacteriol 193:2670–2671

Ardhana MM, Fleet GH (2003) The microbial ecology of cocoa bean fermentations in Indonesia. Int J Food Microbiol 86:87–99

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783

Bartowsky EJ, Henschke PA (2008) Acetic acid bacteria spoilage of bottled red wine: a review. Int J Food Microbiol 125:60–70

Bauer-Petrovska B, Petrushevska-Tozi L (2000) Mineral and water soluble vitamin content in the kombucha drink. Int J Food Sci Technol 35:201–205

Belgisch Ministerie van Economische Zaken (1993) Metrologische Reglementering. Koninklijk Besluit betreffende bier (BS 1993 06 04)

Bergmann RSD, Pereira MA, Veiga SMOM, Schneedorf JM, Oliveira NDS, Fiorini JE (2010) Microbial profile of a kefir sample preparations: grains in natural and lyophilized and fermented suspension. Cien Tecnol Aliment 30:1022–1026

Bertalan M, Albano R, de Padua V, Rouws L, Rojas C, Hemerly A, Teixeira K, Schwab S, Araujo J, Oliveira A, Franca L, Magalhaes V, Alqueres S, Cardoso A, Almeida W, Loureiro M, Nogueira E, Cidade D, Oliveira D, Simao T, Macedo J, Valadao A, Dreschsel M, Freitas F, Vidal M, Guedes H, Rodrigues E, Meneses C, Brioso P, Pozzer L (2009) Complete genome

sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics 10:450

Boesch C, Trcek J, Sievers M, Teuber M (1998) *Acetobacter intermedius*, sp. nov. Syst Appl Microbiol 21:220–229

Bokulich NA, Bamforth CW, Mills DA (2012a) Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. PLoS One 7:e35507

Bokulich NA, Joseph CM, Allen G, Benson AK, Mills DA (2012b) Next-generation sequencing reveals significant bacterial diversity of botrytized wine. PLoS One 7:e36357

Bokulich NA, Thorngate JH, Richardson PM, Mills DA (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci USA 111:E139–E148

Briggs DE, Boulton C, Brookers P, Stevens R (2004) Brewing science and practice. Woodhead, Cambridge

Campanaro S, Treu L, Vendramin V, Bovo B, Giacomini A, Corich V (2014) Metagenomic analysis of the microbial community in fermented grape marc reveals that *Lactobacillus fabifermentans* is one of the dominant species: insights into its genome structure. Appl Microbiol Biotechnol 98:6015–6037

Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, De Vuyst L (2007) Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl Environ Microbiol 73:1809–1824

Camu N, González Á, De Winter T, Van Schoor A, De Bruyne K, Vandamme P, Takrama JS, Addo SK, De Vuyst L (2008) Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. Appl Environ Microbiol 74:86–98

Chen HC, Wang SY, Chen MJ (2008) Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. Food Microbiol 25:492–501

Chen YP, Hsiao PJ, Hong WS, Dai TY, Chen MJ (2012) *Lactobacillus kefiranofaciens* M1 isolated from milk kefir grains ameliorates experimental colitis in vitro and in vivo. J Dairy Sci 95:63–74

Chouaia B, Gaiarsa S, Crotti E, Comandatore F, Degli Esposti M, Ricci I, Alma A, Favia G, Bandi C, Daffonchio D (2014) Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts. Genome Biol Evol 6:912–920

Chu SC, Chen CS (2006) Effects of origins and fermentation time on the antioxidant activities of kombucha. Food Chem 98:502–507

Crafack M, Mikkelsen MB, Saerens S, Knudsen M, Blennow A, Lowor S, Takrama J, Swiegers JH, Petersen GB, Heimdal H, Nielsen DS (2013) Influencing cocoa flavour using *Pichia kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. Int J Food Microbiol 167:103–116

Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, Sacchi L, Bourtzis K, Mandrioli M, Cherif A, Bandi C, Daffonchio D (2010) Acetic acid bacteria, newly emerging symbionts of insects. Appl Environ Microbiol 76:6963–6970

Cui XH, Chen SJ, Wang Y, Han JR (2013) Fermentation conditions of walnut milk beverage inoculated with kefir grains. Food Sci Technol 50:349–352

Currier RW, Goddard J, Buechler K, Quinlisk MP, Wolfe SL, Spencer, Carroll TJ, Bennett T, Stokes J (1996) Unexplained severe illness possibly associated with consumption of kombucha tea. J Am Med Assoc 275:96–98

Daniel H-M, Vrancken G, Takrama JF, Camu N, De Vos P, De Vuyst L (2009) Yeast diversity of Ghanaian cocoa bean heap fermentations. FEMS Yeast Res 9:774–783

De Keersmaecker J (1996) The mystery of lambic beer. Sci Am 275:74–81

de Melo Pereira GV, Miguel MGCP, Ramos CL, Schwan RF (2012) Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast

and bacterial strains to develop a defined starter culture. Appl Environ Microbiol 78:5395–5405

de Melo Pereira GV, Magalhães KT, de Almeida EG, da Silva Coelho I, Schwan RF (2013) Spontaneous cocoa bean fermentation carried out in a novel-design stainless steel tank: influence on the dynamics of microbial populations and physical-chemical properties. Int J Food Microbiol 161:121–133

De Vuyst L, Lefeber T, Papalexandratou Z, Camu N (2010) The functional role of lactic acid bacteria in cocoa bean fermentation. In: Mozzi F, Raya RR, Vignolo GM (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Ames, pp 301–325

Deppenmeier U, Ehrenreich A (2009) Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. J Mol Microbiol Biotechnol 16:69–80

Dobson A, O'Sullivan O, Cotter PD, Ross P, Hill C (2011) High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. FEMS Microbiol Lett 320:56–62

Dufresne C, Farnworth E (2000) Tea, kombucha, and health: a review. Food Res Int 33:409–421

Dutta D, Gachhui R (2006) Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from kombucha tea. Int J Syst Evol Microbiol 56:1899–1903

Dutta D, Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from kombucha tea. Int J Syst Evol Microbiol 57:353–357

Ercolini D (2013) High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl Environ Microbiol 79:3148–3155

Ercolini D, Ferrocino I, Nasi A, Ndagijimana M, Vernocchi P, La Storia A, Laghi L, Mauriello G, Guerzoni ME, Villani F (2011) Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. Appl Environ Microbiol 77:7372–7381

Ercolini D, De Filippis F, La Storia A, Iacono M (2012) "Remake" by high-throughput sequencing of the microbiota involved in the production of water buffalo mozzarella cheese. Appl Environ Microbiol 78:8142–8145

Escalante A, Elena Rodríguez M, Martínez A, López-Munguía A, Bolívar F, Gosset G (2004) Characterization of bacterial diversity in Pulque, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. FEMS Microbiol Lett 235:273–279

Esposito A, Kirschberg M (2014) How many 16S-based studies should be included in a metagenomic conference? It may be a matter of etymology. FEMS Microbiol Lett 351:145–146

Farnworth ER (1999) Kefir: from folklore to regulatory approval. J Nutraceuticals Funct Med Foods 1:57–68

Garcia-Armisen T, Papalexandratou Z, Hendryckx H, Camu N, Vrancken G, De Vuyst L, Cornelis P (2010) Diversity of the total bacterial community associated with Ghanaian and Brazilian cocoa bean fermentation samples as revealed by a 16S rRNA gene clone library. Appl Microbiol Biotechnol 87:2281–2292

Garrote GL, Abraham AG, De Antoni GL (2001) Chemical and microbiological characterisation of kefir grains. J Dairy Res 68:639–652

Ge X, Zhao Y, Hou W, Zhang W, Chen W, Wang J, Zhao N, Lin J, Wang W, Chen M, Wang Q, Jiao Y, Yuan Z, Xiong X (2013) Complete genome sequence of the industrial strain *Gluconobacter oxydans* H24. Genome Announc 1:3–13

Gulitz A, Stadie J, Wenning M, Ehrmann MA, Vogel RF (2011) The microbial diversity of water kefir. Int J Food Microbiol 151:284–288

Gulitz A, Stadie J, Ehrmann MA, Ludwig W, Vogel RF (2013) Comparative phylobiomic analysis of the bacterial community of water kefir by 16S rRNA gene amplicon sequencing and ARDRA analysis. J Appl Microbiol 114:1082–1091

Güzel-Seydim ZB, Seydim AC, Greene AK, Bodine AB (2000) Determination of organic acids and volatile flavor substances in kefir during fermentation. J Food Compos Anal 13:35–43

Hamdouche Y, Guehi T, Durand N, Kedjebo KBD, Montet D, Meile JC (2015) Dynamics of microbial ecology during cocoa fermentation and drying: towards the identification of molecular markers. Food Control 48:117–122

Hartmann AM, Burleson LE, Holmes AK, Geist CR (2000) Effects of chronic kombucha ingestion on open-field behaviors, longevity, appetitive behaviors, and organs in C57-BL/6 mice: a pilot study. Nutrition 16:755–761

Hermann S (1928) Uber die sogenannte "Kombucha.". I Biochem Z 192:176–187

Ho VTT, Zhao J, Fleet G (2013) Yeasts are essential for cocoa bean fermentation. Int J Food Microbiol 174:72–87

Holzapfel WM, Wood BJB (eds) (2014) Lactic acid bacteria: biodiversity and taxonomy. Wiley-Blackwell, Chichester

Hoon LY, Choo C, Watawana MI, Jayawardena N, Waisundara VY (2014) Kombucha 'tea fungus' enhances the tea polyphenol contents, antioxidant activity and alpha-amylase inhibitory activity of five commonly consumed teas. J Funct Foods. doi:10.1016/j.jff.2014.07.010

Illeghems K, De Vuyst L, Papalexandratou Z, Weckx S (2012) Phylogenetic analysis of a spontaneous cocoa bean fermentation metagenome reveals new insights into its bacterial and fungal community diversity. PLoS One 7:e38040

Illeghems K, Weckx S, De Vuyst L (2015) Applying meta-pathway analyses through metagenomics to identify the functional properties of the major bacterial communities of a single spontaneous cocoa bean fermentation process sample. Food Microbiol 50:54–63

Illeghems K, De Vuyst L, Weckx S (2013) Complete genome sequence and comparative analysis of *Acetobacter pasteurianus* 386B, a strain well-adapted to the cocoa bean fermentation ecosystem. BMC Genomics 14:526

Irigoyen A, Arana I, Castiella M, Torre P, Ibanez FC (2005) Microbiological, physicochemical, and sensory characteristics of kefir during storage. Food Chem 90:613–620

Jarrell J, Cal T, Bennet JW (2000) The kombucha consortia of yeasts and bacteria. Mycologist 14:166–170

Jayabalan R, Subathradevi P, Marimuthu S, Sathishkumar M, Swaminathan K (2008) Changes in free-radical scavenging ability of kombucha tea during fermentation. Food Chem 109:227–234

Jayabalan R, Malbasa RV, Loncar ES, Vitas JS, Sathishkumar M (2014) A review on kombucha tea: microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Compr Rev Food Sci Food 13:538–550

Jespersen L, Nielsen DS, Hønholt S, Jakobsen M (2005) Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Res 5:441–453

Jung JY, Lee SH, Kim JM, Park MS, Bae J-W, Hahn Y, Madsen EL, Jeon CO (2011) Metagenomic analysis of kimchi, a traditional Korean fermented food. Appl Environ Microbiol 77:2264–2274

Jung M-J, Nam Y-D, Roh SW, Bae J-W (2012) Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. Food Microbiol 30:112–123

Kebler LF (1921) California bees. J Am Pharm Assoc 10:939–943

Kesenkas H, Dinkci N, Seckin K et al (2011) Antioxidant properties of kefir produced from different cow and soy milk mixtures. J Agric Sci 17:253–259

Kesmen Z, Kacmaz N (2011) Determination of lactic microflora of kefir grains and kefir beverage by using culture-dependent and culture-independent methods. J Food Sci 76:276–283

Kim M-S, Park E-J (2014) Bacterial communities of traditional salted and fermented seafoods from Jeju Island of Korea using 16S rRNA gene clone library analysis. J Food Sci 79:927–934

Kostinek M, Ban-Koffi L, Ottah-Atikpo M, Teniola D, Schillinger U, Holzapfel W, Franz C (2008) Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. Curr Microbiol 56:306–314

Kozaki M, Kitahara K, Koizumi A (1972) Microorganisms of zoogloeal mats formed in tea decoction. Food Hyg Safety Sci 13:89–96

Lagunes-Gálvez S, Loiseau G, Paredes JL, Barel M, Guiraud J-P (2007) Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. Int J Food Microbiol 114:124–130

Laureys D, De Vuyst L (2014) Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation. Appl Environ Microbiol 80:2564–2572

Lefeber T, Janssens M, Camu N, De Vuyst L (2010) Kinetic analysis of strains of lactic acid bacteria and acetic acid bacteria in cocoa pulp simulation media toward development of a starter culture for cocoa bean fermentation. Appl Environ Microbiol 76:7708–7716

Lefeber T, Gobert W, Vrancken G, Camu N, De Vuyst L (2011a) Dynamics and species diversity of communities of lactic acid bacteria and acetic acid bacteria during spontaneous cocoa bean fermentation in vessels. Food Microbiol 28:457–464

Lefeber T, Janssens M, Moens F, Gobert W, De Vuyst L (2011b) Interesting starter culture strains for controlled cocoa bean fermentation revealed by simulated cocoa pulp fermentations of cocoa-specific lactic acid bacteria. Appl Environ Microbiol 77:6694–6698

Leite AMO, Mayo B, Rachid CTCC, Peixoto RS, Silva JT, Paschoalin VMF, Delgado S (2012) Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiol 31:215–221

Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC, Manaia CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P (2014) *Acetobacter sicerae* sp. nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by *dna*K, *gro*EL and *rpo*B sequence analysis. Int J Syst Evol Microbiol 64:2407–2415

Liu JR, Lin CW (2000) Production of kefir from soymilk with or without added glucose, lactose, or sucrose. J Food Sci 65:716–719

Liu CH, Hsu WH, Lee FL, Liao CC (1996) The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. Food Microbiol 13:407–415

Liu JR, Wang SY, Lin YY, Lin CW (2002) Antitumor activity of milk kefir and soy milk kefir in tumor-bearing mice. Nutr Cancer 44:182–187

Liu WJ, Sun ZH, Zhang YB, Zhang CL, Menghebilige, Yang M, Sun TS, Bao QH, Chen W, Zhang HP (2012) A survey of the bacterial composition of kurut from Tibet using a culture-independent approach. J Dairy Sci 95:1064–1072

Lutz ML (1899) Recherches biologiques sur la constitution du Tibi. Bull Soc Mycol France 15:68–72

Lyu C, Chen C, Ge F, Liu D, Zhao S, Chen D (2013) A preliminary metagenomic study of puer tea during pile fermentation. J Sci Food Agric 93:3165–3174

Maeda H, Zhu X, Omura K, Suzuki S, Kitamura S (2004) Effects of an exopolysaccharide (kefiran) on lipids, blood pressure, blood glucose, and constipation. Biofactors 22:197–200

Magalhaes KT, Pereira GVD, Dias DR, Schwan RF (2010) Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J Microbiol Biotechnol 26:1241–1250

Magalhaes KT, Pereira GVD, Campos CR, Dragone G, Schwan RF (2011) Brazilian kefir: structure, microbial communities and chemical composition. Braz J Microbiol 42:693–702

Malbasa R, Loncar E, Djuric M, Klasnja M, Kolarov LJ, Markov S (2006) Scale-up of black tea batch fermentation by kombucha. Food Bioprod Process 84:193–199

Malbasa RV, Loncar ES, Vitas JS, Canadanovic-Brunet JM (2011) Influence of starter cultures on the antioxidant activity of kombucha beverage. Food Chem 127:1727–1731

Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD (2013) Sequence-based analysis of the microbial composition of water kefir from multiple sources. FEMS Microbiol Lett 348:79–85

Marsh AJ, Hill C, Ross RP, Cotter PD (2014a) Fermented beverages with health-promoting potential: past and future perspectives. Trends Food Sci Technol 38:113–124

Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD (2014b) Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. Food Microbiol 38:171–178

Martens H, Dawoud E, Verachtert H (1991) Wort enterobacteria and other microbial populations involved during the first month of lambic fermentation. J Inst Brew 97:435–439

Martens H, Dawoud E, Verachtert H (1992) Synthesis of aroma compounds by wort enterobacteria during the first stage of lambic fermentation. J Inst Brew 98:421–425

Masoud W, Takamiya M, Vogensen FK, Lillevang S, Abu Al-Soud W, Sørensen SJ, Jakobsen M (2011) Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. Int Dairy J 21:142–148

Masoud W, Vogensen FK, Lillevang S, Abu Al-Soud W, Sørensen SJ, Jakobsen M (2012) The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. Int J Food Microbiol 153:192–202

Matsutani M, Hirakawa H, Nishikura M, Soemphol W, Ali IAI, Yakushi T, Matsushita K (2011a) Increased number of arginine-based salt bridges contributes to the thermotolerance of thermotolerant acetic acid bacteria, *Acetobacter tropicalis* SKU1100. Biochem Biophys Res Commun 409:120–124

Matsutani M, Hirakawa H, Yakushi T, Matsushita K (2011b) Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*. FEMS Microbiol Lett 315:122–128

Matsutani M, Hirakawa H, Saichana N, Soemphol W, Yakushi T, Matsushita K (2012) Genome-wide phylogenetic analysis of differences in thermotolerance among closely related *Acetobacter pasteurianus* strains. Microbiology 158:229–239

Matsutani M, Nishikura M, Saichana N, Hatano T, Masud-Tippayasak U, Theergool G, Yakushi T, Matsushita K (2013) Adaptive mutation of *Acetobacter pasteurianus* SKU1108 enhances acetic acid fermentation ability at high temperature. J Biotechnol 165:109–119

Mayser P, Fromme S, Leitzmann C, Grunder K (1995) The yeast spectrum of the 'tea fungus kombucha'. Mycoses 38:289–295

Meersman E, Steensels J, Mathawan M, Wittocx P-J, Saels V, Struyf N, Bernaert H, Vrancken G, Verstrepen KJ (2013) Detailed analysis of the microbial population in Malaysian spontaneous cocoa pulp fermentations reveals a core and variable microbiota. PLoS One 8:e81559

Miguel MGDP, Cardoso PG, Lago LD, Schwan RF (2010) Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods. Food Res Int 43:1523–1528

Miguel MGDP, Cardoso PG, Magalhaes KT, Schwan RF (2011) Profile of microbial communities present in Tibico (sugary kefir) grains from different Brazilian states. World J Microbiol Biotechnol 27:1875–1884

Mikkelsen D, Flanagan BM, Dykes GA, Gidley MJ (2009) Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* strain ATCC 53524. J Appl Microbiol 107:576–583

Moens F, Lefeber T, De Vuyst L (2014) Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. Appl Environ Microbiol 80:1848–1857

Moinas M, Horisberger M, Bauer H (1980) The structural organization of the Tibi grain as revealed by light, scanning and transmission microscopy. Arch Microbiol 128:157–161

Moreira ME, Dos Santos MH, Zolini GP, Wouters AT, Carvalho JC, Schneedorf JM (2008) Anti-inflammatory and cicatrizing activities of a carbohydrate fraction isolated from sugary kefir. J Med Food 11:356–361

Motaghi M, Mazaheri M, Moazami N, Farkhondeh A, Fooladi MH, Goltapeh EM (1997) Kefir production in Iran. World J Microbiol Biotechnol 13:579–581

Nguyen VT, Flanagan B, Gidley MJ, Dykes GA (2008) Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from kombucha. Curr Microbiol 57:449–453

Nielsen DS, Hønholt S, Tano-Debrah K, Jespersen L (2005) Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). Yeast 22:271–284

Nielsen DS, Teniola OD, Ban-Koffi L, Owusu M, Andersson TS, Holzapfel WH (2007) The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. Int J Food Microbiol 114:168–186

Nielsen DS, Jakobsen M, Jespersen L (2010) *Candida halmiae* sp. nov., *Geotrichum ghanense* sp. nov. and *Candida awuaii* sp. nov., isolated from Ghanaian cocoa fermentations. Int J Syst Evol Microbiol 60:1460–1465

Nielsen B, Gurakan GC, Unlu G (2014) Kefir: a multifaceted fermented dairy product. Probiotics Antimicrob Prot 6:123–135

Nieminen TT, Välitalo H, Säde E, Paloranta A, Koskinen K, Björkroth J (2012) The effect of marination on lactic acid bacteria communities in raw broiler fillet strips. Front Microbiol 3:1–8

Ogino H, Azuma Y, Hosoyama A, Nakazawa H, Matsutani M, Hasegawa A, Otsuyama K-I, Matsushita K, Fujita N, Shirai M (2011) Complete genome sequence of NBRC 3288, a unique cellulose-nonproducing strain of *Gluconacetobacter xylinus* isolated from vinegar. J Bacteriol 193:6997–6998

Papalexandratou Z, De Vuyst L (2011) Assessment of the yeast species composition of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis. FEMS Yeast Res 11:564–574

Papalexandratou Z, Camu N, Falony G, De Vuyst L (2011a) Comparison of the bacterial species diversity of spontaneous cocoa bean fermentations carried out at selected farms in Ivory Coast and Brazil. Food Microbiol 28:964–973

Papalexandratou Z, Falony G, Romanens E, Jimenez JC, Amores F, Daniel H-M, De Vuyst L (2011b) Species diversity, community dynamics, and metabolite kinetics of the microbiota associated with traditional Ecuadorian spontaneous cocoa bean fermentations. Appl Environ Microbiol 77:7698–7714

Papalexandratou Z, Vrancken G, De Bruyne K, Vandamme P, De Vuyst L (2011c) Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria. Food Microbiol 28:1326–1338

Papalexandratou Z, Lefeber T, Bahrim B, Lee OS, Daniel H-M, De Vuyst L (2013) *Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process. Food Microbiol 35:73–85

Perron AD, Patterson JA, Yanofsky NN (1995) Kombucha mushroom hepatotoxicity. Ann Emerg Med 26:660–661

Peters B, Junker A, Brauer K, Mühlthaler B, Kostner D, Mientus M, Liebl W, Ehrenreich A (2013) Deletion of pyruvate decarboxylase by a new method for efficient markerless gene deletions in *Gluconobacter oxydans*. Appl Microbiol Biotechnol 97:2521–2530

Pidoux M (1989) The microbial flora of sugary kefir grain (the gingerbeer plant) – biosynthesis of the grain from *Lactobacillus hilgardii* producing a polysaccharide gel. J Appl Microbiol 5:223–238

Pidoux M, Brillouet JM, Quemener B (1988) Characterization of the polysaccharides from a *Lactobacillus brevis* and from sugary kefir grains. Biotechnol Lett 10:415–420

Pogacic T, Sinko S, Zamberlin S, Samarzija D (2013) Microbiota of kefir grains. Mljekarstvo 63:3–14

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Puerari C, Magalhaes KT, Schwan RF (2012) New cocoa pulp-based kefir beverages: microbiological, chemical composition and sensory analysis. Food Res Int 48:634–640

Raspor P, Goranovič D (2008) Biotechnological applications of acetic acid bacteria. Crit Rev Biotechnol 28:101–124

Rauch B, Pahlke J, Schweiger P, Deppenmeier U (2010) Characterization of enzymes involved in the central metabolism of *Gluconobacter oxydans*. Appl Microbiol Biotechnol 88:711–718

Rea MC, Lennartsson T, Dillon P, Drinan FD, Reville WJ, Heapes M, Cogan TM (1996) Irish kefir-like grains: their structure, microbial composition, and fermentation kinetics. J Appl Bacteriol 81:83–94

Rodriguez AV, Denadra MCM (1995) Mixed culture of *Lactobacillus hilgardii* and *Leuconostoc oenos* isolated from Argentine wine. J Appl Bacteriol 78:521–525

Sakurai K, Arai H, Ishii M, Igarashi Y (2011) Transcriptome response to different carbon sources in *Acetobacter aceti*. Microbiology 157:899–910

Sakurai K, Arai H, Ishii M, Igarashi Y (2012) Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. J Biosci Bioeng 113:343–348

Sakurai K, Yamazaki S, Ishii M, Igarashi Y, Arai H (2013) Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. J Biosci Bioeng 115:32–36

Schwan RF, Wheals AE (2004) The microbiology of cocoa fermentation and its role in chocolate quality. Crit Rev Food Sci Nutr 44:205–221

Schwan RF, Rose AH, Board RG (1995) Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. J Appl Bacteriol (Symp Suppl) 79:96–107

Schwan RF, Cooper RM, Wheals AE (1997) Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. Enzyme Microb Technol 21:234–244

Shantha Kumara HMC, Verachtert H (1991) Identification of lambic super-attenuating microorganisms by the use of selective antibiotics. J Inst Brew 97:181–185

Shantha Kumara HMC, De Cort S, Verachtert H (1993) Localization and characterization of α-glucosidase activity in *Brettanomyces lambicus*. Appl Environ Microbiol 59:2352–2358

Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon J-H, Ryu J-H, Lee W-J (2011) *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334:670–674

Sievers M, Lanini C, Weber A, SchulerSchmid U, Teuber M (1996) Microbiology and fermentation balance in a kombucha beverage obtained from a tea fungus fermentation. Syst Appl Microbiol 18:590–594

Silva KR, Rodrigues SA, Xavier L, Lima AS (2009) Antimicrobial activity of broth fermented with kefir grains. Appl Biochem Biotechnol 152:316–325

Soemphol W, Deeraksa A, Matsutani M, Yakushi T, Toyama H, Adachi O, Yamada M, Matsushita K (2011) Global analysis of the genes involved in the thermotolerance mechanism of thermotolerant *Acetobacter tropicalis* SKU1100. Biosci Biotechnol Biochem 75:1921–1928

Spaepen M, Verachtert H (1982) Esterase activity in the genus *Brettanomyces*. J Inst Brew 88:11–17

Spaepen M, Van Oevelen D, Verachtert H (1978) Fatty acids and esters produced during the sponatneous fermentation of lambic and gueuze. J Inst Brew 84:278–282

Spitaels F, Wieme AD, Janssens M, Aerts M, Daniel H-M, Van Landschoot A, De Vuyst L, Vandamme P (2014a) The microbial diversity of traditional spontaneously fermented lambic beer. PLoS One 9:e95384

Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014b) *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. Int J Syst Evol Microbiol 64:1083–1089

Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014c) *Gluconobacter cerevisiae* sp. nov., isolated from the brewery environment. Int J Syst Evol Microbiol 64:1134–1141

Spitaels F, Van Kerrebroeck S, Wieme AD, Snauwaert I, Aerts M, Van Landschoot A, De Vuyst L, Vandamme P (2015a) Microbiota and metabolites of aged bottled gueuze beers converge to the same composition. Food Microbiol 47:1–11

Spitaels F, Wieme A, Janssens M, Aerts M, Van Landschoot A, De Vuyst L, Vandamme P (2015b) The microbial diversity of an industrially produced lambic beer shares members of a

traditionally produced lambic beer and reveals a core microbiota for lambic beer fermentation. Food Microbiol 49:23–32
Stadie J, Gulitz A, Ehrmann MA, Vogel RF (2013) Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. Food Microbiol 35:92–98
Takizawa S, Kojima S, Tamura S, Fujinaga S, Benno Y, Nakase T (1998) The composition of the *Lactobacillus* flora in kefir grains. Syst Appl Microbiol 21:121–127
Tan LL, Ren L, Cao YY, Chen XL, Tang XY (2012) Bacterial cellulose synthesis in kombucha by *Gluconacetobacter* sp. and *Saccharomyces* sp. Adv Mater Res 554–556:1000–1003
Thompson SS, Miller KB, Lopez AS (2007) Cocoa and coffee. In: Doyle MP, Beuchat LR (eds) Food microbiology: fundamentals and frontiers, 3rd edn. American Society for Microbiology, Washington, DC, pp 837–849
Ünal BÜ, Arslanoglu A (2013) Phylogenetic identification of bacteria within kefir by both culture-dependent and culture-independent methods. Afr J Microbiol Res 7:4533–4538
Van Oevelen D, Spaepen M, Timmermans P, Verachtert H (1977) Microbiological aspects of spontaneous wort fermentation in the production of lambic and gueuze. J Inst Brew 83:356–360
Verachtert H (1983) De spontane geuzegisting – La fermentation spontanée de la gueuze. Cerevisia Belg J Brew Biotechnol 8:41–48
Verachtert H, Dawoud E (1984) Microbiology of lambic-type beers. J Appl Bacteriol 57:R11–R12
Verachtert H, Derdelinckx G (2005) Acidic beers: enjoyable reminiscences of the past. Cerevisia Belg J Brew Biotechnol 3:38–47
Verachtert H, Iserentant D (1995) Properties of Belgian acid beers and their microflora. Part I. The production of gueuze and related refreshing acid beers. Cerevisia Belg J Brew Biotechnol 20:37–41
Vitas JS, Malbasa RV, Grahovac JA, Loncar ES (2013) The antioxidant activity of kombucha fermented milk products with stinging nettle and winter savory. Chem Ind Chem Eng Q 19:129–139
Waldherr FW, Doll VM, Meissner D, Vogel RF (2010) Identification and characterization of a glucan-producing enzyme from *Lactobacillus hilgardii* TMW 1.828 involved in granule formation of water kefir. Food Microbiol 27:672–678
Wang Y, Ji BP, Wu W, Wang RJ, Yang ZW, Zhang D, Tian WL (2014) Hepatoprotective effects of kombucha tea: identification of functional strains and quantification of functional components. J Sci Food Agric 94:265–272
Ward HM (1892) The ginger-beer plant and the organisms composing it; a contribution to the study of fermentation: yeasts and bacteria. Philos Trans R Soc Lond 183:125–197
Wei L, Zhu D, Zhou J, Zhang J, Zhu K, Du L, Hua Q (2013) Revealing in vivo glucose utilization of *Gluconobacter oxydans* 621H Δ*mgdh* strain by mutagenesis. Microbiol Res 169:469–475
Witthuhn RC, Schoeman T, Britz TJ (2004) Isolation and characterization of the microbial population of different South African kefir grains. Int J Dairy Technol 57:33–37
Witthuhn RC, Schoeman T, Britz TJ (2005) Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. Int Dairy J 15:383–389
Wood GAR, Lass RA (2001) Cocoa, 4th edn. Blackwell Science, Oxford
Yamada Y, Yukphan P (2008) Genera and species in acetic acid bacteria. Int J Food Microbiol 125:15–24
Yang ZW, Zhou F, Ji BP, Li B, Luo YC, Yang L, Li T (2010) Symbiosis between microorganisms from kombucha and kefir: potential significance to the enhancement of kombucha function. Appl Biochem Biotechnol 160:446–455
Yoshino T, Asakura T, Toda K (1996) Cellulose production by *Acetobacter pasteurianus* on silicone membrane. J Ferment Bioeng 81:32–36
Zeynep G-S, Tugba K-T, Annel KG (2009) Kefir and koumiss. In: Yildiz F (ed) Development and manufacture of yogurt and other functional dairy products. CRC Press, Boca Raton, pp 143–163

Chapter 4
Acetic Acid Bacteria as Plant Growth Promoters

Raúl O. Pedraza

Abstract Different genera and species of the family *Acetobacteraceae* (e.g., *Gluconacetobacter diazotrophicus*, *Gluconacetobacter johannae*, *Gluconacetobacter azotocaptans*, *Swaminathania salitolerans*, *Acetobacter peroxydans*, and *Acetobacter nitrogenifigens*) were found associated with diverse plant species, colonizing the inner tissues and roots. Several of these species are capable of promoting plant growth through special mechanisms such as the biological nitrogen-fixing process, phytohormone production, mineral solubilization, and antagonistic effect against pathogens, among others. Plant growth-promoting bacteria (PGPB) are valuable for agriculture as a tool for improving crop performance and environmental conditions, as they may reduce or avoid the use of chemical fertilizers and pesticides.

From an agricultural point of view, to achieve a sustainable crop production to feed the growing human population, strategic biotechnological approaches should be considered in crop management, including nutritional and phyto-sanitary aspects. Therefore, the use of PGPB is one of the possible approaches. Research and field trials of PGPB over decades in the present and past century have opened up new horizons for their biotechnological application. In this chapter, information on N_2-fixing acetic acid bacteria (AAB), their ecology, their physiological and genetic characteristics, the mode of action of AAB as plant growth promoters, and their biotechnological application is presented.

Keywords *Gluconacetobacter diazotrophicus* • *Gluconacetobacter johannae* • *Gluconacetobacter azotocaptans* • *Gluconacetobacter kombuchae (Komagataeibacter kombuchae)* • *Swaminathania salitolerans* • *Acetobacter peroxydans* • *Acetobacter nitrogenifigens*

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4.1 Introduction

Plant growth-promoting bacteria (PGPB) are free-living soil, rhizosphere, rhizoplane, and phylosphere bacteria that, under some conditions, are beneficial for plants (Bashan and Holguin 1998). Most of the activities of PGPB have been studied in the rhizosphere (the soil area influenced by roots), and to a lesser extent, on leaf surfaces. Presently, endophytic PGPB (those colonizing plant inner tissues without producing damage) constitute an important area of PGPB study also.

PGPB can promote plant growth in two ways: by directly affecting the metabolism of the plants by providing substances that are usually in scant supply, or indirectly by preventing the deleterious effects of phytopathogenic bacteria, fungi, nematodes, and viruses (Bashan et al. 2008). In the first situation, bacteria are capable of fixing atmospheric dinitrogen, solubilizing phosphorus, zinc, or other metals, and enhancing production of plant hormones (e.g., auxins). Furthermore, they can improve plant tolerance to different environmental stresses, such as drought, high salinity of soils, metal toxicity, and pesticide load. One or more of these features may contribute to increase plant growth and development more than that of plants grown under standard cultivation conditions. In addition to the agricultural usefulness of PGPB, there are potential benefits in environmental applications: for example, increased bioremediation of wastewater, prevention of soil erosion in arid zones by improving the growth of desert plants, or extraction of hazardous materials from the soil (Bashan et al. 2008).

Bacteria capable of increasing plant growth and productivity in agriculture have been known for more than a century. Prominent among these organisms are members of the rhizobia whose potential and biotechnological use in agriculture is at present beyond doubt. However, although being not all well known, diverse genera belong to the PGPB, such as *Azospirillum*, *Azotobacter*, *Azoarcus*, *Bacillus*, *Burkholderia*, *Frankia*, *Herbaspirillum*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Sinorhizobium*. Additionally, in the past three decades some members of the family *Acetobacteraceae* have drawn attention to the scientific community as PGPB: *Gluconacetobacter*, *Swaminathania*, and *Acetobacter*. In this chapter, N_2-fixing acetic acid bacteria (AAB), their ecology, physiological and genetic characteristics, mode of action of AAB as plant-growth promoters, and biotechnological application, especially their contribution in agriculture, are described.

4.2 Contribution of Acetic Acid Bacteria as Plant Growth Promoters

4.2.1 Background: Nitrogen-Fixing AAB

In the family *Acetobacteraceae*, only three genera (*Gluconacetobacter*, *Acetobacter*, and *Swaminathania*) include nitrogen-fixing species. Presently,

some of these species are considered as PGPB for harboring different mechanisms to promote plant growth, in addition to the capacity to fix atmospheric dinitrogen.

The first N_2-fixing AAB was described in Brazil by Dr. Johanna Döbereiner and her research team (Cavalcante and Döbereiner 1988); since then, it has been investigated thoroughly with regard to its nitrogen-fixing ability. It was found inside sugarcane plant tissues and was first named *Acetobacter diazotrophicus* (Gillis et al. 1989), then renamed as *Gluconacetobacter diazotrophicus* (Yamada et al. 1997). Later, three new species within the genus *Gluconacetobacter* were described: *Ga. johannae* and *Ga. azotocaptans* associated with coffee plants in Mexico (Fuentes-Ramírez et al. 2001), and *Gluconacetobacter kombuchae*, isolated from Kombucha tea in India (Dutta and Gachhui 2007). However, it was reported that *Ga. kombuchae* (LMG 23726^T) should be reclassified as *Gluconacetobacter hansenii* (now further changed to *Komagataeibacter kombuchae*), according to analyses of amplified fragment length polymorphism (AFLP) DNA fingerprinting (Cleenwerck et al. 2009).

In 2004, *Swaminathania salitolerans* was described (Loganathan and Nair 2004). Then, *Acetobacter peroxydans* and *A. nitrogenifigens*, associated with rice plants and Kombucha tea, respectively, were described as N_2-fixing AAB in India (Dutta and Gachhui 2006; Muthukumarasamy et al. 2005). The identification of *A. peroxydans* as a nitrogen-fixing species was surprising as this species had been already described in 1925 and had thus far never been reported as diazotrophic. Although the type strain and novel isolates of *A. peroxydans* showed low and inconsistent acetylene reduction activity, compared to *Ga. diazotrophicus*, their diazotrophic ability was confirmed by the presence of *nifH* genes (Muthukumarasamy et al. 2005). Some important characteristics of dinitrogen-fixing AAB are shown in Table 4.1.

4.2.2 Ecology

Many reports on N_2-fixing AAB mention that they are mostly associated with sugar- or ethanol-rich environments; however, recent studies show that their distribution is wider.

Gluconacetobacter diazotrophicus was first isolated from sugarcane, colonizing the inner tissues of roots, stems, and leaves (Cavalcante and Döbereiner 1988). It was originally described as an endophytic species, although its natural occurrence in the rhizosphere of different plants has been documented (Jiménez-Salgado et al. 1997; Loganathan et al. 1999; Muthukumarasamy et al. 2005; Santos et al. 2006). Since its first report (Cavalcante and Döbereiner 1988), *G. diazotrophicus* has been isolated from sweet potato (Paula et al. 1991), *Pennisetum purpureum* (Reis et al. 1994), coffee plants in Mexico (Jiménez-Salgado et al. 1997), field-grown pineapples in Mexico (Tapia-Hernández et al. 2000), a grass called "finger millet" (*Eleusine coracana* L. Gaertn) (Loganathan et al. 1999), tea plants (roots), mango (fruits), the rhizosphere of banana plants (Muthukumarasamy et al. 2002), and from wetland rice

Table 4.1 Some important characteristics of dinitrogen-fixing acetic acid bacteria (AAB)*

Characteristics	*Gluconacetobacter diazotrophicus*	*Ga. johannae*	*Ga. azotocaptans*	*K. kombuchae*	*Swaminathania salitolerans*	*Acetobacter peroxydans*	*A. nitrogenifigens*
Cells	Straight rods	Straight rods	Straight rods	Straight rods	Straight rods	Straight rods	Straight rods
	2.0 μm long	1.5–1.9 μm long	1.6–2.0 μm long	2.0–3.0 μm long	1.9–3.1 μm long	2.0–3.0 μm long	1.5–2.0 μm long
	0.7–0.6 μm wide	0.5–0.6 μm wide	0.5–0.6 μm wide	0.5–0.6 μm wide	0.7–0.9 μm wide	0.5–0.6 μm wide	0.1–0.2 μm wide
Motile	+	+	+	+	+	+	+
Gram	–	–	–	–	–	–	–
Colonies on potato agar medium	Dark brown	Brownish	Beige-light-brownish	Light-brown	n.d.	Light-brown	Light-brown
N_2-fixation	+	+	+	+	+	+	+
Optimum growth temperature	30 °C	29 °C	29 °C	30 °C	30 °C	32 °C	30 °C
Optimum pH	5.5	5.5	5.5	4.5	5.5	6.0	4.5
G+C content	61–63 mol%	57.96 mol%	64.01 mol%	55.8 mol%	57.6–59.9 mol%	60.5 mol%	64.1 mol%
First source of isolation	Sugarcane roots and stems	Rhizosphere of coffee plants	Rhizosphere of coffee plants	Kombucha tea	Mangrove-associated wild rice (rhizosphere)	Roots and stems of wet-land rice	Kombucha tea

*Data obtained from Gillis et al. (1989), Fuentes-Ramírez et al. (2001), Dutta and Gachhui (2007), Loganathan and Nair (2004); Muthukumarasamy et al. (2005), and Dutta and Gachhui (2006)
n.d. not determined

(Muthukumarasamy et al. 2005). *Ga. diazotrophicus* was found also in trash of sugarcane (e.g., senescent leaves left on the ground from the last cut) (Reis et al. 1994), and it was also isolated from a mealy bug (*Saccharococcus sacchari*) associated with sugarcane plants (Ashbolt and Inkerman 1990). Recently, the endophytic existence of *Ga. diazotrophicus* in different xerophytic plants of the Sinai desert (Egypt) has been reported (Hanna et al. 2013). This finding speaks well about the possible role of endophytic bacterial populations in the survival, nutrition, and health of existing plants in semiarid environments.

Different plants from locations where *Ga. diazotrophicus* was isolated (e.g., sugarcane, rice, elephant grass, sweet potato, coffee, and pineapple) have a high level of asparagine, which promotes microbial growth and inhibits nitrogenase activity. The regulation of intracellular concentrations of this amino acid is essential for growth and biological nitrogen fixation (BNF) in this diazotroph. Analysis carried out in the asparagine metabolic pathway by Alquéres et al. (2012) showed that intracellular levels of asparagine regulate the expression of nitrogenase *nifD* gene, suggesting that the presence of an alternative route to produce asparagine might give *Ga. diazotrophicus* a tighter control over cell growth and BNF and may be of importance in the regulation of the endophytic plant–microbe interaction.

In contrast to *Ga. diazotrophicus*, which can inhabits inner plant tissues as an endophyte, *Ga. johannae* and *Ga. azotocaptans* were only found colonizing the rhizosphere of coffee plants (Fuentes-Ramírez et al. 2001). Also, *Ga. azotocaptans* was isolated from the rhizosphere of corn (Mehnaz et al. 2006). The last species described within this genus, the N_2-fixing and cellulose-producing *K. kombuchae*, was isolated from Kombucha tea in India (Dutta and Gachhui 2007).

Regarding the other genera and species of N_2-fixing AAB, *Swaminathania salitolerans* was isolated from the rhizosphere, roots, and stems of salt-tolerant, mangrove-associated wild rice (*Porteresia coarctata* Tateoka); as a particular feature, it can solubilize phosphate in the presence of NaCl (Loganathan and Nair 2004). *Acetobacter peroxydans* was found associated with cultivated wetland rice varieties in India (Muthukumarasamy et al. 2005). Then, within this genus, *A. nitrogenifigens*, also isolated from Kombucha tea in India, was described as a novel N_2-fixing acetic acid bacterium (Dutta and Gachhui 2006).

Since the first description of *Ga. diazotrophicus* in 1988 (Cavalcante and Döbereiner 1988), it has been investigated thoroughly, first with regard to its nitrogen-fixing capacity and lately because it has additional abilities to enhance plant growth. Therefore, literature is abundant on this species, compared with the other AAB mentioned herein, and consequently the information in this chapter refers mainly to *Ga. diazotrophicus*.

4.2.3 Physiological Characteristics

The most common physiological characteristics of *Ga. diazotrophicus* are its high sucrose tolerance (10 %), growth and nitrogen fixation at low pH (5.0 or less),

chocolate colonies on potato agar medium with 10 % sucrose, absence of nitrate reductase, nitrogen fixation not affected by high concentrations of NO_3^- (25 mM), and partial inhibition by NH_4^+, especially at high sucrose concentrations (Boddey et al. 1991; Stephan et al. 1991). These characteristics enable *Ga. diazotrophicus* to fix N_2 in the presence of soil nitrogen, thus making it an even more interesting plant-associated diazotroph. For instance, within sugarcane stems, where *Ga. diazotrophicus* occurs in numbers up to 10^7 g^{-1} of plant tissue (Cavalcante and Döbereiner 1988), considerable amounts of nitrogen could be made available to the plant, hence providing a basis for the high estimates of N_2 fixation reported in certain sugarcane genotypes under field conditions in Brazil (Boddey et al. 1991; Urquiaga et al. 1992).

Acid production by *Ga. diazotrophicus* has an additional value as it can solubilize insoluble P and Zn compounds (Muthukumarasamy et al. 2002; Madhaiyan et al. 2004), although this feature is reduced when the organism is in contact with pesticides, as reported by Madhaiyan et al. (2006).

The respiratory system and diazotrophic activity of *Ga. diazotrophicus* PAL5 (the most studied strain) were investigated by Flores-Encarnación et al. (1999). Spectral and high-pressure liquid chromatography analysis of membranes revealed the presence of cytochrome *ba* as a putative oxidase in cells obtained from diazotrophically active cultures, concluding that glucose dehydrogenase and cytochrome *ba* are key components of the respiratory system of *Ga. diazotrophicus* during aerobic diazotrophy.

Levansucrase (LsdA, EC 2.4.1.10) is a constitutive exoenzyme in *Ga. diazotrophicus* that hydrolyzes sucrose to synthesize oligofructans and levan (Hernández et al. 2000). By targeted disruption of the *lsdA* gene it was observed that *Ga. diazotrophicus* utilizes plant sucrose via levansucrase. Arrieta et al. (2004) reported that LsdA, differing from other extracellular levansucrases from gram-negative bacteria, is transported to the periplasm by a signal peptide-dependent pathway: this was the first description of a type II pathway for protein secretion in the *Acetobacteraceae*. Also, the purification and characterization of a levansucrase enzyme produced by *A. nitrogenifigens* have been reported (Paul et al. 2011).

Extracellular glucose oxidation is considered the main route for glucose catabolism in *Ga. diazotrophicus* (Alvarez and Martínez-Drets 1995; Attwood et al. 1991). However, low hexokinase activities have been also reported in this organism; moreover, a nicotinamide adenine dinucleotide-linked glucose dehydrogenase (NADGDH) was found to be actively synthesized in glucose-containing cultures (Alvarez and Martínez-Drets 1995; Attwood et al. 1991). Consequently, two oxidative routes seem to be simultaneously expressed in *Ga. diazotrophicus*, one being intracellular by way of a NAD-GDH, and the other being periplasmic by way of a pyrrolo-quinoline-quinone-linked glucose dehydrogenase (PQQ-GDH). It was reported also that a PQQ-GDH was primarily responsible for the high rates of gluconic acid formation exhibited by *Ga. diazotrophicus* (Attwood et al. 1991). About the regulation of both enzymes, Luna et al. (2006) observed that *Ga. diazotrophicus* metabolizes glucose mainly by way of a PQQ-GDH, particularly under BNF or limited conditions. Studies conducted in batch and continuous

cultures (Luna et al. 2006) showed that glutamate is the central molecule of carbon metabolism in *Ga. diazotrophicus*, and that metabolic flux proceeds mainly by the pentose–phosphate pathway, as has already been reported for other AAB (Matsushita et al. 1994).

It has been demonstrated that exopolysaccharides (EPS) produced by *Ga. diazotrophicus* are important in plant infection. Further studies revealed that, when grown in liquid medium containing mannitol as the sole carbon source, EPS were composed of Glc, Gal, and Man in a molar ratio of 6:3:1, respectively (Serrato et al. 2013). Nuclear magnetic resonance spectroscopy and chemical derivatization showed that the EPS structure has 4-*O*-substituted units of β-glucose, 3-*O*-substituted units of β-galactose, and 2-*O*-substituted units of α-mannose. Glucose and galactose units linked at C6 were also found. The structure proposed by Serrato et al. (2013) is different from EPS produced by other species of *Gluconacetobacter* published so far.

It was determined that reactive oxygen species (ROS) scavenging enzymes of *Ga. diazotrophicus* strain PAL5 are important in the endophytic colonization of rice plants (Alquéres et al. 2013). In that work, they observed that ROS were produced at early stages of rice root colonization, a typical plant defense response against pathogens. The transcription of the pathogen-related-10 gene of the jasmonic acid pathway, but not of the PR-1 gene of the salicylic acid pathway, was activated by the endophytic colonization of rice roots by *Ga. diazotrophicus* strain PAL5. Quantitative polymerase chain reaction analyses showed that, at early stages of colonization, the bacteria upregulated the transcript levels of ROS-detoxifying genes such as superoxide dismutase and glutathione reductase.

The mechanisms of cadmium, cobalt, and zinc resistance were characterized in *Gluconacetobacter diazotrophicus* PAL5 by Intorne et al. (2012). They found that this bacterium was resistant to high concentrations of Cd, Co, and Zn, with minimum inhibitory concentrations of 1.2, 20, and 20 mM, respectively. Therefore, they provided evidence for the high tolerance of *Ga. diazotrophicus* PAL5 to heavy metals and that the *czc* gene (which encodes a protein involved in metal efflux) is a determinant for metal resistance in this bacterium.

4.2.4 Genetic Characteristics

It has been reported that some *Ga. diazotrophicus* strains carry plasmids, and that the *nif* genes (involved in the N_2-fixing process) are not located in plasmids but on the chromosome (Teixeira et al. 1994). Also, Caballero-Mellado and Martínez-Romero (1994) observed that not all *Ga. diazotrophicus* strains harbor plasmids, and that two plasmids were highly conserved among the isolates examined. Although their functions are yet to be identified, the fact that some strains do not harbor plasmids may indicate that fundamental phenotypic characteristics of *Ga. diazotrophicus* such as nitrogen fixation, indole-3-acetic acid (IAA) production, and the use of different carbon substrates are not plasmid encoded. The same

authors have reported that, regardless of the presence of plasmids, all the *Ga. diazotrophicus* isolates analyzed shared a common pattern of *nif* structural gene organization on the chromosome. Different genes involved in the N_2-fixing process and its regulation, such as *nifHDK*, *nifA*, *nifB*, *nifV*, *nifE*, and *ntrBC*, have been identified by Sevilla et al. (1997).

More recently, the complete genome of *Ga. diazotrophicus* PAL5 was reported (Bertalan et al. 2009). It is composed of one circular chromosome of 3,944,163 base pairs (bp) with an average G+C content of 66.19 %, and two plasmids of 38,818 and 16,610 bp, respectively. The circular chromosome has a total of 3864 putative coding sequences, with an overall coding capacity of 90.67 %. Among the predicted genes, 2861 were assigned a putative function, and 1077 encode hypothetical proteins. Regarding noncoding RNA genes, 12 rRNAs (4 rRNA operons) and 55 tRNAs were identified. The larger plasmid (pGD01) has 53 coding sequences; approximately 70 % encode hypothetical or conserved hypothetical proteins, and 5 encode proteins involved in plasmid-related functions. The remaining 11 coding sequences encode putative components of the type IV secretion system. It was found also that the small plasmid (pGD02) has 21 coding sequences, and about 50 % are hypothetical proteins.

The complete genomic sequence of *Ga. diazotrophicus* PAL5 (Bertalan et al. 2009) also revealed the presence of a quorum sensing system, among other features: these are regulatory mechanisms that, through the production of signal molecules or auto-inducers, allow the regulation of the physiology of a microbial population in a coordinated way. In gram-negative bacteria, the *N*-acyl homoserine lactones (AHL) are the most studied auto-inducers, and the presence of AHL-like molecules in cultures of *Ga. diazotrophicus* PAL5 grown in complex and synthetic media has been reported (Nieto-Peñalver et al. 2012). At present, new information on this interesting bacterium is expected after the publication of its genome sequence.

4.3 Mode of Action of AAB as Plant Growth Promoters

It has been reported by different authors that *Ga. diazotrophicus* can promote plant growth through more than a few independent direct or indirect mechanisms besides N_2 fixation, including synthesis of phytohormones, solubilization of nutrients, and antagonistic effects against phytopathogens.

4.3.1 Biological Nitrogen Fixation

The biological reaction that counterbalances the loss of nitrogen from soils or agro-ecosystems is biological nitrogen fixation (BNF), which is the enzymatic reduction of the atmospheric dinitrogen (N_2) to ammonia, catalyzed by the nitrogenase

complex. This process is exclusive to Bacteria and Archaea, and the microorganisms that fix nitrogen are named diazotrophs.

The genetics and biochemistry of BNF and nitrogen utilization by *G. diazotrophicus* have been previously investigated to some extent (revised by Pedraza 2008; Saravanan et al. 2008). Corroborating previous studies through the genome sequence analysis of *G. diazotrophicus* PAL5, Bertalan et al. (2009) have found that the structural genes for nitrogenase *nifHDK* are arranged in a cluster, which also contains other N_2 fixation-related genes, such as *fixABCX*, *modABC*, and *nifAB*. They found also that other related genes (*ntrX*, *ntrY*, and *ntrC*, implicated in regulatory systems) are localized elsewhere in the chromosome in a 5.2-kb cluster. There are three copies of *nifU* homologous genes: one localized in the *nif* cluster and the other two scattered on the bacterial chromosome. No *draT* or *draG* homologues were found in this bacterium, confirming that nitrogenase activity is not regulated at the posttranslational level. It has been suggested that posttranslational modulation in *G. diazotrophicus* might be mediated by a FeSII Shethna protein (Ureta and Nordlund 2002), but no such coding sequence was identified. However, many other FeSII protein genes are present, and they are possible candidates for this function. The apparent absence of *nifL* as a *nifA* activity modulator in response to the cell O_2 status in *Ga. diazotrophicus* (Perlova et al. 2002) is in agreement with the lack of a *nifL* homologue on the genome. The *nifA* protein appears to be inherently sensitive to O_2. In *Ga. diazotrophicus*, the main route for assimilation of ammonia is believed to occur through the glutamine synthetase/glutamate synthase pathway (GS/GOGAT encoded by *glnA* and *gltDB*, respectively) (Dow et al. 2006). However, the genome analysis suggests the existence of alternative routes, wherein the putative enzymes NAD-synthase, aminomethyltransferase, histidine ammonia-lyase, and D-amino acid dehydrogenase would incorporate ammonia into different compounds (Bertalan et al. 2009). The enzymatic activity of GS is known to be regulated by an adenylyltransferase enzyme, which is probably encoded by *glnE*. The glutamate dehydrogenase gene was not found in *Ga. diazotrophicus* PAL5, although its activity was demonstrated for the strain PAL3 (Perlova et al. 2002).

4.3.2 Phytohormones

It is well known that phytohormones are important as signals and regulators of growth and development in plants. The capacity to produce these hormones is frequently considered to be a peculiarity of the plant kingdom. Nevertheless, that characteristic is also extensive among soil- and plant-associated prokaryotes (Costacurta and Vanderleyden 1995). Furthermore, the production of hormonal substances such as auxins and gibberellins by different PGPB has been proposed as one of the mechanisms to explain plant growth promotion.

Indole-3-acetic acid (IAA) is a naturally occurring auxin with broad physiological effects. The production of IAA by *Ga. diazotrophicus* was first reported by

Fuentes-Ramírez et al. (1993). Later, the detection of aromatic amino acid amino-transferase (AAT) activity in *Ga. diazotrophicus*, *Ga. johannae*, and *Ga. azotocaptans*, as well as their IAA production, was reported by Pedraza et al. (2004). The AATs are ubiquitous enzymes that reversibly catalyze the conversion of amino acids to the corresponding α-keto acids; they can participate in multiple metabolic pathways, such as IAA synthesis. The presence of genes encoding enzymes such as aromatic-L-amino-acid decarboxylase, amine oxidase, and aldehyde dehydrogenases in the genome sequence of *Ga. diazotrophicus* suggests that this bacterium might synthesize IAA via the trypamide pathway (Bertalan et al. 2009). Also, the presence of two genes coding for putative nitrilases suggests that IAA might also be produced by the indole-3-acetonitrile pathway.

In addition to IAA, the second hormonal substances detected in *Ga. diazotrophicus* are the gibberellins A1 and A3. They were characterized by capillary gas chromatography–mass spectrometry from chemically defined culture media containing 10 % sucrose (Bastián et al. 1998).

The presence of genes coding for enzymes for the synthesis and secretion of spermidine in the *Ga. diazotrophicus* PAL5 genome sequence suggests that this polyamine may also contribute to promote plant growth (Bertalan et al. 2009).

4.3.3 Mineral Nutrients Solubilization

Mineral phosphate solubilization is generally considered to be a plant growth-promoting characteristic for PGPB. This activity has been observed in different strains of *Ga. diazotrophicus*, including PAL5 recovered from sugarcane and from other crops, in the presence of sucrose or glucose as carbon sources (Fig. 4.1). Also, *Ga. azotocaptans* and *S. salitolerans* are able to solubilize insoluble mineral phosphate (reviewed by Saravanan et al. 2008).

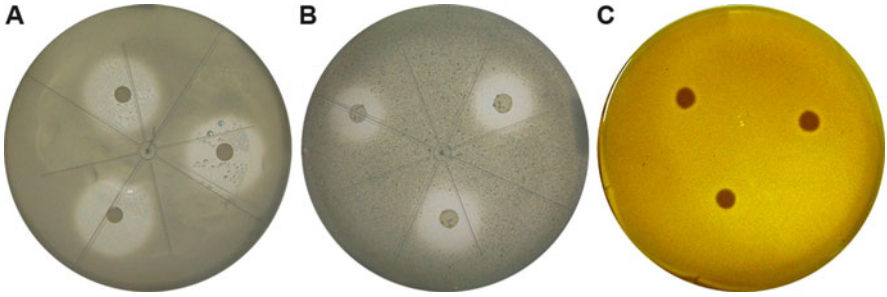

Fig. 4.1 Phosphate solubilization by *Gluconacetobacter diazotrophicus* PAL5 is observed by the formation of a clear halo around the bacterial colony (shown in triplicate in each Petri dish). The growth culture medium was supplemented with different P-sources (5 g l^{-1}): (**a**) tricalcium phosphate [$Ca_3(PO4)_2$]; (**b**) hydroxyapatite [$Ca_5(PO_4)_3OH$]; (**c**) iron-phosphate ($FePO_4$). The solubility of the P-compounds decreases from (**a**) to (**c**)

Zinc is an essential micronutrient in crop production; its deficiency is widespread in arable soils and is also frequent in the host crops of *Ga. diazotrophicus*, such as sugarcane, rice, and coffee. Therefore, solubilization of insoluble Zn compounds by *Ga. diazotrophicus* may enhance Zn nutrition of host crops (reviewed by Saravanan et al. 2008). It was reported that different isolates of *Ga. diazotrophicus* effectively solubilized ZnO over $ZnCO_3$ or $ZnSO_4$ in plate and broth assays, showing variations in the solubilization capacity. After inoculation, those isolates were also able to improve sugarcane growth (Natheer and Muthukkaruppan 2012).

Another essential micronutrient is iron. To maintain growth, bacteria have developed different strategies to obtain iron from the iron-limited environment, but siderophore-mediated iron uptake is probably the most common form of iron acquisition (Braun and Winkelmann 1987). It has been reported that *Ga. diazotrophicus* produces these, being hydroxamate-type siderophores (Logeshwaran et al. 2009). However, their function in the *Ga. diazotrophicus*–plant association was not yet elucidated. Soto Urzúa et al. (2013) have reported the identification and characterization of an ATP-binding cassette (ABC) transport system in *Ga. diazotrophicus* PAL5, comprising three genes (*feuABC*) that encode a periplasmic-binding protein, a permease, and a traffic ATPase, and the involvement of this system in iron acquisition by this bacterium.

4.3.4 Antagonistic Effects Against Pathogens

It has been documented that the ability of *Ga. diazotrophicus* to antagonize diverse plant pathogens, such as fungi and bacteria, contributes to increasing its ability to survive under environmental stress and leads to an improvement in plant fitness, which may have significant consequences for agricultural productivity (Pedraza 2008; Saravanan et al. 2008). On these features, the genome sequence of *Ga. diazotrophicus* encodes a large stock of genes whose products oppose attack from competing microbes, such as drug efflux systems, and acriflavin and fusaric acid resistance proteins (Bertalan et al. 2009). On the other hand, this bacterium may also produce a wide variety of proteins such as lytic enzymes and phospholipases and antibiotic biosynthetic pathways that could be toxic to other organisms. Also, it was observed in the genome sequence that *Ga. diazotrophicus* encodes a putative lysozyme-like bacteriocin, coinciding with data on its secretion of a lysozyme-like bacteriocin that inhibits the growth of *Xanthomonas albilineans* growth, the causal agent of leaf scald disease in sugarcane.

4.4 Biotechnological Applications

From an agricultural point of view, to achieve sustainable crop production to feed the growing human population, strategic biotechnological approaches should be considered in crop management, including nutritional and phyto-sanitary aspects. The use of PGPB is one of the possible approaches. Research and field trials of PGPB over decades in the present and past century have opened up new horizons to their biotechnological application. Even more, in many countries this is a very attractive activity for the agricultural industry of biostimulants. Also, the development of superior or novel PGPB strains with improved plant growth-promoting characteristics, and the development of transgenic crop plants expressing some special PGPB features, is possible by genetic manipulations. Therefore, these PGPB biotechnologies can be now exploited as a low-input, sustainable, and environmental friendly strategy for crop management.

4.4.1 Biostimulants

Recently, the emerging definitions of plant biostimulants (Calvo et al. 2014) were revised. As a general definition, they are diverse substances and microorganisms used to enhance plant growth, including five categories: microbial inoculants, humic acids, fulvic acids, protein hydrolysates and amino acids, and seaweed extracts. Presently, there is growing scientific evidence supporting the use of biostimulants as agricultural inputs on diverse plant species. According to Calvo et al. (2014), the global market for biostimulants is projected to increase 12 % per year and surpass US$ 2200 million by 2018. Also, they reported increased root growth, enhanced nutrient uptake, and stress tolerance as examples of common features in plant responses to different biostimulants.

4.4.2 Inoculants and Inoculation

Bacterial inoculation of plants to enhance yield of crops is a century-old proven technology for rhizobia and a newer venue for PGPB. The two main aspects dominating the success of inoculation are the effectiveness of the bacterial isolate and the proper application technology (Bashan et al. 2014). In general, “bacterial isolates” refer to specific bacterial strains (PGPB) that can promote plant growth after inoculation. “Carrier” refers to the abiotic substrate (e.g., solid, liquid, or gel) that is used in the formulation process. “Formulation” refers to the laboratory or industrial process of unifying the carrier with the bacterial strain. Last, “inoculant” refers to the final product of formulation containing a carrier and bacterial agent or consortium of microorganisms. Presently, there exist different carriers and

formulations of inoculants including liquid, organic, inorganic, polymeric, and encapsulated formulations. Technical aspects of this issue include inoculation techniques (soil and seed application), mass culture production, bulk sterilization, seed coating, shelf life, and effects of moisture (Bashan et al. 2014).

The use of AAB as inoculants in agriculture is something new. In Brazil, studies have been conducted to evaluate maintenance of cell viability and stability, as well as to select cheap carriers to extend the shelf life of plant beneficial bacterial inoculants for agricultural crops (da Silva et al. 2012). Thus, the shelf life and the colonization efficiency of novel liquid- and gel-based inoculant formulations for sugarcane were evaluated. The different inoculant formulations were all composed of a mixture of five strains of diazotrophic bacteria (*Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia tropica*), which are recognized as sugarcane growth promoters. Different inoculant formulations containing as carrier the polymers carboxymethylcellulose (CMC) and cornstarch (60/40 ratio) at five different concentrations (named PIC, for polymeric inoculant carrier) were supplemented, or not, with 2 % MgO, an interfacial stabilizing agent. Laboratory tests showed that in the formulation composed of 0.8 g of the polymeric mixture per 100 g of the final product (PIC 0.8), survival of *Ga. diazotrophicus* and *A. amazonense* was around 10^9 CFU ml^{-1} after 120 days of storage, regardless of supplementation with MgO. The other formulation (2.2 g of polymeric mixture, PIC 2.2) presented survival levels of 10^8 CFU ml^{-1} for up to 60 days of storage for all the individual strains. In the greenhouse, sugarcane seedlings showed a positive growth response 50 days after inoculation when inoculated with the mixture of five bacteria, with and without PIC 2.2. Hence, the polymer carriers used allowed for the long-term survival of the five different bacterial strains tested. Additionally, short-term experiments in the greenhouse showed that their application as part of an inoculant on sugarcane cuttings was at least as effective, in terms of bacterial colonization and the promotion of plant growth, as that of the bacterial mixture without carriers.

Also, the sprouting, survival and growth of young sugarcane var. VMC-86-550 treated with *Ga. diazotrophicus* was investigated in the Philippines (De La Cruz et al. 2012). Three bacterial concentrations (10^8, 10^{10}, and 10^{12} cells ml^{-1}) and three methods of inoculation (spraying, soaking, and dipping) were used. The inoculated plants showed a significant increase in percent survival, plant height, and shoot/root biomass compared with the uninoculated control at 45 days after planting (DAP). However, no significant differences were observed in percent sprouting between inoculated plants and the uninoculated controls at 30 DAP. It was observed that introduction of *Ga. diazotrophicus* at 10^{12} cells ml^{-1} by means of the dipping method had constantly yielded taller plants with greater shoot and root biomass, relative to the other treatments and to the uninoculated controls. This experiment showed the potential use of *Ga. diazotrophicus* in the development of a cost-effective technology in sugarcane production.

4.5 Application in Agriculture

The use of AAB as PGPB was reviewed by Pedraza (2008) and Saravanan et al. (2008), including data on N_2-fixing AAB interacting with agronomically important crops, such as sugarcane, sorghum, rice, maize, and wheat. In most cases, positive plant response after bacterial inoculation was observed; therefore, only recent information on this topic is reported in this chapter.

A field experiment to evaluate the performance of in vitro micropropagated plantlets inoculated with *Ga. diazotrophicus* on the population of diazotrophs, plant growth, yield, quality, and planting material of sugarcane cultivar CoS 96268 was carried out in India (Singh et al. 2012). Maximum cell counts (5.8×10^5 cells g^{-1} fresh weight) were obtained in the inoculated micropropagated plantlets, followed by untreated micropropagated plantlets (4.7×10^4 cells g^{-1} fresh weight), but only 3.7×10^4 cells g^{-1} fresh weight in conventionally grown plants. Bacterial inoculation improved dry matter accumulation, especially in the roots, accompanied by higher uptake of nitrogen and potassium in micropropagated plantlets. There was significant increase in number of millable canes, cane length, number of nodes, cane diameter, and cane yield. Further, treated plantlets produced 5.45 and 1.52 times higher planting material (three budded setts) over conventionally grown plants and untreated micropropagated plantlets, respectively. This result signifies that inoculation with diazotrophic bacteria in micropropagated plantlets for faster multiplication of disease-free healthy seed cane of sugarcane varieties constitutes an interesting biotechnology to be adopted.

Also, a field experiment conducted on sugarcane in India analyzed the effect of pre-planting sett treatment and/or soil application with *Ga. diazotrophicus* or composite culture consisting of *Azotobacter*, *Azospirillum*, *Ga. diazotrophicus*, and phosphate-solubilizing bacteria with varying levels of N fertilizer (Nalawade et al. 2013). They observed that sett treatment with *Ga. diazotrophicus* at the time of planting could save 50 % of chemical N fertilizer with equivalent cane yield and higher monetary returns as compared to only the recommended dose of fertilizers (100 % NPK). In line with this, another field experiment on sugarcane was conducted in India with five PGPB (*Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp., and *Ga. diazotrophicus*) (Chauhan et al. 2013). As the conclusion, the values of plant height, chlorophyll content, total nitrogen, and cane length were significantly higher in almost all inoculated plants compared with the uninoculated control. Particularly, an increase of 42 % in cane yield over the control was obtained after inoculation only with *Ga. diazotrophicus*.

In another trial, three PGPB of diverse habitats (endophytic *Ga. diazotrophicus*, free-living *Azotobacter chrococcum*, and associative *Azospirillum brasilense*) were tested at field conditions during a complete sugarcane crop cycle (plant and a ratoon) (Suman et al. 2013). Different levels of N input for enhancing crop productivity and soil N balance were also considered. In general, the inoculation with PGPB was highly beneficial and, in particular, *Ga. diazotrophicus* was the

most efficient in terms of obtaining a higher cane yield, commercial cane sugar production, efficient N utilization, and maintaining positive soil N balance.

Continuing with experiences in sugarcane, the effects of subsequent sugarcane ratooning on soil quality and the crop yields under four treatments [an absolute control, application of recommended dose of NPK, application of sulphitation press mud (SPM), a sugar factory by-product, and SPM along with *Ga. diazotrophicus*] were evaluated for 7 years (Singh et al. 2013). In the control and NPK-fertilized plots, an increase in soil compaction (5.4 %), decrease in infiltration rate (6.04 %), lower microbial activities, and increased soil phenolic contents (72.4 %) rendered the nutrients unavailable, leading to significant declines in the crop yields at the rate of 5.47 Mg ha^{-1} $year^{-1}$ and 4.67 Mg ha^{-1} $year^{-1}$, respectively. Also, a crop yield decline from 53 kg ha^{-1} in plant crop to 18 kg ha^{-1} in the sixth ratoon crop under the absolute control was observed. However, the rates of yield decline were minimized in plots including SPM and SPM plus *Ga. diazotrophicus* to 3.54 and 3.51 Mg ha^{-1} $year^{-1}$, respectively. It was also reported that *Ga. diazotrophicus* together with the fungus *Trichoderma*, in combination with trash and farmyard manure, were used as recycling amendments to sustain soil quality and sugarcane ratoon yield in an Udic Ustochrep soil of India (Shukla et al. 2012). Recently, the tolerance of *Ga. diazotrophicus* to different herbicides used in the sugarcane crop was also reported (Procópio et al. 2013).

Considering crops other than sugarcane, N_2-fixing AAB, for example, *Ga. diazotrophicus* and *A. peroxydans*, were also found in natural association with rice plants, and they may be considered important for agriculture because both species may supply a part of the nitrogen that is required by rice (Muthukumarasamy et al. 2005).

Concerning the application of *Ga. diazotrophicus* in non-gramineous plants, it was reported by Luna et al. (2012) that inoculation of tomato (*Lycopersicum esculentum*) with this bacterium could confer beneficial effects to this crop after efficient plant colonization. These authors observed that both numbers and weight of fruit production significantly increased in inoculated plants as compared to non-inoculated controls. Although the growth promotion mechanisms involved were not evaluated, *Ga. diazotrophicus* enhanced tomato fruit yield under greenhouse conditions. Also, effective colonization of *Ga. diazotrophicus* in inner tissues of tomato plantlets was observed by Botta et al. (2013).

4.6 Concluding Remarks

This chapter has shown that AAB as plant growth promoters are of beneficial importance to agriculture. Some of these bacteria have more than one mechanism of accomplishing increased plant growth, such as the biological N_2-fixing process, production of phytohormones, and solubilization of mineral nutrients. Although variability of yield in field performance is common, as they are influenced by the environment and plant and bacterial genotypes, PGPB are environmentally

friendly, in contrast to the overuse of the chemical fertilizers and pesticides applied in modern agriculture.

It is well known that chemical fertilizers increase yield in agriculture, but these are expensive, and their inappropriate use may harm the environment. They can deplete nonrenewable energy via side effects, such as leaching out, and polluting water sources, destroying microorganisms and beneficial insects, making the crops more susceptible to diseases, reducing soil fertility and biodiversity, and, consequently, causing irreversible damage to the overall agroecosystem. Therefore, the use of PGPB could be a better alternative to agrochemicals, considering that they are economical, not harmful to the environment, and can easily be found in different habitats.

Regarding the biotechnological application of AAB as plant growth promoters, the formulation and field application of inoculants need further studies. The isolation, characterization, and identification of new bacterial isolates having plant growth-promoting capacities is often not difficult and such are frequently reported. However, most new PGPB strains remain there, without ever reaching the formulation stage. Therefore, new insights for this issue should include not only *Ga. diazotrophicus*, but the other members of the *Acetobacteraceae* family with PGPB characteristics. According to Bashan et al. (2014), the development of PGPB inoculants involves a technological platform with knowledge that is based on fundamental principles of microbiology and material sciences. The unification of these fields, however, presently creates useful products that have important input for sustainable agriculture.

References

Alquéres SM, Cardoso AM, Brito-Moreira J, Baldani JI, Martins OB (2012) Transfer RNA-dependent asparagine biosynthesis in *Gluconacetobacter diazotrophicus* and its influence on biological nitrogen fixation. Plant Soil 356:209–216

Alquéres S, Meneses C, Rouws L, Rothballer M, Baldani I, Schmid M, Hartmann A (2013) The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. Mol Plant Microbe Interact 26: 937–945

Alvarez B, Martínez-Drets G (1995) Metabolic characterization of *Acetobacter diazotrophicus*. Can J Microbiol 41:918–924

Arrieta JG, Sotolongo M, Menéndez C, Alfonso D, Trujillo LE, Soto M, Ramírez R, Hernández L (2004) A type II protein secretory pathway required for levansucrase secretion by *Gluconacetobacter diazotrophicus*. J Bacteriol 186:5031–5039

Ashbolt NJ, Inkerman PE (1990) Acetic acid bacterial biota of the pink sugarcane mealybug, *Saccharococcus sacchari*, and its environs. Appl Environ Microbiol 56:707–712

Attwood MM, van Dijken JP, Pronk JT (1991) Glucose metabolism and gluconic acid production by *Acetobacter diazotrophicus*. J Ferment Bioeng 72:101–105

Bashan Y, Holguin G (1998) Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (Plant Growth-Promoting Bacteria) and PGPB. Soil Biol Biochem 30:1225–1228

Bashan Y, Puente ME, de-Bashan LE, Hernández JP (2008) Environmental uses of plant growth-promoting bacteria. In: Ait Barka E, Clément C (eds) Plant–microbe interactions. Research Signpost, Trivandrum, pp 69–93

Bastián F, Cohen A, Piccoli P, Luna V, Baraldi R, Bottini R (1998) Production of indole-3-acetic acid and gibberellins A1 and A3 by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically defined culture media. Plant Growth Regul 24:7–11

Bertalan M, Albano R, de Padua V, Rouws L, Rojas C, Hemerly A, Teixeira K et al (2009) Complete genome sequence of the sugarcane nitrogenfixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics 10(1):450. doi:10.1186/1471-2164-10-450

Boddey RM, Urquiaga S, Reis VM, Döbereiner J (1991) Biological nitrogen fixation associated with sugar cane. Plant Soil 137:111–117

Botta AL, Santacecilia A, Ercole C, Cacchio P, Del Gallo M (2013) In vitro and in vivo inoculation of four endophytic bacteria on *Lycopersicon esculentum*. N Biotechnol 30:666–674

Braun V, Winkelmann G (1987) Microbial iron transport: structure and function of siderophores. Prog Clin Biochem Med 5:69–95

Caballero-Mellado J, Martínez-Romero E (1994) Limited genetic diversity in the endophytic sugarcane bacterium *Acetobacter diazotrophicus*. Appl Environ Microbiol 60:1531–1537

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383: 3–41

Cavalcante VA, Döbereiner J (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108:23–31

Chauhan H, Bagyaraj DJ, Sharma A (2013) Plant growth-promoting bacterial endophytes from sugarcane and their potential in promoting growth of the host under field conditions. Exp Agric 49:43–52

Cleenwerck I, De Wachter M, González A, De Vuyst L, De Vos P (2009) Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*. Int J Syst Evol Microbiol 59: 1771–1786

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21:1–18

da Silva MF, de Souza Antônio C, de Oliveira PJ, Ribeiro Xavier G, Rumjanek NG, de Barros Soares LH, Massena Reis V (2012) Survival of endophytic bacteria in polymer-based inoculants and efficiency of their application to sugarcane. Plant Soil 356:231–243

De La Cruz CPP, Bird CO, Isulat MD (2012) Sprouting, survival and growth of young sugarcane (*Saccharum officinarum* L.) treated with diazotrophic bacteria (*Gluconacetobacter diazotrophicus*). Philipp Agric Sci 95:106–111

de-Bashan Y, Bashan LE, Prabhu SR, Hernandez JP (2014) Advances in plant growth-promoting bacterial inoculants technology: formulations and practical perspectives (1998–2013). Plant Soil 378:1–33

dos Santos CCR, Perin L, Baldani JI, Reis VM (2006) Isolation of *Gluconacetobacter* spp. in different soil types. Pesq Agropec Bras 41:85–92

Dow JM, Fouhy Y, Lucey JF, Ryan RP (2006) The HD-GYP domain, cyclic di-GMP signaling, and bacterial virulence to plants. Mol Plant Microbe Interact 19:1378–1384

Dutta D, Gachhui R (2006) Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 56:1899–1903

Dutta D, Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 57:353–357

Flores-Encarnación M, Contreras-Zentella M, Soto-Urzúa L, Aguilar GR, Baca BE, Escamilla JE (1999) The respiratory system and diazotrophic activity of *Acetobacter diazotrophicus* PAL5. J Bacteriol 181:6987–6995

Fuentes-Ramírez LE, Jiménez-Salgado T, Abarca-Ocampo IR, Caballero-Mellado J (1993) *Acetobacter diazotrophicus*, an indoleacetic acid producing bacterium isolated from sugarcane cultivars of Mexico. Plant Soil 154:145–150

Fuentes-Ramírez LE, Bustillos-Cristales R, Tapia-Hernández A, Jiménez-Salgado T, Wang ET, Martínez-Romero E, Caballero-Mellado J (2001) Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int J Syst Evol Microbiol 51:1305–1314

Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt M, Stephan MP, Teixeira KRS, Döbereiner J, De Ley J (1989) *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int J Syst Evol Microbiol 39:361–364

Hanna AL, Youssef HH, Amer WM, Monib M, Fayez M, Hegazi NA (2013) Diversity of bacteria nesting the plant cover of north Sinai deserts, Egypt. J Adv Res 4:13–26

Hernández L, Sotolongo M, Rosabal Y, Menéndez C, Ramírez R, Caballero-Mellado J (2000) Structural levansucrase gene (*lsd*A) constitutes a functional locus conserved in the species *Gluconacetobacter diazotrophicus*. Arch Microbiol 174:120–124

Intorne AC, de Oliveira MVV, Pereira LM, de Souza Filho GA (2012) Essential role of the czc determinant for cadmium, cobalt and zinc resistance in *Gluconacetobacter diazotrophicus* PAl5. Int Microbiol 15:69–78

Jiménez-Salgado T, Fuentes-Ramírez LE, Tapia-Hernández A, Mascarúa-Esparza MA, Martínez-Romero E, Caballero-Mellado J (1997) *Coffea arabica* L., a new host plant for *Acetobacter diazotrophicus*, and isolation of other nitrogen-fixing acetobacteria. Appl Environ Microbiol 63:3676–3683

Loganathan P, Nair S (2004) *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). Int J Syst Evol Microbiol 54:1185–1190

Loganathan P, Sunita R, Parida AK, Nair S (1999) Isolation and characterization of two genetically distant groups of *Acetobacter diazotrophicus* from new host plant *Eleusine coracana* L. J Appl Microbiol 87:167–172

Logeshwaran P, Thangaraju M, Rajasundari K (2009) Hydroxamate siderophores of endophytic bacteria *Gluconacetobacter diazotrophicus* isolated from sugarcane roots. Aust J Basic Appl Sci 3:3564–3567

Luna MF, Bernardelli CE, Galar ML, Boiardi JL (2006) Glucose metabolism in batch and continuous cultures of *Gluconacetobacter diazotrophicus* PAL3. Curr Microbiol 52:163–168

Luna MF, Aprea J, Crespo JM, Boiardi JL (2012) Colonization and yield promotion of tomato by *Gluconacetobacter diazotrophicus*. Appl Soil Ecol 61:225–229

Madhaiyan M, Saravanan VS, Bhakiya SSJD, Lee HS, Thenmozhi R, Hari K, Sa TM (2004) Occurrence of *Gluconacetobacter diazotrophicus* in tropical and subtropical plants of Western Ghats, India. Microbiol Res 159:233–243

Madhaiyan M, Poonguzhali S, Hari K, Saravanan US, Sa T (2006) Influence of pesticides on the growth rate and plant-growth promoting traits of *Gluconacetobacter diazotrophicus*. Pestic Biochem Phys 84:143–154

Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301

Mehnaz S, Weselowski B, Lazarovits G (2006) Isolation and identification of Gluconacetobacter azotocaptans from cornrhizosphere. Syst Appl Microbiol 29:496–501

Muthukumarasamy R, Revathi G, Seshadri S, Lakshminarasimhan C (2002) *Gluconacetobacter diazotrophicus* (syn. *Acetobacter diazotrophicus*), a promising diazotrophic endophyte in tropics. Curr Sci 83:137–145

Muthukumarasamy R, Cleenwerck I, Revathi G, Vadivelu M, Janssens D, Hoste B, Gum KU, Park KD, Son CY, Sa T, Caballero-Mellado J (2005) Natural association of *Gluconacetobacter diazotrophicus* and diazotrophic *Acetobacter peroxydans* with wetland rice. Syst Appl Microbiol 28:277–286

Nalawade SV, Pandhare RA, Indi DV, Mali JB, Pawar SM (2013) Combating fertilizer crisis in sugarcane by use of *Gluconacetobacter diazotrophicus*. Ecol Environ Conserv 19:825–828

Natheer SE, Muthukkaruppan S (2012) Assessing the in vitro zinc solubilization potential and improving sugarcane growth by inoculating *Gluconacetobacter diazotrophicus*. Ann Microbiol 62:435–441

Nieto-Peñalver CG, Bertini EV, de Figueroa LIC (2012) Identification of *N*-acyl homoserine lactones produced by *Gluconacetobacter diazotrophicus* PAL5 cultured in complex and synthetic media. Arch Microbiol 194:615–622

Paul A, Samaddar N, Dutta D, Bagchi A, Chakravorty S, Chakraborty W, Gachhui R (2011) Mercuric ion stabilizes levansucrase secreted by *Acetobacter nitrogenifigens* strain RG1^{T}. Protein J 30:262–272

Paula MA, Reis VM, Döbereiner J (1991) Interactions of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato (*Ipomoea batatas*), sugarcane (*Saccharum* spp.), and sweet sorghum (*Sorghum vulgare*). Biol Fertil Soils 11:111–115

Pedraza RO (2008) Recent advances in nitrogen-fixing acetic acid bacteria. Int J Food Microbiol 125:25–35

Pedraza RO, Ramírez-Mata A, Xiqui ML, Baca BE (2004) Aromatic amino acid aminotransferase activity and indole-3-acetic acid production by associative nitrogen-fixing bacteria. FEMS Microbiol Lett 233:15–21

Perlova O, Nawroth R, Zellermann EM, Meletzus D (2002) Isolation and characterization of the *glnD* gene of *Gluconacetobacter diazotrophicus*, encoding a putative uridylyltransferase/uridylyl removing enzyme. Gene (Amst) 297:159–168

Procópio SdeO, Fernandes MF, Teles DA, Sena JG, Cargnelutti A, Vargas L (2013) Tolerance of diazotrophic bacterium *Gluconacetobacter diazotrophicus* to herbicides used in the sugarcane crop. Rev Bras Ciênc Agrár 8:610–617

Reis VM, Olivares FL, Döbereiner J (1994) Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J Microbiol Biotechnol 10: 401–405

Saravanan VS, Madhaiyan M, Osborne J, Thangaraju JM, Sa TM (2008) Ecological occurrence of *Gluconacetobacter diazotrophicus* and nitrogen-fixing *Acetobacteraceae* members: their possible role in plant growth promotion. Microb Ecol 55:130–140

Serrato RV, Meneses CHSG, Vidal MS, Santana-Filho AP, Iacomini M, Sassaki GL, Baldani JI (2013) Structural studies of an exopolysaccharide produced by *Gluconacetobacter diazotrophicus* Pal5. Carbohydr Polym 98:1153–1159

Sevilla M, Meletzus D, Teixeira K, Lee S, Nutakki A, Baldani I, Kennedy C (1997) Analysis of *nif* and regulatory genes in *Acetobacter diazotrophicus*. Soil Biol Biochem 29:871–874

Shukla SK, Singh PN, Chauhan RS, Yadav RL (2012) Recycling of organic wastes amended with *Trichoderma* and *Gluconacetobacter* for sustenance in soil health and sugarcane ratoon yield in Udic Ustochrept. Commun Soil Sci Plant Anal 43:1073–1097

Singh RK, Suman A, Singh J, Singh AK, Kumar S, Singh PK, Singh RK (2012) Effects of *Gluconacetobacter diazotrophicus* on seed cane produced through micro-propagated plantlets in sugarcane (*Saccharum* spp. hybrid complex) under sub-tropics. Indian J Agric Sci 82: 340–344

Singh P, Suman A, Rai RK, Singh KP, Srivastava TK, Arya N, Gaur A, Yadav RL (2013) Bio-organic amendment of Udic Ustochrept soil for minimizing yield decline in sugarcane ratoon crops under subtropical conditions. Commun Soil Sci Plant Anal 44:1849–1861

Soto Urzúa L, Vázquez-Candanedo AP, Sánchez-Espíndola A, Ávila Ramírez C, Baca BE (2013) Identification and characterization of an iron ABC transporter operon in *Gluconacetobacter diazotrophicus* Pal 5. Arch Microbiol 195:431–438

Stephan MP, Oliveira M, Teixeira KRS, Martinez-Drets G, Döbereiner J (1991) Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. FEMS Microbiol Lett 77:67–72

Suman A, Singh P, Lal M (2013) Effects of diverse habitat biofertilizers on yield and nitrogen balance in plant-ratoon crop cycle of sugarcane in subtropics. Sugar Technol 15:36–43

Tapia-Hernández A, Bustillos-Cristales MR, Jiménez-Salgado T, Caballero-Mellado J, Fuentes-Ramírez LE (2000) Natural endophytic occurrence of *Acetobacter diazotrophicus* in pineapple plants. Microb Ecol 39:49–55

Teixeira KRS, Galler R, Kennedy C, Baldani JI (1994) Plasmid contents and *nif* genes detection in *A. diazotrophicus* strains. In: Hegazi NA, Fayez M, Monib M (eds) Nitrogen fixation with non-legumes. The American University in Cairo Press, Cairo, pp 273–281

Ureta A, Nordlund S (2002) Evidence for conformational protection of nitrogenase against oxygen in *Gluconacetobacter diazotrophicus* by a putative FeSII protein. J Bacteriol 184:5805–5809

Urquiaga S, Cruz KH, Boddey RM (1992) Contribution of nitrogen fixation to sugarcane: nitrogen-15 and nitrogen balance estimates. Soil Sci Soc Am J 56:105–114

Yamada Y, Hoshino K, Ishkawa T (1997) The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 61:1244–1251

Chapter 5
Acetic Acid Bacteria as Symbionts of Insects

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Abstract Acetic acid bacteria (AAB) are being increasingly described as associating with different insect species that rely on sugar-based diets. AAB have been found in several insect orders, among them Diptera, Hemiptera, and Hymenoptera, including several vectors of plant, animal, and human diseases. AAB have been shown to associate with the epithelia of different organs of the host, they are able to move within the insect's body and to be transmitted horizontally and vertically. Here, we review the ecology of AAB and examine their relationships with different insect models including mosquitoes, leafhoppers, and honey bees. We also discuss the potential use of AAB in symbiont-based control strategies, such as "Trojan-horse" agents, to block the transmission of vector-borne diseases.

Keywords Symbionts • Host • Sugar-based diet • Symbiotic traits • Developmental influence • Immune system involvement • Symbiont-based control strategies

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5.1 Introduction

Acetic acid bacteria (AAB) are microorganisms widespread in the environment, commonly found in association with plants, flowers, and fruits as well as with fermented food and beverages (Kersters et al. 2006). AAB are gram-negative or gram-variable bacteria, ellipsoidal or rod shaped, from family *Acetobacteraceae* within the order *Rhodospirillales*. They can occur individually, in pairs, or in short chains, and if they have peritrichous or polar flagella they are motile. AAB have an obligate aerobic metabolism with oxygen as a final electron acceptor: they are well known for their ability to oxidize ethanol into acetic acid, which accumulates in the medium, as do aldehydes, ketones, and organic acids, other metabolic products resulting from the partial oxidation of carbohydrates and alcohols (Kersters et al. 2006). Moreover, AAB can produce exopolysaccharides (EPSs), such as cellulose (Raspor and Goranovič 2008), and some, such as *Gluconacetobacter diazotrophicus*, can fix atmospheric nitrogen (Bertalan et al. 2009). These topics are reviewed in other chapters of this book.

Humans have long exploited AAB metabolic properties in so-called oxidative fermentations, for example, in the production of vinegar or other foods (Mamlouk and Gullo 2013). In industrial production, they have been used in biocatalytic processes for the production of different compounds such as vitamin C precursors and dihydroxyacetone (Raspor and Goranovič 2008). Recently, a biotransformation procedure involved the use of AAB for the one-pot preparation of aldoximes (compounds with a wide application in many fields) by combining the first step of the oxidation of primary alcohols to aldehydes, performed enzymatically by AAB, with the second step of the in situ condensation of the aldehydes with hydroxylamine (Zambelli et al. 2012). Certainly, the distinct metabolism of AAB makes them useful for application in different important biotechnological fields (e.g., pharmaceuticals, medicinal biotechnology, alternative energy source production) (Raspor and Goranovič 2008). However, in addition to their use in production of foodstuffs and beverages, AAB could also have a negative impact by spoiling fermented beverages and food (Mamlouk and Gullo 2013). Furthermore, some

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AAB have been described as plant growth-promoting bacteria (please review Chap. 4 in this book for more detail), whereas others have been documented as human pathogens (e.g., *Granulibacter bethesdensis*) or as opportunistic bacteria in immunocompromised patients (Epis et al. 2012).

Recently, an increasing number of reports have documented the detection and isolation of AAB from insect bodies. Insects are the most various and abundant animal taxa, having diverse associations with microorganisms including bacteria, fungi, yeasts, viruses, and protists. Certainly, their microbial partners have had an important part in their evolutionary success, for example, by allowing them considerable adaptability to a broad range of habitats. Microbes can provide essential nutrients to the host such as amino acids or vitamins, contribute to insect physiology, contribute to innate immune system homeostasis, or manipulate insect reproduction (Dale and Moran 2006; Moran 2006). In many cases, insects that rely on nutrient-poor diets can survive thanks to the presence of an associated microbiome which provides them with the lacking resources, thus allowing the insects to exploit and use an otherwise prohibitive food resource (Feldhaar and Gross 2009). Since its first definition as the "living together of unlike organisms" by Anton de Bary in 1879, symbiosis has been considered a fundamental component for evolutionary processes (e.g., consider the intracellular symbiotic events that took part in the formation of eukaryotic cells). Moreover, to emphasize the importance of symbiotic relationships, it is noteworthy to mention that in many cases the elimination of microbial partners and a variation in microbial composition can reduce the fitness of the host (Toft and Andersson 2010).

Since White (1921) described the first isolation of AAB in insects (honey bees), many more publications have characterized the symbiotic relationship between species of AAB and insects. To date, documentation on AAB-associated insects refers to hosts with sugar- or ethanol-rich diets such as insects that belong to the orders Diptera, Hemiptera, and Hymenoptera (Crotti et al. 2010); for example, adult mosquitoes rely on a sugar-based diet, with the exception of adult females, which also feed on blood when they produce eggs to complete the gonotrophic cycle. In addition, the symbiotic relationship between AAB and the experimental model *Drosophila melanogaster*, for which genetic modification tools are available, allow us to study the fundamental mechanisms of symbiosis (Shin et al. 2011; Lee et al. 2013). However, in many cases the role of bacteria in many host–AAB associations remains largely unknown, encouraging research efforts to fill these gaps (Shin et al. 2011; Chouaia et al. 2012).

The aim of this chapter is to review AAB associations with their insect hosts. We begin by discussing the general characteristics of insect symbiosis, followed by a presentation of some known insect species that are colonized by AAB, with particular attention to colonized host organs and transmission routes. Next, we describe how AAB contribute to insect physiology, including information derived from genome sequencing projects. We consider the possibility of using AAB as a tool to control vector-borne pathogens and pest insects in symbiont-based control strategies. Finally, we discuss some prospective practical applications by exploiting host–bacterial relationships.

5.2 General Characteristics of Insect Symbiosis

A number of symbiotic relationships can develop between insects and bacteria that depend on the evolutionary history of each species and the codependence of the interaction (Dale and Moran 2006). Symbiosis can be obligate in nature: this is primary symbiosis, in which the microbial partners are essential for the host's survival. Primary symbionts are vertically transmitted from one generation to the next in cells called bacteriocytes, which may aggregate inside organs called bacteriomes within the host. Typically, this kind of association has a nutritional basis: the primary or obligate symbionts supply the host with essential nutrients such as amino acids or vitamins. Examples of this kind of association include symbioses between the gammaproteobacterium *Candidatus Buchnera aphidicola* and aphids or the gammaproteobacterium *Candidatus Wigglesworthia glossinidia* and tsetse flies (Dale and Moran 2006). Genomic analysis of primary symbionts reveals a reduced genome throughout the course of evolution, indicating a strict dependence of microbial partners on their hosts (Moran and Bennet 2014).

Alternatively, facultative or secondary symbionts refer to the microbial partners that are not essential for the host's survival but might benefit the host through protection against stresses or parasites, or may negatively impact the host's fitness (Dale and Moran 2006). Secondary symbionts can be found in different host tissues and organs; they are typically vertically transmitted but can also be spread horizontally. A few examples include the gammaproteobacteria *Regiella insecticola*, *Hamiltonella defensa*, and *Serratia symbiotica*, which can influence choice of host plant by aphids, resistance against parasitoids and entomopathogenic fungi by the host, and tolerance for high temperatures (Oliver et al. 2010). The group of so-called reproductive manipulators includes bacteria that can induce reproductive alterations in their hosts, such as cytoplasmic incompatibility, parthenogenesis, feminization, and male-killing (Werren et al. 2008): *Wolbachia*, *Cardinium*, *Arsenophonus*, *Rickettsia*, and the wall-less *Spiroplasma* are some members of this group. Some of these, such as *Wolbachia*, may positively affect the host, for instance, for the maturation of oocytes (Pannebakker et al. 2007) and protection against viral DNA or parasitoid wasps (Martinez et al. 2014; Xie et al. 2014).

Recent work has investigated intestinal symbiosis focusing on bacterial effects on host physiology (Engel and Moran 2013). The microbiome of the gut can profoundly influence the host's physiology by affecting nutrition immune system homeostasis (Ryu et al. 2008; Lee et al. 2013), development (Shin et al. 2011; Chouaia et al. 2012), and mating preference (Sharon et al. 2010). Although commensal bacteria do not establish stable, continuous symbiotic relationships with their hosts, they can have a considerable influence on their physiology (Engel and Moran 2013). Because they are acquired directly from the environment where hosts live and feed, the insect bacterial community can vary with sampling locations and diet. However, selective digestive system features in insects restrict the recruitment of bacteria from specific ecological niches. Commensals should efficiently utilize the resources available in the intestine, pass through it, and gain a niche where they

can persist and overcome the competition exerted by other bacteria that are being continually introduced with the food source. Moreover, they should interact positively with the host and its innate immune system without triggering or activating immune response processes (Lee et al. 2013; Engel and Moran 2013).

Symbiotic AAB are gut commensals that may behave like secondary symbionts (Crotti et al. 2010; Engel and Moran 2013). As gut commensals, they are environmentally acquired; they thrive and persist in the insect intestine, without triggering or activating an immune response, and offer benefits to the host. As secondary symbionts, some AAB can spread both vertically and horizontally. These AAB may influence the host's development, metabolism, and homeostasis, although evident only in particular conditions (e.g., when the host is grown on a minimal diet) (Shin et al. 2011), or they may positively affect larval development, as does the vertically and horizontally transmitted mosquito symbiont *Asaia* (Chouaia et al. 2012; Mitraka et al. 2013).

5.3 Insects Colonized by AAB

Nowadays, thanks to high-throughput techniques, such as metabarcoding, metagenomics, metatranscriptomics, and metaproteomics, which can be applied to studying symbiotic assemblages, microbial associations can be characterized more thoroughly than previously (Fukatsu 2012).

Among insects that host AAB, *Drosophila* receives special attention, first, because of its worldwide relevance as an experimental insect model and, second, because it has been proposed as the ideal organism for studying host–symbiont relationships (Valanne and Rämet 2013; Kim and Lee 2014). *Drosophila* hosts a low-complexity gut microbiome compared to other insects (e.g., termites). Its bacterial community is made up mainly of members included in the phyla *Proteobacteria* and *Firmicutes* (Cox and Gilmore 2007; Chandler et al. 2011), as well as fungi such as yeasts belonging to the family Saccharomycetaceae (Chandler et al. 2012). Members of the AAB family *Acetobacteraceae* have been found in both laboratory-reared and wild-caught flies, particularly wild fruit-feeding and mushroom-feeding flies (Chandler et al. 2011; Wong et al. 2011). Using 16S rRNA gene pyrosequencing, different stages of laboratory-reared fruit flies have been shown to harbor sequences predominantly related to the genera *Acetobacter* and *Lactobacillus* (Wong et al. 2011). 16S rRNA gene clone libraries have been used to analyze and compare the bacterial community associated with several *Drosophila* populations from different laboratory-reared and wild species that were collected on different food sources and from different geographic locations (Chandler et al. 2011). The authors found that four bacterial families represented 85 % of the natural bacterial community associated with the flies (*Enterobacteriaceae*, *Lactobacillaceae*, *Enterococcaceae*, and *Acetobacteraceae*). Members of the *Acetobacteraceae* sequences were classified as *Commensalibacter intestini*, *Acetobacter*, and *Gluconobacter* (the latter at low frequency), whereas no

Gluconacetobacter sequences were identified. *C. intestini* have previously been isolated from *D. melanogaster* and described as involved in the host's immune homeostasis (Ryu et al. 2008; Roh et al. 2008). Although a constant microbial core could not be determined, the aforementioned families were hosted by many fly species, independently from the host's taxonomic, ecological, or geographic characteristics or behaviors. Diet has been implicated as the driver of bacterial diversity and, in fact, flies of different species collected on the same food source shared the same bacterial community (Chandler et al. 2011). Staubach et al. (2013) obtained similar results when they analyzed samples of the fruit flies *Drosophila melanogaster* and *Drosophila simulans* reared in the laboratory or from different natural matrices (e.g., rotting fruits). Furthermore, investigations have shown that laboratory-reared flies have a different and less diverse bacterial community than do wild-caught flies (Cox and Gilmore 2007; Chandler et al. 2011), which were dominated by members from *Gluconobacter*, *Acetobacter*, and *Enterobacteriaceae*. Chandler et al. (2011) found that *Acetobacteraceae* represented only 9 % of total sequences, whereas Staubach and colleagues (2013) found that the same family dominated with a relative abundance of 55.3 %. *Gluconobacter* was most prevalent in field-collected flies but was absent from lab-reared flies (Staubach et al. 2013). This study highlights how diet shapes the host's microbiota; in fact, the host effect is expected to play a marginal role, observable only in natural populations. Interestingly, a single operational taxonomic unit (OTU), classified as a *Gluconobacter* species, accounted for 34.7 % of the total sequences from field-collected flies, and it was hypothesized as a candidate for the core microbiome of wild fruit flies. However, using a 16S rRNA gene pyrosequencing approach on lab-reared and field-captured samples, Wong et al. (2013) proposed the absence of a core microbiome in *Drosophila* spp. Nevertheless, AAB were found abundantly and throughout most of the specimens.

Using lab-reared fruit flies (*D. melanogaster*), whose bacterial community comprised *Acetobacter pomorum* (98 %), *Lactobacillus plantarum* (1.9 %), and a member of the *Xanthomonadacae* family (0.1 %), Ridley et al. (2012) highlighted the importance of the resident microbiome for the host. They found that the native microbes (predominantly AAB) were essential for the host's nutrition, because their removal determined a delayed larval development compared to the control. Blum and colleagues (2013) suggested that *Drosophila* can maintain its symbiotic community by continually introducing bacteria associated to the consumed foods rather than as a consequence of the symbiont growth in the host body.

AAB have also been detected in the spotted-wing drosophila *Drosophila suzukii*, an economically important pest because it damages healthy, soft summer fruits in contrast to its fruit fly relatives that attack rotten fruits. Chandler et al. (2014) found a lower frequency of *Gluconobacter* and *Acetobacter* members in the spotted-wing drosophila (*D. suzukii*) microbiota than has been reported in other *Drosophila* flies, and instead they detected a high prevalence of the gammaproteobacterium *Tatumella*.

Another dipteran species described to host AAB, namely *Acetobacter tropicalis*, is the olive fruit fly *Bactrocera oleae*, an appreciable pest that affects olive

production worldwide (Kounatidis et al. 2009). A polymerase chain reaction (PCR)-based examination was used to detect *A. tropicalis* in wild olive fruit flies, sampled in different localities in Greece and in laboratory-reared individuals. Multiple *A. tropicalis* strains were found to inhabit the same olive fruit fly; using an *A. tropicalis* strain labeled with a green fluorescent protein (GFP), its ability to colonize and establish in the digestive system of both larval adult hosts was observed. Within the gut, GFP-labeled *A. tropicalis* cells were restricted in a brown, dense matrix that was likely the peritrophic membrane (Kounatidis et al. 2009).

Asaia is a well-studied acetic acid bacterial symbiont that can establish associations with insects from different orders. Among others, *Asaia* has been found as a major symbiont of mosquitoes, insects highly relevant for public health as vectors of viruses, parasites, and other disease-causing microorganisms. *Asaia* is an abundant bacterial symbiont of malaria-transmitting *Anopheles* and the pathogen-transmitting mosquitoes *Aedes* and *Culex* (Favia et al. 2007; Crotti et al. 2009; De Freece et al. 2014; Zouache et al. 2011). A lack of *Asaia* in the Asian malaria mosquito (*Anopheles stephensi*) larvae was shown to result in developmental delay, whereas adding *Asaia* to the African malaria mosquito (*Anopheles gambiae*) accelerated larval development (Chouaia et al. 2012; Mitraka et al. 2013). *Asaia* dominates the mosquito microbiome at different life stages and is localized to different digestive and reproductive organs and to the salivary glands (Favia et al. 2007; Crotti et al. 2009; Damiani et al. 2010; Capone et al. 2013). Similar to *A. tropicalis* and the olive fruit fly, different *Asaia* strains can be hosted by the same mosquito (Chouaia et al. 2010). In a recent paper devoted to analyzing the colonization resistance phenomenon in the phlebotomine sand fly *Lutzomyia longipalpis*, an isolate identified as *Asaia* prevented the development of the invasive pathogenic hemoflagellate *Leishmania* (Sant'Anna et al. 2014).

Asaia also inhabits the American grapevine leafhopper *Scaphoideus titanus*, vector of the grapevine yellowing phytoplasma, which causes a phytoplasmosis named flavescence dorèe (Marzorati et al. 2006). Quantitative measurements of *Asaia* by real-time PCR showed that *Asaia* 16S rRNA gene copies represented an average of approximately 5 % of the total bacterial 16S rRNA gene copies in this leafhopper (Crotti et al. 2009). In addition, findings of *Asaia* in wine grapes emphasize their presence in the vineyard ecosystem (Bae et al. 2006; Mateo et al. 2014) that constitutes a reservoir of the bacterium.

In Hymenoptera, AAB have been documented in honey bees and bumblebees, insects that provide the crucial ecosystem service of pollination (Gilliam 1997; Mohr and Tebbe 2006; Babendreier et al. 2007; Martinson et al. 2011; Cariveau et al. 2014). The composition of the honey bee microbiota has been assessed by both cultivation-dependent and cultivation-independent methods, demonstrating the association of AAB with the pollinator. Furthermore, through next-generation sequencing, the honey bee's bacterial community could be thoroughly characterized. Eight bacterial phylotypes are described as the microbial core of the honey bee's bacterial community (Alpha-1, Alpha-2, Beta, Gamma-1, Gamma-2, Firm-4, Firm-5, and Bifido) (Crotti et al. 2013). AAB members have been described in one

of the aforementioned phylotypes (the Alpha-2 one, in two clades) with sequences that are related to *C. intestini*, *Saccharibacter floricola*, and the genus *Gluconobacter* (Martinson et al. 2011). AM169, a strain described as a *Saccharibacter* sp., has recently been isolated from the honey bee: its genome has been sequenced and its symbiotic traits have been discussed (Chouaia et al. 2012; see paragraph 6). Interestingly, in 2007 Cox-Foster et al. implicated that an absence of *Firmicutes* and *Alphaproteobacteria* was associated with colony collapse disorder, evidencing the importance of maintaining an appropriate microbial balance to prevent disease (Cox-Foster et al. 2007). Potentially, a connection exists between the recent devastation to honey bee populations reported worldwide and their microbial balance.

Similarly, bumblebee populations have been on a considerable decline. Recently, species of AAB were identified in bumblebees as relevant bacteria of the gut microbiota (Cariveau et al. 2014), and in a recent publication, *Asaia astilbes*, *Commensalibacter intestini*, and the newly described species *Bombella apicola* were isolated from bumblebees (Li et al. 2014). The type strain of *Bombella apicola* isolated from the red-tailed bumblebee *Bombus lapidarius* had a 96.5 % similarity with *S. floricola* on the 16S rRNA gene (Li et al. 2014).

Finally, AAB have been reported in other insect hosts: the pink sugarcane mealybug (*Saccharicoccus sacchari*), the sugar cane leafhopper (*Perkinsiella saccharidica*), a hymenopteran hyperparasitoid wasp (*Marietta leopardina*), and the cabbage white butterfly (*Pieris rapae*) (Crotti et al. 2010).

5.4 Colonized Organs and Transmission Routes

The insect gut is one of the compartments most commonly colonized by bacteria and other microorganisms. In the digestive system, nutrients are degraded by both host enzymes and microbial communities into smaller metabolites that can easily enter the host metabolism (Dillon and Dillon 2004). Here, microbial symbionts can form highly complex or simple consortia. For instance, in the gut of termites, the microbiota is known to be one of the most complex among arthropods, suggesting that likely some of the bacteria convey important functions for the host such as the degradation of cellulose. On the other hand, as outlined earlier, the microbiota associated with *D. melanogaster* is relatively simple, dominated by only a few bacterial species (Cox and Gilmore 2007; Chandler et al. 2011).

Although we lack sufficiently detailed information to generalize the location and characteristics of the indigenous gut strains in arthropods, considering their enormous diversity, most insects known to host symbiotic AAB share one common trait: they feed on sugar-rich diets such as nectars, phloematic sap, and fruits (e.g., the fruit fly *D. melanogaster*, the Asian malaria mosquito *Anopheles stephensi*) (Crotti et al. 2010).

Members of the family *Acetobacteraceae* in the gut of insects have been shown to associate with the epithelium (Kounatidis et al. 2009; Favia et al. 2007), likely favored by their ability to synthesize polysaccharides such as cellulose. Cellulose is

a polymer of β-1,4-linked glucose units, which can be synthesized by different bacterial species, for example, members of *Acetobacteraceae* such as the well-known *Komagataeibacter xylinus*. Cellulose produced by *K. xylinus* has several favorable properties: transparency, tensile strength, fiber-binding ability, adaptability to the living body, and biodegradability (Raspor and Goranovič 2008). Some other *Acetobacteraceae* species known to produce cellulose include *Gluconacetobacter kombuchae* (*Komagataeibacter kombuchae*) (Dutta and Gachhui 2007), *K. swingsii*, *K. rhaeticus*, *K. nataicola*, and some strains of *K. hansenii*, *K. europaeus*, and *K. oboediens* (Dutta and Gachhui 2007; Lisdiyanti et al. 2006). Some cellulose-producing AAB species associate with arthropods, for instance, species of the *Asaia* genus in mosquitoes (Chouaia et al. 2014). Cellulose is valuable for the interaction of the bacterial producers with other prokaryotes and with eukaryotic cells, such as the epithelial cells in the gut of arthropods. In animal pathogens, cellulose has also been shown to participate in biofilm formation, multicellular behavior, adherence to animal cells, and stress tolerance (Barak et al. 2007).

The nature of the open circulatory system of insects may explain the absence of a strict anaerobic environment in the gastrointestinal tract, at least in small insects (Cox and Gilmore 2007). Support for this explanation is provided by studies aiming to characterize the microbiota associated with fruit flies or bees that found no obligate anaerobic phylotypes (Cox and Gilmore 2007; Mohr and Tebbe 2006). Genomic studies have shown that AAB are not only able to thrive in strictly aerobic conditions, but thanks to the presence of fully functional modules of cytochrome oxidases bo_3 and *bd*, these bacteria can grow in low-level O_2 conditions (Chouaia et al. 2014). In fact, functional ubiquinol oxidases can use O_2 as an electron acceptor even at low O_2 concentrations, allowing *Acetobacteraceae* to colonize ecological niches with different gradients of O_2 concentrations, for example, the insect gut and other insect compartments. This study also showed that a common ancestor of AAB has this capacity and that it has been maintained throughout their evolution (Chouaia et al. 2014).

AAB can metabolize sugars to acids and can tolerate a wide range of low pH conditions (Table 5.1). This capability, in addition to their ability to grow at low O_2 concentrations, makes the gut of insects an excellent ecological niche for AAB, as is supported by the recovery of these bacteria from the digestive system of several insects such as fruit flies, mosquitoes, bees, and leafhoppers.

AAB have been shown to colonize insect organs other than the gut. For example, Dong and colleagues (2006) observed that *Asaia* sp. can cross the insect gut epithelial barrier into the hemolymph. In agreement with this observation, Favia et al. (2007) detected *Asaia* cells in the salivary glands of the Asian malaria mosquito (*Anopheles (An.) stephensi*). This observation supports the hypothesis that AAB can actively colonize other compartments of the insect body from their point of entry (i.e., insect gut).

Other studies have shown that AAB can colonize both male and female reproductive organs. Colonization experiments have shown that members of the *Asaia* genus are found in close association with gonoduct epithelium and reproductive organs in some insects including the American grapevine leafhopper (*Scaphoideus*

Table 5.1 Physiological and genomic-inferred features of acetic acid bacteria (AAB) capable of a symbiotic lifestyle in insects

Organism	*bd*	bo_3	Growth on sugars	2,3-Butanediol	Acetoin	Flagellum	T3SS	Production of organic acids
Acetobacter pomorum	+	+	+	−	−	−	−	+
Acetobacter tropicalis	+	+	+	+	+	−	−	+
Asaia platycodi	−	+	+	+	+	+/−	−	+
Commensalibacter intestini	+	+	+	+	+	−	−	+
Gluconacetobacter diazotrophicus	+	+	+	+	−	+	−	+
Gluconobacter morbifer	+	+	+	+	−	+	−	+
Granulibacter bethesdensis	+	+	+	−	−	−	+/−	+
Saccharibacter sp.	+	+	+	−	+	+	−	+

titanus) and malaria-transmitting mosquitoes (*Anopheles*) (Crotti et al. 2009; Damiani et al. 2008, 2010). *Asaia* cells have also been colocalized with *Plasmodium* oocytes during a plasmodial infection (Capone et al. 2013). Evidence proving that AAB are not restricted to the gut of insects but can spread to other organs, especially the salivary glands and reproductive organs, is important in explaining their transmission routes and how they may colonize an insect population.

Favia et al. (2007) described the transmission routes of *Asaia* in malaria-transmitting mosquitoes (*Anopheles*). They demonstrated that the *Anopheles–Asaia* system is as a fringe situation in which environmental intake of the symbiont is most likely a common source of acquisition throughout the developmental stages of the insect, but where transmission from parents to offspring and from male to female mosquitoes is also possible. However, in this case a clear separation between environmental acquisition and vertical transmission of the symbiont is hard to define. The situation is comparable to that of microorganisms found in association with the gut of wood-feeding cockroaches and termites, where vertical and horizontal transmissions may coexist, and their relative importance is not yet clear (Nalepa et al. 2001). Damiani et al. (2008) found that a paternal transmission route of *Asaia* to their progeny occurs in the Asian malaria mosquito (*Anopheles (An.) stephensi*). This mode of transmission is uncommon in arthropods, and these results represent the first demonstration of a paternal transmission of a bacterial symbiont in mosquitoes. Previously, this mode of transmission had been only demonstrated for beneficial symbionts in aphids (Moran and Dunbar 2006).

Evidence of a trans-stadial transfer of *Asaia* from larvae to pupae and from pupae to adults of *An. stephensi* has also been obtained (Damiani et al. 2008); trans-stadial bacterial transfers in mosquitoes have continued to be a subject of controversy (Lindh et al. 2008; Moll et al. 2001; Pumpuni et al. 1996; Jadin et al. 1966). Moreover, experiments using molecular tools presented by Damiani et al. (2008) failed to detect *Asaia* symbionts in the larval breeding water, thus strengthening the belief that symbionts transfer from one mosquito stage to the next. This type of symbiont transfer was supported by Chouaia and colleagues (2012), who detected *Asaia* in Asian malaria mosquito (*An. stephensi*) adults whose L1 larvae had been inoculated with a strain of *Asaia*.

The vertical transmission of *Asaia* to the offspring occurs through an egg-smearing process, in which the symbiotic bacteria are smeared on the egg surface during passage through the mother's gonoduct. This transmission process has been described for *Asaia* symbionts of the African malaria mosquito (*An. gambiae*) and the American grapevine leafhopper (*S. titanus*) (Damiani et al. 2010; Gonella et al. 2012).

The horizontal transmission of *Asaia* symbionts among insects that belong to phylogenetically distant orders indicates another important ecological feature of acetic acid bacterial symbionts of insects (Crotti et al. 2009). *Asaia* has been shown to be present and capable of cross-colonizing other sugar-feeding insects of phylogenetically distant genera and orders in addition to their original hosts, *Anopheles* spp. PCR, real-time PCR, and in situ hybridization experiments have shown *Asaia* in the body of the yellow fever mosquito (*Aedes (Ae.) aegypti*) and the American

grapevine leafhopper (*S. titanus*) (Marzorati et al. 2006), vectors of human viruses and grapevine phytoplasma, respectively. Cross-colonization patterns of the bodies of mosquitoes (*Ae. aegypti* and *An. stephensi*) and the American grapevine leafhopper (*S. titanus*) have been documented with *Asaia* strains isolated from the mosquitoes *An. stephensi* or *Ae. aegypti* and labeled with plasmid- or chromosome-encoded fluorescent proteins.

5.5 The Role of AAB in Insect Physiology

AAB have been found in association with several insect species from different orders. This observation raised the question about the type of interaction between AAB and their corresponding hosts.

Although the presence of several of these bacteria has been investigated in the fruit fly *Drosophila melanogaster* (e.g., *A. pomorum*, *C. intestini*, and *Gluconobacter morbifer*) (Ryu et al. 2008; Shin et al. 2011), and their interactions with their insect host are discussed in detail in the next chapter (Chap. 6), other studies have sought to uncover the role of other AAB (e.g., *Asaia*) in mosquitoes. A study performed on the Asian malaria mosquito (*An. stephensi*) (Chouaia et al. 2012) showed that larvae that have been depleted of their natural microbiota, including *Asaia*, by an antibiotic treatment had a slower developmental rate compared to untreated larvae. The replenishment of the symbiont (using an antibiotic-resistant mutant of the original *Asaia* strain) was sufficient to reestablish a normal developmental rate. Such observations were later confirmed by an independent study that showed that *Asaia*, when administered to a mosquito host (e.g., the African malaria mosquito *An. gambiae*), could stimulate the developmental rate of the larvae and increase their body weight and size (Mitraka et al. 2013). As argued by the authors, this accelerated developmental rate gives the host a selective advantage because it spends a shorter time in the larval stage when it is highly vulnerable to predation. These studies demonstrate the benefits relayed by *Asaia* to host fitness; however, the mechanisms could only be hypothesized as the stimulation of specific pathways on the host (Mitraka et al. 2013) or a supply of lacking nutrients such as vitamin B or amino acids (Chouaia et al. 2012).

Along with the ability to produce cellulose, some members of *Acetobacteraceae* can also fix atmospheric nitrogen. These two metabolic capabilities are noteworthy because they are potentially important for bacterial interaction with insect hosts. Several strains of AAB have been described as nitrogen fixers: *Ga. diazotrophicus* (Yamada et al. 1997), *Ga. johannae*, *Ga. azotocaptans* (Fuentes-Ramírez et al. 2001), *K. kombuchae* (Dutta and Gachhui 2007), *Acetobacter peroxydans* (Muthukumarasamy et al. 2005), *Acetobacter nitrogenifigens* (Dutta and Gachhui 2006), and *Swaminathania salitolerans* (Loganathan and Nair 2004). The potential importance of nitrogen-fixing bacteria as symbionts of arthropods, both for the growth of the arthropods and for their ecosystem, through the processing of carbon and nitrogen has been suggested (Behar et al. 2005; Nardi et al. 2002). The diets of

many arthropods have high carbon-to-nitrogen (C:N) ratios, implying that they do not obtain sufficient nitrogen from their diet and that they must obtain additional nitrogen from other sources (e.g., atmospheric nitrogen fixed by symbiotic partners) (Nardi et al. 2002).

The capacity of some AAB (e.g., *Asaia platycodi*) to synthesize vitamin B could be very important for insects whose diets do not provide sufficient quantities. For instance, the diet of blood-feeding insects lacks vitamin B, which is not carried in the blood; therefore, they must rely on other sources such as their bacterial symbionts for its supply. A well-studied example of nutritional dependence is the tsetse fly *Glossina morsitans*, which relies on its symbionts to supply thiamine (Snyder et al. 2010; Rio et al. 2012). In this case, the loss of the symbionts may cause sterility (Aksoy and Rio 2005).

AAB also interact with the host immune system; this interaction has been widely investigated in different insect models, such as the fruit fly *D. melanogaster* (Ryu et al. 2008; Roh et al. 2008), but also in the African malaria mosquito *An. gambiae*. Using silencing by RNA interference (RNAi), AgDscam, a gene involved in the *An. gambiae* innate immune response, allowed the proliferation of *Asaia bogorensis* in the hemolymph (Dong et al. 2006). In an uncompromised host, *Asaia* has been shown to use the mosquito gut as a primary niche; it is able to line the midgut epithelium and embed in a gelatinous matrix (Favia et al. 2007). The AgDscam immune reaction contains the bacterium in the midgut, and the depletion of this essential hypervariable receptor could allow *Asaia* to initiate a translocation process across the midgut epithelium to the hemolymph, where a massive proliferation occurs.

5.6 Symbiotic Traits of Acetic Acid Bacterial Genomes: An Evolutionary Perspective

Because much of the knowledge needed for a proper understanding of the processes catalyzed by AAB remains unavailable, gaining access to AAB genomes is helping to unravel the relationships between AAB and their insect hosts. In the case of other bacterial symbionts, genome sequencing has successfully uncovered the interactions between some insects and their primary symbionts. For example, a metabolic interdependence between *Candidatus Sulcia muelleri* and *Candidatus Baumannia cicadellinicola* has been deduced from the genome organization and from the reconstruction of the metabolic pathways of both symbionts (McCutcheon and Moran 2007).

To date, 77 acetic acid bacterial genomes have been sequenced, either as a complete genome or in draft form. These genomes belong to 45 species representing 15 genera including *Acetobacter*, *Asaia*, *Commensalibacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, and *Saccharibacter*. Several sequenced genomes belong to species that have been

described in association with arthropods (e.g., *Asaia platycodi*, *Gluconobacter morbifer*, and *C. intestini*) (Table 5.1). The genomic data obtained thus far shed light on the phylogeny and evolution of different members of this group and provide clues to the interactions between AAB and their insect hosts that are beginning to explain their success as symbionts (Chouaia et al. 2014).

Up to now, studies aiming to understand the nature of the symbiosis between AAB and their hosts have focused mostly on their role or potential benefits conveyed by AAB to their hosts (Ryu et al. 2008; Gross et al. 2009; Shin et al. 2011; Chouaia et al. 2012; Lee et al. 2013). These studies have helped to explain interactions within the insect host, including the ability of AAB to colonize host tissues and interact with the innate immunity and developmental pathways of the host. For instance, *Asaia* benefits mosquito larvae during development (Chouaia et al. 2012; Mitraka et al. 2013), and AAB in *Drosophila* have been shown to modulate innate immunity (Ryu et al. 2008; Shin et al. 2011).

Annotation of acetic acid bacterial genomes revealed a series of traits compatible with a symbiotic lifestyle in the gut of insects. For example, *Asia* (*As.) platycodi* and *Saccharibacter* sp. present several secretion systems (e.g., Sec-SRP, Tat, type IV SS) and ABC transporters (in the case of *As. platycodi*) that may be involved in crosstalk with the host (Chouaia et al. 2014). A series of bacterial components for motility and cell-surface structures can be implicated in the colonization of the host gut epithelium: genes for flagellar machinery (e.g., MotA, MotB, FlaA, FlaB, FlgC, FlgD) and genes encoding for fimbriae (sF-Chap, sF-UshP). These features help establish symbiotic relationships by allowing bacteria to actively colonize different compartments of the host. Although these traits are important, they were not specific to insect symbionts because they have been observed among other AAB species (e.g., *Acetobacter aceti*, *Ga. diazotrophicus*, *Gluconobacter frateurii*) (Chouaia et al. 2014).

Both genomes contain the operon for the production of acetoin and 2,3-butandiol: these molecules have been shown to have a role in insect pheromone signaling (Tolasch et al. 2003). 2,3-Butandiol has also been implicated in modulating the innate immunity response of vertebrate hosts, facilitating tissue colonization by pathogenic bacteria (Bari et al. 2011). Therefore, we can speculate that production of metabolites may interfere with insect physiology and innate immunity might have provided AAB with a preadaptive feature toward symbiosis with insect hosts. This trait has been observed in other AAB including most of those described as insect symbionts (i.e., *A. tropicalis*, *C. intestini*, *Ga. diazotrophicus*, *K. europaeus*, *K. oboediens*, *G. frateurii*, *G. morbifer*, *Gluconobacter oxydans*, and *Gluconobacter thailandicus*) (Table 5.1).

The reconstruction of the phylogeny of this group using available genomic data (Chouaia et al. 2014) allowed further understanding of the evolution of AAB. A phylogenic reconstruction was inferred using the amino acidic sequences of 70 proteins common to all the available AAB genomes. Results showed some congruence in taxonomy based on phenotypic traits of AAB. The study found that the genera *Acetobacter* and *Gluconacetobacter* clustered together whereas *Gluconobacter* spp., *Asaia*, and *Saccharibacter* formed a separate group. This result was also

Fig. 5.1 A heatmap analysis performed on the subset of "conserved" orthologue groups based on the work of Chouaia et al. (2014). Analysis of the patterns of gain and loss of different orthologues shows that the clustering of different groups is congruent with the phylogenomic analysis performed on 70 CDS (From Chouaia et al. 2014, Figure 1A)

confirmed by a cluster analysis performed on the conserved orthologous gene groups (e.g., orthologues present in at least 50 % of available AAB genomes), which showed that in terms of gene acquisitions and losses, there was coherence at the genus level and in the topology of the cladogram that resulted from the congruent analysis of phylogeny inferred from the alignments of 70 protein sequences (Fig. 5.1).

The study by Chouaia and colleagues (2014) showed deep branching in the AAB family of pathogens, such as *Granulibacter (Gr.) bethesdensis* (Fig. 5.1), further supporting the prediction that the capacity of AAB to establish intimate associations with animal hosts is an ancestral trait. On the other hand, the association of AAB with phylogenetically diverse insect species (Crotti et al. 2010) can be believed to be relatively recent considering the phylogenetic proximity of symbiont and free-living bacteria (Chouaia et al. 2010).

5.7 Exploitation of AAB as Tools in Symbiont-Based Control Strategies

Nowadays, infectious diseases transmitted by insects are a threat to public health worldwide. For example, mosquitoes can transmit many parasites and viruses to humans and other animals, including malaria plasmodia, which kill more than 1 million people every year with 300–500 million infections recorded per year (Murray et al. 2012). In the absence of vaccines or drug therapies to control some infectious diseases, we rely mainly on chemical insecticides to control vector populations; however, ecosystems saturated with insecticides promote chemically

resistant arthropods. Thus, we urgently need new tools for controlling vector populations (Wang and Jacobs-Lorena 2013).

Symbiotic control refers to the exploitation of microbial symbionts to control an arthropod pest or vector, reduce the vector competence, or improve the health of beneficial insects (Crotti et al. 2012; Ricci et al. 2012). The diversity of interactions between insects and their microbial partners leads to proposing different strategies based on native or modified symbionts (Crotti et al. 2012). Among these is the use of engineered symbionts as vehicles to deliver effector genes encoding for molecules that can impair the transmission of vector-borne pathogens or parasites (i.e., affect vector competence) (Durvasula et al. 1997; Beard et al. 2001). Wang and Jacobs-Lorena (2013) summarize what would be required to exploit the symbiotic relationship in this way. In brief, stably associated symbionts that are easy cultivable and genetically modifiable are engineered. The symbionts must be maintained in the host without altering its function and preserve its fitness comparable to that of the wild type. Next, released effector molecules should interact appropriately with the pathogen, and finally the engineered symbiont must be dispersed into a specific environmental niche.

A well-known example of an engineered symbiont to block the transmission of a pathogen is the blocked transmission of the parasitic protozoan *Trypanosoma cruzi*, which causes Chagas disease and is vectored by the kissing bug *Rhodnius prolixus* (Beard et al. 2001; Sassera et al. 2013). In this case, the symbiotic actinomycete *Rodhococcus rhodnii*, which is an essential symbiont of the kissing bug *R. prolixus* that is involved in its growth and development and which inhabits its host's gut lumen in proximity to *T. cruzi*, was genetically modified to successfully express anti-trypanosomal molecules [e.g., antimicrobial peptides (AMPs), endoglucanases, and transmission-blocking single-chain antibodies] (Hurwitz et al. 2011). Currently, efforts to reduce vector competence of the sand fly *Phlebotomus argentipes* to host the protozoan human blood parasite *Leishmania donovani* are also underway (Hurwitz et al. 2011).

This symbiont-based control strategy is also being investigated and evaluated to control mosquito-borne diseases (Wang and Jacobs-Lorena 2013; Ricci et al. 2012). AAB from the genus *Asaia* have been proposed as a potential "Trojan horse" tool with the aim of controlling malaria parasites transmitted by the malaria-transmitting mosquitoes *Anopheles* (Favia et al. 2007; Damiani et al. 2010; Crotti et al. 2012; Ricci et al. 2012). In fact, in line with the requirements presented here, *Asaia* is stably associated with different developmental stages of mosquitoes from the genera *Anopheles* and *Aedes* that are well known for their ability to transmit *Plasmodium* parasites (*Anopheles* spp.) and viruses (*Aedes* spp.). *Asaia* is a cultivable bacterium that has been engineered for the expression of fluorescent proteins (Favia et al. 2007). GFP-labeled *Asaia* have been used successfully in the laboratory to illustrate recolonization in insects by appearing in different organs and tissues of the host: the salivary glands and the gut, key organs for *Plasmodium* development (Capone et al. 2013), and both male and female reproductive organs. Capone et al. (2013) showed that strains of GFP-labeled *Asaia* colocalized in salivary glands and the gut with a recombinant strain of *Plasmodium* expressing

the fluorescent protein. The authors also showed that in the presence of the parasite cells, *Asaia* followed a bloom, stimulating the transcription of AMPs while remaining tolerated by the immune system (Capone et al. 2013). Furthermore, *Asaia* symbionts are vertically and horizontally transmitted and can cross-colonize mosquitoes of different species and genera. Therefore, using *Asaia* cells engineered for the expression of anti-*Plasmodium* effectors could be a sustainable mechanism for controlling the spread of malaria (Damiani et al. 2008, 2010; Crotti et al. 2009).

To complete our overview of this symbiont-based technique, we wish to highlight that in addition to human and other animal pathogens and parasites, plants can also be a target of insect-transmitted pathogens. For example, the grapevine-affecting Pierce's disease is caused by the gammaproteobacterium *Xylella fastidiosa*, which is transmitted by the glassy-winged sharpshooter, *Homalodisca vitripennis*. In this case, the cultivable and genetically manipulable bacterium *Alcaligenes xylosoxidans* ssp. *denitrificans* isolated from the host's foregut has been proposed as a candidate for delivering *Xylella*-antagonistic factors (Miller 2011). Similarly, *Asaia* symbionts of the American grapevine leafhopper *S. titanus*, vector of *Phytoplasma* pathogens (Marzorati et al. 2006; Gonella et al. 2012; Crotti et al. 2009), could be suitable candidates for delivering anti-*Phytoplasma* molecules (Crotti et al. 2012).

5.8 Conclusions and Remarks

During the past decade, an increasing number of studies have shown the extent of associations between AAB and insects; however, because investigations clarifying the role of these microorganisms have only been performed for a few cases (e.g., on the insect models *Drosophila* and *Anopheles*) (Shin et al. 2011; Chouaia et al. 2012; Mitraka et al. 2013), considerable opportunities remain to fill this knowledge gap. Certainly, genome analysis, transcriptomics, and proteomics could help to delineate symbiotic traits of AAB.

Developments in our understanding of microbial relevance for the function of higher organisms are encouraging an attitude to consider higher animals not as unique organisms but as *metaorganisms*, where microbial partners are included (Bosch and McFall-Ngai 2011). Deciphering the human-associated microbiome, its function, its interaction, and its role with the host is a cutting-edge topic of research. Awareness of microbial involvement in disease and metabolic disorders is directing many current research projects (Turnbaugh et al. 2009). Maintaining the native structure of the host microbiome is essential for preventing pathogenic invasions. Insects, particularly *Drosophila*, represent a model system for studying the symbiotic relationships established between host and microbes (Lee et al. 2013), and, as discussed earlier, AAB are one of the major symbionts in *Drosophila*. Thus, investigations are currently ongoing to uncover the interactions between AAB and their insect hosts and to practically exploit this information (Shin et al. 2011; Lee et al. 2013). Potentially, AAB symbionts could be good candidates for use in

microbial resource management (MRM), an approach that foresees the management and exploitation of microorganisms associated to a specific system, or their metabolic capabilities, to overcome practical problems (Verstraete 2007; Crotti et al. 2012). However, to implement a successful MRM approach, we need to understand the interworkings of the relationship between symbionts and a given host arthropod.

Certain strains of AAB are also of interest for their use as probiotics (Hamdi et al. 2011). Together with lactic acid bacteria and spore-forming bacteria, AAB have been suggested as potential agents to counteract the establishment of pathogens, for instance, in the honey bee, through competitive exclusion, an enhancement of the immune system response, and an acidification of the host gut environment (Hamdi et al. 2011; Evans and Lopez 2004). Lactic acid bacteria are well-known probiotic strains that are widely used in humans and other animals to prevent pathogen invasions, enhance innate immunity, and prevent dysbiosis. AAB in the gut of insects have been reported to modulate host innate immunity (Ryu et al. 2008; Capone et al. 2013); they are efficient colonizers of the insect midgut, tolerate low pH, produce organic acids, and use a wide range of sugars, potentially competing with pathogens for nutrients and physical niches in the gut (Crotti et al. 2013).

The interactions between AAB and their hosts are only beginning to be understood. A considerable amount of work is still necessary to clarify these relationships and potentially exploit this information for practical application.

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References

Aksoy S, Rio RV (2005) Interactions among multiple genomes: tsetse, its symbionts and trypanosomes. Insect Biochem Mol Biol 35:691–698. (Erratum in: Insect Biochem Mol Biol 2008;38:1033)

Babendreier D, Joller D, Romeis J et al (2007) Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol 59:600–610

Bae S, Fleet GH, Heard GM (2006) Lactic acid bacteria associated with wine grapes from several Australian vineyards. J Appl Microbiol 100:712–727

Barak JD, Jahn CE, Gibson DL, Charkowsky AO (2007) The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. Mol Plant Microbe Interact 20:1083–1091

Bari W, Song YJ, Yoon SS (2011) Suppressed induction of proinflammatory cytokines by a unique metabolite produced by *Vibrio cholerae* O1 El Tor biotype in cultured host cells. Infect Immun 79:3149–3158

Beard CB, Dotson EM, Pennington PM et al (2001) Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. Int J Parasitol 31:621–627

Behar A, Yuval B, Jurkevitch E (2005) Enterobacteria-mediated nitrogen fixation in natural population of the fruit fly *Ceratitis capitata*. Mol Ecol 14:2637–2643

Bertalan M, Albano R, de Pádua V et al (2009) Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics 10:450

Blum JE, Fischer CN, Miles J, Handelsman J (2013) Frequent replenishment sustains the beneficial microbiome of *Drosophila melanogaster*. mBio 4:e00860-13

Bosch TC, McFall-Ngai MJ (2011) Metaorganisms as the new frontier. Zoology (Jena) 114:185–190

Capone A, Ricci I, Damiani C et al (2013) Interactions between *Asaia*, *Plasmodium* and *Anopheles*: new insights into mosquito symbiosis and implication in malaria symbiotic control. Parasit Vectors 6:182–194

Cariveau DP, Elijah Powell J, Koch H et al (2014) Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). ISME J 8:2369–2379

Chandler JA, Lang JM, Bhatnagar S et al (2011) Bacterial communities of diverse *Drosophila* species: ecological context of a host–microbe model system. PLoS Genet 7:e1002272

Chandler JA, Eisen JA, Kopp A (2012) Yeast communities of diverse *Drosophila* species: comparison of two symbiont groups in the same hosts. Appl Environ Microbiol 78:7327–7336

Chandler JA, Jospin G, Lang JM (2014) Bacterial communities of *Drosophila suzukii* collected from live cherries. Peer J 2:e474

Chouaia B, Rossi P, Montagna M et al (2010) Typing of *Asaia* spp. bacterial symbionts in four mosquito species: molecular evidence for multiple infections. Appl Environ Microbiol 76:7444–7450

Chouaia B, Rossi P, Epis S et al (2012) Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. BMC Microbiol 12:S2

Chouaia B, Gaiarsa S, Crotti E et al (2014) Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts. Genome Biol Evol 6:912–920

Cox C, Gilmore M (2007) Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Infect Immun 75:1565–1576

Cox-Foster DL, Conlan S, Holmes EC et al (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283–287

Crotti E, Damiani C, Pajoro M et al (2009) *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically-distant genera and orders. Environ Microbiol 11:3252–3264

Crotti E, Rizzi A, Chouaia B et al (2010) Acetic acid bacteria, newly emerging symbionts of insects. Appl Environ Microbiol 76:6963–6970

Crotti E, Balloi A, Hamdi C et al (2012) Microbial symbionts: a resource for the management of insect-related problems. Microb Biotechnol 5:307–317

Crotti E, Sansonno L, Prosdocimi EM et al (2013) Microbial symbionts of honeybees: a promising tool to improve honeybee health. New Biotechnol 30:716–722

Dale C, Moran NA (2006) Molecular interactions between bacterial symbionts and their hosts. Cell 126:453–465

Damiani C, Ricci I, Crotti E et al (2008) Paternal transmission of symbiotic bacteria in malaria vectors. Curr Biol 18:R1087–R1088

Damiani C, Ricci I, Crotti E et al (2010) Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. Microb Ecol 60:644–654

De Freece C, Damiani C, Valzano M et al (2014) Detection and isolation of the α-proteobacterium *Asaia* in *Culex* mosquitoes. Med Vet Entomol 28:438–442

Dillon RJ, Dillon VM (2004) The gut bacteria of insects: non-pathogenic interactions. Annu Rev Entomol 49:71–92

Dong YH, Taylor E, Dimopoulos G (2006) AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. PLoS Biol 4:1137–1146

Durvasula RV, Gumbs A, Panackal A et al (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci USA 94:3274–3278

Dutta D, Gachhui R (2006) Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 56:1899–1903

Dutta D, Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea. Int J Syst Evol Microbiol 57:353–357

Engel P, Moran NA (2013) The gut microbiota of insects: diversity in structure and function. FEMS Microbiol Rev 37:699–735

Epis S, Gaibani P, Ulissi U et al (2012) Do mosquito-associated bacteria of the genus *Asaia* circulate in humans? Eur J Clin Microbiol Infect Dis 31:1137–1140

Evans JD, Lopez DL (2004) Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). J Econ Entomol 97:752–756

Favia G, Ricci I, Damiani C et al (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proc Natl Acad Sci USA 104:9047–9051

Feldhaar H, Gross R (2009) Insects as hosts for mutualistic bacteria. Int J Med Microbiol 299:1–8

Fuentes-Ramírez LE, Bustillos-Cristales R, Tapia-Hernandez A et al (2001) Novel nitrogen fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. Int J Syst Evol Microbiol 51:1305–1314

Fukatsu T (2012) Next-generation sequencing sheds light on intricate regulation of insect gut microbiota. Mol Ecol 21:5908–5910

Gilliam M (1997) Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiol Lett 155:1

Gonella E, Crotti E, Rizzi A et al (2012) Horizontal transmission of the symbiotic bacterium *Asaia* sp. in the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). BMC Microbiol 18: S4

Gross R, Vavre F, Heddi A et al (2009) Immunity and symbiosis. Mol Microbiol 73:751–759

Hamdi C, Balloi A, Essanaa J et al (2011) Gut microbiome dysbiosis and honey bee health. J Appl Entomol 135:524–533

Hurwitz I, Fieck A, Read A et al (2011) Paratransgenic control of vector borne diseases. Int J Biol Sci 7:1334–1344

Jadin J, Vincke IH, Dunjic A et al (1966) Role of *Pseudomonas* in the sporogenesis of the hematozoon of malaria in the mosquito. Bull Soc Pathol Exot Filiales 59:514–525

Kersters K, Lisdiyanti P, Komagata K, Swings J (2006) The family *Acetobacteraceae*: the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The Prokaryotes, vol 5, 3rd edn. Springer, New York, pp 163–200

Kim SH, Lee WJ (2014) Role of DUOX in gut inflammation: lessons from *Drosophila* model of gut–microbiota interactions. Front Cell Infect Microbiol 3:116

Kounatidis I, Crotti E, Sapountzis P et al (2009) *Acetobacter tropicalis* is a major symbiont in the olive fruit fly (*Bactroceraoleae*). Appl Environ Microbiol 75:3281–3288

Lee KA, Kim SH, Kim EK et al (2013) Bacterial-derived uracil as a modulator of mucosal immunity and gut–microbe homeostasis in *Drosophila*. Cell 153:797–781

Li L, Praet J, Borremans W et al (2014) *Bombella intestini* gen. nov., sp. nov., a novel acetic acid bacterium isolated from bumble bee crop. Int J Syst Evol Microbiol pii:ijs.0.068049-0. doi:10.1099/ijs.0.068049-0

Lindh JM, Borg-Karlsonb A-K, Faye I (2008) Transstadial and horizontal transfer of bacteria within a colony of *Anopheles gambiae* (Diptera: Culicidae) and oviposition response to bacteria-containing water. Acta Trop 107:242–250

Lisdiyanti P, Navarro RR, Uchimura T, Komagata K (2006) Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov. Int J Syst Evol Microbiol 6:2101–2111

Loganathan P, Nair S (2004) *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). Int J Syst Evol Microbiol 54:1185–1190

Mamlouk D, Gullo M (2013) Acetic acid bacteria: physiology and carbon sources oxidation. Indian J Microbiol 53:377–384

Martinez J, Longdon B, Bauer S et al (2014) Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. PloS Pathog 10: e1004369

Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. Mol Ecol 20:619–628

Marzorati M, Alma A, Sacchi L et al (2006) A novel *Bacteroidetes* symbiont is localized in *Scaphoideus titanus*, the insect vector of flavescence dorée in *Vitis vinifera*. Appl Environ Microbiol 72:1467–1475

Mateo E, Torija MJ, Mas A, Bartowsky EJ (2014) Acetic acid bacteria isolated from grapes of South Australian vineyards. Int J Food Microbiol 178:98–106

McCutcheon JP, Moran NA (2007) Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci USA 104:19392–19397

Miller T (2011) Paratransgenesis as a potential tool for pest control: review of applied arthropod symbiosis. J Appl Entomol 135:474–478

Mitraka E, Stathopoulos S, Siden-Kiamos I et al (2013) *Asaia* accelerates larval development of *Anopheles gambiae*. Pathog Global Health 107:305–311

Mohr KI, Tebbe CC (2006) Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field. Environ Microbiol 8:258–272

Moll RM, Romoser WS, Modrzakowski MC et al (2001) Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J Med Entomol 38:29–32

Moran NA (2006) Symbiosis. Curr Biol 16:R866–R871

Moran NA, Bennet GM (2014) The tiniest tiny genomes. Annu Rev Microbiol 68:195–215

Moran NA, Dunbar HE (2006) Sexual acquisition of beneficial symbionts in aphids. Proc Natl Acad Sci USA 103:12803–12806

Murray CJ, Rosenfeld LC, Lim SS et al (2012) Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 379:413–431

Muthukumarasamy R, Cleenwerck I, Revathi G et al (2005) Natural association of *Gluconacetobacter diazotrophicus* and diazotrophic *Acetobacter peroxydans* with wetland rice. Syst Appl Microbiol 28:277–286

Nalepa CA, Bignell DE, Bandi C (2001) Detritivory, coprophagy and the evolution of digestive mutualisms in Dictyoptera. Insect Soc 48:194–201

Nardi JB, Mackie RI, Dawson JO (2002) Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? J Insect Physiol 48:751–763

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55:247–266

Pannebakker BA, Loppin B, Elemans CP et al (2007) Parasitic inhibition of cell death facilitates symbiosis. Proc Natl Acad Sci U S A 104:213–215

Pumpuni CB, Demaio J, Kent M et al (1996) Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. Am J Trop Med Hyg 54:214–218

Raspor P, Goranovič D (2008) Biotechnological application of acetic acid bacteria. Crit Rev Biotechnol 28:101–124

Ricci I, Valzano M, Ulissi U et al (2012) Symbiotic control of mosquito-borne disease. Pathog Global Health 106:380–385

Ridley EV, Wong AC-N, Westmiller S, Douglas AE (2012) Impact of the resident microbiota on the nutritional phenotype of *Drosophila melanogaster*. PLoS ONE 7:e36765

Rio RV, Symula RE, Wang J et al (2012) Insight into the transmission biology and species-specific functional capabilities of tsetse (Diptera: Glossinidae) obligate symbiont *Wigglesworthia*. MBio 3.pii: e00240-11

Roh SW, Nam Y-D, Chang H-W et al (2008) Characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. Appl Environ Microbiol 74:6171–6177

Ryu JH, Kim SH, Lee HY et al (2008) Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science 319:777–782

Sant'Anna MR, Diaz-Albiter H, Aguiar-Martins K et al (2014) Colonisation resistance in the sand fly gut: *Leishmania* protects *Lutzomyia longipalpis* from bacterial infection. Parasit Vectors 7:329

Sassera D, Epis S, Pajoro M, Bandi C (2013) Microbial symbiosis and the control of vector- borne pathogens in tsetse flies, human lice, and triatomine bugs. Pathog Glob Health 107:285–292

Sharon G, Segal D, Ringo JM et al (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proc Natl Acad Sci USA 107:20051–20056

Shin SC, Kim SH, You H et al (2011) *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334:670–674

Snyder AK, Deberry JW, Runyen-Janecky L, Rio RV (2010) Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. Proc Biol Sci 277:2389–2397

Staubach F, Baines JF, Künzel S, Bik EM, Petrov DA (2013) Host species and environmental effects on bacterial communities associated with *Drosophila* in the laboratory and in the natural environment. PLoS ONE 8:e70749

Toft C, Andersson SG (2010) Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet 11:465–475

Tolasch T, Sölter S, Tóth M et al (2003) (*R*)-Acetoin-female sex pheromone of the summer chafer *Amphimallon solstitiale* (L.). J Chem Ecol 29:1045–1050

Turnbaugh PJ, Hamady M, Yatsunenko T et al (2009) A core gut microbiome in obese and lean twins. Nature (Lond) 457:480–484

Valanne S, Rämet M (2013) Uracil debases pathogenic but not commensal bacteria. Cell Host Microbe 13:505–506

Verstraete W (2007) Microbial ecology and environmental biotechnology. ISME J 1:1–4

Wang S, Jacobs-Lorena M (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. Trends Biotechnol 31:185–193

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751

White PB (1921) The normal bacterial flora of the bee. J Pathol Bacteriol 24:64–78

Wong A, Ng P, Douglas A (2011) Low diversity bacterial community in the gut of the fruit fly *Drosophila melanogaster*. Environ Microbiol 13:1889–1900

Wong A, Chaston J, Douglas A (2013) The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. ISME J 7:1922–1932

Xie J, Butler S, Sanchez G, Mateos M (2014) Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. Heredity 112:399–408

Yamada Y, Hoshino K-I, Ishikawa T (1997) The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 61:1244–1251

Zambelli P, Pinto A, Romano D et al (2012) One-pot chemoenzymatic synthesis of aldoximes from primary alcohols in water. Green Chem 14:2158–2161

Zouache K, Raharimalala FN, Raquin V et al (2011) Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. FEMS Microbiol Ecol 75:377–389

Chapter 6
Drosophila–Acetobacter as a Model System for Understanding Animal–Microbiota Interactions

Sung-Hee Kim, Kyung-Ah Lee, Do-Young Park, In-Hwan Jang, and Won-Jae Lee

Abstract All metazoans harbor a high number of microorganisms in the gut. It is generally accepted that these gut–microbe interactions modulate a diverse range of host physiology, including immunity, development, and metabolism. Analyses using genetically amenable model animals such as *Drosophila* have provided a key framework to understand the molecular mechanisms underlying gut–microbe interactions. Recent investigations revealed that the acetic acid bacterium is one of the major naturally occurring commensal bacteria in the gut of the *Drosophila* host. The genetic tools available on both the microbial side and the host side provide a unique opportunity to dissect the complex interactions between gut microbes and their hosts. In this chapter, the role of the *Acetobacter* microbiome in animal physiology is described.

Keywords *Drosophila* • Gut commensal bacteria • *Acetobacter* • Immunity • Development • Intestinal stem cell

6.1 Introduction

One of the most intriguing discoveries in biology and medicine recently is that the animal genome itself is not sufficient to control the entirety of animal physiology, but rather additional genetic information such as the gut-associated metagenome of an animal, the so-called "animal gut microbiome," is required for the homeostatic regulation of animal development, metabolism, and immunity (Turnbaugh et al. 2006, 2009; Macpherson and Harris 2004; Maslowski and Mackay 2011;

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O'Hara and Shanahan 2006). Disruption of microbiome-induced regulation is now considered as a cause of many metabolic and inflammatory diseases (Hsiao et al. 2013; Caricilli et al. 2011; Garrett et al. 2010; Henao-Mejia et al. 2012; Ryu et al. 2008; Turnbaugh et al. 2006; Cani et al. 2008; Mazmanian et al. 2008; Vijay-Kumar et al. 2010). The cooperation between the animal genome and its associated microbiome suggests a close coevolution between animals and their environments.

The gut is highly complex and the largest internal organ in the metazoan body. For example, in humans, the absorptive surface area of the small intestine is about 250 m^2, an area equivalent to the size of a tennis court. More importantly, mucosal surfaces covered by a layer of gut epithelia represent the most critical interface between an organism and its environment. It is now commonly accepted that gut epithelial homeostasis influences diverse elements of host physiology including development, metabolism, and immunity. The relationship between gut and host health and diseases is well documented (Hsiao et al. 2013; Caricilli et al. 2011; Garrett et al. 2010; Henao-Mejia et al. 2012; Ryu et al. 2008; Turnbaugh et al. 2006; Cani et al. 2008; Mazmanian et al. 2008; Vijay-Kumar et al. 2010), although the detailed molecular mechanisms remain obscure.

More than a century ago, the Russian scientist Elie Metchnikoff (1845–1916) conceptualized the role of the gut in host physiology and pathology (Lee and Brey 2013). He suggested that gut homeostasis is the key to many components of host physiology, including longevity. He also proposed that gut bacteria are essential modulators influencing homeostasis, and that deregulation of gut homeostasis by certain bacteria leads to a disease state caused by poisoning of the body from bacterial by-products. Unfortunately, Metchnikoff's proposed "relationship between gut homeostasis and host health" remained dormant for nearly a century; his hypothesis has only recently reemerged as one of the most active areas of investigations in modern biology and medicine.

Recent technological advances, such as next-generation sequencing and meta-analyses of transcriptome, proteome, and metabolome data, along with powerful genetic tools available at the organism level, make it possible to revisit Metchnikoff's concepts. In particular, biotic environmental factors such as gut bacteria are found to be critical for the homeostasis of gut epithelia. Numerous investigations have focused on identifying gut bacteria (a specific strain and/or community) that disturb homeostasis (Clemente et al. 2012; Ryu et al. 2008; Lee and Brey 2013). Such findings have successfully, at least in some cases, enabled the phenotyping of myriad host disorders including obesity, chronic inflammatory diseases, diabetes, and even cancers (Hsiao et al. 2013; Caricilli et al. 2011; Garrett et al. 2010; Henao-Mejia et al. 2012; Ryu et al. 2008; Turnbaugh et al. 2006; Cani et al. 2008; Mazmanian et al. 2008; Vijay-Kumar et al. 2010; Tlaskalova-Hogenova et al. 2011). These observations also support the hypothesis that increased incidence of atopic, metabolic, and chronic inflammatory diseases may largely be the result of the deregulation of gut homeostasis.

However, the fundamental mechanisms by which the gut microbiome impacts such a diverse range of host physiology still remain unclear. For instance, how does

an organism sense and integrate signaling inputs from the microbiome to control its immunity, metabolism, and growth? One attractive concept is the "holobiont" theory. The term holobiont was first used to define an animal acting in concert with its associated symbiotic microorganism to adapt to a certain environmental condition (Mindell 1992). Subsequently, the hologenome is defined as genetic information of the holobiont that is the sum of the genetic information of the host and its associated microorganisms (Zilber-Rosenberg and Rosenberg 2008). In this concept, the gut microbiome acts as a part of the hologenome to control host homeostasis by forming a chemical association with an animal body. To prove this intriguing concept, researchers need to discover and validate the signaling circuits between the gut microbe and animal body that govern the organism level of homeostasis. Research in this direction is of paramount importance for understanding a mechanistic link between commensal–animal interactions and animal health. Unfortunately, the biology of commensal–animal interactions is unique in that complex reciprocal crosstalk occurs strictly under in vivo conditions, making validation difficult. Because of these experimental limitations, investigators still face several important hurdles. Customizing an appropriate experimental animal model is essential to deciphering commensal–animal communication. The 2011 Nobel Prize in Physiology or Medicine was awarded for work illuminating host–microbe interactions and innate immunity in *Drosophila* (Lemaitre and Hoffmann 2007), thereby recognizing this animal model as pivotal to solve the most important biological challenges. Indeed, recent findings have demonstrated the value of the *Drosophila* model for understanding the molecular mechanisms underlying commensal–animal interactions (Lee et al. 2013; Buchon et al. 2009a; Shin et al. 2011; Storelli et al. 2011; Ryu et al. 2008). Importantly, gnotobiotic animals such as microbe-free *Drosophila* mono-associated with a single commensal bacterium, *Acetobacter*, have been proposed to be an ideal model system to investigate the molecular circuit controlling bacterially modulated animal physiology (Shin et al. 2011). The present chapter directly addresses key findings observed in the *Acetobacter–Drosophila* model system.

6.2 *Drosophila* and Its Gut Commensal *Acetobacter*

Since Morgan's experiments more than 100 years ago, the fruit fly *Drosophila melanogaster* has been used as a major genetic model organism in modern biology and medicine. At first glance, it seems difficult to readily link the relationship between *Drosophila* and acetic acid-producing bacteria. However, *Drosophila* are often called "vinegar flies," likely because they are always found in vinegar production in nature. It was in fact reported that *Drosophila* has an important function in acetic acid production by acting as a vector to transport microorganisms necessary for acetic acid production. Indeed, several recent metagenome analyses revealed that acetic acid bacteria and lactic acid bacteria are two major commensal microbiota species found in the gut of *Drosophila* (Chandler et al. 2011; Wong

et al. 2011). Therefore, it is likely that *Drosophila* has an already long history of interaction with different members of the *Acetobacteraceae* family.

6.3 Genetic Modeling of Commensal–Animal Interactions

The clearest determination for the role of a commensal bacterium on animal physiology can be observed by comparing a gnotobiotic animal (i.e., a microbe-free animal mono-associated with a specific bacterium) and its microbe-free counterpart (Fig. 6.1). In the same context, the clearest demonstration for the role of a specific gut microbiome can be achieved by studying gnotobiotic animals harboring wild-type bacteria or mutant bacteria having a loss of function of a specific microbiome (Fig. 6.1). The *Drosophila–Acetobacter* model has distinct advantages over other models. *Acetobacter* is easily cultivable in vitro, and tools for genetic manipulation (loss-of-function and gain-of-function) are currently available (Deeraksa et al. 2005). Hundreds of gnotobiotic animals can be easily generated in a single day without sophisticated equipment. In addition, *Drosophila* genetics have available tremendous tools (loss-/gain-of-function as well as tissue and temporal gene expression or knockdown) and resources (large collections of *Drosophila* stocks including publically available mutant, knockdown, and reporter strains) (Dietzl et al. 2007; Drysdale 2008; Perrimon 1998). The versatile tools available for *Drosophila* enable identification of the signaling molecules or pathways by which

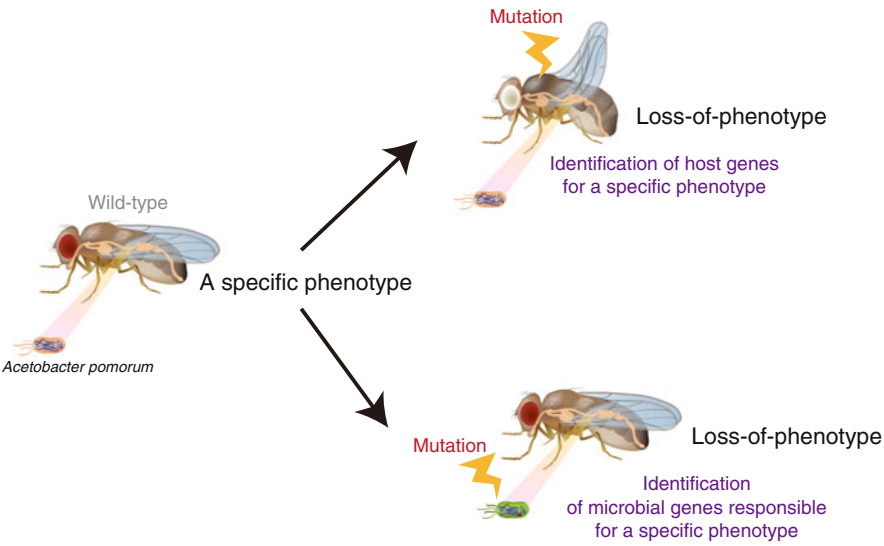

Fig. 6.1 *Drosophila–Acetobacter* interacting model system to decipher the function of the gut microbiota. Genetic tools available in both microbe and host make it possible to assign the role of specific microbiome or host gene in a microbe-modulated animal phenotype

bacteria impact host physiology (Fig. 6.1). All these aspects make the *Drosophila–Acetobacter* connection ideally situated as a genetic model system to decipher complex animal–microbiota interactions.

6.4 Development

6.4.1 Gut Microbiota as an Environmental Factor Affecting Animal Growth

How do animals control critical physiological processes such as final body size? What are the factors controlling animal growth? For biologists, these questions are fundamental. One can easily deduce that genetic (e.g., growth hormones) and nongenetic factors (e.g., nutrition and exercise) greatly influence a diverse range of animal physiology. However, the molecular mechanisms integrating these genetic and nongenetic factors remain to be discovered.

Among environmental factors, gut bacteria were long believed to influence animal organ size. *Drosophila* harbors a relatively simple gut commensal community composed of 5 to 20 different microorganisms (Cox and Gilmore 2007; Ryu et al. 2008; Storelli et al. 2011; Wong et al. 2011). For example, *Drosophila* reared in the author's laboratory harbors five major commensal bacterial species: *Commensalibacter intestini*, *Acetobacter pomorum*, *Gluconobacter morbifer*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (Ryu et al. 2008). However, it should be noted that the members of the gut commensal community vary largely, even in the same laboratory, likely because of different environmental conditions such as temperature and humidity.

By comparing conventional flies (harboring normal commensal microbiota) and their microbe-free counterpart, the effect of gut microbiota on *Drosophila* growth can be examined. The growth phase of *Drosophila* is mainly regulated during the larval stages, wherein body growth can be impressive by increasing about 200 fold during the third-instar larval stage (Storelli et al. 2011). In this system, it has been observed that genetically identical inbred *Drosophila* display different final body sizes based on the presence of gut microbiota (Fig. 6.2). It is important to note that the effect of commensal microbiota on *Drosophila* larval growth is more pronounced when animals are reared in a poor nutrient condition (Fig. 6.2).

6.4.2 Acetobacter pomorum *as a Major Factor Affecting Host Growth in a Specific Diet Condition*

By generating different gnotobiotic animals (by associating individual bacterial species in the *Acetobacteraceae* family with microbe-free *Drosophila* larvae), it has

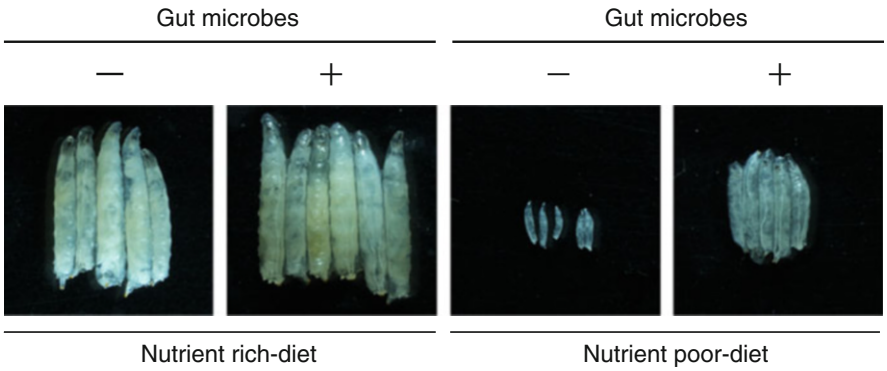

Fig. 6.2 The role of gut microbiota in *Drosophila* larval growth. A nutrient-rich diet containing 5 % yeast or a nutrient-poor diet containing 0.5 % yeast was used in this study. The presence of microbiota enhances larval growth, especially in a nutrient-poor diet

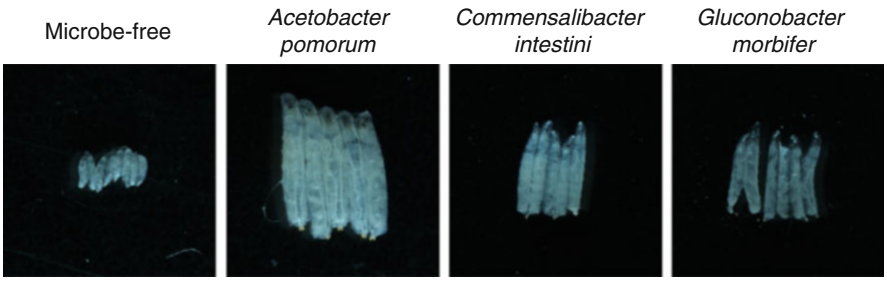

Fig. 6.3 The role of *Acetobacteraceae* in *Drosophila* larval growth. Microbe-free animals were mono-associated with each of three different *Acetobacteraceae* (*Acetobacter pomorum*, *Commensalibacter intestini*, and *Gluconobacter morbifer*). The sizes of animals at the 120th hour of development after egg laying are shown

been found that one component of the gut microbiota, *A. pomorum*, gives the most predominant effect on host growth (Fig. 6.3) (Shin et al. 2011). It is also reported that *Lactobacillus plantarum* also has an important effect in a different poor nutrient condition (Storelli et al. 2011). Therefore, it is likely that different nutritional conditions affects specific microbiome activities that in turn enhance larval growth.

6.4.3 *The* Acetobacter pomorum *Microbiome Affecting* Drosophila *Growth*

Next-generation sequencing was used to generate a draft genome sequence for *A. pomorum*. The draft genome sequence of *A. pomorum* contains 2696 predicted

genes with a total genome size of approximately 2.8 Mb. An *A. pomorum* mutant library containing approximately 3000 independent mutants was then generated using Tn5-based mutagenesis. Each individual mutant was used to generate a gnotobiotic animal, and about 3000 different gnotobiotic animals were generated to screen *A. pomorum* mutants that affect host growth and body size (Fig. 6.4). In total, 23 cohorts of gnotobiotic larvae showed reduced developmental rates. An analysis of 23 mutant strains identified 14 genes. Eleven genes are involved in the periplasmic pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH)-dependent oxidative respiratory chain: PQQ-ADH subunit-I and -II, *PQQ-ADH-I* and *-II*; *cyaA*, ubiquinol oxidase subunit-I; *pqqB*, coenzyme PQQ synthesis protein B; *pqqC*, coenzyme PQQ synthesis protein C; *pqqE*, coenzyme PQQ synthesis protein E; *ccmC*, cytochrome *c*-type biogenesis protein CcmC; *ccmF*, cytochrome *c*-type biogenesis protein CcmF; *ccmH*, cytochrome *c*-type biogenesis protein CcmH; *dsbD*, cytochrome *c*-type biogenesis protein DsbD; and *ctaD*, cytochrome *c* oxidase subunit-I. All of these are involved in the electron transport system that is part of acetic acid production (Fig. 6.4). Three other proteins that have different functions are as follows: *oprM*, secretion system type I outer membrane efflux pump lipoprotein; *shc*, squalene-hopene cyclase; and *hmp*, hypothetical membrane protein (Fig. 6.4).

6.4.4 *PQQ-Dependent Metabolism Induces the Host Insulin Signaling Pathway*

Comparative analyses between gnotobiotic animals carrying wild-type *Acetobacter* and those carrying mutant *Acetobacter* strains (i.e., the ΔPQQ strain bearing a mutation in the PQQ-dependent respiratory chain) provide a unique opportunity to understand the molecular mechanism by which PQQ-dependent metabolism enhances host growth.

It is well known that genetic and nongenetic factors (e.g., nutrition) are major contributors to the phenomenon of host growth. In humans, it is known that environmental factors can contribute as much as 20 % of human height (Silventoinen et al. 2003). In mammals, the hypothalamic–pituitary axis (growth hormone-releasing hormone in hypothalamus; growth hormone secretion in pituitary gland) modulates the production/secretion of insulin-like growth factors (IGF) in a systemic manner (Thissen et al. 1994). In *Drosophila*, growth is mainly dependent on evolutionarily conserved insulin/IGF signaling (IIS) pathways controlled by insulin-like peptides (ILP) produced by specific ILP-producing neurosecretory cells (IPCs) in the brain. Four ILPs, that is, ILP-1, -2, -3, and -5, are known to be produced in brain IPCs (Rulifson et al. 2002).

The reduced developmental rate and small body size observed in the ΔPQQ-mono-associated animals are reminiscent of animals with defective ILP signaling. Indeed, ΔPQQ-mono-associated animals are found to possess reduced insulin

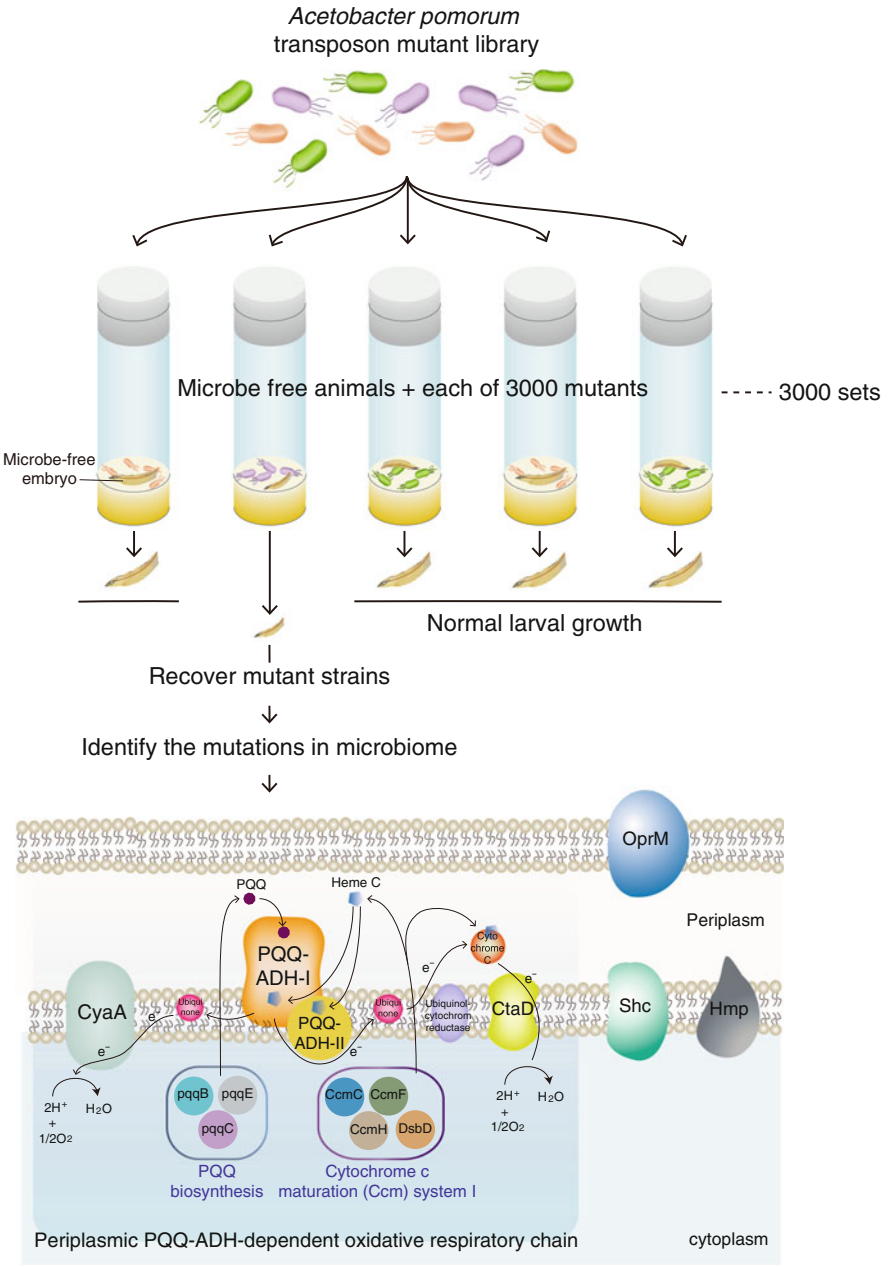

Fig. 6.4 Screening strategy to identify *Acetobacter* microbiome involved in *Drosophila* larval growth. Each of the 3000 *Acetobacter pomorum* mutant bacteria was added to germ-free embryos to generate about 3000 corresponding cohorts of mono-associated-GF animals. The body sizes of animals were compared, and *Acetobacter* mutant strains showing reduced developmental rates were subjected to chromosomal sequencing analysis. A schematic diagram of the screened bacterial gene clusters is shown. Eleven genes found to be involved in periplasmic PQQ-ADH-dependent oxidative respiratory chain are indicated in the diagram: *PQQ-ADH-I* and *-II*,

signaling activity, as evidenced by increased nuclear localization of forkhead transcription factor (dFOXO) (Fig. 6.5) (Shin et al. 2011). As in the case of the *Drosophila* ILP mutant, ΔPQQ-mono-associated animals showed similar metabolic and developmental defects such as reduced organ size, reduced cell number/size, and an increase in sugars and lipid levels (Fig. 6.5). When insulin signaling activity in ΔPQQ-mono-associated animals is boosted genetically, all metabolic and developmental defects observed in ΔPQQ-mono-associated animals are rescued (Fig. 6.5) (Shin et al. 2011), demonstrating that the PQQ-dependent metabolism of *Acetobacter pomorum* affects host physiological homeostasis through systemic activation of the insulin signaling pathway. ILP production/secretion in insulin-producing cells (IPCs) in the brain is known to be involved in the systemic insulin activation. How *Acetobacter*–gut communication contributes to the secretion of ILP in the brain is presently unknown. However, this phenomenon strongly suggests that a distinctive gut environment formed by a specific gut bacterium somehow communicates with the brain IPCs via the gut–brain axis.

6.5 Immunity

Gut commensal bacteria are in constant contact with gut mucosa throughout an animal's life. Therefore, one of the most important characteristics in gut microbiota species is their ability to avoid host gut immunity to prevent chronic inflammation (Kim and Lee 2014; Lee and Lee 2013; You et al. 2014). In *Drosophila*, dual oxidase (DUOX), a member of the NADPH oxidase family, acts as the first line of innate immune defense in the gut epithelia (Ha et al. 2005). When bacteria enter the gut lumen, the DUOX enzyme is activated and produces microbicidal reactive oxygen species (ROS) such as HOCl to antagonize bacterial growth (Ha et al. 2005, 2009a, b). Although most bacteria, including opportunistic pathogens or opportunistic pathobionts (e.g., *Gluconobacter morbifer*, which is normally benign but can be conditionally pathogenic to its host) are able to induce DUOX-dependent ROS generation, major commensal bacteria such as *Acetobacter pomorum* are unable to induce DUOX activation (Lee et al. 2013). It was later found that a bacterial-derived uracil molecule acts as a ligand for DUOX activation. Among members of the *Acetobacteraceae* family found in the *Drosophila* intestine,

Fig. 6.4 (continued) pyrroloquinoline quinone-dependent alcohol dehydrogenase subunit-I and -II; *cyaA*, ubiquinol oxidase subunit-I; *ctaD*, cytochrome *c* oxidase subunit-I; *pqqB*, coenzyme PQQ synthesis protein B; *pqqC*, coenzyme PQQ synthesis protein C; *pqqE*, coenzyme PQQ synthesis protein E; *ccmC*, cytochrome *c*-type biogenesis protein CcmC; *ccmF*, cytochrome *c*-type biogenesis protein CcmF; *ccmH*, cytochrome *c*-type biogenesis protein CcmH; and *dsbD*, cytochrome *c*-type biogenesis protein DsbD. Three other genes of other distinct functions are also indicated: *shc*, squalene-hopene cyclase; *oprM*, secretion system type I outer membrane efflux pump lipoprotein; *hmp*, hypothetical membrane protein

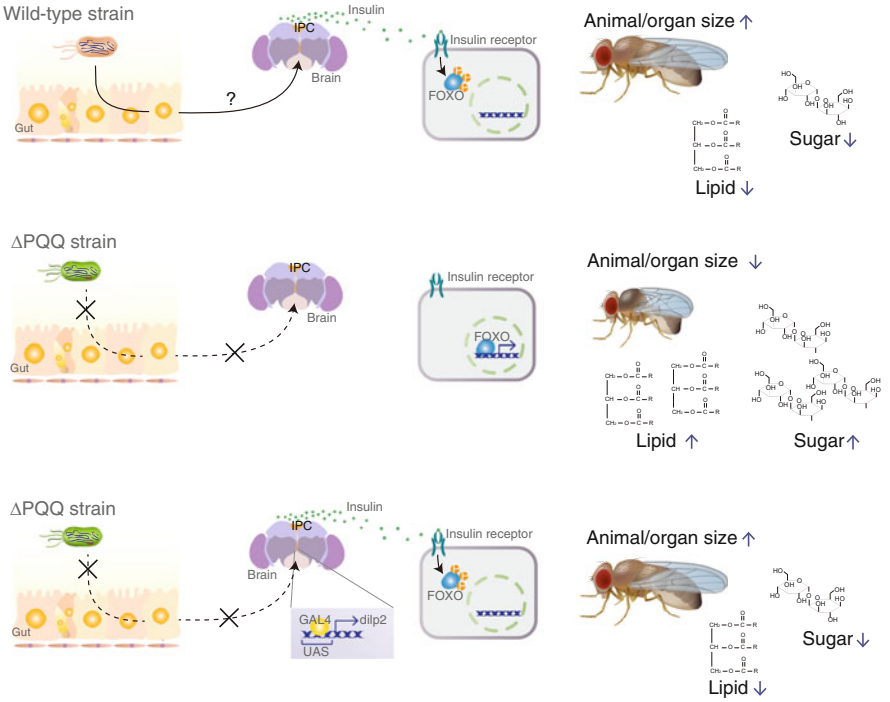

Fig. 6.5 Role of PQQ-dependent metabolism in animal insulin signaling. The PQQ-dependent electron transport system of wild-type *Acetobacter* somehow induces insulin production/secretion in the insulin-producing cells (IPC) in the brain. Bacterially induced insulin activates the systemic insulin signaling pathway to maintain physiological homeostasis. In contrast, animals carrying the ΔPQQ mutant strain (bearing a mutation in the PQQ-dependent electron transport system) are unable to induce systemic insulin activation. See text for further explanation

two symbionts, *Acetobacter pomorum* and *Commensalibacter intestini*, do not release uracil whereas the pathobiont *Gluconobacter morbifer* does release uracil (Lee et al. 2013). Consequently, these two symbionts do not provoke DUOX activation when microbe-free animals are mono associated with one of these bacteria. However, the *G. morbifer* pathobiont provokes chronic DUOX activation by constitutive uracil release. Therefore, it is likely that the uracil excretion ability of a given member of *Acetobacteraceae* may define its potential pathogenicity as a pathobiont. The exact molecular mechanism underlying uracil release in different members of *Acetobacteraceae* will clarify this issue (Fig. 6.6).

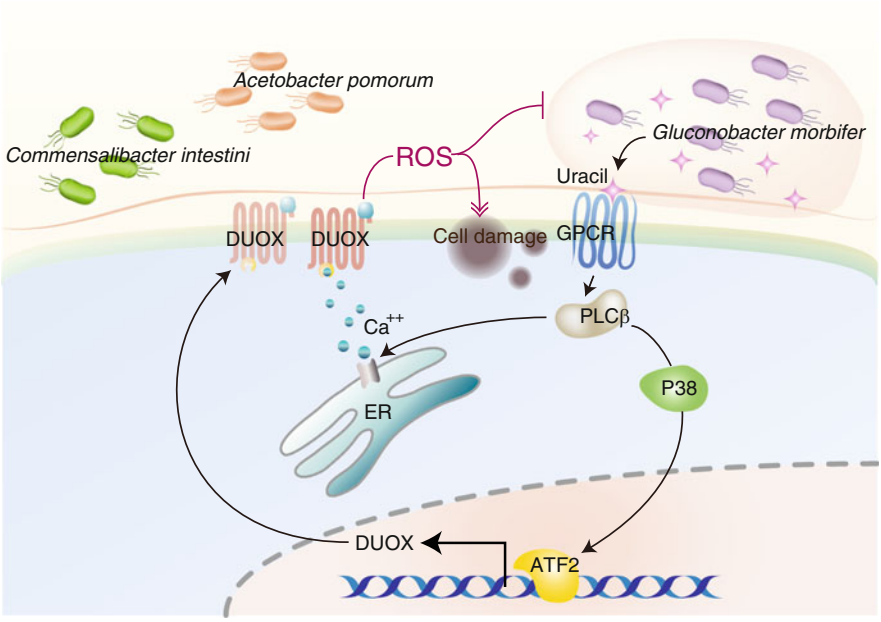

Fig. 6.6 Regulation of dual oxidase (DUOX)-dependent gut immunity by different members of *Acetobacteraceae*. Among the different members of *Acetobacteraceae*, *Acetobacter pomorum* and *Commensalibacter intestini* act as symbionts. These two bacteria do not secrete uracil (or secrete only minimal amounts), thereby avoiding DUOX activation. In contrast, *Gluconobacter morbifer* acts as a pathobiont that provokes chronic DUOX activation consequent to large amounts of released uracil

6.6 Gut Stem Cell Regulation

Epithelial cells lining the intestinal tract are continuously renewed by newly emerging cells to maintain the homeostasis of gut epithelial cells. In normal adult flies, enterocytes of the intestine are replaced about every 5 days. Four different types of cells are found in the adult intestine: intestinal stem cell (ISC), enteroblast (EB), enteroendocrine cells (EEC), and enterocytes (EC). This epithelial cell renewal (ECR) program is maintained by ISC proliferation and differentiation. In this process, each ISC produces one daughter cell that retains the fate of its parent cell and one postmitotic EB that in turn differentiates into either an EC or an EEC (Fig. 6.7) (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007). It is well established that multiple signaling pathways, including Delta-Notch, insulin, growth factor, and JAK-STAT signaling, are involved in the ECR program (Ohlstein and Spradling 2007; Kapuria et al. 2012; Amcheslavsky et al. 2009; Buchon et al. 2009a, 2010; Jiang and Edgar 2009; Jiang et al. 2009; Xu et al. 2011; Cronin et al. 2009). It has been shown that the presence of commensal bacteria is required to maintain the basal rate of the ECR program (Buchon et al. 2009b). Importantly, different members of *Acetobacteraceae* are able to

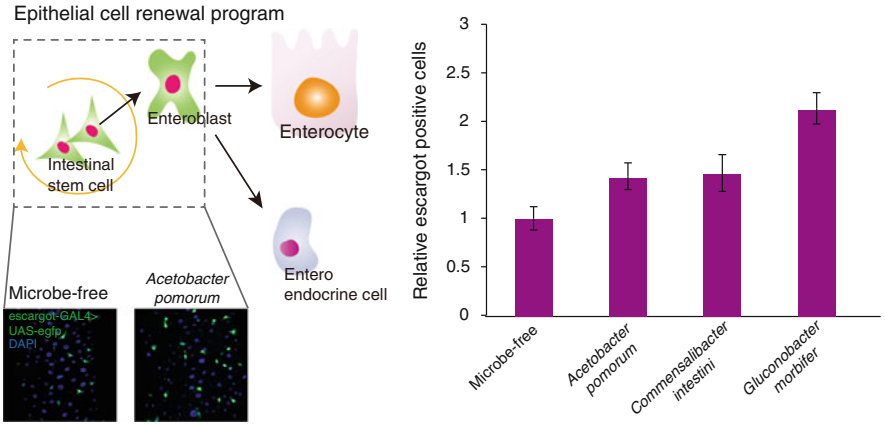

Fig. 6.7 Regulation of intestinal stem cells by different members of *Acetobacteraceae*. A schematic presentation for the epithelial cell renewal program is shown. Microbe-free animals carrying escargot-GAL4>UAS-GFP were mono-associated with each of *Acetobacteraceae* members. Analysis of *escargot*-positive cells using the *escargot-GAL4>UAS-GFP* (marker for ISCs, enteroblasts, and newly synthesized enterocytes) indicates the global epithelial cell renewal rates. The presence of *Acetobacteraceae* accelerates the epithelial cell renewal programs, as evidenced by enhanced escargot-positive cells. Five-day-old adult flies were used in this experiment

accelerate the ECR program (Fig. 6.7). Therefore, it is likely that each bacterial strain may differentially influence the ECR program. At present, it is unclear how *Acetobacteraceae* controls stem cell activity. One interesting possibility is that *Acetobacter*-derived metabolites may act as ligands that induce ISC activation. This important issue can be clarified by clearly establishing the molecular mechanisms by which specific metabolites modulate intracellular signaling pathways involved in ISC proliferation and differentiation.

6.7 Perspective on the *Acetobacter–Drosophila* Model System

Most scientists would agree that the gut and its microbiota are important in host physiology. In fact, this subject has become one of the hottest issues in biology and medicine, as shown by the plethora of literature on this subject during the past 2 years. However, this new field of biology still suffers from an incredible amount of descriptive and speculative data that remain to be proven by a rigorously controlled experimental system. As referenced in this chapter, the *Acetobacter–Drosophila* model has provided a unique opportunity to dissect the role of the gut microbiome in the homeostasis of animal physiology. Many important questions remain to be answered. For example, why are some members of *Acetobacteraceae* mutualistic to their hosts whereas others are pathogenic? Why do different

members of *Acetobacteraceae* produce a distinct effect on host physiology? How many bacterially derived metabolites are involved in host–microbiota crosstalk? What are the function of host receptors that recognize microbiota-derived metabolites? Comparative multiomics analyses in different members of *Acetobacteraceae* together with genetically tractable host animal models will certainly provide even further insights into the molecular dialogue between microbiota and animals.

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References

Amcheslavsky A, Jiang J, Ip YT (2009) Tissue damage-induced intestinal stem cell division in *Drosophila*. Cell Stem Cell 4(1):49–61

Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B (2009a) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe 5(2):200–211

Buchon N, Broderick NA, Chakrabarti S, Lemaitre B (2009b) Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. Genes Dev 23(19):2333–2344

Buchon N, Broderick NA, Kuraishi T, Lemaitre B (2010) Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol 8:152. doi:1741-7007-8-152 [pii] 10.1186/1741-7007-8-152

Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Neyrinck AM, Delzenne NM, Burcelin R (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57(6):1470–1481. doi:10.2337/Db07-1403

Caricilli AM, Picardi PK, de Abreu LL, Ueno M, Prada PO, Ropelle ER, Hirabara SM, Castoldi A, Vieira P, Camara NO, Curi R, Carvalheira JB, Saad MJ (2011) Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol 9(12):e1001212. doi:10.1371/journal.pbio.1001212 PBIOLOGY-D-11-01483 [pii]

Chandler JA, Lang JM, Bhatnagar S, Eisen JA, Kopp A (2011) Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet 7(9):e1002272. doi:10.1371/journal.pgen.1002272 PGENETICS-D-11-00559 [pii]

Clemente JC, Ursell LK, Parfrey LW, Knight R (2012) The impact of the gut microbiota on human health: an integrative view. Cell 148(6):1258–1270. doi:S0092-8674(12)00104-3 [pii] 10.1016/j.cell.2012.01.035

Cox CR, Gilmore MS (2007) Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis pathogenesis*. Infect Immun 75(4):1565–1576

Cronin SJ, Nehme NT, Limmer S, Liegeois S, Pospisilik JA, Schramek D, Leibbrandt A, Simoes Rde M, Gruber S, Puc U, Ebersberger I, Zoranovic T, Neely GG, von Haeseler A, Ferrandon D, Penninger JM (2009) Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. Science 325(5938):340–343. doi:1173164 [pii] 10.1126/science.1173164

Deeraksa A, Moonmangmee S, Toyama H, Yamada M, Adachi O, Matsushita K (2005) Characterization and spontaneous mutation of a novel gene, polE, involved in pellicle formation in *Acetobacter tropicalis* SKU1100. Microbiology 151(pt 12):4111–4120. doi:10.1099/mic.0.28350-0

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature (Lond) 448(7150): 151–156

Drysdale R (2008) FlyBase: a database for the *Drosophila* research community. Methods Mol Biol 420:45–59. doi:10.1007/978-1-59745-583-1_3

Garrett WS, Gordon JI, Glimcher LH (2010) Homeostasis and inflammation in the intestine. Cell 140(6):859–870. doi:S0092-8674(10)00024-3 [pii] 10.1016/j.cell.2010.01.023

Ha EM, Oh CT, Bae YS, Lee WJ (2005) A direct role for dual oxidase in *Drosophila* gut immunity. Science 310(5749):847–850. doi:10.1126/science.1117311

Ha EM, Lee KA, Park SH, Kim SH, Nam HJ, Lee HY, Kang D, Lee WJ (2009a) Regulation of DUOX by the Galphaq-phospholipase Cbeta-$Ca2^{+}$ pathway in *Drosophila* gut immunity. Dev Cell 16(3):386–397. doi:10.1016/j.devcel.2008.12.015

Ha EM, Lee KA, Seo YY, Kim SH, Lim JH, Oh BH, Kim J, Lee WJ (2009b) Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Nat Immunol 10(9):949–957. doi:ni.1765 [pii] 10.1038/ni.1765

Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA (2012) Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482(7384):179–185. doi:nature10809 [pii] 10.1038/nature10809

Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF, Patterson PH, Mazmanian SK (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155(7): 1451–1463. doi:10.1016/j.cell.2013.11.024

Jiang H, Edgar BA (2009) EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. Development 136(3):483–493. doi:136/3/483 [pii] 10.1242/dev.026955

Jiang H, Patel PH, Kohlmaier A, Grenley MO, McEwen DG, Edgar BA (2009) Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the *Drosophila* midgut. Cell 137(7): 1343–1355

Kapuria S, Karpac J, Biteau B, Hwangbo D, Jasper H (2012) Notch-mediated suppression of TSC2 expression regulates cell differentiation in the Drosophila intestinal stem cell lineage. PLoS Genet 8(11):e1003045. doi:10.1371/journal.pgen.1003045 PGENETICS-D-12-00651 [pii]

Kim SH, Lee WJ (2014) Role of DUOX in gut inflammation: lessons from *Drosophila* model of gut–microbiota interactions. Front Cell Infect Microbiol 3:116. doi:10.3389/fcimb.2013.00116

Lee WJ, Brey PT (2013) How microbiomes influence metazoan development: insights from history and *Drosophila* modeling of gut–microbe interactions. Annu Rev Cell Dev Biol 29: 571–592. doi:10.1146/annurev-cellbio-101512-122333

Lee KA, Lee WJ (2013) *Drosophila* as a model for intestinal dysbiosis and chronic inflammatory diseases. Dev Comp Immunol. doi:10.1016/j.dci.2013.05.005

Lee KA, Kim SH, Kim EK, Ha EM, You H, Kim B, Kim MJ, Kwon Y, Ryu JH, Lee WJ (2013) Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in *Drosophila*. Cell 153(4):797–811. doi:10.1016/j.cell.2013.04.009

Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743

Macpherson AJ, Harris NL (2004) Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 4(6):478–485

Maslowski KM, Mackay CR (2011) Diet, gut microbiota and immune responses. Nat Immunol 12(1):5–9. doi:10.1038/Ni0111-5

Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. Nature (Lond) 453(7195):620–625. doi:10.1038/Nature07008

Micchelli CA, Perrimon N (2006) Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. Nature (Lond) 439(7075):475–479

Mindell DP (1992) Phylogenetic consequences of symbioses: Eukarya and Eubacteria are not monophyletic taxa. Biosystems 27(1):53–62

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7(7):688–693. doi:7400731 [pii] 10.1038/sj.embor.7400731

Ohlstein B, Spradling A (2006) The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. Nature (Lond) 439(7075):470–474. doi:10.1038/nature04333

Ohlstein B, Spradling A (2007) Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science 315(5814):988–992. doi:315/5814/988 [pii] 10.1126/science.1136606

Perrimon N (1998) New advances in *Drosophila* provide opportunities to study gene functions. Proc Natl Acad Sci USA 95(17):9716–9717

Rulifson EJ, Kim SK, Nusse R (2002) Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science 296(5570):1118–1120. doi:10.1126/science.1070058296/5570/1118 [pii]

Ryu JH, Kim SH, Lee HY, Bai JY, Nam YD, Bae JW, Lee DG, Shin SC, Ha EM, Lee WJ (2008) Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science 319(5864):777–782. doi:10.1126/science.1149357

Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ (2011) Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334(6056):670-674. doi:334/6056/670 [pii] 10.1126/science.1212782

Silventoinen K, Sammalisto S, Perola M, Boomsma DI, Cornes BK, Davis C, Dunkel L, De Lange M, Harris JR, Hjelmborg JV, Luciano M, Martin NG, Mortensen J, Nistico L, Pedersen NL, Skytthe A, Spector TD, Stazi MA, Willemsen G, Kaprio J (2003) Heritability of adult body height: a comparative study of twin cohorts in eight countries. Twin Res 6(5):399–408. doi:10.1375/136905203770326402

Storelli G, Defaye A, Erkosar B, Hols P, Royet J, Leulier F (2011) *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab 14(3):403–414. doi:10.1016/j.cmet.2011.07.012

Thissen JP, Ketelslegers JM, Underwood LE (1994) Nutritional regulation of the insulin-like growth factors. Endocr Rev 15(1):80–101. doi:10.1210/edrv-15-1-80

Tlaskalova-Hogenova H, Stepankova R, Kozakova H, Hudcovic T, Vannucci L, Tuckova L, Rossmann P, Hrncir T, Kverka M, Zakostelska Z, Klimesova K, Pribylova J, Bartova J, Sanchez D, Fundova P, Borovska D, Srutkova D, Zidek Z, Schwarzer M, Drastich P, Funda DP (2011) The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol 8(2):110–120. doi:10.1038/Cmi.2010.67

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444(7122):1027–1031. doi:nature05414 [pii] 10.1038/nature05414

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI (2009) A core gut microbiome in obese and lean twins. Nature 457(7228):480–484. doi:nature07540 [pii] 10.1038/nature07540

Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz AT (2010) Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 328(5975):228–231. doi:science.1179721 [pii] 10.1126/science.1179721

Wong CN, Ng P, Douglas AE (2011) Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ Microbiol. doi:10.1111/j.1462-2920.2011.02511.x

Xu N, Wang SQ, Tan D, Gao Y, Lin G, Xi R (2011) EGFR, Wingless and JAK/STAT signaling cooperatively maintain Drosophila intestinal stem cells. Dev Biol 354(1):31–43. doi:S0012-1606(11)00182-5 [pii] 10.1016/j.ydbio.2011.03.018

You H, Lee WJ, Lee WJ (2014) Homeostasis between gut-associated microorganisms and the immune system in Drosophila. Curr Opin Immunol 30:48–53. doi:10.1016/j.coi.2014.06.006
Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev 32(5):723–735. doi: FMR123 [pii] 10.1111/j.1574-6976.2008.00123.x

Chapter 7
Distribution, Evolution, and Physiology of Oxidative Fermentation

Kazunobu Matsushita and Minenosuke Matsutani

Abstract Acetic acid bacteria inhabit sugar-rich niches, especially fruits and flowers, and thus have the ability to utilize sugars or sugar alcohols for their energy sources. The strategy of sugar utilization is rather exceptional: they oxidize such the substrates by "oxidative fermentation" and utilize the accumulated products later. The oxidative fermentation is carried out by the respiratory chain comprising periplasmic primary dehydrogenases of quinoproteins or flavoprotein–cytochrome *c* complexes and (terminal) ubiquinol oxidases, both of which seem to be acquired by adaptive evolution in such a sugar-rich niche by interacting with other microbes living at the same habitat. In this chapter, the evolution and physiology of such a respiratory chain related to oxidative fermentation are described.

Keywords Adaptive evolution • Oxidative fermentation • Quinoprotein • Pyroloquinoline quinone • Flavoprotein • Respiratory chain • Ubiquinol oxidase • Cytochrome *c* oxidase

7.1 Introduction: Principle for Oxidative Fermentation

Acetic acid bacteria (AAB) are obligate aerobes and are well known to have a strong ability to oxidize ethanol, sugars, and sugar alcohols to produce the corresponding sugar acids. Such oxidation reactions are traditionally called "oxidative fermentation" because they involve incomplete oxidation of these compounds. Bacteria capable of performing such oxidative fermentation are called "oxidative bacteria," of which the most prominent are AAB. Some other

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pseudomonads and enteric bacteria could perform some specific oxidation reactions similar to AAB (described later). These oxidative bacteria do not oxidize such sugars or alcohols completely to carbon dioxide, at least in their early culture phase, and thus accumulate the corresponding incomplete oxidation products in large amounts in the growth medium. Because these oxidation products, such as acetic acid or L-sorbose, are largely accumulated in the culture media, AAB are also important for fermentation industries to produce useful biomaterials (see Chap. 15).

AAB include 17 genera, including *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*, and are phylogenetically located in the *Acetobacteraceae* family, order *Rhodospirillales*, class *α-Proteobacteria*, of which the details are described in Chap. 1. AAB should have a specified respiratory chain for the oxidative fermentation, because the oxidative reactions such as acetic acid, gluconate, or sorbose production have been shown to be carried out by linking directly to the respiratory chain (Matsushita et al. 1994). The oxidative fermentation is a process of incomplete oxidation where the substrates are oxidized by primary dehydrogenases of the respiratory chain and the oxidized products are then released to the culture medium (see Fig. 7.1). The electrons are abstracted from the substrate by the primary dehydrogenase, and then transferred to the terminal oxidase via ubiquinone. These primary dehydrogenases are specifically located on the periplasmic side of the cytoplasmic membrane, and also consist of a unique group of enzymes in their prosthetic groups. The enzymes are roughly classified into two categories: one is membrane-bound quinoprotein having pyrroloquinoline quinone (PQQ) as the prosthetic group, and the others are a three-subunit complex consisting of dehydrogenase subunit (having different types of the prosthetic group: covalently bound FAD, PQQ, and heme C, and molybdopterin cofactor), the cytochrome *c* subunit (having three hemes C), and a small subunit. The terminal oxidase of the respiratory chain seems also to be specific: ubiquinol oxidase but not cytochrome *c* oxidase is present. The respiratory chain generates a proton motive force only in the terminal oxidase that could be utilized for ATP synthesis or some other energetic work. Therefore, such oxidative fermentation is critical in energy generation for cell growth in addition to accumulation of the oxidative products from the cells, the physiological meaning of which is discussed later.

Such unique periplasmic respirations are involved only in the partial oxidation reactions of these sugars and alcohols, but not in the complete oxidation and the assimilation of the substrates. However, AAB also have an assimilation metabolism, and actually such the assimilation of the oxidized products, for example, acetic acid in *Acetobacter* spp. or *Komagataeibacter* spp., and ketogluconate or dihydroxyacetone in *Gluconobacter* spp., could be observed usually in the late growth phase but not in the early growth phase where oxidative fermentation occurs (Matsushita et al. 2005). Cytosolic enzymes seem to have a role in assimilation of the oxidation products in the later phase, and to further oxidize the substrates to generate NADH, which could be reoxidized via respiration (Matsushita et al. 1994; Richhardt et al. 2012; Sakurai et al. 2013). The cytoplasmic (NADH) respiration competes with the periplasmic respiration (oxidative fermentation) in terms of electron transfer and also in energetics, and thus both respirations seem to not

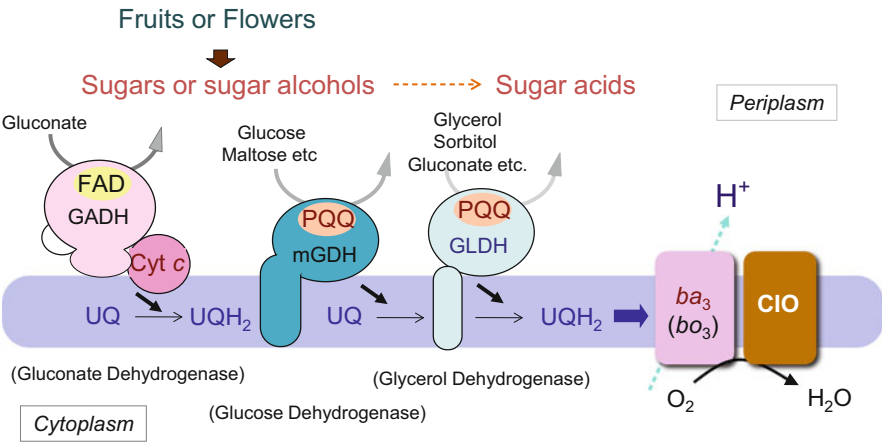

Fig. 7.1 Respiratory chain of acetic acid bacteria. Typical flavoprotein (GADH) and quinoproteins (mGDH and GLDH) are shown. Ubiquinone (UQ) accepts electrons from these dehydrogenases to release the reduced quinone, ubiquinol (UQH_2), which is then oxidized by ubiquinol oxidases, ba_3 UOX, or cyanide-insensitive oxidase (CIO). The ba_3 UOX has H^+ pumping ability

occur at the same growth phase. Assimilation and the related respiration are different in the different genera, even in the species, of AAB, as described in Chaps. 10, 11, and 12.

The unique periplasmic respiration for the oxidative fermentation of AAB is reminiscent of the hydrogen or iron respiration of some chemolithotrophs, and also some specific oxidative fermentation such as glucose oxidation, alcohol oxidation, or gluconate oxidation could be found in some related oxidative bacteria of pseudomonads or enteric bacteria. Thus, in this chapter, it is described, including some speculation, how this unique oxidative fermentation is distributed within the microbial world, and how this system (specific periplasmic dehydrogenase or terminal ubiquinol oxidases) is evolved or acquired in AAB.

7.2 Primary Dehydrogenases for Oxidative Fermentation: Distribution and Adaptive Evolution

Such sugar and alcohol respirations (oxidative fermentation) seem to be somewhat related to a phylogenetic relationship among AAB. *Gluconobacter* species exhibit highly active oxidation reactions on sugars or sugar alcohols such as D-glucose, D-gluconic acid, D-sorbitol, and glycerol, in addition to ethanol. In contrast, *Acetobacter* or *Komagataeibacter* species have a highly active ethanol-oxidizing activity but fewer other sugar or sugar alcohol-oxidizing activities.

Many membrane-bound enzymes have been reported from AAB, as described in Chap. 13: these are separated into quinoproteins (including quinohemoprotein) and flavoproteins as described previously. The former includes glucose dehydrogenase (mGDH), glycerol dehydrogenase (GLDH), and alcohol dehydrogenase (ADH); the latter includes gluconate dehydrogenase (GADH), sorbitol dehydrogenase (SLDH), fructose dehydrogenase (FDH), and 2-ketogluconate dehydrogenase (2KGDH). There is one additional enzyme different from others in terms of the prosthetic group, which is an aldehyde dehydrogenase (ALDH) that is expected to be a molybdoprotein. All these flavoproteins, and a quinohemoprotein ADH and a molybdoprotein ALDH are three- or two-subunit complexes consisting of the cytochrome *c* subunit and an additional small subunit (some enzymes are missing this small subunit).

Bacterial genome sequencing projects have been performed during the past 20 years, and many sequencing projects have been ongoing. In 2005, the first complete genome sequence of AAB was reported in the *Gluconobacter oxydans* 621H strain, of which the quinoproteins and flavoproteins are listed and classified (Prust et al. 2005). In addition to the known quinoproteins ADH, mGDH, and GLDH, *G. oxydans* has four new homologous sequences of the quinoproteins, PQQ-containing dehydrogenase 1 (PQQ1), PQQ-containing dehydrogenase 2 (PQQ2), PQQ-containing dehydrogenase 3 (PQQ3), and PQQ-containing dehydrogenase 4 (PQQ4). Later, Peters and coworkers characterized all the quinoproteins encoded in the 621H genome (Peters et al. 2013). The gene corresponding to mGDH was characterized to have additional oxidation ability for several other substrates such as L-arabinose, D-allose, D-altrose, turanose, and cellobiose in addition to the previously known substrates (Matsushita et al. 2002). That of GLDH was also characterized as polyol dehydrogenase, which can oxidize some important substrates such as glyceraldehydes, D-threose, D-erythrose, D-ribose, D-lyxose, isopropanol, 2-hexanol, 3-hexanol, 2,3-butanediol, 1,3-butanediol, 1,2-pentanediol, 2,4-pentanediol, 1,2-cyclopentanediol, 1,3-cyclopentanediol, 1,2-cyclohexanediol, 1,2-hexanediol, 2,5-hexanediol, L-erythrulose, and L-fucose in addition to the known substrates (Matsushita et al. 2002). PQQ1 was newly characterized as an inositol dehydrogenase (Hölscher et al. 2007), but PQQ3 and PQQ4 still remained uncharacterized.

PQQ2 have very low sequence identities to the other quinoproteins, ranging from 15 % to 18 % with amino acid level, but this gene is homologous to an outer membrane protein coding in *Escherichia coli*, which was shown to be required for assembly of β-barrel protein in the outer membrane (Hagan et al. 2010). Therefore, this gene should be excluded from these quinoprotein families. Another type of PQQ-dependent quinoprotein dehydrogenase, soluble glucose dehydrogenase (sGDH), was also characterized from *Acinetobacter calcoaceticus* (Cleton-Jansen et al. 1989). The homologous sequences of sGDH were not found in the reported complete genome sequences of AAB (Prust et al. 2005; Azuma et al. 2009; Bertalan et al. 2009; Ogino et al. 2011), but instead were found in a wide variety of Bacteria and Archaea. The sGDH sequence has no sequence identities with either membrane-bound quinoproteins or PQQ2 homologues. The crystal structure of

sGDH has already been reported (Oubrie 2003), but the structure, six anti-parallel four-stranded β-sheets, was different from those obtained from quinoprotein methanol dehydrogenase (MDH) or quinoprotein/quinohemoprotein alcohol dehydrogenases (ADH I/ADH II), having eight β-propellers, each of which consists of anti-parallel four-stranded β-sheets (Toyama et al. 2004). As the three-dimensional structures of the two homologues are quite different, we thus focused on the quinoproteins of the eight β-propeller structure.

The whole taxonomic-level phylogenetic tree of such quinoproteins was constructed using quinoprotein homologues involved in protein-coding sequences of all known genera, including six genera in the family *Acetobacteriaceae*, *Gluconobacter*, *Acetobacter*, *Gluconacetobacter*, *Komagataeibacter*, *Granulibacter*, and *Acidiphilium* (Fig. 7.2). Of these, two genera, *Granulibacter* and *Acidiphilium*, are early-diverged ancestors for AAB (Matsutani et al. 2011). As shown in Fig. 7.2, homologous sequences of the quinoprotein exist only in *α-*, *β-*, and *γ-Proteobacteria*, indicating that the quinoproteins have evolved within these narrow classes. The phylogenetic tree is largely or roughly separated into two categories: one is a soluble or alcohol dehydrogenase group, including five clades of MDH, ADH I, ADH II, ADH III, and SDH/SNDH (these abbreviations are described later), and the other membrane-bound quinoproteins, including five clades of mGDH, PQQ3, PQQ4, GLDH, and PQQ1. In the *Gluconobacter* genome, all the latter clades are present but the former are not, except for ADH III, as described later.

MDH homologues (clade MDH) are widely distributed in *α-, β-, and γ-Proteobacteria*. In addition to methylotrophic bacteria, *Methylobacterium extorquens* and *Paracoccus denitrificans*, and AAB ancestors *Acidiphilium (Ac.) cryptum* and *Granulibacter (Gr.) bethesdensis*, also possess MDH homologues. Although the *Ac. cryptum* MDH gene is still functionally unclear, *Gr. bethesdensis* has four MDH (MDH α-subunit) homologous sequences and has been shown to grow on methanol as a sole carbon source (Greenberg et al. 2007). One of the four MDH genes constitutes an operon with downstream genes of a MoxJ precursor, a cytochrome c_L precursor, a MDH β-subunit, and regulatory protein MoxR, all of which are critical to generate a functional MDH complex (Van Spanning et al. 1991) and to share a locational context identical to that of gene clusters in both *M. extorquens* and *P. denitrificans* (Greenberg et al. 2007). One of the AAB, a facultative methylotroph *Acetobacter methanolicus* (*Acidomonas methanolica*), could produce MDH when grown on methanol (Matsushita et al. 1992b). Recently, an MDH gene cluster was found in the draft genome sequence of *A. methanolica* type strain MB58 (Higashiura et al. 2014).

PQQ-ADH is classified into three groups: type I (ADH I), type II (ADH II), and type III (ADH III) (Toyama et al. 2004). Type II and type III have heme C as well as PQQ in the catalytic site, and thus are called quinohemoprotein ADH, whereas type I has only PQQ in the catalytic subunit (quinoprotein ADH). The phylogeny of PQQ-ADH is divided into large two clades, ADH I and ADH II, both of which include functionally validated enzymes of *Pseudomomas aeruginosa* (ADH I), *Pseudomonas fluorescence* (ADH II), and *Comamonas testosteroni* (ADH II)

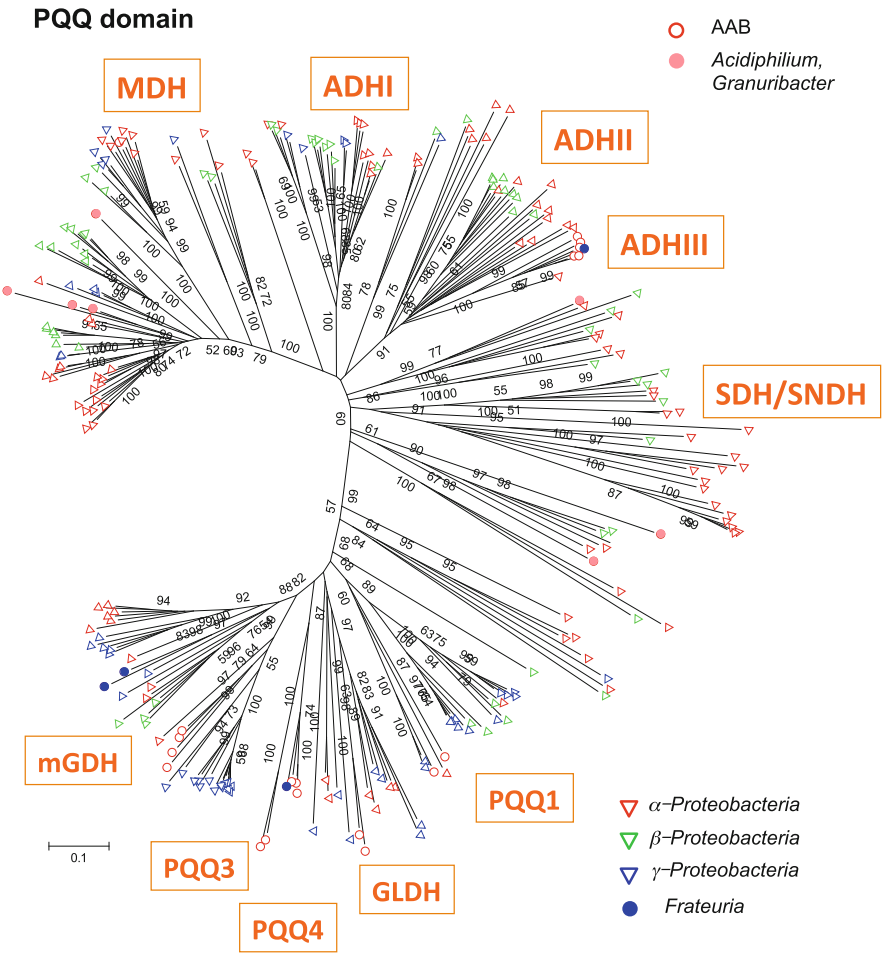

Fig. 7.2 Unrooted neighbor-joining phylogenetic tree of pyrroloquinoline quinone (PQQ) quinoproteins. The phylogenetic tree was constructed using MEGA 5.05 with 1000 bootstrap replicates, and separated into 12 clades, of which 10 clades are recognized as PQQ-dependent methanol dehydrogenase (MDH), type I PQQ-dependent alcohol dehydrogenase (ADHI), type II PQQ-dependent alcohol dehydrogenase (ADHII), type III PQQ-dependent alcohol dehydrogenase (ADHIII), sorbose/sorbosone dehydrogenase (SDH/SNDH), PQQ-dependent dehydrogenase 1 (PQQ1), PQQ-dependent dehydrogenase 3 (PQQ3), PQQ-dependent dehydrogenase 4 (PQQ4), PQQ-dependent glycerol dehydrogenase SldA (GLDH), and membrane-bound PQQ-dependent glucose dehydrogenase (mGDH). *Red open* and *closed circles* indicates the four AAB genera, *Acetobacter*, *Gluconacetobacter*, *Komagataeibacter* and *Gluconobacter*, and two AAB ancestors, *Granulibacter* and *Acidiphilium*, respectively, and are discriminated from other *α-Proteobacteria* (*red open triangles*). *Frateuria* are shown by *blue closed circles*, discriminated from other *γ-Proteobacteria* (*blue open triangles*). Enzymes from *β-Proteobacteria* are shown by *green open triangles*

(Groen et al. 1986; Görisch and Rupp 1989; Toyama et al. 2004). Both types are known as a soluble protein, whereas ADH III is known to have an additional cytochrome *c* subunit as a membrane anchor, thus being membrane bound (Toyama et al. 2004). The ADH III operon exists only in AAB (but not in AAB ancestors), and the quinohemoprotein subunit is located phylogenetically in a sub-clade of the ADH II group, suggesting that the cytochrome subunit of the ADH III group was derived from another group. The ADH II group is mainly distributed in *α-*, *β-Proteobacteria*, whereas ADH III occurs in only four AAB genera, that is, *Gluconobacter*, *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter*, and an exceptional strain, γ-proteobacterial *Frateuria aurantia*, previously known as *Acetobacter aurantia* (Swings et al. 1980). Recently, ADH III was isolated from this strain (Trček and Matsushita 2013). Because these genera are found in the same or similar habitats, it is thought that a horizontal gene transfer (HGT) event occurred among the ancestors of these genera. Although the origin of ADH III is not clear, a gene of ADH II could be transferred from some *α,β-Proteobacteria*, where ADH II is mainly distributed, into an ancestor of AAB to create ADH III. There is an additional clade in the soluble or alcohol dehydrogenase group, SDH/SNDH, which are thought to act as PQQ-dependent L-sorbose dehydrogenases (SDH) or L-sorbosone dehydrogenases (SNDH). This group is known as a soluble quinoprotein having a broad substrate specificity from 1-propanol to D-glucose (Miyazaki et al. 2006). Although there are no homologous sequences in AAB, α-proteobacterial *Ketogulonicigenium vulgare* has seven homologous sequences in the genome (Gao et al. 2013).

Membrane-bound PQQ-quinoproteins such as mGDH and GLDH have five membrane-spanning α-helices in the N-terminal region and in the membrane-anchoring subunit, respectively (Yamada et al. 1993; Miyazaki et al. 2002). The newly identified quinoproteins PQQ1, PQQ3, and PQQ4 also have five membrane-spanning α-helices in the N-terminal region (unpublished data). Therefore, these sequences are classified as mGDH-type quinoprotein. As shown in Fig. 7.2, the mGDH homologues (mGDH clade) are mainly distributed in *α,γ-Proteobacteria*. Of these, *E. coli*, *P. aureginosa*, *A. calcoaceticus*, and species of *Gluconobacter* are already known to have active mGDH (Matsushita and Adachi 1993). The main genera having mGDH in class *γ-Proteobacteria* are present in the orders *Enterobacteriales* (13 genera) and *Pseudomonadales* (4 genera), whereas those in class *α-Proteobacteria* are in the orders *Rhizobiales* (7 genera) and *Rhodospillales* (6 genera, including 4 AAB genera). The mGDH genes in the 4 AAB genera *Gluconobacter*, *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* are very closely related to one another, indicating that these genes diverged from the same ancestor. In contrast, mGDH of *F. aurantia* is located in a different sub-clade, suggesting that the origin of mGDH encoded in *Frateuria* is different from that of AAB. GLDH of AAB has a broad range of substrate specificity and is able to oxidize various polyols to the corresponding ketones (Adachi et al. 2001; Matsushita et al. 2003). The GLDH homologues (GLDH clade) are distributed in *α,γ-Proteobacteria* such as *Sphingomonas*, *Phenylbacterium*, *Serratia*, *Stenotrophomonas*, and *Xanthomonas* and are conserved in several species of

Gluconobacter and *Komagataeibacter*. It has been known that some *Gluconobacter* strains have more than two copies of the GLDH homologues, which may enable AAB strains to utilize the various compounds (Matsutani et al. 2014b). The clade PQQ1 (inositol dehydrogenase), is conserved in *Acetobacter* and *Gluconobacter* and mainly distributed in *γ-Proteobacteria*. The clades PQQ3 and PQQ4 are AAB specific: PQQ3 is only conserved in *Gluconobacter* and *Gluconacetobacter*, whereas PQQ4 is conserved in *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*. In addition to the difference of the repertoire of PQQ-quinoproteins among the genera, it was reported that there is a strain-specific quinoprotein (quinate dehydrogenase) in *Gluconobacter* (Vangnai et al. 2004). Thus, these differences of repertoire of PQQ-quinoproteins may define the species- or strain-specific phenotype. The two clades PQQ3 and PQQ4 are very closely related to each other and belong to almost the same clade: the two groups should be redefined as the same new group. *F. aurantia* also exceptionally has a PQQ4 homologous sequence, suggesting that an HGT event could have occurred between *F. aurantia* and AAB.

In addition to the membrane-bound quinoprotein dehydrogenases, as just described, most AAB strains have several membrane-bound flavoproteins such as GADH, SLDH, FDH, and 2KGDH, all of which are a three-subunit complex (see Chap. 13). These flavoproteins are also unique to be localized in the periplasmic side of cytoplasmic membranes. Similar to PQQ-quinoproteins, these enzymes are also found in many *γ-Proteobacteria* as well as in AAB; especially, GADH has been isolated from species of *Pseudomonas*, *Serratia*, *Klebsiella*, *Erwinia*, and *Pantoea* (Matsushita et al. 1982; Shinagawa et al. 1984; Yum et al. 1997; Pujol and Kado 2000). An additional flavoprotein glucose dehydrogenase (FAD-GDH) was isolated from *β-Proteobacteria* (Inose et al. 2003). These three subunits, flavoprotein–cytochrome *c* complexes, are encoded as an operon in order of the small (S), large (L), and cytochrome *c* (C) subunits, except for FDH, the operon of which is in order of SCL. Although the L-subunit of these flavoprotein complexes contains a FAD-binding domain in the N-terminal regions, which have a relatively high homology, the entire sequence identity is rather low except for the N-terminal regions. The C-subunits have a relatively high sequence identity with each other, but the S-subunits showed almost no sequence identity to each other.

A whole taxonomic-level phylogenetic tree of the L-subunit of these flavoproteins was constructed with all known genera, including four AAB genera, *Gluconobacter*, *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter*, and two AAB ancestors, *Granulibacter* and *Acidiphilium* (see Fig. 7.3). Functionally validated sequences of FAD-GDH (Inose et al. 2003), SLDH (Toyama et al. 2005), two GADHs (Toyama et al. 2007; Saichana et al. 2009), FDH (Kawai et al. 2013), and 2KGDH (Kataoka et al. 2015) were also included in the phylogenetic tree. As shown, the phylogenetic tree of the L-subunit is clearly divided into large two groups (A and B), in which A is separated into four clades (A-1 to A-4) and B into six clades (B-1 to B-6). The largest clade in the A group (A-1) includes AAB strains and also several *γ-Proteobacteria* from which GADH has been isolated, thus reasonably called the GADH clade, and the A-2 clade, mainly consisting of

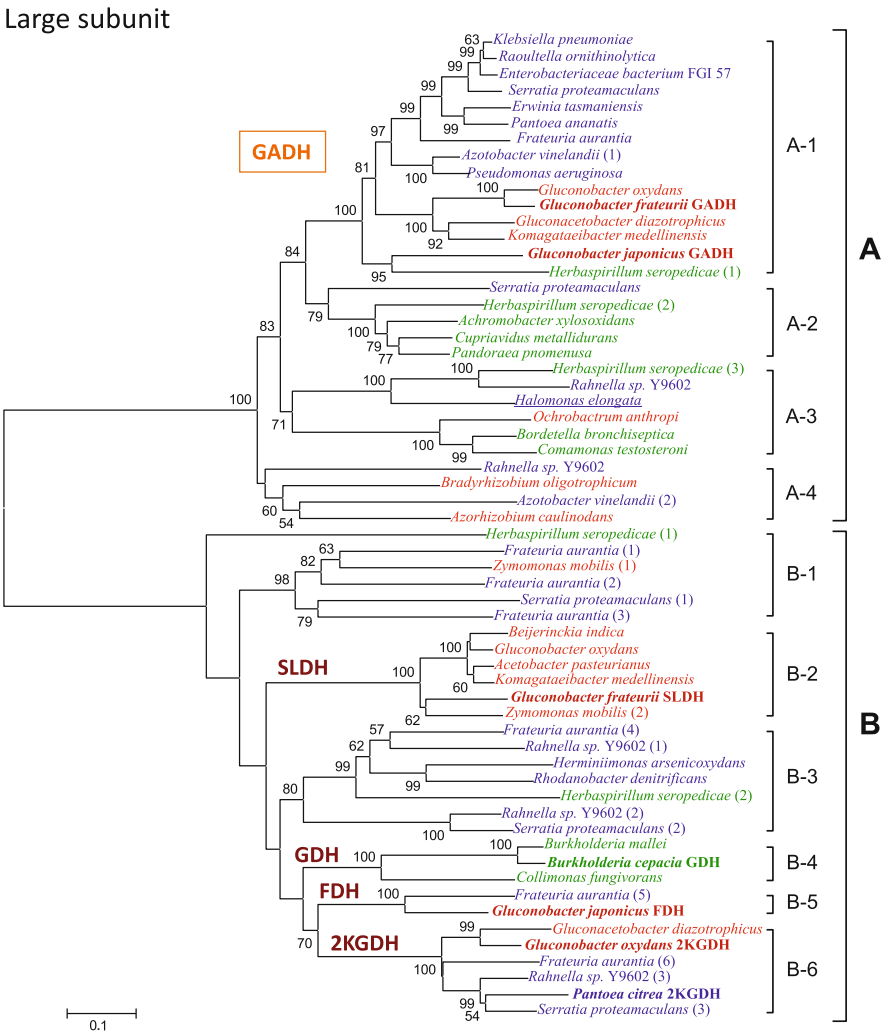

Fig. 7.3 Neighbor-joining phylogenetic tree of large subunit of membrane-bound flavoproteins. The phylogenetic tree was constructed using MEGA 5.05 with 1000 bootstrap replicates. *Bold* species names indicate experimentally validated proteins. Two clades, A and B, indicate gluconate dehydrogenase (GADH) and sorbitol dehydrogenase (SLDH) type enzymes, respectively, and the B clade is further separated into 6 small clades, B1 to B6. Of these, B-2, B-4, B-5, and B-6 could be identified as the clades of SLDH, glucose dehydrogenase (GDH), fructose dehydrogenase (FDH), and 2-ketogluconate dehydrogenase (2KGDH), respectively, based on the experimentally validated enzymes. A *number in parentheses* indicates paralogue gene numbers conserved in the same species. *Red*, *green*, and *blue* colors in species names indicate *α*-, *β*-, and *γ*-*Proteobacteria*, respectively

β-Proteobacteria, may also have GADH in spite of any experimental evidence. The other two (A-3 and A-4) are totally unknown clades consisting of *β,-γ-Proteobacteria* and *α,γ-Proteobacteria*, respectively. The B group is a mixture of several different enzymes from SLDH (B-2), FAD-GDH (B-4), FDH (B-5), 2KGDH (B-6), and unknown clades, B-1 and B-3, which means that the B group of flavoproteins has gained a broad substrate specificity during the divergences of the enzyme, whereas the A group has retained a relatively strict substrate specificity to react with gluconate. These clades consist mainly of *α,γ-Proteobacteria*, except for the SLDH (B-2) and GDH (B-4) clades, which consist of only *α-Proteobacteria* and *β-Proteobacteria*, respectively. AAB species have both GADH and SLDH homologues (SLDH, FDH, and 2KGDH), but not FAD-GDH. In particular, *Gluconobacter* species have many specific flavoproteins (see Fig. 7.3), and their repertoire of membrane-bound flavoproteins is different even within the same species (Matsutani et al., in manuscript), indicating that the HGT event occurred after the divergence of the strains.

It has been known that these membrane-bound flavoproteins share the unique cytochrome *c* subunit with membrane-bound ADH III and ALDH, of which the dehydrogenase subunit is a quinohemoprotein and a molybdoprotein, respectively. These cytochromes are rather unusual in having three hemes C in a single molecule for mediating electron transfer and to seemingly have a membrane-anchoring domain, and have also been shown to have ubiquinone (Q) reaction sites for both Q reduction and QH_2 oxidation, at least in ADH III of *G. oxydans* (Matsushita et al. 2008). Figure 7.4 shows the phylogenetic tree of these cytochrome *c* (C) subunits. The C-subunit of ALDH operon has an identity a little different from those of flavoproteins or a quinohemoprotein enzyme and thus is used as an outgroup. Phylogeny of the C-subunit was almost congruent with that of the L-subunit of group A, including the GADH clade, but not so with that of group B. This finding means that the C-subunit of the GADH operon has evolved together with L- and S-subunits, whereas that of the B group may have evolved randomly, except for the clades of SLDH (B-2) and FDH/2KGDH (B-5, B-6). In ADH III, the L-subunit (quinohemoprotein) is phylogenetically located in the sub-clade of type II PQQ-ADH (ADH II) clade (see Fig. 7.2). Because ADH II is a soluble protein and has no C-subunit in the operon, the C-subunit of ADH III, found only in AAB, was thought to evolve within AAB. Actually, the phylogenetic relationship indicates that the C-subunit of ADH III is most closely related to that of GADH from AAB, far from GADH from *γ-Proteobacteria* (Fig. 7.4), strongly suggesting that the prototype of ADH III has acquired the cytochrome *c* gene from the GADH operon of AAB.

Interestingly, γ-proteobacterial *F. aurantia* also have not only a GADH homologue, of which the L- and C-subunits both are located within γ-proteobacterial clades, but also ADH III, of which both the quinohemoprotein L-subunit and the C-subunit are very close to those of AAB, suggesting that the HGT event may have occurred in the opposite direction, from AAB to *Frateuria* in this case.

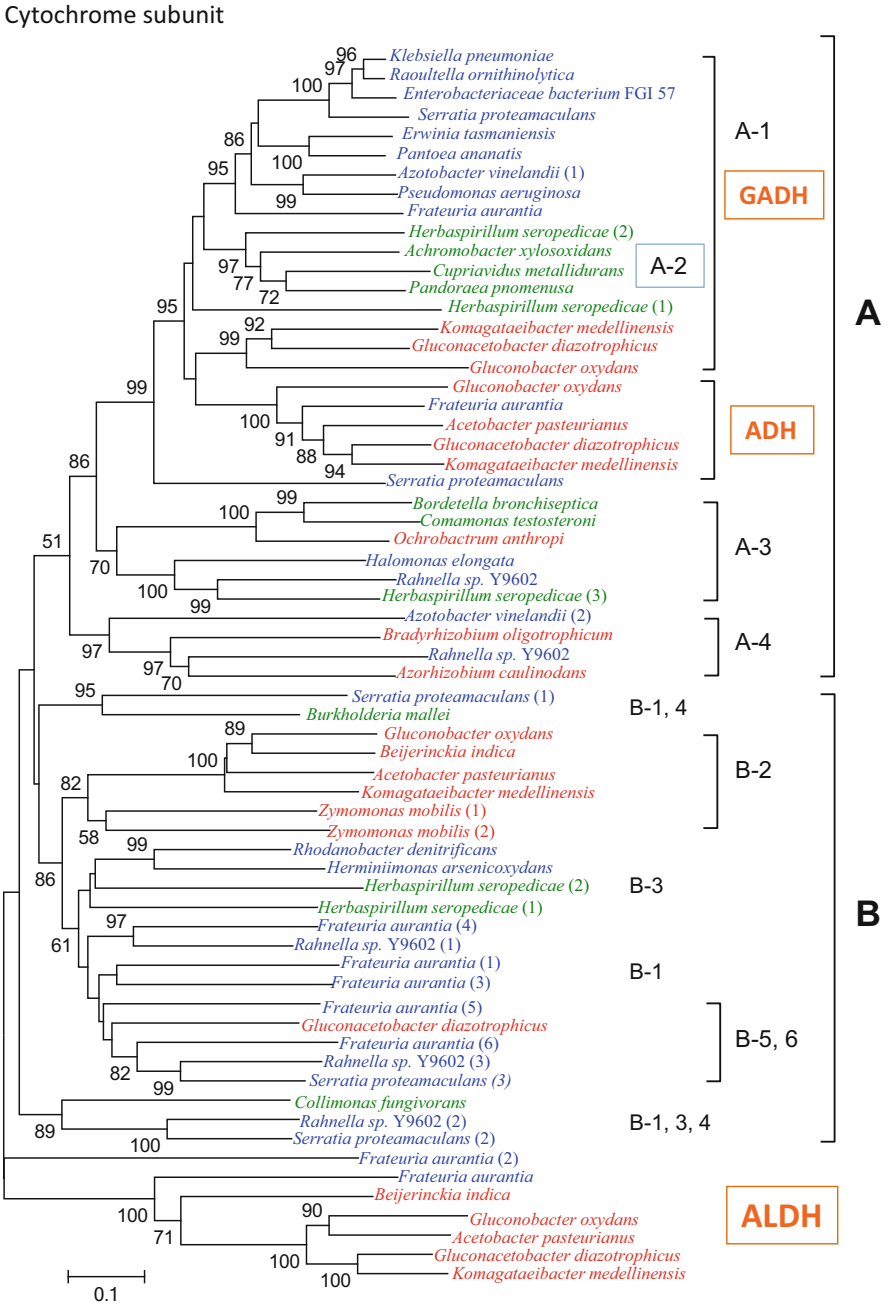

Fig. 7.4 Neighbor-joining phylogenetic tree of cytochrome *c* subunit of membrane-bound flavorproteins. The phylogenetic tree was constructed using MEGA 5.05 with 1000 bootstrap replicates. The cytochrome *c* subunits of type III alcohol dehydrogenase (ADH) and of aldehyde dehydrogenase (ALDH) are also included, and those of ALDH are used as outgroup. Two clades, A and B, indicate GADH and SLDH type clades, respectively, as in the case of the large subunit.

7.3 Respiratory Chain or Terminal Oxidases of Acetic Acid Bacteria: Its Evolutional Aspect

Aerobic organisms have the ability to generate energy by means of a respiratory chain that uses oxygen as the terminal electron acceptor. In the aerobic bacterial respiratory chain, two major terminal oxidases, a heme-copper oxidase and a *bd*-type oxidase, are working. The heme-copper oxidase superfamily has a binuclear O_2-reducing site consisting of a heme (heme A, O, or B) and a copper (Cu_B), and divided into cytochrome *c* oxidase (COX) that receives electrons from cytochrome *c*, and ubiquinol oxidase (UOX) which takes electrons from ubiquinol. The most evolved heme-copper oxidase (called A-type oxidase) has two separate proton channels to generate a higher proton motive force, whereas the *bd*-type oxidase is phylogenetically and also structurally different from the heme-copper oxidase, functioning as UOX. The *bd*-type oxidase has an O_2-reducing site consisting of hemes B and D, and no proton-pumping ability, but retains a scalar proton-releasing ability that generates a partial proton motive force.

In the heme-copper oxidase (A-type), it has been postulated that UOX, widely distributed in *Proteobacteria*, was yielded by a gene duplication event that occurred during the evolution of gram-positive bacterial COX, followed by the HGT event (Castresana et al. 1994); proteobacterial UOX gene (*cyoABCD*/*cyaBACD*) seems to originate from the COX gene (*ctaBCDEG*) of an ancestor of genus *Bacillus* before being transferred into an ancestor of *α*-, *β*-, and *γ*-*Proteobacteria* (Fig. 7.5). The proteobacterial UOX is separated into two major clusters: one formed by *α*- and *β*-*Proteobacteria* (α-type UOX), and the other formed by a single clade of *γ*-*Proteobacteria* (*Enterobacteriaceae*) and a mixed clade consisting of *α*-, *β*-, and *γ*-*Proteobacteria* (γ-type UOX); the UOX genes of AAB did not cluster with the α-type UOX (*α*,*β*-*Proteobacteria*) but within a mixed clade of *α*-, *β*-, *γ*-*Proteobacteria* of the γ-type UOX, where most of the *α*-*Proteobacteria* (five in seven species) were *Acetobacteraceae*, including AAB (Fig. 7.6) (Matsutani et al. 2014a). Thus, because the UOX gene clusters of *Acetobacteraceae* are more closely related to those of the *β*,*γ*-*Proteobacteria* than the *α*-*Proteobacteria*, they may have been acquired by an HGT event. Of the γ-type UOX genes, some groups have an additional *cyoE* gene to form a *cyoABCDE* operon, which is found only in *γ*-*Proteobacteria*, including a large group of *Enterobacteriaceae* and other groups such as *Acinetobacter* or *Pseudomonas* (Fig. 7.6). CyoE has been shown to function as a heme O synthase (HOS) producing heme O from heme B in *E. coli* (Saiki et al. 1992), and thus γ-*Proteobacteria* such as *E. coli* and *Azotobacter vinelandii* express cytochrome bo_3 UOX, which has heme O in the binuclear center (Kita et al. 1984; Matsushita et al. 1984; Yang 1986). The *cyoE* gene is not present in the

Fig. 7.4 (continued) The A-1 to A-4, and B-1 to B-6 clades correspond to those of Fig. 7.3. A number in *parentheses* indicates paralogue genes conserved in the same species. *Red*, *green*, and *blue* colors in species names indicate *α*-, *β*-, and *γ*-*Proteobacteria*, respectively

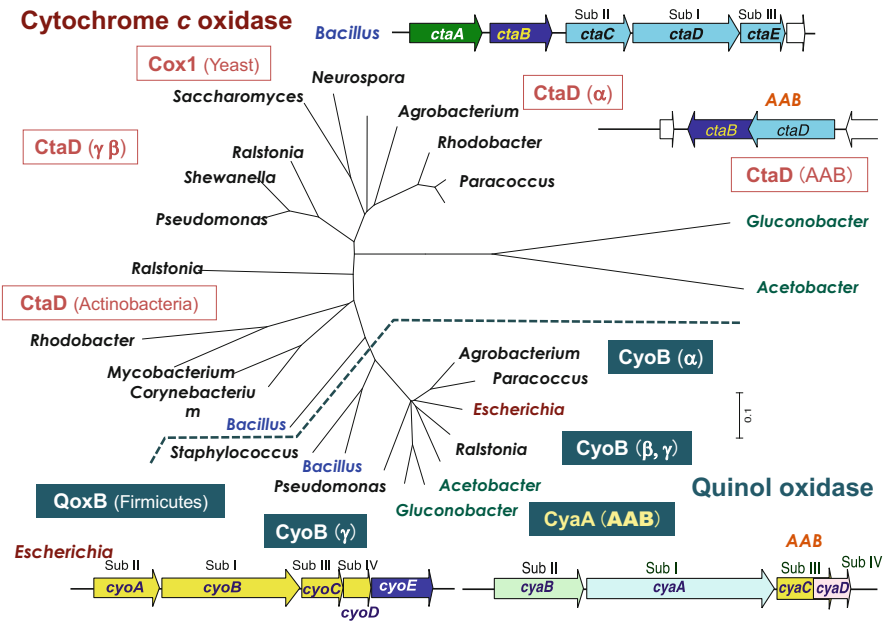

Fig. 7.5 Phylogenetic relationship of A-type cytochrome *c* oxidase and ubiquinol oxidase subunit I. The clades of cytochrome *c* oxidase subunit I (CtaD or Cox1) are separated from those of ubiquinol oxidase subunit I (CyoB, CyaA, or QoxB). In this figure, the clade of cytochrome *c* oxidase includes several microbes from yeast, *α-Proteobacteria (α)*, *β,γ-Proteobacteria (β, γ)*, and Actinobacteria; the clade of ubiquinol oxidase includes several bacteria from *Firmicutes*, *α-Proteobacteria (α)*, *β,γ-Proteobacteria (β, γ)*, and acetic acid bacteria (AAB). The gene organizations for the *Bacillus subtilis cta* gene operon, *Escherichia coli cyo* operon, and *cya* operon and *ctaB*/*ctaD* operon of *Acetobacter pasteurianus* (AAB) are also shown

cyoE-negative γ-type UOX operon, which was found within a mixed clade of *α-, β-, and γ-Proteobacteria*, including *Acetobacteraceae*, and also in the α-type UOX operon. Instead, these bacterial groups all have the COX operon or partial COX genes, where *ctaB* and *ctaA* are present and the products, CtaB and CtaA, work as HOS and a heme A synthase (HAS), respectively. HAS produces heme A from heme O that is produced by HOS. Therefore, these bacteria express cytochrome ba_3 UOX with heme A at the binuclear center. In contrast, almost all bacteria that carry the γ-type UOX (+*cyoE*) lack the COX gene clusters, including *ctaB*. This occurrence clearly shows that *ctaB* in the COX gene cluster was replaced with *cyoE* from the *cyoABCDE* operon. The *cyoE* is most closely related to *ctaB* of *Firmicutes*, suggesting that the ancestor of γ-proteobacterial *cyoE* was also acquired by HGT. Thus, it seems that the distribution of cytochrome bo_3/cytochrome ba_3 UOXs is consistent with two types of UOX.

Acetobacter pasteurianus has a single UOX gene cluster, *cyaBACD*, with no HOS or HAS genes nearby. Instead, *A. pasteurianus* has *ctaB* and *ctaA*, of which the products, CtaB and CtaA, were shown to function as HOS and HAS,

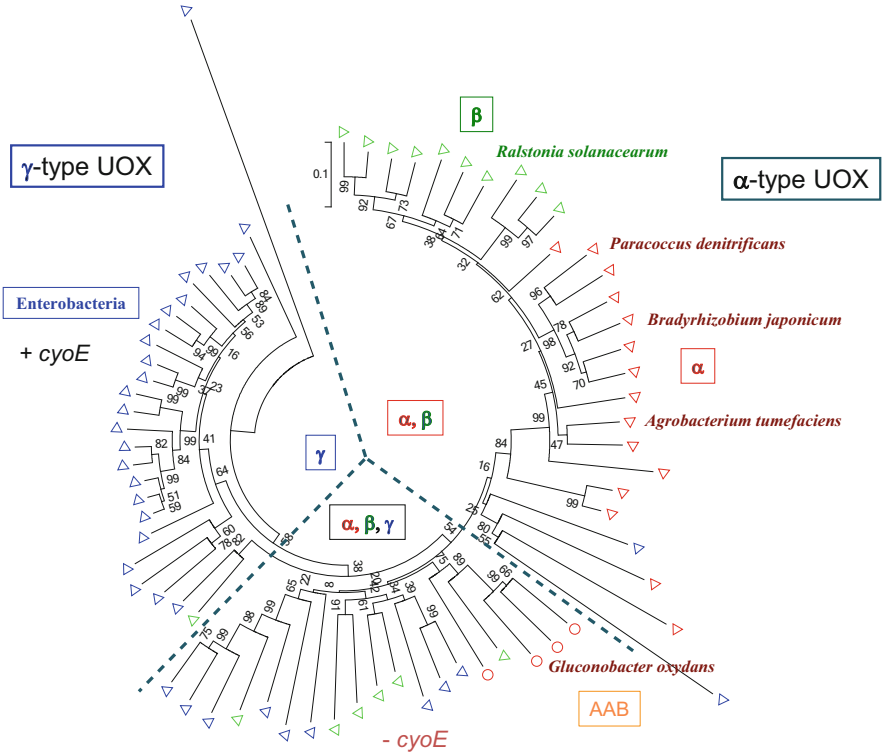

Fig. 7.6 Neighbor-joining phylogenetic tree of UOX subunit I in 72 strains from *α-*, *β-*, and *γ-Proteobacteria*. The tree are divided into three large clades: "α-type UOX" is present in α, and *β-Proteobacteria (α, β)*, "γ-type UOX" only in *γ-Proteobacteria* including a large group of enterobacteria, and the one other clade is a UOX consisting of the oxidases from *β-* or *γ-Proteobacteria*, and *α-Proteobacteria (α, β, γ)*, of which almost all are acetic acid bacteria (AAB). The oxidase operon of the latter clade does not have *cyoE* (−*cyoE*) but the γ-type UOX has *cyoE* in the operon (+*cyoE*). The bacteria species of the tree are shown by *green triangles* (*β-Proteobacteria*), *red triangles* (*α-Proteobacteria*), *blue triangles* (*γ-Proteobacteria*), and *red circles* (AAB)

respectively (Matsutani et al. 2014a). Thus, *A. pasteurianus* seems to produce a cytochrome ba_3 UOX (Matsushita et al. 1992a) by combination with the oxidase proteins from the *cyaBACD* operon and by *ctaB* and *ctaA*. As shown in Table 7.1, all *Acetobacteraceae* have *ctaB* as *cta*(*E*)*BD*(*C*) operon and *ctaA*, except for *G. oxydans*. AAB carry *ctaBD* and *ctaA*, whereas *G. oxydans* has *ctaBD* but not *ctaA*, which is consistent with the production of cytochrome bo_3 with heme O instead of cytochrome ba_3 with heme A. On the other hand, *Ac. cryptum* and *Gr. bethesdensis* genomes have a complete set of COX genes (*ctaABCDE*), in which *Ac. cryptum* was shown to have COX activity with cytochrome *c* (Matsutani et al. 2014a), although the *Ac. cryptum* genome has a partial UOX gene cluster (phylogenetically different from the other AAB *cya* operon) but the *Gr. bethesdensis* genome has the complete UOX genes (Table 7.1). Considering the

phylogenetic divergence of the *Acetobacteraceae* family, *Ac. cryptum* and *Gr. bethesdensis* seem to have retained a complete set of COX genes, but the later-diverged AAB species have *ctaBD* and *ctaA* and are missing *ctaCE*. The *ctaD* genes of AAB have a long branch length (Fig. 7.5), with numerous mutations in almost all essential amino acids for heme and copper ligands at the binuclear center and for H^+ channels (Matsutani et al. 2014a). Thus, CtaD seems to have lost its function in the AAB genera *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*, but not in *Ac. cryptum* and *Gr. bethesdensis*. Despite being nonfunctional, the *ctaD* genes are phylogenetically close to α-proteobacterial *ctaD* (A-type enzymes), similar to functional *ctaB* and *ctaA* of AAB, which are also located in a clade of α-proteobacterial enzymes (Matsutani et al. 2014a). These results suggest AAB have acquired UOX genes (*cyaBACD* operon) from *β-,γ-Proteobacteria*, instead of the ancestral COX, in which the heme O and heme A synthesis genes (*ctaB* and *ctaA*) are retained.

In addition to the UOX, AAB have *bd* oxidase and its homologue, which are known to be alternative low-energy-generating terminal oxidases found in a broad range of bacteria. The cytochrome *bd* oxidase has a high affinity for oxygen; it is used to perform aerobic respiration under low oxygen conditions. On the other hand, a homologue to the *bd* oxidase, cyanide-insensitive oxidase (CIO), was shown only recently to have a low affinity for O_2 but a very high turnover number (Miura et al. 2013). By comparing the phylogenetic relationships between *bd* oxidase and CIO, large four groups were defined and separated into proteobacterial *bd*, gram-positive *bd*, cyanobacterial *bd*, and CIO (Fig. 7.7). The phylogeny of the cytochrome *bd* clade is congruent with the taxonomy of bacterial species. In contrast, CIO is only distributed in *α-, β-, and γ-Proteobacteria*, and their phylogeny is incongruent with the taxonomy of bacterial species. As shown in Table 7.1, all *Acetobacteraceae* have CIO genes, but only *Ac. cryptum* and *A. pasteurianus* have the *bd* oxidase operon (*cydDCAB*): the CIO operon of *A. pasteurianus* and

Table 7.1 Terminal oxidases in *Acetobacteriaceae*

Acetobacteriaceae	COX	COX′	UOX	UOX′	*bd*	CIO
Acidiphilium cryptum	*ctaAA′*, *ctaEBDC*			*cyaCBA*	*cydDCAB*	*cioAB*
Granulibacter bethesdensis	*ctaA*, *ctaE*, *ctaBDC*		*cyaBACD*			*cioAB*
Komagataeibacter medellinensis	*ctaA*, *ctaBD*		*cyaBACD*			*cioAB*, *cioAB′*
Gluconacetobacter diazotrophicus	*ctaA*, *ctaBD*	*ctaEDC*	*cyaBACD*			*cioAB*
Acetobacter pasteurianus	*ctaA*, *ctaBD*		*cyaBACD*		*cydDCAB*	*cioAB*
Gluconobacter oxydans	*ctaBD*		*cyaBACD*			*cioAB*

Cytochrome *c* oxidase (COX), cytochrome *bd* (*bd*), and cyanide-insensitive oxidase (CIO) operons present in the six complete genomes of *Acetobacteriaceae*. COX′ and UOX′ show another group of gene clusters not homologous to COX and UOX, respectively
K. medellinensis = *Komagataeibacter medellinensis* (former name: *Gluconacetobacter xylinus*)

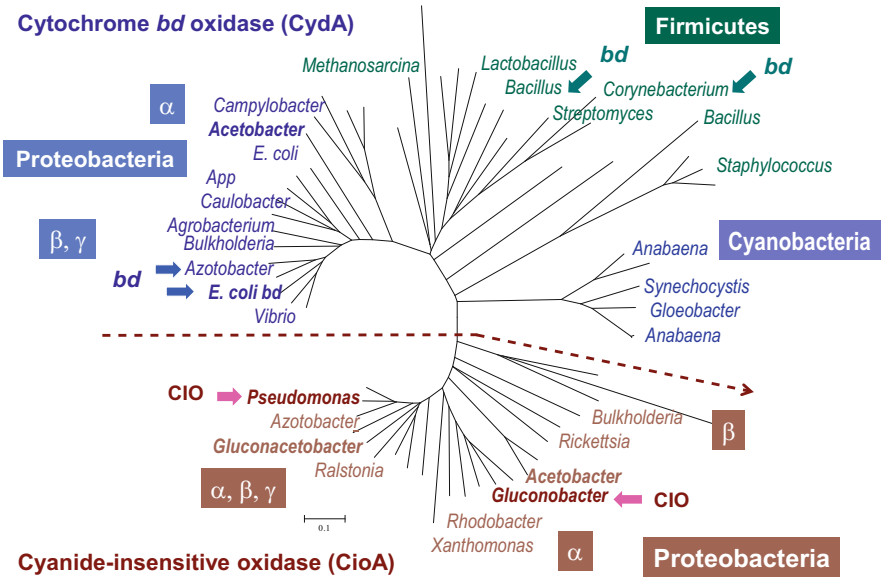

Fig. 7.7 Neighbor-joining phylogenetic tree of cytochrome *bd* subunit I homologues (CydA and CioA). Clades of cytochrome *bd* oxidase subunit I (CydA) could be separated from those of cyanide-insensitive oxidase subunit I (CioA). CydA clades are further separated into three large clades including *Proteobacteria* (α, β, γ), *Firmicutes*, and *Cyanobacteria*; the CIO clade seems to be mixed up. *Arrows* (*Escherichia coli*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Gluconobacter*, and *Pseudomonas*) show the enzymes experimentally validated

G. oxydans formed one cluster together with *Ac. cryptum* and *Gr. bethesdensis*, while the CIO of *Gluconacetobacter diazotrophicus* (known as an endophyte group with nitrogen fixation) was closely related to that of *β,γ-Proteobacteria,* including *Rhizobiae* (Fig. 7.7). Thus, it was strongly suggested that the propagation and evolution of the CIO operon (*cioAB*) had occurred by the HGT event. Although the physiological function is not clear yet, CIO may function as the main alternative terminal oxidase in the respiratory chain of AAB.

7.4 Conclusions

AAB inhabit the surface of flowers (pistil), fruits, and their fermented products such as vinegar, sake, wine, or beer (Asai 1968), and thus seem to be evolved in adapting to such a specific environment where high concentrations of sugars, alcohols, or sugar alcohols exist in highly aerobic conditions. In such a specific environment, there are many oxidative bacteria such as *Burkholderia*, *Ralstonia*, *Xanthomonas*, *Frateuria*, *Sphingomonas*, and *Sinorhizobium*, from *α*- to *γ-Proteobacteria*, which have quinoproteins and flavoprotein–cytochrome *c* complexes. The former

enzymes have a relatively high turnover number and a broad substrate specificity, and the latter enzyme complexes have a narrow substrate specificity but unique electron transfer ability, of which the physiological meaning is still unknown. These primary dehydrogenases, all locating on the outer surface of the cytoplasmic membranes, would be useful for rapidly oxidizing a broad range of useful sugars or sugar alcohols present in the fruits and flowers. In addition, such oxidative bacteria, especially from *β*- and *γ*-*Proteobacteria*, equip a powerful terminal oxidase, γ-type UOX, which could work more actively in terms of the electron transfer rate compared with the usual cytochrome bc_1–cytochrome *c* oxidase respiratory chain.

AAB also equip a specific respiratory chain, consisting of the periplasmic primary dehydrogenases of quinoproteins or flavoprotein–cytochrome *c* complexes, and terminal oxidases of ba_3-type UOX and CIO. The respiratory chain (with a high oxidation and low energy generation ability) enables AAB to perform incomplete oxidation, which contributes to the rapid oxidization of the many sugars or sugar alcohols, and to the extensive accumulation of the acid products in their environment. The oxidative reaction products, such as acetic acid, are harmful to other microorganisms and thus contribute to the fitness of AAB in the competitive microbial world in fruit and flowers. This effect may be the driving force for AAB to acquire such unique periplasmic dehydrogenase genes and also UOX genes by HGT and the loss of COX gene function.

Actually, these primary dehydrogenases (quinoproteins or flavoprotein–cytochrome *c* complexes) and also the terminal oxidases (γ-type UOX or CIO) all are found in only the small microbial world, restricted only in *α*-, *β*-, *and γ*-*Proteobacteria*, and even more concentrated into narrow proteobacterial groups specific to a habitat in fruits or flowers, as described earlier. Thus, these respiratory components seems to be highly evolved or devolved for fitting to such a specific environment, where AAB have a chance to get or to transfer specific genes for these specific respiratory enzymes from or into these bacteria by HGT. Thus, as shown in Fig. 7.1, AAB have established such a unique truncated respiratory chain to generate less energy but higher electron transfer ability. This evolutionary adaptation would enable AAB to survive in fruitful but harsh environments.

References

Adachi O, Fujii Y, Ghaly MF, Toyama H, Shinagawa E, Matsushita K (2001) Membrane-bound quinoprotein D-arabitol dehydrogenase of *Gluconobacter suboxydans* IFO 3257: a versatile enzyme for the oxidative fermentation of various ketoses. Biosci Biotechnol Biochem 65:2755–2762

Asai T (1968) Acetic acid bacteria. Classification and biochemical activities. Tokyo University Press, Tokyo

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783

Bertalan M et al (2009) Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics 10:450

Castresana J, Lübben M, Saraste M, Higgins DG (1994) Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. EMBO J 13:2516–2525

Cleton-Jansen AM, Goosen N, Vink K, van de Putte P (1989) Cloning, characterization and DNA sequencing of the gene encoding the M_r 50,000-quinoprotein glucosedehydrogenase from *Acinetobacter calcoaceticus*. Mol Gen Genet 217:430–436

Gao L, Du G, Zhou J, Chen J, Liu J (2013) Characterization of a group of pyrroloquinoline quinone-dependent dehydrogenases that are involved in the conversion of L-sorbose to 2-keto-L-gluconic acid in *Ketogulonicigenium vulgare* WSH-001. Biotechnol Prog 29:1398–1404

Görisch H, Rupp M (1989) Quinoprotein ethanol dehydrogenase from *Pseudomonas*. Antonie Van Leeuwenhoek 56:35–45

Greenberg DE, Porcella SF, Zelazny AM, Virtaneva K, Sturdevant DE, Kupko JJ 3rd, Barbian KD, Babar A, Dorward DW, Holland SM (2007) Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis*. J Bacteriol 189:8727–8736

Groen BW, van Kleef MA, Duine JA (1986) Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*. Biochem J 234:611–615

Hagan CL, Kim S, Kahne D (2010) Reconstitution of outer membrane protein assembly from purified components. Science 328:890–892

Higashiura N, Hadano H, Hirakawa H, Matsutani M, Takebe S, Matsushita K, Azuma Y (2014) Draft genomic DNA sequence of the facultatively methylotrophic bacterium *Acidomonas methanolica* type strain MB58. FEMS Microbiol Lett 351:9–13

Hölscher T, Weinert-Sepalage D, Görisch H (2007) Identification of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H. Microbiology 153:499–506

Inose K, Fujikawa M, Yamazaki T, Kojima K, Sode K (2003) Cloning and expression of the gene encoding catalytic subunit of thermostable glucose dehydrogenase from *Burkholderia cepacia* in *Escherichia coli*. Biochim Biophys Acta 1645:133–138

Kataoka N, Matsutani M, Yakushi T, Matsushita K (2015) Efficient production of 2,5-diketo-d-gluconate via heterologous expression of 2-keto-gluconate dehydrogenase in Gluconobacter japonicus. Appl Environ Microbiol 81:3552–3560

Kawai S, Goda-Tsutsumi M, Yakushi T, Kano K, Matsushita K (2013) Heterologous overexpression and characterization of a flavoprotein-cytochrome *c* complex fructose dehydrogenase of *Gluconobacter japonicus* NBRC3260. Appl Environ Microbiol 79:1654–1660

Kita K, Konishi K, Anraku Y (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome b_{562}-*o* complex from cells in the early exponential phase of aerobic growth. J Biol Chem 259:3368–3374

Matsushita K, Adachi O (1993) Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase. In: Davidson V (ed) Principles and applications of quinoproteins. Dekker, New York, pp 47–63

Matsushita K, Shinagawa E, Ameyama M (1982) D-Gluconate dehydrogenase from bacteria, 2-keto-D-gluconate-yielding, membrane-bound. Methods Enzymol 89:187–193

Matsushita K, Patel L, Kaback HR (1984) Cytochrome *o* type oxidase from *Escherichia coli*. Characterization of the enzyme and mechanism of electrochemical proton gradient generation. Biochemistry 23:4703–4714

Matsushita K, Ebisuya H, Adachi O (1992a) Homology in the structure and the prosthetic groups between two different terminal ubiquinol oxidases, cytochrome a_1 and cytochrome *o*, of *Acetobacter aceti*. J Biol Chem 267:24748–24753

Matsushita K, Takahashi K, Takahashi M, Ameyama M, Adachi O (1992b) Methanol and ethanol oxidase respiratory chains of the methylotrophic acetic acid bacterium, *Acetobacter methanolicus*. J Biochem (Tokyo) 111:739–747

Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301

Matsushita K, Toyama H, Yamada M, Adachi O (2002) Quinoproteins: structure, function, and biotechnological applications. Appl Microbiol Biotechnol 58:13–22

Matsushita K, Fujii Y, Ano Y, Toyama H, Shinjoh M, Tomiyama N, Miyazaki T, Sugisawa T, Hoshino T, Adachi O (2003) 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl Environ Microbiol 69:1959–1966

Matsushita K, Inoue T, Theeragool G, Trcek J, Toyama H, Adachi O (2005) Acetic acid production in acetic acid bacteria leading to their 'death' and survival. In: Yamada M (ed) Survival and death in bacteria. Research Signpost, Kerala, pp 169–181

Matsushita K, Kobayashi Y, Mizuguchi M, Toyama H, Adachi O, Sakamoto K, Miyoshi H (2008) A tightly bound quinone functions in ubiquinone reaction sites of quinoprotein alcohol dehydrogenase of acetic acid bacteria, *Gluconobacter suboxydans*. Biosci Biotechnol Biochem 72:2723–2731

Matsutani M, Hirakawa H, Yakushi T, Matsushita K (2011) Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*. FEMS Microbiol Lett 315:122–128

Matsutani M, Fukushima K, Kayama C, Arimitsu M, Hirakawa H, Toyama H, Adachi O, Yakushi T, Matsushita K (2014a) Replacement of a terminal cytochrome *c* oxidase by ubiquinol oxidase during the evolution of acetic acid bacteria. Biochim Biophys Acta Bioenerg 1837:1810–1820

Matsutani M, Suzuki H, Yakushi T, Matsushita K (2014b) Draft genome sequence of *Gluconobacter thailandicus* NBRC 3257. Stand Genomic Sci 9:614–623

Miura H, Mogi T, Ano Y, Migita CT, Matsutani M, Yakushi T, Kita K, Matsushita K (2013) Cyanide-insensitive quinol oxidase (CIO) from *Gluconobacter oxydans* is a unique terminal oxidase subfamily of cytochrome *bd*. J Biochem (Tokyo) 153:535–545

Miyazaki T, Tomiyama N, Shinjoh M, Hoshino T (2002) Molecular cloning and functional expression of D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO3255, which requires pyrroloquinoline quinone and hydrophobic protein SldB for activity development in *E. coli*. Biosci Biotechnol Biochem 66:262–270

Miyazaki T, Sugisawa T, Hoshino T (2006) Pyrroloquinoline quinone-dependent dehydrogenases from *Ketogulonicigenium vulgare* catalyze the direct conversion of L-sorbosone to L-ascorbic acid. Appl Environ Microbiol 72:1487–1495

Ogino H, Azuma Y, Hosoyama A, Nakazawa H, Matsutani M, Hasegawa A, Otsuyama K, Matsushita K, Fujita N, Shirai M (2011) Complete genome sequence of NBRC 3288, a unique cellulose-nonproducing strain of *Gluconacetobacter xylinus* isolated from vinegar. J Bacteriol 193:6997–6998

Oubrie A (2003) Structure and mechanism of soluble glucose dehydrogenase and other PQQ-dependent enzymes. Biochim Biophys Acta 1647:143–151

Peters B, Mientus M, Kostner D, Junker A, Liebl W, Ehrenreich A (2013) Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. Appl Microbiol Biotechnol 97:6397–6412

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Pujol CJ, Kado CI (2000) Genetic and biochemical characterization of the pathway in *Pantoea citrea* leading to pink disease of pineapple. J Bacteriol 182:2230–2237

Richhardt J, Bringer S, Bott M (2012) Mutational analysis of the pentose phosphate and Entner–Doudoroff pathways in *Gluconobacter oxydans* reveals improved growth of a Δedd Δeda mutant on mannitol. Appl Environ Microbiol 78:6975–6986

Saichana I, Moonmangmee D, Adachi O, Matsushita K, Toyama H (2009) Screening of thermotolerant *Gluconobacter* strains for production of 5-keto-D-gluconic acid and disruption of flavin adenine dinucleotide-containing D-gluconate dehydrogenase. Appl Environ Microbiol 75:4240–4247

Saiki K, Mogi T, Anraku Y (1992) Heme O biosynthesis in *Escherichia coli*: the *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase. Biochem Biophys Res Commun 189:1491–1497

Sakurai K, Yamazaki S, Ishii M, Igarashi Y, Arai H (2013) Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. J Biosci Bioeng 115:32–36

Shinagawa E, Matsushita K, Adachi O, Ameyama M (1984) D-Gluconate dehydrogenase, 2-keto-D-gluconate yielding, from *Gluconobacter dioxyacetonicus*: purification and characterization. Agric Biol Chem 48:1517–1522

Swings J, Gillis M, Kersters K, De Vos P, Gossle F, De Ley J (1980) *Frateuria*, a new genus for "*Acetobacter aurantius*". Int J Syst Bacteriol 30:547–556

Toyama H, Mathews FS, Adachi O, Matsushita K (2004) Quinohemoprotein alcohol dehydrogenases: structure, function, and physiology. Arch Biochem Biophys 428:10–21

Toyama H, Soemphol W, Moonmangmee D, Adachi O, Matsushita K (2005) Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. Biosci Biotechnol Biochem 69:1120–1129

Toyama H, Furuya N, Saichana I, Ano Y, Adachi O, Matsushita K (2007) Membrane-bound, 2-keto-D-gluconate-yielding D-gluconate dehydrogenase from "*Gluconobacter dioxyacetonicus*" IFO 3271: molecular properties and gene disruption. Appl Environ Microbiol 73:6551–6556

Trček J, Matsushita K (2013) A unique enzyme of acetic acid bacteria, PQQ-dependent alcohol dehydrogenase, is also present in *Frateuria aurantia*. Appl Microbiol Biotechnol 97:7369–7376

Van Spanning RJ, Wansell CW, De Boer T, Hazelaar MJ, Anazawa H, Harms N, Oltmann LF, Stouthamer AH (1991) Isolation and characterization of the *moxJ*, *moxG*, *moxI*, and *moxR* genes of *Paracoccus denitrificans*: inactivation of *moxJ*, *moxG*, and *moxR* and the resultant effect on methylotrophic growth. J Bacteriol 173:6948–6961

Vangnai AS, Toyama H, De-Eknamkul W, Yoshihara N, Adachi O, Matsushita K (2004) Quinate oxidation in *Gluconobacter oxydans* IFO3244: purification and characterization of quinoprotein quinate dehydrogenase. FEMS Microbiol Lett 241:157–162

Yamada M, Sumi K, Matsushita K, Adachi O, Yamada Y (1993) Topological analysis of quinoprotein glucose dehydrogenase in *Escherichia coli* and its ubiquinone-binding site. J Biol Chem 268:12812–12817

Yang T (1986) Biochemical and biophysical properties of cytochrome *o* of *Azotobacter vinelandii*. Biochim Biophys Acta 848:342–351

Yum DY, Lee YP, Pan JG (1997) Cloning and expression of a gene cluster encoding three subunits of membrane-bound gluconate dehydrogenase from *Erwinia cypripedii* ATCC 29267 in *Escherichia coli*. J Bacteriol 179:6566–6572

Chapter 8
Physiology of *Acetobacter* spp.: Involvement of Molecular Chaperones During Acetic Acid Fermentation

Akiko Okamoto-Kainuma and Morio Ishikawa

Abstract Acetic acid bacteria produce acetic acid from ethanol to acquire energy through a process called acetic acid fermentation. These bacteria are inevitably exposed to various stressors during fermentation but have developed resistance to these stressors. These resistance properties have been attributed to the combination of several kinds of mechanisms, including the role of molecular chaperones. In this chapter, we outline the involvement of major molecular chaperones (GroESL, DnaKJ, GrpE, and ClpB) in the stress-resistant abilities of *Acetobacter pasteurianus* NBRC3283, in addition to the role of the regulatory factor RpoH, with reference to our recent studies using proteomic analyses, RNA-seq analyses, and the mutants of these chaperones.

Keywords *Acetobacter pasteurianus* • Molecular chaperone • GroESL • DnaKJ • GrpE • ClpB • RpoH

8.1 Introduction

Acetic acid bacteria acquire adenosine triphosphate (ATP) by oxidative fermentation, which is an aerobic system on the cytoplasmic membrane that produces energy (Matsushita et al. 1992). Substrates outside these cells are oxidized by dehydrogenases connected to the electron transport chain, both of which are present on the membrane. The electrons released by oxidation from these substrates directly flow into the electron transport chain to reduce oxygen to water, which is coupled to the production of ATP. Thus, the products of oxidation accumulate outside cells with the production of energy. When the substrate is ethanol, one of the

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preferred compounds of acetic acid bacteria, this phenomenon is specially termed "acetic acid fermentation" and is applied to the production of vinegar.

Compounds such as ethanol and acetic acid, which generally make stressors for bacterial cells, become abundant around cells as acetic acid fermentation proceeds. Furthermore, increases in temperature also occur from the heat generated by fermentation. Thus, acetic acid bacteria are constantly and inevitably exposed to various stressors during fermentation to acquire energy. To tolerate and thrive under such circumstances, acetic acid bacteria have acquired the ability to resist these stressors.

The mechanisms underlying this resistance ability have been investigated extensively, and various factors, not a single dominant factor, have been implicated in the expression of these resistance properties. The combination of these factors has consequently been considered to cause the characteristic resistance ability of these bacteria, and the involvement of molecular chaperones has been suggested.

We investigated major molecular chaperones in *Acetobacter pasteurianus* NBRC3283 (former name, *Acetobacter aceti* IFO3283), which is a representative acetic acid bacterium used in the production of vinegar with the traditional static fermentation method. The complete genome sequence of this strain was determined and published in 2009 (Azuma et al. 2009). In this chapter, we described the profiles of these molecular chaperones and their relationships to fermentation by this strain, with some additional data of transcriptome analysis that we recently obtained.

8.2 Overview of Molecular Chaperones

Normal cell physiology is expressed by the proper condition of cellular proteins in the aspects of both amount and conformation. Molecular chaperones are important in maintaining the homeostasis of these proteins by conducting quality control.

Most molecular chaperones are involved in heat-shock proteins, which are tentatively induced by the addition of various external stressors such as heat, ethanol, and heavy metals. These proteins are known for their effects on proteins damaged by stressors. Their actions have been roughly classified into two types: the restoration of proteins from a damaged/denatured state to a normal state, and the degradation of denatured/aggregated proteins. Various proteins belong to the heat-shock family of proteins, and molecular chaperones are the main members in the former type.

The functions of molecular chaperones are (1) preventing denatured proteins from aggregation, and refolding them to the proper conformation; (2) resolubilizing aggregated proteins; and (3) facilitating the degradation of seriously damaged proteins by proteases. Differences have been identified in the functions of various kinds of molecular chaperones, and their cooperation conclusively leads to all these actions, which normalize the inner condition of cells exposed to stressors.

Molecular chaperones perform an essential function in some nascent proteins by achieving properly folded structures. Approximately 20 % to 30 % of nascent proteins require the assistance of molecular chaperones for proper folding. Hence,

these chaperones are also expressed constitutively to a certain extent in normal cells that have not been exposed to any stressor.

The GroES-GroEL system, DnaK-DnaJ-GrpE system, and ClpB are representative molecular chaperones in bacterial cells. These chaperones were previously reported to be regulated by sigma32 (RpoH), a bacterial sigma factor. We described the involvement of these chaperones in the process of acetic acid fermentation by acetic acid bacteria.

8.3 Growth Profiles of *Acetobacter pasteurianus* NBRC3283 and Expression of Chaperones on Two-Dimensional Gel Electrophoresis (2-DE)

8.3.1 Growth Behavior Under Ethanol-Containing Conditions

When cells are cultured under ethanol-containing conditions, the first growth phase is achieved by the oxidation of ethanol to acetic acid (the acetic acid fermentation phase), then, via the acetic acid accumulation phase (the transition phase) in which cells temporarily stop increasing in number, cells enter the second growth phase by dissimilating self-produced acetic acid through the tricarboxylic acid (TCA) cycle inside the cells with decreasing acetic acid concentrations outside the cells (the acetate overoxidation phase). These metabolic characteristics clearly indicate that this bacterium has a diauxic growth curve when the initial ethanol concentration is below 1 % (170 mM) (Fig. 8.1a). However, the second growth phase is more likely to disappear when the ethanol concentration is above 3 % (510 mM), which may be

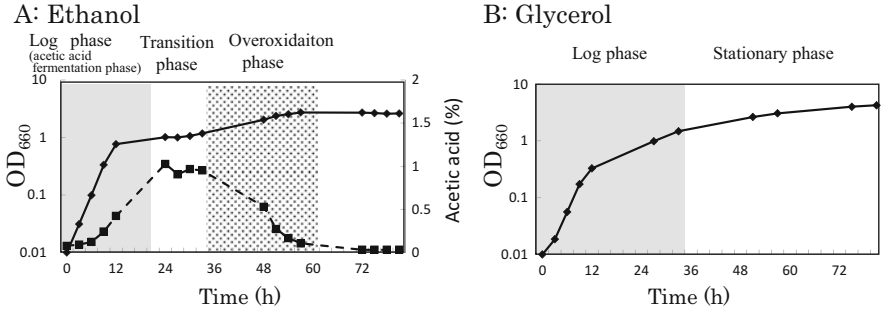

Fig. 8.1 Growth behavior of *Acetobacter pasteurianus* NBRC3283. Cells were cultured in YP basal medium (1 % yeast extract, 1 % polypepton) supplemented with two kinds of carbon sources, respectively. The culturing temperature was 30 °C with shaking at 121 rpm. (**a**) 170 mM ethanol was supplemented as a carbon source. *Solid line*: OD at 660 nm; *broken line*: concentration of acetic acid in culture medium. (**b**) 170 mM glycerol was supplemented as a carbon source

because the high concentrations of stressors such as acetate or acetoaldehyde caused by fermentation have a negative impact on the viability of these cells.

8.3.2 Growth Behavior Under Glycerol-Containing Conditions

Glycerol has no function in oxidative fermentation by this strain but is metabolized via the glycolytic pathway and TCA cycle inside these cells without the production of any acid outside the cells. This path is similar to the normal aerobic metabolism that occurs in many microbes, and these cells have a general one-stage growth curve with log and stationary phases (Fig. 8.1b). This carbon source is considered to generate no stressors during cell growth, which differs from ethanol.

8.3.3 Expression Behavior of Molecular Chaperones on 2-DE

A previous study reported that both GroEL and DnaK in ethanol-cultured cells gave larger protein spots than those in glycerol-cultured cells throughout growth (Okamoto-Kainuma et al. 2012). ClpB also gave larger spots, especially at the transition and acetate overoxidation phases, in ethanol-cultured cells. These findings indicated the involvement of these molecular chaperons in acetic acid fermentation. We describe each chaperone in this strain in detail next.

8.4 Involvement of GroES-GroEL in Acetic Acid Fermentation

8.4.1 General Outline of GroES-GroEL

This chaperone is one of the representative heat-shock proteins that are common in all living bodies, including bacteria. GroEL is a molecular chaperone with a molecular weight of approximately 60 kDa and has also been identified as a chaperonin in the HSP60 family. It functions with the support of the co-chaperone GroES (HSP10), which has a molecular weight of about 10 kDa. This factor prevents the aggregation of denatured or nascent polypeptides and provides the space and time needed for these proteins to form properly folded structures. In the case of nascent proteins, which require the assistance of chaperones for proper folding, GroESL becomes active once the actions of the DnaK system have been completed.

GroEL forms a heptamer that shapes a ring structure, and two layers of this ring, which are formed by back-to-back stacking (14-mer, double-ring structure), make the mature form of this chaperone (Bralg et al. 1994). GroES also forms a heptamer that shapes a lid-like structure, which cooperatively makes a cage-like space with a GroEL ring hole (21-mer) (Mande et al. 1996; Hunt et al. 1996). The space made by the GroEL hole and GroES lid, referred to as the central cavity, is isolated from the outer conditions so that unfolded proteins can be safely folded into their proper conformations.

The detailed functions of this chaperone are as follows (Horwich et al. 2006). (1) The hydrophobic moiety of an unfolded/denatured protein attaches to the ring top (apical domain) of the GroEL ring. (2) ATP attaches to the equatorial domain of the ring, which contains the combining part of two rings, thereby increasing the affinity of the apical domain of the GroEL ring for the GroES-lid. (3) The unfolded/denatured protein is enclosed in the central cavity formed by the GroEL ring and GroES lid (the *cis*-complex). (4) ATP is hydrolyzed and accompanied by proper folding of the protein in the cavity. (5) Another unfolded/denatured protein attaches to the other ring top on the opposite side, which reduces the affinity of the GroES lid for the GroEL ring. (6) The GroES lid dissociates from the GroEL ring, and the folded protein is released.

This chaperone is involved in the folding of proteins with a molecular weight of 20 to 60 KDa and contributes to forming the proper structures of about 10 % to 15 % of all proteins expressed in a cell (Ewalt et al. 1997). Polypeptides with an α/β domain structure have been shown to preferably attach to this chaperone for folding.

The *groEL* and *groES* genes generally exist in the form of an operon on the genome on the order of *groES* and *groEL* (Segal and Ron 1996a, b). In the case of *Proteobacteria*, to which acetic acid bacteria belong, expression of the *groESL* gene is under the control of the sigma 32 factor, which is also called RpoH. Furthermore, a *cis*-element called CIRCE (controlling inverted repeat of chaperone expression, 5′-TTAGCACTC-N_9-GAGTGCTAA-3′) presents upstream of the *groESL* gene, and is the binding region of the repressor protein HrcA, which works cooperatively with RpoH to regulate this gene (Segal and Ron 1998).

8.4.2 Profile of GroES-GroEL in A. pasteurianus *NBRC3283 and Its Relationship with Acetic Acid Fermentation*

8.4.2.1 Profile of the Gene Structure and Transcription Pattern

The genes of *groES* (APA01_17860) and *groEL* (APA01_17870) exist as one copy each in tandem on the genome. The configuration profile of these genes is shown in Fig. 8.2a (Okamoto-Kainuma et al. 2002). The *groES* and *groEL* genes are coded by a 294-bp sequence and a 1641-bp sequence, respectively. The amino acid

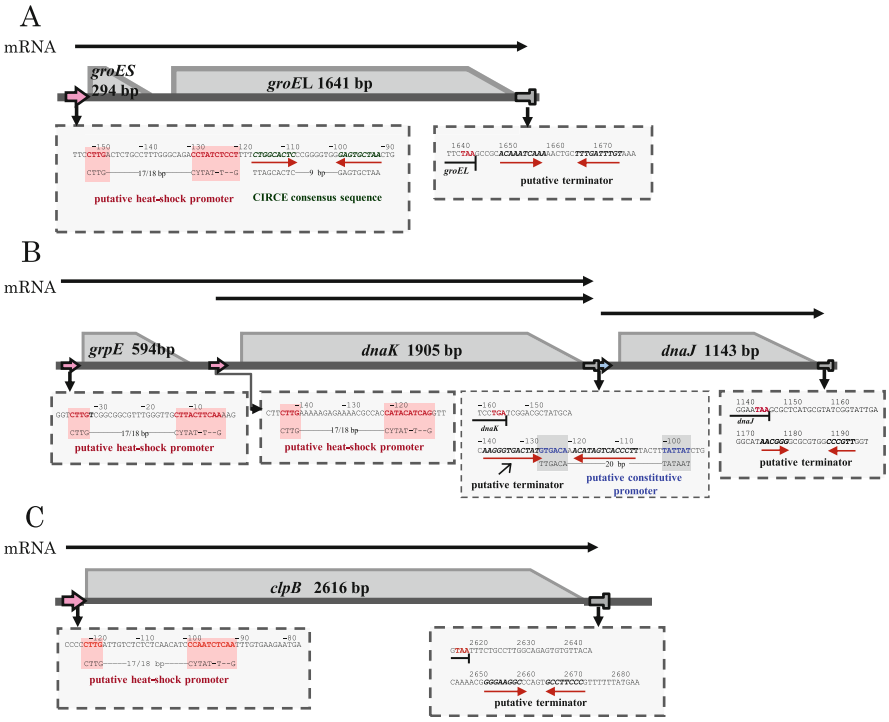

Fig. 8.2 Gene configurations and transcription patterns of various molecular chaperones in *A. pasteurianus* NBRC3283. (**a**) *groES-groEL*; (**b**) *grpE-dnaK-dnaJ*; (**c**) *clpB*

sequences of both GroES and GroEL are well conserved and showed 51 % and 67 % identities with those of *Escherichia coli*, respectively. A putative heat-shock promoter, which is commonly observed in *α-Proteobacteria* as a sequence recognized by RpoH, exists 154 bp upstream of the start codon of *groES* and is followed by the CIRCE sequence 7 bp downstream of the promoter. There is a short interval sequence (54 bp) with no terminator/promoter sequence on it downstream of the *groES* gene, and this is followed by the *groEL* gene. A terminator-like inverted repeat exists downstream of the *groEL* stop codon.

As estimated from this configuration, both *groES* and *groEL* genes are transcribed in one sequence to give one band only in Northern hybridization. The probes designed for the detection of both *groES* and *groEL* mRNA separately gave the same band with the combined size of the two genes, indicating that these two genes form an operon. The transcription initiation site of the *groESL* operon was previously found to be located inside the CIRCE sequence, which is present 114 bp upstream of the *groES*, by a primer extension analysis. This location is just downstream of the estimated heat-shock promoter just described and suggests that this operon is under control of this promoter and RpoH, as reported for other microbes.

8.4.2.2 Relationship to Acetic Acid Fermentation

The expression of the *groESL* gene in this bacterium responds to heat shock, similarly to that in other microbes. When cells grown on glucose were suddenly exposed to a higher temperature (42 °C) than the normal culturing temperature (30 °C) at the log phase, the amount of this gene increased to the maximum level within 20 min of the temperature shift. This behavior was also observed when cells were suddenly exposed to a compound such as ethanol or acetic acid, which are the substances surrounding the cells during acetic acid fermentation being harmful to adjacent cells. The addition of ethanol (ethanol shock) or acetic acid (acetate shock) at a final concentration of 4 % or 3 %, respectively, in the culture medium at the log phase also caused the temporal induction of this gene, as assessed by Northern hybridization. By taking the quick responses of this gene to fermentation-specific stressors into account, the GroES and GroEL chaperone system can be regarded as an important factor that enables cells to perform acetic acid fermentation.

The expression behaviors of the *groES* and *groEL* genes were also investigated in cells during acetic acid fermentation by measuring the amount of mRNAs at various growth phases in 170 mM ethanol-containing culture using an RNA-seq analysis (Fig. 8.3a). The actual relationship between these genes and fermentation, and not the temporary response to each stressor added as a shock, could be estimated by this analysis. As a comparison, the same analysis was conducted on cells grown on 170 mM glycerol.

When cells were cultured on ethanol, the expression levels of the *groES* and *groEL* genes were almost equal throughout the culture. The expression levels of these genes were also generally high, within the top 5 % of all the genes (2700) analyzed. The amount of *groES*/*groEL* mRNAs at the acetic acid fermentation phase was about 3000 RPKM, and this increased with cell growth to approximately 7000 RPKM, which quantitatively ranked as 20th to 30th among all the genes analyzed, at the end of the transition phase in which the accumulation of acetic acid outside the cells reached the maximum level. These levels decreased to about 4500 RPKM during the acetate overoxidation phase, in which acid around the cells gradually diminished as the result of dissimilation by the cells but were still markedly higher than that of the other genes. These findings strongly indicated the involvement of this chaperone system in acetic acid fermentation by this bacterium. However, the high expression levels of both genes were also observed in glycerol-cultured cells, especially at the log phase in which cells actively proliferate with normal aerobic metabolism. The expression patterns observed throughout the cultures were different between cells grown on ethanol and those grown on glycerol, suggesting the different roles of this chaperone system under these conditions.

To confirm the positive role of the GroES/GroEL chaperone system in acetic acid fermentation, we constructed a *groESL* overexpression strain by introducing a plasmid harboring this operon with its heat-shock promoter into the wild-type strain, and compared the growth behavior response to various stressors against

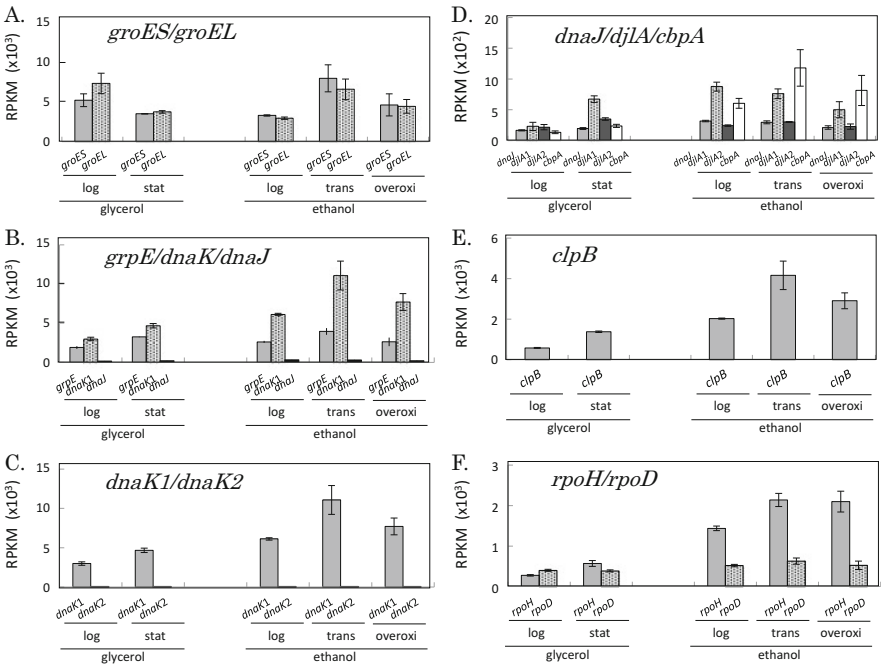

Fig. 8.3 Expression levels of *groES/L*, *grpE-dnaK-dnaJ*, *clpB*, *rpoH*, and related genes depending on the growth phase in the presence of glycerol or ethanol. The expression behavior of each gene was investigated by a RNA-seq analysis using HiSeq 2000 (Illumina): (**a**) *groES* (APA01_17860) and *groEL* (APA01_17870); (**b**) *grpE* (APA01_02890), *dnaK*1 (APA01_02900), and *dnaJ* (APA01_02910); (**c**) *dnaK*1 (APA01_02900) and *dnaK*2 (APA01_13330); (**d**) *dnaJ* (APA01_02910), *djlA*1 (APA01_02160), *djlA*2 (APA01_11120), *cbpA* (APA01_26370); (**e**) *clpB* (APA01_17450); (**f**) *rpoH* (APA01_21370) and *rpoD* (APA01_09910)

that of the parental strain. Both strains were cultured under the conditions of high temperature (42 °C), 5 % ethanol, and 1 % acetic acid, respectively, and under all the conditions tested, the growth behavior of the *groESL* overexpression strain was better than that of the parental strain, suggesting that an increased amount of the GroES/L chaperone endowed the cells with more resistance to the various stressors present during acetic acid fermentation.

The involvement of the GroES/L chaperone system in acetic acid fermentation has been proposed previously. Steiner et al. reported the induction of the GroEL protein in *Acetobacter aceti* DSMZ2002 by 2 % acetate in both batch and continuous cultures when cells were analyzed by 2-DE (Steiner and Sauer 2001). Furthermore, Andrés-Barrao et al. demonstrated the increased expression of GroES/GroEL proteins in *Acetobacter pasteurianus* LMG1262 during acetic acid fermentation by a proteomic analysis (Andrés-Barrao et al. 2012).

8.5 Involvement of DnaK-DnaJ-GrpE in Acetic Acid Fermentation

8.5.1 General Outline of DnaK and Related Co-chaperones

This molecular chaperone is another representative heat-shock protein with a molecular weight of about 70 kDa. This factor is a member of the HSP70 family and is present in all living bodies including bacteria, similar to GroEL. This chaperone has various functions in the quality control of cellular proteins by supporting the proper folding of nascent proteins, refolding of denatured proteins, and degradation of seriously denatured proteins. In many cases, this chaperone expresses its function under the assistance of the co-chaperones DnaJ (HSP40) and GrpE, and prevents nascent/denatured polypeptides from aggregation, which leads them to the properly folded conformation. Among all the chaperones involved in proper folding, DnaK makes the first contact with nascent proteins that need the assistance of chaperones.

DnaK possesses an ATPase domain at the N-terminal side and a substrate-binding domain at the C-terminal side (Genevaux et al. 2007). Not only ATP, but also GrpE, which facilitates nucleotide exchange, binds to the ATPase domain. There are also two portions in the substrate-binding domain: (1) a peptide-binding cleft consisting of a β-sandwich structure and (2) a latch-like part consisting of an α-helical structure (Zhu et al. 1996).

DnaJ is a protein with a molecular weight of about 40 kDa. The N-terminal region with about 70 amino acid residues is a highly conserved region named the J-domain (Hennessy et al. 2005), and two regions called the G/F-rich domain (Fan et al. 2003; Perales-Calvo et al. 2010) and C domain (or central domain) exist downstream of the J-domain (Szabo et al. 1996), which is followed by the C-terminal domain with low conservation. The G/F-rich domain is rich in glycine and phenylalanine residues, whereas the C domain contains four repeats of the motif "CXXCXGXG," consisting of a zinc-finger-like structure. DnaJ is involved in the "DnaJ family," which is characterized by the presence of the J-domain in the protein sequence (Genevaux et al. 2001). This family can be divided into three groups depending on their sequence profiles: (1) the DnaJ type, which has all the foregoing domains; (2) the CbpA type, which has a J-domain at the N-terminal region and following the G/F domain, but no C domain; and (3) the DjlA type, which has a J-domain at the C-terminal region. All these proteins are considered to have some cooperative function with DnaK.

GrpE is a protein with a molecular weight of about 20 kDa that functions in the form of a homodimer. This protein binds to the ATPase domain of DnaK. A long α-helical structure and short α-helical structures have been identified from the position of approximately the 40th amino acid from the N-terminal end, followed by a compact β-sheet structure (Harrison 2003). This β-sheet structure has the ability to wedge the ATP-binding cleft of DnaK ATPase domain open.

The scheme of the DnaK chaperone system, which works cooperatively with DnaJ and GrpE, is considered to be as follows (Hartl and Hayer-Hartl 2002). (1) DnaJ recruits a target polypeptide to DnaK by attaching it to the C-terminal region. (2) A polypeptide attaches to the substrate-binding cleft of DnaK under ATP-binding conditions. Under these conditions, the α- helical latch of DnaK is in the open state. (3) The J-domain of DnaJ binds to DnaK, which promotes the hydrolysis of its ATP. (4) The latch is closed by the hydrolysis of ATP to ADP, which makes DnaK hold the substrate peptide tightly. (5) GrpE attaches to the GrpE binding site in the ATPase domain of DnaK, which promotes the dissociation of ADP from DnaK. Another ATP associates with DnaK, and the latch is opened to release the substrate polypeptide. While being held by DnaK, the substrate polypeptide is prevented from aggregation, which causes the fatal denaturation of proteins.

This DnaK chaperone system preferentially involves proteins with a molecular weight of 20 to 30 kDa; however, it can also interact with proteins larger than 60 kDa, which cannot be settled in the central cavity of GroESL (Teter et al. 1999). A previous study reported that 10 % to 20 % of nascent proteins were treated by the DnaK system for proper folding after translation (Hartl and Heyer-Hartl 2002).

The *dnaK* and *dnaJ* genes are typically organized as an operon on the genome with this order in many kinds of bacteria, although the location of *grpE* varies depending on the genera or species. The expression of this chaperone is also known to be regulated by the sigma 32 factor (RpoH) and a CIRCE sequence located upstream of the *dnaKJ* operon, similar to the expression of *groESL* (Segal and Ron 1996a).

8.5.2 *Profile of DnaK-DnaJ-GrpE in* A. pasteurianus *NBRC3283 and Its Relationship with Acetic Acid Fermentation*

8.5.2.1 Profile of the Gene Structure and Transcription Pattern

Two genes with low mutual identities have been annotated as *dnaK* (APA01_02900, APA01_13330) on the genome because of the presence of some domains characteristic to DnaK. Four genes have also been annotated as *dnaJ* (APA01_02160, APA01_02910, APA01_11120, and APA01_26370). Of these, only APA01_20900 and APA01_02910 can be genuinely regarded as *dnaK* and *dnaJ*, respectively, from their sequence profiles. Concerning *dnaJ* homologues, APA01_02160 and 11120 are considered to be *djlA*s, and APA01_26370 can be assigned as *cbpA*. Concerning *grpE*, a single gene (APA01_02890) is present on the genome with *dnaK* (APA01_02900) and *dnaJ* (APA01_02910) in tandem, which is the same as that observed in other microbes, thereby suggesting functional similarities. Hence, we focused on these genes by regarding them as true *dnaK* and related genes. The order of these genes was *grpE-dnaK-dnaJ* (Fig. 8.2b).

The *grpE*, *dnaK*, and *dnaJ* genes are encoded by 597-bp, 1905-bp, and 1143-bp sequences, respectively. Amino acid sequence identities are 27 % to GrpE, 65 % to DnaK, and 53 % to DnaJ in *E. coli*. The characteristic domains of these proteins are also all observed in GrpE, DnaK, and DnaJ in this strain. A putative heat-shock promoter for *α-Proteobacteria* exists 37 bp upstream of the start codon of the *grpE* gene with no CIRCE sequence following. A 206-bp-interval sequence, on which a short inverted repeat followed by another putative heat-shock promoter can be observed, is found between the *grpE* and *dnaK* genes. A 156-bp-interval sequence is present between the *dnaK* and *dnaJ* genes, with a terminator-like long inverted repeat downstream of *dnaK*, and a constitutive promoter-like sequence upstream of *dnaJ*. A terminator-like long inverted repeat exists downstream of the *dnaJ* gene.

These three genes are transcribed in several ways (Fig. 8.2b). Northern hybridization using a *dnaK*-targeting probe gave two bands with sizes that corresponded to *grpE-dnaK* and *dnaK* only, whereas a *grpE*-targeting probe only gave a single band with the size of *grpE-dnaK*. A *dnaJ* band was not detected by a *dnaJ*-targeting probe or a *dnaK*-targeting probe (Okamoto-Kainuma et al. 2004); however, the expression of this gene was confirmed by a RT-PCR analysis using a primer set for *dnaJ* amplification (Ishikawa et al. 2010a). The primer set for *dnaK–dnaJ* amplification did not give any band in this analysis; therefore, these genes were estimated to be transcribed as *grpE-dnaK*, *dnaK* single, and *dnaJ* single. This finding is consistent with the gene configuration already described; the bicistronic mRNA coding *grpE-dnaK* is transcribed from the first heat-shock promoter upstream of *grpE*, whereas the monocistronic mRNA coding *dnaK* only is from the second heat-shock promoter located between the *grpE* and *dnaK* genes. *dnaJ* is transcribed alone because it cannot be transcribed bicistronically with *dnaK* in the presence of a terminator between *dnaK* and *dnaJ*. In any case, the involvement of RpoH in the expression of this chaperone system must also be considered.

8.5.2.2 Relationship to Acetic Acid Fermentation

The DnaK chaperone system in this bacterium also responds to heat shock. The expression levels of all three genes was tentatively increased by an immediate temperature shift from 30 ° to 42 °C at the log phase in glucose medium, and reached the maximum level within 10 min, which was about tenfold that of the initial state. Although this induction pattern was synchronized among all three genes, the amounts induced differed depending on the genes when measured using the qRT-PCR method. As estimated from the transcription pattern, the expression level of *dnaK* mRNA was the highest among the three genes, followed by that of *grpE* mRNA, which was about two thirds that of *dnaK*. The expression of *dnaJ* was very weak and the amount of *dnaJ* mRNA was less than one tenth that of *dnaK* mRNA. These genes also responded to exposure to 4 % ethanol (ethanol shock), but the expression levels were less than one third those induced by heat shock. The exposure of cells to 3 % acetic acid (acetic acid shock) did not cause clear responses by these genes. Even considering the case of acetic acid shock, the

involvement of these chaperone systems as well as the GroE system in acetic acid fermentation was strongly considered.

The expression behaviors of these three genes based on cell growth with either 170 mM ethanol or 170 mM glycerol were also examined by an RNA-seq analysis (Fig. 8.3b). As shown in the figure, *dnaK* mRNA was characteristically expressed when cultured in ethanol-containing conditions. Its expression levels were high (~6,000 RPKM) even in the acetic acid fermentation phase, but markedly increased toward the transition phase and peaked at the end of this phase with approximately 11,000 RPKM, which placed it 15th from the highest expression levels observed among all the genes analyzed. Its expression levels decreased to about 7,700 RPKM in the acetate overoxidation phase, but were still considered to be high. Another gene, annotated as *dnaK* on the genome (APA01_13330; *dnaK*2), was very weakly expressed throughout cell growth (Fig. 8.3c). The expression behavior of *grpE* was similar to that of *dnaK*, which peaked in the transition phase; however, its expression levels were less than 4,000 RPKM, even at the peak phase. The expression levels of *dnaJ* were characteristically low, at approximately 200 to 300 RPKM throughout cell growth. Among the plural *dnaJ* homologues, the expression behavior of *cbpA* (APA01_26370) was similar to that of *dnaK* (Fig. 8.3d), but its expression levels were not as high as those of *dnaK*, suggesting an association with the DnaK chaperone system under this condition.

The levels of *dnaK* mRNA expressed in the glycerol culture were almost one third to one half that in ethanol culture, whereas no significant differences were observed in the expression levels of *grpE* and *dnaJ* mRNAs between the two conditions. These expression behaviors demonstrated the important role of the DnaK chaperone system, especially DnaK itself, in acetic acid fermentation. A slight increase was observed in the expression of both *grpE* and *dnaK* mRNA toward the stationary phase in the glycerol culture, which is the reverse of that of *groESL* under this condition.

The effects of the overexpression of these genes on the stress-resistant abilities of this strain were also investigated. The strain harboring a plasmid containing the *grpE-dnaK-dnaJ* fragment with their putative promoter regions was constructed and growth behaviors in the presence of various fermentation-oriented stressors were compared with the parental strain. The growth behavior of the overexpression strain was improved at a high temperature (42 °C) or 5 % ethanol, whereas no difference was observed from the parental strain at 1 % acetic acid. These findings also suggested the importance of the DnaK chaperone system in acetic acid fermentation.

Elevations in the expression levels of DnaK and GrpE during acetic acid fermentation have also been reported by Andrés-Barrao et al., who conducted a proteomic analysis on *A. pasteurianus* LMG1262 (Andrés-Barrao et al. 2012).

8.6 Involvement of ClpB in Fermentation

8.6.1 General Outline of ClpB

ClpB is a heat-shock protein with a molecular weight of approximately 100 kDa. This chaperone is a member of the HSP100/Clp family (Schirmer et al. 1996) or AAA+ family (Neuwald et al. 1999), which has a Walker-type ATPase domain. It functions cooperatively with DnaK and assists in the recovery of denatured proteins from an aggregated to a nonaggregated soluble condition, thereby enabling them to express proper activities (Goloubinoff et al. 1999). This chaperone works as a homo-hexamer, which shapes a ring structure (Lee et al. 2003). One subunit consists of four domains, which are the N-terminal domain, AAA-1 domain (ATPase domain), M domain with a coiled-coil structure, and AAA-2 domain (ATPase domain). A denatured polypeptide is recruited to ClpB by DnaK, introduced into the central hole of the ClpB hexamer ring, and then recovered from aggregation with the energy produced from ATP hydrolysis when it passes through the ring hole (Rosenzweig et al. 2013).

ClpB basically exists in its inactive form in cells and is activated when needed. The M domain has been shown to repress the activity of ClpB by interacting with the AAA-1 domain (DeSantis and Shorter 2012; Seyffer et al. 2012). Clearing the interaction between the M domain and AAA-1 domain markedly increases both ATPase activity and suction force into the ring hole, which activates the function of ClpB. This activation has been shown to occur with the support of the DnaK chaperone. The proposed mechanism is as follows. (1) DnaK attaches to an aggregated polypeptide and recruits it to ClpB. (2) DnaK associates with the M domain of ClpB, which clears the interaction between the M domain and AAA-1 domain. (3) ClpB changes to its active form, which exhibits strong ATPase activity and threading power.

ClpB does not take charge in the folding of nascent polypeptides. The role of this chaperone is considered to be disaggregation of polypeptides that were once aggregated by denaturation for recovering their functions.

Expression of the *clpB* gene is also controlled by the sigma 32 factor (RpoH) (Kitagawa et al. 1991), although CIRCE rarely influences this gene in general microbes. However, previous studies suggested a relationship between CIRCE and the regulation of *clpB*, as shown in the case of *Mycoplasma pneumoniae*, which is characterized by the absence of an alternative sigma factor and presence of a CIRCE sequence upstream of *clpB* (Kannan et al. 2008).

8.6.2 *Profile of ClpB in* A. pasteurianus *NBRC3283 and Its Relationship with Acetic Acid Fermentation*

8.6.2.1 Profile of the Gene Structure and Transcription Pattern

Only a single gene is annotated as *clpB* on the genome of this strain (APA01_17450). The coding region of this gene is 2616 bp long and encodes 871 amino acids, showing 58 % identity with that of *E. coli*. There is a putative heat-shock promoter for *α-Proteobacteria* located 93 bp upstream of the start codon of this gene, similar to *groESL*, *grpE*, and *dnaK*, but with no CIRCE sequence around it. A terminator-like inverted repeat can be observed downstream of the stop codon (Fig. 8.2c).

As estimated from the foregoing profile, this gene is transcribed in a monocistronic manner without any peripheral genes on the genome, which was confirmed by only one band with a *clpB*-targeting probe on Northern hybridization.

8.6.2.2 Relationship to Acetic Acid Fermentation

This gene also quickly responded to heat shock at the log phase in glucose culture, and the amount of its mRNA tentatively peaked within 10 min of the shock, with a level about 20 fold that of the initial level (Ishikawa et al. 2010b). Although it also responded to the ethanol shock in a similar induction manner, the amount of its mRNA was markedly lower than that induced by heat shock. The addition of acetic acid did not have any effect on the induction of *clpB*.

The RNA-seq analysis showed the expression behavior of *clpB* mRNA at both 170 mM ethanol and 170 mM glycerol. As shown in Fig. 8.3e, the amount of *clpB* was about 600 RPKM in the log phase and about 1400 RPKM in the stationary phase when cells were grown on glycerol. On the other hand, this amount was about 2000 RPKM even in the first log phase (the acetic acid fermentation phase) when cultured on ethanol. It increased to about 4200 RPKM in the transition phase and then decreased to about 2900 RPKM in the acetate overoxidation phase. The expression profile of *clpB* in the different growth phases was similar to those of the *dnaK* and *grpE* genes in both ethanol- and glycerol-containing cultures, from which the higher level induction of *clpB* on ethanol than on glycerol was observed throughout cell growth. A marked increase was also observed in *clpB* expression levels during acetic acid fermentation to the transition phases, which was similar to that of the other molecular chaperones as already described.

To demonstrate the relationship between this factor and acetic acid fermentation, we constructed a *clpB* disruptant strain and compared its growth behavior with that of the parental strain under the various stress conditions. The disruptant became sensitive to a high temperature (42 °C), whereas no difference was observed under the 5 % ethanol-containing condition. However, the loss of its acetic acid

fermentation ability was prominent even under the 3 % ethanol condition at 40 °C, indicating the importance of this chaperone in acetic acid fermentation.

8.7 Importance of RpoH in Fermentation

8.7.1 General Outline of RpoH (the Sigma 32 Factor)

RpoH (sigma 32) is one of the bacterial sigma factors that binds to the RNA polymerase core enzyme and recruits it to the promoter region on the genome. It recognizes a "heat-shock promoter" to induce the transcription of the genes located downstream of such a promoter. The consensus sequence of the heat-shock promoter of *Escherichia coli* is considered to be TCTC-CCCTTGAA <13/14 bp > CCCCAT-AT, whereas that of *α-Proteobacteria*, to which acetic acid bacteria belong, has been proposed as CTTG <17/18 bp > CYTAT-T – G (Segal and Ron 1995). Many heat-shock proteins including molecular chaperones and proteases are known to be involved in the regulation of RpoH. Hence, the action of RpoH endows cells with resistance under various stress conditions.

Although RpoH exists constitutively to a certain extent in normal cells without any stressors, it is strongly expressed under stress conditions such as heat shock in which intracellular proteins may be damaged. The transcription of *rpoH* is regulated by other sigma factors including sigma 70 (constitutive sigma factor, RpoD) (Erickson et al. 1987) or sigma 24 (extracytoplasmic sigma factor, RpoE) (Wang and Kaguni 1989), and self-regulation has not yet been reported (Tilly et al. 1986). In addition to this transcriptional regulation, the expression of this factor is also controlled at the translational level by the secondary structure of *rpoH* mRNA (Morita et al. 1999). Transcribed *rpoH* mRNA in normal cells has a secondary structure in which the translation initiation site is masked by another part of the same mRNA. This masking is cleaved by external stressors such as heat to initiate translation. The posttranslational regulation of RpoH levels has also been described in the manner of a negative feedback regulation system; this is achieved by molecular chaperones including GroE (Guisbert et al. 2004) and DnaK (Straus et al. 1990) with heat-shock inducible proteases such as FtsH (Tatsuta et al. 1998). A decrease in the amount of denatured proteins in cells promotes the attachment of GroE or DnaK to RpoH, resulting in the inactivation of its function. Furthermore, unnecessary RpoH is considered to be recruited to FtsH at the cytoplasmic membrane by signal recognition particles (SRP) and is rapidly degraded with the support of the chaperones (Lim et al. 2013). The strict regulation of RpoH at various levels allows proper responses to stressors to occur inside the cells.

RpoH has four domains (regions 1, 2, 3, 4) that are commonly observed in sigma factors (Paget and Helmann 2003). Each domain has been further divided into several subdomains. Of these, region 2, which contributes to its activity and regulation, and region 4, which is important for the recognition of promoter

sequences, are well conserved. The characteristic motif of "Q(R/K)(K/R) LFFNLR," called the RpoH box, has been identified downstream of regions 2 and 4 (Nakahigashi et al. 1995).

8.7.2 *Profile of RpoH in* A. pasteurianus *NBRC3283 and Its Relationship with Acetic Acid Fermentation*

8.7.2.1 Profile of the Gene Structure

A single gene is annotated as *rpoH* on the genome (APA01_21370). The coding region of the gene is 891 bp long and encodes 296 amino acids. Sequence identity at the protein level is 41 % with *E. coli*, and the four characteristic domains already described are also observed. A RpoH box can be found as QKKLFFNLR between regions 2 and 3.

8.7.2.2 The Relationship of RpoH to the Expression of Molecular Chaperones and Acetic Acid Fermentation Ability

As discussed in the sequence profiles of *groESL*, *grpE*, *dnaK*, and *clpB*, the putative heat-shock promoters proposed for *α-Proteobacteria* have been detected in all these genes (Fig. 8.2), which strongly suggests their regulation by RpoH in this bacterium. Hence, we constructed a *rpoH* disruptant strain to confirm the relationships between this factor, the expression of these molecular chaperones, and acetic acid fermentation ability.

Figure 8.4 shows the heat-shock responses of the five molecular chaperone genes (Okamoto-Kainuma et al. 2011) described in this chapter in both the parental and *rpoH* disruptant strains, as analyzed by the qRT-PCR method. As shown in Fig. 8.4a, the chaperone genes having putative heat-shock promoters were induced immediately after the shock with various intensities that depended on the genes. The expression level of *groESL* was the highest among the genes analyzed; however, its expression behavior, which peaked 20 min after heat shock, was different from those of the other chaperone genes which peaked 10 min after the shock. Figure 8.4b shows the heat-shock responses of the same genes in the *rpoH* disruptant strain. All the genes, except for the *groESL* gene, very weakly responded to heat shock, as shown in the figure. Even in the case of the *groESL* gene, its expression level was markedly lower in the disruptant strain than in the parental strain. These findings appeared to indicate that RpoH was the causative factor inducing these molecular chaperones as a response to heat shock. The reason for the different behavior of the *groESL* gene currently remains unclear; however, the effects of the synergistic regulation between RpoH and HrcA may be involved because of the presence of the CIRCE sequence, which was observed only on the *groESL* gene.

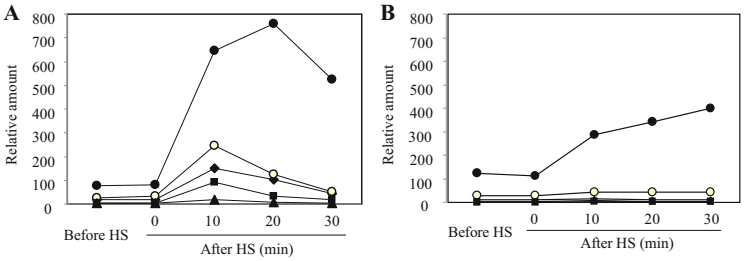

Fig. 8.4 Relative expression levels of the five chaperone genes after heat shock. Levels are indicated in values relative to *mutS* levels. (**a**) Expression behavior by the parental strain. (**b**) Expression behavior by the *rpoH* disruptant strain. *HS* heat shock, *closed circles groEL*, *open circles dnaK*, *closed triangles dnaJ*, *closed diamonds grpE*, *closed squares clpB*. (Figure reprinted from Okamoto-Kainuma et al. 2011)

Figure 8.3f shows the expression behavior of *rpoH* in the wild-type strain during the ethanol or glycerol culture in comparison with that of *rpoD*, which is the gene of the constitutive sigma factor. As shown in this figure, the expression levels of both genes were similar at less than 600 RPKM throughout cell growth in the glycerol culture. On the other hand, the amount of *rpoH* mRNA markedly increased in the ethanol culture and peaked in the transition phase at approximately 2100 RPKM, whereas that of *rpoD* remained at almost the same level as that in the glycerol culture. These findings were considered to reflect the role of RpoH and its regulon, which endow cells with resistance under stress conditions concomitant with acetic acid fermentation. Because similar expression behaviors depending on cell growth were observed among *rpoH* and all the chaperones examined in both cultures, these chaperones were also suggested to be under the control of RpoH. Furthermore, these findings indicated that the expression of RpoH activity was mainly regulated at the transcriptional level under this condition. Only the behavior of *groES*/*groEL* in the glycerol culture differed from those of the others, which is consistent with the heat-shock response already described. Therefore, the involvement of RpoH and its regulating chaperones in acetic acid fermentation was strongly suggested by this analysis.

We examined the growth behavior of the *rpoH* disruptant strain under the conditions with each fermentation-related stressor and compared it to that of the parental strain. As shown in Fig. 8.5, the growth of the *rpoH* disruptant was completely inhibited under a high temperature at 42 °C and was also markedly weaker than that of the parental strain in the presence of 5 % ethanol or 1 % acetic acid. These negative shifts in the growth of the disruptant were all recovered by reintroduction of the *rpoH* gene into cells as a *rpoH*-harboring plasmid (shown as the *rpoH* restoration strain in the figure).

RpoH did not appear to be essential for this strain because its gene could be disrupted; however, the importance of this factor was indicated in the expression of resistance against fermentation-oriented stressors resulting from the induction of

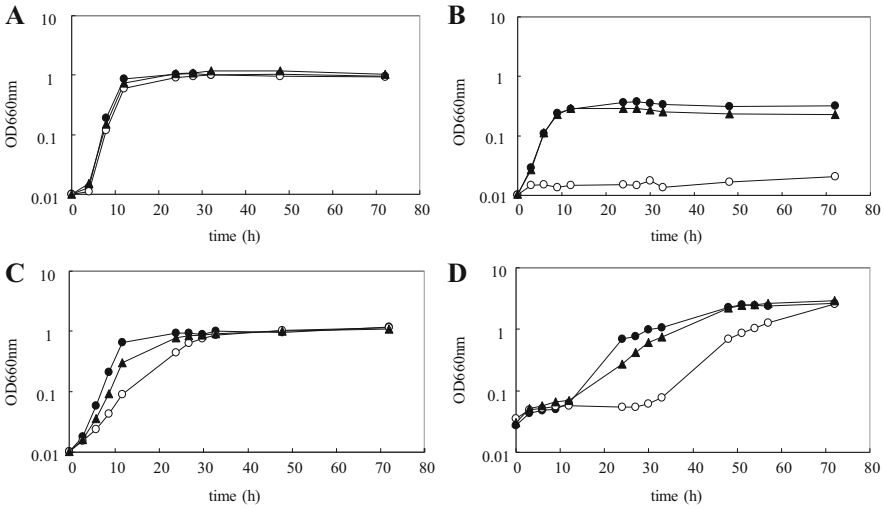

Fig. 8.5 Growth behaviors of the three strains under various stress conditions: in YPD medium (YP + 3 % Glc) at 30 °C (**a**); in YPD medium at 42 °C (**b**); in YPE medium (YP + 5 % ethanol) at 30 °C (**c**); in YPDA medium (YPD + 1 % acetic acid) at 30 °C (**d**). *Closed circles* the parental strain, *open circles* the *rpoH* disruptant strain, *closed triangles* the *rpoH* restoration strain. (Figure reprinted from Okamoto-Kainuma et al. 2011)

proteins such as molecular chaperones, which enabled these cells to perform acetic acid fermentation.

8.8 Conclusion

We herein described the involvement of RpoH and its related molecular chaperones in acetic acid fermentation by *Acetobacter pasteurianus* NBRC3283. These factors are already known to be critical for stress resistance in many other microbes; however, their importance in acetic acid bacteria is considered to be exceptional from the aspect of acetic acid fermentation. Those factors seem to be involved in stress resistance continuously, not tentatively as reported in many cases of chaperone expression, during the entire process of the fermentation. They must function cooperatively during the fermentation for keeping homeostasis of the cellular condition, and thus synergistically and significantly contribute to enable acetic acid bacteria to provide good performance in vinegar production.

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References

Andrés-Barrao C, Saad MM, Chappuis ML, Boffa M, Perret X, Pérez RO, Barja F (2012) Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. J Proteomics 75:1701–1717

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783

Bralg K, Othwlnowsk Z, Hegde R, Bolsvert DC, Joachimiak A, Horwich AL, Sigler PB (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. Nature (London) 371: 578–586

DeSantis ME, Shorter J (2012) The elusive middle domain of Hsp104 and clpB: location and function. Biochim Biophys Acta 1823:29–39

Erickson JW, Vaughn V, Salter WA, Neidhardt FC, Gross CA (1987) Regulation of the promoter and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. Genes Dev 1:419–432

Ewalt KL, Hendrick JP, Houry WA, Hartl FU (1997) In vivo observation of polypeptide flux through the bacterial chaperonin system. Cell 90:491–500

Fan CY, Lee S, Cyr DM (2003) Mechanisms for regulation of Hsp70 function by Hsp40. Cell Stress Chaperones 8:309–316

Genevaux P, Schwager F, Georgopouls D, Kelley WL (2001) The *djlA* gene acts synergistically with *dnaJ* in promoting *Escherichia coli* growth. J Bacteriol 183:5747–5750

Genevaux P, Georgopoulos C, Kelley WL (2007) The Hsp70 chaperone machines of *Escherichia coli*: a paradigm for the repartition of chaperone functions. Mol Microbiol 66:840–857

Goloubinoff P, Mogk A, Zvi APB, Tomoyasu T, Bukau B (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc Natl Acad Sci U S A 96:13732–13737

Guisbert E, Herman C, Lu CZ, Gross CA (2004) A chaperone network controls the heat shock response in *E. coli*. Genes Dev 18:2812–2821

Harrison C (2003) GrpE, a nucleotide exchange factor for DnaK. Cell Stress Chaperones 8: 218–224

Hartle FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858

Hennessy F, Nicoll WS, Zimmermann R, Cheetham ME, Blatch GL (2005) Not all J domains are created equal: implications for the specificity of Hsp40–Hsp70 interactions. Protein Sci 14: 1697–1709

Horwich AL, Farr GW, Fenton WA (2006) GroEL–GroES-mediated protein folding. Chem Rev 106:1917–1930

Hunt JF, Weavr AJ, Landry SJ, Gierasch L, Deisenhofer J (1996) The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. Nature (London) 379:37–45

Ishikawa M, Okamoto-Kainuma A, Jochi T, Suzuki I, Matsui K, Kaga T, Koizumi Y (2010a) Cloning and characterization of *grpE* in *Acetobacter pasteurianus* NBRC3283. J Biosci Bioeng 109:25–31

Ishikawa M, Okamoto-Kainuma A, Matsui K, Takigishi A, Kaga T, Koizumi Y (2010b) Cloning and characterization of *clpB* in *Acetobacter pasteurianus* NBRC3283. J Biosci Bioeng 110: 69–71

Kannan TR, Musatovova O, Gowda P, Baseman JB (2008) Characterization of a unique ClpB protein of *Mycoplasma pneumoniae* and its impact on growth. Infect Immun 76:5082–5092

Kitagawa M, Wada C, Yoshioka S, Yura T (1991) Expression of ClpB, an analog of the ATP-dependent protease regulatory subunit in *Escherichia coli*, is controlled by a heat shock sigma factor (sigma 32). J Bacteriol 173:4247–4253

Lee S, Sowa ME, Watanabe Y, Sigler PB, Chiu W, Yoshida M, Tsai FTF (2003) The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. Cell 115:229–240

Lim B, Miyazaki R, Neher S, Siegele DA, Ito K, Walter P, Akiyama Y, Yura T, Gross CA (2013) Heat shock transcription factor σ^{32} co-opts the signal recognition particle to regulate protein homeostasis in *E. coli*. PLoS Biol 11:e1001735

Mande SC, Mehra V, Bloom BR, Hol WGJ (1996) Structure of the heat shock protein chaperonin-10 of *Mycobacterium leprae*. Science 271:203–207

Matsushita K, Takai Y, Shinagawa E, Ameyama M, Adachi O (1992) Ethanol oxidase respiratory chain of acetic acid bacteria. Reactivity with ubiquinone of pyrroloquinoline quinone-dependent alcohol dehydrogenases purified from *Acetobacter aceti* and *Gluconobacter oxydans*. Biosci Biotechnol Biochem 56:304–310

Morita M, Kanemori M, Yanagi H, Yura T (1999) Heat-induced synthesis of σ^{32} in *Escherichia coli*: structural and functional dissection of *rpoH* mRNA secondary structure. J Bacteriol 181:401

Nakahigashi K, Yanagi H, Yura T (1995) Isolation and sequence analysis of *rpoH* genes encoding σ^{32} homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. Nucleic Acids Res 23:4383–4390

Neuwald AF, Aravind L, Spouge JL, Koonin EV (1999) AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res 9:27–43

Okamoto-Kainuma A, Wang Y, Kadono S, Tayama K, Kozumi Y, Yanagida F (2002) Cloning and characterization of *groESL* operon in *Acetobacter aceti*. J Biosci Bioeng 94:140–147

Okamoto-Kainuma A, Wang Y, Fukaya M, Tukamoto Y, Ishikawa M, Koizumi Y (2004) Cloning and characterization of the *dnaKJ* operon in *Acetobacter aceti*. J Biosci Bioeng 97: 339–342

Okamoto-Kainuma A, Ishikawa M, Nakamura H, Fukazawa S, Tanaka N, Yamagami K, Koizumi Y (2011) Characterization of *rpoH* in *Acetobacter pasteurianus* NBRC3283. J Biosci Bioeng 111: 429–432

Okamoto-Kainuma A, Ishikawa M, Ito K, Koizumi Y (2012) Proteomic study of *Acetobacter pasteurianus* NBRC3283 and analysis of factors possibly related to acetic acid fermentation. Abstract book of third international conference on acetic acid bacteria, vinegar and other products, Cordoba Spain, Apr 17–20, pp 17–18

Paget MSB, Helmann JD (2003) The σ^{70} family of sigma factors. Genome Biol 4:203

Perales-Calvo J, Muga A, Moro F (2010) Role of DnaJ G/F-rich domain in conformational recognition and binding of protein substrates. J Biol Chem 285:34231–34239

Rosenzweig R, Moradi S, Zarrine-Afsar A, Glover JR, Kay LK (2013) Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. Science 339:1080–1083

Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21:289–296

Segal G, Ron EZ (1995) The *dnaKJ* operon of *Agrobacterium tumefaciens*: transcriptional analysis and evidence for a new heat shock promoter. J Bacteriol 177:5952–5958

Segal G, Ron EZ (1996a) Regulation and organization of the *groE* and *dnaK* operons in *Eubacteria*. FEMS Microbiol Lett 138:1–10

Segal G, Ron EZ (1996b) Heat shock activation of the *groESL* operon of *Agrobacterium tumefaciens* and the regulatory roles of the inverted repeat. J Bacteriol 178:3634–3640

Segal G, Ron EZ (1998) Regulation of heat-shock response in bacteria. Ann N Y Acad Sci 851: 147–151

Seyffer FS, Kummer E, Oguchi Y, Winker J, Kumar M, Zahn R, Sourjik V, Bukau B, Mogk A (2012) Hsp70 proteins bind Hsp100 regulatory M domains to activate AAA; disaggregase at aggregate surfaces. Nat Struct Mol Biol 19:1347–1355

Steiner P, Sauer U (2001) Proteins induced during adaptation of *Acetobacter aceti* to high acetate concentrations. Appl Environ Microbiol 67:5474–5481

Straus D, Walter W, Gross CA (1990) DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock synthesis and stability of σ^{32}. Genes Dev 4:2202–2209

Szabo A, Korszun R, Hartl FU, Flanagan J (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. EMBO J 15:408–417

Tatsuta T, Tomoyasu T, Bukau B, Kitagawa M, Mori H, Karata K, Ogura T (1998) Heat shock regulation in the *ftsH* null mutant of *Escherichia coli*: dissection of stability and activity control mechanisms of σ^{32} *in vivo*. Mol Microbiol 30:583–593

Teter SA, Houry WA, Ang D, Tradier T, Rockabrand D, Fischer G, Blum P, Georgopoulos C, Hartl FU (1999) Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. Cell 97:755–765

Tilly K, Erickson J, Sharma S, Georgopoulos C (1986) Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. J Bacteriol 168:1155–1158

Wang Q, Kaguni JM (1989) A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. J Bacteriol 171:4248–4253

Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME, Hendrickson WA (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272: 1606–1614

Chapter 9
Physiology of *Komagataeibacter* spp. During Acetic Acid Fermentation

François Barja, Cristina Andrés-Barrao, Ruben Ortega Pérez, Elena María Cabello, and Marie-Louise Chappuis

Abstract Some species of the genus *Komagataeibacter* (formerly involved in genus *Gluconacetobacter*; see Chap. 1), known for their high acetic acid resistance, are involved in the industrial production of vinegar. Among acetic acid bacteria (AAB), the genus *Acetobacter* is mainly involved in surface static processes, where final acidities may reach a maximum range of 8 % to 9 %; *Komagataeibacter* strains are predominant in submerged processes, where vinegar may show acidities up to 15 % to 20 %. The final acidity that may be obtained in industrial vinegars is also dependent on the alcoholic raw material, so the highest levels may be reached by using distilled alcohol. Their intrinsic tolerance to extreme environmental conditions, such as high acetic acid and ethanol concentrations and low pH, is, among other intracellular molecular mechanisms, the result of a modification of the outer membrane, affecting phospholipids, fatty acids, and polysaccharides, as well as an increased activity and stability of alcohol dehydrogenase (ADH). In this chapter, the characteristics and genomic features of the bacteria implicated in submerged vinegar production are described.

Keywords *Komagataeibacter* • Membrane polysaccharides • LPS–*O*-antigen • Dihydroceramide • *cis*-Vaccenic acid • Heat-shock proteins • Plasmid pJK2-1 • Proline uptake • pH homeostasis

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9.1 General Introduction

Bacteria that convert sugars and alcohols into their corresponding organic acids are generally referred to as acetic acid bacteria (AAB). AAB are widespread and important in numerous natural and industrial processes leading to high-value food and beverage products, such as vinegar, chocolate, kombucha, and fine chemicals of industrial interest such as ascorbic acid (vitamin C), dihydroxyacetone, and polymers such as cellulose and acetan. However, poor understanding of the growth, physiology, and metabolism of AAB hampers rational design and optimization of the numerous biotechnological processes in which these bacteria are involved (Deppenmeier and Ehrenreich 2009).

The genus *Komagataeibacter* (*K.*) was recently proposed, regrouping 14 species previously classified into the genus *Gluconacetobacter* (*Ga.*): *K. swingsii*, *K. europaeus*, *K. nataicola*, *K. xylinus*, *K. sucrofermentans*, *K. intermedius*, *K. oboediens*, *K. kakiaceti*, *K. rhaeticus*, *K. saccharivorans*, *K. kombuchae*, *K. hansenii*, *K. medellinensis*, and *K. maltiaceti* (Yamada et al. 2012; Yamada 2014). Specialized strains from this genus, as well as from the genus *Acetobacter*, are currently used for industrial production of vinegar because they are able to partially oxidize ethanol into acetic acid with high efficiency. Vinegar fermentation, also known as oxidative or acetic acid fermentation, is an oxidative process in which diluted ethanol is first oxidized to the intermediate acetaldehyde that is subsequently converted to acetic acid and water. Although vinegar can be manufactured from almost any product capable of yielding alcohol through fermentation [the raw materials more frequently used are fruits such as apple, grape, plum, mango, date, or coconut; cereals such as rice, malt, or wheat; or animal products (whey or honey)], the commercial production of this condiment relies mostly on two methods: (1) the traditional slow process with *surface culture* (SC), referred as the Orleans or French method, often used for making table vinegar, and (2) a more modern and faster *submerged fermentation* (SF) process. In SC, AAB forms a thin film on the surface of the growth medium, which becomes thicker and more gelatinous with time, as bacterial growth increases. A large number of the heterogeneous bacterial population are embedded into this layer (known as the "mother of vinegar" layer), a fraction of which is used as a starter to inoculate new cultures. Although SC yields vinegars of high quality, with precise and specific organoleptic properties, it is a slow and costly process. Bacterial populations involved in SC are normally heterogeneous, and the most abundant species identified belong to the genus *Acetobacter* (Ilabaca et al. 2008; Vegas et al. 2010, 2013). In contrast to the slow process, SF involves the rapid mixing of AAB starters with forced aeration in specialized bioreactors called acetators. Ecological studies on this kind of fermentation process have revealed a homogeneous population comprising several strains of the genus *Komagataeibacter* (Fernández-Pérez et al. 2010a, b; Andrés-Barrao et al. 2011b; Andrés-Barrao 2012).

The final product of the acetic acid fermentation, acetic acid (AcH), is well known for its ability to inhibit growth of most microorganisms at very low

concentrations (0.5 %) (Conner and Kotrola 1995). AcH is a weak lipophilic molecule that can diffuse easily through the cytoplasmic membrane, and the toxic effect is caused by the dissociation of the molecule when it encounters the higher pH of the cytoplasm. The released protons decrease the internal pH and produce the uncoupling of the oxidative phosphorylation, disrupting the membrane proton gradient, which ultimately results in the poisoning of the cell. The molecular mechanisms that confer resistance or adaptation to acetic acid have not been completely elucidated. Nevertheless, the characterization of these mechanisms conferring acetic acid resistance to AAB is important from the standpoints of both basic and applied microbiology. However, relatively little is known about the general growth, physiology, and metabolism of these bacteria.

Interestingly, although *Komagataeibacter* spp. are known for their high acetic acid resistance, bacterial strains rapidly lose their resistance to high concentrations of acetic acid when cells are grown on synthetic media under less stringent laboratory conditions, indicating that the molecular mechanisms involved are both inducible and transient. Although showing lower resistance to acetic acid, *Acetobacter* strains also share this property.

Evolution must have led to the acquisition of intrinsic resistance to high acetic acid concentrations and low pH through a regulated modification of the outer membrane. Additionally, during growth in alcohol-containing media such as wine, cider, or distilled ethanol, the metabolism of bacteria must adapt step by step to the changing conditions, particularly the increase in acetic acid concentration during acetic acid fermentation. The acquired tolerance by metabolic adaptation must be the result of a sophisticated regulation of the gene expression (activation or repression of important genes needed at every specific moment during the adaptation process), as well as other genomic strategies such as gene transfer of mobile elements (plasmids/transposons) (Zhang et al. 2011). Although a high number of transposable elements and insertion sequences (IS) are present in *Komagataeibacter* as well as in *Acetobacter* species, very little is known about the genetic background of their genome instability (Azuma et al. 2009). Moreover, the number of completely sequenced genomes of *Komagataeibacter* strains is nowadays increasing and will allow the identification and understanding of genes and functions involved in the adaptation mechanisms (see Table 9.1).

The aims of this chapter are to review the main physiological modifications occurring in *Komagataeibacter* spp. during submerged vinegar production, and to describe their characteristics and genomic features.

Table 9.1 Comparison of *Komagataeibacter* genomes

Strain	Genome size (Mb)	G + C (%)	Genes	Proteins	rRNA	tRNA	Pseudogenes	Plasmids	Transposases	Referencess
Komagataeibacter europaeus 5P3	3.99	61.5	3712	3586	20	58	47	ND	94	Andrés-Barrao et al. (2011a)
Komagataeibacter europaeus LMG 18494	3.99	61.2	3654	3494	24	62	73	3	103	Andrés-Barrao et al. (2011a)
Komagataeibacter europaeus LMG 18890^T	4.23	61.2	3957	3792	16	54	94	ND	107	Andrés-Barrao et al. (2011a)
Komagataeibacter xylinus E25	3.45	62.5	3674	3157	15	56	ND	5	133	Kubiak et al. (2014)
Komagataeibacter rhaeticus DST GL02^T	3.94	62.5	3460	3358	19	58	33	ND	120	Dos Santos et al. (2014)
Komagataeibacter hansenii ATCC 23769^T	3.64	59.2	3351	3308	ND	43	ND	ND	23	Iyer et al. (2010)
Komagataeibacter oboediens 174Bp2	4.18	61.3	3755	3601	30	68	55	ND	47	Andrés-Barrao et al. (2011a)
Komagataeibacter kakiaceti JCM 25156^T	3.13	62.1	3170	2314	3	42	810	ND	ND	Iino et al. (2012)
Komagataeibacter medellinensis NBRC 3288^T	3.14	60.9	2886	2811	15	57	ND	7	157	Castro et al. (2013)

Source: Data obtained from NCBI (www.ncbi.nlm.nih.gov) and UniProt (www.uniprot.org) databases

9.2 Population Dynamics of AAB During Acetic Acid Fermentation

Industrial vinegar factories use submerged fermentation for the production of vinegar. This method leads to a rapid acetification rate and high concentration of acetic acid. In modern vinegar plants, the physiochemical parameters such as raw material, temperature, aeration, biomass, and acetic acid as well as ethanol content are controlled during the acetification process (Ebner et al. 1996a, b; Arnold et al. 2002). The bacterial composition in the starter broth is one of the main factors influencing the performance of the fermentation; however, standardized controlled starter cultures for industrial plants have not been yet developed.

Several microbial diversity studies on AAB have been performed for industrial vinegars, on running acetic acid fermentations from different raw materials. Although *Acetobacter* strains are predominant in most SC processes, some species of the genus *Komagataeibacter* have also been observed in this type of process, mainly when the acetic acid concentration is higher than 8 % (Gullo et al. 2009; Hidalgo et al. 2010; Vegas et al. 2010). Because of their specialized metabolism adapted to the high acidities and aeration rate, AAB from the genus *Komagataeibacter* are predominant in SF processes, and strains of *K. europaeus* species are among the most abundant cultivable species identified in a variety of vinegars (cider, wine, and spirit vinegars) produced by this method. Others species found in SF, such as *K. xylinus*, *K. intermedius*, *K. oboediens*, and *Gluconacetobacter entanii*, have been isolated from processes with acidities ranging from 11 to 15 % (Schüller et al. 2000; Sokollek et al. 1998; Jin-Nam et al. 2005; Ilabaca et al. 2008; De Vero et al. 2006; Gullo et al. 2009; Trček et al. 2000; Fernández-Pérez et al. 2010a, b; Haruta et al. 2006; Nanda et al. 2001; Andrés-Barrao et al. 2011b). It is notable that most of the ecological studies on AAB bacterial population performed on surface or submerged industrial processes are based on a culture-dependent approach. Culture of high-acid vinegar bacteria on agar media is difficult mainly because of the drastic decrease in oxygen availability, especially when the bacteria come from submerged cultures and the acidity is higher than 10 % (Entani et al. 1985; Sievers et al. 1992; Trček et al. 2000; Sokollek et al. 1998; Shüller et al. 2000; Fernández-Pérez et al. 2010a). The main implications of this phenomenon are that although species identified using plate culture methods might represent the main AAB involved in a specific process, there is a risk of disregarding other strains that are lost during plating (present in smaller number or having nutritional requirements unfit to the synthetic media used) but which might be important in the acetic acid fermentation process.

The rapid predominance of *Komagataeibacter* spp. in SF was demonstrated by Andrés-Barrao et al. (2011b). In this report, two submerged acetifications of wine were carried out in the laboratory using starters whose bacterial composition was unknown at the onset, but retrieved by applying a culture-dependent approach, and composed of a heterogeneous population of *Acetobacter pasteurianus* and *Komagataeibacter europaeus* in different proportions. The first process was started

with an inoculum containing about 50 % of each species, whereas the inoculum used to start the second process was composed almost completely of *A. pasteurianus* (99 %). The study of the dynamic populations during the processes showed that *A. pasteurianus* was rapidly replaced by *K. europaeus* from the start of both fermentations. The authors concluded that the shorter lag phase (quicker start of the acetification) showed by the second process was favored by the higher proportion of *A. pasteurianus* in the starting mix (when acidity was low, ~4–6 %), but that this species played a secondary role when acidity increased, leaving the main role to *K. europaeus*. The *A. pasteurianus* would be inhibited by the increasing acidity while *K. europaeus* would proliferate under the more favorable conditions. The latter species would be responsible for the high acetic acid concentration of the final product, which ultimately reached 10–11 %. The performance of the first acetification process is shown in Fig. 9.1a. This diagram shows the typical behavior of submerged acetic acid fermentations. The alcoholic raw material, wine in this case, is mixed with active vinegar ("*vinegar inoculum*") and water to obtain an initial mixture containing 4 % ethanol and 6 % acetic acid. After an initial phase of adaptation that may last several days, the acetification process begins when bacteria start producing acetic acid concurrently with ethanol consumption. During the preparation phase, whenever the ethanol content in the fermentation broth reaches 0.5–1.0 %, fresh alcohol mash is added to recover the initial ethanol/acetic acid concentrations. This step is repeated until the desired working volume has reached 8–9 l (in the 10-l acetator used by Andrés-Barrao et al. (2011b); (Fig. 9.1b, c). From that point, an indefinite number of production cycles begins. When the maximum acetic acid content has been reached at the end of a cycle and ethanol content is between the threshold values (0.5–1.0 %), one third of the volume is discharged and replaced by fresh alcoholic mash to begin a new cycle (Fig. 9.1c). The semicontinuous submerged process of the first experiment described by Andrés-Barrao et al. (2011b) and a laboratory-scale acetator are shown in Fig. 9.1a–c. Results similar to those obtained by the authors, showing the favored development of *Komagataeibacter* over *Acetobacter* strains, have been also reported in studies conducted under static conditions on wine vinegar (Vegas et al. 2010; Callejón et al. 2008).

In subsequent experiments, Andrés-Barrao (2012) characterized the microbial population of submerged fermentations using distilled alcohol as a raw material, instead of wine, to reach a maximum acetic acid concentration of up to 14 %. The spirit vinegars used as inoculum in these experiments were withdrawn from the middle stage of a production cycle during industrial submerged processes (in a vinegar factory). The growing conditions provided by the raw material (diluted solution of distilled ethanol) used in these cases are more extreme than those provided by wine. Distilled ethanol is devoid of the diverse compounds and nutrients for bacterial metabolism that give complexity to the wine matrices, such as sugars (glucose, fructose), pigments, and phenolics. Therefore, a small amount of commercial "*nutrients*" (0.5 g/l) was added at the start of experiments. These conditions of spirit vinegar fermentations usually show a very low level of species diversity (Fernández-Pérez et al. 2010a; Trček et al. 2000). Andrés-Barrao (2012)

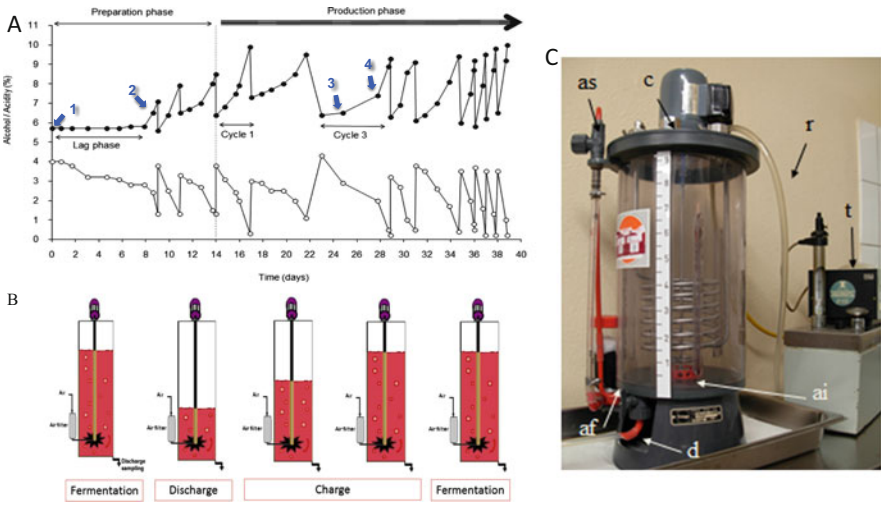

Fig. 9.1 Schematic representation of a submerged fermentation. (**a**) General performance of a red wine acetification process. The *continuous line* corresponds to the acetic acid concentration (% AcH) and the *dotted line* to the residual alcohol (%EtOH). *Circles* represent the time points when vinegar samples where harvested for bacterial identification. Representative time points are indicated by an *arrow*: *1)* 60 % Ap, 40 % Ke; *2)* 4 % Ap, 96 % Ke; *3)* 1 % Ap, 99 % Ke; *4)* 1 % AP, 99 % Ke. *Ap* = *A. pasteurianus*, *Ke* = *K. europaeus* (Andrés-Barrao et al. 2011b). (**b**) Schematic representation of cycles (charge/discharge) of submerged fermentation. (**c**) Pilot acetator used in the laboratory. Charge (*c*), discharge (*d*), regulation valve for air supply (*as*), refrigeration (*r*), thermostat fixed at 30 °C (*t*), air filter using active charcoal (*af*), air injection (*ai*) (Andrés-Barrao 2012)

followed the same culture-dependent approach that the present author and other collaborators used in identifying *K. oboediens* as the predominant AAB species: six different strains of *K. oboediens* and one strain of *K. nataicola* were also isolated from agar plates. Unfortunately, bacterial growth was only observed during the preparation phase and the three first vinegar production cycles, whereas the acidity of the fermentation broth was lower than 10 % (normal threshold allowing the growth of AAB in synthetic media, as pointed out previously). A further spirit vinegar fermentation experiment showing high acidity (8–14 %) during the preparation phase did not show any growth on agar plates, but the analysis of direct vinegar samples, by using a culture-independent approach, succeeded in the identification of a limited and constant AAB population during the entire process. Although it was identified as *Komagataeibacter* spp., the species involved in this case could not be classified into any of the previously described taxonomic groups; being closely related to the group formed by *K. hansenii* and *Ga. entanii*, and it was suggested to be a potential new species.

The use of various molecular tools to identify AAB has led to the elucidation of a direct relationship between specific AAB populations and the concentration of acetic acid.

For the application of both culture-dependent and culture-independent approaches, molecular, phenotypic, and chemotaxonomic data have allowed researchers the unequivocal identification of AAB from many varieties of traditional and industrial vinegar niches (SF and SC acetifications) as well as the classification of new isolates (Cleenwerck and de Vos 2008; Yamada and Yukphan 2008; De Vero et al. 2006; Gullo et al. 2009).

9.3 Cell Morphology and Membrane Modification During Fermentation

The increase in acetic acid concentration during the fermentation process has been shown to cause a change in bacterial morphology. These changes are probably caused by a modification of the protein and lipid composition of the cell membrane, leading to the augmentation of the membrane surface, making the area available for oxygen (O_2) exchange larger; and the increase in the efflux-pump systems, which will favor the elimination of the high excess of dissociated acetate (Ac^-) and protons (H^+) (dissociated form of acetic acid) diffused into the cytoplasm. Electron microscopy has shown that the bacterial cells were reduced in size at the beginning of the acetic acid fermentation, but they started to elongate slightly when bacteria reached the early stationary growth phase and at the end of the process (Andrés-Barrao et al. 2012). Growth on agitated medium during extended time periods might also contribute to the elongation of bacterial cells. The comparison between extended agitated cultures under increasing or controlled acetic acid concentrations will allow researchers to monitor the effect of acetic acid on the cell morphology of AAB.

9.3.1 Membrane Polysaccharides

Komagataeibacter strains synthesize extracellular polysaccharides (EPS) as cellulose or acetan (Ishida et al. 2002; Iyer et al. 2010; Andrés-Barrao et al. 2011a); however, the production of capsular polysaccharides (CPS) has not been described. The capability of producing cellulose has been verified by comparative genome analysis, which has led to the finding that all genes coding for the cellulose synthase operon were only present in the genus *Komagataeibacter* although absent in *Acetobacter* (Andrés-Barrao 2012). For more information about bacterial cellulose production by AAB, refer to Chap. 14 in this book.

Results obtained at our laboratory from scanning electron microscopy (SEM) of bacteria harvested during running wine vinegar fermentations showed the production of cellulose fibers, which is consistent with the capacity of the genus *Komagataeibacter* to produce cellulose or cellulose-like polysaccharides

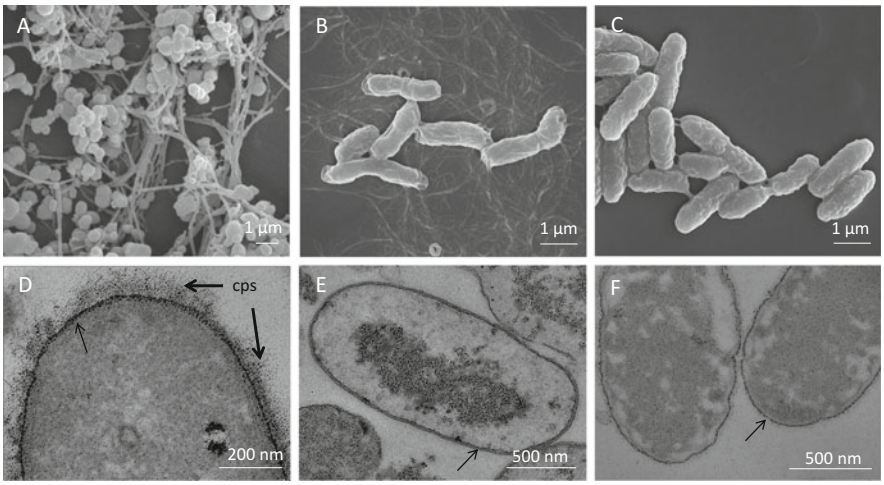

Fig. 9.2 Scanning (**a**–**c**) and transmission (**d**–**f**) electron micrographs. (**a**) Bacteria harvested during submerged wine vinegar fermentation at 8.2 % acetic acid and 1.1 % ethanol (end of a production cycle) showing the production of cellulose-like fibers. (**b**) Bacteria harvested during submerged spirit vinegar fermentation at 14 % acidity, showing the production of a different type of cellulose-like fibers. (**c**) *Komagataeibacter oboediens* 174Bp2 isolated from spirit vinegar sample in **b**, grown on RAE agar plate (3 % glucose, 1 % yeast extract, 1 % peptone, 0.15 % citric acid, 0.34 % $Na_2HPO_4 \bullet 2H_2O$, 1 % acetic acid, 2 % ethanol). The two-layer system was used to pour the agar plate: 2 % agar at the *bottom* and 1 % agar on the *top*. (**d**) *Acetobacter pasteurianus* LMG 1262^T grown on RAE agar plate, showing capsular polysaccharides (CPS) surrounding the cell (*thick arrows*). (**e**–**f**) Same samples as in **b** and **c**, respectively. TEM sections were stained with periodic acid–thiocarbohydrazide-Ag proteinate (PATAg) to highlight polysaccharides (*arrows*)

(Fig. 9.2a). The production of fibers by *Komagataeibacter* was also observed during the submerged production of spirit vinegars, but in this case the cellulose-like fibers are different from those produced by bacteria in wine vinegars (Fig. 9.2b). In contrast, if the same *Komagataeibacter* vinegar samples were cultured on synthetic media containing glucose, the production of the fibers was no longer observed (Fig. 9.2c). Further studies in the nature of these cellulose-like fibers to elucidate the molecular structure and monosaccharide composition are still needed.

Further characterization of the membrane polysaccharides (MP) produced by *Acetobacter* and *Komagataeibacter* strains by specific staining of polysaccharide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels has shown an interesting result. Crude extracts of polysaccharides from reference strains and strains isolated from submerged vinegar that were separated by SDS-PAGE and specifically stained to highlight lipopolysaccharides (LPS) showed that the *O*-antigen is present in *Acetobacter* strains (Fig. 9.3, lines 1–4) but is absent in all *Komagataeibacter* strains, independently of its origin and growing condition (Fig. 9.3, lines 5–9). We also observed that even if this technique is not able to determine the nature of the individual LPS molecules, the LPS synthesized by spirit vinegar bacteria (Fig. 9.3, line 9) were clearly different from those synthesized by

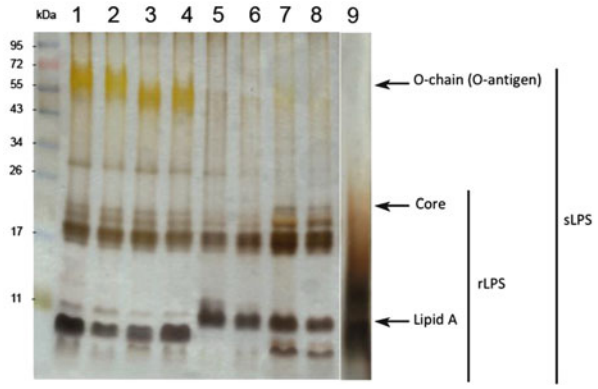

Fig. 9.3 Lipopolysaccharide-stained SDS-PAGE gels. *Acetobacter pasteurianus* LMG 1262^T grown in liquid RAE 0a/1e (basal RAE medium with 1 % ethanol added and no acetic acid) (*line 1*) and liquid RAE 1a/0e (basal RAE medium with 1 % acetic acid added and no ethanol) (*line 2*), *Acetobacter pasteurianus* 3P3 grown in liquid RAE 0a/1e (*line 3*) and liquid RAE 1a/0e (*line 4*), *Komagataeibacter europaeus* 5P3 adapted to liquid RAE 0a/1e (*line 5*) and liquid RAE 1a/0e (*line 6*), *Komagataeibacter hansenii* LMG 1527^T grown in liquid RAE 0a/1e (*line 7*) and liquid RAE 1a/0e (*line 8*), and spirit vinegar bacteria harvested during acetic fermentation at 14 % acetic acid (*line 9*). *sLPS* and *rLPS* are the smooth and rough forms of the lipopolysaccharide molecule, respectively

Komagataeibacter samples grown on synthetic media. The specific staining to highlight capsular polysaccharides (CPS) led to the observation of a specific band in *Acetobacter* strains that was absent in *Komagataeibacter* spp. (data not shown).

These results show that the high-acid *Komagataeibacter* strains synthesized rough LPS (rLPS) with the lack of the *O*-oligosaccharide (*O*-antigen) but failed to synthesize CPS. Although the synthesis of CPS is one of the reported strategies that *Acetobacter* strains use as a mechanical barrier to protect against acetic acid (Moonmangmee et al. 2002; Kanchanarach et al. 2010), *Komagataeibacter* spp. seem not to produce this kind of membrane polysaccharide. Transmission electron micrographs confirmed the previous observation (Fig. 9.2e, f), showing that this species lacked the characteristic polysaccharide layer surrounding the outer membrane that was observed in low-acid *Acetobacter* strains (Fig. 9.2d). Hence, our results suggest that capsular polysaccharides are not involved in acetic acid resistance in *Komagataeibacter* (Andrés-Barrao et al. 2011b; Andrés-Barrao 2012).

CPS has been shown to be essential for pellicle formation and growth of AAB in static cultures, and to confer resistance against exogenous acetic acid stress in *Acetobacter* strains (Deeraksa et al. 2005, 2006; Kanchanarach et al. 2010). When bacteria grow in ethanol-containing medium (acetic acid fermentation conditions), CPS probably serves as a barrier to prevent the diffusion of the produced acetic acid through the cell membrane. This resistance strategy is convenient during the stationary growth phase, when CPS has been shown to increase, coinciding with the acetate resistance phase and a decrease in viability/cultivability of the bacterial cells (Matsushita et al. 2005). However, it is unnecessary during the active

fermentation process, especially under shaking conditions in industrial fermentors. During the production of acetic acid, ethanol and acetic acid must diffuse freely through the outer membrane, and the decrease in the CPS layer surrounding the bacteria can favor the exchange of metabolites between the culture medium and the inner cell.

9.3.2 Lipid Membrane Composition

Changes in the membrane composition are generally recognized as an adaptive response of microbial cells to toxic compounds in the environment (Denich et al. 2003; Andrés-Barrao et al. 2011b), and, in accordance with this response, the change in the membrane lipid composition in AAB when the environmental pH becomes acidic, and the involvement of lipids in tolerance to acidic pH, have been reported (Ogawa et al. 2010).

Trček et al. (2007) studied the influence of acetic acid on the phospholipids and fatty acid composition of the cytoplasmic membranes in *K. europaeus* V3. One of the main phospholipids present in this strain was phosphatidylcholine (PC), which is usually produced by methylation of phosphatidylethanolamine (PE) (Kaneshiro and Law 1964), and phosphatidylglycerol (PG). Although the total phospholipid content decreased when *K. europaeus* V3 was grown in the presence of 3 % acetic acid, the relative content of PC and PG substantially increased, producing a change in the permeability properties of the outer membrane and decreasing the area available for passive transport of lipophilic molecules such as acetic acid. On the other hand, the content of glycolipids increased greatly, which might result in strengthening of the hydrophobic barrier (Trček et al. 2007). *cis*-Vaccenic acid (an unsaturated fatty acid) represents the major fatty acid in most AAB (31–80 % of all fatty acids in PG) (Tahara et al. 1976; Franke et al. 1999; Greenberg et al. 2006; Jojima et al. 2004; Loganathan and Nair 2004; Urakami and Komagata 1987; Yamada et al. 1981). The increase of this fatty acid in the cytoplasmic membrane of *K. europaeus* V3 grown in the presence of acetic acid might result in an increased fluidity of the membrane, being very likely produced by the substantial increase in the relative PG content (Trček et al. 2007). And these results suggest that high-acid vinegar bacteria have a cell membrane that is extremely rich in specific lipid components, which is lost when bacteria are grown in the mild conditions of a synthetic medium.

Sphingolipids such as dihydroceramide are some of the most ubiquitous components in the cell membranes of eukaryotes but are rare in bacterial cells. In contrast, these lipid components are found in the membrane of *Acetobacter* and *Komagataeibacter* strains, suggesting that it may be involved in the tolerance to acidic pH and also to acetic acid. In *Acetobacter malorum* S24, synthesis of the sphingolipid ceramide has been related to the regulation of stress response elements under high temperature and low pH (Ogawa et al. 2010). The idea of this sphingolipid being involved in resistance to acetic acid in AAB was reinforced by

the finding that the acetic acid tolerance in *Gluconacetobacter entanii* (species phylogenetically included in the genus *Komagataeibacter*; see Chap. 1) was enhanced by the overexpression of serine palmitoyltransferase (SPT), an essential enzyme that catalyzes the synthesis of sphinganine (backbone component of ceramide). It was further reported that the synthesis of ceramide is a response connected with the tolerance to acetic acid (Goto and Nakano 2008). Ceramide has also been proposed to stabilize PQQ-alcohol dehydrogenase during acetic acid production (Ogawa et al. 2010). As sphinganine is a toxic compound for yeasts, it is also supposed to be toxic to AAB. It has been suggested that a mechanism to regulate the amounts of sphinganine is necessary in yeast as well as in AAB to overcome this toxicity. In AAB, several enzymes involved in ceramide synthesis have been reported to be induced by heat stress and low pH (Mao et al. 1999), indicating that sphingolipids may serve as a sink to remove sphinganine, while the molecule itself may regulate the expression and translation of genes involved in both heat and low pH stress (Mao et al. 1999).

Recent investigations suggest sphingolipids have an important role as signal mediators to enable enteric bacteria to resist the stressful environment present in the intestine (An et al. 2011; Gerdes 2000). Similarly, these cellular components in AAB, and *Komagataeibacter* spp. more particularly, might be important for its survival in its acidic and toxic natural environment (high-acid vinegar). Further investigation is needed to reveal the exact modification of the lipid components of the highly specialized outer membrane of these bacteria during acetic acid fermentation and its function in acetic acid resistance.

9.4 Amino Acids Involved in the Submerged Fermentation Process

The composition of amino acids of the medium is crucial for growth and active metabolism of AAB. As the initial substrate for vinegar production comes from previous alcoholic fermentations conducted by yeasts, the main sources of nitrogen are the free amino acids in the fermentation broth (Valero et al. 2005). Recently, the presence and quantities of the 22 amino acids contained in the culture broth of submerged and static acetifications from different raw materials (cider, white and red wine) have been measured and analyzed (Valero et al. 2005; Callejón et al. 2008). These reports identified several strains belonging to *Komagataeibacter europaeus* involved in the fermentation processes, that proline was the most concentrated amino acid in vinegars produced by submerged fermentation (between 35 and 69 %), and that its concentration remained constant during the process. This result is in contradiction with the findings of Morales et al. (2001), who described an uptake of proline by AAB during acdetic acid fermentation. This discrepancy could be caused by the diversity of nitrogen needs of various AAB and the substrate used. Additionally, it is important to point out that in spirit submerged

fermentations, amino acids or nitrogen utilization is rather limited, consistent with the limited growth of AAB in this process.

During the acetic acid fermentation process, strains of the genus *Komagataeibacter* might preferentially consume arginine, glutamic acid, and alanine, the most abundant amino acids identified (Valero et al. 2005; Callejón et al. 2008). Their concentration decreased significantly throughout the acetification process. Asparagine, glutamine, cysteine, serine, ornithine, and tryptophan have not been detected during acetic acid fermentation, as they either undergo an important decrease or are totally consumed during the first alcoholic fermentation (Callejón et al. 2008). Although ammonium was not possible to quantify clearly, its relative concentration seemed to decrease during the fermentation process. In contrast, Valero et al. (2005) reported that leucine was the most important amino acid in terms of nitrogen supply and uptake for AAB during submerged fermentation.

9.5 Mechanisms of Acetic Acid Resistance

We have already introduced some of the physiological mechanisms that *Komagataeibacter* spp. may use as strategies to resist the low pH and the high concentration of acetic acid produced by acetic acid fermentation, such as modification of the membrane lipids. We describe next other specific metabolic features of AAB that could be involved in this resistance.

9.5.1 PQQ-Dependent Membrane-Bound Dehydrogenase

The membrane-bound PQQ-alcohol dehydrogenase (ADH) is to be considered the key enzyme in the production of vinegar because of its essential place in the oxidation of ethanol to acetaldehyde, the intermediate that will be later oxidized by the MCD (molybdopterin cytosine dinucleotide)-aldehyde dehydrogenase (ALDH) to acetic acid. This idea is based on the finding that a defect in membrane-bound ADH has been associated with a reduction in acetic acid resistance (Chinnawirotpisan et al. 2003; Okumura et al. 1985; Takemura et al. 1991). Additionally, the elevation in membrane-bound ALDH activity by gene amplification has been reported to enhance the acetic acid concentration finally attained in *Acetobacter* (Fukaya et al. 1989).

A direct link has been established between acetic acid resistance and the capability to oxidize ethanol during the diauxic growth curve in AAB (Ohmori et al. 1982). The PQQ-ADH activity in *Komagataeibacter* strains reached higher values (at least twice as high) than those in *Acetobacter*, under the same growth conditions (Trček et al. 2006, 2007).

9.5.2 Cytosolic Proteins Involved in Resistance

The sophisticated mechanisms developed by AAB to adapt to and resist high concentrations of ethanol and acetic acid, low pH, and, in particular cases, a high oxygenation rate, have been partially elucidated.

Studies on *Acetobacter* strains have reported several mechanisms conferring acetic acid resistance, among them a specialized citric acid cycle in which succinyl-coenzyme A (CoA):acetate CoA-transferase (AarC) replaces succinyl-CoA synthetase (Mullins et al. 2008). Other proteins responsible include aconitase (AcnA), as well as proteins related to the tricarboxylic acid (TCA) cycle such as citrate synthase (CS, AarA), α-ketoglutarate-DH, or isocitrate-DH. Stress-related damage would also be prevented by heat-shock proteins (GroELS, DnaJK, GrpE), as well as by exportation through specialized ABC transporters and a proton motive force-dependent efflux pump (Fukaya et al. 1993; Okamoto-Kainuma et al. 2002, 2004; Matsushita et al. 2005; Ishikawa et al. 2010a; Nakano and Fukaya 2008; Nakano et al. 2004, 2006; Andrés-Barrao et al. 2012; Sakurai et al. 2012). Additionally, cytosolic enzymes (specifically of acid-tolerant bacteria) have been demonstrated to be intrinsically stable to acid (Mullins et al. 2012).

Experimental evidence caused us to suspect that *Komagataeibacter* strains share most of the resistance mechanisms shown by *Acetobacter* sp., although they also must develop specific strategies that enable tolerance to the drastic conditions of submerged vinegar production. We have already addressed the higher activity and stability of PQQ-ADH enzyme compared with that in *Acetobacter* sp., as well as their specialized lipid membrane composition (Trček et al. 2006, 2007).

9.5.3 Genomic Features Involved in Resistance

The main characteristics of the genomes of public access for *Komagataeibacter* strains are shown in the updated Table 9.1. The comparative study of the genome sequences of *Acetobacter* and *Komagataeibacter* strains will provide more information on their inherent differences concerning acetic acid resistance (*Komagataeibacter* strains are more resistant than *Acetobacter* strains). Comparative analysis of the genome sequences of several vinegar-related AAB has shown that the genome size of *Komagataeibacter* strains is greater than that of *Acetobacter* strains, 4.2 Mb and 2.9 Mb, respectively (Azuma et al. 2009; Ogino et al. 2011; Andrés-Barrao et al. 2011b; Kubiak et al. 2014). *Komagataeibacter* spp. also show a higher %G+C (61 %) than *Acetobacter* spp. (51–53 % G+C), suggesting a higher chemical stability of the DNA molecule. Both species have high genetic instability because of the presence of an important number of transposases and insertion sequence elements (IS), which might be important in genome evolution as well as in regulation of the gene expression. The additional genes found in

Komagataeibacter spp. might contribute to increased stability by acquired adaptation to the extreme conditions found in the submerged acetification process.

9.5.4 Role of Plasmids Involved in Acetic Acid Resistance

Several researchers have demonstrated the presence of many plasmids in almost all vinegar-producing AAB. Their presence suggests that some of these plasmids are probably involved in resistance to the extreme conditions of acetic acid fermentation (Krahulec et al. 2003; Mariette et al. 1991; Trček et al. 2000; Trček 2015). Using two-dimensional agarose gel electrophoresis, Trček (2015) found that the plasmid profiles of *K. europaeus* indicate the presence of different topological forms of the same size plasmid. She also identified the plasmid pJK2-1 being involved in the acetic acid-resistant phenotype in AAB. The introduction of this plasmid into *Komagataeibacter oboediens* JK3 as a chimeric plasmid (pJT2) showed a shorter lag phase when bacteria were grown in a medium containing 3 % or 5 % (v/v) acetic acid. Therefore, it is necessary to isolate and characterize the different plasmids present in each phenotype to elucidate the specific genes and molecular component of these plasmids implicated in the resistance of AAB against acetic acid.

9.6 Conclusions and Perspectives

Since molecular methods have been applied to identification and classification of AAB, the number of new genera and species included in this group of microorganisms has increased exponentially. Nevertheless, if we seek thorough ecological study when facing the analysis of the bacterial population during industrial acetic acid fermentations, most particularly when using the submerged methodology, utilization of a metagenomic approach (culture independent) is needed. Additionally, new identification methods able to discriminate between very closely related species, and suitable to be adapted to the high-throughput analysis of a high number of samples, must be applied. In this regard, whole-cell matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has recently been evaluated and seems to be a very promising technique (Andrés-Barrao et al. 2013; Wieme et al. 2014; Li et al. 2014; Spitaels et al. 2014; Trček and Barja 2015).

After about 50 years of investigation, the results of collaborative research on the physiology of these microorganisms have shown the general picture of the metabolic dynamics of AAB during acetic acid fermentation, but the relatively small number of proteins or genes that have been successfully identified indicates that more progress is needed. It appears that different mechanisms are activated for tolerance to ethanol and acetic acid during the exponential growth phase of the

bacteria in submerged processes. The identification of the whole set of modified proteins during the process is necessary as this knowledge will permit understanding the metabolic pathways that confer tolerance to the acetic acid which is produced as a consequence of ethanol oxidation. Because both genomics and proteomic analyses reveal changes in gene expression and protein profiles in specific conditions, a study of metabolomics will allow the characterization and quantification of the primary and secondary metabolites produced by specific AAB strains under a given set of conditions, for example, in media with high acetic acid concentration. In this regard, the most promising tools to allow us to understand and explain the mechanisms that confer acetic acid resistance in acetic acid bacteria are a combination of "-omics" techniques (genomics, transcriptomics, proteomics, metabolomics, lipidomics, etc.). These methodologies could also help to select bacteria with innovative physiological and microbiological properties useful for the improvement of vinegar production as well as other natural fermentation processes, leading to economically important products for consumers.

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References

An D, Na C, Bielawski J, Hannun YA, Kasper DL (2011) Membrane sphingolipids as essential molecular signals for Bacteroides survival in the intestine. Proc Natl Acad Sci USA 108(Suppl 1):4666–4671

Andrés-Barrao C (2012) Characterization of acetic acid bacteria and study of the molecular strategies involved in the resistance to acetic acid during oxidative fermentation. PhD thesis no. 4452. University of Geneva, Switzerland

Andrés-Barrao C, Falquet L, Calderon-Copete SP, Descombes P, Ortega Pérez R, Barja F (2011a) Genome sequences of the high-acetic acid-resistant bacteria *Gluconacetobacter europaeus* LMG 18890T and *G. europaeus* LMG 18494 (reference strains), *G. europaeus* 5P3, and *Gluconacetobacter oboediens* 174Bp2 (isolated from vinegar). J Bacteriol 193(10):2670–2671

Andrés-Barrao C, Weber A, Chappuis ML, Theiler G, Barja F (2011b) Acetic acid bacteria population dynamics and natural imposition of *Gluconacetobacter europaeus* during submerged vinegar production. Arch Sci 64:9–24

Andrés-Barrao C, Saad MM, Chappuis ML, Boffa M, Perret X, Ortega Pérez R, Barja F (2012) Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. J Proteomics 75(6):1701–1717

Andrés-Barrao C, Benagli C, Chappuis M, Ortega Pérez R, Tonolla M, Barja F (2013) Rapid identification of acetic acid bacteria using MALDI-TOF mass spectrometry fingerprinting. Syst Appl Microbiol 36(2):75–81

Arnold S, Becker T, Delgado A, Emde F, Enenkel A (2002) Optimizing high strength acetic acid bioprocess by cognitive methods in an unsteady state cultivation. J Biotechnol 97(2):133–145

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37(17):5768–5783

Callejón RM, Tesfaye W, Torrija MJ, Mas A, Troncoso AM, Morales ML (2008) HPLC determination of aminoacids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota. Eur Food Res Technol 227:93–102

Castro C, Cleenwerk I, Trcek J, Zuluaga R, De Vos P, Caro G, Aguirre R, Putaux JL, Ganan P (2013) *Gluconacetobacter medellinensis* sp. nov., cellulose and non-cellulose-producing acetic acid bacteria isolated from vinegar. Int J Syst Evol Microbiol 63:1119–1125

Chinnawirotpisan P, Theeragool G, Limtong S, Toyama H, Adachi OO, Matsushita K (2003) Quinoprotein alcohol dehydrogenase is involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in *Acetobacter pasteurianus* SKU1108. J Biosci Bioeng 96(6):564–571

Cleenwerck I, De Vos P (2008) Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int J Food Microbiol 125(1):2–14

Conner DE, Kotrola JS (1995) Growth and survival of *Escherichia coli* O157: H7 under acidic conditions. Appl Environ Microbiol 61:382–385

De Vero L, Gala E, Gullo M, Solieri L, Landi S, Giudicci P (2006) Application of denaturing gradient gel electrophoresis (DGGE) analyisis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiol 23:809–813

Deeraksa A, Moonmangmee S, Toyama H, Yamada M, Adachi O, Matsushita K (2005) Characterization and spontaneous mutation of a novel gene, *pol*E, involved in pellicle formation in *Acetobacter tropicalis* SKU1100. Microbiology 151:4111–4120

Deeraksa A, Moonmangmee S, Toyama H, Adachi O, Matsushita K (2006) Conversion of capsular polysaccharide, involved in pellicle formation, to extracellular polysaccharide by *gal*E deletion in *Acetobacter tropicalis*. Biosci Biotechnol Biochem 70:2536–2539

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Methods 52(2):149–182

Deppenmeier U, Ehrenreich A (2009) Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. J Mol Microbiol Biotechnol 16:69–80

Dos Santos RA, Berretta AA, Barud Hda S, Ribeiro SJ, González-García LN, Zucchi TD, Goldman GH, Riano-Pachon DM (2014) Draft genome sequence of *Komagataeibacter rhaeticus* strain AF1, a high producer of cellulose, isolated from kombucha tea. Genome Announc 2(4):e00731–14. doi:10.1128/genomeA.00731-14

Ebner H, Follmann H, Sellmer S (1996a) Vinegar. Ullmann's encyclopaedia of industrial chemistry, vol A27. VCH, Weinheim, pp 403–418

Ebner H, Sellmer S, Follmann H (1996b) Acetic acid. In: Rehm HJ, Reed G (eds) Biotechnology, vol 6. VCH, Weinheim, pp 381–401

Entani E, Ohmori S, Masai H, Suzuki K (1985) *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J Gen Appl Microbiol 31:475–490

Fernández-Pérez R, Torres C, Sanz S, Ruiz-Larrea F (2010a) Strain typing of acetic acid bacteria responsible for vinegar production by the submerged elaboration method. Food Microbiol 27 (8):973–978

Fernández-Pérez R, Torres S, Sanz S, Ruiz-Larrea F (2010b) Rapid molecular methods for enumeration and taxonomical identification of acetic acid bacteria responsible for submerged vinegar production. Eur Food Res Technol 231(5):813–819

Franke IH, Fegan M, Hayward C, Leonard G, Stackebrandt E, Sly LI (1999) Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. Int J Syst Bacteriol 49 (pt 4):1681–1693

Fukaya M, Tayama K, Tamaki T, Tagami H, Okumura H, Kawamura Y, Beppu T (1989) Cloning of the membrane-bound aldehyde dehydrogenase gene of *Acetobacter polyoxogenes* and improvement of acetic acid production by use of the cloned gene. Appl Environ Microbiol 55(1):171–176

Fukaya M, Takemura H, Tayama K, Okumura H, Kawamura Y, Horinouchi S, Beppu T (1993) The aarC gene responsible for acetic acid assimilation confers acetic acid resistance on *Acetobacter aceti*. J Ferment Bioeng 76(4):270–275

Gerdes K (2000) Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. J Bacteriol 182:561–572

Goto H, Handa JP, Nakano S, Chita-gun JP (2008) Gene participating in acetic acid tolerance, acetic acid bacteria bred using the gene, and process for producing vinegar with the use of the acetic acid bacteria. United States Mitsukan Group Corporation (Handa-shi JP). Patent US7446192 (W/O03/078635) [Online]. Available at: http://www.freepatentsonline.com/7446192.html. Accessed on Feb 2014

Greenberg DE, Porcella SF, Stock F, Wong A, Conville PS, Murray PR, Holland SM, Zelazny AM (2006) *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. Int J Syst Evol Microbiol 56(pt 11):2609–2616

Gullo M, De Vero L, Guidicci P (2009) Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. Appl Environ Microbiol 75:2585–2589

Haruta S, Ueno S, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y (2006) Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int J Food Microbiol 109(1-2):79–87

Hidalgo C, Vegas C, Mateo E, Tesfaye W, Cerezo AB, Callejón RM, Poblet M, Guillamón JM, Mas A, Torija MJ (2010) Effect of barrel design and the inoculation of *Acetobacter pasteurianus* in wine vinegar production. Int J Food Microbiol 141(1-2):56–62

Iino T, Suzuki R, Tanaka N, Kosako Y, Ohkuma M, Komagata K, Uchimura T (2012) *Gluconacetobacter kakiaceti* sp. nov., an acetic acid bacterium isolated from a traditional Japanese fruit vinegar. Int J Syst Evol Microbiol 62:1465–1469

Ilabaca C, Navarrete P, Mardones P, Romero J, Mas A (2008) Application of culture culture-independent molecular biology based methods to evaluate acetic acid bacteria diversity during vinegar processing. Int J Food Microbiol 126(1-2):245–249

Ishida T, Sugano Y, Shoda M (2002) Novel glycosyltransferase genes involved in the acetan biosynthesis of *Acetobacter xylinum*. Biochem Biophys Res Commun 295(2):230–235

Ishikawa M, Okamoto-Kainuma A, Jochi T, Suzuki I, Matsui K, Kaga T, Koizumi Y (2010) Cloning and characterization of grpE in *Acetobacter pasteurianus* NBRC 3283. J Biosci Bioeng 109(1):25–31

Iyer PR, Geib SM, Catchmark J, Kao TH, Tien M (2010) Genome sequence of cellulose producing bacterium *Gluconacetobacter hansenii* ATCC 23769. J Bacteriol 192(16):4256–4257

Jin-Nam K, Jong-Sok CH, Young-Jung W, Jong-Sun Y, Hwa-Won R (2005) Culture medium optimization for acetic acid production by a persimmon vinegar-derived bacterium. Appl Biochem Biotechnol 123:1–3

Jojima Y, Mihara Y, Suzuki S, Yokozeki K, Yamanaka S, Fudou R (2004) *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. Int J Syst Evol Microbiol 54(pt 6):2263–2267

Kanchanarach W, Theeragool G, Inoue T, Yakushi T, Adachi O, Matsushita K (2010) Acetic acid fermentation of *Acetobacter pasteurianus*: relationship between acetic acid resistance and pellicle polysaccharide formation. Biosci Biotechnol Biochem 74(8):1591–1597

Kaneshiro T, Law JH (1964) Phosphatidylcholine synthesis in *Agrobacterium tumefaciens*. I. Purification and properties of a phosphatidylethanolamine *N*-methyltransferase. J Biol Chem 239:1705–1713

Krahulec J, Kretová M, Grones J (2003) Characterization of plasmids purified from *Acetobacter pasteurianus* 2374. Biochem Biophys Res Commun 310:94–97

Kubiak K, Kurzawa M, Jędrzejczak-Krzepkowska M, Ludwicka K, Krawczyk M, Migdalski A, Kacprzak MM, Loska D, Krystynowicz A, Bielecki S (2014) Complete genome sequence of

Gluconacetobacter xylinus E25 strain: valuable and effective producer of bacterial nanocellulose. J Biotechnol 176:18–19

Li L, Wieme A, Spitaels F, Balzarini T, Nunes OC, Manaia CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P (2014) *Acetobacter sicerae* sp. nov., isolated from cider and kefir, and identification of species of the genus *Acetobacter* by *dnaK*, *groEL* and *rpoB* sequence analysis. Int J Syst Evol Microbiol 64(Pt 7):2407–2415

Loganathan P, Nair S (2004) *Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka). Int J Syst Evol Microbiol 54(Pt 4):1185–1190

Mao C, Saba JD, Obeid LM (1999) The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. Biochem J 342(pt 3):667–675

Mariette I, Schwarz E, Vogel RF (1991) Characterization by plasmid profile analysis of acetic acid bacteria from wine, spirit and cider acetators for industrial vinegar production. J Appl Microbiol 71:134–138

Matsushita K, Inoue T, Adachi O, Toyama H (2005) *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. J Bacteriol 187(13):4346–4352

Moonmangmee S, Kawabata K, Tanaka S, Toyama H, Adachi O, Matsushita K (2002) A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*. J Biosci Bioeng 93 (2):192–200

Morales ML, Tesfaye W, García-Parrilla MC, Casas JA, Troncoso AM (2001) Sherry wine vinegar: physicochemical changes through the acetification process. J Sci Food Agric 81:611–619

Mullins EA, Francois JA, Kappock TJ (2008) A specialized citric acid cycle requiring succinyl-coenzyme A (CoA):acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. J Bacteriol 190(14):4933–4940

Mullins EA, Starks CM, Francois JA, Sael L, Kihara D, Kappock TJ (2012) Formyl-coenzyme A (CoA):oxalate CoA-transferase from the acidophile *Acetobacter aceti* has a distinctive electrostatic surface and inherent acid stability. Protein Sci 21(5):686–696

Nakano S, Fukaya M (2008) Analysis of proteins responsive to acetic acid in *Acetobacter*: molecular mechanisms conferring acetic acid resistance in acetic acid bacteria. Int J Food Microbiol 125(1):54–59

Nakano S, Fukaya M, Horinouchi S (2004) Enhanced expression of aconitase raises acetic acid resistance in *Acetobacter aceti*. FEMS Microbiol Lett 234:315–322

Nakano S, Fukaya M, Horinouchi S (2006) Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. Appl Environ Microbiol 72(1):497–505

Nanda K, Taniguchi M, Ujike S, Ishihara N, Mori H, Ono H, Murooka Y (2001) Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (komesu) and unpolished rice vinegar (kurosu) produced in Japan. Appl Environ Microbiol 67(2):986–990

Ogawa S, Tachimoto H, Kaga T (2010) Elevation of ceramide in *Acetobacter malorum* S24 by low pH stress and high temperature stress. J Biosci Bioeng 109(1):32–36

Ogino H, Azuma Y, Hosoyama A, Nakazawa H, Matsutani M, Hasegawa A, Otsuyama K, Matsushita K, Fujita N, Shirai M (2011) Complete genome sequence of NBRC 3288, a unique cellulose-nonproducing strain of *Gluconacetobacter xylinus* isolated from vinegar. J Bacteriol 193(24):6997–6998

Ohmori S, Uozumi T, Beppu T (1982) Loss of acetic acid resistance and ethanol oxidizing ability in an *Acetobacter* strain. Agric Biol Chem 46(2):381–389

Okamoto-Kainuma A, Yan W, Kadono S, Tayama K, Koizumi Y, Yanagida F (2002) Cloning and characterization of groESL operon in *Acetobacter aceti*. J Biosci Bioeng 94(2):140–147

Okamoto-Kainuma A, Yan W, Fukaya M, Tukamoto Y, Ishikawa M, Koizumi Y (2004) Cloning and characterization of the dnaKJ operon in *Acetobacter aceti*. J Biosci Bioeng 97(5):339–342

Okumura H, Uozumi T, Beppu T (1985) Construction of plasmid vector and genetic transformation system for *Acetobacter aceti*. Agric Biol Chem 49:1011–1017

Sakurai K, Arai H, Ishii M, Igarashi Y (2012) Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. J Biosci Bioeng 113(3):343–834

Schüller G, Hertel C, Hammes WP (2000) *Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations. Int J Syst Evol Microbiol 50:213–257

Sievers M, Sellmer S, Teuber M (1992) *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in Central Europe. Syst Appl Microbiol 15:386–392

Sokollek SJ, Hertel C, Hammes WP (1998) Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentation. Int J Syst Bacteriol 48:935–940

Spitaels F, Li L, Wieme A, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P (2014) *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. Int J Syst Evol Microbiol 64(Pt 4):1083–1089

Tahara Y, Yamada Y, Kondo K (1976) Phospholipid composition of *Gluconobacter cerinus*. Agric Biol Chem 40:2355–2360

Takemura H, Horinouchi S, Beppu T (1991) Novel insertion sequence IS1380 from *Acetobacter pasteurianus* is involved in loss of ethanol-oxidizing ability. J Bacteriol 173(22):70–76

Trček J (2015) Plasmid analysis of high acetic acid-resistant bacterial strains by two-dimensional agarose gel electrophoresis and insights into the phenotype of plasmid pJK2-1. Ann Microbiol 65(3):1287–1292

Trček J, Barja F (2015) Updates on quick identification of acetic acid bacteria with a focus on the 16S-23S rRNA gene internal transcribed spacer and the analysis of cell proteins by MALDI-TOF mass spectrometry. Int J Food Microbiol 196:137–144

Trček J, Raspor P, Teuber M (2000) Molecular identification of *Acetobacter* isolates from submerged vinegar production, sequence analysis of plasmid pJK2-1 and application in development of a cloning vector. Appl Microbiol Biotechnol 35:1899–1901

Trček J, Toyama H, Czuba J, Misiewicz A, Matsushita K (2006) Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. Appl Microbiol Biotechnol 70(3):366–373

Trček J, Jerneje K, Matsushita K (2007) The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. Extremophiles 11(4):627–635

Urakami T, Komagata K (1987) Cellular fatty acid composition with special references to the existence of hydroxy fatty acids in gram-negative methanol-, methane-, and methylamine-utilizing bacteria. J Gen Appl Microbiol 33:135–165

Valero E, Berlanga TM, Roldán P, Jiménez C, Garcia I, Mauricio J (2005) Free amino acids and volatile compounds in vinegars obtained from different types of substrate. J Sci Food Agric 85:603–608

Vegas C, Mateo E, González A, Jara C, Guillamón JM, Poblet M, Torija MJ, Mas A (2010) Population dynamics of acetic acid bacteria during traditional wine vinegar production. Int J Food Microbiol 138(1–2):130–136

Vegas C, Gonzalez A, Mateo E, Mas A, Poblet M, Torija MJ (2013) Evaluation of representativity of acetic acid bacteria species identified by culture-dependent method during a traditional wine vinegar production. Food Res Int 51(1):404–411

Wieme AD, Spitaels F, Aerts M, De Bruyne K, Van Landschoot A, Vandamme P (2014) Effects of growth medium on matrix-assisted laser desorption-ionization time of flight mass spectra: a case study of acetic acid bacteria. Appl Environ Microbiol 80(4):1528–1538

Yamada Y (2014) Transfer of *Gluconacetobacter kakiaceti*, *Gluconacetobacter medellinensis* and *Gluconacetobacter maltaceti* to the genus *Komagataeibacter* as *Komagataeibacter kakiaceti* comb. nov., *Komagataeibacter medellinensis* comb. nov. and *Komagataeibacter maltaceti* comb. nov. Int J Syst Evol Microbiol 64(Pt 5):1670–1672

Yamada Y, Yukphan P (2008) Genera and species in acetic acid bacteria. Int J Food Microbiol 125:15–24

Yamada Y, Nunoda M, Ishikawa T, Tahara Y (1981) The cellular fatty acid composition in acetic acid bacteria. J Gen Appl Microbiol 27:405–417

Yamada Y, Yukphan P, Lan Vu HT, Muramatsu Y, Ochaikul D, Tanasupawat S, Nakagawa Y (2012) Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). J Gen Appl Microbiol 58(5):397–404

Zhang DF, Li H, Lin XM, Wang SY, Peng XX (2011) Characterization of outer membrane proteins of *Escherichia coli* in response to phenol stress. Curr Microbiol 62:777–783

Chapter 10
Physiology of *Acetobacter* and *Komagataeibacter* spp.: Acetic Acid Resistance Mechanism in Acetic Acid Fermentation

Shigeru Nakano and Hiroaki Ebisuya

Abstract Acetic acid bacteria (AAB) are obligate aerobes that belong to the *α-Proteobacteria* and are used for industrial vinegar production because of their remarkable ability to oxidize ethanol by alcohol dehydrogenase, aldehyde dehydrogenase, and terminal oxidase of respiratory chain members on the cell membrane. Acetic acid tolerance is a crucial ability allowing AAB to stably produce large amounts of acetic acid.

Several molecular machineries responsible for acetic acid tolerance in AAB have been reported, including (1) prevention of acetic acid influx into the cell, (2) acetic acid assimilation, (3) acetic acid efflux by transporter or pump, and (4) protection of cytoplasmic proteins against denaturing by general stress proteins. (1) AAB optimize the lipid component proportion of the membrane and by further forming polysaccharide on the surface of the cells to prevent the influx of acetic acid. (2) AAB acquired the ability to convert the intracellular acetic acid in usable energy effectively via the alternative TCA cycle. (3) AAB possess two types of discharging intracellular acetic acid systems, one of which is a putative ABC transporter, and the other is an efflux pump driven by a proton motive force. (4) AAB adapt to the environmental changes in cells by inducing chaperones that stabilize the structure of proteins from acidification of the cell inside, and by synthesizing the enzymes which decompose reactive oxygen species (ROS) to maintain the intracellular environment in good condition.

Because acetic acid tolerance in AAB is conferred by several mechanisms, these mechanisms of acetic acid tolerance are reviewed here.

Keywords Phosphatidylcholine • Tetrahydoxy-bacteriohopane • Polysaccharide • AarA • AarC • Aconitase • ABC transporter • Efflux pump • Chaperone • Acetic acid tolerance

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10.1 Introduction

As we discuss the acetic acid tolerance of AAB, we first explain the antimicrobial activity mechanism of acetic acid. Vinegar is a fermented food that is well known for its high antimicrobial activity. The antimicrobial activity is the result of acetic acid, which is the main component of vinegar. The antimicrobial activity of acetic acid is partly caused by lowering the pH in the environment, and this is similar to the function of lactic acid, which is produced by lactic acid bacteria in the manufacturing process of fermented foods, such as alcohol and soy sauce. However, the most remarkable difference between acetic acid and lactic acid is that the acid dissociation constant, pK_a, of acetic acid and lactic acid is 4.8 and 3.8, respectively. That is, in a weakly acidic environment, most acetic acid is present as undissociated acid. Undissociated acetic acid is a hydrophobic small molecule easily passing through the phospholipid bilayer of the bacterial membrane; it releases a proton inside the cell, so that the cells are damaged by the decrease in intracellular pH (Russel 1992; Axe and Bailey 1995; Ishii et al. 2012). For this reason, acetic acid has higher antimicrobial activity. It has been confirmed that growth of food poisoning bacteria, such as enterohemorrhagic *Escherichia coli* O-157 and *Staphylococcus aureus*, is inhibited by only 0.1 % acetic acid (Entani et al. 1997).

Acetic acid, which is the main component of vinegar, is produced from ethanol by a respiratory chain consisting of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), and terminal oxidase localized in the cell membrane of AAB (Fig. 10.1) (Adachi et al. 1978; Ameyama et al. 1981). There are two major methods of vinegar production. In "surface fermentation," AAB form a film on the liquid surface of the tub or pot; in "aerobic submerged fermentation," the fermentation liquor is aerated and agitated with stirring blades in the fermentation tank (Fig. 10.2). Acetic acid concentration reached 4–6 % acidity at the surface fermentation and reached 5–20 % acidity in the aerobic submerged fermentation. AAB is capable of growth even in the presence of such a high acetic acid concentration. The acetic acid tolerance mechanism of AAB is very interesting.

Several acetic acid tolerance mechanisms are summarized here.

1. Characteristic membrane structure preventing the inflow of acetic acid into the cell
2. Efficient consumption of intracellular acetic acid
3. Discharge mechanism of intracellular acetic acid
4. Adaptive mechanism to environmental changes in the cell

These four mechanisms of acetic acid tolerance of AAB are reviewed next.

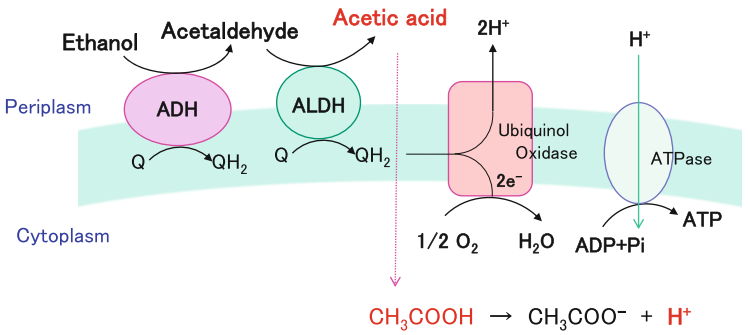

Fig. 10.1 Schematic representation of ethanol respiration of acetic acid bacteria (AAB). Ethanol is oxidized in the periplasm by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which also donate electrons to an ubiquinol oxidase that in turn generates a proton motive force. Acetic acid produced outside the cell is easily passed through the phospholipid bilayer of the bacteria and releases a proton in the cell cytoplasm

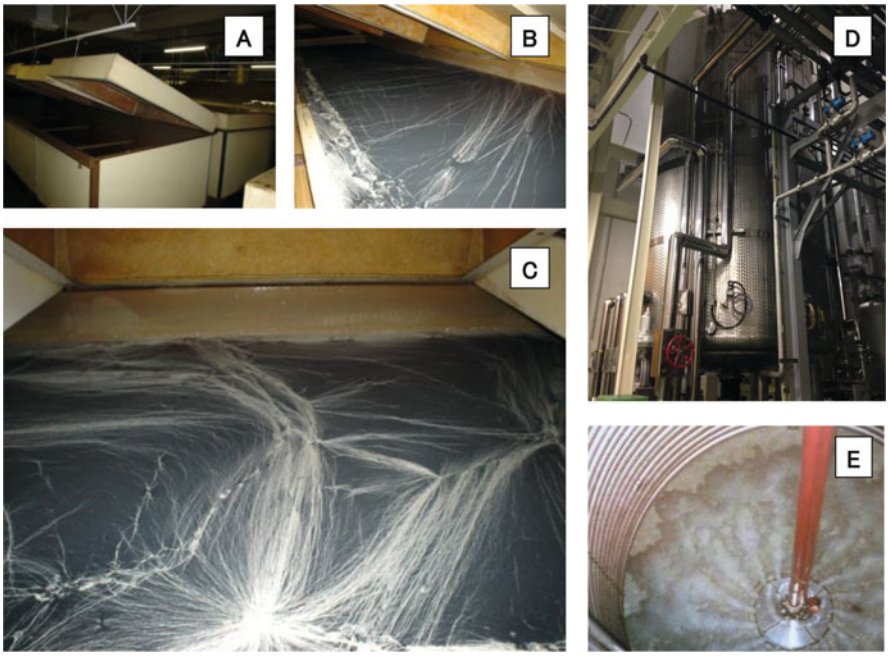

Fig. 10.2 Appearance of the two major methods of vinegar production. (**A**) Surface fermentation is generally carried out in a tub or pot. The volume of the tab is about 4 Kl. (**B**, **C**) Because the surface fermentation proceeds slowly, it takes 1 week or longer to produce vinegar with a film forming on the liquid surface. (**D**) Aerobic submerged fermentation is carried out in a stainless steel tank, which volume is usually from 20 to 100 Kl. (**E**) Sparger air is supplied from the bottom of the tank with stirring blades. One batch of the aerobic fermentation is completed in about 2 days

10.1.1 Characteristic Membrane Structure Preventing the Inflow of Acetic Acid into the Cell

Acetobacter and *Komagataeibacter* are used for industrial vinegar production because of their remarkable ability to oxidize ethanol to acetic acid. *Acetobacter* can produce acetic acid at 12 % concentration, and *Komagataeibacter* can produce up to 20 % acetic acid acidity. In comparison of their phospholipid chemical composition, *Acetobacter polyoxogenes* (presently assigned to genus *Komagataeibacter*) showed a significantly higher phosphatidylcholine (PC) content than *Acetobacter* sp. (Goto et al. 2000). Furthermore, in observation of the PC content of the membrane lipids during acetic acid fermentation of this *Komagataeibacter* strain (vinegar-producing strain), it was shown that the ratio of PC increased and that of phosphatidylglycerol (PG) decreased as the acetic acid concentration increased (Higashide et al. 1996). The phosphatidylethanolamine *N*-methytransferase gene (*pmt*) has been cloned from *Acetobacter aceti* (*Acetobacter pasteurianus*) and sequenced (Hanada et al. 2001). *pmt* catalyzes methylation of phosphatidylethanolamine (PE) to PC. Although the *pmt* disruption mutant could not produce PC and increased PE and PG in the membrane, it showed a reduced growth rate and lowered maximum cell density as compared to the wild-type strain in the medium with acetic acid. Therefore, it was suggested that PC is involved in acetic acid tolerance in *Acetobacter* and *Komagataeibacter*.

The cell membrane lipids of *Komagataeibacter* possess a high content of hopanoids, more than is found in other microorganisms, especially tetrahydoxybacteriohopane (THBH) (Fig. 10.3). THBH is a lipid known as a characteristic component in the cell membranes of *Zymomonas mobilism*, that performs alcohol fermentation of tequila production, and the content of THBH is increased during alcohol fermentation. It is presumed that THBH is involved in stabilization of the cell membrane in the presence of a high concentration of ethanol. Because THBH accounted for 25 % of the membrane lipids of *Komagataeibacter*, it was speculated that THBH is involved in the acetic acid tolerance of AAB. The squalene-hopen cyclase (HpnF) gene, which is involved in the synthesis of THBH precursor, of *A. aceti* (*A. pasteurianus*) was cloned. The *A. pasteurianus* transformant harboring the *hpnF* gene produced THBH of higher ratio than the transformant carrying the vector in the membranes (Fig. 10.4). Although the significant difference in each alcohol tolerance was not confirmed, the acetic acid tolerance of the transformant was higher than that of the vector control.

AAB have the ability to form a pellicle that floats on the liquid medium surface of the static culture. It is thought to synthesize some polysaccharides on the cell surface, as a colony has a rough surface on solid agar medium (Matsushita et al. 1992a). In contrast to the wild-type strain of *A. pasteurianus*, which makes extracellular polysaccharide, intracellular acetate/acetic acid content was confirmed as significantly higher in the strain unable to produce the polysaccharides. This result suggests that pellicle polysaccharide surrounding the bacteria suppresses the influx of acetic acid into the cell (Kanchanarach et al. 2010).

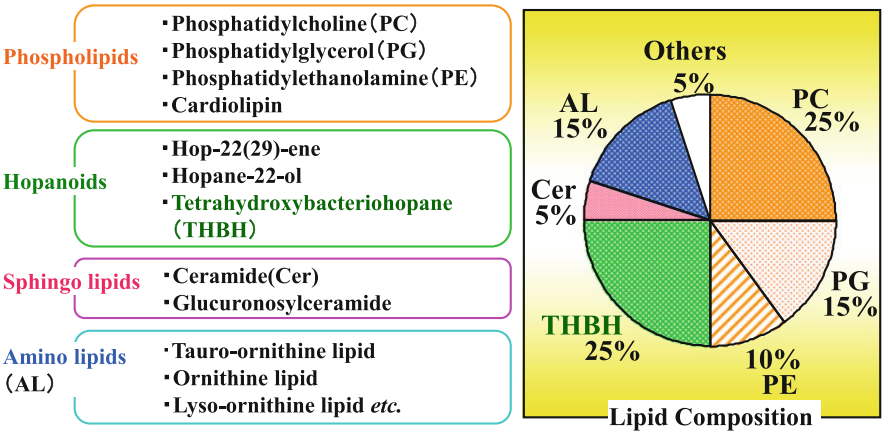

Fig. 10.3 Lipid composition of *Gluconacetobacter* (*Komagataeibacter*). *Komagataeibacter* sp. has a characteristic composition with a high content of tetrahydoxy-bacteriohopane (THBH) and phosphatidylcholine (PC). These lipids are not often found in other microorganisms

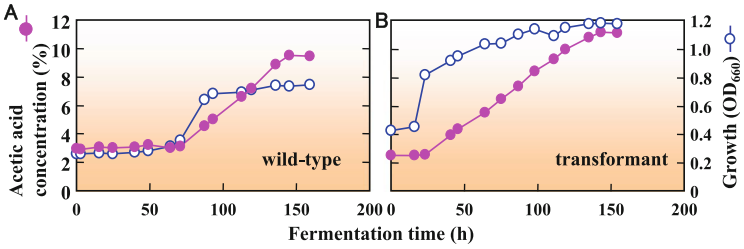

Fig. 10.4 Effect of overexpression of hpnF in *Acetobacter acti*. *A. aceti* strains were cultured at 400 rpm under 0.2 vvm aeration at 30 °C in YPG medium with constant feeding of ethanol at 1 % (v/v) in a jar-fermenter. The *A. aceti* transformant harboring pSHC, which was composed of the *hpnF* coding sequence and *Escherichia coli–Acetobacter* shuttle vector pMV24, produced 11.4 % of acetic acid (**B**), whereas the transformant carrying the vector accumulated 9.5 % of acetic acid (**A**). Specific growth rate (OD_{660}/h) of the transformant harboring pSHC was higher than that of the transformant carrying the vector. OD_{660} optical density at 660 nm

Therefore, it is thought that AAB has specific mechanisms that reduce the influx of acetic acid by optimizing the proportion of the membrane lipid component and further forming polysaccharide on the surface of the cells.

10.1.2 Efficient Consumption of Intracellular Acetic Acid

By a genetic approach using an acetic acid-sensitive mutant of *A. aceti* (*A. pasteurianus*), *aarA* and *aarC* were identified as an acetate-resistant gene cluster, and they were confirmed to be members of enzymes involved in the

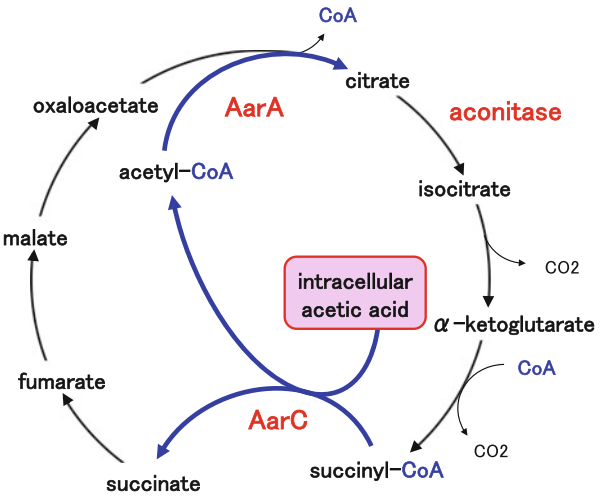

Fig. 10.5 Overoxidation pathway of intracellular acetic acid in *A. aceti*. It was suggested that intracellular acetic acid is consumed by the actions of the enzymes, AarC, AarA and aconitase, which are characteristic of the AAB described in this chapter

tricarboxylic acid (TCA) cycle. AarA acts as citrate synthase, and AarC acts as succinyl CoA:acetate CoA-transferase; thus, they were found to be responsible for acetate assimilation (Fukaya et al. 1990, 1993b; Mullins et al. 2008). AarB actions have not yet been defined.

AAB have the alternative TCA cycle that is modified as part of these enzymes. Although *A. pasteurianus* does not have any succinyl CoA synthase that involves interconversion between succinyl-CoA and succinic acid, AarC complements that function. AarC converts succinyl-CoA to succinate and transfers the CoA to acetic acid in the cell simultaneously; these are converted to acetyl-CoA and then converted to citrate by AarA. Thus, AAB consume acetic acid (Fig. 10.5).

In addition, the surface of AarA protein is more basic than that of *E. coli*, so it is thought that the enzyme functions stably even at lower pH (Francois et al. 2006). Furthermore, citrate synthase purified from *Gluconacetobacter europaeus* (*Komagataeibacter europaeus*) was also shown to be stable in the presence of acetic acid (Sievers et al. 1997). It appears reasonable that the enzymes of acetic acid bacteria are intrinsically resistant to inactivation by low pH and functionally adapt to low pH. It was shown by proteomic analysis that aconitase was induced with acetic acid in a medium (Nakano et al. 2004). The transformant that highly expressed aconitase showed higher productivity of acetic acid than the wild-type strain, which has lower acetic acid tolerance (Fig. 10.6).

In conclusion, AAB acquired the ability to convert the intracellular acetic acid in usable energy effectively by using the AarC. Also, AAB modified the AarA as being more robust. Furthermore, aconitase was induced in the presence of acetic acid. It is thought that AAB can consume intracellular acetic acid efficiently under the high concentrations of acetic acid.

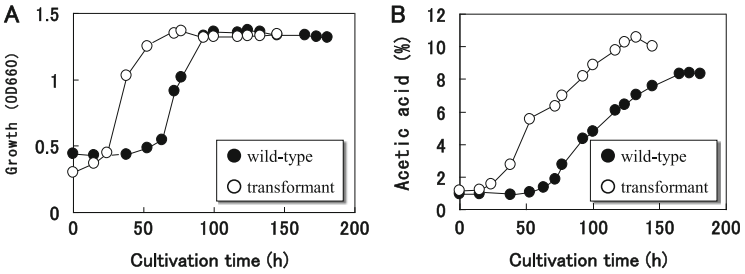

Fig. 10.6 Acetic acid fermentation by *A. aceti* transformants. (Figure from Nakano et al. 2004) Growth (**A**) and acetic acid concentration (**B**). The *A. aceti* transformant was cultured at 400 rpm under 0.2 vvm aeration at 30 °C in YPG medium with constant feeding of ethanol at 1 % (v/v) in a jar-fermenter. *Solid circles* wild-type strain, *open circles aatA*-disrupted strain, OD_{660} optical density at 660 nm

10.1.3 Discharge Mechanism of Intracellular Acetic Acid

It is confirmed that AAB has two types of systems discharging intracellular acetic acid. Proteomic analysis of the membrane fraction in *A. aceti* (*A. pasteurianus*) revealed that several proteins were induced by acetic acid (Nakano et al. 2006). One of these proteins has the structure belonging to the family of macrolide antibiotics transporters, which contain ATP-binding cassette (ABC) sequences and ABC signature sequences (Mendez and Salas 2001). This protein, named AatA, was a putative ABC transporter that possibly functioned as an exporter of intracellular acetic acid. The *aatA* disruption mutant showed reduced resistance to acetic acid as compared to the wild-type strain. Although there was no significant difference with respect to lactic acid and citric acid, formic acid and propionic acid tolerance were reduced as well as acetic acid tolerance in the *aatA* mutant (Fig. 10.7). This finding indicates that AatA is the transporter specific for the linear short-chain fatty acid that had a dissociation constant (pK_a) as high as that of acetic acid. For further investigation on the possibility that *aatA* is involved in acetic acid tolerance, an *E. coli* transformant harboring the *aatA* gene was prepared. The transformant harboring the *aatA* gene acquired higher levels of tolerance to acetic acid, formic acid, and propionic acid as compared to the transformant carrying the vector (Fig. 10.8). These results suggest that the *aatA* gene product is conferring acetic acid tolerance to AAB.

Another efflux system different from the ABC transporter was confirmed in *A. aceti* (*A. pasteurianus*). It was shown that acetate accumulation was low in intact cells in the presence of respiratory substrate and was increased by the addition of protonophores (Matsushita et al. 2005). Right-side-out (RSO) membrane vesicles accumulated a high concentration of intracellular acetic acid by passive diffusion, but accumulation was decreased almost completely by respiration. These phenomena suggest that cells or RSO membrane vesicles actively pump out acetic acid, which penetrates passively into the cell by using energy produced by respiration. On the other hand, in-side-out (ISO) membrane vesicles accumulated a high

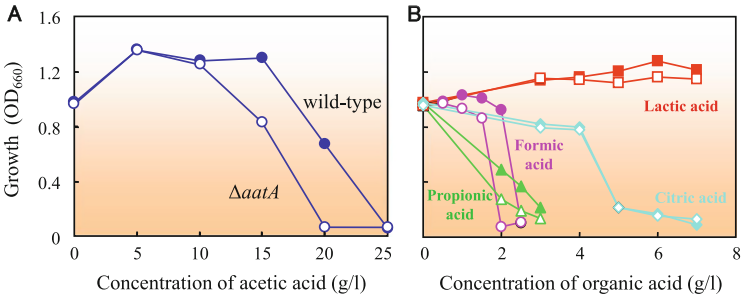

Fig. 10.7 Effects of various organic acids on growth of *A. aceti* strains. (Figure from Nakano et al. 2006) The *A. aceti* transformants were cultured at 30 °C in the presence of acetic acid (**A**), lactic acid, citric acid, formic acid, and propionic acid (**B**) in YPG medium for 120 h. The growth of the strains was followed by measuring optical density at 660 nm (OD_{660}). *Solid circles* wild-type strain carrying the vector, *open circles* transformant harboring multiple copies of the aconitase gene

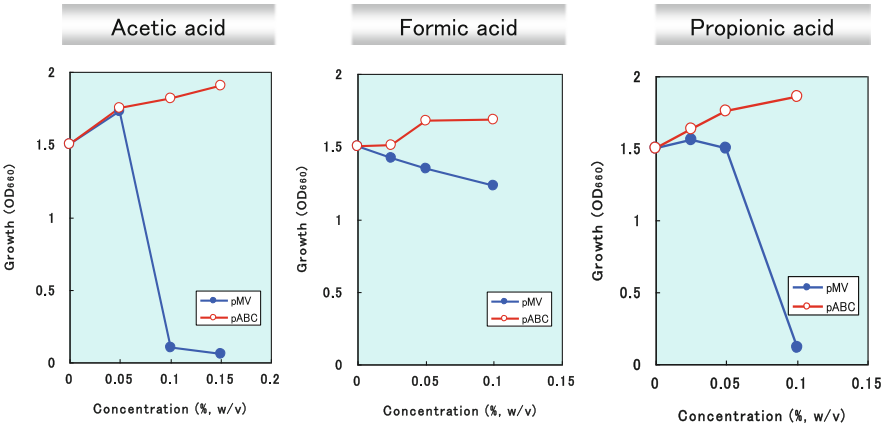

Fig. 10.8 Effect of acetic acids, formic acid, and propionic acid on growth of *E. coli* transformants cultured at 37 °C for 90 h in LB medium (pH 5.0) in the presence of various concentrations of acids. *Solid symbols* transformant harboring pMV24, *open symbols* transformant harboring pABC101, which was composed of the *aatA* coding sequence and *E. coli–Acetobacter* shuttle vector pMV24, OD_{660} optical density at 660 nm

concentration of intracellular acetic acid by respiration. Efflux of acetic acid from RSO membrane vesicles and uptake of acetic acid into ISO membrane vesicles in the presence of the respiratory substrate were largely decreased by the addition of protonophores and a respiratory inhibitor such as cyanide. These results suggest that a proton motive force-dependent acetic acid efflux pump exists in *A. pasteurianus*.

Ethanol oxidation (acetic acid production) with ADH and ALDH linked to the terminal oxidase and the following acetic acid assimilation with the cytoplasmic

enzymes, AarA, AarC and aconitase, generate a proton motive force and then ATP, which in turn is used for the efflux pump and ABC transporter.

Therefore, it is considered that AAB can grow even in an environment of high acetic acid concentration by acquiring multiple types of discharge mechanisms.

10.1.4 Adaptive Mechanism to Environmental Changes in the Cell

AAB that are applied to vinegar production are exposed to various stressors, such as ethanol, acetic acid, or heat generated during the acetic acid fermentation. In the studies of tolerance to various stresses during acetic acid fermentation of AAB, it was shown that plural types of chaperones were induced, and some chaperones were identified (Okamoto-Kainuma et al. 2002). All these chaperones, including GroES and GroEL, have characteristic heat-shock promoter-like sequences for *α-Proteobacteria*, and the expressions of these genes are regulated by RpoH (Okamoto-Kainuma et al. 2011). An *rpoH* disruption mutant of *A. pasteurianus* showed higher sensitivity to ethanol, acetic acid, and temperature as compared to the wild-type strain. Furthermore, the *A. pasteurianus* transformant, which highly expresses GroES, showed higher productivity of acetic acid than the wild-type strain. These results suggest that RpoH is one of the important factors in acetic acid fermentation of AAB.

Acetic acid is generated by oxidation of ethanol, and its reaction is catalyzed by enzymes on the inner membrane in AAB. In this reaction, ADH and ALDH transfer electrons from ethanol and acetaldehyde to ubiquinones, to generate ubiquiols (Matsushita et al. 1990, 1992b). Then, ubiquinol oxidase transfers the electrons from ubiquinol to oxygen to produce water by receiving four electrons (Fukaya et al. 1993a). When these series of reaction are not going well, reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide are generated. In *A. pasteurianus*, the mutant strain of catalase-regulating factor showed higher sensitivity to hydrogen peroxide, and also a more delayed growth, than the wild-type strain in the culture medium containing ethanol or acetic acid (Okamoto-Kainuma et al. 2008). These phenomena suggest that efficient and quick degradation of ROS enables performing stable and effective acetic acid fermentation.

Thus, AAB could adapt to change in the intracellular environment during fermentation by chaperones that refold proteins denatured by acidification and by enzymes which decompose generated ROS.

10.2 Summary of Molecular Basis of Acetic Acid Tolerance in AAB

Previous studies as well as the recent proteomic analysis have revealed several mechanisms of acetic acid tolerance in AAB (Fig. 10.9).

1. Prevention of acetic acid influx: it was suggested that THBH and PC in the membrane lipids and polysaccharide on the surface of the cells are related to acetic acid tolerance.
2. Acetic acid peroxidation: AarA, AarC, and aconitase facilitate the assimilation of intracellular acetic acid.

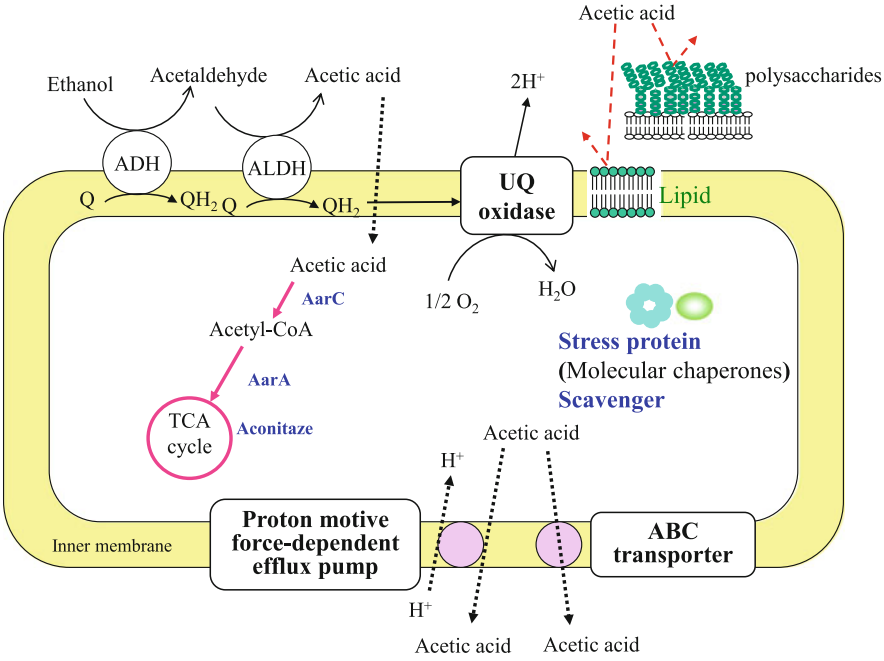

Fig. 10.9 Schematic representation of molecular machineries that confer acetic acid resistance in *Acetobacter* and *Gluconacetobacter*. (Schematic diagram quoted from Nakano and Fukaya 2008) THBH and phosphatidylcholine on the membrane and polysaccharide on the surface of the cells are suggested to be involved in acetic acid resistance. Acetic acid, which penetrates into the cytoplasm, is assumed to be metabolized through the TCA cycle by the actions of enzymes typical for AAB. Furthermore, intracellular acetic acid is possibly pumped out by a putative ABC transporter and proton motive force-dependent efflux pump using energy produced by ethanol oxidation or acetic acid overoxidation. Intracellular cytosolic enzymes are intrinsically resistant to low pH and are protected against denaturation by stress proteins such as molecular chaperones. *ADH* membrane-bound alcohol dehydrogenase, *ALDH* membrane-bound aldehyde dehydrogenase, *CS* citrate synthase, *ACN* aconitase, *PC* phosphatidylcholine

3. Acetic acid efflux by transporter or pump: two types of efflux system, a putative ABC transporter and a proton motive force-dependent efflux pump, function to pump out intracellular acetic acid.
4. Protection of cytoplasmic proteins against denaturing: several kinds of chaperone and ROS scavenger are synthesized to stabilize the cell interior.

These mechanisms will provide clues for breeding a strain having higher tolerance to acetic acid for vinegar fermentation, which may be a target for improving the efficiency of fermentation production.

References

Adachi O, Miyagawa E, Shinagawa E, Matsushita K, Ameyama M (1978) Purification and properties of particulate alcohol dehydrogenase from *Acetobacter aceti*. Agric Biol Chem 42:2331–2340

Ameyama M, Osada K, Shinagawa E, Matsushita K, Adachi O (1981) Purification and characterization of aldehyde dehydrogenase of *Acetobacter aceti*. Agric Biol Chem 45:1889–1890

Axe DD, Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol Bioeng 47:8–19

Entani E, Asai M, Tsujihata S, Tsukamoto Y, Ohta M (1997) Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7. Part 1: Examination of bacteriostatic and bactericidal activities. Jpn Assoc Infect Dis 71:443–450

Francois JA, Starks CM, Sivanuntakorn S, Jiang H, Ransome AE, Nam J-W, Constantine CZ, Kappock TJ (2006) Structure of a NADH-insensitive hexameric citrate synthase that resists acid inactivation. Biochemistry 45:13487–13499

Fukaya M, Takemura H, Okumura H, Kawamura Y, Horinouchi S, Beppu T (1990) Cloning of genes responsible for acetic acid resistance in *Acetobacter aceti*. J Bacteriol 172:2096–2104

Fukaya M, Takemura H, Tayama K, Okumura H, Kawamura Y, Horinouchi S, Beppu T (1993a) The *aarC* gene responsible for acetic acid assimilation confers acetic acid resistance on *Acetobacter aceti*. J Ferment Bioeng 76:270–275

Fukaya M, Tayama K, Tamaki T, Ebisuya H, Okumura H, Kawamura Y, Horinouchi S, Beppu T (1993b) Characterization of cytochrome a_1 that functions as a ubiquinol oxidase in *Acetobacter aceti*. J Bacteriol 175:4307–4314

Goto H, Masuko M, Ohnishi M, Tsukamoto Y (2000) Comparative analysis of phospholipids for two acetobacters producing acetic acid at high and moderate concentrations. J Jpn Oil Chem Soc 49:349–355

Hanada T, Kashima Y, Kosugi A, Koizumi Y, Yanagida F, Udaka S (2001) A gene encoding phosphatidylethanolamine *N*-methyltransferase from *Acetobacter aceti* and some properties of its disruptant. Biosci Biotechnol Biochem 65:2741–2748

Higashide T, Okumura H, Kawamura Y, Teranishi K, Hisamatsu M (1996) Membrane components and cell form of *Acetobacter polyoxogenes* (vinegar producing strain) under high acidic conditions. Nippon Shokuhin Kagaku Kaishi 43:117–123

Ishii S, Kishi M, Yamagami K, Okada S, Abe K, Misaka T (2012) The use of mammalian cultured cells loaded with a fluorescent dye shows specific membrane penetration of undissociated acetic acid. Biosci Biotechnol Biochem 76:523–529

Kanchanarach W, Theeragool G, Inoue T, Yakushi T, Adachi O, Matsushita K (2010) Acetic acid fermentation of *Acetobacter pasteurianus*: relationship between acetic acid resistance and pellicle polysaccharide formation. Biosci Biotechnol Biochem 74:1591–1597

Matsushita K, Shinagawa E, Adachi O, Ameyama M (1990) Cytochrome a_1 of *Acetobacter aceti* is a cytochrome *ba* functioning as ubiquinol oxidase. Proc Natl Acad Sci USA 87:9863–9867

Matsushita K, Ebisuya H, Ameyama M, Adachi O (1992a) Change of the terminal oxidase from cytochrome a_1 in shaking cultures to cytochrome *o* in static cultures of *Acetobacter aceti*. J Bacteriol 174:122–129

Matsushita K, Nakamura Y, Shinagawa E, Ameyama M, Adachi O (1992b) Ethanol oxidase respiratory chain of acetic acid bacteria. Reactivity with ubiquinone of pyrroloquinoline quinine-dependent alcohol dehydrogenases purified from *Acetobacter aceti and Gluconobacter suboxydans*. Biosci Biotechnol Biochem 56:304–310

Matsushita K, Inoue T, Adachi O, Toyama H (2005) *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. J Bacteriol 187:4346–4352

Mendez C, Salas JA (2001) The role of ABC transporters in antibiotic-producing organisms: drug secretion and resistance mechanisms. Res Microbiol 152:341–350

Mullins EA, Francois JA, Kappock TJ (2008) A specialized citric acid cycle requiring succinyl-coenzyme a (CoA):acetate CoA-trasferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. J Bacteriol 190:4933–4940

Nakano S, Fukaya M (2008) Analysis of proteins responsive to acetic acid in *Acetobacter*. Molecular mechanisms conferring acetic acid resistance in acetic acid bacteria. Int J Food Microbiol 125:54–59

Nakano S, Fukaya M, Horinouchi S (2004) Enhanced expression of aconitase raises acetic acid resistance in *Acetobacter aceti*. FEMS Microbiol Lett 235:315–322

Nakano S, Fukaya M, Horinouchi S (2006) Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. Appl Environ Microbiol 72:497–505

Okamoto-Kainuma A, Yan W, Kadono S, Tayama K, Koizumi Y, Yanagida F (2002) Cloning and characterization of *groESL* operon in *Acetobacter aceti*. J Biosci Bioeng 94:140–147

Okamoto-Kainuma A, Ehata Y, Ikeda M, Osono T, Ishikawa M, Kaga T, Koizumi Y (2008) Hydrogen peroxide resistance of *Acetobacter pasteurianus* NBR3283 and its relationship to acetic acid fermentation. Biosci Biotechnol Biochem 72:2526–2534

Okamoto-Kainuma A, Ishikawa M, Nakamura H, Fukazawa S, Tanaka N, Yamagami K, Koizumi Y (2011) Characterization of *rpoH* in *Acetobacter pasteurianus* NBR3283. J Biosci Bioeng 111:429–432

Russel JB (1992) Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J Appl Bacteriol 73:363–370

Sievers M, Stockli M, Teuber M (1997) Purification and properties of citrate synthase from *Acetobacter europaeus*. FEMS Microbiol Lett 146:53–58

Chapter 11
Central Carbon Metabolism and Respiration in *Gluconobacter oxydans*

Stephanie Bringer and Michael Bott

Abstract *Gluconobacter oxydans*, an α-proteobacterial species used for industrial vitamin C production, possesses a number of unusual metabolic features. Because of the absence of phosphofructokinase, succinyl-CoA synthetase, and succinate dehydrogenase, the Embden–Meyerhof–Parnas pathway (EMP) and the tricarboxylic acid (TCA) cycle are interrupted, leaving the pentose phosphate pathway (PPP) and the Entner–Doudoroff pathway (EDP) as the only complete pathways in central metabolism. Mutant and ^{13}C-based carbon flux analysis revealed the PPP to be of prime importance for the cytoplasmic catabolism of sugars and derivatives. Pyruvate is partially converted to the end product acetate by pyruvate decarboxylase and acetaldehyde dehydrogenase. The respiratory chain involves two terminal ubiquinol oxidases, cytochrome bo_3 and a cyanide-insensitive *bd*-type oxidase CIO. Mutant studies disclosed the paramount role of cytochrome bo_3 for growth. In addition, a cytochrome bc_1 complex and cytochrome *c* are present, but presumably no functional cytochrome *c* oxidase. A mutant lacking cytochrome bc_1 showed a growth defect at acidic pH; nevertheless, the precise role of this complex remains to be clarified. Here we present an overview on recent studies concerned with central carbon metabolism and respiration in *G. oxydans* and also discuss corresponding data for species of *Acetobacter* and *Gluconacetobacter*.

Keywords *Gluconobacter oxydans* 621H • Cyclic pentose phosphate pathway • Cytochrome *bd* • Cytochrome bo_3 • Cytochrome bc_1 complex • Genome-wide transcriptome analysis • ^{13}C-Metabolic flux analysis

11.1 Introduction

The strictly aerobic α-proteobacterium *Gluconobacter oxydans* is used for a variety of industrial applications for reasons of its unusual metabolic capabilities, in particular, the incomplete oxidation of organic substrates. The main industrial

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applications of *G. oxydans* are the production of vitamin C, dihydroxyacetone, 6-amino-L-sorbose (a key intermediate for the synthesis of the anti-diabetic drug miglitol), shikimate, and 3-dehydroshikimate (Adachi et al. 2003b; Deppenmeier et al. 2002; Gupta et al. 2001; Macauley et al. 2001; Mamlouk and Gullo 2013; Nishikura-Imamura et al. 2014; Pappenberger and Hohmann 2014; Raspor and Goranovič 2008; Saichana et al. 2015). A set of membrane-integral dehydrogenases enables *G. oxydans* to partially oxidize diverse sugars, sugar alcohols, and other reduced compounds in one or more steps in the periplasm. The resulting products accumulate in the culture medium, often causing its acidification. The catalytic centers of these dehydrogenases are located in the periplasm and deliver electrons or electrons and protons to the respiratory chain (Matsushita et al. 1994, 2004). The rate of these oxidations is usually quite high, resulting in a high demand of *G. oxydans* for oxygen. Only a small fraction of the sugars or sugar alcohols is transported into the cell and catabolized in the cytoplasm via the pentose phosphate pathway (PPP) and the Entner–Doudoroff pathway (EDP). Because of the absence of a gene encoding 6-phosphofructokinase, the Embden–Meyerhof–Parnas pathway (EMP) is nonfunctional (Prust et al. 2005). Similarly, the tricarboxylic acid (TCA) cycle is incomplete because the genes for succinate dehydrogenase and also succinyl-CoA synthetase are lacking. Furthermore, the genome contains neither the key genes of the glyoxylate cycle nor a gene for a gluconeogenetic phosphoenolpyruvate (PEP)-forming enzyme (Deppenmeier and Ehrenreich 2009; Prust et al. 2005).

This chapter summarizes recent studies on central carbon metabolism and respiration in *G. oxydans* 621H (ATCC 621H is identical to DSM2343), including transcriptome analyses with DNA microarrays and ^{13}C-based carbon flux analyses.

11.2 Cytoplasmic Sugar Metabolism

11.2.1 Pentose Phosphate Pathway and the Entner–Doudoroff Pathway

The importance of the PPP and the EDP for cytoplasmic sugar catabolism in *G. oxydans* has long been a matter of interest (Asai 1968; Hauge et al. 1955; Olijve and Kok 1979a, b; Rauch et al. 2010; Shinjoh et al. 1990; Tonouchi et al. 2003). Studies of the dissimilation pathway of L-[U-^{14}C]sorbose by the 2-ketogulonate-producing strain UV10, derived from *Gluconobacter melanogenus* IFO 3293 (now *G. oxydans* NBRC 3293), showed that 40 % of the metabolized substrate was converted to $^{14}CO_2$, which was mainly generated via the PPP (Shinjoh et al. 1990). In vitro characterization of enzymes involved in the central metabolism of *G. oxydans* revealed dual cofactor specificities of the key PPP enzymes glucose 6-phosphate dehydrogenase (GOX0145) and 6-phosphogluconate dehydrogenase (GOX1705). Under physiological conditions, these enzymes were found to be

$NADP^+$- and NAD^+ dependent, respectively (Adachi et al. 1982; Rauch et al. 2010; Tonouchi et al. 2003). Lack of cofactor specificity in the oxidative PPP seemingly is not an uncommon characteristic, as it was also observed in ^{13}C-based metabolic flux analyses of six other bacterial species (Fuhrer and Sauer 2009). Transaldolase (Tal) and glucose 6-phosphate isomerase (Pgi) form a bifunctional enzyme in *G. oxydans* (Sugiyama et al. 2003). Overexpression of the *pgi/tal* gene brought about increased growth rates and final cell densities in strains *G. oxydans* IFO 3293 and *G. oxydans* N44-1 (a derivative of IFO 3293) with sorbitol as the carbon source (Bremus et al. 2008a, b). Furthermore, it was found that the addition of purified Tal-Pgi or ribulokinase increased xylitol production from D-arabitol by a cell-free system of *G. oxydans*, presumably by improving NADH supply via an enhanced activity of the oxidative PPP (Sugiyama et al. 2003).

Recently, an analysis of the cytoplasmic catabolism of fructose formed by oxidation of mannitol, one of the preferred carbon sources of *G. oxydans*, was carried out with a Δ*gnd* mutant lacking 6-phosphogluconate dehydrogenase and thus the oxidative PPP and a Δ*edd-eda* mutant lacking 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, and thus the EDP (Richhardt et al. 2012). Marker-free gene deletion was accomplished by a method based on *G. oxydans* Δ*upp*, which lacks the *upp* gene for uracil phosphoribosyltransferase (Peters et al. 2013). A scheme of the central carbon metabolism of *G. oxydans*, including the reactions involved in mannitol catabolism, is shown in Fig. 11.1. In the first exponential growth phase, mannitol is oxidized to fructose, which accumulates in the medium. In the second growth phase, part of the fructose is taken up by the cells and another part is oxidized to 5-ketofructose (Fig. 11.2). Growth experiments using media with mannitol as an energy and carbon source and yeast extract as supplement revealed that neither of the two pathways, PPP or EDP, is essential for survival of *G. oxydans*. However, the growth characteristics of the two mutants under controlled conditions showed that the PPP is the main route for cytoplasmic fructose catabolism, whereas the EDP is dispensable and even unfavorable (Fig. 11.2). The Δ*edd-eda* mutant formed 24 % more cell mass than the reference strain. The longer period of almost unimpaired exponential growth of the EDP mutant is probably the result of the lack of an adjustment phase during transition from growth phase I to II. This transition is connected with de novo protein synthesis, as was shown in an earlier study (Olijve and Kok 1979a). In contrast, deletion of *gnd* (6-phosphogluconate dehydrogenase) severely inhibited growth and caused a strong selection pressure for secondary mutations inactivating glucose 6-phosphate dehydrogenase, thus also preventing fructose catabolism via the EDP. These Δ*gnd zwf** mutants were almost totally disabled in fructose catabolism but still produced about 14 % of the carbon dioxide of the reference strain, possibly by catabolizing substrates from the yeast extract. The selection pressure of the Δ*gnd* mutant of *G. oxydans* for secondary *zwf* mutations inactivating glucose 6-phosphate dehydrogenase could be caused by increased concentrations of 6-phosphogluconate or KDPG, which were shown to have an inhibitory effect on growth of *Escherichia coli* (Fuhrman et al. 1998) and *Pseudomonas cepacia* (Allenza and Lessie 1982). Overexpression of *gnd* in the reference strain improved

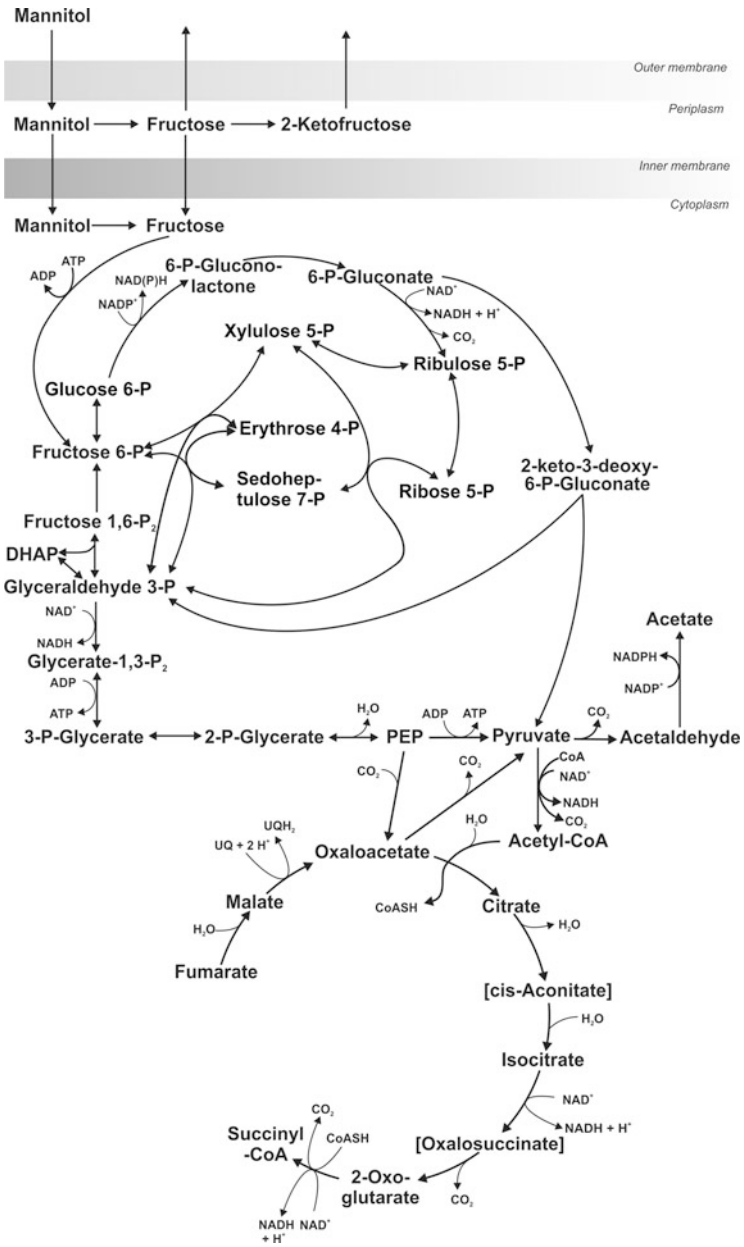


Fig. 11.1 Scheme of the central carbon metabolism of *Gluconobacter oxydans* with the substrate mannitol. *Glucose-6-P* glucose 6-phosphate, *fructose-1,6-P2* fructose 1,6-bisphosphate, *DHAP* dihydroxyacetone phosphate, *UQ* ubiquinone, *PEP* phosphoenolpyruvate

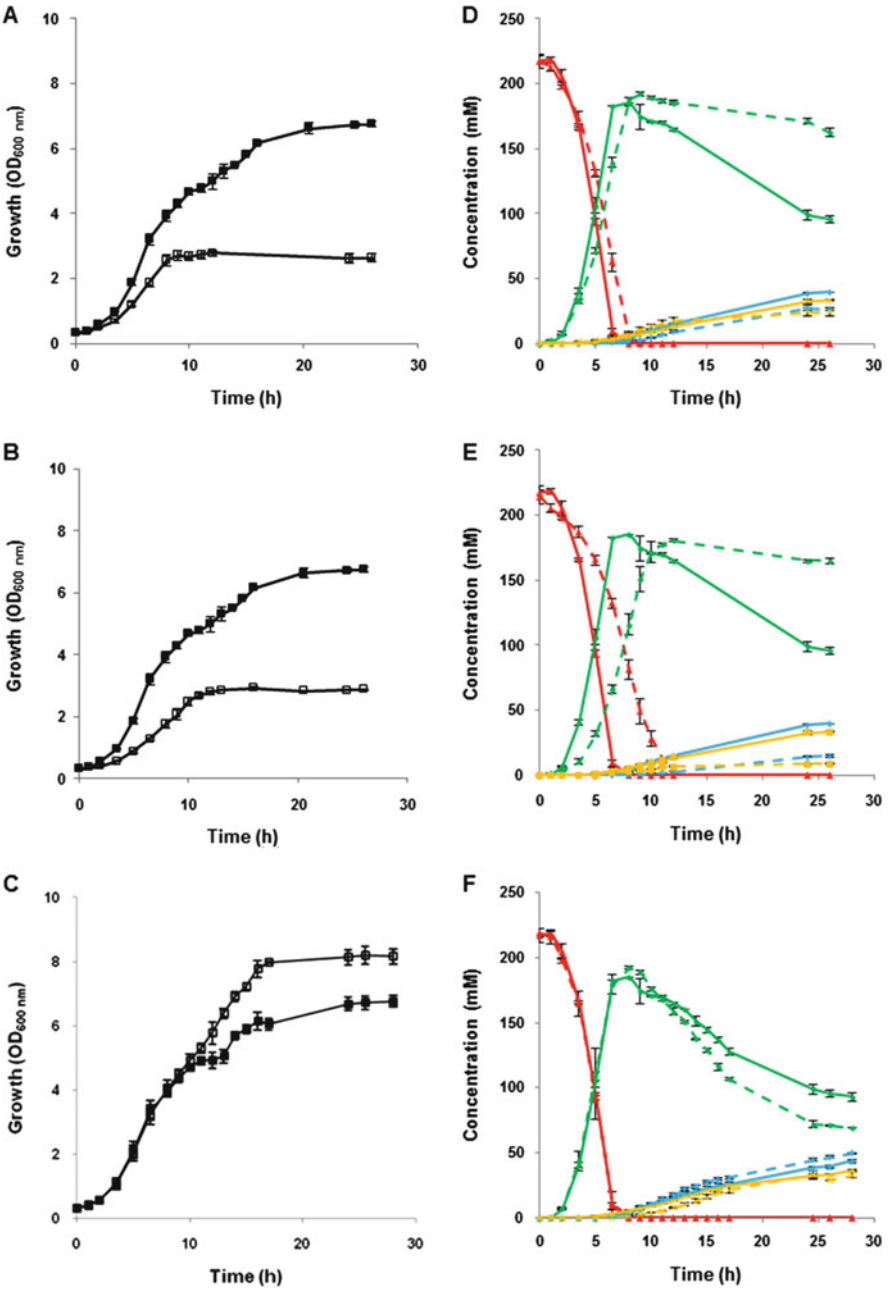


Fig. 11.2 (**a**–**c**) Growth of *G. oxydans* deletion strains (*open symbols*) and the parent strain *G. oxydans* Δ*upp* (*filled symbols*) on mannitol: Δ*gnd* (**a**); Δ*gnd zwf** (**b**); Δ*edd-eda* (**c**). (**d**–**f**) Substrate consumption and product formation of *G. oxydans* deletion strains (*dashed lines*) and the reference strain Δ*upp* (*closed lines*): Δ*gnd* (**d**); Δ*gnd zwf** (**e**); Δ*edd-eda* (**f**). Cells were cultivated in mannitol medium at 15 % dissolved oxygen at pH 6. Mean values and standard deviations of three independent cultures are shown. Mannitol (*red*); fructose (*green*); 5-ketofructose (*blue*); acetate (*yellow*). (Modified from Richhardt et al. 2012)

biomass formation in a manner similar to deletion of *edd-eda*, further confirming the importance of the PPP for cytoplasmic fructose catabolism (Richhardt et al. 2012).

Glucose catabolism by *G. oxydans* proceeds in two phases, comprising rapid periplasmic oxidation of glucose to gluconate together with cytoplasmic 5-ketogluconate production (phase I) followed by periplasmic oxidation of gluconate to 2-ketogluconate and consumption of 5-ketogluconate (phase II) (Fig. 11.3a). Only a small amount of glucose and part of the gluconate are taken up into the cells. To determine the roles of the PPP and the EDP for intracellular glucose and gluconate catabolism, the growth parameters of the mutants Δ*gnd*, Δ*gnd zwf**, and Δ*edd-eda* were determined in a bioreactor at pH 6 and 15 % dissolved oxygen (Richhardt et al. 2013a). In the presence of yeast extract, neither of the two pathways was essential for growth with glucose. However, the PPP mutants showed a reduced growth rate in phase I and completely lacked growth phase II. In contrast, the EDP mutant showed the same growth behavior as the reference strain. These results again demonstrate that the PPP is of major importance for cytoplasmic glucose and gluconate catabolism, whereas the EDP is dispensable. Up to now, the mechanism by which glucose is transported into the cell has not been known for *G. oxydans*. The presence of an incomplete PEP:carbohydrate phosphotransferase system (PTS) lacking the EIIC and EIIB components suggests a regulatory function rather than a transporter function. It was recently shown that the predicted HPr kinase (GOX0816) of *G. oxydans* phosphorylates HPr at Ser54 (Zhang et al. 2014), supporting a regulatory function of the PTS components.

11.2.2 ^{13}C-Metabolic Flux Analyses

The distribution and regulation of periplasmic and cytoplasmic carbon fluxes in *G. oxydans* 621H cultivated on glucose was studied by ^{13}C-based metabolic flux analysis (Hanke et al. 2013). Cells were cultivated with specifically ^{13}C-labeled glucose (Fig. 11.3a), and intracellular metabolites were analyzed for their labeling pattern by liquid chromatography–mass spectrometry (LC-MS). In growth phase I, 90 % of the glucose was oxidized periplasmatically to gluconate and partially further to 2-ketogluconate (Fig. 11.3b). Of the glucose taken up by the cells, 9 % was phosphorylated to glucose 6-phosphate, whereas 91 % was oxidized by cytoplasmic glucose dehydrogenase to gluconate (Fig. 11.3b, c). Of the gluconate formed in this way or taken up into the cells by a gluconate permease (GOX2188), 70 % was oxidized to 5-ketogluconate and 30 % was phosphorylated to 6-phosphogluconate. In growth phase II, 87 % of gluconate was oxidized to 2-ketogluconate in the periplasm and 13 % was taken up by the cells and almost completely converted to 6-phosphogluconate (Fig. 11.3b, c). ^{13}C-Metabolic flux analysis (MFA) showed that 6-phosphogluconate is catabolized primarily via the oxidative PPP in both phase I and II (62 % and 93 %, respectively), and

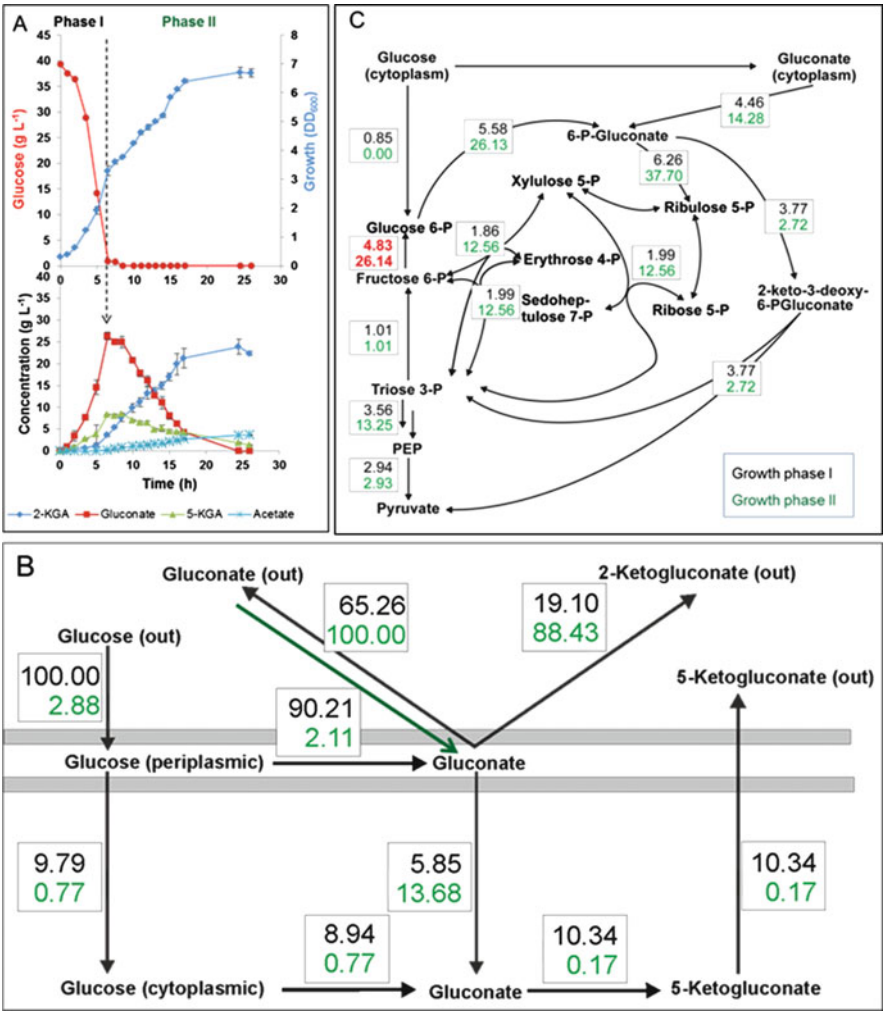


Fig. 11.3 ^{13}C-Metabolic flux analysis of *G. oxydans* during growth on glucose. **a** Growth (optical density at 600 nm) and glucose consumption (*upper graph*), product formation and consumption (*lower graph*). **b** Periplasmic and cytoplasmic carbon fluxes of nonphosphorylated intermediates. **c** Cytoplasmic carbon fluxes in central metabolism during growth phases I and II. Flux values given in **b** and **c** are related to 100 % glucose uptake (growth phase I) or 100 % gluconate uptake (growth phase II)

demonstrated a cyclic carbon flux through the oxidative PPP, as shown by the positive flux from fructose 6-phosphate to glucose 6-phosphate (indicated in red in Fig. 11.3c).

A cyclic operation of the PPP, as shown for *G. oxydans*, was also observed for *Gluconacetobacter (Ga.) oboediens* (recently renamed as *Komagataeibacter oboediens*) and *Gluconacetobacter xylinus* (recently renamed as *Komagataeibacter*

xylinus) (Sarkar et al. 2010; Zhong et al. 2013). Carbon flux analyses with *Ga. oboediens* cultivated on glucose (15 g l^{-1}) and acetate (5.24 g l^{-1}) by ^{13}C-labeling experiments showed that in spite of the presence of a *pfk* gene encoding phosphofructokinase, the activity of this enzyme was very low, leading to a negative carbon flux through the phosphoglucose isomerase (Pgi)-catalyzed reaction (i.e., a positive flux from fructose 6-phosphate to glucose 6-phosphate) and cyclization of the oxidative PPP (Sarkar et al. 2010). The same was observed in another study with *Ga. xylinus*, where negative fluxes through Pgi were determined in cells grown on the carbon sources glucose, fructose, and glycerol (Zhong et al. 2013).

Cocoa bean fermentation is a mixed-culture process, consisting initially of fermentations by yeast and lactic acid bacteria followed by oxidation of the fermentation products ethanol and lactic acid into acetic acid and acetoin by several *Acetobacter* strains, of which *A. pasteurianus* is the prominent one (Moens et al. 2014). A ^{13}C-based carbon flux analysis of *Acetobacter* during cocoa pulp fermentation-simulating conditions revealed a functionally separated metabolism during co-consumption of ethanol and lactate. Acetate was almost exclusively derived from ethanol, whereas lactate served for formation of acetoin and biomass building blocks. This switch was attributed to the lack of phosphoenolpyruvate carboxykinase and malic enzyme activities, which prevents conversion of oxaloacetate and malate formed by acetate metabolism in the TCA cycle to PEP and pyruvate and subsequently to acetoin (Adler et al. 2014). Lactate, on the other hand, can be converted to pyruvate, which is then used for acetoin formation or, after conversion to PEP by pyruvate phosphate dikinase, for gluconeogenesis. The inability of conversion of TCA cycle intermediates to PEP resembles the situation in *G. oxydans*, where in addition no enzyme for conversion of pyruvate to PEP is present.

11.2.3 Transcriptome Analyses

For a better understanding of the two growth phases observed for *G. oxydans* during cultivation on glucose, a transcriptome comparison was performed using RNA isolated from cells harvested in phase I and phase II (Hanke et al. 2013). The DNA microarray analyses revealed 454 genes showing differential expression: 227 genes had an mRNA ratio (phase II/phase I) $\geq$2.0, and 227 genes had an mRNA ratio $\leq$0.5 (Hanke et al. 2013). Several genes encoding proteins that feed electrons into the respiratory chain showed increased mRNA levels in phase II, such as those for a PQQ-containing *myo*-inositol dehydrogenase (GOX1857), for the membrane-bound gluconate 2-dehydrogenase (GOX1230 and GOX1231), and for the type II NADH dehydrogenase (GOX1675). One of the two terminal oxidases of the respiratory chain of *G. oxydans*, the cytochrome *bd* ubiquinol oxidase, renamed "cyanide-insensitive oxidase CIO" (GOX0278 and GOX0279) (Miura et al. 2013), was also upregulated in growth phase II. The genes encoding the membrane-integral pyridine nucleotide transhydrogenase (*pntA1A2B*, GOX0310-0312)

belonged to the most strongly upregulated genes in phase II. The genes downstream of *pntB*, GOX0313 and GOX0314, encode putative alcohol dehydrogenases and showed comparable mRNA ratios as the *pntA1A2B* genes. The *G. oxydans* genome contains three gene clusters coding for subunits of F_1F_o-ATP synthases. The clusters GOX1110 to GOX1113 and GOX1310 to GOX1314 encode the subunits of the F_o part and the F_1 part of an ATP synthase, which is an orthologue of the ATP synthases of *Acetobacter pasteurianus* IFO 3283-01, *Gluconacetobacter diazotrophicus* PAL 5, and other *α-Proteobacteria*. Both these clusters showed decreased expression in phase II. The genes of the third cluster, GOX2167 to GOX 2175, might code for a Na^+-translocating F_1F_o-ATP synthase (Dibrova et al. 2010), and showed an increased expression in phase II. Furthermore, the DNA microarray analysis revealed an increased expression of PPP genes in growth phase II, which correlated with an increased PPP flux in phase II. Moreover, genes possibly related to a general stress response displayed increased expression in growth phase II (Hanke et al. 2013).

Transcriptome studies aimed at understanding the influence of different carbon sources on global gene expression were also performed for *A. aceti* NBRC 14818 cultivated on either ethanol, or acetate, or glucose, or a mixture of ethanol and glucose (Sakurai et al. 2011). In contrast to *G. oxydans*, this species possesses all genes for the TCA cycle as well as the glyoxylate cycle and thus is able to completely oxidize ethanol and acetate and perform anaplerosis. Neither the gene for phosphofructokinase nor that for 2-keto-3-deoxy-6-phosphogluconate aldolase could be identified, suggesting that sugar catabolism presumably proceeds exclusively via the PPP. As genes for acetate kinase and phosphotransacetylase are absent, acetate activation occurs either via acetyl-CoA synthetase, for which two genes are present, or via succinyl-CoA:acetate CoA transferase. Growth on ethanol is diauxic, whereby in the first phase ethanol is oxidized to acetate and in the second phase acetate is oxidized to CO_2. Glucose (40 g/l) was almost completely oxidized to the end product gluconate, and biomass formation was much lower than with 10 g/l ethanol, suggesting that the strain is unable to efficiently utilize glucose and gluconate as carbon and energy sources. The transcriptome studies revealed a variety of carbon source-specific responses. The TCA cycle genes displayed higher mRNA levels during growth on acetate or glucose than on ethanol or ethanol/glucose mixture. The glyoxylate cycle genes showed high expression during growth on ethanol, acetate, and the glucose/ethanol mixture, but were not expressed on glucose alone, in accord with the known function of this pathway. Ethanol triggered increased expression of several stress-responsive genes.

In a further study, the time-dependent transcriptome changes during cultivation of *A. aceti* NBRC 14818 on ethanol were analyzed (Sakurai et al. 2012). In line with the previous data, the TCA cycle genes showed low mRNA levels during oxidation of ethanol to acetate and were significantly upregulated in the transition and acetate oxidation phases. This result suggested that the switch from acetate accumulation to acetate oxidation might be controlled by changes in the metabolic flux through the TCA cycle.

11.3 Respiratory Energy Metabolism

Because its many membrane-bound dehydrogenases incompletely oxidize sugars, sugar alcohols, and other compounds stereo- and regioselectively in the periplasm, *Gluconobacter oxydans* has been used for decades in industrial biotechnology (Adachi et al. 2003a; De Ley et al. 1984; Matsushita et al. 1994, 2002, 2004; Saichana et al. 2015; Yakushi and Matsushita 2010). The membrane-bound dehydrogenases transfer the reducing equivalents to ubiquinone. Two quinol oxidases, cytochrome bo_3 and cytochrome *bd*, then catalyze transfer of the electrons from ubiquinol to molecular oxygen (Fig. 11.4). Based on the cyanide insensitivity of *G. oxydans* cytochrome *bd*, Matsushita and coworkers designated this enzyme as cyanide-insensitive oxidase CIO and the corresponding genes *cioA* and *cioB* instead of *cydA* and *cydB* (Mogi et al. 2009). In a recent study, they purified and characterized the terminal oxidases of *G. oxydans* and for comparison cytochrome *bd* of *Escherichia coli* (Miura et al. 2013) (cf. Chaps. 7 and 13, in this volume). Based on the reported data, a different designation for members of the CIO clade, *cioAB*, is used in the following text (Fig. 11.4).

11.3.1 Roles of the Two Terminal Oxidases Cytochrome bo_3 *and Cyanide-Insensitive Oxidase, CIO*

To elucidate the role of the two terminal oxidases of *G. oxydans*, the in-frame deletion mutants Δ*cioAB* (previously termed Δ*cydAB*) and Δ*cyoBACD* were constructed and characterized with respect to growth, respiratory activity, and H^+/O ratio (Richhardt et al. 2013b). Deletion of the *cioAB* genes had no obvious influence on growth, whereas the lack of the *cyoBACD* genes severely reduced the growth rate and the cell yield. Using a respiration activity monitoring system and adjusting different levels of oxygen availability, hints for a low oxygen affinity of CIO were obtained, which were supported by measurements of oxygen consumption in a respirometer. Our evidence that CIO of *G. oxydans* has a significantly lower oxygen affinity than cytochrome bo_3 oxidase is in agreement with recent biochemical data, wherein the K_m values for oxygen of CIO and cytochrome bo_3 determined with oxymyoglobin were reported to be 21 μM and 3 μM, respectively (Miura et al. 2013).

The H^+/O ratio of the Δ*cyoBACD* mutant with mannitol as substrate was 0.56 ± 0.11, and more than 50 % lower than that of the reference strain (1.26 ± 0.06) and the Δ*cioAB* mutant (1.31 ± 0.16), indicating that cytochrome bo_3 oxidase is the main component for proton extrusion via the respiratory chain (Richhardt et al. 2013b). In previous studies, H^+/O ratios of 1.7–2.2 were reported for *G. oxydans* cells supplied with glycerol, glucose, lactate, or ethanol at pH 6 (Matsushita et al. 1989). The differences could be caused by the use of other substrates or differences in the experimental conditions. For cytochrome bo_3

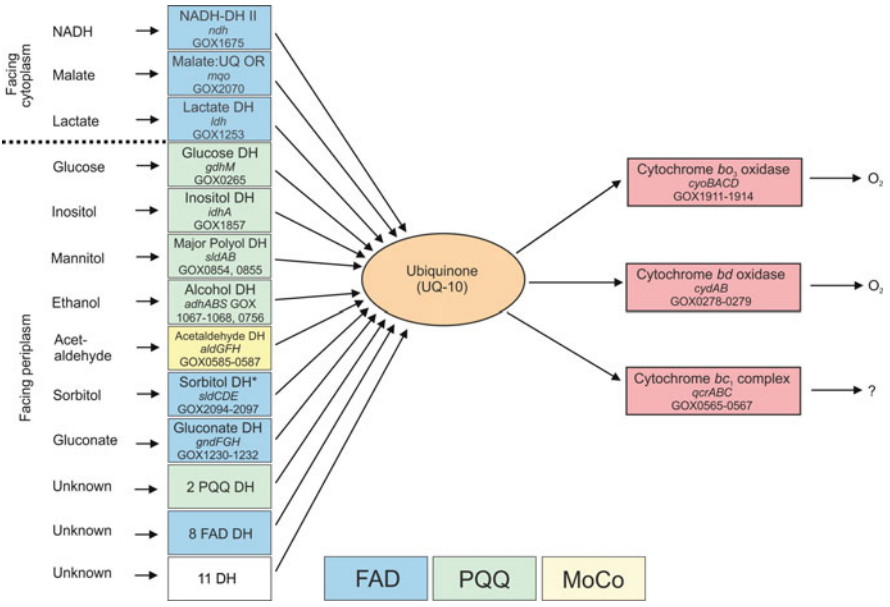


Fig. 11.4 Overview of the components of the respiratory chain of *G. oxydans* 621H. *DH* dehydrogenase, *FAD* flavin adenine dinucleotide, *PQQ* pyrroloquinoline quinone, *MoCo* molybdopterin cofactor, *CIO* cyanide-insensitive oxidase. *In the sequenced strain 621H, the *sldD* gene encoding the flavoprotein subunit of the sorbitol dehydrogenase contains a frameshift mutation in codon 70, resulting in fragmentation (GOX2095, GOX2096) and the absence of 30 amino acids (positions 70–100 in the native SldD protein) (Prust et al. 2005). The functionality of the resulting complex is therefore questionable. (Modified from Richhardt et al. 2013b)

oxidase, an H^+/e stoichiometry of 2 and therefore an H^+/O ratio of 4 can be assumed (Puustinen et al. 1989), and for cytochrome *bd* oxidase an H^+/e stoichiometry of 1 and an H^+/O ratio of 2 (Jasaitis et al. 2000; Puustinen et al. 1991). As the CIO was not relevant for growth of *G. oxydans* and proton translocation under the conditions used by Richhardt et al. 2013b, a H^+/O ratio approaching 4 might be expected for *G. oxydans*. However, both our results as well as those presented by Matsushita et al. 1989 are much lower than a value of 4. There are a number of very speculative hypotheses to explain the low H^+/O ratios determined for *G. oxydans*. (1) The cytoplasmic membrane of *G. oxydans* might be more leaky for protons than that of *E. coli*, causing a non-energy-conserving backflow of protons into the cells (Brown 1992). (2) The respiratory chain could involve a reverse electron transfer coupled to an influx of protons (van der Oost et al. 1995). (3) Cytochrome bo_3 oxidase might not function as a primary proton pump, but as a Na^+ pump, as has been described for cytochrome bo_3 oxidase of *Vitreoscilla* sp. (Kim et al. 2005; Park et al. 1996). In this context it is interesting to note that *G. oxydans* possesses two different F_1F_o-type ATP synthases (Dibrova et al. 2010), one of which might use Na^+ as the coupling ion (Hanke et al. 2012; Prust et al. 2005). The comparative DNA microarray analysis of the *cyoBACD* deletion mutant versus the reference strain showed

an upregulation of genes (GOX2167-2175) coding for the putative Na^+-dependent F_1F_o-ATPase (Richhardt et al. 2013b).

Plasmid-based overexpression of *cyoBACD* led to increased growth rates and growth yields in both the wild type and the Δ*cyoBACD* mutant, suggesting that cytochrome bo_3 might be a rate-limiting factor of the respiratory chain (Richhardt et al. 2013b). Although additional studies are required to clarify the positive effect of cytochrome bo_3 overproduction, the result shows that respiratory chain components are a promising starting point for further optimization of *G. oxydans* for its use in biotechnological applications. Recently, a comparison of the genome sequences of *G. oxydans* 621H and *G. oxydans* DSM 3504 revealed, among others, the presence of an additional type II NADH dehydrogenase gene (*ndh2*) in strain DSM3504, which reaches an optical density almost three times higher than that of *G. oxydans* 621H. Interestingly, plasmid-based expression of *ndh2* from strain DSM3504 in strain 621H led to a significantly increased growth rate (Kostner et al. 2014), explained by a competition between membrane-bound dehydrogenases and NADH dehydrogenase for transferring electrons to ubiquinone, which could be altered in favor of the latter enzyme by increased *ndh* expression.

11.3.2 Influence of Oxygen on Global Gene Expression

G. oxydans is strictly aerobic; nevertheless, in its natural habitat, oxygen deprivation caused by the rapid oxygen consumption by *G. oxydans* itself is likely to occur very often. In a recent study, the genome-wide transcriptional responses of *G. oxydans* 621H to oxygen limitation were analyzed (Hanke et al. 2012). For that purpose, cells were cultivated in a bioreactor system for 6 h at a constant dissolved oxygen concentration (DOC) of 15 %, before gassing was switched to a mixture of 2 % O_2 and 98 % N_2, leading to a DOC of 0 %. Samples for RNA isolation were taken before and 4 h after the switch and used for comparative transcriptome analysis with DNA microarrays. Oxygen deprivation caused expression changes of almost 500 genes, 215 with a $\geq$2.0-fold increased and 271 with a $\geq$2.0-fold decreased mRNA ratio (oxygen limitation/oxygen excess). Accordingly, oxygen limitation triggered a strong response influencing transcription of about 20 % of all chromosomal genes, including many involved in respiration and oxidative phosphorylation.

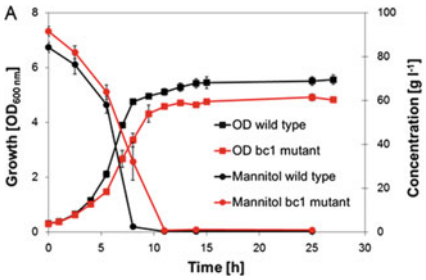
Although several genes coding for respiratory dehydrogenases showed reduced mRNA levels, expression of the terminal oxidase genes was increased. An opposite regulation of the dehydrogenases, several of which are PQQ dependent, and the terminal oxidases represents an adequate response to oxygen limitation, as it probably allows the cells to reduce the electron flux into the respiratory chain on the one hand and to increase the capability to capture the limiting oxygen on the other. The reduced expression of genes involved in PQQ biosynthesis and the increased expression of genes required for heme biosynthesis fits the expression patterns of the dehydrogenases and the terminal oxidases.

The genes for the membrane-integral pyridine nucleotide transhydrogenase PntA1A2B were among the most strongly upregulated genes under oxygen limitation (10- to 15 fold). The enzyme either consumes the electrochemical proton gradient Δp for $NADP^+$ reduction or generates Δp at the expense of NADPH oxidation (Jackson et al. 2002). As observed in another study (see earlier), the genes downstream of *pntA1A2B* (GOX0313, GOX0314) encoding putative zinc-containing alcohol dehydrogenases showed mRNA ratios similar to the *pntA1A2B* genes, suggesting that these five genes might form an operon and a functional connection between the encoded enzymes. Increased levels of these enzymes might allow the cells a rapid exchange between NAD^+/NADH and $NADP^+$/NADPH and reoxidation of NAD(P)H via reduction of aldehydes/ketones to the corresponding alcohols, which could be favorable under oxygen limitation. In fact, the enzyme encoded by GOX0313 was recently shown to function as medium-chain alcohol dehydrogenase oxidizing various primary alcohols, but having a preference for substrate reduction, reducing many aldehydes and α-diketones (Schweiger et al. 2013).

As mentioned previously, the *G. oxydans* genome contains genes for two F_1F_o-ATP synthases, one of which might code for a Na^+-translocating F_1F_o-ATP synthase. The genes for the presumably H^+-translocating F_1F_o-ATP synthase showed a decreased expression under oxygen limitation, whereas those for the putative Na^+-translocating enzyme had increased mRNA levels. Remarkably, an inverse regulation of the two F_1F_o-ATP synthases was also observed in the transcriptome comparison of growth phase I and II during cultivation of *G. oxydans* on glucose (see earlier). Many genes encoding proteins involved in transcription and translation showed lower expression under oxygen limitation, including subunits of the RNA polymerase, elongation factors, and 45 ribosomal proteins. This response probably presents an adaptation to the reduced linear growth observed after shifting the cells from oxygen excess to oxygen limitation. Overall, the transcriptome comparison revealed a complex response of *G. oxydans* to oxygen starvation and raised many interesting questions that deserve more detailed studies, such as the function of the two inversely regulated F_1F_o-ATP synthases.

11.3.3 The Enigmatic Function of the Cytochrome bc_1 *Complex*

G. oxydans possesses the genes *qcrABC* (GOX0565-0567) for a cytochrome bc_1 complex, *cycA* (GOX0258) for a soluble cytochrome c_{552}, GOX1863 for a protein with similarity to subunit I of cytochrome *c* oxidase, and *ctaB* (GOX1864) for heme *o*-synthetase (Matsutani et al. 2014; Prust et al. 2005; Sakurai et al. 2011). As the putative subunit I lacks histidine residues serving as Cu_B and heme a_3 ligands, it is unlikely to be functional. Moreover, genes for subunits II and III of cytochrome



B

Annotation	Gene	I	II
Respiration and ATP synthesis			
NAD(P) transhydrogenase SUα2	*pntA2*	3.14	10.37
NAD(P) transhydrogenase SUα1	*pntA1*	3.80	14.70
NAD(P) transhydrogenase SUβ	*pntB*	3.44	12.04
Alcohol:NAD$^+$oxidoreductase SUα	*adhA*	4.00	13.58
Alcohol:NAD$^+$oxidoreductase SUβ	*adhB*	3.58	14.63
Cytochrome bo_3 ubiquinol oxidase SUII	*cyoB*	2.09	2.82
Cytochrome bo_3 ubiquinol oxidase SUI	*cyoA*	2.28	2.70
Cytochrome bo_3 ubiquinol oxidase SUIII	*cyoC*	2.10	3.56
Cytochrome bo_3 ubiquinol oxidase SUIV	*cyoD*	2.15	3.81

Fig. 11.5 (**a**) Growth and substrate consumption of *G. oxydans* 621H wild type and the cytochrome bc_1 mutant Δ*qcrABC* at pH 4 on 80 g l^{-1} mannitol under oxygen saturation. (**b**) Selected mRNA ratios from two different DNA microarray experiments: *I*, strain Δ*qcrABC* versus wild type, both cultivated at pH 4 under oxygen saturation; *II*, wild type cultivated under oxygen limitation versus wild type cultivated under oxygen saturation, both at pH 6. Genes shown are an excerpt of 51 genes having identical regulation patterns. (**a** was modified from Hanke et al. 2012)

c oxidase are absent in the genome. To understand the function of a cytochrome bc_1 complex in the presumptive absence of a functional cytochrome *c* oxidase, a Δ*qcrABC* mutant of *G. oxydans* was constructed and analyzed (Hanke et al. 2012). Although the mutant showed the same growth behavior as the wild type during cultivation on mannitol at pH 6, a growth defect was observed at pH 4 under oxygen saturation, where the mutant showed a 18 % reduced growth rate and a 13 % lower final optical density. Comparison of the transcriptomes of the Δ*qcrABC* mutant versus the wild type at pH 4 revealed 51 differentially expressed genes. Interestingly, almost all the 45 genes with increased expression in the Δ*qcrABC* mutant at pH 4 (including the cytochrome bo_3 oxidase genes) were also upregulated in the wild type grown at pH 6 under oxygen limitation (Fig. 11.5). The results obtained for the Δ*qcrABC* mutant clearly suggest that the cytochrome bc_1 complex possesses a physiological function in *G. oxydans*, at least at acidic pH, but further studies are required to elucidate this function.

Two sets of genes for the cytochrome bc_1 complex, one of which lacks the gene for the Rieske iron sulfur protein, were identified in *Acetobacter aceti* NBRC 14818 and *A. pasteurianus* NBRC 3283 (Azuma et al. 2009; Sakurai et al. 2011). In both species also a gene encoding a putative subunit I of cytochrome *c* oxidase (*coxA*) was identified, but genes for subunits II and III were absent in the genome (Sakurai et al. 2011). Further organisms containing the genes for a cytochrome bc_1 complex but presumably lacking a functional cytochrome *c* oxidase are *Acetobacter pasteurianus* 386B (Illeghems et al. 2013) and *Zymomonas mobilis* (Balodite et al. 2014; Charoensuk et al. 2011; Sootsuwan et al. 2008), all of which belong to the *α-Proteobacteria*. Accordingly, this situation is not specific for *G. oxydans*, but is widespread in related bacteria.

One idea regarding the function of the cytochrome bc_1 complex is to provide reduced cytochrome *c* for reduction of hydrogen peroxide to water by an enzyme called cytochrome *c* peroxidase. *G. oxydans* (GOX0998) and many other acetic acid bacteria with known genome sequences possess the corresponding *ccpR* gene.

However, both in *G. oxydans* and in *A. aceti* NBRC 14818, the expression level of *ccpR* was low under the conditions tested (Hanke et al. 2012; Sakurai et al. 2011). In other bacteria, these enzymes are usually present in addition to other terminal electron acceptors for reduced cytochrome *c* (Atack and Kelly 2007) and there is currently no experimental evidence for such a function of the cytochrome bc_1 complex in the aforementioned species.

11.4 Concluding Remarks

In the past years, a more detailed understanding of the metabolic features of *G. oxydans* and several other acetic acid bacteria was obtained based on the availability of genome sequences, the application of omics technologies such as transcriptomics and fluxomics, and by the detailed analysis of defined mutants. The genome sequences revealed significant heterogeneity in the equipment of individual species with membrane-bound dehydrogenases, sugar catabolic pathways, the TCA cycle, and the glyoxylate cycle. These differences are reflected in the metabolic properties of the species. *Gluconobacter oxydans* is one of the acetic acid bacteria prominently applied in industrial production processes, which are often whole-cell biotransformations requiring prior biomass production. Because the cell yield of *G. oxydans* (0.09 $g_{cdw}/g_{glucose}$ during bioreactor cultivation is very low (Krajewski et al. 2010; Richhardt et al. 2013a), biomass production is cost intensive and presents a disadvantage for industrial application. The results obtained in the studies summarized here disclose starting points for improving the biomass yield and broadening the application range of *G. oxydans* by metabolic engineering of cytoplasmic sugar catabolism and respiratory energy metabolism.

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References

Adachi O, Osada K, Matsushita K, Shinagawa E, Ameyama M (1982) Purification, crystallization and properties of 6-phospho- D-gluconate dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 46:391–398

Adachi O, Moonmangmee D, Shinagawa E, Toyama H, Yamada M, Matsushita K (2003a) New quinoproteins in oxidative fermentation. Biochim Biophys Acta 1647:10–17

Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K (2003b) New developments in oxidative fermentation. Appl Microbiol Biotechnol 60:643–653

Adler P, Frey LJ, Berger A, Bolten CJ, Hansen CE, Wittmann C (2014) The key to acetate: metabolic fluxes of acetic acid bacteria under cocoa pulp fermentation simulating conditions. Appl Environ Microbiol 80:4702–4716

Allenza P, Lessie TG (1982) *Pseudomonas cepacia* mutants blocked in the Entner-Doudoroff pathway. J Bacteriol 150:1340–1347

Asai T (1968) Acetic acid bacteria: classification and biochemical activities. University of Tokyo Press, Tokyo

Atack JM, Kelly DJ (2007) Structure, mechanism and physiological roles of bacterial cytochrome *c* peroxidases. Adv Microb Physiol 52:73–106

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783

Balodite E, Strazdina I, Galinina N, Mclean S, Rutkis R, Poole RK, Kalnenieks U (2014) Structure of the *Zymomonas mobilis* respiratory chain: oxygen affinity of electron transport and the role of cytochrome *c* peroxidase. Microbiology 160:2045–2052

Bremus C, Bringer-Meyer S, Hermann U, Mouncey NJ, Sahm H (2008a) Biological process using a glucose-6-phosphate isomerase. Google patents EP1873248A1

Bremus C, Bringer-Meyer S, Hermann U, Mouncey NJ, Sahm H (2008b) Biological process using a transaldolase. Google patents EP1873246 A1

Brown GC (1992) The leaks and slips of bioenergetic membranes. FASEB J 6:2961–2965

Charoensuk K, Irie A, Lertwattanasakul N, Sootsuwan K, Thanonkeo P, Yamada M (2011) Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotolerant *Zymomonas mobilis*. J Mol Microbiol Biotechnol 20:70–82

De Ley J, Gillis M, Swings J (1984) The genus *Gluconobacter*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, pp 267–278

Deppenmeier U, Ehrenreich A (2009) Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. J Mol Microbiol Biotechnol 16:69–80

Deppenmeier U, Hoffmeister M, Prust C (2002) Biochemistry and biotechnological applications of *Gluconobacter* strains. Appl Microbiol Biotechnol 60:233–242

Dibrova DV, Galperin M, Mulkidjanian A (2010) Characterization of the N-ATPase, a distinct, laterally transferred Na^+-translocating form of the bacterial F-type membrane ATPase. Bioinformatics 26:1473–1476

Fuhrer T, Sauer U (2009) Different biochemical mechanisms ensure network-wide balancing of reducing equivalents in microbial metabolism. J Bacteriol 191:2112–2121

Fuhrman LK, Wanken A, Nickerson KW, Conway T (1998) Rapid accumulation of intracellular 2-keto-3-deoxy-6-phosphogluconate in an Entner–Doudoroff aldolase mutant results in bacteriostasis. FEMS Microbiol Lett 159:261–266

Gupta A, Singh VK, Qazi GN, Kumar A (2001) *Gluconobacter oxydans*: its biotechnological applications. J Mol Microbiol Biotechnol 3:445–456

Hanke T, Richhardt J, Polen T, Sahm H, Bringer S, Bott M (2012) Influence of oxygen limitation, absence of the cytochrome bc_1 complex and low pH on global gene expression in *Gluconobacter oxydans* 621H using DNA microarray technology. J Biotechnol 157:359–372

Hanke T, Nöh K, Noack S, Polen T, Bringer S, Sahm H, Wiechert W, Bott M (2013) Combined fluxomics and transcriptomics analysis of glucose catabolism via a partially cyclic pentose phosphate pathway in *Gluconobacter oxydans* 621H. Appl Environ Microbiol 79:2336–2348

Hauge JG, King TE, Cheldelin VH (1955) Oxidation of dihydroxyacetone via the pentose cycle in *Acetobacter suboxydans*. J Biol Chem 214:11–26

Illeghems K, De Vuyst L, Weckx S (2013) Complete genome sequence and comparative analysis of *Acetobacter pasteurianus* 386B, a strain well-adapted to the cocoa bean fermentation ecosystem. BMC Genomics 14:526

Jackson JB, White SA, Quirk PG, Venning JD (2002) The alternating site, binding change mechanism for proton translocation by transhydrogenase. Biochemistry 41:4173–4185

Jasaitis A, Borisov VB, Belevich NP, Morgan JE, Konstantinov AA, Verkhovsky MI (2000) Electrogenic reactions of cytochrome *bd*. Biochemistry 39:13800–13809

Kim SK, Stark BC, Webster DA (2005) Evidence that Na^+-pumping occurs through the D-channel in *Vitreoscilla* cytochrome *bo*. Biochem Biophys Res Commun 332:332–338

Kostner D, Luchterhand B, Junker A, Volland S, Daniel R, Büchs J, Liebl W, Ehrenreich A (2014) The consequence of an additional NADH dehydrogenase paralog on the growth of *Gluconobacter oxydans* DSM3504. Appl Microbiol Biotechnol 99(1):375–386. doi:10.1007/s00253-014-6069-9

Krajewski V, Simić P, Mouncey NJ, Bringer S, Sahm H, Bott M (2010) Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation. Appl Environ Microbiol 76:4369–4376

Macauley S, Mcneil B, Harvey LM (2001) The genus *Gluconobacter* and its applications in biotechnology. Crit Rev Biotechnol 21:1–25

Mamlouk D, Gullo M (2013) Acetic acid bacteria: physiology and carbon sources oxidation. Indian J Microbiol 53:377–384

Matsushita K, Nagatani Y, Shinagawa E, Adachi O, Ameyama M (1989) Effect of extracellular pH on the respiratory chain and energetics of *Gluconobacter suboxydans*. Agric Biol Chem 53:2895–2902

Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301

Matsushita K, Toyama H, Yamada M, Adachi O (2002) Quinoproteins: structure, function, and biotechnological applications. Appl Microbiol Biotechnol 58:13–22

Matsushita K, Toyama H, Adachi O (2004) Respiratory chains in acetic acid bacteria: membrane bound periplasmic sugar and alcohol respirations. In: Respiration in Archaea and Bacteria. Advances in photosynthesis and respiration. Springer, Dordrecht, pp 81–99

Matsutani M, Fukushima K, Kayama C, Arimitsu M, Hirakawa H, Toyama H, Adachi O, Yakushi T, Matsushita K (2014) Replacement of a terminal cytochrome *c* oxidase by ubiquinol oxidase during the evolution of acetic acid bacteria. Biochim Biophys Acta 1837:1810–1820

Miura H, Mogi T, Ano Y, Migita CT, Matsutani M, Yakushi T, Kita K, Matsushita K (2013) Cyanide-insensitive quinol oxidase (CIO) from *Gluconobacter oxydans* is a unique terminal oxidase subfamily of cytochrome *bd*. J Biochem (Tokyo) 153:535–545

Moens F, Lefeber T, De Vuyst L (2014) Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. Appl Environ Microbiol 80:1848–1857

Mogi T, Ano Y, Nakatsuka T, Toyama H, Muroi A, Miyoshi H, Migita CT, Ui H, Shiomi K, Omura S, Kita K, Matsushita K (2009) Biochemical and spectroscopic properties of cyanide-insensitive quinol oxidase from *Gluconobacter oxydans*. J Biochem (Tokyo) 146:263–271

Nishikura-Imamura S, Matsutani M, Insomphun C, Vangnai AS, Toyama H, Yakushi T, Abe T, Adachi O, Matsushita K (2014) Overexpression of a type II 3-dehydroquinate dehydratase enhances the biotransformation of quinate to 3-dehydroshikimate in *Gluconobacter oxydans*. Appl Microbiol Biotechnol 98:2955–2963

Olijve W, Kok JJ (1979a) Analysis of growth of *Gluconobacter oxydans* in glucose containing media. Arch Microbiol 121:283–290

Olijve W, Kok JJ (1979b) An analysis of the growth of *Gluconobacter oxydans* in chemostat cultures. Arch Microbiol 121:291–297

Pappenberger G, Hohmann HP (2014) Industrial production of L-ascorbic acid (vitamin C) and D-isoascorbic acid. Adv Biochem Eng Biotechnol 143:143–188

Park C, Moon JY, Cokic P, Webster DA (1996) Na^+-translocating cytochrome *bo* terminal oxidase from *Vitreoscilla*: some parameters of its Na^+ pumping and orientation in synthetic vesicles. Biochemistry 35:11895–11900

Peters B, Junker A, Brauer K, Mühlthaler B, Kostner D, Mientus M, Liebl W, Ehrenreich A (2013) Deletion of pyruvate decarboxylase by a new method for efficient markerless gene deletions in *Gluconobacter oxydans*. Appl Microbiol Biotechnol 97:2521–2530

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Puustinen A, Finel M, Virkki M, Wikström M (1989) Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*. FEBS Lett 249:163–167

Puustinen A, Finel M, Haltia T, Gennis RB, Wikström M (1991) Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry 30:3936–3942

Raspor PP, Goranovič D (2008) Biotechnological applications of acetic acid bacteria. Crit Rev Biotechnol 28:101–124

Rauch B, Pahlke J, Schweiger P, Deppenmeier U (2010) Characterization of enzymes involved in the central metabolism of *Gluconobacter oxydans*. Appl Microbiol Biotechnol 88:711–718

Richhardt J, Bringer S, Bott M (2012) Mutational analysis of the pentose phosphate and Entner–Doudoroff pathways in *Gluconobacter oxydans* reveals improved growth of a Δ*edd* Δ*eda* mutant on mannitol. Appl Environ Microbiol 78:6975–6986

Richhardt J, Bringer S, Bott M (2013a) Role of the pentose phosphate pathway and the Entner–Doudoroff pathway in glucose metabolism of *Gluconobacter oxydans* 621H. Appl Microbiol Biotechnol 97:4315–4323

Richhardt J, Luchterhand B, Bringer S, Büchs J, Bott M (2013b) Evidence for a key role of cytochrome bo_3 oxidase in respiratory energy metabolism of *Gluconobacter oxydans*. J Bacteriol 195:4210–4220

Saichana N, Matsushita K, Adachi O, Frebort I, Frebortova J (2015) Acetic acid bacteria: a group of bacteria with versatile biotechnological applications. Biotechnol Adv 33(6 pt 2):1260–1271. doi:10.1016/j.biotechadv.2014.12.001

Sakurai K, Arai H, Ishii M, Igarashi Y (2011) Transcriptome response to different carbon sources in *Acetobacter aceti*. Microbiology 157:899–910

Sakurai K, Arai H, Ishii M, Igarashi Y (2012) Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. J Biosci Bioeng 113:343–348

Sarkar D, Yabusaki M, Hasebe Y, Ho PY, Kohmoto S, Kaga T, Shimizu K (2010) Fermentation and metabolic characteristics of *Gluconacetobacter oboediens* for different carbon sources. Appl Microbiol Biotechnol 87:127–136

Schweiger P, Gross H, Zeiser J, Deppenmeier U (2013) Asymmetric reduction of diketones by two *Gluconobacter oxydans* oxidoreductases. Appl Microbiol Biotechnol 97:3475–3484

Shinjoh M, Setoguchi Y, Hoshino T (1990) Sorbose dissimilation in 2-keto-L-gulonic acid-producing mutant UV10 derived from *Gluconobacter melanogenus* IFO 3293. Agric Biol Chem 54:2257–2263

Sootsuwan K, Lertwattanasakul N, Thanonkeo P, Matsushita K, Yamada M (2008) Analysis of the respiratory chain in ethanologenic *Zymomonas mobilis* with a cyanide-resistant *bd*-type ubiquinol oxidase as the only terminal oxidase and its possible physiological roles. J Mol Microbiol Biotechnol 14:163–175

Sugiyama M, Suzuki S, Tonouchi N, Yokozeki K (2003) Transaldolase/glucose-6-phosphate isomerase bifunctional enzyme and ribulokinase as factors to increase xylitol production from D-arabitol in *Gluconobacter oxydans*. Biosci Biotechnol Biochem 67:2524–2532

Tonouchi N, Sugiyama M, Yokozeki K (2003) Coenzyme specificity of enzymes in the oxidative pentose phosphate pathway of *Gluconobacter oxydans*. Biosci Biotechnol Biochem 67:2648–2651

van der Oost J, Schepper M, Stouthamer AH, Westerhoff HV, van Spanning RJ, de Gier JW (1995) Reversed electron transfer through the bc_1 complex enables a cytochrome *c* oxidase mutant (Δaa_3/cbb_3) of *Paracoccus denitrificans* to grow on methylamine. FEBS Lett 371:267–270

Yakushi T, Matsushita K (2010) Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. Appl Microbiol Biotechnol 86:1257–1265

Zhang P, Ma Y, Wang F, Wei D (2014) Phosphorylation of HPr by HPr kinase in *Gluconobacter oxydans* 621H. Protein Pept Lett 21:597–601

Zhong C, Zhang GC, Liu M, Zheng XT, Han PP, Jia SR (2013) Metabolic flux analysis of *Gluconacetobacter xylinus* for bacterial cellulose production. Appl Microbiol Biotechnol 97:6189–6199

Chapter 12
Metabolic Features of *Acetobacter aceti*

Hiroyuki Arai, Kenta Sakurai, and Masaharu Ishii

Abstract *Acetobacter aceti* temporarily accumulates acetate as an incomplete oxidation product when cultured in the presence of ethanol. The accumulated acetate is utilized as a carbon and energy source via the tricarboxylic acid (TCA) cycle after the depletion of ethanol by the phenomenon termed acetate overoxidation. In this chapter, we provide an overview of the genomic features and whole-genome transcriptional profiles of *A. aceti* NBRC 14818 when cultured under various growth conditions to understand the molecular genetic basis for the metabolic switching from incomplete ethanol oxidation to acetate overoxidation. The genes encoding enzymes of the TCA cycle and glyoxylate pathway and components of the branched electron transport chain exhibit dynamic changes in expression according to the carbon sources and growth phases. In particular, the TCA cycle genes of *A. aceti* are significantly repressed in the presence of ethanol. The low activity of the TCA cycle in the presence of ethanol may slow the metabolism of acetyl-CoA and lead to the accumulation of acetate. The presence or absence of glyoxylate pathway genes in the genome of acetic acid bacteria is also predicted to affect acetate productivity.

Keywords Acetate • *Acetobacter aceti* • Electron transport chain • Ethanol • Glyoxylate pathway • Incomplete oxidation • Transcriptome • Tricarboxylic acid cycle

12.1 Introduction

Acetobacter aceti is an obligately aerobic α-proteobacterium that inhabits a wide variety of environmental niches where sugar is present, including flowers, fruits, soil, and water. The bacterium has been historically utilized for vinegar production and also causes the spoilage of alcoholic beverages because of its unique ability to incompletely oxidize ethanol to acetate (Asai 1968). The incomplete oxidation of

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ethanol is catalyzed by membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH). The reaction is coupled to oxygen reduction by quinol oxidases via ubiquinone (Yakushi and Matsushita 2010). Therefore, the oxidation of ethanol to acetate in the periplasm by PQQ-dependent ADH leads to the generation of adenosine triphosphate (ATP) by oxidative phosphorylation. Ethanol is mainly utilized as an energy source and is rarely utilized as a carbon source during incomplete oxidation.

The growth of *Acetobacter* species on ethanol results in the accumulation of acetate, which is then completely oxidized by the phenomenon termed acetate overoxidation. The sequential oxidation of ethanol and acetate leads to a diauxic growth profile that is characterized by acetate accumulation in the first exponential growth phase and complete oxidation of the accumulated acetate in the second exponential phase (Saeki et al. 1997). However, in some conditions, ethanol is oxidized to acetate both in the periplasm, by membrane-bound PQQ-dependent ADH and aldehyde dehydrogenase (ALDH), and in the cytoplasm, by soluble NAD(P)$^+$-dependent ADH and ALDH. These conditions are unfavorable for vinegar production because acetate generated in the cytoplasm tends to be rapidly metabolized via the tricarboxylic acid (TCA) cycle.

The enzymatic activities of several TCA cycle enzymes and acetyl-CoA synthetase in *Acetobacter* species are upregulated during the overoxidation of acetate (Jucker and Ettlinger 1985; Saeki et al. 1999). Complete oxidation of acetate to carbon dioxide via the TCA cycle produces NADH, which is utilized as a substrate for aerobic respiration. Several TCA cycle intermediates are also utilized as precursors for the synthesis of amino acids, nucleotides, and cofactors. Therefore, acetate is utilized as both an energy and a carbon source during the overoxidation process. The switching mechanism between the incomplete oxidation of ethanol and overoxidation of acetate, and the control mechanism of energy and carbon metabolism in acetic acid bacteria, are not fully understood. To better understand the molecular genetic basis of these processes, this chapter provides an overview of the genomic features and transcriptome profiles of *A. aceti* NBRC 14818 under various growth conditions.

12.2 Genome Features of *Acetobacter aceti*

12.2.1 Gene Organization for Central Carbon Metabolism

We have reported the draft genome sequence of *A. aceti* NBRC 14818, which is the wild-type strain of *A. aceti* and was isolated from an alcoholic beverage that had turned to vinegar (Sakurai et al. 2011). The NBRC 14818 genome encodes the genes required for the TCA cycle: citrate synthase (*aarA*), aconitase (*acnA*), two isocitrate dehydrogenases (*icd1* and *icd2*), 2-oxoglutarate dehydrogenase (*sucAB*), succinyl-CoA synthetase (*sucCD*), succinate dehydrogenase (*sdhABCD*), and two

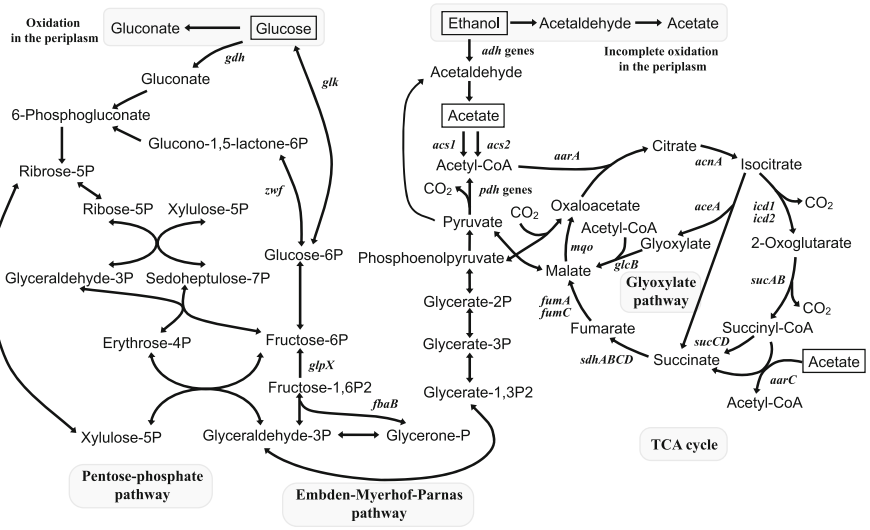

Fig. 12.1 Predicted central carbon metabolic pathway of *Acetobacter aceti* NBRC 14818. (Reproduced from Sakurai et al. 2011)

types of fumarases (*fumA* and *fumC*) (Fig. 12.1). Although the gene for malate dehydrogenase was not identified in the genome, the oxidation of malate to oxaloacetate may be catalyzed by malate:quinone oxidoreductase (*mqo*), as was previously reported for *A. aceti* 1023 (Mullins et al. 2008). It is also possible that succinyl-CoA synthetase activity is bypassed by the succinyl-CoA:acetate CoA transferase (SCACT) encoded by *aarC* (Mullins et al. 2008). The *aceA* and *glcB* genes, encoding isocitrate lyase and malate synthase, respectively, comprise the glyoxylate pathway and are clustered together in the genome.

In contrast to *A. aceti* NBRC 14818, the phylogenetically related bacterium *Acetobacter pasteurianus* NBRC 3283 lacks genes for succinyl-CoA synthetase and the glyoxylate pathway enzymes (Azuma et al. 2009). A vinegar factory isolate, *A. aceti* 1023, was also reported to lack these genes (Mullins et al. 2008), a finding that is consistent with the draft genome sequence of *A. aceti* 1023, which indicates that this strain is more closely related to *A. pasteurianus* NBRC 3283 than *A. aceti* NBRC 14818 (Hung et al. 2014). Among acetic acid bacteria, *Gluconacetobacter diazotrophicus* and *Granulibacter bethesdensis* also possess complete TCA cycle and glyoxylate pathway genes, whereas several TCA cycle and glyoxylate pathway genes are missing in *Gluconobacter oxydans* (Bertalan et al. 2009; Greenberg et al. 2007; Prust et al. 2005). The observed distribution of TCA cycle and glyoxylate pathway genes among acetic acid bacteria indicates that the efficiency of acetate anabolism varies according to the species.

In the genome of NBRC 14818, genes encoding gluconeogenetic Embden–Meyerhof–Parnas (EMP) pathway enzymes were also identified, but the gene for phosphofructokinase, which phosphorylates fructose 6-phosphate during

glycolysis, was not detected, suggesting that glycolysis does not proceed via the EMP pathway in this strain. However, a complete set of genes for the pentose phosphate pathway (PPP) were identified in the NBRC 14818 genome, suggesting that glucose is degraded via PPP (Fig. 12.1). The gene for 2-keto-3-deoxyglucose-phophate aldolase was not found in the genome, indicating that the Entner–Doudoroff pathway might not function in NBRC 14818. A gene encoding pyruvate kinase was identified in the genome of NBRC 14818, which is able to grow on glucose. This feature is in contrast to *A. aceti* NCIB 8554, which reportedly lacks pyruvate kinase activity and does not grow on glucose (Flückiger and Ettlinger 1977).

In acetic acid bacteria, ethanol is oxidized to acetate via acetaldehyde through sequential reactions catalyzed by ADH and ALDH (Fig. 12.1). In addition to genes encoding membrane-bound PQQ-dependent ADH (*adhAB*/*adhS*), the *adh1* and *adh2* genes for soluble NAD^+-dependent ADHs, which share high homology with the genes encoding ADH I and ADH II of *A. pasteurianus* SKU1108, respectively (Chinnawirotpisan et al. 2003), were also identified in the NBRC 14818 genome. Membrane-bound ALDH, which is encoded by *aldFGH*, may be involved in the accumulation of acetate resulting from the incomplete oxidation of ethanol in the periplasm. Several genes encoding a putative soluble $NAD(P)^+$-dependent ALDH, which might be involved in the cytoplasmic generation of acetate, were also identified in the NBRC 14818 genome.

Acetate is converted to acetyl-CoA before becoming completely oxidized in the TCA cycle. In general, two pathways are involved in the conversion of acetate to acetyl-CoA: one pathway is catalyzed by acetyl-CoA synthetase (Acs) and the other is mediated by phosphotransacetylase (Pta) and acetate kinase (Ack) (Saeki et al. 1997). The generation of acetyl-CoA from acetate may also be catalyzed by the aforementioned SCACT, which is encoded by *aarC* in acetic acid bacteria (Mullins et al. 2008). Two genes (*acs1* and *acs2*) encoding putative Acs enzymes have been identified in the NBRC 14818 genome, but genes encoding Pta and Ack are not present, indicating that the Acs pathway and SCACT initiate the metabolism of acetate in NBRC 14818.

12.2.2 Gene Organization for the Respiratory Chain

A. aceti is an obligately aerobic bacterium that uses molecular oxygen as the terminal electron acceptor of the respiratory electron transport chain. The genome sequence of NBRC 14818 indicates that this strain has a highly branched respiratory chain (Fig. 12.2). Notably, three sets of NADH dehydrogenase genes were identified in the genome. The *nuo1* and *nuo2* gene clusters encode multi-component type I enzymes (complex I), whereas the *ndh* gene encodes a single-subunit type II enzyme. In general, the type I enzymes, but not the type II enzymes, have proton-pumping activity (Calhoun et al. 1993; Leif et al. 1995). Four ubiquinol oxidase gene clusters were also identified in the genome (Fig. 12.2). The *cyaBACD*

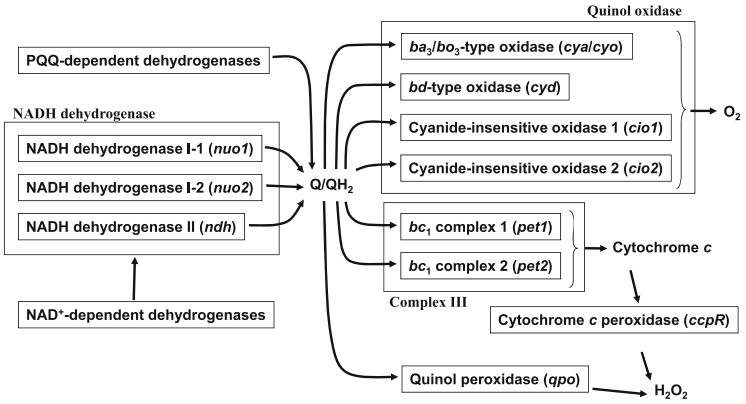

Fig. 12.2 Branched respiratory chain of *A. aceti* NBRC 14818 predicted from the genome sequence. *Arrows* indicate electron transport reactions; Q/QH_2 indicates the ubiquinone-ubiquinol pool. (Reproduced from Sakurai et al. 2011)

(*cyoABCD*) genes encode a ba_3- or bo_3-type quinol oxidase (Ba3/Bo3), which belongs to the heme copper oxidoreductase superfamily, whereas the *cydAB*, *cioA1B1*, and *cioA2B2* gene clusters encode cytochrome *bd* family quinol oxidases. The *cydAB* genes encode a canonical *bd* oxidase (Cyd), whereas the *cioA1B1* and *cioA2B2* genes encode cyanide-insensitive oxidases (CIO1 and CIO2). CIO is similar in amino acid sequence, but phylogenetically distinct, to canonical *bd* oxidases. The conserved sequence of the periplasmic loop (Q-loop), which contains the putative quinol oxidizing site, is significantly shorter in CIOs than in canonical *bd* oxidases (Cunningham et al. 1997). Recent analyses showed that the CIO of *G. oxydans* contains hemes b_{558}, b_{595}, and *d*, as in the case of canonical *bd* oxidases, but has a significantly higher turnover rate (Miura et al. 2013; Mogi et al. 2009). In contrast to the cytochrome *bd* oxidase (*bd*-I) of *Escherichia coli*, which has high affinity for oxygen, the CIOs of *Campylobacter jejuni*, *G. oxydans*, and *Pseudomonas aeruginosa* have low oxygen affinity (Arai et al. 2014; Jackson et al. 2007; Miura et al. 2013).

A gene encoding a protein similar to subunit I of cytochrome *c* oxidase, *ctaD* (*coxA*), was identified in the NBRC 14818 genome. Bacterial cytochrome *c* oxidase is generally composed of three major subunits, but the genes for subunits II and III were not found in the genome. Similarly, a gene corresponding to *ctaD* (*coxA*) was also detected in the genomes of *A. pasteurianus* NBRC 3283 and *G. oxydans* 621H, but no genes encoding subunits II and III of cytochrome *c* oxidase were detected (Azuma et al. 2009; Prust et al. 2005), suggesting that cytochrome *c* oxidase is not active in these acetic acid bacteria. In contrast, *Ga. diazotrophicus* Pal5 also encodes an orphan *ctaD* (*coxA*)-like gene and has a complete set of genes for a putative cytochrome *c* oxidase (Bertalan et al. 2009).

Two gene clusters, *petB1C1A1* and *petB2C2*, encoding subunits of the cytochrome bc_1 complex (complex III), were identified in the NBRC 14818 genome. The *petB1C1A1* genes encode the cytochromes *b* and c_1 subunits and a Rieske Fe-S

protein, and the *petB2C2* genes encode another set of cytochromes *b* and c_1 subunits. The organization of these two *pet* gene clusters was identical to that observed in *A. pasteurianus* NBRC 3283 (Azuma et al. 2009). In contrast, *G. oxydans* 621H and *Ga. diazotrophicus* Pal5 have only one set of cytochrome bc_1 complex genes (Bertalan et al. 2009; Prust et al. 2005). It is not clear why *A. aceti*, *A. pasteurianus*, and *G. oxydans* possess cytochrome bc_1 complex genes, even though these strains do not have a functional cytochrome *c* oxidase. One of the candidates that may receive electrons from the cytochrome bc_1 complex via cytochrome *c* is cytochrome *c* peroxidase, which is encoded by *ccpR* (Fig. 12.2).

12.3 Transcriptome Profiling of *A. aceti*

We have investigated the DNA microarray-based transcriptome profiles of *A. aceti* NBRC 14818 under various growth conditions (Sakurai et al. 2011, 2012). The regulation patterns of the genes involved in central carbon metabolism and respiratory components, and the genes that show significant expression changes according to the growth conditions, are described next.

12.3.1 Repression of TCA Cycle Genes by Ethanol

All genes encoding the enzymes that constitute the complete TCA cycle, namely citrate synthase (*aarA*), aconitase (*acnA*), isocitrate dehydrogenases (*icd1* and *icd2*), 2-oxoglutarate dehydrogenase (*sucAB*), succinyl-CoA synthetase (*sucCD*), succinate dehydrogenase (*sdhABCD*), fumarases (*fumA* and *fumC*), malate:quinone oxidoreductase (*mqo*), and SCACT (*aarC*), exhibit relatively high expression levels when NBRC 14818 is cultivated using glucose or acetate as a carbon source. However, these TCA cycle genes are significantly downregulated when ethanol is present in the culture medium, regardless of the glucose level (Figs. 12.1 and 12.3). *A. aceti* shows a diauxic growth profile when cultured on ethanol as the carbon source. In the first exponential growth phase, ethanol is incompletely oxidized to acetate and the expression levels of TCA cycle genes are very low. However, during the second exponential phase, when the accumulated acetate is consumed, the TCA cycle genes are significantly upregulated. These expression profiles indicate that an unidentified regulatory mechanism represses the TCA cycle genes in response to ethanol utilization and that the metabolic flow through the TCA cycle is significantly lower when ethanol is present in the culture medium.

It was reported that ethanol inhibits the uptake of acetate by *A. aceti* NCBI 8554, and that the activities of several TCA cycle enzymes are lower in cells grown on ethanol (Jucker and Ettlinger 1985). Carbon flux through the TCA cycle was also shown to be reduced in ethanol-grown cells of *Gluconacetobacter xylinus* (*Komagataeibacter medellinensis*) I 2281 (Kornmann et al. 2003). Ethanol also

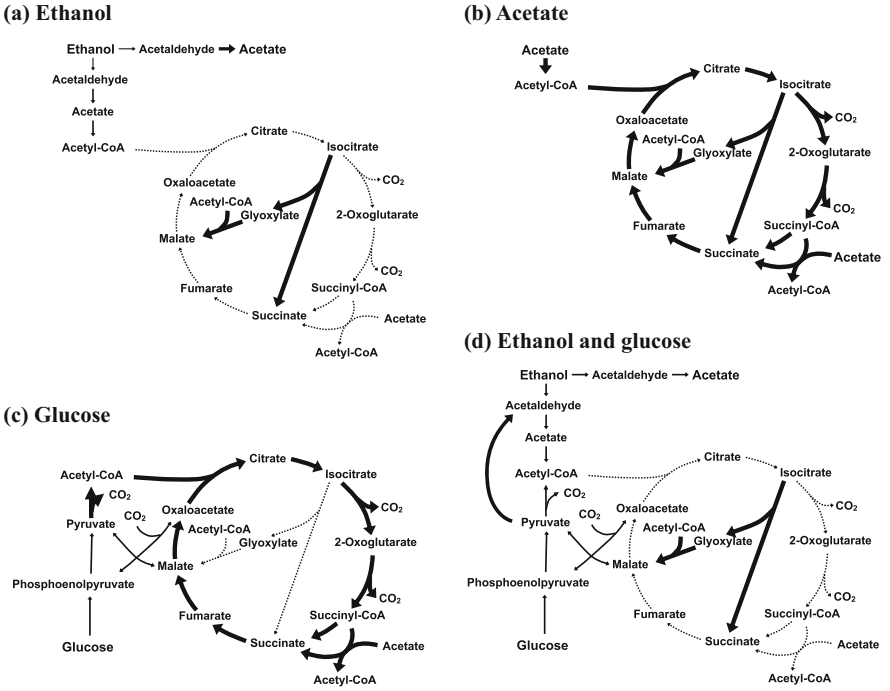

Fig. 12.3 Schematic representations of the transcription profiles of the central carbon metabolism genes in *A. aceti* NBRC 14818 grown on ethanol (**a**), acetate (**b**), glucose (**c**), and ethanol and glucose (**d**). The thickness of the *arrows* indicates the relative expression level of the genes involved in the reactions. *Dotted lines* indicate that the genes involved in the reactions were significantly downregulated under test conditions

significantly impaired the Pta activity of strain I 2281, which was proposed to be the reason for the low carbon flux of the TCA cycle in this strain. Although *A. aceti* NBRC 14818 does not possess genes for the Pta-Ack pathway, the expression levels of *acs2* and *aarC* are lower in cells during the incomplete oxidation of ethanol than in cells during acetate overoxidation or in cells cultured on acetate. Taken together, these findings indicate that carbon flow through the TCA cycle of acetic acid bacteria is generally reduced during the incomplete oxidation of ethanol. The low flux rate of the TCA cycle in the presence of ethanol causes cytoplasmic accumulation of acetate and/or acetyl-CoA, which might inhibit the further production and uptake of acetate in the cytoplasm. The metabolic switch from incomplete ethanol oxidation to acetate overoxidation might be mediated by derepression of the TCA cycle after the depletion of ethanol.

The genes encoding the glyoxylate pathway enzymes isocitrate lyase (*aceA*) and malate synthase (*glcB*) are expressed at high levels when NBRC 14818 is grown on acetate or ethanol as a carbon source (Fig. 12.3). However, these genes are not expressed when cells are grown on glucose. The glyoxylate pathway functions as an anaplerotic shunt of the TCA cycle and supplies oxaloacetate when carbon sources

that are metabolized through acetyl-CoA as an obligate intermediate are utilized. Therefore, the glyoxylate pathway may be important for acetate overoxidation. Elevated expression of the glyoxylate pathway genes during the incomplete oxidation of ethanol suggests that a small fraction of the carbon derived from ethanol is utilized for anabolism, although ethanol is nearly stoichiometrically converted to acetate (Sakurai et al. 2012).

Ethanol is oxidized to acetate via acetaldehyde by the sequential reactions of ADH and ALDH. Unexpectedly, however, the genes encoding PQQ-dependent ADH (*adhA*) and two NAD^+-ADHs (*adh1* and *adh2*) are slightly downregulated in NBRC 14818 cells grown on ethanol. The expression levels of these ADH genes do not significantly change according to the growth phases when cells are grown on ethanol. These results are contradictory to the finding that the PQQ-dependent ADH activity is higher in cells of *A. aceti* NCBI 8554 grown on ethanol (Jucker and Ettlinger 1985). It is likely that PQQ-ADH is post-translationally activated in ethanol-grown cells. In contrast, the genes for membrane-bound ALDH (*aldFGH*) are significantly upregulated during the incomplete oxidation of ethanol. The activities of ADH and ALDH must be coordinately regulated to avoid the accumulation of the toxic intermediate acetaldehyde. Thus, the activity of membrane-bound ALDH appears to be the rate-limiting step of ethanol oxidation in the periplasm.

12.3.2 Inhibition of Glucose Anabolism by Ethanol

Most of the genes involved in glycolysis and gluconeogenesis are expressed in *A. aceti* NBRC 14818 at relatively high levels irrespective of the carbon sources. The genes for fructose-1,6-bisphosphate aldolase (*fbaB*) and fructose-1,6-bisphosphatase (*glpX*) are upregulated in cells grown on acetate, indicating that the EMP pathway is operative for gluconeogenesis. In contrast, the glucose-6-phosphate dehydrogenase gene (*zwf*) and the gene encoding a soluble $NAD(P)^+$-dependent glucose dehydrogenase (*gdh*), which might be involved in the cytoplasmic oxidation of glucose to gluconate, are slightly upregulated in cells grown on glucose. This finding suggests that glucose is utilized through the PPP via either glucose-6-phosphate or gluconate in the cytoplasm (Fig. 12.1), although a large fraction of glucose is converted to gluconate as a dead-end product in the periplasm.

When NBRC 14818 is cultivated on both ethanol and glucose, cell growth is slower compared to growth in medium containing only glucose, but is comparable to that of cells cultured on only ethanol. In addition, the consumption of glucose and accumulation of gluconate are significantly retarded in the presence of both ethanol and glucose. The genes encoding the PPP enzymes transketolase and transaldolase are downregulated by ethanol, irrespective of the presence of glucose, as is observed for TCA cycle genes, although the expression of the PPP genes are maintained at relatively higher levels. These results suggest that ethanol is

preferentially utilized to glucose as a carbon and energy source and inhibits the metabolism of glucose.

The glyoxylate pathway genes *aceA* and *glcB* are highly expressed in NBRC 14818 cells cultured on both ethanol and glucose. This result was unexpected because oxaloacetate can be provided via phosphoenolpyruvate (PEP) or pyruvate from glycolysis. The expression levels of the PEP carboxylase (*ppc*) and malic enzyme (*maeA*) genes do not markedly differ according to the carbon sources. In contrast, the genes encoding pyruvate dehydrogenase (*pdh*) are upregulated when NBRC 14818 is grown on glucose, but not in medium containing both ethanol and glucose (Fig. 12.3). Similarly, the pyruvate decarboxylase gene is significantly upregulated in the presence of both ethanol and glucose, suggesting that pyruvate derived from glucose is converted to acetate via acetaldehyde (Fig. 12.3d). These transcriptome profiles of *A. aceti* indicate that carbon derived from glucose rarely enters the TCA cycle directly from PEP or pyruvate when ethanol coexists with glucose. This difference may also explain why the glyoxylate pathway genes are upregulated in NBRC 14818 cells grown on ethanol and glucose.

Acetate production from glucose is known as overflow metabolism in *E. coli* (el-Mansi and Holms 1989). Such a condition results when *E. coli* is cultivated aerobically in the presence of glucose, as pyruvate and acetyl-CoA are excessively produced because the metabolic flow through the glycolysis pathway is faster than that through the TCA cycle. The excess pyruvate is converted in the cytoplasm to acetate, which is then excreted from cells. The accumulation of acetate from glucose was also reported in *Gluconobacter oxydans* mutants deficient in glucose dehydrogenase genes (Krajewski et al. 2010). It was proposed that acetate was formed from pyruvate via pyruvate decarboxylase and $NADP^+$-dependent ALDH as a result of the high ALDH activity in these mutants.

12.3.3 Differential Expression of the Branched Respiratory Chain

A. aceti NBRC 14818 has a highly branched electron transport chain (Fig. 12.2). Microarray analyses have revealed that the components of the respiratory chain are differentially regulated according to the growth conditions.

Among the three sets of NADH dehydrogenase gene clusters identified in strain NBRC 14818, the *nuo2* genes show relatively high expression levels, suggesting that one of the type I enzymes encoded by the *nuo2* genes is the dominant NADH dehydrogenase under normal growth conditions. The *nuo2* genes are significantly downregulated in cells grown on ethanol, a response that may result from feeding of electrons directly into the respiratory chain from the PQQ-dependent ADH (Fig. 12.2) and the possibility that NADH regeneration activity in the cytoplasm is reduced by the low TCA cycle activity in the presence of ethanol. In contrast, the *nuo1* genes are significantly upregulated under low oxygen conditions, suggesting

that the type I enzyme encoded by the *nuo1* genes functions in microaerobic environments (unpublished data). The conditions influencing the expression of the *ndh* gene encoding the type II enzyme are not presently clear.

NBRC 14818 has four quinol oxidase gene clusters, *cyaBACD* (*cyoABCD*), *cydAB*, *cioA1B1*, and *cioA2B2*, which encode ba_3 or bo_3 oxidase (Ba3/Bo3), *bd* oxidase (Cyd), and two CIO oxidases (CIO1 and CIO2), respectively (Fig. 12.2). In general, bo_3 and CIO oxidases have low affinity for oxygen, whereas canonical *bd* oxidases have high oxygen affinity (Arai 2011; Arai et al. 2014; Jackson et al. 2007; Kita et al. 1984; Miura et al. 2013). The ability to form an electrochemical proton gradient across the cell membrane by consuming oxygen (H^+/O ratio), which correlates with the ATP generation efficiency, is higher for bo_3 oxidases than for *bd*-type or CIO oxidases (Arai et al. 2014; Bekker et al. 2009). Although the enzymatic features of the quinol oxidases of *A. aceti* NBRC 14818 have not been investigated, cells may produce greater amounts of energy when Ba3/Bo3 is used as a terminal oxidase of the respiratory chain compared to Cyd, CIO1, or CIO2. Ba3/Bo3, CIO1, and CIO2 might function under high-oxygen conditions, whereas Cyd might function mainly under low-oxygen conditions.

The *cya* (*cyo*) genes show relatively high expression levels, suggesting that Ba3/Bo3 is the main quinol oxidase under normal growth conditions. The expression levels of the *cya* (*cyo*) genes are downregulated when NBRC 14818 cells are grown on glucose. In contrast, the *cio2* genes show significant upregulation in the presence of glucose. Because glucose is rich in potential energy, these results are in good agreement with the prediction that the proton translocation efficiency of Ba3/Bo3 is higher than that of CIO2. In addition, because the *cio2* genes are significantly downregulated when NBRC 14818 is grown on acetate, which is a relative poor carbon source, or during acetate overoxidation in ethanol medium, CIO2 may be inefficient to produce sufficient energy when cultured on acetate.

The *cio1* genes are constitutively expressed in NBRC 14818 at relatively low levels. A phylogenetically close relative of *A. aceti*, *A. pasteurianus* NBRC 3283, does not have genes corresponding to the *cio1* gene cluster (Azuma et al. 2009). Thus, it is likely that CIO1 has a minor physiological role in NBRC 14818 under normal growth conditions. The *cyd* genes are expressed at relatively low levels when NBRC 14818 is grown on ethanol or glucose but are significantly downregulated when acetate is used as the carbon source. Interestingly, the *cyd* genes are also significantly downregulated in glucose medium when a neutral pH was maintained by the controlled addition of alkaline solution (unpublished data). These results indicate that Cyd is required under low pH conditions, which results when NBRC 14818 cells are grown on ethanol or glucose from the accumulation of acetate or gluconate through the activity of PQQ-dependent dehydrogenases. Under microaerobic conditions (2 % O_2), the *cya* (*cyo*) and *cyd* genes are significantly downregulated and upregulated, respectively (unpublished data), suggesting that Ba3/Bo3 and Cyd have low and high affinities for oxygen, respectively, as has been reported for the corresponding enzymes from other bacterial species.

12.3.4 Regulation of Stress-Responsive Genes

Several stress-responsive genes and genes encoding molecular chaperones, including *recA*, *recN*, *uvrABC*, *dinB*, *dnaJ*, *dnaK*, *clpB*, *hslVU*, and *htpG*, were found to be significantly upregulated when *A. aceti* NBRC 14818 is grown on ethanol (Sakurai et al. 2011). Upregulation of several of these genes was also reported in the ethanol-grown cells of *A. pasteurianus* NBRC 3283 (Ishikawa et al. 2010a, b; Okamoto-Kainuma et al. 2004). The expression levels of these stress-responsive genes in NBRC 14818 were low during the early ethanol oxidation phase, but were upregulated during the transition from the ethanol oxidation phase to the acetate overoxidation phase, indicating that the direct inducer of the expression of these genes is not ethanol but rather the metabolites derived from ethanol (Sakurai et al. 2012). Ethanol is oxidized to acetate via acetaldehyde by the sequential reactions of ADH and ALDH. It is likely that expression of stress-responsive and molecular chaperone genes in *A. aceti* is induced by damage caused by accumulation of the toxic intermediate acetaldehyde.

The genes encoding superoxide dismutase (*sod*) and catalase (*katE*) show high expression levels when NBRC 14818 is grown on glucose. These gene products are involved in the detoxification of reactive oxygen species (ROS). Upregulation of these oxidative stress-responsive genes indicates that ROS are generated during the degradation process of glucose.

12.4 Role of the Glyoxylate Pathway in Acetate Overoxidation

Acetobacter aceti NBRC 14818 carries genes encoding isocitrate lyase (*aceA*) and malate synthase (*glcB*), which constitute the glyoxylate pathway. However, these genes are not present in the genomes of *Acetobacter pasteurianus* NBRC 3283 or *A. aceti* 1023 (Azuma et al. 2009; Mullins et al. 2008). We found that acetic acid bacteria that are utilized for vinegar production also tend to lack the glyoxylate pathway genes. This pathway functions as an anaplerotic shunt of the TCA cycle to supply oxaloacetate when molecules that are metabolized through acetyl-CoA are utilized as a carbon source. For laboratory cultivation of acetic acid bacteria, complex organic substrates such as yeast extract and tryptone are typically added to the medium. Organic substrates are also used for industrial vinegar production. Because oxaloacetate can be supplied from amino acids or other compounds contained in organic substrates, the glyoxylate pathway is not necessarily required for the growth of acetic acid bacteria. This speculation is supported by the fact that *A. pasteurianus* NBRC 3283 and *A. aceti* 1023 are able to grow when acetate is used as a sole carbon source. Despite this ability, NBRC 14818 consumes accumulated acetate faster than *A. pasteurianus* NBRC 3283, indicating that the glyoxylate pathway promotes acetate overoxidation.

To examine this speculation, we constructed an *aceA-glcB* knockout mutant of NBRC 14818, designated strain GP, and examined the effect of glyoxylate pathway deficiency on acetate overoxidation (Sakurai et al. 2013). As expected, knockout of the glyoxylate pathway genes in NBRC 14818 had no detectable effect on the growth rate on glucose, indicating that oxaloacetate could be supplied from PEP when glucose was used as the sole carbon source (Fig. 12.3c). When strain GP was grown on ethanol, the growth rate during the incomplete oxidation of ethanol was similar to that of the wild-type strain. The accumulation rate of acetate from ethanol was also not affected by the mutation. Although the growth rate of the mutant during acetate overoxidation was lower than that of the wild-type strain, the acetate consumption rate was not affected, indicating that the glyoxylate pathway has an effect on acetate anabolism but is not indispensable for acetate consumption (Fig. 12.4a–c).

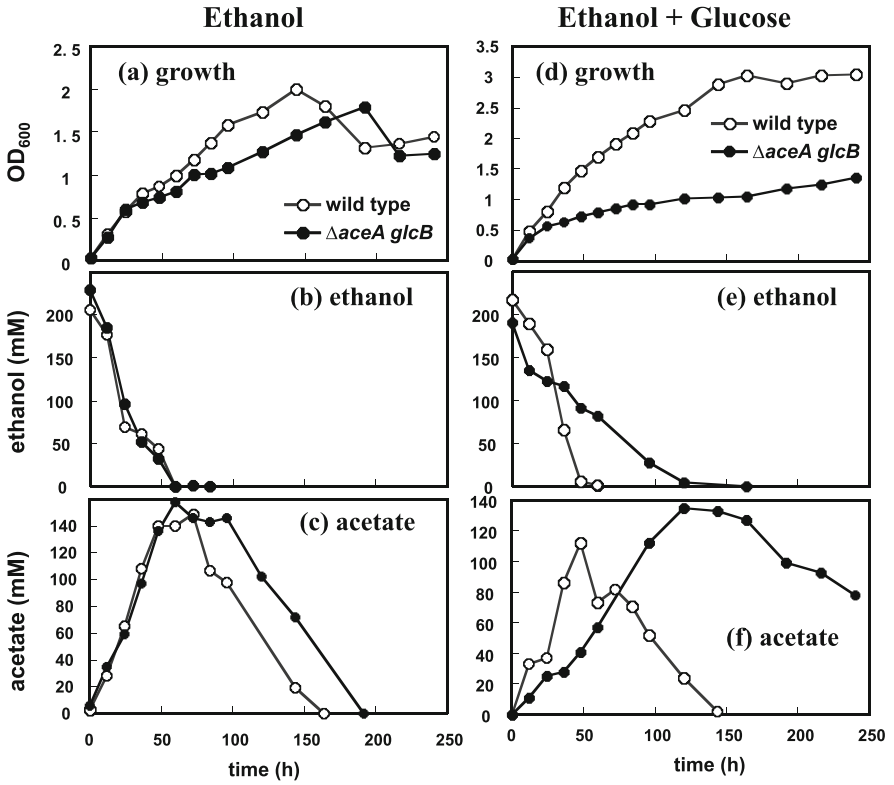

Fig. 12.4 Growth of *A. aceti* NBRC 14818 and its *aceA-glcB* knockout mutant (strain GP) in medium containing 0.2 M ethanol (**a**–**c**) or 0.2 M ethanol and 0.2 M glucose (**d**–**f**). Growth profiles of the two strains (**a**, **d**). Concentrations of ethanol (**b**, **e**) and acetate (**c**, **f**) in the culture medium. *Open* and *closed circles* indicate strains NBRC 14818 and GP, respectively. (Reproduced from Sakurai et al. 2013)

Strain GP exhibited markedly reduced growth compared to that of the wild-type strain in medium containing both ethanol and glucose (Fig. 12.4d–f). Notably, the growth impairment of the mutant was more severe than that observed in medium containing only ethanol or glucose, indicating that ethanol and glucose had synergistic adverse effects on growth. Although strain GP cells displayed poor growth, acetate accumulated in the medium nearly stoichiometrically with the consumption of ethanol and reached a maximum concentration that was comparable to that of the wild-type strain. Notably, however, the accumulated acetate was not consumed immediately in strain GP and remained at higher levels for a longer duration compared to wild-type cells (Fig. 12.4f). Because the media used for industrial vinegar production contain ethanol and various carbohydrates, the glyoxylate pathway-deficient mutant may be suitable for use in acetate production.

As suggested by the aforementioned transcriptome analyses of NBRC 14818, the flux rate of the TCA cycle is low and glucose carbon rarely enters into the TCA cycle via the anaplerotic route from PEP or pyruvate when both ethanol and glucose are present in the culture medium. Microarray analysis of strain GP showed that the TCA cycle genes are significantly downregulated in cell culture in medium containing ethanol and glucose, as is observed for the wild-type strain. The genes encoding malic enzyme (*maeA*), fumarase (*fumC*), a putative $NAD(P)^+$-dependent ALDH, and an Acs enzyme (*acs1*) were upregulated more than two fold in strain GP compared to the wild-type strain (Fig. 12.5a). Because malic enzyme can supply malate from pyruvate, and fumarase generates malate as a reaction product, these two enzymes may be responsible for malate deficiency resulting from disruption of the glyoxylate pathway. The pyruvate decarboxylase gene is significantly upregulated in the wild-type strain in the presence of ethanol and glucose. In addition, the gene was upregulated more than 1.5 fold in the mutant compared to the wild-type strain, suggesting that the lack of the glyoxylate pathway promotes the overflow metabolism of glucose. The low flux of the TCA cycle and upregulation of pyruvate decarboxylase, soluble ALDH, and Acs lead to cytoplasmic accumulation of acetyl-CoA by the overflow metabolism of glucose, which might inhibit the cytoplasmic metabolism of ethanol and uptake of extracellular acetate. This metabolic feature of the glyoxylate pathway mutant may explain the observed retardation of acetate overoxidation in medium containing both ethanol and glucose.

The impaired growth of the GP mutant in the presence of ethanol and glucose is likely caused by the toxic effects of acetaldehyde. Supporting this hypothesis, several stress-responsive genes, including *uvrB*, *clpB*, *hspA*, and *katE*, are significantly upregulated in strain GP when cultured in medium containing ethanol and glucose, indicating that the mutant strain was subjected to stress under these growth conditions. However, the observed growth impairment under these conditions is not observed in *A. pasteurianus* NBRC 3283, even though this strain does not have a functional glyoxylate pathway. NBRC 3283 possesses the genes comprising the Pta-Ack pathway, pyruvate oxidase (*pox*) and pyruvate:ferredoxin oxidoreductase (*por*), which have not been identified in *A. aceti* NBRC 14818 (Fig. 12.5b).

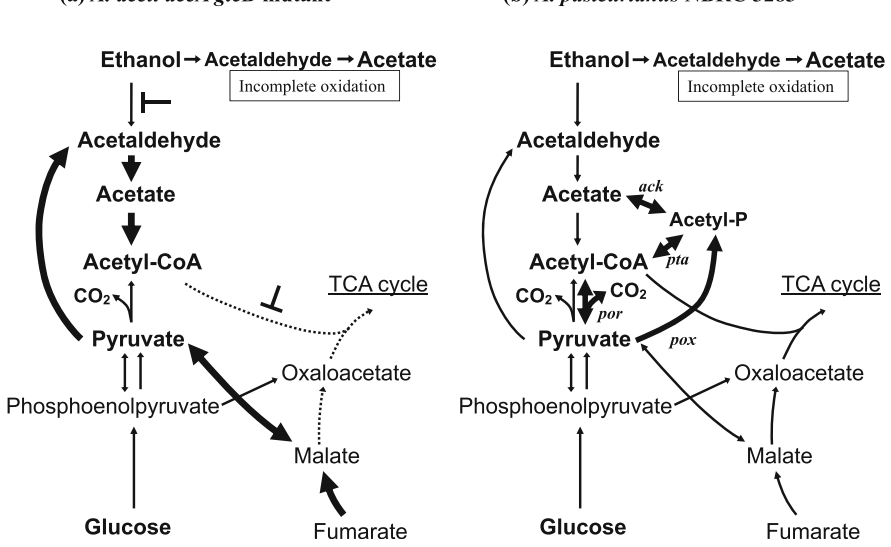

Fig. 12.5 (**a**) Schematic representations of the transcription profiles of the pyruvate metabolism genes in the *aceA-glcB* knockout mutant of *A. aceti* NBRC 14818 grown in the presence of ethanol and glucose. *Thick* and *dotted lines* indicate genes that are significantly upregulated and downregulated, respectively. *Lines* with terminal *bars* indicate reactions that are predicted to be inhibited or restricted. (**b**) Predicted metabolic pathway of pyruvate in *A. pasteurianus* NBRC 3283. *Thick lines* indicate genes for reactions that have not been identified in the genome of *A. aceti* NBRC 14818

The cytoplasmic accumulation of acetaldehyde as a toxic by-product might be avoided by these bypass reactions in *A. pasteurianus* NBRC 3283.

12.5 Conclusions and Future Perspectives

Transcriptome analyses have revealed that ethanol significantly represses the TCA cycle at the transcriptional level in *A. aceti* NBRC 14818 (Sakurai et al. 2011, 2012). The low TCA cycle activity may explain why acetate is accumulated by the incomplete oxidation of ethanol. Because oxidation of ethanol by the PQQ-dependent ADH feeds electrons to quinol oxidases via ubiquinone, ATP may be generated in strain NBRC 14818 by the truncated respiratory chain (Yakushi and Matsushita 2010). During the incomplete oxidation of ethanol by acetic acid bacteria, ethanol is exclusively utilized as an energy source, and the TCA cycle functions only to meet the demand for synthesis of cell materials. Thus, there must be a global transcriptional regulator in *A. aceti* that represses the TCA cycle genes in the presence of ethanol. In *E. coli*, TCA cycle genes are regulated by the ArcAB two-component regulatory system in response to the cellular redox status (Gunsalus

and Park 1994). Although genes corresponding to *arcAB* have not been identified in the genomes of *Acetobacter* species, the regulation of TCA cycle genes might be mediated by functionally similar orthologues.

When ethanol and glucose are both present in the culture medium, the overflow metabolism of glucose might occur in *A. aceti* NBRC 14818 as a consequence of the ethanol-mediated repression of TCA cycle genes and the relatively high expression levels of glycolytic enzyme genes. Such overflow metabolism accelerates the cytoplasmic accumulation of acetate and acetyl-CoA, which inhibit ethanol oxidation in the cytoplasm, and leads to incomplete oxidation of ethanol in the periplasm. A deficiency in the glyoxylate pathway promotes the overflow metabolism of glucose and is beneficial for industrial vinegar production from ethanol.

Most of the metabolic features of *A. aceti* described here are predominantly speculations based on transcriptome profiles. Confirmation of these features by biochemical and metabolomic analyses, and identification of the global regulator that represses the TCA cycle genes in response to ethanol, are required for better understanding of the physiology of *A. aceti*.

References

Arai H (2011) Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. Front Microbiol 2:103

Arai H, Kawakami T, Osamura T, Hirai T, Sakai Y, Ishii M (2014) Enzymatic characterization and *in vivo* function of five terminal oxidases in *Pseudomonas aeruginosa*. J Bacteriol 196:4206–4215

Asai T (1968) Acetic acid bacteria: classification and biochemical activities. University of Tokyo Press, Tokyo

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37:5768–5783

Bekker M, de Vries S, Ter Beek A, Hellingwerf KJ, Teixeira de Mattos MJ (2009) Respiration of *Escherichia coli* can be fully uncoupled via the nonelectrogenic terminal cytochrome *bd*-II oxidase. J Bacteriol 191:5510–5517

Bertalan M, Albano R, de Pádua V, Rouws L, Rojas C, Hemerly A, Teixeira K, Schwab S, Araujo J, Oliveira A et al (2009) Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics 10:450

Calhoun MW, Oden KL, Gennis RB, Teixeira de Mattos MJ, Neijssel OM (1993) Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. J Bacteriol 175:3020–3025

Chinnawirotpisan P, Matsushita K, Toyama H, Adachi O, Limtong S, Theeragool G (2003) Purification and characterization of two NAD-dependent alcohol dehydrogenases (ADHs) induced in the quinoprotein ADH-deficient mutant of *Acetobacter pasteurianus* SKU1108. Biosci Biotechnol Biochem 67:958–965

Cunningham L, Pitt M, Williams HD (1997) The *cioAB* genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome *bd* quinol oxidases. Mol Microbiol 24:579–591

El-Mansi EM, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J Gen Microbiol 135:2875–2883

Flückiger J, Ettlinger L (1977) Glucose metabolism in *Acetobacter aceti*. Arch Microbiol 114:183–187

Greenberg DE, Porcella SF, Zelazny AM, Virtaneva K, Sturdevant DE, Kupko JJ 3rd, Barbian KD, Babar A, Dorward DW, Holland SM (2007) Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis*. J Bacteriol 189:8727–8736

Gunsalus RP, Park SJ (1994) Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. Res Microbiol 145:437–450

Hung JE, Mill CP, Clifton SW, Magrini V, Bhide K, Francois JA, Ransome AE, Fulton L, Thimmapuram J, Wilson RK, Kappock TJ (2014) Draft genome sequence of *Acetobacter aceti* strain 1023, a vinegar factory isolate. Genome Announc 2:e00550–00514

Ishikawa M, Okamoto-Kainuma A, Jochi T, Suzuki I, Matsui K, Kaga T, Koizumi Y (2010a) Cloning and characterization of *grpE* in *Acetobacter pasteurianus* NBRC 3283. J Biosci Bioeng 109:25–31

Ishikawa M, Okamoto-Kainuma A, Matsui K, Takigishi A, Kaga T, Koizumi Y (2010b) Cloning and characterization of *clpB* in *Acetobacter pasteurianus* NBRC 3283. J Biosci Bioeng 110:69–71

Jackson RJ, Elvers KT, Lee LJ, Gidley MD, Wainwright LM, Lightfoot J, Park SF, Poole RK (2007) Oxygen reactivity of both respiratory oxidases in *Campylobacter jejuni*: the *cydAB* genes encode a cyanide-resistant, low-affinity oxidase that is not of the cytochrome *bd* type. J Bacteriol 189:1604–1615

Jucker W, Ettlinger L (1985) The inhibition of acetate oxidation by ethanol in *Acetobacter aceti*. Arch Microbiol 143:283–289

Kita K, Konishi K, Anraku Y (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome b_{558}-*d* complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J Biol Chem 259:3375–3381

Kornmann H, Duboc P, Niederberger P, Marison I, von Stockar U (2003) Influence of residual ethanol concentration on the growth of *Gluconacetobacter xylinus* I 2281. Appl Microbiol Biotechnol 62:168–173

Krajewski V, Simic P, Mouncey NJ, Bringer S, Sahm H, Bott M (2010) Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation. Appl Environ Microbiol 76:4369–4376

Leif H, Sled VD, Ohnishi T, Weiss H, Friedrich T (1995) Isolation and characterization of the proton-translocating NADH: ubiquinone oxidoreductase from *Escherichia coli*. Eur J Biochem 230:538–548

Miura H, Mogi T, Ano Y, Migita CT, Matsutani M, Yakushi T, Kita K, Matsushita K (2013) Cyanide-insensitive quinol oxidase (CIO) from *Gluconobacter oxydans* is a unique terminal oxidase subfamily of cytochrome *bd*. J Biochem (Tokyo) 153:535–545

Mogi T, Ano Y, Nakatsuka T, Toyama H, Muroi A, Miyoshi H, Migita CT, Ui H, Shiomi K, Omura S, Kita K, Matsushita K (2009) Biochemical and spectroscopic properties of cyanide-insensitive quinol oxidase from *Gluconobacter oxydans*. J Biochem (Tokyo) 146:263–271

Mullins EA, Francois JA, Kappock TJ (2008) A specialized citric acid cycle requiring succinyl-coenzyme A (CoA): acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. J Bacteriol 190:4933–4940

Okamoto-Kainuma A, Yan W, Fukaya M, Tukamoto Y, Ishikawa M, Koizumi Y (2004) Cloning and characterization of the *dnaKJ* operon in *Acetobacter aceti*. J Biosci Bioeng 97:339–342

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Saeki A, Taniguchi M, Matsushita K, Toyama H, Theeragool G, Lotong N, Adachi O (1997) Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Biosci Biotechnol Biochem 61:317–323

Saeki A, Matsushita K, Takeno S, Taniguchi M, Toyama H, Theeragool G, Lotong N, Adachi O (1999) Enzymes responsible for acetate oxidation by acetic acid bacteria. Biosci Biotechnol Biochem 63:2102–2109

Sakurai K, Arai H, Ishii M, Igarashi Y (2011) Transcriptome response to different carbon sources in *Acetobacter aceti*. Microbiology 157:899–910

Sakurai K, Arai H, Ishii M, Igarashi Y (2012) Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. J Biosci Bioeng 113:343–348

Sakurai K, Yamazaki S, Ishii M, Igarashi Y, Arai H (2013) Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. J Biosci Bioeng 115:32–36

Yakushi T, Matsushita K (2010) Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. Appl Microbiol Biotechnol 86:1257–1265

Chapter 13
Membrane-Bound Dehydrogenases of Acetic Acid Bacteria

Osao Adachi and Toshiharu Yakushi

Abstract One of the major key features of acetic acid bacteria is their strong oxidation ability of alcohols and sugars, resulting in quantitative production of oxidized compounds. Respiratory chains consisting of ubiquinone, terminal ubiquinol oxidase, and several primary membrane-bound dehydrogenases are responsible for this unique ability. Here, we describe recent progress in the understanding of enzymatic and molecular properties and biogenesis of the membrane-bound dehydrogenases, such as pyrroloquinoline quinone-dependent alcohol dehydrogenase–cytochrome complex, and recent findings on new membrane-bound dehydrogenases. Quinate oxidation by quinate dehydrogenase (QDH) of acetic acid bacteria is a key agent in the vitro shikimate production process composed of the membranes containing QDH and 3-dehydroquinate dehydratase and $NADP^+$-dependent shikimate dehydrogenase. The addition of a catalytic amount of $NADP^+$ and an NADPH-regeneration system drive the process forward to produce shikimate with almost 100 % yield. The pentose oxidation respiratory chain produces 4-keto-D-arabonate or 4-keto-D-ribonate, depending on the substrate. Novel three different membrane-bound enzymes are indicated: D-aldopentose 4-dehydrogenase, 4-keto-D-aldopentose 1-dehydrogenase, and D-pentonate 4-dehydrogenase.

Keywords Flavoprotein • Molybdoprotein • Quinoportein • Quinate • Respiratory chain • Shikimate • 4-Keto-D-aldopentonate • D-Aldopentonate

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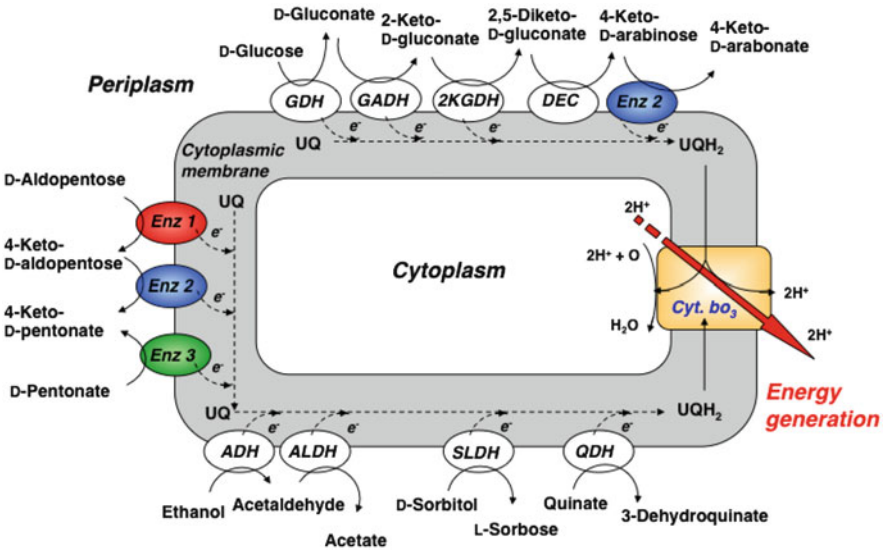

Fig. 13.1 Substance oxidation respiratory chains of acetic acid bacteria. Many kinds of membrane-bound dehydrogenases oxidize the cognate substrates with coupling to reduction of ubiquinone (*UQ*). The reduced ubiquinone (UQH_2) is then directly reoxidized by the terminal ubuquinol oxidase (Cyt. bo_3). The colors of Enz 1, Enz 2, and Enz 3 are related to those in Fig. 13.7. DEC, putative 2,5-diketo-D-gluconate decarboxylase; other abbreviations are referred to the text

13.1 Introduction

Incomplete oxidation with acetic acid bacteria is carried out through oxidation reactions by membrane-bound dehydrogenases located on the periplasmic surface of the cytoplasmic membrane. These dehydrogenases reduce membranous ubiquinone (UQ) coupled with the substrate oxidation. Reduced form of ubiquinone (UQH_2) is then directly re-oxidized by the terminal ubiquinol oxidases. Thus, several kinds of the dehydrogenases function as the primary dehydrogenase in substance-oxidizing respiratory chains (Matsushita et al. 1994) (Fig. 13.1). For example, ethanol- and glucose-oxidizing respiratory chains carry out incomplete oxidations of ethanol and D-glucose, respectively. Although some enzymes are poorly examined in their physiological electron acceptor, all the membrane-bound dehydrogenases mentioned in this chapter are thought to react with membranous ubiquinone (Table 13.1). We briefly review "conventional" membrane-bound dehydrogenases of acetic acid bacteria in the first part. The second part explains new membrane-bound dehydrogenases found recently and their applications.

Table 13.1 Membrane-bound dehydrogenases of acetic acid bacteria

Category	Enzyme	Substrate	Product	Subunit composition	Prosthetic group 1[a]	Prosthetic group 2[a]	Electron acceptor[b]	References
Quinoprotein–cytochrome complex	ADH EC1.1.5.5	Ethanol	Acetaldehyde	αβγ or αβ	PQQ	4 Heme C	UQ	Yakushi and Matsushita (2010)
Molybdoprotein–cytochrome complex	ALDH EC1.2.99.3	Acetaldehyde	Acetic acid	αβγ	MCD	2 [2Fe-2S] 3 Heme C / Heme B	–	Thurner et al. (1997)
Flavoprotein–cytochrome complex	GADH EC1.1.99.3	D-Gluconate	2-Keto-D-gluconate	αβγ	FAD	3 Heme C	UQ	Shinagawa et al. (1984)
	2KGDH EC1.1.99.4	2-Keto-gluconate	2,5-Diketo-D-gluconate	αβγ	FAD	3 Heme C	–	Shinagawa et al. (1981)
	FDH EC1.1.99.11	D-Fructose	5-Keto-D-fructose	αβγ	FAD	3 Heme C	–	Ameyama et al. (1981a)
	SLDH EC1.1.99.21	D-Sorbitol	L-Sorbose	αβγ	FAD	3 Heme C	–	Shinagawa et al. (1982)
Membrane-bound quinoprotein	GDH EC1.1.5.2	D-Glucose	Glucono-δ-lactone	α	PQQ	–	UQ	Ameyama et al. (1981b)
	GLDH EC1.1.99.22	Polyols	Ketones	αβ	PQQ	–	UQ	Matsushita et al. (2003)
	QDH EC1.1.5.8	Quinate	3-Dehydroquinate	α	PQQ	–	UQ	Vangnai et al. (2004)
	IDH	myo-Inositol	2-Keto-myo-inositol	α	PQQ	–	–	Hölscher et al. (2007)

(continued)

Table 13.1 (continued)

Others	SDH EC1.1.99.12	L-Sorbose	L-Sorbosone	α	FAD	–	UQ	Sugisawa et al. (1991)
	SNDH	L-Sorbosone	2-Keto-L-gulonate	α	–	–	–	Shinjoh et al. (1995)

[a]Prosthetic groups 1 and 2 that are involved in substrate oxidation and that are involved in intramolecular electron transfer, respectively

[b]Physiological electron acceptor: those for all the dehydrogenases are assumed to be ubiquinone, but UQ (ubiquinone) was placed in the table based on experimental data

13.2 Fermentation and Membrane-Bound Dehydrogenases

Acetic acid fermentation is carried out by sequential oxidations of ethanol to acetic acid via acetaldehyde. The membrane-bound, pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH) and membrane-bound aldehyde dehydrogenase (ALDH) constitute the ethanol-oxidizing respiratory chain on the outer surface of the cytoplasmic membrane of acetic acid bacteria: *Komagataeibacter*, *Acetobacter*, and *Gluconobacter* (Thurner et al. 1997; Yakushi and Matsushita 2010; Adachi et al. 1980). Unfortunately, it was long misunderstood that these oxidation reactions are mediated by cytoplasmic NAD^+- or $NADP^+$-dependent dehydrogenases. However, explanations for acetic acid fermentation were revised in the textbook as the current understanding in 2003 (Madigan et al. 2003).

Gluconate fermentation is carried out by a single oxidation of D-glucose to produce glucono-δ-lactone, which is converted to D-gluconate spontaneously or with membrane-bound gluconolactonase. 2-Ketogluconate and 5-ketogluconate fermentations are carried out by a single oxidation in position C2 or C5 of D-gluconate, respectively. 2-Ketogluconate (2KGA) is further oxidized at position C5 to produce 2,5-diketogluconate.

13.3 Membrane-Bound Dehydrogenases: An Overview

We take a quick look on the series of membrane-bound dehydrogenase of acetic acid bacteria. We try to categorize them into five groups, that is, quinoprotein–cytochrome complex, molybdoprotein-cytochrome complex, flavoprotein–cytochrome complex, membrane-bound quinoprotein, and others, in terms of their primary structure.

13.4 Quinoprotein–Cytochrome Complex

ADH is an ethanol:ubiquinone oxidoreductase that catalyzes oxidation of ethanol to produce acetaldehyde coupling with reduction of membranous ubiquinone (EC 1.1.5.5). Because the reaction by ADH is carried out in the periplasmic side of the membrane, ADH itself does not produce a proton gradient across the membrane. The enzymatic reaction of ADH was clearly demonstrated by the reconstitution experiments of ethanol-oxidizing respiratory chain in the proteoliposomes consisting of ADH, ubiquinone, and the terminal ubiquinol oxidase (Matsushita et al. 1992). ADH is a peripheral membrane protein localizing on the periplasmic surface of the cytoplasmic membrane without a predictable transmembrane segment. Depending on the bacterial species, ADH consists of at least two subunits: the dehydrogenase subunit, having one molecule each of PQQ and

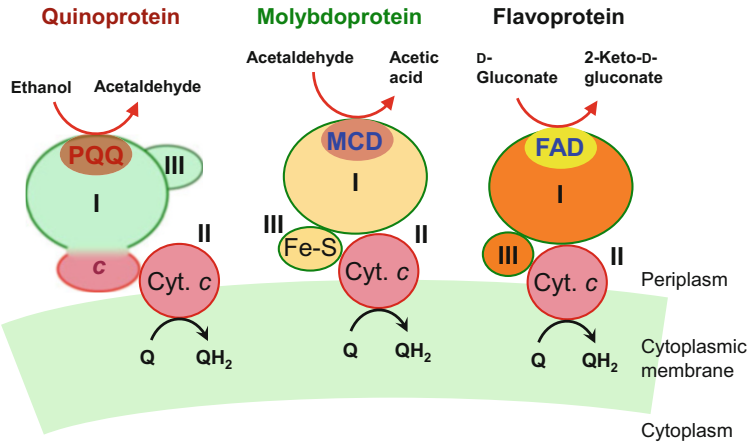

Fig. 13.2 Cartoon for three types of dehydrogenase–cytochrome complex: predicted structures of ADH, ALDH, and GADH. Although the dehydrogenase (*large*) and small subunits (shown as I and III, respectively) are not similar to one another, the cytochrome subunit (II) shows significant homology. See Chap. 7 by Matsushita and Matsutani for detailed phylogenetic analysis and discussion

heme C, and a cytochrome subunit having three hemes C (Fig. 13.2) (Adachi et al. 1978; Tayama et al. 1989). The cytochrome subunit is responsible for reduction of ubiquinone (Matsushita et al. 1996). In the *Acetobacter* and *Gluconobacter* enzymes, an additional small subunit seemingly having no prosthetic group associates with the ADH complex (Kondo et al. 1995). The *adhAB* genes for the large and cytochrome subunits constitute an operon, but the gene for the small subunit is mono-cistronic, apart from the *adhAB* operon.

After protein synthesis on the ribosome in the cytosol, all three subunits are transported to the periplasmic space via the Sec protein translocation system, which is a general protein secretion system in gram-negative bacteria. In the periplasmic space, PQQ and heme C are incorporated into the transported proteins along with the protein folding, because the Sec system translocates proteins in the unfolded state. In *Acetobacter* enzyme, the small subunit binds to the dehydrogenase subunit and assists it in folding properly, assembling the cofactors for constructing an active dehydrogenase subunit (Kondo et al. 1995). Finally, the heterodimeric or heterotrimeric ADH complex is constructed of the active subunits.

The *adh* genes are naturally inactivated by insertion sequences or transposons, whereby the ethanol oxidation ability would be inactivated (Takemura et al. 1991). There is strong genetic evidence that the *adh* genes are involved in acetic acid fermentation. We found recently that the naturally ethanol oxidation-deficient *Gluconobacter* strains have a single nucleotide polymorphism in the *adhB* gene that terminates its translation in the signal sequence. Even if the translation restarts at another possible initiation codon, the polypeptide synthesized would not be directed to the proper subcellular localization and the cofactor assembly. Defects in the ethanol oxidation ability can be restored by transformation with the *adhB*

gene, of which the termination codon has been altered to the codon of a hypothetical parental gene (Charoenyingcharoen et al. 2015).

Inactive forms of ADH were reported for *Gluconobacter* and *Gluconacetobacter* enzymes (Matsushita et al. 1995; Gómez-Manzo et al. 2012). *Gluconobacter* cells grown in acidic or high aeration conditions produce a large amount of ADH protein, but ADH activity is not changed, suggesting the presence of an inactive form of ADH. Purified inactive ADH from *Gluconobacter* shows only a tenth of the activity of the active enzyme. However, it shows higher ubiquinol oxidation activity (see following) than the "active" enzyme (Matsushita et al. 1999). These biochemical features suggest that ADH, as the inactive form particularly, has an unidentified function other than ethanol oxidation. The inactive form of *Gluconacetobacter* ADH is a trimer of hetero-dimer structure (Gómez-Manzo et al. 2012).

ADH shows wide substrate specificity, that is, primary short-chain alcohols up to 1-hexanol except for methanol; 1-propanol can be oxidized in a level similar to ethanol. Glycerol can be a substrate of ADH yielding glyceraldehyde with low affinity; only under high concentration, more than 10 % (w/v), ADH oxidizes glycerol at a significant rate (Habe et al. 2009). Not only alcohols but also aldehydes are oxidized by ADH: some ADHs of acetic acid bacteria oxidize aldehydes with a similar rate to alcohols (Gómez-Manzo et al. 2008), suggesting a possibility that only ADH perform acetic acid fermentation (Gomez-Manzo et al. 2015). ADH oxidizes formaldehyde but not methanol (Shinagawa et al. 2006).

ADH can oxidize ubiquinol in vitro using an artificial electron acceptor, but the physiological acceptor is not yet clarified. Although this activity can be thought to be involved in regulation of redox balance of the membranous ubiquinone, the yet physiological role of this activity is not fully established. ADH has ubiquinone as a bound form in the enzyme (Matsushita et al. 2008). We anticipate that the bound quinone functions in the catalysis of ubiquinone reduction (Yakushi and Matsushita 2010).

13.5 Molybdoprotein–Cytochrome Complex

Although aldehyde dehydrogenase (ALDH) has been purified from a wide variety of acetic acid bacteria (summarized by Gomez-Manzo et al. 2010), there are few reports for systematic research on this enzyme. Therefore, several enzymatic properties are controversial among research groups. Thus, it is rather difficult to determine the structure and function of ALDH of acetic acid bacteria.

ALDH is an acetaldehyde:ubiquinone oxidoreductase. The EC number of ALDH is currently EC 1.2.99.3. ALDH is thought to consist of three subunits, but there are some controversial reports on the subunit composition of purified ALDH complex, possibly because of the instability of the complex integrality. Presumably ALDH has been purified with partial decomposition. Indeed, peculiar activity profiles on column chromatography for the *Acetobacter* enzyme were often obtained: elution profiles for two activities for different artificial electron acceptors

and absorption of cytochrome were different from one another, suggesting the occurrence of decomposed ALDH having partial activity as well as the intact ALDH complex (Shun Nina and T.Y., unpublished). The three subunits are a dehydrogenase subunit having molybdenum-molybdopterin cytosine dinucleotide (MCD), a cytochrome subunit having three hemes C, and a small subunit having two [2Fe-2S] clusters. Because the cytochrome subunit has sequence homology to that of ADH, it is likely responsible for ubiquinone reduction. The *aldFGH* genes for the cytochrome, small, and dehydrogenase subunits constitute an operon (Thurner et al. 1997). As ADH, ALDH is a peripheral membrane protein complex that localizes on the periplasmic surface of the cytoplasmic membrane.

The cytochrome subunit is exported to the periplasm via the Sec pre-protein secretion apparatus, likely that of ADH. On the other hand, the dehydrogenase subunit is exported via the Tat (Twin Arg translocator) system after completion of protein folding as well as MCD incorporation in the cytoplasm. The dehydrogenase subunit has a Tat signal sequence in its N-terminus, although the small subunit does not. Therefore, the small subunit forms a complex with the dehydrogenase subunit in the cytoplasm and is exported by piggybacking on the dehydrogenase subunit being exported. The subcomplex and cytochrome subunit exported via different routes meet in the periplasm to assemble into the ALDH complex.

The prosthetic group of ALDH was reported as PQQ in the 1980s. In the 1990s it was reported that PQQ is not the prosthetic group of ALDH, because a PQQ-auxotroph strain of *Komagataeibacter* sp. (*Acetobacter* sp. in the original report) loses ADH activity completely but does not affect ALDH activity (Takemura et al. 1994). According to the report for cloning of the *aldFGH* genes from *Komagateibacter europaeus* (formerly *Acetobacter europaeus*), the predicted amino acid sequence of the dehydrogenase subunit suggests that MCD is the prosthetic group of ALDH (Thurner et al. 1997). On the other hand, ALDH purified from *Gluconacetobacter diazotrophicus* contains PQQ as the prosthetic group (Gomez-Manzo et al. 2010). The presence of PQQ is supported by detection of the PQQ radical in the substrate-reduced form of ALDH by electron paramagnetic resonance and quantification of PQQ extracted from the purified ALDH.

ALDH oxidizes primary short-chain aldehydes except for formaldehyde. It also oxidizes isobutylaldehyde and glutaraldehyde. Alcohols cannot be a substrate for ALDH. ADH and ALDH are enzymes unique to acetic acid bacteria: these are exclusively found in *Acetobacteraceae* (*Alphaproteobacteria*) and the genus *Frateuria* (*Gammaproteobacteria*).

13.6 Flavoprotein–Cytochrome Complex

Gluconate dehydrogenase (2-keto-D-gluconate-yielding) is D-gluconate:ubiquinone oxidoreductase (EC 1.1.99.3). Because it oxidizes the hydroxyl group of position C2 of D-gluconate to produce 2-keto-D-gluconate, gluconate dehydrogenase (GADH) is often referred as to gluconate 2-dehydrogenase. Thus, its reaction is

different from the catalysis by quinoprotein glycerol dehydrogenase to produce 5-keto-D-gluconate (see next section). GADH is a flavoprotein–cytochrome complex consists of three subunits: a large dehydrogenase subunit having a covalently bound flavin adenine dinucleotide (FAD), a cytochrome subunit responsible for ubiquinone reduction having three heme C moieties, and a small subunit that is indispensable for translocation of dehydrogenase subunit across the cytoplasmic membrane (Toyama et al. 2007). Similar to other flavoprotein dehydrogenase–cytochrome complexes, substrate specificity of GADH is highly restricted to D-gluconate; GADH is inert to sugars, polyols, and organic acids other than D-gluconate (Shinagawa et al. 1984). GADH is found in the genera *Pseudomonas* and *Erwinia* as well as in acetic acid bacteria.

The cytochrome subunit is exported to the periplasm via the Sec preprotein secretion apparatus. On the other hand, the small subunit is exported via the Tat system. Because the dehydrogenase subunit has no signal sequence for translocation across the cytoplasmic membrane, after completion of protein folding as well as FAD incorporation, it is exported by piggybacking on the small subunit being exported. The small subunit is required not only for translocation but also for active enzyme formation (Yamaoka et al. 2004); the biochemical function of the small subunit remains to be clarified. The subcomplex and the cytochrome subunit exported now assemble into the complex in the periplasm.

2-Keto-D-gluconate dehydrogenase (2KGDH), D-sorbitol dehydrogenase (SLDH), and D-fructose dehydrogenase (FDH) are members of the flavoprotein dehydrogenase–cytochrome complex (Toyama et al. 2005; Kawai et al. 2013; Kataoka et al. 2015). All these share the molecular properties and the maturation processes with GADH. 2KGDH and FDH oxidize 2-keto-D-glucose and D-fructose with high specificity to produce 2,5-diketo-D-gluconate and 5-keto-D-fructose, respectively (Shinagawa et al. 1981; Ameyama et al. 1981a). Such specificity of FDH enables this enzyme to be used as a fructose sensor. SLDH oxidizes D-sorbitol to produce L-sorbose, while it weakly oxidizes D-mannitol but does not oxidize pentitols and erythritols (Shinagawa et al. 1982).

13.7 Membrane-Bound Quinoprotein

Glucose dehydrogenase (GDH), a D-glucose:ubiquinone oxidoreductase (EC 1.1.5.2), catalyzes oxidation of hydroxyl group on position C1 of D-glucopyranose to produce glucono-δ-lactone (D-glucono-1,5-lactone) (Ameyama et al. 1981 b). Although glucono-δ-lactone is stable at acidic conditions, lactone of the cyclic structure is spontaneously hydrolyzed to D-gluconic acid under neutral and alkaline conditions. Therefore, GDH is sometimes described to produce D-gluconic acid.

GDH is a quinoprotein containing PQQ as a prosthetic group. GDH consists of a single protomer encoded in the *gdhM* gene and is composed of a N-terminal transmembrane region and C-terminal catalytic region (Fig. 13.3).

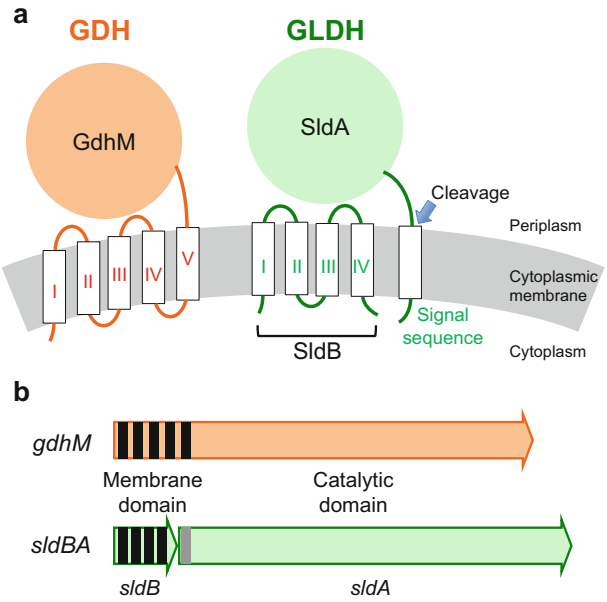

Fig. 13.3 (**a**) Cartoon for GDH and GLDH. Predicted structure of GDH and GLDH. (**b**) Gene organization of *gdhM* and *sldBA* and location of transmembrane segments (*black bars*) and signal sequence (*gray bar*)

PQQ-dependent, soluble glucose dehydrogenase of *Acinetobacter calcoaceticus* or *Archea*, of which the physiological electron acceptor is unknown, is different from GDH in catalytic properties and primary structure (Matsushita et al. 1989a; Sakuraba et al. 2010). Purified GDH oxidizes maltose with 5 % relative activity to that with D-glucose (Ameyama et al. 1981 b). Hexose sugars except for D-glucose and pentoses are not oxidized with *Gluconobacter* GDH. Based on the high substrate specificity, GDH can be expected to be applicable to a construction of glucose sensor. GDH is distributed in a wide variety of *Proteobacteria*, the genera *Pseudomonas* and *Escherichia* (*Gammaproteobacteria*), and *Archaea* as well as acetic acid bacteria.

As described in the ADH section, the glucose oxidase respiratory chain has been reconstituted in proteoliposomes composed of GDH, ubiquinone, terminal oxidase, and phospholipids (Matsushita et al. 1989b). Although GDH has a transmembrane region with five hydrophobic segments, the enzyme itself does not produce membrane potential (Yamada et al. 1993). GDH of *Escherichia coli* is well characterized in biochemical aspects. GDH has bound quinone, which is likely involved in catalysis of ubiquinone reduction (Elias et al. 2004). An intramolecular electron transport pathway is proposed by pulse radiolysis experiments (Mustafa et al. 2008).

Glycerol dehydrogenase (GLDH) is glycerol:ubiquinone oxidoreductase yielding dihydroxyacetone (EC 1.1.99.22). The substrate specificity of GLDH is broad:

polyols, such as arabitol, sorbitol, mannitol, erythritol, and ribitol are oxidized by GLDH to produce the corresponding ketoses (Sugisawa and Hoshino 2002). Interestingly, GLDH oxidizes D-gluconate and produces 5-keto-D-gluconate (Matsushita et al. 2003). Thus, it is also sometimes referred to as sorbitol dehydrogenase or gluconate 5-dehydrogenase. In the industrial aspect, the enzyme is responsible for the L-sorbose, dihydroxyacetone, erythrulose, and 5-keto-D-gluconate production with *Gluconobacter* spp. GLDH oxidizes a secondary hydroxyl group that has the (*R*) configuration. The preferred location of the oxidizing hydroxyl group is the second carbon adjacent to the terminal carbon. GLDH oxidizes 1,2-butanediol with a higher rate than that with 1,3-butanediol, but merely 1-butanol and 1,4-butanediol (Moonmangmee et al. 2001).

The purification of GLDH was reported for the first time by Ameyama et al. (1985). Sugisawa and Hoshino purified it as sorbitol dehydrogenase, and Hoshino's group identified the *sldBA* gene that encodes GLDH (Sugisawa and Hoshino 2002; Miyazaki et al. 2002). GLDH is membrane-bound quinoprotein that consists of two subunits: a PQQ-containing large catalytic subunit encoded in *sldA* and a small membrane-binding subunit encoded in *sldB* (Fig. 13.3). These subunits have significant homology to the catalytic and membrane domains of GDH; GLDH seems to be a separate form of GDH split between the two domains (Fig. 13.3a). The gene organization of the *sldBA* operon is similar to that of GDH; the operon starts at *sldB* and follows *sldA*, and the membrane domain of GDH is encoded in the 5′-region of the *gdhM* gene, supporting as such the split hypothesis (Fig. 13.3b). The precursor form of the large subunit has a signal sequence for the Sec system to travel to the periplasmic space. Then, the large subunit assembles into the enzyme complex with the small subunit in the membrane.

Quinate dehydrogenase (QDH), quinate:ubiquinone oxidoreductase (EC 1.1.5.8), oxidizes the hydroxyl group on the position C3 of quinate to produce 3-dehydroquinate. QDH is a PQQ-dependent quinoprotein consisting of a single protomer having a membrane domain and catalytic domain just as GDH (Vangnai et al. 2010). QDH oxidizes shikimate and protocatechuate with a quarter level of relative activity compared to that of quinate (Vangnai et al. 2004).

Inositol dehydrogenase (IDH) is a myo-inositol:ubiquinone oxide reductase that oxidizes position C2 of inositol to yield 2-keto-myo-inositol. This enzyme is the only dehydrogenase found through a reverse genetic study. Prust et al. (2005) revealed the genome sequence for *G. oxydans* 621H strain and identified four putative PQQ-dependent dehydrogenases through bioinformatics analysis: IDH was one of such orphan dehydrogenases. Hölscher et al. constructed and examined a deletion derivative of the *gox1857* gene to reveal the GOX1857 protein is IDH (Hölscher et al. 2007). IDH is a PQQ-dependent quinoprotein of which the primary sequence is similar to that of GDH. IDH also oxidizes allo- and muco-inositol.

13.8 Others

Sorbose dehydrogenase is an L-sorbose:ubiquinone oxidoreductase (EC 1.1.99.12) that oxidizes position C1 to yield L-sorbosone (Sugisawa et al. 1991). Although the enzyme of *G. oxydans* strain UV10 is thought to function on the periplasmic surface of the cytoplasmic membrane, discrete signature for neither transmembrane region nor signal sequence is found in the protein. The enzyme is a flavoprotein having FAD, but constitutes a monomeric form different from other heterotrimeric flavoprotein-cytochrome complexes. The substrate specificity is high; no sugars and alcohols other than L-sorbose are oxidized.

Sorbosone dehydrogenase (SNDH) is an L-sorbosone:ubiquinone oxidoreductase which oxidizes position C1 of L-sorbosone to yield 2-keto-L-gulonate, which is the important intermediate in vitamin C production industry (Pappenberger and Hohmann 2014). The enzyme likely functions on the periplasmic surface of the cytoplasmic membrane. SNDH of *Gluconacetobacter liquefaciens* (formerly *Acetobacter liquefaciens*) does not have any binding motif for PQQ, FAD, FMN, or MCD)(Shinjoh et al. 1995). In spite of low similarities, by using a recent bioinformatics tool the enzyme likely belongs to a family of the soluble type of PQQ-GDH, which is found in *Acinetobacter* spp. and *Archaea*. Biochemical identification of the cofactor of SNDH is thus an important issue that remains to be clarified.

Sorbose dehydrogenase and SNDH have been intensively studied with the enzymes from the strain DSM4025. These enzymes are different from those of acetic acid bacteria. The DSM4025 strain used to be referred to as *G. oxydans*, but molecular taxonomic study reclassified DSM4025 as *Ketogulonicigenium vulgare*, belonging to *Gammaproteobacteria*, which is phylogenetically distant from acetic acid bacteria.

According to the genomic information of *G. oxydans* 621H strain (Prust et al. 2005), 6 of the membrane-bound dehydrogenases are functionally validated via biochemical and/or genetic studies, but 21 enzymes are not validated, being orphan dehydrogenases of which the substrates are unknown (see Chap. 11 by Bringer and Bott in this volume). Peters et al. (2013) systematically constructed a series of multiple gene deletion mutant derivatives of the 621H strain. The strain having highest number of deletions lacks ADH, IDH, ALDH, GADH, GDH, PQQ-DH3, PQQ-DH4, GLDH, and lactate dehydrogenase, but lactate dehydrogenase faces the cytoplasm. The strains are useful to validate functions of the orphan membrane-bound dehydrogenases. Because genome data of many kinds of acetic acid bacteria are available to the public and the number of genes reported is still growing, many new and useful membrane-bound enzymes will be found in the near future.

13.9 Quinate Dehydrogenase and Shikimate Production

The well-known cytoplasmic shikimate pathway is important to aromatic amino acid synthesis. It takes a long metabolic pathway to reach to an aromatic amino acid from D-glucose. Two metabolic intermediates, phosphoenolpyruvate from the glycolytic pathway and D-erythrose-4-phosphate from the pentose-phosphate pathway, must combine to form 3-deoxy-7-phospho-D-arabinoheptulosonate before formation of 3-dehydroquinate (Fig. 13.4). Thus, there are still several barriers difficult to overcome in the already existing technologies of shikimate fermentation from D-glucose (Draths et al. 1999).

Finding of a membrane-bound quinoprotein quinate dehydrogenase (QDH) in the membrane fraction of acetic acid bacteria is a strong clue of shikimate production without using the cytoplasmic shikimate pathway (Adachi et al. 2003a, b). In the early stage of this study, basic information for quinate oxidation, 3-dehydroquinate dehydration, and 3-dehydroshikimate production were accumulated from experiments with growing cells, resting cells, dried cells, immobilized cells, and the membrane fraction of *G. oxydans* NBRC 3244 (Adachi et al. 2003a). The information indicates shikimate production outside the cells of acetic acid bacteria is independent of the cytoplasmic shikimate pathway. Basic information including mutual separation of NADP-shikimate dehydrogenase (SKDH) (Adachi et al. 2006b), membrane-bound 3-dehydroquinate dehydratase (DQD) (Adachi

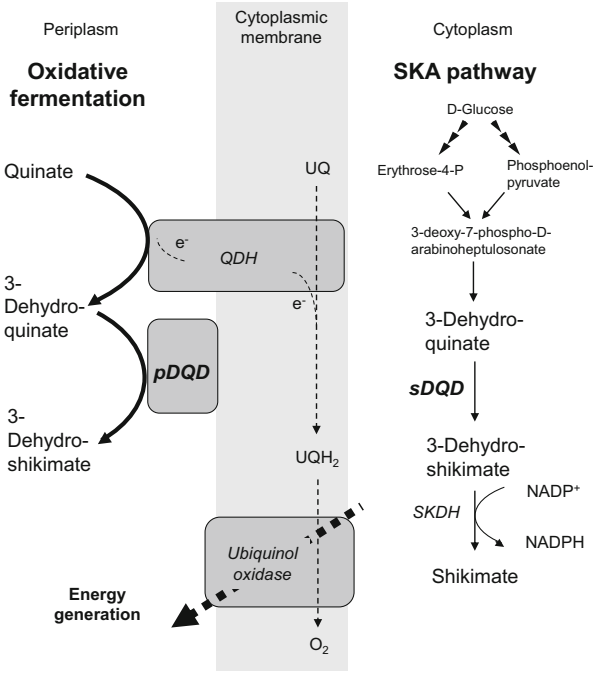

Fig. 13.4 Quinate metabolism in *Gluconobacter oxydans*. *UQ* ubiquinone, UQH_2 reduced form of ubiquinone. [Reprinted with permission by courtesy of *Bioscience, Biotechnology, and Biochemistry* (Adachi et al. 2008b)]

et al. 2008b), and membrane-bound 3-dehydroshikimate dehydratase (Shinagawa et al. 2010) is available.

A method for enzymatic production of 3-dehydroquinate and 3-dehydroshikimate was established by controlling the working pH of enzyme activity of 3-dehydroquinate dehydratase (Adachi et al. 2006a, b). When quinate is incubated with resting cells or membrane fraction of *G. oxydans* NBRC 3244 at pH 5.0, 3-dehydroquinate is formed as the predominant product. On the other hand, 3-dehydroshikimate is exclusively formed when incubated at pH 7.0–8.0. Mutual separation of the metabolic intermediates is easy to perform. Overexpression of 3-dehydroquinate dehydratase enhances bioconversion of quinate to 3-dehydroshikimate in *G. oxydans* NBRC 3244 (Nishikura-Imamura et al. 2014). These two metabolic intermediates are known as the starting materials for several important antibiotics, but they are not commercially available at present. Why are the metabolic intermediates in the shikimate pathway so important? For examples, tuberculosis (TB) is responsible for more than 9 million cases and almost 2 million deaths per year worldwide. The emergency of multidrug-resistant strains of *Mycobacterium tuberculosis* and coinfection with the human immunodeficiency virus has caused an urgent need for the development of new therapeutics against TB. The rational design of new antimycobacterial agents against *M. tuberculosis* is an important approach to contribute to this global health problem. Enzymes in the shikimate pathway are attractive drug targets because this route is absent in mammals, but in *M. tuberculosis*, it was found to be essential for viability of the pathogen. This pathway leads to the biosynthesis of aromatic amino acids, menaquinones, and mycobactin. Future identification of one or more inhibitor compounds of the enzymes might provide a promising route to a new, effective, and shorter treatment for TB (A.L. Bass, personal communications). Similar strategies can be applied to control parasites as an antimalarial agent (Schneider et al. 2008; Ducati et al. 2007). Shikimate production has significance as a starting material for oseltamivir (Tamiflu) synthesis, protecting people from pandemic flu infection. Besides oseltamivir synthesis, production of several metabolic intermediates from the shikimate pathway, including 3-dehydroquinate, 3-dehydroshikimate, shikimate-3-phosphate, 5-enolpyruvyl-shikimate-3-phosphate, and chorismate, becomes easier because shikimate and its derivatives can be used as the starting materials and overcome the barriers preventing bulky production of such compounds.

As shown in Fig. 13.5, a coupling reaction system from quinate to shikimate is constructed. Quinate is produced from chlorogenate in plant origins by the action of fungal chlorogenate hydrolase (Adachi et al. 2008a). Quinate is converted to 3-dehydroshikimate with almost 100 % conversion to 3-dehydroquinate by the action of membrane-bound quinate dehydrogenase and 3-dehydroquinate dehydratase (Adachi et al. 2003b, 2006a). Alternatively, the membrane fraction of *G. oxydans* NBRC 3244 containing membrane-bound quinoprotein quinate dehydrogenase and 3-dehydroquinate dehydratase can be immobilized into calcium alginate beads. The immobilized bacterial membrane catalyzes a sequential reaction of quinate oxidation to 3-dehydroquinate, and its spontaneous conversion to

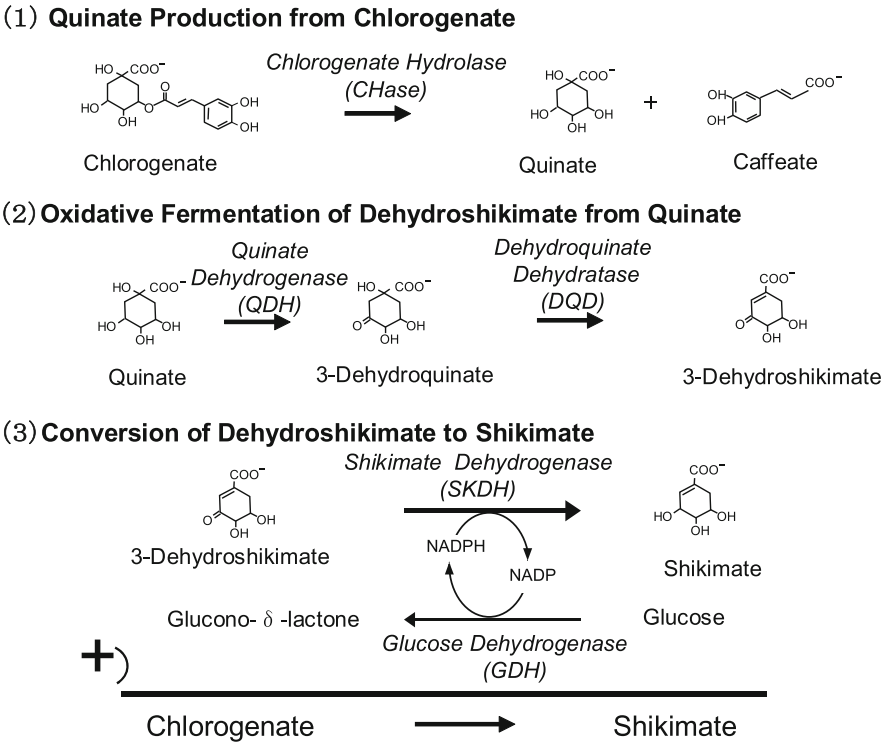

Fig. 13.5 The coupling reaction system from 3-dehydroshikimate to shikimate enables efficient shikimate production from chlorogenate in plant origins. [Reprinted with permission by courtesy of *Bioscience, Biotechnology, and Biochemistry* (Adachi et al. 2010a)]

3-dehydroshikimate is observed (Adachi et al. 2008b). Further conversion of 3-dehydroshikimate to shikimate is conducted by an immobilized coupling enzyme system containing NADP-dependent enzymes, shikimate dehydrogenase, and D-glucose dehydrogenase. They are immobilized together with different carriers as an asymmetrical reduction forming shikimate from 3-dehydroshikimate. Blue Dextran 2000, Blue Dextran Sepharose-4B, DEAE-Sephadex A-50, DEAE-cellulose, and hydroxyapatite are effective carriers for the two cytoplasmic enzymes. When the reaction mixture for asymmetrical reduction is circulated through a column on which shikimate dehydrogenase and D-glucose dehydrogenase are immobilized, 3-dehydroshikimate initially added in the reaction mixture is converted to shikimate at 100 % yield (Adachi et al. 2010a). Only a catalytic amount of $NADP^+$ originally added functions well in the coupling reaction in the presence of excess D-glucose, and regenerated NADPH is confirmed from the fact that 3-dehydroshikimate is totally converted to shikimate.

The following two points are noteworthy about this entirely different shikimate production from the cytoplasmic shikimate pathway. (1) There is no problem if crude enzyme preparations of shikimate dehydrogenase and D-glucose

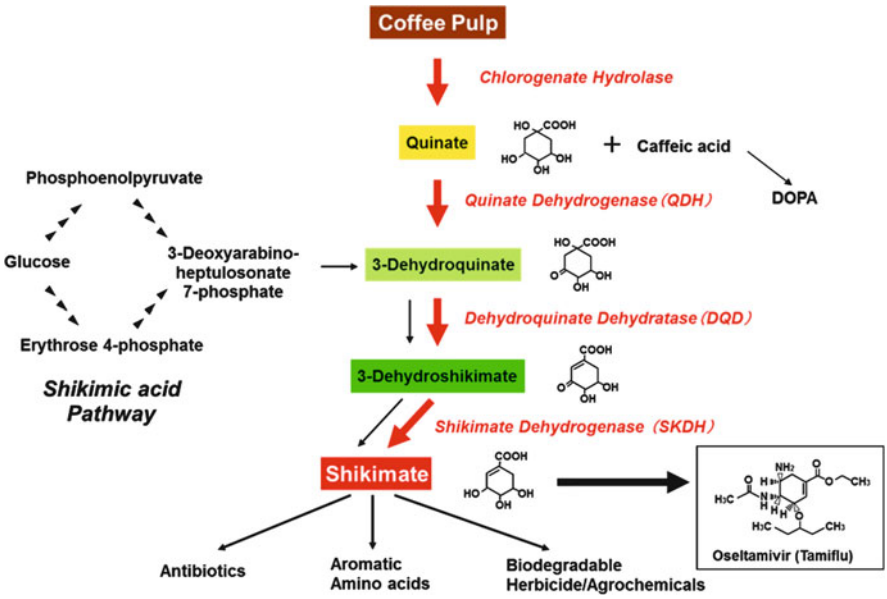

Fig. 13.6 New biochemical production route to shikimate from chlorogenate in plant origins is proposed via 3-dehydroquinate and 3-dehydroshikimate. (Reprinted with permission by courtesy of *Bioscience, Biotechnology, and Biochemistry* (Adachi et al. 2010a))

dehydrogenase are used for the asymmetrical coupling reaction, because 3-dehydroshikimate is the sole substrate and any $NAD(P)^+$ and NAD(P)H-dependent enzymes that contaminate the enzyme reactions have no catalytic function and do not disturb the yield of shikimate. (2) Differing from de novo synthesis of shikimate along with the shikimate pathway from D-glucose, there are no regulatory steps with difficulties to overcome in forming shikimate in the strategy proposed by the oxidative fermentation. Taken together with the process for quinate formation from chlorogenate in plant origins, an entirely new route to shikimate via 3-dehydroquinate and 3-dehydroshikimate is proposed (Fig. 13.6).

13.10 Pentose Oxidation

New membrane-bound dehydrogenases relating to pentose oxidation have been indicated from recent work on oxidative fermentation of acetic acid bacteria (Adachi et al. 2012). As illustrated in Fig. 13.1, enzymes catalyzing pentose oxidation to 4-keto-D-pentose and its further oxidation to 4-keto-D-pentonate, and direct oxidation of D-pentonate to 4-keto-D-pentonate, are involved. No report about the responsible enzymes is available so far. Some reports state that there is no D-ribose-oxidizing enzyme in acetic acid bacteria. Strictly speaking, D-ribose is oxidized by the quinoprotein-GDH to D-ribonate via D-ribonolactone within the

substrate specificity of GDH, as similarly seen with D-xylose oxidation to D-xylonate by GDH. GDH does not catalyze formation of ketogenic reaction product having an intramolecular ketone, such as 4-keto-D-pentose and 4-keto-D-pentonate.

13.11 4-Keto-D-Arabonate from the D-Glucose Oxidation Pathway

A novel pentose oxidation has started by finding the further oxidation of 2,5-diketo-D-gluconate (25DKA) to producing 4-keto-D-arabonate (4KAB) via 4-keto-D-arabinose (upper part in Fig. 13.1). When newly isolated strains from Argentina water kefir are grown on a medium containing D-glucose and D-gluconate, they rapidly oxidize substrates in the medium and accumulate 2,5-diketo-D-gluconate (25DKA) before reaching the stationary phase. 25DKA accumulated is in turn converted to a stable but unidentified oxidation product that is reactive to 2,3,5-tripenyltetrazolium chloride (TTC), suggesting existence of an intramolecular ketone in the compound. The compound was identified to be 4-keto-D-arabonate (4KAB) (Adachi et al. 2010b). The phenotypic characterizations of the isolates were close to those of *Ga. liquefaciens* NBRC 12388. They have been registered as strains *Ga. liquefaciens* RCTMR 9, RCTMR 10, RCTMR 11, and RCTMR 12 at the Research Center for Thermotolerant Microbial Resources (RCTMR), Yamaguchi University. Ameyama and Kondô (1958b) suggested 4KAB in carbohydrate metabolism of acetic acid bacteria about 50 years ago. They obtained lyxuronate as one of the end products from 25DKA and proposed possible formation of 4KAB as an intermediate to lyxuronate, although 4KAB was not obtained.

25DKA figures in the production of 2-keto-L-gulonate, an intermediate in the Reichstein synthesis of L-ascorbate, which is formed stereospecifically by NADPH-dependent 25DKA reductase. In contrast to the industrial significance of 2-keto-L-gulonate, sporadic studies of 25DKA degradation has been done but not followed up extensively. To the best of our knowledge, after sequential oxidation of D-glucose or D-gluconate (GA) to 2-keto-D-gluconate (2KGA), 25DKA is accumulated in the culture medium as an oxidation product of 2KGA, catalyzed by membrane-bound 2KGA dehydrogenase (2KGDH). When RCTMR strains are cultivated in a medium containing 0.5 % D-glucose and 2.0 % GA, the unidentified compound and 25DKA are predominate, both of which are reactive to TTC, giving deep red spots on a TLC plate. After prolonged cultivation, more than 80 % of 25DKA appeared to be converted to the unidentified compound. The unidentified compound is also formed with other strains such as *G. oxydans* NBRC 3244 and NBRC 3294. The productivity of the unidentified compound was far less than that of *Ga. liquefaciens* RCTMR strains.

The unidentified compound from 6-day culture of *Ga. liquefaciens* RCTMR 10 was separated by an ion-exchange chromatography with Dowex 1 × 4 (acetate form) and crystallized. After molecular mass determination, ^{1}H-MNR, IR, and

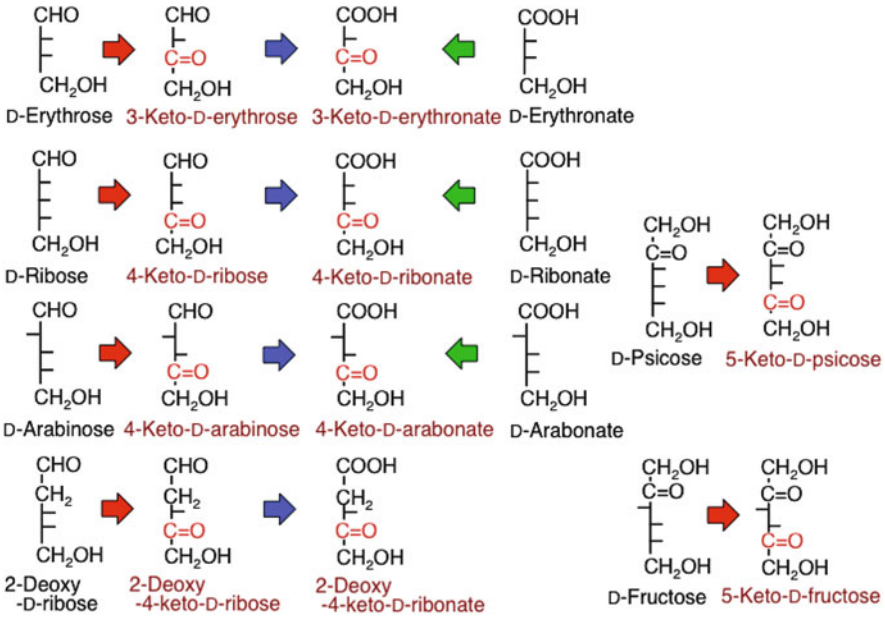

Fig. 13.7 Reactions concerning the new oxidative fermentation involving pentose oxidation. *Red arrows* D-aldopentose 4-dehydrogenase, *blue arrows* 4-keto-D-aldopentose 1-dehydrogenase, *green arrows* pentonate 4-dehydrogenase

2D-NMR such as COSY and HMQC were followed and identified as 4-keto-D-arabonate (4KAB = 4-pentulosonate; Fig. 13.7). It was the same compound as postulated by Ameyama and Kondo in 1958 (Ameyama and Kondô 1958a, b). There was no report about 4KAB formation in oxidative fermentation by acetic acid bacteria as well as in other fields of natural sciences.

13.12 4-Keto-D-Arabonate and 4-Keto-D-ribonate from D-aldopentoses and D-pentonates

Encouraged by the new finding of 4KAB formed from 25DKA via 4-keto-D-arabinose, 4-keto-D-pentonate synthesis is examined from D-aldopentoses and D-pentonate by acetic acid bacteria. D-Ribonate and D-arabonate are oxidized to 4-keto-D-ribonate and 4-keto-D-arabonate by acetic acid bacteria. A membrane fraction from *G. oxydans* NBRC 12528 catalyzed the foregoing reactions and 4-keto-D-pentonate is formed from D-aldopentose as well as D-pentonate by two different pathways (Adachi et al. 2011). In 4KAB formation, addition to the pathway via 25DKA, 4KAB is prepared from D-arabinose via 4-keto-D-arabinose and directly from D-arabonate. 4-Keto-D-ribonate is formed from D-ribose via

4-keto- D-ribose and directly from D-ribonate. 4KAB and 4-keto-D-ribonate are formed by oxidative fermentation using either growing cells, resting cells, or the membrane fraction. The analytical data of 4KAB obtained from D-arabinose and D-arabonate are identical. Based on the IR spectrum and ^{1}H-NMR and ^{13}C-NMR spectra, it is reasonably concluded that the oxidation product is identified as D-*threo*-pent-4-ulosonate (4-keto-D-arabonate, 4KAB; Fig. 13.7).

Oxidation of D-ribose and D-ribonate to 4-keto-D-ribonate can be done in the same manner as that of D-arabinose described earlier by adding either D-ribose or D-ribonate to a culture medium or cell suspension of *G. oxydans* NBRC 12528. The analytical data with the oxidation products from D-ribose and D-ribonate are identical with respect to IR spectra, ^{1}H-NMR, and ^{13}C-NMR. The data suggest that the oxidation product from D-ribose and D-ribonate is identified as D-*erythro*-pent-4-ulosonate (4-keto-D-ribonate). Thus, it is found that 4-keto-D-ribonate is formed from two different pathways of oxidative fermentation (Fig. 13.7).

13.13 2-Deoxy-4-Keto-D-Ribonate from D-ribose and 2-deoxy-D-ribose

D-Ribose and 2-D-deoxy-D-ribose are important sugars in ribonucleic acids and deoxyribonucleic acids, respectively. As stated earlier, D-ribose is well oxidized to 4-keto-D-ribonate via 4-keto-D-ribose by *Gluconobacter* as well as *Gluconacetobacter*. Under the same mechanism of oxidative fermentation, 2-deoxy-D-ribose is also well oxidized to 2-deoxy-4-keto-D-ribonate via 2-deoxy-4-keto-D-ribose (Adachi et al. 2013). Data from the IR and NMR spectra and mass spectrum reveal that the oxidation product is identical to 2-deoxy-D-pent-4-ulosonate (2-deoxy-4-keto-D-ribose). Combining the data here confirm that the oxidation pathways of D-ribose and 2-keto-D-ribose by D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase are involved in the new type of oxidative fermentation of pentose oxidation. Many membrane-bound dehydrogenases have not been assigned to genome or enzymes and, to our best knowledge, particularly the enzyme catalyzing pentose oxidation and yielding a ketone at the position C4 remains to be identified.

It is interesting to check availability of related compounds containing D-ribose and 2-deoxy-D-ribose moieties in the molecules such as ribo- and deoxyribonucleosides and nucleotides to see whether they are oxidized with the membrane fraction of acetic acid bacteria. When various ribo- and deoxyribonucleosides and nucleotides are incubated with the membrane fraction of *Gluconobacter* and *Gluconacetobacter*, TTC positive spots corresponding to 4-keto-D-ribonate and 2-deoxy-4-keto-D-ribonate are observed, but only for a reaction mixture containing purine nucleosides and nucleotides (Adachi et al. 2013). The data clearly indicate acetic acid bacteria have nucleosidase activity in the membrane. There is no report of a nucleosidase occurring in membrane-bound form, and most studies of

nucleosidases from eukaryotes and prokaryotes have considered cytoplasmic soluble enzymes. Different from the primary meaning of oxidative fermentation by acetic acid bacteria, the novel oxidized sugars 4-keto-D-ribonate and 2-deoxy-4-keto-D-ribonate can be produced from nucleic acid resources such as yeast cells.

13.14 D-Fructose Oxidation, D-Psicose Oxidation, and Others

It is noteworthy to make brief additional comments about the oxidation of D-fructose, D-psicose, and others in relationship to D-aldopentose 4-dehydrogenase. As D-aldopentose 4-dehydrogenase recognizes the chemical structure common to D-arabinose, D-ribose, and the C2–C6 moiety of D-fructose and 2-keto-D-gluconate, it recognizes the secondary alcoholic group in the D-*erythro* configuration adjacent to the primary alcolic group. It is known that the oxidation of D-fructose is catalyzed only by FDH of *G. japonicus* NBRC 3260 (formerly *G. industrius* IFO 3260) (Ameyama et al. 1981a). Bacteria possessing FDH, in which a covalently bound flavin is functioning, are limited to only a few *Gluconobacter* strains. FDH activity is not expressed in *G. oxydans* NBRC 12528. Likewise, 2-keto-D-gluconate oxidation is catalyzed only with 2KGDH, in which a covalently bound flavin functions as coenzyme, and is formed only by a few strains of *G. oxydans* (formerly *G. melanogenus*) (Shinagawa et al. 1981) and *Ga. liquefaciens*. FDH and 2KGDH are resistant to EDTA, whereas D-aldopentose 4-dehydrogenase and D-pentonate 4-dehydrogenase, are sensitive to EDTA treatment, resulting in apo-enzyme formation. The enzyme activity of the apo-enzyme is recovered by exogenous addition of PQQ in the presence of Ca^{2+}. At present, it is unclear whether D-aldopentose 4-dehydrogenase and D-pentonate 4-dehydrogenase are identical.

D-Erythrose is also oxidized to 3-keto-D-erythrose and further to 3-keto D-erythronate by the same membrane fraction having D-aldopentose 4-dehydrogenase and D-pentonate 4-dehydrogenase. However, it is necessary to confirm this with highly purified D-erythrose; that commercially available at present does not show high purity.

Intermediate formation observed in the novel oxidative fermentation, such as 4-keto-D-arabinose and 4-keto-D-ribose, has not been confirmed by means of instrumental analysis. D-Aldopentose 4-dehydrogenase is important to identify and purify 4-keto-D-aldopentose 1-dehydrogenase, which links to the intermediate metabolism in pentose oxidation. In Fig. 13.7, typical reactions concerning the new oxidative fermentation involving pentose oxidation are summarized. The red arrows mean D-aldopentose 4-dehydrogenase, the blue arrows indicate 4-keto-D-aldopentose 1-dehydrogenase, and the green arrows show pentonate 4-dehydrogenase.

References

Adachi O, Tayama K, Shinagawa E, Matsushita K, Ameyama M (1978) Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 42(11):2045–2056

Adachi O, Tayama K, Shinagawa E, Matsushita K, Ameyama M (1980) Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 44(3):503–515

Adachi O, Tanasupawat S, Yoshihara N, Toyama H, Matsushita K (2003a) 3-Dehydroquinate production by oxidative fermentation and further conversion of 3-dehydroquinate to the intermediates in the shikimate pathway. Biosci Biotechnol Biochem 67(10):2124–2131

Adachi O, Yoshihara N, Tanasupawat S, Toyama H, Matsushita K (2003b) Purification and characterization of membrane-bound quinoprotein quinate dehydrogenase. Biosci Biotechnol Biochem 67(10):2115–2123

Adachi O, Ano Y, Toyama H, Matsushita K (2006a) High shikimate production from quinate with two enzymatic systems of acetic acid bacteria. Biosci Biotechnol Biochem 70(10):2579–2582. Epub 2006 Oct 7

Adachi O, Ano Y, Toyama H, Matsushita K (2006b) Purification and properties of NADP-dependent shikimate dehydrogenase from *Gluconobacter oxydans* IFO 3244 and its application to enzymatic shikimate production. Biosci Biotechnol Biochem 70(11):2786–2789. Epub 2006 Nov 7

Adachi O, Ano Y, Akakabe Y, Shinagawa E, Matsushita K (2008a) Coffee pulp koji of *Aspergillus sojae* as stable immobilized catalyst of chlorogenate hydrolase. Appl Microbiol Biotechnol 81 (1):143–151. doi:10.1007/s00253-008-1659-z. Epub 2008 Sep 5

Adachi O, Ano Y, Toyama H, Matsushita K (2008b) A novel 3-dehydroquinate dehydratase catalyzing extracellular formation of 3-dehydroshikimate by oxidative fermentation of *Gluconobacter oxydans* IFO 3244. Biosci Biotechnol Biochem 72(6):1475–1482. Epub 2008 Jun 7

Adachi O, Ano Y, Shinagawa E, Yakushi T, Matsushita K (2010a) Conversion of quinate to 3-dehydroshikimate by Ca-alginate-immobilized membrane of *Gluconobacter oxydans* IFO 3244 and subsequent asymmetric reduction of 3-dehydroshikimate to shikimate by immobilized cytoplasmic NADP-shikimate dehydrogenase. Bioscience 74(12):2438–2444. Epub 2010 Dec 7

Adachi O, Hours RA, Akakabe Y, Tanasupawat S, Yukphan P, Shinagawa E, Yakushi T, Matsushita K (2010b) Production of 4-keto-D-arabonate by oxidative fermentation with newly isolated *Gluconacetobacter liquefaciens*. Biosci Biotechnol Biochem 74 (12):2555–2558. Epub 2010 Dec 7

Adachi O, Hours RA, Shinagawa E, Akakabe Y, Yakushi T, Matsushita K (2011) Enzymatic synthesis of 4-pentulosonate (4-keto-D-pentonate) from D-aldopentose and D-pentonate by two different pathways using membrane enzymes of acetic acid bacteria. Biosci Biotechnol Biochem 75(12):2418–2420

Adachi O, Hours RA, Shinagawa E, Akakabe Y, Yakushi T, Matsushita K (2012) 4-Keto-D-aldopentoses and 4-pentulosonates, new products with acetic acid bacteria. Acetic acid bacteria 1(s1):23

Adachi O, Hours RA, Akakabe Y, Shinagawa E, Ano Y, Yakushi T, Matsushita K (2013) Pentose oxidation by acetic acid bacteria led to a finding of membrane-bound purine nucleosidase. Biosci Biotechnol Biochem 77(5):1131–1133

Ameyama M, Kondô K (1958a) Carbohydrate metabolism by *Acetobacter* species. Part III. Isolation and identification of D-lyxuronic acid on glucose oxidation by *A. melanogenum*. Bull Agric Chem Soc Jpn 22(6):380–386

Ameyama M, Kondô K (1958b) Isolation of D-lyxuronic acid as an oxidative metabolite from glucose by *Acetobacter melanogenum*. Bull Agric Chem Soc Jpn 22(4):271–272

Ameyama M, Shinagawa E, Matsushita K, Adachi O (1981a) D-Fructose dehydrogenase of *Gluconobacter industrius*: purification, characterization, and application to enzymatic microdetermination of D-fructose. J Bacteriol 145(2):814–823

Ameyama M, Shinagawa E, Matsushita K, Adachi O (1981b) D-Glucose dehydrogenase of *Gluconobacter suboxydans*: solubilization, purification and characterization. Agric Biol Chem 45(4):851–861

Ameyama M, Shinagawa E, Matsushita K, Adachi O (1985) Solubilization, purification and properties of membrane-bound glycerol dehydrogenase from *Gluconobacter industrius*. Agric Biol Chem 49(4):1001–1010

Charoenyingcharoen P, Matsutani M, Yakushi T, Theeragool G, Yukphan P, Matsushita K (2015) A functionally critical single nucleotide polymorphism in the gene encoding the membrane-bound alcohol dehydrogenase found in ethanol oxidation-deficient *Gluconobacter thailandicus*. Gene (Amst) 567(2):201–207

Draths KM, Knop DR, Frost JW (1999) Shikimic acid and quinic acid: replacing isolation from plant sources with recombinant microbial biocatalysis. J Am Chem Soc 121(7):1603–1604

Ducati RG, Basso LA, Santos DS (2007) Mycobacterial shikimate pathway enzymes as targets for drug design. Curr Drug Targets 8(3):423–435

Elias MD, Nakamura S, Migita CT, Miyoshi H, Toyama H, Matsushita K, Adachi O, Yamada M (2004) Occurrence of a bound ubiquinone and its function in *Escherichia coli* membrane-bound quinoprotein glucose dehydrogenase. J Biol Chem 279(4):3078–3083

Gómez-Manzo S, Contreras-Zentella M, González-Valdez A, Sosa-Torres M, Arreguín-Espinoza R, Escamilla-Marván E (2008) The PQQ-alcohol dehydrogenase of *Gluconacetobacter diazotrophicus*. Int J Food Microbiol 125(1):71–78

Gomez-Manzo S, Chavez-Pacheco JL, Contreras-Zentella M, Sosa-Torres ME, Arreguin-Espinosa R, Perez de la Mora M, Membrillo-Hernandez J, Escamilla JE (2010) Molecular and catalytic properties of the aldehyde dehydrogenase of *Gluconacetobacter diazotrophicus*, a quinoheme protein containing pyrroloquinoline quinone, cytochrome *b*, and cytochrome *c*. J Bacteriol 192(21):5718–5724

Gómez-Manzo S, González-Valdez AA, Oria-Hernández J, Reyes-Vivas H, Arreguín-Espinosa R, Kroneck PMH, Sosa-Torres ME, Escamilla JE (2012) The active (ADHa) and inactive (ADHi) forms of the PQQ-alcohol dehydrogenase from *Gluconacetobacter diazotrophicus* differ in their respective oligomeric structures and redox state of their corresponding prosthetic groups. FEMS Microbiol Lett 328(2):106–113

Gomez-Manzo S, Escamilla JE, Gonzalez-Valdez A, Lopez-Velazquez G, Vanoye-Carlo A, Marcial-Quino J, de la Mora-de la Mora I, Garcia-Torres I, Enriquez-Flores S, Contreras-Zentella ML, Arreguin-Espinosa R, Kroneck PM, Sosa-Torres ME (2015) The oxidative fermentation of ethanol in *Gluconacetobacter diazotrophicus* is a two-step pathway catalyzed by a single enzyme: alcohol-aldehyde dehydrogenase (ADHa). Int J Mol Sci 16(1):1293–1311

Habe H, Shimada Y, Yakushi T, Hattori H, Ano Y, Fukuoka T, Kitamoto D, Itagaki M, Watanabe K, Yanagishita H, Matsushita K, Sakaki K (2009) Microbial production of glyceric acid, an organic acid that can be mass produced from glycerol. Appl Environ Microbiol 75 (24):7760–7766

Hölscher T, Weinert-Sepalage D, Görisch H (2007) Identification of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H. Microbiology 153(Pt 2):499–506

Kataoka N, Matsutani M, Yakushi T, Matsushita K (2015) Efficient production of 2,5-diketo-D-gluconate via heterologous expression of 2-ketogluconate kehydrogenase in *Gluconobacter japonicus*. Appl Environ Microbiol 81(10):3552–3560

Kawai S, Goda-Tsutsumi M, Yakushi T, Kano K, Matsushita K (2013) Heterologous overexpression and characterization of a flavoprotein-cytochrome *c* complex fructose dehydrogenase of *Gluconobacter japonicus* NBRC3260. Appl Environ Microbiol 79(5):1654–1660

Kondo K, Beppu T, Horinouchi S (1995) Cloning, sequencing, and characterization of the gene encoding the smallest subunit of the three-component membrane-bound alcohol dehydrogenase from *Acetobacter pasteurianus*. J Bacteriol 177(17):5048–5055

Madigan MT, Martinko JM, Parker J (2003) Biology of microorganisms, 10th edn. Prentice Hall, Upper Saddle River

Matsushita K, Shinagawa E, Adachi O, Ameyama M (1989a) Quinoprotein D-glucose dehydrogenase of the *Acinetobacter calcoaceticus* respiratory chain: membrane-bound and soluble forms are different molecular species. Biochemistry 28(15):6276–6280

Matsushita K, Shinagawa E, Adachi O, Ameyama M (1989b) Reactivity with ubiquinone of quinoprotein D-glucose dehydrogenase from *Gluconobacter suboxydans*. J Biochem (Tokyo) 105(4):633–637

Matsushita K, Takaki Y, Shinagawa E, Ameyama M, Adachi O (1992) Ethanol oxidase respiratory chain of acetic acid bacteria. Reactivity with ubiquinone of pyrroloquinoline quinone-dependent alcohol dehydrogenases purified from *Acetobacter aceti* and *Gluconobacter suboxydans*. Biosci Biotechnol Biochem 56(2):304–310

Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301

Matsushita K, Yakushi T, Takaki Y, Toyama H, Adachi O (1995) Generation mechanism and purification of an inactive form convertible in vivo to the active form of quinoprotein alcohol dehydrogenase in *Gluconobacter suboxydans*. J Bacteriol 177(22):6552–6559

Matsushita K, Yakushi T, Toyama H, Shinagawa E, Adachi O (1996) Function of multiple heme *c* moieties in intramolecular electron transport and ubiquinone reduction in the quinohemoprotein alcohol dehydrogenase-cytochrome *c* complex of *Gluconobacter suboxydans*. J Biol Chem 271(9):4850–4857

Matsushita K, Yakushi T, Toyama H, Adachi O, Miyoshi H, Tagami E, Sakamoto K (1999) The quinohemoprotein alcohol dehydrogenase of *Gluconobacter suboxydans* has ubiquinol oxidation activity at a site different from the ubiquinone reduction site. Biochim Biophys Acta 1409 (3):154–164

Matsushita K, Fujii Y, Ano Y, Toyama H, Shinjoh M, Tomiyama N, Miyazaki T, Sugisawa T, Hoshino T, Adachi O (2003) 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl Environ Microbiol 69(4):1959–1966

Matsushita K, Kobayashi Y, Mizuguchi M, Toyama H, Adachi O, Sakamoto K, Miyoshi H (2008) A tightly bound quinone functions in the ubiquinone reaction sites of quinoprotein alcohol dehydrogenase of an acetic acid bacterium, *Gluconobacter suboxydans*. Biosci Biotechnol Biochem 72(10):2723–2731

Miyazaki T, Tomiyama N, Shinjoh M, Hoshino T (2002) Molecular cloning and functional expression of D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO3255, which requires pyrroloquinoline quinone and hydrophobic protein SldB for activity development in *E. coli*. Biosci Biotechnol Biochem 66(2):262–270

Moonmangmee D, Fujii Y, Toyama H, Theeragool G, Lotong N, Matsushita K, Adachi O (2001) Purification and characterization of membrane-bound quinoprotein cyclic alcohol dehydrogenase from *Gluconobacter frateurii* CHM 9. Biosci Biotechnol Biochem 65(12):2763–2772

Mustafa G, Migita CT, Ishikawa Y, Kobayashi K, Tagawa S, Yamada M (2008) Menaquinone as well as ubiquinone as a bound quinone crucial for catalytic activity and intramolecular electron transfer in *Escherichia coli* membrane-bound glucose dehydrogenase. J Biol Chem 283 (42):28169–28175

Nishikura-Imamura S, Matsutani M, Insomphun C, Vangnai AS, Toyama H, Yakushi T, Abe T, Adachi O, Matsushita K (2014) Overexpression of a type II 3-dehydroquinate dehydratase enhances the biotransformation of quinate to 3-dehydroshikimate in *Gluconobacter oxydans*. Appl Microbiol Biotechnol 98(7):2955–2963

Pappenberger G, Hohmann H-P (2014) Industrial production of L-ascorbic acid (vitamin C) and D-isoascorbic acid. In: Zorn H, Czermak P (eds) Biotechnology of food and feed additives. Springer, Berlin, pp 143–188

Peters B, Mientus M, Kostner D, Junker A, Liebl W, Ehrenreich A (2013) Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. Appl Microbiol Biotechnol 97(14):6397–6412

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23(2):195–200

Sakuraba H, Yokono K, Yoneda K, Watanabe A, Asada Y, Satomura T, Yabutani T, Motonaka J, Ohshima T (2010) Catalytic properties and crystal structure of quinoprotein aldose sugar dehydrogenase from hyperthermophilic archaeon *Pyrobaculum aerophilum*. Arch Biochem Biophys 502(2):81–88

Schneider CZ, Parish T, Basso LA, Santos DS (2008) The two chorismate mutases from both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*: biochemical analysis and limited regulation of promoter activity by aromatic amino acids. J Bacteriol 190(1):122–134. Epub 2007 Oct 26

Shinagawa E, Matsushita K, Adachi O, Ameyama M (1981) Purification and characterization of 2-keto-D-gluconate dehydrogenase from *Gluconobacter melanogenus*. Agric Biol Chem 45 (5):1079–1085

Shinagawa E, Matsushita K, Adachi O, Ameyama M (1982) Purification and characterization of D-sorbitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. alpha. Agric Biol Chem 46(1):135–141

Shinagawa E, Matsushita K, Adachi O, Ameyama M (1984) D-Gluconate dehydrogenase, 2-keto-D-gluconate yielding, from *Gluconobacter dioxyacetonicus*: purification and characterization. Agric Biol Chem 48(6):1517–1522

Shinagawa E, Toyama H, Matsushita K, Tuitemwong P, Theeragool G, Adachi O (2006) A novel type of formaldehyde-oxidizing enzyme from the membrane of *Acetobacter* sp. SKU 14. Biosci Biotechnol Biochem 70(4):850–857

Shinagawa E, Adachi O, Ano Y, Yakushi T, Matsushita K (2010) Purification and characterization of membrane-bound 3-dehydroshikimate dehydratase from *Gluconobacter oxydans* IFO 3244, a new enzyme catalyzing extracellular protocatechuate formation. Biosci Biotechnol Biochem 74(5):1084–1088

Shinjoh M, Tomiyama N, Asakura A, Hoshino T (1995) Cloning and nucleotide sequencing of the membrane-bound L-sorbosone dehydrogenase gene of *Acetobacter liquefaciens* IFO 12258 and its expression in *Gluconobacter oxydans*. Appl Environ Microbiol 61(2):413–420

Sugisawa T, Hoshino T (2002) Purification and properties of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255. Biosci Biotechnol Biochem 66 (1):57–64

Sugisawa T, Hoshino T, Nomura S, Fujiwara A (1991) Isolation and characterization of membrane-bound L-sorbose dehydrogenase from *Gluconobacter melanogenus* UV10. Agric Biol Chem 55(2):363–370

Takemura H, Horinouchi S, Beppu T (1991) Novel insertion sequence IS1380 from *Acetobacter pasteurianus* is involved in loss of ethanol-oxidizing ability. J Bacteriol 173(22):7070–7076

Takemura H, Tsuchida T, Yoshinaga F, Matsushita K, Adachi O (1994) Prosthetic group of aldehyde dehydrogenase in acetic acid bacteria not pyrroloquinoline quinone. Biosci Biotechnol Biochem 58(11):2082–2083

Tayama K, Fukaya M, Okumura H, Kawamura Y, Beppu T (1989) Purification and characterization of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes* sp. nov. Appl Microbiol Biotechnol 32(2):181–185

Thurner C, Vela C, Thony-Meyer L, Meile L, Teuber M (1997) Biochemical and genetic characterization of the acetaldehyde dehydrogenase complex from *Acetobacter europaeus*. Arch Microbiol 168(2):81–91

Toyama H, Soemphol W, Moonmangmee D, Adachi O, Matsushita K (2005) Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. Biosci Biotechnol Biochem 69(6):1120–1129

Toyama H, Furuya N, Saichana I, Ano Y, Adachi O, Matsushita K (2007) Membrane-bound, 2-keto-D-gluconate-yielding D-gluconate dehydrogenase from "*Gluconobacter dioxyacetonicus*" IFO 3271: molecular properties and gene disruption. Appl Environ Microbiol 73(20):6551–6556

Vangnai AS, Toyama H, De-Eknamkul W, Yoshihara N, Adachi O, Matsushita K (2004) Quinate oxidation in *Gluconobacter oxydans* IFO3244: purification and characterization of quinoprotein quinate dehydrogenase. FEMS Microbiol Lett 241(2):157–162

Vangnai AS, Promden W, De-Eknamkul W, Matsushita K, Toyama H (2010) Molecular characterization and heterologous expression of quinate dehydrogenase gene from *Gluconobacter oxydans* IFO3244. Biochemistry (Moscow) 75(4):452–459

Yakushi T, Matsushita K (2010) Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. Appl Microbiol Biotechnol 86(5):1257–1265

Yamada M, Sumi K, Matsushita K, Adachi O, Yamada Y (1993) Topological analysis of quinoprotein glucose dehydrogenase in *Escherichia coli* and its ubiquinone-binding site. J Biol Chem 268(17):12812–12817

Yamaoka H, Ferri S, Fujikawa M, Sode K (2004) Essential role of the small subunit of thermostable glucose dehydrogenase from *Burkholderia cepacia*. Biotechnol Lett 26(22):1757–1761

Chapter 14
Cellulose and Other Capsular Polysaccharides of Acetic Acid Bacteria

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Abstract Some acetic acid bacteria including *Komagataeibacter xylinus* are known to produce a large amount of cellulose in their culture media. This bacterial cellulose has a unique structure. The fiber of bacterial cellulose is ultrafine, with a width approximately 1/1000th that of pulp cellulose. Although the mechanism of bacterial cellulose synthesis is unclear, recent research, including structure analysis by X-ray diffraction, has revealed the mechanism at the cellulose synthase complex. The biological function of bacterial cellulose, as well as other capsular polysaccharides of acetic acid bacteria, seems to be the formation of biofilms that allow the retention of bacterial cells on the culture surface. This mechanism is favorable for their survival because acetic acid bacteria are strictly oxidative and utilize a large amount of oxygen for their metabolism. To use bacterial cellulose as a commodity material, industrial production systems have been developed using strain improvement and agitation cultures. Bacterial cellulose has many unique properties derived from its structural features. In addition to having been utilized for a long time as a well-known dessert (nata de coco) in Southeast Asia, it has many other possible applications. Some of these have already been commercialized, including speaker diaphragms and artificial skin.

Keywords Bacterial cellulose • Nata de coco • Polysaccharide • *Komagataeibacter xylinus* • Biofilm • Industrial production • Ultrafine fibril

14.1 Introduction

Some strains of acetic acid bacteria produce a gelatinous membrane called a pellicle at the surface of liquid cultures (Fig. 14.1a). The group of cellulose-producing acetic acid bacteria was previously known as *Acetobacter xylinum* (Yamada and Yukphan 2008) and was recently transferred to a newly proposed genus,

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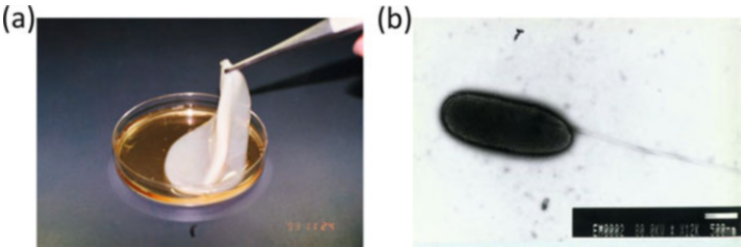

Fig. 14.1 Bacterial cellulose produced by acetic acid bacteria. (**a**) A pellicle produced on the surface of the culture. (**b**) A cellulose ribbon produced by a bacterial cell. (Modified from Tonouchi 2012)

Komagataeibacter, with the type species *Komagataeibacter xylinus* (Yamada et al. 2012, 2013).

Microscopic analysis has revealed that a single ribbon of cellulose is produced from one bacterial cell (Fig. 14.1b). A single ribbon comprises an assembled structure of cellulose microfibrils that are excreted from a row of synthetic sites called terminal complexes (TCs) located along one side of the cell (Kimura et al. 2001). To purify the bacterial cellulose from the culture broth, a gentle treatment using a diluted alkaline solution can be used to separate the medium or the cells. In contrast, for the purification of pulp cellulose, harsh digestion conditions using concentrated alkaline solutions at high temperatures are needed to solubilize the lignin and hemicellulose that are tightly bound to it.

Regarding the structure of cellulose, Brown reported in 1886 that the gelatinous bacterial substance is chemically identical to cellulose. Cellulose is the most abundant biopolymer on earth and has a tremendous global economic importance. Cotton and wood are the major resources for all cellulose and cellulose-derived products. The largest amount of cellulose, however, is produced by unicellular plankton or algae in the oceans, and this represents nature's largest resource of cellulose products.

Although the chemical structures of plant and bacterial cellulose are identical, the physical structure of bacterial cellulose is unique, being composed of ultrafine fibers that form an ultrafine network. The mechanical properties of bacterial cellulose are also unique. In this chapter, the mechanism of acetic acid bacteria cellulose biosynthesis, and its biological functions, properties, and industrial applications, are described.

14.2 Biosynthesis of Bacterial Cellulose

14.2.1 Cellulose Synthesis Metabolic Pathway

The biosynthetic pathway that produces bacterial cellulose from glucose and fructose is shown in Fig. 14.2. Glucose is phosphorylated by glucose hexokinase and not by the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). The resulting glucose-6-phosphate (G6P) is metabolized through the pentose pathway, because the activity of fructose-6-phosphate (F6P) kinase, which phosphorylates F6P to fructose-1,6-diphosphate (FDP), is absent in acetic acid bacteria.

The cellulose pathway branches at G6P where it is metabolized to UGP-glucose (UDPG), the direct precursor of cellulose. The biosynthesis of UDPG from glucose is a two-step enzymatic process involving phosphoglucomutase and UDPG-pyrophosphorylase.

Fructose is metabolized by both fructose hexokinase and PTS (Tonouchi et al. 1996). Fructose is phosphorylated to F6P by hexokinase or to fructose-1-phosphate (F1P) via PTS. F6P is then converted by phosphoglucomutase to G6P, which can subsequently be used for cellulose synthesis or metabolized through the pentose phosphate pathway.

F1P is converted to FDP by F1P kinase, and FDP is subsequently dephosphorylated to F6P. FDP can also be metabolized through the Embden–Myerhoff pathway (EMP).

The activity of phosphoglucose isomerase, which converts F6P to G6P, is very high in a fructose medium compared with a glucose medium. This difference seems to be reasonable because of the metabolism/cellulose synthesis pathway. On the

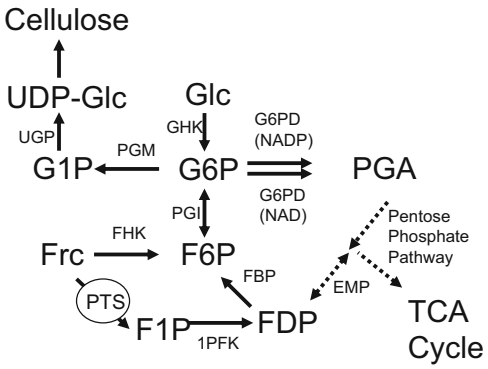

Fig. 14.2 Cellulose biosynthetic pathway in cellulose-producing acetic acid bacteria. *F1P* fructose-1-phosphate, *F6P* fructose-6-phosphate, *FDP* fructose diphosphate, *PGA* phosphogluconate; *GHK* glucose hexokinase, *FHK* fructose hexokinase, *1PFK* fructose-1-phosphate kinase, *FBP* fructose bisphosphatase, *PGI* phosphoglucose isomerase, *PGM* phosphoglucomutase, *UGP* UDP-glucose pyrophosphorylase, *G6PD* glucose-6-phosphate dehydrogenase, *PTS* phosphotransferase system; *EMP* Embden–Myerhoff pathway

other hand, phosphoglucomutase activity is high in a glucose medium compared with a fructose medium. The reason for this is unclear. UDPG-pyrophosphorylase activity is approximately 100 fold higher in the cellulose-producing strain (*Komagataeibacter sucrofermentans* BPR2001) compared with the non-cellulose-producing acetic acid bacteria strain (*Acetobacter aceti* 10-8S2).

In bacteria such as *Escherichia coli*, G6P dehydrogenase (G6PD) activity is NADP dependent. However, in cellulose-producing strains, both NAD-dependent and NADP-dependent G6PD activities are found. The NAD-dependent G6PD reaction shows an unusually low optimum pH of 5.6, and this has been suggested to regulate the flow of G6P into the pentose phosphate pathway (Benziman and Mazover 1973). More recent studies of additional acetic acid bacteria strains have demonstrated that both NAD and NADP activities are the result of a single enzyme that possesses NAD/NADP dual coenzyme specificity (Tonouchi et al. 2003).

The gene for phosphoglucomutase was cloned as a DNA fragment into a cellulose production-deficient mutant before being sequenced (Fjaervik et al. 1991; Brautaset et al. 1994). The gene coding UDPG-pyrophosphorylase was cloned by a similar method (Valla et al. 1989; Brede et al. 1991).

14.2.2 Cellulose Synthase Operon

Cellulose biosynthesis involves three basic steps: binding of glucose molecules to form beta-1,4-glucan (polymerization), extruding it through the membrane (translocation), and assembling the glucan chains (aggregation and crystallization). All these steps require strict organization, and the cellulose synthase complex is thus stringently coordinated. Although most of the world's cellulose is produced by plants, cellulose-producing acetic acid bacteria have been used as a model system for studying its biosynthesis because of established molecular genetic techniques.

The genetic structure of the bacterial cellulose synthase (*bcs*) operon (Fig. 14.3) constitutes a promoter sequence upstream (Nakai et al. 1998a) of four genes (*bcsA*, *bcsB*, *bscC*, *bcsD*). The detailed structure of these genes has been described by Romling (2002) and Valla et al. (2009). Here, the recently described functions of each subunit are discussed.

BcsA is the catalytic subunit of the complex and BcsB is a membrane-anchored periplasmic protein (Morgan et al. 2013). These proteins bind to form a complex, and in some cases, the protein products of these genes are fused as a single polypeptide (Kawano et al. 2002). BcsA contains a conserved family-2 glycosyltransferase (GT) domain, containing the motif D, D, D, Q(Q/R)XRW and FFCGS sequence. The GT domain is located intracellularly. A transmembrane domain forms a narrow channel from the catalytic site to the periplasmic BcsA–BcsB interface, suggesting that the nascent glucan chain is translocated through this channel. The structure of the BcsA-BcsB complex further suggests the possible mechanism of cellulose synthesis. The size of the substrate-binding pocket indicates that the nascent glucan is extended by one glucose molecule, rather than two.

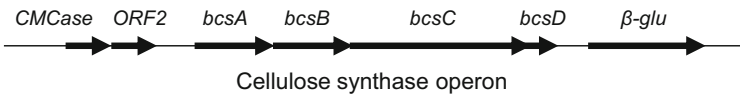

Fig. 14.3 Genetic structure of the cellulose synthase operon and other genes involved in cellulose synthesis

Furthermore, the dimensions of the transmembrane channel require glucopyranose rings to be positioned on the same plane to slide into the pore. Following glycosyl transfer, the newly attached terminal glucose molecule is speculated to rotate around the acetal linkage to align with the glucan in the transmembrane channel. Steric interactions might dictate the rotation direction, resulting in beta-1,4-glucan formation.

The product of *bcsC* is a large protein with transmembrane domains, and its function is to form pores in the outer membrane through which the synthesized beta-glucan chains could be extruded (Whitney et al. 2011). The inactivation of the *bcsC* gene has been reported to lead to the loss of cellulose production activity in vivo, but not in vitro (Wong et al. 1990).

BcsD is a periplasmic protein (Iyer et al. 2011). Bacterial mutants with *bcsD* deficiency produce reduced amounts of cellulose in vivo. However, in vitro, the mutants possessed unchanged cellulose levels, whereas the synthesized cellulose structure has been reported to be altered (Saxena et al. 1994). Recent studies of the X-ray structure revealed their structure and function. The octamer of the *bcsD* gene product forms an exquisite cylindrical shape with a right-twisted dimer interface on its wall, which creates four inner passageways. This structure suggests that four glucan chains are individually extruded through their own passageways (Hu et al. 2010).

14.2.3 Other Genes Involved in Bacterial Cellulose Synthesis

Two open reading flames, *CMCase* (a cellulase) and *CcpAx* (also known as *ORF-2*), exist upstream of the *bcs* operon. In addition, a cellulase (beta-glucosidase) gene exists downstream of the *bcs* operon.

Although carboxymethyl cellulase (CMCase) is a cellulose-degrading enzyme, it is involved in cellulose formation in acetic acid bacteria (Tonouchi et al. 1995). In plants, a membrane-bound endo-1,4-beta-glucanase called Korrigan (KOR) is involved in cellulose formation (Sato et al. 2001). In a previous report, the CMCase gene was also shown to be essential for cellulose production (Standal et al. 1994). Nevertheless, a recent study found that a very small amount of particulate material accumulated in the culture of the disrupted strain (Nakai et al. 2013). The particulate material was shown to contain highly twisted ribbons of cellulose. It was also reported that a portion of CMCase is localized to the cell surface (Yasutake

et al. 2006). CMCase may help to remove the tensional stress of the glucan molecules during the formation of crystalline cellulose microfibrils.

CcpAx gene, or *ORF2* gene, is also required for cellulose production (Standal et al. 1994) as the disruption of the gene results in a deficiency of cellulose production. Interestingly however, the membrane fraction retained the cellulose-producing activity. Although its exact function is still unclear, CcpAx has been suggested to be involved in the higher-order construction of cellulose (Nakai et al. 2002). Recently, microscopic analysis has revealed that the *CcpAx* gene product is approximately 8 kDa in size and is localized along one side of the cell membrane (Sunagawa et al. 2013). This localization pattern is similar to that of BcsD. In addition, pulldown assays have demonstrated that CcpAx interacts with BcsD and is suggested to be a member of the terminal complex.

The function of the beta glucosidase gene remains unclear (Tonouchi et al. 1997). However, it is active during cell culture, and its involvement in cellulose formation has been previously suggested (Tahara et al. 1998).

The cellulose synthase complex is known to require the cofactor cyclic di-GMP (c-di-GMP). This substance was first shown to be an essential factor in cellulose synthesis (Ross et al. 1987), although it is now well known as a second messenger used in signal transduction in a variety of bacteria (Tamayo et al. 2007). The genes responsible for c-di-GMP synthesis in acetic acid bacteria, diguanylate cyclase (cdg), have been cloned, and it was found that there are three homologous genes (Tal et al. 1998). Each *cdg* gene forms an operon with a c-di-GMP degradation enzyme gene (phosphodiesterase A; *pdeA*), which lies upstream of the *cdg* gene. The bcsA subunit contains the c-di-GMP-responsive pilZ domain located adjacent to the GT domain (Fujiwara et al. 2013). The binding of c-di-GMP to bcsA could induce conformational changes that allow UDP-glucose to access its catalytic site.

Recent progress in the structural analysis of cellulose synthase has provided us with the possible model shown in Fig. 14.4.

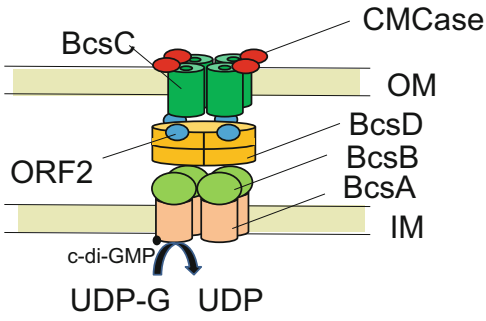

Fig. 14.4 A possible model of cellulose synthase complex. (Modified from Tonouchi 2015)

14.3 Biological Functions of Cellulose Production

14.3.1 Ecological Role of Cellulose in Acetic Acid Bacteria

Some acetic acid bacteria produce a large amount of extracellular cellulose. Interestingly, no selective growth disadvantage for mutant strains lacking cellulose production have been observed in the laboratory, suggesting that this function must have an important role in their natural environment. Cellulose might provide ecological benefits, such as providing a scaffold for cell–cell contact, allowing for their adherence to solid surfaces, or assisting bacteria in localizing at the air–liquid interphase. It may also provide protection against grazing, harmful chemicals, and desiccation.

Cellulose-producing acetic acid bacteria grown in static conditions produces a cellulose pellicle that floats on the liquid surface. The bacteria living in this biofilm have access to nutrients in the liquid phase and also sufficient atmospheric oxygen. When growing on surfaces such as rotting fruits, it has been shown that cellulose enhances the growth of the bacteria, probably by promoting the attachment of the bacteria to the surface, keeping competitors away, and by retaining moisture and protecting the bacteria against UV light.

In some cases, cellulose production is enhanced by co-cultivation with other bacteria (Seto et al. 2006). A microbial colony isolated from vineyard soil was found to contain two bacterial strains: cellulose-forming acetic acid bacteria and a *Lactobacillus* strain. Thus, it was found that cell–cell interactions between the two bacterial species caused the enhancement of cellulose production of the acetic acid bacteria. The *Lactobacillus* mutants deficient in extracellular polysaccharide (EPS) production were unable to promote cellulose production. The addition of EPS to the acetic acid bacterium was also unable to influence its productivity.

14.3.2 Biological Functions of Cellulose Produced by Other Bacteria

Cellulose is produced by many bacteria living in the rhizosphere and sewage sludge other than acetic acid bacteria. In many species, cellulose production seems to be tightly regulated and might occur only under specific growth conditions.

Agrobacterium tumefaciens and *Rhizobium leguminosarium* are cellulose-producing soil bacteria. They bind to plant roots in two steps. In the first step, bacterial proteins bind to plant lectins and acidic surface polymers. Although this is an essential step, the binding is very weak. In the second step, bacterial cellulose fibrils firmly attach the bacterial cells to the plant cell walls. Notably, cellulose-deficient mutants of *A. tumefaciens* are far less infectious than the wild type. Cellulose production in *R. leguminosarium* is induced by plant contact, and it has been suggested that this contact induces the bacteria to produce a cellulose

monolayer. This function ensures the optimal utilization of plant nutrients, and cellulose may assist in water retention, as well as provide protection against temperature changes and UV light.

Several enterobacterial species including *Enterobacter* sp. also produce cellulose (Sunagawa et al. 2012). The cellulose produced by *Salmonella enteritidis* forms part of a biofilm that also contains proteinaceous curli, an O-polysaccharide, and BapA protein. This biofilm protects the bacteria from desiccation and harmful substances such as hypochlorite. The ability to produce the biofilm does not affect virulence, but it is important for bacterial survival outside the animal host.

Cellulose production has also been identified in several species of *Cyanobacteria*. However, here the cellulose forms only a minor part of the cyanobacterial EPS, and there are no reports describing the phenotypic effects of mutants lacking cellulose synthase.

14.3.3 Other Polysaccharides Produced by Acetic Acid Bacteria

The cellulose-producing acetic acid bacteria strains are known to also produce a water-soluble polysaccharide called AM-2 (Tayama et al. 1985) or acetan (Couso et al. 1987). This polysaccharide is composed of D-glucose, D-mannose, L-rhamnose, and D-glucuronic acid in the relative proportions 3/4:1:1:1. It also contains a backbone of cellulose linked to a pentasaccharide residue (Rha-Glc-Glc-GlcA-Man) at the three-position of every other beta-D-glucopyranosyl residue (Jasson et al. 1993). Its biosynthesis pathway is similar to that of other EPSs such as xanthan, with three steps as follows: (1) construction of the lipid-linked oligosaccharide subunit in the cytoplasm, (2) polymerization in the periplasm, and (3) secretion through the outer membrane. The genes involved in acetan biosynthesis are located within an 8.4-kb genomic region that contains six open reading frames. These genes are homologous to the EPS genes of other gram-negative bacteria (Griffin et al. 1995). Acetan appears to affect both the production rate and structure of cellulose because mutant strains differ in their distribution of cellulose polymerization and in their crystallinity (Watanabe et al. 1998). In addition, there are several reports of non-cellulose-producing acetic acid bacteria that produce EPSs with different sugar compositions (Minakami et al. 1984; Grimmecke et al. 1994; MacCormick et al. 1996).

There are some reports regarding the polysaccharides involved in pellicle formation. It is well known that acetic acid bacteria grown in a static culture by forming a pellicle on the surface of the culture medium. Because acetic acid bacteria are strictly oxidative and utilize a large amount of oxygen for their metabolism, these species maintain a high state of aeration by forming a pellicle. *A. pasteurianus* (strain NBRC3284) also produces a pellicle, but the pellicle is not

composed of cellulose. Instead, this polysaccharide is composed of the two monosaccharides, glucose and rhamnose, at an approximately equimolar ratio. It is tightly attached to the cell's outer membrane and keeps the bacteria at the surface of the medium, similar to a crepe membrane in vinegar production (Moonmangmee et al. 2002a). Furthermore, another type of pellicle polysaccharide has been found in a different *Acetobacter* species, *A. tropicalis* (strain SKU1100). This strain produces a thick pellicle at high temperatures. The pellicle polysaccharide is composed of three different monosaccharides: galactose, glucose, and rhamnose, in an approximately equimolar ratio (Moonmangmee et al. 2002b). An operon of five genes (*polABCDE*) has been identified as being responsible for pellicle formation (Deeraksa et al. 2005). Following cultivation of strain SKU1100 in a submerged culture, two types of colony [rough-surfaced (R strain) and smooth-surfaced (S strain)] were observed on agar plates. The R strain is able to produce the pellicle polysaccharide in static culture whereas the S strain cannot. Also, the R and S strains are interconvertible by spontaneous mutation. The terminal oxidase component in *A. aceti* can be changed by culture conditions (Matsushita et al. 1992). Thus, some acetic acid bacteria appear to possess a mechanism for their rapid adaptation to changing environmental conditions.

14.4 Development of Industrial Production Processes with Agitation Cultures

To use bacterial cellulose as a commodity material, the development of an industrial production system is essential. Most of the previously described studies of cellulose production were investigated using the static culture method. However, for the large-scale cultivation required for industrial production, a production system using aeration and agitation cultures is needed. With this aim, a national venture project called the Bio-Polymer Research Co. Ltd. (BPR) was established in Japan, and extensive investigations were performed (Yoshinaga et al. 1997).

14.4.1 Isolation of a Cellulose High-Producing Strain

There have been only a few previous reports of cellulose production using aeration and agitation cultures (Dudman 1960), and in most of these reports cellulose production was decreased by shaking culture. One of the reasons for this decrease is the genetic instability of the producer organism. In some of the strains, cells lacking cellulose-producing ability appeared during the shaking culture. The genetic instability may be caused by insertion sequences (ISs), and they have been detected in cellulose-producing acetic acid bacteria strains (Coucheron 1991). To construct an industrial production system, a strain producing high

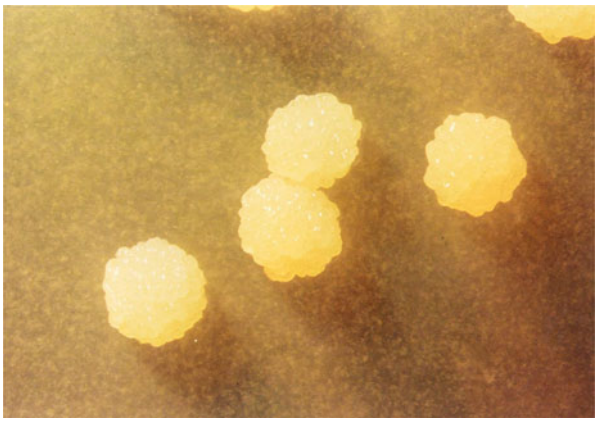

Fig. 14.5 Typical colony morphology of a cellulose-producing acetic acid bacteria (*Komagataeibacter swingsii*)

cellulose levels in agitation culture was isolated. About 3000 cellulose-producing strains were isolated from natural sources, and the best cellulose producer among them, BPR2001, was selected (Toyosaki et al. 1995a) . This strain is capable of achieving high cellulose production in a jar fermenter. The examination of the physiological and biochemical characteristics of this strain indicated that it belonged to a new subspecies, identified as *A. xylinum* subsp. *sucrofermentans* (its present name is *Komagataeibacter sucrofermentans*; Toyosaki et al. 1995b).

Initially, cellulose production was investigated using fructose as the carbon source of the culture medium. However, because of cost constraints when considering an industrial production system, the most suitable carbon source was determined to be sucrose, which is present in large quantities at a low cost. Therefore, cellulose-producing strains using sucrose as the carbon source were screened. The selected strain was identified as *A. xylinum* subsp. *nonacetoxidans* (its present name is also *K. swingsii*) (Seto et al. 1997; Kojima et al. 1997, 1998). A typical colony morphology is shown in Fig. 14.5.

14.4.2 Strain Breeding by Mutation

Improvements to the newly isolated cellulose-producing strains were attempted. One example that we have reported is the disruption of glucose dehydrogenase, which is responsible for the production of gluconate from glucose (Takemura et al. 1994). Another approach that we have examined is obtaining analogue-resistant mutants, which is commonly used for the selection of high producers of amino acids. Cellulose production is known to be associated with cell growth. The addition of *p*-aminobenzoic acid (PABA) to the culture media was found to increase the cell growth and cellulose production. A mutant strain with resistance to sulfaguanidine, which is known to be an analogue of PABA, was selected (Ishikawa et al. 1995). This mutant strain was shown to have a high cell growth rate and a high

level of cellulose production probably with an increased ATP supply (Ishikawa et al. 1998b). Furthermore, to increase the supply of UDP-glucose (the precursor of cellulose), a mutant strain with 5′-fluorouridine resistance was selected: 5′-fluorouridine is a pyrimidine analogue that inhibits the synthesis of UDP-glucose. The intracellular concentration of UDP-glucose in the mutant was found to be increased more than threefold, and its cellulose productivity was also significantly increased (Ishikawa et al. 1998a).

14.4.3 Genetic Engineering

Genetic engineering is also expected to provide a useful approach to increase cellulose production. A new host–vector system has been developed using a cryptic plasmid found in strain BPR2001. Its nucleotide sequence was determined and a novel shuttle vector was constructed (Tonouchi et al. 1994). Strain improvement using the newly developed vector was investigated. Some genes involved in sucrose metabolism, such as invertase (Tonouchi et al. 1998a), sucrose phosphorylase (Tonouchi et al. 1998b), and sucrose synthase (Nakai et al. 1998b), were introduced. The results revealed that sucrose synthase, in particular, is an enzyme for the efficient synthesis of cellulose from sucrose. UDP-glucose, which is the direct precursor of cellulose, is generated directly from sucrose without the need for ATP or UTP, in contrast with the native cellulose synthesis pathway in acetic acid bacteria, where one molecule of ATP and UTP each is consumed. By introducing a mutant sucrose synthase gene (Nakai et al. 1998b), the resultant strain was revealed to have a much higher cellulose productivity (Nakai et al. 1999).

14.4.4 Investigation of the Culture Conditions

Optimal culture conditions are essential if industrial levels of cellulose production are to be achieved. We therefore investigated the culture conditions in a jar fermenter using BPR2001 and its derived mutants. Notably, we also found that the accumulation of cellulose increased culture viscosity, which eventually made controlled agitation and mixing impossible because of the non-Newtonian behavior of the culture broth (Kouda et al. 1996). We also found that the addition of carbonic acids to the culture improved early-phase cell growth and increased cellulose production. The addition of lactic acid was the most effective, and its action has been suggested to be mediated by both the generation of ATP by lactate dehydrogenase and the activation of the TCA cycle by pyruvate generation (Matsuoka et al. 1996).

The study of these and other factors allowed us to produce a scaled-up cellulose-producing culture in a jar fermenter. An additional factor was the development and evaluation of an improved impeller in the jar fermenter (Kouda et al. 1997). With

these culture modifications, we were able to achieve a productivity of approximately 20 g/l accumulated cellulose in 40 h. Moreover, a new production method using an air-lift jar fermenter has also been investigated (Chao et al. 1997).

14.5 Structural Features and Mechanical Properties of Bacterial Cellulose

14.5.1 Structure: Ultrafine Fibrils

In 1886, Brown reported that the gelatinous membrane is composed of a substance that is chemically identical to cellulose (1886). Electron microscopic observations in the 1940s revealed that bacterial cellulose consists of twisting ribbon-shaped fibrils (Muhlethaler 1949). The ribbons are approximately 20–50 nm in width, which is less than 1/1000th the width of plant pulp cellulose (Fig. 14.6). Each ribbon is composed of a number of microfibrils that may be observed under transmission electron microscopy (Brown et al. 1976; Zarr 1979). Acid hydrolyzation experiments have revealed that these microfibrils are composed of regularly repeating units (Hori et al. 1997). Microfibril size correlates with its crystallite size, as measured by X-ray diffraction (Nieduszy and Preston 1970).

Bacterial cellulose has an ultrafine network structure of cellulose fibrils. The width of the microfibrils is approximately 1/1000th that of the pulp fiber. Even the thinnest fiber is a chemically synthetic fiber having a size of approximately 1 μm; bacterial cellulose fibril is 1/10th that of the synthetic fiber.

These structural features vary according to whether a static or agitation culture method was used. In the former method, bacterial cellulose is produced as a gelatinous membrane. However, in the latter method, shear stress seems to inhibit the formation of a gelatinous membrane, resulting in the accumulation of small aggregates of bacterial cellulose. Thus, it accumulates in a dispersed suspension. The microscopic cellulose structure also changes at various phases in the hierarchical structure of the bacterial cellulose. Granular or fibrous aggregates produced

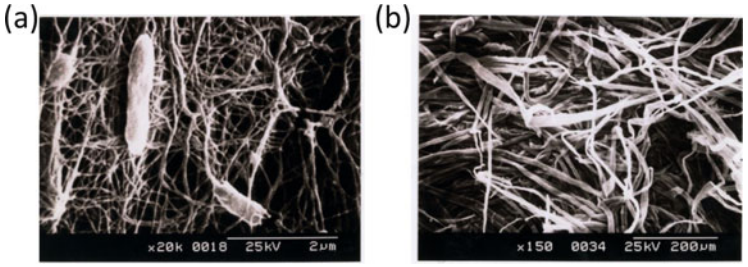

Fig. 14.6 Scanning electron micrograph of bacterial cellulose and wood pulp. (**a**) Bacterial cellulose. (**b**) Wood pulp. *Bars* (**a**) 2 μm; (**b**) 200 μm, respectively

in agitated cultures have a fibril network similar to that observed on the surface of gelatinous membranes in static cultures. However, there are some differences in the structure of cellulose crystals and molecular chains between the two types of bacterial cellulose produced under the different culture conditions. In agitated cultures, the degree of cellulose crystallinity and the cellulose I alpha content are found to be lower than those in static cultures. In addition, the degree of cellulose polymerization is lower. The structural disorder of bacterial cellulose generated in agitated cultures seems to arise from both the physical effects of agitation (Watanabe et al. 1998) and the cellulases excreted by the cells (Tahara et al. 1997a, b).

This unusual cellulose network structure seen in agitated cultures can be regulated by modifying culture conditions such as oxygen tension. These findings could help anticipate the exploitation of the structural design of bacterial cellulose by biotechnology.

14.5.2 Mechanical Properties of "Dried Sheet Cellulose" and "Disintegrated Bacterial Cellulose"

The uses of bacterial cellulose depend on its structural features. A dried sheet prepared from a purified gelatinous membrane grown in a static culture has an extremely high Young's modulus of more than 15 GPa (Yamanaka et al. 1989). This value is higher in the sheets made from other organic polymers. The specific Young's modulus corresponds to that of aluminum (Nishi et al. 1990). The high Young's modulus value for this form of cellulose sheet arises from the strong interfibrillar binding of its ultrafine fibrils. From bacterial cellulose produced in an agitated culture, a dried sheet can be prepared that has a slightly lower Young's modulus because the cellulose has a substantially disordered structure.

By using mechanical homogenization, bacterial cellulose can be processed into a stable suspension containing disintegrated and fragmented fibrils (Ougiya et al. 1998). Such a suspension is called disintegrated bacterial cellulose. Using a homogenizer or disintegrater, disintegrated bacterial cellulose can be obtained from both a gelatinous cellulose membrane grown in a static culture and a cellulose suspension grown in an agitated culture. This disintegrated cellulose shows high water retention characteristics. In addition, by mixing other fibrous materials with disintegrated bacterial cellulose, various composite sheets can be obtained. Bacterial cellulose facilitates the formation of these sheets because of its strong binding ability. A suspension of disintegrated bacterial cellulose can be used as a thickener, disperser, emulsifier, and stabilizer for suspending various materials. It has also been found that these useful properties can be easily controlled by altering various preparative conditions (Watanabe and Yamanaka 1995).

Recently, nanofibrillated cellulose (NFC) has attracted attention as a new cellulose material (Abdul Khalil et al. 2012). NFC is generally produced by the physical

or chemical treatment of cellulose microfibers from plants. Because bacterial cellulose naturally has a nanofiber structure, segmentalized bacterial cellulose (bacterial NFC) is produced by addition of carboxymethylcellulose (CMC) to the culture medium (Kose et al. 2013), which is known to prevent the aggregation of microfibrils during cellulose synthesis (Cheng et al. 2011).

14.6 Applications

Differing from wood pulp cellulose, cellulose produced by acetic acid bacteria is devoid of contaminating polysaccharides such as hemicellulose and lignocellulose. The isolation and purification of bacterial cellulose are therefore relatively simple processes. Because of its high purity, hydrophilicity, structure-forming potential, chirality, and biocompatibility, bacterial cellulose offers many possible applications in medical use.

From an industrial point of view, the potential applications of bacterial cellulose are eagerly anticipated. Although many patent applications have been submitted all over the world, at present only a few commercial applications exist. These are described next.

14.6.1 Nata de Coco

In the Philippines and other countries in Southeast Asia, bacterial cellulose has been utilized as a traditional food for more than 100 years. Known as "nata de coco," it is a chewy, translucent, jelly-like product. Nata de coco is produced by fermenting coconut water. Its production can by summarized by the following steps: (1) extracting coconut water, (2) fermenting it with acetic acid bacteria, (3) separating and cutting the nata de coco cellulose mat, (4) cleaning and washing to remove the acetic acid, and (5) cutting and packaging. The resulting gelatinous pellicle is commonly sweetened and used as a dessert. Although this dessert originated in Southeast Asia, it is now becoming popular throughout the world.

14.6.2 Speaker Diaphragm

The first example of an industrial application for bacterial cellulose is its use as an acoustic transducer diaphragm (Nishi et al. 1990). An acoustic transducer diaphragm requires a high sonic velocity and a high internal loss. The sonic velocity is calculated from the specific Young's modulus, which in a dried sheet of bacterial cellulose is approximately 5000 m/s. Regarding its internal loss, a bacterial cellulose sheet has a high internal loss of 0.03, equal to that of conventional paper.

Generally, a material having high sonic velocity that has a low internal loss is preferred. Thus, bacterial cellulose is a material well suited for these diaphragms, and it has been commercialized in various types of speaker units and headsets.

14.6.3 Artificial Skin

Bacterial cellulose has also been employed as an artificial skin for the temporary covering of wounds (Fontana et al. 1990; Petersen and Gatenholm 2011). Advantages include its high mechanical strength in a wet state, substantial permeability to liquids and gases, and low skin irritation. Biofill and Genfiflex are commercialized bacterial cellulose artificial skin products that have applications in surgery and dental implants and human health care. Many advantages for Biofill have been reported, such as immediate pain relief, close adhesion to the wound bed, diminished post-surgery discomfort, reduced infection rate, easiness of wound inspection (transparency), faster healing rate, improved exudate retention, spontaneous detachment following reepithelization, and reduced treatment time and cost. Its only disadvantage has been reported to be its limited elasticity in areas of great mobility. Similarly, Gengiflex has been developed to treat periodontal tissues.

The first exploratory investigation on the use of bacterial cellulose as a liquid-loaded pad for wound care was performed by Johnson and Johnson in the early 1980s. Bacterial cellulose composites blended with chitosan, polyethylene glycol (PEG), and gelatin were tested for potential biomedical applications, and the products look like a foam in structure. Cell adhesion studies showed that these composite products have a greater biocompatibility than pure bacterial cellulose.

14.6.4 Artificial Blood Vessels

Bacterial cellulose has been investigated for its potential use as artificial blood vessels because it carries a lower risk of blood clots. Native bacterial cellulose has several mechanical properties that are superior to those of many currently used synthetic materials such as polypropylene, polyethylene terephtalate, and cellophane. These properties include shape retention and tear resistance.

Furthermore, the addition of a small amount of bacterial cellulose nanofibrils to anisotropic polyvinyl alcohol produces the nanocomposite material PVA-BC. This material possesses a broad range of mechanical properties, including anisotropy by controlling material and processing parameters. A PVA-BC nanocomposite with a controlled degree of anisotropy that matches the mechanical properties of soft tissue can be created.

14.6.5 Potential Scaffold for Tissue Engineering

It has been reported that bacterial cellulose is capable of supporting the growth of mammalian cells (Watanabe et al. 1993). Bacterial cellulose is also a potential scaffolding material for the tissue engineering of cartilage. In this process it is essential to support cell proliferation and maintain their differentiated functions, in addition to defining the shape of the new growing tissue. Thus far, many natural and synthetic polymers have been evaluated for their scaffolding potential. However, a scaffold possessing the natural mechanical properties of cartilage has not yet been described. Native bacterial cellulose supports bovine chondrocyte proliferation at levels of approximately 50 % of the collagen type II substrate. The use of native bacterial cellulose as a scaffold support has been further explored using human chondrocytes.

14.6.6 Papermaking Binder

Disintegrated bacterial cellulose has been found to have a remarkably high retention aid function for papermaking (Hioki et al. 1995). In the process of papermaking, additives such as calcium carbonate are usually used. These additives are called fillers and are used to increase paper brightness or whiteness. However, a large proportion of the filler is not integrated into the paper and is lost. By adding a small amount of bacterial cellulose, the yield of filler integration was found to increase. Mechanistically, the filler granules are entrapped by the ultrafine bacterial cellulose fibrils, similar to a spider's web, and are consequently retained in the paper. The bacterial cellulose produced in agitated cultures has a higher retention aid function than that produced in static cultures. It is thought that the cellulose formed in agitated cultures has a higher accessibility because of its disordered structure.

14.6.7 Other Possible Applications

Disintegrated bacterial cellulose has been also employed as a functional food additive, a thickener, and a dispersant (Okiyama et al. 1993a, b). It has also been investigated as a novel functional material in fields as diverse as medical supplies, cosmetics, and drilling (Keshk 2014).

Recent reports have also investigated the application of bacterial cellulose for optically transparent composites (Yano et al. 2005) and as a medium for the electrophoretic separation of DNA (Tabuchi and Baba 2005; Tabuchi 2007). In the latter case, small size differences of 10 to 100 bp in DNA fragments were detected, as were single-nucleotide polymorphisms.

14.7 Conclusion

In this chapter, the mechanism of biosynthesis, and the biological functions, properties, and industrial applications of cellulose derived from acetic acid bacteria, were described.

Cellulose production is a very interesting phenomenon in acetic acid bacteria. The biological function of cellulose seems to involve the retention of the bacterial cell on a liquid surface; however, this does not explain why these bacteria produce such vast quantities of cellulose. Further studies on cellulose function are therefore required.

The mechanism of bacterial cellulose production has been extensively studied by many research groups. Notably, some important differences exist between its production in bacteria and in plants. For example, bacterial cellulose is produced in the culture medium, whereas plant cellulose is synthesized as a cell-wall construction. Also, in bacteria the precursor of cellulose is UDP-glucose, whereas in plants it is GDP-glucose.

Considering the unique properties of bacterial cellulose, many aspects regarding its industrial application are promising and show great potential. However, economic feasibility is primarily dependent on its production cost. Many investigations and trials in both application and production systems are needed before large-scale production becomes a reality.

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References

Abdul Khalil HPS, Bhat AH, Ireana Yusra AF (2012) Green composites from sustainable cellulose nanofibrils: a review. Carbohydr Polym 87:963–979

Benziman M, Mazover A (1973) Nicotinamide adenine dinucleotide- and nicotinamide adenine dinucleotide phosphate-specific glucose-6-phosphate dehydrogenase of *Acetobacter xylinum* and their role in the regulation of the pentose cycle. J Biol Chem 248:1603–1608

Brautaset T, Standal R, Fjaervik E, Valla S (1994) Nucleotide sequence and expression analysis of the *Acetobacter xylinum* phosphoglucomutase gene. Microbiology 140:1183–1188

Brede G, Fjaervik E, Valla S (1991) Nucleotide sequence and expression analysis of the *Acetobacter xylinum* uridine diphosphoglucose pyrophosphorylase gene. J Bacteriol 173:7042–7045

Brown AJ (1886) XLIII. On an acetic ferment which forms cellulose. J Chem Soc 49:432–439

Brown RM Jr, Willison JH, Richardson CL (1976) Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the *in vivo* process. Proc Natl Acad Sci USA 73:4565–4569

Chao TP, Sugano Y, Kouda T, Yoshinaga F, Shoda M (1997) Production of bacterial cellulose by *Acetobacter xylinum* with an air-lift reactor. Biotechnol Technol 11:829–832

Cheng KC, Catchmark JM, Demirci A (2011) Effects of CMC addition on bacterial cellulose production in a biofilm reactor and its paper sheets analysis. Biomacromolecules 12:730–736

Coucheron DH (1991) An *Acetobacter xylinum* insertion sequence element associated with inactivation of cellulose production. J Bacteriol 173:5723–5731

Couso RO, Ielpi L, Dankert MA (1987) A xanthan-like, gum-like polysaccharide from *Acetobacter xylinum*. J Gen Microbiol 133:2123–2135

Deeraksa A, Moonmangmee S, Toyama H, Yamada M, Adachi O, Matsushita K (2005) Characterization and spontaneous mutation of a novel gene, *polE*, involved in pellicle formation in *Acetobacter tropicalis* SKU1100. Microbiology 151:4111–4120

Dudman WF (1960) Cellulose production in *Acetobacter* strains in submerged culture. J Gen Microbiol 22:25–30

Fjaervik E, Frydenlund K, Valla S, Huggirat Y, Benziman M (1991) Complementation of cellulose-negative mutants of *Acetobacter xylinum* by the cloned structural gene for phosphoglucomutase. FEMS Microbiol Lett 77:325–330

Fontana JD, de Souza AM, Fontana CK, Torriani IL, Gallotti BJ, de Souza SJ, Narcisco GP, Bichara JA, Farah LF (1990) *Acetobacter* cellulose pellicle as a temporary skin substitute. Appl Biochem Biotechnol 24:253–264

Fujiwara T, Komoda K, Sakurai N, Tajima K, Tanaka I, Yao M (2013) The c-di-GMP recognition mechanism of the PilZ domain of bacterial cellulose synthase subunit A. Biochem Biophys Res Commun 431:802–807

Griffin AM, Edwards KJ, Gasson MJ, Morris VJ (1995) Identification of structural genes involved in bacterial exopolysaccharide production. Biotechnol Gen Eng Rev 13:1–18

Grimmecke HD, Knirel YA, Kiesel G, Voges M, Rietsche ET (1994) Structure of the *Acetobacter methanolicus* MB129 capsular polysaccharide, and of oligosaccharides resulting from degradation by bacteriophage *Acm7*. Carbohydr Res 259:45–58

Hioki N, Hori Y, Watanabe K, Morinaga Y, Yoshinaga F, Hibino Y, Ogura T (1995) Bacterial cellulose: as a new material for papermaking. Jpn TAPPI J 49:718–723

Hori Y, Watanabe K, Morinaga Y, Yoshinaga F (1997) Evidence of the periodically alternating microfibrillar structure of bacterial cellulose. Biosci Biotechnol Biochem 61:1401–1404

Hu SQ, Gao YG, Tajima K, Sunagawa N, Zhou Y, Kawano S, Fujiwara T, Yoda T, Shimura D, Satoh Y, Munekata M, Tanaka I, Yao M (2010) Structure of bacterial cellulose synthase subunit D octamer with four inner passageways. Proc Natl Acad Sci USA 107:17957–17961

Ishikawa A, Matsuoka M, Tsuchida T, Yoshinaga F (1995) Increase in cellulose production by sulfaguanidine-resistant mutants derived from *Acetobacter xylinum* subsp. *nonacetoxidans*. Biosci Biotechnol Biochem 59:2259–2262

Ishikawa A, Tonouchi N, Tsuchida T, Yoshinaga F (1998a) Breeding of a 5-fluorouridine-resistant mutant with increased cellulose production from *Acetobacter xylinum* subsp. *nonacetoxidans*. Biosci Biotechnol Biochem 62:1388–1391

Ishikawa A, Tsuchida T, Yoshinaga F (1998b) Relationship between sulfaguanidine resistance and increased cellulose production in *Acetobacter xylinum* BPR3001E. Biosci Biotechnol Biochem 62:1234–1236

Iyer PR, Catchmark J, Brown NR, Tien M (2011) Biochemical localization of a protein involved in synthesis of *Gluconacetobacter hansenii* cellulose. Cellulose 18:739–747

Jasson PE, Lindberg J, Wimalasin KMS, Dankert MA (1993) Structural studies of acetan, an exopolysaccharide elaborated by *Acetobacter xylinum*. Carbohydr Res 245:303–310

Kawano S, Tajima K, Uemori Y, Yamashita H, Erata T, Munekata M, Takai M (2002) Cloning of cellulose synthesis related genes from *Acetobacter xylinum* ATCC23769 and ATCC53582: comparison of cellulose synthetic ability between strains. DNA Res 9:149–156

Keshk SMAS (2014) Bacterial cellulose production and its industrial applications. J Bioprocess Biotech 4:105. doi:10.4172/2155-9821.100050

Kimura S, Chen HP, Saxena IM, Brown RM Jr, Itoh T (2001) Localization of c-di-GMP-binding protein with the linear terminal complexes of *Acetobacter xylinum*. J Bacteriol 183:5668–5674

Kojima Y, Seto A, Tonouchi N, Tsuchida T, Yoshinaga F (1997) High rate production in static culture of bacterial cellulose from sucrose by a newly isolated *Acetobacter* strain. Biosci Biotechnol Biochem 61:1585–1586

Kojima Y, Tonouchi N, Tsuchida T, Yoshinaga F, Yamada Y (1998) The characterization of acetic acid bacteria efficiently producing bacterial cellulose from sucrose; the proposal of *Acetobacter xylinum* subsp. *nonacetoxidans* subsp. nov. Biosci Biotechnol Biochem 62:185–187

Kose R, Sunagawa N, Yoshida M, Tajima K (2013) One step production of nanofibrillated bacterial cellulose (NFBC) from waste glycerol using *Glulconacetobacter intermedius* NED-01. Cellulose 20:2971–2979

Kouda T, Yano H, Yoshinaga F, Kaminoyama M, Kamiwano M (1996) Characterization of non-Newtonian behavior during mixing of bacterial cellulose in a bioreactor. J Ferment Bioeng 82:382–386

Kouda T, Yano H, Yoshinaga F (1997) Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. J Ferment Bioeng 83:371–374

MacCormick CA, Harris JE, Jay AJ, Ridout MJ, Colquhoun IJ, Morris VJ (1996) Isolation and characterization of a new extracellular polysaccharide from an *Acetobacter* species. J Appl Bacteriol 81:419–424

Matsuoka M, Tsuchida T, Matsushita K, Adachi O, Yoshinaga F (1996) A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* subsp. *sucrofermentans*. Biosci Biotechnol Biochem 60:575–579

Matsushita K, Ebisuya H, Ameyama M, Odachi O (1992) Change of the terminal oxidase from cytochrome a_1 in shaking cultures to cytochrome *o* in static cultures of *Acetobacter aceti*. J Bacteriol 174:122–129

Minakami H, Entani E, Tayama K, Fujiyama S, Masai H (1984) Isolation and characterization of a new polysaccharide-producing *Acetobacter* sp. Agric Biol Chem 48:2405–2414

Moonmangmee S, Toyama H, Adachi O, Theeragool G, Lotong N, Matsushita K (2002a) Purification and characterization of a novel polysaccharide involved in the pellicle produced by a thermotolerant *Acetobacter* strain. Biosci Biotechnol Biochem 66:777–783

Moonmangmee S, Kawabata K, Tanaka S, Toyama H, Adachi O, Matsushita K (2002b) A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*. J Biosci Bioeng 93:192–200

Morgan JL, Strumillo J, Zimmer J (2013) Crystallographic snapshot of cellulose synthesis and membrane translocation. Nature (Lond) 493:181–187

Muhlethaler K (1949) The structure of bacterial cellulose. Biochim Biophys Acta 3:527–535

Nakai T, Moriya A, Tonouchi N, Tsuchida T, Yoshinaga F, Horinouchi S, Sone Y, Mori H, Sakai F, Hayashi T (1998a) Control of expression by the cellulose synthase (bcsA) promoter region from *Acetobacter xylinum* BPR2001. Gene (Amst) 213:93–100

Nakai T, Konishi T, Zhang XQ, Chollet R, Tonouchi N, Tsuchida T, Yoshinaga F, Mori H, Sakai F, Hayashi T (1998b) An increase in apparent affinity for sucrose of Mung bean sucrose synthase is caused by in vitro phosphorylation or directed mutagenesis of Ser^{11}. Plant Cell Physiol 39:1337–1341

Nakai T, Tonouchi N, Konishi K, Kojima Y, Tsuchida T, Yoshinaga F, Sakai F, Hayashi T (1999) Enhancement of cellulose production by expression of sucrose synthase in *Acetobacter xylinum*. Proc Natl Acad Sci USA 96:14–18

Nakai T, Nishiyama Y, Kuga S, Sugano Y, Shoda M (2002) ORF2 gene involved in the construction of high-order structure of bacterial cellulose. Biochem Biophys Res Commun 295:458–462

Nakai T, Sugano Y, Shoda M, Sakakibara H, Oiwa K, Tsuzi S, Imai T, Sugiyama J, Takeuchi M, Yamauchi D, Mineyuki Y (2013) Formation of highly twisted ribbons in a carboxymethyl cellulose gene-disrupted strain of a cellulose-producing bacterium. J Bacteriol 195:958–964

Nieduszy I, Preston RD (1970) Crystallite size in natural cellulose. Nature (Lond) 225:273–275

Nishi Y, Uryu M, Yamanaka S, Watanabe K, Kitamura N, Iguchi M, Mitsuhashi S (1990) The structure and mechanical properties of sheets prepared from bacterial cellulose. 2. Improvement of the mechanical properties of sheets and their applicability to diagrams of electroacoustic transducers. J Mater Sci 25:2997–3001

Okiyama A, Motoki M, Yamanaka S (1993a) Bacterial cellulose III. Development of a new form of cellulose. Food Hydrocoll 6:493–501

Okiyama A, Motoki M, Yamanaka S (1993b) Bacterial cellulose IV. Application to processed foods. Food Hydrocoll 6:503–511

Ougiya H, Watanabe K, Matsumura T, Yoshinaga F (1998) Relationship between suspension properties and fibril structure of disintegrated bacterial cellulose. Biosci Biotechnol Biochem 62:1714–1719

Petersen N, Gatenholm P (2011) Bacterial cellulose-based materials and medical devices: current state and perspectives. Appl Microbiol Biotechnol 91:1277–1286

Romlimg U (2002) Molecular biology of cellulose production in bacteria. Res Microbiol 153:205–212

Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van del Mare GA, van Boom JH, Benziman M (1987) Regulation of a cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature (Lond) 325:278–281

Sato S, Kato T, Kakegawa K, Ishii T, Liu YG, Awano T, Tanabe K, Nishiyama Y, Kuga S, Nakamura Y, Tabata S, Shibata D (2001) Role of the putative membrane-bound endo-1,4-beta glucanase KORRIGAN in cell elongation and cellulose synthesis in *Arabidopsis thaliana*. Plant Cell Physiol 42:251–263

Saxena IM, Kudlicka K, Okuda K, Brown RM Jr (1994) Characterization of genes in the cellulose-synthesizing operon (*acs* operon) of *Acetobacter xylinum*: implications for cellulose crystallization. J Bacteriol 176:5735–5752

Seto A, Kojima Y, Tonouchi N, Tsuchida T, Yoshinaga F (1997) Screening of bacterial cellulose-producing *Acetobacter* strains suitable for sucrose as a carbon source. Biosci Biotechnol Biochem 61:735–736

Seto A, Saito Y, Matsushige M, Kobayashi H, Sasaki Y, Tonouchi N, Tsuchida T, Yoshinaga F, Ueda K, Beppu T (2006) Effective cellulose production by a coculture of *Gluconacetobacter xylinus* and *Lactobacillus mali*. Appl Microbiol Biotechnol 73:915–921

Standal R, Iversen TG, Coucheron DH, Fjaervik E, Blanty JM, Valla S (1994) A new gene required for cellulose production and a gene encoding cellulolytic activity in *Acetobacter xylinum* are co-localized with the *bcs* operon. J Bacteriol 176:665–672

Sunagawa N, Tajima K, Hosoda M, Kawano S, Kose R, Satoh Y, Yao M, Dairi T (2012) Cellulose production by *Enterobacter* sp. CJF-002 and identification of genes for cellulose biosynthesis. Cellulose 19:1989–2001

Sunagawa N, Fujiwara T, Yoda T, Kawano S, Satoh Y, Yao M, Tajima K, Dairi T (2013) Cellulose complementing factor (Ccp) is a new member of the cellulose synthase complex (terminal complex) in *Acetobacter xylinum*. J Biosci Bioeng 115:607–612

Tabuchi M (2007) Nanobiotech versus synthetic nanotech? Nat Biotechnol 25:389–390

Tabuchi M, Baba Y (2005) Design for DNA separation medium using bacterial cellulose fibrils. Anal Chem 77:7090–7093

Tahara N, Tabuchi M, Watanabe K, Yano H, Morinaga Y, Yoshinaga F (1997a) Degree of polymerization of cellulose from *Acetobacter xylinum* BPR2001 decreased by cellulose produced by the strain. Biosci Biotechnol Biochem 61:1862–1865

Tahara N, Yano H, Yoshinaga F (1997b) Two types of cellulase activity produced by a cellulose-producing *Acetobacter* strain. J Ferment Bioeng 83:389–392

Tahara N, Tonouchi N, Yano H, Yoshinaga F (1998) Purification and characterization of exo-1,4-beta-glucosidase from *Acetobacter xylinum* BPR2001. J Ferment Bioeng 85:589–594

Takemura H, Tsuchida T, Yoshinaga F, Matsushita K, Adachi O (1994) Prosthetic group of aldehyde dehydrogenase in acetic acid bacteria not pyrroloquinoline quinone. Biosci Biotechnol Biochem 58:2082–2083

Tal R, Wong HC, Calhoon R, Gelfand D, Fear AL, Volman G, Mayer R, Ross P, Amikam D, Weinhouse H, Cohen A, Sapir S, Ohana P, Benziman M (1998) Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. J Bacteriol 180:4416–4425

Tamayo R, Pratt JT, Camilli A (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu Rev Microbiol 61:131–148

Tayama K, Minakami H, Entani E, Fujiyama S, Masai H (1985) Structure of an acidic polysaccharide from *Acetobacter* sp. NBI1022. Agric Biol Chem 49:959–966

Tonouchi N (2012) Cellulose synthesis. In: Japanese Association of Acetic Acid Bacteria (ed) Function and science of vinegar. Asakura publishing (Tokyo) (in Japanese) pp171–175

Tonouchi N (2015) History and food culture of acetic acid bacteria utilization. Jpn J Lactic Acid Bact 26:6–13 (in Japanese)

Tonouchi N, Tsuchida T, Yoshinaga F, Horinouchi S, Beppu T (1994) A host–vector system in a cellulose-producing *Acetobacter* strain. Biosci Biotechnol Biochem 58:1899–1901

Tonouchi N, Tahara N, Tsuchida T, Yoshinaga F, Beppu T, Horinouchi S (1995) Addition of a small amount of endoglucanase enhances cellulose production by *Acetobacter xylinum*. Biosci Biotechnol Biochem 59:805–808

Tonouchi N, Tsuchida T, Yoshinaga F, Beppu T, Horinouchi S (1996) Characterization of the biosynthetic pathway of cellulose from glucose and fructose in *Acetobacter xylinum*. Biosci Biotechnol Biochem 60:1377–1379

Tonouchi N, Tahara N, Kojima Y, Nakai T, Sakai F, Hayashi T, Tsuchida T, Yoshinaga F (1997) A beta-glucosidase gene downstream of the cellulose synthase operon in cellulose-producing *Acetobacter*. Biosci Biotechnol Biochem 61:1789–1790

Tonouchi N, Yanase H, Kojima Y, Tsuchida T, Yoshinaga F, Horinouchi S (1998a) Increased cellulose production from sucrose with reduced levan accumulation by an *Acetobacter* strain harboring a recombinant plasmid. Biosci Biotechnol Biochem 62:833–835

Tonouchi N, Horinouchi S, Tsuchida T, Yoshinaga F (1998b) Increased cellulose production from sucrose by *Acetobacter* after introducing the sucrose phosphorylase gene. Biosci Biotechnol Biochem 62:1778–1780

Tonouchi N, Sugiyama M, Yokozeki K (2003) Coenzyme specificity of enzymes in the oxidative pentose phosphate pathway of *Gluconobacter oxidans*. Biosci Biotechnol Biochem 67:2648–2651

Toyosaki H, Naritomi T, Seto A, Matsuoka M, Tsuchida T, Yoshinaga F (1995a) Screening of bacterial cellulose-producing *Acetobacter* strains suitable for agitated culture. Biosci Biotechnol Biochem 59:1498–1502

Toyosaki H, Kojima Y, Tsuchida T, Hoshino K, Yamada Y, Yoshinaga F (1995b) The characterization of an acetic-acid bacterium useful for producing bacterial cellulose in agitation cultures: the proposal of *Acetobacter xylinum* subsp. *sucrofermentans* subsp. nov. J Gen Appl Microbiol 41:307–314

Valla S, Coucheron DH, Fjaervik E, Kjosbakken J, Weinhouse H, Ross P, Amikam D, Benziman M (1989) Cloning of a gene involved in cellulose biosynthesis in *Acetobacter xylinum*: complementation of cellulose-negative mutants by the UDPG pyrophosphorylase structural gene. Mol Gen Genet 217:26–30

Valla S, Ertesvag H, Tonouchi N, Fjaervik E (2009) Bacterial cellulose production: biosynthesis and applications. In: Rehm BH (ed) Microbial production of biopolymers and polymer precursors: applications and perspectives. Caister Academic Press (Norfolk) pp 43–77

Watanabe K, Yamanaka S (1995) Effects of oxygen-tension in the gas-phase on production and physical properties of bacterial cellulose formed under static culture conditions. Biosci Biotechnol Biochem 59:65–68

Watanabe K, Eto Y, Takano S, Nakamori S, Shibai H, Yamanaka S (1993) A new bacterial cellulose substrate for mammalian cell culture. Cytotechnology 13:107–114

Watanabe K, Tabuchi M, Morinaga Y, Yoshinaga F (1998) Structural features and properties of bacterial cellulose produced in agitation culture. Cellulose 5:187–200

Whitney JC, Hay ID, Li C, Eckford PDW, Robinson H, Amaya MF, Wood LF, Ohman DE, Bear CE, Rehm BH, Howell PL (2011) Structural basis for alginate secretion across the bacterial outer membrane. Proc Natl Acad Sci USA 108:13083–13088

Wong HC, Fear AL, Calhoon RD, Eichinger GH, Mayer R, Amikam D, Benziman M, Gelfand DH, Meade JH, Emerick AW, Bruner R, Ben-Bassat A, Tal R (1990) Genetic organization of the cellulose synthase operon in *Acetobacter xylinum*. Proc Natl Acad Sci USA 87:8130–8134

Yamada Y, Yukphan P (2008) Genera and species in acetic acid bacteria. Int J Food Microbiol 125:15–24

Yamada Y, Yukphan P, Vu HTL, Muramatsu Y, Ochaikul D, Tanasupawat S, Nakagawa Y (2012) Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). J Gen Appl Microbiol 58:397–404

Yamada Y, Yukphan P, Vu HTL, Muramatsu Y, Ochaikul D, Tanasupawat S, Nakagawa Y (2013) *Komagataeibacter* gen. nov. in list of new names and new combinations previously effectively, but not validly, published. Validation List no. 149. Int J Syst Evol Microbiol 63:1–5

Yamanaka S, Watanabe K, Kitamura N, Iguchi M, Mitsuhashi S, Nishi Y, Uryu M (1989) The structure and mechanical properties of sheets prepared from bacterial cellulose. J Mater Sci 24:3141–3145

Yano H, Sugiyama J, Nakagaito AN, Nogi M, Matsuura T, Hikita M, Handa K (2005) Optically transparent composites reinforced with networks of bacterial nanofibers. Adv Mater 17:153–155

Yasutake Y, Kawano S, Tajima K, Yao M, Satoh Y, Munekata M, Tanaka I (2006) Structural characterization of the *Acetobacter xylinum* endo-beta-1,4-glucanase CMCax required for cellulose biosynthesis. Proteins 64:1069–1077

Yoshinaga F, Tonouchi N, Watanabe K (1997) Research progress in production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material. Biosci Biotechnol Biochem 61:219–224

Zaar K (1979) Visualization of pores (export sites) correlated with cellulose production in the envelope of the gram negative bacterium *Acetobacter xylinum*. J Cell Biol 80:773–777

Chapter 15
Industrial Application of Acetic Acid Bacteria (Vitamin C and Others)

Masako Shinjoh and Hirohide Toyama

Abstract Acetic acid bacteria have been commercially used for more than vinegar production for a long time; especially, strains of genus *Gluconobacter* are applied for the regio- and stereo-specific oxidation of sugars and sugar alcohols. The most notable example is the production of L-sorbose from D-sorbitol in vitamin C production. *Gluconobacter* strains have been also developed for production of 2-keto-L-gulonic acid, an intermediate of vitamin C. Recently, a direct production of vitamin C by *Gluconobacter* was reported. In this chapter, the history and current situation of vitamin C production are explained. Moreover, possibilities of production of other value-added materials including gluconic acid, keto-gluconic acid, tartaric acid, glyceric acid, and lactobionic acid by acetic acid bacteria are also explained.

Keywords Vitamin C • L-Sorbose • 2-Keto-L-gulonic acid • L-Sorbosone • Gluconic acid • Tartaric acid • Glyceric acid • Lactobionic acid

15.1 Vitamin C (L-Ascorbic Acid) Production: Contribution of Acetic Acid Bacteria

15.1.1 History of Vitamin C Discovery and Establishment of the Production Process

The year 2012 celebrated the 100th anniversary of the vitamin concept (Pappenberger and Hohmann 2014). The name "vitamin" had been given in 1912 by the Polish-American biochemist Casimir Funk (Funk 1912). Originally the name

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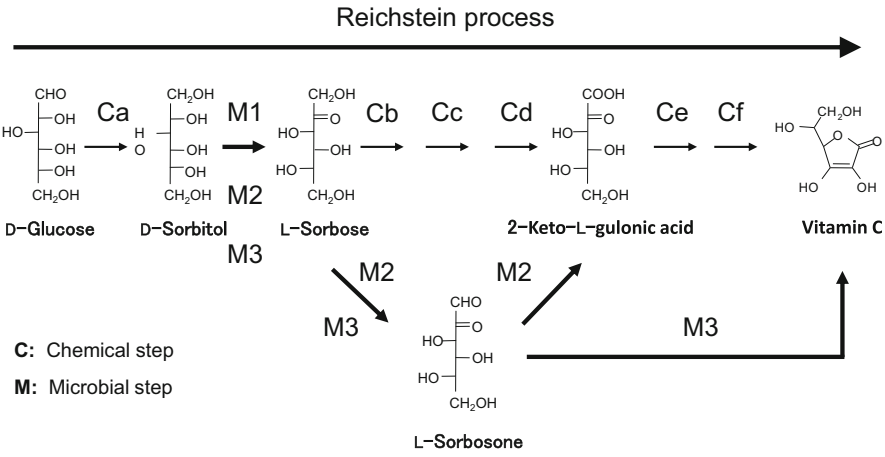
had been "Vitamine," which means *Vit*al + *amine*, but it was later renamed as "Vitamin" according to its wider use for compounds without *amine*.

Among the vitamins, vitamin C has a long medical history. The Egyptian Ebers papyrus from 1550 BC already describes the avitaminose symptoms of scurvy. Scurvy is caused by a dietary lack of vitamin C, which the human as well as guinea pigs cannot synthesize.

In 1928, the Hungarian biochemist Albert Szent-Györgyi isolated vitamin C, which was first designated as hexuronic acid, from the adrenal glands of animals and later from Hungarian paprika peppers. Between 1933 and 1934, the British chemists Sir Walter Norman Haworth and Sir Edmund Hirst and the Polish-Swiss chemist Tadeusz Reichstein succeeded in synthesizing vitamin C. The Reichstein process was adopted by F. Hoffmann-La Roche to produce vitamin C from 1934. The process includes one microbial oxidation step, from D-sorbitol to L-sorbose, and has been used commercially for about 80 years with many chemical and technical modifications to improve the efficiency of each step (Fig. 15.1). Currently more than 100,000 tons per year of pure vitamin C are produced worldwide.



M1: Reichstein process: L-sorbose fermentation
M2: Fermentation of 2KGA from L-sorbose or D-sorbitol
M3: Direct fermentation of vitamin C from L-sorbosone, L-sorbose, or D-sorbitol

Fig. 15.1 The Reichstein process and microbial fermentation processes for L-ascorbic acid production. Three pathways for vitamin C production are shown: The Reichstein process includes six chemical steps (*Ca–Cf*) and one microbial step (M1). *Ca* catalytic hydrogenation, *Cb* protection of hydroxyl groups with acetone, *Cc* oxidation, *Cd* deprotection of acetone, *Ce* esterification with methanol, *Cf* lactonization. *M2* microbial process includes three chemical steps (*Ca*, *Ce*, *Cf*) and three microbial steps shown as *M2* in the figure. *M3* microbial process includes one chemical step (*Ca*) and three microbial steps (shown as *M3*)

15.1.2 The Reichstein Process

In 1898, Gabriel Bertrand reported the formation of L-sorbose from D-sorbitol for the first time. In 1934, this reaction was adopted to produce vitamin C in the so-called "Reichstein process" (Reichstein and Grüssner 1934):

D-glucose →D-sorbitol →L-sorbose → diacetone-L-sorbose → diacetone-2-keto-L-gulonic acid → 2-keto-L-gulonic acid (2KLGA) → methyl-2-keto-L-gulonic acid → vitamin C (Fig. 15.1).

In the Reichstein's initial experiments, the one microbial step (oxidation "D-sorbitol →L-sorbose") was performed by *Komagataeibacter xylinus* (*Acetobacter aceti* subsp. *xylinum*), a mother of vinegar (a biofilm similar to nata de coco consisting of cellulose gel) formed during a vinegar fermentation (Pappenberger and Hohmann 2014). Then, *G. oxydans* (*Gluconobacter suboxydans*) was introduced because of its higher oxidation ability and has been used up to now for this reaction.

15.1.3 Microbial Processes of 2KLGA Production

Once the Reichstein process was established, alternative processes for vitamin C production have been studied to produce 2KLGA, an intermediate of vitamin C production, mainly from three starting substrates via corresponding intermediates (Fig. 15.2):

[1a] L-sorbose via L-sorbosone;
[1b] D-sorbitol via L-sorbose and L-sorbosone;
[2] D-glucose via 2,5-diketo-D-gluconic acid (2,5-DKGA).

There were further routes for 2KLGA production, via L-idonic acid, 5-keto-D-gluconic acid, etc., by microorganisms including *Pseudomonas* strains, as studied since the 1940s, as well summarized in several reviews (Boudrant 1990; Delić and Vlasic 1989). In this chapter, only processes by using acetic acid bacteria are introduced.

Various strains of acetic acid bacteria have been selected and studied for the vitamin C production processes since the 1960s. The strains used for the processes from three substrates ([1a: L-sorbose], [1b: D-sorbitol], [2: D-glucose]) in the initial series of experiments are briefly summarized here.

[1a: L-sorbose]: mainly *G. oxydans* including *G. oxydans* NBRC 3293 (*G. melanogenus* IFO 3293) (Tsukada and Perlman 1972; Kitamura and Perlman 1975; Hoshino et al. 1990) and its mutants, such as UV10 and U-13 strains (Sugisawa et al. 1990; Manning and Kahn 1987), were investigated.

Sugisawa et al. improved the wild-type strain NBRC 3293, producing 2.8 g/l 2KLGA from 25 g/l L-sorbose by random mutagenesis and screening using UV and chemical mutagens to have the 2KLGA high producer U-13, producing 60 g/l 2KLGA from 100 g/l L-sorbose. Hoshino et al. confirmed the metabolic pathway

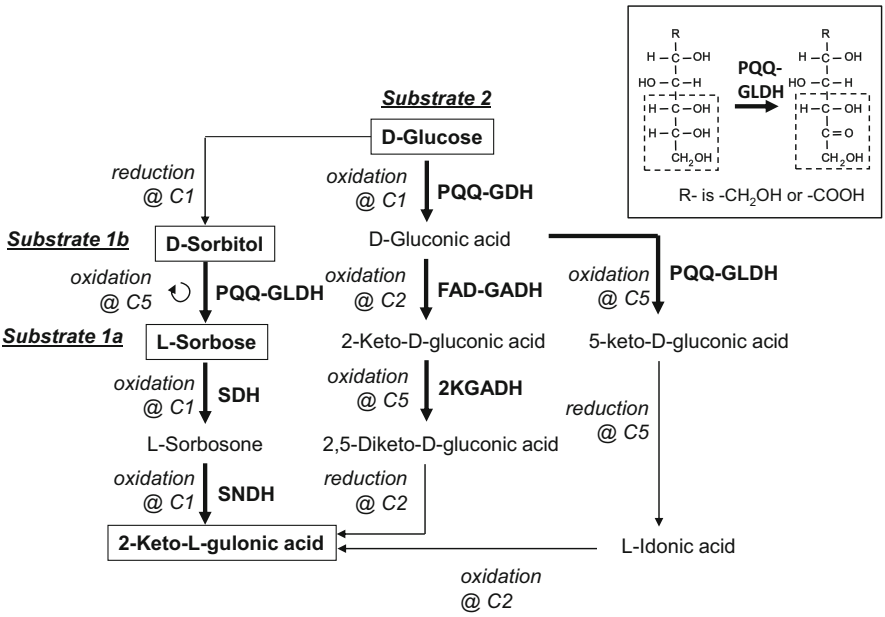


Fig. 15.2 Proposed routes from L-sorbose, D-sorbitol, or D-glucose to 2KLGA in *Gluconobacter* strains. Three major routes from different substrates to 2KLGA are shown: (*1a*) L-sorbose as the substrate; (*1b*) D-sorbitol; (*2*) D-glucose. In addition, other routes from D-glucose to 2KLGA via 5-keto-D-gluconic acid (see text in Sect. 15.2) and L-idonic acid are shown. Enzyme reactions described in the text are shown in *thick lines*. *Inserted panel* shows substrate recognition by PQQ-GLDH (Bertland-Hudson's rule)

for 2KLGA formation from L-sorbose via L-sorbosone, which had previously been proposed (Makover et al. 1975). Manning and Kahn (1987) cloned a DNA region comprising a gene encoding L-sorbose dehydrogenase, which was identified by Tn5 mutagenesis.

[1b: D-sorbitol]: mainly *G. oxydans* including *G. oxydans* NBRC 3292 (Isono et al. 1968; Okazaki et al. 1969) and the strains UV10 and Z-84 (Sugisawa et al. 1990) were investigated. Isono et al. first found that *G. oxydans* NBRC 3292 produced 2KLGA from D-sorbitol. Sugisawa et al. improved strain NBRC 3293 to generate strain Z-84 producing 60 g/l 2KLGA from 100 g/l D-sorbitol;

[2: D-glucose]: *G. oxydans* (*G. melanogenus*) ATCC9937 (Stroshane and Perlman 1977) was used to find 93 % conversion of 100 g/l D-glucose to 2,5-DKGA. Later, 2KLGA production processes via 2,5-DKGA were intensively studied by using *Erwinia* sp. in combination with *Corynebacterium* sp. strain to have 84.6 % conversion yield from D-glucose to 2KLGA (Sonoyama et al. 1982) .

The dissimilation pathway of L-sorbose in *G. oxydans* UV10 (*G. melanogenus* UV10; 2KLGA-producing mutant of IFO 3293) was studied with isotopically labeled substrates by measuring the $^{14}CO_2$ evolved. This study showed that the

Gluconobacter strain used mainly a pentose phosphate pathway (Shinjoh et al. 1990). The enzymological and genetic studies together with strain and process development for the processes from L-sorbose and D-sorbitol are described later in this chapter.

It is reasonable to use *Gluconobacter* strains for oxidation of D-sorbitol or L-sorbose. Actually, *Gluconobacter* strains have often been isolated from fruits such as persimmons, dried persimmons, and strawberries in addition to flowers and fermentation products (NBRC Catalogue Bacteria Section, http://www.nbrc.nite.go.jp/doc/catalogue/NBRC2010_613-934bacteria.pdf). Ripe fruits (apples, pears, etc.) or dried fruits are generally known to contain D-sorbitol at a high concentration. *Gluconobacter* strains can utilize such accumulated D-sorbitol by converting it to L-sorbose, which is hardly assimilated by other microorganisms. Then, *Gluconobacter* strains utilize L-sorbose gradually as an exclusive carbon source for themselves. In China, sorbitol and sorbose are written in Chinese characters as 山梨糖醇 and 山梨糖, respectively. 山梨糖 literally means mountain pear sugar.

In China, the first commercially implemented biotechnology process of 2KLGA production (M2 route in Fig. 15.2) was developed with a mixed culture of two bacteria, *Ketogulonicigenium vulgare* DSM 4025 and *Bacillus megaterium* DSM4026 (Ning et al. 1987) to produce 2KLGA from L-sorbose, which is used in China for commercial production of vitamin C. Also in this process, a *Gluconobacter* strain is used to produce L-sorbose from D-sorbitol.

Currently, commercial production is done by DSM as the sole Western vitamin C producer and by four leading Chinese producers, Northeast Pharmaceutical Group Co., Ltd., Weisheng Pharmaceutical Company, Welcome Pharmaceutical Company, and Jiangsu Jiangshan Pharmaceutical (now as Aland Nutraceutical Co., Ltd.) (Pappenberger and Hohmann 2014).

15.1.4 Enzymes Responsible for 2KLGA Production in Acetic Acid Bacteria and Genetic Tools

D-Sorbitol Dehydrogenase (PQQ-GLDH): PQQ-Dependent Membrane-Bound from *G. oxydans* This enzyme is the key enzyme for L-sorbose production from D-sorbitol. It was first purified from *G. thailandicus* NBRC3255 (*G. oxydans* IFO3255) and characterized to have a homogeneous 80-kDa subunit and a broad substrate specificity toward D-sorbitol (L-sorbose as product), D-mannitol (D-fructose), D-arabitol (D-xylulose), meso-erythritol (erythrulose), D-adonitol (D-ribulose), glycerol (dihydroxyacetone), and D-gluconic acid (not detected) (Sugisawa and Hoshino 2002). PQQ-GLDH obeys the so-called Bertrand-Hudson's rule (Adachi et al. 2007): the polyols with a cis-arrangement of two secondary hydroxyl groups in D-configurations to the adjacent primary alcohol group are oxidized to the corresponding ketoses (Fig. 15.2). In other words, the enzyme oxidizes secondary alcohols with *R*-configuration in C2 position in the sugar alcohols, and the bulkiness

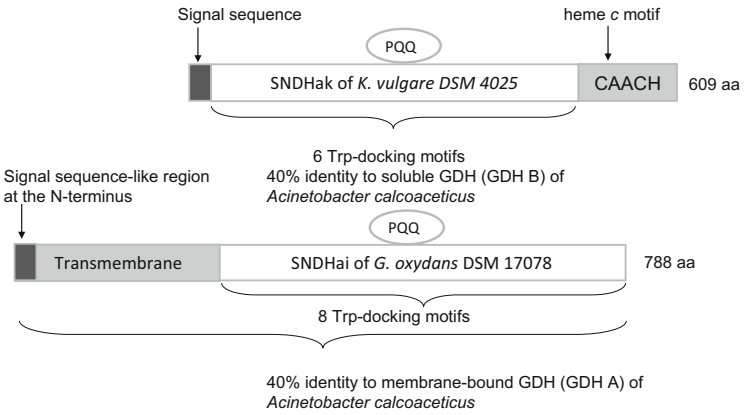


Fig. 15.3 Structure comparison of the two L-ascorbic acid-forming SNDHs (L-sorbosone dehydrogenases). SNDHak from *Ketogulonicigenium vulgare* and SNDHai from *G. oxydans* (from Pappenberger and Hohmann 2014, figure 12). Both are PQQ-dependent enzymes, having six and eight Trp-docking motifs, respectively. SNDHak is localized in a periplasmic space as a soluble enzyme, whereas SNDHai is a membrane-bound enzyme with transmembrane region at the N-terminus. Only SNDHak has a cytochrome domain with a heme motif

and configuration of C4 are not important. The gene cloning was done by using the peptide sequences obtained from the purified enzyme, and identified two genes, *sldA* and *sldB* genes, encoding a polypeptide of 740 residues including a 24-residue signal sequence, and a polypeptide of a 126-residue hydrophobic sequence. The SldA sequence has eight Trp-docking motifs and shows a significant sequence homology to the membrane-bound quinoprotein D-glucose dehydrogenase from *G. oxydans*, *Escherichia coli*, and *Acinetobacter calcoaceticus* (Miyazaki et al. 2002) (Fig. 15.3). The enzyme depends on pyrroloquinoline quinone (PQQ) as the prosthetic group and was characterized as a main polyol dehydrogenase. Gene disruption conducted on the *sldA* and *sldB* separately resulted in no conversion of D-sorbitol, D-mannitol, or glycerol to the corresponding ketose products (Shinjoh et al. 2002b). The *sldA* gene was functionally expressed in *E. coli* only when the *sldB* gene was coexpressed. SldB was found to function as a chaperon-like component that assists signal processing and folding of the SldA polypeptide to form an active catalytic subunit. This enzyme was, via this disruption experiment, confirmed to be identical to glycerol dehydrogenase (PQQ-GLDH), which is described next in Sect. 15.2.

D-Sorbitol Dehydrogenase (FAD-SLDH): FAD-Dependent Membrane-Bound (Shinagawa et al. 1982) The enzyme was purified from *Gluconobacter thailandicus* NBRC3254 (*G. suboxydans* var. *α* IFO 3254) and characterized to have a flavoprotein (63 kDa), a cytochrome *c* (51 kDa), and an uncharacterized small (17-kDa) subunit. Later, the oxidation product of D-sorbitol was confirmed to be L-sorbose. The corresponding genes *sldSLC* were cloned from *G. frateurii* (Toyama et al. 2005). *sldS* encodes the small subunit with a *tat* signal sequence,

and *sldL* encodes the large subunit without a signal sequence. Thus, it is suggested that the SldL peptide is translocated into periplasm together with the SldS peptide after the cofactor FAD is incorporated in cytoplasm. *sldC* encodes the cytochrome *c* subunit, the amino acid sequence of which is similar to those of the cytochrome *c* subunits of D-gluconate dehydrogenase (GADH), 2-keto-D-gluconate dehydrogenase (FAD-2KGADH), and alcohol dehydrogenase (ADH-III), discussed in Chap. 7.

L-Sorbose Dehydrogenase (SDH): Membrane-Bound (Sugisawa et al. 1991) The enzyme was purified from *G. oxydans* UV10 strain. The SDH enzyme (58 kDa) shows high substrate specificity for L-sorbose but is also active on L-sorbosone to give 2KLGA as the oxidation product (Pappenberger and Hohmann 2014). The presence of FAD as a prosthetic group was shown by fluorescence on SDS-PAGE gels. The corresponding gene was first cloned from *G. oxydans* UV10 via transposon mutagenesis using Tn5 (Manning and Kahn 1987), and then from *G. oxydans* T-100 and characterized (ORF of 1497 nucleotide DNA encoding 498 amino acid residues) (Saito et al. 1997).

L-Sorbosone Dehydrogenase (SNDH): NADP-Dependent (Hoshino et al. 1991) The enzyme was purified from *G. oxydans* UV10 strain. The SNDH (about 50 kDa) required NAD or NADP as a cofactor and shows a broad substrate specificity on various aldehyde compounds including glyoxal, glycolaldehyde, and glutaraldehyde besides L-sorbosone as the best substrate. The corresponding gene was cloned from *G. oxydans* T-100 and characterized (ORF of 1596 bp encoding 531 amino acid residues) (Saito et al. 1997). The *sdh* and *sndh* genes were found to form an operon, and the polypeptides show homologies to choline dehydrogenase (FAD-dependent) and betainaldehyde dehydrogenase (NAD(P)-dependent) enzymes, which were reported to be involved in osmotic tolerance. A recombinant *Gluconobacter* overexpressing the *sdh* gene together with the NAD(P)-SNDH gene with *E. coli tufB* promoter achieved a high-level production of 2KLGA (130 g/l) by a single fermentation with 13 % D-sorbitol and 6 % glycerol (Saito et al. 1997).

In addition to the NADP-dependent SNDH already mentioned, a membrane-bound SNDH was found from *Gluconacetobacter liquefaciens* NBRC 12258 (*A. liquefaciens* IFO 12258). The gene was cloned and expressed in *G. oxydans strains*, resulting in a high-yield (about 80 %) conversion of L-sorbosone to 2KLGA in a resting cell reaction (Shinjoh et al. 1995).

L-Sorbose Reductase (SR): NADPH-Dependent (Shinjoh et al. 2002a) The gene was cloned from *G. thailandicus* NBRC3291 (*Gluconobacter suboxydans* IFO 3291) and characterized as involved in intracellular L-sorbose assimilation. The gene disruptant showed no SR activity and did not assimilate the L-sorbose once produced, indicating that the SR functions as the main L-sorbose-reducing enzyme in vivo. This finding is confirmed also in *G. frateurii*, and the gene *sboA* is found to be in the same transcriptional unit with *sboR*, which is a repressor for the *sboRA* operon (Soemphol et al. 2007). Disruption of *sboR* resulted in higher expression of the SR activity.

Vectors and Gene Introduction Methods A shuttle vector pGE1 (11.9 kb) that can replicate in both *G. oxydans* and *E. coli* was constructed from the cryptic *Gluconobacter* plasmid pGO3293S (9.9 kb) and *E. coli* plasmid pSUP301 (5 kb, Km^r, Ap^r)

(Shinjoh and Hoshino 1995). The vector is very stable in *Gluconobacter* strains in the absence of selective antibiotics. Similarly, a shuttle vector pFG15A (7.3 kb) was constructed by using a cryptic plasmid from *G. oxydans* T-100 for cloning of the *sdh*/*sndh* genes (Saito et al. 1997). Besides the shuttle vectors, broad host range plasmids such as pVK100 have been used. Gene introduction methods widely used are transconjugation and electroporation-aided transformation.

15.1.5 Direct Microbial Production Processes of Vitamin C and the Enzyme Profiles

Direct Production Process of Vitamin C from L-Gulono-γ-Lactone (Sugisawa et al. 1995)

Sugisawa et al. reported the isolation, purification, and characterization of vitamin C-producing L-gulonic-γ-lactone dehydrogenase from *Ketogulonicigenium vulgare* DSM4025. In addition to the strain DSM4025, some acetic acid bacteria including *G. oxydans* ATCC621 also produced up to 1 g/l vitamin C from 10 g/l L-gulono-γ-lactone compared with the 2.5 g/l produced by strain DSM4025 under the same conditions. This is the first report for the microbial production of vitamin C.

Direct Production Process of Vitamin C from L-Sorbosone, L-Sorbose, and D-Sorbitol

Sugisawa et al. reported a direct production of vitamin C from D-sorbitol, L-sorbose, L-gulose, and L-sorbosone by *Ketogulonicigenium (Ke.) vulgare* (Sugisawa et al. 2005). This was the first finding of direct microbial production of vitamin C from the substrates that had been known as those for 2KLGA production. The responsible vitamin C-producing sorbosone dehydrogenase enzyme was cloned and characterized as a dimer of 75-kDa (609 amino acid residues) subunits consisting of two domains, having PQQ and heme *c* as the prosthetic groups, respectively (Miyazaki et al. 2006) (Fig. 15.3). It has a signal sequence that is removed, resulting in a soluble periplasmic enzyme with a six-bladed β-propeller structure that shows a significant sequence similarity to the soluble GDH of *Acinetobacter calcoaceticus*.

Then, another direct production of vitamin C from D-sorbitol, L-sorbose, and L-sorbosone was reported (Berry et al. 2003) from *G. oxydans* N44-1, a 2KLGA-producing derivative (Sugisawa et al. 1990) of *G. oxydans* NBRC 3293. The responsible vitamin C-forming sorbosone dehydrogenase was abbreviated as SNDHai because of the profile, SNDH yielding ascorbic acid from *G. oxydans* NBRC 3293(IFO 3293); L-sorbosone dehydrogenase was found in *G. oxydans* N44-1, whereas the former SNDH was called as SNDHak because of its profile,

SNDH yielding ascorbic acid from *Ke. vulgare* (Pappenberger and Hohmann 2014). The SNDHai was cloned via a Tn5 mutagenesis, identifying the responsible gene. The gene encodes a polypeptide (788 amino acid residues) having PQQ as the prosthetic group. SNDHai is a membrane-bound periplasmic polypeptide consisting of two domains: the N-terminal part presumably is a membrane anchor with five predicted transmembrane regions and the C-terminal part is the catalytic domain with an eight-bladed β-propeller structure (Fig. 15.3). The amino acid sequence is nearly identical to the "PQQ-containing dehydrogenase 1" (GOX1857 of *G. oxydans* ATCC621H) (Prust et al. 2005), which is also characterized as membrane-bound quinoprotein inositol dehydrogenase (Hölscher et al. 2007). The catalytic domain shows a significant sequence similarity to the membrane-bound PQQ-dependent GDHs of *G. oxydans*, *E. coli*, *A. calcoaceticus*, etc., and to the membrane-bound PQQ-dependent GLDH of *G. oxydans* (already mentioned), entirely different from the catalytic domain of SNDHak. SNDHai shows no activity on D-glucose or D-sorbitol, whereas SNDHak is active on D-glucose. PQQ dependency was proven using *E. coli* as a PQQ-deficient host. Only upon addition of PQQ does the *E. coli* transformant expressing the SNDHai gene convert L-sorbosone to vitamin C in a resting cell reaction (Berry et al. 2003).

G. oxydans N44-1 expressing SNDHai gene also converts D-sorbitol and L-sorbose to vitamin C, via L-sorbosone as the intermediate. SNDHai enzymes were also detected enzymatically and genetically in other acetic acid bacteria strains such as *Gluconobacter* strains NBRC 3292, 3244, 3287, and ATCC 15164 (Berry et al. 2003).

15.1.6 Outlook

Since the Reichstein process of vitamin C production was established 80 years ago, much R&D for improving the process and strains has been conducted. As described in this Sect 15.1, acetic acid bacteria have very important roles in this long history. Starting from the famous L-sorbose fermentation from D-sorbitol by membrane-bound quinoprotein GLDH in 1934, further oxidation of L-sorbose has been investigated by using *Gluconobacter* strains that have SNDHs (NAD(P)-dependent, and PQQ-dependent) to produce 2KLGA since the 1970s, and also to directly produce vitamin C from around the year 2000. In this line, direct vitamin C production from D-sorbitol via L-sorbose and L-sorbosone has become one of the candidates for a future ideal vitamin C production process. Currently, the extension of microbial steps for vitamin C production has been realized in China by using *Gluconobacter* for L-sorbose production together with the other bacteria, *Ketogulonicigenium* and *Bacillus*, for 2KLGA production from L-sorbose. Alternatively, D-glucose oxidation to produce 5-KGA by *Gluconobacter* strains has been investigated even from the 1940s and for producing 2,5-DKGA since the 1970s, but further conversion to 2KLGA did not reach a performance level sufficient for commercialization.

Recent genomic- and related -omics data accumulation together with the biochemical and fermentation data obtained during the past 80 years opens a new era to further improve enzymes, strains, and production processes. We would expect the acetic acid bacteria to have very attractive enzymes and respiratory systems for establishing a next-generation eco-friendly vitamin C production process.

15.2 Production of Materials Other Than Vitamin C

Biotechnological application of acetic acid bacteria and their enzymes were reviewed previously by Adachi et al. (2003) and recently by Saichana et al. (2014). Other topics than those described next are described in these reviews.

15.2.1 Gluconic Acid and Keto-Gluconic Acid Production

Acetic acid bacteria are proposed for use for production of several compounds in industry other than vinegar and L-sorbose productions, which is mostly done by unique membrane-bound enzymes only found in *Gluconobacter* (Adachi et al. 2007). D-Gluconic acid (GA), 5-keto D-gluconic acid (5-KGA), 2-keto-D-gluconic acid (2-KGA), and 2,5-diketo-D-gluconic acid (2,5-DKGA) are possible to produce by using some strains of *Gluconobacter*. GA is produced by membrane-bound glucose dehydrogenase (PQQ-GDH, Fig. 15.2), which possesses PQQ as the prosthetic group and is found in several bacteria besides *Gluconobacter*. 2-KGA is produced by membrane-bound GA dehydrogenase (FAD-GADH; Fig. 15.2), which has FAD and heme *c* as prosthetic groups and consists of three subunits. The calcium salt of 2-KGA is used for grinding powder. 5-KGA is produced by glycerol dehydrogenase (PQQ-GLDH; Fig. 15.2), which is also involved in the production of dihydroxyacetone from glycerol and L-sorbose from D-sorbitol (see Sect. 15.1.4) (Matsushita et al. 2003), having PQQ as the prosthetic group. 2,5-DKGA is produced by 2-KGA dehydrogenase (2KGADH; Fig. 15.2), which is similar to FAD-GADH. Both 5-KGA and 2,5-DKGA are possible precursor of 2-keto-L-gulonic acid, which is converted to vitamin C (Fig. 15.2) (Gray 1947; Sonoyama et al. 1982).

5-KGA is also a possible precursor of vitamin C in industry. 5-KGA is reduced to L-idonic acid, and then oxidized to 2KLGA (Fig. 15.2) (Gray 1947). 5-KGA can also be converted to L-tartaric acid, as mentioned next.

Production of 5-KGA by *Gluconobacter* is examined by using *G. oxydans* NBRC12528 (*G. suboxydans* IFO12528), which has low capability to produce 2-KGA. However, its optical temperature for 5-KGA production is 15 °C, and as temperature is higher, the productivity becomes lower and production of the by-product 2-KGA increases (Shinagawa et al. 1999). Elfari et al. (2005) constructed a gene-disrupted mutant of *G. oxydans* 621H in the structural gene of

FAD-GADH and successfully produced 5-KGA from glucose at the yield of 84 % at 30 °C. For production at higher temperature, Saichana et al. screened *Gluconobacter* strains with high ability to produce 5-KGA among thermotolerant strains isolated in Thailand; they constructed a mutant deficient in the structural gene of FAD-GADH, and showed that the mutant produced 5-KGA at 37 °C, with 92 % yield from D-glucose and D-gluconic acid (Saichana et al. 2009). Hoshino and Toyama also developed a method to produce 5-KGA from D-glucose by using this mutant with controlling pH to 5.5 or above by adding KOH and recovering 5-KGA potassium salt from culture broth (unpublished results).

High yield (87 %) production of 5KGA from D-glucose is also achieved by using nongenetically modified *G. oxydans* NBRC12528 with strict pH control from 3.5 to 4.0 (Ano et al. 2011).

15.2.2 Tartaric Acid Production

Tartaric acid is a major organic acid that is found in sour fruits and is one of the main acids found in wine. It is useful for a food additive, such as an acidulant in soft drinks and a conditioning agent for pH adjustment, and is also useful for baking soda to function as a leavening agent in recipes. It has two chiral centers; and there are three isomers: L-(−)-tartaric acid (2*R*, 3*R*) or *levo*-tartaric acid, D-(+)-tartaric acid (2*S*, 3*S*) or *dextro*-tartaric acid, and *meso*-tartaric acid (2*R*, 3*S*). The major isomer found in nature is the L-form. Racemic DL-tartaric acid is industrially produced in a multistep chemical reaction from maleic acid or fumaric acid. L-Tartaric acid used for a food additive is obtained from naturally occurring component of lees, a solid by-product of fermentations of red wine remaining on the inside of aging barrels, which mostly consist of potassium bitartrate. The demand for L-tartaric acid has been recently increasing, and the global tartaric acid market is expected to grow at a compounded annual rate of approximately 2.25 % over the period 2001–2010. Worldwide demand for tartaric acid in food and beverages is expected to be around 28,000 tons in 2010, and expected to reach 58,000 tons by 2012.L-Tartaric acid was used for adjusting sourness of red wines, especially in 2011 because the sugar content in grapes was particularly high. Moreover, the use of tartar for balsamic vinegar production is also increasing; the price of L-tartaric acid increased because of shortage of tartar, the major source for industrial production of L-tartaric acid. In Japan, tartar is mostly imported from the EU; therefore, the price of L-tartaric acid has been affected by the price of tartar in EU.

Thus, in Japan, studies on L-tartaric acid production by fermentation have been conducted. In the 1970s, Yamada et al. (1971) found that the genus *Gluconobacter* was capable of producing L-tartaric acid from D-glucose. They also found that its production increased by the addition of ammonium vanadate, and one of the intermediate compounds was 5KGA (Kotera et al. 1972). Later Klasen et al. (1992) reported that L-tartaric acid is produced by ammonium vanadate even in the absence of cells of *Gluconobacter* if the starting material is 5KGA,

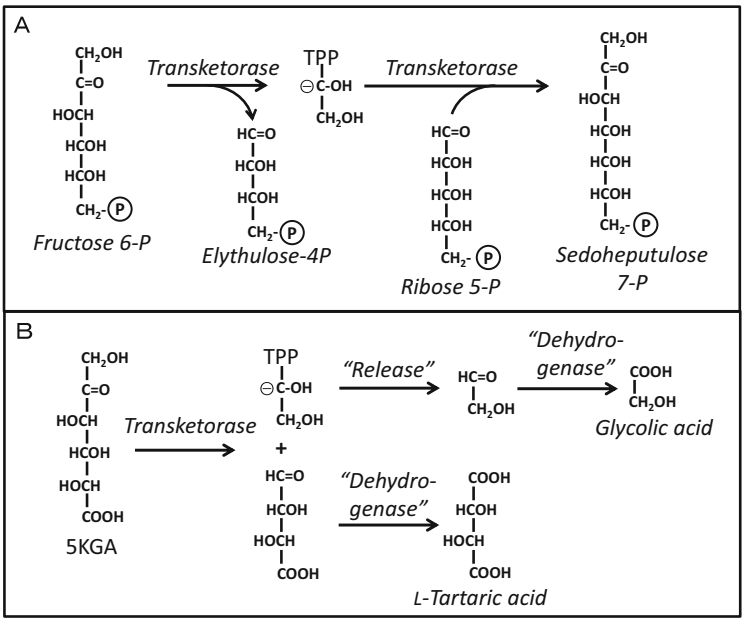


Fig. 15.4 Reaction of transketolase. (**a**) Typical transketolase reaction. TPP is thiamine pyrophosphate bound in the enzyme. C-2 unit from fructose 6-phosphate (C6) is transferred to aldose phosphate (ribose 5-phosphate in the figure, C5) via TPP to produce shorter ketose (erythulose 4-phosphate, C4) and longer chain length ketose product (sedoheptulose 7-phosphate, C7). (**b**) Proposed reaction with 5KGA. C2 unit of 5KGA might be transferred to TPP in the enzyme as described in **a**. The remaining part of 5KGA is tartaric semialdehyde, which is oxidized by a certain enzyme in acetic acid bacteria to become L-tartaric acid. C2 unit attached on TPP might be transferred to aldose phosphate as in **a**, or released to form glycol aldehyde, which is oxidized to become glycolic acid

and that effective production of L-tartaric acid is achieved with ammonium vanadate at alkaline pH in the presence of phosphate or carbonate ions (Matzeratha et al. 1995). However, vanadium is a heavy metal and toxic; therefore, using vanadate for the production of L-tartaric acid for food additives is not desirable, and development of production by fermentation is required. Recently, Salusjärvi et al. (2004) reported the involvement of transketolase in L-tartaric acid production from 5KGA. In general, transketolase catalyzes the reaction as in Fig. 15.4a, where a two-carbon unit is transferred to another aldose substrate (ribose 5-phosphate in the figure). Similarly, 5KGA may be reacted and the two-carbon unit is transferred to TPP in the enzyme (Fig. 15.4b). The remaining part should be tartaric semialdehyde, which is probably oxidized by an oxidizing enzyme in *Gluconobacter* cells and converted to L-tartaric acid. The two-carbon unit may be transferred to another substrate or released as glycolaldehyde and oxidized, probably by an oxidizing enzyme in *Gluconobacter* cells, and converted to glycolic acid (Fig. 15.4), which was reported as a by-product from 5KGA (Kotera et al. 1972).

15.2.3 Glyceric Acid Production

Glycerol is a by-product from the production of biodiesel fuel (BDF), which is released from triacylglycerol by an ester exchange reaction with methanol under alkaline conditions. Because there is a considerable increase in BDF production worldwide, surplus waste glycerol has become an important material for the production of chemicals and fuels (Habe et al. 2009b).

Glyceric acid (GLA) can be prepared by oxidation of glycerol and is reported to have a variety of biological activities. GLA is produced by bacteria under aerobic conditions, and the only known producers investigated so far belong to the family of acetic acid bacteria (*Acetobacteriaceae*), such as *Gluconobacter* sp., *Acetobacter* sp., and *Gluconacetobacter* sp. *Gluconobacter* is known to produce dihydroxyacetone from glycerol by PQQ-dependent GLDH. GLA was originally known as a by-product of DHA production from glycerol by *G. oxydans*, but little work has focused on the biotechnological production of GLA as a target compound. A high glycerol concentration is known to enhance GLA production and to inhibit DHA production. The oxidation of glycerol to GLA should be done by ADH III (Chap. 7), because disruption of the structural gene diminishes the production of GLA (Habe et al. 2010). The enzyme shows no activity to glycerol at the millimolar level but reacts with glycerol at molar level (Habe et al. 2009d).

Recently, Habe et al. intently investigated GLA production by acetic acid bacteria from glycerol, and found that the enantiomeric composition of the GLA produced by *Gluconobacter* sp. NBRC3259 is a mixture of DL-forms with a 77 % enantiomeric excess (ee) of D-GA (Habe et al. 2009c). On the other hand, they also investigated *Acetobacter tropicalis* NBRC16470 for its GA productivity (Habe et al. 2009a), and the enantiomeric composition of the GLA produced was obviously D-GLA. When raw crude glycerol derived from the BDF and oleochemical industries was used for GLA production, production of GLA was very small because of the methanol in the crude glycerol preparation (Sato et al. 2013). They found that the *adhC* gene encoding NAD- and glutathione-dependent formaldehyde dehydrogenase was upregulated in *G. frateurii* NBRC103465 during GLA production from glycerol in the presence of methanol (Sato et al. 2014), indicating that formaldehyde produced by the oxidation of methanol should be removed efficiently to obtain better growth and GLA production from crude glycerol.

15.2.4 Lactobionic Acid Production

Lactobionic acid (β-D-galactopyranosyl-(1→4)D-gluconic acid, LBA) is a relatively new product and an aldonic acid obtained from the oxidation of lactose, with high potential application as an ingredient in foods and pharmaceutical products, because of its antioxidant, chelating, and humectant properties (Gutiérrez

et al. 2012). Recently, Kiryu et al. (2009) reported that LBA was found in traditional Caucasian fermented milk, so-called Caspian Sea yogurt, in Japan, and *Acetobacter orientalis* KYG22, one of the main bacteria involved in fermentation of the yogurt, was involved in LBA production. They optimized the conditions for the preparation of the resting cells and lactose oxidation and successfully produced LBA from 2 % to 10 % lactose at 97.2–99.7 mol% yield. The enzyme involved in the LBA production seems to be membrane-bound PQQ-GDH (Kiryu et al. 2012).

15.2.5 Dextran and Fructan Production

Certain AAB were known to be associated with the type of spoilage of beer known as "ropiness," producing slime from dextrin (Naessens et al. 2005). Dextran is produced by the enzyme dextrin dextranase (DDase, EC2.4.1.2), a transglucosidase converting maltodextrins (α(1,4)-linkage) into (oligo)dextran (α(1,6)-linkage). DDase from *Gluconobacter oxydans* is studied extensively (Naessens et al. 2005). Dextran produced by *G. oxydans* displayed lower viscosity than that produced by *Leuconostoc mesenteroides* as a consequence of a higher degree of branching, which is supposed to be suitable for certain food use applications not associated with thickening functionality, such as a source of dietary fiber, as a cryostabilizer, as a fat substitute, or as a low-calorie bulking agent for sweetening.

Jakob et al. (2012) recently showed several strains of AAB (*Gluconobacter frateurii* TMW2.767, *Gluconobacter cerinus* DSM 9533T, *Neoassaia chiangmaiensis* NBRC101099, and *Kozakia baliensis* DSM14400) can produce high amounts of fructans from sucrose, which are identified as levan (β(2,6)-linkage) (Jakob et al. 2013). Levan are built up by levansucrase (EC2.4.1.10) from sucrose, but the gene of this enzyme is actually found in all *Gluconobacter* genome sequences known so far, but in restricted strains of other AAB (reported in AAB4 in Taiyuan, China 2015).

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References

Adachi O, Moonmangmee D, Shinagawa E, Toyama H, Yamada M, Matsushita K (2003) New quinoproteins in oxidative fermentation. Biochim Biophys Acta 1647:10–17

Adachi O, Ano Y, Toyama H, Matsushita K (2007) Biooxidation with PQQ- and FAD-dependent dehydrogenases. In: Schmid RD, Urlacher VB (eds) Modern biooxidation. Enzymes, reactions and applications. Wiley-VCH, Weinheim, pp 1–41

Ano Y, Shinagawa E, Adachi O, Toyama H, Yakushi T, Matsushita K (2011) Selective, high conversion of D-glucose to 5-keto-D-glucoconate by *Gluconobacter suboxydans*. Biosci Biotechnol Biochem 75:586–589

Berry A, Lee C, Mayer AF, Shinjoh M (2003) Microbial production of L-ascorbic acid. EP2348113
Boudrant J (1990) Microbial process for ascorbic acid biosynthesis: a review. Enzyme Microb Technol 12:322–329
Delić V, Vlasic SD (1989) Microbial reactions for the synthesis of vitamin C (L-ascorbic acid). In: Vandamme EJ (ed) Biotechnology of vitamins, pigments and growth factors. Elesevier Applied Science, London, New York, pp 299–333
Elfari M, Ha SW, Bremus C, Merfort M, Khodaverdi V, Herrmann U, Sahm H, Görisch H (2005) A *Gluconobacter oxydans* mutant converting glucose almost quantitatively to 5-keto-D-gluconic acid. Appl Microbiol Biotechnol 66(6):668–674
Funk C (1912) The etiology of the deficiency diseases. Beri-beri, polyneuritis in birds, epidemic dropsy, scurvy, experimental scurvy in animals, infantile scurvy, ship beri-beri, pellagra. J State Med (Lond) 20:341–368
Gray BE (1947) Preparation of 2-ketogulonic acid and its salts. U.S. Patent 2,421,611
Gutiérrez L-F, Hamoudia S, Belkacemi K (2012) Lactobionic acid: a high value-added lactose derivative for food and pharmaceutical applications. Int Dairy J 26(2):103–111
Habe H, Fukuoka T, Kitamoto D, Sakaki K (2009a) Biotransformation of glycerol to D-glyceric acid by *Acetobacter tropicalis*. Appl Microbiol Biotechnol 81:1033–1039
Habe H, Fukuoka T, Kitamoto D, Sakaki K (2009b) Biotechnological production of D-glyceric acid and its application. Appl Microbiol Biotechnol 84:445–452
Habe H, Shimada Y, Fukuoka T, Kitamoto D, Itagaki M, Watanabe K, Yanagishita H, Sakaki K (2009c) Production of glyceric acid by *Gluconobacter* sp. NBRC3259 using raw glycerol. Biosci Biotechnol Biochem 73:1799–1805
Habe H, Shimada Y, Yakushi T, Hattori H, Ano Y, Fukuoka T, Kitamoto D, Itagaki M, Watanabe K, Yanagishita H, Matsushita K, Sakaki K (2009d) Microbial production of glyceric acid, an organic acid that can be mass produced from glycerol. Appl Environ Microbiol 75:7760–7766
Habe H, Fukuoka T, Morita T, Kitamoto D, Yakushi T, Matsushita K, Sakaki K (2010) Disruption of the membrane-bound alcohol dehydrogenase-encoding gene improved glycerol use and dihydroxyacetone productivity in *Gluconobacter oxydans*. Biosci Biotechnol Biochem 74:1391–1395
Hölscher T, Weinert-Sepalage D, Görisch H (2007) Identification of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H. Microbiology 153:499–506
Hoshino T, Sugisawa T, Tazoe M, Shinjoh M, Fujiwara A (1990) Metabolic pathway for 2-keto-L-gulonic acid formation in *Gluconobacter melanogenus* IFO 3293. Agric Biol Chem 54:1211–1218
Hoshino T, Sugisawa T, Fujiwara A (1991) Isolation and characterization of NAD(P)-dependent L-sorbosone dehydrogenase from *Gluconobacter melanogenus* UV10. Agric Biol Chem 55:665–670
Isono M, Nakanishi I, Sasajima K, Motizuki K, Kanzaki T, Okazaki H, Yoshino H (1968) 2-Keto-L-gulonic acid fermentation. Part I. Paper chromatographic characterization of metabolic products from sorbitol and L-sorbose by various bacteria. Agric Biol Chem 35:424–431
Jakob F, Steger S, Vogel RF (2012) Influence of novel fructans produced by selected acetic acid bacteria on the volume and texture of wheat breads. Eur Food Res Technol 234:493–499
Jakob F, Pfaff A, Novoa-Carballal R, Rübsam H, Becker T, Vogel RF (2013) Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. Carbohydr Polym 92:1234–1242
Kiryu T, Kiso T, Nakano H, Ooe K, Kimura T, Murakami H (2009) Involvement of *Acetobacter orientalis* in the production of lactobionic acid in Caucasian yogurt ("Caspian Sea yogurt") in Japan. J Dairy Sci 92:25–34
Kiryu T, Yamauchi K, Masuyama A, Ooe K, Kimura T, Kiso T, Nakano H, Murakami H (2012) Optimization of lactobionic acid production by *Acetobacter orientalis* isolated from Caucasian fermented milk, "Caspian Sea yogurt". Biosci Biotechnol Biochem 76:361–363

Kitamura I, Perlman D (1975) Metabolism of L-sorbose by enzymes from *Gluconobacter melanogenus* IFO 3293. Eur J Appl Microbiol 2:1–7

Klasen R, Bringer-Meyer S, Sahm H (1992) Incapability of *Gluconobacter oxydans* to produce tartaric acid. Biotechnol Bioeng 40:183–186

Kotera U, Kodama T, Minoda Y, Yamada K (1972) Isolation and chemical structure of new oxidation product of 5-ketogluconic acid, and a hypothetical pathway from glucose to tartaric acid through this new compound. Agric Biol Chem 36:1315–1325

Makover S, Ramsey G, Vane F, Witt C, Wright R (1975) New mechanisms for the biosynthesis and metabolism of 2-keto-L-gulonic acid in bacteria. Biotechnol Bioeng 17:1485–1514

Manning R, Kahn, M (1987) Biosynthesis of 2 keto-L-gulonic acid. US 5082785

Matsushita K, Fujii Y, Ano Y, Toyama H, Shinjoh M, Tomiyama N, Miyazaki T, Sugisawa T, Hoshino T, Adachi O (2003) 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl Environ Microbiol 69:1959–1966

Matzeratha I, Kläui W, Klasenb R, Sahm H (1995) Vanadate catalysed oxidation of 5-keto-D-gluconic acid to tartaric acid: the unexpected effect of phosphate and carbonate on rate and selectivity. Inorg Chim Acta 237:203–205

Miyazaki T, Tomiyama N, Shinjoh M, Hoshino T (2002) Molecular cloning and functional expression of D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IF03255, which requires pyrroloquinoline quinone and hydrophobic protein SldB for activity development in *E. coli*. Biosci Biotechnol Biochem 66:262–270

Miyazaki T, Sugisawa T, Hoshino T (2006) Pyrroloquinoline quinone-dependent dehydrogenases from *Ketogulonicigenium vulgare* catalyze the direct conversion of L-sorbosone to L-ascorbic acid. Appl Environ Microbiol 72:1487–1495

Naessens M, Cerdobbel A, Soetaert W, Vandamme EJ (2005) Dextran dextrinase and dextran of *Gluconobacter oxydans*. J Ind Microbiol Biotechnol 32:323–334

Ning W, Tao Z, Wang C, Wang S, Yan Z, Yin G (1987) Fermentation process for producing 2-keto-L-gulonic acid. EP 0278447B1

Okazaki H, Kanzaki T, Sasajima K, Terada Y (1969) 2-Keto-L-gulonic acid fermentation. Part III. Evaluation of the pathway of sorbitol metabolism in *Gluconobacter melanogenus*. Agric Biol Chem 33:207–211

Pappenberger G, Hohmann HP (2014) Industrial production of L-ascorbic acid (vitamin C) and D-isoascorbic acid. Adv Biochem Eng Biotechnol 143:143–188

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200

Reichstein T, Grüssner A (1934) Eine ergiebige Synthese der L-Ascorbinsäure (C-Vitamin). Helv Chim Acta 17:311–328

Saichana I, Moonmangmee D, Adachi O, Matsushita K, Toyama H (2009) Screening of thermotolerant *Gluconobacter* strains for production of 5-keto-D-gluconic acid and disruption of flavin adenine dinucleotide-containing D-gluconate dehydrogenase. Appl Environ Microbiol 75:4240–4247

Saichana N, Matsushita K, Adachi O, Frébort I, Frébortová J (2014) Acetic acid bacteria: a group of bacteria with versatile biotechnological applications. Biotechnol Adv 33(6 pt 2):1260–1271

Saito Y, Ishii Y, Hayashi H, Imao Y, Akashi T, Yoshikawa K, Noguchi Y, Soeda S, Yoshida M, Niwa M, Hosoda J, Shimomura K (1997) Cloning of genes coding for L-sorbose and L-sorbosone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-L-gulonate, a precursor of L-ascorbic acid, in a recombinant *G. oxydans* strain. Appl Environ Microbiol 63:454–460

Salusjärvi T, Povelainen M, Hvorslev N, Eneyskaya EV, Kulminskaya AA, Shabalin KA, Neustroev KN, Kalkkinen N, Miasnikov AN (2004) Cloning of a gluconate/polyol dehydrogenase gene from *Gluconobacter suboxydans* IFO 12528, characterisation of the enzyme and

its use for the production of 5-ketogluconate in a recombinant *Escherichia coli* strain. Appl Microbiol Biotechnol 65:306–314

Sato S, Morita N, Kitamoto D, Habe H (2013) Expression and characterization of a class III alcohol dehydrogenase gene from *Gluconobacter frateurii* in the presence of methanol during glyceric acid production from glycerol. J Oleo Sci 62:835–842

Sato S, Kitamoto D, Habe H (2014) Chemical mutagenesis of *Gluconobacter frateurii* to construct methanol-resistant mutants showing glyceric acid production from methanol-containing glycerol. J Biosci Bioeng 117:197–199

Shinagawa E, Matsushita K, Adachi O, Ameyama M (1982) Purification and characterization of D-soribitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. α. Agric Biol Chem 46:135–141

Shinagawa E, Matsushita K, Toyama H, Adachi O (1999) Production of 5-ketogluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase. J Mol Catal B Enzymatic 6:341–350

Shinjoh M, Hoshino T (1995) Development of stable shuttle vector and a conjugative transfer system for *Gluconobacter oxydans*. J Ferment Bioeng 79:95–99

Shinjoh M, Setoguchi Y, Hoshino T, Fujiwara A (1990) L-Sorbose dissimilation in 2-keto-L-gulonic acid-producing mutant UV10 derived from *Gluconobacter melanogenus* IFO 3293. Agric Biol Chem 54:2257–2263

Shinjoh M, Tomiyama N, Asakura A, Hoshino T (1995) Cloning and nucleotide sequencing of the membrane-bound L-sorbosone dehydrogenase gene of *Acetobacter liquefaciens* IFO 12258 and its expression in *Gluconobacter oxydans*. Appl Environ Microbiol 61:413–420

Shinjoh M, Tazoe M, Hoshino T (2002a) NADPH-dependent L-sorbose reductase is responsible for L-sorbose assimilation in *Gluconobacter suboxydans* IFO 3291. J Bacteriol 184:861–863

Shinjoh M, Tomiyama N, Miyazaki T, Hoshino T (2002b) Main polyol dehydrogenase of *Gluconobacter suboxydans* IFO 3255, membrane-bound D-sorbitol dehydrogenase, that needs product of upstream gene, sldB, for activity. Biosci Biotechnol Biochem 66:2314–2322

Soemphol W, Toyama H, Moonmangmee D, Adachi O, Matsushita K (2007) L-Sorbose reductase and its transcriptional regulator involved in L-sorbose utilization of *Gluconobacter frateurii*. J Bacteriol 189:4800–4808

Sonoyama T, Tani H, Matsuda K, Kageyama B, Tanimoto M, Kobayashi K, Yagi S, Kyotani H, Mitsushima K (1982) Production of 2-keto-L-gulonic acid from D-glucose by two-stage fermentation. Appl Environ Microbiol 43:1064–1069

Stroshane RM, Perlman D (1977) Fermentation of glucose by *Acetobacter melanogenus*. Biotechnol Bioeng 19:459–465

Sugisawa T, Hoshino T (2002) Purification and properties of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255. Biosci Biotechnol Biochem 66:57–64

Sugisawa T, Hoshino T, Masuda S, Nomura S, Setoguchi Y, Tazoe M, Shinjoh M, Someha S, Fujiwara A (1990) Microbial production of 2-keto-L-gulonic acid from L-sorbose and D-sorbitol by *Gluconobacter melanogenus*. Agric Biol Chem 54:1201–1209

Sugisawa T, Hoshino T, Nomura S, Fujiwara A (1991) Isolation and characterization of membrane-bound L-sorbose dehydrogenase from *Gluconobacter melanogenus* UV10. Agric Biol Chem 55:363–370

Sugisawa T, Ojima S, Matzinger P, Hoshino T (1995) Isolation and characterization of a new vitamin C producing enzyme (L-gulono-γ-lactone dehydrogenase) of bacterial origin. Biosci Biotechnol Biochem 59:190–196

Sugisawa T, Miyazaki T, Hoshino T (2005) Microbial production of L-ascorbic acid from D-sorbitol, L-sorbose, L-gulose, and L-sorbosone by *Ketogulonicigenium vulgare* DSM 4025. Biosci Biotechnol Biochem 69:659–662

Toyama H, Soemphol W, Moonmangmee D, Adachi O, Matsushita K (2005) Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. Biosci Biotechnol Biochem 69:1120–1129

Tsukada Y, Perlman D (1972) The fermentation of L-sorbose by *Gluconobacter melanogenus*. III. Investigation of the metabolic pathway from sorbose to 2-keto-L-gulonic acid. Biotechnol Bioeng 14:1035–1038

Yamada K, Kodama T, Obata T, Takahashi N (1971) Microbial formation of tartaric acid from glucose: (I) Isolation and identification of tartaric acid producing microorganisms. Hakko Kougaku Kaishi 49:85–92 (in Japanese)

Appendix

Summary of changes in names of Acetic Acid Bacteria (AAB) in each chapter

	Strain names that appeared in each chapter: those not used now			Strain names that appeared in each chapter: those presently used	
	Names originally used	Names used previously but further changed	Names presently used		
Chapter 2	*Acetobacter liquefaciens*		*Gluconacetobacter liquefaciens*	*Acetobacter cerevisiae*	*Acetobacter fabarum*
	'*Acetobacter polyoxogenes*'		'*Komagataeibacter polyoxogenes*'	*Acetobacter ghanensis*	*Acetobacter indonesiensis*
	Acetobacter xylinum	*Gluconacetobacter xylinus*[a]	*Komagataeibacter xylinus*	*Acetobacter lambici*	*Acetobacter malorum*
	Acetobacter europaeus	*Gluconacetobacter europaeus*	*Komagataeibacter europaeus*	*Acetobacter orientalis*	*Acetobacter orleanensis*
	Acetobacter hansenii	*Gluconacetobacter hansenii*	*Komagataeibacter hansenii*	*Acetobacter pasteurianus*	*Acetobacter pomorum*
	Gluconacetobacter intermedius		*Komagataeibacter intermedius*	*Acetobacter senegalensis*	*Acetobacter sicerae*
	Gluconacetobacter kakiaceti		*Komagataeibacter kakiaceti*	*Acetobacter syzygii*	*Acetobacter tropicalis*
	Gluconacetobacter maltaceti		*Komagataeibacter maltaceti*	'*Acetobacter rancens*'	*Acetobacter lovaniensis*
	Gluconacetobacter medellinensis		*Komagataeibacter medellinensis*	*Gluconobacter frateurii*	*Gluconacetobacter sacchari*
	Gluconacetobacter oboediens		*Komagataeibacter oboediens*	'*Gluconacetobacter intermedius* subsp. *tamanoi*'	
	Gluconobacter suboxydans		*Gluconobacter oxydans*		
	Gluconobacter oxydans subsp. *sphaericus*		*Gluconobacter sphaericus*		

(continued)

K. Matsushita et al. (eds.), *Acetic Acid Bacteria*,
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	Strain names that appeared in each chapter: those not used now			Strain names that appeared in each chapter: those presently used	
	Names originally used	Names used previously but further changed	Names presently used		
Chapter 3	*Gluconobacter melanogenus* IFO3293		*Gluconobacter oxydans* NBRC3293	*Gluconobacter oxydans*	*Gluconobacter oxydans* 621H
	Gluconacetobacter oboediens		*Komagataeibacter oboediens*	*Gluconobacter oxydans* H24	*Komagataeibacter xylinus*
	Gluconacetobacter saccharivorans		*Komagataeibacter saccharivorans*	*Komagataeibacter medellinensis*	*Komagataeibacter intermedius*
	Gluconacetobacter kombuchae	*Gluconacetobacter hansenii*[b]	*Komagataeibacter kombuchae*	*Komagataeibacter hansenii*	*Gluconacetobacter diazotrophicus*
				Acetobacter aceti	*Gluconobacter oxydans* DSM3504
				Acetobacter aceti NBRC14818	*Acetobacter pasteurianus* NBRC3283
				Acetobacter pasteurianus 386B	*Acetobacter pasteurianus*
				Acidiphilium cryptum	*Acidiphilium multivorum*
				Gluconacetobacter sacchari	*Gluconobacter japonicus*
				Acetobacter pasteurianus NBRC3191	*Acetobacter pasteurianus* NBRC101655
				Acetobacter pasteurianus SKU1108	*Acetobacter tropicalis* SKU1100
				Acetobacter nitrogenifigens	*Acetobacter syzygii*
				Acetobacter lovaniensis	*Acetobacter fabarum*
				Acetobacter cerevisiae	*Acetobacter ghanensis*
				Acetobacter sicerae	*Acetobacter malorum*
Chapter 4	*Gluconacetobacter kombuchae* (LMG23726T)	*Gluconacetobacter hansenii*[b]	*Komagataeibacter kombuchae*	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter azotocaptans*
				Gluconacetobacter johannae	*Acetobacter nitrogenifigens*
				Swaminathania salitolerans	*Acetobacter peroxydans*

(continued)

	Strain names that appeared in each chapter: those not used now			Strain names that appeared in each chapter: those presently used	
	Names originally used	Names used previously but further changed	Names presently used		
Chapter 5	*Gluconacetobacter kombuchae*	*Gluconacetobacter hansenii*[b]	*Komagataeibacter kombuchae*	*Acetobacter aceti*	*Acetobacter nitrogenifigens*
				Acetobacter peroxydans	*Acetobacter pomorum*
				Acetobacter tropicalis	*Asaia astilbes*
				Asaia bogorensis	*Asaia platycodi*
				Commensalibacter intestini	*Gluconacetobacter diazotrophicus*
				Gluconobacter frateurii	*Gluconobacter morbifer*
				Gluconobacter oxydans	*Gluconobacter thailandicus*
				Granulibacter bethesdensis	*Komagataeibacter europaeus*
				Komagataeibacter hansenii	*Komagataeibacter nataicola*
				Komagataeibacter oboediens	*Komagataeibacter rhaeticus*
				Komagataeibacter swingsii	*Komagataeibacter xylinus*
				Saccharibacter floricola	*Swaminathania salitolerans*
Chapter 6				*Commensalibacter intestini*	*Gluconobacter morbifer*
				Acetobacter pomorum	
Chapter 7	*Acetobacter methanolicus*		*Acidomonas methanolica*	*Acetobacter pasteurianus*	*Acidiphilium cryptum*
	Acetobacter aurantius		*Frateuria aurantia* (not AAB)	*Gluconacetobacter diazotrophicus*	*Gluconobacter oxydans*
				Granulibacter bethesdensis	
Chapter 8	*Acetobacter aceti* IFO 3283	*Acetobacter* sp. NBRC 3283	*Acetobacter pasteurianus* NBRC 3283	*Acetobacter pasteurianus* LMG 1262	
	Acetobacter aceti DSMZ 2002		*Acetobacter aceti* DSM 2002		

(continued)

	Strain names that appeared in each chapter: those not used now			Strain names that appeared in each chapter: those presently used	
	Names originally used	Names used previously but further changed	Names presently used		
Chapter 9	*Gluconacetobacter europaeus*		*Komagataeibacter europaeus*	*Acetobacter pasteurianus*	*Acetobacter malorum* S24
	Gluconacetobacter hansenii		*Komagataeibacter hansenii*	*Gluconacetobacter entanii*	
	Gluconacetobacter intermedius		*Komagataeibacter intermedius*		
	Gluconacetobacter kakiaceti		*Komagataeibacter kakiaceti*		
	Gluconacetobacter kombuchae	*Gluconacetobacter hansenii*[b]	*Komagataeibacter kombuchae*		
	Gluconacetobacter maltaceti		*Komagataeibacter maltaceti*		
	Gluconacetobacter medellinensis		*Komagataeibacter medellinensis*		
	Gluconacetobacter nataicola		*Komagataeibacter nataicola*		
	Gluconacetobacter oboediens		*Komagataeibacter oboediens*		
	Gluconacetobacter rhaeticus		*Komagataeibacter rhaeticus*		
	Gluconacetobacter saccharivorans		*Komagataeibacter saccharivorans*		
	Gluconacetobacter sucrofermentans		*Komagataeibacter sucrofermentans*		
	Gluconacetobacter swingsii		*Komagataeibacter swingsii*		
	Gluconacetobacter xylinus		*Komagataeibacter xylinus*		
Chapter 10	*Acetobacter aceti* (IFO3283)	*Acetobacter* sp. NBRC 3283 (= IFO 3283)	*Acetobacter pasteurianus* NBRC 3283 (= IFO 3283)		
	'*Acetobacter polyoxogenes*'		'*Komagataeibacter polyoxogenes*'		
	Acetobacter europaeus	*Gluconacetobacter europaeus*	*Komagataeibacter europaeus*		
Chapter 11	*Gluconobacter melanogenus* IFO3293		*Gluconobacter oxydans* NBRC3293	*Gluconobacter oxydans*	*Gluconobacter oxydans* 621H
	Gluconacetobacter oboediens		*Komagataeibacter oboediens*	*Acetobacter pasteurianus* IFO3283-01	*Gluconacetobacter diazotrophicus* PAl5
	Gluconacetobacter xylinus		*Komagataeibacter xylinus*	*Acetobacter aceti*	*Gluconobacter oxydans* DSM3504
				Acetobacter aceti NBRC14818	*Acetobacter pasteurianus* NBRC3283
				Acetobacter pasteurianus 386B	

(continued)

	Strain names that appeared in each chapter: those not used now			Strain names that appeared in each chapter: those presently used	
	Names originally used	Names used previously but further changed	Names presently used		
Chapter 12	*Gluconacetobacter xylinus* I 2281		*Komagataeibacter medellinensis*	*Acetobacter aceti* NBRC 14818	*Acetobacter pasteurianus* NBRC 3283
				Gluconacetobacter diazotrophicus	*Gluconobacter oxydans*
				Granulibacter bethesdensis	
Chapter 13	*Acetobacter europaeus*	*Gluconacetobacter europaeus*	*Komagataeibacter europaeus*	*Gluconacetobacter diazotrophicus*	
	Acetobacter liquefaciens		*Gluconacetobacter liquefaciens*	*Gluconacetobacter liquefaciens* RCTMR 9	
	Gluconobacter industrius IFO 3260		*Gluconobacter japonicus* NBRC 3260	*Gluconobacter oxydans* IFO 3244	
	Gluconobacter melanogenus		*Gluconobacter oxydans*	*Gluconobacter oxydans* NBRC 12528	
	Gluconobacter oxydans DSM4025		*Ketogulonicigenium vulgare* (not AAB)		
Chapter 14	*Acetobacter xylinum* subsp. *sucrofermentans*		*Komagataeibacter sucrofermentans* BPR2001	*Acetobacter pasteurianus* (strain NBRC3284)	
	Acetobacter xylinum subsp. *nonacetooxidans*		*Komagataeibacter swingsii*		
Chapter 15	*Acetobacter aceti* subsp. *xylinum*		*Komagataeibacter xylinus*	*Gluconobacter oxydans* NBRC 3292	*Ketogulonicigenium vulgare* DSM4025
	Acetobacter liquefaciens IFO 12258		*Gluconacetobacter liquefaciens* NBRC 12258	*Acetobacter orientalis* KYG22	
	Gluconobacter melanogenus IFO 3293		*Gluconobacter oxydans* NBRC 3293		
	Gluconobacter melanogenus UV10		*Gluconobacter oxydans* UV10		
	Gluconobacter melanogenus ATCC9937		*Gluconobacter oxydans* ATCC9937		
	Gluconobacter oxydans IFO3255		*Gluconobacter thailandicus* NBRC3255		
	Gluconobacter suboxydans		*Gluconobacter oxydans*		
	Gluconobacter suboxydans IFO 3291		*Gluconobacter thailandicus* NBRC3291		
	Gluconobacter suboxydans IFO12528		*Gluconobacter oxydans* NBRC12528		

[a]In some references, mistakenly used as *Ga. xylinum*
[b]*Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii* Cleenwerck et al. (2009)

Index

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