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TITLE: Characterization of Acetic Acid Adapted Strains and Analysis of Alcohol Dehydrogenase Genes From *Acetobacter syzygii* SKU19

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THESIS

CHARACTERIZATION OF ACETIC ACID ADAPTED STRAINS AND ANALYSIS OF ALCOHOL DEHYDROGENASE GENES FROM *ACETOBACTER SYZYGII* SKU19

WILAWAN SINTUPRAPA

A Thesis Submitted in Partial Fulfillment of
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Wilawan Sintuprapa 2008: Characterization of Acetic Acid Adapted Strains and Analysis of Alcohol Dehydrogenase Genes from *Acetobacter syzygii* SKU19. Doctor of Philosophy (Microbiology), Major Field: Microbiology, Department of Microbiology. Thesis Advisor: Assistant Professor Gunjana Theeragool, D.Agr. 199 pages.

Acetobacter sp. is one of the most attractive bacteria for proteomic analysis of proteins involved in stress response or adaptation. To elucidate an adaptive response to acetic acid, acetic acid adapted strains were isolated from sequential cultivations of acetic acid sensitive strain, *Acetobacter syzygii* SKU19 (wild type), in a medium containing 1.0% acetic acid. The adapted variants could be divided into two groups based on growth and ability to further oxidize acetate. The first group consisted of cells with increased overoxidation or rapid acetate oxidizer, while the second group contained cells with increased stability to acetate or slow acetate oxidizer. The randomly amplified polymorphic DNA (RAPD) profiles of the genomic DNA showed no obvious difference in genetic background among these adapted strains. In contrast, quinoprotein alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH) activities of these adapted strains were higher than those of the wild type. The result corresponded well with the increased amount of protein with molecular mass of 72-80 (subunit I) and 44-54 kDa (subunit II) when the organism was cultivated in acetic acid containing medium. Three *adh* genes, *adhA*, *adhB* and *adhS* encoding for PQQ-ADH subunit I, II and III, from *A. syzygii* SKU19 were cloned and analyzed for nucleotide sequences. These three genes encoded for 743, 472, and 205 amino acids, respectively. Subunit I contained one additional amino acid and subunit III possessed 3 different amino acids compared with subunit III of an acetic acid resistant strain *A. pasteurianus* SKU1108. Surprisingly, subunit III protein of this strain could not be detected in both membrane and soluble fractions by immunoblot analysis although its gene was cloned and sequenced. Transfer of pCM*adhS* plasmid carrying *adhS* gene from *A. pasteurianus* SKU1108 into *A. syzygii* SKU19 enhanced growth on media containing various concentrations of acetic acid. Expression of *adhS* gene from *A. pasteurianus* SKU1108 in *A. syzygii* SKU19 could be induced by ethanol but it seemed to be that it was unable to bind with subunit I from *A. syzygii* SKU19.

Wilawan Sintuprapa. Gunjana Theeragool 05/06/2008

Student's signature Thesis Advisor's signature

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Wilawan Sintuprapa

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LIST OF ABBREVIATIONS

rpm	=	rotations per minute
°C	=	degree Celsius
h	=	hour
µl	=	microlitre
µg/ml	=	microgram per millilitre
v/v	=	volume by volume
ng	=	nanogram
pmol	=	picomole
µmol	=	micromole
mM	=	millimolar
min	=	minute
sec	=	second
kv	=	kilovolts
µF	=	microflux
ml	=	millilitre
N	=	normality
kDa	=	kilodaltons
µg	=	microgram
bp	=	base pairs

CHARACTERIZATION OF ACETIC ACID ADAPTED STRAINS AND ANALYSIS OF ALCOHOL DEHYDROGENASE GENES FROM *ACETOBACTER SYZYGII* SKU19

INTRODUCTION

Acetic acid bacteria are recognized as one of the most important industrial microorganisms especially *Acetobacter* sp. which has been used in vinegar fermentation for thousands of years due to its strong ability to oxidize ethanol to acetaldehyde and acetic acid, respectively. In addition to those industrial applications, this bacterium is one of the most attractive bacteria for studying response or adaptation to acid, ethanol and thermal stress when the fermentation process is carried out under high temperature or without precise cooling system. The microenvironment surrounding the cells during acetic acid fermentation contain various stressors as follow: (i) high temperature due to energy generated during fermentation, (ii) ethanol, an acetic acid fermentation-initiating compound that is always present around the cells and (iii) acetic acid, which is a product of fermentation and whose level gradually increases as the fermentation proceeds (Okamoto-Kainuma *et al.*, 2002). Ethanol is a source of ATP for *Acetobacter* sp. and is oxidized to acetic acid by quinoprotein alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH). In PQQ-ADH deficient mutant two NAD-ADHs were induced and the defect in PQQ-ADH led to a global metabolic change from ethanol oxidation to ethanol assimilation as adaptive response to ethanol stress (Chinnawirotpisan *et al.*, 2003a, 2003b).

Acetobacter species are food-grade microorganisms, strongly oxidize ethanol to acetic acid and thus being used as a vinegar producer. In many countries, hot summer can bring indoor temperature increase beyond 30°C even at night time. That is a serious problem not only to vinegar fermentation but also other fermentation industries, since they need a large amount of cooling water to maintain the optimum fermentation temperature. Normally, domestic vinegar production by acetic acid

bacteria is performed at 30°C. The temperature is controlled strictly for the static or submerged culture. The increasing of temperature only 2-3°C causes a serious failure in both fermentation rate and fermentation efficiency. In submerged culture, a large amount of heat is generated during fermentation so the cooling costs have become expensive. Therefore, if the favorable strains of acetic acid bacteria for oxidative fermentation in industries that can work optimally at 37-40°C were available, the cooling expenses would be reduced greatly. The 129 isolates of thermotolerant acetic acid bacteria, which were isolated from various fruits in Thailand (Theeragool *et al.*, 1996) were of interest for this purpose. These thermotolerant acetic acid bacteria would allow a reduction in energy costs, including heating-cooling power, as exemplified by vinegar fermentation at higher temperature. Many advantages of vinegar fermentation cultures at higher temperatures with thermotolerant acetic acid bacteria have been reported. (Saeki *et al.*, 1997a).

In case of acetic acid stress, it is well known for its cytotoxicity including retardation of growth and product fermentation. However, there are few reports on investigation of stress response proteins in this bacterium even though many reports have been obtained from several species. The aim of this study was to elucidate an adaptive response to acetic acid in acetic acid-adapted strains, which were isolated from sequential cultivations of acetic acid sensitive strain, *Acetobacter syzygii* SKU19, in the medium containing 1.0% (v/v) acetic acid. This strain would be a good model for preliminary study of acid stress response due to its ability to produce acetic acid from ethanol, possessing low toleration but high adaptation to acetic acid and high toleration to ethanol. The adapted strains were characterized for their genetic background, alcohol dehydrogenase and possible mechanism for acetic acid adaptation. Molecular cloning and nucleotide sequencing of three *adh* genes (*adhA*, *adhB* and *adhS*) were also performed.

OBJECTIVES

1. To isolate acetic acid sensitive and adapted strains from thermotolerant acetic acid bacteria.

2. To identify the selected strains by 16S rDNA sequence analysis.

3. To characterize the acetic acid adapted strains by genetic analysis, enzyme assay, heme staining and immunoblotting analysis of alcohol dehydrogenase.

4. To clone and determine nucleotide sequences of three *adh* genes (*adhA*, *adhB* and *adhS*).

LITERATURE REVIEW

1. Acetic Acid Bacteria

1.1 General characteristics and distribution

Acetic acid bacteria are gram-negative or gram variable obligate aerobes, non-spore forming, ellipsoidal to rod-shaped cells. They are classified in the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α-subdivision of the *Proteobacteria* (De Ley *et al.,* 1984; Sievers *et al.,* 1994). These bacteria are commonly found associated with various kinds of fruits, flowers and ethanol-containing habitats. The acetic acid bacteria show relatively high tolerance to acidic conditions. Members of the family are characterized phenotypically by their ability to grow at low pH and their ability to oxidize ethanol to acetic acid in neutral and acidic (pH 4.5) media by two sequential catalytic reactions of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). These two enzymes are localized in the cytoplasmic membrane and their function linked to the respiratory chain (De Ley *et al.,* 1984; Swings *et al.,* 1992; Matsushita *et al.,* 1994).

Acetobacteraceae family is composed of eight genera namely, *Acetobacter, Gluconobacter* (De Ley *et al.,* 1984; Swings *et al.,* 1992), *Acidomonas* (Urakami *et al.,* 1989), *Gluconacetobacter* (Yamada *et al.,* 1997), *Asaia* (Yamada *et al.,* 2000a), *Kozakia* (Lisdiyanti *et al.,* 2002), *Swaminathania* (Loganathan and Nair, 2004) and *Saccharibacter* (Jojima *et al*., 2004). The taxonomic studies on acetic acid bacteria have been mainly carried out on the isolates obtained from sources in temperate regions, such as Europe, North America, and Japan. However, a few reports have been made on isolates from tropical regions, such as Indonesia and Thailand (Theeragool *et al.,* 1996; Yamada *et al.,* 1999; Lisdiyanti *et al.,* 2000, 2001; Moonmangmee *et al.,* 2000; Tanasupawat *et al.,* 2004; Yukphan *et al.,* 2004a, 2004b). Acetic acid bacteria are widespread and occur mainly in sugary, acid, and/or ethanol-containing habitats. *A. polyoxogenes* is the first isolation of acetic acid bacteria from high-acid vinegar production (Entani *et al.,* 1985). Later on, Sievers *et al.* (1992) and

Yamada (2000b) described "*Ga. europaeus*" as the dominant species of acetic acid bacteria in industrial vinegar fermentation in Europe. In addition, *Ga. entanii* was isolated from submerged high-acid industrial vinegar fermentations (Schüller *et al.*, 2000). "*Ga. intermedius*" was isolated from Kombucha beverage, cider vinegar and spirit vinegar as well as from industrial acetators (Boesch *et al.*, 1998; Yamada, 2000b). Moreover, the endophytic nitrogen-fixing species, *"Ga. diazotrophicus"* was first isolated from sugar cane tissue, such as root and stem vessels (Jimenez-Salgado *et al.*, 1997) and pineapple plant. It has several characteristics that are suitable for its endophytic growth (Tapia-Hernandez *et al.*, 2000). In Mexico, the novel nitrogen fixing acetic acid bacteria, which are associated with coffee plants, *Ga. johannae* and *Ga. azotocaptans* have been reported (Fuentes-Ramirez *et al.*, 2001). In Queensland and Northern New South Wales, Australia, *Ga. sacchari* could be isolated from the leaf sheath of sugar cane and the pink sugar cane mealy bug (Franke *et al.*, 1999).

Gluconobacter strains can be found naturally in garden soils, fruits (Gupta *et al.*, 2001), alcoholic beverages (wines and beers) and soft drinks, where they cause off-favors and spoilage (Battey and Schaffner, 2001). Four different species belong to the genus *Gluconobacter*, namely *G. asaii, G. cerinus, G frateurii* and *G. oxydans* (Sievers *et al.*, 1995). Lisdiyanti *et al.* (2001) identified 46 *Acetobacter* strains newly isolated from flowers, fruits, and traditional fermented foods collected in Indonesia and proposed three species, *A. syzygii*, *A. cibinogensis* and *A. orientalis*. A number of strains belonging to the genera *Acetobacter, Gluconobacter* and *Gluconacetobacter* were isolated in Indonesia (Lisdiyanti *et al.*, 2002) and two new species of *Acetobacter, A. indonesiensis* and *A. tropicalis* were proposed (Lisdiyanti *et al.*, 2000).

The genus *Asaia* was introduced as the fifth genus in the family *Acetobacteraceae*, with a single species, *Asaia bogorensis* isolated from flowers of the orchid tree (Bauhinia purpurea), plumbago (Plumbago auriculata) and fermented glutinous rice collected in Indonesia (Yamada *et al*., 2000a). The second species, *A. siamensis*, was isolated from tropical flowers collected in Thailand (Katsura *et al.*,

2001) and the third species, *A. krungthepensis* sp. nov. was described by Yukphan *et al.* (2004a). In Thailand, thermotolerant acetic acid bacteria have been isolated from various kinds of fruits and flowers (Theeragool *et al.,* 1996; Moonmangmee *et al.,* 2000) some of which were identified as *Acetobacter* and *Gluconobacter* strains (Tanasupawat *et al.,* 2004; Yukphan *et al.,* 2004b).

1.2 Thermotolerant acetic acid bacteria

Temperature is one of the most environmental factors affecting growth and survival of microorganisms. At both too low and too high temperature, microorganisms will not be able to grow. Temperature can affect living organisms in either of two opposing ways. As the temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates, and growth becomes faster. However, above a certain temperature, particular proteins may be irreversibly damaged. Thus, as the temperature is increased within a given range, growth and metabolic function increase up to a point where inactivation reactions set in. Above this point, cell functions fall sharply to zero. Normally, the optimum temperature is always nearer the maximum than the minimum (Madigan *et al.,* 2000).

Today, the industrial vinegar production process is carried out by continuous submerged culture in a fermentor which gives higher fermentation rate and yield of acetic acid. However, it requires precise control of fermentation temperature for the efficient vinegar production. Optimum temperature in the process is about 30°C and slight temperature increase by 2-3°C causes a serious failure in both fermentation rate and fermentation efficiency. Hot summer in the past couple of years has brought indoor temperature increases beyond 30°C even at night time in many countries, which is a serious problem. In submerged culture, a large amount of heat is generated during fermentation and thus cooling costs become rather expensive. It can be readily suggested that if favorable strains of acetic acid bacteria that can work optimally at 37-40°C were available, the cooling expenses would be very much reduced. However, little has so far been reported about vinegar fermentation by

thermotolerant acetic acid bacteria. Ohmori *et al.* (1980) isolated three *Acetobacter* strains with the ability to produce acetic acid in continuous submerged culture at 35°C and produced 45% of activity at 38°C, while the usual strain of *A. aceti* completely lost its activity at 35°C.

In Thailand, isolation, identification and characterization of thermotolerant acetic acid bacteria were set in progress to develop new microbial resources for oxidative fermentation. Saeki *et al.* (1997a) screened some thermotolerant acetic acid bacteria showing the same fermentation efficiency at 38-40°C as that of mesophilic strains at 30°C. Moonmangmee *et al.* (2000) isolated and screened eight thermotolerant *Gluconobacter* strains with the ability to produce D-fructose and L-sorbose at 37°C and the result obtained from 16s rRNA sequence analysis showed that those isolated strains were almost identical to *G. frateurii* with homology scores of 99.31-99.79%.

1.3 Oxidative and acetic acid fermentation

During the last decade, new discoveries in oxidative fermentation by acetic acid bacteria have led to a new perspective on oxidative fermentation, which is attractive from both academic and practical aspects. The oxidation of substrates has been identified and characterized in acetic acid bacteria (Figure 1). It was clearly shown that acetic acid bacteria are used for several biotechnological processes for example gluconic and ketogluconic acid production, L-sorbose fermentation for vitamin C production, dihydroxyacetone production, synthesis of 1-deoxynojirimycin and miglitol, vinegar fermentation and other oxidative fermentations (Macauley *et al.*, 2001; Deppenmeier *et al.*, 2002; Adachi *et al.*, 2003).

For vinegar fermentation, it has been known for centuries by natural fermentation of ethanol-containing solution. The first description of vinegar fermentation was made by Pasteur in 1862. He recognized that mother of vinegar was naturally occurred in vinegar products. It caused acetic acid fermentation. In 1898

and 1924, Beijerinck and Kluyver and de Leeuw discovered acetic acid bacteria, *A. aceti* and *G. suboxydans*. Vinegar has been produced from many other materials including molasses, sorghum, fruits, berries, melons, coconut, honey, beer, potatoes, beets, malt, grains and whey of nearly any other liquid containing sugar. The varieties of vinegar were classified according to materials from which they were made and the methods of manufacturing. The label will describe starting materials for example “apple cider vinegar”, “wine vinegar”, “rice vinegar”, “white vinegar”, “cane vinegar”, raisin vinegar”, “beer vinegar” or “balsamic vinegar” were made from apple juice, wine, rice, distilled alcohol, sugar cane, raisin, beer, or trebbiano grapes juice, respectively (Conner and Allgeier, 1976).

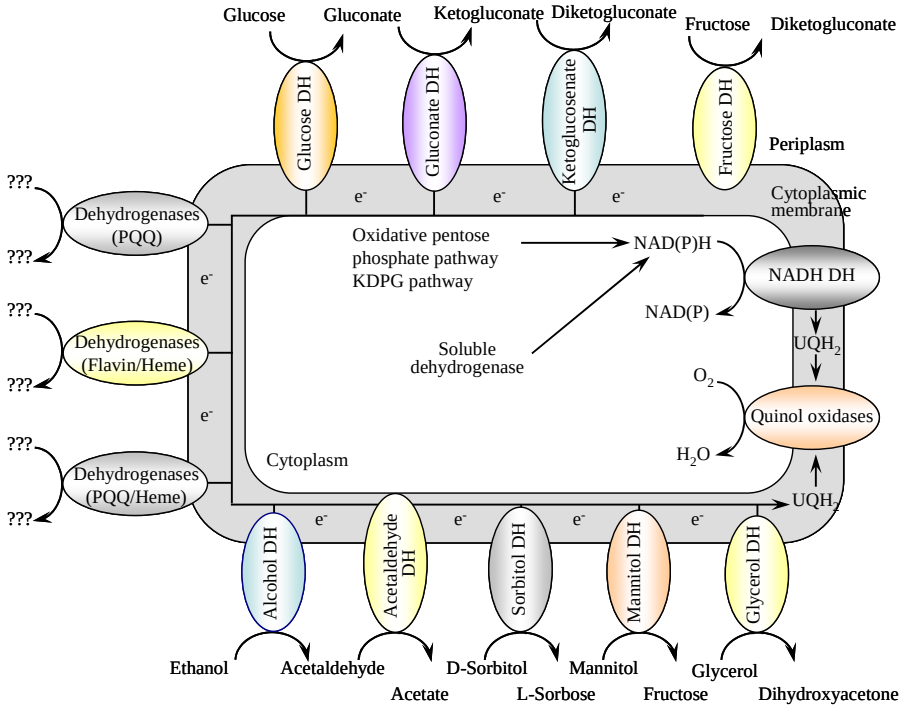


Figure 1 Alcohol- and sugar oxidizing systems of *Gluconobacter* species. Question marks = uncharacterized dehydrogenase, UQH_2 = ubiquinol, KDPG = 2-keto-3-deoxy-6-phosphogluconate.

Source: Deppenmeier *et al.* (2002: 234)

Vinegar was made by two distinct biochemical processes with the action of microorganisms. The first process is called the alcoholic fermentation; yeasts change natural sugars to alcohol under controlled conditions. The second process is acetic acid fermentation. A group of acetic acid bacteria converts alcohol to acetic acid or vinegar. These two reactions make clear that acetic acid bacteria produce vinegar from ethanol by two sequential oxidation reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Matsushita *et al.,* 1994). Vinegar is typically three to five percent by volume of acetic acid but natural vinegar also contains smaller amounts of tartaric acid, citric acid, and others. The most known acetic acid bacteria which produced vinegar are genera *Acetobacter* (*A. aceti*) and *Gluconobacter* (*G. suboxydans*) (Lasko *et al.,* 1997, 2000). *A. aceti* can grow in the presence of up to 60 g/l acetic acid (Steiner and Sauer, 2003) and accumulate acetic acid exceeding 140 g/l in semi-continuous process (Steiner and Sauer, 2001). *Gluconobacter* strains, which is the most known acetic acid bacteria same as *Acetobacter*, were used in vinegar manufacture, since they do not overoxidize acetic acid to CO_2 and H_2O (Macauley *et al.,* 2001). In fact, it seems that species of the genera *Gluconacetobacter* and *Acetobacter* are more important in vinegar fermentation than *Gluconobacter* (Deppenmeier *et al.,* 2002).

Vinegar can be produced by both chemical and biological processes. There are three chemical processes: methanol carbonylation, butane oxidation and acetaldehyde oxidation. The world wide biological production of vinegar was in the range of $1x10^6$ l/year (Lusta and Reshetilov, 1998). The United States was the world's largest producer of acetic acid with about 2.3 billion pounds in 2000. About 80 percent of the US production of acetic acid was produced by methanol carbonylation. However, this synthetic process requires relatively high temperature and pressure, a toxic/corrosive environment, and extensive safety-related equipment so this process had high capital cost. Therefore, fermentation process was developed for acetic acid production. The advantage of producing acetic acid by fermentation was lower cost feedstock, low energy membrane-based purification and lower temperature and pressure requirements. The production of acetic acid could be

operated in a small scale. They are four different biological processes for the production of vinegar. The first is film culture system or static surface fermentation. It was not popular because they were slow and expensive but this process is still used for vinegar production in Japan. This is an original and traditional Japanese method (Nanda *et al.,* 2001). The second system is the open vat method (Orleans method). Wine was placed in the bottom of vats which air was exposured. The acetic acid bacteria developed as a slime layer on the top of the liquid. The third system is the trickled generator process. The alcoholic liquid is trickled over beech-wood shavings where the acetic acid bacteria grow. Air enters the generators at the bottom and passes upwards so that the bacteria can contact oxygen. The last system is based on submerged fermentation in aerated and agitated vessels. This system has become widely used at industrial scale. In fermentor, the biomass is suspended in the medium and it is stirred and aerated. Likewise, this fermentor is usually fitted with a thermal jacket for the maintenance of the optimum temperature in fermentation process. The efficiency of this process is very high; 90-98% of ethanol is converted to acetic acid at final concentration of 12-17% (Deppenmeier *et al.,* 2002). A very popular submerged culture system is the Frings acetifier (Ebner *et al.,* 1991).

1.4 Enzymes responsible for acetic acid fermentation

Acetic acid fermentation is the most distinguished characteristic of acetic acid bacteria in which ethanol is oxidized to acetic acid (Conner and Allgeier, 1976). The recent understanding of acetic acid fermentation can be summarized as follows: alcohol dehydrogenase oxidizes ethanol by transferring electrons to ubiquinone embedded in the membranous phospholipids. The resulting ubiquinol is further oxidized by the terminal ubiquinol oxidase, cytochrome *o* or *a1* generating proton gradient across the cytoplasmic membranes yielding bioenergy (Matsushita *et al.,* 1992a, 1992b, 1994). The dehydrogenases in acetic acid bacteria are divided into two groups, $NAD(P)^+$ dependent dehydrogenase located in the cytoplasm and $NAD(P)^+$ independent dehydrogenase located in the membrane (Matsushita *et al.,* 1994), as shown in Figure 2. Pyrroloquinoline quinone alcohol dehydrogenase (PQQ-

ADH) and aldehyde dehydrogenase (ALDH) which involved in acetic acid fermentation are $NAD(P)^+$ independent dehydrogenase (Matsushita *et al.,* 1992a, 1994, 1995). The membrane bound dehydrogenases can be divided into quinoproteins and flavoproteins that have pyrroloquinoline quinone (PQQ) and covalently-bound flavinadenine dinucleotide (FAD) as prosthetic groups, respectively. These quinoprotein and flavoprotein dehydrogenases have been shown to function by linking to the respiratory chain which transfers electrons to the final electron acceptor, oxygen and generating energy for growth (Shinagawa *et al.*, 1990; Matsushita *et al.,* 1991, 1994). The quinoprotein alcohol and aldehyde dehydrogenases the play main role in vinegar production (Matsushita *et al.,* 1994).

ADH activity was reported to be largely decreased in *A. pasteurianus* when cultivated without ethanol which seemed to be required for the correct localization of the dehydrogenase subunit in the membrane (Takemura *et al.*, 1993). The incorrect localization of dehydrogenase subunit responsible for the lower ADH activity in the absence of ethanol. It has been reported that the decreasing in ADH activity of *G. suboxydans* was due to reversible change from active to inactive form during cultivation under certain conditions (Matsushita *et al.,* 1995).

1.5 Enzyme involved in acetate assimilation

According to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994), all species in the genus *Acetobacter* oxidize acetate into carbon dioxide and water. "Overoxidation of acetate" or "acetate peroxidation" has been used synonymously with acetate oxidation to designate the phenomenon of aerobic acetate catabolism by acetic acid bacteria. The addition of a small amount of energy source increased acetate usage allowing the bacteria to grow on the medium. It could be suggested that ethanol plays an important role as oxidizable substrate generating energy that supports the initial part of microbial growth in a stage where the tricarboxylic acid (TCA) cycle and NADH dehydrogenase system are not predominant (Matsushita *et al.,* 1994). For oxidation of acetate to water and carbon

dioxide while cell biomass increases, the TCA cycle must be driven smoothly, as enzymes in TCA cycle make citrate with acetyl-CoA. The cells oxidizing acetate may have increased enzyme activities in the TCA cycle because of the flux of citrate from acetyl-CoA and oxaloacetate. The enzyme activities of acetyl-CoA synthase, isocitrate lyase and malate synthase also increased significantly in the cells when acetate was consumed. These results indicated that acetic acid is converted to acetyl-CoA by acetyl-CoA synthase to put acetate into the TCA cycle as well as to the glyoxylate cycle allowing the bacteria to grow rapidly on acetic acid after ethanol exhaustion (Saeki *et al.*, 1997b). Furthermore, isocitrate dehydrogenase and fumarase activities were confirmed to be increased during acetate oxidation. The mechanisms of acetate oxidation by acetic acid bacteria had been clarified (Saeki *et al.*, 1999; Matsushita *et al.*, 2004, 2005a) as shown in Figure 2. The finding suggested that strong acetate oxidation caused by acetyl-CoA synthase or phosphotransacetylase activity, together with phosphoenolpyruvate carboxylase, increased the biomass.

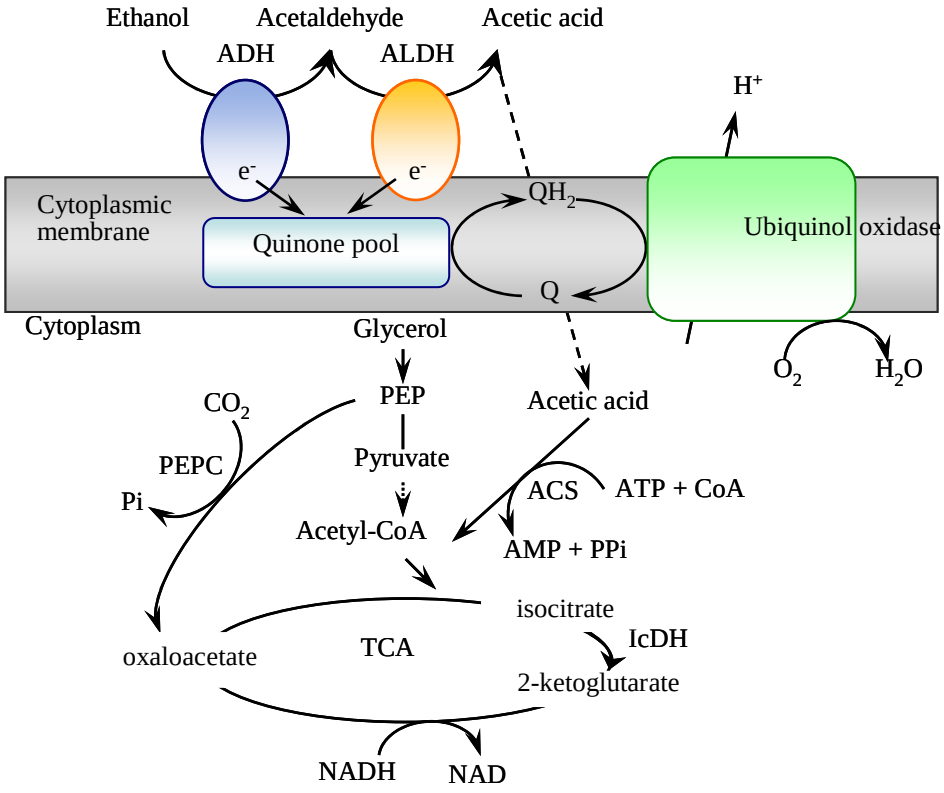


Figure 2 Ethanol respiration and metabolic pathway responsible for the overoxidation of *Acetobacter* species. The ethanol respiration consists of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) located on the outer surface of the cytoplasmic membrane as primary dehydrogenases, ubiquinone pool (Q and QH_2), and ubiquinol oxidase, which generates a proton motive force. Acetic acid overoxidation is carried out by acetyl CoA synthase (ACS) and TCA cycle enzymes such as isocitrate dehydrogenase (IcDH), which are driven *via* NADH reoxidation by NADH dehydrogenase linked to the respiratory chain. Phosphoenol pyruvate carboxylase (PEPC) is also working to supply oxaloacetate via phosphoenol pyruvate (PEP).

Source: Matsushita *et al.* (2005a: 175)

Extensive acetate consumption is always accompanied by an increase in cell biomass. When all available carbon and energy sources were exhausted and only acetic acid remained in the late stationary phase, the bacteria started to consume the acetic acid that had been accumulated in the culture medium for vinegar fermentation. In ethanol culture of *A. aceti*, there were three growth phases (Figure 3): *A. aceti* first grew by oxidizing ethanol completely to acetic acid (ethanol oxidation phase), then the growth stopped and remained for a long time with the viable cell number being decreased (first stationary phase) and finally started to grow again by utilizing the accumulated acetic acid, the phase of which was called as "overoxidation of acetate". It was thus conceivable that *Acetobacter* species have two different phases related to acetic acid resistance, the ethanol oxidation and the first stationary phases where the strains resist against acetic acid accumulated in the culture medium without utilizing the acetate, and the overoxidation phase where the strains just utilize the acetate for cell growth. Acetic acid was administrated as sole carbon and energy sources. They grew rapidly and a typical biphasic growth curve was observed. However, no distinct acetate oxidation took place when oxidizable ethanol and other available carbon sources still remained in the culture medium. The growth curve became biphasic when the initial ethanol concentration was limited below 3.0%. This means that acetate oxidation must take place if the amount of acetic acid accumulated is controlled to be less than 3.7%, though the length of the first stationary phase depends on the initial ethanol concentration. Thus, beginning of the second growth was delayed more with 3.0% ethanol than with 1.0 or 2.0% of initial ethanol concentration. Growth curves observed with 4.0 and 5.0% of ethanol showed no second stationary phase. Moreover, no apparent acetate oxidation was observed in vinegar mash in which more than 4.5% of acetic acid was allowed to accumulate. Also, the critical point for acetate oxidation exists between 3.7 and 4.5% of acetic acid accumulated. At the moment, it is unclear what kind of regulation occurs in the presence of more than 4.5% acetic acid. One speculation can be proposed that, the acetic acid accumulated in the culture medium can exist as two forms, dissociated and undissociated. If the undissociated form of acetic acid increases, it can be out of the

mediated transport system and diffuses through the bacterial membrane, causing inhibition to bacterial respiration (Saeki *et al.*, 1997b).

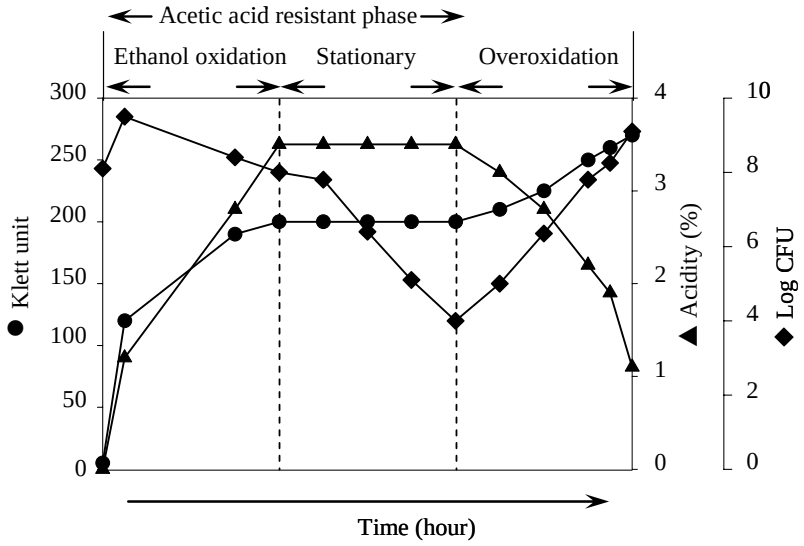


Figure 3 Typical growth patterns of *Acetobacter* species in ethanol culture. *Acetobacter* strain exhibits a biphasic growth curve in ethanol culture, where the first phase has an ethanol oxidation to produce acetic acid, and the second phase, an overoxidation of acetic acid (assimilation).

Source: Matsushita *et al.* (2004: 172)

1.6 Genetic instability in acetic acid bacteria

Bacterial mutation is a heritable change in the sequence of nucleotide in the DNA that typically resulted in the generation of a new genotype and/or phenotype. Mutation can be divided into two types, spontaneous and induced mutations. Spontaneous mutation can occur in nature without any external mutagenic agent, but occurs at low frequencies. Induced mutation occurs by mutagens such as chemical and physical mutagens. Acetic acid bacteria has been frequently observed for genetic instability which causes spontaneous mutations at high frequencies leading to

deficiencies in various physiological properties, such as ethanol oxidation (Ohmori *et al.,* 1982; Okumura *et al.,* 1985b; Takemura *et al.,* 1991), acetic acid resistance (Ohmori *et al.,* 1982) and cellulose formation (Cook and Colvin, 1980; Coucheron, 1991). Prolonged cultivation of thermotolerant strains of *A. aceti* caused the appearance of acetic acid sensitive mutants which are deficient in ethanol oxidizing ability with high frequency as well as lost of acetic acid resistance in *A. pasteurianus* (Takemura *et al.,* 1991). The genetic instability has significantly influenced on the industrial use of acetic acid bacteria. In contrast, sometimes when mutation is induced by mutagen, (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, NTG), in acetic acid bacteria, the obtained mutants showed greater ability to produce acetic acid from ethanol than the parental strain (Harada and Mori, 1971).

Many investigations have revealed that multiple insertion sequences play a major role in genetic instability leading to deficiencies in various physiological properties. Insertion sequence IS*1380* was first reported as a mobile DNA involving in the loss of ethanol oxidizing ability in *A. pasteurianus* by insertion in the cytochrome *c* subunit (subunit II) of ADH (Takemura *et al.,* 1991). The second insertion sequence, IS*1452* was also found to be responsible for the inactivation of ADH by insertion in the *adhS* gene encoding subunit III of ADH in *A. pasteurianus* (Kondo and Horinouchi, 1997a). Moreover, another insertion sequence IS*12528* was found to be associated with the inactivation of the ADH by insertion in the *adhA* gene which encodes the primary dehydrogenase subunit (subunit I) of ADH complex in *G. suboxydans* (Kondo and Horinouchi, 1997b).

2. Acid Stress Response

For centuries, man has applied natural preservatives and preservation methods. The naturally occurring preservatives are weak organic acids. They include acetic, benzoic, lactic, propionic and sorbic acids which are widely used in large-scale food and beverage preservation. However, several weak acid-tolerant microorganisms can adapt to even higher concentrations of these compounds and can utilize them as carbon and energy sources. The toxicity of weak acids is dependent on the pH of the environment and the pK_a of the acid. At low pH, acetic acid (pK_a 4.75), sorbic acid (pK_a 4.76) or benzoic acid (pK_a 4.19) are mainly present in the undissociated form (XCOOH; Figure 4), which are potent growth inhibitors, and can diffuse across the cell membrane by passive diffusion. As shown in Figure 4, inside the cell these acids rapidly dissociate into protons and the acid anion ($XCOO^-$) in the higher pH of the cytosol. These anions will accumulate in the cell membrane to very high levels, it cannot very readily diffuse from the cell. This high anion accumulation may generate an abnormally high turgor pressure. The proton released can lead to a decrease of intracellular pH which interferes with several metabolic pathways (Abee and Wouters, 1999; Augstein *et al.,* 2003; Axe and Bailey, 1995; Booth *et al.,* 2002; Foster, 1999; van de Guchte *et al.,* 2002; Kashket, 1987; Piper *et al.,* 1998, 2001). Organic acid then affects cell growth in at least two ways, by lowering pH_i and by increasing turgor pressure through anion accumulation. Weak acids cause several strong changes in intracellular processes, for example cell division, DNA metabolism, ion transport, membrane structure, membrane perturbing (Brul and Coote, 1999; Diez-Gonzalez and Russell, 1997; Roe *et al.,* 1998) in fatty acid and phospholipids composition (Chang and Cronan Jr, 1999; Quivey Jr. *et al.,* 2000) as well as in protein syntheses.

Several species of bacteria are known to be relatively tolerated to acetate. The gram-negative genera *Acetobacter* and *Gluconobacter*, known as acetic acid bacteria because they are widely used in vinegar fermentation, are the best known examples of these. These organisms grow in the presence of up to 70 g/l acetate, more than a full

order of magnitude greater than the inhibitory levels reported for *E. coli*, and accumulate levels up to 150 g/l in vinegar fermentation (Lasko *et al.*, 2000). The previous report on acetate resistance genes (*aarABC*), of a thermophilic *A. aceti* strain, was important for acetate resistance on solid media (Fukaya *et al.*, 1990). They identified functions of the *aarA* and *aarC* gene products in citrate synthase (Fukaya *et al.*, 1990) and acetate uptake (Fukaya *et al.*, 1993), respectively. The result showed that these proteins confer resistance by acetate assimilation *via* a local reduction of acetate concentrations on solid media. Previous proteome analysis of acetic acid bacteria revealed eight acetate stress proteins (Asps) that were induced specifically by challenging unadapted *A. aceti* and *G. suboxydans* cultures with 10 g/l acetate (Lakso *et al.*, 1997). Steiner and Sauer (2001) investigated the changes in global protein expression levels during long-term adaptation of *A. aceti* to high acetate concentrations by two-dimensional electrophoresis (2-DE). They reported a complex proteome response with at least 50 proteins that are specifically induced by adaptation to acetate but not by other stress conditions, such as heat or oxidative or osmotic stress. Membrane-associated processes appear to be of major importance for adaptation, because some of the Asps bear N-terminal sequence homology to membrane proteins for example AatA, a putative ATP-binding cassette (ABC) transporter, which possibly functioned as an exporter of acetic acid (Nakano *et al.*, 2006). One of protein in cytoplasm whose production was enhanced in response to acetic acid was identified as aconitase (Nakano *et al.*, 2004). Recently, Matsushita *et al.* (2005b) reported that the efflux pump mechanism was responsible for acetic acid resistance in acetic acid bacteria. Recently, Trcek *et al.* (2006) reported the relationship between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria.

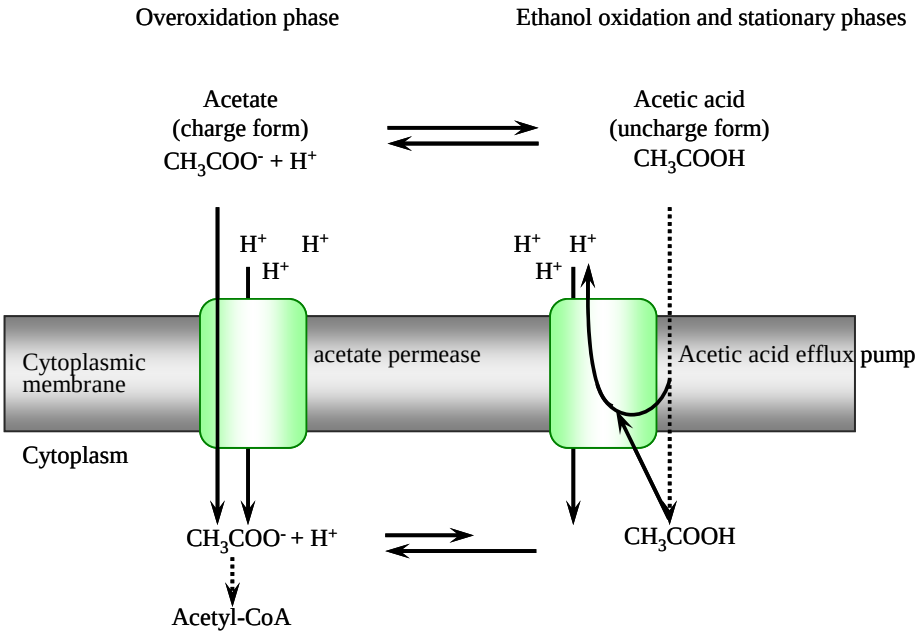


Figure 4 Acetic acid or acetate efflux and uptake in acetic acid bacteria. XCOOH is the protonated or uncharged form of the weak acid. $XCOO^-$ is the acid dissociates to anion.

Source: Matsushita *et al.* (2005b: 177)

3. Principles and Techniques of DNA Manipulation

3.1 Polymerase chain reaction (PCR) and its applications

The polymerase chain reaction (PCR) is a procedure for generating large amounts of a specific DNA target *in vitro* by an enzymatic reaction. In 1983, this technique was invented by Kary Mullis. It has rapidly become one of the most widely used techniques for the molecular biologist and revolutionized molecular biology. This technique is used in the laboratory every day. This is due to its rapid, inexpensive, and simple means of producing relatively large numbers of copies of a specific DNA fragment from minute quantities of DNA material in a test-tube. PCR

is a complicated process with many reactants. A typical amplification reaction consists of target DNA, thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs: dATP, dTTP, dGTP, dCTP), reaction buffer, magnesium chloride ($MgCl_2$), and optimum additives.

The initial version of this technique used the Klenow polymerase to make repeated copies of a DNA template. After each round of DNA synthesis, the mixture had to be heated to >90°C to detach the newly synthesized strands from the original templates. As the Klenow polymerase was inactivated during the heat cycle, it was necessary to replenish the reaction continually with fresh enzyme, which made the procedure costly and laborious to carry out. Two important innovations were responsible for automatic PCR. The first, a heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus*, isolated from hot springs. This enzyme was called "*Taq*" polymerase. This polymerase was capable of remaining active throughout the high denaturation temperatures required at the beginning of each amplification cyclers despite of repeated heating during many cycles of amplification. Second, DNA thermal cycles were invented that use a computer to control the precise repetitive temperature changes require for PCR.

The PCR similarly replicates DNA by repeated cycles of three steps. The first step is template denaturation. This initial step in a cycle denatures, the target gene sequence was separated from double strand to single strands at high temperature (95°C or higher for 15 seconds to 2 minutes). This step is necessary because it can produce two single-stranded of the target DNA strands to serve as the template and the oligonucleotides serve as primers. The second step is primer annealing. The temperature was reduced to approximately 40-60°C for 30-60 seconds. At this temperature, the oligonucleotide primers can form stable association (hybridization or anneal) with the separated target DNA strands and serve as primers for DNA synthesis by a thermostable DNA polymerase. The last step is primer extension. Finally, the synthesis of new DNA begins when the reaction temperature is raised to the optimum for the thermostable DNA polymerase by the addition of new bases to

the free-3' ends of the primers. This results in the synthesis of new DNA strands which are complementary to the parent template strands. The optimum temperature of the thermostable DNA polymerase is 72-74°C for 1-2 minutes. This step completes one cycle and the next cycle begins with a return to 95°C for denaturation by the newly synthesized strands which can serve as templates in the next cycle. The cycle is repeated some 20-40 times leading to amplification of the target sequence by up to 10^5 fold. The amplification is complete within a few hours. The ability of PCR to amplify single sequences from a background of many others means that sequences in the original sample which were too rare to be detected by other methods become major species after amplification. PCR can be used to facilitate cloning the productions of amplification, generate mutations, detect mutations and compare the DNA in different individuals. It is not only used as a tool in basic research, but also for applications ranging from prenatal diagnosis and analysis of carried status in genetic diseases to forensic analyses, pathogen detection and archeological studies (Brown, 1998).

3.2 Molecular taxonomy by 16S rDNA sequences and phylogenetic tree construction

The 16S rRNA technique is widely used as a biomarker and for microbial ecology studies. 16S rDNA gene is the gene encoding for 16S rRNA which makes up the bulk of the 30S subunit. It is important for subunit association and translational accuracy. It consists of 1,542 bases and contains the substrate binding A-, P- and E-sites. rRNA represents only a small part approximately 0.3-0.4% of the genome. The primary structure of 16S rRNA is highly conserved.

In 1970s, Carl R. Woese and coworkers, who began to investigate the phylogeny of prokaryotes by using 16S rRNA, found that organisms can be divided into three major lines of descent, named the domains *Archaea, Bacteria* and *Eucarya* (Busse *et al.,* 1996). 16S rDNA based molecular could achieve identification due to region of rRNA sequences which are highly conserved among all organisms and

others regions that vary to different degrees (Nakatsu *et al.,* 2000). Bacterial identification based on percentage similarity of 16S rDNA has been using PCR technique, DNA sequencing and similarity analysis of rRNA genes. 16S rDNA was amplified and sequenced by using two oligonucleotide primers (Table 1), which are complementary to highly conserved regions of bacterial rRNA coding gene, to obtain a stretch of 16S rDNA with more than 1,300 nucleotides.

Table 1 Oligonucleotide primers for amplified 16S rDNA gene

Oligonucleotide pimer	Sequence (5'--------3')
Forward-sequencing primers	
27f	-AGAGTTTGATCCTGGCTCAG-
357f	-CTCCTACGGGAGGCAGCAG-
530f	-GTGCCAGCAGCCGCGC-
704f	-GTAGCGGTGAAATGCGTAGA-
926f	-AAACTCAAAGGAATTGACGG-
1114f	-GCAACGAGCGCAACCC-
1242f	-CACACGTGCTACAATGG-
1406f	-TGTACACACCTCCCGTG-
Reverse-sequencing primers	
321r	-AGTCTGGACCGTGTCTCAGT-
519r	-G(AT)ATTACCGCGGC(GT)GCTG-
685r	-TCTACGCATTTCACCGCTAC-
907r	-CCGTCAATTCCTTTGAGTTT-
1069r	-CCAACAT(TC)TCACA(AG)CACGAG-
1100r	-GGGYYGCGCTCGTTG-
1392r	-ACGGGCGGTGTGT(AG)C-
1492r	-TACGGCTACCTTGTTACGACTT-
1522r	-AAGGAGGTGATCCA(AG)CCGCA-

Source: Johnson (1994)

3.3 Randomly amplified polymorphic DNA (RAPD)

In the past two decades, molecular marker techniques have been developed as a direct result of the needs of genomic analysis. These techniques range from molecular assays for genetic mapping, gene cloning and marker assisted plant breeding to genome fingerprinting and for the investigation of genetic relatedness. Genetic markers are based on DNA polymorphisms in the nucleotide sequences of genomic regions either defined by restriction enzymes, or two priming sites.

PCR-based techniques for detecting DNA markers require the development of specific DNA primers as a start site for amplification. Randomly amplified polymorphic DNA (RAPD) is a polymorphism assay or a commonly used molecular marker in genetic diversity studies, which was described by Williams *et al.* (1993). This technique is based on the amplification of random DNA segments using sets of primers of arbitrary nucleotide sequence. Nanogram amounts of total genomic DNA are subjected to amplification using short oligonucleotides of random sequence. In theory, the primer anneals to many regions of the genome simultaneously. This means that the amplified fragments generated by PCR depend on the length and size of both primer and target genome. However, genometric amplification only occurs in those regions in which the 3' end of the annealed primers face one another on opposite strands and are no more than 3 kb apart. These conditions suggest that the primer annealing sites must be inverted repeats. Moderate and highly repetitive DNA segments in centromeric, telomeric and heterochromatic genomic regions are rich in inverted repeats, as are various classes of dispersed repetitive and mobile elements. RAPD is biased in its amplification of these repetitive regions, but amplified unique regions as well. Thus, the technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA sequences of variable length. Therefore, this technique requires single 10-mer oligonucleotides in order to promote the generation of several discrete DNA products and a small amount of total genomic DNA as mentioned above. Polymorphisms result from mutations or rearrangements

either at or between the primer binding sites and detected as the presence or absence of a particular RAPD band (Figure 5).

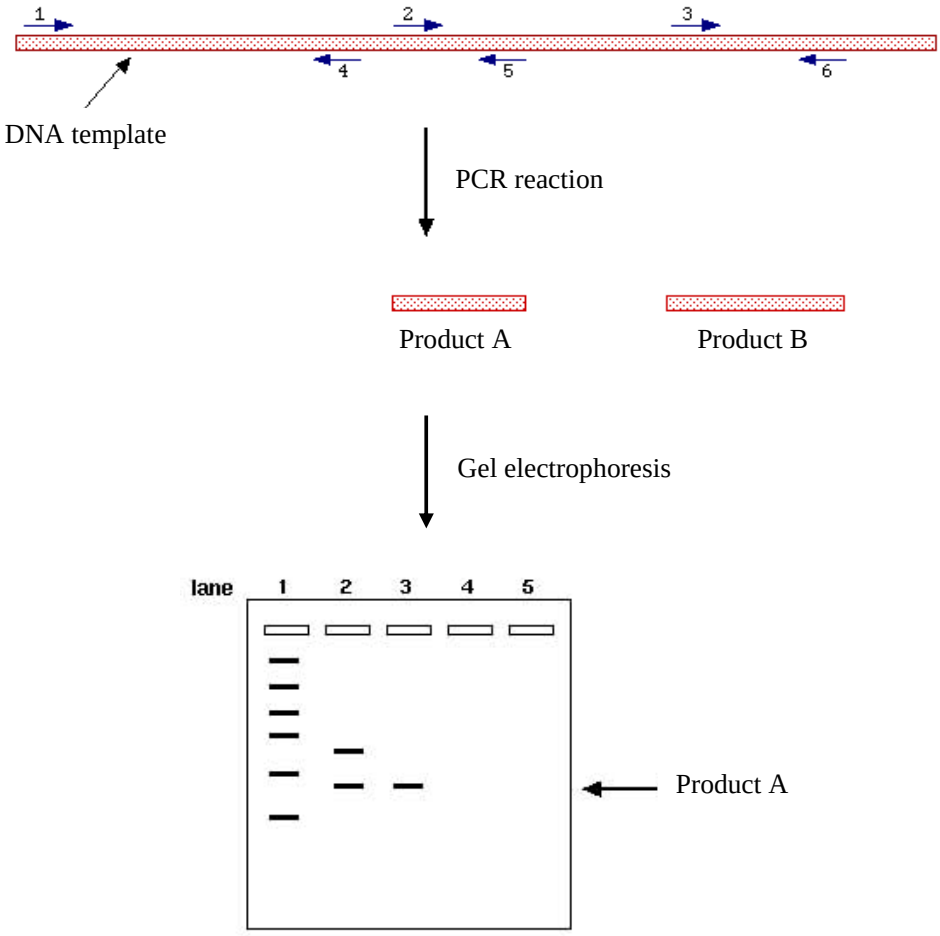


Figure 5 Schematic representation of randomly amplified polymorphic DNA (RAPD)

Source: Sofer (2005)

RAPDs have become widely used in molecular biology laboratories because of the following advantages: the first, RAPDs require no DNA probes and previous sequence information or molecular knowledge. Second, they do not require for the design of specific primers. Third, the procedure involves no blotting or

hybridization steps as restriction fragment length polymorphisms (RFLPs). Therefore, this technique is quick, easy, simple and efficient. Finally, RAPDs require small amounts of DNA (about 10 ng per reactions) and the procedure can be automated. In addition, they have proved to be a powerful technique when a variety of samples have to be checked for species and differentiation. RAPDs have also been reported to detect higher levels of polymorphism compared. However, RAPDs have some disadvantages. This technique is sensitive to reaction conditions and slight changes in the conditions may affect the reproducibility of shape of the temperature profile, type of polymerase used, magnesium chloride ($MgCl_2$) concentration, template DNA and primer size.

3.4 Nucleotide sequencing

Nucleotide sequencing is the determination of the sequence of DNA. The detailed information of DNA fragment can be obtained after this DNA fragment was analyzed by nucleotide sequencing. This information is useful, for example, for identifying gene sequences and regulatory sequences within the fragment and for comparing the sequences of homologous genes from different organisms.

The most popular method for doing this is called the dideoxy sequencing or the chain termination method, which was devised by Fred Sanger in 1975. All of DNA sequencing reactions use a primer to initiate DNA synthesis. This primer will determine the starting point of the sequences being read. This method gets its name from the critical role played by synthetic nucleotides. The 2, 3-dideoxynucleotide triphosphates (ddNTPs) were used to stop DNA synthesis at specific nucleotide. They lack the –OH at the 3’ carbon atom so they cannot form a phosphodiester bond with the next deoxynucleotide. For this reason, the dideoxy method is also called the chain termination method.

Today, the automated DNA sequencing method has been developed from Fred Sanger method. This system uses fluorescently-labeled primers or dideoxy

terminators instead of radioactivity. Sequencing reactions prepared by thermocycle sequencing are resolved and read in a single lane, rather than four separate lanes, enabling a much higher throughput of DNA sequence data. Several automated DNA sequencers are now commercially available and becoming increasingly popular (Alphey, 1997).

3.5 Southern blot hybridization

Hybridization is the artificial construction of a double stranded nucleic acid by complementary base pairing of two single stranded nucleic acids. When a solution of DNA has been heated and allowed to cool slowly, many of the complementary strands reassociate and the original double stranded complex reforms reannealing. The reannealing occurs only if the base sequences of the two strands are complementary. Thus, nucleic acid hybridization permits the formation of artificial double stranded hybrids of DNA, RNA or DNA and RNA. This procedure is a powerful one for studying the genetic relatedness between nucleic acids. It also permits the detection of pieces of nucleic acid that are complementary to a single stranded molecule of known sequence. Such a single stranded molecule of known sequence is called a probe. For example, a radioactive nucleic acid probe can be used to locate, in an unknown mixture, a nucleic acid sequence complementary to the probe.

Detection of nucleic acid hybridization is usually done with membrane filters in a format known as either dot blot or slot dot. Single stranded DNA is first bound to the membrane, the probe is added that which does not form hydrogen bonds *via* complementary base pairing to the DNA on the membrane is washed off. The probe binding to the attached DNA on the membrane is of course kept with the membrane. Then the detection of the probe can be carried out depending upon the method of labeling. Hybridization conditions can be manipulated to favor the formation of either DNA:DNA or DNA:RNA hybrids. However, hybridization can also be done after gel electrophoresis. The nucleic acid molecules are transferred by

blotting from the gel to a membrane and the probe is added to the filter. The procedure when DNA is in the gel and RNA or DNA is the probe is called Southern blotting. When RNA is in the gel and DNA or RNA is the probe, the procedure is called Northern blotting. A Western blot can also be done and this involves protein-antibody binding rather than nucleic acid hybridization.

The Southern blot hybridization combines with conventional agarose or polyacrylamide gel electrophoresis and is commonly used for characterization of a gene or a specific DNA segment. The procedure was first performed by E.M. Southern in 1976 and named after the inventor. The entire approach is conducted in two major phases. First, the DNA is purified from the target organisms and treated with restriction enzyme(s), and the DNA fragments are separated by the gel electrophoresis method, the DNA fragments in the gel are then denatured by treatment with alkaline followed by immobilization onto solid support, i.e., nylon or nitrocellulose membrane by capillary transfer. The DNA can also be transferred onto the membrane by electroblotting or vacuum blotting. Second, the membrane is exposed to an appropriate labeled probe for hybridization under hybridization conditions. The absorbent material above the filter draws the transfer solution in the reservoir up through the gel carrying the DNA with it. When the DNA reaches the filter, it binds to the filter. The composition and ionic strength of the transfer solution are important for binding and are determined by the type and properties of the filter. Transfer is usually carried out overnight, but if the depth of the gel is sufficiently small, transfer may be complete in a few hours (Anderson, 1999). Generally, a known DNA complementary to the probe DNA is used as a positive control, and another DNA that is unrelated to the probe is used as a negative control. If the probe is radiolabeled, an autoradiogram is performed on a X-ray film. Positive hybridization signals represent the specific restriction digested DNA fragment(s) complementary to the probe (Dangler, 1996).

Long DNA fragment (>8 kb) does not migrate efficiently through agarose, so its size is first reduced by depurination under carefully controlled

conditions. The gel is exposed to dilute acid at room temperature such that about 1 in every 500 purines is removed. Since sites of depurination are sensitive to hydrolysis by alkaline, exposure of depurination DNA to alkaline causes the DNA to fragment at these sites. The gel is then neutralized and capillary transfer carried out as above. Depurination does not affect the position of DNA in the gel, but simply improves the efficiency of transfer to the filter (Anderson, 1999).

The Southern blot hybridization method is useful in determining the presence of a gene or a specific DNA sequence in a pool of DNA. Changes in the molecular weight of a gene due to mutation such as deletion, and identification of a single base mutation of a gene fragment, which may be an indication of a disease state, can be determined. The disadvantages of this method are that it may sometimes take days to perform unless a relatively expensive, commercially available, partially automated device is used. Most of the time, the Southern hybridization is used for qualitative analyses of DNA or a gene of interest (Dangler, 1996).

4. Analysis of Nucleotide Sequences

4.1 Homology search analysis

Analysis of nucleotide sequence is important step after finishing nucleotide sequencing step. The nucleotide sequence determined the function of the DNA sequence obtained. The analysis of an unknown sequence is performed to homology search, which is a search for sequence similarity with known sequence in the database. There are three international DNA databases available as shown in Table 2. These databases exchanged their data daily to update the sequence information.

Table 2 Major sequence databases accessible through the internet

Databases	Sponsor	Location
DNA Data Bank of Japan	National Institute of Genetics	http://www.ddbj.nig.ac.jp
EMBL Data Library	European Molecular Biology Laboratory	http://ebi.ac.uk/embl.html/
Genebank	National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/

Source: Higgins and Taylor (2001: 180)

In addition, comparison of unknown and known protein sequences should be performed. It has proven to be a much more effective tool. Protein-level searches are valuable for detecting evolutionary related genes (Table 3).

4.2 Open reading frame analysis

Regions of DNA encoding proteins are first transcribed into messenger RNA and then translated into protein. Examining the DNA sequence will determine the sequence of amino acid which will appear in the final protein. Therefore, an open reading frame (ORF) is any sequences of DNA or RNA that can be translated into a protein. In a gene, ORFs are located between the start-code sequence (initiation codon: ATG, GTG, TTG) and the stop-code sequence (termination codon: TAA, TAG, TGA). It is important to determine the correct open reading frame. Every region of DNA sequence can be read in six reading frames, three in the forward and three in the reverse direction. Typically only reading frame is used in translating a gene and its corresponding amino acid sequence.

Table 3 Comparison program and the types of comparison

Programs	Query	Database	Comparison	Common use
Blastn, fasta search	DNA	DNA	DNA-level	Identical DNA sequences and splicing patterns
Blastp, fasta, search	protein	Protein	Protein-level	Homologous proteins
Blastx, fastx	DNA	Protein	Protein-level	Query new DNA to find genes and homologous proteins
Tblastn, tfasta, tfastx	Protein	DNA	Protein-level	Search for genes in unannotated DNA
Tblastx	DNA	DNA	Protein-level	Discover gene structure

Source: Higgins and Taylor (2001: 179)

The determination of an ORF as an unknown sequence can be accomplished either by using commercial software tools or from the database search tools available on the internet. For example, the ORF Finder (Open Reading Frame Finder) program is a graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes. This program available at the WWW site at http://www.ncbi.nlm.nih.gov/gorf/gorf.html.

4.3 Phylogenetic tree analysis

Phylogenetic tree analysis is the technique of methodically showing the evolutionary interrelationships among various species or other entities that are believed to be a common ancestor. Today, this technique has become an important

tool for studying the evolutionary history of bacteria to humans. In addition, it is also important for clarifying the evolutionary of multigene families as well as for understanding the process of adaptive evolution at the molecular level. There are two steps to create a phylogenetic tree: aligning the DNA sequences and using the aligned DNA sequences to generate a tree by phylogenetic program. DNA sequence is used more than amino acid sequence because the pattern of mutation, insertion and deletion at the nucleotide level is definitive. Silent mutation at the DNA level does not result in an amino acid substitution at the protein level. There are three main methods of construction phylogenetic trees: distance-based methods such as neighbour-joining, parsimony-based methods such as maximum parsimony, and character-based methods such as maximum likelihood or Bayesian inference. Phylogenetic trees are often represented graphic, either in the form of phylogenetic trees or dendrograms.

5. Principles and Techniques of Immunoblotting Analysis

Proteins are the products of genes and provide cellular structure and function. Analysis of proteins is therefore central to understand biological processes. Cells are composed of complex mixtures of many proteins, some with similar molecular weights. Assessments of protein expression, quantity, and tissue distribution are only some of the analyses required to more fully understand a specific protein's function (Scheppler *et al.,* 2000). The specificity with which antibodies react with their targets form the basis of a number of analytical methods that can be used to confirm the identity of a protein. Two of the most common immunochemical methods for protein analysis are western blots and antibody capture assays (Copeland, 1994).

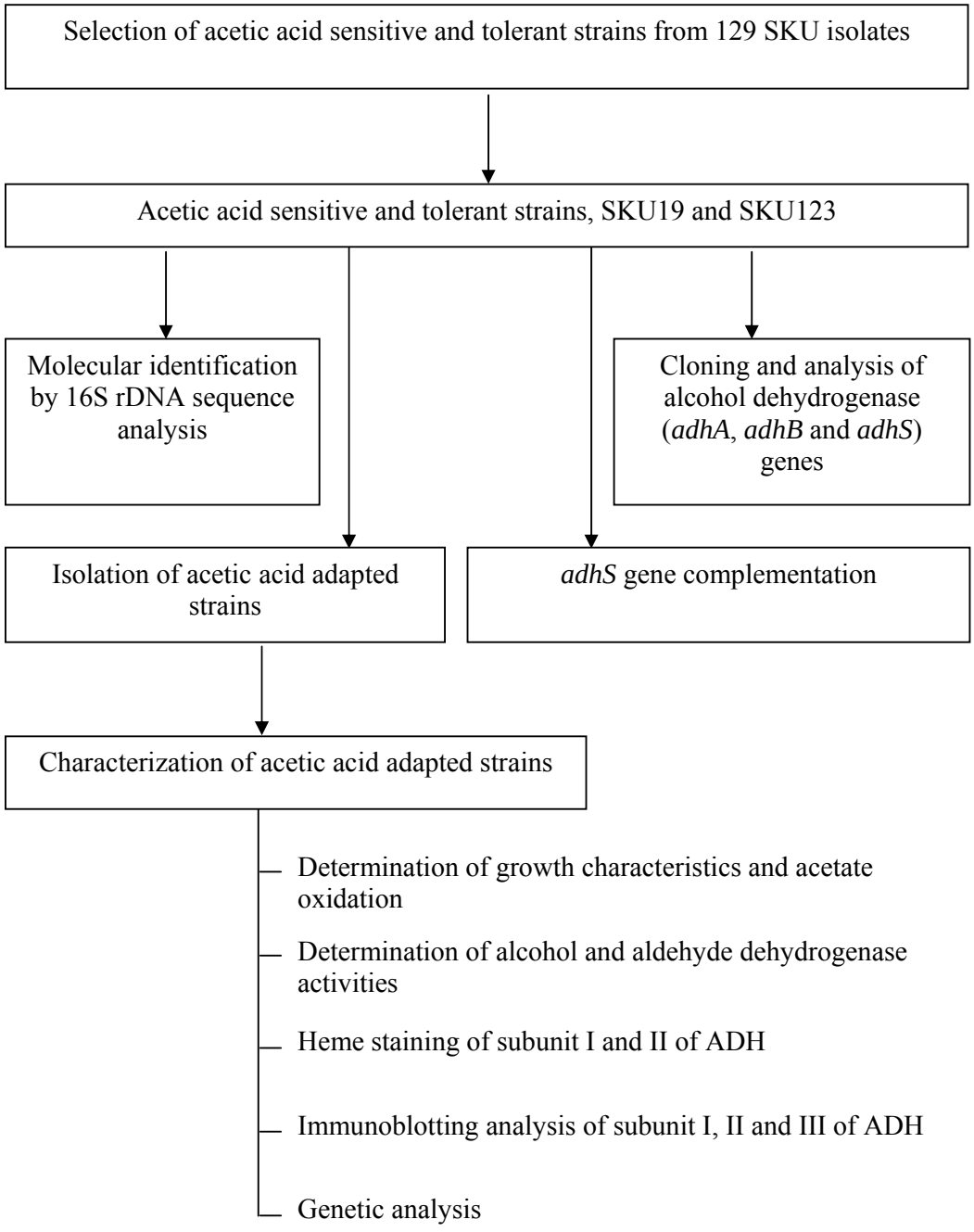
Western blotting is an electrophoretic technique that allows one to test the cross reactivity of individual protein bonds on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or an isoelectric focusing (IEF) gel, with an antibody raised against a specific antigen. This method is often used to verify the identity of a protein band as the target protein (Burnette, 1981; Copeland, 1994; Scheppler *et al.,* 2000). Blotting can be used to ascertain a number of important

characteristics of protein antigens, including the presence and quantity of an antigen, the molecular weight of the antigen, and the efficiency of antigen extraction. This method is especially helpful when dealing with antigens that are insoluble, difficult to label, or easily degraded, and thus not amenable to procedure such as immunoprecipitation.

The goal of "western blotting" and related techniques (e.g. "Northern blot" and "Southern blot" experiments) is to separate proteins as a function of MW to blot or electroblot to a membrane, nitrocellulose, which binds protein well. The rest of the membrane is saturated (blocked) with non-antigen protein, usually bovine serum albumin (BSA) or non-fat dry milk, to prevent non-specific immunoglobulin binding to the membrane. The membrane is treated with a "probe" then with an antibody. When this antibody recognizes an "antigenic determinant" on the blotted protein pattern, the corresponding band (or bands) binds a secondary antibody that carries an attached conjugated moiety (an "immunoconjugate"). The conjugate is then supplied with a substrate that reacts to yield a colored (or otherwise detectable) product. Color only develops in the vicinity of the blot-bound antigen and attached primary antibody (Hardin *et al.,* 2001). The basic blotting procedure can be divided into six steps: 1) preparation of the antigen sample, 2) resolution of the sample by gel electrophoresis, 3) transfer of the separated polypeptides to a membrane support, 4) blocking nonspecific binding sites on the membrane, 5) addition of the antibody and 6) detection.

MATERIALS AND METHODS

The overall procedures followed in this study are shown in the following diagram:



Materials

1. Chemicals

Yeast extract, polypeptone, tryptone, glucose and glycerol were obtained from Merck & Co., Inc. (New Jersey, USA). Restriction enzymes: *Eco*RI, *Hind*III, *Pst*I and *Sal*I were purchased from New England Biolabs, Inc., (Massachusetts, USA). Substrate for enzyme assays: acetaldehyde, acetic acid and ethanol were supplied by Wako Inc., (Osaka, Japan). 3, 3', 5, 5'-tetramethylbenzidine (TMBZ) was obtained from Dojindo Ltd. (Kumamoto, Japan). Standard DNA marker and 6X loading dye were obtained from Fermentas Inc., (Maryland, USA). Pre-stained protein marker was purchased from Bio-Rad Laboratories, Inc., (California, USA). All other chemicals used in this study were reagent grade and obtained from commercial sources.

2. Bacterial Strains, Culture Medium, and Culture Conditions

2.1 Acetic acid bacteria

A total of 129 isolates of acetic acid bacteria (SKU1-129) previously isolated from fruits in Thailand (Theeragool *et al.,* 1996) were screened for acetic acid sensitive (*Acetobacter syzygii* SKU19) and tolerant strains (*A. pasteurianus* SKU123 and *A. pasteurianus* SKU1108). The acetic acid adapted strains were obtained from *A. syzygii* SKU19 (Table 4) by sequential cultivation of acetic acid sensitive strain in the medium containing 1.0% acetic acid.

Two different culture media were used in this study according to the purpose of individual experiments; the first medium, potato medium was used for stock cultures prepared by adding 1.5% agar to a potato medium (Appendix A) and the second medium, seed culture medium (SCM, Appendix A) was for the study of growth characterization and enzyme activity measurements.

All isolates of acetic acid bacteria were preserved in potato medium containing 50% glycerol at –80°C and were maintained on a potato agar slant. The inoculum was prepared by inoculating one loopful of cells from a culture grown on the potato agar into 5 ml of potato medium and cultivated at 30°C, on a shaker at 200 rpm for 24 h. Then, 5 ml of this inoculum was inoculated to 100 ml of SCM broth in 500 ml Elenmeyer flask with a side-arm. Cultivation was done at the same condition of the inoculum preparation. The growth was measured with a Klett Summerson photometer or spectrophotometer.

2.2 *Escherichia coli*

Escheriachia coli DH5α was used as the host cell for 16S rDNA, *adhA*, *adhB* and *adhS* gene and the *adhA-adhB* junction region of *adhAB* gene of acetic acid adapted strain no. 112 cloning into plasmid pGEM®-T Easy vector. *E. coli* S17-1 was used as the host cell for subcloning of 2 kb *Eco*RI DNA fragment carrying *adhS* from *A. pasteurianus* SKU1108. The genotype of *E. coli* DH5α and S17-1 strains were showed in Table 4. Both *E. coli* DH5α and S17-1 were cultured and maintained in Luria-Bertani (LB) medium (Appendix A) containing appropriate antibiotic and incubated at 37°C.

Table 4 Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Source
Bacterial strains		
A. pasteurianus SKU1108	Acetic acid tolerant strain	Theeragool *et al.* (1996)
A. pasteurianus SKU123	Acetic acid tolerant strain	Theeragool *et al.* (1996)
A. syzygii SKU19	Wild type	Theeragool *et al.* (1996)
Acetic acid adapted strains		
No.108	Acetic acid adapted strain	This work
No.112	Acetic acid adapted strain	This work
No.187	Acetic acid adapted strain	This work
No.217	Acetic acid adapted strain	This work
No.258	Acetic acid adapted strain	This work
No.264	Acetic acid adapted strain	This work
E. coli		
DH5α	*supE*44 Δ*lacU*169 *(Φ*80*lacZ*ΔM15*)* *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	
S17-1	Tp^r Sm^r *recA*, *thi*, *pro*, *hsdR*$^-$$M^+$RP4: 2-Tc:Mu: Km Tn7 λpir	
Plasmids		
pGEM®-T Easy vector	Amp^r, *lacZ*	Anonymous (1997)
pUC119	Amp^r, *lacZ*	
pCM62	Tet^r. *lacZ*, *Acetobacter-E. coli* shuttle vector	Marx and Lidstrom (2001)
pGEM®-T16SSKU19	Amp^r, *lacZ* containing 1.5 kb DNA fragment carrying 16S rDNA gene from *Acetobacter* sp. SKU19	This work

Table 4 (Continued)

Bacterial strains and plasmids	Relevant characteristics	Source
pGEM®-T16SSKU123	Amp^r, *lacZ* containing 1.5 kb DNA fragment carrying 16S rDNA gene from *Acetobacter* sp. SKU123	This work
pUC119-16S600	Amp^r, *lacZ* containing 600 bp DNA fragment carrying 16S rDNA gene from *A. syzygii* SKU19 and *A. pasteurianus* SKU123	This work
pUC119-16S900	Amp^r, *lacZ* containing 900 bp DNA fragment carrying 16S rDNA gene from *A. syzygii* SKU19 and *A. pasteurianus* SKU123	This work
pGEM®-T*adhAB*3.8	Amp^r, *lacZ* carrying 3.8 kb DNA fragment in the opposite orientation from P*lac*	This work
pGEM®-T*adhS*	Amp^r, *lacZ* carrying 618 bp DNA fragment in the opposite orientation from P*lac*	This work
pGEM®-TCAA	Amp^r, *lacZ* carrying 398 bp DNA fragment	This work
pCM*adhS* (←)	Tet^r, *lacZ* containing 2 kb *Eco*RI DNA fragment carrying *adhS* from *A. pasteurianus* SKU1108 in the same orientation from P*lac*	This work
pCM*adhS* (→)	Tet^r, *lacZ* containing 2 kb *Eco*RI DNA fragment carrying *adhS* from *A. pasteurianus* SKU1108 in the opposite orientation from P*lac*	This work

Methods

1. Selection of Acetic Acid Sensitive and Tolerant Strains and Molecular Identification by 16S rDNA Sequencing and Analysis

1.1 Selection of acetic acid sensitive and tolerant strains

The inoculum was prepared from one loopful of each isolate cultured in potato medium at 30°C, 200 rpm for 18-24 h. For selection of acetic acid sensitive and acetic acid tolerant strains, 5 µl of the inoculum was inoculated on SCM agar containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0% (v/v) of acetic acid and was incubated at 30°C for 5 days. The growth of all isolates was observed and the acetic acid sensitive strains were selected from the isolates exhibited the poorest growth on those selective media. In contrast, the acetic acid tolerant strains were selected from the isolates exhibited the best growth on those selective media.

1.2 Identification by 16S rDNA sequencing and analysis

Chromosomal DNA from acetic acid bacteria was isolated by the method described by Okumura *et al*. (1985a). The bacterial cells were harvested and suspended in 10 ml of sterile TNE buffer, pH 7.9 (Appendix B). Then 10 mg of lysozyme was added, the cell suspension was incubated at 37°C for 30 min with gentle shaking. After the incubation, 1/10 volume of 10% sodium dodecylsulfate (SDS) was added to the suspension. It was incubated at 50°C for 10 min without shaking followed by adding 1/10 volume of 10% N-cetyl-N, N, N-trimethyl ammonium bromide (CTAB) in 0.7 M NaCl and statically incubated at 65°C for 10 min. After the mixture was cooled down, it was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vigorously shaken. After shaking, the mixture was centrifuged at 12,000 rpm for 20 min at room temperature. The upper layer was collected and 1/10 volume of 3 M sodium acetate (pH 5.0) was added. As described above, the solution was extracted again with the

phenol:chloroform:isoamyl alcohol solution. Then, 2.5-fold of cold absolute ethanol was added to the upper layer and DNA was wound with a sterile Pasteur pipette. The DNA was washed with 70% ethanol and resuspended with 1 ml of TE buffer (pH 8.0) containing 3 μg/ml RNase H and incubated at 37°C for 1 h. After incubation, an equal volume of phenol:chlorofom:isoamyl alcohol solution was added to the mixture, and it was mixed and centrifuged at 12,000 rpm for 20 min at room temperature. The upper layer was collected and mixed with 2.5-fold volume of cold absolute ethanol. Extracted DNA was dried up and finally resuspended with 200 μl of TE buffer. The concentration of chromosomal DNA was determined by measuring absorbance at 260 nm.

The DNA primers for amplification of 16S rRNA gene were designed from highly conserved regions of nucleotide sequence of 16S rDNA of α-Proteobacteria as shown in Table 5. Forward primer, 27f (20 mers), was 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer, 1525r (18 mers), was 5'-AAAGGAGGTGATCCAGCC-3'. These primers were used as described by Devereux and Wills (1995). The desired product was about 1.5 kb DNA fragment. The PCR was performed in a total volume of 25 μl. The chromosomal DNA template (50 ng) and 25 pmol each of DNA primers were added into the Ready-To-Go™ PCR Beads (Amersham pharmacia biotech Inc., USA) containing 200 μmol of each deoxynucleotide triphosphates (dNTPs; dATP, dTTP, dCTP and dGTP), 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1.5 unit of *Taq* DNA polymerase, and the final volume was adjusted to 25 μl with sterile distilled water. The PCR conditions for amplification of 16S rDNA were shown in Table 6. Amplification reactions were performed on a Thermal cycler (Perkin-Elmer GeneAmp PCR system 2400). The 1.5 kb PCR product was analyzed by 0.8% (w/v) agarose gel in TAE buffer at 50 volts, and purified by using QIAquick Gel Extraction Kit (QIAGEN, Germany, Appendix C). The purified DNA fragment from PCR product was confirmed by agarose gel electrophoresis before used. This purified DNA fragment was ligated with pGEM®-T Easy vector system (Promega, USA). The vector is prepared by cutting with *Eco*RV and adding a 3' terminal thymidine to end. This

single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products. The high copy number pGEM®-T Easy vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide by the insert DNA allows recombinant clones to be directly screened by color screening (blue/white) on indicator plates. The physical map of pGEM®-T Easy vector was shown in Figure 6.

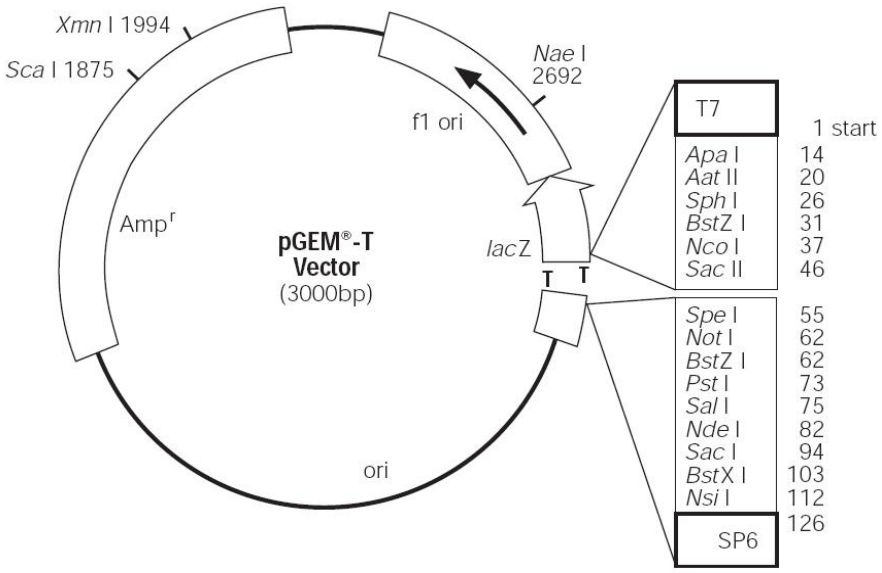
Table 5 Nucleotide sequences of primers used in this study

Primer designation	Sequence (5'---3')	Purpose
27f	-AGAGTTTGATCCTGGCTCAG-	16S rDNA sequencing
1525r	-AAAGGAGGTGATCCAGCC-	16S rDNA sequencing
70%G+C	-AGCGGGCGTA-	RAPD profile
80%G+C	-CGCGTGCCCA-	RAPD profile
$(GTG)_{5x}$	-GTGGTGGTGGTGGTG-	RAPD profile
AD01	-CAAAGGGCGG-	RAPD profile
FadhAB-*Hind*III	-ccaagcttggATGACCCGCCCCGCCTCC-	*adhA* and *adhB* amplification
RadhAB-*Sal*I	-cggtcgaccgTTACTGGGCTTCATCCAC-	*adhA* and *adhB* amplification
FadhS	-ATGAAACTGATTGCCGTA-	*adhS* amplification
RadhS	-TTACGAAACAGAACTGGT-	*adhS* amplification
Universal T7	-TAATACGACTCACTATAGGG-	*adhAB* and *adhS* sequencing
adhAB6F	-CAAAGCCTACAAGACCTG-	*adhAB* sequencing
UniADHF	-TGGYWCGGYATYCCSGG-	*adhAB* sequencing
adhAB13F	-CCTGCCCGTCAAGCCGCC-	*adhAB* sequencing
adhAB23F	-GCAATCAAAACCCCCATC-	*adhAB* sequencing
1.2R	-AGTGCGACAATGCTGCC-	*adhAB* sequencing
UniADHR	-GTVGCGTCRTARGCRTGGAA-	*adhAB* sequencing
adhAB16R	-CAGTTGGTAGGCGGCAGA-	*adhAB* sequencing
Universal SP6	-ATTTAGGTGACACTATAG-	*adhAB* and *adhS* sequencing
adhAB24F	-ATCGCTTTGACACGAGC-	The *adhA*-*adhB* junction region of *adhAB* amplification and *adhAB* sequencing
adhAB22R	-TTTGATTGCCAGCCCACC-	The *adhA*-*adhB* junction region of *adhAB* amplification

Table 6 PCR conditions

Primer	PCR condition					
	Temperature profile	Denaturing	Annealing temperature	Polymerization	Final extension	Cycles
1. 27f and 1525r	94°C, 5 min	94°C, 1 min	50°C, 1 min	72°C, 2 min	72°C, 10 min	35
2. 70%G+C	94°C, 5 min	94°C, 30 sec	41°C, 45 sec	72°C, 45 sec	72°C, 7 min	35
3. 80%G+C	94°C, 5 min	94°C, 30 sec	41°C, 45 sec	72°C, 45 sec	72°C, 7 min	35
4. $(GTG)_{5x}$	94°C, 5 min	94°C, 30 sec	56°C, 45 sec	72°C, 45 sec	72°C, 7 min	35
5. AD01	95°C, 12 min	94°C, 1 min	36°C, 2 min	72°C, 3 min	72°C, 7 min	45
6. FadhAB-*Hind*III and RadhAB-*Sal*I	95°C, 5 min	94°C, 1 min	49°C, 3 min	72°C, 3 min	72°C, 10 min	30
7. FadhS and RadhS	95°C, 5 min	94°C, 1 min	50°C, 1 min	72°C, 1 min	72°C, 10 min	30
8. adhAB24F and adhAB22R	95°C, 5 min	94°C, 1 min	45°C, 1 min	72°C, 1 min	72°C, 10 min	30

(A)



(B)

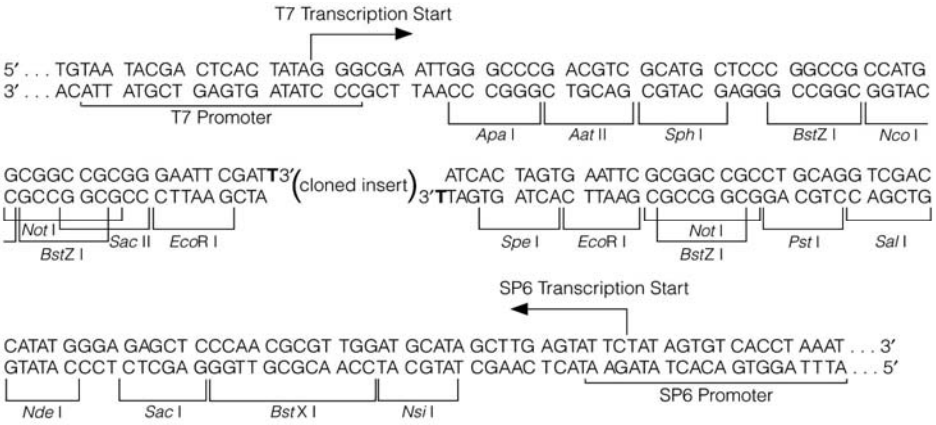
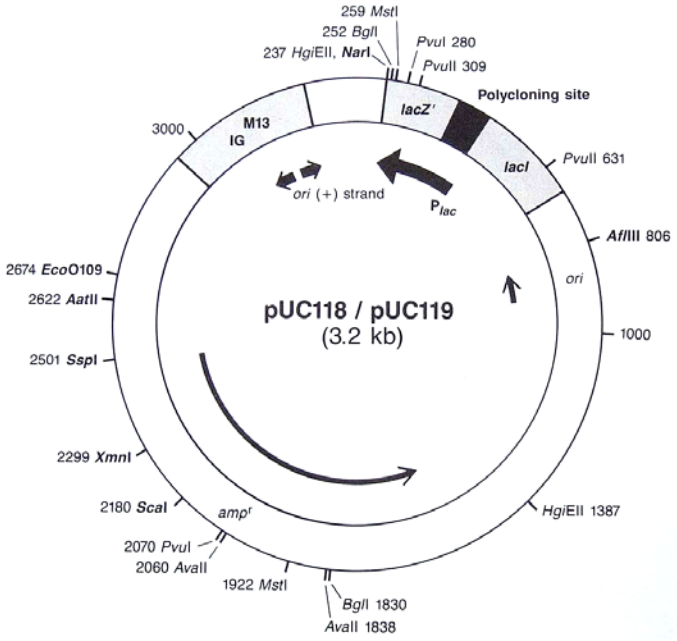


Figure 6 The physical map (A) and multiple cloning sequence of pGEM®-T Easy vector (B).

Source: Anonymous (1997)

The ligation mixtures contain 3 μl of 75 ng 1.5 kb purified DNA fragment, 1 μl of pGEM®-T Easy vector, 10x ligation buffer, T_4 DNA ligase and 5 μl of sterile deionized water to give the total volume of 10 μl. The ligation reaction was carried out at 16°C overnight. Then, 2 μl of the reaction mixture was used to transform competent *E. coli* DH5α (Appendix C) by electroporation using *E. coli* Pulser™ Transformation Apparatus (Bio-Rad Laboratories Inc., USA) at 1.8 kV (resistance 200 ohms, capacitance 25 μF). After adaptation with shaking at 37°C for 1-1.5 h, the solution was spreaded onto LB agar containing 50 μg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The plates were incubated at 37°C overnight. White colonies were picked up onto LB agar containing 50 μg/ml of ampicillin, and incubated at 37°C overnight. These white colonies were analyzed by size screening method (Appendix C). The interested clones were cultured in 5 ml of LB medium containing 50 μg/ml of ampicillin, and incubated at 37°C, 200 rpm for 12-18 h. The cells were harvested and recombinant plasmids were isolated by alkaline lysis method (Sambrook *et al.,* 1989, Appendix C). The insertion of the 1.5 kb PCR product was confirmed by electrophoresis after digested with *EcoR*I. The 1.5 kb PCR product was subcloned into pUC119 before sequencing. The physical map of pUC119 vector was shown in Figure 7. The ligation reaction was set up as described previously and incubated at 16°C overnight. The nucleotide sequencing of the cloned 16S rDNA fragment was determined by applying the chain termination method using an ABI PRISM 310 genetic analyzer. The obtained nucleotide sequences were analyzed for homology search by using the standard BLAST sequence similarity searching program located at http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequences at Genebank database. The nucleotide sequences of the 16S rDNA will appear in the DDBJ, EMBL and Genebank sequences databases with the accession number AB264094.

(A)



(B)

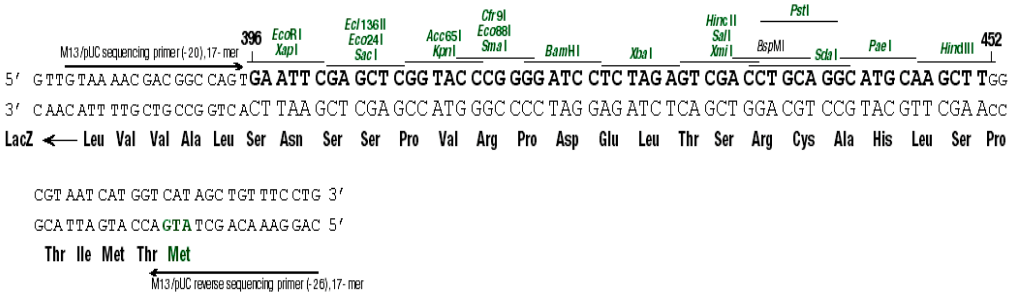


Figure 7 The physical map (A) and multiple cloning sequence of pUC119 cloning vector (B).

Source: Sambrook *et al.* (1989)

2. Isolation of Acetic Acid Adapted Strains

2.1 Isolation of acetic acid adapted strains

Isolation of acetic acid adapted strains was performed by sequential cultivations of acetic acid sensitive strain (*A. syzygii* SKU19) in the SCM broth containing 1.0% acetic acid. The 5% inoculum of *A. syzygii* SKU19, which could not grow well in the medium containing 1.0% acetic acid, was inoculated into SCM broth containing 1.0% acetic acid. The culture was incubated at 30°C, 200 rpm for 7 days and 250 µl was transferred to a 5 ml fresh SCM broth containing 1.0% acetic acid. This subculture was sequential performed for 8 times and the culture was sampling and checked for its growth on SCM agar containing 1.0% acetic acid. The culture was incubated at 30°C for 5-7 days. The colonies with adaptation to grow on this medium were selected as the acetic acid adapted strains.

2.2 Determination of growth characteristics and acetate oxidation

All of the acetic acid adapted strains were precultured in 5 ml of potato broth containing 1.0% acetic acid, incubated at 30°C, 200 rpm and their growth was measured by Klett Summerson photometer until to log phase. Then, the 1% inoculum was inoculated into glucose free-SCM broth containing various concentrations of acetic acid. The culture was incubated at 30°C, 200 rpm. The growth of all strains was observed by measuring with a Klett Summerson photometer. Acidity of the culture medium was measured by alkaline-titration with 0.8 N NaOH (Appendix C). The pH was measured by using pH meter.

In addition, to investigate acetate oxidation, the 1% inoculum was inoculated into glucose free-SCM broth containing 2.0% (v/v) of ethanol. The culture was incubated at 30°C, 200 rpm. The growth, acidity, residual ethanol, and pH were determined.

2.3 Determination of alcohol and aldehyde dehydrogenase activities

The 1% inoculum was inoculated into glucose free-SCM broth containing 2.0% (v/v) of ethanol. The culture was incubated at 30°C, 200 rpm for 2 days or until late-log phase. The cells were harvested by centrifugation at 9,000 rpm for 5-10 min. The cell pellets were washed with 10 mM phosphate buffer (KPB) pH 7.0 (Appendix B) and resuspended in the same buffer. The cell suspension was used to prepare membrane fraction by using a French Cell Press as described in Appendix C. The Membrane-bound ADH (EC 1.1.99.8) and ALDH (EC 1.2.99.3) were measured colorimetrically with potassium ferricyanide as an electron acceptor described by Adachi *et al.* (1978) with slight modifications by Matsushita *et al.* (1995, Appendix C). The rate of reduction of ferricyanide to ferrocyanide gives a quantitative amount of ethanol oxidized. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ethanol per min. These enzyme assays were performed at 25°C.

2.4 Heme staining of membrane fraction

The protein samples containing 100 μg of protein were mixed with sample buffer for heme staining and then heated at 60°C for 30 min. The samples were applied on a slab gel composed of 5% acrylamide stacking gel and 12.5% acrylamide separating gel. Electrophoresis was performed for 4-5 h with running buffer. Pre-stained molecular weight markers (low range, Bio-Rad Laboratories Inc., USA) were used for standard marker proteins which consisting of phosphorylase b (108 kDa), bovine serum albumin (90 kDa), ovalbumin (50.7 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (28.6 kDa), and lysozyme (21.2 kDa). After electrophoresis, the gel was stained in the staining buffer containing 9 mg of TMBZ in 6 ml of methanol and 14 ml of 0.25 M acetate buffer (pH 5.0) with gentle shaking at room temperature for 1-2 h. The blue color was developed by adding 60 μl of hydrogen peroxide into the staining buffer. The reaction was stopped by adding 6 ml of isopropanol and 14 ml of 0.25 M acetate buffer (pH 5.0) into gel.

2.5 Immunoblotting analysis of ADH protein

Western immunoblotting of ADH was modified from Burnette (1981). Preparation of antibody was performed by the method of Toyama *et al.* (1995). 100-200 μg of the proteins samples were separated by 12.5% SDS-PAGE, and electroblotted onto a methanol treated polyvinylidene difluoride (PVDF) membrane (Figure 8). One side of the gel was placed in contact with a piece of PVDF membrane. The gel and its attached filter were sandwiched between Whatman 3MM paper, two porous pads, and two plastic supports. The entire construction was immersed in an electrophoresis tank that contained transferring buffer (pH 8.3) for immunoblotting analysis (Appendix B). The membrane filter was placed toward the anode. The transferring was done in transferring buffer at 100 mM for 4 h. Then, the membrane was blocked with 3% gelatin in Tris-buffered saline (TBS, Appendix B) overnight and washed three times with washing buffer (TBS containing 0.05% Tween 20) with shaking at room temperature for 10 min. The membrane was incubated with 1% gelatin in TBS containing antibody raised against ADH for 2 h. Then, the membrane was washed and incubated with 1% gelatin in TBS containing protein A-peroxidase conjugate for 2 h. The membrane was washed again with washing buffer. The ADH protein band was visualized by the addition of diaminobenzidine (DAB) as a color reagent and hydrogen peroxide in TBS. The color was developed in less than 5 min. The reaction was stopped by washing the membrane with distilled water for several times.

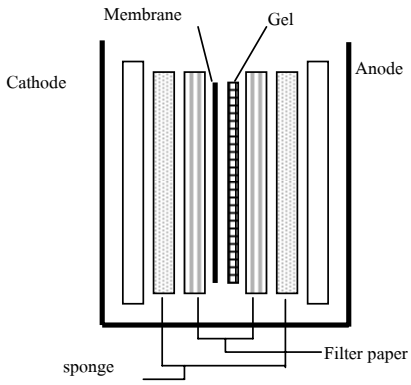


Figure 8 Diagram of electrically protein transfer to polyvinylidene difluoride (PVDF) membrane filter for immunoblotting analysis.

Source: Anonymous (2005)

3. DNA Manipulation Techniques

3.1 Plasmid profiles analysis

Plasmid DNA was isolated from wild type and the acetic acid adapted strains by the modified alkaline lysis method (Sambrook *et al.,* 1989). The cells grown in potato broth containing 1% acetic acid at the late-log phase were harvested by centrifugation at 12,000 rpm for 5 min at 4°C, and washed twice with 50 mM Tris-HCl buffer (pH 7.0). The pellet was resuspended in 60 µl of solution I followed by adding 40 µl of 10 mg/ml of lysozyme. The cell suspension was incubated at 37°C for 30 min with gentle shaking. After the incubation, 200 µl of freshly prepared solution II was added to the suspension and gently mixed by inverting the tube until the solution was clear and viscous. Then, it was stored on ice for 5 min. The 150 µl of cold solution III was added. It was mixed and stored on ice. The mixture was centrifuged at 12,000 rpm for 5 min. The supernatant was collected by transferring to a new tube and mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by vigorous shaking. The mixture was centrifuged at 12,000 rpm

for 20 min at room temperature. If the sample contained the cellulose, 1/10 volume of CTAB was added to the suspension. Then, it was incubated at 65°C for 10 min. It was extracted again by adding with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vigorous shaking. Then, the upper layer was collected to a new eppendorf and 2.5-fold of cold absolute ethanol was added to the upper layer. The suspension was incubated at –80°C for 30 min and then, it was centrifuged at 4°C, 12,000 rpm for 20 min. The plasmid DNA was washed twice with 70% ethanol, dried and resuspended with TE buffer containing 10 μg/ml RNase H.

3.2 Polymerase chain reaction and randomly amplified polymorphic DNA (RAPD)

Chromosomal DNA of all acetic acid adapted strains was isolated from cells grown to the late-log phase in potato broth containing 1.0% acetic acid. The sequences of random oligonucleotide primers used in this study were listed in Table 5. The PCR was performed in a total volume of 20 μl. The DNA template and DNA primers were added into the Ready-To-Go™ PCR Beads and the reaction was carried out by a Perkin-Elmer GeneAmp PCR system 2400. The PCR conditions for RAPD from acetic acid adapted strains were shown in Table 6.

3.3 Southern hybridization of chromosomal DNA of acetic acid adapted strains with IS*1380* DNA

3.3.1 DNA probe preparation

The recombinant plasmid, pUCIS*1380*, carrying IS*1380* sequence from *A. pasteurianus* NCI1380, was digested by *Eco*RI and *Bgl*II and electrophored in 0.8% agarose gel electrophoresis. The IS*1380* DNA fragment was purified by QIAquick Gel Extraction Kit for labeling with the enzyme horseradish peroxidase. The labeling reaction was done as described in the protocol provided by Amersham Pharmacia Biotech Co.

3.3.2 Southern hybridization

The chromosomal DNA was digested by *Hinc*II and hybridized with IS*1380* DNA probe. The agarose gel containing DNA was placed in 200 ml of 0.25 N HCl solution for 5-10 min. Then, the denaturation solution was added and incubated at room temperature for 1 h with gentle shaking. The denaturation solution was poured off. The neutralization solution was added and incubated at room temperature for 1 h with gentle shaking. After the gel had been treated with the neutralization solution, the transfer was performed. The strip of Whatman 3MM paper was cut to the same size as the gel. This strip was soaked with 2x SSC solution. It was placed on the bottom of the tray containing 10x SSC solution. The prepared gel was put up on the Whatman paper. Then, the Hybond-N membrane was cut to the same size as the agarose gel and carefully placed on the gel. The air bubbles trapped between membrane and the gel surface were completely removed. The double layers of Whatman paper were placed on the membrane. Then, the desired dimensions paper towels were placed on the Whatman paper. The transfer was allowed to proceed 18-24 h. Transfer membrane into UV transilluminator and crosslink DNA to the membrane for 5 min. The transfer membrane was placed in a screw cap tube containing 20 ml of hybridization solution and incubated at 42°C, 20 rpm for 1 h (prehybridization). Then, the labeled IS*1380* DNA fragment was added to hybridization solution and incubated at 42°C, 20 rpm for 9-13 h (hybridization). After the incubated period, the transfer membrane was washed twice with washing buffer solution I (Appendix B) at 55°C for 10 min and then washed twice with washing solution II (Appendix B) for 5 min at room temperature. The membrane was allowed to air dry. The detection was done as described in the protocol provided by Amersham Pharmacia Biotech Co.

3.4 Cloning and analysis of alcohol dehydrogenase genes (*adhA*, *adhB* and *adhS*)

3.4.1 PCR cloning and nucleotide sequencing of *adhA* and *adhB*

In order to amplify *adhA* and *adhB* genes by PCR, forward and reverse primers were designed from conserved region of *adhAB* gene of *A. aceti* K6033, *A. aceti*, *A. pasteurianus* NCI1193, *A. pasteurianus*, *A. polyoxogenes*, *Acetobacter* sp. and *A. pasteurianus* SKU1108 (Appendix Figure C1). Analysis of the designed primers for hairpin and palindrome structure, number of bases, annealing and melting temperatures and G/C content was performed by using CybergeneAB and Oligo Toolkit web server available an URL http://www.cybergene.se/primer.html and http://www.operon.com/oligos/toolkit.php, respectively. The forward primer, FadhAB-*Hind*III (28 mers with *Hind*III linker) and the reverse primer, RadhAB-*Sal*I (29 mers with *Sal*I linker) were shown in Table 5. These primers started at base pair number 127 and 3,808 of *adhAB* of *A. pasteurianus* SKU1108. The desired product was 3.8 kb DNA fragment.

The PCR was performed by using the Ready-To-Go™ PCR Beads, in total volume of 25 µl. The reaction was carried out by a Perkin-Elmer GeneAmp PCR system 2400. A DNA thermal cycler was shown in Table 6. The PCR amplified product was analyzed by running on 0.8% agarose gel electrophoresis. This 3.8 kb PCR amplified product was purified by QIAquick Gel Extraction Kit. The purified 3.8 kb DNA fragment was ligated with pGEM®-T Easy vector system. The physical map of pGEM®-T Easy vector was shown in Figure 6. The ligation reaction was incubated at 16°C overnight. 2 µl of the reaction mixture was used to transform *E. coli* DH5α competent cells by electroporation as previously described. The interested clones were selected and digested with restriction enzyme to detect the size of inserted DNA. The nucleotide sequences of *adhAB* were determined by applying the chain termination method using an ABI PRISM 310 genetic analyzer. The obtained nucleotide sequences were analyzed for homology by

using the standard BLAST sequence similarity searching program. The nucleotide sequences of the *adhAB* will appear in the DDBJ, EMBL and Genebank sequences databases with the accession number AB264315.

The obtained nucleotide sequences were searched for an open reading frame (ORF) using translate tool (ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics) available *via* an URL: http://expasy.ch /tools/dna.html and ORF finder (National Center for Biotechnology Information; NCBI) at http://www.ncbi.nlm.nih.gov/gorf.html. Homology search was performed using the standard BLAST sequence similarity searching program. The hydropathy profile of the deduced amino acids was analyzed using a classification and secondary structure prediction of membrane proteins by SOSUI signal result program at Department of Biotechnology, Tokyo University of Agriculture and Technology at SOSUI WWW server *via* the URL: http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/ sosuisignal_submit.html. Binding motifs were searched using Motif Scan and PPsearch at URL: http://www.ebi.ac.uk/ppsearch, respectively.

3.4.2 PCR cloning and nucleotide sequencing of *adhS* gene

In an attempt to amplify *adhS* gene, PCR primers were designed from conserved region of *adhS* gene of *A. pasteurianus* NCI1193 and NCI1452. The forward primer, FadhS (18 mers) was 5'-ATGAAACTGATTGCCGTA-3' started at base pair number 1 and the reverse primer, RadhS (18 mers) was 5'-TTACGAAACA GAACTGGT-3' started at base pair number 618 (Appendix Figure C2). The desired product was 618 bp DNA fragment.

The PCR reaction was performed by using the Ready-To-Go™ PCR Beads in total volume of 25 µl. Thermal cycler was shown in Table 6. The PCR amplified product was analyzed by running on 0.8% agarose gel electrophoresis.

This 618 bp PCR amplified product was purified by QIAquick Gel Extraction Kit. The purified 618 bp DNA fragment was ligated with pGEM®-T Easy vector system and transformed into *E. coli* DH5α competent cells by electroporation method as previously described. The selected clones were digested with *Eco*RI and *Pst*I. The nucleotide sequences were determined by applying the chain termination method. The nucleotide sequences of the *adhS* will appear in the DDBJ, EMBL and Genebank sequences databases with the accession number AB264314.

3.4.3 PCR cloning and nucleotide sequencing of the *adhA-adhB* junction region of acetic acid adapted strain no. 112.

In order to confirm the nucleotide sequences in the *adhA-adhB* junction region, PCR primers were designed from nucleotide sequence of *adhAB* gene of *A. syzygii* SKU19. The forward primer, adhAB24F (17 mers), was 5'-ATCGCT TTGACACGAGC-3' and started at base pair number 2,078. The reverse primer, adhAB22R (18 mers), was 5'-TTTGATTGCCAGCCCACC-3' and started at base pair number 2,484 as shown in Table 5 (Appendix Figure C1). Chromosomal DNA of acetic acid adapted strain no. 112 was used as template DNA. The desired product was 398 bp DNA fragment.

PCR amplification was performed by using the Ready-To-Go™ PCR Beads, in total volume of 25 µl. Thermal cycler was shown in Table 6. The PCR amplified product was analyzed by running on 0.8% agarose gel electrophoresis. This 398 bp PCR amplified product was purified and ligated with pGEM®-T Easy vector system. The purified 398 bp DNA fragment was transformed into *E. coli* DH5α competent cells by electroporation method as previously described. The interested clones were digested with *Eco*RI to detect the size of inserted DNA. The nucleotide sequences were determined by applying the chain termination method. The obtained nucleotide sequences were analyzed for homology by using the standard

BLAST sequence similarity searching program against previously reported sequences at Genebank database.

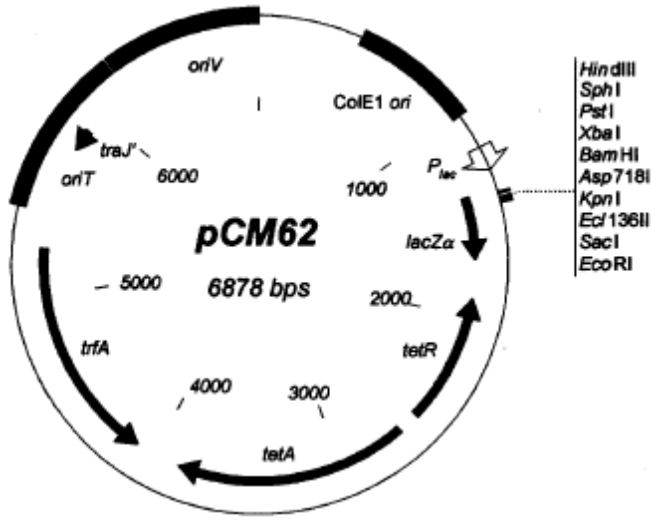
3.4.4 Transformation of *A. syzygii* SKU19 with a plasmid carrying *adhS* gene from *A. pasteurianus* SKU1108

2 kb *Eco*RI DNA fragment carrying *adhS* gene from *A. pasteurianus* SKU1108 (Vanittananon, 2005) was inserted into pCM62, which is a broad-host-range cloning vector, as shown in Figure 9 and transformed into *E. coli* S17-1. The recombinant plasmid pCM*adh*S was further introduced into *A. syzygii* SKU19 by conjugation. *A. syzygii* SKU19 carrying pCM*adhS* was selected on SCM agar containing 50 μg/ml of tetracycline and 0.6% acetic acid.

All of the transformants carrying pCM*adhS* in the same orientation P*lac*, pCM*adhS* (←), and in the opposite orientation from P*lac*, pCM*adhS* (→), were precultured in 5 ml of potato broth containing 25 μg/ml of tetracycline, and incubated at 30°C, 200 rpm for 24 h. Then, 5 μl of the inoculum was dropped on SCM agar containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) of acetic acid and was incubated at 30°C for 5 days. The growth of each transformant was observed.

The 1% inoculum of the selected transformant was inoculated into glucose free-SCM broth containing 0, 0.5, 1.0, 1.5, and 2.0% (v/v) of acetic acid. The culture was incubated at 30°C, 200 rpm. The growth and acidity of the culture were observed. Expression of *adhS* gene and localization of subunit III in *A. syzygii* SKU19 carrying pCM*adhS* were determined by immunoblotting.

(A)



(B)

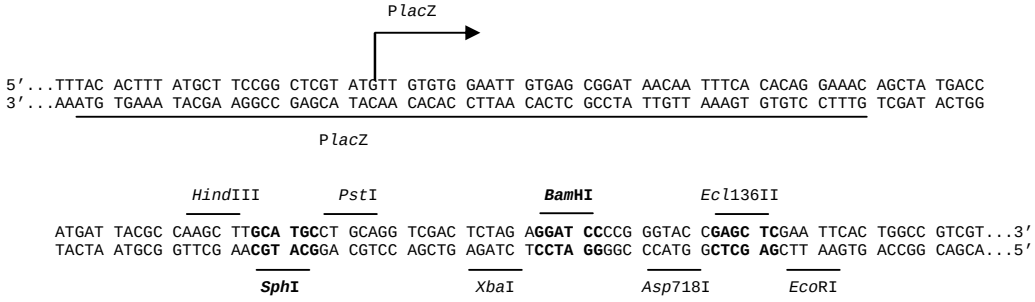


Figure 9 The physical map (A) and multiple cloning sequence of pCM62 broad-host-range cloning vector (B).

Source: Marx and Lidstrom (2001)

RESULTS AND DISCUSSION

1. Selection and Molecular Taxonomy of Acetic Acid Sensitive and Tolerant Strains

1.1 Selection of acetic acid sensitive and tolerant strains

A total of 129 isolates of thermotolerant acetic acid bacteria (SKU1-129) previously isolated from fruits in Thailand (Theeragool *et al*., 1996) were selected for acetic acid sensitive and tolerant strains. The 5 μl of the inoculum was inoculated on SCM agar containing 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) of acetic acid and was incubated at 30°C for 5 days. Comparison of the growth of all isolates was observed. Finally, among the 129 isolates of thermotolerant acetic acid bacteria, *Acetobacter* sp. SKU19 could not grow on SCM agar containing 1.0, 1.5 and 2.0% acetic acid. This strain was analyzed for Gram's staining and ability to overoxidize acetic acid to CO_2 and H_2O on YPG agar containing 2.0 or 4.0% ethanol and 0.03% bromcresol purple. *Acetobacter* sp. SKU19 was rod-shaped and able to overoxidize acetic acid to CO_2 and H_2O and was selected as an acetic acid sensitive strain (Figure 10A). In contrast, *Acetobacter* sp. SKU123 was selected as an acetic acid tolerant strain (Figure 10B). *Acetobacter* sp. SKU19 could grow well on the SCM agar containing 0.25 and 0.5% acetic acid but it could not grow on the medium containing 1.0% acetic acid whereas *Acetobacter* sp. SKU123 grew well even in the presence of 3.0% acetic acid.

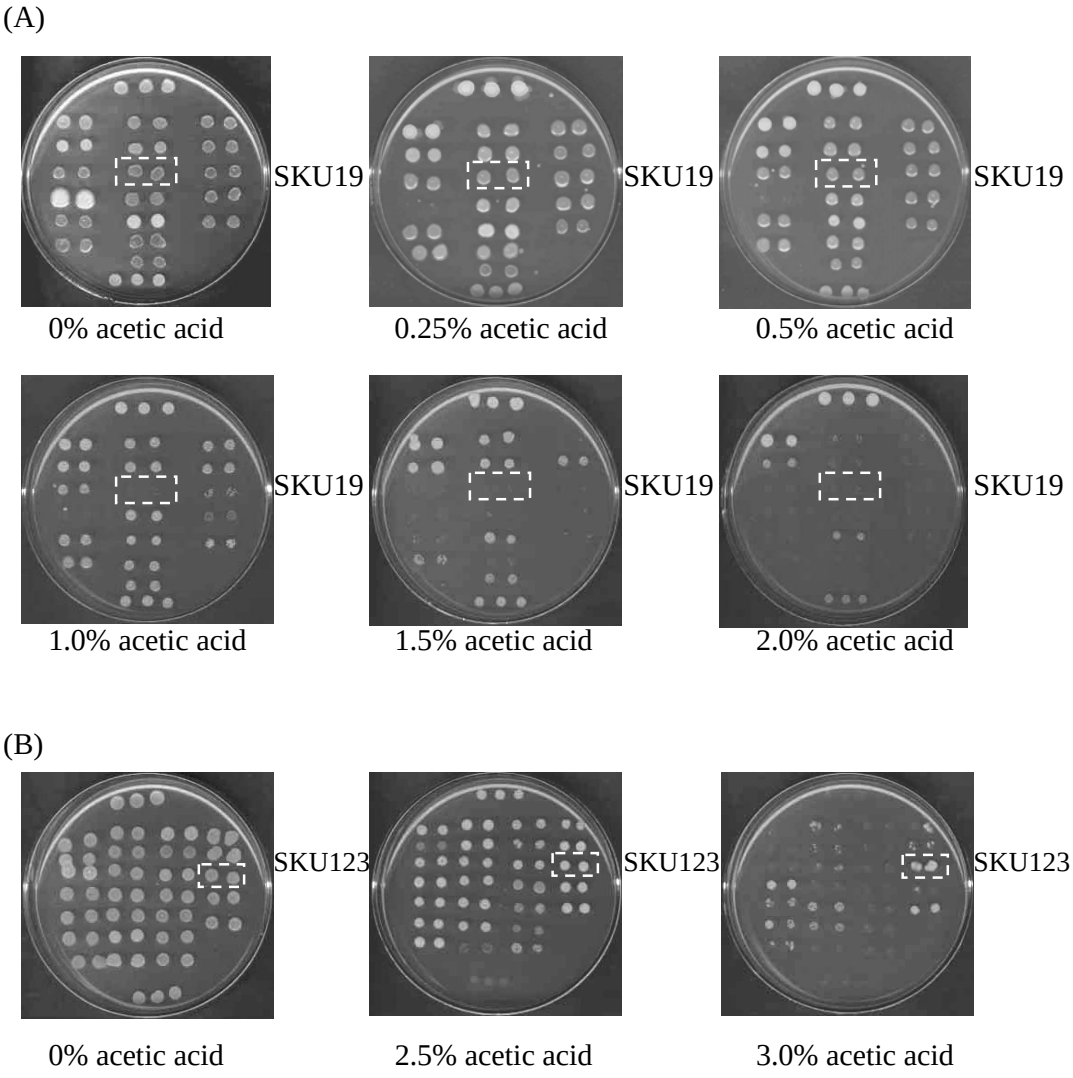


Figure 10 Growth of acetic acid sensitive strain, *Acetobacter* sp. SKU19 (A) and acetic acid tolerant strain, *Acetobacter* sp. SKU123 (B) on SCM agar containing various concentrations of acetic acid; 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (v/v).

1.2 Molecular taxonomy of acetic acid sensitive and tolerant strains by 16S rDNA sequences

Acetobacter sp. SKU19 and SKU123 isolated from guava and longkon, respectively, showed a typical colony with circular, convex, and smooth with cream color. Molecular taxonomy was performed by using 16S rDNA sequences. The chromosomal DNA from *Acetobacter* sp. SKU19 and SKU123 were used as DNA template for 16S rDNA amplification by PCR with two primers, 27f primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525r primer (5'-AAAGGAGGTGATCCA GCC-3'). The size of desired PCR product is 1.5 kb as shown in Figure 11A. The PCR product was recovery from 0.8% agarose gel and ligated with pGEM®-T Easy vector. The ligation mixture was used to transform *E. coli* DH5α by electroporation. The positive recombinant plasmids, designated as pGEM®-T16SSKU19 and pGEM®-T16SSKU123, was digested with *Eco*RI as shown in Figure 11B and each DNA fragment was subcloned into pUC119. These positive recombinant clones were designated as pUC119-16S600 and pUC119-16S900 carrying 600 and 900 bp of 16S DNA fragment in the opposite orientation from P*lac* as shown in Figure 12. The positive recombinant plasmids were determined for their nucleotide sequences by using ABI PRISM™ 310 Genetic Analyzed (PE Applied Biosystems) at BSU Bioservice Unit, Thailand. The obtained nucleotide sequences were shown in Figure 13.

The nucleotide sequences of 16S rDNA amplified from chromosomal DNA of *Acetobacter* sp. SKU19 and SKU123 were analyzed for sequence identity percentage and the obtained results were summarized in Table 7. They were found that the highest identity percentages (99%) were observed when compared with *A. syzygii* and *A. pasteurianus*, respectively. The alignment of these nucleotide sequences with other acetic acid bacteria was shown in Figure 14. The phylogenetic tree was also constructed by using the neighbour-joining method and shown in Figure 15. Therefore, *Acetobacter* sp. SKU19 and SKU123 were identified as *Acetobacter syzygii* SKU19 and *Acetobacter pasteurianus* SKU123, respectively.

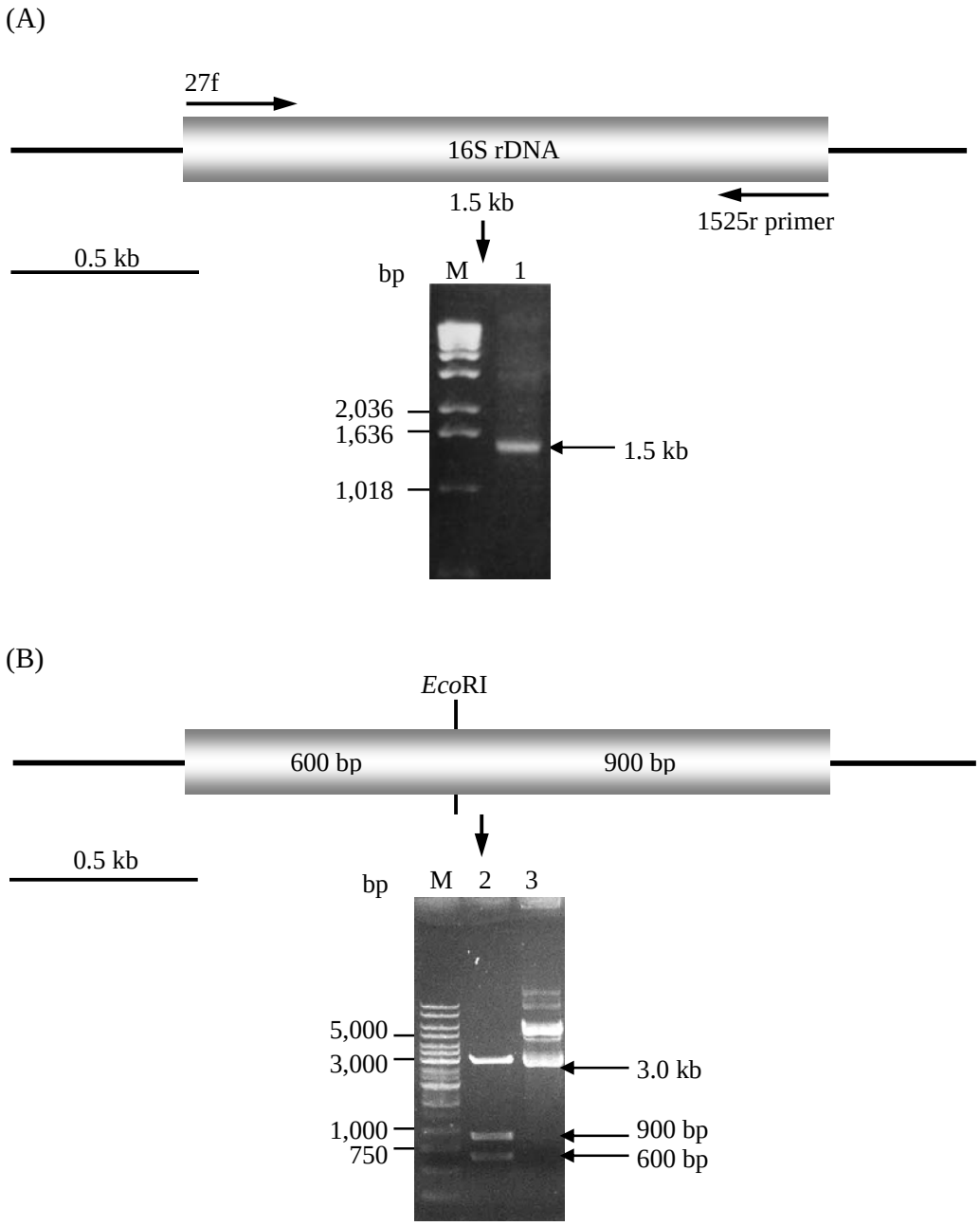
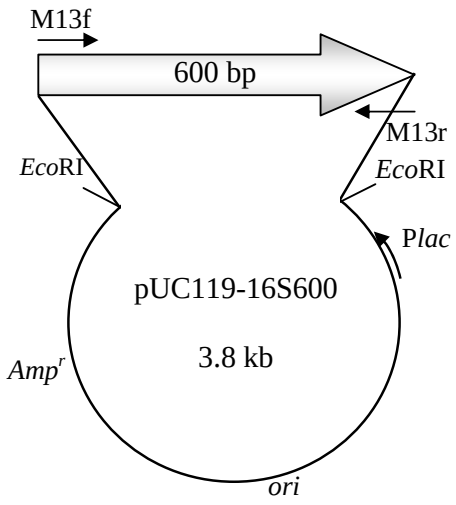
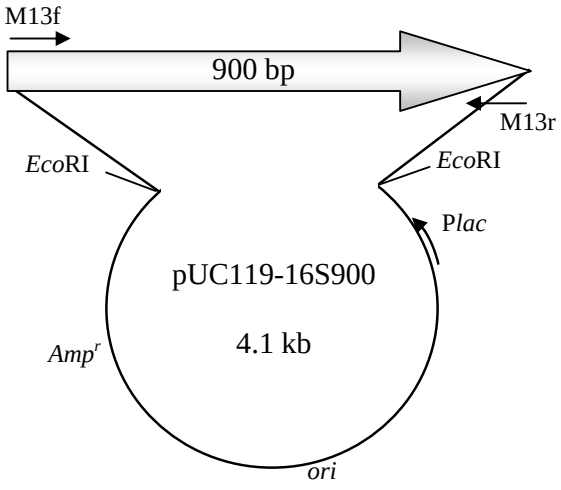


Figure 11 Agarose gel electrophoresis of 1.5 kb PCR product of 16S rDNA from *Acetobacter* sp. SKU19 (A) and pGEM®-T16SSKU19 digested with *Eco*RI (B). Lane M = 1 kb ladder DNA marker, 1 = 1.5 kb PCR product, 2 = pGEM®-T16SSKU19/*Eco*RI, 3 = pGEM®-T16SSKU19.

(A)



(B)



100 bp

Figure 12 The structure of pUC119-16S600 (A) and pUC119-16S900 (B).

(A)

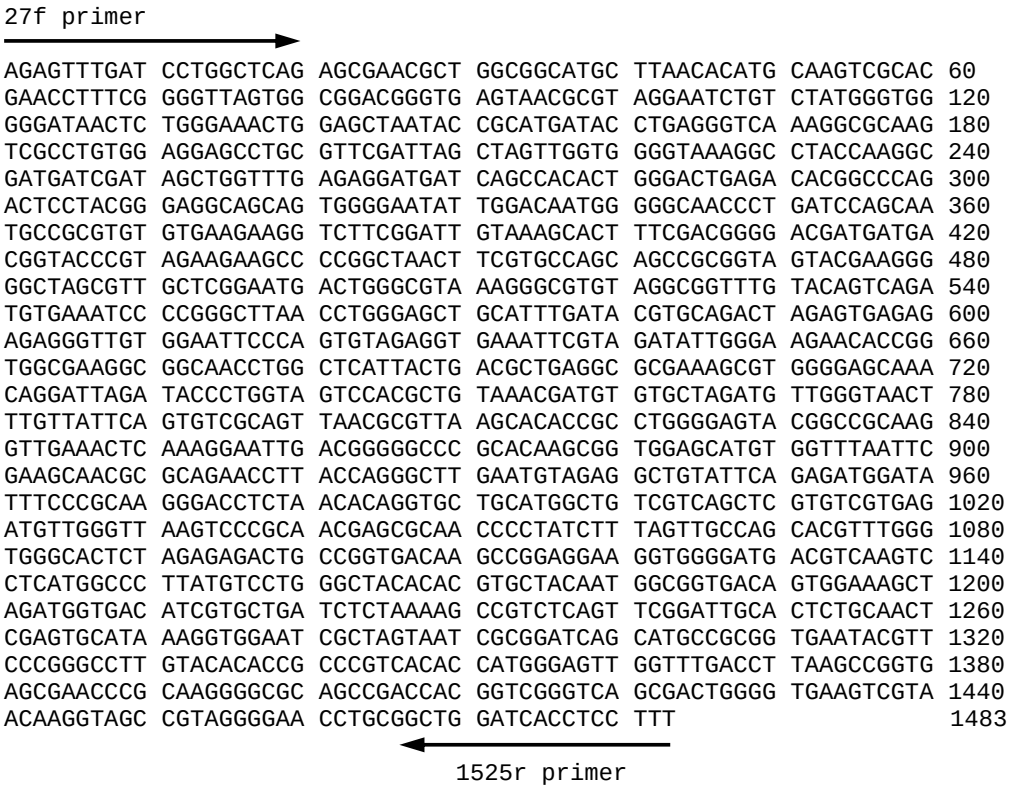


Figure 13 Nucleotide sequences of 1.5 kb PCR product of 16S rDNA from *Acetobacter* sp. SKU19 (A) and SKU123 (B).

(B)

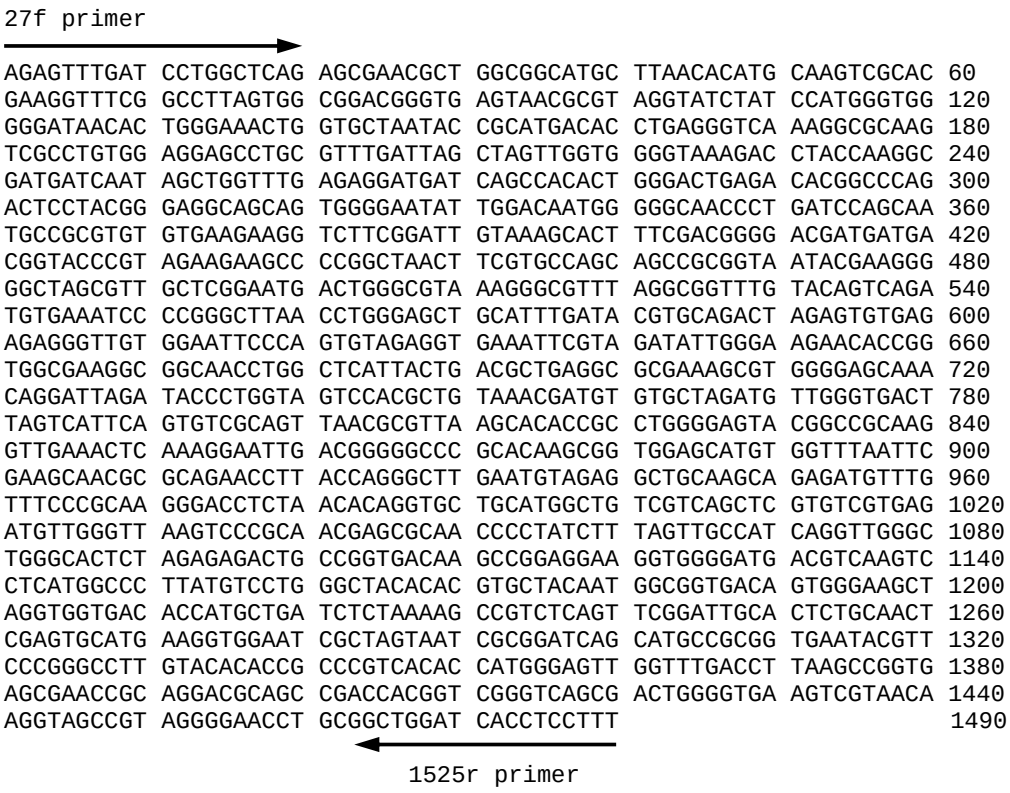


Figure 13 (Continued)

Table 7 Comparison of 16S rDNA sequence identity percentage between *Acetobacter* sp. SKU19 and SKU123 with other acetic acid bacteria and archaebacteria as out group control.

Strain	%16S rDNA sequence identity	
	Acetobacter sp. SKU19	*Acetobacter* sp. SKU123
AB052714 *A. syzygii*	99	97
AJ419837 *A. lovaniensis*	99	97
AJ419835 *A. pomorum*	97	99
AB086016 *A. pasteurianus*	97	99
AJ419841 *A. indonesiensis*	97	96
AJ419840 *A. aceti*	97	97
A. pasteurianus SKU1108	96	98
AB025929 *Asaia bogorensis*	96	95
AB056319 *Kozakia baliensis*	95	94
AY180960 *Gluconacetobacter swingsii*	95	94
AB110714 *Acidomonas methanolica*	94	95
AB074566 *Haloferax volcanii* (archaebacteria)	81	81

```
AB025929 Asaia bogorensis           AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGGACCTTT 48
AB052714 A. syzygii                 AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAACCTTT 48
AB056319 Kozakia baliensis          AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGGACCTTT 48
AB086016 A. pasteurianus            AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGTTT 48
AB110714 Acidomonas methanolica     AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAGGGTTT 48
AJ419835 A. pomorum                 AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGTTT 48
AJ419837 A. lovaniensis             AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAACCTTT 48
AJ419840 A. aceti                   AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGCTT 48
AJ419841 A. indonesiensis           AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGTTT 48
AY180960 Gluconacetobacter swingsii -GCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAACCTTT 47
A. pasteurianus SKU1108             AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGTTT 48
Acetobacter sp. SKU19               AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAACCTTT 48
Acetobacter sp. SKU123              AGCGAACGCTGGCGGCATGC--TTAACACATGCAAGTCGCACGAAGGTTT 48
AB074566 Haloferax volcanii         GGTCATTGCTATTGGGGTCCGATTTAGCCATGCTAGTTGCACGAG---TT 47
                                     *  *  ***   **  * *  ** *  ***** *** *****     **

AB025929 Asaia bogorensis           CGGGGTGAGTGGCGGACGGGTGAGTATCGCGTAGG-GATCTATCCANGGG 97
AB052714 A. syzygii                 CGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG-AATCTGTCCATGGG 97
AB056319 Kozakia baliensis          CGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG-GATCTATCCATGGG 97
AB086016 A. pasteurianus            CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-TATCTATCCATGGG 97
AB110714 Acidomonas methanolica     CGGCCCTAGTGGCGGACGGGTGAGTAGCGCGTAGG-GATCTATCCATGGG 97
AJ419835 A. pomorum                 CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-TATCTATCCATGGG 97
AJ419837 A. lovaniensis             CGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG-AATCTGTCCACGGG 97
AJ419840 A. aceti                   CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-AATCTATCCATGGG 97
AJ419841 A. indonesiensis           CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-AATCTATCCGTGGG 97
AY180960 Gluconacetobacter swingsii CGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG-GATCTGTCCATGGG 96
A. pasteurianus SKU1108             CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-TATCTATCCATGGG 97
Acetobacter sp. SKU19               CGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGG-AATCTGTCTATGGG 97
Acetobacter sp. SKU123              CGGCCTTAGTGGCGGACGGGTGAGTAACGCGTAGG-TATCTATCCATGGG 97
AB074566 Haloferax volcanii         CATACTC-GTGGCGAAAAGCTCAGTAACACGTGGCCAAACTACCCTACAG 96
                                    *       ****** *  * * **** * *** *   * **  *     *

AB025929 Asaia bogorensis           TGGGGGATAACATCGGGAAACTGGTGCTAATACCGCATG--------ATA 139
AB052714 A. syzygii                 TGGGGGATAACTCTGGGAAACTGGAGCTAATACCGCATG--------ATA 139
AB056319 Kozakia baliensis          TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ATG 139
AB086016 A. pasteurianus            TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ACA 139
AB110714 Acidomonas methanolica     TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ATG 139
AJ419835 A. pomorum                 TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ACA 139
AJ419837 A. lovaniensis             TGGGGGATAACTCTGGGAAACTGGAGCTAATACCGCATG--------ATA 139
AJ419840 A. aceti                   TGGGGGATAACTCCGGGAAACTGGAGCTAATACCGCATG--------ATA 139
AJ419841 A. indonesiensis           TGGGGGATAACTCTGGGAAACTGGAGCTAATACCGCATG--------ATA 139
AY180960 Gluconacetobacter swingsii TGGGGGATAACTTTGGGAAACTGAAGCTAATACCGCATG--------ACA 138
A. pasteurianus SKU1108             TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ACA 139
Acetobacter sp. SKU19               TGGGGGATAACTCTGGGAAACTGGAGCTAATACCGCATG--------ATA 139
Acetobacter sp. SKU123              TGGGGGATAACACTGGGAAACTGGTGCTAATACCGCATG--------ACA 139
AB074566 Haloferax volcanii         AGAACGATAACCTCGGGAAACTGAGGCTAATAGTTCATACGGGAGTCATG 146
                                     *   ******   *********  *******   ***         *
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Figure 14 Alignment of 16S rDNA sequence from *Acetobacter* sp. SKU19 and SKU123 with other acetic acid bacteria and archaebacteria. AB025929: *Asaia bogorensis*, AB052714: *A. syzygii*, AB056319: *Kozakia baliensis*, AB086016: *A. pasteurianus*, AB110714: *Acidomonas methanolica*, AJ419835: *A. pomorum*, AJ419837: *A. lovaniensis*, AJ419840: *A. aceti*, AJ419841: *A. indonesiensis*, AY180960: *Gluconacetobacter swingsii*, *A. pasteurianus* SKU1108, AB074566 *Haloferax volcanii* (archaebacteria). Asterisks indicate identity.

```
AB025929 Asaia bogorensis           CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AB052714 A. syzygii                 CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AB056319 Kozakia baliensis          CCTGAGGGCCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AB086016 A. pasteurianus            CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AB110714 Acidomonas methanolica     CCTGAGGGCCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AJ419835 A. pomorum                 CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AJ419837 A. lovaniensis             CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AJ419840 A. aceti                   CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AJ419841 A. indonesiensis           CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGC-GGAGGAGCCTGCG 181
AY180960 Gluconacetobacter swingsii CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAACCTGCG 180
A. pasteurianus SKU1108             CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
Acetobacter sp. SKU19               CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
Acetobacter sp. SKU123              CCTGAGGGTCAAAGGCGCAAGTCGC-------CTGT-GGAGGAGCCTGCG 181
AB074566 Haloferax volcanii         CTGGAATGCCGACTCCCCGAAACGCTCAGGCACTGTAGGATGTGGCTGCG 196
                                    *  **  * * *   * * *  ***       ***  *** *   *****

AB025929 Asaia bogorensis           TTCGATTAGCTTGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
AB052714 A. syzygii                 TTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
AB056319 Kozakia baliensis          TTCGATTAGCTTGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
AB086016 A. pasteurianus            TTTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAATA 231
AB110714 Acidomonas methanolica     TTCGATTAGCTTGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
AJ419835 A. pomorum                 TTTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAATA 231
AJ419837 A. lovaniensis             TTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
AJ419840 A. aceti                   TTTGATTAGCTTGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAATA 231
AJ419841 A. indonesiensis           TTTGATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATCAATA 231
AY180960 Gluconacetobacter swingsii TTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 230
A. pasteurianus SKU1108             TTTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAATA 231
Acetobacter sp. SKU19               TTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATA 231
Acetobacter sp. SKU123              TTTGATTAGCTAGTTGGTGGGGTAAAGACCTACCAAGGCGATGATCAATA 231
AB074566 Haloferax volcanii         GCCGATTAGGTAGACGGTGGGGTAACGGCCCACCGTGCCGATAATCGGTA 246
                                       ****** * *  ********** * ** ***  * **** ***  **

AB025929 Asaia bogorensis           GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AB052714 A. syzygii                 GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AB056319 Kozakia baliensis          GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AB086016 A. pasteurianus            GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AB110714 Acidomonas methanolica     GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AJ419835 A. pomorum                 GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AJ419837 A. lovaniensis             GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AJ419840 A. aceti                   GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AJ419841 A. indonesiensis           GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AY180960 Gluconacetobacter swingsii GCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 280
A. pasteurianus SKU1108             GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
Acetobacter sp. SKU19               GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
Acetobacter sp. SKU123              GCTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA 281
AB074566 Haloferax volcanii         CGGGTTGTGAGAGCAAGAGCCCGGAGACGGAATCTGAGACAAGATTCCGG 296
                                       * * ****** * ** *    * ** *   ******** *   * *

AB025929 Asaia bogorensis           CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG 331
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AB056319 Kozakia baliensis          CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG 331
AB086016 A. pasteurianus            CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AB110714 Acidomonas methanolica     CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AJ419835 A. pomorum                 CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AJ419837 A. lovaniensis             CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AJ419840 A. aceti                   CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTG 331
AJ419841 A. indonesiensis           CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AY180960 Gluconacetobacter swingsii CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG 330
A. pasteurianus SKU1108             CTCCTACGGGAGGCAGCAGTGGGGGATATTGGACAATGGGGGCAACCCTG 331
Acetobacter sp. SKU19               CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
Acetobacter sp. SKU123              CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTG 331
AB074566 Haloferax volcanii         GCCCTACGGGGCGCAGCAGGCGCGAAACCTTTACACTGCACGCAAGTGCG 346
                                      ********  *******  * * *   *  *** **   * **    *
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
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AB056319 Kozakia baliensis          ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AB086016 A. pasteurianus            ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AB110714 Acidomonas methanolica     ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AJ419837 A. lovaniensis             ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AJ419835 A. pomorum                 ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AJ419840 A. aceti                   ATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTGTAAAGCACTT 381
AJ419841 A. indonesiensis           ATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTGTAAAGCACTT 381
AY180960 Gluconacetobacter swingsii ATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTGTAAAGCACTT 380
A. pasteurianus SKU1108             ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
Acetobacter sp. SKU19               ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
Acetobacter sp. SKU123              ATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT 381
AB074566 Haloferax volcanii         ATAAGGGGACCCCAAGTGCG------AGGGCATATAGTCCT----CGCTT 386
                                    **   *  *  **  *** *      ***   *    *  *    * ***

AB025929 Asaia bogorensis           TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AB052714 A. syzygii                 TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AB056319 Kozakia baliensis          TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AB086016 A. pasteurianus            TCGACGGGGACGATGATGACGGTACCCGTAGAAGGAGCCCCGGCTAACTT 431
AB110714 Acidomonas methanolica     TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AJ419835 A. pomorum                 TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AJ419837 A. lovaniensis             TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AJ419840 A. aceti                   TCGGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTT 431
AJ419841 A. indonesiensis           TCGGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTT 431
AY180960 Gluconacetobacter swingsii TCAGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTT 430
A. pasteurianus SKU1108             TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
Acetobacter sp. SKU19               TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
Acetobacter sp. SKU123              TCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTT 431
AB074566 Haloferax volcanii         TTCTCG---ACTGTGA-GGCGGT---CGAGGAATAAGAGCTGGGCAAGAC 429
                                    *   **   **  *** * ****   **  ***  **  * **  **

AB025929 Asaia bogorensis           CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AB052714 A. syzygii                 CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AB056319 Kozakia baliensis          CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATA 480
AB086016 A. pasteurianus            CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AB110714 Acidomonas methanolica     CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AJ419835 A. pomorum                 CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AJ419837 A. lovaniensis             CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AJ419840 A. aceti                   CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AJ419841 A. indonesiensis           CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AY180960 Gluconacetobacter swingsii CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCAAGCGTTGCTCGGAATG 479
A. pasteurianus SKU1108             CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
Acetobacter sp. SKU19               CG-TGCCAGCAGCCGCGGTAGTACGAAGGGGGCTAGCGTTGCTCGGAATG 480
Acetobacter sp. SKU123              CG-TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATG 480
AB074566 Haloferax volcanii         CGGTGCCAGCCGCCGCGGTAATACCGGCAGCTCAAGTGATGACCGATATT 479
                                    ** ******* ********* ***     *  * ** * **  **  **

AB025929 Asaia bogorensis           ACTGGGCGTAAAGGGCGCGTAGGCGGTTTACACAGTCAGATGTGAAATTC 530
AB052714 A. syzygii                 ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AB056319 Kozakia baliensis          ACTGGGCGTAAAGGGCGCGTAGGCGGTTTGGACAGTCAGATGTGAAATTC 530
AB086016 A. pasteurianus            ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AB110714 Acidomonas methanolica     ACTGGGCGTAAAGGGCGCGTAGGCGGTTGACACAGTCAGATGTGAAATTC 530
AJ419835 A. pomorum                 ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AJ419837 A. lovaniensis             ACTGGGCGTAAAGGGCGTGTAGGCGGTTTACACAGTCAGATGTGAAATCC 530
AJ419840 A. aceti                   ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AJ419841 A. indonesiensis           ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AY180960 Gluconacetobacter swingsii ACTGGGCGTAAAGGGCGCGTAGGCGGTTGACACAGTCAGATGTGAAATTC 529
A. pasteurianus SKU1108             ACTGGGCGTAAAGGGCGTGTAGGCGGTATGTACAGTCAAGTGTGAAATCC 530
Acetobacter sp. SKU19               ACTGGGCGTAAAGGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
Acetobacter sp. SKU123              ACTGGGCGTAAAGGGCGTTTAGGCGGTTTGTACAGTCAGATGTGAAATCC 530
AB074566 Haloferax volcanii         ATTGGGCCTAAAGCGTCCGTAGCCGGCCACGAAGGTTCATCGGGAAATCC 529
                                    * ***** ***** *    *** ***     *  **     * ***** *
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           CAGGGCTTAACCTTGGGGCTGCATTTGATACGTGT-AGACTAGAGTGTGA 579
AB052714 A. syzygii                 CCGGGCTTAACCTGGGAGCTGCATTTGATACGTAC-AGACTAGAGTGTGA 579
AB056319 Kozakia baliensis          CTGGGCTTAACCTGGGGGCTGCATTTGATACGTAC-AGACTAGAGTGTGA 579
AB086016 A. pasteurianus            CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC-AGACTAGAGTGTGA 579
AB110714 Acidomonas methanolica     CAGGGCTTAACCTTGGGGCTGCATTTGAGACGTGT-TGACTGGAGTTCGA 579
AJ419835 A. pomorum                 CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC-AGACTAGAGTATGA 579
AJ419837 A. lovaniensis             CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGT-AGACTAGAGTGTGA 579
AJ419840 A. aceti                   CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC-AGACTAGAGTGTGA 579
AJ419841 A. indonesiensis           CCGGGCTTAACCTGGGAGCTGCATTTGAGACGTGC-AGACTAGAGTGTGA 579
AY180960 Gluconacetobacter swingsii CTGGGCTTAACCTGGGGGCTGCATTTGATACGTGG-CGACTAGAGTGTGA 578
A. pasteurianus SKU1108             CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGCGAGACTAGAGTGTGA 580
Acetobacter sp. SKU19               CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC-AGACTAGAGTGAGA 579
Acetobacter sp. SKU123              CCGGGCTTAACCTGGGAGCTGCATTTGATACGTGC-AGACTAGAGTGTGA 579
AB074566 Haloferax volcanii         GCCAGCTCAACTGGCGGGCGTCCGGTGAAAACCACGTGGCTTGGGACCGG 579
                                        *** ***    * **  *   *** *       * ** * *   *

AB025929 Asaia bogorensis           GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AB052714 A. syzygii                 GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AB056319 Kozakia baliensis          GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AB086016 A. pasteurianus            GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AB110714 Acidomonas methanolica     GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AJ419835 A. pomorum                 GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AJ419837 A. lovaniensis             GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AJ419840 A. aceti                   GAGAGGGTTGTGGAATTCTCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AJ419841 A. indonesiensis           GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AY180960 Gluconacetobacter swingsii GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 628
A. pasteurianus SKU1108             GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGG- 629
Acetobacter sp. SKU19               GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
Acetobacter sp. SKU123              GAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG 629
AB074566 Haloferax volcanii         AAGGCTCGAGGGGTACGTCCGGGGTAGGAGTGAAATCCCGTAATCCTGGA 629
                                     **      * ** *    * * ****  ******* *    **  ***

AB025929 Asaia bogorensis           AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AB052714 A. syzygii                 AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AB056319 Kozakia baliensis          AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTGAGG 679
AB086016 A. pasteurianus            AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AB110714 Acidomonas methanolica     AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCGATACTGACGCTGAGG 679
AJ419835 A. pomorum                 AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AJ419837 A. lovaniensis             AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTGAGG 679
AJ419840 A. aceti                   AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AJ419841 A. indonesiensis           AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AY180960 Gluconacetobacter swingsii AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTGAGG 678
A. pasteurianus SKU1108             AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
Acetobacter sp. SKU19               AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
Acetobacter sp. SKU123              AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATTACTGACGCTGAGG 679
AB074566 Haloferax volcanii         CGGGCCACCGATGGCGAAAGCACCTCGAGAAGACGGATCCGACGGTGAGG 679
                                      *  ***** ******* **  *         *    * **** *****

AB025929 Asaia bogorensis           CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AB052714 A. syzygii                 CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AB056319 Kozakia baliensis          CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AB086016 A. pasteurianus            CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AB110714 Acidomonas methanolica     CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AJ419835 A. pomorum                 CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AJ419837 A. lovaniensis             CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AJ419840 A. aceti                   CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AJ419841 A. indonesiensis           CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AY180960 Gluconacetobacter swingsii CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 728
A. pasteurianus SKU1108             CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
Acetobacter sp. SKU19               CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
Acetobacter sp. SKU123              CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT 729
AB074566 Haloferax volcanii         GACGAAAGCTAGGGTCTCGAACCGGATTAGATACCCGGGTAGTCCTAGCT 729
                                      *******  ***   * *** ************* ********  ***
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           GTAAACGATGTGTGCTGGATGT----TGGGTAACTTAGTTACTCAGTGTC 775
AB052714 A. syzygii                 GTAAACGATGTGTGCTAGATGT----TGGGTGACTTTGTCATTCAGTGTC 775
AB056319 Kozakia baliensis          GTAAACGATGTGTGCTGGATGT----TGGGCAACTTAGTTGCTCAGTGTC 775
AB086016 A. pasteurianus            GTAAACGATGTGTGCTAGATGT----TGGGTGACTTAGTCATTCAGTGTC 775
AB110714 Acidomonas methanolica     GTAAACGATGTGTGCTGGATGT----TGGGTGACTTTGTCATTCAGTGTC 775
AJ419835 A. pomorum                 GTAAACGATGTGTGCTAGATGT----TGGGTAACTTAGTTATTCAGTGTC 775
AJ419837 A. lovaniensis             GTAAACGATGTGTGCTAGATGT----TGGGTAACTTTGTTATTCAGTGTC 775
AJ419840 A. aceti                   GTAAACGATGTGTGCTGGATGT----TGGGTAACTTAGTTACTCAGTGTC 775
AJ419841 A. indonesiensis           GTAAACGATGTGTGCTGGATGT----TGGGTAACTTAGTTATTCAGTGTC 775
AY180960 Gluconacetobacter swingsii GTAAACGATGTGTGCTGGATGT----TGGGTGACTTTGTCATTCAGTGTC 774
A. pasteurianus SKU1108             GTAAACGATGTGTGCTAGATGT----TGGGTGACTTAGTCATTCAGTGTC 775
Acetobacter sp. SKU19               GTAAACGATGTGTGCTAGATGT----TGGGTAACTTTGTTATTCAGTGTC 775
Acetobacter sp. SKU123              GTAAACGATGTGTGCTAGATGT----TGGGTGACTTAGTCATTCAGTGTC 775
AB074566 Haloferax volcanii         GTAAACGATGCTCGCTAGGTGTGACACAGGCTACGAGCCTGTGTTGTGCC 779
                                    **********   *** * ***      **  **           *** *

AB025929 Asaia bogorensis           GAAGCTAACGCGCTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AB052714 A. syzygii                 GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AB056319 Kozakia baliensis          GTAGCTAACGCGCTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AB086016 A. pasteurianus            GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AB110714 Acidomonas methanolica     GAAGCTAACGCGCTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AJ419835 A. pomorum                 GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AJ419837 A. lovaniensis             GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AJ419840 A. aceti                   GTAGCTAACGCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AJ419841 A. indonesiensis           GTAGCTAACGCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AY180960 Gluconacetobacter swingsii GTAGTTAACGCGATAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 824
A. pasteurianus SKU1108             GCAGTTAACGCGTTAAGCACACCGCCTGGGGATGACGGCCGCAAGGTTGA 825
Acetobacter sp. SKU19               GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
Acetobacter sp. SKU123              GCAGTTAACGCGTTAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGA 825
AB074566 Haloferax volcanii         GTAGGGAAGCCGAGAAGCGAGCCGCCTGGGAAGTACGTCCGCAAGGATGA 829
                                    * **  **  **  ****   ********* *  *** ******** ***

AB025929 Asaia bogorensis           AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AB052714 A. syzygii                 AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AB056319 Kozakia baliensis          AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AB086016 A. pasteurianus            AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AB110714 Acidomonas methanolica     AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AJ419835 A. pomorum                 AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AJ419837 A. lovaniensis             AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AJ419840 A. aceti                   AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AJ419841 A. indonesiensis           AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AY180960 Gluconacetobacter swingsii AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 873
A. pasteurianus SKU1108             AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
Acetobacter sp. SKU19               AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
Acetobacter sp. SKU123              AACTCAAAGGAATTGACGGGGGCCCGC-ACAAGCGGTGGAGCATGTGGTT 874
AB074566 Haloferax volcanii         AACTTAAAGGAATTGGCGGGGGAGCACTACAACCGGAGGAGCCTGCGGTT 879
                                    **** ********** ******  * * **** *** ***** ** ****

AB025929 Asaia bogorensis           TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGACATGGGGAGGCT 924
AB052714 A. syzygii                 TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
AB056319 Kozakia baliensis          TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGACATGGGGAGGCT 924
AB086016 A. pasteurianus            TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
AB110714 Acidomonas methanolica     TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGACATGGGGAGGCT 924
AJ419835 A. pomorum                 TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
AJ419837 A. lovaniensis             TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
AJ419840 A. aceti                   TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGT-ATGGAGAGGCT 923
AJ419841 A. indonesiensis           TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGT-ATGGGTAGGCT 923
AY180960 Gluconacetobacter swingsii TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGACATGCGGAGGCC 923
A. pasteurianus SKU1108             TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
Acetobacter sp. SKU19               TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
Acetobacter sp. SKU123              TAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGA-ATGTAGAGGCT 923
AB074566 Haloferax volcanii         TAATTGGACTCAACGCCGGACATCTCACCAG--CTCCGACTACAGTGATG 927
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           GTATCCAGAGATGGGTATTTCCCGCAAGGGACCTCCTGCACAGGTGCTGC 974
AB052714 A. syzygii                 GTATTCAGAGATGGATATTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
AB056319 Kozakia baliensis          GTAGCCAGAGATGGTTATTTCCCGCAAGGGACCTCCTGCACAGGTGCTGC 974
AB086016 A. pasteurianus            GCAAGCAGAGATGTTTGTTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
AB110714 Acidomonas methanolica     GTGTCCAGAGATGGGCATTTCCCGCAAGGGACCTCCTGCACAGGTGCTGC 974
AJ419835 A. pomorum                 GCAAGCAGAGATGTTTGTTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
AJ419837 A. lovaniensis             GTATTCAGAGATGGATATTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
AJ419840 A. aceti                   GTATTCAGAGATGGATATTTCCCGCAAGGGACCTCTTGCACAGGTGCTGC 973
AJ419841 A. indonesiensis           GTATTCAGAGATGGATATTTCCCGCAAGGGACCTACCGCACAGGTGCTGC 973
AY180960 Gluconacetobacter swingsii GTGTCCAGAGATGGGCATTTCTCGCAAGAGACCTCCAGCACAGGTGCTGC 973
A. pasteurianus SKU1108             GCAAGCAGAGATGTTTGTTTCCCGCAAGG-ACCTCTAACACAGGTGCTGC 972
Acetobacter sp. SKU123              GCAAGCAGAGATGTTTGTTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
Acetobacter sp. SKU19               GTATTCAGAGATGGATATTTCCCGCAAGGGACCTCTAACACAGGTGCTGC 973
AB074566 Haloferax volcanii         ACGATCAGGTTGATGACCTTATCACGACG---CTGTAG-AGAGGAGGTGC 973
                                         ***          **  * * *     **     * *** * ***

AB025929 Asaia bogorensis           ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1023
AB052714 A. syzygii                 ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AB056319 Kozakia baliensis          ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1023
AB086016 A. pasteurianus            ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AB110714 Acidomonas methanolica     ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1023
AJ419835 A. pomorum                 ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AJ419837 A. lovaniensis             ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AJ419840 A. aceti                   ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AJ419841 A. indonesiensis           ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AY180960 Gluconacetobacter swingsii ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
A. pasteurianus SKU1108             ATGGCTGTCGTCAAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC-GCAAC 1021
Acetobacter sp. SKU19               ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
Acetobacter sp. SKU123              ATGGCTGTCGTCA-GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC 1022
AB074566 Haloferax volcanii         ATGGCCGCCGTCA-GCTCGTACCGTGAGGCGTCCTGTTAAGTCAGGCAAC 1022
                                    ***** * ***** ******  ******  **   ********  *****

AB025929 Asaia bogorensis           GAGCGCAACCCTCGCCTTTAGTTGCCAGCA-------CGTTTGGGTGGGC 1066
AB052714 A. syzygii                 GAGCGCAACCCCTATCTTTAGTTGCCAGCA-------TGTTTGGGTGGGC 1065
AB056319 Kozakia baliensis          GAGCGCAACCCTCGCCTTTAGTTGCCAGCA-------CGTTTGGGTGGGC 1066
AB086016 A. pasteurianus            GAGCGCAACCCCTATCTTTAGTTGCCATCA-------GGTTGGGCTGGGC 1065
AB110714 Acidomonas methanolica     GAGCGCAACCCTTGCCTTTAGTTGCCAGCA-------TGATCGGGTGGGC 1066
AJ419835 A. pomorum                 GAGCGCAACCCCTATCTTTAGTTGCCATCA-------GGTTGGGCTGGGC 1065
AJ419837 A. lovaniensis             GAGCGCAACCCCTATCTTTAGTTGCCAGCA-------TGTTTGGGTGGGC 1065
AJ419840 A. aceti                   GAGCGCAACCCTTATCTTTAGTTGCCAGCA-------TGTTTGGGTGGGC 1065
AJ419841 A. indonesiensis           GAGCGCAACCCCTATCTTTAGTTGCCAGCA-------TGTTTGGGTGGGC 1065
AY180960 Gluconacetobacter swingsii GAGCGCAACCCTCGCCTTTAGTTGCCATCA-------CGTCTGGGTGGGC 1065
A. pasteurianus SKU1108             GAGCGCAACCCCTATCTTTAGTTGCCATCA-------GGTTGGGCTGGGC 1064
Acetobacter sp. SKU19               GAGCGCAACCCCTATCTTTAGTTGCCAGCA-------CGTTTGGGTGGGC 1065
Acetobacter sp. SKU123              GAGCGCAACCCCTATCTTTAGTTGCCATCA-------GGTTGGGCTGGGC 1065
AB074566 Haloferax volcanii         GAGCGAGACCCGCACTTCTAATTGCCAGCAGCAGTTTCGACTGGCTGGGT 1072
                                    *****  ****     * ** ****** **        *   ** ****

AB025929 Asaia bogorensis           ACTCTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1116
AB052714 A. syzygii                 ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AB056319 Kozakia baliensis          ACTCTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1116
AB086016 A. pasteurianus            ACTCTAAAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AB110714 Acidomonas methanolica     ACTCTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1116
AJ419835 A. pomorum                 ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AJ419837 A. lovaniensis             ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AJ419840 A. aceti                   ACTCTAAAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AJ419841 A. indonesiensis           ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AY180960 Gluconacetobacter swingsii ACTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
A. pasteurianus SKU1108             ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1114
Acetobacter sp. SKU19               ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
Acetobacter sp. SKU123              ACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTC 1115
AB074566 Haloferax volcanii         ACATTAGAAGGACTGCCGCTGCTAAAGCGGAGGAAGGAACGGGCAACGGT 1122
                                    **  ** *   ******* **  **  **********   **   ***
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1166
AB052714 A. syzygii                 AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AB056319 Kozakia baliensis          AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1166
AB086016 A. pasteurianus            AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AB110714 Acidomonas methanolica     AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1166
AJ419835 A. pomorum                 AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AJ419837 A. lovaniensis             AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AJ419840 A. aceti                   AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AJ419841 A. indonesiensis           AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AY180960 Gluconacetobacter swingsii AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
A. pasteurianus SKU1108             AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGCCGG 1164
Acetobacter sp. SKU19               AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
Acetobacter sp. SKU123              AAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGG 1165
AB074566 Haloferax volcanii         AGGTCAGTATGCCCCGAATGAGCTGGGCTACACGCGGGCTACAATGGTCG 1172
                                    * ***   *** ***  ***  *********** ** *********   *

AB025929 Asaia bogorensis           TGACAGTGGGAAGCTAGATGGTGACATCGTGCCGATCTCTAAAAGCCG-T 1215
AB052714 A. syzygii                 TGACAGTGGGAAGCTAGATGGTGACATCGTGCTGATCTCTAAAAGCCG-T 1214
AB056319 Kozakia baliensis          TGACAGTGGGAAGCTAGACAGCGATGTCATGCCGATCTCTAAAAACCG-T 1215
AB086016 A. pasteurianus            TGACAGTGGGAAGCTAGGTGGTGACACCATGCTGATCTCTAAAAGCCG-T 1214
AB110714 Acidomonas methanolica     TGACAGTGGGAAGCCAGGCAGTGATGCTGAGCTGATCTCAAAAAGCCG-T 1215
AJ419835 A. pomorum                 TGACAGTGGGAAGCTAGGTGGTGACACCATGCTGATCTCTAAAAGCCG-T 1214
AJ419837 A. lovaniensis             TGACAGTGGGAAGCTAGATGGTGACATCATGCTGATCTCTAAAAGCCG-T 1214
AJ419840 A. aceti                   TGACAGTGGGAAGCTAGATGGTGACATCATGCCGATCTCTAAAAACCG-T 1214
AJ419841 A. indonesiensis           TGACAGTGGGAAGCTAGATGGCGACATCGTGCTGATCTCTAAAAGCCG-T 1214
AY180960 Gluconacetobacter swingsii TGACAGTGGGAAGCCAGGTAGCGATACCGAGCCGATCTCAAAAAGCCG-T 1214
A. pasteurianus SKU1108             TGACAGTGGGAAGCTAGGTGGTGACACCATGCTGATCTCTAAAAGCCG-T 1213
Acetobacter sp. SKU19               TGACAGTGGAAAGCTAGATGGTGACATCGTGCTGATCTCTAAAAGCCG-T 1214
Acetobacter sp. SKU123              TGACAGTGGGAAGCTAGGTGGTGACACCATGCTGATCTCTAAAAGCCG-T 1214
AB074566 Haloferax volcanii         AGACAATGGGTTGCTATCTCGAAAGAGAACGCTAATCTCCTAAACTCGAT 1222
                                     **** ***   ** *    *  *      **  *****  ***  ** *

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AB056319 Kozakia baliensis          CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1265
AB086016 A. pasteurianus            CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AB110714 Acidomonas methanolica     CTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGGTGGAATCGCT 1265
AJ419835 A. pomorum                 CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AJ419837 A. lovaniensis             CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AJ419840 A. aceti                   CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AJ419841 A. indonesiensis           CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AY180960 Gluconacetobacter swingsii CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
A. pasteurianus SKU1108             CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCC 1263
Acetobacter sp. SKU19               CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATAAAGGTGGAATCGCT 1264
Acetobacter sp. SKU123              CTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCT 1264
AB074566 Haloferax volcanii         CGTAGTTCGGATTGAGGGCTGAAACTCGCCCTCATGAAGCTGGATTCGGT 1272
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AB052714 A. syzygii                 AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AB056319 Kozakia baliensis          AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1315
AB086016 A. pasteurianus            AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AB110714 Acidomonas methanolica     AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1315
AJ419835 A. pomorum                 AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AJ419837 A. lovaniensis             AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AJ419840 A. aceti                   AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AJ419841 A. indonesiensis           AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AY180960 Gluconacetobacter swingsii AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
A. pasteurianus SKU1108             AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1313
Acetobacter sp. SKU19               AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
Acetobacter sp. SKU123              AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1314
AB074566 Haloferax volcanii         AGTAATCGCATTTCAATAGAGTGCGGTGAATACGTCCCTGCTCCTTGCAC 1322
                                    *********   ***  *    ************* ** *  ***** **
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Figure 14 (Continued)

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AB025929 Asaia bogorensis           ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCCGAAGCCGGTGAGCG 1365
AB052714 A. syzygii                 ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AB056319 Kozakia baliensis          ACACCGCCCGTCACACCATGGGAGTTGGTTCGACCTTAAGCCGGTGAGCG 1365
AB086016 A. pasteurianus            ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AB110714 Acidomonas methanolica     ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1365
AJ419835 A. pomorum                 ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AJ419837 A. lovaniensis             ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AJ419840 A. aceti                   ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AJ419841 A. indonesiensis           ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AY180960 Gluconacetobacter swingsii ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
A. pasteurianus SKU1108             ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1363
Acetobacter sp SKU19                ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
Acetobacter sp. SKU123              ACACCGCCCGTCACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCG 1364
AB074566 Haloferax volcanii         ACACCGCCCGTCAAAGCACCCGAGTGAGGTCCGGATGAGGCCACCACACG 1372
                                    ************* * **   ****  * *       * ***      **

AB025929 Asaia bogorensis           AACC--GCAA--GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1405
AB052714 A. syzygii                 AACCC-GCAAG-GGGCGCAGCCGACCACGGTCGGGTCAGCGACT 1406
AB056319 Kozakia baliensis          AACCCAGCAATGGGGCGCAGCCGATCACGGTCGGGTTAGCGACT 1409
AB086016 A. pasteurianus            AACC--GCAA--GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1404
AB110714 Acidomonas methanolica     AACC--GCAA--GGACGCAGCCGACCACGGTCAGGTCAGCGACT 1405
AJ419835 A.pomorum                  AACC--GCAA--GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1404
AJ419837 A. lovaniensis             AACCC-GCAAG-GGGCGCAGCCGACCACGGTCGGGTCAGCGACT 1406
AJ419840 A. aceti                   AACC--GCAAG-GA-CGCAGCCGACCACGGTCGGGTCAGCGACT 1404
AJ419841 A. indonesiensis           AACCC-GCAAG-GGGCGCAGCCGACCACGGTCGGGTCAGCGACT 1406
AY180960 Gluconacetobacter swingsii AACC--GCAA--GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1404
A. pasteurianus SKU1108             AACC--GCAA--GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1403
Acetobacter sp. SKU19               AACCC-GCAAG-GGGCGCAGCCGACCACGGTCGGGTCAGCGACT 1406
Acetobacter sp. SKU123              AACC--GCA---GGACGCAGCCGACCACGGTCGGGTCAGCGACT 1403
AB074566 Haloferax volcanii         GT----------GGTCGAATCTGGCTTCGCAAGGGGCTTAAGTC 1406
                                              *  ** * * *    **    **
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Figure 14 (Continued)

(A)

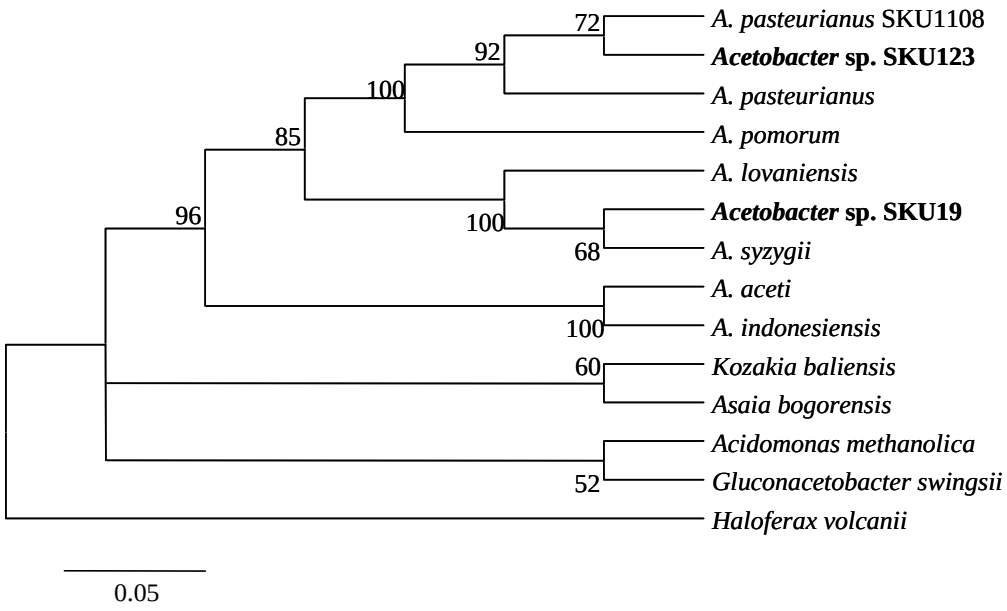


Figure 15 16S rDNA-based tree reflecting the phylogenetic position of *Acetobacter* sp. SKU19, SKU123, related acetic acid bacteria and archaebacteria. The analysis was done by using the following sequence from Genebank: AB025929: *Asaia bogorensis*, AB052714: *A. syzygii*, AB056319: *Kozakia baliensis*, AB086016: *A. pasteurianus*, AB110714: *Acidomonas methanolica*, AJ419835: *A. pomorum*, AJ419837: *A. lovaniensis*, AJ419840: *A. aceti*, AJ419841: *A. indonesiensis*, AY180960: *Gluconacetobacter swingsii*, *A. pasteurianus* SKU1108, AB074566 *Haloferax volcanii* (archaebacteria).

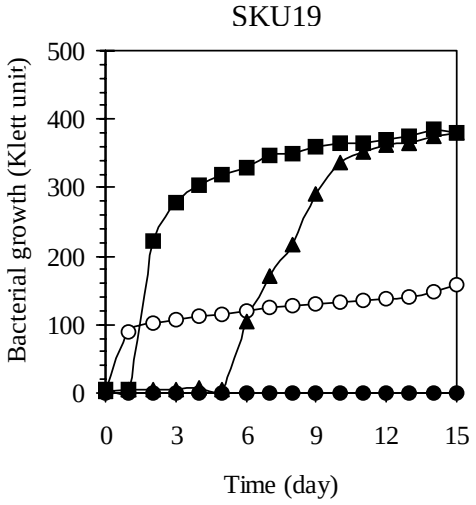
2. Growth Characterization of Acetic Acid Sensitive and Tolerant Strains

2.1 Time-course of growth of acetic acid sensitive and tolerant strain in the medium containing various concentrations of acetic acid

In an attempt to study growth characteristics of acetic acid sensitive and tolerant strains on the medium containing various concentrations of acetic acid. *A. syzygii* SKU19 and *A. pasteurianus* SKU123 were precultured in potato broth and incubated at 30°C for 18-24 h. The 1% inoculum was inoculated into glucose free-SCM broth containing 0, 0.5, 1.0, 1.5 and 2.0% acetic acid as shown in Figure 16A and 16B.

In order to confirm the sensitivity and tolerancy to acetic acid in *A. syzygii* SKU19 and *A. pasteurianus* SKU123, respectively, both strains were precultured in SCM broth containing various concentrations of acetic acid. The culture was collected when their growth was until log phase. Then, this culture broth was spreaded onto SCM agar with and without 1.0% acetic acid. The viable counts of those strains were shown in Figure 17. It was found that *A. syzygii* SKU19 is sensitive to acetic acid but can adapt to survive on the SCM agar containing 1.0% acetic acid if it has been precultured in SCM broth containing 0.5 and 1.0% acetic acid. In contrast, preculture in medium containing various concentrations of acetic acid for *A. pasteurianus* SKU123 was not necessary. This strain tolerates acetic acid without any adaptation. However, it seemed to be that this strain contains a mixed population of acetic acid sensitive and tolerant strains. This was due to the fact that only 0.2% of viable count on SCM agar containing 1.0% acetic acid was observed when it was precultured in SCM broth without 0% acetic acid.

(A)



(B)

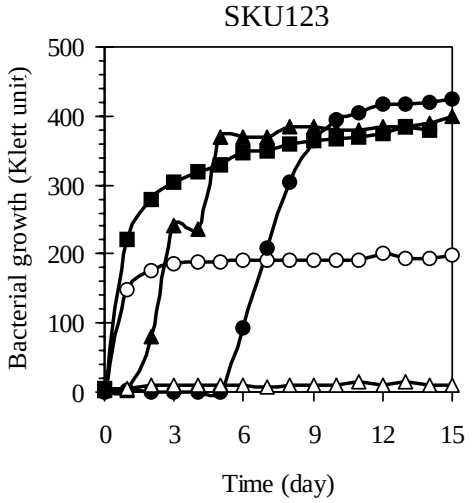


Figure 16 Time-course of growth of acetic acid sensitive (A) and tolerant strains (B) in glucose free-SCM broth containing various concentrations of acetic acid. Both strains were precultured in potato broth, at 30°C for 18-24 h. The 1% inoculum was inoculated in glucose free-SCM broth containing 0 (○), 0.5 (■), 1.0 (▲), 1.5 (●) and 2.0 (△) % (v/v) of acetic acid, and incubated at 30°C, 200 rpm.

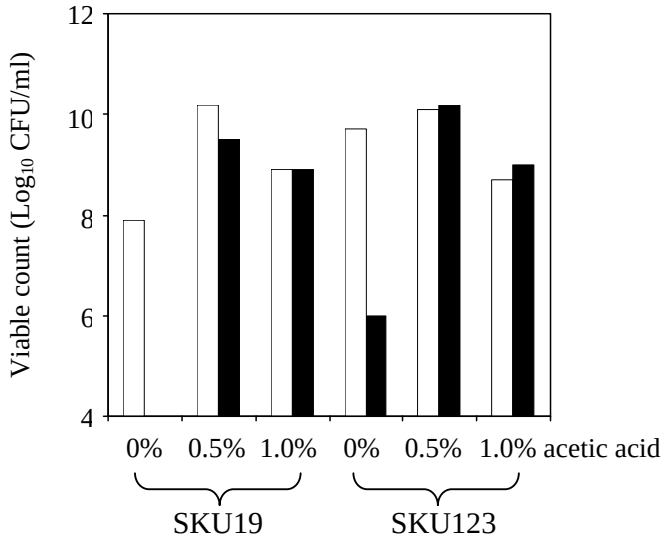


Figure 17 Viable count of acetic acid sensitive strain, *A. syzygii* SKU19, and acetic acid tolerant strain, *A. pasteurianus* SKU123 on SCM agar without (□) or with (■) 1.0% (v/v) acetic acid.

2.2 Acetate overoxidation

Acetate oxidation, which causes the loss of acetic acid in the medium, was observed in many strains; especially *Acetobacter* sp. which oxidizes acetic acid to carbon dioxide and water to increase cell biomass. This was an undesirable phenomenon, so called "acetate overoxidation" (Saeki *et al.*, 1997b, 1999). To examine the ethanol and acetate oxidation ability in *A. syzygii* SKU19 and *Acetobaceter* sp. SKU123, both of them were grown in SCM broth containing 2.0% ethanol at 30°C, 200 rpm.

Growth of *A. syzygii* SKU19 and *A. pasteurianus* SKU123 in glucose free-SCM broth containing 2.0% ethanol were shown in Figure 18. *A. syzygii* SKU19 showed a phasic growth curve whereas *A. pasteurianus* SKU123 showed a typical biphasic growth curve. The first growth phase was accompanied with ethanol oxidation and the second growth phase was accompanied with acetate oxidation of

A. syzygii SKU19 could not start acetate overoxidation phase. It still in remained a longer stationary phase period. For *A. pasteurianus* SKU123 started overoxidation immediately after ethanol conversion into acetic acid, and thus almost no stationary phase occurred. The first growth phase of *A. syzygii* SKU19 and *A. pasteurianus* SKU123, viable cell number measured as the colony forming unit (CFU/ml) increased during the ethanol oxidation phase and reached a maximum in the early stationary phase. Further, viable cell number of *A. syzygii* SKU19 decreased. It could not survive. In contrast, the viable cell number of a tolerant strain *A. pasteurianus* SKU123 increased during the acetate overoxidation due to the ability to immediately utilized acetic acid after acetic acid production. In *Acetobacter* sp., variations in the growth phase-pattern are different from species to species such as 1) some strains do not have appreciable stationary phase because acetic acid overoxidation start during the ethanol oxidation phase; they immediately oxidize acetic acid after production or 2) some strains do not have appreciable overoxidation so it remains in the stationary phase for a longer period of time or 3) several species exhibit a stationary phase intermediate between case 1 and case 2 (Matsushita *et al.*, 2005a).

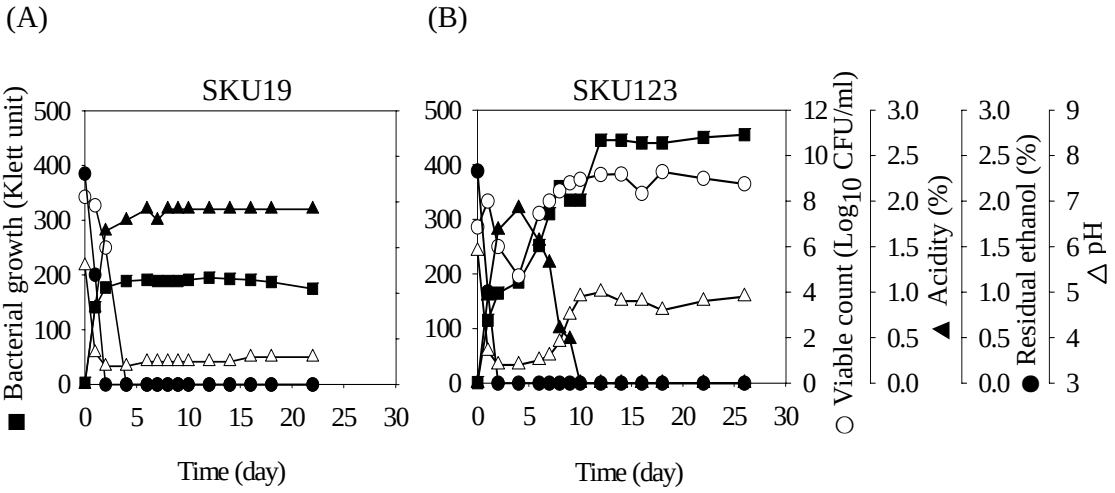


Figure 18 Time-course of growth and acetate oxidation of *A. syzygii* SKU19 (A) and *A. pasteurianus* SKU123 (B) in glucose free-SCM broth containing 2.0% (v/v) ethanol. Both strains were precultured in potato broth incubated at 30°C, 200 rpm. The 1% inoculum was cultivated in glucose free-SCM containing 2.0% (v/v) ethanol. Bacterial growth (■), viable count (○), acidity (%) (▲), residual ethanol (%) (●) and pH (△).

Recently, Matsushita *et al.* (2005a) studied the change of *A. aceti* IFO3284 cell viability in ethanol culture by using fluorescent microscopy technique. The results clearly showed that active cell and inactive cell numbers were decreased and increased, respectively, concominant with the accumulation of acetic acid, while large portions of the cell exhibited an intermediate color during the stationary phase. However, the number of active cells turned to increase and the number of intermediate cells decreased after the initiation of acetate overoxidation. In contrast, the numbers of inactive cells were very low in the ethanol oxidation phase, but increased in the mid stationary phase. These results indicated that despite increasing number of inactive cells or dead cells with accumulation of acetic acid, the numbers of the adapted cells were also increased. Therefore, this result showed clearly that the adaptation to acetic acid increased during the stationary phase. Both types of cells, adapted and unadapted to acetic acid appear during the ethanol oxidation phase,

indicating that despite the increasing cell number of inactive cells with the accumulation of acetic acid the ratio of adapted cells to acetic acid also increased.

A. syzygii SKU19 and *A. pasteurianus* SKU123 did not exhibit acetate overoxidation phenomenon in glucose free-SCM broth containing 4.0% ethanol (not show data). Both strains could only oxidize ethanol to acetic acid but acetic acid was not further oxidized to carbon dioxide and water. These results corresponded well with the viable count. The viable count increased to the maximum level in the ethanol oxidation phase and decreased in late ethanol oxidation until stationary phase. In the culture medium containing 4.0% ethanol, *A. syzygii* SKU19 lost its viability because it could only oxidized 4.0% ethanol to 1.8% acidity and 2.2% ethanol was still remained. This ethanol concentration inhibited cell growth and acetic acid was toxic to the cells. Therefore, these concentrations of both ethanol and acetic acid may be the critical concentration to inhibit growth of this strain. Similarly, *A. pasteurianus* SKU123 oxidized 4.0% ethanol to 3.2% acidity and 0.8% ethanol was remained. The growth of this strain was also inhibited by the above concentration of ethanol and acetic acid.

3. Isolation and Characterization of Acetic Acid Adapted Strains

3.1 Isolation of acetic acid adapted strains

Isolation of acetic acid adapted strains was performed by sequential cultivations of acetic acid sensitive strain, *A. syzygii* SKU19, in the SCM broth containing 1.0% acetic acid. The inoculum of this *A. syzygii* SKU19 was cultured in SCM broth containing 1.0% acetic acid and incubated at 30°C, 200 rpm for 7 days. Then, this culture broth was transferred to a fresh SCM broth containing 1.0% acetic acid. This culture condition was repeated 8 times and six acetic acid adapted strains (no. 108, 112, 187, 217, 258 and 264) were isolated. All of acetic acid adapted strains were further characterized.

3.2 Growth of acetic acid adapted strains on the medium containing various concentrations of acetic acid

To determine acetic acid response in acetic acid adapted strains, they were precultured in potato broth containing 1.0% acetic acid and incubated at 30°C for 4 days and then 5 μl of the preculture was inoculated on SCM agar containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) of acetic acid and incubated at 30°C for 5-10 days. All of the acetic acid adapted strains can grow very well in SCM agar containing 0.5, 1.0, 1.5 and 2.0% acetic acid, especially no. 108, 217 and 258 can grow very well even on SCM agar containing 2.0 and 2.5% acetic acid (Figure 19, Table 8).

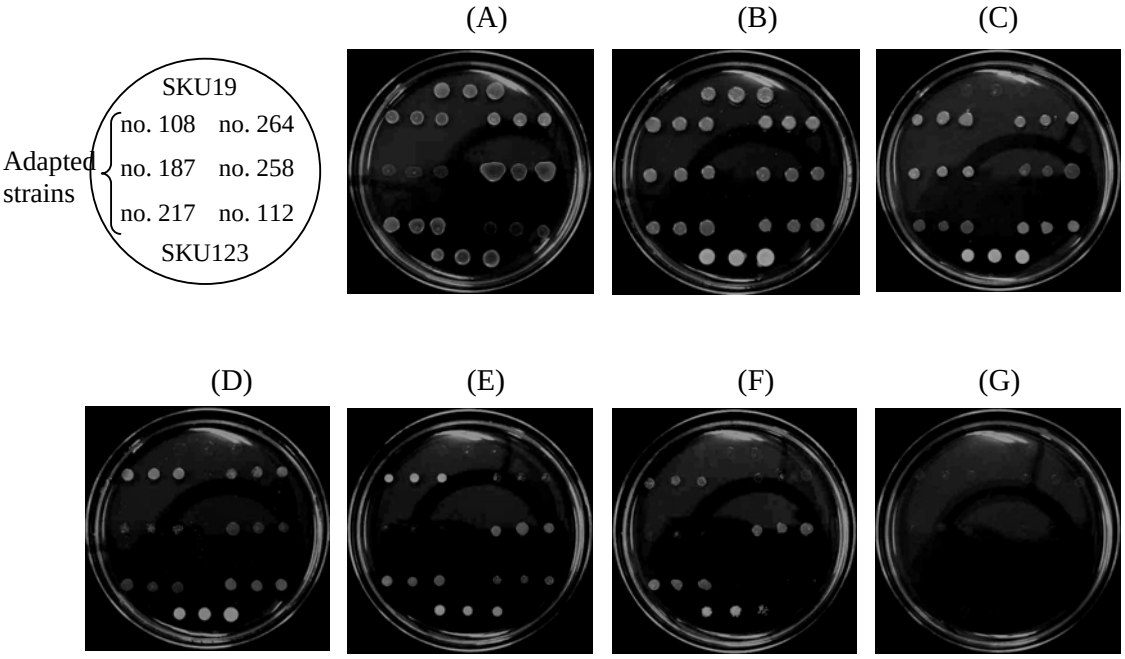


Figure 19 Growth of acetic acid adapted strains from *A. syzygii* SKU19 on SCM agar containing various concentrations of acetic acid; 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 2.0 (E), 2.5 (F), and 3.0% (G) (v/v) acetic acid. All strains were precultured in potato broth containing 1.0% acetic acid and incubated at 30°C for 4 days. 5 μl of the preculture was inoculated on SCM agar containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) of acetic acid and incubated at 30°C for 5 days.

Table 8 The growth of acetic acid adapted strains derived from *A. syzygii* SKU19 on SCM agar containing various concentrations of acetic acid.

Strains	Acetic acid concentrations						
	0%	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%
Acetic acid tolerant strain							
A. pasteurianus SKU123	+++	+++	+++	+++	+++	-	-
Acetic acid sensitive strain							
A. syzygii SKU19	+++	+++	+/-	-	-	-	-
Acetic acid adapted strains							
No. 108	+++	+++	+++	+++	+++	++	+
No. 112	+	+++	+++	+++	+++	-	-
No. 187	+	+++	+++	+++	-	-	-
No. 217	+++	+++	+++	+++	+++	+++	++
No. 258	+++	+++	+++	+++	+++	+++	++
No. 264	+++	+++	+++	+++	+++	-	-

Notes: -, no growth; +, low growth; ++, medium growth; +++, high growth.

In order to investigate that adapted strains possess acetic acid resistance, they were precultured in potato broth containing 1.0% acetic acid. After incubation at 30°C for 4 days, the culture broth was spreaded on SCM agar containing various concentrations of acetic acid. The viable count obtained was shown in Table 9. The viable count of adapted strains were decreased when they were grown on SCM agar containing 0, 1.0, 1.5, 2.0, 2.5, and 3.0% acetic acid, respectively. However, some adapted strains could grow at high concentration of acetic acid so the population of the adapted strain seemed to be a mixed population between sensitive and resistant strains.

Table 9 Viable count of acetic acid adapted strains derived from *A. syzygii* SKU19 on SCM agar containing various concentrations of acetic acid.

Strains	Viable count (CFU/ml)				
	0%	1.0%	2.0%	2.5%	3.0%
Acetic acid tolerant strain *A. pasteurianus* SKU123	$2.9x10^7$	$8.6x10^6$	$8.9x10^3$	0	0
Acetic acid sensitive strain *A. syzygii* SKU19	$4.8x10^8$	10	0	0	0
Acetic acid adapted strains					
No. 108	$1.1x10^9$	$7.1 x10^8$	$8.7x10^6$	$3.9x10^4$	0
No. 112	$6.5x10^8$	$4.5x10^8$	$1.2x10^8$	0	0
No. 187	$1.1x10^9$	$1.4x10^9$	$1.8x10^8$	0	0
No. 217	$1.4x10^9$	$1.0x10^6$	$1.0x10^6$	$1.0x10^3$	0
No. 258	$1.5x10^9$	$1.0x10^6$	$1.0x10^6$	$1.0x10^4$	0
No. 264	$2.7x10^9$	$1.4x10^9$	$3.1x10^7$	$1.0x10^3$	0

The resistance to acetic acid and ethanol are crucial factors to determine the fermentation activity of the acetic acid bacteria, but very little has been revealed about their mechanism. The results showed that the parent strain ceased to grow at 1.0% (v/v) acetic acid while all acetic acid adapted strains were able to grow at higher than 1.0% (v/v) acetic acid. Therefore, six acetic acid adapted strains were clearly more resistant than their wild type strain. Thus, the acquired acetic acid resistance appeared to be an inheritable phenotype acquired by mutation that is more stable than would be expected for a transient physiological adaptation. If the acquired acetate resistance resulted from a transient physiological adaptation, which would be a regulatory phenomenon analogous to the adaptive pH response of certain bacteria, one would expect a rapid loss of resistance upon cultivation under non-inducing conditions. Steiner and Sauer (2003) showed that the evolved culture was harvested at 30 g/l acetate for several generations under non-selective conditions in YPD medium without acetate supplementation. After 15 generations of nonselective

cultivation, resistance to acetate concentrations exceeding 30 g/l was lost, while resistance to lower acetate concentrations was still significantly higher than in the wild type strain. In principle, the acquired resistance of *A. aceti* may result from physiological or genetic (evolutionary) adaptation. While the first is transient in nature, as seen for example, in low pH adaptation, the latter is a more stable, inheritable feature. However, these adapted strains were isolated from short-term sequential cultivations in acetic acid. Therefore, the resistance to acetic acid of adapted strain was probably the result of a transient physiological adaptation not genetic evolutionary adaptation which may be exclude the possibility of a mixed population with co-existing resistant and sensitive cells. This was similar to the previously reported short-term conditioning to acetic acid that occurred within three generations (Steiner and Sauer, 2001). In contrast, Steiner and Sauer (2003) reported that their evolved *A. aceti* seems to result from an evolutionary adaptation because the acquired acetate resistance persists for more than 15 generations without selective pressure. The generation dependent reduction of resistance may be due to the genetic instability of acetic acid bacteria. It appears that acetic acid adapted strains exhibit a relatively short-term physiological adaptation to acetate that confer moderate resistance by activation of the acetate adaptation regulon, while high-level resistance requires genetic evolution.

3.3 Growth of acetic acid adapted strains in the medium containing various concentrations of acetic acid

Six of acetic acid adapted strains were studied for their growth in the glucose free-SCM broth containing various concentration of acetic acid as shown in Figure 20. Almost all of the adapted strains could grow very well in glucose free-SCM broth containing 1.0% to 2.5% (v/v) acetic acid except the adapted strain no. 217 which could grow in the same medium containing 2.5% acetic acid. The obtained results clearly showed that acetic acid adapted strains exhibited enchanced acetic acid toleration.

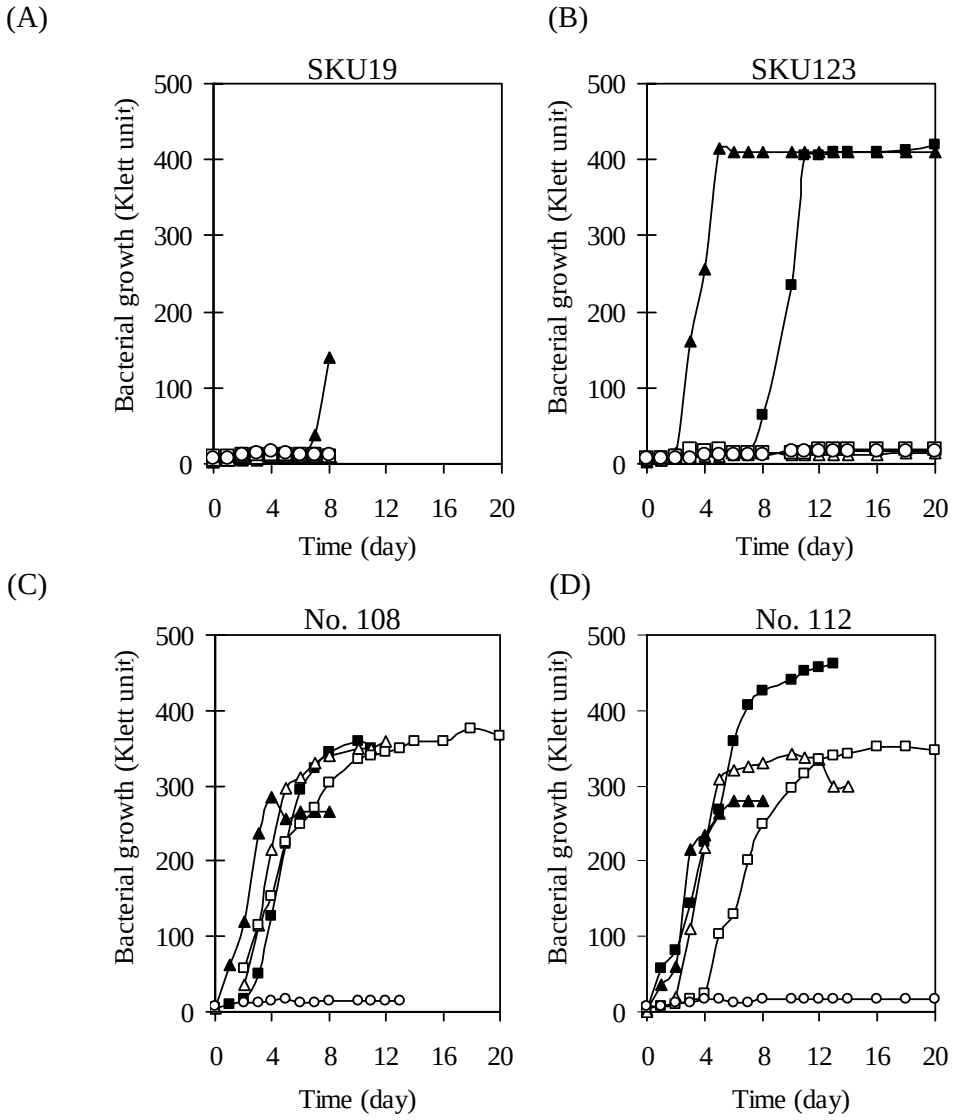


Figure 20 Growth of acetic acid adapted strains from *A. syzygii* SKU19 in glucose free-SCM broth containing various concentrations of acetic acid. *A. syzygii* SKU19 (A), *A. pasteurianus* SKU123 (B) and adapted strains from *A. syzygii* SKU19 no. 108 (C), 112 (D), 187 (E), 217 (F), 258 (G), and 264 (H). All strains were precultured in potato broth containing 1.0% acetic acid, incubated at 30°C for 4 days. 1% of the preculture was inoculated in glucose free-SCM broth containing 1.0 (▲), 1.5 (■), 2.0 (△), 2.5 (□) and 3.0 (○) % (v/v) of acetic acid, and incubated at 30°C, 200 rpm.

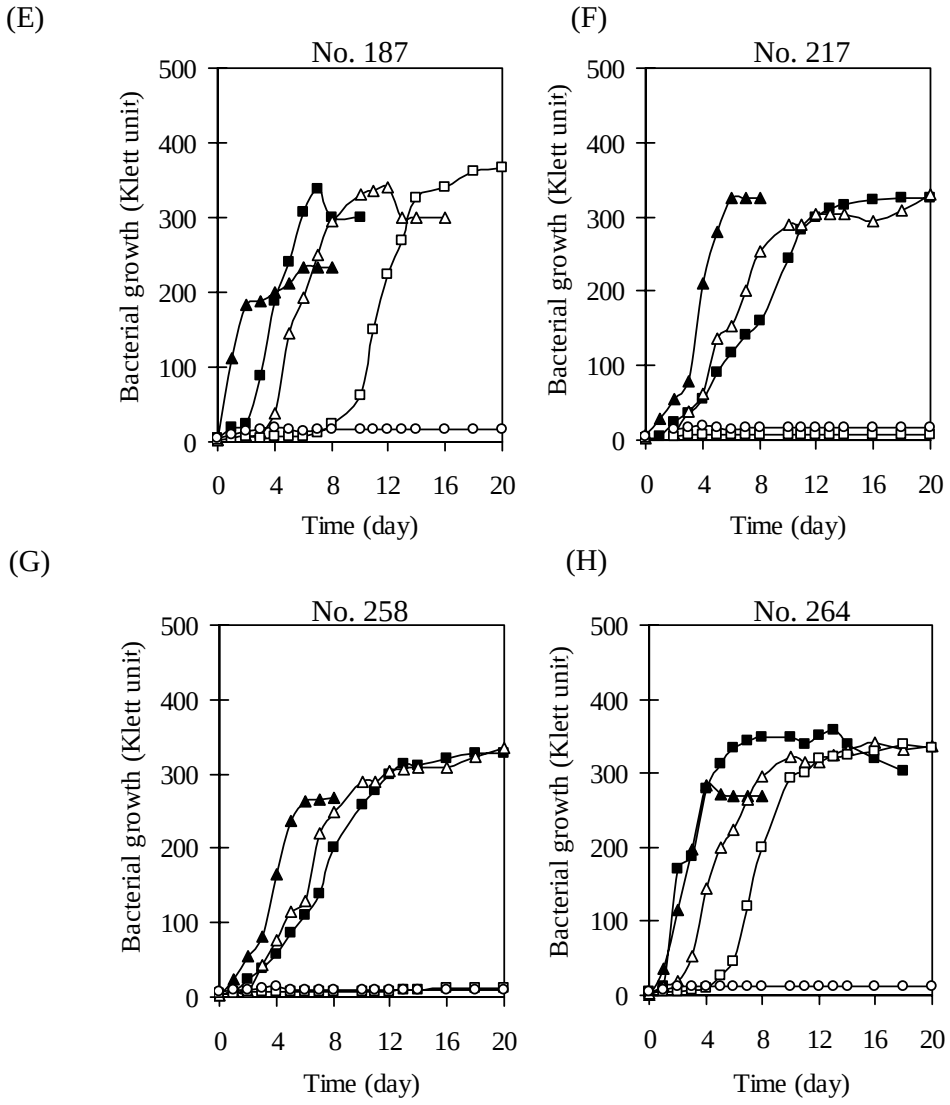


Figure 20 (Continued)

3.4 Acetate overoxidation of acetic acid adapted strains

The ethanol oxidation and acetate overoxidation were studied in order to determine the ethanol oxidation ability and acetic acid resistant ability of adapted strains. They were cultured in potato broth containing 1.0% acetic acid and incubated at 30°C for 4 days. The 1% inoculum was inoculated in glucose free-SCM broth containing 2.0% ethanol as shown in Figure 21. The growth and ability to oxidize acetate were compared between unadapted *A. syztgii* SKU19 strain and adapted strains, the results were categorized into 2 groups. The first group was those with increased cells and overoxidation or rapid acetate oxidizer, which included the adapted strains no. 108 and 187. The second group was those with increased cells and increased stability to acetate or slow acetate oxidizer, which included the adapted strains no. 217, 112, 258, and 264. They can oxidize ethanol at 2.0% and can slowly oxidize acetate to carbondioxide and water as the unadapted *A. syzygii* SKU19. The result accords to the report of Matsushita *et al.* (2005a) however, there are some variations in the growth phase-pattern which are different from species to species.

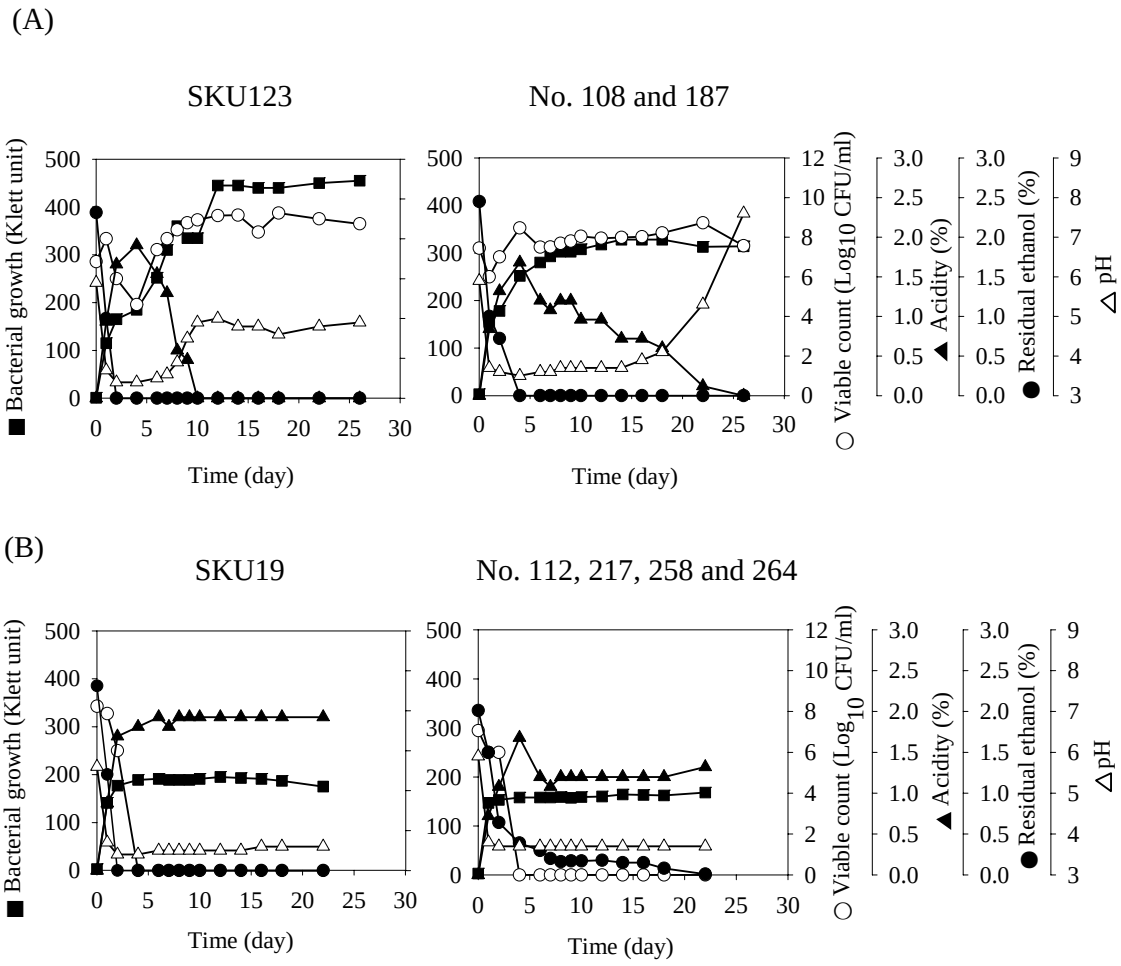


Figure 21 Time-course of the growth and acetate oxidation of acetic acid adapted strains from *A. syzygii* SKU19 in glucose free-SCM broth containing 2.0% (v/v) ethanol. Rapid acetate oxidizer (*A. pasteurianus* SKU123, no. 108 and 187, A) and slow acetate oxidizer (*A. syzygii* SKU19, no. 112, 217, 258 and 264, B). Bacterial growth (■), viable count (○), acidity (%) (▲), residual ethanol (%) (●), and pH (△).

3.4 Enhancement of alcohol and aldehyde dehydrogenase activities, heme staining and immunoblotting analysis in acetic acid adapted strains

Many researchers are interested in the mechanism of acetic acid resistance, which has been studied for a long time because many physiological questions remain to be solved regarding acetic acid production and the resistance to acetic acid of acetic acid bacteria. Several ideas have been proposed on this resistance mechanism. One of proposed idea was the relationship between acetic acid resistance and some functions of alcohol dehydrogenase (ADH). Ohmori *et al.* (1982) and Takemura *et al.* (1991) reported that ADH-deficient mutants of *A. aceti* 1031 showed sensitivity to acetic acid. Moreover, when an ADH gene of *Acetobacter* sp. was introduced to *A. pasteurianus* NP2503, the transformant were resistance to acetic acid. Chinnawirotpisan *et al.* (2003b) showed that the PQQ-ADH deficient mutant obtained from thermotolerant strain, *A. pasteurianus* SKU1108, by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment and *adhA* gene disruptant exhibited a complete loss in ethanol oxidation and acetic acid resistance. In these mutants, unlike the wild strain, growth was observed in medium containing 0.5% acetic acid but with a delay of 2-3 days, and no growth occurred in the presence of 1.0% acetic acid.

Acetic acid resistance is a crucial factor for acetic acid bacteria to stably produce large amount of acetic acid. Acetic acid is produced by a typical periplasmic oxidation system. All acetic acid bacteria except for the genus *Asaia* contain unique NAD(P)-independent, membrane-bound ADH and ALDH as the primary dehydrogenase responsible for ethanol oxidation. Membrane-bound ADH, which catalyzes the first step of ethanol oxidation, is a quinohemoprotein-cytochrome *c* complex bound to the periplasmic side of the cytoplasmic membrane. The next step, ALDH catalyzes the oxidation from aldehyde to acetic acid. The ethanol oxidation is linked with respiratory chain reaction in order to generate energy for cell growth. Acetic acid production is closed relationship between acetic acid resistance and some functions of ADH. To determine the relationship between acetic acid resistance and ADH and ALDH activities of acetic acid resistance strain, acetic acid adapted strains

were cultured in glucose free-SCM broth containing 2.0% ethanol and harvested in the first stationary phase as a cell resistant to acetic acid. The membrane fraction of adapted strains was prepared and then, both enzymes were measured by the method of Adachi *et al.* (1978). The results are shown in Figure 22. The specific activities of ADH and ALDH of acetic acid adapted strains were higher than those of *A. syzygii* SKU19 especially adapted strains no. 112 and 187. The result was confirmed by heme staining which showed higher intensity of ADH in acetic acid adapted strains than *A. syzygii* SKU19 which was the same as in *A. pasteurianus* SKU123 (Figure 23). From the results, it seems that acetic acid adapted strains were more resistance to acetic acid than the wild type. The results were also supported by growth of acetic acid adapted strains in glucose free-SCM broth containing various concentrations of acetic acid.

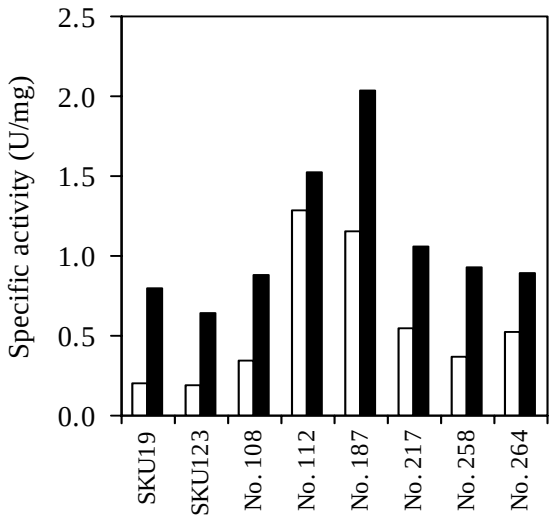


Figure 22 Enzyme activities of alcohol (ADH) and aldehyde dehydrogenase (ALDH) in membrane fraction of acetic acid adapted strains from *A. syzygii* SKU19. ADH (□) and ALDH (■). All strains were cultured in glucose free-SCM broth containing 2.0% (v/v) ethanol, and incubated at 30°C, 200 rpm. The cells were harvested from stationary-phase growth.

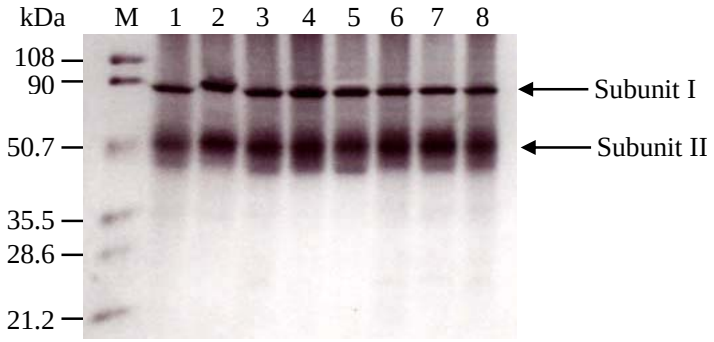
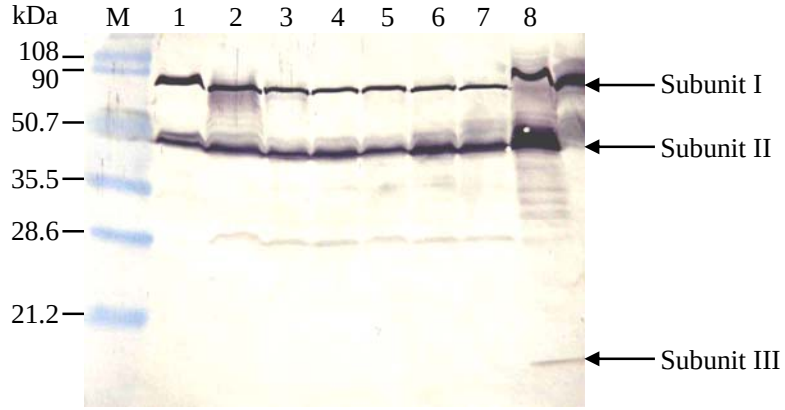


Figure 23 Heme staining of acetic acid adapted strains from *A. syzygii* SKU19. Lane M = standard protein marker, 1 = *A. syzygii* SKU19, 2 = *A. pasteurianus* SKU123, 3 to 8 = acetic acid adapted strains from *A. syzygii* SKU19, no. 108, 112, 187, 217, 258, and 264, respectively. All acetic acid adapted strains were precultured in potato broth containing 1.0% acetic acid, incubated at 30°C for 4 days. The 1% inoculum was inoculated in glucose free-SCM broth containing 2.0 % (v/v) ethanol, and incubated at 30°C, 200 rpm. The cells were harvested from stationary-phase growth. 100 μg of total protein from membrane fraction was loaded in each lane.

The localization of PQQ-ADH in *A. syzygii* SKU19 and acetic acid adapted strains was also determined by using immunoblotting analysis with both membrane and soluble fractions of *A. syzygii* SKU19 and acetic acid adapted strains. The positive bands of PQQ-ADH subunits were performed by using anti-sera of PQQ-ADH purified from *A. aceti*. Two of three subunits of PQQ-ADH were found in the membrane and soluble fractions of wild strain and adapted strains, no significantly appreciable bands for subunit I and II were detected in the membrane and soluble fraction of adapted strains. Interestingly, subunit III were not found both in the membrane and soluble fractions of *A. syzygii* SKU19 and adapted strains (Figure 24). Therefore, in the further study, three *adh* genes, *adhA*, *adhB* and *adhS* encoding for PQQ-ADH subunit I, II and III, from *A. syzygii* SKU19 were cloned and analyzed for nucleotide sequences.

(A)



(B)

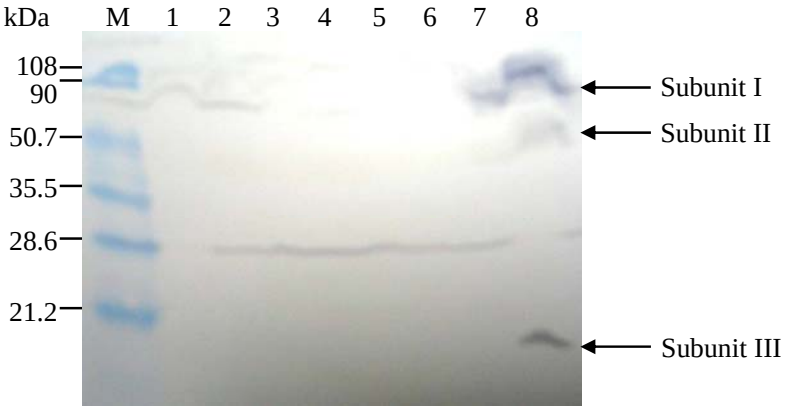


Figure 24 Immunoblot analysis of membrane (A) and soluble fractions (B) prepared from acetic acid adapted strains of *A. syzygii* SKU19. Lane M = standard protein marker, 1 = *A. syzygii* SKU19, 2 to 7 = acetic acid adapted strains from *A. syzygii* SKU19, no. 108, 112, 187, 217, 258, and 264, respectively, 8 = *A. pasteurainus* SKU1108. All acetic acid adapted strains were precultured in potato broth containing 1.0% acetic acid, incubated at 30°C for 4 days. 100 μg (A) and 300 μg (B) of total protein from membrane and soluble fractions were loaded in each lane.

3.6 Comparison of plasmid profiles, randomly amplified polymorphic DNA (RAPD) and distribution of IS element

The study of plasmid was important for understanding the genetic background of acetic acid bacteria because there were suggested to be responsible for their genetic variability. Most of them contained many plasmids ranging from 1 to over 17 megadaltons in size. In order to determine that those plasmids are involved in acetic acid adaptation or not, comparison of plasmid profiles were performed. The plasmid DNA was isolated from the cells grown in potato broth containing 1.0% acetic acid by the alkaline lysis method. The plasmid profiles were shown in Figure 25 and no obvious difference in plasmid patterns between acetic acid adapted strains and *A. syzygii* SKU19 were detected. The results implicated that there was no relation between plasmid profiles and acetic acid adaptation.

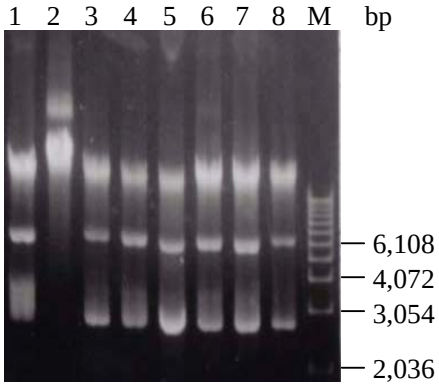
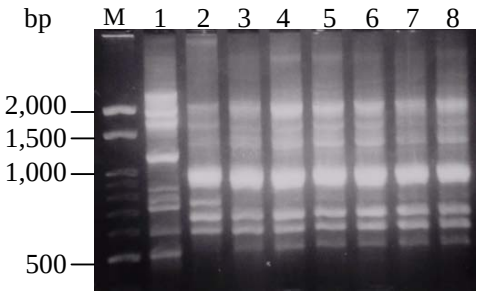


Figure 25 Plasmid profiles of genomic DNAs from acetic acid adapted strains *A. syzygii* SKU19. Lane M = 1 kb ladder DNA marker, 1 = *A. syzygii* SKU19, 2 = *A. pasteurianus* SKU123, 3 to 8 = acetic acid adapted strains from *A. syzygii* SKU19; no. 108, 112, 187, 217, 258, and 264, respectively.

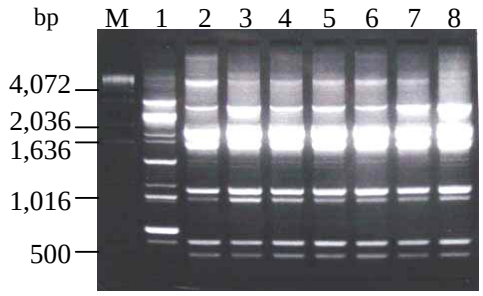
In addition to the reported relationship between plasmids and genetic variability, many researchers have revealed that multiple insertion sequences play a major role in genetic instability leading to deficiencies in various physiological properties. To investigate the possible change of DNA sequence involved in acetic acid adaptation in acetic acid adapted strains, their genomic DNAs were examined for the deficiency of some genetic element (s) by randomly amplified polymorphic DNA (RAPD). The results were shown in Figure 26. The RAPD analysis did not show any obvious difference in genetic background so these acetic acid adapted strains might not contain any insertion sequences in their genomic DNA.

In addition to RAPD analysis, the distribution of one insertion sequence, IS*1380*, was investigated by Southern hybridization with IS*1380* probe. The chromosomal DNA was isolated and digested with *Hinc*II and Southern hybridization was performed by using the 1.1-kb *Eco*RI-*Bgl*II fragment carrying IS*1380* sequence. The results indicated that both *A. syzygii* SKU19 and its acetic acid adapted strains did not contain IS*1380* in their chromosome (Figure 27).

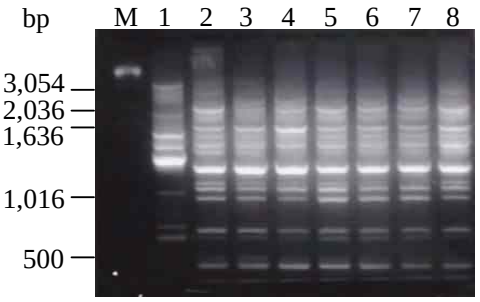
(A) 80% G+C primer



(B) 70% G+C primer



(C) $(GTG)_{5x}$ primer



(D) AD01 primer

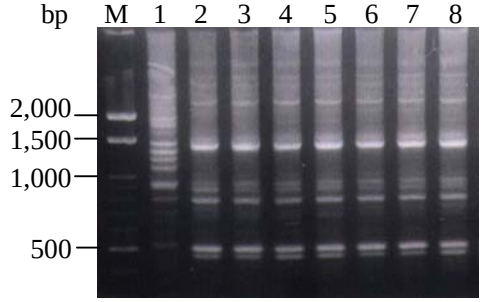


Figure 26 RAPD profiles of genomic DNAs from acetic acid adapted strains from *A. syzygii* SKU19 with 80%G+C (A), 70% G+C (B), $(GTG)_{5x}$. (C) and AD01 (D) primer. Lane M = 1 kb ladder DNA marker; 1 = *A. pasteurianus* SKU123, 2 = *A. syzygii* SKU19, 3 to 8 = acetic acid adapted strain from *A. syzygii* SKU19; no. 108, 112, 187, 217, 258, and 264, respectively.

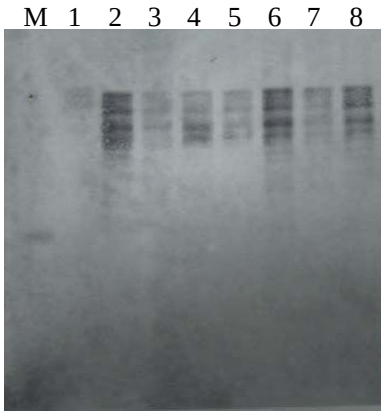


Figure 27 Southern hybridization of *Hinc*II-digested genomic DNAs from acetic acid adapted strains of *A. syzygii* SKU19 with IS*1380* DNA probe. Lane M = 1 kb ladder DNA marker, 1 = *A. pasteurianus* SKU123, 2 = *A. syzygii* SKU19, 3 to 8 = acetic acid adapted strains from *A. syzygii* SKU19, no. 108, 112, 187, 217, 258, and 264, respectively.

4. Amplification and Cloning of Alcohol Dehydrogenase Genes (*adhA*, *adhB*, and *adhS* gene) from *A. syzygii* SKU19

4.1 Amplification of *adhAB* gene by Polymerase Chain Reaction (PCR)

In order to determine the function of PQQ-ADH from *A. syzygii* SKU19, two DNA primers were designed for *adhAB* gene amplification from nucleotide sequence alignment of *adhAB* gene from other acetic acid bacteria. The *adhAB* from chromosomal DNA of *A. syzygii* SKU19 was amplified by PCR method with the forward primer with *Hind*III linker (FadhAB-*Hind*III primer: 5'-ccaagcttggATGACC CGCCCCGCCTCC-3') and the reverse primer with *Sal*I linker (RadhAB-*Sal*I primer: 5'-cggtcgaccgTTACTGGGCTTCATCCAC-3') (Table 5). The PCR reaction was performed by using the Ready-To-Go™ PCR Bead. The size of desired PCR product was 3.8 kb as shown in Figure 28A. This PCR product was digested with *Bam*HI and two DNA fragments of 1.6 and 2.2 kb (Figure 28B) were observed similar to restriction map of *adhAB* from *A. pasteurianus* SKU1108.

The 3.8 kb DNA fragment was further cloned into pGEM®-T Easy vector system, which is a convenient system for the cloning of PCR products. The ligation mixture was introduced into *E. coli* DH5α competent cells by electroporation as mentioned in Materials and Methods.

Two recombinant clones were obtained and the recombinant plasmids were isolated by alkaline lysis method. One of them was further analyzed for the inserted DNA fragment by digested with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I and *Hind*III-*Sal*I. The digested DNAs were carrying analyzed in 0.8% agarose gel electrophoresis and the result was shown in Figure 29. This positive recombinant clone was designated as pGEM®-T*adhAB*3.8 carrying 3.8 kb DNA fragment in the opposite orientation from P*lac* as shown in Figure 30.

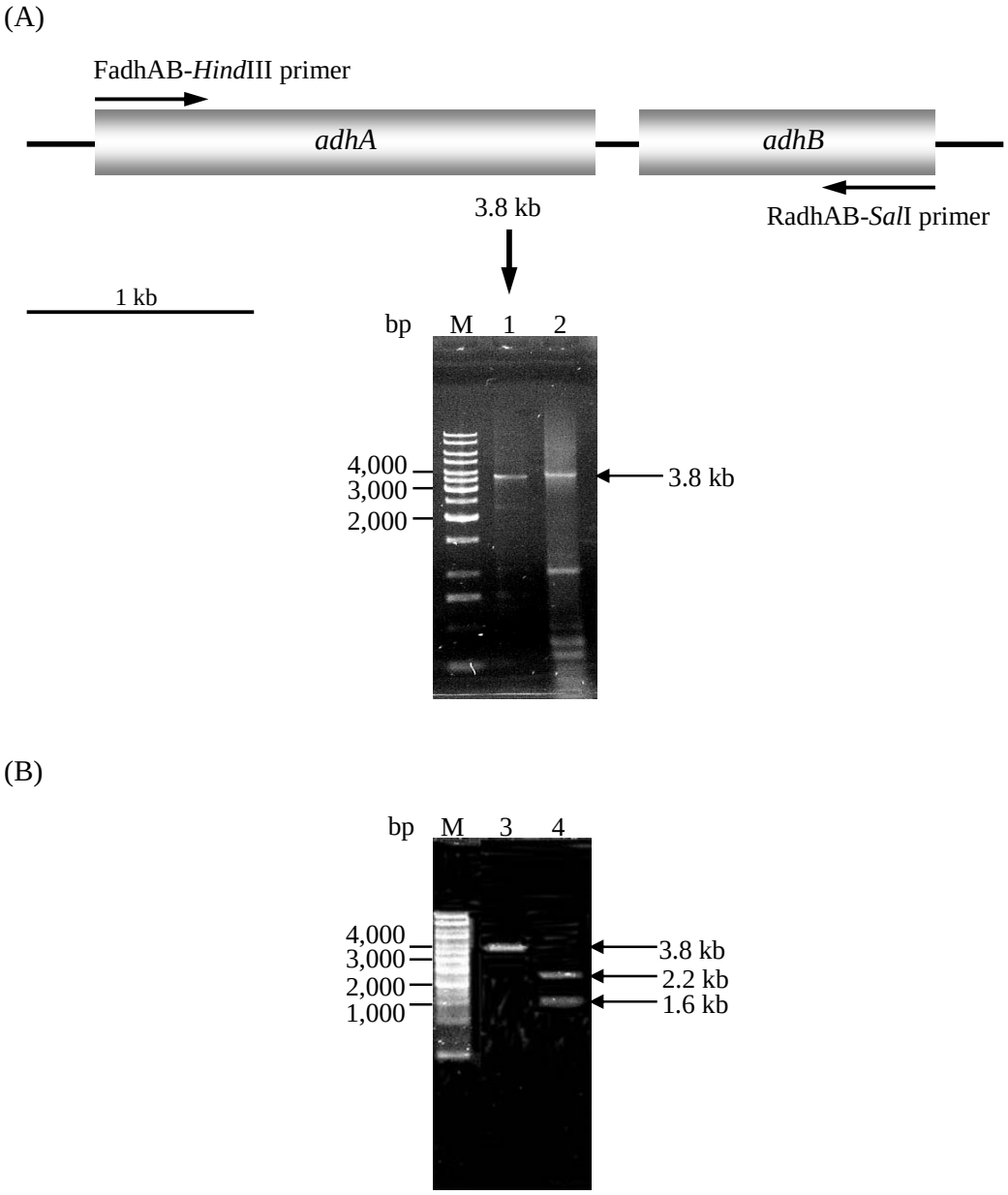


Figure 28 Agarose gel electrophoresis of 3.8 kb PCR product (A) and PCR product digested with *Bam*HI. Lane M = 1 kb DNA Ladder, 1 = 3.8 kb of *A. pasteurianus* SKU1108, 2 = 3.8 kb PCR product of *A. syzygii* SKU19, 3 = purified 3.8 kb PCR product and 4 = 3.8 kb PCR product digested with *Bam*HI.

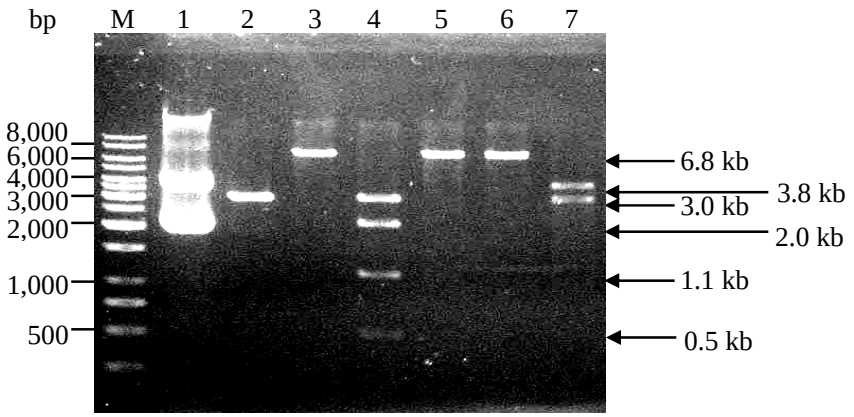


Figure 29 Agarose gel electrophoresis of 3.8 kb insert DNA fragment from the recombinant clone. Lane M = 1 kb DNA Ladder, 1 = pGEM®-T Easy vector, 2 = pGEM®-T Easy vector digested with *Eco*RI, 3 to 7 = pGEM®-T*adhAB*3.8 digested with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I and *Hind*III-*Sal*I, respectively.

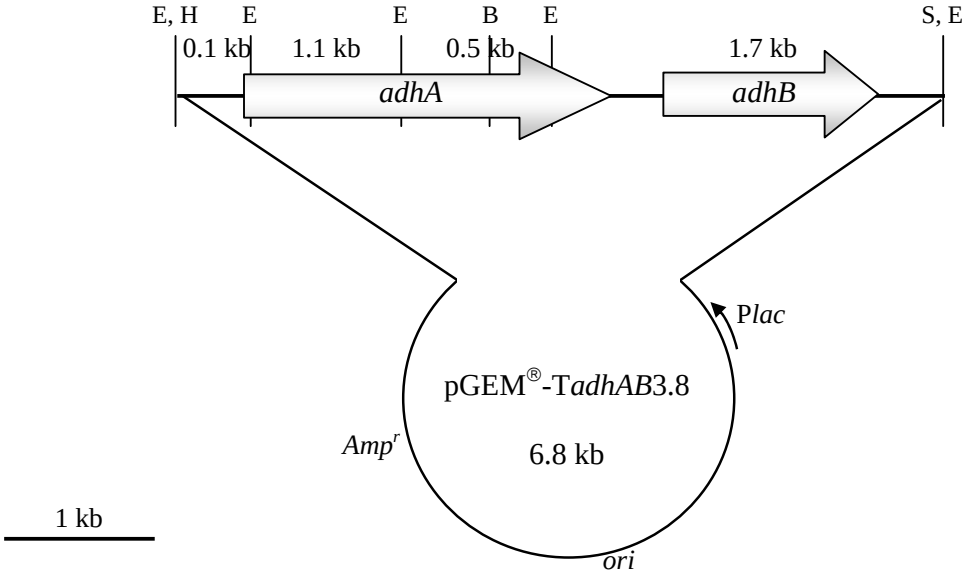
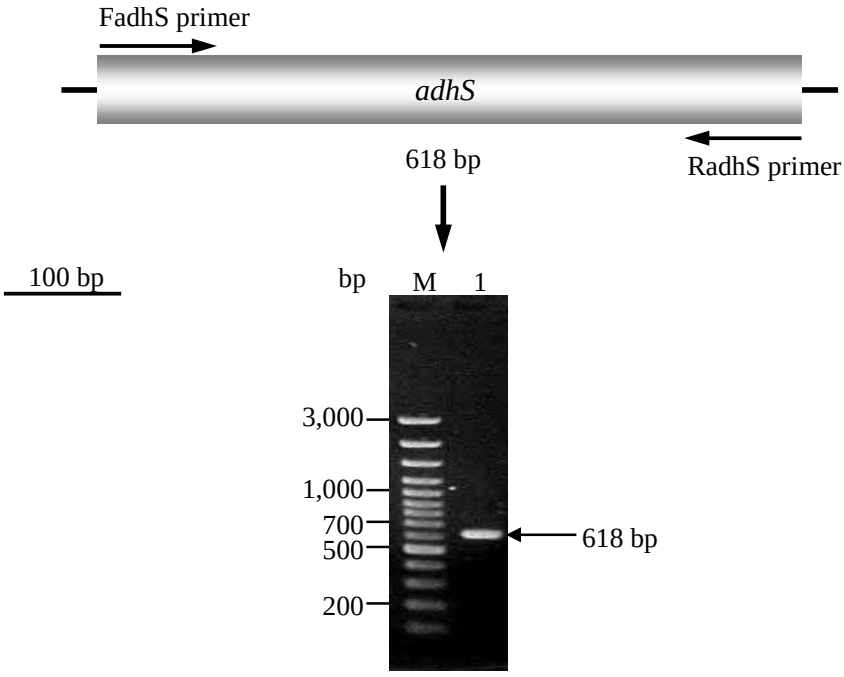


Figure 30 The structure of pGEM-T*adhAB*3.8. The 3.8 kb PCR product from *A. syzygii* SKU19 was cloned into pGEM®-T Easy vector system. B = *Bam*HI, E = *Eco*RI, H = *Hind*III and S = *Sal*I.

4.2 Amplification of *adhS* gene by Polymerase Chain Reaction (PCR)

For amplification of *adhS* gene, two specific DNA primers were designed from conserve region of *adhS* gene from *A. pasteurianus* NCI1193 and NCI1452. The nucleotide sequences of the forward primer (FadhS,18 mers) are 5'-ATGAAACTGATTGCCGTA-3' started at base pair number 1 and the reverse primer (RadhS, 18 mers) are 5'-TTACGAAACAGAACTGGT-3' started at base pair number 618 (stop codon). Chromosomal DNA from *A. syzygii* SKU19 was used as DNA template for PCR reaction which was performed by using the Ready-To-Go™ PCR Bead as previously described in Materials and Methods. The PCR product was analyzed in 0.8% agarose gel electrophoresis. The single band of 618 bp PCR product was compared to 100 bp DNA ladder marker as shown in Figure 31A. This 618 bp DNA fragment was cloned into pGEM®-T Easy vector and the ligation mixture was introduced into *E. coli* DH5α competent cells. The recombinant clone, designated as pGEM®-T*adhS* carrying 618 bp PCR product was selected from white colonies grown on the LB agar containing 50 μg/ml ampicillin and X-Gal. The recombinant plasmid was screened by size screening method and isolated by alkaline lysis method. The insert DNA was checked by digested with *Eco*RI and *Pst*I, and then analyzed in 0.8% agarose gel electrophoresis as shown in Figure 31B. The obtained 253 and 365 bp DNA fragments when pGEM®-T*adhS* was digested with *Eco*RI-*Pst*I indicated that *adhS* gene was inserted into pGEM®-T Easy vector in the opposite orientation from P*lac* (Figure 32)

(A)



(B)

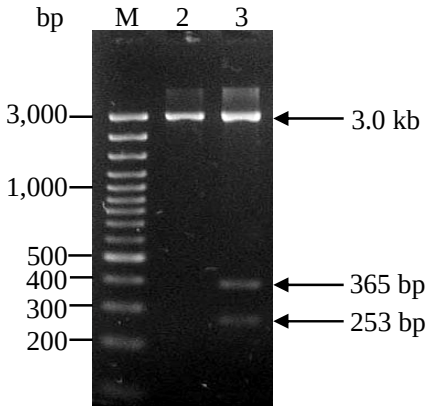


Figure 31 Agarose gel electrophoresis of 618 bp PCR product from *A. syzygii* SKU19 (A) and pGEM®-T*adhS* digested with *Eco*RI and *Pst*I (B). Lane M = 100 bp DNA ladder, 1 = 618 bp PCR product, 2 = pGEM®-T Easy vector/*Eco*RI, 3 = pGEM®-T *adhS*/*Eco*RI-*Pst*I.

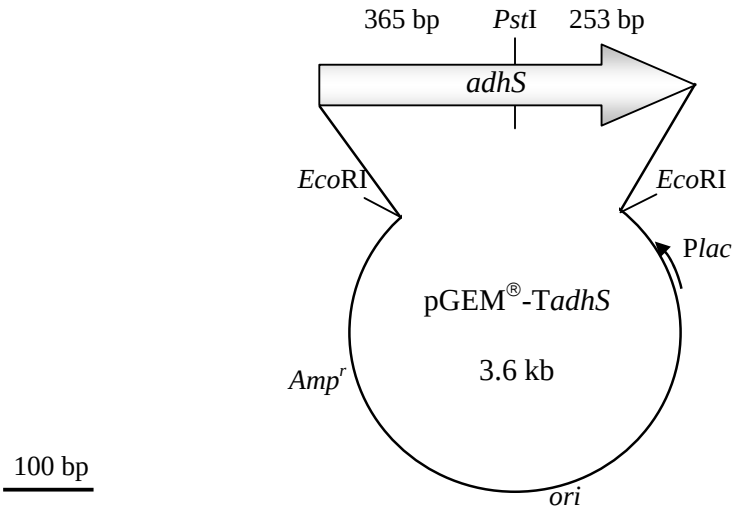


Figure 32 The structure of pGEM®-T*adhS* from *A. syzygii* SKU19. The 618 bp PCR product was cloned into pGEM®-T Easy vector in the opposite orientation from P*lac*.

4.3 Nucleotide sequencing and analysis of 3.8 kb *adhAB* gene

The nucleotide sequence of the pGEM®-T*adhAB*3.8 was determined by applying the chain termination method. The complete nucleotide sequence of 3.8 kb PCR product was obtained with the sequencing reaction using several primers as follows; universal T7, adhAB6F, uniADH$_F$, adhAB13F, adhAB23F, universal SP6, adhAB16R, adhAB22R, uniADH$_R$ and 1.2R1, respectively (Figure 33 and Table 10).

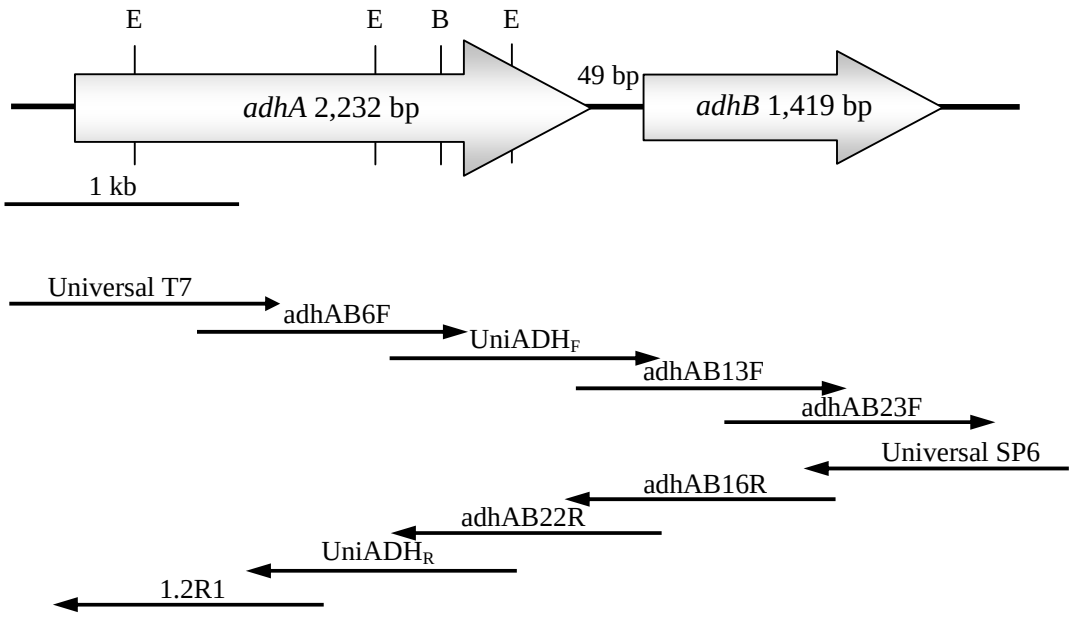


Figure 33 Sequencing strategy used to determine the nucleotide sequence of 3.8 kb PCR product.

Table 10 Sequencing primers used to determine the nucleotide sequences of 3.8 kb PCR product.

Sequencing primer	Length (bp)	Nucleotide sequence (5'---3')	Position of *adhAB* *A. syzygii* SKU19
Forward primer			
universal T7	20	-TAATACGACTCACTATAGGG-	-
adhAB6F	18	-CAAAGCCTACAAGACCTG-	774-791
uniADH$_F$	17	-TGGYWCGGYATYCCSGG-	1,261-1,277
adhAB13F	18	-CCTGCCCGTCAAGCCGCC-	1,872-1,889
adhAB23F	18	-GCAATCAAAACCCCCATC-	2,467-2,484
Reverse primer			
1.2R1	17	-AGTGCGACAATGCTGCC-	952-968
uniADH$_R$	20	-GTVGCGTCRTARGCRTGGAA-	1,618-1,637
adhAB22R	18	-TTTGATTGCCAGCCCACC-	2,461-2,475
adhAB16R	18	-CAGTTGGTAGGCGGCAGA-	3,396-3,413
universal SP6	18	-ATTTAGGTGACACTATAG-	-

The complete nucleotide sequences of 3.8 kb PCR product (3,699 bp) and deduced amino acid sequences are shown in Figure 34. Open reading frames (ORFs) analysis by using ORFs finder at URL: http://www.ncbi.nlm.nih.gov/gorf/gorf.himl. revealed two possible open reading frame (ORFs), designated as ORF1 and ORF2, corresponding to the dehydrogenase subunit and the cytochrome *c* subunit. The ORF1 corresponding to the dehydrogense subunit consisted of 2,232 bp, started at ATG initiation codon at position 1 and terminated at TAA stop codon at position 2,230 to 2,232. ORF1 of *adhA* gene from other acetic acid bacteria consist of 742 amino acid residues except *A. polyoxogenes* (Fukaya *et al.*, 1989), *A. europaeus* and *G. suboxydans* (Kondo and Horinouchi, 1997b) possess subunit I consist of 738, 739 and 757 amino acid, respectively. The hydropathy profile of ORF1 was predicted by using SOSUI www server. The deduced amino acid sequences and hydropathy profile of this ORF are shown in Figure 35A and 35B. The SOSUI signal indicated that the NH_2-terminal amino acid sequence at position 1 to 33 was a possible transmembrane region or signal peptide as illustrated in Figure 35B and 35C. This sequence of 33 amino acids has the typical characteristics of a signal sequence i.e., positively charged residues at the N-terminus as arginine and lysine, followed by an Ala-X-Ala sequence and a stretch of hydrophobic residues at C terminus. These 33 amino acid residues at NH_2-terminus served as a signal sequence for translocation of the mature dehydrogenase subunit across the cytoplasmic membrane. This finding agreed with the localization of this subunit in periplasm. The results from the Motif Scan (http://hits.isb-sib.ch/cgi-bin/PSFSCAN), the PPSearch: Protein functional analysis (www.ebi.ac.uk/ppsearch/), and the NCBI Consensus Domain Search (www.ncbi.nlm.nih.gov/Blast/structure) revealed that two putative pyrroloquinoline quinone (PQQ) binding motifs were observed at amino acid position 54 to 82 (NWLSYGRTYSEQRYSPLDQINRSNVGNLK) and 280 to 301 (DSIVYDPVSDL VYLAVGNGSP) and one heme *c* binding motif (C-X-X-H) at amino acid position 649 to 653 (CQTCH) as shown in Figure 35A. These motifs were previously found in the gene cluster encoding three subunits of membrane-bound alcohol dehydrogenase from *A. pasteurianus* SKU1108 (Tuasakul, 2004) and *G. suboxydans* (Kondo and Horinouchi, 1997b) and the genes encoding two-component membrane-bound alcohol

dehydrogenase from *A. polyoxogenase* (Tamaki *et al.*, 1991). One molecule of PQQ is thought to be bound by two PQQ-binding motifs. PQQ-binding motifs are also present in other quinoprotein dehydrogenases, such as glucose dehydrogenase and methanol dehydrogenase (Matsushita *et al.*, 1992a; Reid and Fewson, 1994; Toyama *et al.*, 2004). Therefore, the presence of these motifs supported the idea that this ORF1 was the dehydrogenase subunit of ADH. Homology search using BLASTP showed that this ORF was similar to subunit I of ADH of *A. aceti* K6033 (88%), *A. pasteurianus* SKU1108 (89%), *A. pasteurianus* NCI1193 (88%), *A. pasteurianus* NCI1380 (89%) and *A. polyoxogenase* (77%). Alignment of amino acid sequences of ORF1 with ADHI from other acetic acid bacteria is shown in Figure 36.

```
***ATG***ACCCGCC CCGCCTCCGC CAAGAGACGT TCGCTGCTAG GAATTCTCGC GGCTGGAACA 60
M  T  R  P     A  S  A     K  R  R     S  L  L  G     I  L  A     A  G  T
ATCTGTGCCG CTGCCCTACC TTACGCAGCA GCCCCTGCCC GCGCCGATAG TCAGGGTGAC 120
I  C  A  A     A  L  P     Y  A  A     A  P  A  R     A  D  S     Q  G  D
ACGGGACAAG CCGTCATCCA CGCTGATGAG CACCCCGAAA ACTGGCTGTC CTATGGTCGC 180
T  G  Q  A     V  I  H     A  D  E     H  P  E  N     W  L  S     Y  G  R
ACCTACTCTG AACAGCGCTA CAGCCCGCTG GATCAGATCA ACCGCTCCAA CGTTGGAAAC 240
T  Y  S  E     Q  R  Y     S  P  L     D  Q  I  N     R  S  N     V  G  N
CTGAAGCTGG CCTGGTACTT CAACCTGGAC AGGAACCGCG GTCAGGAAGG CACGCCTCTG 300
L  K  L  A     W  Y  F     N  L  D     S  N  R  G     Q  E  G     T  P  L
ATTGTGGACG GCATTATGTA TGCCACGACC AACTGGTCCA AAATGAAGGC CCTTGATGCA 360
I  V  D  G     I  M  Y     A  T  T     N  W  S  K     M  K  A     L  D  A
GCAACCGGTA AGCTGCTGTG GGAATACGAC CCGAAGGTGC CGGGTAACAT TGCCGACAAA 420
A  T  G  K     L  L  W     E  Y  D     P  K  V  P     G  N  I     A  D  K
GGCTGCTGCG ATACCGTAAA CCGCGGTGCT GGCTACTGGA ACGGCAAGGT CTATTTTGGC 480
G  C  C  D     T  V  N     R  G  A     G  Y  W  N     G  K  V     Y  F  G
ACGTTTGATG GCCGCCTGAT TGCACTGGAC GCCAAGACCG GCAAAAAGGC GTGGGAAGTC 540
T  F  D  G     R  L  I     A  L  D     A  K  T  G     K  K  A     W  E  V
AACACCATTC CCGCCGATGC CTCCCTGGGC AAACAGCGCT CTTACACGGT TGACGGTGCA 600
N  T  I  P     A  D  A     S  L  G     K  Q  R  S     Y  T  V     D  G  A
GTCCGCATTG CCAAAGGCTT GGTCCTGATT GGTAATGGTG GTGCAGAATT TGGCGCCCGT 660
V  R  I  A     K  G  L     V  L  I     G  N  G  G     A  E  F     G  A  R
GGCTTTGTGT CCGCTTTTGA TGCGGAAACC GGCAAGCTGA AATGGCGCTT CTACACGGTT 720
G  F  V  S     A  F  D     A  E  T     G  K  L  K     W  R  F     Y  T  V
CCCAACAACA AGAACGAGCC TGACCACGCT GCGTCGGACA ACGTTCTGAT GACCAACGCC 780
P  N  N  K     N  E  P     D  H  A     A  S  D  N     V  L  M     T  N  A
TACAAGACCT GGGGCCCCAA CGGGGCATGG GTCCGTCAGG GCGGTGGTGG CACCGTGTGG 840
Y  K  T  W     G  P  N     G  A  W     V  R  Q  G     G  G  G     T  V  W
GACTCCATTG TCTATGACCC CGTGTCCGAT CTGGTCTATC TGGCCGTTGG TAACGGTTCC 900
D  S  I  V     Y  D  P     V  S  D     L  V  Y  L     A  V  G     N  G  S
CCTTGGAACT ACAAATACCG CTCCGAAGGT ATCGGGAACA ACCTGTTCCT GGGCAGCATT 960
P  W  N  Y     K  Y  R     S  E  G     I  G  N  N     L  F  L     G  S  I
GTGGCGGTCA AGCCGGAAAC GGGCGAATAC GTCTGGCACT TCCAGGCCAC CACCATGGAC 1020
V  A  V  K     P  E  T     G  E  Y     V  W  H  F     Q  A  T     T  M  D
CAGTGGGACT ACACCTCTGT TCAGCAGGTC ATGACGCTTG ACATGCCGAT CAATGGTGAA 1080
Q  W  D  Y     T  S  V     Q  Q  V     M  T  L  D     M  P  I     N  G  E
ATGCGCCACG TTGTTGTGCA GGCCCCCAAG AACGGCTTCT TCTATGTTCT GGACGCCAAG 1140
M  R  H  V     V  V  Q     A  P  K     N  G  F  F     Y  V  L     D  A  K
ACGGGTGAAT TCCTGGCTGG CAAAAACTAC GTTTACGAAA ACTGGGCCAG TGGCCTTGAT 1200
T  G  E  F     L  A  G     K  N  Y     V  Y  E  N     W  A  S     G  L  D
CCGCTGACCG GTCGCCCGAT CTACAAGCCC GAAGGCCTGT GGACCCTTAA CGGCAACTTC 1260
P  L  T  G     R  P  I     Y  K  P     E  G  L  W     T  L  N     G  N  F
TGGTACGGCA TTCCCGGCCC GCTGGGTGCT CATAACTTCA TGGCTATGGC GTACAGCCCC 1320
W  Y  G  I     P  G  P     L  G  A     H  N  F  M     A  M  A     Y  S  P
AAGACCCATC TGGTCTACCT GCCTGCGCAC CAGATTCCGT TTGGTTACCA GAACCAGGTT 1380
K  T  H  L     V  Y  L     P  A  H     Q  I  P  F     G  Y  Q     N  Q  V
GGCGGCTTCA AACCGCATCC GGATTCATGG AACATCGGTC TGGACATGAC CAAGACCGGC 1440
G  G  F  K     P  H  P     D  S  W     N  I  G  L     D  M  T     K  T  G
```

Figure 34 Nucleotide sequences and predicted amino acid sequences of 3,699 kb *adhA* and *adhB* gene from *A. syzygii* SKU19. Possible ORF, start, stop codon, and ribosome-binding site are indicated by bold, bold-italic, italic-underline letter and bold-italic-underline, respectively. The deduced amino acid sequences of the possible ORFs are shown below the nucleotide sequences.

```
CTGCCTGATA CCCCTGAAGC CCGCTCCGCT TACCTGAAGG ACCTGCACGG CGAACTGCTC 1500
L  P  D  T   P  E  A   R  S  A   Y  L  K  D   L  H  G   E  L  L
GCATGGGATC CGGTGAAAAT GGAAACCGTG TGGAAGATCG ACCACAAGGG CCCATGGAAC 1560
A  W  D  P   V  K  M   E  T  V   W  K  I  D   H  K  G   P  W  N
GGTGGTGTTC TGGCAACTGG TGGTGACCTG CTGTTCCAGG GCCTCGCCAA CGGTGAATTC 1620
G  G  V  L   A  T  G   G  D  L   L  F  Q  G   L  A  N   G  E  F
CACGCCTACG ACGCAACCAA CGGTGCTGAC CTTTACAAGT TTGACGCACA GAGCGGCATT 1680
H  A  Y  D   A  T  N   G  A  D   L  Y  K  F   D  A  Q   S  G  I
ATTGCTCCGC CTGTGACCTA CAGCGTCAAC GGCAAGCAGT ACGTTGCGGT TGAAGTGGGC 1740
I  A  P  P   V  T  Y   S  V  N   G  K  Q  Y   V  A  V   E  V  G
TGGGGCGGCA TCTACCCAAT CTCCATGGGT GGTATGGGCC GTACGTCCGG CTGGACCGTC 1800
W  G  G  I   Y  P  I   S  M  G   G  M  G  R   T  S  G   W  T  V
AACCATTCCT ACATTGCCGT GTTCTCTCTG GATGGCAAAG CGCAGCTGCC GACCATGAAC 1860
N  H  S  Y   I  A  V   F  S  L   D  G  K  A   Q  L  P   T  M  N
GAACTGGGCT TCCTGCCCGT CAAGCCGCCA GCGGAATATG ACACGAAGGA AGCTGCCAAG 1920
E  L  G  F   L  P  V   K  P  P   A  E  Y  D   T  K  E   A  A  K
GGCTACTTCC AGTATCAGAC CTATTGCCAG ACCTGCCACG GTGACAACGG TGAAGGGGCC 1980
G  Y  F  Q   Y  Q  T   Y  C  Q   T  C  H  G   D  N  G   E  G  A
GGTGTGCTCC CTGACCTGCG TTGGTCCGGT TCCATCCGTC ATCAGGACGC GTTCTACAAT 2040
G  V  L  P   D  L  R   W  S  G   S  I  R  H   Q  D  A   F  Y  N
GTTGTGGGCC GCGGCGCGCT GACCGCTTAC GGCATGGATC GCTTTGACAC GAGCATGAAG 2100
V  V  G  R   G  A  L   T  A  Y   G  M  D  R   F  D  T   S  M  K
CCTGAAGAAA TCGAATCCAT TCGTCAGTAC CTCATTAAGA GGGCGAACGA GACCTATCAG 2160
P  E  E  I   E  S  I   R  Q  Y   L  I  K  R   A  N  E   T  Y  Q
CGCGAAGTGG ACGCCCGAAA GAACGATCAG GGGGTTCCGC AGGTCCCGGT CGTGGGCATT 2220
R  E  V  D   A  R  K   N  D  Q   G  V  P  Q   V  P  V   V  G  I
ACGCCCCAAT AAAGCGGCAG TCATGACGTC ATTCGGTACA CAAGCGATAC AGTGGTAAAA 2280
T  P  Q  *
ATGATGATGA ACAGACTAAA AGCCGCTCTT GGAGCGGTCA CTGTCGGGCT TCTGGCAGGA 2340
M  M  M  N   R  L  K   A  A  L   G  A  V  T   V  G  L   L  A  G
ACCTCCCTGG CACACGCACA GGGAGCGGAT GAAGACCTGA TCAAAAAGGG CGAATACGTT 2400
T  S  L  A   H  A  Q   G  A  D   E  D  L  I   K  K  G   E  Y  V
GCCCGTCTTG GTGACTGTGT GGCTTGCCAC ACAGCACTCA ACGGTCAGAA ATTTGCAGGT 2460
A  R  L  G   D  C  V   A  C  H   T  A  L  N   G  Q  K   F  A  G
GGGCTGGCAA TCAAAACCCC CATCGGCATG ATTTATTCGA CCAACATTAC GCCTGACCCC 2520
G  L  A  I   K  T  P   I  G  M   I  Y  S  T   N  I  T   P  D  P
ACCTACGGGA TTGGCACCTA TACGTTGCAG GAGTTTGATG AAGCCGTGCG CCACGGTGTC 2580
T  Y  G  I   G  T  Y   T  L  Q   E  F  D  E   A  V  R   H  G  V
CGCAAGGACG GCAGCACGCT TTATCCGGCC ATGCCGTATC CGTCCTTTGC TCGTATGTCT 2640
R  K  D  G   S  T  L   Y  P  A   M  P  Y  P   S  F  A   R  M  S
CAGGACGATA TCAAATCACT CTATGCTTAC TTCATGCATG GTGTGAAACC GATCGCCCAG 2700
Q  D  D  I   K  S  L   Y  A  Y   F  M  H  G   V  K  P   I  A  Q
AAAAACCGGG AAACGGGCAT TAGCTGGCCG CTGTCCATGC GCTGGCCGCT GTCCATCTGG 2760
K  N  R  E   T  G  I   S  W  P   L  S  M  R   W  P  L   S  I  W
CGCTCCATGT TTGCCCCCAC ACCCAAGGAC TTTACGCCTG CTCCGGGTAC GGATGCAGAC 2820
R  S  M  F   A  P  T   P  K  D   F  T  P  A   P  G  T   D  A  D
ATTGCCCGCG GTGAATACCT TGTAACGGGT GCCGGACATT GCGGTGCGTG CCATACACCC 2880
I  A  R  G   E  Y  L   V  T  G   A  G  H  C   G  A  C   H  T  P
CGTGGCTTTG CCATGCAGGA AAAGGCGCTG GATGCCTCCG GTGGTCCTGA CTTCCTTGCT 2940
R  G  F  A   M  Q  E   K  A  L   D  A  S  G   G  P  D   F  L  A
GGTGGTGCGC CGATCGACAA CTGGATTGCG CCCAGCCTGC GCAACGACCC GGTTGTGGGC 3000
G  G  A  P   I  D  N   W  I  A   P  S  L  R   N  D  P   V  V  G
CTTGGCCGCT GGTCTGAAGA TGACATCTAC CTGTTCCTGA AGTCCGGCCG TACAGACCAC 3060
L  G  R  W   S  E  D   D  I  Y   L  F  L  K   S  G  R   T  D  H
```

Figure 34 (Continued)

```
TCCGCCGTAT TTGGTGGCAT GGCTGACGTG GTTGGCTGGA GCACCCAGTA CTTCACCGAC 3120
S  A  V  F     G  G  M     A  D  V     V  G  W  S     T  Q  Y     F  T  D
TCTGACCTGC ACGCCATTGC CAAGTATCTG AAGTCCATGC CGCCGGTTCC GCCGTCACGG 3180
S  D  L  H     A  I  A     K  Y  L     K  S  M  P     P  V  P     P  S  R
GGTGACTACA CATACGATCC GTCCACGGCT CAGGCTCTGG ATTCAGGCAA CACGGCCAAC 3240
G  D  Y  T     Y  D  P     S  T  A     Q  A  L  D     S  G  N     T  A  N
AACCCCGGCG CTCGGGTCTA TGTTGAACAG TGCGCAGCCT GCCATCGCAA CGATGGTGGT 3300
N  P  G  A     R  V  Y     V  E  Q     C  A  A  C     H  R  N     D  G  G
GGTGTAGCCC GCATGTTCCC GCCGCTGGCT GGTAACCCGG TTGTTGTTGG TGATGACCCG 3360
G  V  A  R     M  F  P     P  L  A     G  N  P  V     V  V  G     D  D  P
ACCTCCATTG CCCACATTGT TATGGCCGGT GGTGTTCTGC CGCCTACCAA CTGGGCACCG 3420
T  S  I  A     H  I  V     M  A  G     G  V  L  P     P  T  N  W  A  P
TCTGCCGTTG CCATGCCGGA CTACCCGAAC ATCCTGTCCG ACCAGCAGAT GGCTGATGTG 3480
S  A  V  A     M  P  D     Y  K  N     I  L  S  D     Q  Q  M     A  D  V
GTCAACTTCA TCCGCTCCGC ATGGGGCAAC AAGGCTCCGG CTAACGTGAC GGCCGCTGAC 3540
V  N  F  I     R  S  A     W  G  N     K  A  P  A     N  V  T     A  A  D
GTTCAGAAAC TCCGTCTGGA CCACGCTCCG ATCCCGACCA CCGGCTGGGC CGACCCGACC 3600
V  Q  K  L     R  L  D     H  A  P     I  P  T  T     G  W  A     D  P  T
TCTGCTACGT CAACATGGGG CCTGTTTATG CCGCAGCCTT ACGGCTCTGG CTGGACCTTT 3660
S  A  T  S     T  W  G     L  F  M     P  Q  P  Y     G  S  G     W  T  F
GCGCCGCAGA CCCATACCGG TGTGGATGAA GCCCAGTAA                         3699
A  P  Q  T     H  T  G     V  D  E     A  Q  *
```

Figure 34 (Continued)

(A)

```
MTRPASAKRR SLLGILAAGT ICAAALPYAA APARADSQGD TGQAVIHADE HPENWLSYGR 60
TYSEQRYSPL DQINRSNVGN LKLAWYFNLD SNRGQEGTPL IVDGIMYATT NWSKMKALDA 120
ATGKLLWEYD PKVPGNIADK GCCDTVNRGA GYWNGKVYFG TFDGRLIALD AKTGKKAWEV 180
NTIPADASLG KQRSYTVDGA VRIAKGLVLI GNGGAEFGAR GFVSAFDAET GKLKWRFYTV 240
PNNKNEPDHA ASDNVLMTNA YKTWGPNGAW VRQGGGGTVW DSIVYDPVSD LVYLAVGNGS 300
PWNYKYRSEG IGNNLFLGSI VAVKPETGEY VWHFQATTMD QWDYTSVQQV MTLDMPINGE 360
MRHVVVQAPK NGFFYVLDAK TGEFLAGKNY VYENWASGLD PLTGRPIYKP EGLWTLNGNF 420
WYGIPGPLGA HNFMAMAYSP KTHLVYLPAH QIPFGYQNQV GGFKPHPDSW NIGLDMTKTG 480
LPDTPEARSA YLKDLHGELL AWDPVKMETV WKIDHKGPWN GGVLATGGDL LFQGLANGEF 540
HAYDATNGAD LYKFDAQSGI IAPPVTYSVN GKQYVAVEVG WGGIYPISMG GMGRTSGWTV 600
NHSYIAVFSL DGKAQLPTMN ELGFLPVKPP AEYDTKEAAK GYFQYQTYCQ TCHGDNGEGA 660
GVLPDLRWSG SIRHQDAFYN VVGRGALTAY GMDRFDTSMK PEEIESIRQY LIKRANETYQ 720
REVDARKNDQ GVPQVPVVGI TPQ*                                        743
```

(B)

(C)

No.	N terminal	Transmembrane region	C terminal	Type	Length
0	1	MTRPASAKRRSLLGILAAGTICAAALPYAAAPA	33	Signal peptide	33

Figure 35 Predicted amino acid sequence of the possible ORF1 (A), hydropathy profile (B), and SOSUI signal analysis of ORF1 (C). The signal peptide and two putative PQQ-binding motifs are indicated by dotted line and bold letter, respectively. The heme-binding motif and the Ala-X-Ala sequence are indicated by underline and double underline, respectively.

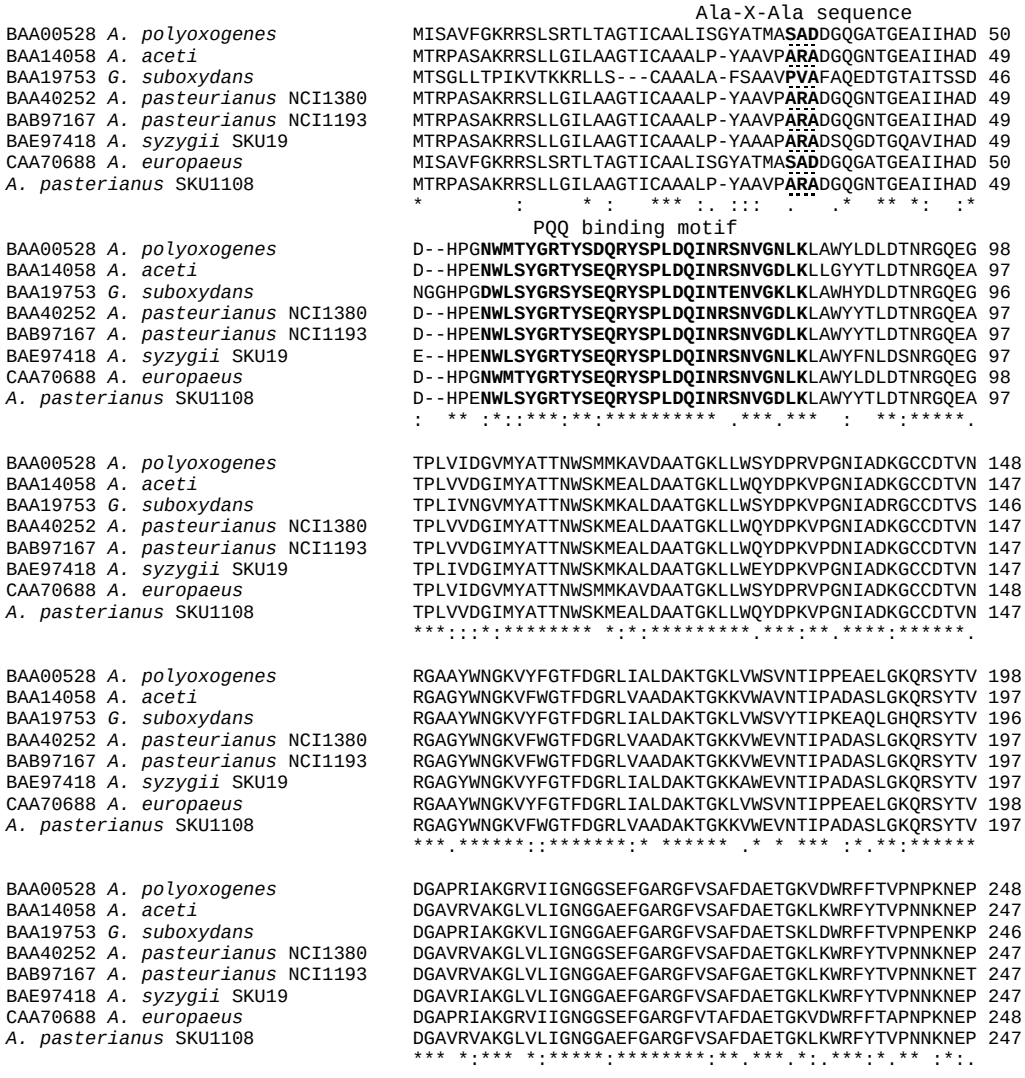


Figure 36 Alignment of amino acid sequences of ORF1 from *A. syzygii* SKU19 and other acetic acid bacteria. The two putative PQQ-binding motifs are indicated by bold letter. The heme *c* binding motif, the Ala-X-Ala sequence and the one additional amino acid in subunit I from *A. syzygii* SKU19 are indicated by underline, bold letter-dotted line and bold letter-double underline, respectively. (*) = single, fully conserved residue, (:) = conservation of strong groups, (.) = conservation of weak groups, and ( ) = no consensus.

```
                                                                PQQ binding motif
BAA00528 A. polyoxogenes            D-AASDSVLMNKAYQTWSPTGAWTRQGGGGTV**WDSIVYDPVADLVYLGVG** 297
BAA14058 A. aceti                   DHAASDNILMNKAYKTWGPKGAWVRQGGGGTV**WDSLVYDPVSDLIYLAVG** 297
BAA19753 G. suboxydans              DGAASDDILMSKAYPTWGKNGAWKQQGGGGTV**WDSLVYDPVTDLVYLGVG** 296
BAA40252 A. pasteurianus NCI1380    DHAVADNVLMSKAYKTWGPKGAWVRQGGGGTV**WDSLVYDPVSDLIYLAVG** 297
BAB97167 A. pasteurianus NCI1193    DHAASDNILMKKAYKTWGPKGAWERQGGGGTV**WDFLVYDPVSDLIYLAVG** 297
BAE97418 A. syzygii SKU19           DHAASDNVLMTNAYKTWGPNGAWVRQGGGGTV**WDSIVYDPVSDLVYLAVG** 297
CAA70688 A. europaeus               DHTASDSVLMNKAYQTWSPTGAWTRQGGGGTV**WDSIVYDPVADLVYLGVG** 298
A. pasterianus SKU1108              DHAASDNILMNKAYKTWGPKGAWVRQGGGGTV**WDSLVYDPVSDLIYLAVG** 297
                                    * :.:*.:**.:** **. .*** :********* :*****:**:**.**

BAA00528 A. polyoxogenes            NGSPWNYKYRSEGKGDNLFLGSIVALKPETGEYVWHFQETPMDQWDFTSD 347
BAA14058 A. aceti                   NGSPWNYKYRSEGIGSNLFLGSIVALKPETGEYVWHFQATPMDQWDYTSV 347
BAA19753 G. suboxydans              NGSPWNYKFRSEGKGDNLFLGSIVAINPDTGKYVWHFQETPMDEWDYTSV 346
BAA40252 A. pasteurianus NCI1380    NGSPWNYKYRSEGIGSNLFLGSIVALKPETGEYVWHFQATPMDQWDYTSV 347
BAB97167 A. pasteurianus NCI1193    NGFPWNYKYRSEGIGSNLFLGSIVALKPETGEYVWHFQATPMDQWDYTSV 347
BAE97418 A. syzygii SKU19           NGSPWNYKYRSEGIGNNLFLGSIVAVKPETGEYVWHFQATTMDQWDYTSV 347
CAA70688 A. europaeus               NGSPWNYKYRSEGKGDNLFLGSIVALKPETGEYVWHFQETPMDQWDFTSV 348
A. pasterianus SKU1108              NGSPWNYKYRSEGIGSNLFLGSIVALKPETGEYVWHFQATPMDQWDYTSV 347
                                    ** *****:**** *.*********::*:**:****** *.**:**:**

BAA00528 A. polyoxogenes            QQIMTLDLPINGETRHVIVHARKNGFFYIIDAKTGEFISGKNYVYVNWAS 397
BAA14058 A. aceti                   QQIMTLDMPVKGEMRHVIVHAPKNGFFYVLDAKTGEFLSGKNYVYQNWAN 397
BAA19753 G. suboxydans              QQIMTLDMPVNGEMRHVIVHAPKNGFFYIIDAKTGKFITGKPYTYENWAN 396
BAA40252 A. pasteurianus NCI1380    QQIMTLDMPVNGEMRHVIWHAPKNGFFYVLDAKTGEFLAGKNYVYQNWAN 397
BAB97167 A. pasteurianus NCI1193    QQIMTLDMPVNGEMRHVIVHAPKNGFFYVLDAKTGEFLSGKNYVYQNWAN 397
BAE97418 A. syzygii SKU19           QQVMTLDMPINGEMRHVVVQAPKNGFFYVLDAKTGEFLAGKNYVYENWAS 397
CAA70688 A. europaeus               QQIMTLDLPINGETRHVIVHAPKNGFFYIIDAKTGEFISGKNYVYVNWAS 398
A. pasterianus SKU1108              QQIMTLDMPVNGEMRHVIVHAPKNGFFYVLDAKTGEFLSGKNYVYQNWAN 397
                                    **:****:*::** ***: :* ******::*****:*::** *.* ***.

BAA00528 A. polyoxogenes            GLDPKTGRPIYNPDALYTLTGKEWYGIPGDLGGHNFAAMAFSPKTGLVYI 447
BAA14058 A. aceti                   GLDPLTGRPMYNPDGLYTLNGKFWYGIPGPLGAHNFMAMAYSPKTHLVYI 447
BAA19753 G. suboxydans              GLDPVTGRPNYVPDALWTLTGKPWLGIPGELGGHNFAAMAYSPKTKLVYI 446
BAA40252 A. pasteurianus NCI1380    GLDPLTGRPIYNPDGLYTLTGKFWYGIPGPLGAHNFMGMAYSPKTHLVYL 447
BAB97167 A. pasteurianus NCI1193    GLDPLTGRPIYNPDGLYTLNGKFWYGIPGPLGAHNFMAMAYSPKTHLVYI 447
BAE97418 A. syzygii SKU19           GLDPLTGRPIYKPEGLWTLNGNFWYGIPGPLGAHNFMAMAYSPKTHLVYL 447
CAA70688 A. europaeus               GLDPKTGRPIYNPDALYTLTGKEWYGIPGDLGGHNFAAMAFSPKTGLVYI 448
A. pasterianus SKU1108              GLDPLTGRPIYNPDGLYTLNGKFWYGIPGPLGAHNFMAMAYSPKTHLVYI 447
                                    **** **** * *:.*:**.*: * **** **.*** .**:**** ***:

BAA00528 A. polyoxogenes            PAQQVPFLYTNQVGGFTPHPDSWNLGLDMNKVGIPDSPE-----AKQAFV 492
BAA14058 A. aceti                   PAHQIPFGYKNQVGGFKPHADSWNVGLDMTKNGLPDTPE-----ARTAYI 492
BAA19753 G. suboxydans              PAQQIPLLYDGQKGGFKAYHDAWNLGLDMNKIGLFDDNDPEHVAAKKDFL 496
BAA40252 A. pasteurianus NCI1380    PAHQIPFGYKNQVGGFKPHPDAWNVGLDMTKNGLPDTPE-----ARTAYI 492
BAB97167 A. pasteurianus NCI1193    PAHQIPFGYKNQVGGFKPHADSWNVGLDMTKNGLPDTPE-----ARTAYI 492
BAE97418 A. syzygii SKU19           PAHQIPFGYQNQVGGFKPHPDSWNIGLDMTKTGLPDTPE-----ARSAYL 492
CAA70688 A. europaeus               PAQQVPFLYTNQVGGFTPHPDSWNLGLDMNKVGIPDSPE-----AKQAFV 493
A. pasterianus SKU1108              PAHQIPFGYKNQVGGFKPHADSWNVGLDMTKNGLPDTPE-----ARTAYI 492
                                    **:*:*: * .* ***..: *:**:****.* *: *  :     *:  ::

BAA00528 A. polyoxogenes            KDLKGWIVAWDPQKQAEAWRVDHKGPWNGGILATGGDLLFQGLANGEFHA 542
BAA14058 A. aceti                   KDLHGWLLAWDPVKMETVWKIDHKGPWNGGILATGGDLLFQGLANGEFHA 542
BAA19753 G. suboxydans              KVLKGWTVAWDPEKMAPAFTINHKGPWNGGLLATAGNVIFQGLANGEFHA 546
BAA40252 A. pasteurianus NCI1380    KDLHGWLLAWDPVKMETVWKIDHKGPWNGGVLATGGDLLFQGLANGEFHA 542
BAB97167 A. pasteurianus NCI1193    KDLHGWLLAWDPVKMETVWKIDHKGPWNGGILATGGDLLFQGLANGEFHA 542
BAE97418 A. syzygii SKU19           KDLHGELLAWDPVKMETVWKIDHKGPWNGGVLATGGDLLFQGLANGEFHA 542
CAA70688 A. europaeus               KDLKGWIVAWDPQKQAEAWRVDHKGPWNGGILATGGDLLFQGLANGEFHA 543
A. pasterianus SKU1108              KDLHGWLLAWDPVKMETVWKIDHKGPWNGGILATGGDLLFQGLANGEFHA 542
                                    * *:*  :**** *   .: ::********:***.*:::***********

BAA00528 A. polyoxogenes            YDATNGSDLFHFAADSGIIAPPVTYLANGKQYVAVEVGWGGIYPFFLGGL 592
BAA14058 A. aceti                   YDATNGSDLYKFDAQSGIIAPPMTYSVNGKQYVAVEVGWGGIYPISMGGV 592
BAA19753 G. suboxydans              YDATNGNDLYSFPAQSAIIAPPVTYTANGKQYVAVEVGWGGIYPFLYGGV 596
BAA40252 A. pasteurianus NCI1380    YDATNGSDLYKFDAQSGIIASPMTYSVNGKQYVAVEVGWGGIYPISMGGV 592
BAB97167 A. pasteurianus NCI1193    YDATNGSDLYKFDAQSGIIAPPMTYSVNGKQYVAVEVGWGGIYPISMGGV 592
BAE97418 A. syzygii SKU19           YDATNGADLYKFDAQSGIIAPPVTYSVNGKQYVAVEVGWGGIYPISMGGM 592
CAA70688 A. europaeus               YDATNGSDLFHFAADSGIIAPPVTYLANGKQYVAVEVGWGGIYPFFLGGL 593
A. pasterianus SKU1108              YDATNGSDLYKFDAQSGIIAPPMTYSVNGKQYVAVEVGWGGIYPISMGGV 592
                                    ****** **: * *:*.***.*:** .***************:  **:
```

Figure 36 (Continued)

```
BAA00528 A. polyoxogenes            ARTSGWTVNHSRIIAFSLDGKSGPLPKQNDQGFLPVKPPAQFDSKRTDNG 642
BAA14058 A. aceti                   GRTSGWTVNHSYIAAFSLDGKAK-LPALNNRGFLPVKPPAQYDQKVVDNG 641
BAA19753 G. suboxydans              ARTSGWTVNHSRVIAFSLDGKDS-LPPKNELGFTPVKPVPTYDEARQKDG 645
BAA40252 A. pasteurianus NCI1380    GRTSGWTVNHSYIAVFSLDGKAK-LPALNNRGFLPVKPPAQYDQKVVDNG 641
BAB97167 A. pasteurianus NCI1193    GRTSGWTVNHSYIAAFSLDGKAK-LPALNNRGFLPVKPPAQYDQKVVDNG 641
BAE97418 A. syzygii SKU19           GRTSGWTVNHSYIAVFSLDGKAQ-LPTMNELGFLPVKPPAEYDTKEAAKG 641
CAA70688 A. europaeus               ARTSGWTVNHSRIIAFSLDGKSGPLPKQNDQGFLPVKPPAQFDSKRTDNG 643
A. pasterianus SKU1108              GRTSGWTVNHSYIAAFSLDGKAK-LPALNNRGFLPVKPPAQYDQKVVDNG 641
                                    .********** : .******   **  *: ** **** . :*     .*

                                Heme c binding motif
BAA00528 A. polyoxogenes            YFQFQTYCAACHGDNAEGAGVLPDLRWSGSIRHEDAFYNVVGRGALTAYG 692
BAA14058 A. aceti                   YFQYQTYCQTCHGDNGEGAGMLPDLRWAGAIRHQDAFYNVVGRGALTAYG 691
BAA19753 G. suboxydans              YFMYQTFCSACHGDNAISGGVLPDLRWSGRPRGRESFYKLVGRGALTAYG 695
BAA40252 A. pasteurianus NCI1380    YFQYQTYCQTCHGDNGEGAGMLPDLRWAGAIRHQDAFYNVVGRGALTAYG 691
BAB97167 A. pasteurianus NCI1193    YFQYQTYCQTCHGDNGEGAGMLPDLRWAGAIRHQDAFYNVVGRGALTAYG 691
BAE97418 A. syzygii SKU19           YFQYQTYCQTCHGDNGEGAGVLPDLRWSGSIRHQDAFYNVVGRGALTAYG 691
CAA70688 A. europaeus               YFQFQTYCAACHGDNAEGAGVLPDLRWSGSIRHEDAFYNVVGRGALTAYG 693
A. pasterianus SKU1108              YFQYQTYCQTCHGDNGEGAGMLPDLRWAGAIRHQDAFYNVVGRGALTAYG 691
                                    ** :**:* :*****. ..*:******:*  * .::**::********** 

BAA00528 A. polyoxogenes            MDRLHGNMNPTEIEDIRQFLIKRANETYQREVDARKNADGIPEQLP---- 738
BAA14058 A. aceti                   MDRFDTSMTPDEIEAIRQYLIKRANDTYQREVDARKNDKNIPENPTLGIN 741
BAA19753 G. suboxydans              MDRFDTSMTPEQIEDIRNFIVKRANESYDDEVKARENSTGVPNDQFLNVP 745
BAA40252 A. pasteurianus NCI1380    MDRFDTSMTPDEIEAIRQYLIKRANDTYQREVDARKNDKDIPENPTLGIN 741
BAB97167 A. pasteurianus NCI1193    MDRFDTSMTPDEIEAIRQYLIKRANDTYQREVDARKNDKNIPENPTLGIN 741
BAE97418 A. syzygii SKU19           MDRFDTSMKPEEIESIRQYLIKRANETYQREVDARKNDQGVPQVPVVGIT 741
CAA70688 A. europaeus               MDRFDGNMNPTEIEDIRQFLIKRANETYQREVDARKNADGIPEQLP---- 739
A. pasterianus SKU1108              MDRFDTSMTPDEIEAIRQYLIKRANDTYQRVVDARKNDKNIPENPTLGIN 741
                                    ***:. .*.* :** **::::****::*:  *.**:*  .:*:

BAA00528 A. polyoxogenes            ------------
BAA14058 A. aceti                   P----------- 742
BAA19753 G. suboxydans              QSTADVPTADHP 757
BAA40252 A. pasteurianus NCI1380    P----------- 742
BAB97167 A. pasteurianus NCI1193    P----------- 742
BAE97418 A. syzygii SKU19           PQ---------- 743
CAA70688 A. europaeus               ------------
A. pasterianus SKU1108              P----------- 742
```

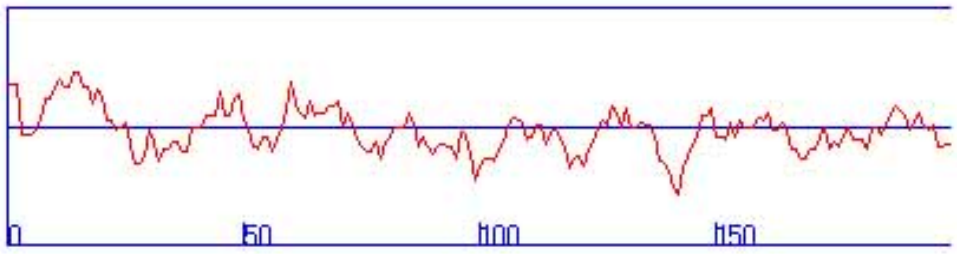
Figure 36 (Continued)

The *adhB* gene corresponding to the cytochrome *c* subunit was located 49 bp downstream of the termination codon of *adhA* gene. The ORF started at ATG (nucleotide no. 2,281 to 2,283) and terminated at TAA (nucleotide no. 3,697 to 3,699), encoded a 472 amino acids. A possible ribosome-binding site, AGTGG, was present 5 nt upstream of the ATG codon. The deduced amino acid sequences and hydropathy profile of this ORF were shown in Figure 37A and 37B. The hydropathy profile revealed that this amino acid sequence is a membrane protein. The SOSUI signal analysis indicated that the extra 24 amino acids at the NH_2 terminal of this ORF also appeared to be a signal peptide (Figure 37B and 37C). Many researchers suggested that subunit I and II translocate through the cytoplasmic membrane to its outer surface or into the periplasmic space (Kondo and Horinouchi, 1997b). The results from the Motif Scan, the PPSearch: Protein functional analysis and the NCBI Consensus Domain Search showed that three C-X-X-C-H sequences motifs at position 46 to 50, 194 to 198 and 331 to 335, which may serve as heme *c* binding sites were present in the amino acid sequence of this ORF as shown in Figure 37A. The cytochrome *c* domain was found at amino acid no. 321 to 404. The presence of these heme *c* binding motif and cytochrome *c* domain was supported the idea that ORF2 is subunit II of ADH. Comparison of amino acid of *adhB* also shows significant similarity in amino acid sequence to the cytochrome *c* subunits of ADH composed of two components and three components i.e. *A. pasteurianus* SKU1108 (85%), *A. pasteurianus* NCI1193 (84%), *A. pasteurianus* NCI1380 (84%), *A. polyoxogenase* (76%) as shown in Figure 38.

(A)

```
MMMNRLKAAL GAVTVGLLAG TSLAHAQGAD EDLIKKGEYV ARLGDCVACH TALNGQKFAG 60
GLAIKTPIGM IYSTNITPDP TYGIGTYTLQ EFDEAVRHGV RKDGSTLYPA MPYPSFARMS 120
QDDIKSLYAY FMHGVKPIAQ KNRETGISWP LSMRWPLSIW RSMFAPTPKD FTPAPGTDAD 180
IARGEYLVTG AGHCGACHTP RGFAMQEKAL DASGGPDFLA GGAPIDNWIA PSLRNDPVVG 240
LGRWSEDDIY LFLKSGRTDH SAVFGGMADV VGWSTQYFTD SDLHAIAKYL KSMPPVPPSR 300
GDYTYDPSTA QALDSGNTAN NPGARVYVEQ CAACHRNDGG GVARMFPPLA GNPVVVGDDP 360
TSIAHIVMAG GVLPPTNWAP SAVAMPDYKN ILSDQQMADV VNFIRSAWGN KAPANVTAAD 420
VQKLRLDHAP IPTTGWADPT SATSTWGLFM PQPYGSGWTF APQTHTGVDE AQ*        472
```

(B)



(C)

No.	N terminal	Transmembrane region	C terminal	Type	Length
0	1	MMMNRLKAALGAVTVGLLAGTSL	24	Signal peptide	24

Figure 37 Predicted amino acid sequence of the possible ORF2 (A), hydropathy profile (B), and SOSUI signal analysis of the ORF2 (C). The signal peptide and the heme *c* binding motifs are indicated by dotted line and underline, respectively. The cytochrome *c* domain is indicated by bold letter.

```
BAA00529 A. polyoxogenes            -----MINRLKVTFS-----AAAFSLLAGTA-LAQTPDADSALVQKGAYV 39
BAA19754 G. suboxydans              MLNALTRDRLVSEMKQGWKLAAAIGLMAVSFGAAHAQDADEALIKRGEYV 50
BAA02993 A. pasteurinaus NCI138     ----MMMNRLKTALG-----AVAVGLLAGTS-LAYAQNADEDLIKKGEYV 40
BAB97168 A. pasteurianus NCI1193    ----MMINRLKAALG-----AVAVGLLAGTS-LAHAQNADEDLIKKGEYV 40
BAE97419 A. syzygii SKU19           ----MMMNRLKAALG-----AVTVGLLAGTS-LAHAQGADEDLIKKGEYV 40
CAA70689 A. europaeus               -----MINRLKVTFS-----AAAFSLLAGTA-LAQTPDADSALVQKGAYV 39
A. pasterianus SKU1108              ----MMINRLKAALG-----AVAVGLLAGTS-LAHAQNADEDLIKKGEYV 40
                                          :**   :      *.:..*:* :   * : .**. *:::* **
                                      Heme c motif
BAA00529 A. polyoxogenes            ARLGDCVACHTALHGQSYAGGLEIKSPIGTIYSTNITPDPTYGIGRYTFA 89
BAA19754 G. suboxydans              ARLSDCIACHTALHGQPYAGGLEIKSPIGTIYSTNITPDPEHGIGNYTLE 100
BAA02993 A. pasteurinaus NCI1380    ARLGDCVACHTALNGQKYAGGLSIKTPIGTIYSTNITPDPTYGIGTYTFK 90
BAB97168 A. pasteurianus NCI1193    ARLGDCVACHTSLNGQKYAGGLSIKTPIGTIYSTNITPDPTYGIGTYTFK 90
BAE97419 A. syzygii SKU19           ARLGDCVACHTALNGQKFAGGLAIKTPIGMIYSTNITPDPTYGIGTYTLQ 90
CAA70689 A. europaeus               ARLGDCVACHTALHGQSYAGGLEIKSPIGTIYSTNITPDPTYGIGRYTFA 89
A. pasterianus SKU1108              ARLGDCVACHTSLNGQKYAGGLSIKTPIGTIYSTNITPDPTYGIGTYTFK 90
                                    ***.**:****:*:** :**** **:*** ********** :*** **:

BAA00529 A. polyoxogenes            EFDEAVRHGIRKDGSTLYPAMPYPSFSRMTKEDMQALYAYFMHGVKPVAQ 139
BAA19754 G. suboxydans              DFTKALRKGIRKDGATVYPAMPYPEFARLSDDDIRAMYAFFMHGVKPVAL 150
BAA02993 A. pasteurinaus NCI1380    EFDEAVRHGVRKDGATLYPGMPYPSFARMTQDDMKALYAYFMHGVQPIAE 140
BAB97168 A. pasteurianus NCI1193    EFDEAVRHGVRKDGATLYPAMPYPSFARITQDDMKALYAYFMHGVQPIAQ 140
BAE97419 A. syzygii SKU19           EFDEAVRHGVRKDGSTLYPAMPYPSFARMSQDDIKSLYAYFMHGVKPIAQ 140
CAA70689 A. europaeus               EFDEAVRHGIRKDGSTLYPAMPYPSFSRMTKEDMQALYAYFMHGVKPVAQ 139
A. pasterianus SKU1108              EFDEAVRHGVRKDGATLYPAMPYPSFARMTQDDMKALYAYFMHGVQPIAQ 140
                                    :* :*:*:*:*:****:*:**.****.*:*::.:*::::**:*****:*:*

BAA00529 A. polyoxogenes            PDKQPDISWPLSMRWPLGIWRMMFSPSPKDFTPAPGTDPEIARGDYLVTG 189
BAA19754 G. suboxydans              QNKAPDISWPLSMRWPLGMWRAMFVPSMTPGVDKSISDPEVARGEYLVNG 200
BAA02993 A. pasteurinaus NCI1380    KNHPTDISWPMSMRWPLSIWRSVFAPAPKDFTPAPGTDAETARGEYLITG 190
BAB97168 A. pasteurianus NCI1193    KNHPTDISWPMSMRWPLSIWRSVFAPAPKDFTPATGTDAEIARGEYLVTG 190
BAE97419 A. syzygii SKU19           KNRETGISWPLSMRWPLSIWRSMFAPTPKDFTPAPGTDADIARGEYLVTG 190
CAA70689 A. europaeus               PDKQPDISWPLSMRWPLGIWRMMFSPSPKDFTPAPGTDPEIARGDYLVTG 189
A. pasterianus SKU1108              KNHPTDISWPMSMRWPLSIWRSVFAPAPKDFTPAPGTDAEIARGEYLVTG 190
                                     :: ..****:******.:** :* *: .  .  . :*.: ***:**:.*
                                   Heme c motif
BAA00529 A. polyoxogenes             PGHCGACHTPRGFAMQEKALDAAGGPDFLSGGAPIDNWVAPSLRNDPVVG 239
BAA19754 G. suboxydans               PGHCGECHTPRGFGMQVKAYGTAGGNAYLAGGAPIDNWIAPSLRSNSDTG 250
BAA02993 A. pasteurinaus NCI1380     PGHCGACHTPRGFGMQEKALDGAGGPDFLAGGGVIDNWIAPSLRNDPVLG 240
BAB97168 A. pasteurianus NCI1193     PGHCGACHTPRGFGMQEKALDASGGPDFLGGGGVIDNWIAPSLRNDPVLG 240
BAE97419 A. syzygii SKU19            AGHCGACHTPRGFAMQEKALDASGGPDFLAGGAPIDNWIAPSLRNDPVVG 240
CAA70689 A. europaeus                PGHCGACHTPRGFAMQEKALDAAGGPDFLSGGAPIDNWVAPSLRNDPVVG 239
A. pasterianus SKU1108               PGHCGACHTPRGFGMQEKALDASGGPDFLGGGGVIDNWIAPSLRNDPVLG 240
                                     .**** *******.** ** . :**  :*.**. ****:*****.:.  *

BAA00529 A. polyoxogenes             LGRWSEDDIYTFLKSGRIDHSAVFGGMGDVVAWSTQYFTDDDLHAIAKYL 289
BAA19754 G. suboxydans               LGRWSEDDIVTFLKSGRIDHSAVFGGMADVVAYSTQHWSDDDLRATAKYL 300
BAA02993 A. pasteurinaus NCI1380     LGRWSDEDLFLFLKSGRTDHSAAFGGMADVVGWSTQYFTDADLHAMVKYL 290
BAB97168 A. pasteurianus NCI1193     LGRWSDEDLFLFLKSGRTDHSAAFGGMADVVGWSTQYFTDADLHAMVKYI 290
BAE97419 A. syzygii SKU19            LGRWSEDDIYLFLKSGRTDHSAVFGGMADVVGWSTQYFTDSDLHAIAKYL 290
CAA70689 A. europaeus                LGRWSEDDIYTFLKSGRIDHSAVFGGMGDVVAWSTQYFTDDDLHAIAKYL 289
A. pasterianus SKU1108               LGRWSDEDLFLFLKSGRTDHSAAFGGMADVVGWSTQYYTDADLHAMVKYI 290
                                     *****::*:  ****** ****.****.***.:***:::* **:* .**:
```

Figure 38 Comparison of amino acid sequences of ORF2 from other acetic acid bacteria. The heme-binding motif is indicated by underline. The cytochrome *c* domain is indicated by bold italic letter. (*) = single, fully conserved residue, (:) = conservation of strong groups, (.) = conservation of weak groups, and ( ) = no consensus.

```
                                                                  Heme c motif
BAA00529 A. polyoxogenes          KSLPPVPPSQGNYTYDPSTANMLASGNTASVPGADTYVKECAICHRNDGG 339
BAA19754 G. suboxydans            KSMPAVPEGKNLGQDDGQTTALLNKGG-QGNAGAEVYLHNCAICHMNDGT 349
BAA02993 A. pasteurinaus NCI1380  KSLPPVPPARGDYSYDASTAQMLDSNNFSGNAGAKTYVEQCAICHRNDGG 340
BAB97168 A. pasteurianus NCI1193  KSLPPVPPARGDYSYDASTAQMLDSNNFSGNAGAKTYVEQCAICHRNDGG 340
BAE97419 A. syzygii SKU19         KSMPPVPPSRGDYTYDPSTAQALDSGNTANNPGARVYVEQCAACHRNDGG 340
CAA70689 A. europaeus             KSLPPVPPSQGNYTYDPSTANMLASGNTASVPGADTYVKECAICHRNDGG 339
A. pasterianus SKU1108            KSLPPVPPARGDYSYDASTAQMLDSNNFSGNAGAKTYVEQCAICHRNDGG 340
                                  **:*.** .:.    * .*:  * ...  . .** .*:.:** ** ***
                                                   The cytochrome c domain
BAA00529 A. polyoxogenes          GVARMFPPLAGNPVVVTENPTSLVNVIAHGGVLPPSNWAPSAVAMPGYSK 389
BAA19754 G. suboxydans            GVNRMFPPLAGNPVVITDDPTSLANVVAFGGILPPTNSAPSAVAMPGFKN 399
BAA02993 A. pasteurinaus NCI1380  GVARMFPPLAGNPVVVSDNPTSVAHIVVDGGVLPPTNWAPSAVAMPDYKN 390
BAB97168 A. pasteurianus NCI1193  GVARMFPPLAGNPVVVSDNPTSVAHIVVDGGVLPPTNWAPSAVAMPDYKN 390
BAE97419 A. syzygii SKU19         GVARMFPPLAGNPVVVGDDPTSIAHIVMAGGVLPPTNWAPSAVAMPDYKN 390
CAA70689 A. europaeus             GVARMFPPLAGNPVVVTENPTSLVNVIAHGGVLPPSNWAPSAVAMPGYSK 389
A. pasterianus SKU1108            GVARMFPPLAGNPVVVSDNPTSVAHIVVDGGVLPPTNWAPSAVAMPDYKN 390
                                  ** ************: ::***:.:::  **:***:* ********.:.:

BAA00529 A. polyoxogenes          SLSAQQIADVVNFIRTSWGNKAPGTVTAADVTKLRDTGAPVSSSGWN--- 436
BAA19754 G. suboxydans            HLSDQEMADVVNFMRKGWGNNAPGTVSASDIQKLRTTGAPVSTAGWN--- 446
BAA02993 A. pasteurinaus NCI1380  ILSDQQIADVVNFIRSAWGNRAPANTTAADIQKLRLDHTPLPTPGWANAT 440
BAB97168 A. pasteurianus NCI1193  ILSDQQIADVVNFIRSAWGNRAPANTTAADIQKLRLDHTPLPTPGWANAT 440
BAE97419 A. syzygii SKU19         ILSDQQMADVVNFIRSAWGNKAPANVTAADVQKLRLDHAPIPTTGWADPT 440
CAA70689 A. europaeus             SLSAQQIADVVNFIRTSWGNKAPGTVTAADVTKLRDTGAPVSSSGWN--- 436
A. pasterianus SKU1108            ILSDQQIADVVNFIRSAWGNRAPANTTAADIQKLRLDHTPLPTPGWANAT 440
                                   ** *::******:*..***.**...:*:*: ***   :*:.:.**

BAA00529 A. polyoxogenes          SVSSGWSVFLPQPYGSGWTFAPQTHTGQDAAQ 468
BAA19754 G. suboxydans            VSSKGWMAYMPQPYGEDWTFSPQTHTGVDDAQ 478
BAA02993 A. pasteurinaus NCI1380  EDSATWGLFMPQPYGAGWSFAPQTHAGVDEAQ 472
BAB97168 A. pasteurianus NCI1193  EESATWGLFMPQPYGAGWTFAPQTHAGVDEAQ 472
BAE97419 A. syzygii SKU19         SATSTWGLFMPQPYGSGWTFAPQTHTGVDEAQ 472
CAA70689 A. europaeus             SVSSGWSVFLPQPYGSGWTFAPQTHTGQDAAQ 468
A. pasterianus SKU1108            EESATWGLFMPQPYGAGWTFAPQTHAGVDEAQ 472
                                    :  *  ::***** .*:*:****:* * **
```

Figure 38 (Continued)

4.4 Determination and analysis of nucleotide sequence of 618 bp

The complete nucleotide sequence of 618 bp PCR product of *A. syzygii* SKU19 was obtained with the sequencing reaction using T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and SP6 promoter primer (5'-ATTTAGGTG ACACTATAG-3') as forward and reverse sequencing primer, respectively. Nucleotide sequence of 618 bp PCR products and deduced amino acid sequences of both are shown in Figure 39.

Possible open reading frame (ORF) was observed by using ORF finder. The ORF corresponding to subunit III of ADH of *A. syzygii* SKU19 started with ATG at nucleotide no. 1 to 3 and terminated with TAA at nucleotide no. 616 to 618. This ORF encodes a polypeptide composed of 205 amino acid residues. The deduce amino acid sequences and hydropathy profile of this ORF are illustrated in Figure 40A and 40B. The hydropathy profile indicated that this ORF are a soluble protein. The SOSUI signal analysis indicated that the NH_2-terminal amino acid sequence at position 1 to 26 are a possible transmenbrane region or signal peptide as shown in Figure 40B and 40C. No homologous proteins have been registered in any protein data bank. Homology search using BLASTP revealed that the amino acid sequence ORF from *A. syzygii* SKU19 was similar to subunit III of ADH of *A. pasteurianus* NCI1193 (99%) and *A. pasteurianus* NCI1452 (91%). Comparision of amino acid sequences from *A. syzygii* SKU19 between *A. pasteurianus* SKU1108 (98%) (Vanittananon, 2005), *A. pasteurianus* NCI1193 (98%), *A. pasteurianus* NCI1452 (89%) (Kondo *et al.*, 1995) and *A. pasteurianus* SKU123 (98%) is shown in Figure 41.

The deduced primary structures of the three subunits of ADH of *A. syzygii* SKU19 from the nucleotide sequences of the cloned genes indicated that two subunit of *adhAB* gene were aligned with the same transcriptional polarity and may be no possible transcriptional termination signal or inverted repeat sequence were found in the space between ORF1 and ORF2. Therefore, these two ORFs form

the same operon and a co-transcribed, like the ones in *A. polyoxogenes* (Tamaki *et al.*, 1991), *A. pasteurianus* (Takemura *et al.*, 1993; Tuasakul, 2004), and *Gluconobacter suboxydans* (Kondo and Horinouchi, 1997b). Comparison of the primary translation products predicted from the nucleotide sequences showed that a typical signal sequence was present at their NH_2 termini. *adhA* showed similarity in amino acid sequence to the dehydrogenase subunits of both two-component- and three-component-type ADHs from others *Acetobacter* spp. In addition, two PQQ-binding consensus motifs were found in *adhA* gene. *adhB*, which has been found to be identical to cytochrome *c*-533, shows significant similarity in amino acid sequence to the cytochrome *c* subunits (subunit II) of the ADH complexes in acetic acid bacteria (Kondo and Horinouchi, 1997b). All of these data showed that *adhA* serving as the primary dehydrogenase subunit and *adhB* serving as the cytochrome for ubiquinone reduction comprise a membrane-bound quinohemoprotein-cytochrome *c* system very similar in electron transfer and ubiquinone reduction.

The smallest subunit, *adhS* gene, showed same similarity in amino acid sequence to *adhS* gene of three-component ADHs from several acetic acid bacteria. Kondo *et al.* (1995) suggested that *adhS* gene belongs to a transcriptional unit different from that for the other subunits because it was not located near the *adhAB* gene. No homologous proteins have been registered in any protein data bank. However, *adhS* gene from *A. syzygii* SKU19 showed variations in 3 amino acids when it was compared with *adhS* gene from *A. pasteurianus* SKU1108 at amino acid no. 75 (Ala to Val), no. 134 (Gly to Arg) and no. 155 (Thr to Ile), respectively, as shown in Figure 41.

```
***ATG***AAACTGA TTGCCGTACG TGCCCTGTCG GCGCTTGCCC TCACCACATC TCTGCTGGCA 60
M  K  L  I   A  V  R   A  L  S   A  L  A  L   T  T  S   L  L  A
GGGGCTGCTG TGTCTGCCCA TGCGCAGGAA AACACAGATG CGCCCGTAAC ACGCGCCGGA 120
G  A  A  V   S  A  H   A  Q  E   N  T  D  A   P  V  T   R  A  G
GATACGTCCC GCCTGACAGA TGTAGATCCG TCTGGCTTTG TTGGTTCCAT TGATCCGGCA 180
D  T  S  R   L  T  D   V  D  P   S  G  F  V   G  S  I   D  P  A
GAAAACGCTG GCCTGCTGAA CTACTGTGTG CAGAACGAAT ACGCTGATTA CGACGATGCT 240
E  N  A  G   L  L  N   Y  C  V   Q  N  E  Y   A  D  Y   D  D  A
            PstI
GGCGCACGCT GCAGGAATAC AACAAAAAGA CCAACGCCGT GCCAGAAGGG CAGGAAGGCA 300
G  A  R  C   R  N  T   T  K  R   P  T  P  C   Q  K  G   R  K  A
ATATGTCTTA CGCCAATGGT TCTGCCGGTC TGCTGCATGC CAACAACCGC ACCTACACCA 360
I  C  L  T   P  M  V   L  P  V   C  C  M  P   T  T  A   P  T  P
TTGCCATGGC TATTCTGCCA GTGCGTCAGA AAACCTGTAG GGCTGTGCTG GAACGGGCCA 420
L  P  W  L   F  C  Q   C  V  R   K  P  V  G   L  C  W   N  G  P
AGGCTTCTCT GTAAGCACAA GGCTGTTATT CCGGTTTTGT TGACAAGGCC GGATCACGCT 480
R  L  L  C   K  H  K   A  V  I   P  V  L  L   T  R  P   D  H  A
GAACACGGCA TGATGGAAGG GTCAGTCACG CAAAGTGGCT GGCCCTTTTT CTGTATGAAC 540
E  H  G  M   M  E  G   S  V  T   Q  S  G  W   P  F  F   C  M  N
GCACTTTATT TGGTGTTCAT GCGGTGCAGG GTCTTTCACG CAGGCGTGCA ATATGGTCAA 600
A  L  Y  L   V  F  M   R  C  R   V  F  H  A   G  V  Q   Y  G  Q
ACCAGTTCTG TTTCG***TAA***                                          618
T  S  S  V   S  *
```

Figure 39 Nucleotide sequences and predicted amino acid sequences of *adhS* from *A. syzygii* SKU19. Start and stop codons are indicated by bold-italic and bold-italic underline letters. The deduced amino acid sequence of the possible ORF is shown below the nucleotide sequences.

(A)

```
MKLIAVRALS ALALTTSLLA GAAVSAHAQE NTDAPVTRAG DTSRLTDVDP SGFVGSIDPA 60
ENAGLLNYCV QNEYADYDDA GARCRNTTKR PTPCQKGRKA ICLTPMVLPV CCMPTTAPTP 120
LPWLFCQCVR KPVGLCWNGP RLLCKHKAVI PVLLTRPDHA EHGMMEGSVT QSGWPFFCMN 180
ALYLVFMRCR VFHAGVQYGQ TSSVS*                                     205
```

(B)

(C)

No.	N terminal	Transmembrane region	C terminal	Type	Length
0	1	MKLIAVRALSALALTTSLLAGAAVSA	26	Signal peptide	26

Figure 40 Predicted amino acid sequences of the possible ORF (A), hydropathy profile (B) and SOSUI signal analysis of this ORF of *adhS* gene from *A. syzygii* (C). The signal peptide is indicated by dotted underline and the signal sequence is indicated in the box.

```
BAA06528 A. pasteurianus NCI1452      MKLIAVRALSALALTTSLLAGAAVSAHAQENTDAPVTRAGDTSRLTDVDP 50
BAB97169 A. pasteurianus NCI1193      MKLIAVRALSALALTTSLLAGAAVSAHAQENTDAPVTRAGDTSRLTDVDP 50
BAE97417 A. syzygii SKU19             MKLIAVRALSALALTTSLLAGAAVSAHAQENTDAPVTRAGDTSRLTDVDP 50
A. pasteurianus SKU1108               MKLIAVRALSALALTTSLLAGAAVSAHAQENTDAPVTRAGDTSRLTDVDP 50
A. pasteurianus SKU123                MKLIAVRALSALALTTSLLAGAAVSAHAQENTDAPVTRAGDTSRLTDVDP 50
                                      **************************************************

                                                    75
BAA06528 A. pasteurianus NCI1452      SGFVGSIDPAENAGLLNYCVQNEYVDYDDAGARCRNTTKRPTPCQKGRKA 100
BAB97169 A. pasteurianus NCI1193      SGFVGSIDPAENAGLLNYCVQNEYVDYDDAGARCRNTTKRPTPCQKGRKA 100
BAE97417 A. syzygii SKU19             SGFVGSIDPAENAGLLNYCVQNEYADYDDAGARCRNTTKRPTPCQKGRKA 100
A. pasteurianus SKU1108               SGFVGSIDPAENAGLLNYCVQNEYVDYDDAGARCRNTTKRPTPCQKGRKA 100
A. pasteurianus SKU123                SGFVGSIDPAENAGLLNYCVQNEYVDYDDAGARCRNTTKRPTPCQKGRKA 100
                                      ************************.************************

                                                          134
BAA06528 A. pasteurianus NCI1452      TCLTPMVLLACCTPTTTPTPLPWLFCQCARKPVRLFWNGPRLPCKHKAVI 150
BAB97169 A. pasteurianus NCI1193      ICLTPMVLPVCCMPTTAPTPLPWLFCQCVRKPVRLCWNGPRLLCKQKAVI 150
BAE97417 A. syzygii SKU19             ICLTPMVLPVCCMPTTAPTPLPWLFCQCVRKPVGLCWNGPRLLCKHKAVI 150
A. pasteurianus SKU1108               ICLTPMVLPVCCMPTTAPTPLPWLFCQCVRKPVRLCWNGPRLLCKHKAVI 150
A. pasteurianus SKU123                ICLTPMVLPVCCMPTTAPTPLPWLFCQCVRKPVRLCWNGPRLLCKHKAVI 150
                                       ******* .** ***:***********.****.* ****** **:****

                                        155
BAA06528 A. pasteurianus NCI1452      PVLLIRPDHAERGMMEGSVTQGGWPFFCMRAVYLRCMPDQGFHAGVQYGQ 200
BAB97169 A. pasteurianus NCI1193      PVLLIRPDHAEHGMMEGSVTQSGWPFFCMNALYLVFMRCRVFHAGVQYGQ 200
BAE97417 A. syzygii SKU19             PVLLTRPDHAEHGMMEGSVTQSGWPFFCMNALYLVFMRCRVFHAGVQYGQ 200
A. pasteurianus SKU1108               PVLLIRPDHAEHGMMEGSVTQSGWPFFCMNALYLVFMRCRVFHAGVQYGQ 200
A. pasteurianus SKU123                PVLLIRPDHAEHGMMEGSVTQSGWPFFCMNALYLVFMRCRVFHAGVQYGQ 200
                                      ****.******:*********.*******.*:**  *  : *********

BAA06528 A. pasteurianus NCI1452      TSSVS 205
BAB97169 A. pasteurianus NCI1193      TSSVS 205
BAE97417 A. syzygii SKU19             TSSVS 205
A. pasteurianus SKU1108               TSSVS 205
A. pasteurianus SKU123                TSSVS 205
                                      *****
```

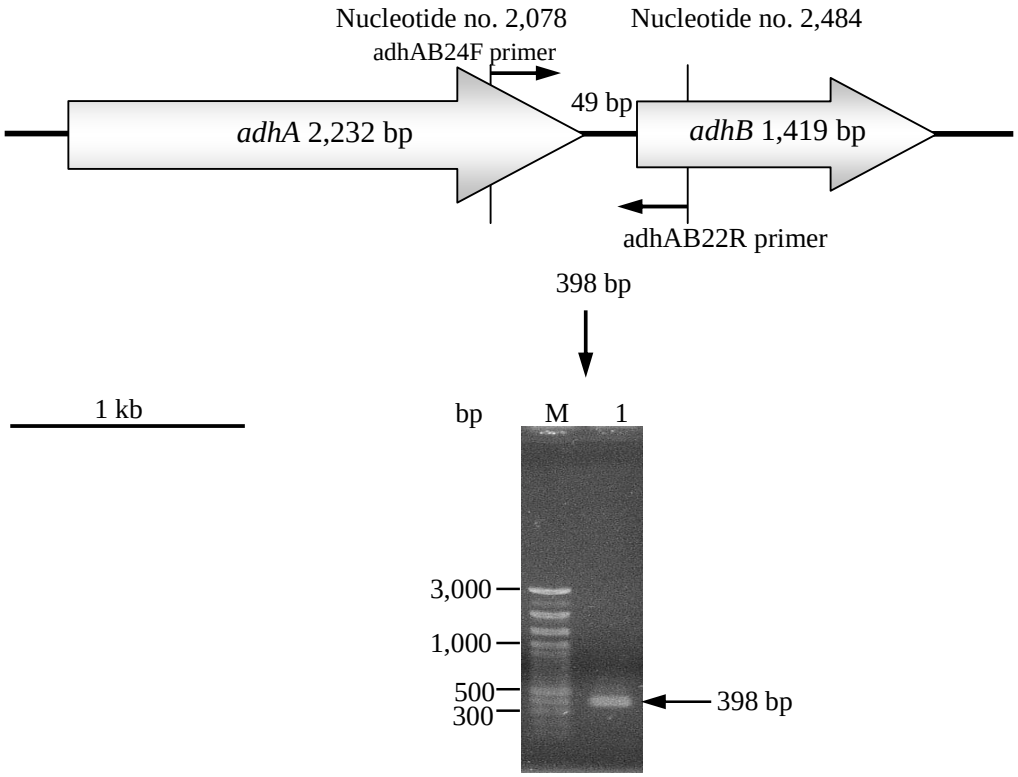
Figure 41 Alignment of amino sequences of *adhS* gene from *A. syzygii* SKU19 and other acetic acid bacteria. * = single, fully conserved residue, (:) = conservation of strong groups, (.) = conservation of weak groups and ( ) = no consensus. The variations in 3 amino acids between subunit III of *A. syzygii* SKU19 and *A. pasteurianus* SKU1108 are indicated by bold-underline letter

4.5 Amplification of the *adhA-adhB* junction region in acetic acid adapted strain no. 112 by Polymerase Chain Reaction (PCR)

Comparison of nucleotide and amino acid sequences of *adhA* gene from *A. pasteurianus* NCI1380 (Takemura *et al.,* 1993), *A. pasteurianus* SKU1108 (Tuasakul, 2004), and *A. aceti* K6033 (Inoue *et al.,* 1989) showed that *adhA* gene of *A. syzygii* SKU19 have 743 amino acid residues, which is different from acetic acid bacteria as mentioned above (Figure 36). At stop codon, it had slided three nucleotide base pairs or one amino acid when it was compared with *adhA* gene or subunit I of the other acetic acid bacteria. In order to find some difference in acetic acid adapted strains compared to wild type strain (*A. syzygii* SKU19) because of these adapted strains can grow very well in the medium containing acetic acid concentration. Therefore, adapted strain no. 112 was selected for this study due to its ability to oxidize 2.0% ethanol but cannot oxidize acetate in the medium. However, this strain could still survive in the medium.

Two primer (adhAB24F: 5'-ATCGCTTTGACACGAGC-3') and a reverse primer (adhAB22R: 5'-TTTGATTGCCAGCCCACC-3') as shown in Table 5, were designed for amplification of the *adhA-adhB* junction region from nucleotide sequence of *adhAB A. syzygii* SKU19 at nucleotide no. 2,230 to 2,232 as shown in Appendix Figure C1. PCR was performed by using the Ready-To-Go™ PCR Bead with a standard condition as previously described. The desired PCR product was 398 bp as shown in Figure 42A. This PCR amplified product 398 bp was cloned into pGEM®-T Easy vector. The recombinant clone, designated as pGEM®-TCAA carrying 398 bp DNA fragment, was selected from white colonies growing on the LB agar containing 50 μg/ml ampicillin and X-Gal. The recombinant plasmid was extracted by alkaline lysis method. The insert DNA was digested by *Eco*RI and analyzed in 0.8% agarose gel electrophoresis as shown in Figure 42B. The recombinant plasmid was further analysis by nucleotide sequencing.

(A)



B)

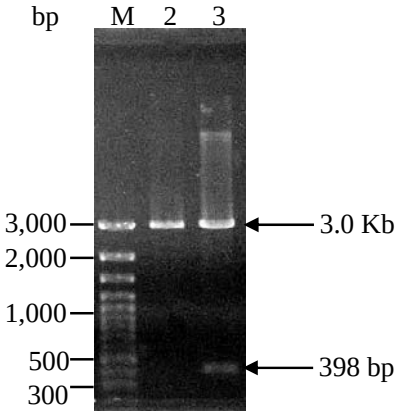


Figure 42 Agarose gel electrophoresis of 398 bp PCR product from acetic acid adapted strain no. 112 from *A. syzygii* SKU19 (A) and pGEM®-TCAA digested with *Eco*RI (B). Lane M = 100 bp DNA ladder, 1 = PCR product 398 bp, 2 = pGEM®-T/*Eco*RI, and 3 = pGEM®-TCAA/*Eco*RI.

Nucleotide sequence of 398 bp PCR product is shown in Figure 43. Comparison of nucleotide sequence of the *adhA-adhB* junction region from acetic acid bacteria is shown in Figure 44. The result from nucleotide sequence indicated that both of *adhA* gene of *A. syzygii* SKU19 and adapted strain no. 112 still has 743 amino acid residues. At stop codon, it had slided three base pairs or one amino acid when it was compared with *adhA* gene or subunit I of other acetic acid bacteria. Therefore, this position may not be related to resistance to acetic acid of adapted strains.

adhAB24F


```
ATCGCTTTGA CACGAGCATG AAGCCTGAAG AAATCGAATC CATTCGTCAG TACCTCATTA 60
AGAGGGCGAA CGAGACCTAT CAGCGCGAAG TGGACGCCCG AAAGAACGAT CAGGGGGTTC 120
CGCAGGTCCC GGTCGTGGGC ATTACGCCCC AATAAGCGG CAGTCATGAC GTCATTCGGT 180
ACACAAGCGA TACAGTGGTA AAAATGATGA TGAACAGACT AAAAGCCGCT CTTGGAGCGG 240
TCACTGTCGG GCTTCTGGCA GGAACCTCCC TGGCACACGC ACAGGGAGCG GATGAAGACC 300
TGATCAAAAA GGGCGAATAC GTTGCCCGTC TTGGTGACTG TGTGGCTTGC CACACAGCAC 360
TCAACGGTCA GAAATTTGCA GGTGGGCTGG CAATCAAA                        398
```


adhAB22R

Figure 43 Nucleotide sequences of 398 bp PCR product from acetic acid adapted strain no. 112 of *A. syzygii* SKU19. Stop codon of *adhA* are indicated by bold-underline letter.

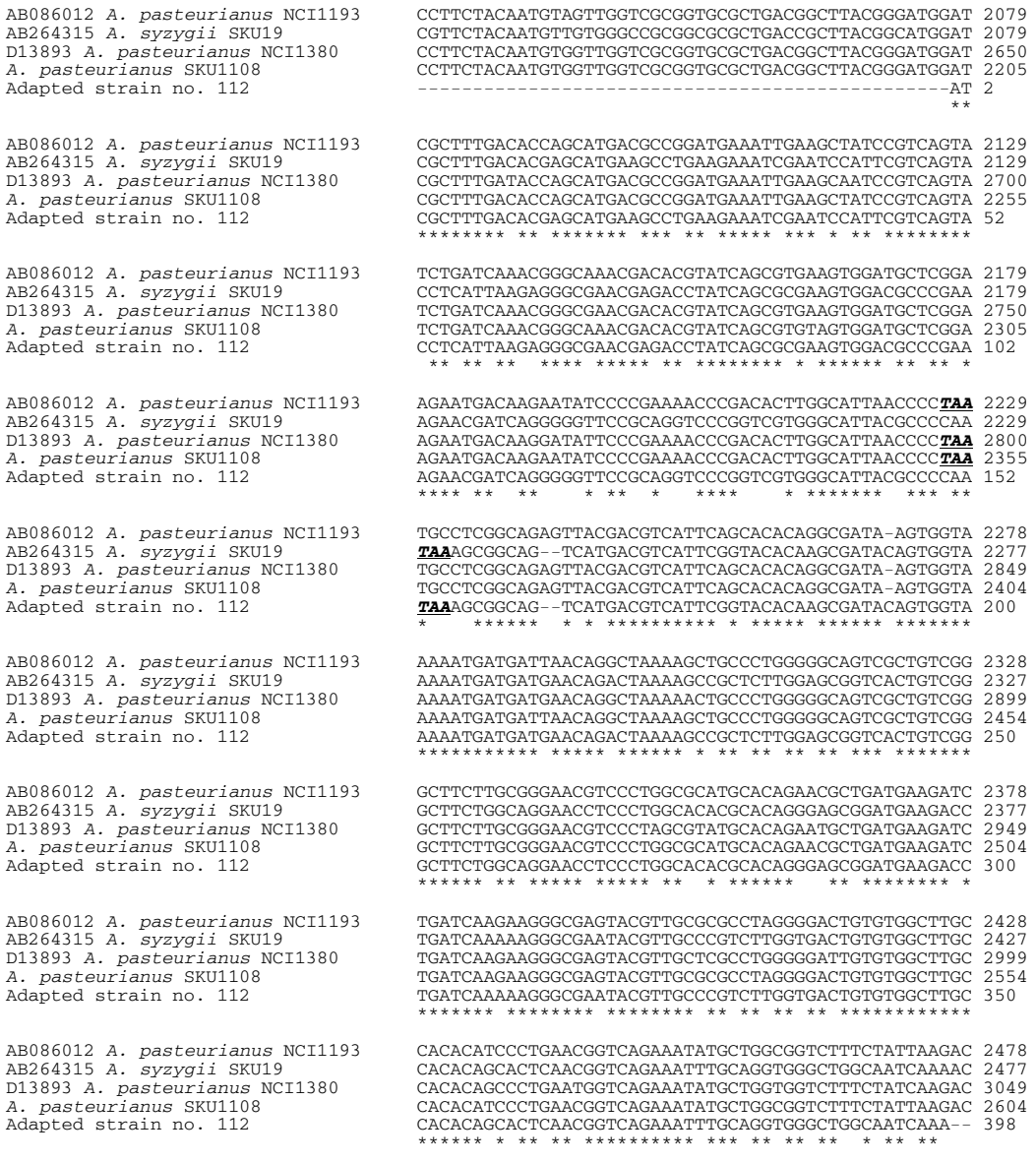


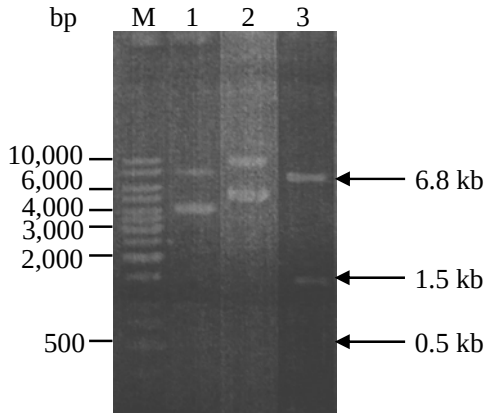
Figure 44 Alignment of nucleotide sequences of the *adhA-adhB* junction region from acetic acid adapted strain no. 112 from *A. syzygii* SKU19 and other acetic acid bacteria. The stop codon of *adhA* is indicated by bold-italic and underline letter. * = single, fully conserved residue and ( ) = no consensus.

5. *adhS* Gene Complementation

5.1 Transformation of *A. syzygii* SKU19 by a plasmid carrying *adhS* from *A. pasteurianus* SKU1108

To elucidate the function of quinoprotein ADH, 2 kb *Eco*RI fragment carrying *adhS* gene from *A. pasteurianus* SKU1108 (Vanittananon, 2005) was introduced into pCM62, which is a broad-host-range cloning vector, and transformed into *E. coli* S17-1. The recombinant clone was analyzed by digested with *Eco*RI, as shown in Figure 45. The positive clones were designated as pCM*adhS* (8.8 kb) carrying 2 kb *Eco*RI DNA fragment carrying *adhS* gene from *A. pasteurianus* SKU1108 in the same orientation from P*lac* (pCM*adhS*, ←) and in the opposite orientation from P*lac* (pCM*adhS*, →) as shown in Figure 46. Both of recombinant plasmids were introduced into *A. syzygii* SKU19 by conjugation. *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) were selected on SCM agar containing 50 μg/ml of tetracycline and 0.6% acetic acid. These transformants were confirmed by modified alkaline lysis method, as shown in Figure 47.

(A)



(B)

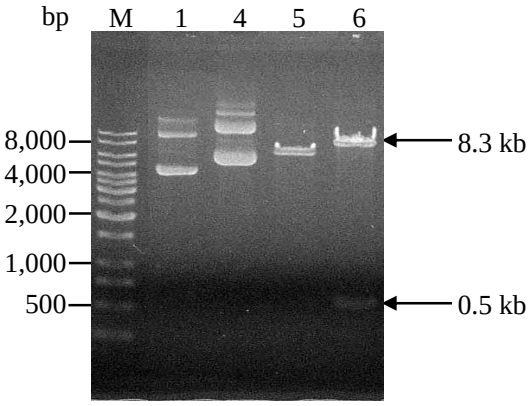
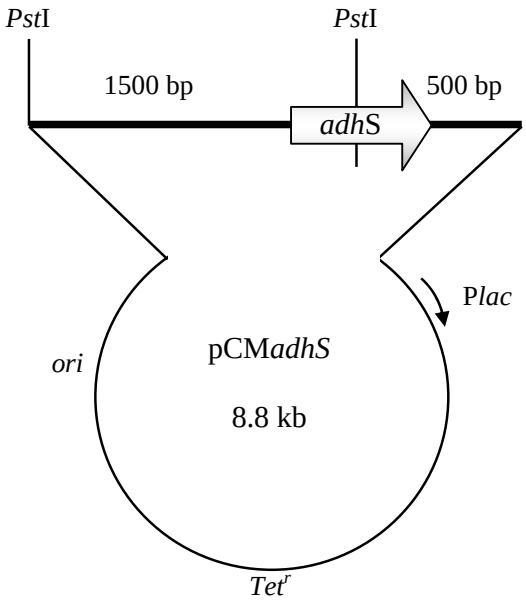


Figure 45 Agarose gel electrophoresis of pCM62 carrying *adhS* gene from *A. pasteurianus* SKU1108 in the same (pCM*adhS* (←), A) and opposite orientation from P*lac* (pCM*adhS* (→), B). Lane M = 1 kb DNA ladder, 1 = pCM62, 2 = pCM*adhS* (←), 3 = pCM*adhS* (←)/PstI, 4 = pCM*adhS* (→), 5 = pCM62/*Pst*I, and 6 = pCM*adhS* (→)/*Pst*I.

(A)



(B)

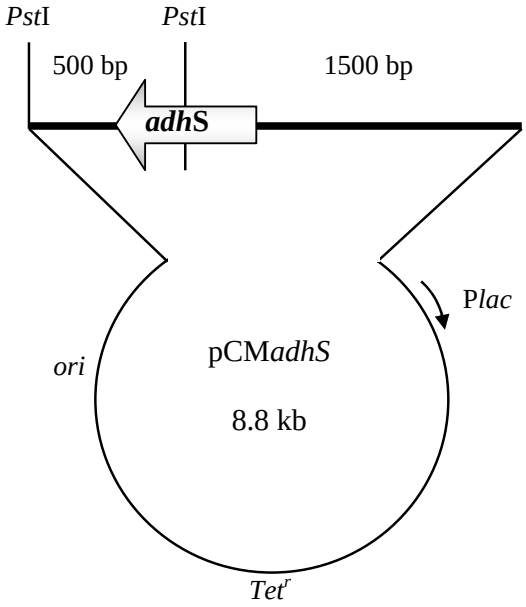
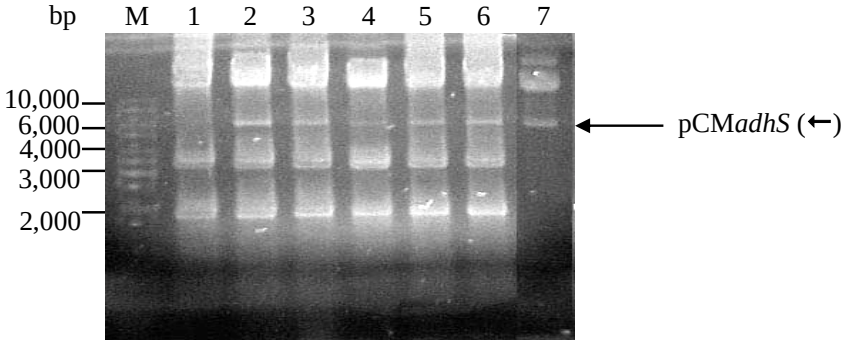


Figure 46 The structure of pCM*adhS* in the same (pCM*adhS* (←), A) and opposite orientation from P*lac* (pCM*adhS* (→), B). The 2 kb *Eco*RI DNA fragment carrying *adhS* gene from *A. pasteurianus* SKU1108 was cloned into pCM62 broad-host-range cloning vector.

(A)



(B)

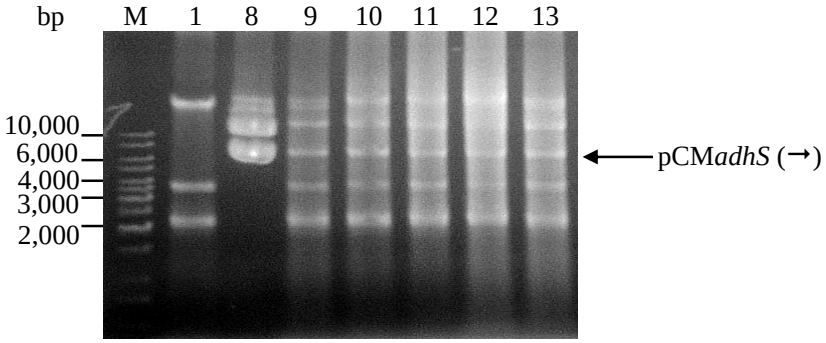
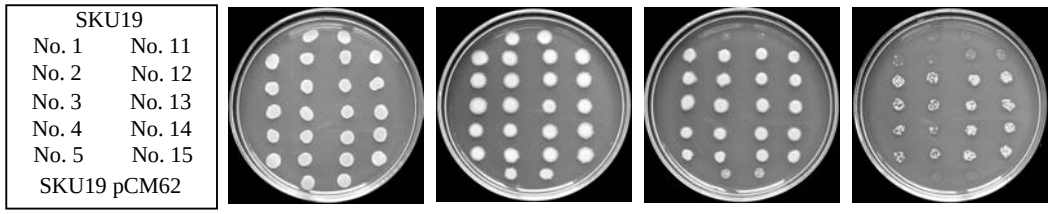


Figure 47 Plasmid profiles of recombinant clones carrying pCM*adhS* (←, A) and pCM*adhS* (→, B). Lane M = 1 kb DNA ladder, 1 = *A. syzygii* SKU19, 2 to 6 = recombinant clone carrying pCM*adhS* (←) no. 1, 2, 3, 4 and 5, 7 = pCM*adhS* (←), 8 = pCM*adhS* (→), 9 to 13 = recombinant clone carrying pCM*adhS* (→) no. 11, 12, 13, 14 and 15.

5.2 Investigation of growth characteristics of the *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→)

To investigate the growth of *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) from *A. pasteurianus* SKU1108, Both of *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) were cultured on SCM and glucose free-SCM agar with or without 25 μg/ml tetracycline compared with *A. syzygii* SKU19 and *A. syzygii* SKU19 carrying pCM62. Both media were added 0, 0.5, 1.0, 1.5, and 2.0% (v/v) of acetic acid, respectively. The growth of all transformants was observed. The results are shown in Figure 48 and 49. The results showed different growth on both media between all of *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) from *A. pasteurianus* SKU1108 and *A. syzygii* SKU19 carrying pCM62 at 1.0 to 1.5 and 1.0% acetic acid on SCM agar without and with 25 μg/ml tetracycline, respectively.

(A)



(B)

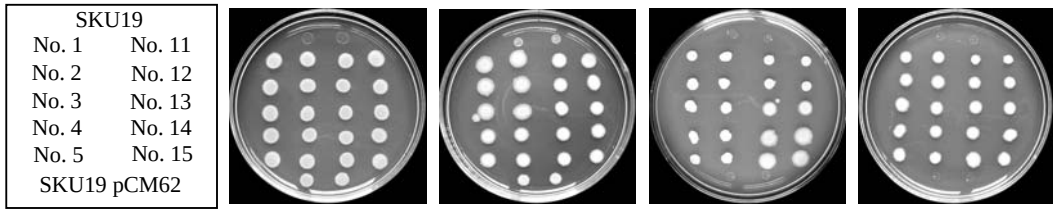
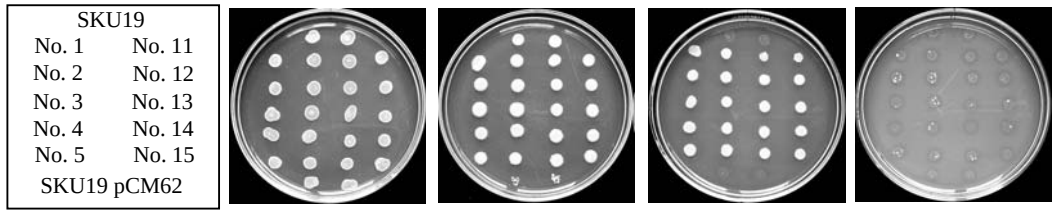


Figure 48 Growth of *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) from *A. pasteurianus* SKU1108 on SCM agar without (A) or with 25 μg/ml tetracycline (B). Both media were added various acetic acid concentrations; 0, 0.5, 1.0 and 1.5% (v/v) acetic acid and incubated at 30°C for 5 days. No. 1 to 5 = *A. syzygii* SKU19 carrying pCM*adhS* (←) from *A. pasteurianus* SKU1108 in the same orientation from P*lac*, no. 11 to 15 = *A. syzygii* SKU19 carrying pCM*adhS* (→) from *A. pasteurianus* SKU1108 in the opposite orientation from P*lac* and SKU19 pCM62 = *A. syzygii* SKU19 carrying pCM62.

(A)



(B)

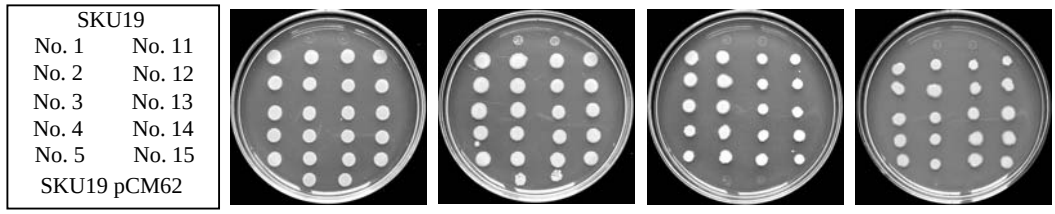


Figure 49 Growth of *A. syzygii* SKU19 carrying pCM*adhS* (←) and pCM*adhS* (→) from *A. pasteurianus* SKU1108 on glucose free-SCM agar without (A) or with 25 μg/ml tetracycline (B). Both media were added various acetic acid concentrations; 0, 0.5, 1.0 and 1.5% (v/v) acetic acid and incubated at 30°C for 5 days. No. 1 to 5 = *A. syzygii* SKU19 carrying pCM*adhS* (←) from *A. pasteurianus* SKU1108 in the same orientation from P*lac*, no. 11 to 15 = *A. syzygii* SKU19 carrying pCM*adhS* (→) from *A. pasteurianus* SKU1108 in the opposite orientation from P*lac* and SKU19 pCM62 = *A. syzygii* SKU19 carrying pCM62.

To investigate the growth of *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 in glucose free-SCM broth, *A. syzygii* SKU19 carrying pCM*adhS* was cultured in glucose free-SCM broth containing 25 μg/ml tetracycline and supplied 0.5, 1.0, 1.5, and 2.0% (v/v) of acetic acid, respectively and incubated at 30°C and 200 rpm. The growth of all transformants was observed. The results are shown in Figure 50A. No differences in growth between *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 and *A. syzygii* SKU19 carrying pCM62 in the medium containing 0 to 1.5% acetic acid but *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 at 2.0% acetic acid showed more rapid growth than *A. syzygii* SKU19 carrying pCM62 which was in accordance with the reduction of acetic acid (Figure 50B).

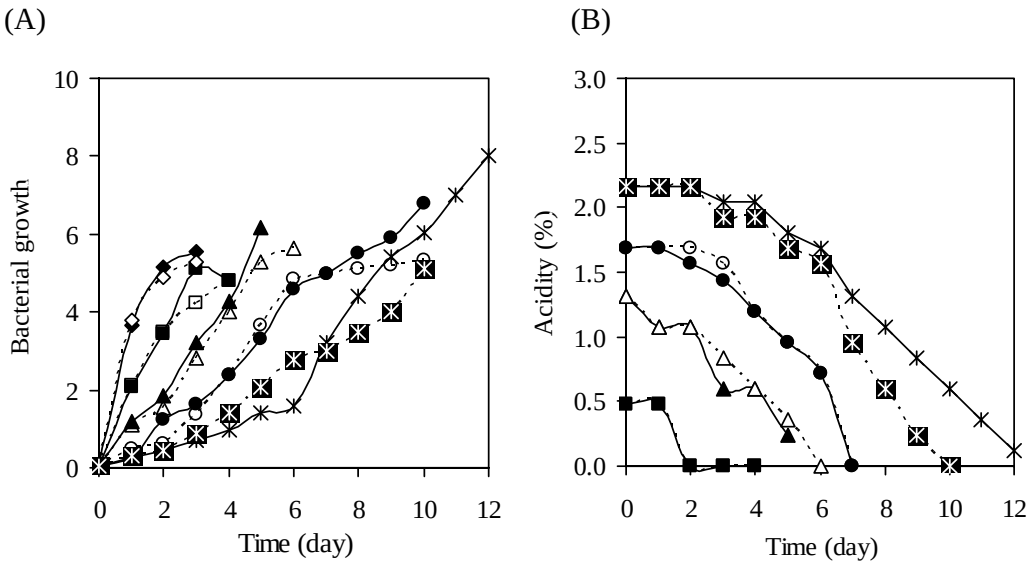


Figure 50 Growth of *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 and *A. syzygii* SKU19 carrying pCM62, respectively, in glucose free-SCM broth containing 50 μg/ml tetracycline and 0, 0.5, 1.0, 1.5, and 2.0% (v/v) acetic acid and incubated at 30°C for 12 days. Bacterial growth (A), acetic acid (%) (B) and various concentrations of acetic acid; 0 (◆ and ◇), 0.5 (■ and □), 1.0 (▲ and △), 1.5 (● and ○), and 2.0% (✠ and *) (v/v) acetic acid.

5.3 Expression of *adhS* gene and localization of subunit III in *A. syzygii* SKU19 carrying pCM*adhS*

The experiment to investigate the expression of *adhS* gene and localization of subunit III from *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 was performed in potato medium containing 4.0% (v/v) ethanol and incubated at 30°C and 200 rpm. Growth, acetic acid production and ADH activity of *A. syzygii* SKU19 harboring *adhS* gene from *A. pasteurianus* SKU1108 was observed (Figure 51). However, subunit III from *A. pasteurianus* SKU1108 was dramatically increased in the soluble fraction when the cells were grown in the medium containing 4.0% (v/v) ethanol as shown in Figure 52.

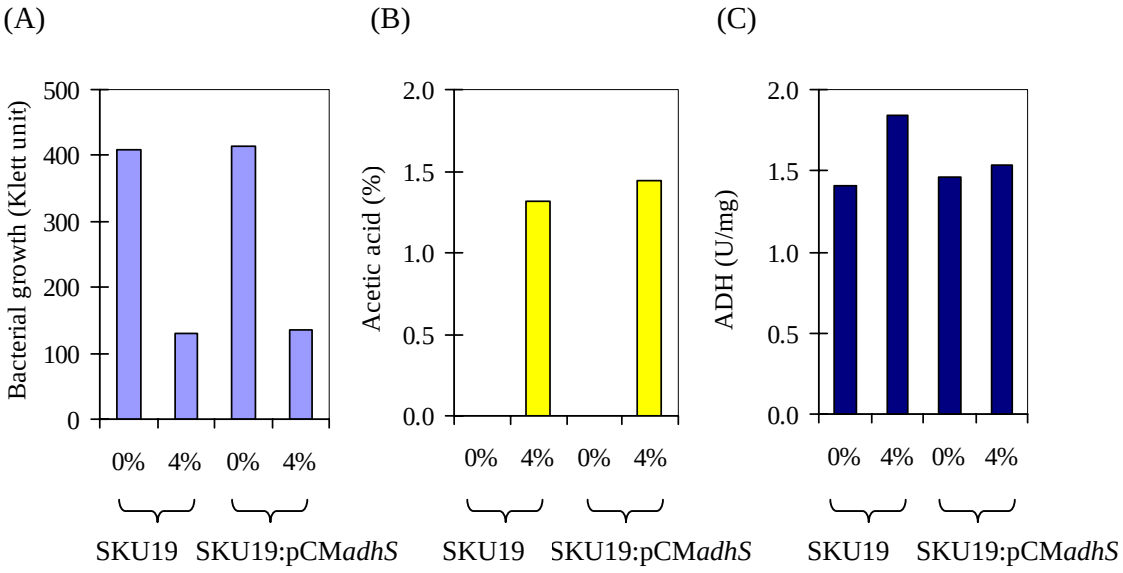


Figure 51 Growth (A), acetic acid production (B) and ADH activity (C) of *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 in potato medium.

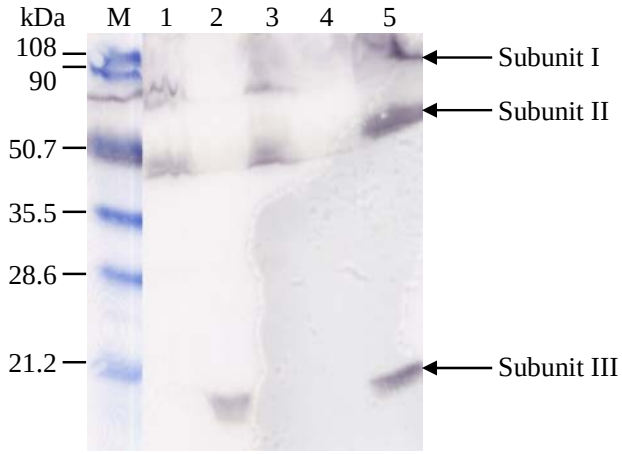


Figure 52 Immunoblot analysis of membrane and soluble fractions from *A. syzygii* SKU19 carrying pCM*adhS* from *A. pasteurianus* SKU1108 in potato medium containing 4.0% (v/v) ethanol. Lane M = standard protein marker, 1, 3 and 5 = membrane fraction from *A. syzygii* SKU19 carrying pCM*adhS*, *A. syzygii* SKU19 and *A. pasteurianus* SKU1108, respectively, and 2, 4 = soluble fraction from *A. syzygii* SKU19 carrying pCM*adhS* and *A. syzygii* SKU19.

So, we proposed that subunit III from *A. pasteurianus* SKU1108 might be unable to bind with subunit I from *A. syzygii* SKU19 as shown in Figure 53. This might be due to two possibilities, binding region on subunit I was occupied by subunit III from *A. syzygii* SKU19 or variations in 3 amino acids occurred between subunit III of *A. syzygii* SKU19 and *A. pasteurianus* SKU1108.

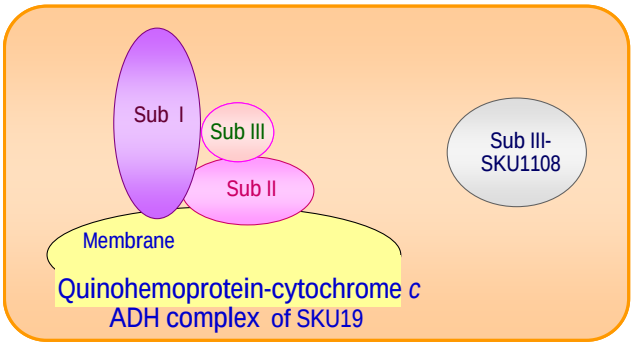


Figure 53 Speculative model for localization of subunit III from *A. pasteurianus* SKU1108 in *A. syzygii* SKU19.

CONCLUSION AND RECOMMENDATION

Conclusion

Thermotolerant acetic acid bacteria, *Acetobacter* sp. SKU19 and SKU123 isolated from guava and longkon were selected as acetic acid sensitive and tolerant strains, respectively. Both strains were identified by 16S rDNA analysis. Comparison of 16S rDNA sequence identity showed that *Acetobacter* sp. SKU19 and SKU123 contain the highest sequence identity percentage (99%) with *Acetobacter syzygii* and *Acetobacter pasteurianus*, respectively. Therefore, both of them were identified as *A. syzygii* SKU19 and *A. pasteurianus* SKU123. *A. syzygii* SKU19 could not grow on seed culture medium (SCM) agar containing 1.0% (v/v) acetic acid at 30°C for 5 days whereas other strains including *A. pasteurianus* SKU123 grew very well on the same medium. However, growth of *A. syzygii* SKU19 could be observed in glucose free-SCM broth after 5 days incubation and under the same condition, growth of *A. pasteurianus* SKU123 could be observed after only 1 day incubation. Moreover, pre-cultivation of both strains in SCM broth containing 0.5 and 1.0% acetic acid could promote their viable count on SCM agar containing 1% acetic acid. Investigation of ethanol and acetate oxidation of both strains in glucose free-SCM broth containing 2% ethanol indicated that both of them could oxidize ethanol to acetate but only *A. pasteurianus* SKU123 could further oxidize acetate to CO_2 and H_2O. Based on those results, it has been purposed that acetate oxidation may be involved in acetic acid toleration or adaptive response.

In order to elucidate an adaptive response to acetic acid, acetic acid adapted strains were isolated from sequential cultivations of acetic acid sensitive strain, *A. syzygii* SKU19, in the medium containing 1.0% acetic acid. Six acetic acid adapted strains (no. 108, 112, 187, 217, 258 and 264) were isolated from sequential cultivations. These adapted strains showed enhanced growth in acetic acid-containing medium compared to *A. syzygii* SKU19. However, when the acetic acid adapted strains were examined by plating method on the same media containing different concentrations of acetic acid, the number of colony forming unit (CFU) revealed that

the culture of adapted strains contained mixed populations of acetic acid sensitive and resistant cells. It seemed to be that these adapted strains were not considered as mutants because wild type cells could be observed when they were re-cultured. The adapted variants could be divided into two groups based on growth and ability to further oxidize acetate. The first group was the cells with increased overoxidation or rapid acetate oxidizer similar to *A. pasteurianus* SKU123, the second group with increased stability to acetate or slow acetate oxidizer same as *A. syzygii* SKU19. Comparison of the plasmid and RAPD profiles of the genomic DNA showed no obvious differences in genetic background among these adapted strains. In contrast, the enzyme activities of pyrroloquinoline quinone dependent alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH) in these adapted strains were higher than those in *A. syzygii* SKU19. The results were corresponded well with the protein profiles in membrane fractions prepared from those cells. The amount of proteins with molecular mass of 72-80 and 44-54 kDa were increased when those strains were cultivated in acetic acid-containing medium. These two proteins were confirmed by heme staining and immunoblotting analysis, and were considered as PQQ-ADH subunit I (dehydrogenase subunit) and II (cytochrome *c*), respectively. Surprisingly, ADH subunit III could not be detected by immunoblotting analysis with anti-ADH subunit III from *A. aceti*. Based on these evidences, it is worthy to characterize the genes coding for PQQ-ADH subunit I, II and III.

Three *adh* genes, *adhA*, *adhB* and *adhS* encoding for PQQ-ADH subunit I, II and III, respectively, from *A. syzygii* SKU19 were cloned and characterized. The *adhA* gene consisted of 2,232 bp coding for 743 amino acid residues whereas *adhA* gene from other *Acetobacter* spp. coding for 742 amino acid residues. These amino acid residues composed of two putative pyrroloquinoline quinone (PQQ) binding motif and one cytochrome *c* family heme *c* binding motif and showed 88, 89, 88, 87, and 77% identity with alcohol dehydrogenase subunit I from *A. aceti* K6033, *A. pasteurianus* SKU1108, *A. pasteurianus* NCI1193, *A. pasteurianus* NCI1452, and *A. polyoxogenase*, respectively. Comparison of nucleotide sequence of the end of *adhA* gene between acetic acid adapted strain No. 112 and *A. syzygii*, SKU19 showed that *adhA* gene of both from *A. syzygii* SKU19 and adapted strain no. 112 consisted of

743 amino acid residues. Thus, this one amino acid addition may not be involved in acetic acid toleration in adapted strains. The second gene, *adhB* consisted of 1,419 bp coding for 472 amino acid residues. These amino acid residues composed of three heme-binding sites and showed 85, 84, 84, and 76% identity with alcohol dehydrogenase subunit II, as a cytochrome *c* subunit, from *A. pasteurianus* SKU1108, *A. pasteurianus* NCI1380, and *A. polyoxogenase*, respectively. In addition, *adhS* consisted of 618 bp coding for 205 amino acid residues. These amino acid residues showed 98, 98, 89 and 98% identity with alcohol dehydrogenase subunit III from *A. pasteurianus* SKU1108, *A. pasteurianus* NCI1193, *A. pasteurianus* NCI1452, and *A. pasteurianus* SKU123, respectively. However, it showed variations in 3 amino acids when it was compared with *adhS* gene from *A. pasteurianus* SKU1108. In addition, subunit III of *A. syzygii* SKU19 could not be detected in both membrane and soluble fractions by immunoblotting with anti-ADH subunit III of *A. aceti*. There were two possibilities to explain this evidence. Firstly, there was no expression from *adhS* gene from *A. syzygii* SKU19. Secondly, subunit III from this strain tightly bound with subunit I and permanently located in membrane fraction. Regarding its low molecular weight, so it was hardly to distingue between free subunit I and subunit I-subunit III complex by immunoblotting. However, transferring of *adhS* gene from *A. pasteurianus* SKU1108 into *A. syzygii* SKU19 could enhance acetic acid toleration when it was grown in the medium containing high concentration of acetic acid. Subunit III from *A. pasteurianus* SKU1108 could be detected in soluble fraction of *A. syzygii* SKU19 by immunoblotting when the cells were grown in the medium containing ethanol. It seemed to be that this subunit III could not bind with subunit I from *A. syzygii* SKU19. This may be due to the variations in three amino acid residues between subunit III of *A. pasteurianus* SKU1108 and *A. syzygii* SKU19. Those three amino acids might be located in the binding region or involved in binding with subunit I. Further investigation to clarify those evidences is recommended.

Recommendation

Based on those conclusions, there are several points should be clarified. The first point is the relationship between acetate oxidation and acetic acid adaptation. It is recommended to compare activities of certain enzymes involved in acetate oxidation from both *A. syzygii* SKU19 and *A. pasteurianus* SKU123.

The second point is effect of three amino acid variations found in subunit III from acetic acid sensitive strain, *A. syzygii* SKU19 and acetic acid tolerant strain, *A. pasteurianus* SKU1108. The variation of these three amino acid residues may associate with binding between subunit I and subunit III. To further clarify this point, site directed mutagenesis of these three amino acid residues should be performed. Finally, we hope that the results obtained from this study will benefit to understanding function of subunit III, mechanism of acetic acid adaptation and strategy to develop industrial vinegar production.

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APPENDICES

Appendix A

Bacterial Media and Antibiotics

1. Potato medium

Glucose	5	g
Yeast	10	g
Polypeptone	10	g
Glycerol	20	ml
Potato extract	15	ml
H_2O	1	L

Sterilization was performed by autoclaving at 121°C for 15 min. For agar, 15 g of agar was added to the liquid media before autoclaving.

2. Seed culture medium (SCM)

Glucose	5	g
Yeast extract	5	g
Polypeptone	5	g
Glycerol	5	ml
H_2O	1	L

Sterilization was performed by autoclaving at 121°C for 15 min. For agar, 15 g of agar was added to the liquid media before autoclaving.

3. YPG medium

Yeast extract	2	g
Polypeptone	2	g
Glycerol	2	g
H_2O	1	L

Sterilization was performed by autoclaving at 121°C for 15 min.

4. LB (Luria-Bertani) medium

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
H_2O	1	L

The pH was adjusted to 7.0 with 5 N NaOH. The volume of the solution was adjusted to 1 L with H_2O. Sterilization was performed by autoclaving at 121°C for 15 min.

For preparation of LB agar containing 50 µg/ml ampicillin, after sterilization, LB medium was allowed to cool down to 50°C and then 100 µl of 50 mg/ml ampicillin stock solution was added. The medium can be stored at 4°C for up to one month or at room temperature for up to one week.

5. 50 mg/ml Ampicillin

Ampicillin stock solution was prepared by dissolving 50 mg of ampicillin in 1 ml of distilled water. This stock solution was sterilized by filtration through a 0.22 µm filter. The solution was stored at -20°C.

6. 50 mg/ml Kanamycin

Kanamycin stock solution was prepared by dissolving 50 mg of kanamycin in 1 ml of distilled water. This stock solution was sterilized by filtration through a 0.22 µm filter. The solution was stored at -20°C.

7. 25 mg/ml Tetracycline

Tetracycline stock solution was prepared by dissolving 25 mg of tetracycline in 1 ml of 70% ethanol. This stock solution was sterilized by filtration through a 0.22 µm filter. The solution was stored in light-tight container at -20°C.

Appendix B

Preparation of reagents

1. Preparation of commonly reagents

1.1 20 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)

A stock X-gal solution was prepared by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at -20°C. It is not necessary to sterilize X-gal solution by filtration.

1.2 1 M Tris

A Tris HCl solution was prepared by dissolving 121.1 g of Tris base in 800 ml of double distilled water. The solution was adjusted the pH to the desired value by adding concentrated HCl as described below and allowed the solution to cool down to room temperature before making final adjustment to the desired pH. The volume of the solution was adjusted to 1 L with double distilled water, and then was sterilized by autoclaving.

pH	Volume of HCl (ml)
7.4	70
7.6	60
8.0	42

1.3 5 M NaCl

A 292.2 g of NaCl was dissolved in 800 ml of double distilled water, and adjusted the volume to 1 L with double distilled water. The solution was sterilized by autoclaving.

1.4 10% SDS

The stock solution of 10% SDS was prepared by dissolved 10 g of sodium dodecyl sulphate in 100 ml of sterilized distilled water. Sterilization is not required for the preparation of this stock solution.

1.5 1 M Potassium acetate (pH 7.5)

A 9.82 g of potassium acetate was dissolved in 60 ml of double distilled water. The solution was adjusted pH to 7.5 with glacial acetate acid and added double distilled water to adjust volume to 100 ml.

1.6 3 M Sodium acetate (pH 5.2)

A 3 M Sodium acetate pH 5.2 was prepared by dissolving 408.1 g of sodium acetate.$3H_2O$ in 800 ml of double distilled water. The solution was adjusted the pH to 5.2 with glacial acetic acid, and then adjusted volume to 1 L with double distilled water. The solution was sterilized by autoclaving at 121°C for 15 min.

1.7 0.5 M EDTA (pH 8.0)

A 186.1 g of disodium ethylenediaminetetra-acetate.$2H_2O$ was added to 800 ml of double distilled water. The pH was adjusted to 8.0 with pellet of NaOH. This stock solution was sterilized by autoclaving.

1.8 10 mg/ml Ethidium bromide

The ethidium bromide solution was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water. The solution was stored in light-tight container at room temperature.

1.9 Saline-EDTA

NaCl	0.1	M
EDTA.2Na (pH 8.0)	50	mM

1.10 Phenol:chloroform:isoamyl alcohol

Crystalline phenol was liquided in water bath at 65°C and mixed with chloroform and isoamyl alcohol in the ratio of 25:24:1. The solution was stored under 100 mM Tris HCl (pH 8.0) in light-tight bottle at 4^{o}C.

1.11 TE buffer

Tris HCl (pH 8.0)	10	mM
EDTA.2Na (pH 8.0)	1	mM

1.12 TE buffer with 10 µg/ml RNase H

TE buffer	990	µl
10 mg/ml of RNase H	10	µl

1.13 Electrophoresis buffer (1xTAE)

The working solution of 1X TAE buffer was prepared as followed.

Tris base	4.84	g
Glacial acetic acid	1.15	ml
0.5 M EDTA.2Na (pH 8.0)	3	ml
H_2O	1	L

1.14 Gel loading buffer

Bromophenol blue	0.025	g
Glycerol	3	ml

1.15 TNE buffer

Tris-HCl pH 8.0	30	mM
NaCl	50	mM
EDTA	5	mM

2. Lysis buffer for size screening method

EDTA	5	mM
Sucrose	10%	
SDS	0.25%	
NaOH	100	mM
KCl	60	mM
Bromophenol blue	0.05%	

3. Alkaline lysis buffers for minipreparation of plasmid DNA

3.1 Solution I

Glucose	50	mM
Tris HCl (pH 8.0)	25	mM
EDTA (pH 8.0)	10	mM

Solution I can be prepared in batches of approximately 100 ml, autoclaved at 121°C for 15 min and stored at 4°C.

3.2 Solution II

NaOH	0.2	N
SDS	1%	

The solution II was freshly prepared from solution of 2 N NaOH and 10% SDS.

3.3 Solution III

Potassium acetate	60	ml
Glacial acetic acid	11.5	ml
H_2O	28.5	ml

4. Reagents and buffers for Southern hybridization

4.1 Depurination solution (0.25 N HCl)

Concentrated HCl	11	ml
H_2O	989	ml

The solution was mixed and stored at room temperature for up to 1 month.

4.2 Denaturation solution (0.5 M NaOH and 1.5M NaCl)

NaOH	20	g
NaCl	87.7	g

The solution was adjusted volume to 1L with double distilled water. Stored at room temperature for up to 3 months.

4.3 Neutralization solution (0.5 M Tris and 1.5M NaCl)

Tris base	60.6	g
NaCl	87.7	g

The solution was adjusted volume to 1L with double distilled water. Stored at room temperature for up to 3 months.

4.4 20x SSC solution (0.3 M Na_3citrate.$2H_2O$ and 3 M NaCl)

Na_3citrate .$2H_2O$	88.23	g
NaCl	175.32	g

The solution was adjusted pH to 7.2 with 1 N NaOH and adjusted volume to 1 L with double distilled water. Stored at room temperature for up to 3 months.

4.5 Hybridization solution (5%Blocking reagent and 0.5 M NaCl in hybridization buffer)

Blocking reagent	1	g
NaCl	0.58	g
Denhardt's hybridization buffer	20	ml

1 g of blocking reagent and 0.58 g of NaCl was dissolved into 20 ml of Denhardt's hybridization buffer. The hybridization solution was preheated at 42°C for 20 min.

4.6 Washing buffer solution I (0.5x SSC and 0.4% SDS)

20x SSC stock solution	25	ml
SDS	4	g

The solution was made up to a final volume of 1 L.

4.7 Washing solution II (2x SSC)

20x SSC stock solution	100	ml

The solution was made up to a final volume of 1 L.

4.8 Detection solution

Detection reagent I	2	ml
Detection reagent II	2	ml

5. Reagents for determination of protein concentration

5.1 Solution A

2% Na_2CO_3	20	g/l
0.1 N NaOH	4	g/l
0.5% SDS	5	g/l

5.2 Solution B

0.5% $CuSO_4.5H_2O$	4	g
1% Potassium sodium tartrate	2	g

The solution was adjusted volume to 400 ml with distilled water and mixed gently by stirring. The solution was filtrated by filter paper. Working reagent was prepared by mixing solution A and solution B in the ration 50:1.

5.3 Solution C

The solution of phenol reagent was diluted double times with distilled water and stored in refrigerator.

6. Reagents and buffers for Enzyme activity assay

6.1 0.1 M Potassium ferricyanide ($K_3[Fe(CN)_6]$)

A 32.9 g of potassium ferricyanide was dissolved in distilled water and adjusted volume to 1 L. Keep in light-tight container at 4°C.

6.2 1 M Ethanol

A 2.9 ml of absolute ethanol was diluted in distilled water and adjusted volume to 50 ml. Keep in light-tight container at 4°C.

6.3 1 M Acetaldehyde

A 2.79 ml of acetaldehyde was diluted in distilled water and adjusted volume to 50 ml. Keep in light-tight container at 4°C.

6.4 Ferricsulfated-dupanol reagent

$Fe(SO_4)_3. xH_2O$	5	g
SDS	3	g
85% Phosphoric	95	ml

The solution was adjusted volume to 1 L with distilled water and mixed gently by stirring. Keep in light-tight container at room temperature.

7. Reagents and buffers for Sodium dodecylsulfate acrylamide gel electrophoresis (SDS-PAGE) for CBR and heme staining

7.1 25% Acrylamide bis stock solution

25% (w/v) acrylamide and 1% (w/v) N, N'-methylenebisacrylamide was resuspended in deionized water. The pH of the solution should be 7.0 or less. The solution should be stored in dark bottle at room temperature.

7.2 10% Ammonium persulfate

10 g of Ammonium persulfate was dissolved in deionized water, and kept at -20°C.

7.3 Preparation of SDS-polyacrylamide gels

7.3.1 Stacking gel (3.003 ml)

25% Acrylamide bis stock solution	600	µl
0.5 M Tris-HCl (pH 6.8)	750	µl
10% SDS	30	µl
Distilled water	1.6	ml

10% Ammonium persulfate	20	µl
TEMED	3	µl

7.3.2 Separating gel (8.048 ml)

25% Acrylamide bis stock solution	4	ml
1.5 M Tris-HCl (pH 8.8)	2	ml
10% SDS	80	µl
Distilled water	1.9	ml
10% Ammonium persulfate	54	µl
TEMED	14	µl

7.4 Sample buffer for heme staining

0.5 MTris-HCl (pH 6.8)	125	mM
SDS	6%	
Dithiothreitol (DTT)	200	mM
Phenylmethlsulfonyl fluoride (PMSF)	3	mM
Glycerol	20%	

7.5 Running buffer

Tris base	30	g
Glycine	140	g
SDS	5	g

7.6 CBR staining

Coomassie Brilliant Blue R-250 (CBR)	0.25	g
Methanol	45	ml
Distilled water	45	ml
Glacial acetic acid	10	ml

7.7 Destaining solution for CBR

Methanol	5	ml
Glacial acetic acid	7	ml
Distilled water	100	ml

8. Immunoblotting analysis of ADH protein

Immunoblot method was described in Material and Methods. The reagents and buffers in immunoblot were shown belows.

8.1 Transferring buffer (pH 8.5)

Tris-glycine buffer (pH 8.5)	100	ml
Methanol	160	ml
Distilled water	540	ml

8.2 Tris-glycine buffer (pH 8.5)

Tris base (0.2 M)	24.228	g/l
Glycine (1.2 M)	90.084	g/l

8.3 2x Tris-buffered saline (TBS)

Tris base	4.84	g
NaCl	58.44	g

The pH of solution buffer was adjusted to 7.5 with conc. HCl, and then the volume of the solution was adjusted to 1 L with distilled water. The solution has to be diluted double times before used.

8.4 Tris-buffered saline with 0.05% Tween 20

2x TBS	50	ml
Tween 20	25	µl
Distilled water	50	ml

8.5 3% Gelatin in TBS solution (blocking solution)

Gelatin powder	3	g
2x TBS	50	ml
Distilled water	50	ml

8.6 1% Gelatin in TBS solution (buffer for antibody)

3% Gelatin in TBS	50	ml
2x TBS	50	ml
Distilled water	50	ml

8.7 Anti-ADH primary antibody

1% Gelatin in TBS solution	75	ml
Anti-ADH antibody	100	µl

8.8 Second antibody against anti-ADH primary antibody

1% Gelatin in TBS solution	75	ml
Protein A-horse raddish peroxidase solution	10	µl

8.9 Detection solution

TBS	50	ml
Hydrogen peroxide	30	µl
Color reagent	10	ml

8.10 Color reagent

Diaminobenzidine (DAB)	30	mg
Cold methanol	10	ml

9. Preparation of buffers

9.1 Preparation of potassium phosphate buffer (KPB)

50 mM KPB (pH 6.0)	1 M K_2HPO_4	5 ml
	1 M KH_2PO_4	45 ml
50 mM KPB (pH 6.5)	1 M K_2HPO_4	15.5 ml
	1 M KH_2PO_4	34.5 ml
50 mM KPB (pH 7.0)	1 M K_2HPO_4	29 ml
	1 M KH_2PO_4	21 ml
50 mM KPB (pH 7.5)	1 M K_2HPO_4	40.8 ml
	1 M KH_2PO_4	9.2 ml

The solution was adjusted to 1 L with distilled water. Alternatively, preparation of 1 M KPB in various pH and then diluted to the used concentration with distilled water.

9.2 Preparation of McIIvaine buffer

McIIvaine buffer in various pH	0.1 M Citric acid (ml)	0.2 M Na_2HPO_4 (ml)
2.6	17.82	2.18
3.0	15.89	4.11
3.6	13.56	6.44
4.0	12.90	7.71
4.6	10.65	9.35
5.0	9.70	10.30
5.6	8.40	11.60
6.0	7.37	12.63
6.6	5.45	14.55
7.0	3.53	16.47
7.6	1.27	18.73
8.0	0.55	19.45

Appendix C

General methods

1. Size screening method

A bacterial colony was picked by sterile toothpick and resuspended in the prewarmed lysis buffer (at 37°C, Appendix B). The suspension was incubated at 37°C for 5 min, placed on ice for 5 min and spin down for 1 min. Then, 20 µl of supernatant was loaded on 0.8% agarose gel.

2. Alkaline lysis method for *E. coli*

Alkaline lysis method was described by Sambrook *et al*. (1989). *E. coli* harboring plasmid was grown in LB broth containing appropriate antibiotic at 37°C for 12-24 h. The cells were harvested by centrifugation at 9000 rpm for 2 min. The cells were resuspended in 100 µl of solution I (Appendix B). Then, 200 µl of a freshly prepared solution II (Appendix B) was added into the cells suspension and gently mixed by inverting the tube until the solution is clear and viscous. The tube was stored on ice for 5 min and 150 µl of ice-cold solution III (Appendix B) was added into this tube followed by vortex and stored the tube on ice for 5 min. The supernatant was transferred into a new tube after centrifuged at 10,000 rpm for 10 min. Phenol extraction was carried out by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the sample. The upper layer of the mixture was transferred to a fresh tube after centrifuged at 10,000 rpm for 15-20 min. The plasmid DNA was precipitated with 2 volume of ice-cold absolute ethanol, further stored at -20°C overnight or -80°C for 30 min and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was discarded and kept the pellet of nucleic acid to dry in the air before redissolved in 20 µl of TE buffer containing 3 µg/ml RNase H. The solution was mixed and incubated at 37°C overnight and stored at -20°C. Plasmid DNA was analyzed by running in 0.8% agarose gel electrophoresis.

3. Isolation of plasmid DNA by QIAprep spin column

The bacterial cell pellet was resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. 250 µl of buffer P2 was added and gently inverted the tube 4-6 times to mix. 350 µl of buffer N3 was added and inverted the tube immediately but gently 4-6 times and centrifuged at 13,000 rpm for 10 min in a table-top microcentrifuge. The supernatant was applied to the QIAprep spin column by pipetting and centrifuged for 1 min, The flow-through was discarded. The QIAprep spin column was washed by adding 0.5 ml buffer PB and centrifuged for 1 min. The flow-through was discarded again. The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 1 min. The flow-through was discarded again and centrifuged for 1 min. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 50 µl of EB buffer or H_2O to the center of the QIAprep membrane and centrifuged the column for 1 min. Alternatively, for increased DNA concentration, 30 µl of elution buffer was added to the center of the QIAprep membrane, let the column stand for 1 min, and then centrifuged.

4. Purification of DNA fragment by QIAquick Gel Extraction Kit

A DNA fragment was cut from the agarose gel with a clean and sharp scalpel. The weighed gel slice was placed in a colorless tube and added 3 volumes of QC buffer to 1 volume of gel (100 µl/100 mg). The mixture was incubated at 50°C for 10 min or until the gel slice has completely dissolved. After the gel slice has dissolved completely, check that the color of the mixture is yellow. A one gel volume of isopropanol was added to the sample and mixed. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min. The supernatant was discarded and placed QIAquick column back in the same collection tube. A 0.5 ml of QG Buffer was added to QIAquick column and centrifuged for 1 min. QIAquick column was washed by adding 0.75 ml of PE buffer to QIAquick column and centrifuged for 1 min. The supernatant was discarded and placed QIAquick column back in the same collection tube and centrifuged for 1 min again. The QIAquick column was placed into a clean 1.5

ml microcentrifuge tube. DNA was eluted by adding 50 µl of EB buffer or H_2O to the center of the QIAquick membrane and centrifuged the column for 1 min. Alternatively, for increased DNA concentration, added 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and the centrifuge for 1 min.

5. Preparation of *E. coli* DH5α competent cells for electroporation

Competent cells were prepared by the Operating Instruction and Application Guide for *E. coli* Pulser™ (Bio-Rad Laboratories Inc., USA). A 200 µl of a fresh overnight culture was inoculated into 50 ml of LB broth. The culture was grown at 37°C with vigorous shaking to an ABS_{600} of approximately 0.5-0.7 (the best results are obtained with cells that are harvested at early-to mid-log phase). Throughout their preparation, the cells were kept as close to 0^oC as possible (in an ice/water bath). The culture was harvested and centrifuged in a cold centrifuge bottle in a cold rotor at 4000 x g for 15 min. Remove a much of the supernatant as possible. The pellets were gently resuspended in a total of 40 ml of ice-cold 10% glycerol and centrifuged as previously described. The pellets were resuspended again in 20 ml of ice-cold 10% glycerol, and centrifuged again. Finally the pellets were resuspended in 1 ml of ice-cold 10% glycerol. Competent cells were aliquoted into 1.5 ml microcentrifuge tube 40 µl per tube and ready for electro-transformation. The competent cells were frozen in Liquid-Nitrogen and stored at -80°C.

6. Preparation of membrane fractions

The cells were harvested from late-log phase growth by centrifugation at 9,000 rpm for phosphate buffer (KPB) pH 7.0 (Appendix B) and resuspended in the same buffer. The cell suspension was passed twice through a French Cell Press at 16,000 psi (Thermo Electron Co., USA). The remaining intact cells were removed by centrifugation at 6,000 rpm for 10 min. The supernatant was collected and ultracentrifuged at 40,000 rpm for 90 min. The precipitate (membrane fraction) was homogenized in 0.5 ml of 10 mM KPB pH 7.0.

7. Enzyme activities assay of membrane-bound ADH (EC 1.1.99.8) and ALDH (EC 1.2.99.3)

The membrane fraction was used for determination of ADH and ALDH activities by colorimetrically method with potassium ferricyanide as an electron acceptor described Adachi *et al*. (1978) with slight modifications by Matsushita *et al*. (1995). All operations were done at 25°C. ADH and ALDH activities were determined in a 1 ml of reaction volume. 600 µl 0f McIIvaine buffer pH 5.0 (Appendix B), 100 µl of enzyme solution and 100 µl of 1 M substrate (ethanol for ADH and acetaldehyde for ALDH activities, Appendix B) were mixed. The reaction mixtures were pre-incubated at 25°C for 5 min and 200 µl of 0.1 M potassium ferricyanide (Appendix B) was added to start the reaction. After incubation for 5 min, the reaction was stopped by adding 0.5 ml of ferricsulfated-dupanol reagent (Appendix B). After standing at 25°C for 20 min, the reaction mixtures were diluted with 3.5 ml of distilled water and mixed well. The absorbance was measured at 660 nm. One unit of the enzyme activity was expressed as 1 µmol of substrate oxidized per min, which was equivalent to 4.0 absorbance units. The enzyme activity was calculated as follows.

Enzyme activity (U/ml) = A_{660} x 1/4x 1/5 x 1/Enzyme (ml) x Dilution factor

Specific activity (U/mg of protein) = Enzyme activity (U/ml)/Protein concentration (mg/ml)

8. Determination of protein concentration

Protein concentration was determined by the modified method of Lowry (Dully and Grieve, 1975). The reaction mixture was shown as follows.

1 mg/ml BSA (μl)	-	10	20	30	40	50	60
Distilled water (μl)	400	390	380	370	360	350	340

Each assay was carried out by mixing 0.4 ml of protein solution with 2.0 ml of Folin reagent (solution A: solution B = 50 : 1), mixed by vortex, and incubated at 35°C for 10 min. After standing incubation, 0.2 ml of phenol reagent (solution C) was added with rapid mixing. After further incubation for at least 30 min at 35°C, the absorbance was measured at 750 nm (Hitachi Model U-2000 spectrophotometer) against reagent blank. The concentration of protein was determined by calculating from a standard curve prepared by using 1 mg/ml of bovine serum albumin (BSA) as a standard. The protein concentration was calculated as follows.

Protein concentration (mg/ml) = Concentration from spectrophotometer (mg/ml) x Dilution factor/Sample (μl)

9. Determining of acetic acid in the solution by alkaline-titration method

Acidity or acetic acid concentration in the culture media was determined by modified method of Saeki *et al*. (1997a). A 1 ml of culture media was titrated with 25 µl of 0.8 N NaOH using 10 µl of phenolphthalein as a pH indicator. %Acetic acid was calculated as described as bellows.

$$\%\text{Acetic acid} = 0.12 (X - 1)$$

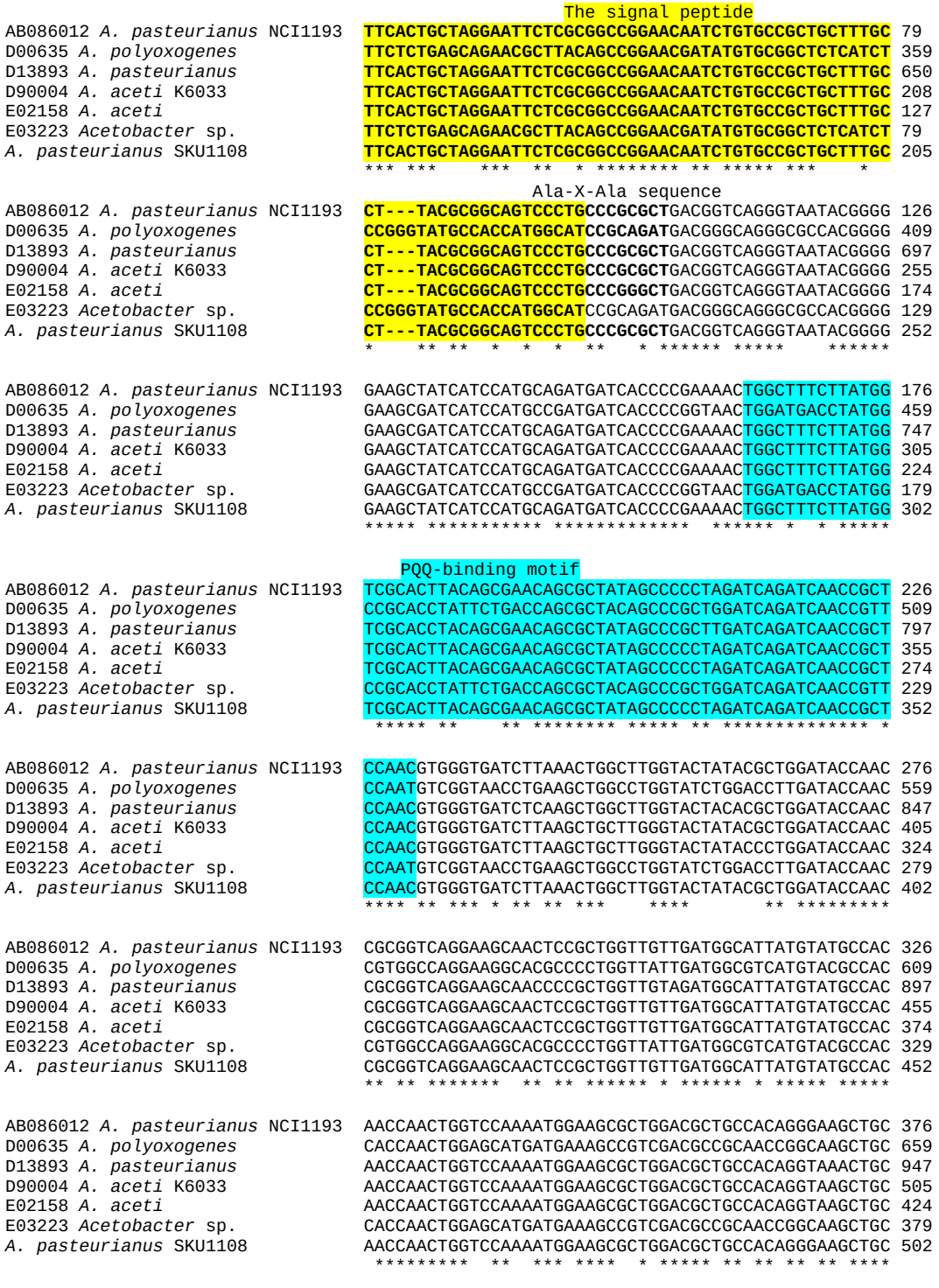
When; X = number of drop of 0.8 N NaOH

```
AB086012 A. pasteurianus NCI1193  --------------------------------------------------
D00635 A. polyoxogenes            --------------------------------------------------
D13893 A. pasteurianus            GCACTCGTTCATTCCGGGTTGGACGTTGCGCAACAAGGCGAGCGAATCAT 50
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           --------------------------------------------------


AB086012 A. pasteurianus NCI1193  --------------------------------------------------
D00635 A. polyoxogenes            --------------------------------------------------
D13893 A. pasteurianus            TCTCACGGCCAAGATTCTGTCCGCTGTATGTGATCACCTTTCCGTGAAAA 100
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           --------------------------------------------------


AB086012 A. pasteurianus NCI1193  --------------------------------------------------
D00635 A. polyoxogenes            --------------------------------------------------
D13893 A. pasteurianus            TCAAACACCAGGCGCAGCGGGCGGGTGTGAAGATAAAGCGCATCTGCCAC 150
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           --------------------------------------------------


AB086012 A. pasteurianus NCI1193  --------------------------------------------------
D00635 A. polyoxogenes            --------------------------------------------------
D13893 A. pasteurianus            CCACTCAGCGTACCCCTAGCCACGATCTCAACTGTTCCACCAGAAGAGGT 200
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           --------------------------------------------------


AB086012 A. pasteurianus NCI1193  --------------------------------------------------
D00635 A. polyoxogenes            --------------------------------------------------
D13893 A. pasteurianus            CCATAATTGGTTGCGCATCCACCACAGCCTGCGTGCGCAAGATGAAGATG 250
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           --------------------------------------------------
```

Appendix Figure C1 Alignment of nucleotide sequences of *adhAB* gene from other acetic acid bacteria. The forward primers used for amplification of *adhAB* and the *adhA-adhB* junction region from *A. syzygii* SKU19 were indicated as (>>>>>>) and (#####) symbol, respectively. The reverse primers used for amplification of *adhAB* and the *adhA-adhB* junction region from *A. syzygii* SKU19 were indicated as (<<<<<<) and (+++++), respectively; is single, fully conserved residue and ( ) is no consensus.

```
AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            ----------------------------------------GAATTCCGAAC 11
D13893 A. pasteurianus            TTTATGAATTCACCCTGTTCAGTTCTTGGAAAAATCGTGAAACGACAGAT 300
D90004 A. aceti K6033             ------------------------------------------------
E02158 A. aceti                   ------------------------------------------------
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           ------------------------------------------------


AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            TATCCGTTTCATTGCTTATGCGACAGCATGTTCACTTTTTAGTGAGGCTG 61
D13893 A. pasteurianus            ATGGAACTTCTGGCTGATTATGCGTTTATACCAAGTTGCATAATACCATC 350
D90004 A. aceti K6033             ------------------------------------------------
E02158 A. aceti                   ------------------------------------------------
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           ------------------------------------------------


AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            AACACTAAAATGTCAGGAGACGAGCGTGCTAGCCTCAGTATGTTGCCATG 111
D13893 A. pasteurianus            CTGATTGTTATAACAAACATATAGCGCCCTTATCCTATCTTAATTGTGAG 400
D90004 A. aceti K6033             ------------------------------------------------
E02158 A. aceti                   ------------------------------------------------
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           ------------------------------------------------


AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            AAACGGACCACCTGCTTTGTCTTTCCTGCCTGAAGCCGGTTTCTGTCTGG 161
D13893 A. pasteurianus            CATAAATTCCGGCTCTACACGCCTGTAAGCACGGGAAATTATCTCGCAAA 450
D90004 A. aceti K6033             ----------------------------------------CCCGCAGA 8
E02158 A. aceti                   ------------------------------------------------
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           ----------------------------------------CTGCG 5


                                                  -35 promoter
AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            CCGGAAAAGAAGCGCTAGCGCGTTTTTTTGCCGGATACATTCAGAAAGCT 211
D13893 A. pasteurianus            ATGGAAGCTGCTATTCCTTCCTCGTGATTGACAGACAGCGTTCCCCTTGG 500
D90004 A. aceti K6033             ATGGAAGCTGCTATTCCTTCCCCGTGATTGACAGACAGCGTTCCCCTTGG 58
E02158 A. aceti                   ------------------------------------------------
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           GTCGACGCTGCTATTCCTTCCTCGTGATTGACAGACAGCGTTCCCCTTGG 55


                                                                              -10
AB086012 A. pasteurianus NCI1193  ------------------------------------------------
D00635 A. polyoxogenes            GCTCCGGGCAGAAAGTTGCAGCGGCGGC-ATCCTGAATTCGAAACCGTTA 260
D13893 A. pasteurianus            CTGGTGTGTGAGAAACCGGCCTGCCGGCTATCTTTTTCCTCACCGTATAT 550
D90004 A. aceti K6033             CTGTTGTGTGAGAAACCGGCCTGCCGGCTATCTTTTTCCTCACCGTATAT 108
E02158 A. aceti                   ----------------------CCGGCTATCTTTTTCCTCACCGTATAT 27
E03223 Acetobacter sp.            ------------------------------------------------
A. pasteurianus SKU1108           CTGTTGTGTGAGAAACCGGCCTGCCGGCTATCTTTTTCCTCACCGTATAT 105


                                        RBS          start point of ADHI
AB086012 A. pasteurianus NCI1193  --------------------ATGACCCGCCCCGCCTCCGCCAAGAGACG 29
D00635 A. polyoxogenes            GTTTTCTGAGGACATCA-CATATGATTTCTGCCGTTTTCGGAAAAAGACG 309
D13893 A. pasteurianus            AATAGTCGAGGACATGAACGCATGACCCGCCCCGCCTCCGCCAAGAGACG 600
D90004 A. aceti K6033             AATAGTCGAGGACATGAACGCATGACCCGCCCCGCCTCCGCCAAGAGACG 158
E02158 A. aceti                   AATAGTCGAGGACATGAACGCATGACCCGCCCCGCCTCCGCCAAGAGACG 77
E03223 Acetobacter sp.            --------------------ATGATTTCTGCCGTTTTCGGAAAAAGACG 29
A. pasteurianus SKU1108           AATAGTCGAGGACATGAACGCATGACCCGCCCCGCCTCCGCCAAGAGACG 155
                                                      ****      ***  * **  ** *****
                                                        >>>>>>>>>>>>>>>>>
```

Appendix Figure C1 (Continued)



Appendix Figure C1 (Continued)

```
AB086012 A. pasteurianus NCI1193  TTTGGCAGTATGACCCCAAAGTGCCAGACAACATTGCTGACAAAGGCTGC 426
D00635 A. polyoxogenes            TGTGGTCCTATGACCCGCGCGTGCCCGGCAACATTGCCGACAAGGGCTGC 709
D13893 A. pasteurianus            TTTGGCAGTATGACCCCAAAGTGCCAGGCAACATTGCTGACAAAGGCTGC 997
D90004 A. aceti K6033             TTTGGCAGTATGACCCCAAAGTGCCAGGCAACATTGCTGACAAAGGCTGC 555
E02158 A. aceti                   TTTGGCAGTATGACCCCAAAGTGCCAGGCAACATTGCTGACAAAGGCTGC 474
E03223 Acetobacter sp.            TGTGGTCCTATGACCCGCGCGTGCCCGGCAACATTGCCGACAAGGGCTGC 429
A. pasteurianus SKU1108           TTTGGCAGTATGACCCCAAAGTGCCAGGCAACATTGCTGACAAAGGCTGC 552
                                  * ***   ********    ***** * ********* ***** ******

AB086012 A. pasteurianus NCI1193  TGTGATACGGTTAACCGTGGTGCAGGCTATTGGAACGGCAAGGTTTTCTG 476
D00635 A. polyoxogenes            TGTGACACGGTCAACCGTGGCGCGGCATACTGGAATGGCAAGGTCTATTT 759
D13893 A. pasteurianus            TGTGATACGGTTAACCGTGGTGCAGGCTATTGGAACGGCAAGGTTTTCTG 1047
D90004 A. aceti K6033             TGTGATACGGTTAACCGTGGTGCAGGCTATTGGAACGGCAAGGTTTTCTG 605
E02158 A. aceti                   TGTGATACGGTTAACCGTGGTGCAGGCTATTGGAACGGCAAGGTTTTCTG 524
E03223 Acetobacter sp.            TGTGACACGGTCAACCGTGGCGCGGCATACTGGAATGGCAAGGTCTATTT 479
A. pasteurianus SKU1108           TGTGATACGGTTAACCGTGGTGCAGGCTATTGGAACGGCAAGGTTTTCTG 602
                                  ***** ***** ******** ** *  ** ***** ******** *  *

AB086012 A. pasteurianus NCI1193  GGGCACGTTTGATGGCCGTCTGGTCGCTGCCGATGCAAAAACCGGTAAAA 526
D00635 A. polyoxogenes            CGGCACGTTCGACGGTCGCCTGATCGCGCTGGACGCCAAGACCGGCAAGC 809
D13893 A. pasteurianus            GGGCACGTTTGATGGCCGCCTGGTTGCTGCCGATGCCAAAACCGGTAAAA 1097
D90004 A. aceti K6033             GGGCACGTTTGATGGCCGTCTGGTCGCTGCCGATGCAAAAACCGGTAAAA 655
E02158 A. aceti                   GGGCACGTTTGATGGCCGTCTGGTCGCTGCCGATGCAAAAACCGGTAAAA 574
E03223 Acetobacter sp.            CGGCACGTTCGACGGTCGCCTGATCGCGCTGGACGCCAAGACCGGCAAGC 529
A. pasteurianus SKU1108           GGGCACGTTTGATGGCCGTCTGGTCGCTGCCGATGCAAAAACCGGTAAAA 652
                                   ******** ** ** ** *** * **    ** ** ** ***** **

AB086012 A. pasteurianus NCI1193  AAGTGTGGGAAGTTAATACCATCCCAGCCGATGCCTCTCTGGGCAAGCAG 576
D00635 A. polyoxogenes            TGGTCTGGAGCGTCAACACCATTCCGCCCGAAGCGGAACTGGGCAAGCAG 859
D13893 A. pasteurianus            AGGTTTGGGAAGTTAACACCATCCCAGCCGATGCCTCTCTGGGCAAGCAG 1147
D90004 A. aceti K6033             AGGTGTGGGCAGTTAACACCATCCCAGCCGATGCCTCTTTGGGCAAGCAG 705
E02158 A. aceti                   AGGTGTGGGCAGTTAACACCATCCCAGCCGATGCCTCTTTGGGCAAGCAG 624
E03223 Acetobacter sp.            TGGTCTGGAGCGTCAACACCATTCCGCCCGAAGCGGAACTGGGCAAGCAG 579
A. pasteurianus SKU1108           AGGTGTGGGAAGTTAATACCATCCCAGCCGATGCCTCTCTGGGCAAGCAG 702
                                    ** ***   ** ** ***** **  **** **     ***********

AB086012 A. pasteurianus NCI1193  CGCTCCTACACAGTGGATGGCGCGGTACGCGTAGCGAAGGGCCTGGTGCT 626
D00635 A. polyoxogenes            CGTTCCTATACGGTTGACGGCGCGCCCCGTATCGCCAAGGGCCGCGTGAT 909
D13893 A. pasteurianus            CGCTCCTACACAGTGGATGGCGCGGTACGTGTAGCAAAAGGCCTTGTGCT 1197
D90004 A. aceti K6033             CGCTCCTACACAGTGGATGGCGCGGTACGCGTAGCGAAGGGCCTGGTCCT 755
E02158 A. aceti                   CGCTCCTACACAGTGGATGGCGCGGTACGCGTAGCGAAGGGCCTGGTCCT 674
E03223 Acetobacter sp.            CGTTCCTATACGGTTGACGGCGCGCCCCGTATCGCCAAGGGCCGCGTGAT 629
A. pasteurianus SKU1108           CGCTCCTACACAGTGGATGGCGCGGTACGCGTAGCGAAGGGCCTGGTGCT 752
                                  ** ***** ** ** ** ******   **  * ** ** ****  **  *

AB086012 A. pasteurianus NCI1193  GATTGGTAACGGTGGTGCCGAGTTCGGCGCACGGGGTTTTGTTTCCGCTT 676
D00635 A. polyoxogenes            CATCGGTAACGGTGGTTCCGAATTCGGTGCCCGTGGCTTCGTCAGCGCGT 959
D13893 A. pasteurianus            GATTGGTAACGGTGGCTCTGAATTTGGTGCCCGTGGCTTTGTTTCTGCTT 1247
D90004 A. aceti K6033             GATTGGTAACGGTGGTGCCGAATTTGGTGCCCGTGGCTTTGTTTCCGCTT 805
E02158 A. aceti                   GATTGGTAACGGTGGTGCCGAATTTGGTGCCCGTGGCTTTGTTTCCGCTT 724
E03223 Acetobacter sp.            CATCGGTAACGGTGGTTCCGAATTCGGTGCCCGTGGCTTCGTCAGCGCGT 679
A. pasteurianus SKU1108           GATTGGTAACGGTGGTGCCGAATTTGGTGCCCGTGGCTTTGTTTCCGCTT 802
                                   ** ***********  * ** ** ** ** ** ** ** **    ** *

AB086012 A. pasteurianus NCI1193  TTGGTGCCGAAACCGGCAAGCTGAAATGGCGCTTCTACACCGTTCCGAAC 726
D00635 A. polyoxogenes            TCGATGCGGAAACCGGCAAGGTCGACTGGCGCTTCTTCACGGTTCCGAAC 1009
D13893 A. pasteurianus            TTGATGCCGAAACCGGCAAGCTGAAATGGCGCTTCTACACTGTTCCCAAC 1297
D90004 A. aceti K6033             TTGATGCCGAAACCGGCAAGCTGAAATGGCGCTTCTACACCGTTCCGAAC 855
E02158 A. aceti                   TTGATGCCGAAACCGGCAAGCTGAAATGGCGCTTCTACACCGTTCCGAAC 774
E03223 Acetobacter sp.            TCGATGCGGAAACCGGCAAGGTCGACTGGCGCTTCTTCACGGTTCCGAAC 729
A. pasteurianus SKU1108           TTGATGCCGAAACCGGCAAGCTGAAATGGCGCTTCTACACCGTTCCGAAC 852
                                  * * *** ************ *  * ********** *** ***** ***
```

Appendix Figure C1 (Continued)

```
AB086012 A. pasteurianus NCI1193  AACAAGAACGAAACCGATCACGCTGCATCAGATAATATTCTGATGAAAAA 776
D00635 A. polyoxogenes            CCCAAGAACGAACCGGAC---GCTGCATCCGACAGCGTGCTGATGAACAA 1056
D13893 A. pasteurianus            AACAAGAACGAACCTGACCATGCGGTAGCAGACAATGTTCTGATGAGCAA 1347
D90004 A. aceti K6033             AATAAGAACGAACCCGACCACGCCGCATCAGATAATATTCTGATGAACAA 905
E02158 A. aceti                   AATAAGAACGAACCCGACCACGCCGCATCAGATAATATTCTGATGAACAA 824
E03223 Acetobacter sp.            CCCAAGAACGAACCGGAC---GCTGCATCCGACAGCGTGCTGATGAACAA 776
A. pasteurianus SKU1108           AACAAGAACGAACCCGATCACGCTGCATCAGATAATATTCTGATGAACAA 902
                                     ********* * **    ** * * * ** *   * *******  **

AB086012 A. pasteurianus NCI1193  AGCCTACAAAACCTGGGGCCCCAAAGGCGCTTGGGAACGCCAAGGTGGCG 826
D00635 A. polyoxogenes            GGCCTACCAGACCTGGAGCCCGACCGGCGCCTGGACCCGCCAGGGTGGCG 1106
D13893 A. pasteurianus            AGCCTACAAAACCTGGGGCCCCAAAGGCGCTTGGGTGCGTCAGGGCGGTG 1397
D90004 A. aceti K6033             AGCCTACAAAACCTGGGGCCCCAAAGGCGCTTGGGTACGCCAGGGTGGCG 955
E02158 A. aceti                   AGCCTACAAAACCTGGGGCCCCAAAGGCGCTTGGGTACGCCAGGGTGGCG 874
E03223 Acetobacter sp.            GGCCTACCAGACCTGGAGCCCGACCGGCGCCTGGACCCGCCAGGGTGGCG 826
A. pasteurianus SKU1108           AGCCTACAAAACCTGGGGCCCCAAAGGCGCTTGGGTACGCCAAGGTGGCG 952
                                   ****** * ****** **** *  ***** ***   ** ** ** ** *

AB086012 A. pasteurianus NCI1193  GTGGCACCGTATGGGATTTTCTGGTTTATGATCCGGTCAGTGACCTGATC 876
D00635 A. polyoxogenes            GCGGCACGGTATGGGATTCCATCGTGTATGACCCCGTGGCCGACCTGGTC 1156
D13893 A. pasteurianus            GTGGCACCGTATGGGATTCGCTGGTTTATGATCCGGTGAGCGACCTGATC 1447
D90004 A. aceti K6033             GTGGCACCGTATGGGATTCTCTGGTTTATGATCCGGTCAGTGACCTCATC 1005
E02158 A. aceti                   GTGGCACCGTATGGGATTCTCTGGTTTATGATCCGGTCAGTGACCTCATC 924
E03223 Acetobacter sp.            GCGGCACGGTATGGGATTCCATCGTGTATGACCCCGTGGCCGACCTGGTC 876
A. pasteurianus SKU1108           GTGGCACCGTATGGGATTCTCTGGTTTATGATCCGGTCAGTGACCTGATC 1002
                                  * ***** **********   * ** ***** ** **    *****  **

AB086012 A. pasteurianus NCI1193  TATCTGGCTGTTGGTAACGGGTTTCCCTGGAACTATAAGTATCGTTCCGA 926
D00635 A. polyoxogenes            TACCTGGGCGTTGGCAACGGTTCGCCGTGGAACTACAAGTACCGTTCCGA 1206
D13893 A. pasteurianus            TATCTGGCTGTTGGTAACGGATCTCCTTGGAACTATAAGTATCGTTCCGA 1497
D90004 A. aceti K6033             TATCTGGCTGTTGGTAACGGATCTCCCTGGAACTATAAGTATCGTTCCGA 1055
E02158 A. aceti                   TATCTGGCTGTTGGTAACGGATCTCCCTGGAACTATAAGTATCGTTCCGA 974
E03223 Acetobacter sp.            TACCTGGGCGTTGGCAACGGTTCGCCGTGGAACTACAAGTACCGTTCCGA 926
A. pasteurianus SKU1108           TATCTGGCTGTTGGTAACGGGTCTCCCTGGAACTATAAGTATCGTTCCGA 1052
                                  ** ****  ***** ***** *  ** ******** ***** ********

AB086012 A. pasteurianus NCI1193  AGGCATTGGTTCCAACCTGTTCCTGGGCAGCATTGTCGCACTGAAGCCTG 976
D00635 A. polyoxogenes            AGGCAAGGGCGACAACCTGTTCCTGGGCAGCATCGTCGCACTGAAGCCGG 1256
D13893 A. pasteurianus            AGGCATTGGTTCCAACCTGTTCCTGGGCAGCATTGTTGCACTGAAACCTG 1547
D90004 A. aceti K6033             AGGCATTGGTTCCAACCTGTTCCTGGGCAGCATTGTCGCACTGAAGCCTG 1105
E02158 A. aceti                   AGGCATTGGTTCCAACCTGTTCCTGGGCAGCATTGTCGCACTGAAGCCTG 1024
E03223 Acetobacter sp.            AGGCAAGGGCGACAACCTGTTCCTGGGCAGCATCGTCGCACTGAAGCCGG 976
A. pasteurianus SKU1108           AGGCATTGGTTCCAACCTGTTCCTGGGCAGCATTGTCGCACTGAAGCCTG 1102
                                  *****  **   ********************** ** ******** ** *

AB086012 A. pasteurianus NCI1193  AAACCGGTGAATATGTCTGGCACTTCCAGGCGACCCCGATGGATCAGTGG 1026
D00635 A. polyoxogenes            AAACCGGCGAATACGTCTGGCATTTCCAGGAAACGCCGATGGACCAGTGG 1306
D13893 A. pasteurianus            AAACCGGTGAATATGTCTGGCATTTCCAGGCAACCCCAATGGATCAGTGG 1597
D90004 A. aceti K6033             AAACCGGTGAATATGTCTGGCACTTCCAGGCAACCCCGATGGATCAGTGG 1155
E02158 A. aceti                   AAACCGGTGAATATGTCTGGCACTTCCAGGCAACCCCGATGGATCAGTGG 1074
E03223 Acetobacter sp.            AAACCGGCGAATACGTCTGGCATTTCCAGGAAACGCCGATGGACCAGTGG 1026
A. pasteurianus SKU1108           AAACCGGTGAATATGTCTGGCACTTCCAGGCAACCCCGATGGATCAGTGG 1152
                                  ******* ***** ******** *******  ** ** ***** ******

AB086012 A. pasteurianus NCI1193  GATTACACCTCGGTTCAGCAGATCATGACCCTTGATATGCCAGTGAATGG 1076
D00635 A. polyoxogenes            GACTTCACCTCGGACCAGCAGATCATGACGCTTGACCTGCCGATCAATGG 1356
D13893 A. pasteurianus            GATTACACCTCGGTTCAGCAGATCATGACGCTGGATATGCCTGTTAACGG 1647
D90004 A. aceti K6033             GATTACACCTCGGTTCAGCAGATCATGACACTGGATATGCCAGTGAAAGG 1205
E02158 A. aceti                   GATTACACCTCGGTTCAGCAGATCATGACACTGGATATGCCAGTGAAAGG 1124
E03223 Acetobacter sp.            GACTTCACCTCGGACCAGCAGATCATGACGCTTGACCTGCCGATCAATGG 1076
A. pasteurianus SKU1108           GATTACACCTCGGTTCAGCAGATCATGACACTGGATATGCCAGTGAATGG 1202
                                  ** * ********  ************** ** **  ****  * ** **
```

Appendix Figure C1 (Continued)

```
AB086012 A. pasteurianus NCI1193  CGAAATGCGCCACGTGATCGTGCATGCACCTAAAAACGGCTTCTTCTACG 1126
D00635 A. polyoxogenes            TGAAACCCGCCACGTCATCGTCCATGCGCGCAAGAACGGCTTCTTCTACA 1406
D13893 A. pasteurianus            TGAAATGCGCCACGTGATCTGGCATGCACCTAAAAACGGCTTCTTCTACG 1697
D90004 A. aceti K6033             CGAAATGCGCCACGTGATCGTGCATGCACCTAAAAACGGCTTCTTCTACG 1255
E02158 A. aceti                   CGAAATGCGCCACGTGATCGTGCATGCACCTAAAAACGGCTTCTTCTACG 1174
E03223 Acetobacter sp.            TGAAACCCGCCACGTCATCGTCCATGCGCGCAAGAACGGCTTCTTCTACA 1126
A. pasteurianus SKU1108           CGAAATGCGCCACGTGATCGTGCATGCACCTAAAAACGGCTTCTTCTACG 1252
                                   ****  ******** ***   ***** *  ** ***************

AB086012 A. pasteurianus NCI1193  TTCTGGATGCCAAAACCGGTGAATTCCTGTCCGGCAAAAACTACGTTTAC 1176
D00635 A. polyoxogenes            TCATCGATGCGAAGACCGGTGAGTTCATCTCGGGCAAGAACTACGTCTAT 1456
D13893 A. pasteurianus            TGCTGGATGCCAAGACCGGTGAATTCCTGGCTGGCAAAAACTATGTTTAC 1747
D90004 A. aceti K6033             TTCTGGATGCCAAAACCGGTGAATTCCTGTCCGGCAAAAACTACGTTTAC 1305
E02158 A. aceti                   TTCTGGATGCCAAAACCGGTGAATTCCTGTCCGGCAAAAACTACGTTTAC 1224
E03223 Acetobacter sp.            TCATCGATGCGAAGACCGGTGAGTTCATCTCGGGCAAGAACTACGTCTAT 1176
A. pasteurianus SKU1108           TTCTGGATGCCAAAACCGGTGAATTCCTGTCCGGCAAAAACTACGTTTAC 1302
                                  *  * ***** ** ******** *** *  * ***** ***** ** **

AB086012 A. pasteurianus NCI1193  CAGAACTGGGCCAATGGCCTGGACCCTCTGACAGGTCGCCCGATCTACAA 1226
D00635 A. polyoxogenes            GTGAACTGGGCCAGCGGCCTTGATCCCAAGACCGGCCGTCCGATCTACAA 1506
D13893 A. pasteurianus            CAGAACTGGGCTAACGGCCTTGACCCGTTGACCGGCCGCCCGATCTACAA 1797
D90004 A. aceti K6033             CAGAACTGGGCCAATGGTCTGGACCCTCTGACAGGTCGCCCGATGTACAA 1355
E02158 A. aceti                   CAGAACTGGGCCAATGGTCTGGACCCTCTGACAGGTCGCCCGATGTACAA 1274
E03223 Acetobacter sp.            GTGAACTGGGCCAGCGGCCTTGATCCCAAGACCGGCCGTCCGATCTACAA 1226
A. pasteurianus SKU1108           CAGAACTGGGCCAATGGCCTGGACCCTCTGACAGGTCGCCCGATCTACAA 1352
                                    ********* *  ** ** ** **   *** ** ** ***** *****

AB086012 A. pasteurianus NCI1193  TCCGGATGGTCTGTATACCCTGAACGGCAAGTTCTGGTACGGTATCCCCG 1276
D00635 A. polyoxogenes            CCCCGATGCGCTCTACACCCTTACGGGCAAGGAATGGTACGGCATTCCGG 1556
D13893 A. pasteurianus            CCCGGATGGGCTGTACACCCTCACCGGCAAGTTCTGGTATGGTATCCCAG 1847
D90004 A. aceti K6033             CCCGGATGGTCTGTATACCCTGAACGGCAAGTTCTGGTACGGTATCCCCG 1405
E02158 A. aceti                   CCCGGATGGTCTGTATACCCTGAACGGCAAGTTCTGGTACGGTATCCCCG 1324
E03223 Acetobacter sp.            CCCCGATGCGCTCTACACCCTTACGGGCAAGGAATGGTACGGCATTCCGG 1276
A. pasteurianus SKU1108           TCCGGATGGTCTGTATACCCTGAACGGCAAGTTCTGGTACGGTATCCCCG 1402
                                   ** ****  ** ** ***** *  ******   ***** ** ** ** *

AB086012 A. pasteurianus NCI1193  GCCCACTGGGTGCGCATAACTTTATGGCCATGGCCTATAGCCCCAAAACG 1326
D00635 A. polyoxogenes            GTGACCTTGGCGGCCATAACTTCGCGGCCATGGCGTTCAGCCCCAAGACC 1606
D13893 A. pasteurianus            GCCCACTGGGTGCACATAACTTCATGGGAATGGCCTATAGCCCCAAGACG 1897
D90004 A. aceti K6033             GCCCACTGGGTGCGCATAACTTTATGGCCATGGCCTACAGCCCCAAAACG 1455
E02158 A. aceti                   GCCCACTGGGTGCGCATAACTTTATGGCCATGGCCTACAGCCCCAAAACG 1374
E03223 Acetobacter sp.            GTGACCTTGGCGGCCATAACTTCGCGGCCATGGCGTTCAGCCCCAAGACC 1326
A. pasteurianus SKU1108           GCCCACTGGGTGCGCATAACTTTATGGCCATGGCCTATAGCCCCAAAACG 1452
                                  *    ** ** *  ********   **  ***** *  ******** **

AB086012 A. pasteurianus NCI1193  CACCTGGTCTATATCCCGGCTCACCAAATTCCGTTTGGTTATAAAAACCA 1376
D00635 A. polyoxogenes            GGGCTGGTCTATATTCCGGCGCAGCAGGTTCCGTTCCTGTACACCAATCA 1656
D13893 A. pasteurianus            CACTTGGTCTATCTGCCAGCACACCAGATTCCGTTTGGTTATAAAAACCA 1947
D90004 A. aceti K6033             CATCTGGTTTATATCCCGGCTCACCAGATTCCGTTTGGTTATAAAAATCA 1505
E02158 A. aceti                   CATCTGGTTTATATCCCGGCTCACCAGATTCCGTTTGGTTATAAAAATCA 1424
E03223 Acetobacter sp.            GGGCTGGTCTATATTCCGGCGCAGCAGGTTCCGTTCCTGTACACCAATCA 1376
A. pasteurianus SKU1108           CACCTGGTCTATATCCCGGCTCACCAGATTCCGTTTGGTTATAAAAACCA 1502
                                      **** *** * ** ** ** **  *******    ** *  ** **

AB086012 A. pasteurianus NCI1193  GGTTGGTGGCTTTAAGCCGCATGCGGATTCCTGGAACGTGGGTCTGGACA 1426
D00635 A. polyoxogenes            GGTCGGTGGCTTCACGCCGCACCCCGACAGCTGGAACCTGGGTCTGGACA 1706
D13893 A. pasteurianus            GGTTGGTGGCTTTAAACCGCATCCGGATGCCTGGAACGTTGGTTTGGACA 1997
D90004 A. aceti K6033             GGTTGGTGGCTTTAAGCCGCATGCGGATTCCTGGAACGTTGGTCTGGACA 1555
E02158 A. aceti                   GGTTGGTGGCTTTAAGCCGCATGCGGATTCCTGGAACGTTGGTCTGGACA 1474
E03223 Acetobacter sp.            GGTCGGTGGCTTCACGCCGCACCCCGACAGCTGGAACCTGGGTCTGGACA 1426
A. pasteurianus SKU1108           GGTTGGTGGCTTTAAGCCGCATGCGGATTCCTGGAACGTGGGTCTGGACA 1552
                                  *** ******** *  *****  * **   ******* * *** ******
```

Appendix Figure C1 (Continued)

```
AB086012 A. pasteurianus NCI1193  TGACCAAGAACGGTCTGCCTGATACCCCAGAAGCCCGTACCGCTTACATC 1476
D00635 A. polyoxogenes            TGAACAAGGTCGGTATTCCCGACTCGCCTGAAGCCAAGCAGGCCTTCGTG 1756
D13893 A. pasteurianus            TGACCAAAAACGGTCTGCCCGACACCCCAGAAGCCCGTACGGCTTACATC 2047
D90004 A. aceti K6033             TGACCAAGAACGGTCTGCCTGATACCCCAGAAGCCCGTACCGCTTACATC 1605
E02158 A. aceti                   TGACCAAGAACGGTCTGCCTGATACCCCAGAAGCCCGTACCGCTTACATC 1524
E03223 Acetobacter sp.            TGAACAAGGTCGGTATTCCCGACTCGCCTGAAGCCAAGCAGGCCTTCGTG 1476
A. pasteurianus SKU1108           TGACCAAGAACGGTCTGCCTGATACCCCAGAAGCCCGTACCGCTTACATC 1602
                                  *** ***   **** * ** **  * ** ******      ** * * *

AB086012 A. pasteurianus NCI1193  AAGGATCTGCACGGGTGGCTGCTGGCATGGGATCCGGTCAAGATGGAAAC 1526
D00635 A. polyoxogenes            AAGGACCTGAAGGGCTGGATCGTGGCCTGGGATCCGCAGAAGCAGGCTGA 1806
D13893 A. pasteurianus            AAGGATCTGCACGGCTGGCTGCTGGCCTGGGATCCGGTGAAGATGGAAAC 2097
D90004 A. aceti K6033             AAGGATCTGCACGGCTGGCTGCTGGCATGGGATCCGGTCAAGATGGAAAC 1655
E02158 A. aceti                   AAGGATCTGCACGGCTGGCTGCTGGCATGGGATCCGGTCAAGATGGAAAC 1574
E03223 Acetobacter sp.            AAGGACCTGAAGGGCTGGATCGTGGCCTGGGATCCGCAGAAGCAGGCTGA 1526
A. pasteurianus SKU1108           AAGGATCTGCACGGCTGGCTGCTGGCATGGGATCCGGTCAAGATGGAAAC 1652
                                  ***** *** * ** *** *  **** *********   ***  **

AB086012 A. pasteurianus NCI1193  GGTCTGGAAGATTGACCACAAAGGTCCGTGGAACGGCGGCATTCTGGCCA 1576
D00635 A. polyoxogenes            AGCATGGCGCGTGGACCACAAGGGGCCGTGGAACGGCGGTATCCTGGCAA 1856
D13893 A. pasteurianus            GGTCTGGAAGATTGACCATAAAGGTCCGTGGAACGGCGGCGTTCTGGCCA 2147
D90004 A. aceti K6033             GGTCTGGAAGATTGACCACAAAGGTCCGTGGAACGGCGGCATCCTGGCCA 1705
E02158 A. aceti                   GGTCTGGAAGATTGACCACAAAGGTCCGTGGAACGGCGGCATCCTGGCCA 1624
E03223 Acetobacter sp.            AGCATGGCGCGTGGACCACAAGGGGCCGTGGAACGGCGGTATCCTGGCAA 1576
A. pasteurianus SKU1108           GGTCTGGAAGATTGACCACAAAGGTCCGTGGAACGGCGGCATTCTGGCCA 1702
                                   *  ***    * ***** ** ** **************  * ***** *

AB086012 A. pasteurianus NCI1193  CCGGTGGTGATCTTCTGTTCCAGGGCTTGGCCAATGGTGAATTCCACGCC 1626
D00635 A. polyoxogenes            CTGGCGGCGACCTGCTGTTCCAGGGCTTGGCGAACGGCGAATTCCATGCC 1906
D13893 A. pasteurianus            CCGGTGGTGATCTCCTGTTCCAGGGGCTGGCAAATGGTGAATTCCACGCC 2197
D90004 A. aceti K6033             CCGGCGGTGATCTTCTGTTCCAGGGCTTGGCCAATGGTGAATTCCACGCC 1755
E02158 A. aceti                   CCGGCGGTGATCTTCTGTTCCAGGGCTTGGCCAATGGTGAATTCCACGCC 1674
E03223 Acetobacter sp.            CTGGCGGCGACCTGCTGTTCCAGGGCTTGGCGAACGGCGAATTCCATGCC 1626
A. pasteurianus SKU1108           CCGGTGGTGATCTTCTGTTCCAGGGCTTGGCCAATGGTGAATTCCACGCC 1752
                                  * ** ** ** ** ***********  **** ** ** ******** ***

AB086012 A. pasteurianus NCI1193  TATGACGCCACGAACGGTAGCGACCTTTACAAGTTTGACGCACAGAGCGG 1676
D00635 A. polyoxogenes            TATGACGCGACGAACGGTTCCGACCTGTTCCACTTCGCGGCGGACAGCGG 1956
D13893 A. pasteurianus            TATGACGCTACAAACGGGAGCGACCTTTACAAGTTTGACGCACAGAGCGG 2247
D90004 A. aceti K6033             TATGACGCCACGAACGGTAGCGACCTTTACAAGTTTGACGCACAGAGCGG 1805
E02158 A. aceti                   TATGACGCCACGAACGGTAGCGACCTTTACAAGTTTGACGCACAGAGCGG 1724
E03223 Acetobacter sp.            TATGACGCGACGAACGGTTCCGACCTGTTCCACTTCGCGGCGGACAGCGG 1676
A. pasteurianus SKU1108           TATGACGCCACGAACGGTAGCGACCTTTACAAGTTTGACGCACAGAGCGG 1802
                                  ******** ** *****   ****** * * * ** *  **  * *****

AB086012 A. pasteurianus NCI1193  CATTATCGCTCCGCCTATGACCTATAGCGTCAACGGCAAGCAGTATGTTG 1726
D00635 A. polyoxogenes            CATCATCGCACCGCCTGTGACCTACCTTGCCAATGGCAAGCAGTATGTTG 2006
D13893 A. pasteurianus            CATTATTGCCTCGCCTATGACCTATAGCGTCAACGGCAAGCAGTATGTTG 2297
D90004 A. aceti K6033             CATTATCGCTCCGCCTATGACCTATAGCGTCAACGGCAAGCAGTATGTTG 1855
E02158 A. aceti                   CATTATCGCTCCGCCTATGACCTATAGCGTCAACGGCAAGCAGTATGTTG 1774
E03223 Acetobacter sp.            CATCATCGCACCGCCTGTGACCTACCTTGCCAATGGCAAGCAGTATGTTG 1726
A. pasteurianus SKU1108           CATTATCGCTCCGCCTATGACCTATAGCGTCAACGGCAAGCAGTATGTTG 1852
                                  *** ** **  ***** *******    * *** ****************

AB086012 A. pasteurianus NCI1193  CCGTCGAAGTGGGCTGGGGCGGCATCTACCCAATTTCCATGGGTGGTGTA 1776
D00635 A. polyoxogenes            CGGTTGAAGTGGGCTGGGGCGGCATCTATCCGTTCTTCCTTGGTGGCCTG 2056
D13893 A. pasteurianus            CTGTTGAAGTGGGTTGGGGCGGCATTTACCCGATTTCCATGGGGGGTGTG 2347
D90004 A. aceti K6033             CTGTTGAAGTGGGCTGGGGCGGCATTTACCCAATTTCCATGGGTGGTGTA 1905
E02158 A. aceti                   CTGTTGAAGTGGGCTGGGGCGGCATTTACCCAATTTCCATGGGTGGTGTA 1824
E03223 Acetobacter sp.            CGGTTGAAGTGGGCTGGGGCGGCATCTATCCGTTCTTCCTTGGTGGCCTG 1776
A. pasteurianus SKU1108           CTGTTGAAGTGGGCTGGGGCGGCATCTACCCAATTTCCATGGGTGGTGTA 1902
                                  * ** ******** ********** ** **  * * * * ** **  *
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Appendix Figure C1 (Continued)

```
AB086012 A. pasteurianus NCI1193  GGCCGTACTTCTGGCTGGACAGTTAACCACTCCTACATTGCTGCGTTCTC 1826
D00635 A. polyoxogenes            GCCCGTACCAGCGGCTGGACCGTCAACCACTCGCGCATCATTGCCTTCTC 2106
D13893 A. pasteurianus            GGCCGTACTTCCGGCTGGACAGTTAACCACTCCTACATCGCTGTGTTCTC 2397
D90004 A. aceti K6033             GGCCGTACTTCTGGCTGGACAGTTAACCACTCCTACATTGCTGCGTTCTC 1955
E02158 A. aceti                   GGCCGTACTTCTGGCTGGACAGTTAACCACTCCTACATTGCTGCGTTCTC 1874
E03223 Acetobacter sp.            GCCCGTACCAGCGGCTGGACCGTCAACCACTCGCGCATCATTGCCTTCTC 1826
A. pasteurianus SKU1108           GGCCGTACTTCTGGCTGGACAGTTAACCACTCCTACATTGCTGCGTTCTC 1952
                                  * ******    ******** ** ********   ***   **  *****

AB086012 A. pasteurianus NCI1193  TCTGGATGGCAAAGCCAAGCTGCCGGCT---CTGAACAACCGCGGCTTCC 1873
D00635 A. polyoxogenes            GCTCGATGGCAAGTCCGGCCCGCTGCCCAAGCAGAATGACCAGGGCTTCC 2156
D13893 A. pasteurianus            TCTGGACGGCAAAGCCAAGCTGCCGGCT---CTGAACAACCGTGGCTTCC 2444
D90004 A. aceti K6033             TCTGGATGGCAAAGCCAAGCTGCCGGCT---CTGAACAACCGCGGCTTCC 2002
E02158 A. aceti                   TCTGGATGGCAAAGCCAAGCTGCCGGCT---CTGAACAACCGCGGCTTCC 1921
E03223 Acetobacter sp.            GCTCGATGGCAAGTCCGGCCCGCTGCCCAAGCAGAATGACCAGGGCTTCC 1876
A. pasteurianus SKU1108           TCTGGATGGCAAAGCCAAGCTGCCGGCT---CTGAACAACCGCGGCTTCC 1999
                                   ** ** *****  **   * ** * *    * ***  ***  *******

AB086012 A. pasteurianus NCI1193  TGCCCGTAAAACCACCTGCACAGTATGACCAGAAGGTCGTTGATAACGGT 1923
D00635 A. polyoxogenes            TGCCCGTCAAGCCGCCGGCACAGTTCGACAGCAAGCGTACCGATAACGGT 2206
D13893 A. pasteurianus            TGCCCGTAAAACCACCAGCACAGTATGACCAGAAGGTCGTTGATAATGGA 2494
D90004 A. aceti K6033             TGCCCGTAAAACCACCTGCACAGTATGACCAGAAGGTCGTTGATAACGGT 2052
E02158 A. aceti                   TGCCCGTAAAACCACCTGCACAGTATGACCAGAAGGTCGTTGATAACGGT 1971
E03223 Acetobacter sp.            TGCCCGTCAAGCCGCCGGCACAGTTCGACAGCAAGCGTACCGATAACGGT 1926
A. pasteurianus SKU1108           TGCCCGTAAAACCACCTGCACAGTATGACCAGAAGGTCGTTGATAACGGT 2049
                                  ******* ** ** ** *******  ***   ***      ***** **

                                                  Heme c binding motif
AB086012 A. pasteurianus NCI1193  TACTTCCAGTATCAGACCTACTGCCAGACCTGCCATGGTGATAACGGCGA 1973
D00635 A. polyoxogenes            TACTTCCAGTTCCAGACCTATTGCGCCGCCTGTCATGGCGATAACGCAGA 2256
D13893 A. pasteurianus            TACTTCCAGTATCAGACCTACTGCCAGACCTGTCATGGTGATAACGGCGA 2544
D90004 A. aceti K6033             TACTTCCAGTATCAGACCTACTGCCAGACCTGCCATGGTGATAACGGCGA 2102
E02158 A. aceti                   TACTTCCAGTATCAGACCTACTGCCAGACCTGCCATGGTGATAACGGCGA 2021
E03223 Acetobacter sp.            TACTTCCAGTTCCAGACCTATTGCGCCGCCTGTCATGGCGATAACGCAGA 1976
A. pasteurianus SKU1108           TACTTCCAGTATCAGACCTACTGCCAGACCTGCCATGGTGATAACGGCGA 2099
                                  **********  ******** ***    **** ***** *******  **

AB086012 A. pasteurianus NCI1193  AGGTGCGGGCATGCTGCCTGATCTGCGTTGGGCCGGTGCTATCCGCCACC 2023
D00635 A. polyoxogenes            AGGTGCCGGTGTGCTGCCTGACCTGCGCTGGTCCGGGTCCATCCGTCATG 2306
D13893 A. pasteurianus            AGGTGCAGGCATGCTGCCTGATCTGCGTTGGGCCGGTGCCATCCGCCACC 2594
D90004 A. aceti K6033             AGGTGCAGGCATGCTGCCTGATCTGCGTTGGGCCGGTGCTATCCGCCACC 2152
E02158 A. aceti                   AGGTGCAGGCATGCTGCCTGATCTGCGTTGGGCCGGTGCTATCCGCCACC 2071
E03223 Acetobacter sp.            AGGTGCCGGTGTGCTGCCTGACCTGCGCTGGTCCGGGTCCATCCGTCATG 2026
A. pasteurianus SKU1108           AGGTGCGGGCATGCTGCCTGATCTGCGTTGGGCCGGTGCTATCCGCCACC 2149
                                  ****** **  ********** ***** *** ****  * ***** **

AB086012 A. pasteurianus NCI1193  AGGATGCCTTCTACAATGTAGTTGGTCGCGGTGCGCTGACGGCTTACGGG 2073
D00635 A. polyoxogenes            AGGACGCGTTCTACAATGTTGTCGGCCGCGGCGCGCTTACCGCCTACGGT 2356
D13893 A. pasteurianus            AGGATGCCTTCTACAATGTGGTTGGTCGCGGTGCGCTGACGGCTTACGGG 2644
D90004 A. aceti K6033             AGGATGCCTTCTACAATGTAGTTGGTCGCGGTGCGCTGACGGCTTACGGG 2202
E02158 A. aceti                   AGGATGCCTTCTACAATGTAGTTGGTCGCGGTGCGCTGACGGCTTACGGG 2121
E03223 Acetobacter sp.            AGGACGCGTTCTACAATGTTGTCGGCCGCGGCGCGCTTACCGCCTACGGT 2076
A. pasteurianus SKU1108           AGGATGCCTTCTACAATGTGGTTGGTCGCGGTGCGCTGACGGCTTACGGG 2199
                                  **** ** *********** ** ** ***** ***** ** ** *****

                                          adhAB24F
AB086012 A. pasteurianus NCI1193  ATGGATCGCTTTGACACCAGCATGACGCCGGATGAAATTGAAGCTATCCG 2123
D00635 A. polyoxogenes            ATGGATCGCTTGCACGGTAACATGAACCCGACCGAGATTGAGGACATCCG 2406
D13893 A. pasteurianus            ATGGATCGCTTTGATACCAGCATGACGCCGGATGAAATTGAAGCAATCCG 2694
D90004 A. aceti K6033             ATGGATCGCTTTGACACCAGCATGACGCCGGATGAAATTGAAGCAATCCG 2252
E02158 A. aceti                   ATGGATCGCTTTGACACCAGCATGACGCCGGATGAAATTGAAGCAATCCG 2171
E03223 Acetobacter sp.            ATGGATCGCTTGCACGGTAACATGAACCCGACCGAGATTGAGGACATCCG 2126
A. pasteurianus SKU1108           ATGGATCGCTTTGACACCAGCATGACGCCGGATGAAATTGAAGCTATCCG 2249
                                  ****################****  ***   ** ***** *  *****
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Appendix Figure C1 (Continued)

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AB086012 A. pasteurianus NCI1193  TCAGTATCTGATCAAACGGGCAAACGACACGTATCAGCGTGAAGTGGATG 2173
D00635 A. polyoxogenes            CCAGTTCCTGATCAAGCGTGCGAACGAGACCTATCAGAGGGAAGTTGATG 2456
D13893 A. pasteurianus            TCAGTATCTGATCAAACGGGCGAACGACACGTATCAGCGTGAAGTGGATG 2744
D90004 A. aceti K6033             TCAGTATCTGATCAAACGGGCAAACGACACGTATCAGCGTGAAGTGGATG 2302
E02158 A. aceti                   TCAGTATCTGATCAAACGGGCAAACGACACGTATCAGCGTGAAGTGGATG 2221
E03223 Acetobacter sp.            CCAGTTCCTGATCAAGCGTGCGAACGAGACCTATCAGAGGGAAGTTGATG 2176
A. pasteurianus SKU1108           TCAGTATCTGATCAAACGGGCAAACGACACGTATCAGCGTGTAGTGGATG 2299
                                   ****  ******** ** ** ***** ** ****** * * *** ****

AB086012 A. pasteurianus NCI1193  CTCGGAAGAATGACAAGAATATCCCCGAAAACCCGACACTTGGCATTAAC 2223
D00635 A. polyoxogenes            CCCGGAAGAACGCTGACGGTATCCCCGAG-------CAGCTGCCGTAATC 2499
D13893 A. pasteurianus            CTCGGAAGAATGACAAGGATATTCCCGAAAACCCGACACTTGGCATTAAC 2794
D90004 A. aceti K6033             CTCGGAAGAATGACAAGAATATTCCCGAAAACCCGACACTTGGCATTAAC 2352
E02158 A. aceti                   CTCGGAAGAATGACAAGAATATTCCCGAAAACCCGACACTTGGCATTAAC 2271
E03223 Acetobacter sp.            CCCGGAAGAACGCTGACGGTATCCCCGAG-------CAGCTGCCG----- 2214
A. pasteurianus SKU1108           CTCGGAAGAATGACAAGAATATCCCCGAAAACCCGACACTTGGCATTAAC 2349
                                  * ******** *   *   *** *****        **  ** *

                                   stop point of ADHI
AB086012 A. pasteurianus NCI1193  CCCTAATGCCTCGGCAGAGTTACGACGTCATTCAGCACACAGGCGATAAG 2273
D00635 A. polyoxogenes            TCCCGGT--TTTGACAATTTTTCGCATCAACGCGACGCATGA-------- 2539
D13893 A. pasteurianus            CCCTAATGCCTCGGCAGAGTTACGACGTCATTCAGCACACAGGCGATAAG 2844
D90004 A. aceti K6033             CCCTAATGCCTCGGCAGAGTTACGACGTCATTCAGCACACAGGCGATAAG 2402
E02158 A. aceti                   CCCTAATGCCTCGGCAGAGTTACGACGTCATTCAGCACACAGGCGATAAG 2321
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CCCTAATGCCTCGGCAGAGTTACGACGTCATTCAGCACACAGGCGATAAG 2399

                                   RBS start point of ADHI
AB086012 A. pasteurianus NCI1193  TGGTAAAAATGATGA-TTAACAGGCTAAAAGCTGCCCT-GGGGGCAGTCG 2321
D00635 A. polyoxogenes            TGGTGAACATAATGA-TCAACAGACTTAAGGTGACATT-CAGCGCGGCAG 2587
D13893 A. pasteurianus            TGGTAAAAATGATGA-TGAACAGGCTAAAAACTGCCCT-GGGGGCAGTCG 2892
D90004 A. aceti K6033             TGGTAAAAATGATGA-TTAACAGGCTAAAGCTGCCCT-GGGGGCAGTCG 2450
E02158 A. aceti                   TGGTAAAAATGATGAATTAACAGGCTAAAGCTGCCCTTGGGGGCAGTCG 2371
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TGGTAAAAATGATGA-TTAACAGGCTAAAGCTGCCCT-GGGGGCAGTCG 2447

                                  The transmembrane helix
AB086012 A. pasteurianus NCI1193  CTGTCGGGCTTCTTGCGGGAACGTCCCTGGCGCATGCACAGAACGCTGAT 2371
D00635 A. polyoxogenes            CGTTTAGTCTGCTGGCAGGGACGGCATTGGCACAGACGCCAGATGCTGAC 2637
D13893 A. pasteurianus            CTGTCGGGCTTCTTGCGGGAACGTCCCTAGCGTATGCACAGAATGCTGAT 2942
D90004 A. aceti K6033             CTGTCGGGCTTCTTGCG---------------------------------- 2467
E02158 A. aceti                   CTGTCGGGCTTCTTGCG---------------------------------- 2388
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CTGTCGGGCTTCTTGCGGGAACGTCCCTGGCGCATGCACAGAACGCTGAT 2497

AB086012 A. pasteurianus NCI1193  GAAGATCTGATCAAGAAGGGCGAGTACGTTGCGCGCCTAGGGGACTGTGT 2421
D00635 A. polyoxogenes            TCCGCGCTGGTCCAGAAGGGGGCATATGTCGCGCGACTGGGTGACTGCGT 2687
D13893 A. pasteurianus            GAAGATCTGATCAAGAAGGGCGAGTACGTTGCTCGCCTGGGGGATTGTGT 2992
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GAAGATCTGATCAAGAAGGGCGAGTACGTTGCGCGCCTAGGGGACTGTGT 2547

                                 Heme c binding motif                          adhAB22R
AB086012 A. pasteurianus NCI1193  GGCTTGCCACACATCCCTGAACGGTCAGAAATATGCTGGCGGTCTTTCTA 2471
D00635 A. polyoxogenes            AGCATGTCATACCGCTCTCCATGGACAGTCGTACGCAGGCGGGCTTGAAA 2737
D13893 A. pasteurianus            GGCTTGCCACACAGCCCTGAATGGTCAGAAATATGCTGGTGGTCTTTCTA 3042
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GGCTTGCCACACATCCCTGAACGGTCAGAAATATGCTGGCGGTCTTTCTA 2597
                                                                   ++++++++++++
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Appendix Figure C1 (Continued)

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AB086012 A. pasteurianus NCI1193  TTAAGACCCCCATCGGCACAATTTACTCCACCAACATCACGCCAGACCCC 2521
D00635 A. polyoxogenes            TCAAGAGCCCGATCGGTACGATCTACTCCACGAACATCACACCGGACCCG 2787
D13893 A. pasteurianus            TCAAGACCCCCATCGGCACAATTTACTCCACCAATATTACGCCAGACCCC 3092
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TTAAGACCCCCATCGGCACAATTTACTCCACCAACATCACGCCAGACCCC 2647
                                  +++++

AB086012 A. pasteurianus NCI1193  ACGTACGGGATTGGCACCTACACATTTAAAGAGTTTGATGAAGCCGTGCG 2571
D00635 A. polyoxogenes            ACCTACGGTATCGGTCGCTACACCTTCGCCGAATTCGACGAAGCCGTGCG 2837
D13893 A. pasteurianus            ACGTACGGGATTGGCACCTACACATTTAAAGAGTTTGATGAAGCCGTGCG 3142
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           ACGTACGGGATTGGCACCTACACATTTAAAGAGTTTGATGAAGCCGTGCG 2697


AB086012 A. pasteurianus NCI1193  CCATGGCGTGCGTAAGGATGGTGCTACGCTGTATCCAGCAATGCCCTACC 2621
D00635 A. polyoxogenes            CCATGGTATCCGCAAGGACGGTTCCACGCTGTATCCGGCCATGCCGTATC 2887
D13893 A. pasteurianus            CCATGGCGTGCGTAAGGATGGTGCAACGCTGTATCCGGGAATGCCCTACC 3192
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CCATGGCGTGCGTAAGGATGGTGCTACGCTGTATCCAGCAATGCCCTACC 2747


AB086012 A. pasteurianus NCI1193  CATCCTTCGCCCGCATAACGCAGGATGACATGAAGGCTCTGTACGCATAC 2671
D00635 A. polyoxogenes            CCTCCTTCTCGCGCATGACGAAGGAAGACATGCAGGCGCTGTATGCGTAC 2937
D13893 A. pasteurianus            CATCCTTCGCCCGCATGACGCAGGATGACATGAAGGCTCTGTATGCATAC 3242
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CATCCTTCGCCCGCATGACGCAGGATGACATGAAGGCTCTGTACGCATAC 2797


AB086012 A. pasteurianus NCI1193  TTCATGCATGGCGTGCAGCCGATTGCGCAGAAAAATCATCCAACTGATAT 2721
D00635 A. polyoxogenes            TTCATGCATGGGGTGAAGCCGGTCGCGCAGCCGGACAAGCAGCCGGACAT 2987
D13893 A. pasteurianus            TTCATGCATGGTGTGCAGCCGATTGCTGAGAAAAATCACCCCACGGATAT 3292
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TTCATGCATGGCGTGCAGCCGATTGCGCAGAAAAATCATCCAACTGATAT 2847


AB086012 A. pasteurianus NCI1193  TTCCTGGCCGATGTCCATGCGTTGGCCGCTGTCCATCTGGCGCTCTGTAT 2771
D00635 A. polyoxogenes            CTCCTGGCCCTTGTCCATGCGCTGGCCGCTGGGCATCTGGCGCATGATGT 3037
D13893 A. pasteurianus            TTCTTGGCCAATGTCCATGCGCTGGCCGCTGTCCATCTGGCGTTCTGTAT 3342
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TTCCTGGCCGATGTCCATGCGTTGGCCGCTGTCCATCTGGCGCTCTGTAT 2897


AB086012 A. pasteurianus NCI1193  TTGCTCCGGCTCCCAAGGATTTCACACCAGCCACTGGCACGGATGCTGAA 2821
D00635 A. polyoxogenes            TCTCGCCTTCGCCGAAGGACTTCACGCCGGCGCCAGGCACGGATCCTGAA 3087
D13893 A. pasteurianus            TTGCGCCAGCACCCAAGGACTTCACACCAGCTCCTGGCACAGATGCTGAA 3392
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TTGCTCCGGCTCCCAAGGATTTCACACCAGCCCCTGGCACGGATGCTGAA 2947
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Appendix Figure C1 (Continued)

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                                                                Heme c binding motif
AB086012 A. pasteurianus NCI1193  ATTGCACGTGGTGAATACCTTGTCACCGGCCCTGGCCACTGTGGTGCATG 2871
D00635 A. polyoxogenes            ATCGCACGTGGCGATTATCTGGTTACCGGCCCCGGGCATTGCGGTGCGTG 3137
D13893 A. pasteurianus            ACAGCACGTGGTGAATACCTTATAACTGGCCCAGGCCATTGCGGGGCATG 3442
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           ATTGCACGTGGTGAATACCTTGTCACCGGCCCTGGCCACTGTGGTGCATG 2997


AB086012 A. pasteurianus NCI1193  TCATACACCGCGCGGCTTCGGTATGCAGGAAAAAGCTCTGGATGCATCTG 2921
D00635 A. polyoxogenes            TCATACCCCGCGTGGCTTCGCCATGCAGGAAAAGGCGCTGGACGCTGCCG 3187
D13893 A. pasteurianus            TCATACACCACGCGGCTTTGGTATGCAGGAAAAAGCTCTGGACGGCGCCG 3492
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TCATACACCGCGCGGCTTCGGTATGCAGGAAAAAGCTCTGGATGCATCTG 3047


AB086012 A. pasteurianus NCI1193  GCGGGCCAGACTTCCTTGGCGGCGGTGGCGTGATCGACAACTGGATTGCC 2971
D00635 A. polyoxogenes            GTGGTCCTGACTTCCTGTCCGGTGGCGCACCGATCGACAACTGGGTCGCG 3237
D13893 A. pasteurianus            GTGGGCCAGACTTCCTTGCTGGCGGTGGCGTTATCGACAACTGGATTGCA 3542
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GCGGGCCAGACTTCCTTGGCGGCGGTGGCGTGATCGACAACTGGATTGCC 3097


AB086012 A. pasteurianus NCI1193  CCAAGCCTGCGTAACGATCCGGTTCTCGGCCTTGGCCGCTGGTCTGATGA 3021
D00635 A. polyoxogenes            CCGAGCCTGCGCAACGATCCTGTCGTTGGTCTGGGCCGCTGGTCCGAGGA 3287
D13893 A. pasteurianus            CCAAGCCTGCGTAACGATCCGGTTCTCGGCCTTGGGCGCTGGTCTGATGA 3592
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CCAAGCCTACGTAACGATCCGGTTCTGGGCCTTGGCCGCTGGTCTGATGA 3147


AB086012 A. pasteurianus NCI1193  AGACCTGTTCCTGTTCCTGAAATCCGGTCGTACCGATCACTCCGCAGCCT 3071
D00635 A. polyoxogenes            TGACATCTACACCTTCCTGAAGTCCGGCCGTATCGACCACTCCGCCGTGT 3337
D13893 A. pasteurianus            GGACCTGTTCCTGTTCCTGAAATCTGGTCGTACCGATCACTCCGCAGCCT 3642
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           AGACCTGTTCCTGTTCCTGAAATCCGGTCGTACCGATCACTCCGCAGCCT 3197


AB086012 A. pasteurianus NCI1193  TTGGTGGCATGGCAGACGTGGTTGGCTGGAGCACCCAGTACTTCACCGAT 3121
D00635 A. polyoxogenes            TCGGTGGCATGGGCGATGTGGTGGCATGGAGCACCCAGTACTTCACCGAT 3387
D13893 A. pasteurianus            TTGGTGGCATGGCAGACGTAGTTGGCTGGAGCACCCAGTACTTCACCGAT 3692
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TTGGTGGCATGGCAGACGTGGTTGGCTGGAGCACCCAGTACTACACCGAT 3247


AB086012 A. pasteurianus NCI1193  GCAGATCTGCACGCCATGGTGAAATACATTAAATCCCTGCCGCCGGTTCC 3171
D00635 A. polyoxogenes            GACGACCTGCACGCCATCGCGAAGTACCTGAAGAGCCTGCCGCCGGTGCC 3437
D13893 A. pasteurianus            GCTGACCTGCATGCCATGGTGAAATACCTTAAATCCCTGCCGCCGGTTCC 3742
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GCAGATCTGCACGCCATGGTGAAATACATTAAATCCCTGCCGCCGGTTCC 3297
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Appendix Figure C1 (Continued)

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AB086012 A. pasteurianus NCI1193  GCCTGCACGTGGTGATTACAGCTACGATGCCTCTACGGCTCAGATGCTGG 3221
D00635 A. polyoxogenes            GCCGTCACAGGGCAACTACACCTACGATCCGTCCACCGCGAACATGCTGG 3487
D13893 A. pasteurianus            GCCTGCACGCGGTGATTACAGCTACGATGCCTCTACGGCTCAGATGCTGG 3792
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GCCTGCACGTGGTGATTACAGCTACGATGCCTCTACGGCTCAGATGCTGG 3347


AB086012 A. pasteurianus NCI1193  ATTCCAACAACTTCTCCGGCAATGCTGGTGCAAAAACATATGTGGAACAG 3271
D00635 A. polyoxogenes            CTTCGGGTAATACCGCCAGCGTTCCGGGTGCTGATACGTATGTGAAGGAA 3537
D13893 A. pasteurianus            ATTCCAACAACTTCTCCGGTAATGCTGGCGCCAAAACGTATGTGGAACAG 3842
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           ATTCCAACAACTTCTCCGGCAATGCTGGTGCAAAAACATATGTGGAACAG 3397


                                  Heme c binding motif
AB086012 A. pasteurianus NCI1193  TGCGCAATCTGCCATCGTAACGATGGTGGTGGTGTAGCCCGTATGTTCCC 3321
D00635 A. polyoxogenes            TGCGCCATCTGTCACCGTAACGACGGTGGTGGCGTGGCCCGCATGTTCCC 3587
D13893 A. pasteurianus            TGCGCAATCTGCCATCGTAACGATGGTGGTGGTGTAGCCCGTATGTTCCC 3892
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TGCGCAATCTGCCATCGTAACGATGGTGGTGGTGTAGCCCGTATGTTCCC 3447


                                    The cytochrome c domain
AB086012 A. pasteurianus NCI1193  GCCGCTGGCTGGTAACCCGGTTGTTGTTTCCGACAACCCGACATCTGTTG 3371
D00635 A. polyoxogenes            GCCGCTGGCTGGCAACCCGGTTGTCGTGACCGAGAACCCGACCTCGCTGG 3637
D13893 A. pasteurianus            GCCGCTGGCAGGTAACCCGGTTGTTGTTTCTGACAACCCGACATCTGTTG 3942
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           GCCGCTGGCTGGTAACCCGGTTGTTGTTTCCGACAACCCGACATCTGTTG 3497


AB086012 A. pasteurianus NCI1193  CCCATATCGTTGTTGATGGTGGCGTTCTGCCTCCCACCAACTGGGCACCT 3421
D00635 A. polyoxogenes            TGAACGTGATTGCGCATGGTGGCGTGCTGCCGCCGAGCAACTGGGCACCG 3687
D13893 A. pasteurianus            CCCATATTGTTGTTGATGGTGGTGTTCTGCCGCCCACCAACTGGGCACCT 3992
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CCCATATCGTTGTTGATGGTGGCGTTCTGCCTCCCACCAACTGGGCACCT 3547


AB086012 A. pasteurianus NCI1193  TCTGCAGTGGCCATGCCGGATTACAAAAACATCCTGTCTGACCAGCAGAT 3471
D00635 A. polyoxogenes            TCCGCAGTGGCAATGCCGGGTTACAGCAAGTCGCTGTCCGCCCAGCAGAT 3737
D13893 A. pasteurianus            TCTGCCGTGGCCATGCCGGATTACAAGAATATCCTGTCTGACCAGCAGAT 4042
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TCTGCAGTGGCCATGCCGGATTACAAAAACATCCTGTCTGACCAGCAGAT 3597


AB086012 A. pasteurianus NCI1193  TGCAGATGTTGTAAACTTCATCCGTTCTGCATGGGGTAACCGCGCACCGG 3521
D00635 A. polyoxogenes            TGCTGATGTGGTCAACTTCATCCGCACCAGCTGGGGCAACAAGGCGCCCG 3787
D13893 A. pasteurianus            TGCAGATGTTGTAAACTTCATCCGTTCTGCATGGGGTAACCGTGCACCGG 4092
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           TGCAGATGTTGTAAACTTCATCCGTTCTGCATGGGGTAACCGCGCACCGG 3647
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Appendix Figure C1 (Continued)

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AB086012 A. pasteurianus NCI1193  CTAACACAACAGCAGCAGATATCCAGAAACTCCGTCTGGATCATACGCCG 3571
D00635 A. polyoxogenes            GCACCGTTACGGCTGCGGATGTTACCAAGCTGCGCGACACGGGCGCCCCG 3837
D13893 A. pasteurianus            CGAATACAACAGCAGCAGATATCCAGAAACTCCGTCTGGATCATACGCCG 4142
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CTAACACAACAGCAGCAGATATCCAGAAACTCCGTCTGGATCATACGCCG 3697


AB086012 A. pasteurianus NCI1193  CTGCCGACACCGGGTTGGGCTAATGCGACTGAAGAATCCGCTACATGGGG 3621
D00635 A. polyoxogenes            GTTTCCAGCTCTGGCTGGAACAGCGTGAGCAGCGGCT---------GGTC 3878
D13893 A. pasteurianus            CTGCCAACGCCGGGCTGGGCAAATGCAACGGAAGACTCCGCAACATGGGG 4192
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CTGCCGACACCGGGTTGGGCTAATGCAACTGAAGAATCCGCTACATGGGG 3747


AB086012 A. pasteurianus NCI1193  CCTCTTCATGCCGCAGCCTTACGGCGCTGGCTGGACCTTCGCACCGCAGA 3671
D00635 A. polyoxogenes            GGTCTTCCTGCCGCAGCCTTACGGCTCGGGCTGGACGTTTGCCCCGCAGA 3928
D13893 A. pasteurianus            CCTGTTCATGCCGCAGCCTTATGGTGCTGGCTGGTCCTTCGCACCGCAGA 4242
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CCTCTTCATGCCGCAGCCTTACGGCGCTGGCTGGACCTTCGCACCGCAGA 3797

                                                    stop point of ADHII
AB086012 A. pasteurianus NCI1193  CCCATGCTGGTGTGGATGAAGCCCAGTAA---------------------- 3700
D00635 A. polyoxogenes            CGCACACCGGTCAGGACGCCGCACAGTAAGG---CGGATGGGTGGTTCCC 3975
D13893 A. pasteurianus            CCCATGCTGGTGTGGATGAAGCCCAGTAAGCTTCCGCCTAATTAACGCCC 4292
D90004 A. aceti K6033             --------------------------------------------------
E02158 A. aceti                   --------------------------------------------------
E03223 Acetobacter sp.            --------------------------------------------------
A. pasteurianus SKU1108           CACATGCTGGTGTGGATGAAGCCCAGTAAGC------------------- 3828
                                            <<<<<<<<<<<<<<<<<<
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Appendix Figure C1 (Continued)

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AB086013 A. pasteurianus NCI193    --------------------------------------------------
D31730 A. pasteurianus NCI1452     GCATGCGCTTATGCCCCGGCCAAGGCCAGTCCGCGCCGGGGCATCTGGCA 50


AB086013 A. pasteurianus NCI1193   --------------------------------------------------
D31730 A. pasteurianus NCI1452     CGCTGTGGGGAAAAACCCTGATATATCTGTGGTCTGCCATGTGTGGGGTG 100


AB086013 A. pasteurianus NCI1193   --------------------------------------------------
D31730 A. pasteurianus NCI1452     CGCAATGGGGCTGCTTGATCTTTCGGGCAGATACAGGGCAGTTTGAGGCA 150


AB086013 A. pasteurianus NCI1193   ----------------------ATGAAACTGATTGCCGTACGTGCCCTGT 28
D31730 A. pasteurianus NCI1452     TATTCCGCTAGGGAGATTCTGAATGAAACTGATTGCCGTACGTGCCCTGT 200
                                                          ****************************
                                                         >>>>>>>>>>>>>>>>>>>

AB086013 A. pasteurianus NCI1193   CGGCGCTTGCCCTCACCACATCTCTGCTGGCAGGGGCTGCTGTGTCTGCC 78
D31730 A. pasteurianus NCI1452     CGGCGCTTGCCCTCACCACATCTCTGCTGGCAGGGGCTGCTGTGTCTGCC 250
                                   **************************************************

AB086013 A. pasteurianus NCI1193   CATGCGCAGGAAAACACAGATGCGCCCGTAACACGCGCCGGAGATACGTC 128
D31730 A. pasteurianus NCI1452     CATGCGCAGGAAAATACAGATGCGCCCGTAACACGCGCCGGAGATACATC 300
                                   ************** ********************************** **

AB086013 A. pasteurianus NCI1193   CCGCCTGACAGATGTAGATCCGTCTGGCTTTGTTGGTTCCATTGATCCGG 178
D31730 A. pasteurianus NCI1452     CCGCCTGACAGATGTAGATCCGTCCGGCTTTGTTGGCTCCATTGATCCGG 350
                                   ************************ ************ **************

AB086013 A. pasteurianus NCI1193   CAGAAAATGCTGGCCTGCTGAACTACTGTGTGCAGAACGAATACGTTGAT 228
D31730 A. pasteurianus NCI1452     CAGAAAACGCCGGCCTGCTGAACTACTGTGTGCAGAACGAATACGTTGAT 400
                                   ******* ** ****************************************

AB086013 A. pasteurianus NCI1193   TACGACGATGCTGGCGCACGCTGCAGGAATACAACAAAAAGACCAACGCC 278
D31730 A. pasteurianus NCI1452     TATGACGACGCTGGCGCACGCTGCAGGAATACAACAAAAAGACCAACGCC 450
                                   ** ***** ******************************************

AB086013 A. pasteurianus NCI1193   GTGCCAGAAGGGCAGGAAGGCAATATGTCTTACGCCAATGGTTCTGCCGG 328
D31730 A. pasteurianus NCI1452     GTGCCAGAAGGGCAGGAAGGCAACATGTCTTACGCCAATGGTTCTGCTGG 500
                                   ********************** ********************** **

AB086013 A. pasteurianus NCI1193   TCTGCTGCATGCCAACAACCGCACCTACACCATTGCCATGGCTATTCTGC 378
D31730 A. pasteurianus NCI1452     CCTGCTGCACGCCAACAACCACACCTACACCATTGCCATGGCTATTCTGC 550
                                   ******** ********** ******************************

AB086013 A. pasteurianus NCI1193   CAGTGCGTCAGAAAACCTGTAAGGCTGTGCTGGAACGGGCCAAGGCTTCT 428
D31730 A. pasteurianus NCI1452     CAGTGCGCCAGAAAACCTGTAAGGCTGTTCTGGAACGGGCCAAGGCTTCC 600
                                   ******* ******************** ********************

AB086013 A. pasteurianus NCI1193   CTGTAAGCAAAAGGCTGTTATTCCGGTTTTGTTGATAAGGCCGGATCACG 478
D31730 A. pasteurianus NCI1452     CTGTAAGCACAAGGCTGTTATTCCGGTTTTGTTGATAAGGCCGGATCACG 650
                                   ********* ****************************************

AB086013 A. pasteurianus NCI1193   CTGAACACGGCATGATGGAAGGGTCAGTCACGCAAAGTGGCTGGCCCTTT 528
D31730 A. pasteurianus NCI1452     CTGAACGCGGCATGATGGAAGGGTCAGTCACACAAGGTGGCTGGCCCTTT 700
                                   ****** ********************** *** ***************

AB086013 A. pasteurianus NCI1193   TTCTGTATGAACGCACTTTATTTGGTGTTCATGCGGTGCAGGGTCTTTCA 578
D31730 A. pasteurianus NCI1452     TTCTGTATGCGTGCGGTTTATTTGCGGTGCATGCCGGATCAGGGCTTTCA 750
                                   *********   **  ********  ** ***** *     ** ******

AB086013 A. pasteurianus NCI1193   CGCAGGCGTGCAATATGGTCAAACCAGTTCTGTTTCGTAA---------- 618
D31730 A. pasteurianus NCI1452     CGCAGGCGTGCAATATGGTCAAACCAGTTCTGTTTCGTAACAAGAACAAC 800
                                   ****************************************
                                                    <<<<<<<<<<<<<<<<<
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Appendix Figure C2 Alignment of nucleotide sequences of *adhS* gene from *A. pasteurianus* NCI1193 and NCI1452. The forward and reverse primers used for amplification of *adhS* gene from *A. syzygii* SKU19 were indicated as (>>>>>>) and (<<<<<<) arrows, respectively; (*) is single, fully conserved residue and ( ) is no consensus.

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