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TITLE: Functional Analysis of *adhS* Gene Encoding Quinoprotein Alcohol Dehydrogenase Subunit III and Characterization of NAD^+-*adh* Genes from *Acetobacter pasteurianus* SKU1108

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THESIS

FUNCTIONAL ANALYSIS OF *adhS* GENE ENCODING QUINOPROTEIN ALCOHOL DEHYDROGENASE SUBUNIT III AND CHARACTERIZATION OF NAD^{+}-*adh* GENES FROM *Acetobacter pasteurianus* SKU1108

URAIWAN MASUD

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Uraiwan Masud 2011: Functional Analysis of *adhS* Gene Encoding Quinoprotein Alcohol Dehydrogenase Subunit III and Characterization of NAD^+-*adh* Genes from *Acetobacter pasteurianus* SKU1108. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Gunjana Theeragool, D.Agr. 179 pages.

The role of the *adhS* gene product, alcohol dehydrogenase subunit III, encoded from *adhS* gene, on the function of PQQ-ADH was investigated by construction of the *adhS* gene disruptant and mutants of *Acetobacter pasteurianus* SKU1108. The *adhS* gene disruptant completely lost its PQQ-ADH activity and acetate-producing ability but retained acetic acid toleration. In contrast, this disruptant grew well, even better than the wild type, in the ethanol containing medium even though its ethanol oxidizing ability was completely lost, while the NAD^+-dependent ADH (NAD^+-ADH) was induced. The tentative promoter region of *adhS* gene is located at approximately 118-268 bp upstream from an initiation codon. The essential amino acids for functional subunit III are 104Thr and 73, but not the 22 amino acids at the C-terminal. However, the over-expressed *adhS* gene did not enhance acetic acid production. To elucidate the genes expression of cytosolic NAD^+-ADHs, *adhI* and *adhII* genes were amplified and cloned into pGEM-T® Easy Vector. The Open Reading Frame of *adhI* and *adhII* consist of 1,029 and 762 bp, respectively. The deduced amino acids of ADH I and ADH II were 342 and 253 amino acids exhibit 99.71% and 99.60% homology, respectively with the same proteins from *A. pasteurianus* IFO 3283. The ADH I belongs to group I Zn-dependent long chain ADHs, while the ADH II belongs to group II short-chain dehydrogenase/reductase of NAD^+-ADHs. The reduction of growth was observed when the NAD^+-*adh* gene disruptants grown in ethanol medium. In *E.coli*, ethanol remarkably induced *adhI* and *adhII* promoter activities about 1.5 and 2.0 times, respectively. The possible promoter region of *adhI* and *adhII* genes are located at around 81-105 and 74-92 bp, in order, from its ATG start codon and the repressor binding regions might be located closed to these promoters.

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Student's signature Thesis Advisor's signature

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Uraiwan Masud

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LIST OF ABBREVIATIONS

bp	=	base pairs
d	=	day
°C	=	degree Celsius
h	=	hour
kDa	=	kilodaltons
kv	=	kilovolts
µF	=	microflux
µg	=	microgram
µg/ml	=	microgram per milliliter
µl	=	microlitre
µmol	=	micromole
MW	=	molecular weight
ml	=	milliliter
mM	=	millimolar
min	=	minute
ng	=	nanogram
N	=	normality
rpm	=	revolution per minute
pmol	=	picomole
psi	=	pounds per square inch
Q	=	ubiquinone
sec	=	second
v/v	=	volume by volume
w/v	=	weight by volume

FUNCTIONAL ANALYSIS OF *adhS* GENE ENCODING QUINOPROTEIN ALCOHOL DEHYDROGENASE SUBUNIT III AND CHARACTERIZATION OF NAD^+-*adh* GENES FROM *Acetobacter pasteurianus* SKU1108

INTRODUCTION

Acetic acid bacteria are obligate aerobes that belong to α-Proteobacteria, and have the strong ability to oxidize ethanol, sugar alcohols and sugars into their corresponding organic acids. Acetic acid fermentation, in which ethanol is oxidized to acetic acid by acetic acid bacteria, is the most characteristic process in vinegar production. This ethanol oxidation is catalyzed by two membrane bound enzymes, quinoprotein alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH) localized on the periplasmic side of the cytoplasmic membrane (Matsushita *et al*., 1994). The PQQ-ADH has been shown to have pyrroloquinoline quinone (PQQ) as the prosthetic group. PQQ-dependent ADH has been found in several other aerobic bacteria such as *Pseudomonas*, *Comamonas*, or *Ralstonia* species. Among such PQQ-ADHs, PQQ-ADH in acetic acid bacteria is rather unique because of consisting of three different subunits. It contains additional prosthetic groups for multiple heme *c*, and being present in the membrane, and thus termed as type III quinoprotein ADH, distinguished from other soluble type I and type II ADHs. Two genes, *adhA* and *adhB* encoding subunit I and II form an operon and thus are co-transcribed, while *adhS* encoding subunit III is located elsewhere. It has previously reported on cloning, sequencing and characterization of *adhS* gene encoding subunit III of the three-component membrane-bound alcohol dehydrogenase from *A. pasteurianus* NCI 1452 (Kondo *et al*., 1995). They found that some spontaneous mutants which lost subunit III showed a deficient phenotype of ADH activity deficient phenotype. It has been suggested that this smallest subunit may involve in stabilizing dehydrogenase subunit I and acts as a molecular coupler of subunit I and II on the cytoplasmic membrane (Kondo *et al*., 1995). Laster, the *adhS* gene encoding subunit III from *A. pasteurianus* SKU1108 was cloned and its determined nucleotide sequence was compared with the previous cloned *adhS* gene (Vanittananon, 2005).

Acetic acid bacteria also have an additional ADH, which is more general NAD $(P)^+$-dependent enzyme, in the cytoplasm at lower level. The NAD$(P)^+$-ADHs can be divided into three groups: Group I, zinc-dependent long chain ADHs; Group II, zinc-independent short chain ADHs; Group III, "iron-activated" ADHs (Jörnvall *et al*., 1987). Many organisms have been reported to contain multiple NAD^+-dependent ADHs, the physiological roles of which have sometimes been proven to be difficult to disentangle (Ismaiel *et al*., 1993). In acetic acid bacteria, ethanol is oxidized extensively by the membrane-bound quinoprotein ADH and only low NAD^+-ADH activity is detected in the cytoplasm (Matsushita *et al*., 1994).

Despite the long history of alcohol dehydrogenase in acetic acid bacteria, the certain function of the PQQ-ADH subunit III is still unknown. In this study, in order to elucidate the function(s) of subunit III of the three-component membrane bound ADH from *A. pasteurianus* SKU1108; the *adhS* gene disruptant was constructed. This strain was characterized comparing with wild type strain and the PQQ-ADH deficient mutant CN6-2, which was previously isolated by NTG treatment (Chinnawirotpisan *et al*., 2003b). Moreover, analysis of the regulation of *adhS* gene expression and the effect of its mutagenized gene on PQQ-ADH activity, subunit localization and ethanol oxidizing ability were performed. It has been proposed that PQQ-ADH is a key enzyme involved in ethanol oxidation, whereas NAD^+-ADH is involved in ethanol assimilation in acetic acid bacteria (Chinnawirotpisan *et al*., 2003b). In PQQ-ADH deficient mutant (CN6-2 and *adhS* gene disruptant), two NAD^+-ADHs were induced and the defect in PQQ-ADH leads to a global metabolic change from ethanol oxidation to ethanol assimilation as adaptive response to ethanol stress (Chinnawirotpisan *et al*., 2003b; Masud *et al*., 2010). There has been no report on the genetic level of NAD^+-ADHs so far. Therefore, the NAD^+-*adhI* and *adhII* gene were cloned and characterized. The ethanol induction and analysis of the possible promoter region of NAD^+-*adh* genes were also analyzed.

OBJECTIVES

1. To construct and characterize the *adhS* gene disruptant from *Acetobacter pasteurianus* SKU1108.

2. To investigate the function(s) of alcohol dehydrogenase subunit III in *Acetobacter pasteurianus* SKU1108 and its *adhS* gene disruptant.

3. To clone, sequence and characterize the NAD^+-*adh* genes, *adhI* and *adhII*, from *Acetobacter pasteurianus* SKU1108.

4. To construct and characterize the NAD^+-*adh* genes disruptants from *Acetobacter pasteurianus* CN6-2 strain.

LITERATURE REVIEW

1. Acetic Acid Bacteria

1.1 General characteristics and classification

Acetic acid bacteria have been intensively studied for more than one century. These bacteria are gram negative or gram variable, ellipsoidal to rod-shaped cells, obligate aerobic bacteria that have the ability to incompletely oxidize alcohols or sugar to organic acids as end products using oxygen as the terminal electron acceptor. They are widespread in nature and most of them are capable to oxidize ethanol as substrate to acetic acid and are particularly well adapted to sugar or alcoholic solutions. These characteristics make that acetic acid bacteria are often involved in foods and beverages, for example, beer, wine, cider, vinegar, honey and fermented products. Acetic acid bacteria produce acetic acid from ethanol by two sequential oxidation reactions of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) localized at the outer surface of cytoplasmic membrane and their function linked to the respiratory chain (De Ley *et al*., 1984; Matsushita *et al*., 1994). The optimum growth temperature of these bacteria is 25 to 30°C and no growth at 37°C for *Gluconobacter.*

Acetic acid bacteria are classified in the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α-subdivision of the *Proteobacteria* based on 16S rDNA sequence analysis and conventional classification. Taxonomic studies of acetic acid bacteria were historically considered. A single species of genus *Acetobacter, A. aceti,* was first exposed in 1898. The genus *Gluconobacter* was proposed in 1935 for strains with intense oxidation of glucose to gluconic acid rather than oxidation of ethanol to acetic acid and no further oxidation of acetate. In 1984, the genus *Acetobacter* was divided into two subgenera, *Acetobacter* and *Gluconacetobacter*. The latter was elevated to the genus *Gluconacetobacter* in 1998. The genus *Gluconacetobacter* was phylogenetically divided into two groups: the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group. The

two groups were discussed taxonomically (Yamada and Yukphan, 2008). Currently, the family *Acetobacteraceae* is composed of thirteen genera namely, *Acetobacter*, *Gluconobacter* (De Ley *et al.*, 1984), *Acidomonas* (Urakami *et al*., 1989), *Gluconacetobacter* (Yamada *et al*., 1997), *Asaia* (Yamada *et al*., 2000), *Kozakia* (Lisdiyanti *et al*., 2002), *Swaminathania* (Loganathan and Nair, 2004), *Saccharibacter* (Jojima *et al*., 2004), *Neoasaia* (Yukphan *et al*., 2005), *Granulibacter* (Greenberg *et al*., 2006), *Tanticharoenia* (Yukphan *et al*., 2008), *Commensalibacter* (Roh *et al*., 2008) and *Ameyamaea* (Yukphan *et al*., 2009).

In addition to ethanol oxidation, acetic acid bacteria have a pronounced tendency for incomplete oxidation converting alcohols to acids or secondary alcohols to ketones. With the exception of *Asaia*, the most prominent process is the oxidation of ethanol to acetic acid (Yamada *et al*., 2000; Katsura *et al*., 2001). Based on a further utilization of acetate, the acetic acid bacteria can be divided in two physiologic groups. The first group composes of the members of the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter* and *Kozakia*, which are able to oxidize acetate and lactate completely to CO_2 (over oxidation) *via* the TCA cycle and the glyoxylic acid shunt (Leisinger, 1965; Chinnawirotpisan *et al*., 2003b). The second one is formed by members of the genus *Gluconobacter*. These organisms lack the glyoxylic acid shunt and the TCA cycle is incomplete (Greenfield and Claus, 1972; Prust *et al*., 2005). Therefore, they are not able to completely oxidize acetate to CO_2.

1.2 Oxidative fermentation in acetic acid bacteria

Acetic acid bacteria, especially *Acetobacter* and *Gluconobacter*, are well known for their potential to incompletely oxidize a wide variety of sugars, alcohols and polyols. The corresponding products (aldehydes, ketones and organic acids) are accumulated almost completely in the medium (Figure 1). Due to their capability of rapid and nearly quantitative stero- and regiosepecifc incomplete oxidation reactions they can be used in a wide variety of biological processes (Gupta *et al*., 2001; Deppenmeier *et al*., 2002; Adachi *et al*., 2003a, 2003b). In this aspect, acetic acid bacteria can be seen as ‘living oxidative catalysts’ that perform very specific oxidation reactions and released electrons to molecule oxygen (Deppenmeier and Ehrenreich,

2009). These biological processes are called ‘oxidative fermentation’ (Adachi *et al*., 2003a).

Oxidative fermentations have been well established for a long time, especially in vinegar and in L-sorbose production. A wide variety of oxidation products in acetic acid bacteria is carried out by two different types of membrane-bound enzymes, quinoproteins and flavoproteins; no cytosolic $NAD(P)^+$-dependent dehydrogenase is involved in oxidation (Adachi *et al*., 2003a, 2003b; Raspor and Goranovič, 2008). These enzymes have PQQ or FAD as the primary coenzyme, respectively, and directly linked to the respiratory chain and generate bioenergy for growth and thus they form the specific machinery in substrate oxidation as a membrane-bound dehydrogenase-dependent oxidase system (Matsushita *et al*., 1994, 2002). Vinegar production and L-sorbose production are the most-typical examples of oxidative fermentation and can be regarded as the classic microbial bioconversion. This due to the individual enzymes from acetic acid bacteria catalyzes an incomplete one-step oxidation reaction and equivalent amounts of oxidized substrates are accumulated in the culture media. Moreover, a series of study on the shikimate pathway revealed that there are many membrane-bound dehydrogenases involved in the shikimate production by oxidative fermentation of *Gluconobacter* strains including quinoprotein quinate dehydrogenase, 3-dehydroquinate dehydrogenase (Adachi *et al*., 2003c, 2006a, 2006b, 2006c, 2008), and 3-dehydroshikimate dehydratase (Shinagawa *et al*., 2010).

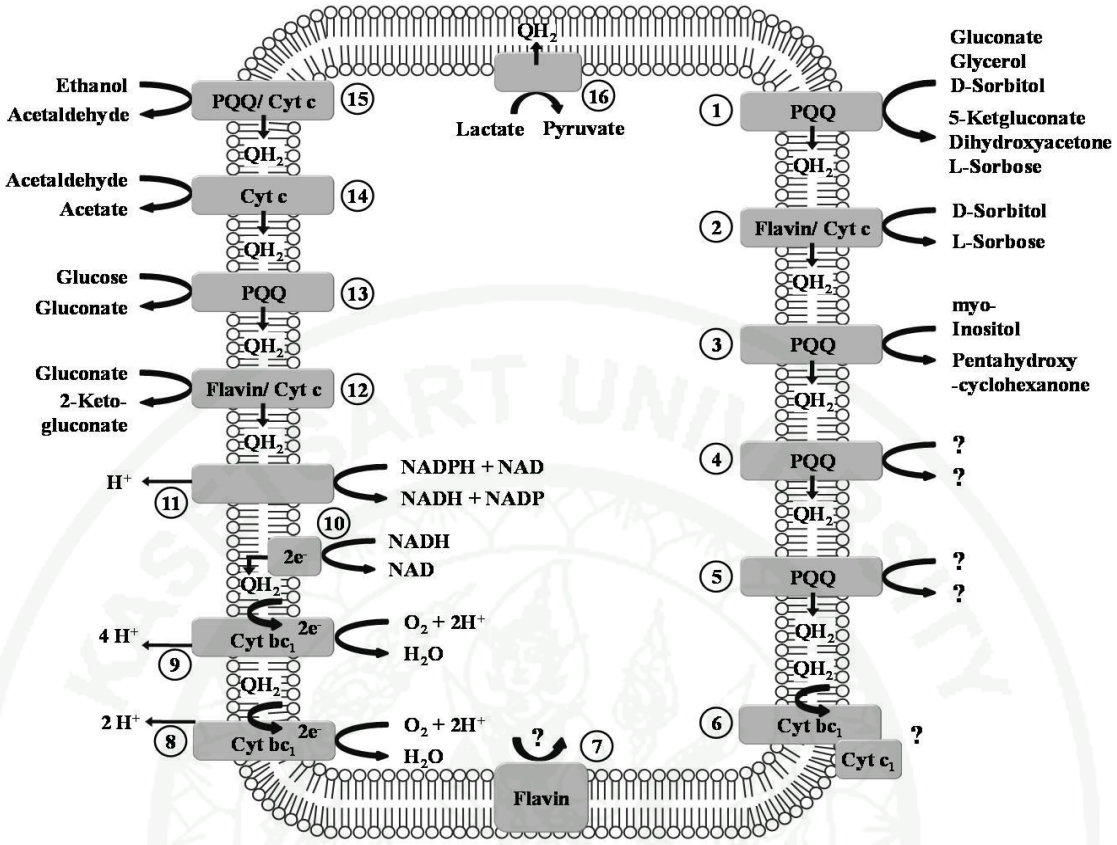


Figure 1 Respiratory chain of *G. oxydans* 621H. (1) Glycerol/sorbitol/gluconate dehydrogenase (SldAB), (2) sorbitol dehydrogenase, (3) PQQ-dependent myoinositol dehydrogenase, (4) uncharacterized membrane-bound quinoprotein, (5) uncharacterized membrane-bound quinoprotein, (6) ubiquinol:cytochrome *c* oxidoreductase, (7) uncharacterized flavin containing dehydrogenase, (8) quinol oxidase (*bd* type), (9) quinol oxidase (bo_3-type), (10) NADH-dehydrogenase (type II), (11) membrane-bound transhydrogenase, (12) membrane-bound gluconate-2-dehydrogenase, (13) PQQ-dependent glucose dehydrogenase, (14) membrane-bound acetal-dehyde dehydrogenase, (15) PQQ-dependent alcohol dehydrogenase, (16) membrane-bound lactate dehydrogenase. The membrane-bound dehydro-genases transfer electrons to ubiquinone to form the reduced cofactor ubiquinol (UQH_2). Question marks indicate that the substrates and products of the enzyme reaction are not known.

Source: Deppenmeier and Ehrenreich (2009)

1.3 Vinegar fermentation and thermotolerant acetic acid bacteria

It has been well established that acetic acid fermentation (or vinegar production) is the most distinguish characteristics of acetic acid bacteria especially *Acetobacter* species. Vinegar production has been known for centuries by natural fermentation of alcoholic beverages. In 1862, the first explanation of vinegar fermentation was emerged by Pasteur. He recognized that mother of vinegar was naturally occurred in vinegar products and caused acetic acid fermentation. Then, in 1989 and 1924, Beijerinck and Kluyver and de Leeuw discovered acetic acid bacteria, *A. aceti* and *G. suboxydans*. Vinegar was made by two distinct biochemical processes with the action of microorganisms. The first step is called the alcoholic fermentation; yeasts produce ethanol from carbohydrate source such as glucose under controlled conditions. The second step is the biooxidation of ethanol to acetic acid or vinegar by acetic acid bacteria. 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 (Conner and Allgeier, 1976). Commercial vinegar is produced either by fast or slow fermentation processes. Slow methods generally are used with traditional vinegars and fermentation proceeds slowly over the course of weeks or months. The longer fermentation period allows the accumulation of a nontoxic slime composed of acetic acid bacteria and soluble cellulose, known as the mother of vinegar.

The recent global warming has raised indoor temperatures beyond 30°C, even at night time, in many countries. This is a serious problem for vinegar fermentation and other fermentation industries, since they have to pay increased energy costs to keep the fermentation at the optimum temperature. 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).

Domestic vinegar production by acetic acid bacteria is usually taken place at 30°C; temperature control is necessary irrespective of whether the culture is static or submerged. A temperature increase of 2-3°C causes a serious deterioration in both the fermentation rate and efficiency. Industrial acetic acid production by a continuous submerged culture requires precise control of fermentation temperature. A slight increase in the temperature causes a remarkable decrease in fermentation rate and yield of acetic acid. If favorable strains of acetic acid bacteria that can work optimally at 37-40°C were available, the cooling expenses would be therefore reduced greatly (Adachi *et al*., 2003b). The thermotolerant strains with high resistances to ethanol and acetic acid are of interest. However, there have been few reports of vinegar fermentation by thermotolerant acetic acid bacteria (Ohmori *et al*., 1980; Saeki *et al*. 1997b; Kanchanarach *et al*., 2010). 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. Then, isolation, identification, and characterization of thermotolerant acetic acid bacteria were investigated to develop new microbial resources for oxidative fermentation (Saeki *et al*. 1997a). The course of vinegar fermentation at higher temperatures with the isolated strains, which able to grow at 37-40°C, was compared with mesophilic strains. Fermentation efficiency of vinegar production with the thermotolerant strains at 38-40°C was almost the same as that of mesophilic strains at 30°C. However, the thermotolerant strains worked rapidly, with a higher fermentation rate at higher temperatures, at which the mesophilic strains were unable to work. Thermotolerant acetic acid bacteria are thus useful for vinegar fermentation at higher temperatures, allowing a possible reduction in cooling expenses and other costs (Saeki *et al*., 1997a). Moreover, thermoresistant strains of *A. tropicalis* and *A. pasteurianus* have been isolated and selected for their ability to grow and produce acetic acid at high temperatures (Ndoye *et al*., 2006, 2007). Recently, Kanchanarach *et al*. (2010) isolated and characterized several thermotolerant *A. pasteurianus* strains and their quinoprotein ADHs were purified. The fermentation ability at 37°C of these thermotolerant strains was higher than that of mesophilic

strain. In addition, PQQ-ADH of the thermotolerant strains had a little higher optimal temperature and heat stability than that of mesophilic strain. More critically, PQQ-ADHs from thermotolerant strains exhibited a higher resistance to ethanol and acetic acid than that from mesophilic strain at elevated temperature.

1.4 Enzymes responsible to acetic acid fermentation

In spite of the long history of vinegar production, biochemical and molecular studies on responsible enzymes involved in this biological manner, ADH and ALDH, were only started in the early 1980s (Adachi *et al*. 1978a, 1978b). This biological process is carried out by two sequential oxidation reactions of membrane-bound quinohemoprotein alcohol dehydrogenase, PQQ-ADH and membrane-bound quinohemoprotein aldehyde dehydrogenase, ALDH (Matsushita *et al*., 1994). PQQ-ADH and ALDH have been extensively investigated and are well characterized (Matsushita *et al*., 1992a, 1992b, 1994; Matsushita and Adachi 1993a, 1993b). The two enzymes are bound to the periplasmic side of the cytoplasmic membrane and catalyze oxidation reactions in the periplasmic space. These two reactions are described below.

1) The oxidation of ethanol to acetaldehyde by alcohol dehydrogenase.

$$\underset{\text{ethanol}}{CH_3CH_2OH} + [O] \longrightarrow \underset{\text{acetaldehyde}}{CH_3CHO} + H_2O$$

2) The production of acetic acid by acetaldehyde dehydrogenase.

$$CH_3CHO + [O] \longrightarrow \underset{\text{acetic acid}}{CH_3COOH}$$

The dehydrogenase 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 cytoplasmic membrane (Matsushita *et al.*, 1994). Alcohol dehydrogenase involved in acetic acid fermentation is $NAD(P)^+$-

independent dehydrogenase (Matsushita *et al*., 1992c, 1994, 1995). The membrane bound dehydrogenase 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 play main role in vinegar production. In addition, several quinoproteins have been newly found in acetic acid bacteria, all of which can be applied to fermentative or enzymatic production of useful materials by means of oxidative fermentation process (Adachi *et al.*, 2003a).

ADH activity was reported to be largely decreased in *A. pasteurianus* when cultivated without ethanol which seem to be required for the correct localization of the dehydrogenase subunit in the membrane (Takemura *et al*., 1993). The incorrect localization of dehydrogenase subunit responded for the lower ADH activity in the absence of ethanol. The addition of acetic acid into the medium caused rapid decrease in both ADH and ALDH activities (Theeragool *et al*., 1996). Another investigation reported the decreasing in ADH activity of *G. suboxydans* was due to reversible change from active to inactive form during cultivation under certain condition (Matsushita *et al*., 1995). Moreover, in *A. pasteurianus* IFO 3283, membrane-bound ADH and ALDH activities dramatically decrease during the stationary phase after the complete consumption of ethanol, and these enzymes activities slightly increase again during the overoxidation phase (Matsushita *et al*., 2005a).

The respiratory chain of acetic acid bacteria branches into the cytochrome bo_3 ubiquinol oxidase and a cyanide-insensitive bypass oxidase at the membranous ubiquinone (Q) site, both of which reduce oxygen to water (Figure 2). Thus, PQQ-ADH functions as the primary dehydrogenase in the ethanol oxidation respiratory chain. Oxidation of ethanol takes place at the PQQ site by transferring electrons to PQQ, heme *c* I site in subunit I, hemes *c* II_1 and II_2 sites in subunit II and then to tightly membranous-bound Q of subunit II, which acts as the electron transfer mediators. Finally, the enzyme reduces bulk Q and is further oxidized by the terminal

uniquinol oxidase, cytochrome *o* or a_1 generating proton gradient across the cytoplasmic membranes yielding bioenergy as shown in Figure 3 (Yakushi and Matshushita, 2010).

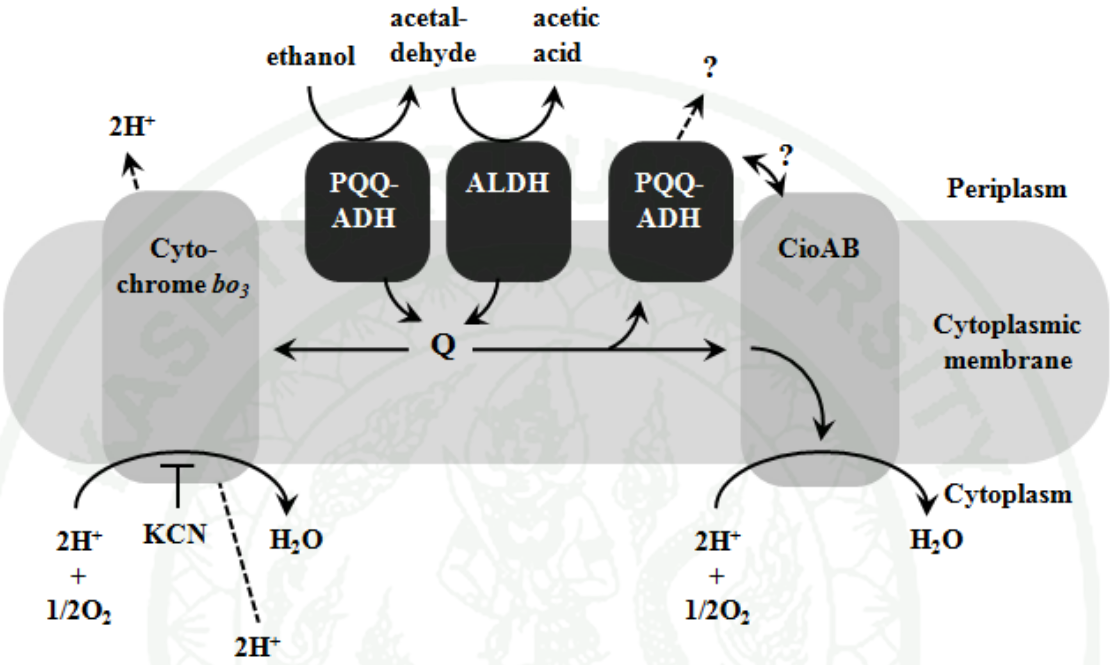


Figure 2 An overview of the ethanol oxidation respiratory chain of acetic acid bacteria. Ethanol is oxidized to acetic acid by a sequential action of PQQ-ADH and membrane-bound aldehyde dehydrogenase (ALDH), reducing Q in the cytoplasmic membrane. Then, the respiratory chain branches to energy-producing cytochrome bo_3 ubiquinol oxidase and less energy-producing cyanide insensitive oxidase CioAB, both of which reduce oxygen to water. PQQ-ADH also participates in a cyanide insensitive respiratory chain, but its electron acceptor remains unknown. Double arrow indicates a possible interaction between PQQ-ADH and CioAB.

Source: Yakushi and Matsushita (2010)

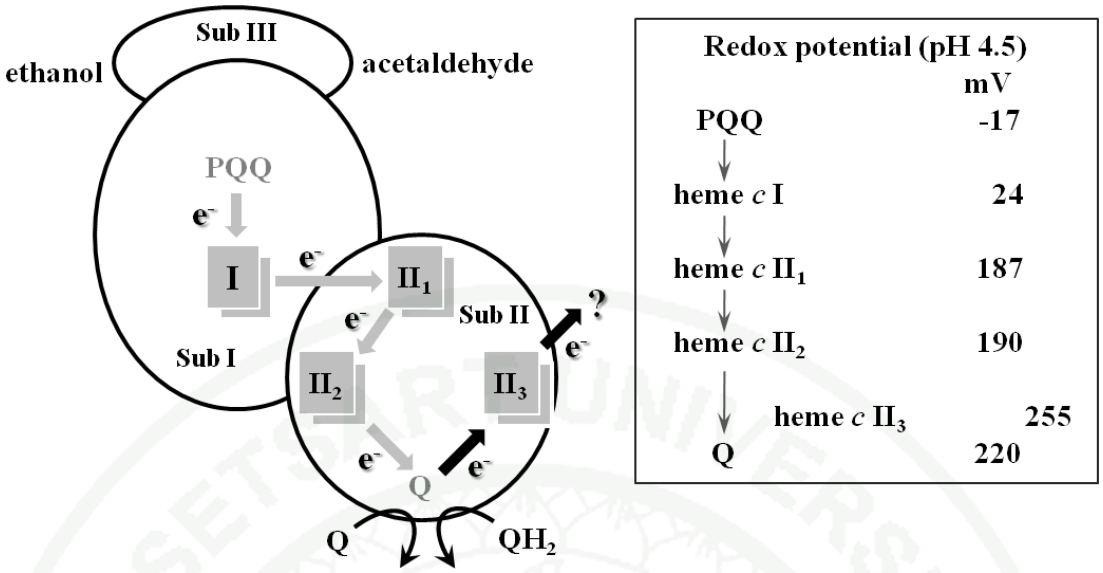


Figure 3 Model for the intramolecular electron transport of PQQ-ADH. Left panel: Electrons from ethanol are transported to PQQ and likely along the gray arrows to bound Q through multiple hemes *c*. On the other hand, electrons from ubiquinol are transported along the black arrows to the heme *c* II_3 site and then to unknown electron acceptor shown by question marks. Light gray Q, bound Q; black Q, bulk Q. Right panel: Redox potentials at pH 4.5 for the redox cofactors in PQQ-ADH are shown in millivolts. The arrows indicate a proposed intramolecular electron transport path upon ethanol:Q oxidoreduction.

Source: Yakushi and Matsushita (2010)

1.5 Enzyme involved in acetate assimilation

Among twelve genera of acetic acid bacteria, the genus *Acetobacter* and *Gluconacetobacter* exhibit their ability to further oxidize acetic acid into carbon dioxide and water, a phenomenon called “acetate oxidation” which is a nuisance for vinegar fermenters. “Over-oxidation of acetate” or “acetate peroxidation” has been used synonymously with acetate oxidation designing the phenomenon of aerobic acetate catabolism by acetic acid bacteria (De Ley *et al*., 1984). An intensive consumption of acetic acid is always accompanied by a corresponding increase in cell biomass. Moreover, 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 acetate overoxidation while cell biomass increases, the TCA cycle must be driven smoothly, as enzymes in TCA cycle make citrate with acetyl-CoA. Enzymes involved in the acetate overoxidation are not strengthened while ethanol remains in the culture medium and the oxidative fermentation yielding acetic acid becomes predominant. 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, 2005b) as shown in Figure 4. The finding suggested that strong acetate oxidation caused by acetyl-CoA synthase or phosphotransacetylase activity, together with phosphoenolpyruvate carboxylase, accompany with increased the biomass. Recently, a specialized citric acid cycle of *A. aceti* 1023, which lacks malate dehydrogenase (Mdh) and succinyl-CoA synthase (SCS) genes, was discovered (Mullins *et al*., 2008). They proposed that *A. aceti*

contains a complete but unorthodox citric acid cycle in which Mdh and SCS are functionally replaced by malate:quinine oxidoreductase (Mqo) and succinyl-CoA:acetate CoA-transferase (SCACT), respectively. This bypass facilitates the metabolic activation and oxidation of acetate and constitutes part of a conditionally essential detoxification pathway that is required for survival at high acetate concentrations at a low pH but is not required for growth at low acetate concentrations, even at same pH. This citric acid cycle is shown in figure 5.

In ethanol culture, *Acetobacter* and *Gluconacetobacter* species show three growth phases; they first grew by completely oxidizing ethanol to acetic acid (ethanol oxidation phase), then stop growing and remained for a while in the stationary phase where their viable cell number is decreased (first stationary phase) and finally start growing again by utilizing the accumulated acetic acid, a growth phase called "overoxidation of acetate". A typical growth pattern of *Acetobacter* species in ethanol culture is presented in Figure 6. 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 (Matsushita *et al*., 2005a).

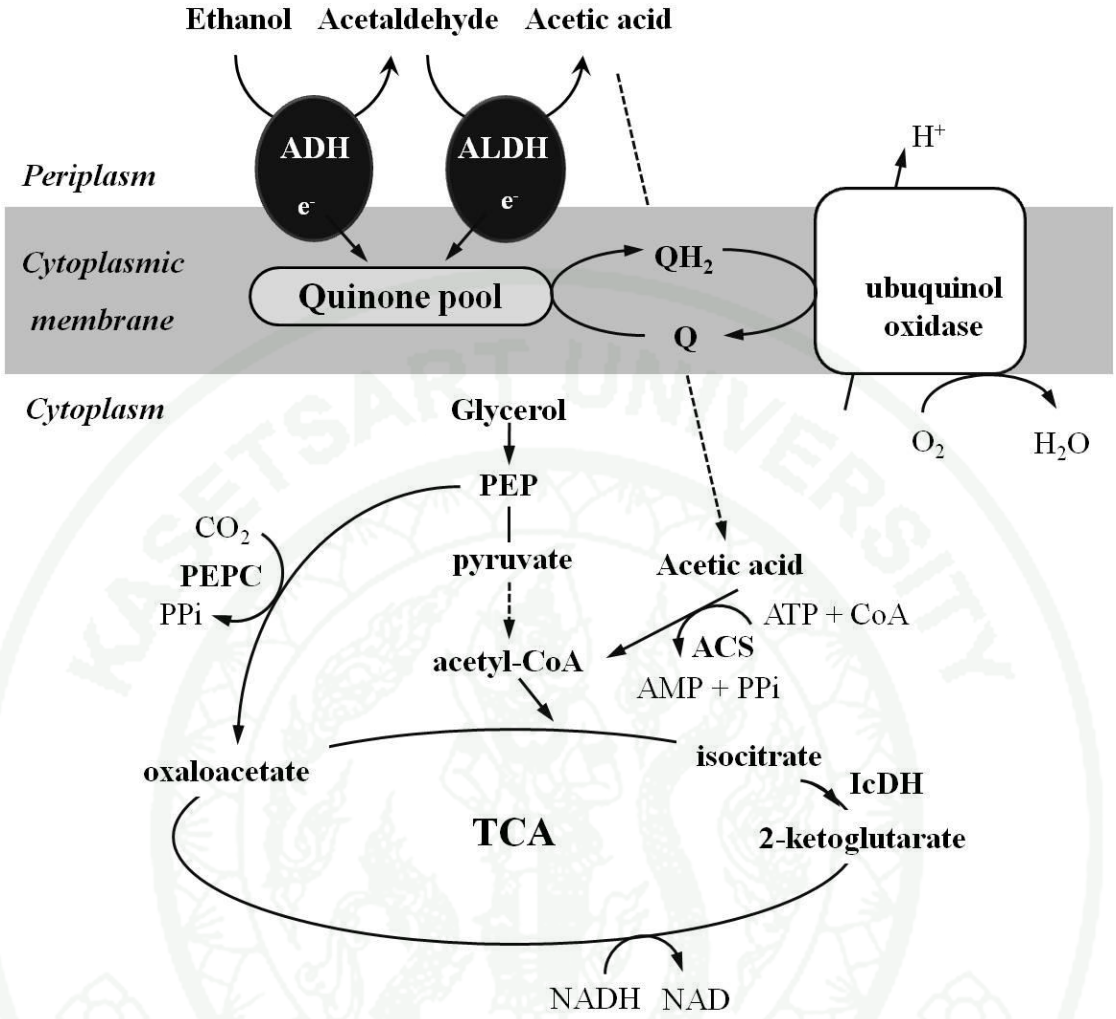


Figure 4 Ethanol respiration and the metabolic pathway responsible for the overoxidation of *Acetobacter* species. The ethanol respiration consists of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) located on the outer surface of the cytoplasmic membrane as primary dehydrogenases, ubiquinone 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 (LcDH), 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)

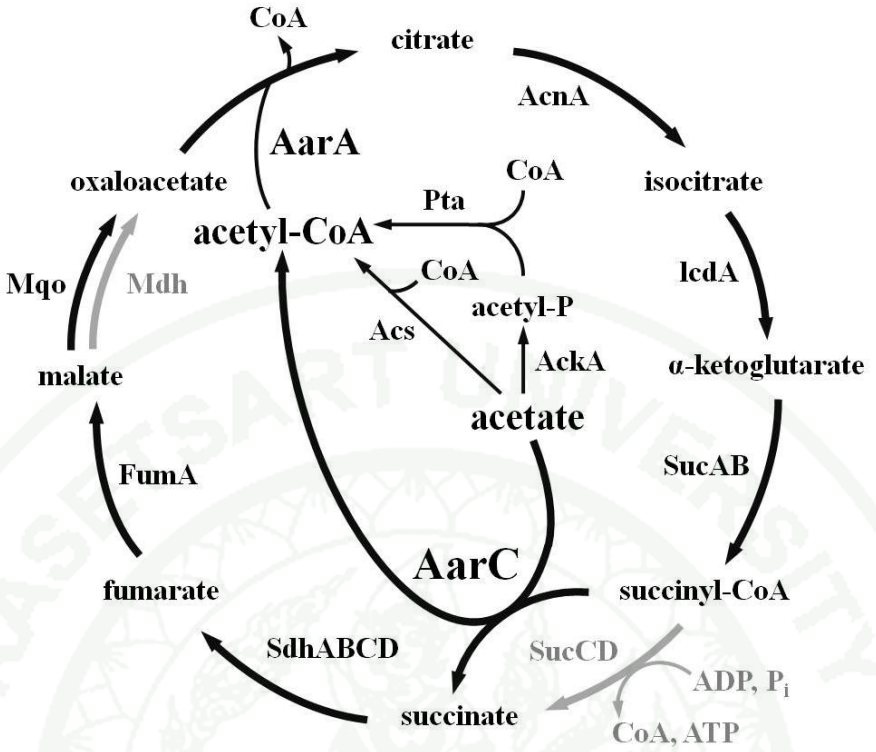


Figure 5 An unorthodox *A. aceti* CAC oxidizes acetate. The gray arrows indicate CAC genes that are not found in a draft *A. aceti* 1023 genome sequence (Kappock *et al*., unpublished). Each has a functional replacement. Using periplasmic dehydrogenases, *A. aceti* produces large quantities of acetic acid from ethanol and must contend with a constant influx of the former. Together with a complete oxidative phosphorylation pathway, this variant CAC functions in the eight electron oxidation of acetic acid: $CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$. This pathway skips AckA, Acs, and SCS (*sucCD*), the only steps that would be directly influenced by cytoplasmic nucleotide pools, and reduces the number of enzymes that have free CoA substrates from two to one. The initial electron acceptors, two NAD^+, one FAD, and one ubiquinone (presumed to be Q9 in *A. aceti*), would give a lower energy yield than a canonical CAC but have an additional irreversible step, the Mqo-mediated quinone reduction. Genes for the glyoxylate shunt enzymes isocitrate lyase and malate synthase are not found in *A. aceti* 1023.

Source: Mullins *et al*. (2008)

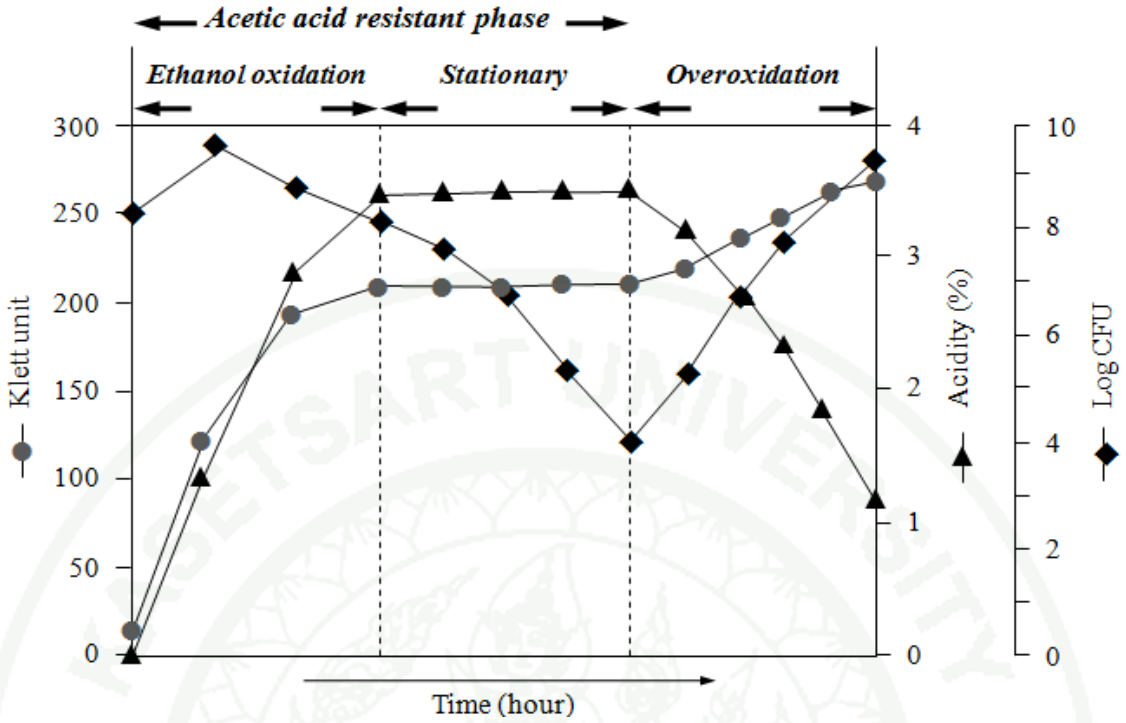


Figure 6 Typical growth pattern of *Acetobacter* species in ethanol culture. *Acetobacter* species exhibits a biphasic growth curve in ethanol culture, where during the first phase ethanol oxidation produces acetic acid, and after the following stationary phase the overoxidation of acetic acid occurs as the final phase. The ethanol oxidation and stationary phases correspond to the acetic acid resistant phase.

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

1.6 Enzyme involved in ethanol assimilation

ADHs are involved in wide range of metabolism, especially in microorganism. In the reductive reaction (or reverse reaction), NAD^+-ADH results in the production of alcohol and oxidized cofactor (NAD^+). This occurs, for instance, in the formation of alcohols during fermentation by anaerobic bacteria and yeasts where the regeneration of NAD^+ is essential for other oxidative and energy-yielding metabolic processes to continue (Clark, 1989, 1992). Oxidation of alcohols results in the production of aldehydes or ketones and reduced cofactor (NADH) and may be involved in feeding alcohols into the central metabolism as carbon and energy sources for growth. Such roles are common among alcohol oxidoreductase and they are involved in functions as diverse as the growth of methylotrophs, and the oxidation of aromatic alcohols that occurs in species of *Pseudomonas* and *Acinetobacter calcoaceticus* (Reid and Fewson, 1994). The ethanol oxidizing system in *P. aeruginosa* was reported that *P. aeruginosa* ATCC 17933 grows aerobically with ethanol or acetic acid as sole carbon source by using a soluble quinoprotein alcohol dehydrogenase, a periplasmic cytochrome *c550*, NAD^+-dependent acetaldehyde dehydrogenase (NAD^+-ALDH), acetyl-CoA synthetase and malate:quinine oxidoreductase (Kretzschmar *et al*., 2002). The mutant of *Salmonella enterica* serovar Typhimurium is capable of growing in air on ethanol as sole carbon and energy source by high expression level of novel alcohol dehydrogenase (*Adh I*) that uses ethanol, 1-propanol and 2-propanol as substrates (Dailly *et al*., 2001). Moreover, it has been reported that the regeneration of NADH is essential for the production of xylitol at the second step by NAD^+-dependent xylitol dehydrogenase in *G. oxydans* ATCC 621 (Suzuki *et al*., 2002).

The ethanol assimilation in thermotolerant acetic acid bacterium, *A. pasteurianus* SKU1108, has been clarified (Chinnawirotpisan *et al*., 2003b). In the PQQ-ADH deficient mutant of this strain, the activities of two NAD^+-ADHs, ADH I and ADH II, present in a small amount in the wild-type strain, markedly increased in the cytoplasm when cultured in a medium containing ethanol, concomitant to the increase in the activities of the key enzymes in TCA and glyoxylate cycles, as shown in Figure 7. Ethanol in the culture medium penetrates through cytoplasm of the cells

of this mutant and induces NAD^+-ADH I to oxidize ethanol at the initial stage, and then induce NAD^+-ADH II at the second stage. The acetaldehyde produced in the cytoplasm seems to be oxidized to acetate by the $NADP^+$-ALDH induced by the substrate. Finally, acetate accumulated in the cytoplasm may induce acetate assimilation, where acetate is converted into acetyl-CoA by acetyl-CoA synthetase or acetate kinase, and then acetyl-CoA is metabolized *via* the TCA or glyoxylate cycle.

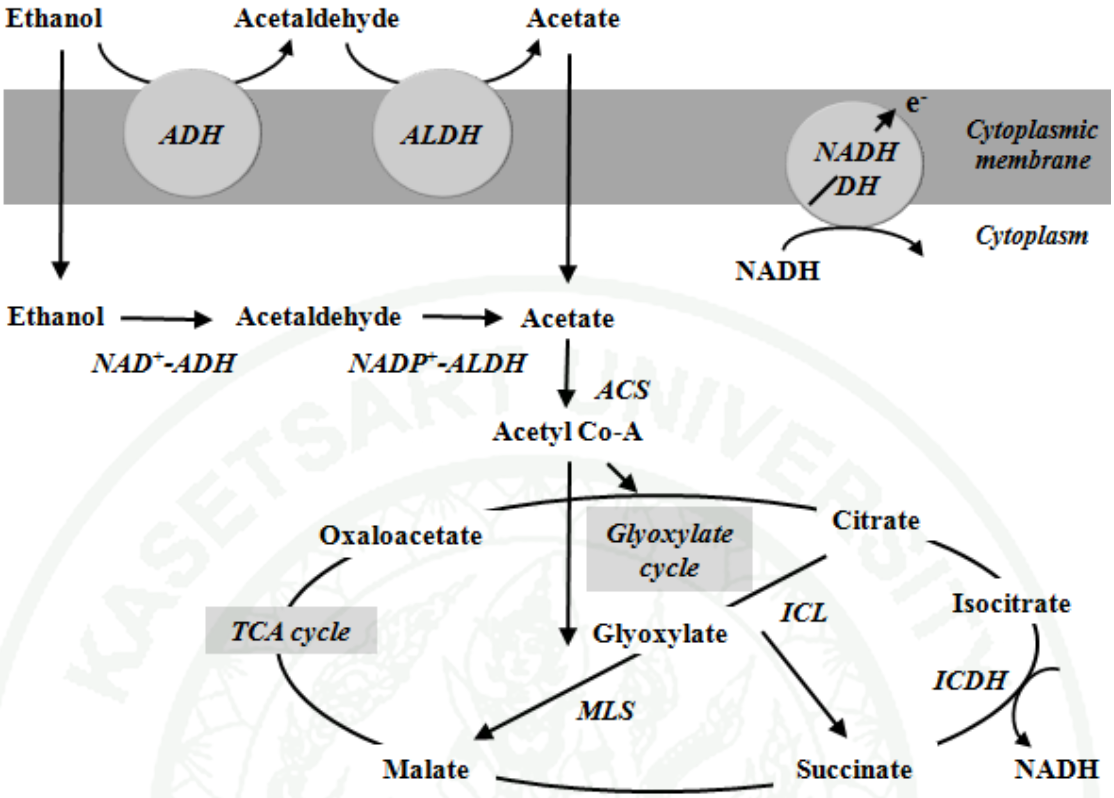


Figure 7 Metabolic pathways of oxidative assimilation of ethanol into TCA and glyoxylate cycles *via* acetyl-CoA. The enzymes involved in ethanol assimilation are abbreviated in the figure: NAD-ADH, NAD-dependent alcohol dehydrogenase; NADP-ALDH, NADP-dependent aldehyhe dehydrogenase; ACS, acetyl-CoA synthetase; ICL, isocitrate lyase; and ICDH, isocitrate dehydrogenase.

Source: Chinnawirotpisan *et al*. (2003b)

1.7 Acetic acid stress response

The microenvironment during acetic acid fermentation exposes the acetic acid bacteria to various stresses such as high temperature, ethanol and acetic acid. Despite the adverse microenvironment, acetic acid bacteria exhibited relatively normal growth patterns with a resistance to the unfavorable conditions. In respect to acetic acid resistance, proteomic analysis of acetic acid bacteria revealed that 8 acetate stress proteins (Asps) were induced specifically by exposure of unadapted *A. aceti* and *G. suboxydans* cultures to 10 g/l acetate (Lakso *et al*., 1997). The acetate resistance genes, *aarABC*, of a thermophilic *A. aceti* strain, were important for resistance to acetate 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. In addition, a succinyl-CoA:acetate CoA-transferase (SCACT), AarC homologue, was proposed (Mullins *et al*., 2008). SCACT is encoded by *aarC* gene and involved in a specialized citric acid cycle conferring acetic acid resistance on *A. aceti*. Further investigation of the changes in global protein expression levels during long term adaptation of *A. aceti* to high acetate concentrations by two dimension electrophoresis (2-DE) indicated that at least 50 proteins are specifically induced by adaptation to acetate but not by other stress conditions (Steiner and Sauer, 2001). It was further clarified that enhanced expression of aconitase raise acetic acid resistance in *A. aceti* (Nakano *et al*., 2004). It has been later reported that *A. aceti* possesses a proton motive force-dependent efflux system for acetic acid. It has been suggested that this phenomenon is involved in acetic acid resistance. The most plausible mechanism responsible for acetic acid-resistance is an efflux pump localized in the cytoplasmic membrane which pumps out acetic acid and enables cells grow in the presence of high acetic acid concentration (Matsushita *et al*., 2005a, 2005b), as shown in Figure 8. Nakano *et al*. (2006) proposed that the putative ABC transporter is responsible for acetic acid resistance in *A. aceti*. They analyzed its membrane fraction by 2-DE and determined that 60 kDa protein, designated as AatA, was induced by acetic acid. Further investigation of the responsible gene suggested that this protein is a putative ABC transporter. It has also been suggested that overexpression of *aatA* gene could

increase final yield of acetic acid production. In addition, Trcek *et al.* (2006) reported the correlation between acetic acid resistance and characteristics of PQQ-ADH in acetic acid bacteria. They suggested that higher ADH activity can produce a higher energy pool for exporting acetate to the exterior of the cell which results in higher acetic acid resistance. They also studied the adaptation against high acetic acid concentration in *Gluconacetobacter europaeus* by changes in lipid composition, morphological properties and PQQ-ADH expression (Trcek *et al*., 2007). The intrinsic stability of intracellular proteins and general stress proteins such as chaperonins also contribute to the phenotypic expression of acetic acid resistance (Nakano and Fukaya, 2008). In addition, Sintuprapa *et al*. (2008a, 2008b) elucidated an adaptive response to acetic acid by isolation of acetic acid adapted strains from sequential cultivations of an acetic-acid sensitive strain, *A. syzygii* SKU19, in a medium containing 1% acetic acid. They suggested that these adapted strains exhibit a higher PQQ-ADH and ALDH activities than the wild type strain and there are several mechanisms involved in the acetic acid resistance in acetic acid bacteria, such as acetate assimilation and PQQ-ADH functions.

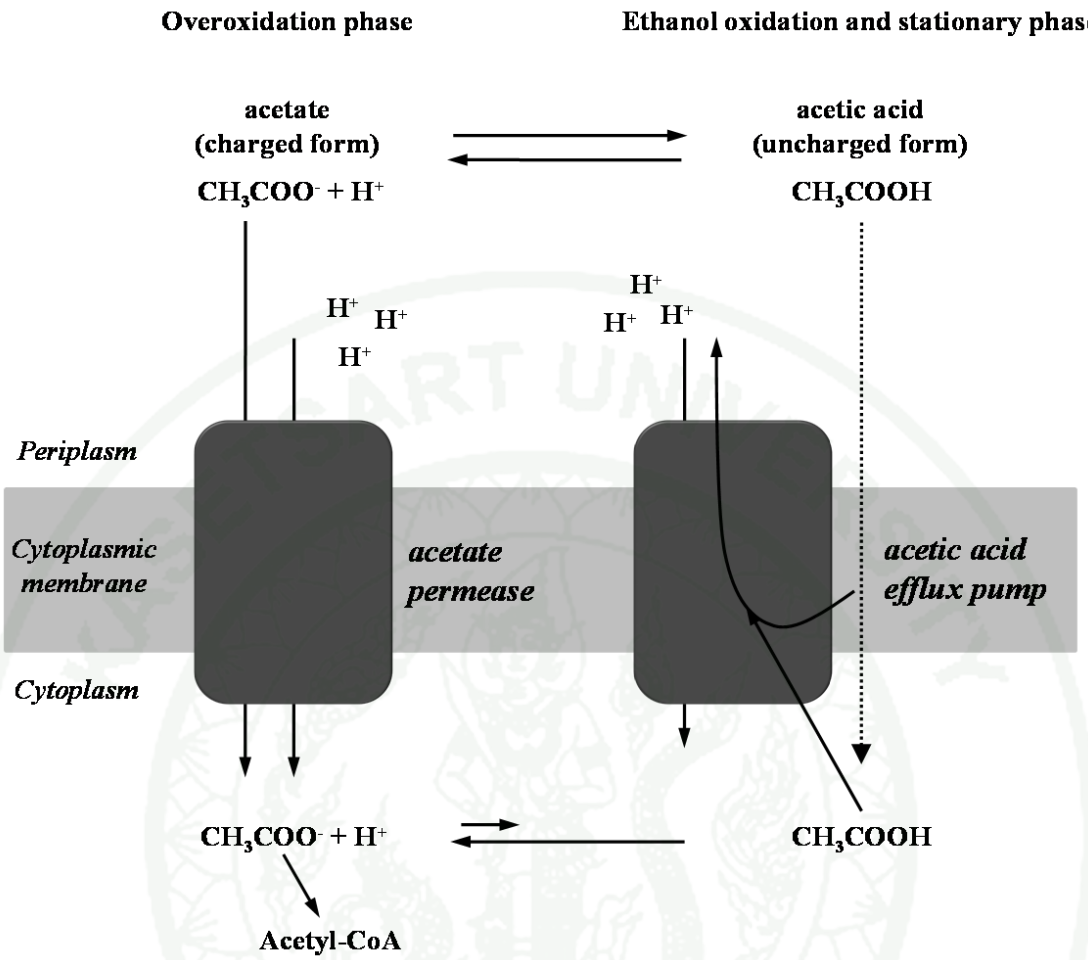


Figure 8 Acetic acid or acetate efflux and uptake in acetic acid bacteria. During the ethanol oxidation and stationary phases, an acetic acid efflux pump may work to pump acetic acid out by consuming a proton motive force, while a proton motive force dependent acetate permease may work to uptake acetate during the overoxidation phase.

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

1.8 Mutation and genetic instability in acetic acid bacteria

Mutation provides decisive evidence that DNA is the genetic material. It has been widely known that change in the sequence of DNA causes an alteration in the consequent effects on the organisms, which typically resulted in the generation of a new genotype and-or phenotype. The existence of many mutations in a gene may allow many variant forms of a protein to be compared, and a detailed analysis can be used to identify regions of the protein responsible for individual enzymatic or other functions (Lewin, 1998). Mutation is divided into two types: spontaneous and induced mutation. 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. Normally, spontaneous mutation, which causes heritable change, occurs at low frequencies. However, it has been frequently observed that acetic acid bacteria show considerable genetic instability which causes high frequency spontaneous mutations leading to deficiencies in various physiological properties including ethanol oxidation (Ohmori *et al*., 1982; Okumura *et al*., 1985; Takemura *et al*., 1991, Kondo and Horinouchi, 1997b), acetic acid resistance (Ohmori *et al*., 1982) and cellulose formation (Cook and Colvin, 1980; Coucheron, 1991; Nguyen *et al*., 2010). Prolonged cultivation of thermotolerant strain, *A. aceti*, was reported to lead to 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). Recently, Nguyen *et al.* (2010) proposed that a novel type of spontaneous cellulose production mutation associated with *Ga. xylinus* which is generated under conditions of nutrient limitation. They suggested that different nutrient conditions may play a role in the selection of the spontaneous mutant. 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).

Insertion of DNA fragment into a gene will usually result in the inactivation of that gene by the loss of its function. A number of types of genetic

elements, including some types of both viruses and plasmid, can be inserted into the bacterial chromosome. The simplest of these genetic elements are known as insertion sequences or IS (Dale, 1994). 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). In *A. xylinum* ATCC 23769 losing ability to produce cellulose was found to be associated with the transposition of an indigenous insertion sequence, IS*1031* (Coucheron, 1991). The further insertion sequence, IS*1452*, was 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. Alcohol Dehydrogenase in Acetic Acid Bacteria

Alcohol dehydrogenases (ADH) are a group of dehydrogenase enzymes that occur in many organisms, human, plant, and animal. They display a wide variety of substrate specificities and they fulfill several vital but quite different physiological functions. Some of these enzymes are involved in the production of alcoholic beverages and of industrial solvents, others are important in the production of vinegar, and still others participate in the degradation of naturally occurring and xenobiotic aromatic compounds as well as in the growth of bacteria and yeasts on methanol. They can be divided into three major categories: (1) The NAD^+- or $NADP^+$-dependent dehydrogenases; (2) $NAD(P)^+$-independent enzymes that use pyrroloquinoline quinone (PQQ), heme or cofactor F_{420} as cofactor; (3) FAD-dependent alcohol oxidases that catalyze an essentially irreversible oxidation of alcohols (Reid and Fewson, 1994). ADH is most often an $NAD(P)^+$-dependent enzyme present in the cytoplasm. In contrast, PQQ-dependent or $NAD(P)^+$-independent ADHs are rather unique and are found in only a narrow range of species of bacteria, the α, β, and γ-

proteobacteria, and are localized only in the periplasmic fraction (Toyama *et al*., 2004).

In acetic acid bacteria, ADH has been studied since the early of 1980s and revealed that it belongs to type III of ADH containing PQQ and heme *c* as cofactor (Adachi *et al*. 1978a, 1978b). Recently, in *A. pasteurianus* SKU1108; a thermotolerant acetic acid bacterium, two types of alcohol dehydrogenases, quinoprotein ADH and NAD^+-dependent ADH, were observed. The quinoprotein ADH is extensively involved in acetic acid production, while NAD^+-dependent ADH only in ethanol assimilation through the TCA and glyoxylate cycles in acetic acid bacteria (Chinnawirotpisan *et al*., 2003b, 2003c; Masud *et al*., 2010).

2.1 PQQ-dependent alcohol dehydrogenase (PQQ-ADH)

ADHs whose reaction is $NAD(P)^+$- independent have been found in many aerobic bacteria. Most of these enzymes contain PQQ as a prosthetic group. The PQQ has been found in as many as 10 different species of enzymes working mainly on the dehydrogenation of the primary or secondary hydroxyl group in alcohol or sugar. They are a new class of oxidoreductase and called quinoproteins. In these enzymes, PQQ is non-covalently bound to the apoenzyme. Some of these quinoproteins have, together with PQQ, an additional prosthetic group, heme *c* within a single polypeptide; these are called quinohemoproteins (Matsushita *et al*., 2002).

PQQ-dependent ADH is the largest enzyme group within the quinoprotein family. Its members consist of 18 different enzymes (Table 1) and are divided in to 3 groups, type I, II, and III ADHs according to their molecular properties, catalytic properties, and localization. Type I ADH is a simple quinoprotein having PQQ as the only prosthetic group, while type II and type III ADHs are quinohemoprotein having heme *c* as well as PQQ in the catalytic polypeptide. Type I ADH found in a limited number of Proteobacteria is very similar to quinoprotein methanol dehydrogenase (MDH) in methylotrophs. These are all soluble enzymes and seeming work in the periplasm. Type II ADH is a soluble enzyme in the periplasm and has a relatively wide distribution among several Proteobacteria such as *Pseudomonas*, *Ralstonia*,

Comamonas, etc. In contrast, type III ADH is a membrane-bound, working on the periplasmic surface and is unique to acetic acid bacteria (Toyama *et al*., 2004).

Type III ADH of acetic acid bacteria is a quinohemoprotein-cytochrome *c* complex and has been purified from different genera of acetic acid bacteria, *Acetobacter, Gluconobacter, Gluconacetobacter* and *Acidomonas* (Adachi *et al*., 1978a, 1978b; Tayama *et al*., 1989; Matsushita *et al*., 1992c; Kondo *et al*., 1995; Frébortová *et al*., 1997; Trcek *et al*., 2006; Gómez-Manzo *et al*., 2008; Kanchanarach *et al*., 2010), as summarized in Table 2. The purified ADH has been shown to consist of three different subunits; subunit I, II, and III, except for PQQ-ADHs purified from *Gluconacetobacter* species, which consist of only the subunits I and II. The enzyme complex contains one mole of pyrroloquiniline quinone (PQQ) and four moles of heme *c*. The largest subunit (subunit I) is a quinoprotein catalytic subunit of approximately 80 kDa in the molecular size containing one mole each of PQQ and heme *c* moiety as redox cofactors, functions as the catalytic site for ethanol. The second subunit (subunit II), a triheme cytochrome *c* subunit of approximately 50 kDa in the molecular size, contains three heme *c* moieties (Inoue *et al*., 1992; Tamaki *et al*., 1991) and presumably a Q, which is tightly bound to the protein (Matsushita *et al*., 2008). The bound Q seems to work as the redox mediator to the bulk Q, the physiological electron acceptor in the cytoplasmic membrane. Thus, the subunit II works as the electron mediator from the subunit I to the membranous Q. The smallest subunit (subunit III) of approximately 15 kDa in the molecular size is a peptide and suggested to have no prosthetic groups related to redox reaction from biochemical experiments and its predicted amino acid sequences (Kondo *et al*., 1995). Indeed, PQQ-ADHs purified from *Gluconacetobacter* species do not contain the subunit III (Tayama *et al*., 1989). The subunit III seems to work as a molecular chaperone for folding and/or maturation of the subunit I, which is speculated from the following findings. The subunit III exists freely in the periplasmic space besides in the PQQ-ADH complex on the cytoplasmic membrane. Mutant strains defective in the *adhS* gene encoding subunit III of *A. pasteurianus* lose ADH activity because they produce only the subunit II but fail to produce the subunit I as well as the subunit III (Kondo *et al*., 1995; Masud *et al*., 2010). All three subunits are indispensable for the ADH activity except for the smallest subunit of ADH from *G. oxydans* IFO 12528, formerly

G. suboxydans IFO 12528, which is not essential for ADH activity (Kondo and Horinouchi, 1997a).

The PQQ-ADH deficient strain of *A. pasteurianus* SKU1108 grow even better than the wild type in ethanol-containing medium, where two NAD^+-dependent ADHs, present in only a small amount in the wild strain, are dramatically increased in the cytoplasm, concomitant to the increase of the key enzyme activities in TCA and glyoxylate cycles (Chinawirotpisan *et al*., 2003b; Masud *et al*., 2010). Thus, it is clearly shown that the quinoprotein ADH is extensively involved in acetic acid production, while NAD^+-dependent ADH is used only for ethanol assimilation through the TCA and glyoxylate cycles.

Table 1 Quinoprotein and quinohemoprotein alcohol dehydrogenases and related enzymes

Enzymes	Location[a] (S or M)	Sources
Quinoproteins able to oxidize alcohols		
Methanol dehydrogenase (MDH)	S	Methylotrophs
Type I ADH (PQQ)		
Ethanol dehydrogenase (qEDH)	S	*Pseudomonas aeroginosa*
Alcohol dehydrogenase (ADH I)	S	*Pseudomonas putida*
1-Butanol dehydrogenase (BOH)	S	*Pseudomonas butanovora*
Polypropyleneglycol dehydrogenase (PPGDH)	S	*Stenotrophomonas maltophilia*
Other Quinoproteins able to oxidized alcohols (PQQ)		
Sorbose/Sorbosone dehydrogenase	S	*Gluconobacter* species *Pseudogluconobacter saccharoketogenes*
Glycerol dehydrogenase (GLDH)	M	*Gluconobacter* species
Quinohemoproteins able to oxidize alcohols		
Type II ADH (PQQ/heme *c*)		
Vanillyl alcohol or polyethyleneglycol dehydrogenase (PEGDH)	S	*Rhodopseudomonas acidophila*
Ethanol dehydrogenase (qhEDH)	S	*Comamonas testosterone*
Alcohol dehydrogenase (ADH IIB)	S	*Pseudomonas putida*
Alcohol dehydrogenase (ADH IIG)	S	*Pseudomonas putida*
Polyvinylalcohol dehydrogenase (PVADH)	S	*Pseudomonas* sp.
Tetrahydrofurfuryl alcohol dehydrogenase (THF ADH)	S	*Ralstonia eutropha*
1-Butanol dehydrogenase (BDH)	S	*Pseudomonas butanovora*
Type III ADH (PQQ/heme *c*/3 hemes *c*)		
Alcohol dehydrogenase	M	*Acetobacter aceti*
Alcohol dehydrogenase	M	*Acetobacter pasteurianus*

Table 1 (Continued)

Enzymes	Location[a] (S or M)	Sources
Alcohol dehydrogenase	M	*Gluconacetobacter polyoxogenes*
Alcohol dehydrogenase	M	*Acidomonas methanolicus*
Alcohol dehydrogenase	M	*Gluconobacter suboxydans*
Other related quinohemoproteins		
Lupanine hydroxylase (PQQ/heme *c*)	S	*Pseudomonas* sp.
Amine dehydrogenase (CTQ/heme *c*)	S	*Pseudomonas putida* *Paracoccus denitrificans*

[a] Soluble (S) or Membrane-bound (M)

Source: Toyama *et al*. (2004)

Table 2 Molecular organizations of PQQ-ADHs from various acetic acid bacteria

Species	Strains	Molecular sizes of subunits (kDa)			References
		I	II	III	
A. lovaniensis	IFO3284	72	50	15	(Matsushita *et al*., 1992c)
A. pasteurianus	NCI1452	72	44	20	(Kondo *et al*., 1995)
	KKP584	74	44	16	(Trcek *et al*., 2006)
	IFO3191	76	55	16	(Kanchanarach *et al*., 2010)
	SKU1108	76	55	16	(Kanchanarach *et al*., 2010)
	MSU10	76	55	16	(Kanchanarach *et al*., 2010)
Ga. polyoxogenes[a]	NBI1028	72	44	-	(Tayama *et al*., 1989)
Ga. europaeus	V3	72	45	-	(Trcek *et al*., 2006)
Ga. intermedius	JK3	72	45	-	(Trcek *et al*., 2006)
Ga. diazotrophicus	PAL5	71	44	-	(Gómez-Manzo *et al*., 2008)
Ga. xylinus[b]	-	71	44	-	(Gómez-Manzo *et al*., 2008)
G. oxydans	IFO12528	85	49	14	(Adachi *et al*., 1978b)
A. methanolicus	JCM6891	80	54	8	(Frébortová *et al*., 1997)

[a] Formerly *Acetobacter polyoxogenes*

[b] *Gluconacetobacter xylinus*

Source: Yakushi and Matsushita (2010)

2.2 NAD^+-dependent alcohol dehydrogenase (NAD^+-ADH)

Many different $NAD(P)^+$-dependent ADHs from a wide variety of animals, plants, and microorganisms show certain structural and functional similarities. The alcohol dehydrogenase superfamily can be divided into three groups: Group I, zinc-dependent long chain ADHs (containing approximately 350 residues per subunit); Group II, zinc-independent short chain ADHs (containing approximately 250 residues per subunit); Group III, "iron-activated" ADHs of subunit size of about 385 residues (Jörnvall *et al*., 1987).

Many different group I ADHs have been characterized and they exist in dimeric and tetrameric forms, represented by horse liver alcohol dehydrogenase (HLADH) and the yeast *Saccharomyces cerevisiae* alcohol dehydrogenase I (SADHI), respectively. HLADH is a dimer of subunit size of 40 kDa, each subunit containing 374 residues arranged in a catalytic domain and a coenzyme binding domain, divided by a long cleft and zinc atom bind to each catalytic domain at the active site. The GXGXXG fingerprint pattern of the coenzyme binding domain has been found to be conserved in HLADH as well as in other NAD(H)-binding ADHs (Wierenga *et al*., 1985). Group II ADHs, as represented by *Drosophila* spp. alcohol dehydrogenase (Villarroya *et al*., 1989), have been characterized from both prokaryotes and eukaryotes. Microbial polyol/sugar dehydrogenases were first suggested to be related to this group (Jörnvall *et al.*, 1981). Amino acid alignment of the group II alcohol dehydrogenase revealed that the GXXXGXG pattern is presented in all but not in sorbitol-6-phosphate dehydrogenase from *E. coli* and the NodG protein from *Rhizobium meliloti* (Debelle and Sharma, 1986; Yamada and Saier, 1987; Persson *et al*., 1991). The active enzymes are dimers or tetramers, each subunit size is approximately 27 kDa, with around 250 residues in the polypeptide chain (Peltoketo *et al*., 1988). There is no evidence for a metal requirement in any of the enzymes so far characterized. The group III ADHs represented by the second isozyme from *Zymomonas mobilis* (Conway *et al*., 1989). The subunit size for the group III ADHs is approximately 40 kDa except for the multifunctional fermentative alcohol dehydrogenase of *E. coli*, which is a 96 kDa protein of 891 residues (Goodlove *et al*.,

1989) and the similar enzyme from *Clostridium acetobutylicum* that contains 862 residues (Fischer *et al*., 1993).

The NAD(P)$^+$-ADHs have been isolated and characterized from many microorganisms. They are for examples, the short-chain NAD(H)-dependent alcohol dehydrogenase belonging to group II of NAD(P)$^+$-ADHs from *Thermus thermophilus*, a thermophilic microorganism (Pennacchio *et al*., 2008), two novel metal-independent long-chain alkyl alcohol dehydrogenases from *Geobacillus thermodenitrificans* NG80-2 (Liu *et al*., 2009), and a novel alcohol dehydrogenase (ADH) that belongs to the medium chain dehydrogenase/reductase (MDR) superfamily from the hyperthermophilic archaeon, *Pyrobaculum aerophilum* (Vitale *et al*., 2010).

On the other hand, in acetic acid bacteria, only NAD$^+$-ADHs from *A. pasteurianus* SKU1108 have been characterized (Chinnawirotpisan *et al*., 2003c). There has been no report on the genetic level of NAD$^+$-ADH in acetic acid bacteria so far. Two NAD$^+$-ADHs, ADH I and ADH II, were found in the cytoplasm when a membrane-bound ADH-deficiency mutant of this strain was grown on ethanol. Characterization of the two ADHs indicated that ADH I is a member of the long-chain family and shows a preference for primary alcohols and ADH II is a member of the short-chain family and shows a preference for secondary alcohols. The relative molecular masses obtained from SDS-PAGE revealed that ADH I is a trimer consisting of an identical subunit of 42 kDa, while ADH II is a homodimer having a subunit of 31 kDa.

2.3 Cloning and analysis of gene encoding quinoprotein alcohol dehydrogenase

The PQQ-ADHs have been purified and characterized from several acetic acid bacteria. The purified PQQ-ADHs are composed of two or three subunits contingent on the bacterial species. The gene coding for the largest subunit (subunit I) of the PQQ-ADH complex known as *adhA* has been cloned and sequenced from *A. aceti* (Inoue *et al*., 1989) and *Ga. polyoxygenes,* formerly *A. polyoxogenes* (Tamaki *et al*., 1991). The predicted amino acid sequences of the 72 kDa of *A. aceti* has about

80% identity with the same protein from *Ga. polyoxogenes* and about 30% identity with methanol dehydrogenase (MDH) from methylotrophic bacteria. Both of the PQQ-ADHs were predicted to have a longer than normal N-terminal signal sequence of 35 amino acids, which was thought to be cleaved from the preprotein to generate the mature protein. Later, cloning and characterization of the *adhA* gene from *G. oxydans* was reported (Kondo and Horinouchi, 1997a). The amino acid sequences of this subunit I showed significant similarity (approximately 70%) with subunit I from *Acetobacter* sp. Two putative pyrroloquinoline quinone (PQQ)-binding motifs and a heme-binding motif were observed. One molecule of PQQ is thought to be bound at two PQQ-binding motifs. Recently, the expression of the *adhA* gene in *A. aceti*, *A. pasteurianus*, *Ga. hansenii* and *G. oxydans* in different growth conditions with glucose or ethanol as the carbon sources was reported. The *adhA* gene expression in all the species was higher when grown in a medium containing ethanol, with the exception of *G. oxydans* which did show any growth in such a medium. The increased expression of the *adhA* gene was clearly observed in two *Acetobacter* species grown in a medium containing ethanol. On the other hand, *Ga. hansenii* displayed strong growth in such a medium without any significant changes in the *adhA* gene expression (Quintero *et al*., 2009).

The *adhB* gene encoding subunit II was cloned from *Ga. polyoxogenes* (Tamaki *et al*., 1991) and *G. oxydans* (Kondo and Horinouchi, 1997a). This *adhB* is located in the same orientation and just downstream of *adhA* with no obvious transcriptional signal. This phenomenon indicates that these two genes are within the same operon and are co-transcribed (Tamaki *et al*., 1991). It was also observed that in subunit II-deficient mutant of *A. pasteurianus* NCI 1452, larger amount of subunit I and III were present in the soluble fraction than in the membrane fraction. These observations suggested that subunit II serves as an anchor for subunit I and III to be fractionated in the membrane (Kondo *et al*., 1995).

The cloning, sequencing and characterization of the gene which encodes the smallest subunit of ADH from *A. pasteurianus* NCI 1452 (Kondo *et al*., 1995), *G. oxydans* IFO 12528, formerly *G. oxydans* IFO 12528 (Kondo and Horinouchi, 1997a), *A. pasteurianus* NCI 1193 (Takamura *et al*., 2002) and *A. pasteurianus* SKU1108

(Masud *et al*., 2010) were reported. The restriction map around the *adhS* gene revealed that this gene was not located very close to the *adhA* and *adhB* gene cluster (Takemura, *et al*., 1993). The nucleotide sequence of *adhS* gene showed that the 22 kDa protein was synthesized as a preprotein, with the 28 amino acids NH_2-terminal probably serving as its signal sequence for secretion from cytoplasm to periplasm. The disruption of this gene resulted in the loss of PQQ-ADH activity in *A. pasteurianus* NCI 1452. The absence of subunit III led to a decrease in the amount of subunit I in both membrane and soluble fractions, especially in the stationary phase. An additional speculative function of subunit III may involve in stabilization of the dehydrogenase subunit and acts as a molecular coupler of subunit I to subunit II on the cytoplasmic membrane (Kondo *et al*., 1995). However, *adhS* gene of *G. oxydans* could not complement a defect in the subunit III of *A. pasteurianus*. Cloning and sequencing of the *adhS* gene from *A. pasteurianus* NCI 1193 was later reported and its deduced amino acid sequences exhibited a 90.7% homology with the same protein from *A. pasteurianus* NCI 1452 (Takakuwa *et al.*, 2002).

Recently, *adhS* gene from *A. pasteurianus* SKU1108 was cloned and sequenced. The *adhS* gene consisted of 618 bp for 205 amino acid residues with 99% and 91% homology with alcohol dehydrogenase subunit III from *A. pasteurianus* NCI 1193 and *A. pasteurianus* NCI 1452, respectively (Vanittananon, 2005; Masud *et al*., 2010). Moreover, *adhA*, *adhB* and *adhS* genes from *A. pasteurianus* MSU10, SKU1108; thermotolerant strains, and IFO 3191; mesophilic strain, were cloned and the amino acid sequences were compared (Kanchanarach *et al*., 2010). Interestingly, the 94th C common residue was observed in the enzyme of thermotolerant strain, MSU10 and SKU1108, but not in the enzymes of mesophilic strain IFO 3191.

3. Gene Manipulation Principles

3.1 Polymerase chain reaction (PCR)

In 1984, Kary Mullis devised an ingenious method called the polymerase chain reaction (PCR) for amplifying specific DNA sequences. PCR is a procedure for generating large amounts of a specific DNA target *in vitro* by an enzymatic reaction.

Millions of the target sequences can be readily obtained by PCR if the flanking sequences of the target are known. It has rapidly become one of the most widely used techniques for the molecular biologist and revolutionized molecular biology. PCR provides a sensitive, selective, and extremely rapid means of amplifying a desired sequence of DNA. 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), reaction buffer, magnesium chloride ($MgCl_2$), and optimum additives. Early PCR reactions used an *E. coli* DNA polymerase that was destroyed by each heat denaturation cycle. Substitution of a heat-stable DNA polymerase from *Thermus aquaticus*, called "*Taq*" polymerase, (or the corresponding DNA polymerase from other thermophilic bacteria), an organism that lives and replicates at 70-80 °C, obviates this problem and has made possible automation of the reaction, since the polymerase reactions can be run at 70°C. This has also improved the specificity and the yield of PCR product. This polymerase was capable of remaining active throughout the high denaturation temperatures required at the beginning of each amplification cycles despite of repeated heating during many cycles of amplification. Moreover, 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: denaturation, annealing and extension. PCR specificity is based on the use of two oligonucleotide primers that hybridize to complementary sequences on opposite strands of DNA and flank the target sequence. The DNA sample is first heated to separate the two strands; the primers are allowed to bind to the DNA; and each strand is copied by a DNA polymerase, starting at the primer site. The two DNA strands each serve as a template for the synthesis of new DNA from the two primers. Heat denaturation, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase are repeated and result in the exponential amplification of DNA segments of defined length.

3.2 Nucleotide sequencing

The term of nucleotide sequencing refers to sequencing methods for determining the order of the nucleotide bases, adenine (A), guanine (G), cytosine (C),

and thymine (T), in a molecule of DNA. Knowledge of DNA sequences has become indispensable for basic biological research, other research branches utilizing DNA sequencing, and in numerous applied fields such as diagnostic, biotechnology, forensic biology and biological systematics. The advent of DNA sequencing has significantly accelerated biological research and discovery. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of the human genome, in the Human Genome Project. Related projects, often by scientific collaboration across continents, have generated the complete DNA sequences of many animal, plant, and microbial genomes.

The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of dye-based sequencing methods with automated analysis (Olsvik *et al*., 1993), DNA sequencing has become easier and orders of magnitude faster (Pettersson *et al*., 2009). RNA sequencing was one of the earliest forms of nucleotide sequencing. The major landmark of RNA sequencing is the sequence of the first complete gene and the complete genome of Bacteriophage MS2, identified and published by Walter Fiers and his coworkers at the University of Ghent (Ghent, Belgium), between 1972 and 1976 (Min Jou *et al*., 1972; Fiers *et al*., 1976). Prior to the development of rapid DNA sequencing methods in the early 1970s by Frederick Sanger at the University of Cambridge, in England and Walter Gilbert and Allan Maxam at Harvard (Maxam and Gilbert, 1977), a number of laborious methods were used. For instance, in 1973, Gilbert and Maxam reported the sequence of 24 bp using a method known as wandering-spot analysis (Gilbert and Maxam, 1973). The chain-termination method developed by Sanger and coworkers in 1975 soon became the method of choice, owing to its relative ease and reliability (Sanger and Coulson, 1975; Sanger *et al*., 1977).

3.3 Mutagenesis of the cloned gene

Genes and other genetic elements are frequently characterized by correlating specific changes in the DNA sequence with effects on function. The classical genetic approach to this problem has been to obtain mutations by selecting

for organisms that have new properties. The relevant wild-type and mutant genes can then be cloned and subjected to DNA sequence analysis. The advent of recombinant DNA technology has made it possible to reverse the procedure of classical mutagenesis. Mutations are first generated in cloned segments of DNA by using a variety of chemical and enzymatic methods. This methods can produce mutations at an extremely high frequency (approaching 100% in some cases), and essentially all possible mutations can be generated. Once generated, the mutations can be obtained in a systematic manner without regard to their phenotype. The end result is that the functions of a given region of DNA can be investigated in much more detail.

In addition, over three decades ago, a variety of efficient and reliable methods have been developed for the construction of site-directed mutations in DNA using synthetic oligonucleotides. This has revolutionized the study of gene regulation and protein structure and function. All of these mutagenesis methods fall into one of three broad strategies. One approach uses an oligonucleotide complementary to part of a single-stranded(ss) DNA template but containing an internal mismatch(es) to direct the desired mutation. A second strategy is to replace the region of interest with a synthetic mutant fragment generated by annealing complementary oligonucleotides, or by hybridization and ligation of a number of oligonucleotides. The third and most recent strategy has been to harness the power of the polymerase chain reaction (PCR) to generate a mutant fragment starting from a double-stranded(ds) DNA template using mismatched oligonucleotides (Carter, 1991).

Most of the PCR-based methods of mutagenesis in current use are direct descendants of techniques originally described in the late 1980s, soon after the introduction of thermostable DNA polymerase to PCR (Higuchi *et al*., 1988, Ho *et al*., 1989, Kadowaki *et al*., 1989, Vallette *et al*., 1989). At that time, it was already known that centrally located single-base mismatches in hybrids between oligonucleotide primers and the target DNA did not affect the efficiency of amplification. Early investigators shows that these mismatches could be converted into mutations located in the primer regions of PCR products, which, in turn, could be substitututed for the homologous wild-type segment in, for example, a recombinant plasmid. Examples of

the important advantages of PCR-based methods for site-directed mutagenesis are high rates of recovery of mutants and availability of commercial kits.

Initially, PCR has been used mostly for the accurate amplification of known DNA sequences, but PCR based gene manipulation has become invaluable for the alteration of genetic information at the molecular level. This tool is permitting site-directed mutagenesis of PCR products at an arbitrary distance from the ends of the template sequence. Random mutagenesis of a defined region of DNA is performed by error-prone PCR (EP-PCR), which takes advantage of the inherently low fidelity of *Taq* DNA polymerase to introduce mutations. Using the optimized conditions, which is decreased further by adding Mn^{2+}, increasing Mg^{2+} concentration, and using unequal dNTP concentrations, this enzyme can incorporate incorrect nucleotides at a frequency of ~0.07% per position. Errors accumulate by performing exponential amplification by PCR. The boundaries of the mutated region are defined by the choice of PCR primers, making it possible to mutagenize an entire gene or any defined segment of a gene. The average number of mutations per DNA fragment can be controlled as a function of the number of EP-PCR doublings performed. EP-PCR is extremely simple, fast, and versatile. It can be used to generate wide spectrum of nucleotide substitutions. It is the method of choice for introducing random mutations into a defined segment of DNA that is too long to be chemically synthesized as a degenerate sequence.

3.4 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.

A Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane in a format known as either dot blot or slot dot and subsequent fragment detection by probe hybridization. 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. The method is named after its inventor, the British biologist Edwin Southern (Southern, 1975). Other blotting methods (i.e., northern, western, eastern, southwestern blotting) that employ similar principles, but using RNA or protein, have later been named in reference to Edwin Southern's name. The northern blotting is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample (Alwine *et al*., 1977). The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract (Burnette, 1981). The eastern blotting is a technique to analyze proteins, lipids, or glycoconjugates, and is most often used to detect carbohydrate epitopes (Towbin *et al*., 1979). The southwestern blotting, based along the lines of Southern blotting and first described by Bowen *et al*. (1980), is a lab technique which involves identifying and characterizing DNA-binding proteins by their ability to bind to specific oligonucleotide probes. Proteins are separated by electrophoresis and blotted onto a nitrocellulose membrane similar to Western blotting but the proteins are identified when they bind labeled DNA Probe (as with Southern blotting) instead of antibodies.

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 the last and may be the most important step in nucleotide sequencing. The goal is to determine the function of the sequence obtained. The most frequent analysis of an unknown sequence is to perform homology search, which is a search for sequence similarity with known sequence in the database. These are three international DNA databases available, which provide sequence information as no cost over the internet (Table 3). All databases exchange their data daily to update the sequence information. The database search is necessary to decide what types of sequences will be compared: DNA, protein, or DNA as protein. A homology search can be carried out either with nucleotide sequence or amino acid sequence. However, homology search with nucleotide sequence rarely gives valuable information (Brown, 1998). Since the DNA sequence information is so simple, just four different bases (A, T, G, and C). As a result, unrelated sequence can have high degree of similarity and the identification for biologically significant homology is very difficult. On the contrary, amino acid sequence information is relatively complex (20 different amino acid), and amino acid similarity between proteins often indicates a corresponding similarity in the structure and function. By the comparison of protein sequences have proven to be much more effective tool. Thus, the determination for the structure and function of unknown proteins is easily achieved by comparing amino acid sequence of an unknown protein with previously characterized proteins. Protein-level searches are valuable for detecting evolutionary related genes, while DNA searches are best for locating nearly identical regions of sequence (see Table 4 for available comparison programs and the corresponding types of comparison) (Higgins and Tayler, 2001).

Table 3 List of international primary sequence database

Database	Sponsor	Location (URL)
DNA Data Bank of Japan	National Institute of Genetics	http://www.ncbi.nlm.nih.gov/
EMBL Data Library	European Molecular Biology Laboratory	http://ebi.ac.uk/embl.html/
GenBank	National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/

Source: Higgins and Taylor (2001)

Table 4 Comparison programs and the type's comparison

Program	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, tfasx	Protein	DNA	Protein-level	Search for genes in unannotated DNA
tblastx	DNA	DNA	Protein-level	Discover gene structure

Source: Higgins and Taylor (2001)

4.2 Open reading frame analysis

DNA sequence can potentially be translated into protein sequence in no less than six different ways. The triplet genetic code allows for six possible reading frames. An open reading frame (ORF) is defined as a sequence stretch that begins with an initiation codon, usually ATG (alternatively GTG, TTG or any codon), and ends with a termination codon (TAA, TAG or TGA). In addition, homology search is more useful when carried out with an amino acid sequence translated from an ORF located in the DNA sequence. Because of the similarity nature of DNA sequence information, unrelated sequences can have degrees of similarity and the identification of significant homologies is very difficult. The determination of an ORF as an unknown sequence can be accomplished either by using computational software tools or from the database search tools available on the internet.

4.3 Sequence alignment

Sequence alignment is the procedure of comparing two (pair-wise alignment) or more (multiple sequence alignment) sequences by searching for a series of individual characters or character patterns that are in the same order in the sequences. Two sequences are aligned by writing them across a page in two rows. Computational approaches to sequence alignment generally fall into two categories: global alignments and local alignments. Calculating a global alignment is a form of global optimization that forces the alignment to span the entire length of all query sequences. By contrast, local alignments identify regions of similarity within long sequences that are often widely divergent overall. Local alignments are often preferable, but can be more difficult to calculate because of the additional challenge of identifying the regions of similarity.

Pairwise sequence alignment methods are used to find the best-matching piecewise (local) or global alignments of two query sequences. Pairwise alignments can only be used between two sequences at a time, but they are efficient to calculate and are often used for methods that do not require extreme precision (such as searching a database for sequences with high similarity to a query). Multiple sequence

alignment is an extension of pairwise alignment to incorporate more than two sequences at a time. Multiple alignment methods try to align all of the sequences in a given query set. These methods are often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionarily related. Alignments are also used to aid in establishing evolutionary relationships by constructing phylogenetic trees (Higgins and Taylor, 2001; Mount, 2004). A more complete list of available software categorized by algorithm and alignment type is available at sequence alignment software, but common software tools used for general sequence alignment tasks include ClustalW and T-coffee for alignment, and BLAST and FASTA3x for database searching.

4.4 Phylogenetic tree analysis

A phylogenetic tree or evolutionary tree is a branching diagram or "tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from 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. Phylogenetic trees among a nontrivial number of input sequences are constructed using computational phylogenetics methods. Distance-matrix methods such as neighbor-joining or UPGMA, which calculate genetic distance from multiple sequence alignments, are simplest to implement, but do not invoke an evolutionary model. Many sequence alignment methods such as ClustalW also create trees by using the simpler algorithms (i.e. those based on distance) of tree construction. Maximum parsimony is another simple method of estimating phylogenetic trees, but implies an implicit model of evolution. More advanced methods use the optimality criterion of maximum likelihood, often within a Bayesian Framework, and apply an explicit model of evolution to phylogenetic tree estimation. Identifying the optimal tree using many of these techniques is NP-hard, so heuristic search and optimization methods are

used in combination with tree-scoring functions to identify a reasonably good tree that fits the data.

5. Principles and Techniques of Immunoblotting Analysis

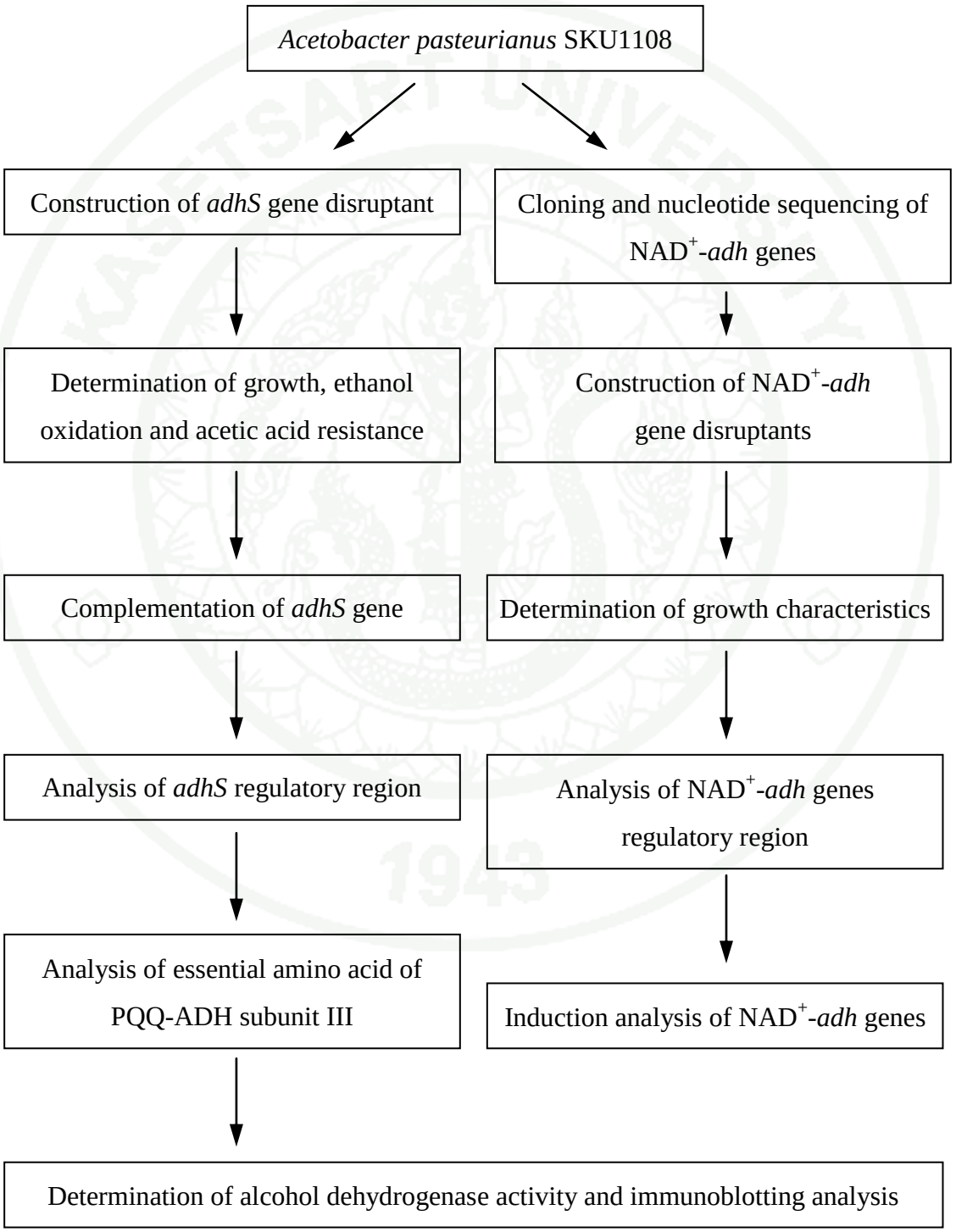
Immunoblotting (also known as Western Blotting) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or polyvinylidene fluoride**;** PVDF), where they are probed (detected) using antibodies specific to the target protein (Towbin *et al*., 1979; Renart *et al*., 1979). This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. Other related techniques include using antibodies to detect proteins in tissues and cells by immunostaining and enzyme-linked immunosorbent assay (ELISA). The method originated from the laboratory of George Stark at Stanford. The name western blot was given to the technique by W. Neal Burnette and is a play on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern. Detection of RNA is termed northern blotting and the detection of post-translational modification of protein is termed eastern blotting.

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:



1. Chemicals

NAD^+, NADH were obtained from Sigma. Yeast extract, polypeptone, tryptone, glucose and glycerol were obtained from Merck & Co., Inc. (New Jersey, USA). Restriction enzymes were purchased from Takara Bio Inc. (Tokyo, Japan) or Toyobo Co., Ltd. (Osaka, Japan). 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). *ortho*-Nitrophenyl-β-galactoside (ONPG) was obtained from Nacalai Tesque Co. (Kyoto, Japan). All other chemicals used in this study were reagent grade and obtained from commercial sources.

2. Bacterial Strains, Culture Media and Culture Conditions

2.1 Acetic acid bacteria

Acetobacter pasteurianus SKU1108, a strain with potential for acetic acid production (Saeki *et al*., 1997a), CN6-2 (*adhAB* mutant); which was previously constructed (Chinnawirotpisan *et al*., 2003b), DPS (*adhS* gene disruptant) derived from *A. pasteurianus* SKU1108 and NAD^+-*adh* disruptants derived from CN6-2 were used in this study. The procedure for construction of the DPS and NAD^+-*adh* genes disruptants are described in 3.1 and 4.2, respectively, of Materials and Methods section. The stock cultures were maintained on a potato medium (Appendix A) containing 0.5% $CaCO_3$ and 1.5% agar. 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. YPGD or Seed Culture Medium (SCM, Appendix A) and YPG medium (Appendix A) were used for general cultivation to determine bacterial growth, acetic acid production and enzyme activity. Ethanol and/or acetic acid were added aseptically when indicated. Five ml of overnight culture in potato medium was inoculated into 100 ml of YPGD or YPG medium in a 500-ml Erlenmeyer flask. Cultivation was performed at 30°C on a rotary shaker at 200 rpm. The growth was

periodically measured with a Klett Summerson photometer or Spectrophotometer. The acidity of the culture medium was measured by titration with 0.8 N NaOH using phenolphthalein as the pH indicator and expressed as acetate concentration according to the analytical method described by Saeki *et al*. (1997a). The genotype of acetic acid bacteria used in this study is shown in Table 5.

2.2 *Escherichia coli*

Escherichia coli DH5α and S17-1 were routinely cultured and maintained in Luria-Bertani (LB) broth (Sambrook *et al*., 1989) containing appropriate antibiotic and incubated at 37°C. The ingredients of LB medium are shown in Appendix A. The genotype of *E. coli* DH5α and S17-1 strains are shown in Table 5. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml) and gentamicin (12.5 μg/ml) were added to the medium when necessary to maintain plasmids.

Table 5 Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	**Relevant characteristics**	**Reference**
Strains		
A. pasteurianus SKU1108	Wild type	Theeragool *et al*. (1996) Saeki *et al*. (1997a)
CN6-2	*adhAB* mutant of SKU1108 isolated from NTG treatment	Chinnawirotpisan *et al*. (2003b)
DPS	*adhS* gene disruptant of SKU1108 with insertion by Km^r cassette from pUC4K	This study
DPI	*adhI* gene disruptant of CN6-2 with insertion by Tc^r cassette from pKRP12	This study
DPII	*adhII* gene disruptant of CN6-2 with insertion by a non-polar Km^r cassette from pTKm	This study
DPI-II	*adhI-adhII* genes double disruptant of CN6-2; *adhI*::Tc^r, *adhII*::Km^r	This study
E. coli		
DH5α	*supE*44 Δ*lacU*169 *(*Φ80*lacZ*ΔM15*)* *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	
S17-1	Tpr Smr *recA*, *thi*, *pro*, *hsdR*-M+RP4: 2-Tc:Mu: Km Tn7 λpir	
Plasmids		
pGEM®-T Easy vector	Cloning vector, Ap^r, *lacZ*	Promega
pCM62	*Acetobacter-E. coli* shuttle vector, Tc^r, *lacZ*	Marx and Lidstrom (2001)
pUC4K	Km^r cassette	Taylor and Rose (1988)
pRP2012	Tc^r cassette	Reece and Phillips (1995)
pTKm	Non-polar Km^r cassette	Yoshida *et al.* (2003)

Table 5 (Continued)

Bacterial strains and plasmids	**Relevant characteristics**	**Reference**
pCMadhS10	Tc^r, *lac*Z containing 2 kb *Eco*RI DNA fragment carrying *adhS* from *A. pasteurianus* SKU1108 in the opposite orientation from P*lac*	This study
pCMadhS14	Tc^r, *lac*Z containing 2 kb *Eco*RI DNA fragment carrying *adhS* from *A. pasteurianus* SKU1108 in the same orientation from P*lac*	This study
pGEM®-TadhI	Ap^r, *lac*Z carrying a 2.1 kb *adhI* gene which encodes NAD^+-ADHI from *A. pasteurianus* SKU1108	This study
pGEM®-TadhII	Ap^r, *lac*Z carrying a 1.86 kb *adhII* gene which encodes NAD^+-ADHII from *A. pasteurianus* SKU1108	This study
pGEM®-TadhI::Tc^r	Ap^r, Tc^r, *lacZ* carrying a Tc^r disrupted *adhI* gene at *Hin*dIII site	This study
pGEM®-TadhII::Km^r	Ap^r, Tc^r, *lacZ* carrying a Km^r disrupted *adhII* gene at *Hin*dIII site	This study

3. Functional Analysis of *adhS* Gene Encoding Membrane-Bound Alcohol Dehydrogenase Subunit III

3.1 Construction of *adhS* disruptant

The *adhS* disruptant (DPS) was constructed by replacing the internal part of *adhS* gene of *A. pasteurianus* SKU1108 with Km^r cassette (*npt*) from pUC4K (Figure 9) using the homologous recombination strategy. The procedures are described briefly as follows: The plasmid pUC18 was first digested with *Pst*I, followed by end-blunting with Blunting High (Toyobo, Osaka, Japan) and self-ligation. The 2 kb *Eco*RI DNA fragment with the *adhS* gene obtained from the *Eco*RI-digested pUCadhS1 (Vanittananon, 2005) was inserted into pUC18 (with blunted *Pst*I site) at the *Eco*RI location. Then, a 1.2 kb *Pst*I DNA fragment with kanamycin cassette from the *Pst*I-digested pUC4K was inserted at the *Pst*I site located in the coding sequence of *adhS* gene. The subsequent plasmid pUCadhS::Km^r was electroporated into *A. pasteurianus* SKU1108 by gene pluse II as described by Mostafa *et al.* (2002) and *adh*-deficient mutants were selected on a YPGD medium containing 50 μg/ml kanamycin, 0.5% $CaCO_3$ and 4% ethanol. Colonies deficient in ethanol oxidizing ability were screened on the basis of the clear zone absence and confirmed by PCR amplification and Southern hybridization, compared with wild type strain. PCR amplification was performed by using PCR Kit (Ready-To-Go™ PCR Beads of Amersham Pharmacia Biotech Inc., USA.) The forward primer or FadhS is 5'-ATGAAACTGATTGCCG TA-3' and the reverse primer or RadhS is 5'-TTACGAAACAGAACTGGT-3'. A Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 5 min at 95°C followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min and a final extension at 72°C for 10 min. The PCR amplification products were analyzed by 1.2% agarose gel electrophoresis in 1x TAE buffer. Agarose gel was stained in ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator (UVP Inc.) to visualize PCR products. A 1 kb DNA ladder and λ/*Hind*III fragment was used as DNA size marker. The procedure of Southern hybridization was described below. The obtained disruptant was further analyzed for their abilities to produce ADH subunit I, II and III by heme staining and immonoblotting analysis as well as subunit localization and ADH activity.

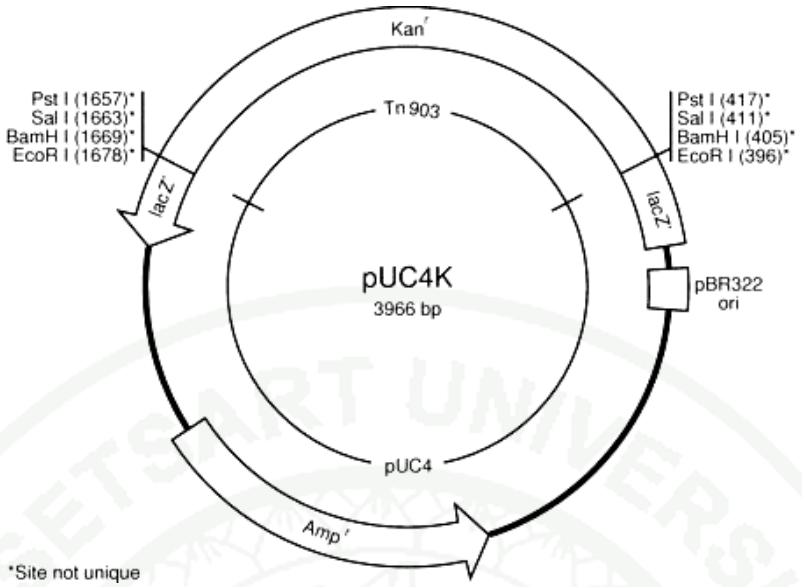


Figure 9 Structure and physical map of plasmid pUC4K.

Source: Amersham biosciences (2004)

3.2 Southern hybridization of *adhS* gene

The chromosomal DNA of *A. pasteurianus* SKU1108 and DPS strains were digested by *Eco*RI restriction enzyme and hybridized with the 618 bp *adhS* gene DNA probe. The agarose gel containing DNA was placed in 200 ml of 0.25 N HCl solution (Appendix B) for 5-10 min twice. The gel was then washed with nuclease free distilled water. Then, the denaturation solution (Appendix B) was added and incubated at room temperature for 45 min with gentle shaking. The denaturation solution was poured off and the gel was rinsed twice with nuclease free distilled water. The neutralization solution (Appendix B) was added and incubated at room temperature with gentle shaking for 30 min twice. After the gel had been treated with the neutralization solution for the indicated time, 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 (Appendix B). It was placed on the bottom of the tray containing 10x SSC solution. The prepared gel was put up side down 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, five to ten cm high of the desired dimensions paper towels were placed on the Whatman paper. A weight (~500 g) was put on the top of the paper. The transfer was allowed to proceed for 18-24 h. Then, the transfer set was disassembled and the membrane was washed in 2x SSC solution and allowed to dry at room temperature. The membrane was transferred into UV transilluminator to crosslink DNA by irradiation both side of membrane, each for 5 min. All following incubation were performed with shaking or mixing unless state otherwise. The membrane was placed in a sealable bag containing 1-2 ml of prehybridization solution (Appendix B) and incubated at 42°C at least 1-2 h (prehybridization) and transferred the membrane to a new bag. The hybridization solution containing the labeled 618 bp *adhS* gene DNA probe or 1.2 kb Km^r probe was added and incubated at 42°C for 18-24 h (hybridization). After the incubated period, the membrane was washed with shaking at 65°C with the solutions as follows: 2x SSC for 10 min twice, 2x SSC containing 0.1% SDS for 30 min, 0.1x SSC for 10 min. Then, the membrane was soaked in 2x SSC at 65°C for 5 min. Finally, the hybridization product was detected as follows: the nylon membrane was soaked in a small volume of buffer II (Appendix B) at 65°C for 30 min. The membrane was then incubated for 10 min at room temperature in diluted Digoxigenin-AP conjugate solution (1:5000 in buffer I). After the incubation period, unbound antibody conjugate was removed by washing in 50 ml of buffer I for 15 min twice and in 50 ml of buffer III for 5-10 min. The membrane was transferred into the sealable bag containing a freshly prepared color-substrate solution (NBT/BCIP solution in buffer III, Appendix B) and incubated in the dark until the purple/blue precipitates was formed. The reaction was stopped by washing the membrane in buffer IV (Appendix B). The membrane was dried at 80°C for 1-2 min and stored in the sealed plastic bag.

3.3 Complementation of *adhS* gene in *adhS* disruptant

A 2 kb *Eco*RI DNA fragment with the *adhS* gene from *A. pasteurianus* SKU1108 was obtained from the *Eco*RI-digested pCMadhS1 and further inserted into a broad host range vector pCM62 (Marx and Lidstrom, 2001) as shown in Figure 10 at the applicable site. The three types of constructed plasmids were pCMadhS10 (*adhS*

gene inserted in an opposite orientation from *Plac*), pCMadhS14 (*adhS* gene inserted in the same orientation with *Plac*) and pCMadhS11 (two copies of *adhS* gene inserted in the same orientation with *Plac*). Each plasmid as well as pCM62 was individually transferred conjugationally (Appendix C) into the *adhS* disruptant and the desired clones were selected on a YPGD medium containing 4% ethanol, 25 µg/ml tetracycline, 50 µg/ml kanamycin and 0.5% $CaCO_3$ for an incubation period of two to three days at 30°C. The conjugants were designated as DPS-62, DPS-10 and DPS-14. The conjugants with *adhS* gene complementation produced a clear zone surrounding their colonies. Among those *adhS* complemented strains, DPS-10 was selected for further studies because it possessed its own transcriptional signal for gene expression.

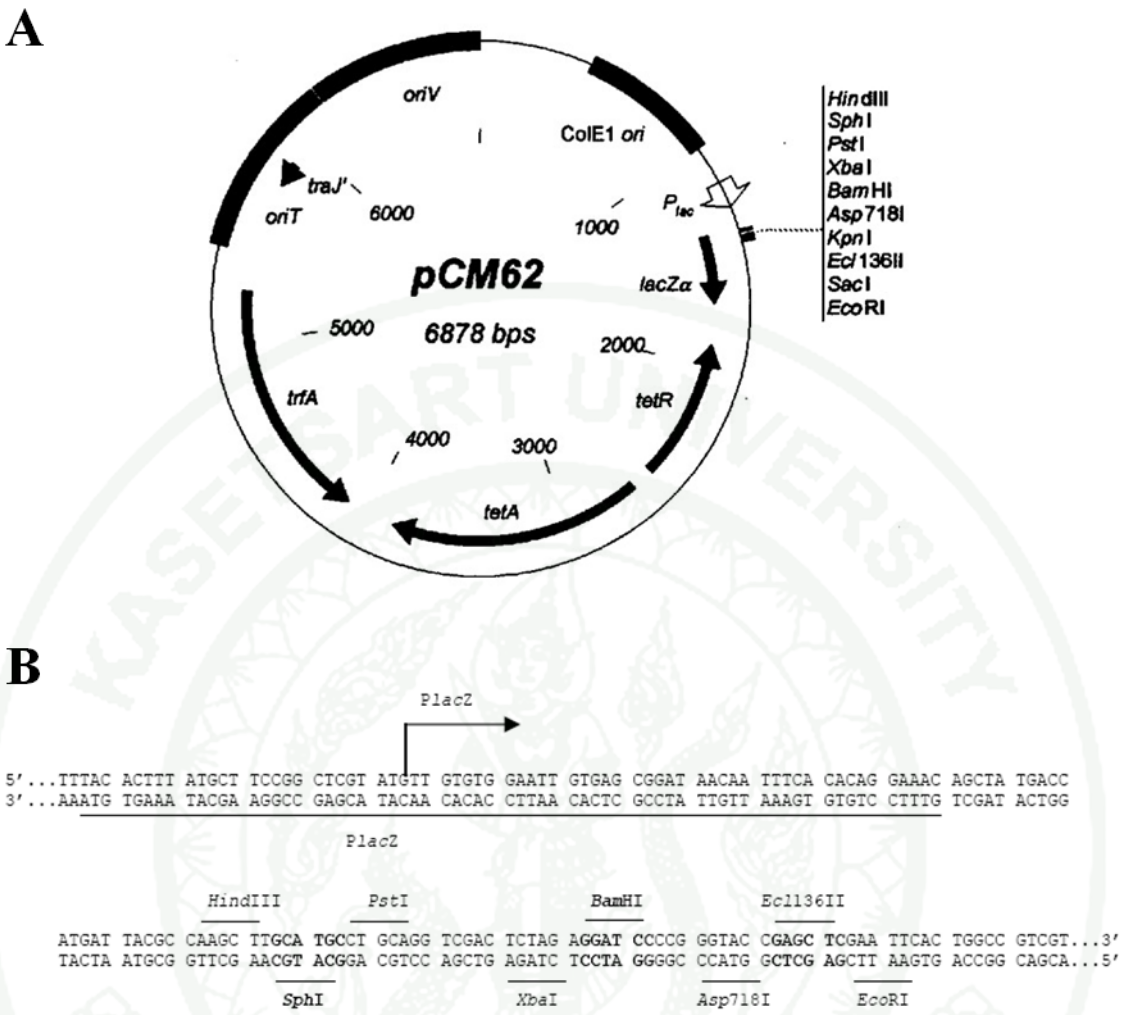


Figure 10 Plasmid maps depicting the relevant features (A) and multiple cloning sequence (B) of the versatile broad-host range vector pCM62.

Source: Marx and Lidstrom (2001)

3.4 Deletion analysis of *adhS* gene

In order to analyze the regulatory region of *adhS* gene, three constructions of the *adhS* gene were obtained by PCR amplification using oligonucleotide primers as follows: The three forward primers composed of the *Eco*RI restriction site (underlined); FEPadhS1: 5′-CCGGAATTCTCCCGCTTGTCGTTTC-3′, FEPadhS2: 5′-CCGGAATTCTTTTCGTGTTGTGGTG-3′ and FEPadhS3: 5′-CCGGAATTCTTG CGGAAAAACCCG-3′ and the reverse primer with the *Bam*HI restriction site (underlined), RBPadhS: 5′-GGCGGATCCCTTCTGTTGTTCTT-3′, were designed to cover the 532nd, 268th and 118th nucleotide upstream from the ATG start codon, respectively, to the 15th nucleotide downstream from TAA stop codon of the *adhS* gene. These three sets of primers, RBPadhS and FEPadhS1 or FEPadhS2 or FEPadhS3, produced PCR products of 0.75, 0.9 and 1.17 kb, respectively. Each of them was digested with *Eco*RI and *Bam*HI and introduced into the appropriate sites of pCM62 in an opposite direction from *Plac*, resulting in plasmid pCMadhS10/1, pCMadhS10/2 and pCMadhS10/3. The *adhS* derivative plasmids were separately transferred to *adhS* disruptant by conjugation and the desired conjugants were selected on a YPGD medium containing 4% ethanol, 25 µg/ml tetracycline, 50 µg/ml kanamycin and 0.5% $CaCO_3$.

3.5 Random mutagenesis of *adhS* gene

In order to analyze the essential amino acids of subunit III which involved in molecular coupling with subunit I, random mutagenesis based on error-prone PCR was performed by application of the GeneMorph® II Random Mutagenesis Kit (Stratagene, La Jolla, CA). Two oligonucleotide primers composed of certain restriction enzymes recognition sequences, FEPadhS2 and RBPadhS, were synthesized. The 2 kb *Eco*RI DNA fragment with the *adhS* gene was used as a DNA template for PCR amplification. To produce a low mutation rate (0-4.5 mutations/kb), low amount of target DNA (100 ng) was amplified for 20-25 cycles. The 50-µl PCR mixture contains 100 ng of target *adhS* gene (or 220 ng of 2 kb *Eco*RI DNA fragment), 1x Mutazyme® II reaction buffer, 200 µM dNTP, 0.25 µM primers and 2.5 U Mutazyme® II DNA polymerase. The PCR amplification was carried with a

temperature profile of 5 min at 95°C followed by 25 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 45 sec and a final extension at 72°C for 10 min. The obtained PCR products were analyzed by 0.8% agarose gel electrophoresis and a 0.9 kb PCR product was recovered by GeneJET™ GelExtraction Kit (Fermentas Inc., Maryland, USA) as described in Appendix C. The mutagenized *adhS* genes were digested with *Eco*RI and *Bam*HI and inserted into pCM62 and further conjugationally transferred into *adhS* gene disruptant. The conjugants were selected on the YPGD medium containing 4% ethanol, 25 µg/ml tetracycline, 50 µg/ml kanamycin and 0.5% $CaCO_3$. The plasmid profiles of those transconjugants were analyzed. The nucleotide sequences of the mutagenized *adhS* gene were determined. The desired conjugants harboring mutagenized *adhS* gene were analyzed for ethanol oxidizing abilities, PQQ-ADH activity and immunoblotting of subunit III.

3.6 Characterization of growth characteristics

3.6.1 Time course of growth and acetic acid production

A. pasteurianus SKU1108, CN6-2 and DPS strains were precultured in 5 ml of the potato medium and incubated at 30°C under vigorous shaking (200 rpm) and their growth was measured by Klett Summerson photometer until reached to log phase. Then, the 5% of seed culture was inoculated into 100 ml of YPGD medium containing 0 or 4% ethanol in a 500-ml Erlenmeyer flask. Cultivation was performed at 30°C on a rotary shaker at 200 rpm. The growth was periodically measured with a Klett Summerson photometer. The acidity of the culture medium was measured by titration with 0.8 N NaOH using phenolphthalein as the pH indicator and expressed as acetate concentration (Appendix C).

3.6.2 Analysis of ethanol oxidizing ability and acetic acid resistance

A. pasteurianus SKU1108, CN6-2 and DPS strains were cultured in 5 ml of the potato medium and incubated at 30°C under vigorous shaking (200 rpm). Overnight culture was measured the turbidity by spectrophotometer and adjusted to an initial absorbance of approximately 1.0 (OD 540 nm). Then, serial 10-fold dilution

was performed to dilute the culture at 10^{-1}, 10^{-2} and 10^{-3}. Three µl of each dilution was spot on the YPGD agar containing 4% ethanol and 0.5% $CaCO_3$ or 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 2.75% acetic acid. The cultures were incubated overnight at 30°C to determine the acetate-producing ability or for 7 days to determine the acetic acid resistance.

3.7. Preparation of crude enzyme solution

Cells of the wild-type and mutant strains were cultivated for 48 h in YPGD medium supplemented with or without ethanol. The cells were harvested by centrifugation at 9000 rpm for 10 min, and washed twice with ice-cold 50 mM potassium phosphate buffer (KPB) pH 7.5 (Appendix B), containing 1 mM ditriothreitol (DTT) and 1 mM $MgCl_2$. The washed cells were resuspended at about 1 g of wet cells per 4 ml in the same buffer and passed twice through a French pressure cell press (American Instruments, Silver Spring, MD., USA) at 16,000 psi. Cell debris was removed by centrifugation at 9000 rpm for 10 min; the supernatant was centrifuged at 40,000 rpm for 90 min to separate the soluble fraction from the membrane fraction. The supernatant was used directly as a soluble fraction, while the precipitate was homogenized with pre-chilled 10 mM KPB pH 6.0 (Appendix B) and used as a membrane fraction.

3.8 Heme staining of membrane and soluble fractions

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. Pre-stained molecular weight markers (low range, Bio-Rad Laboratories Inc., USA) were used for standard marker proteins. 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 (Appendix B) 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.

3.9 Immunoblotting analysis of ADH subunit III

Immunoblotting of ADH subunit III was modified from Burnette (1981). Preparation of antibody was performed by the method of Toyama *et al.* (1995). One hundred µg of the proteins samples were separated by 12.5% SDS-PAGE in a vertical electrophoresis system with a standard molecular size protein. The gel was immersed in the anode buffer II solution (Appendix B) and allowed it to equilibrate for 15 min. A piece of Immobilon^{TM}-P membrane (Millipore Co., Massachusetts, USA) was cut and wet in 100% methanol for 15 seconds. The membrane was then transferred to a container of Milli-Q water for 2 min and equilibrated for at least 5 min in the anode buffer II solution. The transfer stack for a semi-dry system was assembled as follows: two sheets of filter paper were wet in the anode buffer I solution (Appendix B) and placed in the center of the graphite anode electrode plate. A sheet of filter paper was wet in the anode buffer II and placed on top of the first two filter paper sheets followed by the membrane, the gel and three pieces of filter paper which was wet in the cathode buffer solution (Appendix B). The cathode electrode plate was then covered on top of the assembled transfer stack (Figure 11). The transferring was done at 2.5 mA/cm^2 for 30-45 min.

Once the electrotransfer complete, the membrane was completely dried by incubated at 37°C for 1 hour or placed on a lab bench at room temperature for 2 hours before continuing to rapid immunodetection procedure. In immunodetection, a specific antibody identifies the location of a membrane-bound antigen. Immunodetection on Immobilon^{TM}-P membrane does not require blocking or lengthy wash steps if the membrane was thoroughly dried after protein transfer; drying the membrane returns it to hydrophobic state. Antibodies can then bind specifically to the proteins on the membrane surface, but not to the membrane itself, eliminating the blocking step. After drying, the blot was incubated for 1 hour with an anti-ADH subunit III antibody from *A. aceti* diluted in blocking buffer (Appendix B) which contains 0.05% Tween$^{®}$-20 wetting agent and washed twice in PBS (Appendix B) for 10 seconds. Then, the blot was incubated for 30 min with a protein A-peroxidase conjugant diluted in blocking buffer containing 0.05% Tween$^{®}$-20 and washed twice for 10 sec in PBS. The protein band was visualized by the addition of the freshly prepared solution of 50 ml PBS

which contains 30 µl of hydrogen peroxide and 10 ml of cold methanol containing 30 mg of a color reagent (diaminobenzidine, DAB). The color was developed until the signal reached to the desired contrast. The reaction was stopped by washing the membrane with distilled water for several times.

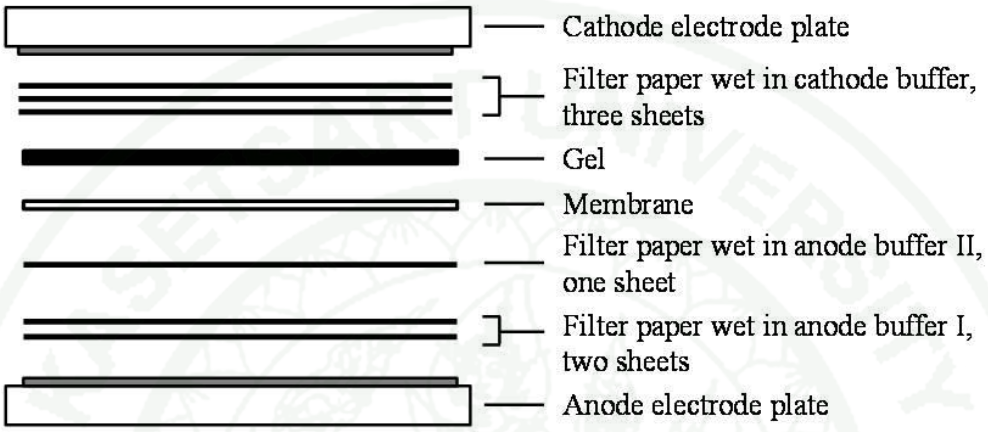


Figure 11 The transfer stack for a semi-dry system.

Source: Millipore Coorperation (2005)

3.10 Enzyme activity assays

All enzyme assays of the membrane and soluble fractions were performed at 25°C as follows: Membrane-bound PQQ-ADH (EC 1.1.99.8) activity was measured colormetrically with potassium ferricyanide according to the standard method described by Adachi *et al*. (1978a, 1978b) with slight modifications (Matsushita *et al*., 1995). NAD^+-ADH (E.C. 1.1.1.1) activity was measured according to the method described by Bernt and Gutmann (1974). One unit of enzyme activity was defined as the amount of enzyme catalysis of the oxidation of 1.0 µmol of substrate per minute under the conditions indicated. The procedures of enzymatic activity assay are described in Appendix C.

3.11 Other analytical procedures

SDS-PAGE was performed on a 12.5% slab gel in Tris–glycine running buffer, pH 8.5 (Laemmli, 1970). The samples were treated with 3% SDS and 50 mM DTT at 60°C for 30 min before application. Protein content was measured by a modified Lowry method with bovine serum albumin as the standard (Appendix C).

4. Cloning, Sequencing and Characterization of the NAD^+-*adh* Genes

4.1 Cloning and nucleotide sequencing of the NAD^+-*adh* genes

Molecular cloning of *adhI* and *adhII* genes encoding NAD^+-ADH I and ADH II, respectively, were performed by PCR cloning into pGEM®-T Easy Vector (Promega, Medison, WI, USA). The physical map of pGEM®-T Easy Vector is shown in Figure 12. Two specific primer sets were designed from nucleotide sequences of a draft *A. pasteurianus* SKU1108 genome sequence (unpublished data). FadhI (5'-GCG AATTCGTCAGCGTATTTTTAATGCC-3') and RadhI (5'-ATCTGCAGGTCTTCA CCTGCTCTTCG-3') were designed to cover the 1,001th nucleotide upstream from the ATG start codon to the 86th nucleotide downstream from the TGA stop codon of the *adhI* gene. FadhII (5- ATGAATTCCCATCCGTTGGTCAATGTGG-3') and RadhII (5'-ATCTGCAGGATCAAATCCTCACGTGTCG-3') were designed to cover the 999th nucleotide upstream from the ATG start codon to the 94th nucleotide downstream from the TAA stop codon of the *adhII* gene. PCR was performed in a 25 µl of reaction volume of illustra™ puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Buckinghamshire, UK) and the PCR conditions are shown in Table 6. The obtained 2.0 and 1.86 kb PCR products responsible to the expected sizes of *adhI* and *adhII*, respectively, were recovered from 0.8% agarose gel by the application of a MagExtractor-Plasmid-kit (Toyobo, Osaka, Japan) and introduced into a pGEM®-T Easy Vector for PCR cloning and determination of the nucleotide sequences.

The ligation mixture contains 3 µl of 100 ng purified DNA fragment, 1 µl of pGEM®-T Easy Vector (50 ng), 2x Rapid Ligation Buffer, T4 DNA ligase (5 unit)

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 and used to transform into *E. coli* DH5α by heat shock (Appendix C) and selected on LB agar containing 50 µg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and incubated at 37°C overnight. The recombinant clones were selected from white colonies formed on the plate. The recombinant clones were further used for recombinant plasmid isolation by using alkaline lysis method (Sambrook *et al.,* 1989, Appendix C) and MagExtractor-Plasmid-kit (Toyobo, Osaka, Japan). The resulting plasmid designated as pGEM-TadhI and pGEM-TadhII were confirmed by the PCR amplification. The nucleotide sequences were subsequently determined by applying the chain termination method using an ABI PRISM 310 genetic analyzer. The obtained nucleotide sequences were searched for an open reading frame (ORF) using Clone Manager (Scientific and Education Software, Cary, NC, USA) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf.html). Homology search and alignment analyses were performed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (www.ebi.ac.uk/clustalw/), respectively.

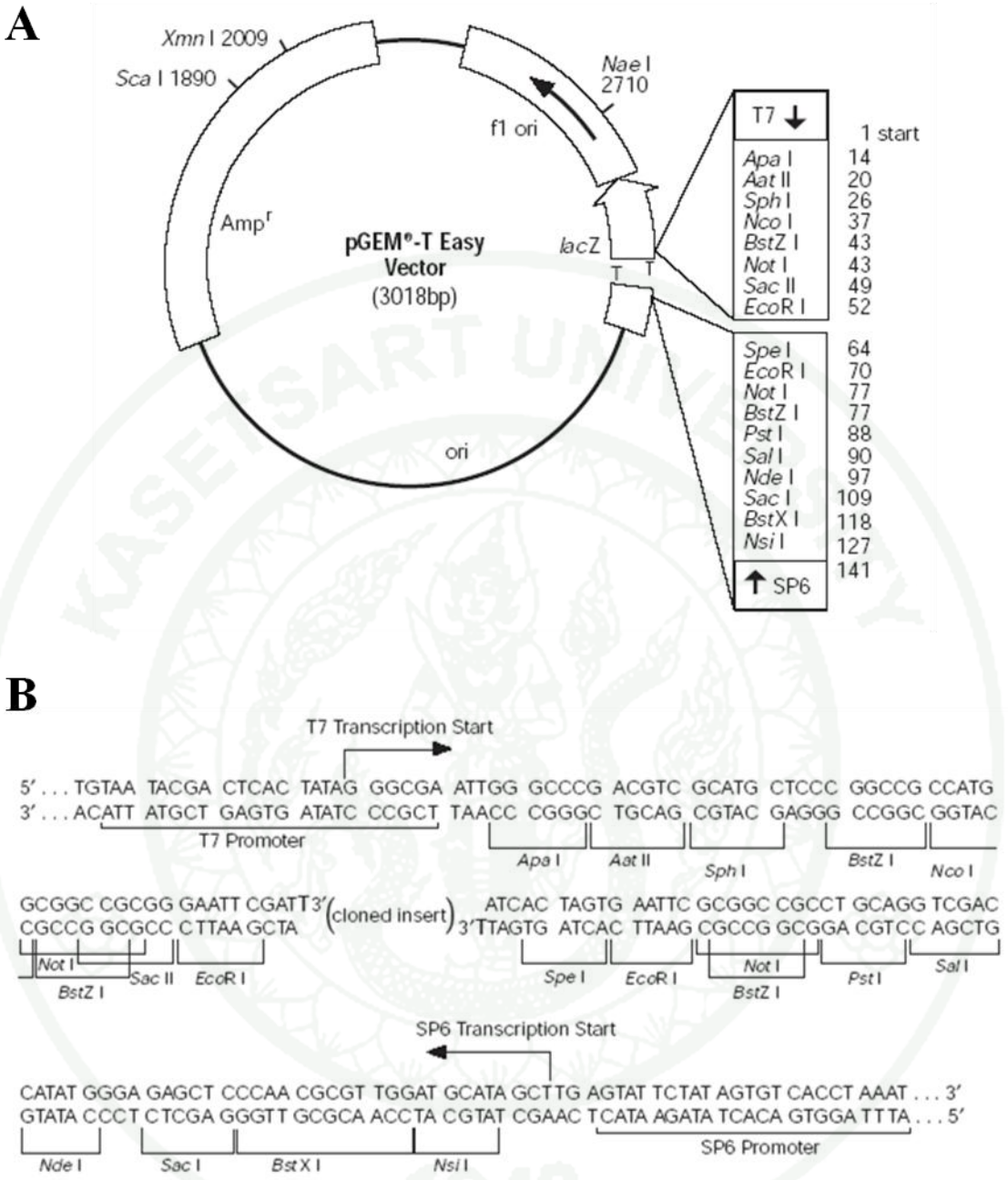


Figure 12 The physical map (A) and multiple cloning sequence of pGEM®-T Easy vector (B).

Source: Promega corporation (2010)

Table 6 PCR conditions

Primer	PCR condition					
	Temperature profile	**Denaturing**	**Annealing**	**Extension**	**Final extension**	**Cycles**
FadhI and RadhI	95°C, 5 min	94°C, 1 min	57°C, 1 min	72°C, 1 min	72°C, 10 min	30
FadhII and RadhII	95°C, 5 min	94°C, 1 min	61°C, 1 min	72°C, 1 min	72°C, 10 min	30

4.2 Construction of NAD^+-*adh* disruptants

The NAD^+-*adh* gene disruptants were constructed by replacement of the internal parts of the *adhI* and *adhII* genes of CN6-2 parental strain with tetracycline and kanamycin resistant cassettes by the homologous recombination strategy. The procedures are briefly described as follows: The plasmid pGEM-TadhI was first digested with *Hin*dIII and further inserted with a 1.9 kb *Hin*dIII digested pRP2012 which carried the Tc^r cassette (Reece and Phillips, 1995), resulting in plasmid pGEM-TadhI::Tc^r. In order to construct the *adhII* gene disrupted plasmid, the plasmid pGEM-TadhII was digested with *Hin*dIII and followed by end-blunting with Blunting High (Toyobo, Osaka, Japan). The 900 bp *Eco*RV DNA fragment which carried a non-polar Km^r cassette from plasmid pTKm (Yoshida *et al*., 2003) was inserted into the blunted pGEM-TadhII, which yielded a plasmid pGEM-TadhII::Km^r. The subsequent plasmids pGEM-TadhI::Tc^r and pGEM-TadhII::Km^r were separately electroporated into CN6-2. The NAD^+-*adh* gene disruptants were selected on a YPGD medium composed of 25 µg/ml tetracycline or 50 µg/ml kanamycin. Tetracycline or kanamycin resistant colonies were collected and confirmed with PCR amplification. Moreover, an *adhI-adhII* double disruptant was also constructed by insertion of the Km^r cassette into the internal part of *adhII* gene on the chromosome of *adhI* gene disruptant. The double disruptant was selected on a YPGD medium containing 25 µg/ml tetracycline and 50 µg/ml kanamycin and confirmed by PCR amplification.

4.3 Determination of growth characteristics

A. pasteurianus SKU1108 PQQ-ADH deficient mutant, CN6-2 and its NAD^+-*adh* gene disrupted strains were precultured in 5 ml of the potato medium and incubated at 30°C under vigorous agitation (200 rpm) for 18 h. The turbidity of the cells was measured by a spectrophotometer at a wave length of 540 nm. The cell cultures were inoculated into 100 ml of YPG or YPGD medium (Appendix A) containing 0 or 4% ethanol in a 500-ml Erlenmeyer flask at an initial absorbance of approximately 0.05. Cultivation was performed at 30°C on a rotary shaker (200 rpm). The growth was periodically measured and the acidity of the culture medium was assessed by titration with 0.8 N NaOH using phenolphthalein as the pH indicator and expressed as acetate concentration as previously described by Saeki *et al*. (1997a; Appendix C).

4.4 Induction Analysis of NAD^+-*adh* genes by ethanol

A promoter probe vector, pGSA, with the promoterless *lacZ* gene (Soemphol, 2008) was used for the construction of the transcriptional *adh-lacZ* gene fusion. This plasmid was constructed by the combination of pGG9 (Petruschka *et al*., 2002) at the *Hind*III site with a 4-kb DNA fragment from pSA19, a shuttle vector for acetic acid bacteria (Tsuchida and Yoshinaga, 1997). Two DNA fragments with a 500 bp upstream region and a part of structural NAD^+-*adh* genes, *adhI* and *adhII*, were amplified with PCR amplification. FPadhI-P (5´-GCCTGCAGGCACAACAGAAA CTTTACG-3´) and RPadhI-B (5´-GCGGATCCTTCTTCAATGGTCAGTGG-3´) were designated to cover the 513rd nucleotide upstream to the 63rd nucleotide downstream from the ATG start codon of the *adhI* gene. FPadhII-P (5´-GCCTGCAG ATATGGTGATTGCACAGG-3´) and RPadhII-B (5´-GAGGATCCCTTGCCAATG CCATTAGC-3´) were designated to cover the 520th nucleotide upstream to the 60th nucleotide downstream from the ATG start codon of the *adhII* gene. The *Pst*I and *Bam*HI restriction sites in the forward and reverse primers, respectively, are underlined. The 0.58 kb PCR products were digested with *Pst*I and *Bam*HI and further ligated into the *Pst*I-*Bam*HI digested pGSA. The resulting plasmids, pGSAPadhI and

pGSAPadhII, which comprised pGSA were separately introduced into *E. coli* DH5α by heat shock and selected on a LB medium composed of 12.5 µg/ml gentamicin and 20 µg/ml X-gal. The recombinant plasmids were confirmed with PCR amplification and nucleotide sequencing. *E. coli* DH5α harboring the transcriptional *adh-lacZ* fusion plasmids were precultured in 5 ml of LB medium for 18 h and measured for turbidity with a spectrophotometer at a wave length of 600 nm. The cell cultures were inoculated into 100 ml of LB medium (Appendix A) containing 0, 2 or 4% ethanol in a 500-ml Erlenmeyer flask at an initial absorbance of approximately 0.05. Cultivation was performed at 37°C on a rotary shaker at 200 rpm for 48 h. The growth was periodically measured with a spectrophotometer. Five ml of cell cultures were harvested and centrifuged at 12,000 rpm for 1 min using a refrigerated centrifuge MR-150 (Tomy Seiko Co., Ltd., Tokyo, Japan). The cell pellets were washed with deionized water and stored at -20°C for further determination of β-galactosidase activity according to the standard method described by Miller *et al*. (1972) with slight modifications (Appendix C).

4.5 Deletion Analysis of NAD^+-*adh* genes

In order to analyze the regulatory region of NAD^+-*adh* genes, eight constructions of the NAD^+-*adh* genes were obtained by PCR amplification with specific oligonucleotide primers. The forward and reverse primers composed of *Pst*I and *Bam*HI restriction sites, respectively. Nucleotide sequences and position in the *adhI* or *adhII* genes of those primers are described in Table 7. The obtained PCR products were digested with *Pst*I and *Bam*HI and separately introduced into the appropriate sites of pGSA. The resulting plasmids are described in Table 8. Each of the *adh-lacZ* fusion plasmid was separately transferred to *E. coli* DH5α by heat shock and selected on a LB medium containing 12.5 µg/ml gentamicin and 20 µg/ml X-gal. The recombinant plasmids were confirmed with PCR amplification and nucleotide sequencing. Schematic representation of the resulting plasmids was described in Figure 12. *E. coli* DH5α harboring the transcriptional *adh-lacZ* plasmids inclusive the promoter probe vector pGSA were cultured in 100 ml of a LB medium in a 500-ml Erlenmeyer flask and cultivated at 37°C on a rotary shaker at 200 rpm for 18 h. Five

ml of cell cultures were harvested; the cell pellets were collected and stored at -20°C for further β-galactosidase activity assay (Appendix C).

Table 7 Nucleotide sequences and position of oligonucleotide primers used in this study

Primer designation	Sequence (5´→3´)[a]	Position
adhI		
FPadhI-1-P	GCCTGCAGATGATAGTTTCGCTACAACG	The 356th nucleotide upstream from the ATG start codon
FPadhI-2-P	GCCTGCAGCAGGCGTTGGATATTCTG	The 203rd nucleotide upstream from the ATG start codon
FPadhI-3-P	GCCTGCAGGAAGCTGTCAAAAGTCTTGG	The 105th nucleotide upstream from the ATG start codon
FPadhI-4-P	GCCTGCAGTCCTGAGCATGACAAGG	The 81st nucleotide upstream from the ATG start codon
RPadhI-B	GCGGATCCTTCTTCAATGGTCAGTGG	The 63rd nucleotide downstream from the TGA start
adhII		
FPadhII-1-P	GCCTGCAGCACAGTTACCCTTAATGG	The 348th nucleotide upstream from the ATG start codon
FPadhII-2-P	GCCTGCAGAATTGTTGGATACGG	The 228th nucleotide upstream from the ATG start codon
FPadhII-3-P	GCCTGCAGTTGCAAGAATTGATGCAGG	The 92nd nucleotide upstream from the ATG start codon
FPadhII-4-P	GCCTGCAGGTCATGGTTGGGATATCTG	The 74th nucleotide upstream from the ATG start codon
RPadhII-B	GAGGATCCCTTGCCAATGCCATTAGC	The 60th nucleotide downstream from the TAA start

[a] *Pst*I and *Bam*HI restriction sites are underlined

Table 8 Plasmids used in this study

Plasmid	**Relevant characteristics**[a]	**Reference**
pGSA	Gm^r, promoter probe vector carrying the promoterless *lacZ* gene	Soemphol (2008)
pGSAPadhI-1	Gm^r, carrying a 356 bp promoter region of *adhI* gene	This study
pGSAPadhI-2	Gm^r, carrying a 203 bp promoter region of *adhI* gene	This study
pGSAPadhI-3	Gm^r, carrying a 105 bp promoter region of *adhI* gene	This study
pGSAPadhI-4	Gm^r, carrying a 81 bp promoter region of *adhI* gene	This study
pGSAPadhII-1	Gm^r, carrying a 348 bp promoter region of *adhII* gene	This study
pGSAPadhII-2	Gm^r, carrying a 228 bp promoter region of *adhII* gene	This study
pGSAPadhII-3	Gm^r, carrying a 92 bp promoter region of *adhII* gene	This study
pGSAPadhII-4	Gm^r, carrying a 74 bp promoter region of *adhII* gene	This study

[a] The indicates length excluded some part of structural gene, *adhI* (63 bp) and *adhII* (60 bp)

RESULTS AND DISCUSSION

1. Functional Analysis of Alcohol Dehydrogenase Subunit III

1. 1 Disruption of the *adhS* gene

The genomic *adhS* gene was disrupted by homologous recombination using a kanamycin resistant cassette in order to examine the function of subunit III on the PQQ-ADH activity. One kanamycin resistant colony designated as DPS, was isolated and confirmed by PCR amplification and Southern hybridization analysis (Figure 13) compared with the wild-type strain. For PCR amplification using two specific primers of *adhS* gene, FadhS and RadhS, the 1.8 and 3.6 kb PCR products were obtained when chromosomal DNA of DPS strain was used as a template compared with the 618 bp of that from SKU1108. The result from Southern hybridization using the 618 bp *adhS* gene DNA probe showed that the positive hybridization signals were detected with 2.0 and 5.1 kb from the *Eco*RI-digested chromosome from *A. pasteurianus* SKU1108 and DPS, respectively. In addition, 1.8 and 3.6 kb positive signals were detected from PCR products of DPS strain compared with the positive signal of the 618 bp *adhS* gene of wild type. In case of the 1.2 kb Km^r cassette was used as DNA probe, the same positive signal size, 5.1 kb from digested chromosome and 1.8 and 3.6 kb from PCR amplification, were detected. In contrast, there is no positive signal of wild type when used this probe. These results suggested that the *adhS* gene of DPS strain was inactivated with two copies of *adhS*::Km^r DNA fragment. The schematic representation of the inactivated *adhS* gene of DPS strain by the 1.2 bp Km^r cassette is shown in Figure 14. Acetate-producing ability of wild type SKU1108 and DPS were compared on YPGD agar containing 4% ethanol and the result showed that DPS was completely lost ethanol oxidizing ability (Figure 15). It has been suggests that the smallest subunit is essential for the PQQ-ADH activity similar to the previous observations of the disruption of the corresponding gene in *A. aceti* (Kondo *et al*., 1995) and the inclusion of the insertion sequence element, IS*1452*, in the *adhS* gene of *A. pasteurianus* (Kondo and Horinouchi, 1997b), which were found to be associated with the inactivation of the alcohol dehydrogenase.

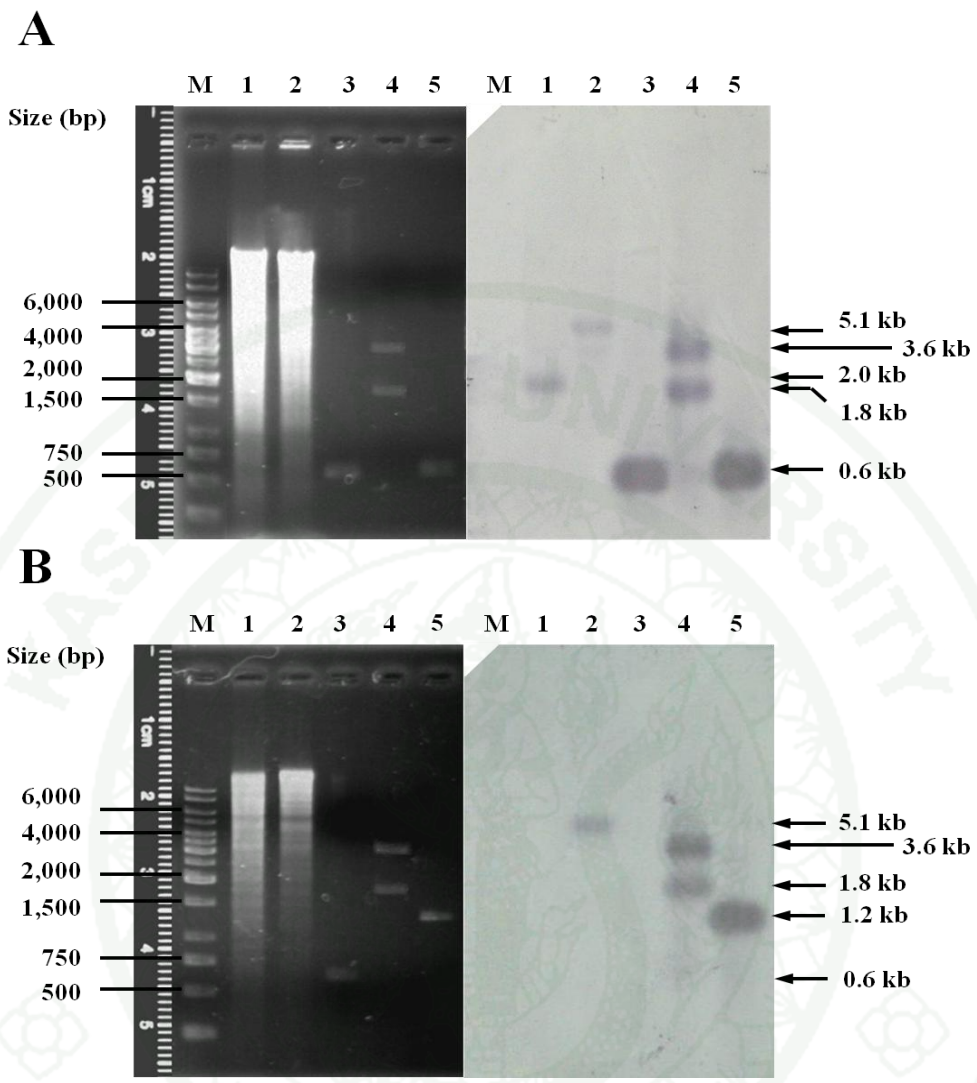


Figure 13 Agarose gel electrophoresis (left) and Southern hybridization pattern (right) of the *Eco*RI-digested genomic DNA and *adhS* gene PCR products form *A. pasteurianus* SKU1108 and DPS strains. The 618 bp *adhS* gene and the 1.2 kb Km^r cassette were used as DNA probe (A and B, respectively). Lane M = 1 kb DNA ladder, 1 = *Eco*RI-digested genomic DNA of SKU1108, 2 = *Eco*RI-digested genomic DNA of DPS strain, 3 and 4 = PCR products using chromosome of SKU1108 and DPS as templates, respectively, 5 = the 618 bp *adhS* probe or the 1.2 kb Km^r probe. For PCR amplification, FadhS and RadhS primers were used.

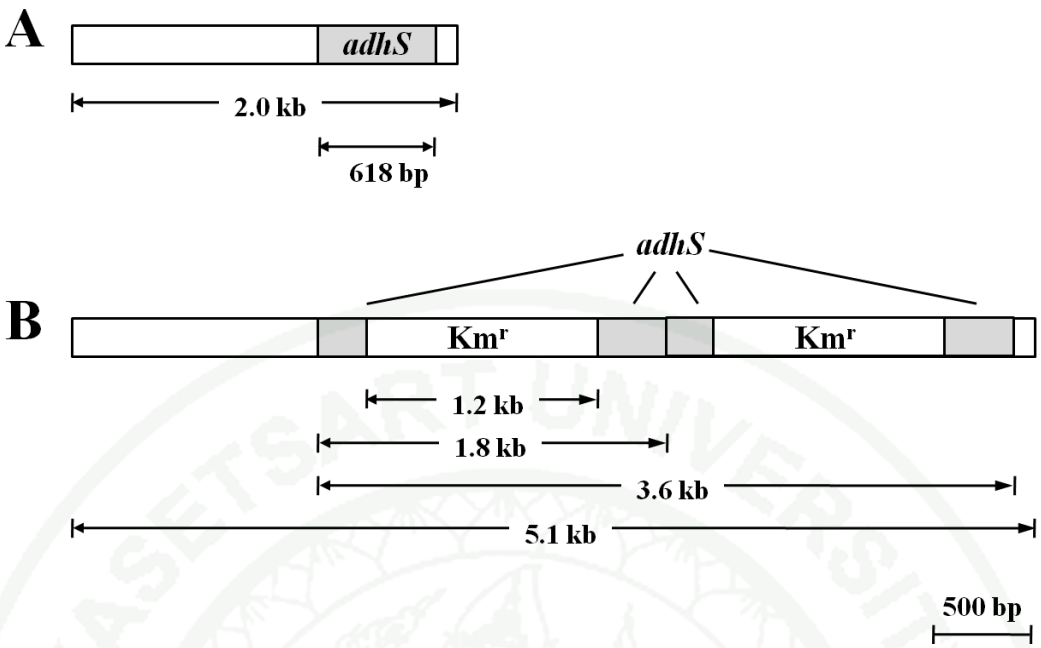


Figure 14 Schematic representation of the inactivated *adhS* gene of DPS strain by the 1.2 kb Km^r cassette. A 2.0 kb *Eco*RI DNA fragment carrying *adhS* gene of wild type SKU1108 (A) and a Km^r cassette inserted *adhS* gene of DPS strain (B) were shown. Distance of each fragment was indicated.

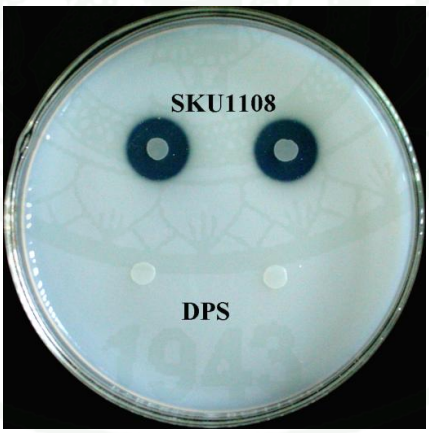


Figure 15 Comparison of ethanol oxidizing ability between *A. pasteurianus* SKU1108 and *adhS* gene disruptant (DPS). These strains were spot-inoculated on YPGD medium containing 0.5% $CaCO_3$ and 4% ethanol and incubated for 24 h at 30°C. The clear zone surrounding bacterial colony indicated a hydrolysis of $CaCO_3$ to H_2CO_3 by acetic acid which is a product obtained from ethanol oxidation.

1.2 Restoration of the *adhS* gene

Three derivative plasmids were constructed by insertion of the 2 kb *Eco*RI DNA fragment carrying the *adhS* gene into a broad host range plasmid, pCM62. The resulting plasmids were designated as pCMadhS10, pCMadhS11 and pCMadhS14. One copy of the 2 kb *Eco*RI fragment was observed in pCMadhS10 and pCMadhS14, while two copies of that were detected in pCMadhS11. The *adhS* gene of pCMadhS10 was inserted in an opposite orientation from *Plac*, in contrast, the *adhS* gene of pCMadhS11 and pCMadhS14 were inserted in the same orientation with *Plac*. Agarose gel electrophoresis of the *adhS* complemented plasmids digested with *Pst*I restriction enzyme and physical map of these plasmids are shown in Figure 16. Each plasmid as well as pCM62 was individually transferred conjugationally into the *adhS* disruptant. The conjugants were designated as DPS-62, DPS-10, DPS-11 and DPS-14 and plasmid profiles of these strains were analyzed (Figure 17). The ethanol oxidizing ability of *A. pasteurianus* SKU1108, DPS and those transconjugants grown on a YPGD medium supplemented with 4% ethanol and 0.5% $CaCO_3$ were compared. All of the conjugants with *adhS* gene complementation produced a clear zone surrounding their colonies (Figure 18). Among those *adhS* complemented strains, DPS-10 was selected for further studies because it possessed its own transcriptional signal for gene expression.

The membrane and soluble fractions were prepared from *A. pasteurianus* SKU1108, DPS harboring pCM62 (DPS-62) and *adhS* complemented strain (DPS-10) grown in a YPGD medium containing the appropriate antibiotics and 4% ethanol at 30°C for 48 h. Heme staining and immunoblot analysis with anti-ADH subunit III from *A. aceti* of both membrane and soluble fractions are shown in Figure 19A and 19B, respectively. All of the three subunits of PQQ-ADH were found in the membrane and soluble fractions of the wild type and DPS-10 (*adhS* complemented strain), whereas no band corresponding to subunit III was detected in both fractions of the DPS-62 strain. The pale band correlative to subunit II detected in soluble fraction may be a result of the leakage of membrane-bound proteins or a nascent protein that yet has to be secreted to the periplasmic space and persisted in the cytoplasm. A pale band of subunit I in soluble fraction was discerned and no bands in both fractions of

DPS-62 strain grown in a YPGD medium supplemented either without and with 4% ethanol, respectively, was distinguished. These results imply that the *adhS* defect caused the instability of subunit I and hence might be easily degraded, while no effect on subunit II was detected. The result is a clear indication that the DPS strain is deficient in the production of subunit III. The PQQ-ADH activities of those strains grown at the same conditions as mentioned above were compared (Figure 19C). It was discovered that no PQQ-ADH activity was detected in the membrane fraction prepared from DPS strain grown in any medium. The complete loss in PQQ-ADH activity of DPS indicated that subunit III was essential for formation of the active ADH complex. In contrast, the PQQ-ADH activity in the membrane fraction was dramatically increased about 3-4 times when the cells of *A. pasteurianus* SKU1108 and DPS-10 were grown in the medium of 4% ethanol content. In addition, a dark band of subunit III was detected in soluble fractions of those strains when the cells were grown in the medium without ethanol supplementation. This result corresponded well with the previous report of Takemura *et al*. (1993) showing that PQQ-ADH is produced as an inactive form in the absence of ethanol and the activity was activated by ethanol.

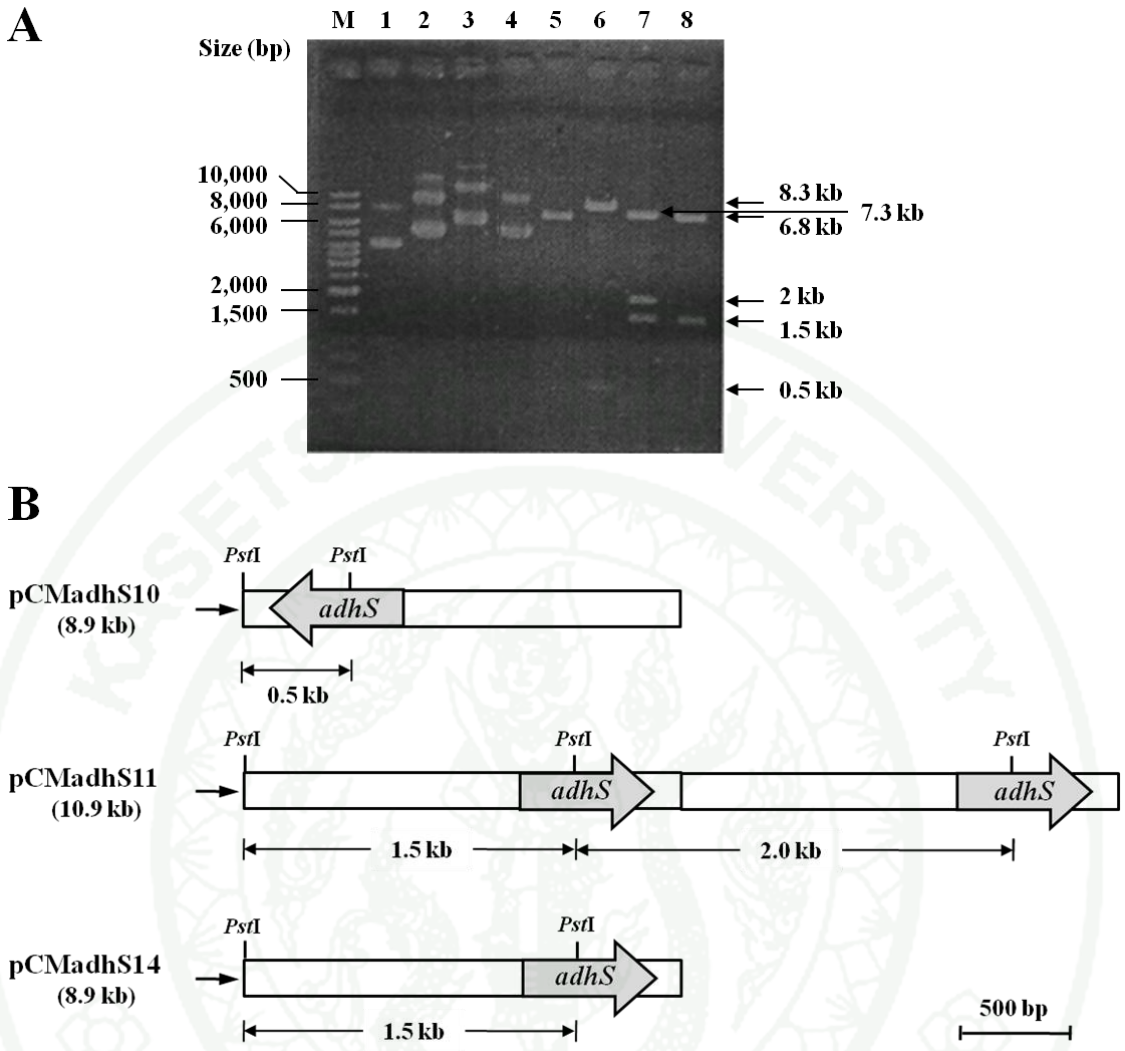


Figure 16 Agarose gel electrophoresis and structure of the *adhS* complemented plasmids. A, Lane M = 1 kb DNA ladder, 1-4 = uncut plasmid, 5-8 = *Pst*I-digested plasmid; 1 and 4 = pCM62, 2 and 5 = pCMadhS10, 3 and 6 = pCMadhS11, 4 and 7 = pCMadhS14. B, the pCMadhS10 and pCMadhS14 contain one copy of a 2 kb *Eco*RI fragment carrying *adhS* gene in pCM62, while the pCMadhS11 holds two copies of that fragment. Bold arrow and light arrow indicated the direction of *adhS* gene and *Plac* of plasmid, respectively.

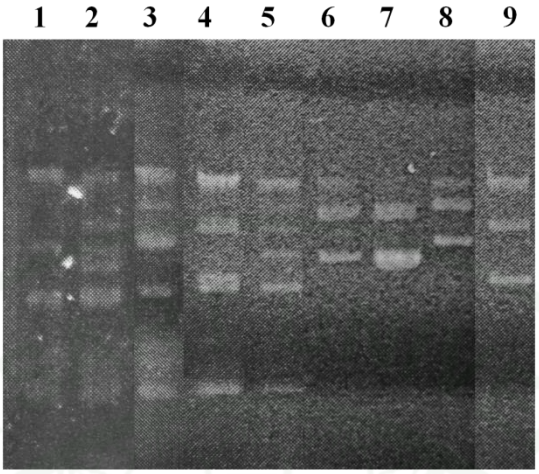


Figure 17 Plasmid profiles of transconjugants harboring a broad host range plasmid pCM62 and *adhS* complemented plasmids. Lane 1 = *adhS* gene disruptant (DPS), 2-4 = *adhS* complemented strains; DPS-10, DPS-11 and DPS-14, respectively, 5 = DPS carrying pCM62 (DPS-62), 6 = pCMadhS10, 7 = pCMadhS14, 8 = pCMadhS11, 9 = pCM62.

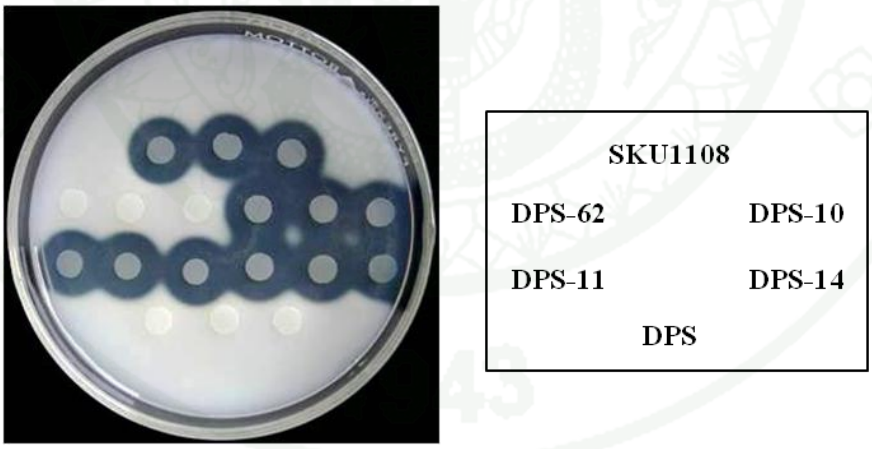


Figure 18 Comparison of ethanol oxidizing ability between *A. pasteurianus* SKU1108, *adhS* gene disruptant (DPS), DPS harboring pCM62 (DPS-62) and *adhS* complemented strain (DPS-10, DPS-11 and DPS-14). These strains were spot-inoculated on YPGD medium containing 0.5% $CaCO_3$ and 4% ethanol and incubated for 24 h at 30°C.

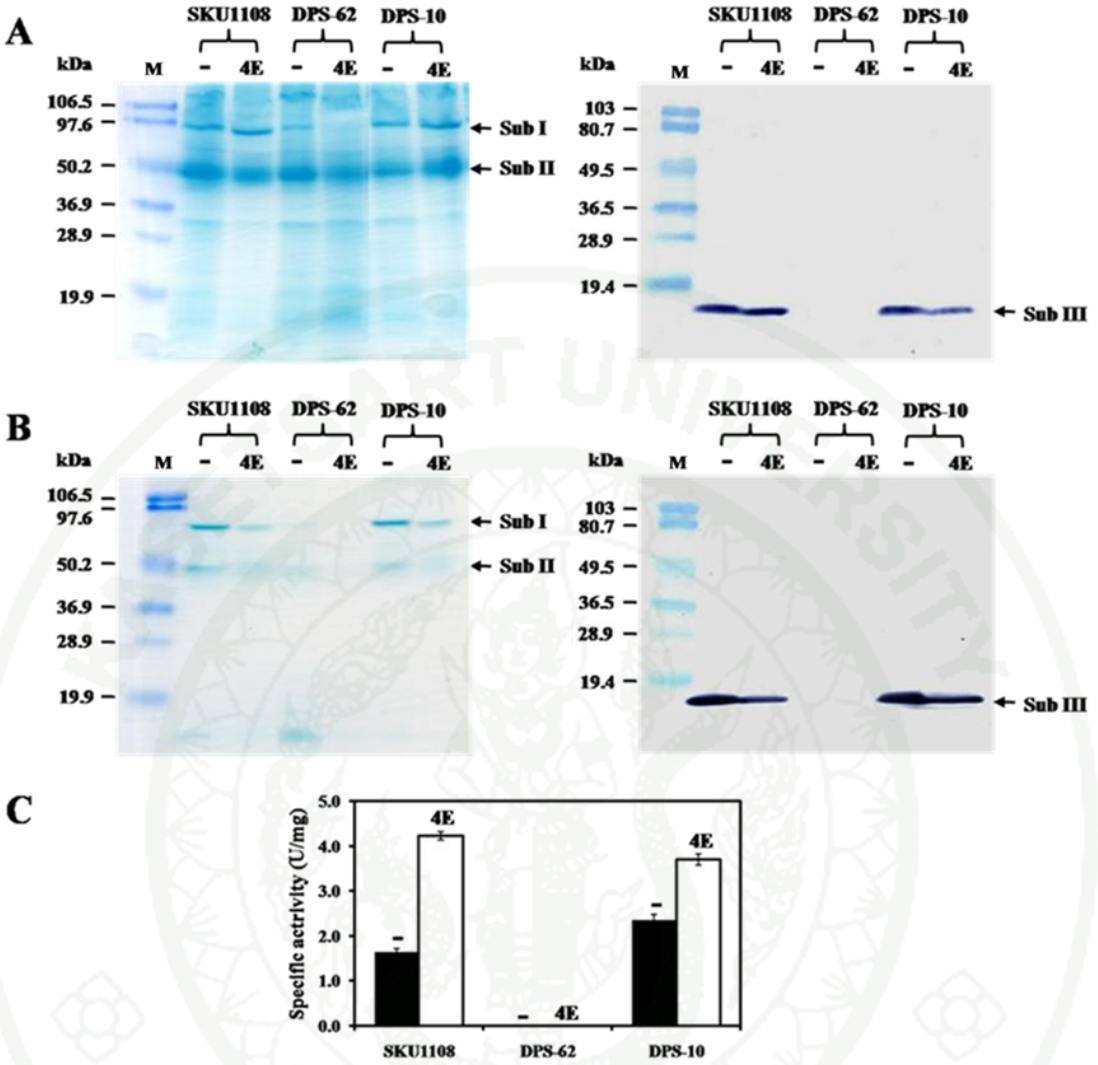


Figure 19 Heme staining (left) and immunoblot analysis (right) of PQQ-ADH with the membrane (A) and soluble fractions (B) and PQQ-ADH activity (C) of *A. pasteurianus* SKU1108, *adhS* gene disruptant (DPS-62) and *adhS* complemented strain (DPS-10). These strains were grown in YPGD medium containing 0% (black) or 4% ethanol (white). The antisera used were anti-ADH subunit III from *A. aceti*.

1.3 Effect of PQQ-ADH defect on growth, acetic acid production and acetic acid resistance

Time course of growth and acidity in culture broth of the cells grown in the YPGD medium supplemented with 4% ethanol and/or 1% acetic acid were compared with *A. pasteurianus* SKU1108 and its derivative mutants, CN6-2 and DPS (Figure 20). In the medium of 4% ethanol content, SKU1108 rapidly oxidized ethanol without any lag time and accumulated nearly 4% acetic acid in the culture medium. Surprisingly, the DPS strain grew well, even better than wild-type strain SKU1108, in the medium containing 4% ethanol even though it completely lost the ethanol oxidizing ability. This phenomenon was also observed in the CN6-2 strain, with the inactivated *adhAB* gene cluster (Chinnawirotpisan *et al*., 2003b). It has been suggested that the addition of ethanol to the culture medium significantly enhances its growth, concomitant with ethanol consumption instead of ethanol oxidation. In addition, growth and acetic acid production of these strains grown in a medium containing 1% acetic acid or 1% acetic acid plus 4% ethanol indicates that most strains assimilated acetic acid *via* the TCA or glyoxylate cycle. These results implicate that one of the acetic acid resistant mechanism is acetate assimilation similar to those previously reported on enhanced expression of aconitase (an enzyme in TCA cycle) raise acetic acid resistance in *A. aceti* (Nakano *et al*., 2004). It was further observed that the acetate assimilation is inhibited or delayed by ethanol which was clearly detected in a YPGD medium of ethanol and acetic acid contents.

In addition, the toleration to acetic acid of *A. pasteurianus* SKU1108, NTG mutant CN6-2 and DPS strains were compared on YPGD agar supplemented with 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0% acetic acid grown at 30°C for 1-7 d. As shown in Figure 21, the wild type SKU1108 exhibited the highest resistance to acetic acid (up to 3%) among those strains. DPS could able to tolerate up to 2.0% acetic acid concentration, whereas CN6-2 could able to grow only at 1.0% acetic acid. The result suggested that a PQQ-ADH defect not only affected on the ethanol oxidizing ability but also influenced in the acetic acid resistance. It corresponded well with the observation in the spontaneous mutants of *A. aceti* (Okumura *et al*., 1985) and *A. pasteurianus* (Takemura *et al*., 1991). They reported that a reduction of growth in the

presence of acetic acid was associated with the loss of membrane-bound ADH. However, not much alteration of acetic acid resistance was observed in the *adhS* gene disruptant of *A. pasteurianus* SKU1108.

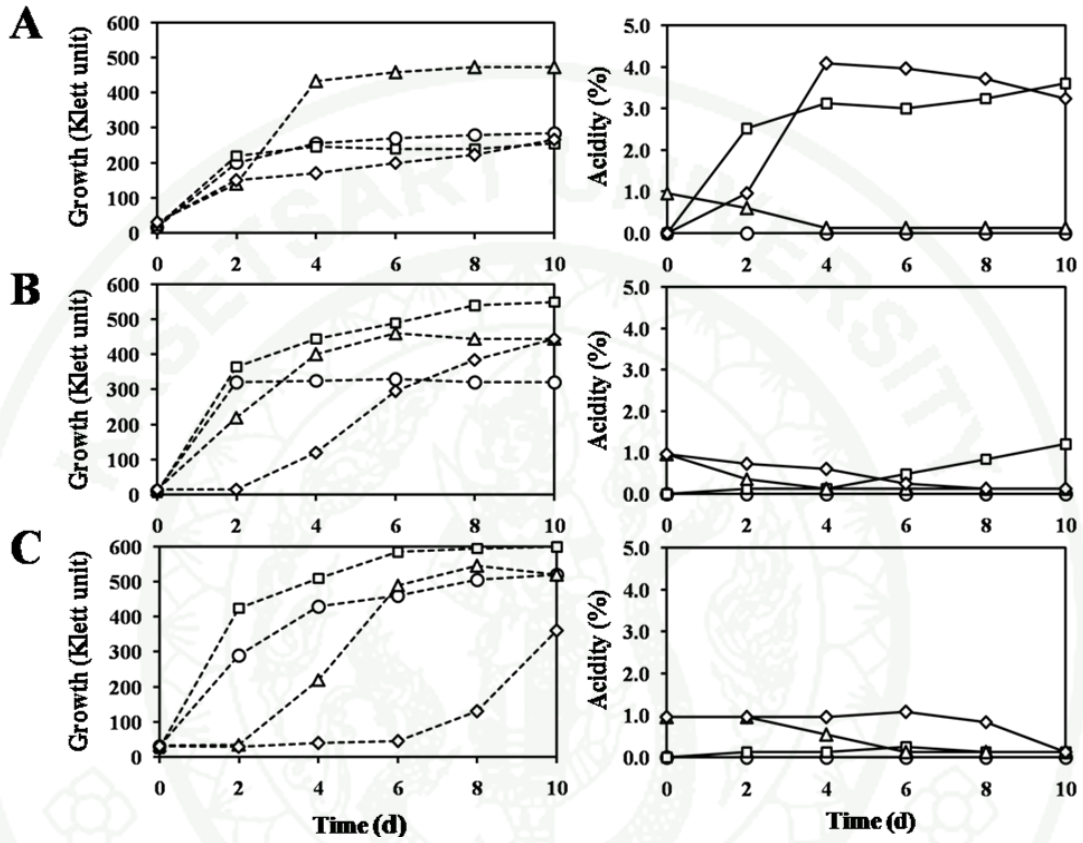


Figure 20 Time course of growth (left) and acidity in culture broth (right) of *A. pasteurianus* SKU1108 (A), *adhS* gene disruptant DPS (B) and NTG mutant CN6-2 (C). These strains were cultured in 100 ml of (○) YPGD medium supplemented with (□) 4% ethanol or (Δ) 1% acetic acid or (◇) 1% acetic acid and 4% ethanol. Bacterial growth was measured on the basis of turbidity (Klett unit). Acetic acid concentration was titrated under the standard conditions as described in Materials and Methods.

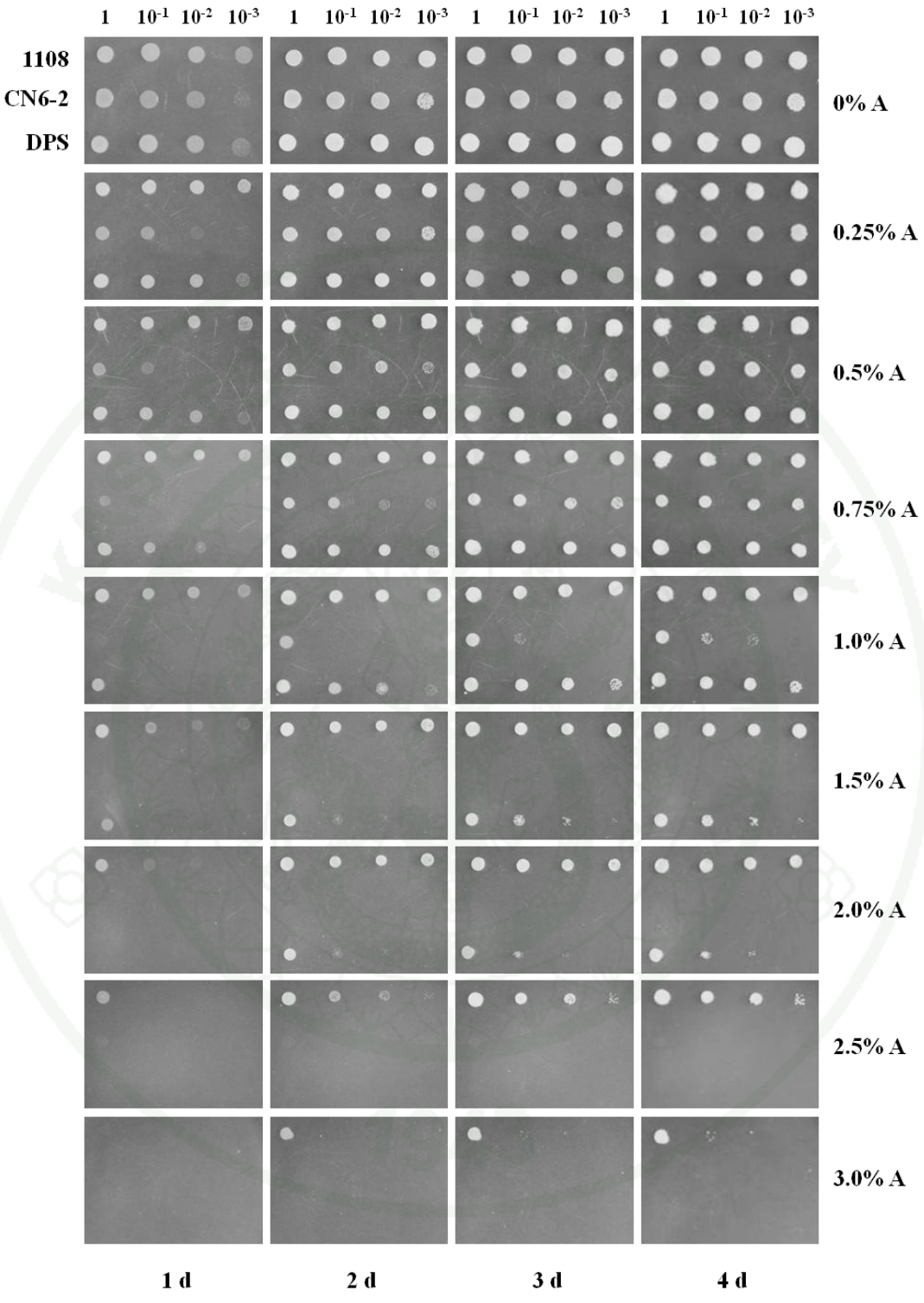


Figure 21 Comparison of acetic acid resistance capacity of the PQQ-ADH deficient mutants. *A. pasteurianus* SKU1108, NTG mutant CN6-2 and DPS were spot-inoculated on YPGD agar supplemented with various acetic acid concentrations as indicated.

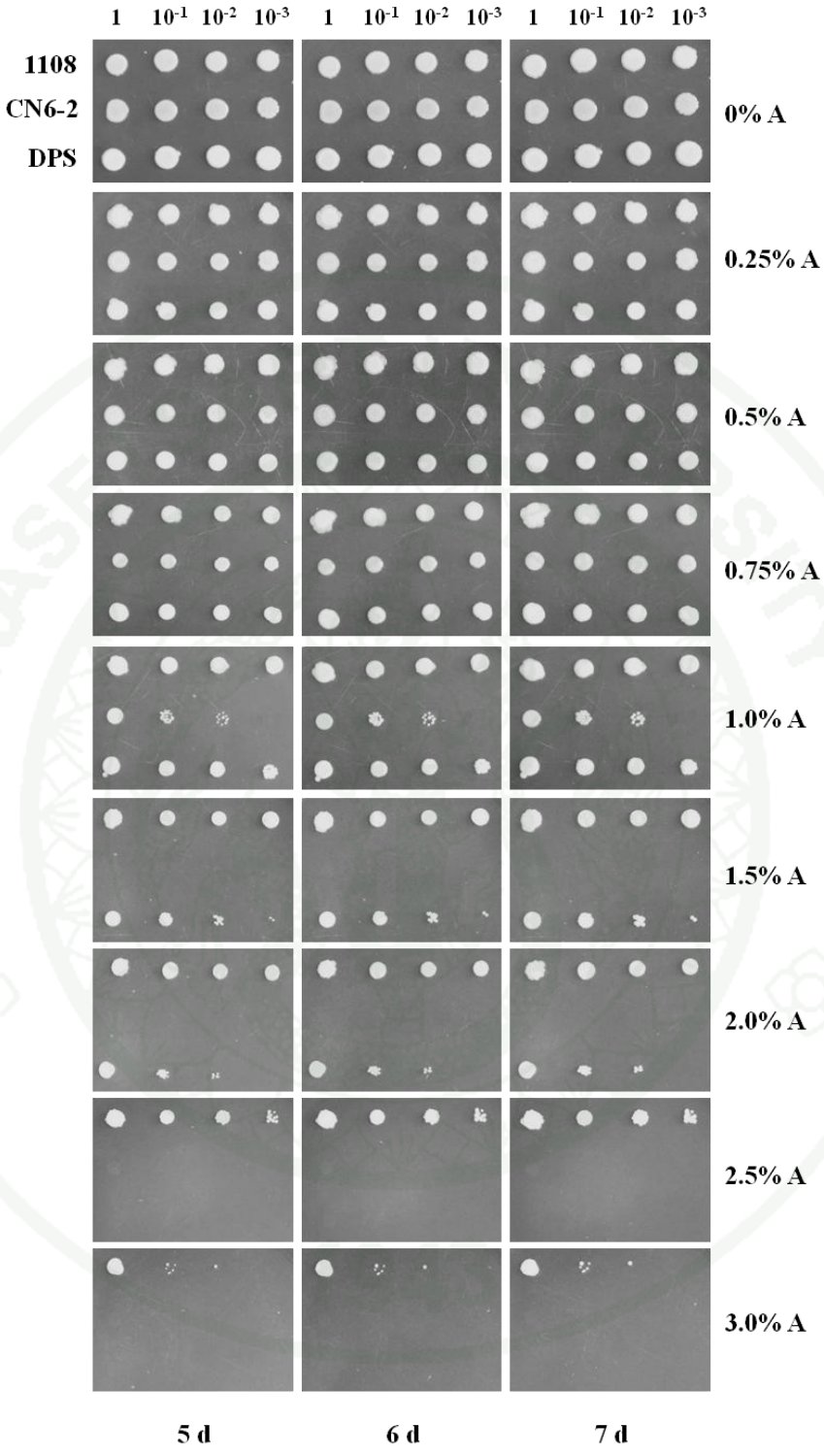


Figure 21 (Continued)

1.4 Comparison of enzyme activity involved in ethanol oxidation

To understand the defect of PQQ-ADH activity in the mutant strains, the activities of the enzymes involved in ethanol oxidation were assayed in both membrane and soluble fractions of the cells grown in the YPGD medium supplemented either with or without ethanol (Figure 22). It was established that the PQQ-ADH activity was remarkably induced by the addition of ethanol to the culture medium in SKU1108 and was completely lost in DPS and CN6-2 mutant strains. On the other hand, NAD^+-ADH activities in those mutants including DPS strain were increased when they were grown in media containing ethanol. This suggests that a defect in the PQQ-ADH activity at the *adhS* gene may change the global metabolic pathway from ethanol oxidation to ethanol assimilation; this is concurrent with our previous report in CN6-2 (Chinnawirotpisan *et al*., 2003b).

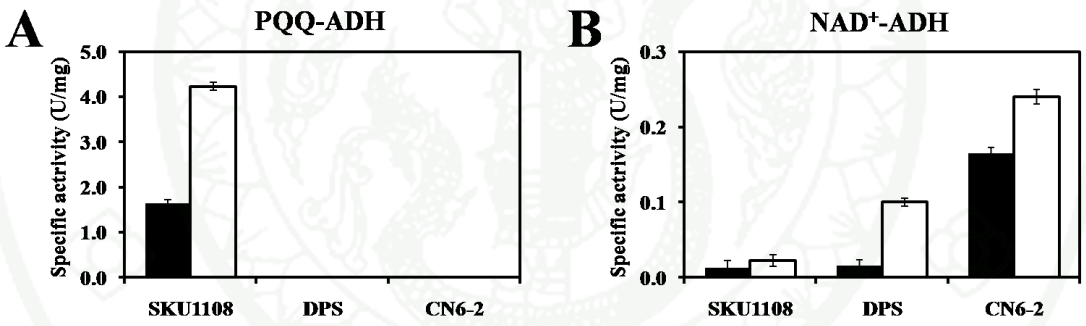


Figure 22 Comparison of PQQ-ADH activity in membrane fractions (A) and NAD^+-ADH activity in soluble fractions (B). *A. pasteurianus* SKU1108, NTG mutant CN6-2, and *adhS* gene disruptant DPS were cultured in YPGD medium containing 0% (black) or 4% (white) ethanol. Enzymatic analysis was described in Materials and Methods.

1.5 Deletion analysis of the regulatory region of *adhS* gene

In order to analyze the regulatory region of *adhS* gene expression, three derivatives of the *adhS* gene were obtained by PCR amplification using three sets of oligonucleotide primers. The three forward and reverse primers were designed to cover the 532nd, 268th and 118th nucleotide upstream of ATG start codon, respectively, to the 15th nucleotide downstream of the TAA stop codon of the *adhS* gene of *A. pasteurianus* SKU1108. Three sets of primers generated PCR products of 0.75, 0.9 and 1.17 kb, respectively (Figure 23). Each of them was subcloned into the pCM62 in an opposite direction of *Plac*, resulting in plasmid pCMadhS10/1, pCMadhS10/2 and pCMadhS10/3 (Figure 24A). The *adhS* derivative plasmids were individually transferred to *adhS* gene disruptant by conjugation and selected on a YPGD medium containing 4% ethanol, 25 µg/ml tetracycline, 50 µg/ml kanamycin and 0.5% $CaCO_3$. It was discerned that only pCMadhS10/1 and pCMadhS10/2 with 532 and 268 bp upstream of the ATG start codon of the *adhS* gene could restore the PQQ-ADH activity in the *adhS* disruptant. The partial restoration of ethanol oxidizing ability and the PQQ-ADH activity were observed in pCMadhS10/3 with 118 bp upstream region of the ATG start codon (Figure 24B and 24C). Immunoblotting analysis of subunit III of DPS which displayed those *adhS* deletions indicated that less amount of subunit III was synthesized by pCMadhS10/3 (Figure 24D). The result suggested that the tentative promoter region of *adhS* gene might be located at around 118 to 268 bp upstream of the initiation codon of the *adhS* gene. In addition, heme staining of membrane and soluble fractions of those strains was also analyzed (Figure 25). A normal amount of PQQ-ADH subunit I and II were detected in the membrane and soluble fractions of the *adhS* derivative mutants, except in the soluble fraction of DPS strain carrying pCMadhS10/3 grown in the ethanol condition. In the latter fraction, less amount of subunit I was found, but higher than occurred in the *adhS* gene disruptant at the same condition. It has been implied that lower amount of PQQ-ADH subunit III in this strain may cause an unstabilized subunit I and could degrad easiler.

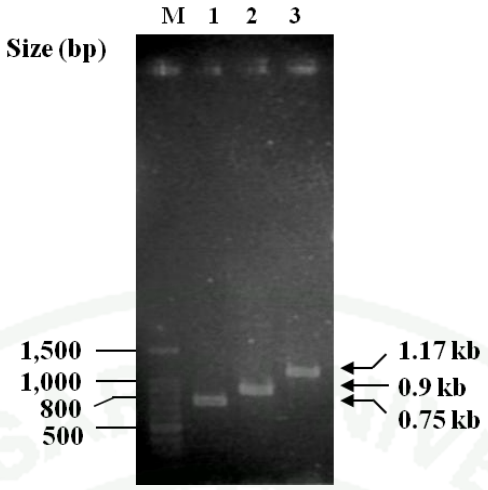


Figure 23 Agarose gel electrophoresis of PCR products obtained from primers designed for deletion analysis of *adhS* gene. Lane M = 100 bp DNA ladder, 1 = *adhS*10/3, 2 = *adhS*10/2, 3 = *adhS*10/1.

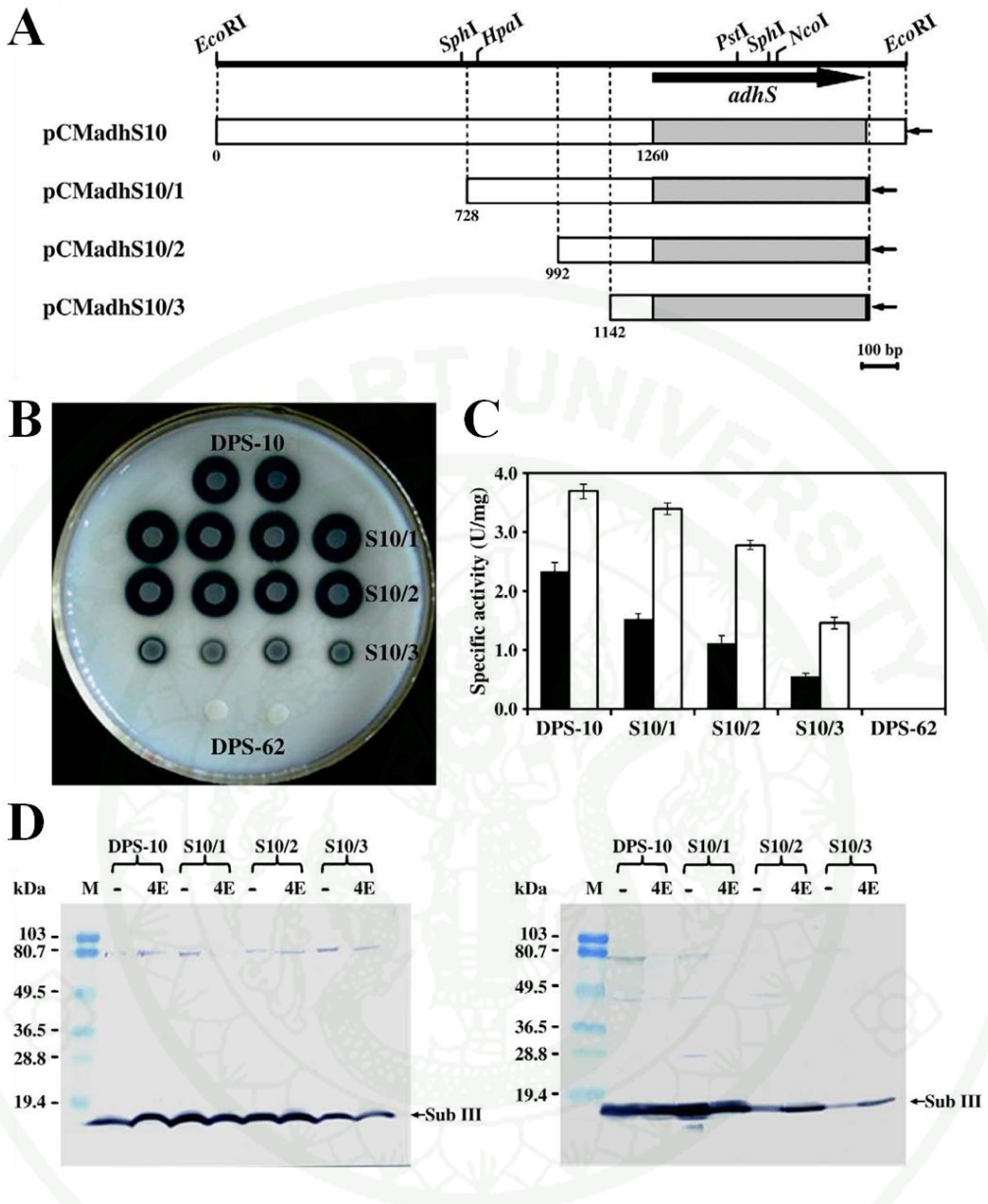


Figure 24 Structures of *adhS* derivatives (A), ethanol oxidizing ability (B) PQQ-ADH activity in membrane fractions (C) and immunoblot analysis with membrane (left) and soluble (right) fractions (D) of DPS harboring various pCMadhS. pCMadhS10 contains 2 kb *Eco*RI DNA fragment in pCM62. pCMadhS10/1, pCMadhS10/2 and pCMadhS10/3 contain various sizes of DNA fragments carrying *adhS* gene which constructed by PCR amplification. These strains were spot-inoculated on YPGD agar containing 0.5% $CaCO_3$, 4% ethanol, 50 µg/ml kanamycin and 25 µg/ml tetracycline and incubated at 30 °C for 24 h. The antisera used were anti-ADH subunit III from *A. aceti*.

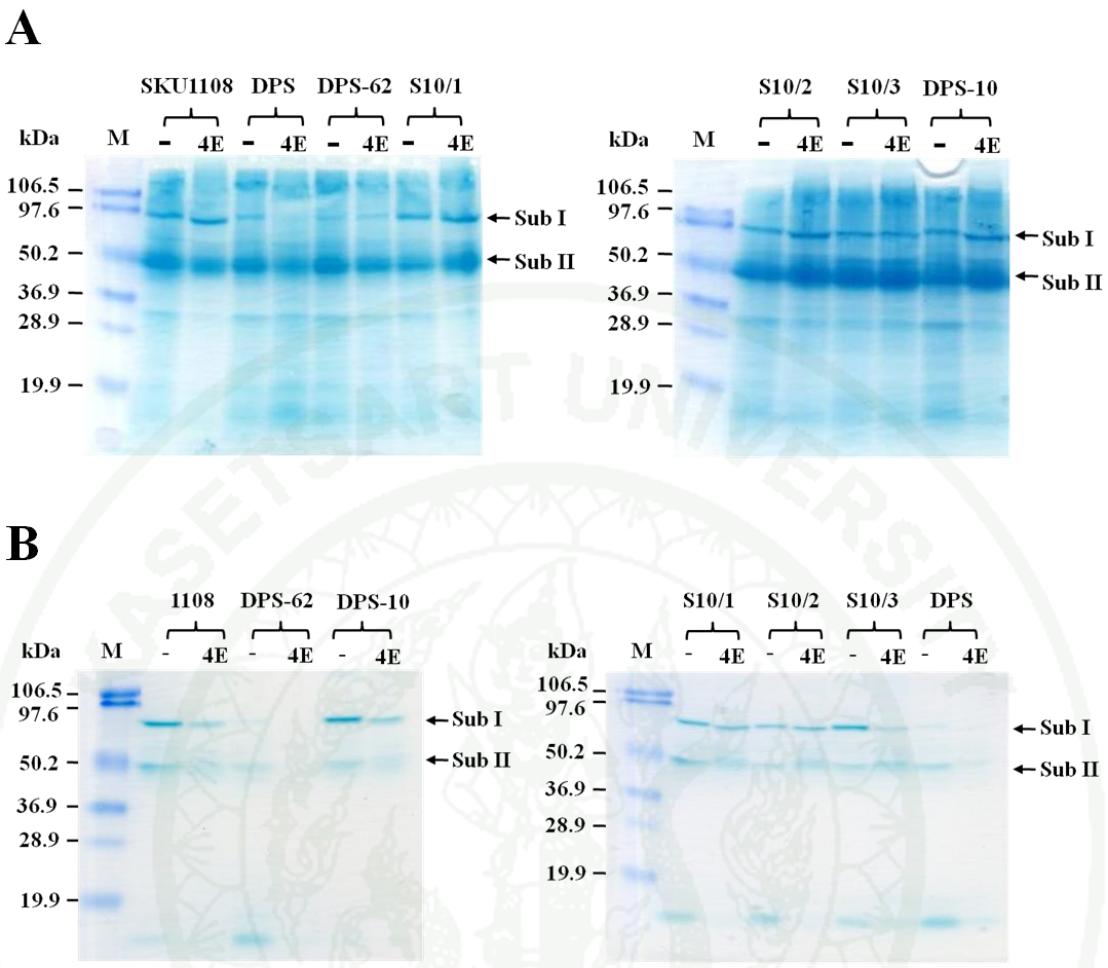


Figure 25 Heme staining of membrane (A) and soluble fractions (B) of *A. pasteurianus* SKU1108, *adhS* gene disruptant (DPS), DPS harboring pCM62 (DPS-62) and DPS harboring various pCMadhS. The membrane and soluble fractions were prepared from the cells grown in YPGD medium supplemented 0 or 4% ethanol.

1.6 Random mutagenesis of *adhS* gene

Random mutagenesis based on error-prone PCR was used to analyze the essential amino acids involved in the molecular coupling of subunit I. The amino acid and nucleotide substitutions in mutagenized *adhS* gene are summarized in Figure 26A and B, respectively. The effect of random mutagenized *adhS* genes on the ethanol oxidizing ability on YPGD agar comprised of 0.5% $CaCO_3$, 4% ethanol, 50 µg/ml kanamycin and 25 µg/ml tetracycline and incubated at 30°C for 24 h and their PQQ-ADH activities are shown in Figure 27A and B. It was ascertained that a complete loss of ethanol oxidizing ability and the PQQ-ADH activities were caused by mutants devoid of 73 (Δ2) and 140 (Δ46) amino acid residues at C-terminal on pCMΔadhS2 and pCMΔadhS46, respectively. The nucleotide sequencing indicated a single deletion of one base, 268^{th}A, in *adhS* gene on pCMΔadhS2, with the ramification of a frame shift and nonsense mutation by the TAA stop codon at 397^{th} nucleotide. This mutagenized *adhS* gene generated 43 different amino acids at 90^{th} to 132^{nd} and stop at 133^{th} amino acid. Moreover, the nucleotide sequence of the mutagenized *adhS* gene on pCMΔadhS46 revealed the deletion of one base, A, at 184^{th} nucleotide caused a frame shift and nonsense mutation by TGA stop codon at 196^{th} nucleotide, similar to pCMΔadhS2. This phenomenon provided a short polypeptide consisting of 65 amino acid residues with 4 different residues at the C-terminal. In addition, some alteration of amino acid residues such as Leu18Gln, Ala26Val, Val36Ile, Val54Ile, Gly55Asp, Val70Ala and Val107Ala did not show any affect on the ethanol oxidizing ability. Interestingly, amino acid alteration at the 104^{th} position from Thr to Lys in the mutant m18 on pCMmadhS18 led to complete loss of ethanol oxidizing ability. Furthermore, the mutant devoid of the 22 amino acid residues at C-terminal of subunit III in the mutant Δ14 on pCMΔadhS14 exhibited no effect on the ethanol oxidizing ability and its nucleotide sequences revealed that there were three points of mutation in the *adhS* structural gene; 234Ala→Thr, 378Cys→Thr and 551Thr→Ala, and one point at the position 45 bp upstream of the initiation codon, C→T. The alteration of 551Thr led to nonsense mutation by the TAG stop codon and resulted in a shorter protein. This corollary infers that the 22 amino acid residues at the C-terminal of subunit III might not be involved in the PQQ-ADH activity, while the amino acid at the 104^{th} position is an indication for the correlation of the molecular coupling of subunit I and subunit III.

Nevertheless, an alteration of nucleotide sequences at the position 150Gly→Ala (m8), 460Thr→Cys and 513Ala→Gly (m12) produced a silent mutation, with no change in amino acid residues. However, the mutant with a point mutation at the tentative promoter region from 15C to T (m5) and 117T to C (m53) exhibited low ethanol oxidizing ability. In contrast, alteration of 96G→A and 186C→T (m61) retained the ethanol oxidizing ability. This result corresponded well with the analysis of *adhS* regulatory sequences revealed that the possible promoter region might be located at approximately 118 to 268 bp upstream the initiation start site. In addition, immunoblotting was used to analyze the PQQ-ADH subunit III in those mutagenized strains (Figure 28). The result corresponded to the result of PQQ-ADH activity as mentioned above. Interestingly, little amounts of PQQ-ADH subunit III was observed in mutant m18, which lost PQQ-ADH activity. It has been suggested that only one amino acid alteration from 104Thr to Lys in this mutant may cause a misfold protein and easily degraded by protease enzyme in cytoplasm of the cell. Unfortunately, no report about the three dimensional structure of the PQQ-ADH, especially subunit III, is available, therefore, the actual functions of this residue is still unknown.

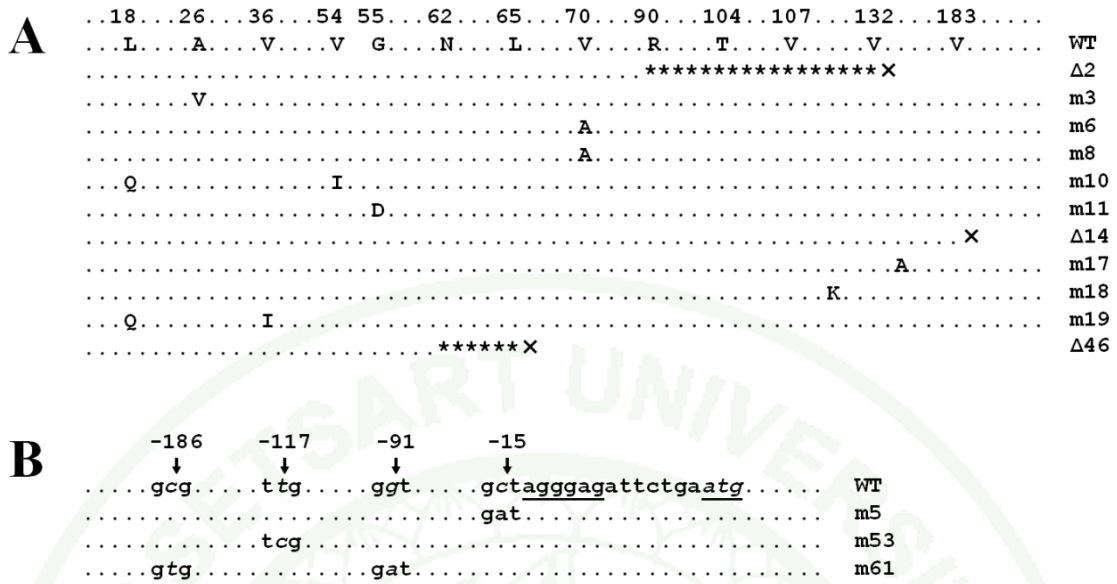


Figure 26 Summarization of amino acid substitution in mutagenized *adhS* gene (A) and nucleotide substitution at upstream region of *adhS* gene (B). A, different amino acid residues between wild type and mutant indicated by asterisk, nonsense mutations indicated by crosses and the position of amino acid residue indicted by number above the wild type sequence. B, a possible ribosome binding site (RBS) indicated by underline, start codon indicated by italic and underline, nucleotide substitution indicated by italic letters and position of nucleotide sequence indicated by numbers.

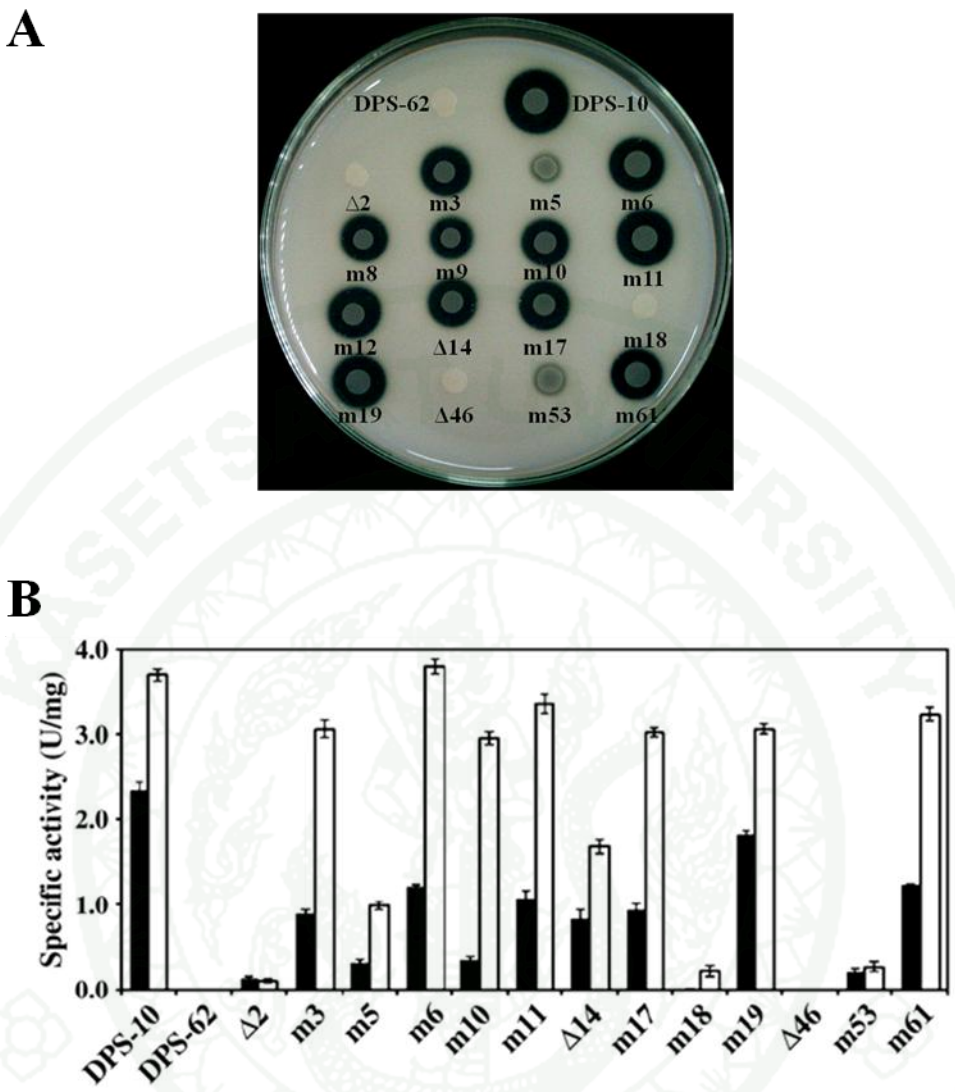


Figure 27 Comparison of ethanol oxidizing ability (A) and PQQ-ADH activity (B) of *adhS* disruptant carrying mutagenized genes (B). These strains were spot-inoculated on YPGD agar containing 0.5% $CaCO_3$, 4% ethanol, 50 µg/ml kanamycin and 25 µg/ml tetracycline and incubated at 30°C for 24 h. The PQQ-ADH activity was measured with membrane fraction prepared from cell grown in YPGD medium containing 0 or 4% ethanol at late log phase.

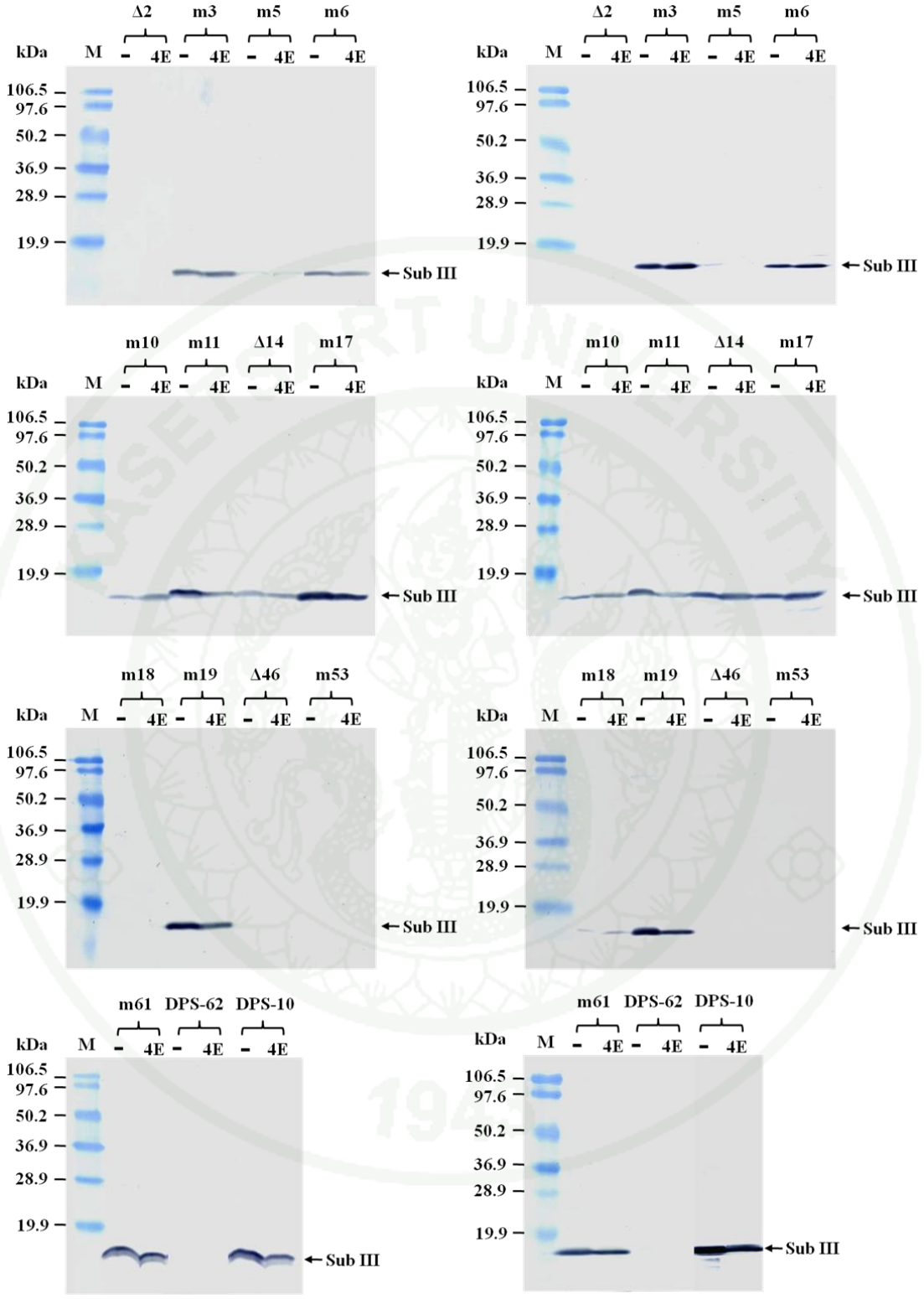


Figure 28 Immunoblotting analysis of membrane (left) and membrane fractions (right) of DPS harboring mutagenized *adhS* gene. These strains were grown in 100 ml of YPGD medium supplemented with or without 4% ethanol. The antisera used were anti-ADH subunit III from *A. aceti*.

2. Cloning, Nucleotide Sequencing and Characterization of NAD^+-*adh* Genes

2.1 PCR cloning of NAD^+-*adh* genes

The *adhI* gene was amplified by Polymerase Chain Reaction (PCR) using two specific primers, FadhI (5'-GCGAATTCGTCAGCGTATTTTTAATGCC-3') and RadhI (5'-ATCTGCAGGTCTTCACCTGCTCTTCG-3'). For amplification of *adhII* gene, the forward and reverse primers are FadhII (5-ATGAATTCCCATCCGTTGGT CAATGTGG-3') and RadhII (5'-ATCTGCAGGATCAAATCCTCACGTGTCG-3'). Chromosomal DNA of *A. pasteurianus* SKU1108 was used as template and PCR reaction was performed by using the illustra™ puReTaq Ready-To-Go PCR Beads as described in Material and Methods. The obtained 2.1 and 1.86 kb PCR products of *adhI* and *adhII* genes, respectively, were cloned into pGEM-T®-Easy Vector. The resulting plasmids were designated as pGEM-TadhI and pGEM-TadhII. Both genes were inserted in an opposite orientation from *Plac* of pGEM-T®-Easy vector. Agarose gel electrophoresis of the obtained PCR products and the *Eco*RI-digested recombinant plasmid are shown in Figure 29. The complete nucleotide sequences of 2.1 and 1.86 kb DNA fragment carrying *adhI* and *adhII* genes, respectively, were obtained from sequencing reaction using four forward and reverse primers. The sequencing strategy is shown in Figure 30. The nucleotide sequences and the position of primers used for sequencing are shown in Table 9.

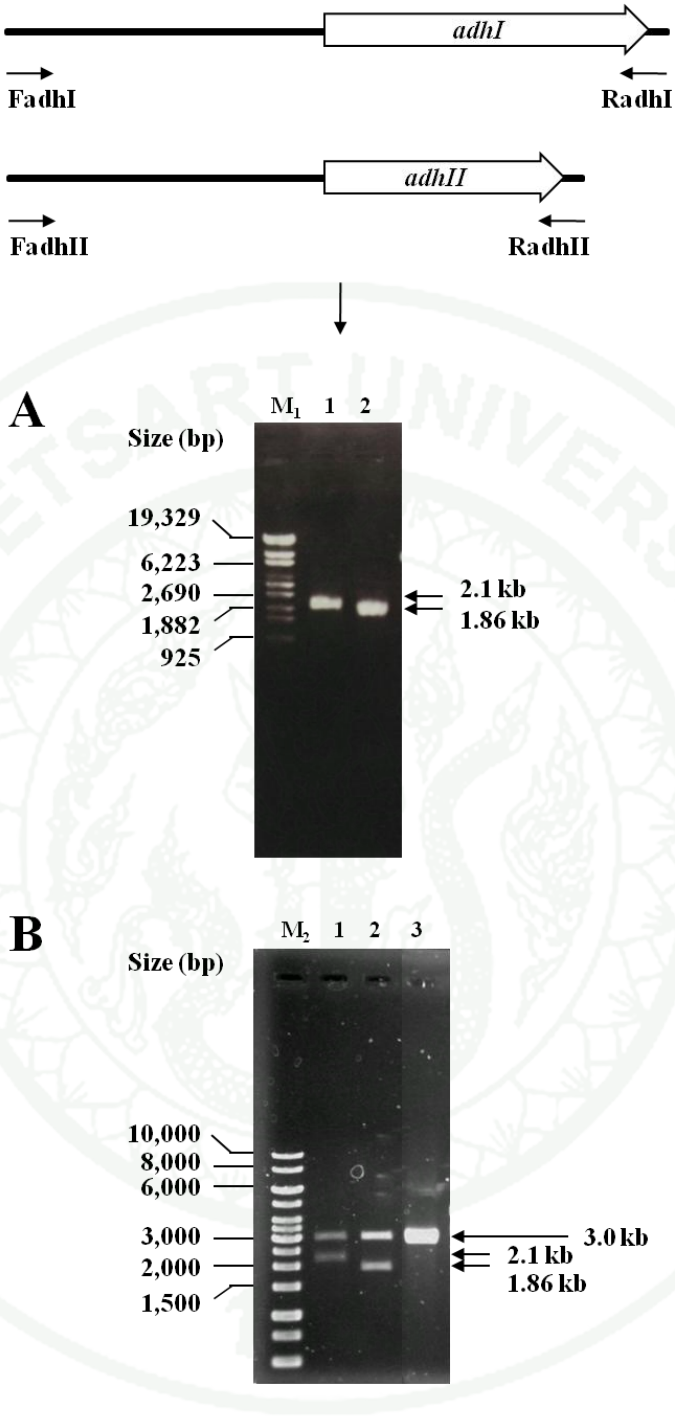


Figure 29 Agarose gel electrophoresis of the *adhI* and *adhII* genes PCR products (A) and the *Eco*RI-digested recombinant plasmids carrying *adhI* and *adhII* genes (B) from *A. pasteurianus* SKU1108. A, lane M_1 = *Sty*I-digested λ DNA marker, 1 = *adhI* gene, 2 = *adhII* gene. B, lane M_2 = 1 kb DNA ladder, 1-3 = pGEM-TadhI, pGEM-TadhI and pGEM-T digested with *Eco*RI, respectively.

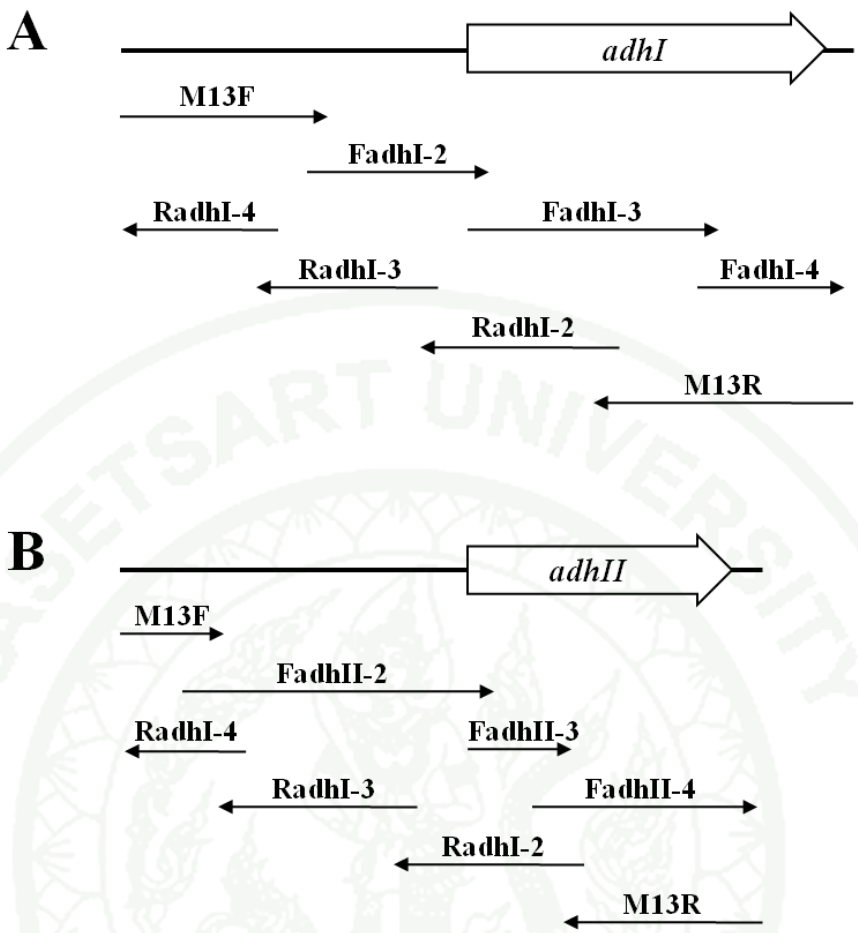


Figure 30 Sequencing strategy used to determine the nucleotide sequence of 2.1 and 1.86 kb PCR products of *adhI* (A) and *adhII* genes (B), respectively.

Table 9 Nucleotide sequences of primers used for sequencing of *adhI* and *adhII* genes

Primer designation	**Sequence (5′→3′)**	**Position**
adhI gene sequencing		
Universal M13F	GTAAAACGACGGCCAGT	-
FadhI-2	GATGTTAAGTTTGCCATCC	534-552
FadhI-3	ATGTCCGGAAAAATGAAAGC	1,002-1,021
FadhI-4	GCCAGAAATACAGATCCG	1,565-1,673
Universal M13R	CAGGAGACAGCTATGAC	-
RadhI-2	ACTTTTACAGGGTCTACG	1,424-1,441
RadhI-3	CCAAGACTTTTGACAGC	900-916
RadhI-4	ACGCTAGATGCATAACG	429-445
adhII gene sequencing		
Universal M13F	GTAAAACGACGGCCAGT	-
FadhII-2	ATATGGTGATTGCACAGG	480-497
FadhII-3	ATGGCACGTGTAGCAGGCAAG	1,000-1,020
FadhII-4	ATCAATGCCTGAATACGG	1,489-1,507
Universal M13R	CAGGAGACAGCTATGAC	-
RadhII-2	GATTGATAGACTGAACGC	1,325-1,342
RadhII-3	ACGTTTGCCATGAACAC	846-862
RadhII-4	ATCAATGCCTGAATACGG	349 -366

2.2 Nucleotide and deduced amino acid sequences of *adhI* gene

The complete nucleotide sequences of the DNA fragment carrying *adhI* gene and its deduced amino acid sequence is shown in Figure 31. Based on *E. coli* sigma 70 promoter recognition program (BPROM tool of Softberry), the predicted -35 and -10 sequences of sigma 70, TTTATT and TGTTTGTAAAAT, were located at 961st to 966th and 976th to 987th nucleotide (nt) of *adhI* gene, respectively.The possible open reading frame (ORF) corresponding to ADH I consisted of 1,029 bp, started with ATG at the 1,002nd to the 1,004th nt and terminated with TGA at the 2,028th to the 2,030th nt. The possible ribosome-binding sequence, AGGAAG, was presented at the 989th to the 994th nucleotide (6 nt upstream from the start codon). The deduced amino acid sequence of *adhI* gene was 342 residues. Although the complete sequences of NAD^+-ADH in acetic acid bacteria have not been published, the genome sequences, for instance *Acetobacter pasteurianus* IFO 3283 (Azuma *et al*., 2009), *G. oxydans* (Prust *et al*., 2005), *Ga. hansenii* (Iyer *et al*., 2010) and *Ga. diazotrophicus* (Bertalan *et al*., 2009) are distributed in the GenBank database. In addition, the BLAST search sequences were compared with the published data on *Sulfolobus solfataicus* (Ammendola *et al*., 1992), *Thermus thermophilus* (Henne *et al*., 2004), *Gluconobacter oxydans* (Prust *et al*., 2005), *Rhodobacter sphaeroides* (Lim *et al*., 2009), *Comamonas testosterone* (Ma *et al*., 2009) and some annotations in the GenBank database. Homology search using tblastX and blastP revealed that ADH I is identical to a member of Zn-dependent alcohol dehydrogenases, which is group I NAD^+-dependent alcohol dehydrogenase (Reid and Fewson, 1994). ADH I showed high similarity to the same proteins as follows: *A. pasteurianus* IFO 3283 (99.71%), *Ga. hansenii* ATCC 23769 (78.07%), *Ga. diazotrophicus* PAl 5 (78.08%), *G. oxydans* 621H (76.38%), *Comamonas tertosteroni* CNB-2 (67.26%), *Ralstonia eutropha* JMP134 (67.07%), *Pseudomonas aeruginosa* PAO1 (63.39%) and *Acinetobacter johnsonii* SH046 (60.53%). This protein showed a lesser similarity (31.70%) to Zn-dependent ADH of the thermoacidophilic archaebacterium *Sulfolobus solfataricus* DSM 1617. The summary of the sequence similarity percentages is shown in Table 10. Amino acid sequence alignments of the deduced polypeptides of Zn-dependent ADHs found in blastP search was performed with ClustalW program (Figure 32). The results from the PPSearch (http://www.ebi.ac.uk/Tools/ppsearch/index.html), the NCBI Conserved

Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the ScanProsite (http://expasy.org/tools/scanprosite/) revealed that a zinc-containing alcohol dehydrogenase signature was observed. This motif was found as Gly-His-Glu-Gly-Val-Gly-Thr-Val-Val-Gln-Val-Gly-Ser-Glu-Val in ADH I of *A. pasteurianus* (position 64 to 78) which contains the conserved second ligand (65His) of the catalytic zinc atom (Jörnvall *et al*., 1987; Reid and Fewson, 1994). In addition, the conserved sequence of glycine residues directly involved in the cofactor-binding domain (Gly-X-Gly-X-X-Gly) and a highly conserved aspartate (Jörnvall *et al*., 1987; Lesk, 1995; Reid and Fewson, 1994, Sun and Plapp, 1992) were found as Gly-Val-Gly-Gly-Leu-Gly (position 176 to 181) and 199Asp, respectively. In addition to the zinc-containing ADH signature, the Pfam search (www.sanger.ac.uk/resources/software/) showed that ADH I contains an alcohol dehydrogenase GroES-like domain at N-terminus. This is the catalytic domain of ADHs and has a GroES-like structure. Taken together, all these results indicated that the ADH I of *A. pasteurianus* SKU1108 is a member of the group I NAD^+-dependent ADHs (Reid and Fewson, 1994). This group is the Zn-dependent long chain ADHs which form dimers (typically higher plants, mammals) or tetramers (yeast, bacteria). The predicted molecular mass of the deduced polypeptide of *adhI* gene was calculated to be 36.3 kDa with the application of the Molecular Weight Calculation of Protein Information Resource (PIR) *via* URL: http://pir.georgetown.edu/pirwww/search/comp_mw.shtml. Chinnawirotpisan *et al.* (2003c) showed that molecular mass of ADH I of *A. pasteurianus* SKU1108 analyzed with SDS-PAGE and gel filtration is 42 and 130 kDa, respectively. They proposed this enzyme to exist as a trimer consisting of three identical subunits, which is different from other Zn-dependent long chain ADHs which form a dimer or tetramer. On the other hand, the molecular mass prediction of ADH I obtained from the present study is 36.3 kDa which implied the active form of this enzyme has the potential to form a tetramer (typical form of this enzyme) instead of a trimer. The difference in MW of ADH I derived from SDS-PAGE analysis and MW prediction is likely the result of its hydrophobicity or several impurities which can retard mobility of the protein (Lin *et al*., 1999).

```
GTCAGCGTAT TTTTAATGCC GGGGTGTTTC TTATTGCTCT GGTGTTGGGC GCACTTACGG 60
TTATGCAGTA TGCCACACCG ATGGGGGAAC TCCCGCGGGA TCTGTTCTTC CTAGCGGCTC 120
TGCTGTTTGG CGTGTGCATG ACACTGCCTA TCGGTGGTGC CGATATGCCG GTCGTTATTT 180
CGCTCTACAA CGCATTTACC GGGCTGCCTG TTGGGCTTGA AGGGTATGTG ATGAACAACC 240
CCGCCCTGAT GATTGCAGGC ATGGTTGTAG GGTCTGCTGG CACGTTGCTT ACTGTGCTGA 300
TGGCCAAGGC CATGAACCGC TCACTCACCA ACGTGTTGTT TAGCAACTTT GGTGATAGCG 360
CTAGCAGCGC CAAAGGCCCG CAAGGTGAAA TGCATTCTGT TGATCCAGCA GATGCTGCCA 420
CCACCATGCG TTATGCATCT AGCGTGATCA TTATTCCCGG TCACGGGCTG GCCGTGGCAC 480
AGGCACAACA GAAACTTTAC GAATTTGTCA AAATTCTTGT TGCCGATGGT GTAGATGTTA 540
AGTTTGCCAT CCACCCCGTT GCTGGCCGTA TGCCTGGACA TATGAACGTT CTGCTGGCCG 600
AAGCTGGCGT GCCATATGAT ATGATTTATG ACATGGATGA CATAAATGAT AGTTTCGCTA 660
CAACGGATGT TGCGCTGGTT ATTGGCGCGA ACGATGTGGT GAACCCGGAA GCCCTGACCG 720
ATAAATCCTC CCCCATCTAT GGCATGCCTA TTTTGAATGC TTACAAGGCG CATCAGGTCT 780
TTGTCATTAA ACGTGGTACA GGCGTTGGAT ATTCTGGCGT GCAGAATCCG CTCTTCTTCC 840
AGAAGAACTG CACAATGGTA TTTGGAGATG CACAGGCCGT GCTTTCCAAA ATGGTTGAAG 900
CTGTCAAAAG TCTTGGCGGC TCCTGAGCAT GACAAGGGCG AGGCTTATCC CTCGCCCTTT 960
TTTATTTCTC ATATCTGTTT GTAAAATAAA GGAAGCATAT CATGTCCGGA AAAATGAAAG 1020
 -35                -10          RBS          M  S  G  K  M  K
CCGCTGTGGT TCATGAATTT GGCAAACCAC TGACCATTGA AGAACTGGAC ATTCCCACCA 1080
A  A  V  V   H  E  F   G  K  P   L  T  I  E   E  L  D   I  P  T
TTAAACCCAC CCAGATTTTG GTTAAAATGA TTGCCTGCGG CGTGTGCCAT ACAGATTTGC 1140
I  K  P  T   Q  I  L   V  K  M   I  A  C  G   V  C  H   T  D  L
ATGCTGCCAG CGGGGATTGG CCCAAAAAAC CACATCTGCC GTTTATTCCC GGCCACGAAG 1200
H  A  A  S   G  D  W   P  K  K   P  H  L  P   F  I  P   G  H  E
GCGTTGGCAC GGTTGTGCAG GTTGGCAGCG AAGTAGACTG GGTAAAGGAA GGCGACGTTG 1260
G  V  G  T   V  V  Q   V  G  S   E  V  D  W   V  K  E   G  D  V
TTGGCGTGCC TTGGCTCTAT TCTGCCTGCG GCCATTGCGA GCATTGTCTG GCTGGGTGGG 1320
V  G  V  P   W  L  Y   S  A  C   G  H  C  E   H  C  L   A  G  W
AAACCCTTTG TGCGAAGCAA GAAGAAACCG GCTATTCCGT AAACGGCTGC TTTGCCGAAT 1380
E  T  L  C   A  K  Q   E  E  T   G  Y  S  V   N  G  C   F  A  E
ACGTGGTAGC AGACCCTAAC TACATTGCTC ATCTTCCCAA AGGCGTAGAC CCTGTAAAAG 1440
Y  V  V  A   D  P  N   Y  I  A   H  L  P  K   G  V  D   P  V  K
TTGCCCCCGT ACTGTGCGCA GGCCTGACCG TTTATAAGGG CCTGAAAATG ACAGACACGC 1500
V  A  P  V   L  C  A   G  L  T   V  Y  K  G   L  K  M   T  D  T
GGGCAGGGAA CTGGGTTGCC ATTTCCGGCG TTGGGGGGTT AGGCCAGATG GCTGTGCAGT 1560
R  A  G  N   W  V  A   I  S  G   V  G  G  L   G  Q  M   A  V  Q
ACGCCGTAGC AATGGGGCTG AATGTTGTGG CGGTAGATAT TGATGATGAA AAACTGGCAA 1620
Y  A  V  A   M  G  L   N  V  V   A  V  D  I   D  D  E   K  L  A
CCGCCAAAAA GCTTGGTGCA ACGTACACTG TTAACGCCAG AAATACAGAT CCGGCAGCAT 1680
T  A  K  K   L  G  A   T  Y  T   V  N  A  R   N  T  D   P  A  A
TTATGCAGGA AAAAGTTGGT GGCGTACACG GTGGATTGAT CACCGCTGTT TCCACCAAAG 1740
F  M  Q  E   K  V  G   G  V  H   G  G  L  I   T  A  V   S  T  K
CATTTTCTCA GGCTATGGGT TATGTGCGGG CAGGGGGCAC TTTGGTGCTA AATGGGCTGC 1800
A  F  S  Q   A  M  G   Y  V  R   A  G  G  T   L  V  L   N  G  L
CGCCGGGTGA TTTCCCAATT TCTATCTTTG ACGTGGTCAT GAATGCCATT ACCATTCGCG 1860
P  P  G  D   F  P  I   S  I  F   D  V  V  M   N  A  I   T  I  R
GCTCCATAGT AGGCACACGG CTGGACATGA TTGAAGCTCT TTCCTTCTTT GCGGAAGGGA 1920
G  S  I  V   G  T  R   L  D  M   I  E  A  L   S  F  F   A  E  G
AGGTAACATC TGTCACCACA ACGGATCGGA TTGATAACAT CAACGCAATT TTTGATGCGC 1980
K  V  T  S   V  T  T   T  D  R   I  D  N  I   N  A  I   F  D  A
TCAAGAACGG TCGGGTGGAA GGCCGCGTGG TTCTGGACTT CCGCAACTGA GCAACTCCTA 2040
L  K  N  G   R  V  E   G  R  V   V  L  D  F   R  N  *
ATCTCAGAAA CAAAAGAAGG GTTATTCTGA TGGCTTATGC AACAATCAAT CCCTTCACCG 2100
AAGAGCAGGT GAAGAC
```

Figure 31 Nucleotide and deduced amino acid sequences of 2.1 kb DNA fragment carrying the *adhI* gene from *A. pasteurianus* SKU1108. The possible ribosome-binding site is in a box. The predicted -10 and -35 sequences are underline.

Table 10 Comparison of the identity percentage of deduced amino acid sequence of *adhI* gene of *A. pasteurianus* SKU1108 with Zn-dependent ADH proteins from related bacteria.

Strain[a]	% identity SKU1108
Acetobacter pasteurianus IFO 3283 (YP_003186562)	99.71
Gluconacetobacter hansenii ATCC 23769 (ZP_0683484829)	78.07
Gluconacetobacter diazotrophicus PAI 5 (YP_002275125)	78.07
Gluconobacter oxydans 621H (YP_190752)	76.38
Comamonas tertosteroni CNB-2 (YP_003280308)	67.26
Ralstonia eutropha JMP134 (YP_297879)	67.06
Pseudomonas aeruginosa PAO1 (NP_254114)	63.39
Acinetobacter johnsonii SH046 (ZP_006064662)	60.53
Sulfolobus solfataricus DSM 1617 (AAB24546)	31.70

[a] GenBank Accession No. are in the parentheses

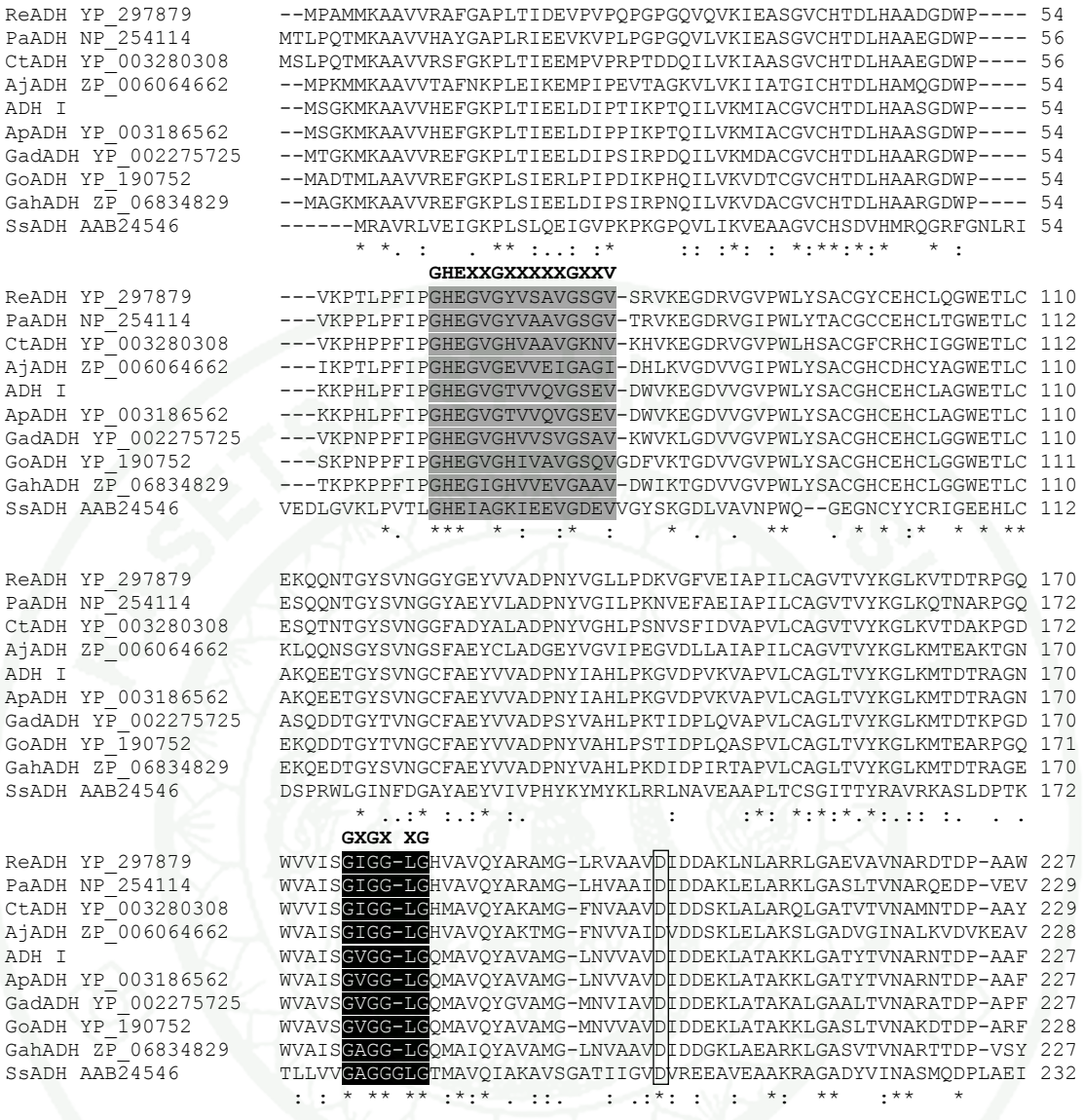


Figure 32 Amino acid sequence alignment of Zn-dependent ADH. ReADH, *Ralstonia eutropha* JMP134; ADH I, *A. pasteurianus* SKU1108 ADH; ApADH, *A. pasteurianus* IFO 3283, GadADH, *Ga. diazotrophicus* PAl 5; GoADH, *G. oxydans* 621H; GahADH, *Ga. hansenii* ATCC 23769; CtADH, *Comamonas testosteroni* CNB-2; PaADH, *Pseudomonas aeruginosa* PAO1; AjADH, *Acinetobacter johnsonii* SH046; SsADH, *Sulfolobus solfataricus* DSM 1617. The sequence alignment was performed with the application of ClustalW. NAD^+ and catalytic Zn binding motifs are enclosed by black and grey boxes, respectively. Aspartate conserved residue for NAD^+ binding is indicated by a box. Genbank accession no. is indicated.

```
ReADH YP_297879       LQKEIG-GAHGVLVTAVSPKAFSQAIGMVRRGGTIALNGLPPGDFGTPIFDVVLKGITIR 286
PaADH NP_254114       LQRDIG-GAHGVLVTAVSNSAFGQAIGMARRGGTIALVGLPPGDFPTPIFDVVLKGLHIA 288
CtADH YP_003280308    LQREIE-GAHGVLVTAVSPKAFEQALGMVRRGGTISLNGLPPGDFPLPIFSMVLKGITVR 288
AjADH ZP_006064662    LAATGE-GCHGVLVTAVSPKAFEQAVAIVRRGGTMVLNGLPPGKFDLSIFDMVLDGITVR 287
ADH I                 MQEKVG-GVHGGLITAVSTKAFSQAMGYVRAGGTLVLNGLPPGDFPISIFDVVMNAITIR 286
ApADH YP_003186562    MQEKVG-GVHGGLITAVSTKAFSQAMGYVRAGGTLVLNGLPPGDFPISIFDMVMNAITIR 286
GadADH YP_002275725   IQSQVG-GAHGALVTAVSRSAFSQAMGFARRGGTIVLNGLPPGEFPISIFDMVMAGTTVR 286
GoADH YP_190752       IQQQIG-GAHGALVTAVGRTAFSQAMGYARRGGTIVLNGLPPGDFPVSIFDMVMNGTTIR 287
GahADH ZP_06834829    IRQKTG-GTHGVLVTAVSNTAFTQAIGYARRGGTIVLNGLPPGEFPISIFDMVMNGTTVR 286
SsADH AAB24546        RRITESKGVDAVIDLNNSEKTLSVYPKALAKQGKYVMVGLFGADLHYHAPLITLSEIQFV 292
                           * .. :    . .::          *.  : **  ..:      :.:    .

ReADH YP_297879         GSIVGTRSDLQESLDFAAHGDVKATVS-TAKLDDVNDVFGRLREGKVEGRVVLDFSR 342
PaADH NP_254114        GSIVGTRADLQEALDFAGEGLVKATIH-PGKLDDINQILDQMRAGQIEGRIVLEM-- 342
CtADH YP_003280308     GSIVGTRLDLQESLDFAAQGKVKATVS-TDKLENINDIFARMHEGKIEGRVVLDIAA 344
AjADH ZP_006064662     GSIVGTRLDLKEALDIAAKGKVKAHIS-VEPIENINDIFSRMEHGKIDGRIVVDMRL 343
ADH I                  GSIVGTRLDMIEALSFFAEGKVTSVTT-TDRIDNINAIFDALKNGRVEGRVVLDFRN 342
ApADH YP_003186562     GSIVGTRLDMIEALSFFAEGKVTSVTT-TDRIDNINAIFDALKNGRVEGRVVLDFRN 342
GadADH YP_002275725    GSIVGTRLDMIEAVSFFADGKVKTVIE-PDRLENINQIFTDLEEGKIQGRKVLDFRQ 342
GoADH YP_190752        GSIVGTRLDMIEAMDFFARGKVKSVVT-PGKLENINTIFDDLQNGRLEGRTVLDFRS 343
GahADH ZP_06834829     GSIVGTRLDMIEALSFFTEGKVSTVVA-TDKLENINDIFDRLQHGQVAGRIVLDFRN 342
SsADH AB24546          SLVGNQSDFLGIMRLAEAGKVKPMITKTMKLEEANEAIDNLENFKAIGRQVLIP-- 347
                       **:**.: *:   : :   * *..      ::: *  :  :.  :  ** *:
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Figure 32 (Continued)

2.3 Nucleotide and deduced amino acid sequences of *adhII* gene

The complete nucleotide sequences of the DNA fragment carrying *adhII* gene and its deduced amino acid sequence is shown in Figure 33. Based on *E. coli* sigma 70 promoter recognition program (BPROM tool of Softberry), the predicted -35 and -10 sequences of sigma 70, TTGAGA and TGCAAGAAT, were located at 885st to 890th and 909th to 917th nt of *adhII* gene, respectively. The possible ribosome-binding sequence, AGGGAAG, was presented at the 989th to the 995th nt (4 nt upstream from the start codon). The complete nucleotide sequence of the DNA fragment carrying *adhII* gene contains one ORF with the length of 762 bp, encoding 253 amino acid residues. The *adhII* gene started with ATG at the 1,000th to the 1,002nd nt and terminated with TAA at the 1,759th to the 1,761st nt. The predicted molecular mass of the deduced polypeptide was calculated to be 26.3 kDa by using Molecular Weight Calculation of Protein Information Resource (PIR) *via* URL: http://pir. georgetown.edu/pirwww/search/comp_mw.shtml. The deduced amino acid sequence of ADH II was analyzed using BLAST search tool (http://www.ncbi.nlm.nih.gov/ BLAST/). The similarity search results revealed that ADH II was identical to a short-chain dehydrogenase/reductase (SDR), which is a group II NAD^+-dependent alcohol dehydrogenase (Reid and Fewson, 1994). ADH II showed high similarity to the same proteins of other acetic acid bacteria and related bacteria as follows: *A. pasteurianus* IFO 3283 (99.60%), *Ga. hansenii* ATCC 23769 (78.16%), *Ga. diazotrophicus* PAl 5 (77.87%), *Lactobacillus reuteri* DSM 20016 (55.33%), *Geobacter metallireducens* GS-15 (54.90%), *Bacillus tusciae* DSM 2912 (53.75%) and *Rhodobacter sphaeroides* KD131 (51.16%). Surprisingly, only 27.57% identity was observed with a short-chain dehydrogenase in the genome of *G. oxydans* 621H. In addition, ADH II showed 31.10% similarity to a short-chain dehydrogenase of the thermophilic bacterium *Thermus thermophilus* HB27. The summary of the sequence similarity percentages is shown in Table 11. Amino acid sequence alignments of the deduced polypeptides with SDR proteins available from GenBank database was performed with ClustalW program (Figure 34). The results from the PPSearch (http://www.ebi.ac.uk/Tools/ ppsearch/index.html) and the ScanProsite (http://expasy.org/tools/scanprosite/) revealed that a short-chain dehydrogenases/reductases family signature was observed. This motif was found as Ser-Ile-Glu-Gly-Leu-Ile-Gly-Asp-Pro-Met-Leu-Ala-Ala-Tyr-

Asn-Ala-Ser-Lys-Gly-Gly-Vla-Arg-Leu-Phe-Thr-Lys-Ser-Ala-Ala (position 142 to 170) and 155Tyr is an active site residue in ADH II of *A. pasteurianus* SKU1108. In addition to that the SDR signature, a Rossmann-fold NAD(P)H/NAD(P)$^+$ binding (NADB) domain was found from the result of the NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The NADB domain is found in numerous dehydrogenases of metabolic pathways such as glycolysis, and many other redox enzymes. The conserved sequences of the SDR superfamily, i.e., the N-terminal cofactor-binding pattern Gly-X-X-X-Gly-X-Gly (Rossmann-fold motif) and a common Ser-X_n-Tyr-X-X-X-Lys sequence in active sites were found. These two motifs were Gly-Ala-Ala-Asn-Gly-Ile-Gly (position 13 to 19) and Ser-Ile-Glu-Gly-Leu-Ile-Gly-Asp-Pro-Met-Leu-Ala-Ala-Tyr-Asn-Ala-Ser-Lys (position 142 to 159), respectively (Jörnvall *et al*., 1995; Kallberg *et al*., 2002). An aspatate residue at position 37 of ADH II is a critical role in discriminating NAD(H) from NADP(H) (Filling *et al*., 2002; Kallberg *et al*., 2002). This preference corresponded well with the results from Chinnawirotpisan *et al*. (2003c) that ADH II was NAD$^+$-specific but not NADP$^+$-specific. These highly conserved sequences indicated that ADH II belongs to the SDR superfamily or group II of NAD$^+$-dependent ADHs (Reid and Fewson, 1994) and extended to the cD2 subfamily of the classical SDRs (Kallberg *et al*., 2002).

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CCATCCGTTG GTCAATGTGG TGTCTTTTAC CGGATCAACA CAGGCGGGCA TTGCCGTGGC 60
GCAGGCCGCA GCGCCTACTG TTAAACGTGT TTTGCAGGAA CTGGGCGGCA AATCTGCCAA 120
TATCATTTTC CCTGATACGG ATTTTGCAGA TGCAGTAGAA CGGGGTGCCC AAGAGTGTTT 180
TGGAAATTCC GGCCAGTCCT GTGATGCGCC AACCCGCATG TTGGTGCCGC ATGAGCTGAT 240
GGAAAAGGCC ATGGATATTG CAGCTAAAGT TGCGAGTGGT CTGCGTGTGG GCAATCCGGC 300
CGATAGCATG ACGGAGCTTG GCCCCGTTGT AAGTACCAGC CAGTTTAACC GTATTCAGGC 360
ATTGATTGAA AAAGGTACAG CCCAAGGAGC CATTCTGGCG GCTGGCGGGG CAGGGCGTCC 420
AGAAGGGCTT ACGCAAGGGT ATTACGTGCG CCCCACCGTG TTTGGTCACG TTACATCGGA 480
TATGGTGATT GCACAGGAAG AAATCTTCGG TCCGGTTTTG TCCATCATGG GGTATGCGGA 540
TGAAGATGAT GCCATCCGAC TGGCAAATAG CACGCCATAT GGTTTGGCCG GATATGTGCA 600
ATCTGGCAGC ATGGAGCGTG CTCGTCGGGT CGCACGTAAG CTCCGGGCTG GCACAGTTAC 660
CCTTAATGGT GCTGCGTGGA CCGTAAAAGC TCCATTTGGT GGGTATGGCC TGTCTGGCAA 720
TGGCCGTGAA TGCAGCCGTT TTGGAATTGC AGAGTTTTTG GAAACCAAAG CAATTGTTGG 780
ATACGGAGCA GACTCAAAAA AGCACTGAAC AGTTCAGAAA GAAACTATCG TCGTCTCAAA 840
ACATTGTGTT CATGGCAAAC GTTTGTCCCA ATCTTTTCAT CATTTTGAGA TGTCCAAAAA 900
                                                  -10
            -35
TACACATTTG CAAGAATTGA TGCAGGTCAT GGTTGGGATA TCTGATTCAG GCTGGGATCA 960
GTGCGCAATG CAACAGAATG TATAGGTAAG GGAAGTATTA TGGCACGTGT AGCAGGCAAG 1020
                                RBS               M  A  R  V   A  G  K
GTTGCCATTG TTTCTGGGGC CGCTAATGGC ATTGGCAAGG CAACCGCACA GCTTTTGGCC 1080
 V  A  I   V  S  G  A   A  N  G   I  G  K   A  T  A  Q   L  L  A
AAGGAAGGCG CAAAAGTTGT TATTGGTGAT TTAAAAGAAG AAGATGGGCA GAAAGCTGTT 1140
 K  E  G   A  K  V  V   I  G  D   L  K  E   E  D  G  Q   K  A  V
GCAGAAATTA AGGCAGCAGG TGGTGAAGCC GCATTTGTCA AACTGAATGT AACAGATGAG 1200
 A  E  I   K  A  A  G   G  E  A   A  F  V   K  L  N  V   T  D  E
GCTGCATGGA AAGCCGCTAT TGAGCAAACG CTTAAGCTTT ATGGGCGGCT GGATATTGCA 1260
 A  A  W   K  A  A  I   E  Q  T   L  K  L   Y  G  R  L   D  I  A
GTGAACAATG CAGGCATTGC GTATTCTGGC AGTGTAGAAA GCACATCTCT GGAAGATTGG 1320
 V  N  N   A  G  I  A   Y  S  G   S  V  E   S  T  S  L   E  D  W
CGGCGCGTTC AGTCTATCAA TCTGGATGGC GTGTTTTTGG GCACACAGGT GGCTATTGAG 1380
 R  R  V   Q  S  I  N   L  D  G   V  F  L   G  T  Q  V   A  I  E
GCCATGAAGA AGTCGGGCGG TGGATCCATT GTCAATCTGT CTTCCATTGA AGGACTGATT 1440
 A  M  K   K  S  G  G   G  S  I   V  N  L   S  S  I  E   G  L  I
GGGGACCCAA TGTTGGCCGC CTATAACGCC AGTAAAGGTG GGGTAAGGCT GTTTACAAAA 1500
 G  D  P   M  L  A  A   Y  N  A   S  K  G   G  V  R  L   F  T  K
TCTGCGGCCC TACATTGTGC CAAATCTGGA TACAAAATTC GGGTAAACTC AGTGCATCCC 1560
 S  A  A   L  H  C  A   K  S  G   Y  K  I   R  V  N  S   V  H  P
GGCTATATCT GGACACCTAT GGTGGCCGGT TTAACAAAGG AAGATGCTGC TGCACGCCAA 1620
 G  Y  I   W  T  P  M   V  A  G   L  T  K   E  D  A  A   A  R  Q
AAGCTGGTGG ATCTGCACCC CATTGGCCAC TTGGGTGAGC CCAACGATAT TGCTTACGGT 1680
 K  L  V   D  L  H  P   I  G  H   L  G  E   P  N  D  I   A  Y  G
ATTTTGTATC TTGCCTCTGA TGAATCCAAG TTTGTTACAG GGAGCGAACT GGTCATTGAT 1740
 I  L  Y   L  A  S  D   E  S  K   F  V  T   G  S  E  L   V  I  D
GGTGGGTACA CCGCACAATA AGCAATCAGG TGTAAGGGAG TAAAATTTTA TGTCCGGTAA 1800
 G  G  Y   T  A  Q  *
AAAGCATTTC TCTCCAAGGC GGGAGAAAAA GCATGCGACA CGTGAGGATT TGATC
```

Figure 33 Nucleotide and deduced amino acid sequences of 1.86 kb DNA fragment carrying the *adhII* gene from *A. pasteurianus* SKU1108. The possible ribosome-binding site is in a box. The predicted -10 and -35 sequences are underline.

Table 11 Comparison of the identity percentage of deduced amino acid sequence of *adhII* gene of *A. pasteurianus* SKU1108 with short-chain dehydrogenase/reductase proteins from related bacteria.

Strain[a]	% identity SKU1108
Acetobacter pasteurianus IFO 3283 (YP_003188543)	99.60
Gluconacetobacter hansenii ATCC 23769 (ZP_06835581)	78.16
Gluconacetobacter diazotrophicus PAI 5 (YP_002275223)	77.87
Lactobacillus reuteri DSM 20016 (YP_001272235)	55.33
Bacillus tusciae DSM 2912 (YP_003589160)	53.75
Rhodobacter sphaeroides KD131 (YP_002521229)	51.16
Geobacter metallireducens GS-15 (YP_385169)	54.90
Thermus thermophilus HB27 (YP_004276)	31.01
Gluconobacter oxydans 621H (YP_191035)	27.57

[a] GenBank Accession No. are in the parentheses

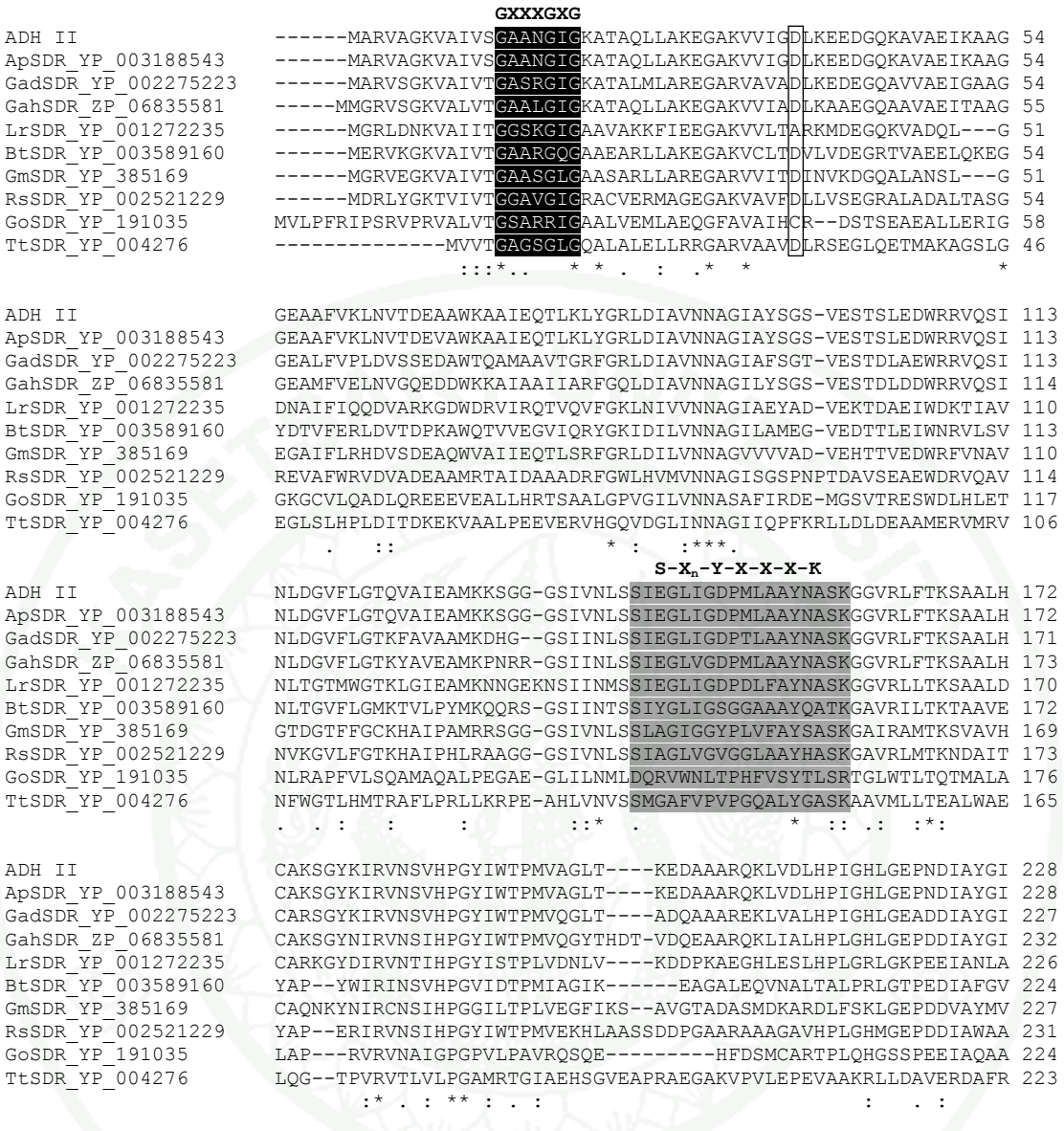


Figure 34 Amino acid sequence alignment of short-chain dehydrogenase/reductase. ADH II, *A. pasteurianus* SKU1108 ADH; ApADH, *A. pasteurianus* IFO 3283, GadADH, *Ga. diazotrophicus* PAl 5; GahADH, *Ga. hansenii* ATCC 23769; LrSDR, *Lactobacillus reuteri* DSM 20016; BtSDR, *Bacillus tusciae* DSM 2916; GmSDR, *Geobacter metallireducens* GS-15; RsSDR, *Rhodobacter sphaeroides* KD131; GoSDR, *G. oxydans* 621H; TtSDR, *Thermus thermophilus* HB27. The sequence alignment was performed with the application of ClustalW. NAD^+ binding motif and a common sequence in active site are enclosed by black and grey boxes, respectively. The major determinant of the coenzyme specificity is indicated by a box. Genbank accession no. is indicated.

```
ADH II SKU1108          LYLASDESKFVTGSELVIDGGYTAQ--------- 253
ApSDR_YP003188543       LYLASDESKFVTGSELVIDGGYTAQ--------- 253
GadSDR_YP002275223      LYLASDESKFMTGSELVIDGGYTAQ--------- 252
GahSDR_ZP06835581       VFLASDESKFMTGSELVIDGGYTAQ--------- 257
LrSDR_YP001272235       LYLASDESSFSTGSEFVADGGYTAQ--------- 251
BtSDR_YP003589160       LYLASDESSFVTGSELVIDGGYTTR--------- 249
GmSDR_YP385169          LYLASDESKFVSGSEFIIDNTATITEGVVPT--- 258
RsSDR_YP002521229       VYLASDEAKFVTGAELVVDGGYTAR--------- 256
GoSDR_YP191035          LALFALPS--VTGQMLALDGGQHLNWSPAS---- 252
TtSDR_YP004276          VLLGRDAQTMDLLYRLSPALAARIVQRRMAHLLT 257
                        : *           :
```

Figure 34 (Continued)

2.4 Phylogenetic tree analysis

The phylogenetic tree of ADH I and ADH II of *A. pasteurianus* SKU1108 was analyzed with the application of the MEGA 4 software based on the neighbour-joining method. As shown in Figure 35, ADH I and ADH II were compared with 22 sequences which cover the Zn-dependent ADHs, SDRs, and Fe-dependent ADHs available from the GenBank database. The results show the Zn-dependent ADHs from acetic acid bacteria to be clustered together and clearly separated from the Zn-dependent ADHs of other bacteria. Moreover, the SDRs from acetic acid bacteria were in the same cluster with the exception of the SDR of *G. oxydans* 621H, which is phylogenetically only remotely related to this group. These results were in line with the protein similarity search aforementioned described.

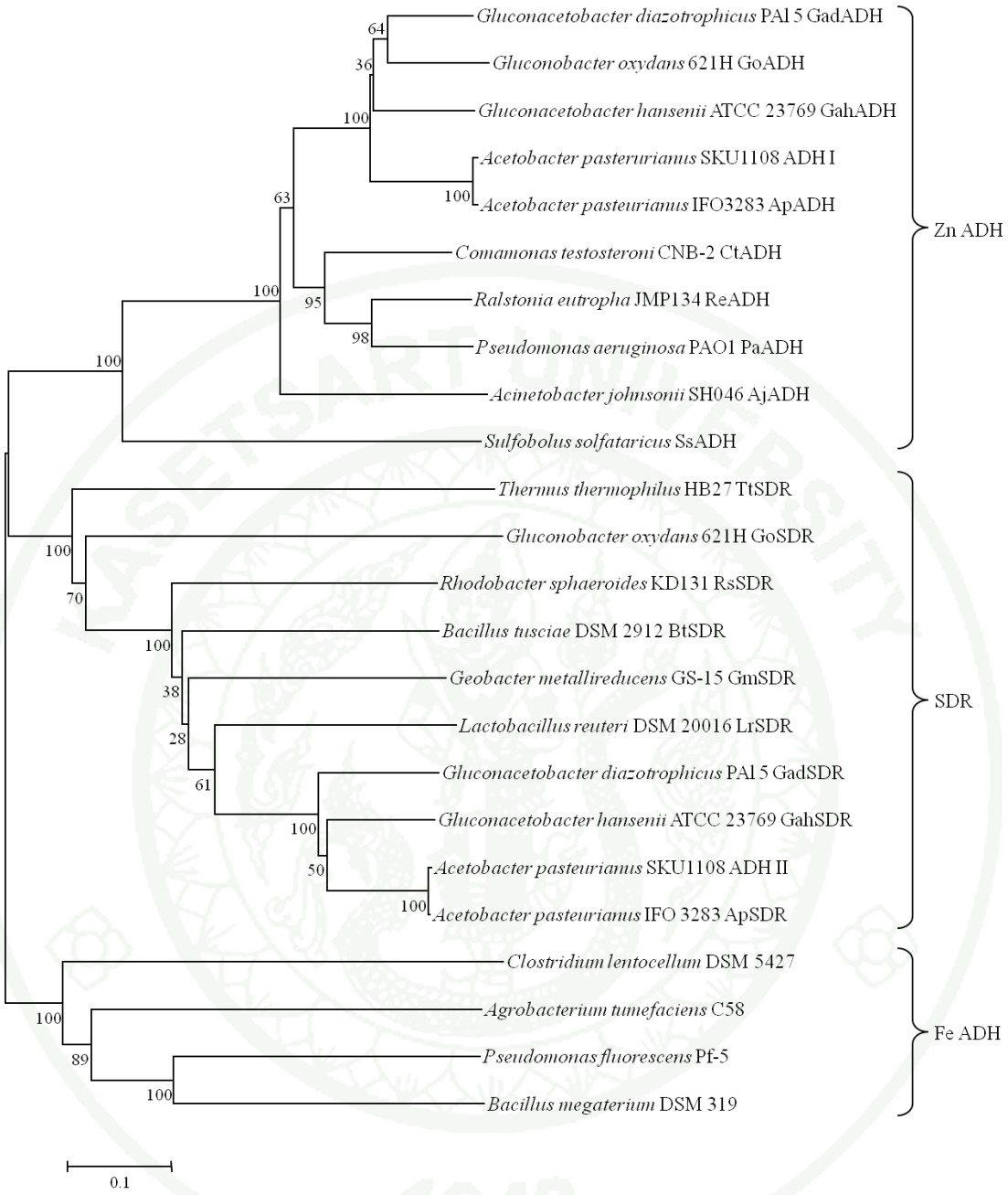


Figure 35 Phylogenetic tree derived from amino acid sequences of NAD(P)$^+$-dependent ADH family of *A. pasteurianus* SKU1108 and related bacteria. Zn-ADH, Zn-dependent alcohol dehydrogenase; SDR, short-chain dehydrogenase/reductase; Fe-ADH, iron-containing alcohol dehydrogenase. The analysis based on the neighbor-joining method was performed with the MEGA 4 software. The amino acid sequences were obtained from GenBank database.

2.5 Disruption of NAD^+-*adh* genes

The recombinant plasmids carrying the NAD^+-*adh* disrupted genes were constructed with the insertion of an antibiotic resistant cassette into the genomic NAD^+-*adh* genes of the CN6-2 strain, as previously described in Materials and Methods. Two recombinant plasmids were isolated and the resulting plasmids were designated as pGEM-TadhI::Tc^r and pGEM-TadhII::Km^r. PCR amplification was used to confirm as shown in Figure 36. The results showed that 1.9 kb Tc^r and 0.95 kb Km^r cassettes were inserted at the internal part of *adhI* and *adhII* genes, respectively. These plasmids were used to individually introduce into CN6-2 strain (NTG mutant of *A. pasteurianus* SKU1108) in order to construct the NAD^+-*adh* gene disruptants. The *adhI*, *adhII* and double disruptants were designated as DPI, DPII, and DPI-II, respectively, and the PCR amplification was used to confirm the genotype of these disruptants. The 2.9 kb PCR product was found from DPI and DPI-II strains by using primers for *adhI* gene, which showed 1.9 kb larger than parental strain and corresponded with the insertion of Tc^r cassette at *adhI* gene (Figure 37A). On the other hand, the PCR product amplified with two primers for *adhII* gene from DPII and DPI-II (1.65 kb) exhibited the insertion of 0.95 kb Km^r cassette compared with parental strain (Figure 37B).

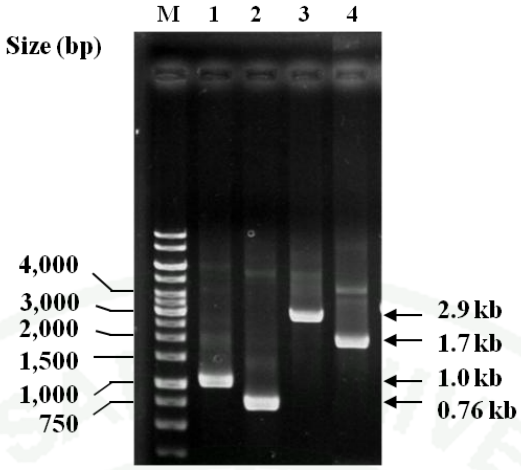


Figure 36 Agarose gel electrophoresis of PCR products amplified from recombinant plasmids carrying NAD$^+$-*adh* genes. PCR amplification was carried out by using two specific primer sets, FadhI-3 and RadhI (lane 1 and 3) or FadhII-3 and RadhII (lane 2 and 6). Lane M = 1 kb DNA ladder, 1 = pGEM-TadhI, 2 = pGEM-TadhII, 3 = pGEM-TadhI::Tc^r, 4 = pGEM-TadhII::Km^r.

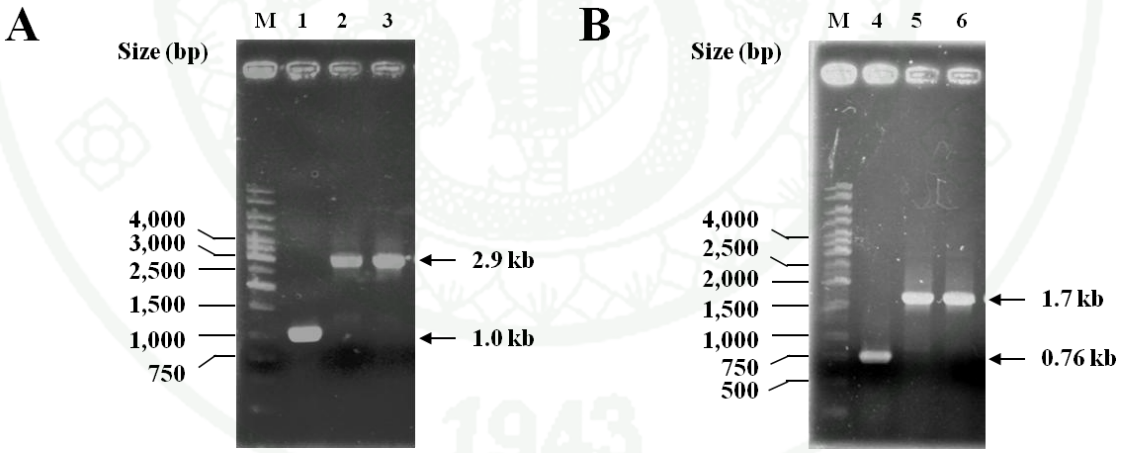


Figure 37 Agarose gel electrophoresis of PCR products amplified from NAD$^+$-*adh* genes disruptants. PCR amplification was carried out by using two specific primer sets, FadhI-3 and RadhI (A) or FadhII-3 and RadhII (B). Lane M = 1 kb DNA ladder, 1 and 4 = CN6-2, 2 = DPI, 3 and 6 = DPI-II, 5 = DPII.

2.6 Growth characteristics of NAD^+-*adh* gene disruptants

Time course of growth and acidity in the culture broth of the cells grown in the YPGD medium both with and without 4% ethanol supplement were compared between the CN6-2 strain and its derivative disruptants (Figure38). The parental strain, CN6-2, grew well even in the absence of any capacity to produce acetic acid in the YPGD medium containing the 4% ethanol supplement. Unlike the parental strain, growth of all NAD^+-*adh* gene disruptants in the same medium significantly decreased. Conversely, acetic acid was detected at a concentration of approximately 0.4% in the *adhI* gene disruptant DPI but not in the other disruptants. Furthermore, growth and acetic acid production in a YPG medium containing 0 or 4% ethanol of those strains were compared (Figure 39). The result was similar to the observation when the cells were grown in YPGD medium except for the *adhII* gene disruptant DPII. This mutant could not grow in YPG medium but grew well when ethanol occurred. Furthermore, the results from the present study showed that the inactivation of NAD^+-*adhII* genes caused a severe reduction in growth in YPG medium, but not in YPG medium supplemented with ethanol. This phenomenon is explained by the absence of appropriate carbon source in YPG medium for growth of the *adhII* gene disruptant. It has been suggested that ADH I, which still active in the *adhII* gene disruptant, cannot oxidize glycerol in that medium. In ethanol medium, the result suggested that ADH I can use ethanol as its substrate hence the *adhII* gene disruptant exhibited normal growth comparison to the parental strain. The results implied that ADH II not only important in ethanol assimilation but also involved in glycerol utilization. In addition, the decrease of growth was observed in the *adhI* gene disruptant grown in ethanol medium indicating that ADH II acts instead of ADH I in the initial stage of ethanol assimilation (Chinnawirotpisan *et al*., 2003c). This phenomenon is dissimilar to the inactivation of *adhA* gene encoding Zn-dependent ADH of *Corynebacterium glutamicum*, which displayed no growth in ethanol medium (Arndt and Eikmanns, 2007). However, homologue proteins of ADH I and ADH II with low similarity were observed in the draft genome of *A. pasteurianus* SKU1108. The possibility of some homologous proteins may work instead of ADH I and ADH II in NAD^+-*adh* gene disruptants in ethanol medium.

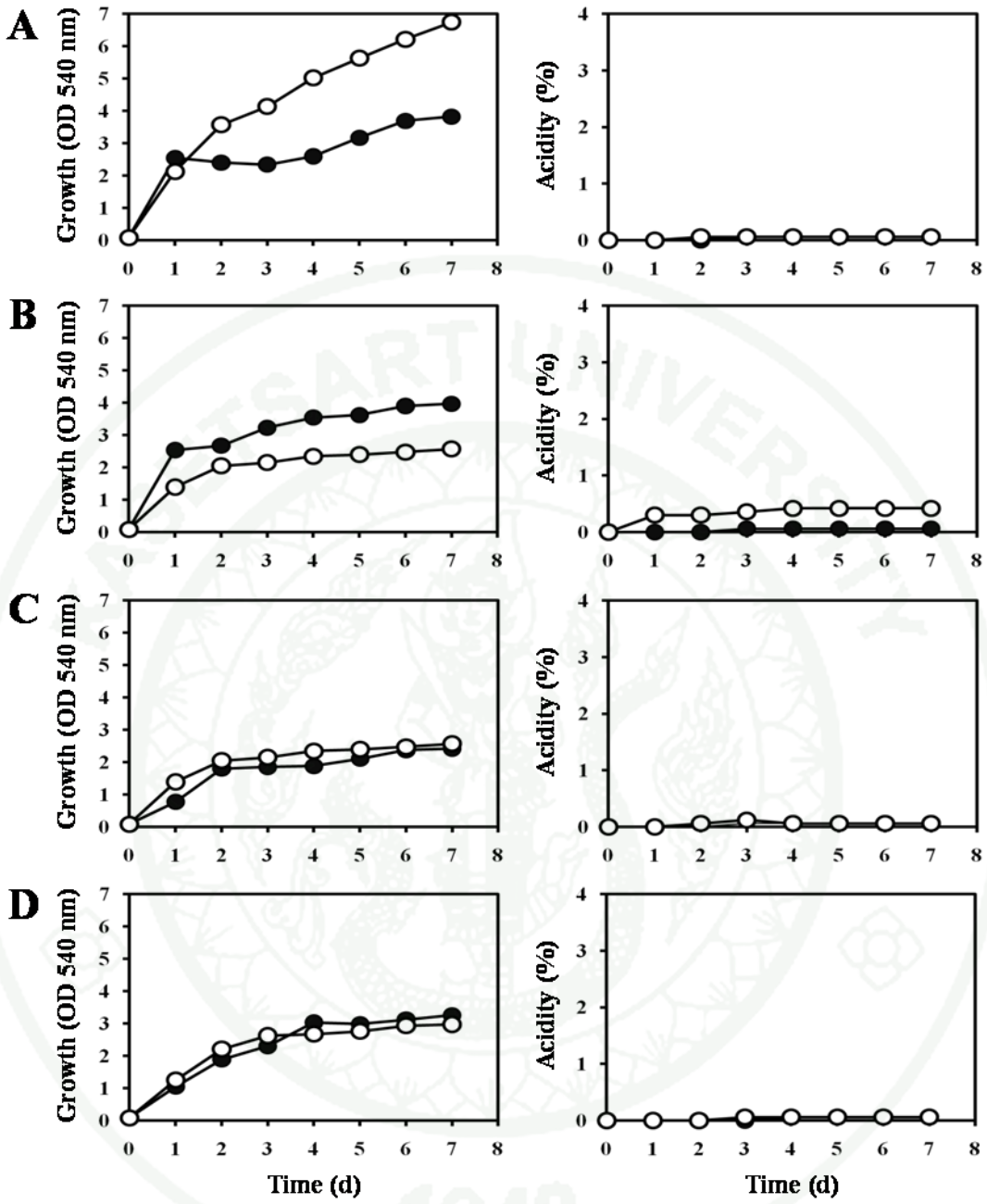


Figure 38 Time course of growth (left) and acidity in culture broth (right) of the cells grown in YPGD medium. CN6-2 (A), *adhI* gene disruptant (B), *adhII* gene disruptant (C) and *adhI-adhII* double disruptant (D) were cultured in 100 ml of YPGD medium (●) supplemented with 4% ethanol (○). Bacterial growth was measured by spectrophotometer at 540 nm. Acetic acid concentration was titrated under the standard conditions as described in Materials and Methods.

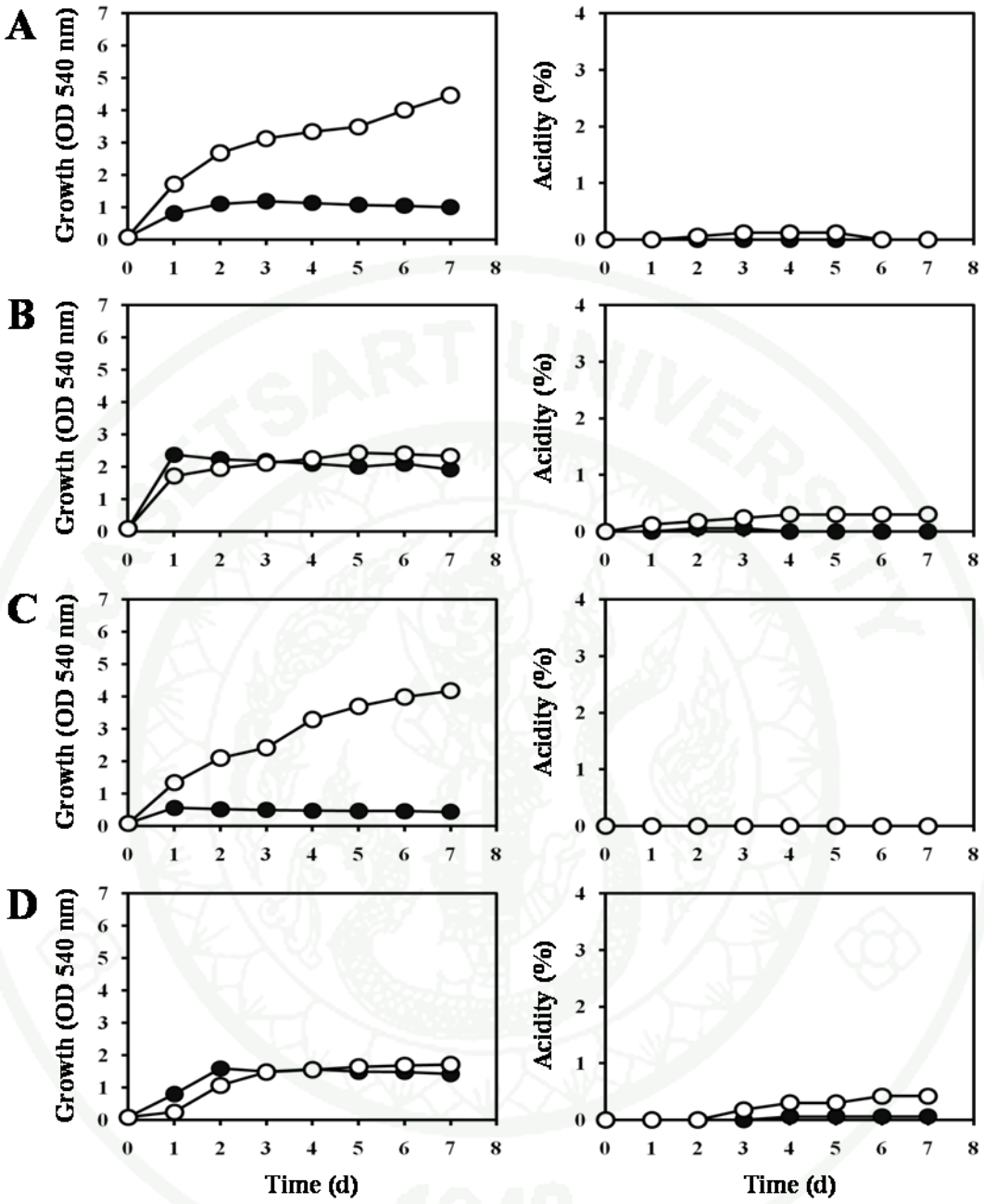
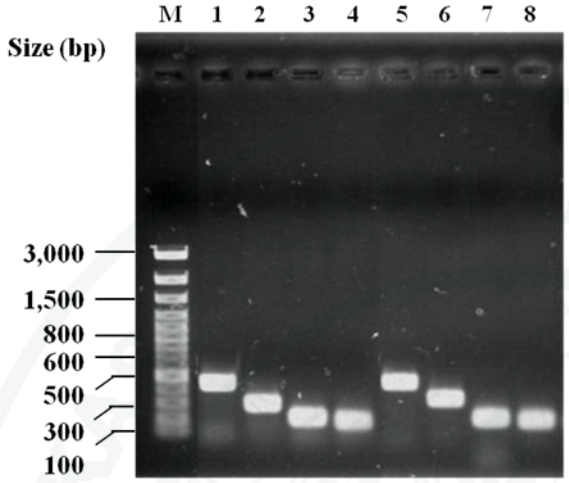


Figure 39 Time course of growth (left) and acidity in culture broth (right) of the cells grown in YPG medium. CN6-2 (A), *adhI* gene disruptant (B), *adhII* gene disruptant (C) and *adhI-adhII* double disruptant (D) were cultured in 100 ml of YPG medium (●) supplemented with 4% ethanol (○). Bacterial growth was measured by spectrophotometer at 540 nm. Acetic acid concentration was titrated under the standard conditions as described in Materials and Methods.

2.7 Deletion analysis of the regulatory region of NAD^+-*adh* genes

Eight DNA fragments of the different lengths of the upstream region of the NAD^+-*adhI* and *adhII* genes were obtained by PCR amplification (Figure 40) and inserted into the promoter probe vector pGSA in order to analyze the regulatory region of the genes expression. The recombinant plasmids were confirmed with nucleotide sequencing. The structure of the obtained DNA fragments is shown in Figure 41A and 41B. Each of them was individually introduced into *E. coli* DH5α and determined for the β-galactosidase activity. The transformants were grown for 18 h with shaking (200 rpm) at 37ºC in 100 ml of a LB medium containing 0, 2 or 4% ethanol and 12.5 μg/ml gentamicin. The result showed that β-galactosidase activity was enhanced both in the pGSAPadhI-2 and pGSAPadhI-3 compared to pGSAPadhI-1 (Figure 41C). The increase of β-galactosidase activity was determined by the plasmid harboring shorten upstream region from the ATG codon with the exception of the pGSAPadhI-4 which possesses 81 bp upstream from the ATG start codon of the *adhI* gene. An analogous result was observed in the transformants harboring pGSAPadhII-1 to 4 (Figure 41D), with the highest β-galactosidase activity detected in pGSAPadhII-3 carrying 92 bp upstream from the ATG start codon of the *adhII* gene. In addition, a considerable decrease was detected in pGSAPadhII-4 with 74 bp in the upstream region. These results suggested that some elements important for transcription exist at the position 81-105 bp upstream from the ATG start codon of *adhI* gene, and in the region of 74-92 bp upstream from the ATG start codon of the *adhII* gene. In addition, there are some elements which repress the transcription of the genes were suggested to be located at the position 105-203 bp and 92-228 bp upstream from the ATG start codon of the *adhI* and *adhII* genes, respectively. However, the NAD^+-*adh* genes seem to be induced by ethanol with the similar degree in pGSAPadhI-1, I-2, I-3 and pGSAPadhII-1, II-2, II-3 (Fig. 6C and D) suggesting that the elements concerning the ethanol regulation do not locate around the regions. The results from ethanol induction and promoter analysis suggested that the promoter of the both NAD^+-*adh* genes from *A. pasteurianus* SKU1108 can function also in *E. coli*. The decrease of growth in *E. coli* grown in ethanol medium due to it is unable to grow well in the high concentration of ethanol. The results obtained from *E. coli* reflect physiology of *E. coli*, not of *A. pasteurianus*. Because of the cytoplasmic ADHs in acetic acid bacteria

have been hardly studied up to the present, thus, no further matter can be mentioned concerning the physiology of *A. pasteurianus*.



Lane	Distance from ATG (bp)
1	356
2	203
3	105
4	81
5	348
6	228
7	92
8	74

Figure 40 Agarose gel electrophoresis of PCR products containing the possible promoter region of NAD^+-*adh* genes. PCR amplification was carried out by using eight specific primer sets as previously described in Materials and Methods. Distance of the start position from ATG start codon of each fragment was explained. These DNA fragments composed of a different length of upstream region and the 63 nt or 60 nt downstream of *adhI* (lane 1-4) and *adhII* genes (lane 5-8), respectively.

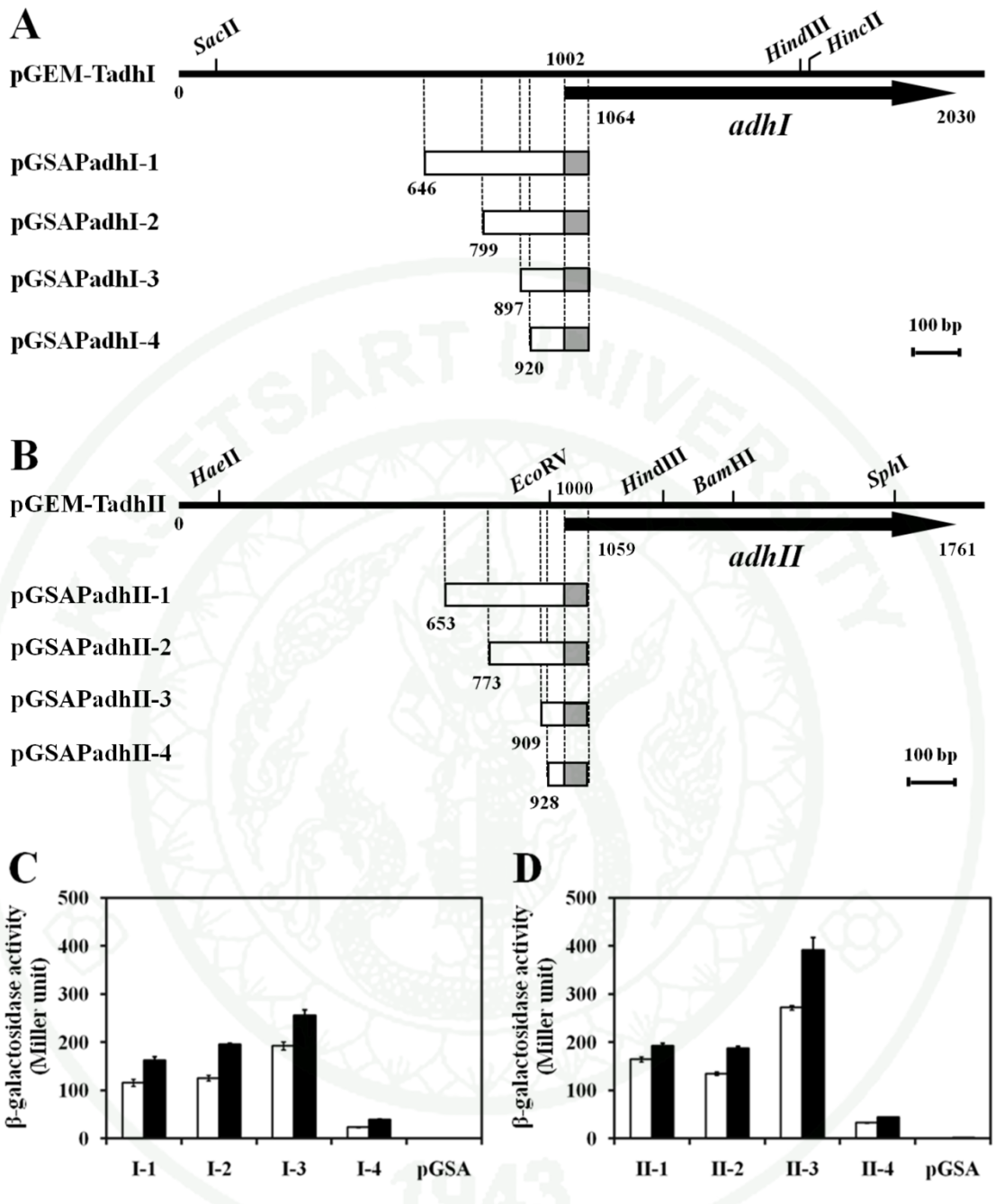


Figure 41 Structure of DNA fragments carrying the possible promoter region of NAD^+-*adhI* (A) and *adhII* genes (B) and comparison of β-galactosidase activity in *E. coli* DH5α harboring the various transcriptional *adhI-lacZ* (C) and *adhII-lacZ* fusion plasmids (D). These strains were cultured with shaking at 37ºC in 100 ml of LB medium containing 0% (white) or 4% (black) ethanol and 12.5 μg/ml gentamicin. Construction procedures and enzymatic analysis were described in Materials and Methods

2.8 Induction analysis of NAD^+-*adh* genes

It was speculated that ethanol might be involved in the induction of NAD^+-*adh* genes expression. In order to investigate the ethanol induction, the expression of NAD^+-*adh* genes were examined in different growth phases by monitoring the β-galactosidase activity in *E. coli* DH5α harboring transcriptional *adh-lacZ* fusion plasmids. The 0.58 kb PCR products which contained the possible promoter region of *adhI* and *adhII* genes were amplified (Figure 42) and inserted upstream of the *lacZ* structural gene on a promoterless *lacZ* vector pGSA. The *adhI* gene promoter (*PadhI*) DNA fragment carried a 520 nt upstream to 63 nt downstream of the ATG start codon of *adhI* gene, while the *PadhII* DNA fragment held a 519 nt upstream to 60 nt downstream of ATG start site of *adhII* gene. The procedures for construction of transcriptional fusion plasmids were described in the materials and methods above and the recombinant plasmids were confirmed with nucleotide sequencing. The β-galactosidase activity was found to be significantly higher than that when the cells grown in ethanol medium (Figure 43). The highest activity was detected at an early stationary phase of cultivation (~24 h). The results showed that ethanol could induce *adhI* and *adhII* genes expression approximately 1.5 and 2.0 times, respectively, and *adhII* gene promoter activity exceeded that of the *adhI* promoter about 3.5 fold. Thus, the significantly elevated promoter activity of *adhII* gene in comparison to the *adhI* gene under ethanol induction condition implied that ADH II is a dominant enzyme involved in the ethanol assimilation rather than ADH I.

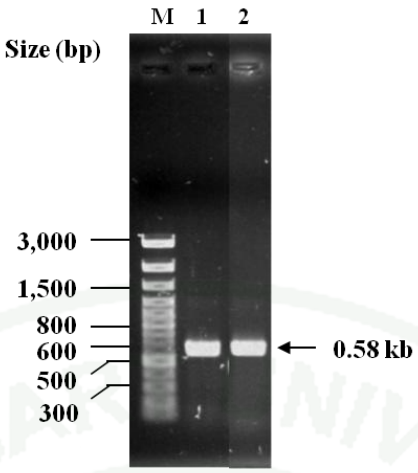


Figure 42 Agarose gel electrophoresis of PCR products carrying the 0.58-kb NAD^+-*adh* genes. PCR amplification was carried out by using two specific primer sets, FPadhI-P and RPadhI-B (lane 1) or FPadhII-P and RPadhII-B (lane 2). Lane M = 100 bp DNA ladder, 1 = *PadhI*, 2 = *PadhII*.

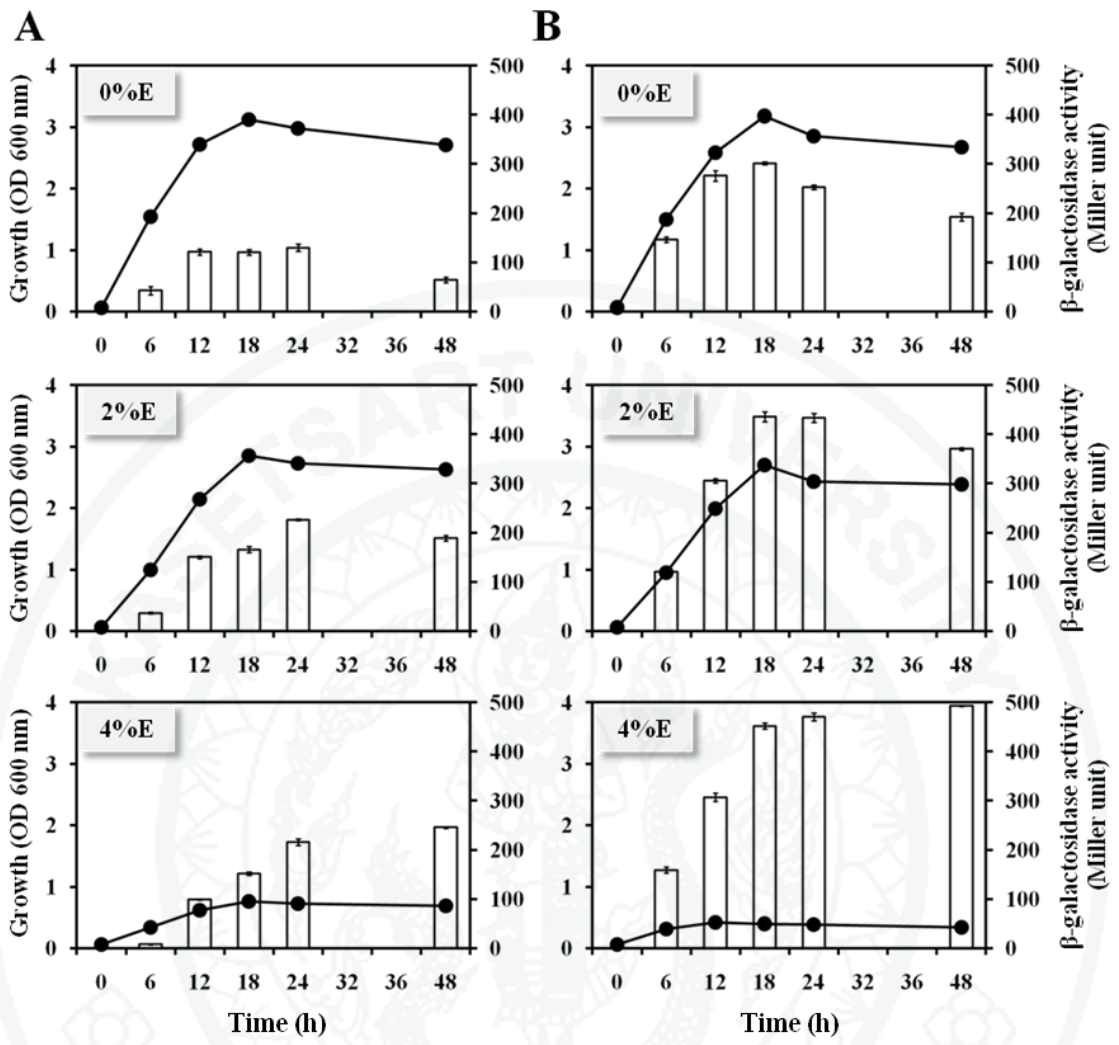


Figure 43 Time course of growth (-●-) and β-galactosidase activity (□) of *E. coli* DH5α harboring pGSAPadhI (A) and pGSAPadhII (B) fusion plasmids. These strains were culture with shaking at 200 rpm at 37ºC in 100 ml of LB medium supplemented 0, 2 or 4% ethanol and 12.5 μg/ml gentamicin. β-galactosidase activity was measured enzymatically as described in Materials and Methods.

CONCLUSION AND RECOMMENDATIONS

Conclusion

In this study, the function of subunit III of quinoprotein alcohol dehydrogenase (PQQ-ADH) and the functional correlation between PQQ-ADH and NAD^+-ADH from *Acetobacter pasteurianus* SKU1108 were clarified. The role of subunit III on the function of PQQ-ADH was investigated by construction of *adhS* gene disruptant with insertion of Km^r cassette into the *adhS* gene on genomic DNA of *A. pasteurianus* SKU1108. The *adhS* gene disruptant completely lost its PQQ-ADH activity and acetate-producing ability but retained acetic acid toleration. In addition to PQQ-ADH, NAD^+-ADHs detected in small amounts in cytoplasm of *A. pasteurianus* SKU1108 were induced by ethanol in a PQQ-ADH deficient mutant. Heme staining and immunoblot analysis of both membrane and soluble fractions with anti-ADH subunit III suggested that ethanol did not affect the *adhS* gene expression but induced PQQ-ADH activity. Over-expressed *adhS* did not enhance acetic acid production in both the wild type and the *adhS* gene disruptant. The defect of the PQQ-ADH activity in the *adhS* gene disruptant suggested that the smallest subunit is essential for ethanol oxidation. It has been proposed that mutation in PQQ-ADH led a global metabolic change from ethanol oxidation to ethanol assimilation as an adaptive response to ethanol. With regard to acetic acid toleration, it appears that acetic acid assimilation or over-oxidation is involved in acetic acid toleration. The study revealed that only *adhA* and *adhB* but not *adhS* are involved in acetic acid toleration. In addition, deletion analysis of upstream region of *adhS* gene suggested that its tentative promoter(s) might be located at around 118-268 bp upstream from an initiation codon. Random mutagenesis of *adhS* gene revealed that complete loss of PQQ-ADH activity and ethanol oxidizing ability were observed in the mutants' lack of the 140 and 73 amino acid residues at the C-terminal, whereas the lack of 22 amino acid residues at the C-terminal affected neither the PQQ-ADH activity nor ethanol oxidizing ability. In addition, some amino acid substitutions such as Leu18Gln, Ala26Val, Val36Ile, Val54Ile, Gly55Asp, Val70Ala and Val107Ala did not show any affect on PQQ-ADH activity and ethanol oxidizing ability. Interestingly, alteration of Thr104Lys led to a complete loss of ethanol oxidizing ability. However, point mutation at the possible

promoter region also exhibited low PQQ-ADH activity and ethanol oxidizing ability. This result suggests that 104Thr might be involved in molecular coupling with subunit I in order to construct active ADH complex, whereas 22 amino acid residues at C-terminal may be not necessary for PQQ-ADH activity.

Moreover, functional analysis of PQQ-ADH and NAD^+-ADHs indicated that PQQ-ADH was intensively involved in acetic acid production, while NAD^+-ADHs worked for ethanol assimilation through TCA and glyoxylate cycles. Moreover, NAD^+-ADHs were inducible enzymes in PQQ-ADH deficient mutant when it was cultured in the medium containing ethanol. Therefore, it is worthwide to clarify this indication mechanism. In this study, the *adhI* and *adhII* genes encoding NAD^+-ADH I and ADH II, respectively, were cloned into pGEM-T®-Easy Vector and characterized, in order to investigate the regulation of these genes expression. The 2.1 and 1.86 kb DNA fragment carrying *adhI* and *adhII* genes, respectively, were obtained. Open Reading Frame (ORF) analysis revealed that the *adhI* gene consists of 1,029 bp encoding 342 amino acid residues, whereas the *adhII* gene composes of 762 bp encoding 253 amino acid residues. ADH I and ADH II share 99.71 and 99.60% identity, respectively, with the same proteins of *A. pasteurianus* IFO 3283. The deduced amino acid sequence of ADH I contains the zinc-containing alcohol dehydrogenase signature which contains the conserved second ligand (65His) of catalytic zinc atom. In addition, the conserved sequence of glycine residues directly involved in the cofactor-binding domain and a highly conserved aspatate for NAD^+ binding were found as Gly-Val-Gly-Gly-Leu-Gly (position 176 to 181) and 199Asp, respectively. The deduced amino acid sequence of ADH II consists of the short-chain dehydrogenase/reductase (SDR) family signature and the Rossmann-fold NAD(P)H/ $NADP^+$ binding (NADB) domain. The Rossmann-fold motif (Gly-Ala-Ala-Asn-Gly-Ile-Gly) and a common Ser-X_n-Tyr-X-X-X-Lys sequence in active site were found at position 13 to 19 and 142 to 159, respectively. In addition, the discriminated Asp for NAD(H) was found at position 37. Taken together, all results indicated that the ADH I of *A. pasteurianus* SKU1108 belongs to group I, Zn-dependent long chain ADHs, while the ADH II is identical to group II, SDRs, of NAD^+-dependent ADHs and extended to the cD2 subfamily of the classical SDRs. The defect of NAD^+-ADH II caused a severe reduction of growth in an ethanol medium, whereas a significant

decrease was observed in a NAD^+-ADH I deficient mutant. It has been proposed that ADH II rather than ADH I plays a dominant role in the ethanol assimilation of acetic acid bacteria, in addition, ethanol is likely to induce the transcription of NAD^+-*adh* genes. The possible promoter regions of *adhI* and *adhII* genes might be located at approximately 81-105 bp and 74-92 bp, respectively, from its ATG start codon. There are some elements which repress the transcription of the genes were suggested to be located at the position 105-203 bp and 92-228 bp upstream from the ATG start codon of the *adhI* and *adhII* genes, respectively, and caused the repression of those genes expression in wild type carrying normal PQQ-*adh* gene.

Recommendations

From this study, the function and the essential amino acids involved in molecular coupling with subunit I of PQQ-ADH subunit III were clarified. The 104Thr and 73, but not 22 amino acids at C-terminal are essential for PQQ-ADH activity. In order to elucidate this phenomenon precisely, prediction of three dimensional structure of the PQQ-ADH is recommended. In addition to PQQ-ADH, an additional alcohol dehydrogenase (NAD^+-ADH) was also investigated. The growth of NAD^+-ADH genes disruptants in the ethanol medium was remarkably decreased. To further clarify this observation, the key enzyme activities in TCA and glyoxylate cycles should be analyzed. Finally, it is hoped that the results obtained from this study will not only provide basic knowledge in understanding function of *adhS* gene encoding subunit III and the gene expression of NAD^+-*adh* genes in the thermotolerant acetic acid bacteria but also benefit to the basic knowledge implicated to strain improvement for industrial vinegar production.

LITERATURE CITED

Adachi, O., E. Miyagawa, E. Shinagawa, K. Matsushita and M. Ameyama. 1978a. Purification and properties of particulate alcohol dehydrogenase from *Acetobacter aceti*. **Agric. Biol. Chem.** 42: 2331-2340.

_____, K. Tayama, E. Shinagawa, K. Matsushita and M. Ameyama. 1978b. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans.* **Agric. Biol. Chem.** 42: 2045-2056.

_____, D. Moonmangmee, E. Shinagawa, H. Toyama., M. Yamada and K. Matsushita. 2003a. New quinoproteins in oxidative fermentation. **Biochim. Biophys. Acta** 1647: 10-17.

_____, _____, H. Toyama., M. Yamada, E. Shinagawa and K. Matsushita. 2003b. New developments in oxidative fermentation. **Appl. Microbiol. Biotechnol.** 60: 643-653.

_____, S. Tanasupawat, N. Yoshihara, H. Toyama and K. Matsushita. 2003c. 3-Dehydroquinate production by oxidative production and further conversion of 3-dehydroquinate to the intermediates in the shikimate pathway. **Biosci. Biotechnol. Biochem.** 67: 2124-2131.

_____, Y. Ano, H. Toyama and K. Matsushita. 2006a. High shikimate production from quinate from two enzymatic systems of acetic acid bacteria. **Biosci. Biotechnol. Biochem.** 70: 2579-2582.

_____, _____, _____, and _____. 2006b. Purification and properties of NADP-dependent shikimate dehydrogenase from *Gluconobacter oxydans* IFO 3244 and its application to enzymatic shikimate production. **Biosci. Biotechnol. Biochem.** 70: 2786-2789.

Adachi, O., Y. Ano, H. Toyama and K. Matsushita. 2006c. Enzymatic preparation of metabolic intermediates, 3-dehydroquinate and 3-dehydroshikimate, in the shikimate pathway. **Biosci. Biotechnol. Biochem.** 70: 3081-3083.

_____, _____, _____, and _____. 2008. A novel 3-dehydroquinate dehydrogenase catalyzing extracellular formation of 3-dehydroshikimate by oxidative fermentation of *Gluconobacter oxydans* IFO 3244. **Biosci. Biotechnol. Biochem.** 72: 1475-1482.

Alwine, J.C., D.J. Kemp and G.R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. **Proc. Natl. Acad. Sci. U.S.A.** 74: 5350-5354.

Ammendola, S., C.A. Raia, C. Caruso, L. Camardella, S. D'Auria, M. De Rosa and M. Rossi. 1992. Thermostable NAD(+)-dependent alcohol dehydrogenase from *Sulfolobus solfataricus*: gene and protein sequence determination and relationship to other alcohol dehydrogenases. **Biochemistry** 31: 12514-12523.

Anonymous. 2010. **Technical Manual: pGEM®-T and pGEM®-T Easy Vector Systems.** Available Source: http://www.promega.com/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol/, March 19, 2011.

Arndt, A. and B.J. Eikmanns. 2007. The alcohol dehydrogenase gene *adhA* in *Corynebacterium glutamicum* is subject to carbon catabolite repression. **J. Bacteriol.** 189: 7408-7416.

Azuma,Y., A. Hosoyama, M. Matsutani, N. Furuya, H. Horikawa, T. Harada, H. Hirakawa, S. Kuhara, K. Matsushita, N. Fujita and M. Shirai. 2009. Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. **Nucleic Acids Res.** 37: 5768-5783

Bernt, E. and I. Gutmann. 1974. Determination with alcohol dehydrogenase and NAD, pp. 1499. *In* H.U. Bergmeyer, ed. **Methods of enzymatic analysis.** Vol. III, Academic Press, New York.

Bowen, B., J. Steinberg, U.K. Laemmli and H. Weintraub. 1980. The detection of DNA-binding proteins by protein blotting. **Nucleic Acids Res.** 8: 1-20.

Brown, T. A. 1998. **Gene Cloning.** 2nd ed. Chapman & Hall, New York.

Burnette, W.N. 1981. Western blotting: electrophoresis transfer of proteins from sodium dodecyl sulfate -polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. **Anal. Biochem.** 112: 195-203.

Carter, P. 1991. Mutagenesis facilitated by the removal or introduction of unique restriction sites. *In* M.J. McPherson, ed. **Directed Mutagenesis: A Practical Approach.** Oxford University Press, New York. pp. 282.

Chinnawirotpisan, P. 2003a. **Functional Analysis and Characterization of the Purified Quinoprotein Alcohol Dehydrogenase and Inducible NAD-Dependent Alcohol Dehydrogenases from A*cetobacter pasteurianus* SKU1108 and Its Mutants**. Ph.D. Thesis, Kasetsart University.

_____, G. Theeragool, S. Limtong, H. Toyama, O. Adachi and K. Matsushita. 2003b. Quinoprotein alcohol dehydrogenase is involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in *Acetobacter pasteurianus* SKU1108. **J. Biosci. Bioeng.** 96: 564-571.

Chinnawirotpisan, P., K. Matsushita, H. Toyama, O. Adachi, S. Limtong and G. Theeragool. 2003c. Purification and characterization of two NAD-dependent alcohol dehydrogenase (ADHs) induced in the quinoprotein ADH-deficient mutant of *Acetobacter pasteurianus* SKU1108. **Biosci. Biotechnol. Biochem.** 67: 958-965.

Clark, D.P. 1989. The fermentation pathways of *Escherichia coli.* **FEMS Microbiol Rev.** 63: 223-234.

_____. 1992. Evolution of bacterial alcohol metabolism, pp. 105-114. *In* R.P. Mortlock, ed. **The Evolution of Metabolic Function**. CNC Press, Canada.

Conner, H.A. and R.J. Allgeier. 1976. Vinegar: its history and development. **Adv. Appl. Microbiol.** 20: 81-133.

Conway, T. and L.O. Ingram. 1989. Similarity of *Escherichia coli* propanediol oxidoreductase (*fucO* product) and an unusual alcohol dehydrogenase from *Zymomonas mobilis* and *Saccharomyces cerevisiae.* **J. Bacteriol.** 171: 3744-3759.

Cook, K.E. and J.R. Colvin. 1980. Evidence for a beneficial influence of cellulose production on growth of *Acetobacter xylinum* in liquid medium. **Curr. Microbiol.** 3: 203-205.

Coucheron, D.H. 1991. An *Acetobacter xylinum* insertion sequence element associated with inactivation of cellulose production. **J. Bacteriol.** 173: 5723-5731.

Dailly, Y., F. Mat-Jan and D.P. Clark. 2001. Novel alcohol dehydrogenase activity in a mutant of *Salmonella* able to use ethanol as sole carbon source. **FEMS Microbiol. Lett.** 201: 41-45.

Dale, J.W. 1994. **Molecular Genetics of Bacteria.** John Wiley & Sons, Chicheter. pp. 287.

Dangler, C.A. 1996. **Nucleic Acid Analysis: Principles and Bioapplications.** John Wiley & Sons, Inc., New York.

Debelle, F. and S.B. Sharma. 1986. Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specificity of nodulation. **Nucl. Acids Res.** 14: 7453-7472.

De Ley, J., J. Swing and F. Gosselé. 1984. Genus *Acetobacter*, pp. 268-274. *In* N.R. Krieg and J.G. Holt, eds., **Bergey's Manual of Systematic Bacteriology.** Vol. I, Williams & Wilkins Co., Baltimore.

Deppenmeier, U., M. Hoffmeister and C. Prust. 2002. Biochemistry and biotechnological applications of *Gluconobacter* strains. **Appl. Microbiol. Biotechnol.** 59: 1513-1533.

_____ and A. Ehrenreich. 2009. Physiology of Acetic Acid Bacteria in Light of the Genome Sequence of *Gluconobacter oxydans*. **J. Mol. Microbiol. Biotechnol.** 16: 69-80.

Dully, J.R. and P.A. Grieve. 1975. A simple technique for eliminating interference by detergents in Lowry method of protein determination. **Ann. Biochem**. 64: 136-141.

Fiers, W., R. Contreras, F. Duerinck, G. Haegeman, D. Iserentant, J. Merregaert, W. Min Jou, F. Molemans, A. Raeymaekers, A. Van den Berghe, G. Volckaert and M. Ysebaert. 1976. Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. **Nature** 260: 500-507.

Filling, C., K.D. Berndt, J. Benach, S. Knapp, T. Prozorovski, E. Nordling, R. Ladenstein, H. Jörnvall and U. Oppermann. 2002. Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. **J. Biol. Chem.** 227: 25677-25684.

Fischer, R.J., J. Helms and P. Diirre. 1993. Cloning, sequencing, and molecular analysis of the sol operon of *Closnidium acetobutylicum*, a chromosomal locus involved in solventogenesis. **J. Bacteriol.** 175: 6959-6969.

Frébortová, J., K. Matsushita, T. Yakushi, H. Toyama and O. Adachi. 1997. Quinoprotein alcohol dehydrogenase of acetic acid bacteria: kinetic study on the enzyme purified from *Acetobacter methanolicus*. **Biosci. Biotechnol. Biochem.** 61: 459-465.

Fukaya, H. Takemura, H. Okumura, Y. Kawamura, S. Horinouchi and T. Beppu. 1990. Cloning of genes responsible for acetic acid resistance in *Acetobacter aceti*. **J. Bacteriol.** 172: 2096-2104.

_____, _____, K. Tayama, H. Okumura, Y. Kawamura, S. Horinouchi and T. Beppu. 1993. The *aarC* gene responsible for acetic acid assimilation confers acetic acid resistance on *Acetobacter aceti*. **J. Ferment. Bioeng.** 76: 270- 275.

Gilbert, W. and A. Maxam. 1973. The nucleotide sequence of the *lac* operator. **Proc. Natl. Acad. Sci. U.S.A.** 70: 3581-3584.

Gómez-Manzo, S., M. Contreras-Zentella, A. González-Valdez, M. Sosa-Torres, R. Arreguín-Espinoza and E. Escamilla-Marván. 2008. The PQQ-alcohol dehydrogenase of *Gluconacetobacter diazotrophicus*. **Int. J. Food Microbiol.** 125: 71-78.

Goodlove, P.E, P.R. Cunningham, J. Parker and D.P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. **Gene.** 85: 209-214.

Greenberg, D.E., S.F. Porcella, F. Stock, A. Wong, P.S. Conville, P.R. Murray, S.M. Holland and A.M. Zelazny. 2006. *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*. **Int. J. Syst. Evol. Microbiol.** 56: 2609-2616.

Greenfield, S. and G.W. Claus. 1972. Nonfunctional tricarboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans*. **J. Bacteriol.** 112: 1295-1301.

Gupta, A., V.K. Singh, G.N. Qazi and A. Kumar. 2001. *Gluconobacter oxydans*: its biotechnological applications. **J. Mol. Microbiol. Biotechnol.** 3: 445-456.

Harada, I. and H. Mori. 1971. Mutants of acetic acid bacteria used in vinegar production. **J. Ferment. Technol.** 49: 836-841.

Hardin, C., J. Pinczes, A. Riell, D. Presutti, W. Miller and D. Robertson. 2001. **Cloning, Gene Expression, and Protein Purification: Experimental Procedures and Process Rationale.** Oxford University Press, Oxford.

Henne, A., H. Bruggemann, C. Raasch, A. Wiezer, T. Hartsch, H. Liesegang, A. Johann, T. Lienard, O. Gohl, R. Martinez-Arias, C. Jacobi, V. Starkuviene, S. Schlenczeck, S. Dencker, R. Huber, H. P. Klenk, W. Kramer, R. Merkl, G. Gottschalk and H.J. Fritz. 2004. The genome sequence of the extreme thermophile *Thermus thermophilus*. **Nat. Biotechnol.** 22: 547-553.

Higgins, D. and W. Taylor. 2001. **Bioinformatics: Sequence Structure and Databanks.** 2nd ed. Oxford University Press, Oxford.

Higuchi, R., B. Krummel and R. Saiki. 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. **Nucleic Acids Res.** 16: 7351-7367.

Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. **Gene.** 77: 51-59.

Inoue, T., M. Sunagawa, A. Mori, C. Imai, M. Fukuda, M. Takagi, K. Yano. 1989. Cloning and sequencing of the gene encoding the 72-kilodalton dehydrogenase subunit of alcohol dehydrogenase form *Acetobacter aceti*. **J. Bacteriol.** 171: 3115-3122.

_____, M. Sunagawa, A. Mori, C. Imai, M. Fukuda, M. Takagi and K. Yano. 1992. Cloning and sequencing of the gene encoding the 45-kilodalton subunit of alcohol dehydrogenase from *Acetobacter aceti*. **J. Ferment. Bioeng.** 73: 419-424.

Ismaiel, S., S. Doe, T. Yorifuji, M. Takagi and K. Yano. 1993. Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. **J. Bacteriol.** 175: 5097-5105.

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

Jörnvall, H., B. Person and J. Jeffery. 1981. Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. **Proc. Natl. Acad. Sci. USA.** 78: 4226-4230.

_____, _____ and _____. 1987. Characteristics of alcohol/polyo1 dehydrogenases. **Eur. J. Biochem.** 167: 195-201.

Kadowaki, H., T. Kadowaki, F.E. Wondisford and S.I. Taylor. 1989. Use of polymerase chain reaction catalyzed by *Taq* DNA polymerase for site-specific mutagenesis. **Gene.** 76: 161-166.

Kallberg, Y., U. Oppermann, H. Jörnvall and B. Persson. 2002. Short-chain dehydrogenases/reductases (SDRs) coenzyme-based functional assignments in completed genomes. **Eur. J. Biochem.** 269: 4409-4417.

Kanchanarach, W., G. Theeragool, T. Yakushi, H. Toyama, O. Adachi and K. Matsushita. 2010. Characterization of thermotolerant *Acetobacter pasteurianus* strains and their quinoprotein alcohol dehydrogenases. **Appl. Microbiol. Biotechnol.** 85: 741-751.

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

Kondo, K., T. Beppu, S. Horinouchi. 1995. Cloning, sequencing, and characterization of the gene encoding the smallest subunit of the three-component membrane-bound alcohol dehydrogenase from *Acetobacter pasteurianus*. **J. Bacteriol.** 177: 5048-5055.

_____ and S. Horinuochi. 1997a. Characterization of the genes encoding the three component membrane bound alcohol dehydrogenase from *Gluconobacter suboxydans* and their expression in *Acetobacter pasteurianus*. **Appl. Environ. Microbiol.** 63: 1131-1138.

_____ and _____. 1997b. A new insertion sequence IS*1452* from *Acetobacter pasteurianus*. **Microbiol.** 143: 539-546.

Kretzschmar, U., A. Rückert, J. Jeoung and H. Görisch. 2002. Malate:quinone oxidoreductase is essential for growth on ethanol or acetate in *Pseudomonas aeruginosa*. **Microbiology** 148: 3839–3847.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembling of the head of bacteriophage T4. **Nature** 227: 680–685.

Lakso, D.R., C. Schwerdel, J.E. Bailey and U. Sauer. 1997. Acetate-specific stress response in acetate-resistant bacteria: an analysis of protein patterns. **Biotechnol. Prog.** 13: 519-523.

Leisinger, T. 1965. Untersuchungen zur Systematik und Stoffwechsel der Essigsäurebakterien. **Zentbl. Bakteriol. Parasitenkd. Infektionskrankh. Hyg. Abt. II.** 119: 329-376 (in German).

Lewin, B. 1998. **Gene VI.** Oxford University Press, New York. pp. 1260.

Lim, S.K., S.J. Kim, S.H. Cha, Y.K. Oh, H.J. Rhee, M.S. Kim and J.K. Lee. 2009. Complete genome sequence of *Rhodobacter sphaeroides* KD 131. **J. Bacteriol.** 191: 1118-1119.

Lin, S., A. Gangloff, Y. Huang and B. Xie. 1999. Electrophoresis of hydrophobic proteins. **Anal. Chim. Acta** 383: 101-107.

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

Liu, X., Y. Dong, J. Zhang, A. Zhang, L. Wang and L. Feng. 2009. Two novel metal-independent long-chain alkyl alcohol dehydrogenases from *Geobacillus thermodenitrificans* NG80-2. **Microbiolgy** 155: 2078-2085.

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

Ma,Y.F., Y. Zhang, J.Y. Zhang, D.W. Chen, Y. Zhu, H. Zheng, S.Y. Wang, C.Y. Jiang, G.P. Zhao and S.J. Liu. 2009. The complete genome of *Comamonas testosteroni* reveals its genetic adaptations to changing environments. **Appl. Environ. Microbiol.** 75: 6812-6819.

Madigan, M.T., J.M. Martinko and J. Parker. 2000. **Brock Biology of Microorganisms.** 9th ed. Prentice Hall International, Inc., New Jersey.

Marx, C.J. and M.E. Lidstrom. 2001. Development of improved versatile broad host-range vectors for use in methylotrophs and other gram-negative bacteria. **Microbiology.** 147: 2065-2075.

Masud, U., K. Matsushita and G. Theeragool. 2010. Cloning and functional analysis of adhS gene encoding quinoprotein alcohol dehydrogenase subunit III from *Acetobacter pasteurianus* SKU1108. **Int. J. Food Microbiol.** 139: 39-49.

Matsushita, K., Y.I. Nagatani, E. Shinagawa, O. Adachi and M. Ameyama. 1991. Reconstitution of the ethanol oxidase respiratory chain in membranes of quinoprotein alcohol dehydrogenase-deficient *Gluconobacter suboxydans* subsp. α strains. **J. Bacteriol.** 173: 3440-3445.

_____, H. Ebisuya, M. Ameyama and O. Adachi. 1992a. Change of the terminal oxidase from cytochrome a_1 in shaking cultures to cytochrome *o* in static cultures of *Acetobacter aceti*. **J. Bacteriol.** 174: 122-129.

_____, K. Takahashi, M. Takahashi, E. Shinagawa, M. Ameyama and O. Adachi. 1992b. Methanol and ethanol oxidase respiratory chains of the methylotrophic acetic acid bacterium, *Acetobacter methanolicus*. **J. Biochem.** 111: 739-747.

Matsushita, K., Y. Takaki, E. Shinagawa, M. Ameyama and O. Adachi. 1992c. Ethanol oxidase respiratory chain of acetic acid bacteria. Reactivity with ubiquinone of pyrroloquinoline quinone-dependent alcohol dehydrogenases purified from *Acetobacter aceti* and *Gluconobacter suboxydans*. **Biosci. Biotechnol. Biochem.** 56: 304-310.

_____ and O. Adachi. 1993a. Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase, pp. 47-63. *In* V.L. Davidson, ed. **Applications and Principles of Quinoproteins.** Dekker, New York.

_____ and _____. 1993b. Quinoprotein aldehyde dehydrogenases in microorganisms, pp. 65-71. *In* V.L. Davidson, ed. **Applications and Principles of Quinoproteins.** Dekker, New York.

_____, H. Toyama and O. Adachi. 1994. Respiratory chains and bioenergetics of acetic acid bacteria, pp. 247-301. *In* A.H. Rose and D.W. Tempest, eds. **Advances in Microbial Physiology.** Vol. 36. Academic Press, London.

_____, T. Yakushi, Y. Takaki, H. Toyama and O. Adachi. 1995. Generation mechanism and purification of an inactive form convertible *in vivo* to the active form of quinoprotein alcohol dehydrogenase in *Gluconobacter suboxydans*. **J. Bacteriol.** 177: 6552-6559.

_____, H. Toyama, M. Yamada and O. Adachi. 2002. Quinoproteins: structure, function, and biotechnological applications. **Appl. Microbiol. Biotechnol.** 58: 13-22.

_____, _____ and O. Adachi. 2004. Respiratory chains in acetic acid bacteria: membrane-bound periplasmic sugar and alcohol respirations, pp 1-19. *In* D. Zannoni, ed. **Respiration in Archaea and Bacteria.** Vol. 2**,** Kluwer Academic Publishers, the Netherland.

Matsushita, K., T. Inoue, G. Theeragool, J. Trcek, H. Toyama and O. Adachi. 2005a. Acetic acid production in acetic acid bacteria leading to their "death" and survival, pp. 169-181. *In* M. Yamada, ed. **Survival and Death in Bacteria.** Research Signpost Publishers, Kerala, India.

_____, _____, O. Adachi and H. Toyama. 2005b. *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. **J. Bacteriol.** 187: 4346-4352.

_____, Y. Kobayashi, M. Mizuguchi, H. Toyama, O. Adachi, K. Sakamoto and H. Miyoshi. 2008. A tightly bound quinine functions in the ubiquinone reaction sites of quinoprotein alcohol dehydrogenase of an acetic acid bacterium, *Gluconobacter suboxydans*. **Biosci. Biotechnol. Biochem.** 72: 2723-2731.

Maxam, A.M. and W. Gilbert. 1977. A new method for sequencing DNA. **Proc. Natl. Acad. Sci. U.S.A.** 74: 560-564.

Miller, J. H. 1972. **Experiments in Molecular Genetics: a Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria.** Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. pp. 61, 73-75.

Min Jou, W., G. Haegeman, M. Ysebaert and W. Fiers. 1972. Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. **Nature** 237: 82-88.

Mostafa, H.E., K.J. Heller and A. Geis. 2002. Cloning of *Escherichia coli lacZ* and *lacY* Genes and Their Expression in *Gluconobacter oxydans* and *Acetobacter liquefaciens.* **Appl. Environ. Microbiol.** 68: 2619–2623.

Mount, D.M. 2004. **Bioinformatics: Sequence and Genome Analysis.** 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Mullins, E.A., A.F. Julie and T.J. Kappock. 2008. A specialized citric acid cycle requiring succinyl-coenzyme A (CoA): acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. **J. Bacteriol.** 190: 4933-4940.

Murooka, Y., N. Takizawa and T. Harada. 1981. Introduction of bacteriophage Mu into bacteria of various genera and intergeneric gene transfer by RP4::Mu**. J. Bacteriol.** 145: 358-368.

Nakano, S., M. Fukaya and S. Horinouchi. 2004. Enhanced expression of aconitase raise acetic acid resistance in *Acetobacter aceti*. **FEMS Microbiol. Lett.** 235: 315-322.

_____, _____ and _____. 2006. Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. **Appl. Environ. Microbiol.** 72: 497-505.

_____ and _____. 2008. Analysis of proteins responsive to acetic acid in *Acetobacter*: Molecular mechanism conferring acetic acid resistance in acetic acid bacteria. **Int. J. Food Microbiol.** 125: 54-59.

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

_____, F. Weekers, B. Diawara, A.T. Guiro and P. Thonart. 2007. Survival and preservation after freeze-drying process of thermoresistant acetic acid bacteria isolated from tropical products of Subsaharan Africa. **Africa. J. Food Eng.** 79: 1374-1382.

Nguyen, V.T., B. Flanagan, D. Mikkelsen, S. Ramirez, L. Rivas, M.J. Gidley and G.A. Dykes. 2010. Spontaneous mutation results in lower cellulose production by a *Gluconacetobacter xylinus* strain from Kombucha. **Carbohyd. Polym.** 80: 338-344.

Ohmori, S., H. Masai, K. Arima and T. Beppu. 1980. Isolation and identification of acetic acid bacteria for submerged acetic acid fermentation at high temperature. **Agric. Biol. Chem.** 44: 2901-2906.

_____, T. Uozumi and T. Beppu. 1982. Loss of acetic acid resistance and ethanol oxidizing ability in an *Acetobacter* strain. **Agric. Biol. Chem.** 46: 381-389.

Okumura, H., T. Uozumi and T. Beppu. 1985. Biochemical characteristics of spontaneous mutants of *Acetobacter aceti* deficient in ethanol oxidation. **Agric. Biol. Chem.** 49: 2485-2487.

Olsvik, O., J. Wahlberg, B. Petterson, M. Uhlén, T. Popovic, I.K. Wachsmuth and P.I. Fields. 1993. Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. **J. Clin. Microbiol.** 31: 22-25.

Peltoketo, H., V. Isomaa, O. Maentausta and R. Vihko. 1988. Complete amino acid sequence of human placental 17β-hydroxysteroid dehydrogenase from cDNA. **FEBS Lett.** 239: 73-77.

Pennacchio, A., B. Pucci, F. Secundo, F. La Cara, M. Rossi and C.A. Raia. 2008. Purification and characterization of a novel recombinant highly enantioselective, short-chain NAD(H)-dependent alcohol dehydrogenase from *Thermus thermophilus.* **Appl. Environ. Microbiol.** 74: 3949-3958.

Persson, B., M. Krook and H. Jörnvall. 1991. Characteristics of short-chain alcohol dehydrogenases and related enzymes. **Eur. J. Biochem.** 200: 537-543.

Petruschka, L., K. Adolf, G. Burchhardt, J. Jurgensen and H. Herrmann. 2002. Analysis of the *zwf-pgl-eda*-operon in *Pseudomonas putida* strains H and KT2440. **FEMS Microbiol. Lett.** 215: 89-95.

Pettersson, E., J. Lundeberg and A. Ahmadian. 2009. Generations of sequencing technologies. **Genomics** 93: 105-111.

Prust, C., M. Hoffmeister, H. Liesegang, A. Wiezer, W.F. Fricke, A. Ehrenreich, G. Gottschalk and U. Deppenmeier. 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. **Nat. Biotechnol.** 23: 195-200.

Quintero, Y., M. Poblet, J.M. Guilamón and A. Mas. 2009. Quantification of the expression of reference and alcohol dehydrogenase genes of some acetic acid bacteria in different growth conditions. **J. Appl. Microbiol.** 106: 666-674.

Raspor, P. and D. Goranovič. 2008. Biotechnological applications of acetic acid bacteria. **Crit. Rev. Biotechnol.** 28: 101-124.

Reece, K.S. and G.J. Phillips. 1995. New plasmids carrying antibiotic-resistance cassettes. **Gene.** 165: 141-142.

Reid, M.F. and C.A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. **Crit. Rev. Biotechnol.** 20: 13-56.

Renart, J., J. Reiser and G.R. Stark. 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. **Proc. Natl. Acad. Sci. U.S.A** 76: 3116-3120.

Roh, S.W., Y. Nam, H. Chang, K. Kim, M. Kim, J. Ryu, S. Kim, W. Lee and J. Bae. 2008. Phylogenetic Characterization of Two Novel Commensal Bacteria Involved with Innate Immune Homeostasis in *Drosophila melanogaster*. **Appl. Environ. Microbiol.** 74: 6171-6177.

Saeki, A., G. Theeragool, K. Matsushita, H. Toyama, N. Lotong and O. Adachi. 1997a. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. **Biosci. Biotechnol. Biochem.** 61: 138-154.

Saeki, A., M. Taniguchi, K. Matsushita, H. Toyama, G. Theeragool, N. Lotong and O. Adachi. 1997b. Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. **Biosci. Biotechnol. Biochem.** 61: 317-323.

_____, K. Matsushita, S. Takeno, M. Taniguchi, H. Toyama, G. Theeragool, N. Lotong and O. Adachi. 1999. Enzyme responsible for acetate oxidation by acetic acid bacteria. **Biosci. Biotechnol. Biochem.** 63: 2102-2109.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. **Molecular cloning: a laboratory manual.** 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sanger, F. and A.R. Coulson. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. **J. Mol. Biol.** 94: 441-448.

_____, S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. **Proc. Natl. Acad. Sci. U.S.A.** 74: 5463-5467.

Shinagawa, E., K. Matsushita, O. Adachi and M. Ameyama. 1990. Evidence for electron transfer *via* ubiquinone between quinoproteins D-glucose dehydrogenase and alcohol dehydrogenase of *Gluconobacter suboxydans*. **J. Biochem.** 107: 863-867.

_____, O. Adachi, Y. Ano, H. Toyama and K. Matsushita. 2010. Purification and characterization of membrane-bound 3-dehydroshikimate dehydratase from *Gluconobacter oxydans* IFO 3244, a new enzyme catalyzing extracellular protocatechuate formation. **Biosci. Biotechnol. Biochem.** 74: 1084-1088.

Sintuprapa, W. 2008a. **Characterization of Acetic Acid Adapted Strains and Analysis of Alcohol Dehydrogenase Genes from A*cetobacter syzygii* SKU19.** Ph.D. Thesis, Kasetsart University.

Sintuprapa, W., G. Theeragool, W. Yongmanitchai, P. Srifah-Huehne and K. Matshushita. 2008b. Molecular taxonomy of *Acetobacter syzygii* SKU19 and characterization of its acetic acid adapted strains. **Kasetsart J. (Nat. Sci.)** 42: 701-714.

Soemphol, W. 2008. **Molecular Biology Study of Sugar Alcohol Oxidation Systems in Thermotolerant *Gluconobacter* Strain.** D.Eng. Thesis, Yamaguchi University, Japan.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **J. Mol. Biol.** 98: 503-517.

Steiner, P. and U. Sauer. 2001. Protein induced during adaptation of *Acetobacter aceti* to high acetate concentrations. **Appl. Environ. Microbiol.** 67: 5474-5481.

Suzuki, S., M. Sugiyama, Y. Mihara, K. Hashiguchi and K. Yokozeki. 2002. Novel enzymatic method for the production of xylitol from D-arabitol by *Gluconobacter oxydans*. **Biosci. Biotechnol. Biochem.** 66: 2614-2620.

Takakuwa, N., K. Yamane, H. Goto, U. Oda, M. Fukaya, Y. Tsukamoto and M. Ohnishi. 2002. Identification of acetic acid bacterium NCI 1193 and nucleotide sequences of the gene encoding enzymes related to ethanol oxidation. **J. Brew. Soc. Japan** 97: 645-650 (in Japanese).

Takemura, H., S. Horinouchi and T. Beppu. 1991. Novel insertion sequence IS*1380* from *Acetobacter pasteurianus* is involved in loss of ethanol-oxidizing ability. **J. Bacteriol.** 173: 7070-7076.

Takemura, H., K. Kondo, S. Horinuochi and T. Beppu. 1993. Induction by ethanol of alcohol dehydrogenase activity in *Acetobacter pasteurianus*. **J. Bacteriol.** 175: 6857-6866.

Tamaki, T., M. Fukaya, H. Takemura, K. Tayama, H. Okumura, Y. Kawamura, M. Nishiyama, S. Horinouchi and T. Beppu. 1991. Cloning and sequencing of the gene cluster encoding two subunits of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxygenes*. **Biochim. Biophys. Acta** 1088: 292-300.

Tayama, K., M. Fukaya, Y. Kawamura and T. Beppu. 1989. Purification and characterization of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes* sp. nov. **Appl. Microbiol. Biotechnol.** 32: 181-185.

Taylor, L.A. and R.E. Rose. 1988. A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. **Nucleic Acids Res.** 16: 358.

Theeragool, G., N. Lotong, O. Adachi, A. Saeki and K. Matsushita. 1996. **Characterization of Thermotolerant Acetic Acid Bacteria Isolated in Thailand and IFO (Institute for Fermentation Osaka) Strains.** A report submitted to Monbusho International Scientific Research Program: Kasetsart University-Yamaguchi University.

Towbin, H., T. Staehelin and J. Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proc. Natl. Acad. Sci. U.S.A** 76: 4350-4354.

Toyama, H., A. Fujii, K. Matsushita. E. Shinagawa. M. Ameyama and O. Adachi. 1995. Three distinct quinoprotein alcohol dehydrogenases are expressed when *Pseudomonas putida* is grown on different alcohols. **J. Bacteriol.** 177: 2442-2450.

Toyama, H., F.S. Mathews, O. Adachi and K. Matsushita. 2004. Quinohemoprotein alcohol dehydrogenases: structure, function, and physiology. **Arch. Biochem. Biophys.** 428: 10-21.

Trcek, J., K. Toyama, J. Czuba, A. Misiewicz and K. Matsushita. 2006. Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. **Appl. Microbiol. Biotechnol.** 70: 366–373.

_____, K. Jernejc and K. Matsushita. 2007. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. **Extremophiles** 11: 627-635.

Tsuchida, T. and F. Yoshinaga. 1997. Production of bacterial cellulose by agitation culture systems. **Pure Appl. Chem.** 69: 2453-2458.

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

Vallette, F., E. Mege, A. Reiss and M. Adesnik. 1989. Construction of mutant and chimeric genes using the polymerase chain reaction. **Nucleic Acids Res.** 17: 723-733.

Vanittananon, N. 2005. **Cloning and Nucleotide Sequencing of *adh*S Gene Encoding Alcohol Dehydrogenase Subunit III From *Acetobacter pasteurianus* SKU1108.** M.S. Thesis, Kasetsart University.

Villarroya, A., E. Juan, B. Egestad and H. Jörnvall. 1989. The primary structure of alcohol dehydrogenase from *Drosophila lebanonensis.* **Eur. J. Biochem.** 180 191-197.

Vitale, A., F. Rosso, A. Barbarisi, T. Labella and S. D'Auria. 2010. Properties and evolution of an alcohol dehydrogenase from the Crenarchaeota *Pyrobaculum aerophilum*. **Gene.** 461: 26-31.

Wierenga, R. K., M.C.H. DeMaeyer, and W.G.J. Hol. 1985. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. **Biochemistry** 24: 1346-1357.

Yakushi, T. and K. Matsushita. 2010. Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. **Appl. Microbiol. Biotechnol.** 86: 1257-1265.

Yamada, M. and M.H. Saier. 1987. Glucitol-specific enzymes of the phosphotransferase system in *Escherichia coli*. **J. Biol. Chem.** 262: 5455-5463.

Yamada, Y., K. Hoshino and T. Ishikawa. 1997. The Phylogeny of Acetic Acid Bacteria Based on the Partial Sequences of 16S Ribosomal RNA : The Elevation of the Subgenus *Gluconoacetobacter* to the Generic Level**. Biosci. Biotechnol. Biochem.** 61: 1244-1251.

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

_____ and P. Yukphan. 2008. Genera and species in acetic acid bacteria. **Int. J. Food Microbiol.** 125: 15-24.

Yoshida, T., Y. Ayabe, M. Yasunaga, Y. Usami, H. Habe, H. Nojiri and T. Omori. 2003. Genes involved in the synthesis of the exopolysaccharide methanolan by the obligate methylotroph *Methylobacillus* sp. strain 12S. **Microbiology** 149: 431-444.

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

_____, _____, Y. Muramatsu, M. Takahashi, M. Kaneyasu, S. Tanasupawat, Y. Nakagawa, K. Suzuki, W. Potacharoen and Y. Yamada. 2008. *Tanticharoenia sakaeratensis* gen. nov., sp. nov., a New Osmotolerant Acetic Acid Bacterium in the α-*Proteobacteria*. **Biosci. Biotechnol. Biochem.** 72: 672-676.

_____, _____, _____, _____, _____, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, K. Hamana, Y. Tahara, K. Suzuki, M. Tanticharoen and Y. Yamada. 2009. *Ameyamaea chiangmaiensis* gen. nov., sp. nov., an Acetic Acid Bacterium in the α-*Proteobacteria.* **Biosci. Biotechnol. Biochem.** 73: 2156-2162.

APPENDICES

Appendix A

Culture media and antibiotics

1. Potato medium

Glucose	5	g
Yeast extract	5	g
Polypeptone	5	g
Glycerol	20	ml
Potato extract	15	ml
H_2O	1000	ml

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. YPGD medium

Glucose	5	g
Yeast extract	5	g
Polypeptone	5	g
Glycerol	5	ml
H_2O	1000	ml

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	5	g
Polypeptone	5	g
Glycerol	5	ml
H_2O	1000	ml

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

4. YPG medium (for preparation of competent cell of acetic acid bacteria)

Yeast extract	5	g
Polypeptone	5	g
Glycerol	20	ml
H_2O	1000	ml

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

5. LB (Luria-Bertani) medium

Yeast extract	5	g
Tryptone	10	g
NaCl	10	g
H_2O	1000	ml

The pH was adjusted to 7.0 with 5 N NaOH. The volume of the solution was adjusted to 1000 ml with tap water. Sterilization was performed by autoclaving at 121°C for 15 min.

For preparation of agar media containing antibiotics, after sterilization, those media were allowed to cool down to 50°C and then the appropriated antibiotic stock solution was added.

6. Ampicillin (50 mg/ml)

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.

7. Kanamycin (50 mg/ml)

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.

8. Tetracycline (25 mg/ml)

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.

9. Gentamicin (12.5 mg/ml)

Gentamicin stock solution was prepared by dissolving 12.5 mg of gentamicin 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.

Appendix B

Preparation of reagents

1. Preparation of commonly used reagents

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

A stock X-gal solution was prepared by dissolving a 20 mg of X-gal powder in 1 ml of dimethylformamide. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and stored at -20°C. It is not necessary to sterilize X-gal solution by filtration.

1.2 Tris (1 M)

A Tris-Cl solution was prepared by dissolving 121.1 g of Tris base in 800 ml of double distilled water. The solution was adjusted the desired pH 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 1,000 ml 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 NaCl (5 M)

A 292.2 g of NaCl was dissolved in 800 ml of double distilled water, and adjusted the volume to 1000 ml with double distilled water. The solution was sterilized by autoclaving.

1.4 NaOH (0.8 N)

The stock solution of 0.8 N NaOH was prepared by dissolved 32 g of sodium hydroxide (NaOH) in 100 ml of distilled water. Sterilization is not required for the preparation of this stock solution and stored at room temperature in a plastic bottle.

1.5 Phenolphthalein indicator

The stock solution of phenolphthalein indicator was prepared by dissolved 0.5 g of phenolphthalein in 100 ml of 70% ethanol. The solution was stored at room temperature in a light-tight bottle.

1.6 SDS (10%)

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.

1.7 Potassium acetate (1 M, pH 7.5)

A 9.82 g of potassium acetate was dissolved in 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. Sterilization was performed by autoclaving at 121°C for 15 min.

1.8 Sodium acetate (3 M, pH 5.2)

A solution was prepared by dissolving 408.1 g of sodium acetate.$3H_2O$ in double distilled water and adjusted the pH to 5.2 with glacial acetic acid. Then, the solution was adjusted volume to 1000 ml with double distilled water and sterilized by autoclaving at 121°C for 15 min.

1.9 EDTA (0.5 M, pH 8.0)

A 186.1 g of disodium ethylenediaminetetra-acetate.$2H_2O$ was added to double distilled water. The pH was adjusted to 8.0 with pellet of NaOH and added distilled water to adjust volume to 1000 ml. This stock solution was sterilized by autoclaving.

1.10 Ethidium bromide (10 mg/ml)

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.

1.11 Saline-EDTA

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

1.12 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-Cl (pH 8.0) in a light-tight bottle at 4°C.

1.13 CTAB solution (10%)

A 4.1 g of NaCl was slowly added into 80 ml of distilled water. The dissolved NaCl solution was further added with 10 g of N-cetyl-N, N, N-trimethyl ammonium bromide (CTAB) and subsequently heated at 65°C with stirring. The solution was sterized by autoclaving at 121°C for 15 min and stored at room temperature.

1.14 TE buffer

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

1.15 TE buffer with 20 µg/ml RNase A

TE buffer	998	µl
RNase A (10 mg/ml)	2	µl

1.16 Gel loading buffer

Bromphenol blue	0.025	g
Glycerol	3	ml

1.17 Electrophoresis buffer (1xTAE)

Tris base	4.84	g
Glacial acetic acid	1.15	ml
EDTA.2Na (0.5 M, pH 8.0)	3	ml
H_2O	1000	ml

1.18 NaOH (0.8 N)

The stock solution of 0.8 N NaOH was prepared by dissolved 32 g of sodium hydroxide (NaOH) in 100 ml of distilled water. Sterilization is not required for the preparation of this stock solution and stored at room temperature in a plastic bottle.

1.19 Phenolphthalein indicator

The stock solution of phenolphthalein indicator was prepared by dissolved 0.5 g of phenolphthalein in 100 ml of 70% ethanol. The solution was stored at room temperature in a a light-tight bottle.

2. Alkaline lysis buffers for preparation of plasmid DNA

2.1 Solution I

Glucose	50	mM
Tris-Cl (pH 8.0)	25	mM
EDTA (pH 8.0)	10	mM

2.2 Solution II

NaOH	0.2	N
SDS	1	% (w/v)

The solution II was freshly prepared before used.

2.3 Solution III

Potassium acetate (1 M, pH 7.5)	60	ml
Glacial acetic acid	11.5	ml
H_2O	28.5	ml

3. Reagents and buffers for Southern hybridization

3.1 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.

3.2 Denaturation solution

NaOH	0.5	M
NaCl	1.5	M

The solution was adjusted volume to 1,000 ml with double distilled water and stored at room temperature for up to 3 months.

3.3 Neutralization solution

Tris-Cl (pH 7.4)	0.5	M
NaCl	1.5	M

The solution was adjusted volume to 1,000 ml with double distilled water and stored at room temperature for up to 3 months.

3.4 SSC solution (20x, pH 7.0)

tri-sodium citrate	0.3	M
NaCl	3	M

The solution was adjusted pH to 7.0 with 1 N NaOH, autoclave and stored at room temperature.

3.5 Prehybridization solution

Formamide	45	% (v/v)
SSC solution	6	x
Denhardt’s hybridization buffer	2	x
SDS	0.5	% (w/v)

The hybridization solution was preheated at 42°C for 20 min.

3.6 Buffer I

SSC solution	2	x

3.7 Buffer II

Tris-Cl (pH 7.5)	0.1	M
NaCl	1.5	M
Blocking powder	0.5	% (w/v)

3.8 Buffer III

Tris-Cl (pH 9.5)	0.1	M
NaCl	0.1	M
$MgCl_2$	50	mM

3.9 Color-substrate solution

NBT-BCIP solution	100	µl
Buffer III solution	4900	µl

4. Reagents for determination of protein concentration

4.1 Solution A

Na_2CO_3	2	% (w/v)
SDS	0.5	% (w/v)
NaOH	0.1	N

4.2 Solution B

$CuSO_4.5H_2O$	0.5	% (w/v)
Potassium sodium tartrate	1	% (w/v)

Both solutions were filtrated by filter paper. Working reagent was prepared by mixing solution A and B in the ratio 50:1.

4.3 Solution C

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

4.4 BSA solution (1 mg/ml)

A 0.1 g of bovine serum albumin (BSA) was dissolved in 100 ml of distilled water containing 0.9% (w/v) NaCl and 0.05% (w/v) sodium azide (NaN_3). The stock solution was stored at -20°C.

5. Reagents and buffers for enzyme activity assay

5.1 Ferricsulfated-dupanol reagent

$Fe(SO4)_3.H_2O$	5	g
SDS	3	g
85% Phosphoric	95	ml

The solution was adjusted volume to 1,000 ml with distilled water and mixed gently by stirring. Keep in a light-tight container at room temperature.

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

A 32.9 g of potassium ferricyanide was dissolved in distilled water and adjusted volume to 1,000 ml. Keep in light-tight container at 4°C.

5.3 NAD^+ solution (50 mM)

A 0.0341 g of beta-nicotinamide adenine dinucleotide (NAD^+) sodium salt was dissolved in 1 ml of distilled water. The solution was stored in a light-tight container at -20°C.

5.4 NADH solution (50 mM)

A 0.0355 g of beta-nicotinamide adenine dinucleotide (NADH) disodium salt was dissolved in 1 ml of distilled water. The solution was stored in a light-tight container at -20°C.

5.5 Ethanol (1 M)

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.

5.6 Acetaldehyde (1 M)

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. Reagents and buffers for sodium dodecylsulfate acrylamide gel electrophoresis (SDS-PAGE), coomassie brilliant blue (CBR) and heme staining

6.1 Bis-acrylamide stock solution (25% w/v)

Acrylamide (25% w/v) and N, N'-methylenebisacrylamide (1% w/v) were resuspended in deionized water. The pH of the solution should be 7.0 or less. The solution should be stored in dark bottle at 4°C.

6.2 Ammonium persulfate (APS, 10%)

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

6.3 Preparation of SDS-polyacrylamide gels

6.3.1 Stacking gel

Bis-acrylamide solution (25%)	0.6	ml
Tris-Cl (0.5 M, pH 6.8)	0.75	ml
SDS (10%)	30	µl
Distilled water	1.6	ml
APS (10%)	20	µl
TEMED	3	µl

6.3.2 Separating gel

Bis-acrylamide solution (25%)	4.0	ml
Tris-Cl (1.5 M, pH 8.8)	2.0	ml
SDS (10%)	80	µl
Distilled water	1.9	ml
APS (10%)	20	µl
TEMED	14	µl

6.4 Sample buffer for CBR staining

Tris-Cl (0.5 M, pH 6.8)	125	mM
SDS	6	% (w/v)
Dithiothreitol (DTT)	200	mM
Phenylmethlsulfonyl fluoride (PMSF)	3	mM
Glycerol	20	% (v/v)

6.5 Sample buffer for heme staining

Tris-Cl (0.5 M, pH 6.8)	125	mM
SDS	6	% (w/v)
Phenylmethlsulfonyl fluoride (PMSF)	3	mM
Glycerol	20	% (v/v)

6.6 Running buffer

Tris base	30	g
Glycine	140	g
SDS	5	g

6.7 CBR staining buffer

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

6.8 Destaining solution for CBR

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

6.9 Heme staining buffer

Methanol	6	ml
Acetate buffer (pH 5.0)	14	ml
TMBZ	9	mg

7. Buffers for immunoblotting analysis of PQQ-ADH subunit III

7.1 Cathode buffer

Tris base	25	mM
Glycine	40	mM
Methanol	10	% (v/v)

7.2 Anode buffer I

Tris base	0.3	mM
Methanol	10	% (v/v)

The solution was adjusted pH to 10.4 with 1 M HCl.

7.3 Anode buffer II

Tris base	25	mM
Methanol	10	% (v/v)

The solution was adjusted pH to 10.4 with 1 M HCl.

7.4 Blocking buffer

A 1 g of skim milk powder was dissolved in 100 ml of phosphate-buffered saline and cooled down to room temperature before used.

7.5 Phosphate-buffered saline (PBS)

Na_2PO_4	10	mM
NaCl	0.9	% (w/v)

The solution was adjusted pH to 7.2 with 1 M NaOH.

7.6 Detection solution

PBS (pH 7.2)	50	ml
Hydrogen peroxide	30	µl
Color reagent	10	ml

7.7 Color reagent solution

Diaminobenzidine (DAB)	30	mg
Cold methanol	10	ml

8. Reagents and buffers for determination of β-galactosidase activity

8.1 Z buffer

Na_2HPO_4	60	mM
NaH_2PO_4	40	mM
KCl	10	mM
$MgSO_4$	1	mM
β-mercaptoethanol	50	mM

The solution was adjusted pH to 7.0 with 1 N NaOH.

8.2 *o*-nitrophenyl-β-D-galactoside solution (ONPG, 4 mg/ml)

A 40 mg of ONPG was dissolved in 100 ml of distilled water and stored at -20°C.

8.3 SDS (0.1%)

A 0.1 g of SDS was dissolved in 100 ml of distilled water and stored at room temperature.

8.4 Na_2CO_3 (1M)

A 105.99 g of sodium carbonate (Na_2CO_3) in 1,000 ml of distilled water and stored at room temperature.

9. Preparation of buffers

9.1 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 1000 ml with distilled water. Alternatively, preparation of 1 M KPB in various pH and then diluted to the used concentration with distilled water.

9.2 Glycine-NaOH (100 mM, pH 8.5)

A 7.507 g of glycine was dissolved in distilled water and adjusted the pH to 8.5 with 5 N NaOH. The distilled water was added to adjust the volume to 1000 ml.

9.3 Good buffer

Good buffer		0.1 mol/l NaOH				
		0 ml	5 ml	10 ml	15 ml	20 ml
0.1 mol/l MES	25 ml	3.7*	5.6*	6.0*	6.4*	8.4*

*Indicated the pH value

9.4 McIlvaine buffer

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. The modified Lowry method

Protein content was measured by a modified method of Lowry (Dully and Grieve, 1975) with bovine serum albumin (1 mg/ml BSA) as the standard protein. The reaction mixture was shown as follows.

Distilled water (µl)	400	390	380	370	360	350
BSA (µl)	0	10	20	30	40	50

Each assay was carried out by mixing 0.4 ml of protein solution with 2 ml of Folin reagent (Solution A: Solution B = 50: 1). The solution was mixed by vortex and incubated at 35°C for 10 min and then 0.2 ml of phenol reagent (Solution C) was added with rapidly mixing. Incubation was done by standing at 35°C for 30 min and the absorbance was measured at 750 nm with a 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 BSA as a standard. The protein concentration was calculated as follows.

$$\text{Protein concentration (mg/ml)} = \frac{\text{Concentration from Spectrophotometer x Dilution}}{\text{Sample (µl)}}$$

2. 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 assay at 25°C with potassium ferricyanide as an electron acceptor according to the standard method described by Adachi *et al*. (1978a, 1978b) with slight modifications (Matsushita *et al*., 1995). The enzyme activities were determined in a 1 ml of reaction volume. The 0.6 ml of McIlvaine buffer pH 5.0 (Appendix B) and 0.1 ml of 1 M substrate (ethanol for ADH and acetaldehyde for ALDH, Appendix B) were mixed. The reaction mixtures were incubated at 25°C for 5 min and then 0.1 ml of 0.1 M potassium ferricynide was added to start the reaction. After further incubation for 5 min, the reaction was stopped by the addition of 0.5 ml of ferricsulfate-dupanol reagent (Appendix B). After incubation at 25°C for 20 min,

the reaction mixtures were diluted with 3.5 ml of distilled water and the absorbance was measured at 660 nm. One unit of the enzyme activity was expressed as 1 µmol of substrate oxidized per min, which is equivalent to 4.0 absorbance units. The enzyme activity was calculated as follows.

$$\text{Enzyme activity (U/ml)} = \frac{1}{4} \times \frac{1}{5} \times \frac{1}{\text{vol (µl)}} \times Abs_{660} \times 1000 \times \text{Dilution}$$

$$\text{Specific activity (U/mg of protein)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

3. NAD^+-dependent ADH (EC 1.1.1.1)

The soluble fraction was measured for the NAD^+-dependent ADH activity at 25°C spectrophotometrically at 340 nm (Hitachi Model U-2000) by measuring the formation or disappearance of NADH. The formation of NADH follows the oxidation of ethanol to acetaldehyde (forward reaction), while the disappearance of NADH is the reduction of acetaldehyde to ethanol (reverse reaction).

Reaction mixture for forward reaction	
100 mM Glycine-NaOH (pH 8.5)	888 µl
1 M Ethanol	100 µl
50 mM NAD^+	2 µl
Soluble fraction	10 µl
Total	1000 µl
Reaction mixture for reverse reaction	
100 mM MES buffer (pH 6.0)	888 µl
1 M Ethanol	100 µl
50 mM NADH	2 µl
Soluble fraction	10 µl
Total	1000 µl

The reaction was started by the addition of enzyme solution. The enzyme activity was calculated with a millimolar extinction coefficient of 6.22 at 340 nm and expressed as 1 µmol of NADH oxidized or reduced per min.

$$\text{Specific activity (U/mg)} = \frac{Abs_{340} \times 1000 \times \text{dilution}}{250 \times 6.22 \times \text{time (min)} \times \text{vol (µl)} \times \text{protein (mg/ml)}}$$

3. Isolation of chromosomal DNA of acetic acid bacteria

Chromosomal DNA from acetic acid bacteria was isolated by the method described by Okumura *et al*. (1985). The bacterial cells were harvested and suspended in 10 ml of sterile saline-EDTA 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% CTAB/NaCl solution 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 5.2) was added. The 2.5-fold of cold absolute ethanol was then added to the upper layer and DNA was wound with a sterile Pasteur pipette. The DNA was washed with 70% ethanol and dried up. Finally, the extracted DNA was resuspended with TE buffer containing 20 mg/ml RNase A. The concentration of chromosomal DNA was determined by measuring absorbance at 260 nm.

4. Analysis of plasmid profile of acetic acid bacteria

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 at the late-log phase were harvested by centrifugation at 12,000 rpm for 5 min at 4°C, and washed twice with 0.5 M 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 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, mixed and stored on ice for 5 min. 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 10% 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. 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 was then centrifuged at 4°C, 12,000 rpm for 30 min. The plasmid DNA was washed twice with 70% ethanol, dried and resuspended with TE buffer containing 20 µg/ml RNase A.

5. Alkaline lysis method for isolation of plasmid DNA from *E. coli*

Alkaline lysis method was described by Sambrook *et al*. (1989) with slight modification. *E. coli* harboring plasmid was grown in LB broth containing appropriate antibiotic at 37°C for 18-24 h. The cells were harvested by centrifugation at 12,000 rpm for 1 min. The cell pellet was resuspended in 100 µl solution I (Appendix B). Then, 200 µl of a freshly prepared solution II (Appendix B) was added into the cell suspension and gently mixed by inverting the tube until the solution is clear and viscous. The 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 12,000 rpm for 5 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 12,000 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 12,000 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 TE buffer containing 20 µg/ml RNase A. The solution was mixed, incubated at 37°C overnight and stored at -20°C. Plasmid DNA was analyzed by running in 0.8% agarose gel electrophoresis.

6. Isolation of plasmid DNA by GeneJET™ Plasmid Miniprep Kit

The bacterial cell pellet was resuspended in 250 µl of the Resuspension Solution. The cell suspension was transferred to a microcentrifuge tube and resuspended completely by vortexing or pipetting up and down until no cell clump remains. The 250 µl of the Lysis Solution was added and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. The 350 µl of the Neutralization Solution was then added, mixed immediately and thoroughly by inverting the tube 4-6 times. The suspension was centrifuged for 5 min to remove cell debris and chromosomal DNA. The supernatant was transfer to the supplied GeneJET™ spin column by decanting or pipetting, avoid disturbing or transferring the white precipitate and centrifuge for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. The 500 µl of the Wash Solution was added to the GeneJET™ spin column and centrifuged at the maximum speed for 30-60 sec. The flow-through was discarded and the column was placed back into the same collection tube. The wash procedure was repeated using 500 µl of the Wash Solution. The GeneJET™ spin column was transferred into a fresh 1.5 ml microcentrifuge tube and 30 µl of the Elution Buffer was added to the center of GeneJET™ spin column membrane to elute the plasmid DNA and incubated for 2 min at room temperature. The purified plasmid DNA was eluted by centrifugation for 2 min at maximum speed.

7. Purification of DNA fragment by GeneJET™ GelExtraction Kit

A gel slice containing DNA fragment was excised with a clean and sharp scalpel or razor blade. The weighted gel slice was placed in a colorless tube and added 1 volume of Binding Buffer to the gel (e.g., add 100 µl of Binding Buffer for every 100 mg of agarose gel). The gel mixture was incubated at 50-60°C for 10 min or until

the gel slice is completely dissolved. The gel mixture was mixed by inversion the tube every few min to facilitate the melting process. After the gel slice has dissolved completely, the color of the mixture was checked that it is yellow. If the DNA fragment is <500 bp, add a 1:2 volume of 100% isopropanol to the solubilized gel solution (e.g. 100 µl of isopropanol should be added to 100 mg gel slice solubilized in 100 µl of Binding Buffer). If the DNA fragment is >10 kb, add a 1:2 volume of water to the solubilized gel solution (e.g. 100 µl of water should be added to 100 mg gel slice solubilized in 100 µl of Binding Buffer). The solubilized gel solution was transferred up to 800µl to the GeneJET™ purification column and centrifuged for 1 min. The flow-through was discarded and placed back into the same collection tube. The 700 µl of the Wash Buffer was then added to the GeneJET™ purification column and centrifuged for 1 min. The flow-through was discarded and placed back into the same collection tube. The empty GeneJET™ purification column was centrifuged for an additional 1 min to completely remove residual wash buffer. The column was transferred into a clean 1.5 ml microcentrifuge tube. The 30 µl of the Elution Buffer was then added to the center of the purification column membrane. The purified plasmid DNA was eluted by centrifugation for 1 min at maximum speed.

8. Preparation of competent cell from acetic acid bacteria

Acetic acid bacteria were cultured in YPG medium for 18-24 h or an optical density at 540 nm reached to 0.8-1.0. All operations were done aseptically at 4°C. The culture medium was transferred to a centrifuge tube and incubated on ice for 15 min. The bacterial cells were harvested by centrifugation at 6,000 rpm for 5 min. The precipitate was washed with cold 0.1 M $MgCl_2$ and centrifuged at 6,000 rpm for 5 min. The cell pellet was washed with cold distilled water and further washed twice with cold 10% glycerol by centrifugation at the same condition. The cell paste was suspended in 1 ml of cold 10% glycerol. The suspension was dispensed into 65 µl aliquots, shocked frozen in liquid nitrogen and stored at -80°C until used.

9. Preparation of *E. coli* competent cell for heat-shock transformation

Competent cells were prepared by the procedure described by Sambrook *et al*. (1989) with slightly modification. A fresh overnight culture of *E. coli* was inoculated into 40 ml of LB broth at 37°C with vigorous shaking (200 rpm) until an Abs_{600} of approximately 0.4-0.6 (the best results are obtained with cells that are harvested at early to mid-log phase). Throughout the preparation, the cells were kept as close to 0°C as possible (on ice). The culture was transferred to a prechilled sterile tube and centrifuged at 5,000 rpm, 4°C for 5 min. Remove a much of the supernatant as possible. The pellets were gently resuspended in 20 ml of 50 mM $CaCl_2$ and placed on ice for 45 min. The suspension was centrifuged as previously described. The pellets were resuspended again in 2 ml of 50 mM $CaCl_2$ containing 20% glycerol, and stored on ice for 30 min. Finally, the competent cell was dispensed into 100 µl aliquots and ready for transformation. The competent cells were frozen in Liquid-Nitrogen and stored at -80°C.

10. Heat-shock transformation

The procedure for heat-shock transformation was described by Sambrook *et al*. (1989) with slightly modification. A 2 µl of the ligation mixture was added into 100 µl of *E. coli* competent cells and stored on ice for 10-30 min. The cell suspension was heat-shocked at 42°C for 2 min and immediately put on ice for 2 min. The cell suspension was transferred into a test tube containing 1 ml of LB medium and incubated at 37°C for 30 min with no shaking and further gentle shaking for 30 min. After adaptation, the solution was spreaded on LB agar containing an appropriate antibiotic and incubated overnight at 37°C.

11. Conjugal transfer

Transformation of plasmid DNA by conjugation was performed according to the procedure described by Murooka *et al*. (1981) with slightly modification. Acetic acid bacterium was used as a recipient strain and *E. coli* S17-1 harboring a recombinant plasmid was used as a donor strain. The acetic acid bacterium was

cultured in 5 ml potato medium at 30°C for overnight and *E. coli* S17-1 was cultured in 5 ml of LB broth at 37°C for overnight. Exponentially growing donor strain was mixed with an equal volume of recipient cultured in the late exponential phase in an eppendorf tube. The cell mixture was centrifuged at 12,000 rpm for 5 min. The cell pellet was resuspended in 100 µl of potato medium and 50 µl of mixed cell suspension was dropped onto a potato agar. The mixed culture was incubated at 30°C for overnight. One loopful of mixed culture was resuspended in 500 µl potato medium. The cell suspension was diluted, spreaded on potato agar containing an appropriate antibiotic and incubated at 30°C until a colony forming occurred.

12. Determination of acetic acid in the solution by alkaline-titration method

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

Acetic acid concentration (% w/v) = 0.12 x (X – 1)

When; X = number of drop of 0.8 N NaOH

13. Determination of β-galactosidase activity

The β-galactosidase activity assay was measured according to the standard method described by Miller (1972) with slightly modification. The cell pellet was resuspended in Z buffer (without β-mercaptoethanol) and measured the optical density at 600 nm. The 100 µl of cell suspension was transferred into a test tube. The 600 µl of Z buffer containing 0.05 M β-mercaptoethanol was added to the cell suspension. Ten drops of 0.1% SDS and three drops of chloroform were added, mixed by vortex vigorously for 10-15 seconds and equilibrated at 28°C for 2 min. Th reaction was started by adding 200 µl substrate (4 mg/ml ONPG), mixed well by vortexing. The adding time was precisely noted. The cell was incubated at28°C until a sufficient yellow color has developed. The reaction was stopped by adding 500 µl of 1 M

Na_2CO_3 and mixed well. The stop time was precisely recorded. The reaction mixture was transferred to an eppendorf tube and centrifuged at maximum speed for 5 min to remove cell debris and chloroform. The supernatant then was measured the optical density at 420 and 550 nm. The following equation is used to calculate the units of β-galactosidase activity.

$$\text{β-galactosidase activity (Miller unit)} = \frac{[Abs_{420} - (1.75 \times Abs_{550})] \times 1000}{T \times V \times Abs_{600}}$$

When; T = time of the reaction (min)

V = volume of culture used in the assay (ml)

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