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NAME: Miss Nittaya Pitiwittayakul

THIS THESIS HAS BEEN ACCEPTED BY

.... **THESIS ADVISOR**

(Associate Professor Gunjana Theeragool, D.Agr.)

.... **THESIS CO-ADVISOR**

(Ms. Wilawan Sintuprapa, Ph.D.)

.... **THESIS CO-ADVISOR**

(Mrs. Pattaraporn Rattanawaree, Ph.D.)

.... **GRADUATE COMMITTEE CHAIRMAN**

(Assistant Professor Siriwan Prapong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON ....

.... **DEAN**

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THESIS

MOLECULAR CHARACTERIZATION OF *groESL* OPERON FROM *Acetobacter pasteurianus* SKU1108 AND ITS TAXONOMIC APPLICATIONS

NITTAYA PITIWITTAYAKUL

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Genetic Engineering)
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2014

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Nittaya Pitiwittayakul 2014: Molecular Characterization of *groESL* Operon from *Acetobacter pasteurianus* SKU1108 and Its Taxonomic Applications. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Gunjana Theeragool, D.Agr. 201 pages.

The objectives of this study were to clarify the potential roles of the *groESL* gene of acetic acid bacteria (AAB), the effects of the disruption of the *groEL* gene, and the induction of the *groESL* gene expression into the thermotolerant acetic acid bacterium, *Acetobacter pasteurianus* SKU1108. When the *groEL* gene was disrupted, its rate of growth decreased and it exhibited a complete loss of toleration of stressors. The presence of the *A. pasteurianus* SKU1108 *groESL* gene in the *groEL* gene disruptant restored significant acceptance of stressors. A heat shock promoter was induced by stressors, as was shown by a steady increase in the transcription level of the *groESL* gene, along with elevated temperatures and a heightened concentration of acetic acid and ethanol. A CIRCE element which was found in the upstream region was examined and compared with all known genome sequences of AAB. This revealed that the sequences of all of these strains are very well preserved, and have evolved phylogenetically. Moreover, the *groEL* gene, which encodes 60-kDa heat shock proteins, was found to be conserved to a very significant extent among AAB. The *groEL* gene analysis was further used for classification and identification of AAB, together with 16S rRNA gene analysis. Twenty-four strains of AAB, which were isolated from fruits, flowers, mushrooms, and fermented rice products collected in Thailand by an enrichment culture approach and assigned to the genus *Acetobacter*, were taxonomically examined for the 16S rRNA gene and *groEL* gene sequences. Based on analysis of the 16S rRNA gene and *groEL* gene sequences, the strains were divided into ten groups: Group 1 comprised four isolates identified as *A. tropicalis*, Group 2 consisted of three isolates identified as *A. indonesiensis*, Group 3 was made up of two isolates identified as *A. persici*, Group 4 was composed of two isolates (*A. orientalis*), Group 5 possessed one isolate (*A. cibinongensis*), Group 6 contained three isolates (*A. pasteurianus*), Group 7 was constituted of one isolate of *A. papayae*, Group 8 held two isolates identified as *A. fabarum*, Group 9 consisted of one isolate of *A. okinawensis*, and Group 10 was made up of four isolates identified as *A. ghanensis*. Besides the 10 groups, one strain, BCC 15839, constituted an independent cluster which was taken from the type strains of other *Acetobacter* species in phylogenetic trees, based on 16S rRNA and *groEL* gene sequences. The polyphasic approach was implemented. Based on the results obtained in physiological, biochemical and genotypic analysis of the differences between the isolate and the type strains of the genus *Acetobacter*, the isolate was classified as a novel species of *Acetobacter*. The name *A. thailandicus* sp. nov., was introduced.

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Student's signature Thesis Advisor's signature

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Nittaya Pitiwittayakul
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LIST OF ABBREVIATIONS

bp	=	base pairs
d	=	day
ºC	=	degree Celsius
h	=	hour
kDa	=	kilodaltons
µg	=	microgram
µg/ml	=	microgram per milliliter
µl	=	microlitre
ml	=	milliliter
mM	=	millimolar
min	=	minute
N	=	normality
rpm	=	revolution per minute
Q	=	ubiquinone
sec	=	second
v/v	=	volume by volume
w/v	=	weight by volume

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MOLECULAR CHARACTERIZATION OF *groESL* OPERON FROM *Acetobacter pasteurianus* SKU1108 AND ITS TAXONOMIC APPLICATIONS

INTRODUCTION

Acetobacter pasteurianus SKU1108, isolated from grape in Thailand, is a thermotolerant acetic acid bacterium showing high ethanol oxidizing ability and acetic acid production at high temperature. These bacteria produce acetic acid from ethanol by an oxidative pathway employing two enzymes; pyrroloquinoline quinone alcohol dehydrogenase (PQQ-ADH) and acetaldehyde dehydrogenase (ALDH) located at the surface of cell membrane. The microenvironment surrounding acetic acid bacteria during acetic acid fermentation contains various stressors such as high temperature, acetic acid and ethanol. Although there are many stressors, acetic acid bacteria show relatively normal growth with high resistance to those stressors.

GroEL and GroES, which are members of the heat shock proteins, HSP60 and HSP10 protein families, respectively, are among the most extensively investigated stress proteins. These proteins are believed to protect cells from various stresses resulted from changes in the surrounding environments. They mediate the correct folding and assembly of many cellular proteins and consequently, prevent misfolding and promote the refolding and proper assembly of unfolded proteins. In the light of these functions, GroEL and GroES are believed to be associated with the resistance against various stresses. Hence, elucidating the mechanism underlying the resistance of acetic acid bacteria to stressors is important for understanding the mechanism of vinegar production. The obtained results will be helpful in developing strains with more sophisticated acetic acid fermentation abilities.

It has previously reported on cloning and characterization of *groESL* operon from *A. pasteurianus* NBRC 3283 (formally known as *A. aceti* IFO 3283) (Okamoto-Kainuma

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et al., 2002). They found that the *groESL* gene expression was induced by stressors such as heat, acetic acid or ethanol, which exist during vinegar fermentation, and *groESL* overexpression made the NBRC 3283 strain more resistant than the control strain to stressors. In this study, the *groESL* gene from thermotolerant acetic acid bacterium, *A. pasteurianus* SKU1108 was cloned and its nucleotide sequences were determined. Moreover, to elucidate the function of the heat shock protein, GroES/EL; the *groEL* gene disruptant was constructed and then investigated for its contribution to the bacterial survival mechanism under stress conditions. We also analyzed the regulatory region, the CIRCE heat shock element, including the effect of stressors to induce *groESL* gene expression and also its distribution in acetic acid bacteria.

The *groEL* gene, which encodes 60-kDa (GroEL) heat shock proteins, is ubiquitous and evolutionarily highly conserved among bacteria. In recent years, the housekeeping gene, *groEL* gene, has been considered as useful phylogenetic marker. Commonly, acetic acid bacterial species are identified by polyphasic analysis, combining with phenotypic, chemotaxonomic and genotypic data (Colwell, 1970; Cleenwerck and De vos, 2008). For genotypic methods of acetic acid bacteria identification has been based on 16S rRNA gene. The use of 16S rRNA alone is not sufficient for discrimination between species that have very high similar 16S rRNA sequences (Stackebrandt and Goebel, 1994). Due to the lack of variability of 16S rRNA gene sequence did not allow identification of closely related species or subspecies. Cleenwerck *et al.* (2010) and Huang *et al.* (2014) have previously observed that evolutionary trees constructed from the nucleotide sequences of *groEL* gene in *Gluconacetobacter* and *Acetobacter*, respectively, demonstrated remarkable similarity to those derived from 16S rRNA genes. Therefore, the combination of *groEL* gene and 16S rRNA gene analysis was performed for classification and identification of the genus *Acetobacter.*

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OBJECTIVES

1. To clone and determine nucleotide sequences of *groESL* genes encoding heat shock protein (GroES and GroEL) from *A. pasteurianus* SKU1108 and characterize the role of *groESL* operon in acetic acid fermentation at high temperature and their effect on physiological properties of acetic acid bacteria

2. To investigate the variation of *groESL* gene in thermotolerant and thermosensitive acetic acid bacteria by PCR and Southern hybridization

3. To identify and classify acetic acid bacteria by 16S rRNA gene sequence and *groEL* gene sequence analyses

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LITERATURE REVIEWS

1. Acetic Acid Bacteria

1.1 General characteristics

Acetic acid bacteria are classified in the family *Acetobacteraceae* as a branch of the acidophilic bacteria in the α-subdivision of the *Proteobacteria* (De Ley *et al*., 1984a; Sievers *et al.,* 1994). These bacteria are gram negative or gram variable, ellipsoidal to rod-shaped cells that have an obligate aerobic metabolism with oxygen as the terminal electron acceptor. Their optimal temperature for growth is 25 to 30°C. In nature, acetic acid bacteria are particularly well adapted to sugar or alcoholic solutions. Therefore, the natural habitats of acetic acid bacteria are fruits, flowers, beer, wine, cider, vinegar, honey, and fermented products. Acetic acid bacteria produce acetic acid from ethanol by two sequential oxidation reactions of pyrroloquinoline quinone alcohol dehydrogenase (PQQ-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*., 1984b; Swings, 1992; Matsushita *et al*., 1994).

1.2 Genera of acetic acid bacteria

Recently, the family *Acetobacteraceae* is composed of eighteen genera. The first reported genus was *Acetobacter* (Beijerinck, 1898). *Acetobacter* strains are also frequently recovered from fruits and flowers (De Ley *et al.,* 1984a; Lisdiyanti *et al.,* 2003; Seearunruangchai *et al.,* 2004; Sievers and Swings, 2005; Kommanee *et al.,* 2008; Tanasupawat *et al.,* 2009). The second reported genus was *Gluconobacter* (Asai, 1935). *Gluconobacter* are mainly isolated from sugar-rich habitats, such as fruits and flowers (Lisdiyanti *et al.,* 2003; Sievers and Swings, 2005) except *G. morbifer* sp. nov. that was isolated from the gut of *Drosophila melanogaster* (Roh *et al.,* 2008). They have the ability to intensely oxidize glucose to gluconic acid rather than ethanol to acetate and no oxidation of acetate, which were different from strains of the genus *Acetobacter* (Asai, 1934; 1935). The third reported genus was *Acidomonas* (Urakami *et al.,* 1989). The main characteristics

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of *Acidomonas* are methanol assimilation and oxidation of acetate but not lactate. The fourth reported genus was *Gluconacetobacter* (Yamada *et al.,* 1997). In 1984, the genus *Acetobacter* was divided in two subgenera, *Acetobacter* and *Gluconacetobacter.* In 1998, the genus *Gluconacetobacter* was elevated. The genera of *Gluconacetobacter* and *Acetobacter* can be differentiated on the basis of their ubiquinone content. Ubiquinone Q9 is found in *Acetobacter*, and ubiquinone Q10 in *Gluconacetobacter* (Trček and Teuber, 2002). The genus *Asaia* was introduced as the fifth genus in family *Acetobacteraceae*. Firstly, the genus *Asaia* had been assumed as an acetic acid bacterium with specific niches in the tropical region because the *Asaia* strains have been isolated from flowers and fruits in the tropical countries, Indonesia (*Asaia bogorensis* and *Asaia siamensis*) (Yamada *et al.,* 2000; Katsura *et al.,* 2001) and Thailand (*Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannensis*) (Katsura *et al.,* 2001; Yukphan *et al.*, 2004b; Malimas *et al.,* 2008). In 2010, the three novel species, *Asaia astilbes* sp. nov., *Asaia platycodi* sp. nov., and *Asaia prunellae* sp. nov., isolated from flowers collected in several prefectures in Japan were reported (Suzuki *et al.,* 2010). Therefore, the genus *Asaia* is not an acetic acid bacterium of which distribution is limited to tropical countries, and can be regarded as a cosmopolitan bacterium. The sixth reported genus was *Kozakia* (Lisdiyanti *et al.,* 2002). The genus *Kozakia* consists of only one species. *Kozakia baliensis* strains have been isolated from ragi (starter for fermented foods) and palm brown sugar. The seventh reported genus was *Swaminathania* (Loganathan and Nair, 2004). The genus *Swaminathania* also consists of only one species. *Swaminathania salitolerans* strains were isolated from wild rice. The genus *Saccharibacter* was introduced as the eighth genus in the family *Acetobacteraceae*, with a single species, *Saccharibacter floricola* isolated from the pollen of Japanese flowers (Jojima *et al.,* 2004). The ninth reported genus was *Neoasaia* with a single species, *Neoasaia chiangmaiensis* isolated from tropical flowers (Yukphan *et al.,* 2005). The tenth reported genus was *Granulibacter* with a single species, *Gr. bethesdensis* (Greenberg *et al.,* 2006). It was isolated from the patients with chronic granulomatous disease and appears to be genuine pathogens. The eleventh reported genus was *Tanticharoenia* (Yukphan *et al.,* 2008). *Tanticharoenia sakaeratensis* gen. nov., sp. nov. was isolated from soil collected in Thailand. The twelfth reported genus was *Commensalibacter* (Roh *et al.,* 2008). *Commensalibacter intestini* gen. nov., sp. nov. was isolated from *Drosophila* midgets. The thirteenth reported genus was *Ameyamaea* (Yukphan *et al.,* 2009). *Ameyamaea*

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chiangmaiensis gen. nov., sp. nov. was isolated from the flowers of red ginger collected in Chiang Mai, Thailand. The fourteenth reported genus was *Neokomagataea* (Yukphan *et al.,* 2011). This genus contains only two species, *Neokomagataea thailandica* sp. nov. and *Neokomagataea tanensis* sp. nov., that were isolated from flowers of lantana and candle bush, respectively at Tan Island, Nakhon-Si-Thammarat, Thailand. In 2012, the fifteenth new genus *Komagataeibacter* gen. nov. was proposed with 13 new species combinations on the basis of taxonomic characteristics. This genus was elevated from the *Gluconacetobacter xylinus* group but not from *Gluconacetobacter liquefaciens* group (Yamada *et al.,* 2012). The sixteenth reported genus was *Endobacter* gen. nov. (Ramîrez-Bahena *et al.,* 2013). The only one species, *Endobacter medicaginis* gen. nov., sp. nov. is proposed. It was isolated from a surface-sterilized nodule of Medicago sativa in Zamora (Spain). The seventeenth reported genus was *Swingsia* gen. nov. (Malimas *et al.,* 2013). Nowaday, this genus consists of only one species as *Swingsia samuiensis* that was isolated from a flower of golden trumpet collected at Samui Island, Surathani, Thailand. The last reported genus was *Nguyenibacter* gen. nov. (Thi Lan Vu *et al.,* 2013). This genus comprises only one species as *Nguyenibacter vanlangensis* gen. nov., sp. nov. which was isolated from the rhizosphere of Asian rice collected at Long Thanh Trung Commune, Hoa Thanh District, Tay Ninh Province, Vietnam.

1.3 Species of genus *Acetobacter*

Nowadays, the taxonomy of genus *Acetobacter* is composed of twenty-five species. These *Acetobacter* species are phylogenetically divided into two major groups (Yamada and Yukphan, 2008). The first group corresponded to the *Acetobacter aceti* group, which included fifteen closely related species: *Acetobacter aceti*, *Acetobacter estunensis*, *Acetobacter malorum*, *Acetobacter cerevisiae*, *Acetobacter orleanensis*, *Acetobacter cibinongensis*, *Acetobacter orientalis*, *Acetobacter indonesiensis*, *Acetobacter tropicalis*, *Acetobacter senegalensis*, *Acetobacter oeni*, *Acetobacter nitrogenifigens*, *Acetobacter farinalis*, *Acetobacter persici* and *Acetobacter sicerae*. The second group corresponded to the *Acetobacter pasteurianus* group, which included ten closely related species: *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Acetobacter peroxydans*, *Acetobacter papayae*, *Acetobacter lovaniensis*, *Acetobacter syzygii*, *Acetobacter fabarum*,

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Acetobacter ghanensis, Acetobacter okinawensis and *Acetobacter lambici* (Skerman *et al.,* 1980; Sokollek *et al.,* 1998; Lisdiyanti *et al.,* 2000, 2001, 2002; Cleenwerck *et al.,* 2002, 2007, 2008; Dutta and Gachhui, 2006; Silva *et al.,* 2006; Ndoye *et al.,* 2007; Tanasupawat *et al.,* 2011a; Iino *et al.,* 2012; Li *et al.,* 2014; Spitaels *et al.,* 2014).

1.4 Acetic acid fermentation and alcohol dehydrogenase

Acetic acid fermentation (or vinegar production) is a process of oxidation in which alcohol is converted into acetic acid by acetic acid bacteria under aerobic condition. It is the most distinguished characteristics of acetic acid bacteria. This process consists of two sequential reactions (Ameyama and Adachi, 1982a, b). The first reaction is oxidation of ethanol to acetaldehyde that carried out by pyrroloquinoline quinone alcohol dehydrogenase (PQQ-ADH). The second reaction is the conversion of acetaldehyde to acetic acid by acetaldehyde dehydrogenase (ALDH) (Figure 1).

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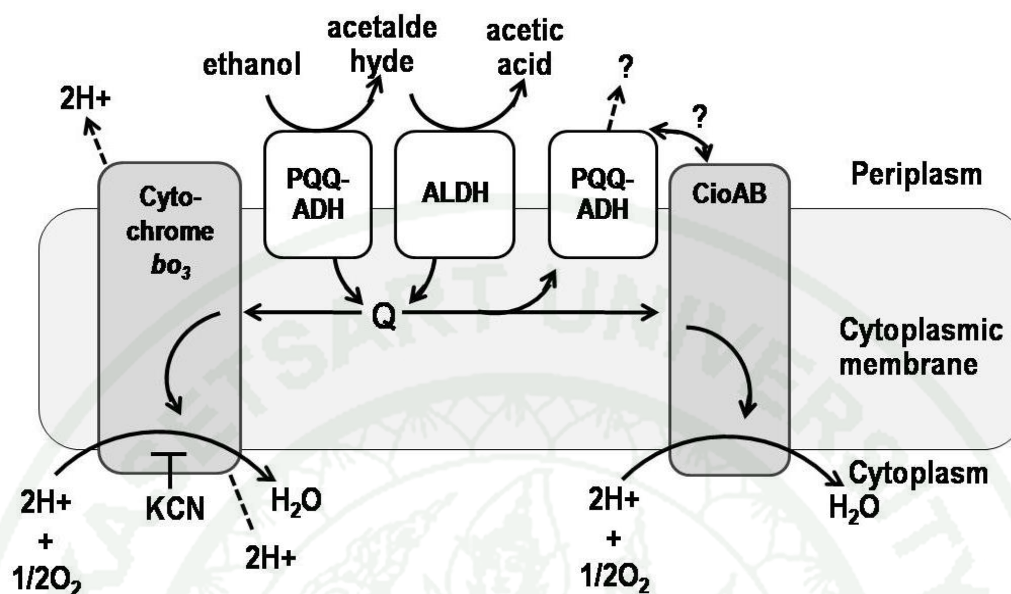

Figure 1 An overview of the ethanol oxidation respiratory chain of acetic acid bacteria. Ethanol is oxidized to acetic acid by a sequential action of PQQ-ADH and membrane-bound aldehyde dehydrogenase (ALDH), reducing Q in the cytoplasmic membrane. Then, the respiratory chain branches to energy-producing 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)

In acetic acid bacteria, there are two groups of dehydrogenase enzyme. The first group, NAD^+-dependent dehydrogenase, is located in the cytoplasm of acetic acid bacteria (Chinnawirotpisan *et al.,* 2003). Another one is $NAD(P)^+$-independent dehydrogenase located at the cytoplasmic membrane (Matsushita *et al.*, 1994). The latter alcohol dehydrogenase is associated with acetic acid fermentation (Matsushita *et al.*, 1992a, 1992b, 1994, 1995). The membrane bound dehydrogenase can be divided into quinoproteins and flavoproteins that have pyrroloquinoline quinone (PQQ) and covalently bound flavin adenine dinucleotide (FAD) as prosthetic groups, respectively. The functions of these

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quinoprotein and flavoprotein dehydrogenases link to the respiratory chain by transferring electrons to the final electron receptor, oxygen and generating energy for growth (Shinagawa *et al.*, 1990; Matsushita *et al.,* 1991, 1994). The quinoprotein alcohol and aldehyde dehydrogenase play main role in vinegar production (Matsushita *et al.*, 1994). Beside the PQQ-ADH type, acetic acid bacteria have NAD^+-dependent dehydrogenase (NAD^+-ADH) that has the different biochemical characteristics when comparing with PQQ-ADH. Chinnawirotpisan *et al.* (2003) studied about the relationship between PQQ-ADH and NAD^+-ADH by constructing the PQQ-ADH deficient mutant from *A. pasteurianus* SKU1108. It was revealed that NAD^+-ADH involved only in ethanol assimilation through the TCA and glyoxylate cycles but not acetic acid production (Figure 2).

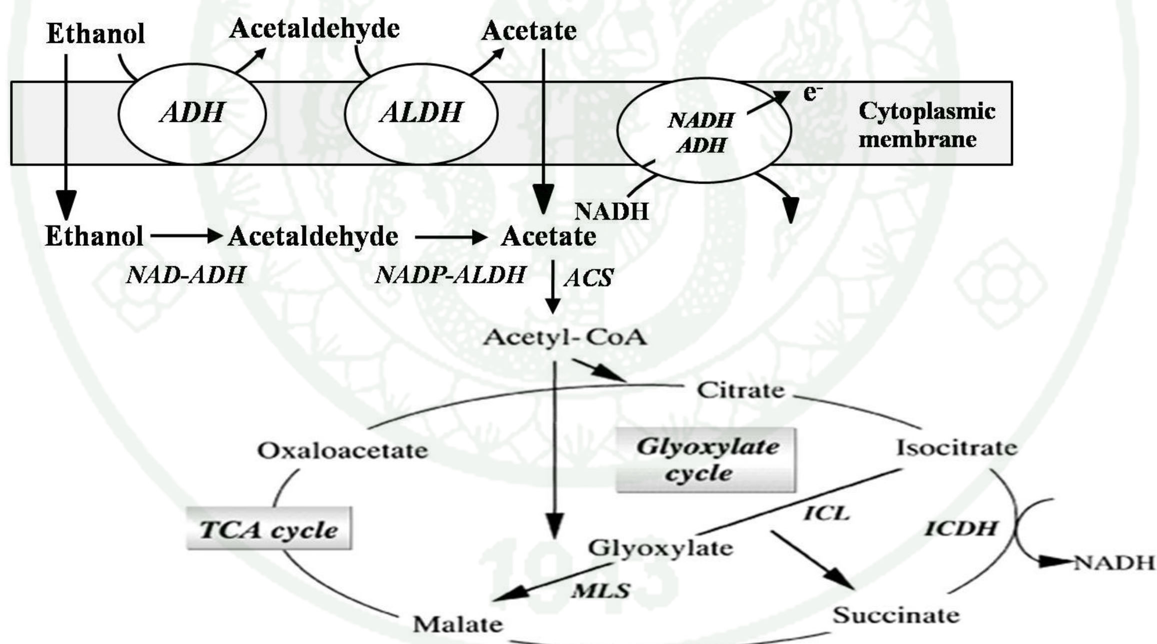

Figure 2 Metabolic pathways of oxidative assimilation of ethanol into TCA and glyoxylate cycles *via* acetyl-CoA. The enzymes involved in ethanol assimilation are abbreviated in the figure: NAD^+-ADH, NAD^+-dependent alcohol dehydrogenase; NADP-ALDH, NADP-dependent aldehyde dehydrogenase; ACS, acetyl-CoA synthetase; ICL, isocitrate lyase; and ICDH, isocitrate dehydrogenase.

Source: Chinnawirotpisan *et al.* (2003)

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PQQ-ADH consists of three subunits (only two subunits in genus *Gluconacetobacter* and *Frateuria*) (Table 1) and has a pyrroloquinoline quinone, 4 heme C moieties, and a tightly bound Q as the electron transfer mediators (Yakushi and Matsushita, 2010). The subunit I (approx. 80 kDa in the molecular size) encoded by *adhA* gene is a quinoprotein containing pyrroloquinoline quinone (PQQ) and a heme *c* moiety as the prosthetic groups (Inoue *et al.,* 1989). The function of this subunit is the catalytic site for ethanol oxidation (Matsushita *et al.,* 1996). The subunit II (approx. 50 kDa in the molecular size) encoded by *adhB* gene is quinohemoprotein containing three heme C moieties and presumably a Q (Matsushita *et al.,* 2008). The function of subunit II is the electron mediator from subunit I to the membranous Q. The last subunit, subunit III encoded by *adhS* gene (approx. 15 kDa in the molecular size) is a peptide and suggested to have no prosthetic groups (Kondo *et al.,* 1995). The subunit III was found existing freely in periplasmic space in the PQQ-ADH complex on the cytoplasmic membrane. The mutant strains defective in the *adhS* gene encoding subunit III were constructed. The results revealed that the mutants lose ADH activity because they produce only the subunit II but fail to produce subunit I and subunit III. From the results, it can be speculated that the subunit I is unstable, and destroyed by proteolytic degradation therefore subunit III formed a complex with subunit I for protecting it from proteolysis. It can be concluded that the function of subunit III seems to be as molecular chaperone for folding or maturation of the subunit I (Kondo *et al.,* 1995; Masud *et al.,* 2010).

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Table 1 Molecular organization of PQQ-ADHs from various acetic acid bacteria

Species	Strains	Molecular sizes of subunits (kDa)			References
		I	II	III	
A. lovaniensis	IFO 3284	72	50	15	(Matsushita *et al.,* 1992c)
A. pasteurianus	NCI 1452	72	44	20	(Kondo *et al.,* 1995)
	KKP584	74	44	16	(Trček *et al.,* 2006)
	IFO 3191	76	55	16	(Kanchanarach *et al.,* 2010)
	SKU1108	76	55	16	(Kanchanarach *et al.,* 2010)
	MSU10	76	55	16	(Kanchanarach *et al.,* 2010)
G. polyoxogenes[a]	NBI 1028	72	44	-	(Tayama *et al.,* 1989)
G. europaeus	V3	72	45	-	(Trček *et al.,* 2006)
G. intermedius	JK3	72	45	-	(Trček *et al.,* 2006)
G. diazotrophicus	PAL5	71	44	-	(Gómez-Manzo *et al.,* 2008)
G. xylinus[b]	-	71	44	-	(Gómez-Manzo *et al.,* 2008)
G. oxydans	IFO 12528	85	49	14	(Adachi *et al.,* 1978)
A. methanolica	JCM 6891	80	54	8	(Frébortová *et al.,* 1997)
F. aurantia[c]	LMG 1558	72	45	-	(Trček *et al.,* 2013)

[a] Formerly *Acetobacter polyoxogenes*

[b] *Gluconacetobacter xylinus*

[c] *Frateuria aurantia* has been identified as a pseudo-acetic acid bacterium.

Source: Modified from Yakushi and Matsushita (2010)

Moreover, Takemura *et al.* (1993) reported that PQQ-ADH activity of *A. pasteurianus* was evaluated more than ten fold in the medium containing ethanol whereas the activity largely decreased when cultivated without ethanol. They speculated that ethanol was the important factor required for the correct localization of dehydrogenase subunits in the membrane. The cause of low ADH activity in the absence of ethanol was the incorrect localization of dehydrogenase subunits. Another investigation also reported that both ADH and ALDH activities were decreased when cultivated in the medium containing acetic acid (Theeragool *et al*., 1996). Moreover, during the stationary phase

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after the complete consumption of ethanol, membrane-bound ADH and ALDH activities of *A. pasteurianus* IFO 3283 decrease whereas slightly increase again in the overoxidation phase (Matsushita *et al.,* 2005a). In *Gluconobacter suboxydans*, ADH activity was decreased in some culture conditions such as high aeration condition, that because ADH level was mainly produced as inactive form (Matsushita *et al.,* 1995).

1.5 Stressors and adaptive responses during acetic acid fermentation

During acetic acid fermentation process, there are various stressors in the microenvironment surrounding acetic acid bacteria as follows: (i) high temperature which occurs during fermentation, (ii) ethanol, an acetic acid fermentation initiating compound that is always present around the cells. Although ethanol is a source of ATP for acetic acid bacteria, it is considered to be a stressor for these cells as well as other microorganisms and (iii) acetic acid, which is a fermentation product and whose level gradually increases as the fermentation proceeds. This compound is acidic and lowers the pH around cells to a level inadequate for cell growth. Although there are many stressors, acetic acid bacteria show relatively normal growth with high resistance to these stressors.

1.5.1 High temperature

It is well known that temperature is an important physical stress factor related to acetic acid fermentation. In 2007, Schumann concluded that in eubacteria, temperature is one of the physical parameters sensed by bacteria and relates to regulate some gene expression. Three different macromolecules that are DNA, mRNA and proteins, have been found to be able to sense by this stressor. In bacterial cells, there are two way for response to the temperature as follows: (i) the heat shock response and (ii) the high temperature response. The heat shock response is induced by temperature increment and is transient; the high temperature response needs a specific temperature to become induced and proceeds as long as cells are exposed to that temperature.

In tropical countries, acetic acid fermentation by mesophilic acetic acid bacteria is not efficient because the fermentation process requires the cooling system for

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controlling the fermentation temperature to be 25-30°C. Nowadays, global warming is the serious problem that makes more difficult to control the temperature in large scale fermentation. Thus thermotolerant acetic acid bacteria are very interesting for vinegar fermentation at higher temperatures, allowing a possible reduction in cooling expenses and other costs (Saeki *et al*., 1997; Adachi *et al*., 2003). Ndoye *et al*. (2006) isolated 2 strains of thermotolerant acetic acid bacteria from tropical products of Sub-Saharan Africa and identified as *A. tropicalis* and *A. pasteurianus*. These strains can produce a high level of acetic acid at 35 and 38°C, respectively. In Sub-Saharan Africa, the use of these strains for industrial vinegar production would be possible to reduce cooling expenses.

In 2011, Soemphol *et al.* studied about the genes involved in the thermotolerant mechanism of thermotolerant *Acetobacter tropicalis* SKU1100. They found that 24 genes, related to thermotolerance, were required for growth at high temperature. All of the 24 genes were categorized as (i) heat shock or stress response genes that play major role in the folding repair and degradation of denatured proteins (ii) gene involved in the cell cycle and cell division, which may be related to DNA replication errors and damage at high temperatures (iii) the transport system; Na^{+}/H^{+} antiporter involved in intracellular pH regulation with K^{+} but not with Na^{+}. In addition, they found that some genes were required for both thermotolerance and acetic acid resistance. Another investigation reported about the ADH properties of thermotolerant *A. pasteurianus* comparing with mesophilic strain (Kanchanarach *et al.,* 2010). The results showed that ADH of thermotolerant strain had slightly higher optimal temperature and heat stability than the same protein of the mesophilic strain. Furthermore, ADH from thermotolerant strains showed a higher resistance to ethanol and acetic acid than ADH from mesophilic strain at elevated temperature. Matsutani *et al.* (2012) investigated the relationship between phylogeny and thermotolerance of the three closely related strains of *A. pasteurianus* by genome-wide phylogenetic analysis. The results revealed that the more tolerant strains, SKU1108 and NBRC 3283, are more closely related to each other than to thermosensitive strain, IFO 3191. They also found several genes with increased mutation rate in thermosensitive strains, IFO 3191 when compared with the thermotolerant strains, SKU1108 and NBRC 3283. In 2013, Matsutani *et al.* constructed the thermo-adapted strain from *A. pasteurianus* SKU1108 by performing *in vitro* adaptation. The results showed that the obtained thermo-

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adapted strains, especially TH-3 strain, have the ability for acetic acid fermentation at higher temperature more than the wild-type strain. Genomic analysis by next generation sequencing identified the mutation or disruption site from two adapted strains. There were three mutated genes as follows; amino acid transporter, transcription regulator MarR and C_4-dicarboxylate transporter. From the results, it could be concluded that the mutation of these genes may play the crucial role in thermotolerance and fermentation at higher temperature. Another investigation of the mutated genes during performing thermal adaptation of *A. pasteurianus* revealed that a 92 kb deletion and three single nucleotide mutations occurred in the genome (Azuma *et al.,* 2009). The mutated gene caused an amino acid mutation of "two component hybrid sensor histidine kinase and regulator" and a 92-kb deletion region included a homolog of "C_4-dicarboxylate transporter". The mutated gene that were found in this investigation, were phylogenetically identical to the mutated genes of two thermo-adapted strains as described above (Matsutani *et al.*, 2013).

1.5.2 Ethanol

When *Acetobacter* and *Gluconacetobacter* species were cultured in the medium containing ethanol, they showed three growth phases; (i) ethanol oxidation phase; in this phase, they grew by completely oxidize ethanol to acetic acid (ii) first stationary phase or transition phase; they stop growing and remained for a while in the stationary phase where their viable cell number is decreased (iii) overoxidation of acetate or acetate oxidation phase; they start growing again by utilizing the accumulated acetic acid (Matsushita *et al.,* 2005a; Sakurai *et al.,* 2012). DNA microarray was performed for analyzing the time-dependent transcriptome changes in cells of *Acetobacter aceti* during growth on ethanol (Sakurai *et al.,* 2011, 2012). They found the genes encoding PQQ-ADH and PQQ-ALDH including NAD^+-ADH were constitutively expressed throughout the culture period. Whereas the expression of the genes encoding tricarboxylic acid (TCA) cycle enzymes were quit low in during acetate accumulation, but were upregulated when cells start to oxidize the accumulated acetate. Moreover, after cells start oxidizing ethanol, the genes encoding enzymes of glyoxylate pathway were found to be upregulated. They suggested that this pathway is important for ethanol utilization as a carbon source. Isocitrate lyase (*aceA*) and malate synthase (*glcB*), related with the glyoxylate pathway

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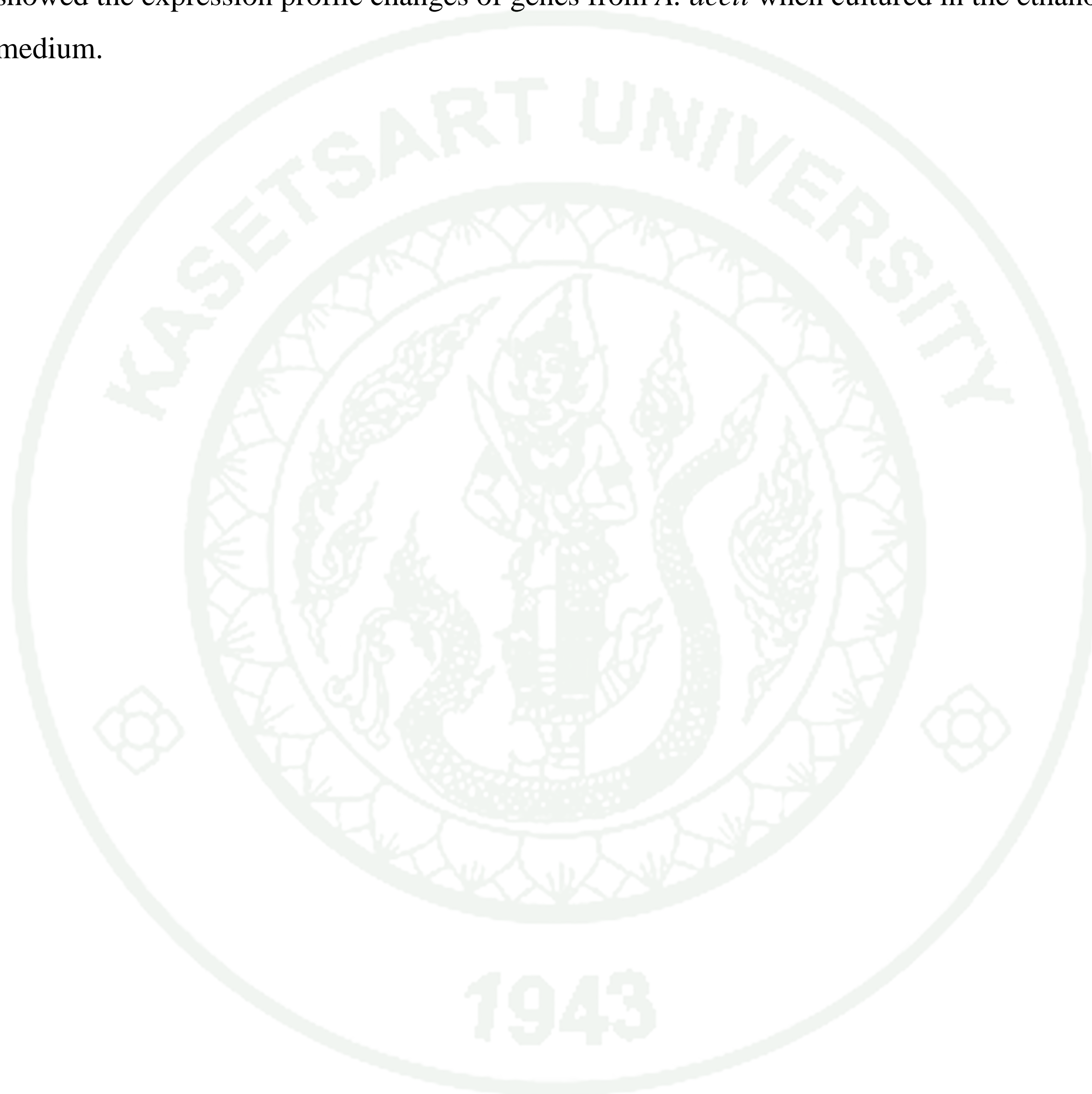
were mutated by performing gene knockout (Sakurai *et al.,* 2013). The results revealed that the mutant could grow normally during ethanol oxidation to acetate, but exhitibited slower growth than the wild type strain when the accumulated acetate was oxidized. Figure 3 showed the expression profile changes of genes from *A. aceti* when cultured in the ethanol medium.



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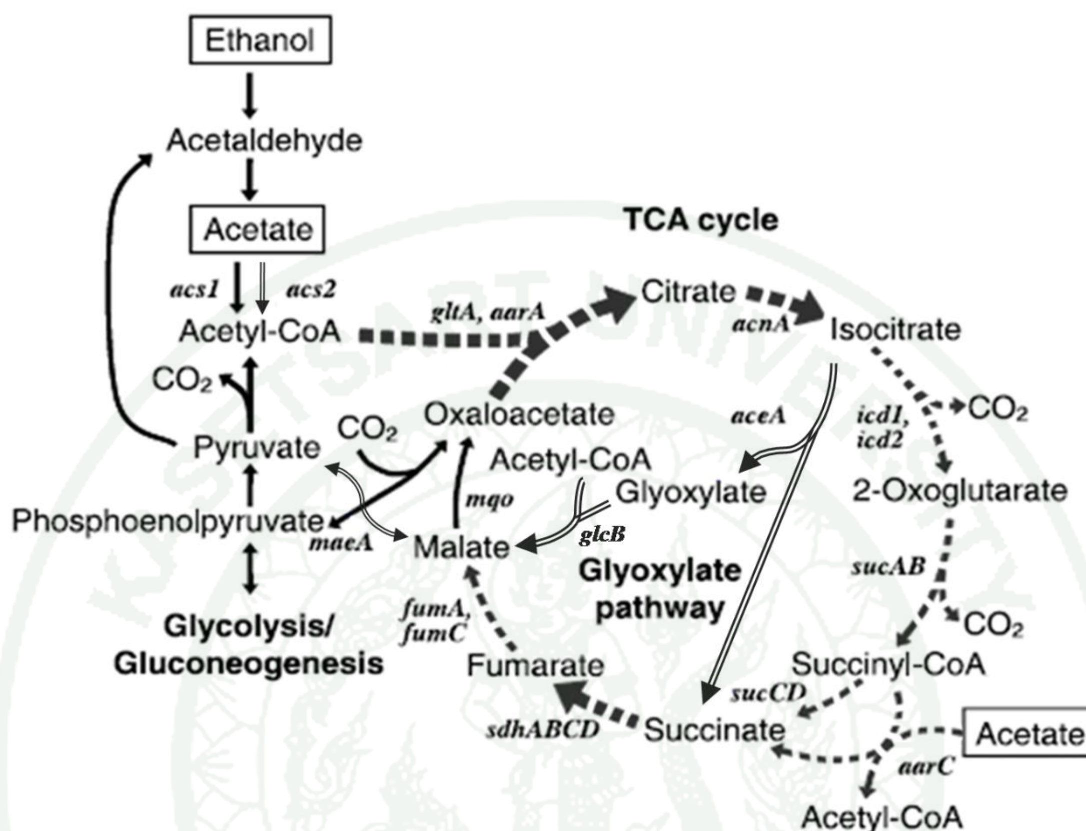

Figure 3 The predicted central metabolic pathway of *A. aceti* NBRC 14818. The thickness of the arrows indicates the relative expression level of the genes involved in the respective reactions. Dash arrows indicate that the genes mediating the reactions were upregulated in cells after the transition phase. Double arrows indicate that the genes involved in the reactions were upregulated in cells after the early-ethanol-oxidation phase. *acs1* and *acs2*, gene encoding acetyl-CoA synthetase; *gltA* and *aarA*, citrate synthase; *maeA*, malic enzyme; *fumA* and *fumC*, fumarate hydratase; *mqo*, malate:quinone oxidoreductase; *acnA*, aconitase; *icd1* and *icd2*, isocitrate dehydrogenases; *sucAB*, 2-oxoglutarate dehydrogenase; *sucCD*, succinyl-CoA synthetase; *sdhABCD*, succinate dehydrogenase; *aceA*, isocitrate lyase; *glcB*, malate synthase; *aarC*, succinyl-CoA: acetate CoA transferase

Source: Sakurai *et al.* (2012)

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Aerobic metabolism causes the cellular oxidative stress that induces DNA damage and protein misfolding. When *Acetobacter* species are cultured on ethanol, the gene related with SOS response system and several molecular chaperones are induced. Many genes encoding oxidative stress-resistant proteins were found in the genome of *A. aceti* NBRC 14818. The *katE* gene encoding catalase was time-dependent upregulated until cells start the transition phase (Sakurai *et al.,* 2012). The same gene was disrupted in *A. pasteurianus* NBRC 3283 (Okamoto-Kainuma *et al.,* 2008). The results revealed that the *katE* gene disruption caused the delayed growth in the acetate-oxidation phase whereas the growth rate of the wild type and the disruptant were not different during the ethanol-oxidation phase. Beside *katE* gene, there was another oxidative stress-resistant protein, superoxide dismutase encoded by *sod* gene. The expression level of *sod* gene was slightly upregulated after the transition phase (Sakurai *et al.,* 2012). Moreover, several stress-responsive genes related in SOS system and molecular chaperones are upregulated when *Acetobacter* were cultured on the ethanol (Okamoto-Kainuma *et al.,* 2004; Ishikawa *et al.,* 2010a, 2010b). Moreover, these genes, *recA*, *uvrABC* (excinuclease UvrABC), *dnaJ* and *clpB*, were induced during the early-to mid-ethanol-oxidation phase but were lower at the early ethanol oxidation phase. It could be suggested that ethanol is not the direct inducer for SOS responsive gene and molecular chaperones genes expression. The inducer might be the damage caused by acetaldehyde, the toxic intermediate (Sakurai *et al.,* 2012, 2013).

1.5.3 Acetic acid

Several species of acetic acid bacteria are known to be relatively tolerated to acetate. The gram-negative genera *Acetobacter* and *Gluconobacter* are widely used in vinegar fermentation. In order to understand about acetic acid resistance, Lasko *et al.* (1997) performed proteomic analysis of acetate-resistant bacteria, *Acetobacter aceti* and *Gluconobacter suboxydans*. The results showed that when cells grew in the medium containing acetate, many stress response proteins were elevated. Moreover, they found only eight proteins designated as acetate-specific stress proteins (Asps), are overexpressed in response to acetate. In 1990, Fukaya *et al.* constructed acetic acid sensitive mutant from *A. aceti* by using chemical mutation and proposed the acetate resistance genes (*aarABC*) that were responsible for expression of acetic acid resistance. After that the functions of the

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aarA and *aarC* genes were identified as citrate synthase involved in TCA cycle and the protein involved in acetate assimilation (Fukaya *et al.,* 1990, 1993). The results suggested that these genes are responsible for acetic acid assimilation conferring resistance. In 2008, Mullins *et al.* proposed AarC homologue, a succinyl-CoA: acetate CoA-transferase (SCACT). SCACT encoded by *aarC*, involved in citric acid cycle conferring acetic acid resistance in *A. aceti*. Steiner and Sauer (2001) investigated the changes in global protein expression levels during long-term adaptation of *A. aceti* to high acetate concentrations by two-dimensional electrophoresis (2-DE). They reported complex proteome responses with at least 50 proteins are specifically induced by adaptation to acetate but not by other stress conditions, such as heat or oxidative or osmotic stress. One of protein in cytoplasm whose production was enhanced in response to acetic acid was identified as aconitase (Nakano *et al.,* 2004). In addition, they found that the *A. aceti* harboring multiple copies of aconitase gene could produce high concentration of acetic acid when compared with wild type. In 2005, Matsushita *et al.* proposed that the efflux pump mechanism was responsible for acetic acid resistance in acetic acid bacteria. Acetic acid/acetate was uptaken in the inside-out membrane vesicles depending on the proton motive force but not on ATP. Thus, *A. aceti* possesses a proton motive force-dependent efflux pump for acetic acid (Matsushita *et al.,* 2005a, 2005b). It has been another reported that *A. aceti* possesses a putative ATP-binding cassette (ABC) transporter or AatA that was induced by acetic acid (Nakano *et al.,* 2006). They also found that the *aatA* mutant strains showed decrease level of acetic acid resistance whereas the enhancement of acetic acid resistance was found in the *aatA* gene overexpressed strains. The results clearly suggested that AatA is involved in acetic acid resistance (Nakano *et al.,* 2006). Moreover, Trček *et al.* (2006) reported the relationship between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. They compared the growth properties and molecular characteristics of PQQ-ADH from three acetic acid bacteria as follows; *Gluconacetobacter europapeus* (the highest resistance to acetic acid 10%), *Gluconacetobacter intermedius* and *Acetobacter pasteurianus* (resisted up to 6%). The results suggested that the species that could grow in the extreamly high concentrations of acetic acid, should have two unique features as follows: (i) high ADH activity and (ii) high acetic acid stability of the purified enzyme. They also studied the adaptive response against high acetic acid concentration in *Gluconacetobacter europapeus.* The results showed that acetic acid changes the total fatty

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acid composition, morphological properties including PQQ-ADH expression (Trček *et al.,* 2007). In 2008, Nakano and Fukaya summarized the mechanisms related to acetic acid resistance in acetic acid bacteria. There are several mechanisms as follows; alcohol oxidation-related mechanism, acetate assimilation-related mechanism, a putative ABC transporter, structural stability of cytoplasmic enzymes at low pH, and protection of cytoplasmic proteins against denaturation by general stress proteins (Figure 4). Another investigation reported about the acetic acid adapted strains that were isolated by performing the sequential cultivations of an acetic acid sensitive strain, *A. syzygii*, in the medium containing 1% acetic acid (Sintuprapa *et al.,* 2008a; 2008b). The results revealed that these adapted strains showed higher PQQ-ADH and ALDH activities than the wild type strain. Moreover, the results supported the previous reports that there are several mechanisms relating with the acetic acid resistance in acetic acid bacteria, such as acetate assimilation and PQQ-ADH functions.

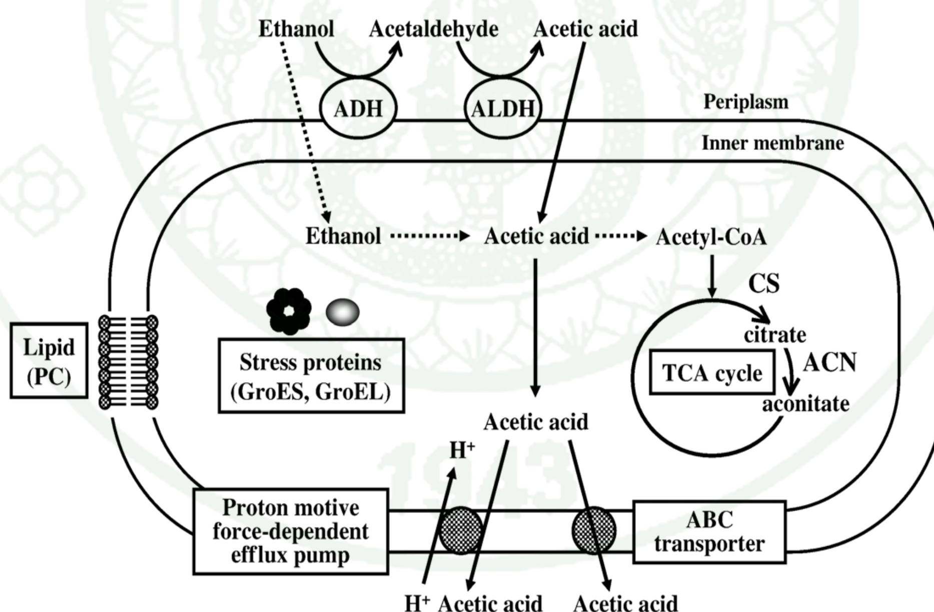

Figure 4 Schematic representations of molecular machineries that confer acetic acid resistance in *Acetobacter* and *Gluconacetobacter*. Intracellular cytosolic enzymes are intrinsically resistant to low pH and are protected against denaturation by stress proteins such as GroES and GroEL.

Source: Nakano and Fukaya (2008)

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2. Molecular Chaperone in Bacteria

There are two major families of molecular chaperones that have been recognized; Heat shock protein (Hsp) 70 family, and the Hsp60 family or the chaperonin families (Table 2). Major Hsp70 family is composed of DnaK, DnaJ and GrpE (both co-chaperones of DnaK) which form the DnaK chaperone machine working in cooperation with the ClpB chaperone (Georgopoulos and Welch, 1993; Zolkiewski, 1999). Additionally, Hsp60 family consists of GroEL (chaperonin) and GroES (co-chaperonin), which constitute the GroE chaperone machine (Georgopoulos and Welch, 1993).

Table 2 Components of Hsp70 and chaperonin system

Bacteria	Properties
Hsp 70 system	
DnaK	70 kDa protein with ATPase activity, binds extended peptides, interacts with Hsp40 or its homologues and GrpE, required for post-translational protein import
DnaJ	40 kDa protein, binds unfolded proteins, interacts with Hsp70 or its homologues, stimulates their ATPase activity
GrpE	20 kDa protein, nucleotide exchange factor for Hsp70 or its homologues
Chaperonin system	
GroEL-GroES	Homo-oligomer, two rings of 60 kDa subunits, ATPase activity, binds folding intermediates, mediates protein folding together with co-chaperonin GroES (Hsp10)

Source: Modified from Braig (1998)

They are essential for growth of all organisms under all conditions. In case of DnaK system, the local interaction of nascent peptide occurs in a co-translational manner. In case of GroEL system, the entrapment of substrates is essential for folding by GroEL complex

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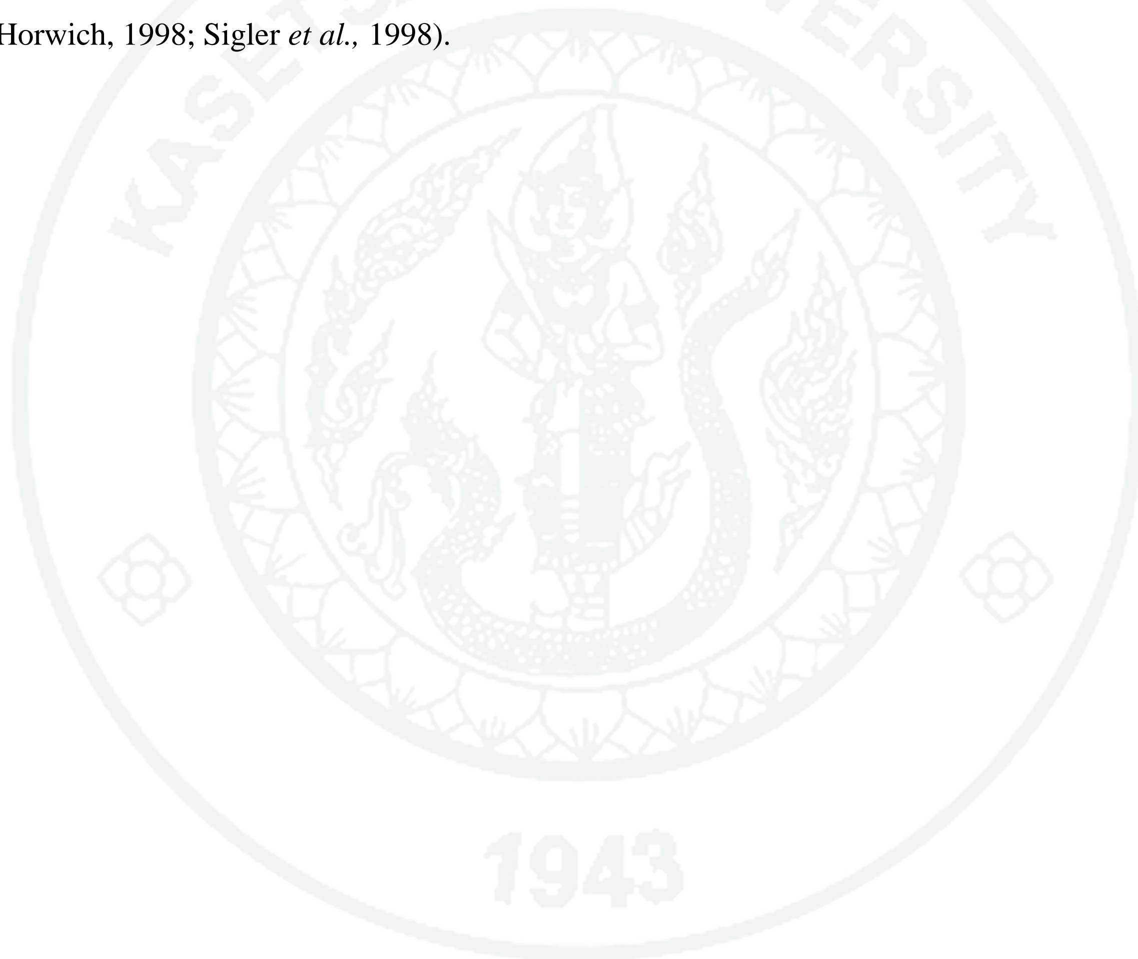
and occurs at post translational manner (Figure 5). Most of these are associated with the bacterial stress-response mechanism. The most intensively studied HSPs are those of the 60 kDa-GroEL families, which are designated as molecular chaperones according to their functions. The GroES and GroEL are essential for growth at all temperatures and are constitutively translated. In cooperation with the 10 kDa-GroES protein, GroEL mediates the correct folding and assembly of many cellular proteins and consequently, prevents misfolding, promotes the refolding and proper assembly of unfolded protein (Bukau and Horwich, 1998; Sigler *et al.,* 1998).



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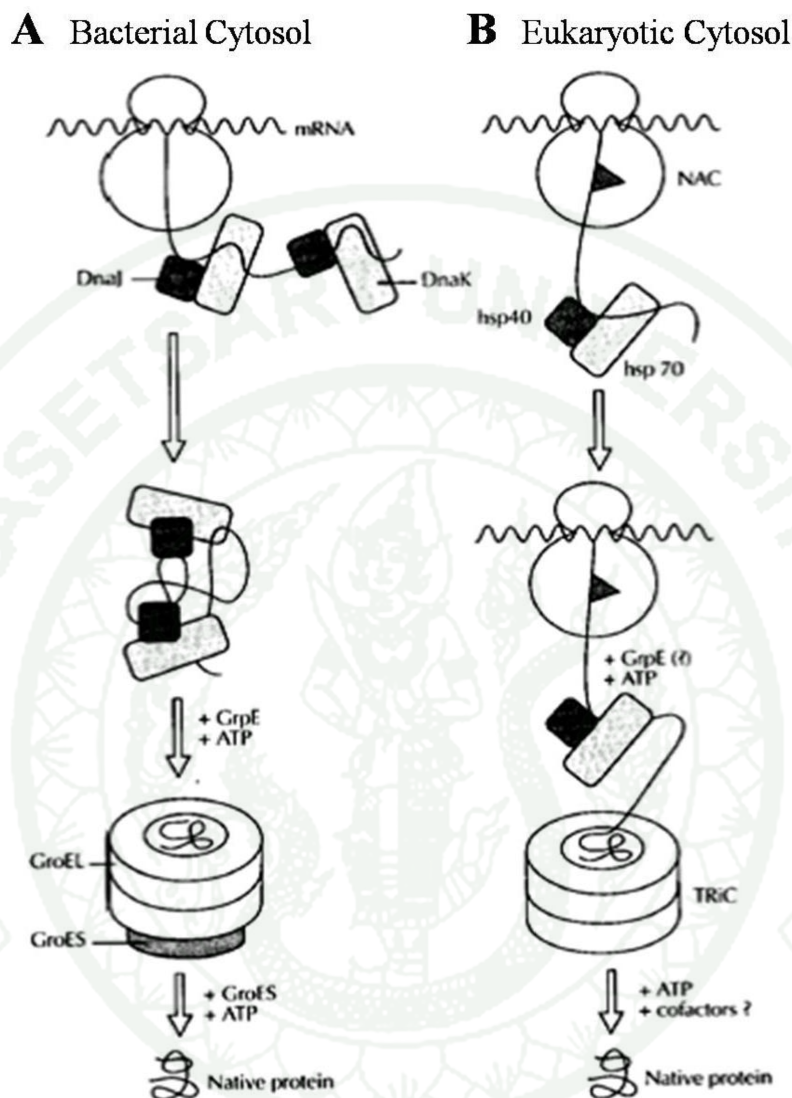

Figure 5 Pathways of chaperone mediated protein folding in the cytosol of (A) bacterial cells and (B) eukaryotic cells. TRiC, TCP-1 ring complex; NAC, nascent polypeptide associated complex; Hsp40, a DnaJ homologue in the mammalian cytosol. TRiC has the ability to interact with ribosome bound polypeptides, whereas binding of GroEL to nascent chains has not been observed. Alternative folding pathways involving different sets of chaperone components are likely to exist.

Source: Hartl and Martin (1995)

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The substrates of GroEL are characterized by presence of less stable native-like secondary structure in the absence of tertiary interactions. Cylindrical GroEL consists of two heptameric rings of 57 kDa subunits that have a 3-domain structure. The apical domain forms the opening of the cylinder and exposes a number of hydrophobic amino acid residues towards the center, which are thought to interact with the complementary surfaces of the non-native substrate, an intermediate domain that joins the apical to the equatorial domain and the large equatorial domain that contains a nucleotide-binding site. GroES is a heptamer of 10 kDa subunits and fits like a lid on the GroEL cylinder (Martin *et al.,* 1997). Mechanistically, substrate binding to one ring of GroEL is closely followed by binding of seven molecules of ATP and GroES, resulting in substrate encapsulation. Upon GroES binding, GroEL undergoes major conformational changes consisting of an enlargement of the cavity and a shift in the surface to a more hydrophilic lining. This so-called Anfinsen cage provides an environment for a single protein molecule to correctly fold. After ATP hydrolysis and binding of ATP to the opposite ring, GroES and substrate are released. Several such cycles may be necessary before a polypeptide is completely folded (Gilbert, 1994; Hartl, 1996).

2.1 Molecular chaperone in acetic acid bacteria

One of the interesting microbial strains for the study of adaptation under environmental stresses is acetic acid bacteria, especially *Acetobacter* sp. This is a food grade microorganism which has been used as a vinegar producer because of its strong ability to oxidize ethanol to acetic acid as well as its high resistance to acetic acid. During acetic acid fermentation, acetic acid bacteria are exposed to unfavorable conditions such as high temperature, high concentrations of acetic acid and ethanol. Although there are many stressors, acetic acid bacteria show relatively normal growth with high resistance to these stressors. The adaptation to such various stressors involves the induction of the synthesis of a large number of highly conserved heat shock proteins (HSPs).

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2.1.1 GroEL/GroES chaperonin in acetic acid bacteria

Okamoto-Kainuma *et al*. (2002) studied about *groESL* operon in *Acetobacter pasteurianus* NBRC 3283 (formerly known as *Acetobacter aceti* IFO 3283). The *groESL* operon was cloned and sequenced. They observed that the amino acid sequence of GroES and GroEL of *A. aceti* had high homologies to the same proteins of *Escherichia coli* and *Bacillus subtilis.* Transcription of this operon in *A. aceti* was induced by heat shock as well as by exposure to ethanol and acetic acid, which are present during acetic acid fermentation process. In *A. pasteurianus,* the expression of *groESL* operon is transcriptionally regulated by the heat shock transcription activator, alternative sigma factor, which recognizes specific heat shock promoter (Segal and Ron, 1995; Okamoto-Kainuma *et al.,* 2002).The upstream region of the *groESL* operon contained the heat shock promoter, which is similar to the sequence of the new heat shock promoter in *Agrobacterium tumefaciens* proposed by Segal and Ron (1995) and the highly conserved inverted repeat sequence (Controlling Inverted Repeat of Chaperone Expression, CIRCE). The configuration of this promoter-CIRCE region of *A. aceti* is quite similar to that of other genera in α-purple proteobacteria, such as *Agrobacterium*, *Rhizobium*, *Zymomonas*, *Brucella* and *Caulobacter* (Okamoto-Kainuma *et al.,* 2002; Azuma *et al.,* 2009). The highly conserved CIRCE element has been found in numerous phylogenetically distant bacteria. The CIRCE element is involved in negative regulation at the RNA level by affecting mRNA synthesis and/or stability or at the DNA level by acting as an operator for the repressor protein, HrcA (Zuber and Schumann, 1994; Segal and Ron, 1996a). The existence of a conserved CIRCE in the upstream regulatory region of the two most highly conserved proteins GroEL and DnaK suggests that the corresponding genes are also regulated in a conserved way (Segal and Ron, 1996b). Moreover, the *groESL* gene overexpressed strain from *A. pasterianus* showed more resistant to stressors; heat, ethanol and acetic acid than wild type strain (Okamoto-Kainuma *et al.,* 2002).

2.1.2 Other chaperones in acetic acid bacteria

Beside GroEL/GroES chaperonin, it has been reported about other chaperones in *A. pasteurianus*. In 2004, Okamoto-Kainuma *et al.* studied about *dnaKJ*

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operon in *A. pasteurianus* NBRC 3283. They found that the profile of the *dnakJ* gene configuration was similar to other *α-Proteobacteria*. Moreover, DnaK and DnaJ proteins of *A. pasteurianus* were conserved with other microorganisms. The temperature shift as well as exposure to ethanol or acetic acid could induce the expression of this *dnaKJ* operon. The *dnaKJ* operon overexpression in *A. pasteurianus* could improve growth at high temperature or in the presence of ethanol but not in the presence of acetic acid. It could be concluded that this *dnaKJ* operon correlates to the resistance against stressors that occur during acetic acid fermentation, except acetic acid. Another investigation studied the heat shock protein, GrpE in *A. pasteurianus* NBRC 3283 (Ishikawa *et al.,* 2010a). The function of GrpE encoded by *grpE* gene works as co-chaperone in the functional cycle of the DnaK chaperone system for the proper chaperone activities. They investigated the relationship between GrpE and DnaK/J and the mechanism underlying stress resistance during acetic acid fermentation. The *grpE* gene was found to be located in tandem with *dnaK* and *dnaJ* gene in the order *grpE-dnaK-dnaJ*. There was a σ^{32}-type promoter sequence in the upstream region of *grpE*. The genes were transcribed as *grpE-dnaK*, *dnaK*, and *dnaJ* in the ratio 1:2:0.1. The heat shock and treatment with ethanol could induce the expression of *grpE*. Co-overexpression of GrpE with DnaK/J in *A. pasteurianus* could improve growth at high temperature or ethanol-containing conditions when compared to the single overexpression of DnaK/J. From the results supported that GrpE acts as co-chaperone and work cooperative with DnaK/J for exhibiting resistance to the stressors that exist during acetic acid fermentation. The latter investigation reported about another chaperone, ClpB that was found in *A. pasteurianus* (Ishikawa *et al.,* 2010b). ClpB is a kind of chaperone that is able to solubilize and refold aggregated protein by work cooperatively with DnaK/J and GrpE. It has been reported that the function of ClpB is protein disaggregation relating for resistance to stress in α-proteobacteria (Squires *et al.,* 1991; Ezaka *et al.,* 2001). The *clpB* gene of *A. pasteurianus* was found at downstream of the *sodB* gene. This gene was transcribed in response to a temperature shift and exposure to ethanol. Furthermore, the *clpB* gene disruptant was only affected by high temperature. From the results suggested that the ClpB protein is closely related with only the heat resistance (Ishikawa *et al.,* 2010b).

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In *A. pasteurianus*, several major chaperones; GroES, GroEL, DnaK, DnaJ, GrpE, and ClpB were reported as described above. All of the genes of these chaperones were found to possess heat-shock promoter-like sequences for *α-Proteobacteria*, putative recognition sequences for RhoH (σ^{32}) at upstream of the coding regions, as purposed by Segal and Ron (1995). RpoH is one of the alternative sigma factors for RNA polymerase. It acts as a regulation factor of heat-shock response and regulates many genes relating to stress responses in other kinds of stresses beside heat stress (Zhao *et al.,* 2005; Martinez-Salazar *et al.,* 2009). In 2011, Okamoto-Kainuma *et al.* investigated the relationship of RpoH to the molecular chaperone in acetic acid bacteria. The *rpoH* gene disruptant was constructed. The disruptant became to be affected by heat, ethanol and acetic acid. The results revealed that the *rpoH* controls the expression of *groEL*, *dnaKJ*, *grpE*, and *clpB* however the expression level varied depending on the chaperones.

2.2 The GroES/GroEL chaperonin in other bacteria

2.2.1 GroES/GroEL chaperonin in psychrophilic bacterium, *Colwellia maris*

In 2003, Yamauchi *et al.* studied about *groESL* gene structure and transcriptional regulation from the psychrophilic bacterium, *Colwellia maris*. The *groESL* operon of *C. maris*, was cloned and sequenced. One specific feature of *C. maris groESL* was that in the putative untranslated region, the G+C content was about 24 mol%, which is much lower than that of mesophilic bacteria such as *E. coli*. The low G+C content may be important for maintaining transcription at low temperatures. They found that *groESL* mRNA level was increased when they increased the temperature from 10ºC to 20ºC. The *groESL* operon from *C. maris* was expressed at low temperature than *groESL* operon of mesophilic bacteria. Moreover, they suggested that *C. maris groESL* operon is regulated by σ^{32}-heat shock element, as in *E. coli*. By contrast, no CIRCE-like element was detected at upstream region of *groESL*, suggesting that there is no HrcA-CIRCE system in this bacterium.

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2.2.2 GroES/GroEL chaperonin in lactic acid bacterium, *Lactobacillus plantarum*

In 2006, Cristiana *et al.* studied about the heat shock response in *Lactobacillus plantarum*. The mutant strain, carrying a null mutation in the *ccpA* gene, encoding the catabolic control protein A (CcpA), showed a lower percentage of survival at high temperature when compared with the wild type strain. In addition, the GroEL protein appears to be more abundant in the wild type strain comparing with the mutant strain. The target of CcpA protein is found in the regulation region of the *dnaK* and *groESL* operons.The results suggested that the CcpA protein involved with the positive regulation of the *dnaK* and *groESL* operons. Furthermore, the sequence analysis of the *groESL* promoter region revealed the presence of the *cis* acting sequence CIRCE, suggesting a negative regulation by the HrcA/CIRCE system. The HrcA/CIRCE system is a common type for controlling the class I heat shock operons of Gram-positive bacteria.

2.2.3 GroES/GroEL chaperonin in Mycoplasmas

Chang *et al.* (2008) reported the regulation of heat-shock protein expression from Mycoplasmas. Genomic analysis indicated that *Mycoplasma hyopneumoniae* possesses the genes of a single sigma factor and the HrcA repressor for negative regulation of the heat-shock response. A perfect inverted repeat sequence (5′-CTGGCACTT-N_9-AAGTGCCAA-3′) was found at upstream region of the *dnaK* gene. Most HSP genes such as *dnaK*, *lon*, *clpB* and *groES/groEL* appear to have CIRCE-like elements which shared very high similarities. However, the distance of the predicted CIRCE element from the start codons of the HSP genes vary from -20 to102 nt. From this result implied that the DNA sequence fragments involved in the HrcA-CIRCE interaction may be looped to interfere with the actions of RNA polymerase. A multiple alignment of the mycoplasmal HrcA sequences indicated that the protein was highly conserved. The presence of the unique sigma factor, HrcA repressor, and the CIRCE-like sequences revealed that mycoplasmal species may use the negative regulatory mechanism in the heat-shock response. It is likely that mycoplasmas have evolved a single HrcA repressor-based

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mechanism which might be the most simple and economical way of controlling HSP gene expression.

3. Taxonomy of acetic acid bacteria

Nowadays, acetic acid bacteria are identified by polyphasic analysis, combining with phenotypic, chemotaxonomic and genotypic data (Colwell, 1970; Cleenwerck and De vos, 2008). In 1970, Colwell defined the term of polyphasic taxonomy that the biological diversity cannot be encoded in a single molecule and that variability of characters is group dependent. Polyphasic taxonomy stimulates the collection and integration of as much genotypic and phenotypic information as possible (Cleenwerck and De vos, 2008). There are several available techniques for polyphasic taxonomy (Table 3), some are essential, some are applicable but not necessary, some are interchangeable with each other, and others are in some cases unsuitable. In case of acetic acid bacteria taxonomy, some useful techniques are selected to use depending on the taxonomic level as described below.

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Table 3 Techniques for studying acetic acid bacteria at different taxonomic levels

Method	Notes
PHENOTYPIC	
Morphological, physiological and biochemical features	Basis for formal description of taxa from family to strain level Provides descriptive information needed to recognize taxa Highly standardized procedures are required to obtain reproducible results
Serotyping	Used for species and strain characterization The need for living bacteria limits the power resolution
CHEMOTAXONOMIC	
Cell wall composition, cellular fatty acids, isoprenoid quinones, whole cell protein analysis	Robust tools to describe phylogenetically closely related species as genera
GENOTYPIC	
Genomic G+C content	Considered part of the standard description of bacterial taxa
DNA-DNA hybridization	Acknowledged as the reference method for establishing relationships within and between species Drawbacks: (1) differences in genome size and DNA concentration influence results; (2) plasmid, chromosomal, housekeeping gene and DNA acquired by horizontal gene transfer are not discriminated; (3) results depend on the experimental parameters and are not cumulative; (4) different experiments cannot be compared directly; (5) the respective references (type strains) have to be included in each individual experiment
RFLP (Restriction Fragment Length Polymorphism)	The method is concerned with differentiation rather than identification of closely related strains.
RAPD (Random Amplified Polymorphic DNA)	Used for species and strain level identification Strict standardization of experimental conditions is needed. Fragments are amplified with all ranges of efficiency, and the resulting patterns are very complex and difficult to interpret.

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Table 3 (Continued)

Method	Notes
DGGE (Denaturing Gradient Gel Electrophoresis)	Short PCR products (<500 bp) are separated, which limits the phylogenetic information obtained by band sequencing. When applied to complex bacterial communities do not provide reasonable estimates of diversity.
AFLP (Amplified Fragment Length Polymorphism)	Irregular amplification of fragments can hamper the ability to reliably assign bands over multiple gels. Very laborious inspection of manual and computer-assisted band analyses
ARDRA (Amplified Ribosomal DNA Restriction Analysis)	The main drawback is misinterpretation of pattern. Overcome by modern rapid sequencing rDNA
rRNA comparative sequence analysis	Gold standard for comprehensive phylogenetic analysis Large availability of sequences in public databases
rRNA FISH (rRNA Fluorescence in Situ Hybridization)	Effective identification of individual cells Three dimensional localization of cells inside samples
DNA microarrays	Allow profiling of differential gene expression The main current limitation is the high cost.
MLST/MLSA (Multilocus Sequence typing/ Multilocus sequence analysis)	Allow intraspecific level identification Database inconsistent with respect to other molecular markers

Source: Gullo and Guidici (2009: 58)

3.1 Phenotypic characteristics

Acetic acid bacteria are characterized by determination of colony and cell morphology, Gram staining and tests to determine oxidase and catalase activity. In 2008, Cleenwerck and de Vos summarized about the more general characteristics used for the phenotypic classification of acetic acid bacteria. These tests are very useful for the identification and differentiation of acetic acid bacteria in genera as well as species level as described in the following: (1) the production of acetic acid from ethanol; (2) overoxidation of lactate and acetate to carbon dioxide and water; (3) growth in the presence of 0.35% acetic acid; (4) growth in 1% nitric acid; (5) formation of 2-ketogluconic, 5-ketogluconic

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and 2,5-diketogluconic acid from glucose; (6) ketogenesis of glycerol; (7) growth on different carbon sources (e.g. methanol); (8) formation of brown water-soluble pigments; (9) formation of γ-pyrones from glucose or fructose; (10) production of acids from sugar; (11) production of cellulose; (12) growth in 30% glucose; (13) presence and positions of flagella; (14) motility. These characteristics can be used to differentiate the different genera of acetic acid bacteria. However, only phenotypic tests are not enough for the reliable classification of acetic acid bacteria because many phenotypic features are shared within genera *Acetobacter*, *Gluconacetobacter* and *Asaia* (Gosselé *et al.,* 1983a, 1983b; Navarro and Komagata, 1999; Lisdiyanti *et al.,* 2000, 2001; Cleenwerck *et al.,* 2002). Moreover, there are none and too few differential phenotypic characteristics that were found at species level of some acetic acid bacterial strains such as *A. tropicalis*, *A. indonesiensis*, *A. orleanensis* and *As. bogorensis*, *As. siamensis*, *As. krugthepensis*, respectively.

3.2 Chemotaxonomic characteristics

Chemotaxonomy was defined as the study of chemical constituents of cells (peptidoglycan structure, isoprenoid quinones, lipid and fatty acid composition of cells, polyamines, pigments and mycolic acids). The determinations of chemical characters were used for classification and identification of the bacterial groups at genus level. Chemotaxonomic study is the important part that is combined in the polyphasic approach. Acetic acid bacteria have been characterized by using chemotaxonomical methods that are quinone and fatty methyl ester (FAME) analyses. Isoprenoid quinones have the important function relating with electron transport. In generally, strictly aerobic bacteria that are Gram-negative have only ubiquinone type whereas menaquinones and /or demethylmenaquinones belong to the other types of bacteria. Ubiquinones have been detected in acetic acid bacteria which have been phylogenetically described by Stackebrandt *et al.* (1988). In case of acetic acid bacteria, the method of isoprenoid quinones extraction and purification was reported by Yamada *et al.* (1969). Moreover, the quantitatively determination by using reverse-phase high performance liquid chromatography (HPLC) was described by Tamaoka *et al.* (1983) or Komakata and Suzuki (1987). Only *Acetobacter* strains have Q9 as the major ubiquinone whereas other acetic acid bacteria possess Q10 (the ubiquinone content of *Granulibacter* was not determined). The analysis of fatty acid

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patterns is used for classification and identification of acetic acid bacteria because the fatty acids determination is rather easily and quickly. However, the fatty acid patterns depend on the conditions that are used to cultivate the bacteria such as growth temperature, pH composition of the medium, or age of the culture. In case of acetic acid bacteria, the C18:1ω7 straight chain unsaturated acid in major amounts and 2-hydroxy acids were generally detected (Yamada *et al.,* 1981).

3.3 Genotypic characteristics

3.3.1 Whole-genome DNA-DNA hybridization

Although, whole genome DNA-DNA hybridization values play a key role in species delineation, the method applied are rather time consuming and only laboratories highly specialized in bacterial systematic perform any of them. Moreover, the results of DNA-DNA hybridization might not be as stable and comparable as those involving nucleotide sequences, because of the different methods and conditions involved. There are several different hybridization methods that have been developed based on the use of radio-labelled DNA for the detection of the heteroduplex formation such as the hydroxyapatite method (Brenner *et al.,* 1969), S1 nuclease method (Crosa *et al.,* 1973) and the membrane filter method (Legault-Démare *et al.,* 1967). Moreover, the new methods based on free of radioactivity are developed. The optical method (De ley *et al.,* 1970), the DNA similarity is calculated from the renaturation rate of the melted of two DNA mixture. In case of acetic acid bacteria, the hybridization method in microdilution wells with photobiotin-labelled DNA, reaction with streptovidin-beta-D-galactosidase was used (Ezaki *et al.,* 1989). Wayne *et al.* (1987) suggested that a DNA similarity above 70% indicating a relationship on the species level while the value between 20% and 60% give evidence for genus (Johnson, 1985). In addition, Stackbrandt and Goebel (1994) recommended the determination of the DNA similarity by DNA-DNA hybridization for a clear allocation of new strains to the species level.

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3.3.2 Determination of the guanine + cytosine content

A classical method for the description of taxa is the determination of the guanine and cytosine content of the DNA. The G+C content is often still considered a part of the standard description of bacterial taxa. The use of the G+C approach does not provide any phylogenetic information and does not allow an organism to be assigned to a particular taxon; instead it shows discriminating capacity. Generally, the variation in the G+C content should not increase more than 5% with in species and 10% within the genus (Schleifer and Sackbrandt, 1983). For acetic acid bacteria, the G+C content was included in *Bergey's Manual of Systematic Bacteriology* (De Ley *et al.,* 1984a). There are several methods for the determination of G+C content as follows; the HPLC (Tamaoka and Komagata, 1984), the thermal denaturation method (Marmur and Doty, 1962). Although the G+C content of acetic acid bacteria ranges from 52-67 mol%, most species include strains with percentages around the middle of this range (59%) (Cleenwerck and De Vos, 2008).

3.3.3 Direct sequence analysis of 16S rRNA gene, 16S-23S rRNA gene internally transcribed spacer (ITS) regions, *recA* gene and *groEL* gene

Comparison of the 16S rRNA sequences from closely related species, the conserved region and highly variable fragments are also presented in this molecule. The sequences of these variable fragments in the 16S rRNA can be used for differentiation between closely related species and as a target for highly specific probe. Greenberg *et al.* (2006), Kersters *et al.* (2006) and Cleenwerck and De vos (2008) constructed the phylogenetic tree based on almost complete 16S rRNA sequences reflecting the distant relationships of acetic acid bacteria among members of the *Alphaproteobacteria*. On the other hand, even the nearly complete 16S rRNA sequence is not suitable for identification at the species level that the species are phylogenetically very close related, sharing more than 99.5% 16S rRNA sequence similarity such as *A. cerevisiae*, *A. malorum* and *A. orleanensis*; *As. bogorensis*, *As. krungthepensis* and *As. siamensis* etc. Furthermore, Stackbrandt and Goebel (1994) have shown that it is unlikely that strains showing < 97% similarity between 16S rRNA genes belong to the same species. Strains sharing more > 97% 16S rRNA sequence similarity do not necessarily belong to the same species.

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Therefore, additional data obtained from the investigation features are needed for a reliable taxonomic description of bacterial isolates.

Beside 16S rRNA gene, 16S-23S rRNA internally transcribed spacer (ITS) region sequences have been used to analyse the phylogenetic relationships between acetic acid bacteria (Tanasupawat *et al.,* 2004; Greenberg *et al.,* 2006; Takahashi *et al.,* 2006; Yukphan *et al.,* 2006). The 16S-23S rRNA gene ITS region exhibits more variation in sequence and length, so it has a higher discriminatory power than 16S or 23S rRNA genes (Ruiz *et al.,* 2000). In 2011, González and Mas proposed about the usefulness of the 16S-23S rRNA gene ITS for the classification and identification of acetic acid bacteria at the species level. They constructed the phylogenetic tree based on 16S-23S rRNA gene ITS from sixty-four strains belonging to the different acetic acid bacteria genera. The results showed that the topologies of the 16S-23S rRNA gene ITS based trees were in accordance with the 16S rRNA gene based trees. It can be concluded that the phylogenetic analysis by using 16S-23S rRNA gene ITS is a useful criteria in taxonomic polyphasic studies of acetic acid bacteria.

Another gene that was used for comparative sequence analysis in acetic acid bacteria is *recA* gene (Greenberg *et al.,* 2006). The conservative *recA* gene encoded the DNA repair protein RecA. The *recA* gene has been used in multilocus sequence analysis for taxonomic studies of novel bacterial isolates because this gene is found to be a conserved gene (Eisen, 1995). In 2006, Greenberg *et al.* proposed the novel genus in the family *Acetobacteraceae* as *Granulibacter bethesdensis* gen. nov., sp. nov. They analysed the *recA* gene together with 16S rRNA and 16S-23S rRNA gene ITS sequences of this bacterial strain. The results showed that the topology of phylogenetic tree based on *recA* gene was similar to the 16S rRNA-based tree and 16S-23S rRNA gene ITS based tree.

In recent years, some housekeeping proteins such as 60 kDa heat shock protein (HSP60) have been considered as useful phylogenetic markers. The *groEL* genes, which encode 60-kDa heat shock proteins (GroEL), are ubiquitous and evolutionarily highly conserved among bacteria. In 2010, the *groEL* gene was used for performing multilocus sequence analysis for species differentiation in family *Acetobacteraceae*

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(Cleenwerck *et al.,* 2010). The phylogenetic tree based on *groEL* gene confirmed the existence of two phylogenetic groups in *Gluconacetobacter.* In 2014, Huang *et al*. proved about the *groEL* gene (*hsp60*) can be used for species discrimination in genus *Acetobacter.* The results showed that the phylogenetic relationships in the *Acetobacter* genus can be resolved by using *groEL* (*hsp60*) gene sequencing.

3.3.4 Multilocus Sequence Typing/Analysis (MLST/MLSA)

Multilocus sequence typing (MLST) refers to a method for the genotypic characterization of prokaryotes at the intraspecific level by using the allelic mismatches of a small number of housekeeping genes. In contrast, multilocus sequence analysis (MLSA) is a method for the genotypic characterization of a more diverse group of prokaryotes (including entire genera) by using the sequences of single-copy and ubiquitous protein-coding genes, which evolve faster than rRNA (Gevers *et al.,* 2005). In case of acetic acid bacteria, ten genes (*recA*, *dnaK*, *rpoB*, *atpD*, *groEL*, *glnA*, *thrC*, *gyrA*, *gltA*, *infB*) were evaluated for the construction of a multilocus sequence analysis (MLSA) framework (Cleenwerck *et al.,* 2010). The genes *atpD*, *glnA*, *gyrA* and *infB* were found in multiple copies in the complete genome sequence of acetic acid bacteria available at the start of this study (*Gluconobacter oxydans* and *Granulibacter bethesdensis*), and were for this reason discarded from further analyses. Only three genes (*dnaK*, *groEL* and *rpoB*) could be amplified by using primers that were designed from two genome sequences of acetic acid bacteria. It can be concluded that three housekeeping genes; *dnaK* (encoded a DnaK heat-shock protein), *groEL* (encoded a chaperonin protein) and *rpoB* (encoded the β-subunit of bacterial RNA polymerase) were used for species identification of genus *Gluconacetobacter*. The results revealed that the multilocus of the three housekeeping genes can be useful for species differentiation in the genus *Gluconacetobacter.*

3.3.5 DNA profiling method

There are several methods for generating and visualizing DNA fragments that can be used for DNA based differentiation. These methods include indirect, rapid and simple techniques currently popular in many laboratories. RFLP (restriction fragment

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length polymorphisms), RAPD (random amplification of polymorphic DNA), AFLP (amplified fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis) and DGGE (denaturing gradient gel electrophoresis) are the most widespread techniques which allow the subdivision of species into a number of district types. In general, the procedures can be divided in two groups as follows; site specific fragmentation and primer directed PCR amplification of purified DNA. Several general and specific biases are associated with these approaches (Table 3) and often they provide only differentiation information; for these reasons, in the modern taxonomy era, most of them have been replaced by rapid sequencing methods providing much more information for identification and phylogeny.

In case of acetic acid bacteria, RFLP analysis of 16S rRNA and 16S-23S rRNA gene ITS have been used for multiple taxonomy. Poblet *et al.* (2000) performed RFLP of 16S rRNA with *TaqI* restriction enzyme. They found that the obtained banding patterns could discriminate between the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* and some *Acetobacter* species. Moreover, the usefulness of RFLP of 16S rRNA and 16S-23S rRNA gene ITS for characterization and identification of acetic acid bacteria were investigated by Ruiz *et al.* (2000). The result showed that RFLP analysis of 16S rRNA gene by using *TaqI* and *RsaI* was a reliable method to identify acetic acid bacteria at the species-level or species group. On the other hand, RFLP analysis of 16S-23S rRNA gene ITS was not useful to identify isolates of acetic acid bacteria at the species-level but it is useful to detect at intraspecific variations. In 2001, Fuentes-Ramírez *et al.* used the RFLP analysis of 16S rRNA for differentiation among *Ga. johannae*, *Ga. azotocaptans* and *Ga. diazotrophic.* Trček and Teuber (2002) evaluated RFLP of 16S-23S rRNA gene ITS for species delineation of acetic acid bacteria and validated the PCR-RFLP data by 16S rRNA sequence analysis. They compared the 16S-23S rRNA gene ITS sequence similarities of some type strains of the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* in order to find the optimal restriction enzymes. The results of 12 distinct groups of banding patterns from the digestion of amplified spacers by *Hae*III and *Hpa*II were found either representing a species or a species group. Finally, the collection of banding patterns was used as a reference frame to compare unknown acetic acid bacteria from vinegar and the isolates could be assigned to the one of 12 groups. Later, Trček

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(2005) successfully characterized bacterial populations from high acid containing wine vinegar and alcohol vinegar by using these finding as described above. Moreover, RFLP of 16S-23S rRNA gene ITS was used for investigate the diversity among *Gluconobacter* strains. The discrimination at the species and below species level in some case depend on the restriction enzymes that were used such as *Mbo*II, *Bsp*12861 and *Ava*II (Yukphan *et al.,* 2004a, c, d; Tanasupawat *et al.,* 2004). In 2006, González *et al.* used RFLP of 16S rRNA for species differentiation. RFLP of 16S rRNA digested with *Alu*I was used for grouping the strains with the combination of the patterns that were generated with other endonucleases. *A. cerevisiae* and *A. orleanensis* are phenotypically very similar, could be differentiated by using this technique (Cleenwerck *et al.,* 2002). In addition, RFLP of 16S-23S rRNA gene ITS digested with *Pvu*II was used for discriminate *Ga. xylinus* from *Ga. europaeus.* Another investigation reported about RFLP analysis of 16S-23S rRNA gene ITS with *Rsa*I was used for identification of isolates from traditional balsamic vinegar production (Gullo *et al.,* 2006).

RAPD fingerprinting and ERIC-PCR were used for characterize the flora of Japanese rice vinegar (komesu) and unpolished rice vinegar (kurosu) (Nanda *et al.,* 2001). In 2003, Bartowsky *et al.* successfully used RAPD fingerprinting to determine the bacterial species associated with an outbreak of spoilage in commercially bottled red wine. González *et al.* (2005) investigated the species and strain evolution of acetic acid bacteria population during wine production by using three techniques as follows; RFLP analysis of 16S rRNA, ERIC-PCR and REP-PCR. In the previous report (González *et al.,* 2004), they compared the results among ERIC-PCR and REP-PCR techniques and found the similar results. Another technique, RFGE using *Xha*I was used for differentiation of the strains of acetic acid bacteria (Sievers and Swings, 2005). In 1987, Teuber *et al.* used the plasmid profiling to characterize cultures producing high percentage spirit, wine and cider vinegar for allow the conclusion regarding diversity and stability of isolates from submerged fermentations carried out in acetators and trickle generator

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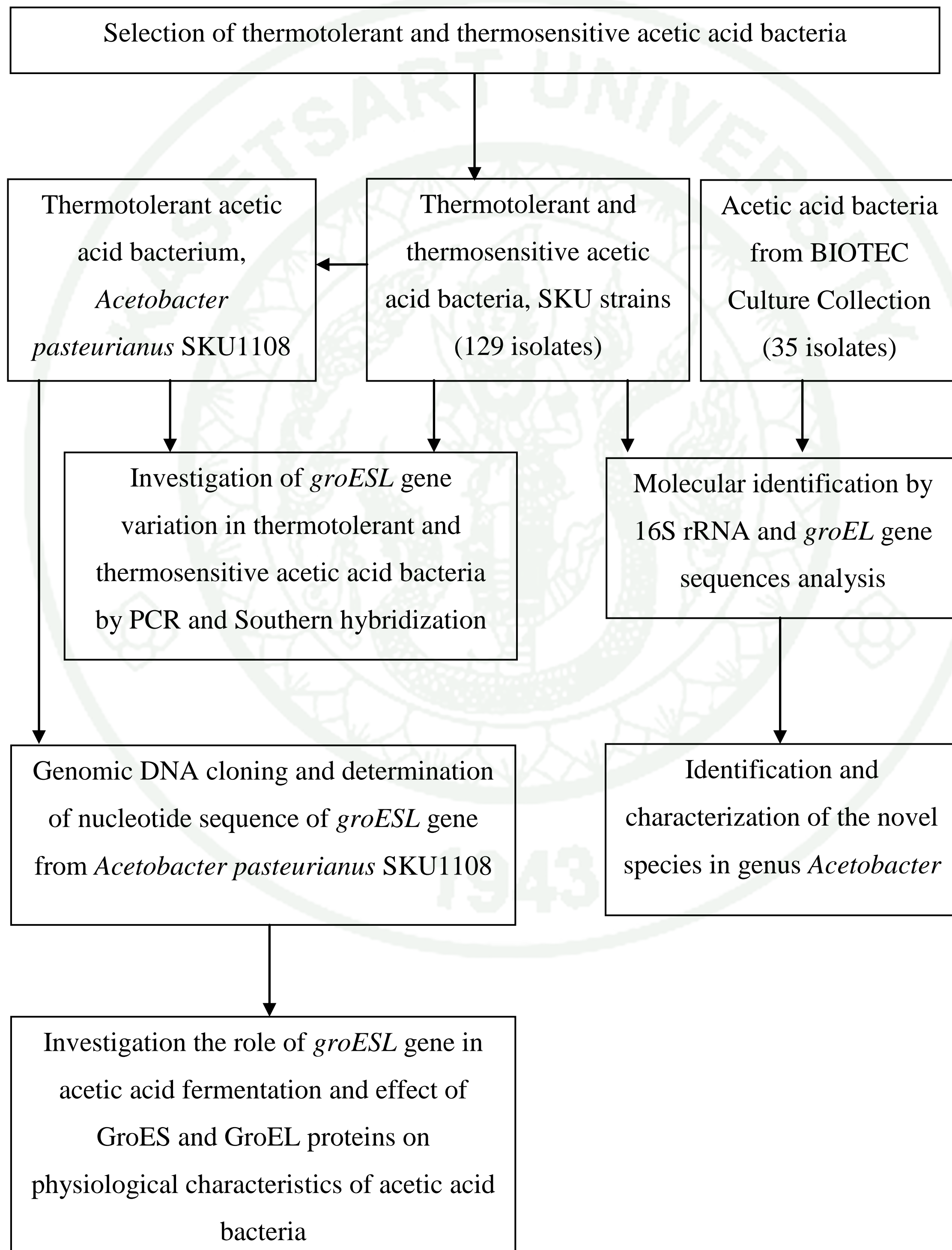
MATERIALS AND METHODS

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



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1. Chemicals

Yeast extract, polypeptone and glucose were obtained from Difco^{TM} ; Voigt Global Distribution Inc. (KS, USA). Glycerol was purchased from J.T. Baker® Chemicals; Avantor Performance Materials, Inc. (PA, USA). Restriction enzymes, Standard DNA marker and 6X loading dye were obtained from Thermo Fisher Scientific Inc. (CO, 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

Bacterial strains used in this study consisted of 23 reference strains of various *Acetobacter* species obtained from BCC: BIOTEC Culture Collection, Pathumthani, Thailand; LMG: Laboratorium voor Microbiologie Gent Culture Collection, Ghent, Belgium and JCM: Japan Collection of Microorganisms, Ibaraki, Japan (Table 4), thermotolerant acetic acid bacteria isolated in Thailand (Theeragool *et al*., 1996) including *Acetobacter* strains from BCC: BIOTEC Culture Collection (Table 5). Among those strains, *Acetobacter pasteurianus* SKU 1108 (Saeki *et al.,* 1997) isolated from grape, was used as a source for *groESL* gene cloning. This strain is a promising strain for acetic acid fermentation at higher temperature. DGL strains (*groEL* gene disruptant) and SKU1108/pCMgroESL (overexpressed strains) derived from *A. pasteurianus* SKU1108 were performed in this study. The procedures for construction of the *groESL* overexpressed strains and *groEL* gene disruptant are described in 3.5 and 3.7, 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 -80ºC and were maintained on a potato agar slant. YPGD or Seed Culture Medium (SCM, Appendix A) was used for general cultivation to determine bacterial growth, acetic acid production. Ethanol and/or acetic acid were added aseptically when indicated. Five ml of overnight culture in potato

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medium was inoculated into 100 ml of YPGD in a 500-ml Erlenmeyer flask. Cultivation was performed at 30ºC on a rotary shaker at 200 rpm. The growth was periodically measured with a 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.* (1997). The genotype of acetic acid bacteria used in this study is shown in Table 6.

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 37ºC. The ingredients of LB medium are shown in Appendix A. The genotype of *E. coli* DH5α and S17-1 strains are shown in Table 6. Ampicillin (50 μl/ml), kanamycin (50 μl/ml), tetracycline (25 μl/ml) and gentamicin (12.5 μl/ml) were added to the medium when necessary to maintain plasmids.

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Table 4 The reference strains used in the present study

Strain	Original bacterial no.	Accession no. of 16S rRNA
Acetobacter pasteurianus BCC 6320^T	TISTR 1056	X71863
Acetobacter aceti BCC 12455^T	IFO 14818	AB680674
Acetobacter estunensis BCC 23120^T	NBRC 13751	AB680505
Acetobacter orleanensis BCC 23121^T	NBRC13752	NR028614
Acetobacter lovaniensis BCC 23122^T	NBRC 13753	AB680507
Acetobacter tropicalis BCC 23123^T	NBRC 16470	AB681066
Acetobacter indonesiensis BCC 23124^T	NBRC 16471	AB681067
Acetobacter syzygii BCC 23125^T	NBRC 16604	AB681084
Acetobacter cibinongensis BCC 23126^T	NBRC 16605	AB681085
Acetobacter orientalis BCC 23127^T	NBRC 16606	AB681086
Acetobacter nitrogenifigens BCC33377^T	NBRC 105050	AB682235
Acetobacter cerevisiae BCC 40086^T	LMG 1625	AJ419843
Acetobacter peroxydans BCC 40087^T	LMG 1635	JF793969
Acetobacter malorum BCC 40088^T	LMG 1746	AJ419844
Acetobacter pomorum BCC 40089^T	LMG 18848	AJ419835
Acetobacter senegalensis BCC 40091^T	LMG 23690	AM748710
Acetobacter farinalis BCC 44845^T	G360-1	AB602333
Acetobacter oeni BCC 40090^T	LMG 21952	JF793961
Acetobacter ghanensis LMG 23848^T	LMG 23848	EF030713
Acetobacter fabarum LMG 24244 ^{T}	LMG 24244	AM905849
Acetobacter papayae JCM 25143^T	JCM 25143	AB665066
Acetobacter okinawensis JCM 25146^T	JCM 25146	AB665068
Acetobacter persici JCM 25330^T	JCM 25330	AB665070

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Table 5 Bacterial strains from BIOTEC Culture Collection used in this study

Strain	BCC	Original code	Accession no. of 16S rRNA
A. pasteurianus	6290	TISTR 102	AB906396
A. indonesiensis	15762	AC 27	AB906398
A.orientalis	15812	AC 79	AB906399
A.orientalis	15813	AC 80	AB906400
A. fabarum	15845	AD 38	AB906401
A. fabarum	15846	AD 39	AB906402
A. papayae	15884	AD 87	AB906404
A. cibinongensis	24363	TR5S2	AB906405
A. persici	24365	TR5SU1	AB906406
A. persici	15756	AC 17	AB906397
A. thailandicus	15839^{T}	AD 25^{T}	AB937775
A. okinawensis	15851	AD 46	AB906403

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Table 6 Bacterial strains and plasmids used in this study

Bacterial strain and plasmid	Relevant characteristic	Source or reference
Strains		
SKU isolates	Thermotolerant and thermosensitive acetic acid bacteria	Theeragool *et al.* (1996)
A. pastaurianus SKU1108	Wild type	Theeragool *et al.* (1996) Saeki *et al.* (1997)
DGL5	*groEL* gene disruptant of SKU1108 with insertion by Km^r cassette from pTKm	This study
Escherichia coli		
DH5α	*supE*44 Δ*lacU*169 (Φ80*lac*ZΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Toyobo
S17-1	Tpr Smr *recA, thi, pro, hrdR*-M+RP4: 2-Tc:Mu: Km Tn7 λpir	
Plasmids		
pGEM-T® Easy vector	Cloning vector, Ap^r, *lacZ*	Promega
pGEM-T groESL1.4	Ap^r, a 1.4kb DNA fragment carrying *groESL* gene	This study
pUC119 vector	Ap^r, *lacZ*	Takara
pUCgroESL3.2	Ap^r, a 3.2 kb DNA fragment carrying *groESL* gene	This study
pUCgroEL::Km	Ap^r, Km^r , *lacZ* carrying a Km^r disrupted *groEL* gene at *Msc*I site	This study
pCM62	*Acetobacter-E. coli* shuttle vector, Tc^r, *lacZ*	Marx and Lidstrom (2001)
pCMgroESL	Tc^r, a 3.2 kb DNA fragment carrying *groESL* gene	This study
pTKm	Non-polar Km^r cassette	Yoshida *et al.* (2003)
pGSA	Gm^r, promoter probe vector carrying the promoterless *lacZ* gene	This study
pGSAgroESL	Gm^r, carrying a 239 bp promoter region of *groES* gene[a]	This study

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3. Cloning, Sequencing and Characterization of the *groESL* Genes

3.1 Cloning and nucleotide sequencing of the partial 1.4 kb *groESL* genes

Molecular cloning of *groESL* genes encoding GroES and GroEL, 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 6. The specific forward and reverse primers were designed from conserved region of *groESL* gene of *A. pasteurianus* NBRC 3283 (formally known as *A. aceti* IFO 3283) (Okamoto-Kainuma *et al.,* 2002) and other related microorganisms. Analysis of the designed primers for hairpin and palindrome structure, annealing and melting temperature and G/C content was performed by using Oligo Toolkit web server available an URL http://www.operon.com/tools/oligo-analysis-tool.aspx. The forward primer GF1, (5'-AGG AAA AGC CTA TGG AAG-3') and the reverse primer GR1, (5'-GCT GTG GAA GAA GGC AT-3') were designed to start at 97th nucleotide from initiation codon of *groES* and at 1,216th nucleotide from initiation codon of *groEL*. The desired PCR product was 1.4 kb DNA fragment. The PCR was performed by using the Ready-To-Go™ PCR Beads (Amersham Biosciences, Buckinghamshire, UK), in the total volume of 25 µl. The PCR thermal cycling conditions were as follows: 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 54ºC for 1 min and 72ºC for 2 min. A final extension was performed at 72ºC for 10 min. The obtained 1.4 kb PCR product was recovered from 0.8% agarose gel and purified by the application of a HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience Corp., Taipei, Taiwan, Appendix C). The purified 1.4 kb PCR product was sent to the First Base Laboratory (Selangor, Malaysia) for determination of the nucleotide sequence.

The ligation mixture contains 3 µl of 100 ng purified DNA fragment, 1µl of pGEM®-T Easy Vector (50 ng), 2x Rapid Ligation Buffer, T4 DNA ligase (5 unit) and 5µl of sterile deionized water to give the total volume of 10 µl. The ligation reaction was carried out at 16ºC overnight and used to transform into *E. coli* DH5α by heat shock transformation (Appendix C). The blue/white colonies were selected on LB agar containing 50 µg/ml of ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and

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incubated at 37ºC overnight. The recombinant clones were selected from white colonies formed on the agar plate. The recombinant clones were further used for recombinant plasmid isolation by using alkaline lysis method (Sambrook *et al.,* 1989, Appendix C) and GeneJet™ Plasmid Miniprep Kit (Thermo scientific, USA). The resulting plasmid designated as pGEM-TgroESL1.4 was confirmed by PCR amplification. The purified recombinant plasmid, pGEM-TgroESL1.4 was sent to First Base Laboratory (Malaysia) for the DNA sequencing analysis. The obtained nucleotide sequences were performed for homology search by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

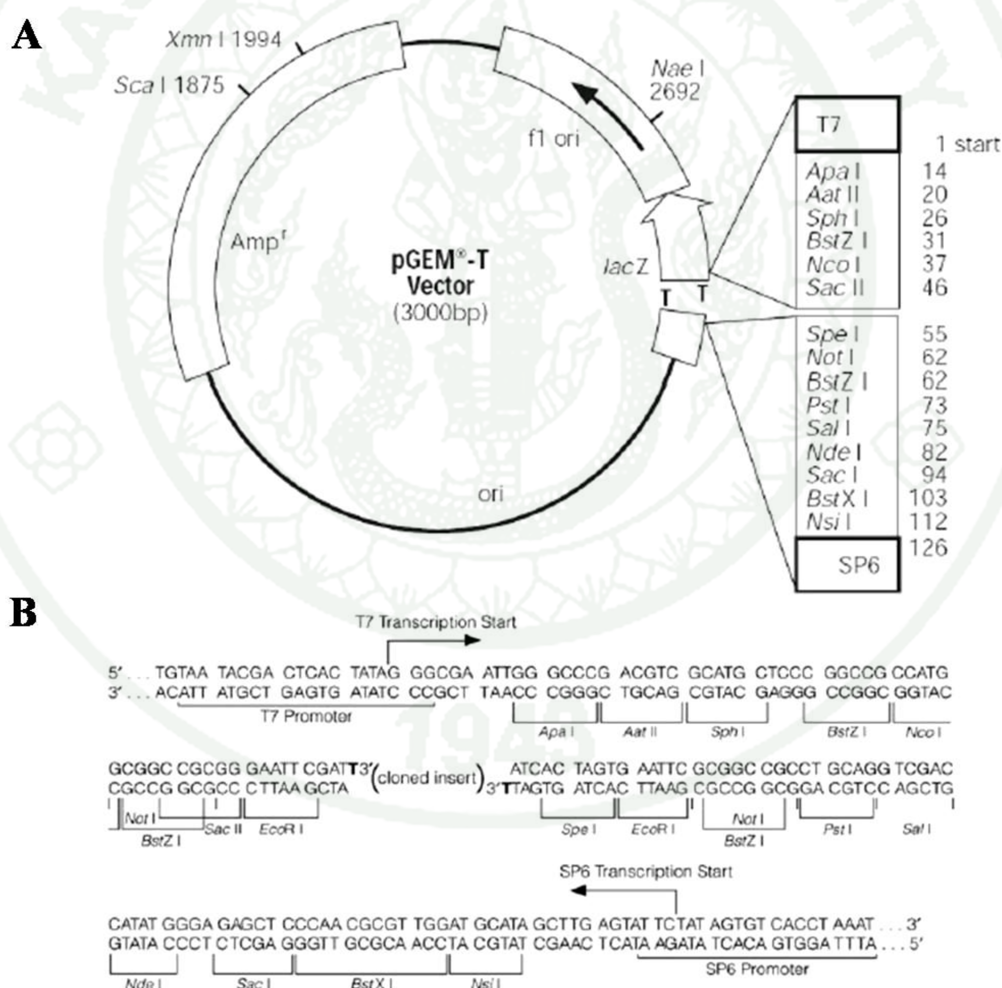

Figure 6 The physical map (A) and multiple cloning sequence of pGEM®-T Easy vector (B).

Source: Promega technical manual for pGEM®-T Easy vector system (1989)

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3.2 Southern hybridization of genomic DNA from *A. pasteurianus* SKU1108 by 1.4 kb *groESL* gene DNA probe

The chromosomal DNA from *A. pasteurianus* SKU1108 was completely digested with several enzymes; *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Sma*I (Thermo scientific, USA). The purified 1.4 kb *groESL* gene was labeled with digoxigenin-11-dUTP (Dig-11-dUTP; Roche Applied Science, Penzberg, Germany) by random primed labeling and used as DNA probe for Southern hybridization. The digested chromosomal DNAs were hybridized with the 1.4 kb *groESL* 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 has 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 3MM 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 the membrane and the gel surface were completely removed. The double layers of Whatman 3MM paper were placed on the membrane. Then, five to ten cm high of the desired dimensions paper towels were placed on the Whatman 3MM paper. A weight (approximately 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 5 ml of prehybridization solution (Appendix B) and incubated at 42ºC at least 1-2 h (prehybridization) and transferred the membrane to a new bag. The hybridization solution containing the labeled 1.4 kb *groESL* gene DNA probe was added

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and incubated at 42ºC for 18-24 h (hybridization). After the incubation period, the membrane was washed with shaking at 65ºC 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 65ºC 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 65ºC 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 80ºC for 1-2 min and stored in the sealed plastic bag.

3.3 Cloning of 3.2 kb *groESL* fragments into pUC119 vector

The chromosomal DNA isolated from *A. pasteurianus* SKU1108 was digested with *Hind*III and then loaded on 0.8% agarose gel. The 3.2 kb *Hind*III fragment was recovered by HiYield^{TM} Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience Corp., Taiwan). The purified 3.2 kb DNA fragment was ligated into *Hind*III digested vector. The physical map of pUC119 vector was shown in Figure 7. The ligation mixture contains 6 µl of 300 ng purified DNA fragment, 2µl of *Hind*III digested pUC119 vector (300 ng), 10x Rapid Ligation Buffer, T4 DNA ligase (5 units) and 9 µl of sterile deionized water to give the total volume of 20 µl. The ligation reaction was carried out at 16ºC overnight and used to transform into *E. coli* DH5α by heat shock transformation (Appendix C) and the blue/white colonies were 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 37ºC overnight. The recombinant white colonies were further analyzed by colony hybridization with 1.4 kb *groESL* gene DNA probe.

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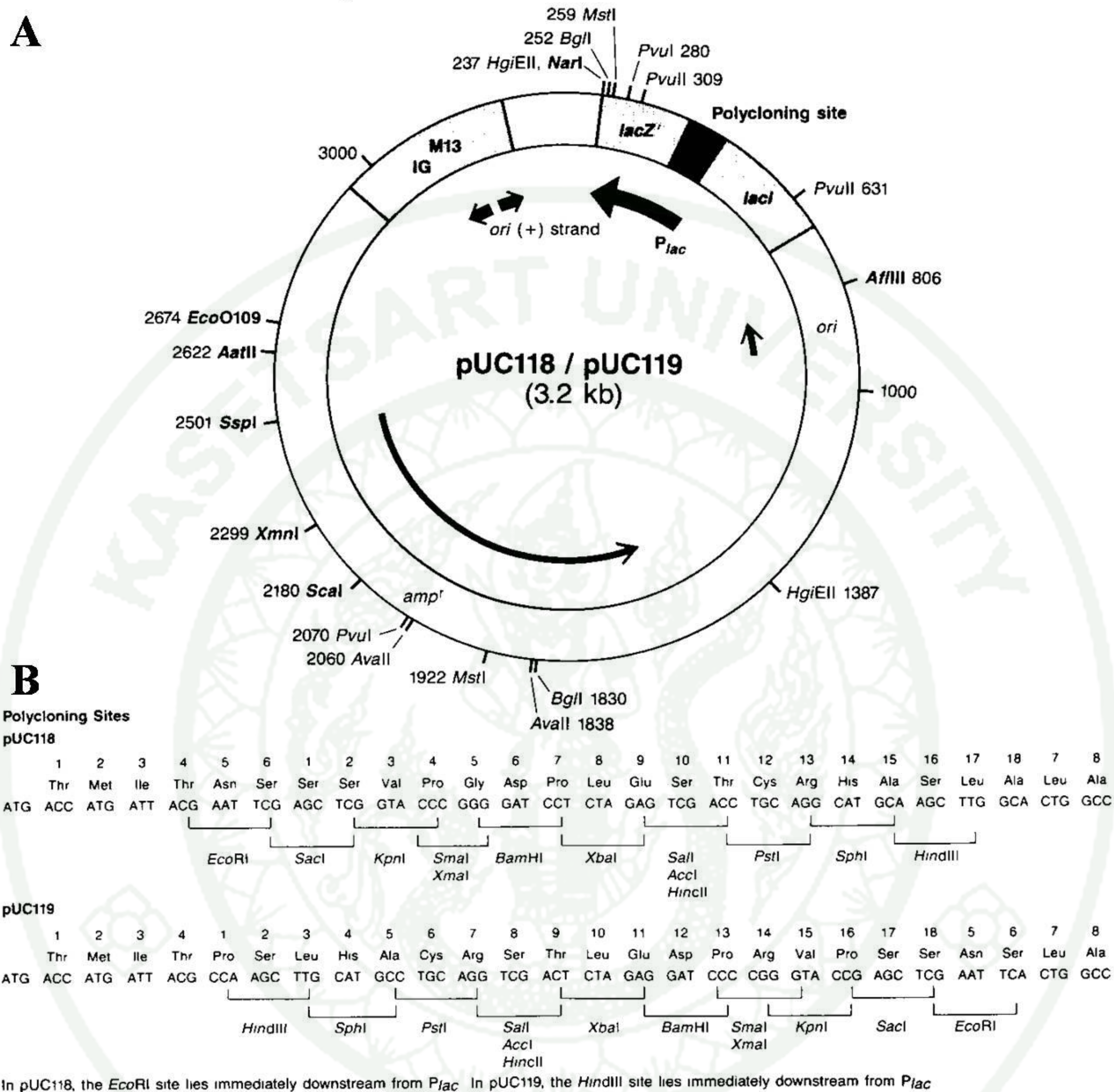

Figure 7 The physical map (A) and multiple cloning sequence of pUC119 cloning vector (B)

Source: Sambrook *et al.* (1989)

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3.4 Screening for *groESL* gene from the recombinant clones by colony hybridization with 1.4 kb *groESL* gene DNA probe

All of the white colonies of transformed *E. coli* DH5α were subjected to analysis by colony hybridization. Recombinant colonies were grown on the LB agar containing 50 μg/ml of ampicillin at 37°C for 18-24 h. The nylon membrane filter was placed on the surface of an agar plate and allowed to direct contact with the tested colonies for 10 min. The membrane was transferred from the plate and places them, colony side up, on the sheet of Whatman 3MM paper saturated with denaturation solution twice, each for 10 min and then transferred it to a dry Whatman 3MM paper. The membrane was transferred to the sheet of Whatman 3MM paper saturated with neutralization solution twice, each for 10 min followed by transferred them on a dry Whatman 3MM paper. Then, the membrane was rinsed in 2x SSC solution and transferred them, colony side up, on a dry Whatman 3MM paper allowed them to dry at room temperature for at least 10 min. Usually this treatment also results in denaturation of the DNA molecules, so that the hydrogen bonds between individual strands in the double helix are broken. These single-stranded molecules can then be bound tightly to the membrane by fixed under UV light for 5 min. The detection of the hybridization signals was performed by the procedure as described in Southern hybridization. The hybridization signals were observed from colonies that hybridize specifically to the probe. The positive clones were further confirmed by PCR amplification of 1.4 kb *groESL* gene prior to determination of nucleotide sequences.

The recombinant plasmid, pUC119 carrying 3.2 kb *groESL* gene designated as pUCgroESL3.2 was isolated from the positive clone. The purified recombinant plasmid, pUCgroESL3.2 was sent to First Base Laboratory (Malaysia) for the DNA sequencing analysis. The obtained nucleotide sequences were searched for an open reading frame (ORF) using Clone Maneger (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.

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3.5 Overexpression of *groESL* gene in *A. pasteurianus* SKU1108

A 3.2 kb *Hind*III DNA fragment with the complete *groESL* gene from *A. pasteurianus* SKU1108 was obtained from the *Hind*III-digested pUCgroESL3.2 and further inserted into a broad host range vector pCM62 (Marx and Lidstrom, 2001) as shown in Figure 8 at the applicable site. The *groESL* gene was inserted in the same orientation with P*lac*. The recombinant plasmid, pCMgroESL as well as control vector, pCM62 were transformed into *E. coli* S17-1. Both plasmids, pCM62 and pCMgroESL, were individually transferred into *A. pasteurianus* SKU1108 by conjugal transformation and selected on YPGD medium containing 25µg/ml tetracycline and 0.1% acetic acid. The obtained tetracycline resistant conjugants were designated as SKU1108/pCM62 and SKU1108/pCMgroESL. Both strains were used for growth determination under stress conditions. For growth determination under stress conditions, the *groESL*-overexpressed strain, SKU1108/pCMgroESL and control strain, SKU1108/pCM62 were precultured in 5 ml of the potato medium and incubated at 30°C with vigorous agitation (200 rpm) for 18 h. The turbidity of the bacterial cell cultures were measured by a spectrophotometer at 540 nm. The cell cultures were inoculated into 100 ml of YPGD medium containing 0%, 4% acetic acid or 5% ethanol in a 500-ml Erlenmeyer flask at an initial absorbance of approximately 0.1. Cultivation was performed at 30°C or 40°C on a rotary shaker (200 rpm). Bacterial growth was periodically measured for 7 d.

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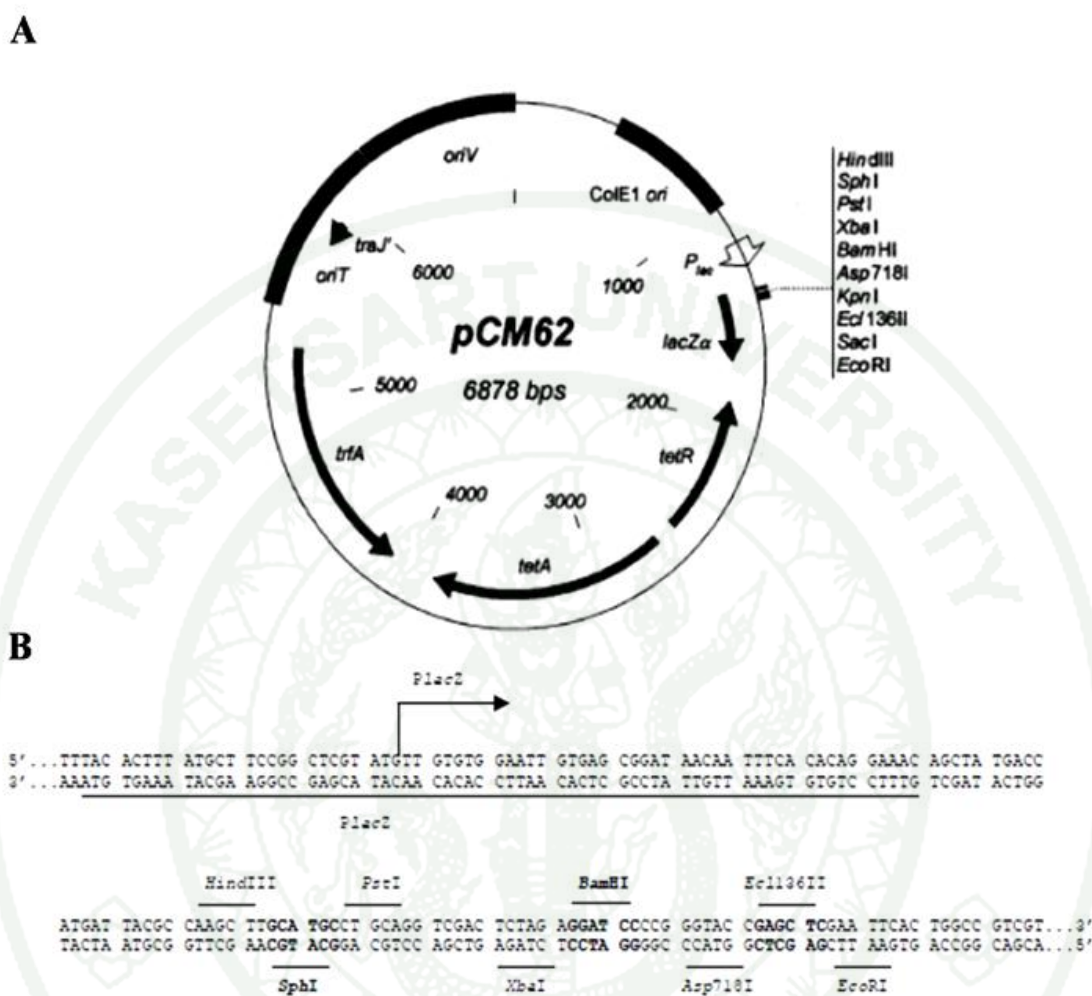

Figure 8 The physical map (A) and multiple cloning sequence of pCM62 broad-host-range cloning vector (B)

Source: Marx and Lidstrom (2001)

3.6 Reverse transcriptase PCR analysis

Cells were grown until early exponential phase and subsequently induced with stressors such as high temperature, high concentration of acetic acid or ethanol. After incubation at various times, total RNA was prepared by the hot phenol method (Aiba *et al.,* 1981, Appendix C) and purified by using RNeasy® mini kit (QIAGEN, Germany, Appendix C). The concentration of RNA was measured by spectrophoto-meter at 260 nm.

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RT-PCR analysis was performed by using an mRNA Selective RT-PCR Kit (Takara Shuzo, Kyoto, Japan) with 0.1 µg of RNA as template and primer set that was described below. The *groEL* gene was amplified by two specific primers, GroEL-F (5'-GCA GAA AGT TGG CTC GA-3') and GroEL-R (5'-TTC AGG GTA ACG GTT TCC-3') started at 498th nucleotide and 926th nucleotide from initiation codon of *groEL*, respectively. The RT-PCR reaction was performed at 40°C for 15 min, PCR was consisted of denaturing at 82°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min. The 447 bp *groEL* gene PCR products after 20, 25, 30 and 35 cycles were analyzed by 0.8% agarose gel electrophoresis. Under these conditions, no band was found when reverse transcriptase was omitted so the RNA-selective RT-PCR was able to detect specifically with mRNA.

3.7 Construction of *groEL* gene disruptant

The *groEL* gene disruptant was constructed by replacing the internal part of *groEL* gene with non polar Km^r cassette using homologous recombination strategy. The procedures were described briefly as following. The *Msc*I restriction site, the 679th nucleotide downstream from the ATG start codon of the *groEL* gene on pUCgroESL3.2, was blunt digested and further inserted with a 0.9 kb *Eco*RV DNA fragment carrying a kanamycin resistant cassette from a pTKm plasmid (Yoshida *et al.,* 2003) at the same site. The resulting plasmid, pUCgroEL::Km^r was electroporated into *A. pasteurianus* SKU1108 and the *groEL* gene-deficient mutants were selected on YPGD medium containing 50 µg/ml kanamycin. Kanamycin resistant colonies were collected and confirmed with PCR amplification as well as Southern hybridization.

3.8 Characterization of growth characteristics of *groEL* gene disruptants

3.8.1 Analysis of temperature sensitive determination, acetic acid and ethanol resistance

A. pasteurianus SKU1108, DGL strains no. 1-7 were cultured in 5 ml of the potato medium and incubated at 30ºC under vigorous shaking (200 rpm). Overnight culture was measured the turbidity by spectrophotometer and adjusted to an initial

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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 serially diluted (10 times) cells were spot-inoculated on YPGD agar plate and incubated at various temperatures for determination of temperature sensitivity. Their abilities to grow were observed after incubation for 3 d. To check sensitivity to acetic acid or ethanol, growth behaviors were also observed on YPGD with and without acetic acid or on YPGD containing 0.5% $CaCO_3$ with and without ethanol addition at 30°C incubated for 3 d and 1 d, respectively.

3.8.2 Time course of growth

A. pasteurianus SKU1108, DGL5 strain were precultured in 5 ml of the potato medium and incubated at 30ºC under vigorous shaking (200 rpm) and their growth was measured by spectrophotometer at 540 nm until reached to log phase. Then, the 5% of seed culture was incoculated into 100 ml of YPGD medium containing 0% or 2% acetic acid and 8% ethanol in a 500-ml Erlenmeyer flask. Cultivation was performed at 30ºC and 40ºC on a rotary shaker at 200 rpm. The growth was periodically measured with a spectrophotometer at 540 nm.

3.9 Complementation of *groESL* gene in *groEL* gene disruptant

The pCMgroESL plasmid and pCM62 plasmid were individually transferred into the *groEL* gene deficient mutant by conjugation method. The obtained conjugants were selected on YPGD medium containing 25µg/ml tetracycline and 50 µg/ml kanamycin. The conjugants were designated as DGL5/ pCMgroESL and DGL5/pCM62. For plate assay, cells grown on potato liquid medium at 30°C for 18 h were measured the turbidity and adjusted them to the same concentration (absorbance of approximately 1.0), and then cells were subjected to 10-fold dilution with sterilized water. Three µL of serially diluted (10 times) cells was spotted on YPGD plate and incubated at various temperatures for temperature sensitive determination. Their growth abilities were observed after incubation for 3 d. To check sensitivity to acetic acid or ethanol, growth behaviors were also observed on YPGD with and without acetic acid or YPGD containing 0.5% $CaCO_3$ with and without ethanol addition at 30°C incubating for 3 d and 1 d, respectively.

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3.10 Induction analysis of *groESL* genes

A promoter probe vector, pGSA, carrying the promoterless *lacZ* gene (Soemphol, unpublished data) was used for construction of transcriptional *PgroESL-lacZ* gene fusion. The DNA fragment carrying 239 bp upstream region and a part of structural *groESL* gene was amplified by PCR amplification. FgroESL-P (5´GCT GCA GGC AGC CTG AAG TGT TGT G-3´) and RgroESL-P (5´-GCG GAT CCA GCG GTC TTC TGT TCG CC -3´) were designed to cover the 239th nucleotide upstream to the 69th nucleotide downstream from the ATG start codon of the *groESL* gene. The *Pst*I and *Bam*HI restriction sites in the forward and reverse primers, respectively, are underlined. The 308 bp PCR product was digested with *Pst*I and *Bam*HI and consequently ligated into the *Pst*I-*Bam*HI digested pGSA. The resulting plasmid, pGSAPgroESL including pGSA were individually transformed into *E. coli* DH5α by heat shock transformation and the recombinant clones were selected on LB medium containing 12.5 µg/ml gentamicin and 20 µg/ml X-gal. The recombinant plasmids were confirmed by PCR amplification and nucleotide sequencing. *E. coli* DH5α harboring the transcriptional *PgroESL-lacZ* fusion plasmid were precultured in 5 ml of LB medium for 18 h and measured the turbidity by spectrophotometer at a wave-length of 600 nm. The cell cultures were inoculated into 100 ml of LB medium containing 0%, 0.05% acetic acid or 5% ethanol in a 500-ml Erlenmeyer flask at an initial absorbance of approximately 0.05. Cultivation was performed at 37°C and 40°C on a rotary shaker at 200 rpm for 48 hours. The bacterial growth was periodically measured by a spectrophotometer. Five ml of cell cultures were harvested and centrifuged at 12,000 rpm for 1 min. The cell pellets were washed with deionized water and stored at -20°C for determination of β-galactosidase activity according to the standard method described by Miller (1972) with slightly modification.

3.11 Sequence retrieval and sequence alignment of CIRCE element in 20 genomes of acetic acid bacteria

Four complete genome sequences of acetic acid bacteria, *A. pasteurianus* IFO 3283-01 (NC_013209), *Gluconobacter oxydans* 621H (NC_006677), *Gluconacetobacter xylinus* NBRC 3288 (NC_016027) and *Gluconacetobacter diazotrophicus* Pal 5

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(NC_010125) were obtained from the NCBI FTP website at ftp.ncbi.nlm.nih.gov/genomes/ Bacteria/ (Prust *et al.,* 2005; Azuma *et al.,* 2009; Bertalan *et al.,* 2009; Ogino *et al.,* 2011). Sixteen draft genome sequences of acetic acid bacteria, *A. aceti* NBRC 14818 (BABW01000001-BABW01001488), *A. pasteurianus* 3P3 (CADQ01000001-CADQ01000101), *A. pasteurianus* IFO 3191 (BACG01000001-BACG01000306), *A. pasteurianus* NBRC 101655 (SKU1108) (BACF01000001-BACF01000294), *A. pasteurianus* subsp. *pasteurianus* LMG 1262 (CADO01000001-CADO01000141), *A. pomorum* DM001 (AEUP01000001-AEUP01000066), *A. tropicalis* NBRC 101654 (BABS01000001-BABS01000773), *Gluconacetobacter europaeus* 5P3 (CADS01000001-CADS01000256), *Gluconacetobacter europaeus* LMG 18494 (CADR01000001-CADR01000216), *Gluconacetobacter europaeus* LMG 18890 (CADP01000001-CADP01000321), *Gluconacetobacter hansenii* ATCC 23769 (ADTV01000001-ADTV01000071), *Gluconacetobacter oboediens* 174Bp2 (CADT01000001-CADT01000200), *Gluconacetobacter* sp. SXCC-1 (AFCH01000001-AFCH01000064), *Gluconobacter frateurii* NBRC 101659 (BADZ01000001- BADZ01000145), *Gluconobacter morbifer* G707 (AGQV01000001-AGQV01000019), and *Gluconobacter oxydans* WSH-003 (AHKI01000001-AHKI01000179), were obtained from the DDBJ FTP website at ftp.ddbj.nig.ac.jp/ddbj_database/wgs/ (Iyer *et al.,* 2010; Andrés-Barrao *et al.,* 2011; Du *et al.,* 2011; Matsutani *et al.,* 2011; Sakurai *et al.,* 2011; Hattori *et al.,* 2012; Matsutani *et al.,* 2012). CIRCE elements of all acetic acid bacteria genomes were identified by a homology search of known CIRCE element sequence using the BLASTN (Altschul *et al.,* 1997). Homologous regions were collected and CIRCE element regions were manually assigned. Nucleotide sequence alignment was carried out using CLUSTALW (Larkin *et al.,* 2007). We used the MEGA version 5.05 package to generate phylogenetic trees to study the phylogenetic relationship based on CIRCE element nucleotide sequence with the neighbor-joining (NJ) approach and 1000 bootstrap replicates (Tamura *et al.,* 2011). Consensus of CIRCE elements were analyzed and displayed in the WebLogo format (Crooks *et al.,* 2004).

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4. Investigation of the *groESL* Gene Variation in Thermotolerant and Thermo-sensitive Acetic Acid Bacteria by PCR and Southern Hybridization

4.1 Growth determination of SKU strains

The inoculums were prepared from one loopful of each isolates cultured in potato medium at 30ºC, 200 rpm for 18-24 h. For selection of thermotolerant and thermosensitive acetic acid bacterial strains, 5 µl of the inoculum was spot-inoculated on YPGD agar and incubated at 30, 37 and 40ºC for 5 days. The growths of all isolates were observed and the thermotolerant strains were selected from the isolates exhibited the best growth at 37 and 40ºC. In contrast, the thermosensitive strains were selected from the isolates exhibited no growth at high temperature.

4.2 PCR amplification of the partial *groESL* gene from the strains of genus *Acetobacter*

For partial *groESL* gene amplification, primer GF1 (5'-AGG AAA AGC CTA TGG AAG-3') and the reverse primer GR1, (5'-GCT GTG GAA GAA GGC AT-3') were used. PCR condition was conducted, as described in 3.1 of Materials and Methods section. The obtained 1.4 kb PCR product was recovered from 1.0% agarose gel and purified by the application of a HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience Corp., Taipei, Taiwan). After that, the purified DNA was visualized on 0.8% agarose gel after electrophoresis at 100V in TAE buffer approximately 30 min, ethidium bromide staining, and illumination with UV light. Then, the purified 1.4 kb PCR product was sent to the First Base Laboratory (Selangor, Malaysia) for determination of the nucleotide sequences.

4.3 Southern hybridization of genomic DNA from thermotolerant and thermosensitive acetic acid bacterial strains by using 1.4 kb *groESL* gene DNA probe

The chromosomal DNAs from thermotolerant and thermosensitive acetic acid bacterial strains were completely digested with *Hin*dIII restriction enzyme. The purified 1.4

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kb *groESL* gene PCR product from *A. pasteurianus* SKU1108 was labeled with digoxigenin-11-dUTP (Dig-11-dUTP; Roche Applied Science, Penzberg, Germany) by random primed labeling and used as DNA probe for Southern hybridization. The digested chromosomal DNAs were hybridized with the 1.4 kb *groESL* gene DNA probe. The process of membrane preparation, hybridization and detection were conducted, as described in 3.2 of Materials and Methods section.

5. Identification and Phylogenetic Analysis of Genus *Acetobacter* Based on *groEL* Gene Sequences

5.1 PCR amplification of 16S rRNA gene from the strains of genus *Acetobacter*

Chromosomal DNA from acetic acid bacteria was isolated by the method described by Okumura *et al.* (1985). In this part, the twenty-four *Acetobacter* isolates (12 isolates of BCC strains and 12 isolates of SKU strains) and the twenty-three type strains were used for 16S rRNA gene amplification. The DNA primers for 16S rRNA gene amplification were designed from highly conserved regions of nucleotide sequence of 16S rRNA of α-*Proteobacteria.* Forward primer, 27f (20 mers), was 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer, 1525r (18 mers), was 5'-AAA GGA GGT GAT CCA GCC-3'. These primers were used as described by Devereux and Wills (1995). The desired PCR product was about 1.5 kb DNA fragment. PCR amplifications were conducted by performing reaction mixture contained 0.2 μM of each primer, 100 μM deoxynucleoside triphosphate (dNTPs), 1U *Taq* DNA polymerase (Thermo scientific, USA) in 1x PCR buffer. Amplification was carried out on a thermal cycler (Labnet, USA). The PCR thermal cycling conditions were as follows: 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 54ºC for 1 min and 72ºC for 2 min. A final extension was performed at 72ºC for 10 min. The 1.5 kb PCR product was analyzed by 0.8% agarose gel in TAE buffer at 100V, and purified by using HiYield^{TM} Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taiwan). The purified DNA fragment was confirmed by agarose gel electrophoresis before determine nucleotide sequences.

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5.2 PCR amplification of *groEL* gene from the strains of genus *Acetobacter*

In this part, the twenty-four *Acetobacter* isolates (12 isolates of BCC strains and 12 isolates of SKU strains) and the twenty-three type strains were used for *groEL* gene amplification. The *groEL*-specific primers for PCR amplification and sequencing of *groEL* genes were designed on the basis of the genome sequences of *A. pasteurianus* IFO 3283 (Azuma *et al.,* 2009) and *A. pomorum* DM001 (Shin *et al.,* 2011) except for primers groEL-10-F and groEL-11-R (Cleenwerck *et al.,* 2010) as shown in Table 7. PCR amplifications were conducted by performing reaction mixture contained 0.2 µM of each primer, 100 µM deoxynucleoside triphosphate (dNTPs), 1U *Taq* DNA polymerase (Thermo scientific, USA) in 1x PCR buffer. Amplification was carried out on a thermal cycler (Labnet, USA). The PCR thermal cycling conditions were as follows: 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 54ºC for 1 min and 72ºC for 2 min. A final extension was performed at 72ºC for 10 min. The amplified DNA was visualized on 1% agarose gel after electrophoresis at 100V in TAE buffer for approximately 30 min, ethidium bromide staining, and illumination with UV light. The amplified PCR products were purified for determination of the nucleotide sequences.

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Table 7 Primers for *groEL* gene amplification used in this study

Primer	Sequence (5' to 3')	Bacterial species to which primer applies	Size (bp)	Source or reference
FgroEL	CAA TGG CTG CCA AAG ACG	*A. pasteurianus, A. orleanensis, A. lovaniensis, A. tropicalis, A. indonesiensis, A. syzygii, A. cibinongensis, A. orientalis, A. ghanensis, A. malorum, A. senegalensis, A. fabarum, A. farinalis, A. okinawensis, A. papayae* and *A. persici*	1,600	The present study
RgroEL	GAA GGA CTT AGA AGT CCA T			The present study
FgroELnew	CTG GAC AAG AGC TTC GGC	*A. cerevisiae* and *A. peroxydans*	1,400	The present study
RgroELnew	GGA TAA CGG CAA CAC CGC	*A. thailandicus* isolate AD25^{T}	1,000	The present study
FgroEL89	CCG TGC GGA CAA CCT TGG	*A. pomorum*	1,200	The present study
RgroEL89	CGG CAC CAC AAC GGC TAC			The present study
FgroELcenter	GGT TGA AGA AGC CAA GCA	All species except for *A. aceti, A. nitrogenifigens*, *A. estunensis* and *A. oeni*		The present study
groEL-10-F	ACA AGT TCG AGA ACA TGG GC	*A. aceti, A. nitrogenifigens*, *A. estunensis* and *A. oeni*	900	Cleenwerck *et al.*, 2010
groEL-11-R	TCC TTG CGC TCC TTC ACC TC			Cleenwerck *et al.*, 2010

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5.3 Analysis of sequence data and phylogenetic tree construction

The purified PCR products were sent to The First Base Laboratory (Selangor, Malaysia) for DNA sequencing. The 16S rRNA gene sequences and the *groEL* gene sequences obtained were edited by using the Chromas 2.33 program. (http://www.technelysium.com.au/chromas.html). The DNA sequences of the isolate and the type strains of all the known species of the genus *Acetobacter* were aligned using CLUSTAL W (version 1.83; Thompson *et al.,* 1994). Gaps in the sequences were deleted using the BioEdit program (Hall, 1999). The phylogenetic relationships among species using 16S rRNA gene and *groEL* gene sequences were analyzed by the neighbor-joining approach (Saitou and Nei, 1987) listed in the MEGA (Molecular Evolutionary Genetic Analysis, version-5.1 software; Tamura *et al.,* 2011). For the neighbor-joining analysis, the distance between the sequences was calculated by Kimura's two-parameter model (Kimura, 1980). Bootstrap values were obtained for 1,000 randomly generated trees (Felsenstein, 1985). The pair-wise sequence similarity values (%) of 16S rRNA genes and *groEL* genes were calculated, respectively with 1,357 and 870 bases, among the type strains of the species.

6. Identification and Characterization of the Novel Species in Genus *Acetobacter*

6.1 Bacterial isolation, reference strains, culture medium and culture conditions

Isolate AD25^T (= BCC 15839^T = NBRC 103583^T), which obtained from BIOTEC Culture Collection, was selected for further characterization to propose as a novel species due to this isolate constituting an independent cluster with long phylogenetic branch in the phylogenetic trees based on 16S rRNA and *groEL* gene sequences. The isolate was isolated from a flower of blue trumpet vine by an enrichment culture approach using glucose/ethanol/ yeast extract (GEY) medium (Appendix A), as briefly described below (Yamada *et al.,* 1976, 1999; Kommanee *et al.,* 2008; Muramatsu *et al.,* 2009; Tanasupawat *et al.,* 2011a). A sample source was incubated at pH 4.5 and 30ºC for 3-5 days in a liquid GEY medium (15 ml/tube). When microbial growth was observed, the culture was streaked onto a GEY-agar plate containing 0.3% $CaCO_3$ (w/v). The acetic acid

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bacteria were selected as acid-producing bacterial strains that formed a clear zone around the colony on GEY-agar plate containing 0.3% $CaCO_3$ (w/v).The reference strains of the genus *Acetobacter* were *A. orientalis* BCC 23127^T, *A. cibinongensis* BCC 23126^T and *A. tropicalis* BCC 23123^T. Isolate AD25^T and the reference strains used in this study were grown in a GEY broth on a rotary shaker (150-200 rpm) at 30ºC for 24 h.

6.2 PCR amplification of 16S rRNA, 16S-23S rRNA gene ITS and *groEL* gene sequences

Genomic DNA was extracted by the method described by Okumura *et al.* (1985). The primer sequences and PCR condition for the amplification and sequencing of the 16S rRNA and *groEL* genes were described in 5.1 and 5.2 of Materials and Methods section, respectively. For 16S-23S rRNA gene ITS (internal transcribed spacer region) amplification, primer 1522f (5'-TGC GGY TGG ATC ACC TCC T-3') and primer 38r (5'-GTG CCW AGG CAT CCA CCG-3') were used (Ruiz *et al.,* 2000). PCR amplifications of 16S-23S rRNA gene ITS were conducted by performing reaction mixture contained 0.2 µM of each primer, 100 µM deoxynucleoside triphosphate (dNTPs), 1U *Taq* DNA polymerase (Thermo scientific, USA) in 1x PCR buffer. Amplification was carried out on a thermal cycler (Labnet, USA). The PCR thermal cycling conditions were as follows: 94ºC for 5 min, followed by 25 cycles of 92ºC for 30 sec, 56ºC for 45 sec and 72ºC for 1 min. A final extension was performed at 72ºC for 7 min. The 0.7 kb PCR product was analyzed by 0.8% agarose gel in TAE buffer at 100V, and purified by using HiYield^{TM} Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taiwan). The purified DNA fragment was confirmed by agarose gel electrophoresis before determine the nucleotide sequences. The purified PCR products were sent to The First Base Laboratory (Selangor, Malaysia) for DNA sequencing.

6.3 Analysis of sequence data and phylogenetic tree construction

Analysis of the obtained sequence data and phylogenetic tree construction were conducted as described in 5.3 of Materials and Methods section. Moreover, the pair-wise 16S rRNA genes, 16S-23S rRNA gene ITS and *groEL* gene sequence similarity values (%)

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of the strain BCC 15839 were calculated without considering gaps in the sequences, respectively with 1,343, 413 and 866 bases, among the type strains of the species.

6.4 Morphological characteristics

6.4.1 Colony morphology

The bacterial culture was grown on glucose/ethanol/calcium carbonate agar (GECA) medium (Appendix A) and incubated at 30ºC for 24-48 h. The characteristics of colony on GECA such as color, size, surface, margin and texture were recorded.

6.4.2 Cellular morphology

The bacterial culture was grown on GECA medium for 24-48 h. Microscopy observation was performed after wet mount. The results, such as cell shape and cell size were recorded.

6.5 Physiological characteristics

Isolate $AD25^T$ (BCC 15839^T) was examined for phenotypic features (Hucker and Conn, 1923; Asai *et al.,* 1964; Gosselé *et al.,* 1980; Tanasupawat *et al.,* 2011a). The phenotypic features of the isolate were determined as follows.

6.5.1 Catalase test

The active culture were streaked on GECA medium and incubated at 30ºC for 18-24 h. The small amount of bacterial cell was collected by using the steriled inoculating loop and placed on the microscope slide. Then one drop of 3% H_2O_2 was placed on the bacterial cell on microscope slide. The positive result was observed by bubble formation. No bubble formation was negative for catalase.

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6.5.2 Oxidase test

The active culture were streaked on GECA medium and incubated at 30ºC for 18-24 h. The filter paper was wet with the Kovac's oxidase reagent (Appendix B). Then small amount of bacterial cell was picked up by using the steriled inoculating loop and placed on the filter paper saturated with Kovac's oxidase reagent. The positive result was observed by the development of a blue color on the bacterial cell placed on the filter paper within 10 sec. No development of blue color was negative for oxidase.

6.5.3 Growth and acid formation from carbon sources

The suspension of the bacterial cells was prepared in the steriled normal saline (0.85% NaCl). Then 50 µl of bacterial cell suspension was inoculated into basal medium (Appendix A) with various carbon sources and incubated at 30ºC for 7 d. The growth was observed daily as turbidity of culture broth by placing the test tube on a white paper on which 0.75 mm thick lines drawn with India ink are present. The degree of growth was recorded as 3+ (lines are completely obscured), 2+ (lines observed are diffuse band), +1 (lines are indistinguishable but have blurred edges), +/- (the results of these are doubtful) and – (lines are completely cleared). The acid formation was examined daily as the change of color of bromcresol purple (indicator) from purple to yellow. The carbon compounds used in this study consisted of D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-xylose, D-arabinose, L-arabinose, L-rhamnose, D-mannitol, D-sorbitol, ducitol, meso-erythriol, glycerol, maltose, lactose, mellibiose, sucrose, raffinose, ethanol, D-arabitol, L-arabitol, methanol, meso-ribitol, meso-inositol, D-ribose, D-sorbose, trehalose, starch, 1-propanol, 1-butanol and 2-butanol.

6.5.4 Growth on ammoniac nitrogen

The suspension of the bacterial cells was prepared in the steriled normal saline (0.85% NaCl). Then 50 µl of bacterial cell suspension was inoculated into the Frateur modified Hoyer medium (Appendix A) with 3% glucose, with ethanol and with mannitol and incubated at 30ºC for 7 d. The growth was observed daily as turbidity of

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culture broth. The growth ability of the isolate was confirmed by transferring the culture broth to the fresh medium for two times. The positive result was observed by the turbidity of culture after transferred the culture to fresh medium for two times.

6.5.5 Growth on the presence of 0.35% acetic acid

The suspension of the bacterial cells was prepared in the steriled normal saline (0.85% NaCl). Then 50 µl of bacterial cell suspension was inoculated into the glucose-ethanol medium (Appendix A) containing 0.35% acetic acid and incubated at 30ºC for 7 d. The growth was observed daily as turbidity of culture broth.

6.5.6 Ketogenesis (dihydroxyacetone formation) from glycerol

The active culture was inoculated into the medium containing 3% glycerol (w/v), 0.5% yeast extract (w/v) and 1% peptone (w/v) and incubated in shaking condition at 30ºC for 24 h. After the incubation, the 200µl of Fehling solution (Appendix B) was added into the culture broth. The positive result was observed by the formation of red color. The absence of the red color formation was negative for ketogenesis.

6.5.7 Growth on 10% ethanol

The active bacterial cells were streaked on the GECA medium containing 10% ethanol (v/v) (Appendix A) and incubated at 30ºC for 7 d. The growth was daily observed and recorded. The positive result was examined by bacterial growth on GECA medium with 10% ethanol. No growth on GECA medium with 10% ethanol was negative.

6.5.8 Growth on 30% D-glucose

The active bacterial cells were streaked on the medium containing 30% D-glucose (w/v), 0.5% yeast extract (w/v) (Appendix A) and incubated at 30ºC for 7 d. The growth was daily observed and recorded. The positive result was examined by bacterial

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growth on the medium containing 30% glucose. No growth on the medium containing 30% glucose was negative.

6.5.9 Production of ketogluconic acid from D-glucose

The isolate was cultured in the medium containing 3% D-glucose and 0.3% yeast extract and incubated in shaking condition at 30ºC for 3-5 d. The culture broth was centrifuged and the supernatant was collected. Then the supernatant including the standard of 2- keto-gluconic acid (0.1 M), 5-keto-gluconic acid (0.1 M) and 2, 5 diketo-gluconic acid were spot on Cellulose plate Merck TLC (1.05552) (TLC, 0.1 mm). After that, the TLC plate was applied into ethyl acetate: formic acid: acetic acid: water (18: 1: 3: 4 v/v) and was completely dried the plate in cold airflow (repeat this step for three times). Finally, the TLC plate was sprayed with the mixed solution containing 0.5 g of o-phenylenediamine, 0.81 ml of HCL, 3.75 of water and ethanol (added up to 25 ml) and dried at 105ºC. The color development was immediately observed as follows.

Blue	= 5-keto-gluconic acid
Green	= 2-keto-gluconic acid
Yellow	= 2,5-diketo-gluconic acid
Brown-red color	= D-glucose

6.6 Chemotaxonomic characteristic: Ubiquinone system

The isoprenoid quinone of the isolate was determined by the method of Yamada *et al.* (1969). Acetic acid bacteria were grown in potato broth on a rotary shaker (150-200 rpm) at 30°C for 24 h. Ubiquinone was extracted from freeze-dried cells by shaking with a mixture of chloroform: methanol (2:1, v/v). Cells were removed by filtration, and the combined filtrates were evaporated to dryness under a reduced pressure on a rotary evaporator. The resulting residue was dissolved in a small volume of acetone, followed by thin-layer chromatography on a silica gel plate (20x 20 cm, silica gel $60F_{254}$, Art 5715, E. Merck, Darmstdt, Germany) with a solvent system of pure benzene. The purified ubiquinone preparation was applied to reversed-phase paper chromatography (Tamaoka *et*

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al., 1983) and to high performance liquid chromatography (HPLC) for its homologues. The ubiquinone type was identified by HPLC using a Cosmosil (5C18; Waters) 4.6 x 250 mm column and methanol/2-propanol (2:1) at 1 ml/min as the elution system, with spectrophotometric detection (275 nm wavelength).

6.7 DNA base composition

Chromosomal DNA was prepared by the method of Saito and Miura (1963). DNA base composition was determined by the method of Tamaoka and Komagata (1984). The chromosomal DNA was incubated at 60ºC for completely dissolve DNA. 2-25 µg of DNA was added into microcentrifuge tube. Then sterile water was added into the microcentrifuge tube to adjust the volume to 15 µl. DNA was kept in boiling water for 5 min, and immediately cooled in ice bath for 5 min. Then 15 µl of nuclease P1 (Appendix B) was added into the microcentrifuge tube containing DNA and incubated at 50ºC for 2 h. After the indicated time, 15 µl of alkaline phosphatase (Appendix B) was added and incubated at 37ºC for 2 h or overnight. The sample was applied to high performance liquid chromatography (HPLC) for analyse the quantification of nucleosides using a Cosmosil (5C18; Waters) 4.6 x 250 mm column and 0.02 M $NH_4H_2PO_4$: acetonitrile (20:1) at 1 ml/min as the elution system, with spectrophotometric detection (270 nm wavelength).

6.8 DNA-DNA hybridization

DNA-DNA hybridization was performed by the photobiotin-labeling method with microplate wells as described by Ezaki *et al.* (1989).

6.8.1 Fixing DNA on microplate

The DNA samples of tested strains for all wells of microplate (1 µg of DNA/well) were added into microcentrifuge tube. Then, 1x SSC solution (Appendix B) was added to dilute DNA (100 µg/ml). After that, the microcentrifuge tube containing diluted DNA was kept in boiling water for 5 min, and immediately cooled in ice bath. The PBSM (Appendix B) was prepared and used to dilute the DNA solution (10 µg/ml DNA

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solution). The DNA solution was added into each well of microplate. The microplate containing DNA solution was incubated at 28ºC (or room temperature) for 3 h. Then the solution was discarded from microplate. After that, the microplate fixing with DNA was incubated at 45ºC for overnight (or more than 3 h).

6.8.2 DNA probe labeling

The amount of DNA probe for all wells was added to 1.5 ml microcentrifuge tube (1 µg of DNA probe/well). Then, 1x SSC solution (Appendix B) was added to adjust the volume to 10 µl in total (the final concentration should not exceed 0.5 µg/µl). The equal volume of photobiotin (10 µl) was added in to the microcentrifuge tube containing DNA probe. Then the cap of microcentrifuge tube was opened and the tube was kept under the opened mercury vapur lamp for 20 min. The 0.1 M of Tris-Cl, 1 mM EDTA (pH 9) (Appendix B) were added to make the total volume to 100 µl. After that, 1-butanol (n-butanol) was added in the tube. The solution was mixed and centrifuged at max speed for 10 sec. The upperphase of solution was discarded. The DNA probe was kept in dark until use.

6.8.3 Hybridization

The pre-hybridization solution (Appendix B) was added into each well of microplate and incubated at 37ºC for 30 min. After the indicated time, the pre-hybridization solution was discared. DNA probe was prepared by boiling for 5 min and immediately keeping cool in ice bath. The hybridization solution (Appendix B) containing the prepared DNA probe was added into all wells of microplate (100 µl/well). The microplate was sealed with plate seal and incubated at 45ºC for 15 h.

Hybridization temperature (50% formamide) = (Tm-45) ºC

Tm = 69.3+ (%G+C) x 0.41

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6.8.4 Detection by colorimetric reaction

Levels of DNA-DNA relatedness (%) were determined colorimetrically (Verlander, 1992). The hybridization solution was discarded. The 0.2x SSC was used for washing (three times) the microplate well (200µl/well). After that, the 200 µl of solution I (Appendix B) was added into each well and incubated at 30ºC for 10 min. Then the solution I was removed. The 100 µl of solution II was added into each well (each well should be equal volume) and incubated at 37ºC for 30 min. After the indicated time, the solution II was removed. Then the microplate well was washed with PBS solution (Appendix B) for three times. The 100 µl of solution III was added into the each well and incubated at 37ºC for 10 min. After that, 100 µl of 2M H_2SO_4 was added into each well. The color intensity was measured at A_{450} on a model Versa Max microplate reader (Molecular Devices, Sunnyvale, CA, USA).

$$\%\ \text{DNA-DNA relatedness} = \frac{100 \times (\text{Sample-Calf})}{(\text{Probe-Calf})}$$

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RESULTS AND DISCUSSION

1. Cloning, Nucleotide Sequencing and Characterization of *groESL* Gene from *A. pasteurianus* SKU1108

1.1 PCR cloning and sequencing of the partial *groESL* gene

The partial *groESL* gene was amplified by Polymerase Chain Reaction (PCR) using two specific primers, GF1 (5'-AGG AAA AGC CTA TGG AAG-3') and GR1 (5'-GCT GTG GAA GAA GGC AT-3'). These primers were designed from conserved region of *groESL* gene from *A. pasteurianus* NBRC 3283 (formally known as *A. aceti* IFO 3283) and other related microorganisms. Chromosomal DNA of *A. pasteurianus* SKU1108 was used as template and PCR reaction was performed by using the Ready-To-Go™ PCR Beads as described in Materials and Methods. Schematic representation of the position of 1.4 kb DNA fragment carrying the partial *groESL* gene comparing with complete *groESL* gene from *A. aceti* IFO 3283 and agarose gel electrophoresis of the obtained PCR product are shown in Figure 9A and 9B. The obtained 1.4 kb PCR product of *groESL* gene was cloned into pGEM-T®-Easy Vector. The resulting plasmid designated as pGEM-TgroESL1.4 and the *Eco*RI-digested recombinant plasmid are shown in Figure 9C. The complete nucleotide sequence of 1.4 kb PCR product was obtained with the sequencing reaction using T7 promoter primer (5'-GTA ATA CGA CTC ACT ATA GGG C-3') and SP6 promoter primer (5'-GAT TTA GGT GAC ACT ATA G-3') as forward and reverse sequencing primers, respectively. Homology search using BLASTN revealed that 1.4 kb PCR product was similar to *groESL* gene of *A. pasteurianus* IFO 3283 (99%) and *A. pasteurianus* NBRC 3283 (98%) (Okamoto-Kainuma *et al.,* 2002). Thus, the result from homology search using BLASTN revealed that 1.4 kb PCR amplified product by using two specific primers, GF1 and GR1 was *groESL* gene. The purified 1.4 kb *groESL* gene was used as probe for Southern hybridization.

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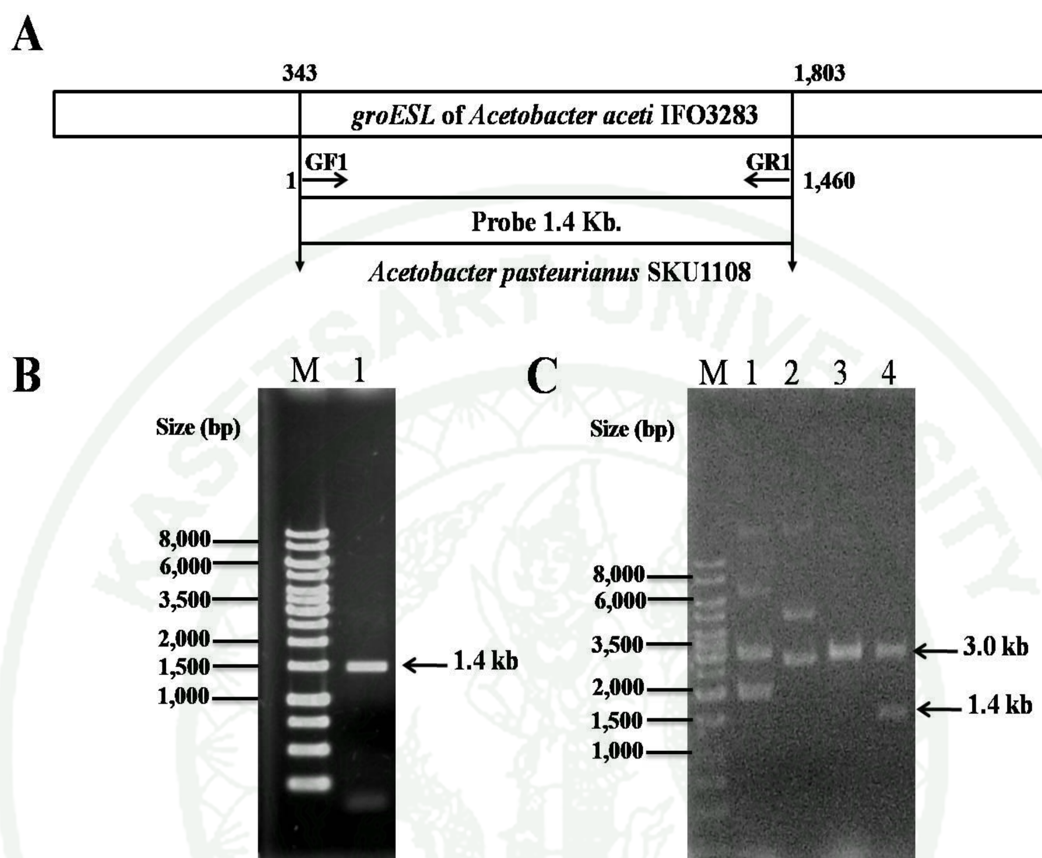

Figure 9 Schematic representation of the position of 1.4 kb DNA fragment carrying the partial *groESL* gene comparing with complete *groESL* gene from *A. aceti* IFO 3283 (A) agarose gel electrophoresis of the obtained PCR product (B) the resulting plasmid designated as pGEM-TgroESL1.4 and the *Eco*RI-digested recombinant plasmid (C). B, Lane M = 1 kb DNA ladder and Lane 1 = 1.4 kb DNA fragment carrying *groESL* gene. C, Lane M = 1 kb DNA ladder, Lane 1 = pGEM-T®-Easy vector, Lane 2 = pGEM-TgroESL1.4, Lane 3 = pGEM-T®-Easy vector digested with *Eco*RI and Lane 4= pGEM-TgroESL1.4 digested with *Eco*RI.

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1.2 Southern hybridization of genomic DNA from *A. pasteurianus* SKU1108 by 1.4 kb *groESL* gene DNA probe

The 1.4 kb PCR amplified *groESL* gene was analyzed in 0.8% agarose gel electrophoresis and recovered from the gel. The purified PCR product was labeled with digoxogenin-11-dUTP (Dig-11-dUTP) by random primed labeling and used as DNA probe for Southern hybridization. The genomic DNA from *A. pasteurianus* SKU1108 was completely digested by several enzymes; *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Sma*I and double digested by *Bam*HI-*Eco*RI, *Eco*RI-*Hind*III, *Hind*III-*Kpn*I, *Sma*I-*Bam*HI, *Bam*HI-*Hind*III, *Bam*HI-*Kpn*I, *Eco*RI-*Kpn*I, *Eco*RI-*Sma*I and *Sma*I-*Hind*III. These restriction enzymes were selected due to the predicted that *groESL* gene cluster of *A. pasteurianus* SKU1108 may harbor similar restriction map with *A. pasteurianus* NBRC 3283 (Okamoto-Kainuma *et al.,* 2002).These digested DNA were chosen to hybridize with 1.4 kb *groESL* gene DNA probe by Southern hybridization. As shown in Figure 10A, the positive hybridization signals were detected with several sizes of DNA fragment. The restriction mapping was performed as shown in Figure 10B. From the results, the 3.2 kb *Hind*III DNA fragment was selected for subclone into pUC119 vector. The reason for selection of 3.2 kb *Hind*III DNA fragments for *groESL* gene cloning was its smaller size with complete *groESL* structural gene which may be able to be inserted into pUC119 vector more easily.

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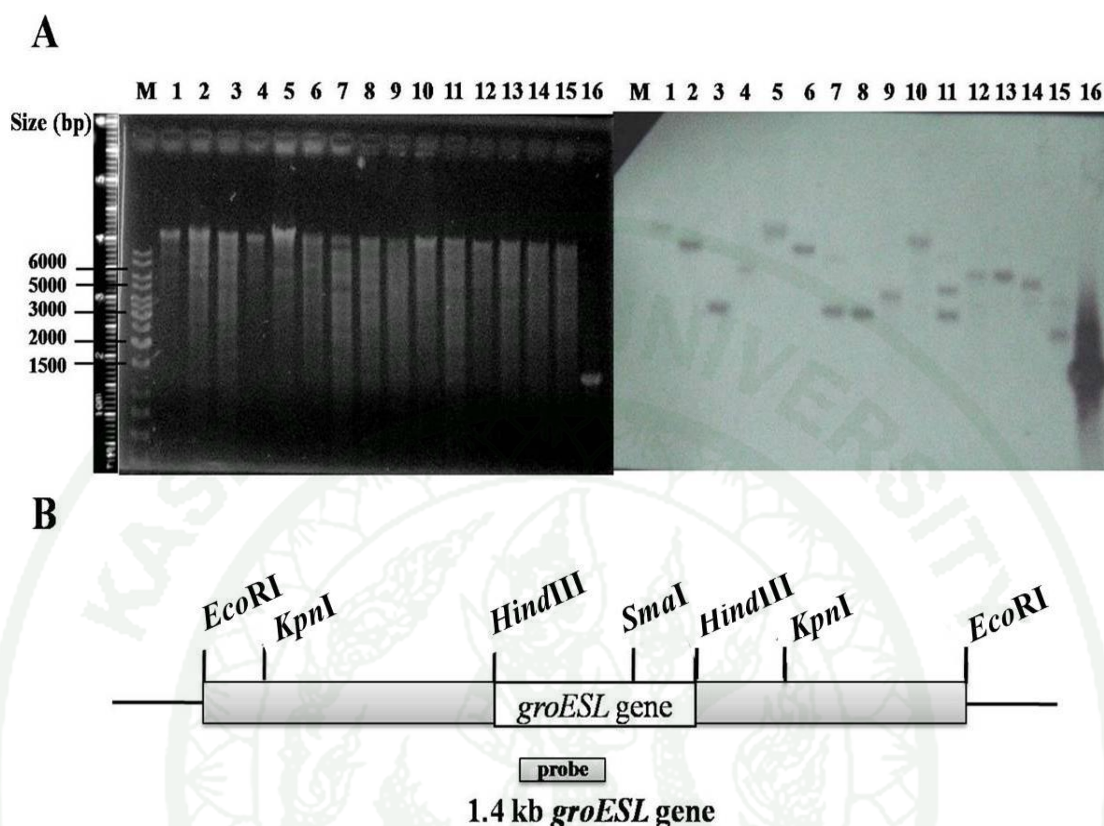

Figure 10 Agarose gel electrophoresis (left) and Southern hybridization pattern (right) of the digested genomic DNA and 1.4 kb *groESL* gene product from *A. pasteurianus* SKU1108. The 1.4 kb *groESL* gene was used as DNA probe (A) and the schematic representation of restriction mapping (B). A, Lane M = 1kb DNA ladder, 1 = *Bam*HI digested genomic DNA, 2 = *Eco*RI digested genomic DNA, 3 = *Hind*III digested genomic DNA, 4 = *Kpn*I digested genomic DNA, 5 = *Sma*I digested genomic DNA, 6 = *Bam*HI-*Eco*RI digested genomic DNA, 7 = *Eco*RI-*Hind*III digested genomic DNA, 8 = *Hind*III-*Kpn*I digested genomic DNA, 9 = *Kpn*I-*Sma*I digested genomic DNA, 10 = *Sma*I-*Bam*HI digested genomic DNA, 11 = *Bam*HI-*Hind*III digested genomic DNA, 12 = *Bam*HI-*Kpn*I digested genomic DNA, 13 = *Eco*RI-*Kpn*I digested genomic DNA, 14 = *Eco*RI-*Sma*I digested genomic DNA, 15 = *Sma*I-*Hind*III digested genomic DNA and 16 = the 1.4 kb *groESL* probe.

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1.3 Purification and cloning of 3.2 kb *Hind*III DNA fragments carrying *groESL* gene

The results from Southern hybridization of genomic DNA digested with *Hind*III from previous experiment indicated that *groESL* gene is localized on 3.2 kb *Hind*III DNA fragments. The 3.2 kb *Hind*III DNA fragments were recovered from 0.8% agarose gel by HiYield™ Gel/PCR Fragments Extraction Kit before ligated into *Hind*III digested pUC119. The agarose gel electrophoresis patterns of the purified 3.2 kb *Hind*III DNA fragments were shown in Figure 11A. In order to confirm that purified DNA fragment carrying *groESL* gene, it was used as DNA template for amplification of *groESL* gene by using GF1 and GR1 primers. The PCR reaction was performed by using the illustra™ puReTaq Ready-To-Go PCR Beads and PCR products were analyzed in 0.8% agarose gel electrophoresis. The 1.4 kb PCR amplified products were shown in Figure 11B indicated that the purified *Hind*III DNA fragments carry *groESL* gene from *A. pasteurianus* SKU1108. These purified DNA fragments were then ligated into *Hind*III digested pUC119. Ligation products were used to transform into *E. coli* DH5α competent cells. The pUC119 vector contains a multiple cloning site within the α-coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide by the insert DNA allows recombinant clones to be directly identified by color screening (blue/white) on the LB plates containing 50 μg/ml amplicillin and 50 μg/ml X-gal.

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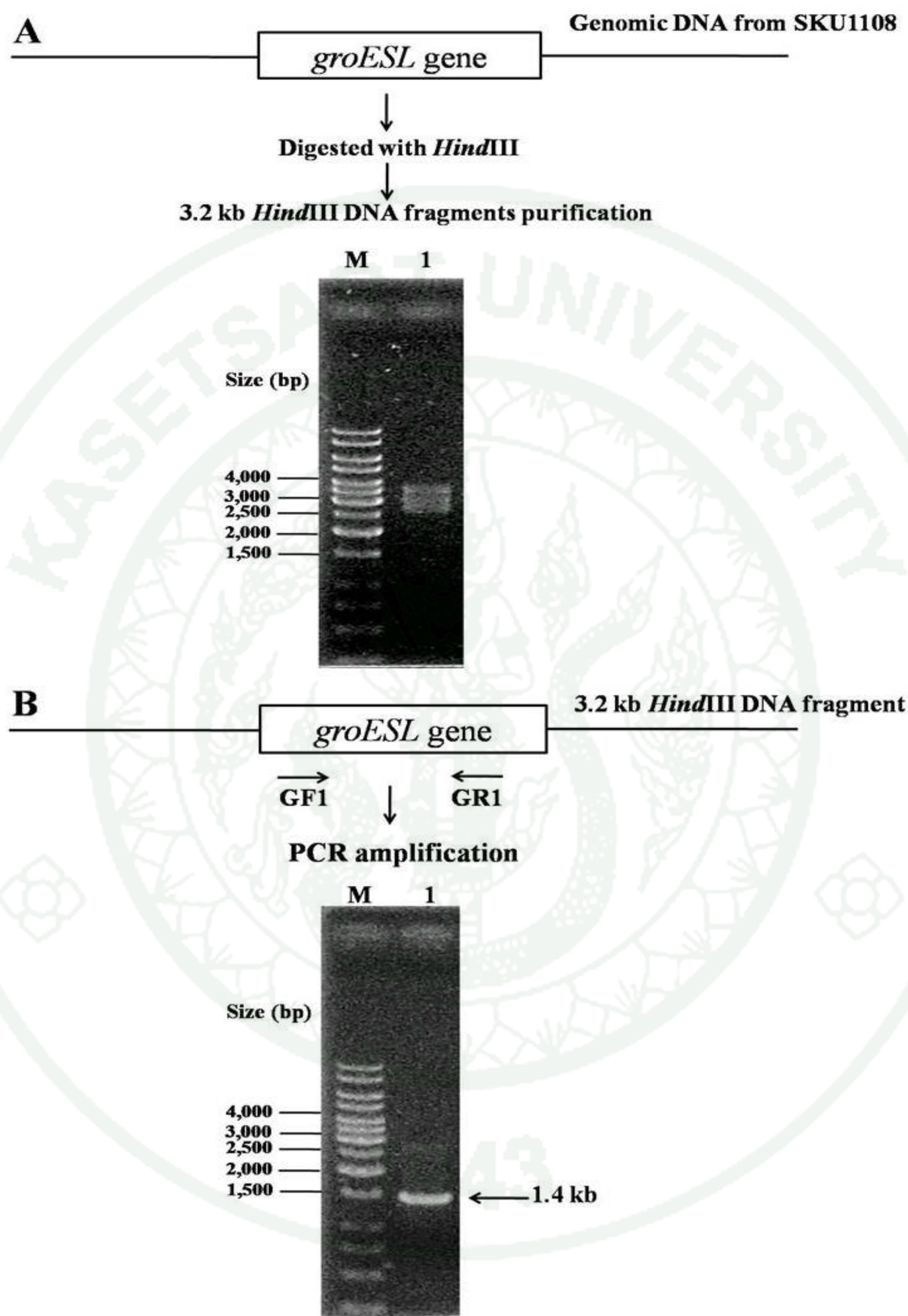

Figure 11 Agarose gel electrophoresis of the purified 3.2 kb *Hind*III fragment product and PCR amplification of 1.4 kb *groESL* gene from 3.2 kb *Hind*III fragment. A, Lane M = 1 kb DNA ladder and 1 = 3.2 kb *Hind*III fragments. B, Lane M = 1 kb DNA Ladder and 1 = 1.4 kb *groESL* gene PCR product.

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1.4 Screening and identification of recombinant clones

As a result obtained from competent cells transformation, 200 white colonies were collected and analyzed by colony hybridization with 1.4 kb *groESL* DNA probe. The three positive clones obtained from colony hybridization were designated as pUCgroESL no.66, pUCgroESLno.72 and pUCgroESLno.89, respectively (Figure 12). The recombinant plasmids from those three positive clones were isolated and digested with *Hind*III to detect the size of insertional DNA by agarose gel electrophoresis. As shown in Figure 13A, the recombinant plasmids, pUCgroESLno.66, pUCgroESLno.72 and pUCgroESLno.89 were bigger than pUC119 and 3.2 kb *Hind*III DNA fragment was clearly detected when those plasmids were digested with *Hind*III. Further confirmation that those plasmids carrying *groESL* gene were used as DNA template for PCR amplification of 1.4 kb *groESL* gene. The obtained result was shown in Figure 13B, the 1.4 kb PCR amplified product was detected from all of the three recombinant plasmids. The recombinant plasmid pUCgroESLno.89 was selected for nucleotide sequencing of *groESL* gene. The complete nucleotide sequence of 3.2 kb DNA fragment carrying *groESL* gene were obtained from sequencing reaction using seven forward and reverse primers. The sequencing strategy is shown in Figure 14. The nucleotide sequences and the position of primers used for sequencing are shown in Table 8.

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Figure 12 Nylon membrane obtained from colony hybridization of 200 white colonies with 1.4 kb *groESL* DNA probe. The three positive clones are indicated by open circular. C = positive control (*E. coli* DH5α harboring pGEM-TgroESL1.4); N= negative control (*E. coli* DH5α harboring pUC119).

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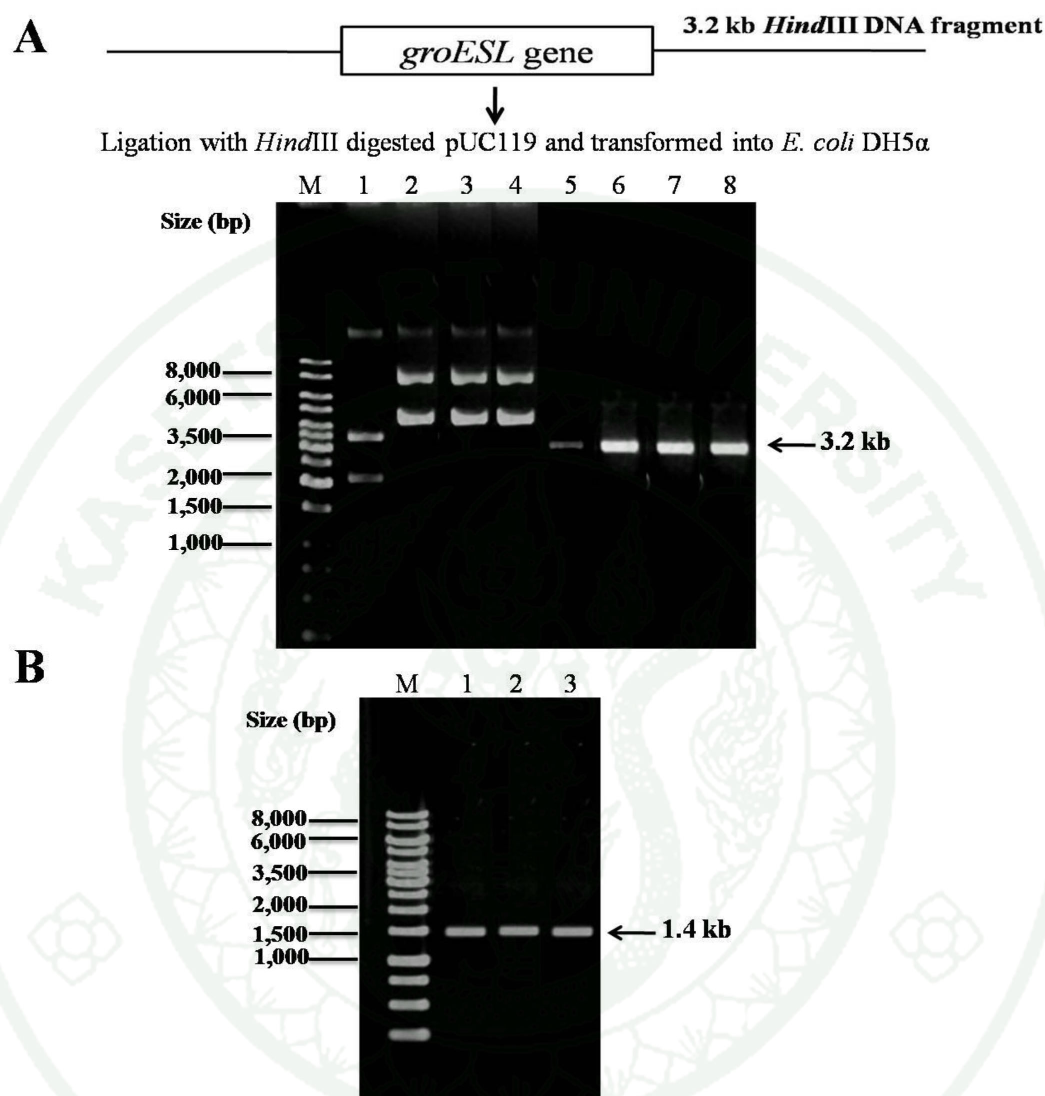

Figure 13 Agarose gel electrophoresis of recombinant plasmids from three positive clones and digested with *Hind*III (A). The 1.4 kb PCR amplified *groESL* gene from three positive clones by using two specific primers, GF1 and GR1 (B). A, Lane M = 1 kb DNA ladder, 1 = pUC119 vector, 2 = pUCgroESL no.66, 3 = pUCgroESL no.72, 4 = pUCgroESL no.89, 5-8 = pUC119 vector, pUCgroESL no.66, pUCgroESLno.72 and pUCgroESLno.89 digested with *Hind*III, respectively. B, Lane M = 1 kb DNA ladder, 1-3 = 1.4 kb PCR amplified product from pUCgroESLno.66, pUCgroESLno.72 and pUCgroESLno.89, respectively.

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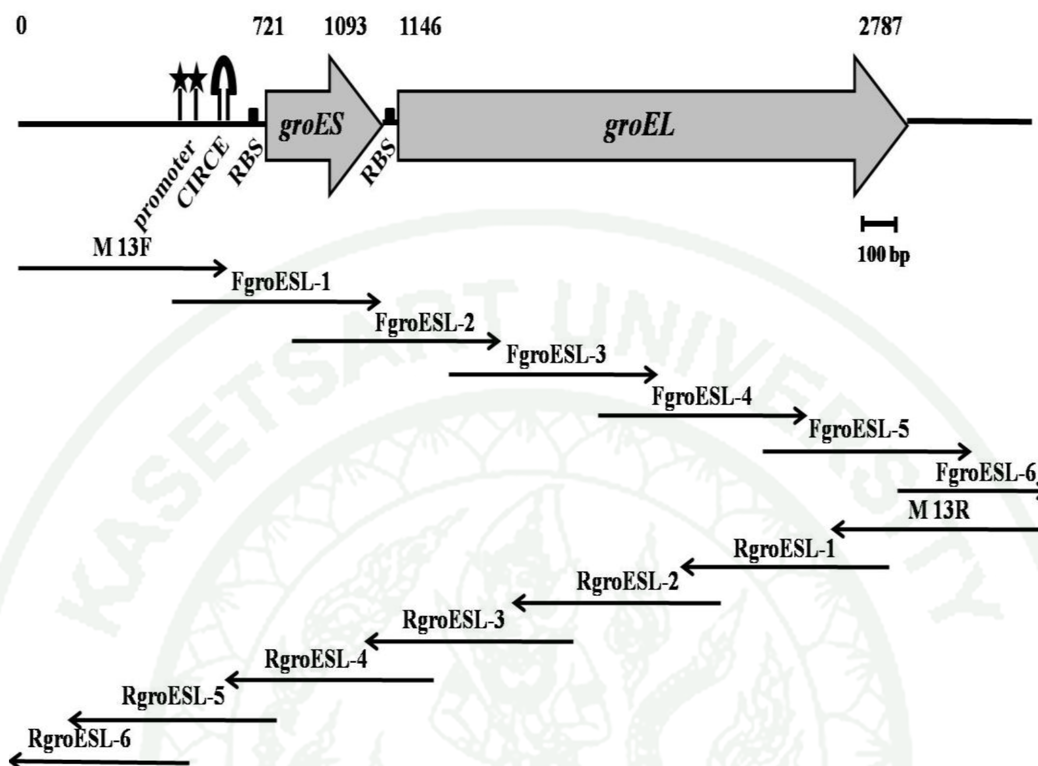

Figure 14 Sequencing strategy used to determine the nucleotide sequence of 3.2 kb PCR products of *groESL* genes

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Table 8 Nucleotide sequences of primers used for sequencing of *groESL* gene

Primer designation	Sequence (5'→3')	Position
Forward Primer		
Universal M 13F	GTG AAA ACG ACG GCC AGT	-
FgroESL-1	GTG TGT AAG GAT GAT GGC	504-521
FgroESL-2	CCT GTT CGG CAA ATG GTC	996-1,013
FgroESL-3	CAA GGC TGT TGC CGT TGT	1,507-1,525
FgroESL-4	GGC TAT GCT GGA AGA CAT	2,003-2,020
FgroESL-5	CAC AAC GCT GGT GAA GAC	2,510-2,528
FgroESL-6	TCC TCT CAC GAC TTC TCT	3,032-3,050
Reverse primer		
Universal M 13R	GTA AAA CGA CGG CCA GT	-
RgroESL-1	GTT GGA AGG ACT TAG AAG	2,779-2,797
RgroESL-2	GCG TTC TTT CAC TTC CAC	2,304-2,321
RgroESL-3	TGT AGG GGT TTT CCA GAT	1,786-1,803
RgroESL-4	CGA TTT CCT TGG CAA CGG	1,309-1,326
RgroESL-5	CAT TTT TGG GAT GGT GCC	763-780
RgroESL-6	GGG CAT TAT CGG CTA TTG	245-262

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1.5 Nucleotide and deduced amino acid sequences of *groESL* gene

The complete nucleotide sequences of the DNA fragment carrying *groESL* gene and its deduced amino acid sequences are shown in Figure 15. The *groES* gene is preceded by the new heat shock promoter sequence based on similarity to known promoter in α-purple *Proteobacteria* (Segal and Ron, 1995) that was found at the upstream region of the *groESL* operon (Figure 16). As shown in Figure 16A, the sequence is highly similar to the CIRCE heat shock element, an inverted repeat relating to regulate gene expression, was observed at downstream of promoter (Okamoto-Kainuma *et al.,* 2002). Sequencing analysis revealed two contiguous Open Reading Frame (ORFs) of 372 nucleotides for *groES* and 1,641 nucleotides for *groEL*. The *groES* gene encoded a polypeptide of 123 amino acid residues (Figure 16B). Homology search using tblastX and blastP revealed that GroES showed high identity to the same proteins of other acetic acid bacteria and related bacteria as follows: *A. pasteurianus* IFO 3283 (100%) (Azuma *et al.,* 2009), *A. aceti* (100%) (Okamoto-Kainuma *et al.,* 2002) *Gluconobacter oxydans* 621H (89%) (Prust *et al.*, 2005), *Gluconacetobacter diazotrophicus* PAl5 (88.54%) (Bertalan *et al.,* 2009) and *E. coli* (52.08%). The *groEL* gene encoded a polypeptide of 546 amino acid residues (Figure 16C). Homology search using tblastX and blastP revealed that GroEL showed high identity to the same proteins of other acetic acid bacteria and related bacteria as follows: *A. pasteurianus* IFO 3283 (100%) (Azuma *et al.,* 2009), *A. aceti* (99.26%) (Okamoto-Kainuma *et al.,* 2002), *G. diazotrophicus* Pal5 (90.73%) (Bertalan *et al.,* 2009), *G. oxydans* 621H (88.46%) (Prust *et al.*, 2005) and *E. coli* (67.60%). The summary of the sequence identity percentages is shown in Table 9. The predicted molecular mass of the deduced polypeptide of *groES* and *groEL* gene were calculated to be 13.4 kDa and 58.1 kDa, respectively with the application of the Molecular Weight Calculation of Protein Information Resource (PIR) *via* URL:
http://pir.georgetown.edu/pirwww/search/comp_mw.shtml.

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```
CTTTCGGGCA CCAAATCCGT GCCCTGCATC GTCGTACACG TAAAGTTCAA CTTCCGGATG CGCCTGCCGA ATAGCCGCAA TATCGGTATG CGGGATACTT   100
GCATCCTGTT CACCAAAATG AAGCTGCACC GGGCAGCGCG GAGTCTCGTT TCGGCTGGCG GCAATACCGC CGCCATACCA ACCAACAGCA GCCGCAAAAT   200
CATCGGTTCT GCACGCAGCT TCCCACGCCA ATGTGCCGCC CCAGCAATAG CCGATAATGC CCACCTTACG GGGTTTGAAT ACTGAGGCCG CCGCCTGAAT   300
ATCCAGTAAT GTTTTGGCAA GCGGAATCTG TGCCCGCAGT GCTAGGCCTT TTTTCACACC TTCTGCTGAA TAATCCAGCT CTACTCCACG TTTCACACGG   400
TCAAACAAAG CTGGCGCAAT GACGTGAAAT CCTGCCTTGG CAAAGTTTTC ACAAACCTGC CGGATATGGT GGTTCACACC AAAAATTTCC TGCACCACCA   500
CAAGTGTGTA AGGATGATGG CTGCCTTCGG TTTCCCATGC AGAAAACTCA TGTCCATCTG CAGCCTGAAG TGTTGTGATA TGACCCATGT AAGCACCTCC   600
TATGTGCTCT CAGGTTACAG CAAAAAGAAA CTTTATCCAC ATTCCTTGAC TCTGCCTTTG GGCAGACCTA TCTCCTTTTC TGGCACTCCC GGGGTGGGAG   700
TGCTAACGTA ACGCGGCGTT GGCAGACCTA TCTCCTTTTC TGGCACTCCC GGGGTGGGAG TGCTAACGTA ACGCGGCGTT ATGTTACGCG CGACAAAGCG   800
                                                                                       M L R  A T K
GATGGGGCTG CTTTCTGTTA AGGGCACCAT CCCAAAAATG AATGTGGAGC GATCCATAAT GACGAAGTTT CGTCCCTTAC ACGACCGGGT AGTTGTCCGT   900
R M G L  L S V  K G T  I P K M  N V E  R S I  M T K F  R P L  H D R  V V V R
CGCCTTGAAG GCGAACAGAA GACCGCTGGC GGAATCATTA TTCCTGACAC AGCTCAGGAA AAGCCTATGG AAGGCGAAGT GGTTGCAGTT GGTCCGGGTG   1000
 R L E  G E Q  K T A G  G I I  I P D  T A Q E  K P M  E G E  V V A V  G P G
CCCGAAATGA ACAGGGCCAG ATTGTGGCGC TTGATGTTAA GGCTGGTGAC CGCGTCCTGT TCGGCAAATG GTCCGGCACG GAAGTGAAGA TCGACGGCGA   1100
 A R N  E Q G Q  I V A  L D V  K A G D  R V L  F G K  W S G T  E V K  I D G
AGAGCTGCTG ATCATGAAGG AAAGCGACAT CATGGGCGTG GTAACCGCCT GATTTTGCGC TGATCCGCAT TTTTATTTCA GAACGATCTT CACAGGAGAA   1200
E E L L  I M K  E S D  I M G V  V T A  *
ATTCAATGGC TGCCAAAGAC GTAAAGTTTG GTGCAGACGC ACGCCAGCGT ATGCTGCGCG GTGTGGATAT TCTGGCTGAC GCTGTAAAAG TAACGCTGGG   1300
     M  A A K D  V K F  G A D  A R Q R  M L R  G V D  I L A D  A V K  V T L
CCCCAAAGGC CGTAACGTGG TGCTGGACAA GAGCTTCGGT GCTCCCCGTA TCACCAAGGA CGGTGTTTCC GTTGCCAAGG AAATCGAACT GGCTGACAAG   1400
G P K G  R N V  V L D  K S F G  A P R  I T K  D G V S  V A K  E I E  L A D K
TTCGAAAACA TGGGCGCTCA GATGCTGCGT GAAGTAGCAT CCAAAACCAA CGACATTGCT GGTGACGGCA CCACAACGGC TACGGTTCTG GCTCAGGCTA   1500
 F E N  M G A  Q M L R  E V A  S K T  N D I A  G D G  T T T  A T V L  A Q A
TCGTGCGTGA AGGCCATAAG GCAGTTGCCG CTGGCATGAA CCCGATGGAT CTGAAGCGCG GGATCGACAA GGCTGTTGCC GTTGTGATCG AAGAGCTGAA   1600
 I V R  E G H K  A V A  A G M  N P M D  L K R  G I D  K A V A  V V I  E E L
GAAAAACGCC AAGAAAGTAA CCACCCCGGC GGAAACCGCT CAGGTTGGTA CGATTTCTGC AAACGGTGAA CTCGAAATCG GTCAGATGAT CTCCGAAGCC   1700
K K N A  K K V  T T P  A E T A  Q V G  T I S  A N G E  S E I  G Q M  I S E A
ATGCAGAAAG TTGGCTCCGA AGGCGTGATC ACGGTTGAAG AAGCCAAGCA CTTCCAGACA GAACTGGATG TTGTTGAAGG CATGCAGTTT GACCGCGGCT   1800
 M Q K  V G S  E G V I  T V E  E A K  H F Q T  E L D  V V E  G M Q F  D R G
ACATCTCTCC GTACTTCGTG ACGAACCCGG AAAAGATGAC AGCGGATCTG GAAAACCCCT ACATCCTGAT CCATGAAAAG AAGCTGTCTT CCCTGCAGCC   1900
 Y I S  P Y F V  T N P  E K M  T A D L  E N P  Y I L  I H E K  K L S  S L Q
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P M L P  L L E  S V V  Q S G R  P L L  I I A  E D V D  G E A  L A T  L V V N
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 K L R  G G L  K I A A  V K A  P G F  G D R R  K A M  L E D  I A I L  T G G
AGGTCATCAG CGAAGATCTG GGCATCAAGC TGGAAACCGT TACCCTGAAC ATGCTTGGCA CGGCCAAGAA GGTGCACATC GACAAAGAAA ACACCACCAT   2200
 Q V I  S E D L  G I K  L E T  V T L N  M L G  T A K  K V H I  D K E  N T T
TGTTGATGGT GCTGGCAAAG CCGATGACAT CAAGGGTCGT GTTAAGCAGA TTCGTGCACA GATCGAAGAA ACCTCTTCCG ACTACGACCG CGAAAAGCTG   2300
I V D G  A G K  A D D  I K G R  V K Q  I R A  Q I E E  T S S  D Y D  R E K L
CAGGAACGTC TGGCCAAACT GGCTGGCGGT GTTGCCGTGA TCCGCGTTGG TGGTTCCACC GAAGTGGAAG TGAAAGAACG CAAAGACCGC GTTGACGATG   2400
 Q E R  L A K  L A G G  V A V  I R V  G G S T  E V E  V K E  R K D R  V D D
CCCTGCACGC AACCCGCGCT GCTGTGGAAG AAGGCATTGT TCCGGGTGGT GGCACGGCTC TGGCCCGCGC TACGCTGAAG CTGGAAGGCC TGCACTACCA   2500
 A L H  A T R A  A V E  E G I  V P G G  G T A  L A R  A T L K  L E G  L H Y
CAACGATGAC CAGCGCGTTG GTGGTGACAT CATCCGCCGC GCTCTGCAGG CTCCTCTGCG CCAGATCGCT CACAACGCTG GTGAAGACGG CGCTGTGATC   2600
H N D D  Q R V  G G D  I I R R  A L Q  A P L  R Q I A  H N A  G E D  G A V I
GCAAACAAGG TGCTGGAAAA CAGCGACTAC AACTTCGGGT TCGACGCTCA GGCAGGCGAA TACAAGAACC TTGTTGAAGC CGGTATTATC GACCCGGCCA   2700
 A N K  V L E  N S D Y  N F G  F D A  Q A G E  Y K N  L V E  A G I I  D P A
AGGTTGTCCG CACGGCTCTG CAGGATGCAG CATCTGTTGC TGGTCTGCTG ATCACCACGG AAGCCATGGT TGCTGAACGC CCGGAAAAGA AAGCCGCTCC   2800
K V V  R T A L  Q D A  A S V  A G L L  I T T  E A M  V A E R  P E K  K A A
GGCTGGCGGC CCTGACATGG GTGGCATGGG TGGTATGGAC TTCTAAGTCC CTGAAAGGAG GGCAGTTTTT TTTTGAAACT TTCAGTCTCT CCTGAATTAC   2900
P A G G  P D M  G G M  G G M D  F *
AGTATACTAA CTATAATATC GATATTATGA AAAACATTGG CTCAGATGAG AGGTAACCGT GGCAGTTCAT TTTAGCGTAA CCAAAGCATT ACTTGAATCC   3000
ACTGTATGGA CTTGGGGACC TCTTAATCAC CCTCCTCTCA CGACTTCTCT TCGATTTGGG GCTGATGGTC TGATCAAAGA CTATACACAT TCCAACGAAC   3100
ACTCATGGAA ACTGACCGGA AATTTATTAG AAATTTTCGA TATCCACGGG AAAATGATCT GGAAATTCGA AGGTATATTT AACAATCAAG ATTCCATTAC   3200
CCTCGTAAGC TTGCATGCCT GCAGGTCGAC TCTAGAGGAT CCCCGGGTAC CGAGCTCG                                               3300
```

Figure 15 Nucleotide and deduced amino acid sequences of 3.2 kb DNA fragment carrying the *groESL* gene from *A. pasteurianus* SKU1108. The possible ribosome binding site is in the boxes. The predicted -10 and -35 sequences are underline.

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Figure 16 Regulatory region of *groESL* gene (A), alignment of the amino acid sequences deduced from the *groES* (B) and *groEL* (C) genes. The putative -35 and -10 binding sites for *sigma32* (Sig32) subunits of RNA polymerase, and CIRCE consensus sequence in the promoter region of *groESL* gene are located at 76th and 41st nucleotide upstream from initiation codon of *groES* gene, respectively. The sequence alignment was performed by using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2). Asterisks indicate amino acid identical in all sequences and dots represent similar amino acids. The different deduced amino acids are indicated by boxes.

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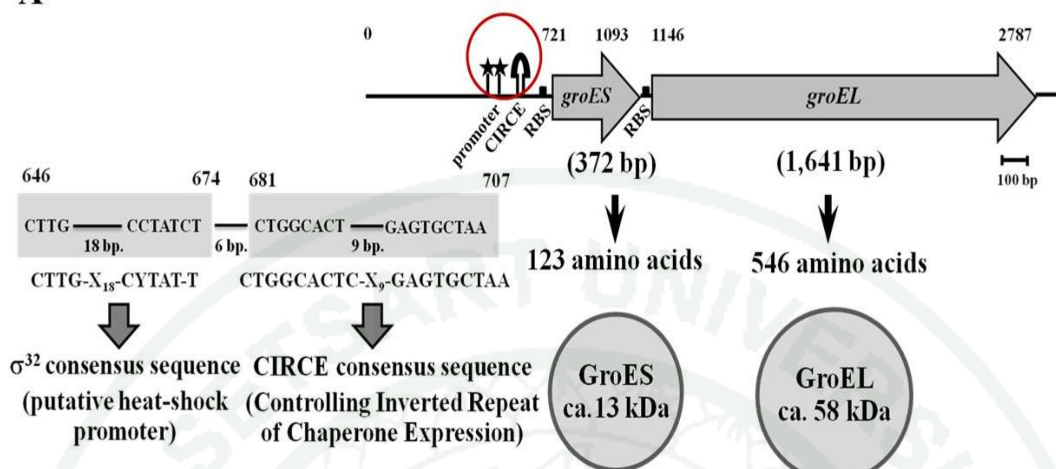
A



B

```
groES_A_pasteurianusIFO3283-01    MLRATKRMGLLSVKGTIPKMNVERSIMTKFRPLHDRVVVRRLEGEQKTAG 50
groES_A_pasteurianusSKU1108       MLRATKRMGLLSVKGTIPKMNVERSIMTKFRPLHDRVVVRRLEGEQKTAG 50
groES_A_aceti                     --------------------------MTKFRPLHDRVVVRRLEGEQKTAG 24
                                                            ************************

groES_A_pasteurianusIFO3283-01    GIIIPDTAQEKPMEGEVVAVGPGARNEQGQIVALDVKAGDRVLFGKWSGT 100
groES_A_pasteurianusSKU1108       GIIIPDTAQEKPMEGEVVAVGPGARNEQGQIVALDVKAGDRVLFGKWSGT 100
groES_A_aceti                     GIIIPDTAQEKPMEGEVVAVGPGARNEQGQIVALDVKAGDRVLFGKWSGT 74
                                  **************************************************

groES_A_pasteurianusIFO3283-01    EVKIDGEELLIMKESDIMGVVTA 123
groES_A_pasteurianusSKU1108       EVKIDGEELLIMKESDIMGVVTA 123
groES_A_aceti                     EVKIDGEELLIMKESDIMGVVTA 97
                                  ***********************
```

C

```
groEL_A_pasteurianusIFO3283-01    MAAKDVKFGADARQRMLRGVDILADAVKVTLGPKGRNVVLDKSFGAPRIT 50
groEL_A_pasteurianusSKU1108       MAAKDVKFGADARQRMLRGVDILADAVKVTLGPKGRNVVLDKSFGAPRIT 50
groEL_A_aceti                     MAAKDVKFGADARQRMRAGVDILADAVKVTLGPKGRNVVLDKSFGAPRIT 50
                                  ****************  ********************************

groEL_A_pasteurianusIFO3283-01    KDGVSVAKEIELADKFENMGAQMLREVASKTNDIAGDGTTTATVLAQAIV 100
groEL_A_pasteurianusSKU1108       KDGVSVAKEIELADKFENMGAQMLREVASKTNDIAGDGTTTATVLAQAIV 100
groEL_A_aceti                     KDGVSVAKEIELADKFENMGAQMLREVASKTNDIAGDGTTTATVLAQAIV 100
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    REGHKAVAAGMNPMDLKRGIDKAVAVVIEELKKNAKKVTTPAETAQVGTI 150
groEL_A_pasteurianusSKU1108       REGHKAVAAGMNPMDLKRGIDKAVAVVIEELKKNAKKVTTPAETAQVGTI 150
groEL_A_aceti                     REGHKAVAAGMNPMDLKRGIDKAVAVVIEELKKNAKKVTTPAETAQVGTI 150
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    SANGESEIGQMISEAMQKVGSEGVITVEEAKHFQTELDVVEGMQFDRGYI 200
groEL_A_pasteurianusSKU1108       SANGESEIGQMISEAMQKVGSEGVITVEEAKHFQTELDVVEGMQFDRGYI 200
groEL_A_aceti                     SANGESEIGQMISEAMQKVGSEGVITVEEAKHFQTELDVVEGMQFDRGYI 200
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    SPYFVTNPEKMTADLENPYILIHEKKLSSLQPMLPLLESVVQSGRPLLII 250
groEL_A_pasteurianusSKU1108       SPYFVTNPEKMTADLENPYILIHEKKLSSLQPMLPLLESVVQSGRPLLII 250
groEL_A_aceti                     SPYFVTNPEKMTADLENPYILIHEKKLSSLQPMLPLLESVVQSGRPLLII 250
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    AEDVDGEALATLVVNKLRGGLKIAAVKAPGFGDRRKAMLEDIAILTGGQV 300
groEL_A_pasteurianusSKU1108       AEDVDGEALATLVVNKLRGGLKIAAVKAPGFGDRRKAMLEDIAILTGGQV 300
groEL_A_aceti                     AEDVDGEALATLVVNKLRGGLKIAAVKAPGFGDRRNVMLEDIAILTGGQV 300
                                  ***********************************:.*************

groEL_A_pasteurianusIFO3283-01    ISEDLGIKLETVTLNMLGTAKKVHIDKENTTIVDGAGKADDIKGRVKQIR 350
groEL_A_pasteurianusSKU1108       ISEDLGIKLETVTLNMLGTAKKVHIDKENTTIVDGAGKADDIKGRVKQIR 350
groEL_A_aceti                     ISEDLGIKLETVTLNMLGTAKKVHIDKENTTIVDGAGKADDIKGRVKQIR 350
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    AQIEETSSDYDREKLQERLAKLAGGVAVIRVGGSTEVEVKERKDRVDDAL 400
groEL_A_pasteurianusSKU1108       AQIEETSSDYDREKLQERLAKLAGGVAVIRVGGSTEVEVKERKDRVDDAL 400
groEL_A_aceti                     AQIEETSSDYDREKLQERLAKLAGGVAVIRVGGSTEVEVKERKDRVDDAL 400
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    HATRAAVEEGIVPGGGTALARATLKLEGLHYHNDDQRVGGDIIRRALQAP 450
groEL_A_pasteurianusSKU1108       HATRAAVEEGIVPGGGTALARATLKLEGLHYHNDDQRVGGDIIRRALQAP 450
groEL_A_aceti                     HATRAAVEEGIVPGGGTALARATLKLEGLHYHNDDQRVGGDIIRRALQAP 450
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    LRQIAHNAGEDGAVIANKVLENSDYNFGFDAQAGEYKNLVEAGIIDPAKV 500
groEL_A_pasteurianusSKU1108       LRQIAHNAGEDGAVIANKVLENSDYNFGFDAQAGEYKNLVEAGIIDPAKV 500
groEL_A_aceti                     LRQIAHNAGEDGAVIANKVLENSDYNFGFDAQAGEYKNLVEAGIIDPAKV 500
                                  **************************************************

groEL_A_pasteurianusIFO3283-01    VRTALQDAASVAGLLITTEAMVAERPEKKAAPAGGPDMGGMGGMDF 546
groEL_A_pasteurianusSKU1108       VRTALQDAASVAGLLITTEAMVAERPEKKAAPAGGPDMGGMGGMDF 546
groEL_A_aceti                     VRTALQDAASVAGLLITTEAMVAERPEKKAAPAGGPDMGGMGGMDF 546
                                  **********************************************
```

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Table 9 Comparison of the identity percentage of deduced amino acid sequence of *groES* and *groEL* gene from *A. pasteurianus* SKU1108 with the same proteins, GroES and GroEL proteins from other related bacteria

GroES protein	
Strains [a]	% identity SKU1108
Acetobacter pasteurianus IFO 3283 (YP_003188291)	100
Acetobacter aceti IFO 3283 (BAC16232)	
(Okamoto-Kainuma *et al.,* 2002)	100
Gluconobacter oxydans 621H (YP_192295)	89.00
Gluconacetobacter diazotrophicus PAl 5 (YP_001602295)	88.54
Escherichia coli strain. K-12 substr. MG1655 (NP_418566)	52.08
GroEL protein	
Strains [a]	% identity SKU1108
Acetobacter pasteurianus IFO 3283 (YP_003188292)	100
Acetobacter aceti IFO 3283 (BAC16232)	
(Okamoto-Kainuma *et al.,* 2002)	99.26
Gluconobacter oxydans 621H (YP_192296)	88.46
Gluconacetobacter diazotrophicus PAl 5 (YP_001602294)	90.73
Escherichia coli strain. K-12 substr. MG1655 (NP_418566)	67.60

[a] GenBank Accession No. are in the parentheses.

1.6 Overexpression of *groESL* gene in *A. pasteurianus* SKU1108

To investigate the effect of *groESL* gene on growth of *A. pasteurianus* SKU1108 under various stress conditions, the *groESL* gene was subcloned into the expression vector. The recombinant plasmid was constructed by insertion of the 3.2 kb *Hind*III fragment carrying complete *groESL* gene into a broad host range plasmid, pCM62. The resulting plasmid was designated as pCMgroESL. One copy of the 3.2 *Hind*III DNA fragment was inserted in the same orientation with *Plac*. Agarose gel electrophoresis of the *groESL* complemented plasmid digested with *Hind*III restriction enzyme is shown in Figure 17.The recombinant plasmid, pCMgroESL as well as pCM62 were individually

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conjugational transferred into *A. pasteurianus* SKU1108. The conjugants were designated as SKU1108/pCMgroESL and SKU1108/ pCM62 and plasmid profiles of these strains were analyzed (Figure 18). The growth of those transconjugants grown under various stress conditions were compared as shown in Figure 19. The growth curve under standard condition (30°C in YPGD medium) is shown in Figure 19A. Under this condition, the growth patterns of SKU1108/ pCMgroESL and SKU1108/pCM62 were nearly identical. When the cells were cultured at 42°C, the growth pattern of SKU1108/ pCMgroESL was quite better than control strain (Figure 19B). Under acetic acid and ethanol stress conditions, the growth patterns of SKU1108/pCMgroESL were significant enhanced compared to the control strain as shown in Figure 19C and 19D, respectively. In this study, the *groESL* gene overexpression in *A. pasteurianus* SKU1108 resulted in enhanced stress toleration and promoted growth when compared with control strain and similar to *A. aceti* IFO 3283 (Okamoto-Kainuma *et al.,* 2002).

It has been revealed that the *groESL* gene overexpressed strain from *Clostridium acetobutylicum* (Tomas *et al.,* 2003), *Lactobacillus paracasei* and *Lactobacillus lactis* (Desmond *et al.,* 2004) exhibited increased solvent tolerance. Furthermore, GroESL-overproducing *L. paracasei* NFBC 338 (Corcoran *et al.,* 2006) showed higher rate of survival after dying process comparable with wild type. In *Anabaena* sp. strain (Chaurasia and Apte, 2009), the enhancement of heat and salinity stress tolerance as well as promote the essential activities of nitrogen fixation and photosynthesis were observed in the *groESL* overexpressed strain compared with wild type. From these results, it can be concluded that *groESL* gene is correlated to resistance against those stressors.

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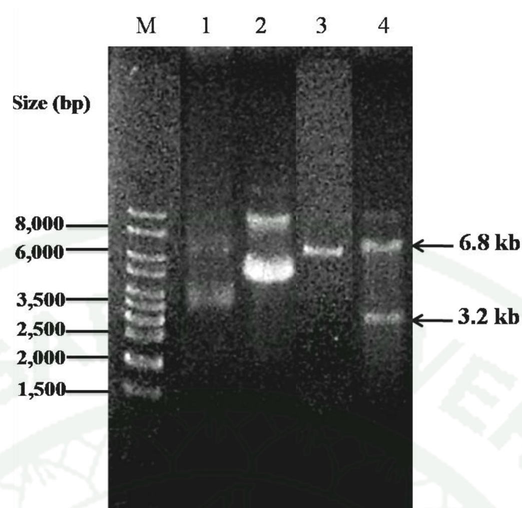

Figure 17 Agarose gel electrophoresis of recombinant plasmid, pCMgroESL and *Hind*III-digested pCMgroESL. Lane M = 1 kb DNA ladder, 1 = pCM62 (uncut plasmid), 2 = pCMgroESL (uncut plasmid), 3 = *Hind*III-digested pCM62 and 4 = *Hind*III-digested pCMgroESL.

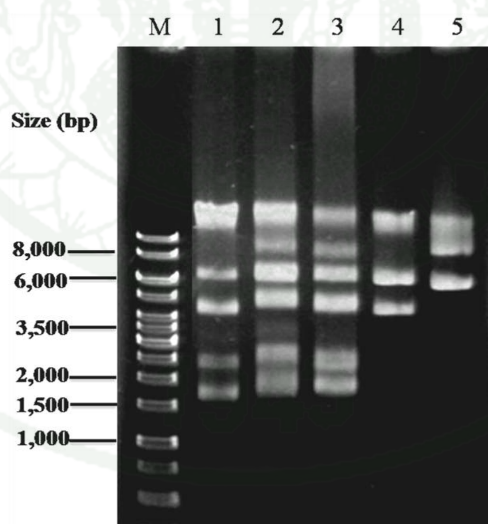

Figure 18 Plasmid profile of transconjugants harboring a broad host range plasmid, pCM62 and *groESL* complemented plasmid. Lane M = 1 kb DNA ladder, 2 = *A. pasteurianus* SKU1108, 2 = SKU1108/pCM62, 3 = SKU1108/pCMgroESL, 4 = pCM62 vector and 5 = pCMgroESL recombinant plasmid.

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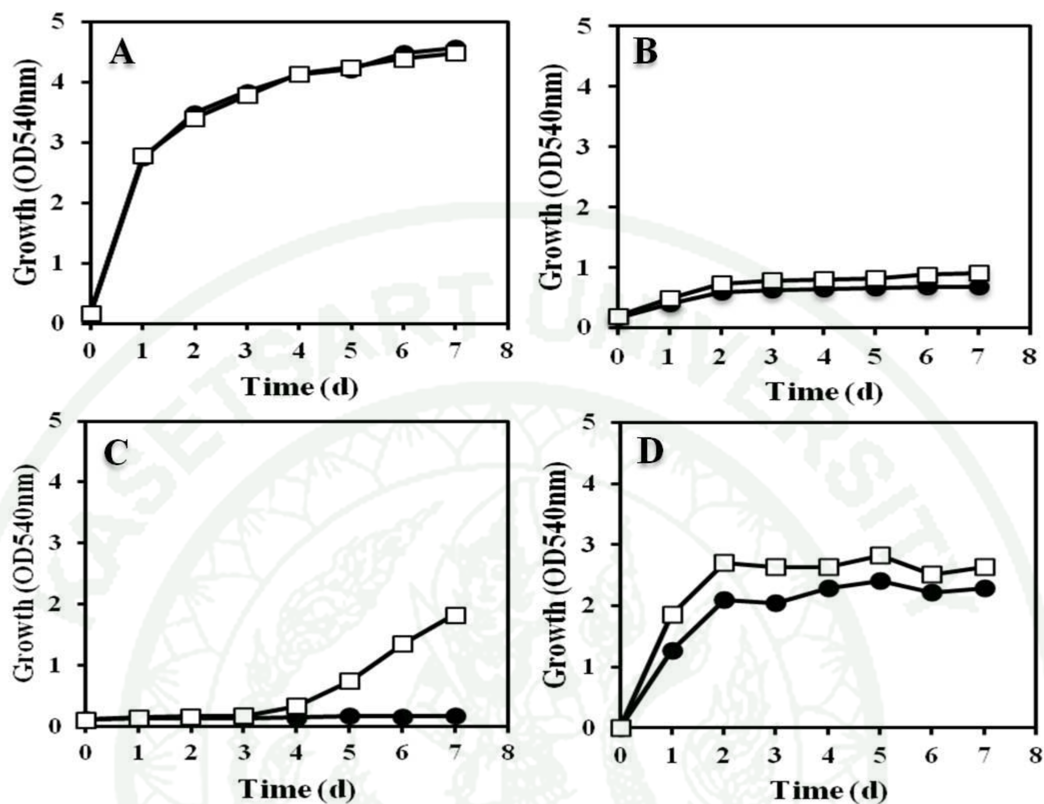

Figure 19 Time course of growth of *A. pasteurianus* SKU1108 harboring pCM62 (●) and pCMgroESL (□) grown in YPGD medium at 30°C (A), 42°C (B), YPGD supplemented with 4% acetic acid (C) or 5% ethanol (D) at 30°C. Bacterial growth was determined by using a spectrophotometer at 540 nm.

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1.7 Analysis of the *groESL* gene transcripts under various stress conditions

To establish the expression of *groESL* gene in *A. pasteurianus* SKU1108 in transcriptional level, RT-PCR was performed with total RNA from cells at different stress conditions with various time intervals. The structure of *groESL* gene of *A. pasteurianus* SKU1108 and the schematic representation of amplification of 447 bp *groEL* gene PCR products with primer GroEL-F and GroEL-R are shown in Figure 20A. The agarose gel electrophoresis of 447 bp *groEL* gene PCR product as shown as *groESL* gene expression under stress conditions at various time intervals are shown in Figure 20B. *A. pasteurianus* SKU1108 were cultured at 30°C until early exponential phase and then cells were transferred to 42°C. RT-PCR was carried out as described in Materials and Methods. The appearance of a band in each cycle and its intensity suggested that the level of *groESL* mRNA expression gradually increased with time after temperature shift and reached the maximum at 20 min (Figure 20B). Moreover, Figure 20B shows the change in mRNA expression levels after the addition of acetic acid (final concentration of 4%). The *groESL* mRNA gradually increased with time after acetic acid addition. In case of ethanol (Figure 20B), final concentration of 5% ethanol was added to culture at the early exponential phase. The *groESL* mRNA was largely expressed after ethanol addition and was reduced after 20 min. In *A. pasteurianus* SKU1108, GroES/GroEL was transiently synthesized, quickly turned over similar to present in *E. coli* (Lemaux *et al.,* 1978). Cells were unable to accumulate this heat shock proteins hence these genes were not related with thermotolerance trait of these bacteria (VanBogelen *et al.,* 1987). On the other hand, GroEL of *Anabaena* sp. are strongly synthesized and accumulated in cells during heat stress, resulting in the superior thermotolerance of *Anabaena* sp., compared to the transient expression in *E. coli* (Rajaram and Apte, 2003). Moreover, it has been reported about genes involving in thermotolerance mechanism from *A. tropicalis* SKU1100 (Soempol *et al.,* 2011). Totally 24 genes, excluding the *groESL* gene, were revealed as necessary genes for growth at high temperature. It can be concluded that the *groESL* gene might play the importance role for heat shock response or other stress response but not for thermotolerance.

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Furthermore, the effect of mild to strong stressors to induce the putative σ^{32}-type promoter for *groESL* gene expression in *A. pasteurianus* SKU1108 were determined by performing RT-PCR. The various temperatures, various concentrations of acetic acid and ethanol were used for the induction of *groESL* gene. The appearance of a band in each cycle and its intensity suggested that the level of *groESL* mRNA expression gradually increased with elevated temperature, acetic acid and ethanol concentration and that their expression levels were largely expressed in the elevated temperature condition when comparing with others (Figure 20C). *A. pasteurianus* belonging to the α-subdivision of proteobacteria, contain a unique heat-shock promoter, was presumably activated by a sigma 32-like transcription factor (Segal and Ron, 1995; Okamoto-Kainuma *et al.,* 2002). In this study, the stress induction and *groESL* gene expression level under various stress conditions were analyzed by RT-PCR. Expression analysis revealed that the *groESL* mRNA levels steadily increased with elevated temperature, acetic acid and ethanol concentration. The results suggested that all stressors occurring during acetic acid fermentation could induce the putative heat shock promoter of *A. pasteurianus* SKU1108 for *groESL* gene expression.

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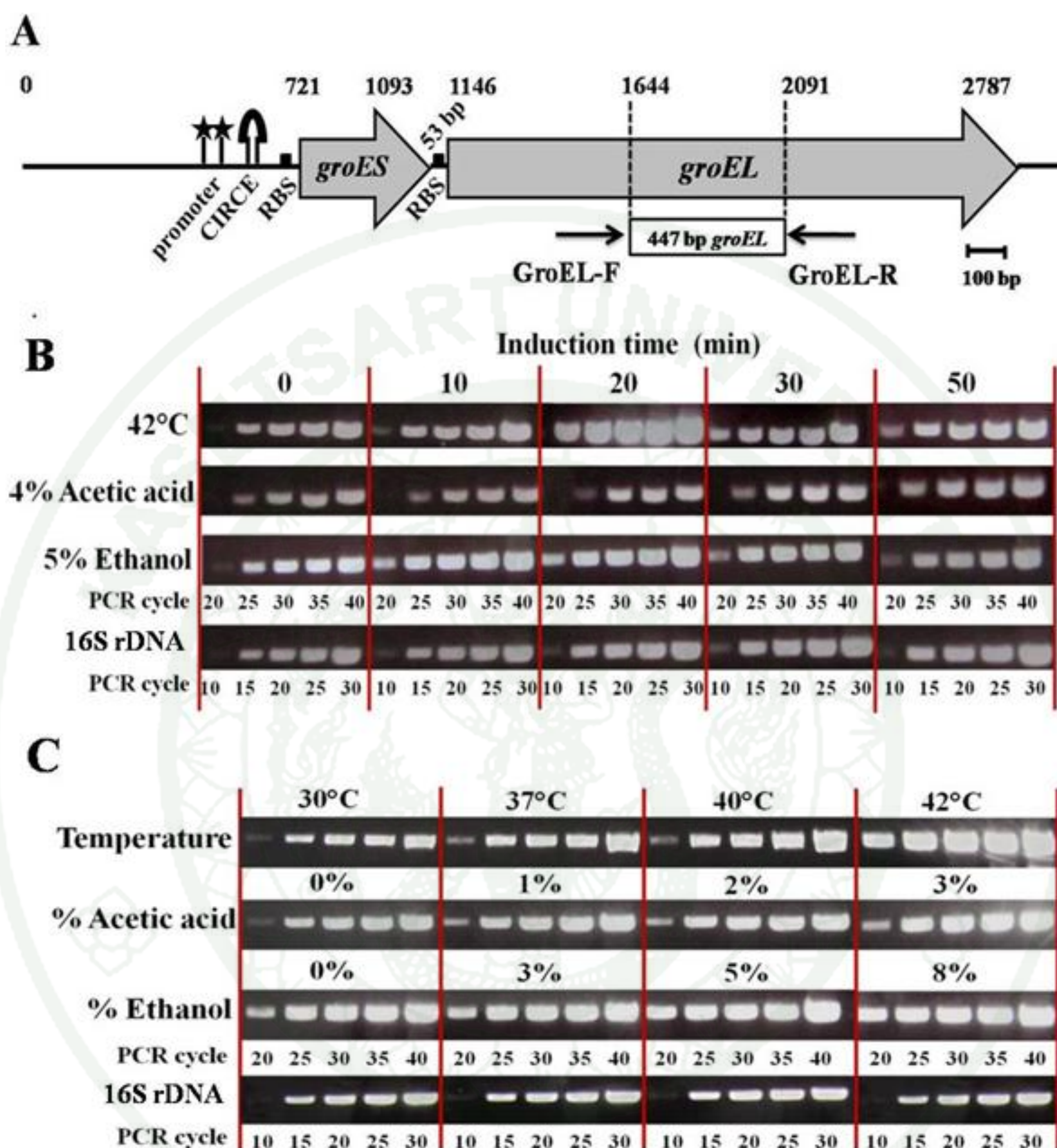

Figure 20 Structure of DNA fragment carrying the promoter region, structural gene of *groESL* and schematic representation of amplification of *groEL* gene PCR products by using primer GroEL-F and GroEL-R (A) and the *groESL* gene expression under stress conditions at various time intervals (B) and at various stress conditions (C) *A. pasteurianus* SKU1108 cells were cultured in YPGD medium incubating at 30°C until early exponential phase and then cells were induced with various stressors. Total RNA was isolated and subjected to RT-PCR as described in Materials and Methods.

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1.8 Disruption of *groEL* gene

The genomic *groEL* gene was disrupted by homologous recombination using a kanamycin resistant cassette in order to more clearly analyze the effects of different stresses on the survival of *A. pasteurianus* SKU1108 cells lacking the chaperones GroEL. The recombinant plasmids carrying the disrupted *groEL* gene were constructed with the insertion of an antibiotic resistant cassette into the genomic *groEL* gene of *A. pasteurianus* SKU1108, as previously described in Materials and Methods. The recombinant plasmid was isolated and the resulting plasmid were designated as pUCgroEL::Km^r. PCR amplification was used to confirm the insertion of 0.9 kb Km^r cassettes at the internal part of *groEL* gene. This plasmid was used to introduce into *A. pasteurianus* SKU1108 in order to construct the *groEL* gene disruptants. Seven kanamycin resistant colonies designated DGL1-7, were isolated and confirmed by PCR amplification (Figure 21) and Southern hybridization analysis (Figure 22) compared with the wild-type strain. For PCR amplification, when chromosomal DNA of DGL strains were used as template by using primer F3.5groESL and R3.5groESL (Figure 21A) and FgroEL and RgroEL (Figure 21B), the extra band as 3.1 kb and 2.6 kb were obtained, respectively compared with the 2.2 kb and 1.7 kb of that from SKU1108. The results from Southern hybridization using 900 bp Km^r cassette DNA probe showed that the positive hybridization signals were detected with 4.1 kb from the *Hind*III-digested chromosome from all DGL strains but not *A. pasteurianus* SKU1108 (Figure 22). From the results suggested that the *groEL* gene was inactivated by the 0.9 kb Km^r cassette. Figure 23 shows the schematic representation of the inactivated *groEL* gene of DGL strains by the 0.9 kb Km^r cassette.

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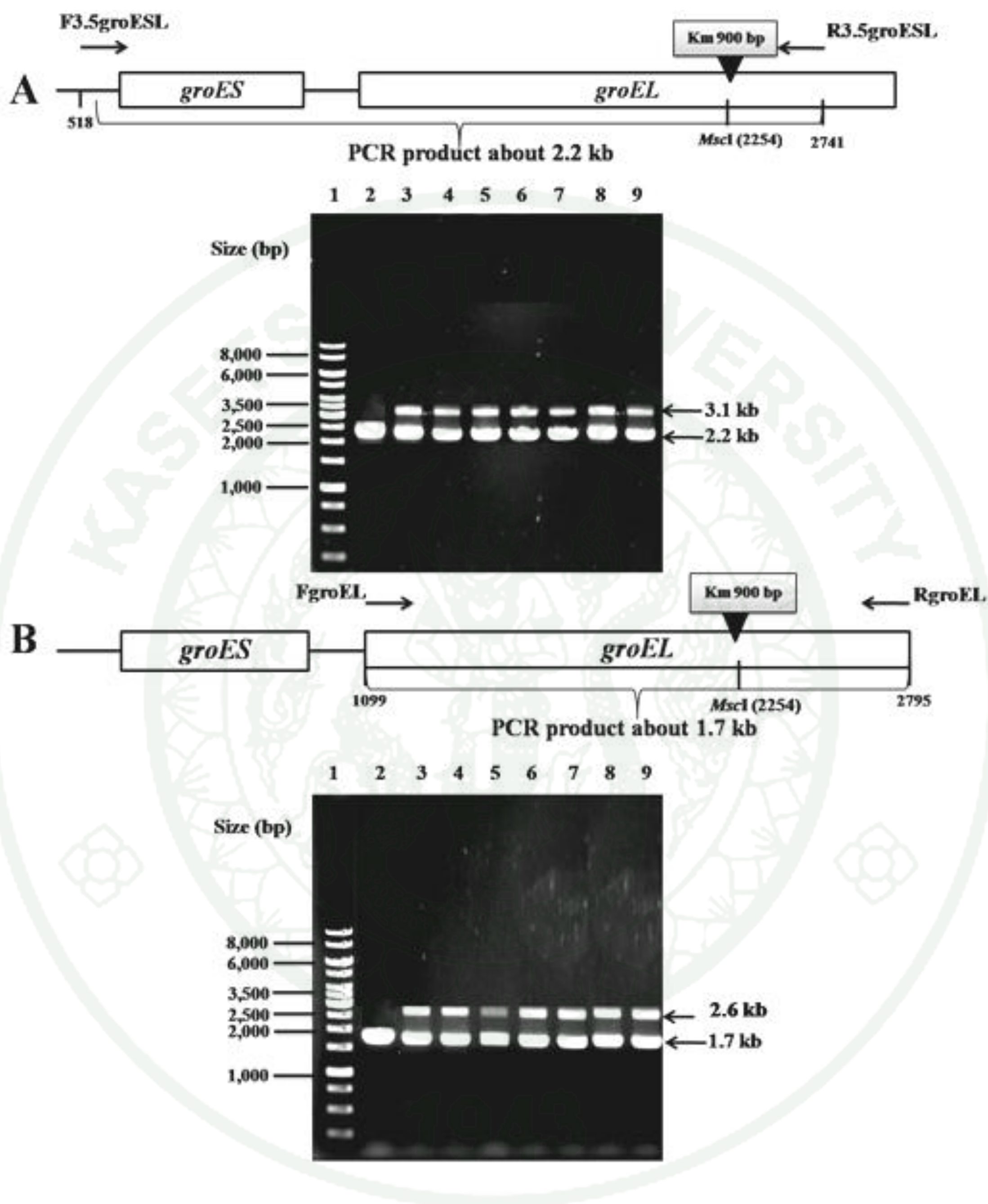

Figure 21 Structure of the *groESL* gene and the position of primers used for confirmation the *groEL* gene disruptant and agarose gel electrophoresis (A and B). Lane M = 1 kb DNA ladder, 1 = SKU1108, 2-8 = *groEL* gene disruptant; DGL1, DGL2, DGL3, DGL4, DGL5, DGL6 and DGL7, respectively.

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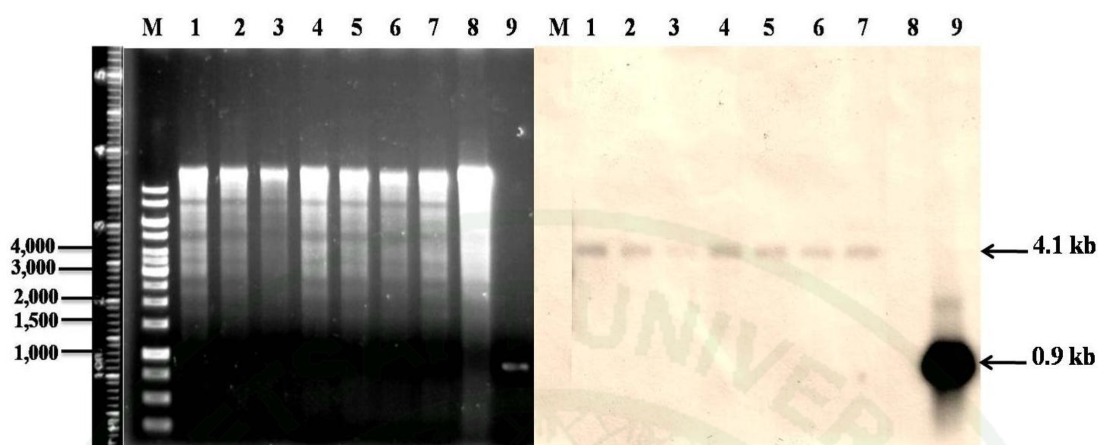

Figure 22 Agarose gel electrophoresis (left) and Southern hybridization pattern (right) of the *Hind*III-digested genomic DNA from *A. pasteurianus* SKU1108 and DGL strains. The 900 bp Km^r cassette was used as DNA probe. Lane M = 1 kb DNA ladder, 1 = *Hind*III-digested genomic DNA of DGL1, 2 = *Hind*III-digested genomic DNA of DGL2, 3 = *Hind*III-digested genomic DNA of DGL3, 4 = *Hind*III-digested genomic DNA of DGL4, 5 = *Hind*III-digested genomic DNA of DGL5, 6 = *Hind*III-digested genomic DNA of DGL6, 7= *Hind*III-digested genomic DNA of DGL7, 8 = *Hind*III-digested genomic DNA of SKU1108 and 9 = 900 bp Km^r cassette probe.

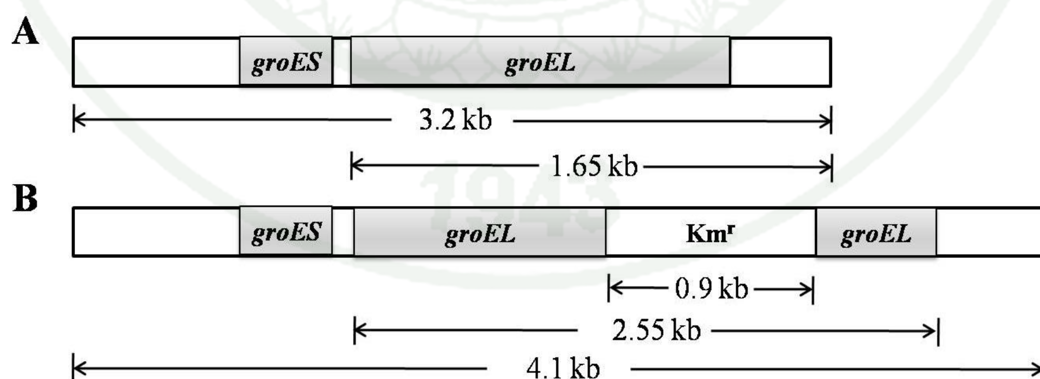

Figure 23 Schematic representation of the inactivated *groEL* gene of DGL strains by the 0.9 kb Km^r cassette. A 3.2 kb *Hind*III DNA fragment carrying *groESL* gene of wild type SKU1108 (A) and a Km^r cassette inserted *groEL* gene of DGL strain (B) were shown. Distance of each fragment was indicated.

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1.9 Growth characteristics of *groEL* gene disruptant

To more clearly analyze the effects of different stresses on the survival of *A. pasteurianus* SKU1108 cells lacking the chaperones GroEL, the genomic *groEL* gene was disrupted by homologous recombination using kanamycin resistant cassette. Seven kanamycin resistant colonies designated as DGL1-7, were isolated and confirmed by PCR amplification as well as Southern hybridization compared to the wild type strain as described above. The growths of *A. pasteurianus* SKU1108 and *groEL* gene disruptant, DGL1-7 were compared on YPGD agar incubating at various temperatures for temperature sensitive determination as shown in Figure 24. On agar plate, the wild type SKU1108 could grow well when incubating at all temperature, while almost DGL strains except DGL1 and DGL6, showed some growth defect at high temperature even at 37°C.

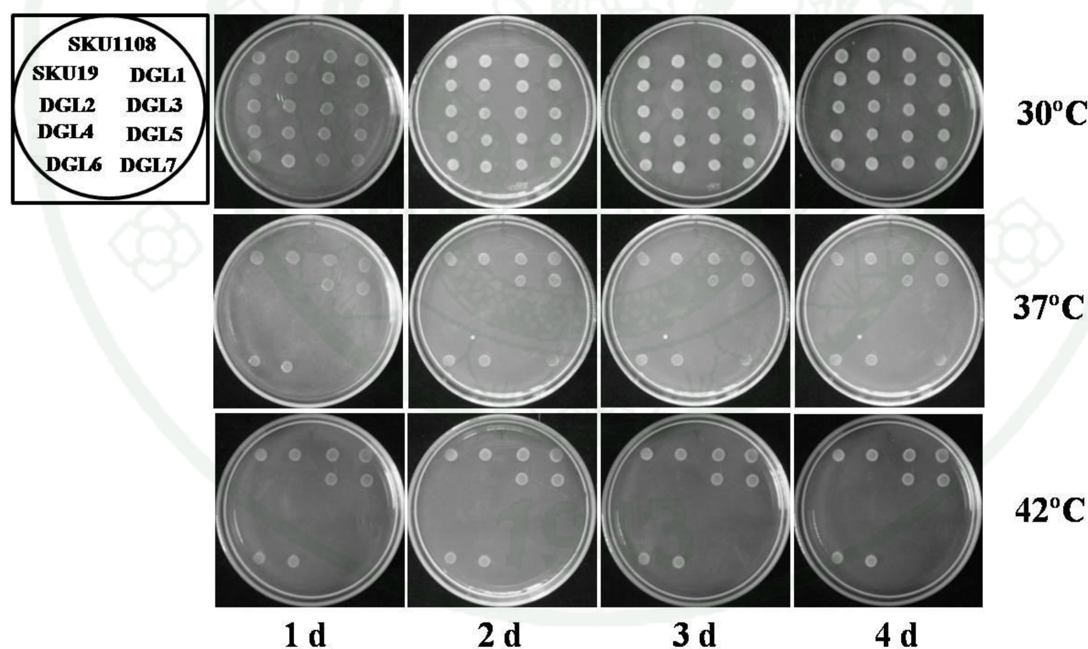

Figure 24 Comparison of temperature sensitive of the *groEL* gene disruptants. *A. pasteurianus* SKU1108 and DGL strains no.1-7 were spot-inoculated on YPGD agar incubating at 30°C, 37°C and 42°C.

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In addition, the toleration to acetic acid and ethanol of *A. pasteurianus* and DGL strains were compared on YPGD agar supplemented with 0, 0.5, 1, 1.5, 2 and 3% acetic acid or 0, 2, 4, 6 and 8% ethanol (Figure 25). As shown in Figure 25A, the wild type SKU1108 exhibited the highest resistance to acetic acid (up to 3%) similar to DGL1 and DGL6. The remaining DGL strains (DGL2, DGL3, DGL4, DGL5 and DGL7) could able to grow up to 2% acetic acid concentration. In case of ethanol toleration (Figure 25B), the wild type SKU1108 showed the normal growth at all concentrations of ethanol similar to DGL1 and DGL6 whereas the other DGL strains showed some growth defect at high concentration of ethanol (8%). The results showed that the *groEL* gene disruptants were divided in two groups. The first one composed of DGL1 and DGL6 that exhibited the growth at any conditions similar to the wild type SKU1108. In contrary to the second group composing of DGL2, DGL3, DGL4, DGL5 and DGL7 showed the growth defect under stress condition as high temperature as well as high concentration of acetic acid and ethanol. For confirmation the results that described above, the strain DGL5 was selected for determine time couse of growth under stress conditions and *groESL* gene complementation.

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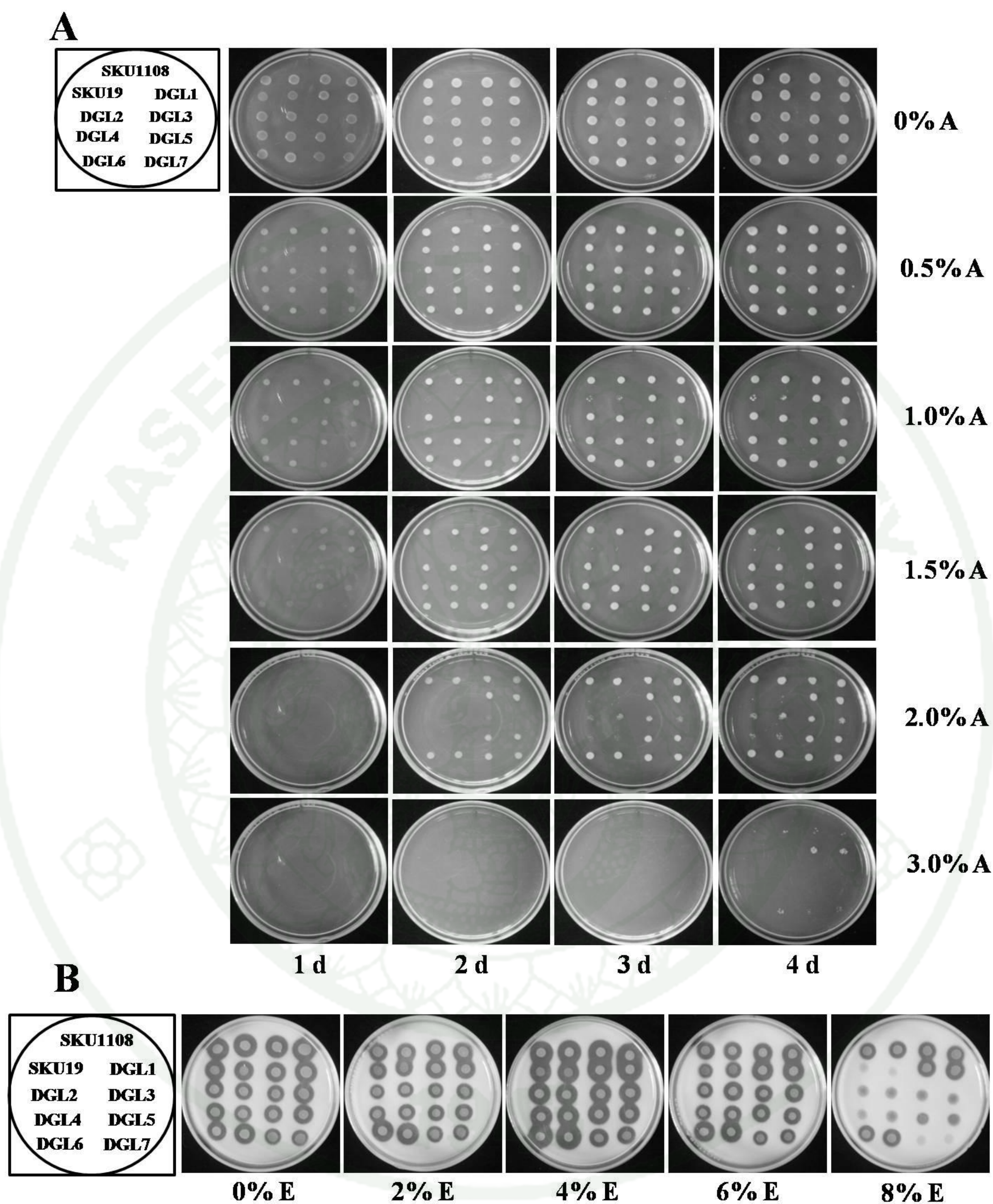

Figure 25 Comparison of acetic acid resistance capacity (A) and ethanol resistance capacity (B) of the *groEL* gene disruptants. *A. pasteurianus* SKU1108 and DGL strains no.1-7 were spot-inoculated on YPGD agar supplemented with various acetic acid and ethanol concentrations as indicated incubating at 30ºC.

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The growth curve of *A. pasteurianus* SKU1108 and the *groEL* gene disruptant, DGL5 grown in YPGD medium under various conditions were compared as shown in Figure 26. The growth curve under standard condition (30°C in YPGD medium) is shown in Figure 26A. Under this condition, the growth pattern of SKU1108 grew better than DGL5. Moreover, the growth was compared between SKU1108 and *groEL* gene disruptant, DGL5, at 40°C (Figure 26B), of 2% acetic acid (Figure 26C), and in the presence of 8% ethanol (Figure 26D). Under these conditions, SKU1108 exhibited normal growth whereas DGL5 could not grow. The results suggested that the *groEL* gene disruptant entirely lost the toleration activity to these stressors. The *groEL* gene disruptant clearly exhibited defective response to all stressors such as high temperature, high concentration of acetic acid and ethanol. Chapman *et al.* (2006) have constructed the *groEL* gene deficient strain from *E. coli*, where extensive aggregations of newly translated proteins were observed after temperature shift. This observed phenomenon in *E. coli* might occur similarly in *groEL* gene disruptant from *A. pasteurianus* SKU1108.

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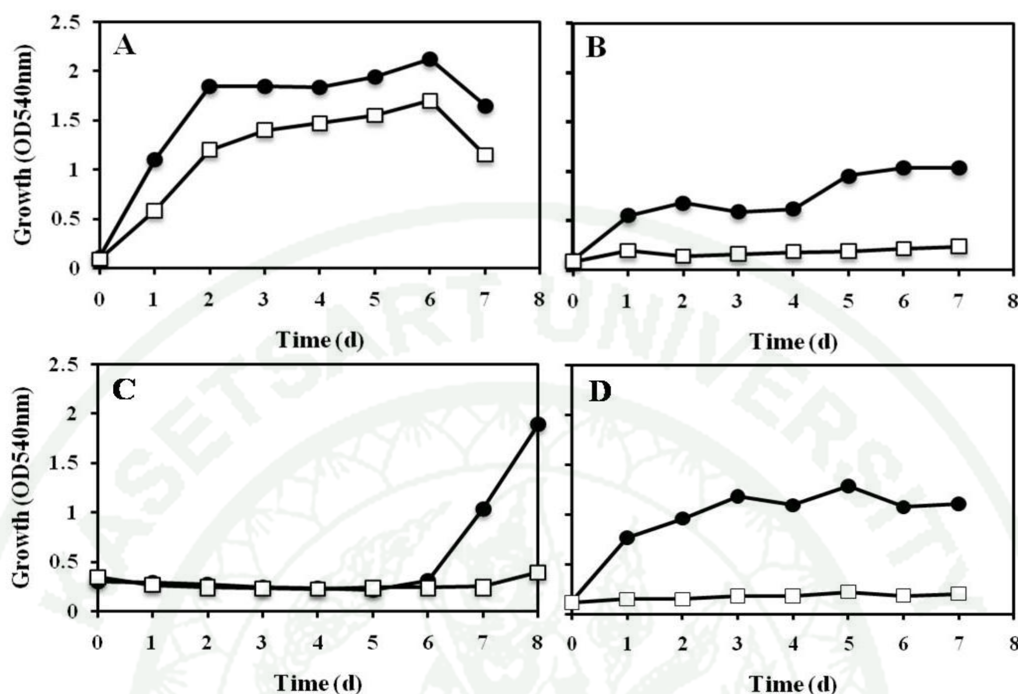

Figure 26 Time course of growth of *A. pasteurianus* SKU1108 (●) and DGL5 (*groEL* gene disruptant) (□) grown in YPGD medium. Cells were cultured in 100 ml of YPGD medium incubating at 30°C (standard condition) (A), 40°C (B), YPGD supplemented with 2% acetic acid (C) and 8% ethanol (D) incubating at 30°C. Bacterial growth was determined by using a spectrophotometer at 540 nm.

1.10 Complementation of *groESL* gene in *groEL* gene disruptant

From the above described results, the *groEL* gene disruptant seems to be deficient in their tolerant ability against high temperature, acetic acid and ethanol. In order to confirm these findings, restoration of *groEL* gene disruption was examined by the conjugational transfer of pCMgroESL carrying *groESL* gene into the *groEL* gene disruptant (DGL5). The recombinant plasmid, pCMgroESL as well as pCM62 were individually transferred into the *groEL* gene disruptant. The conjugants were designated as DGL5/pCMgroESL and DGL5/pCM62 and plasmid profiles of these strains were analyzed (Figure 27). The growth of *A. pasteurianus* SKU1108, DGL5, DGL5 harboring pCM62 (vector control), and DGL5 harboring pCMgroESL were compared on YPGD medium at various temperatures and also on the same medium supplemented with acetic acid or

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ethanol as shown in Figure 28. The results showed that the growth of the complemented strain, DGL5/pCMgroESL, was similar to that of wild type at any condition, which is contrary to the *groEL* gene disruptant (DGL5). On agar plate, DGL5 could not grow well over 37˚C while the wild type and complemented strain grew well at 42˚C. DGL5 could not grow well under high concentration of acetic acid over 2.0% but the complemented strain did well even at 2.5% acetic acid. In the presence of ethanol, DGL5 showed some growth defect even at 2% ethanol, while the wild type and complemented strain did well even at 8% ethanol. The restoration strain and the parental strain showed the same growth pattern under stress conditions. The results indicated that the *groESL* gene from *A. pasteurianus* SKU1108 was able to complement the stress defect of the *groEL* gene disruptant. From these data strongly suggested that the *groESL* gene is essential for survival when acetic acid bacteria were attacked with stressors during acetic acid fermentation such as high temperature, high concentration of acetic acid and ethanol.

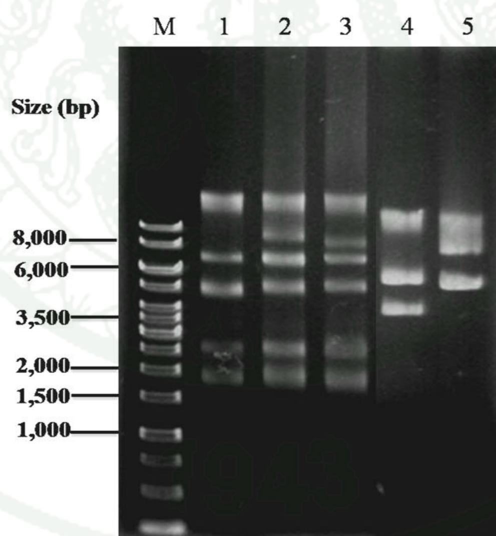

Figure 27 Plasmid profile of transconjugants harboring a broad host range plasmid, pCM62 and *groESL* complemented plasmid. Lane M = 1 kb DNA ladder, 2 = DGL5 (*groEL* gene disruptant), 2 = DGL5/pCM62, 3 = DGL5/ pCMgroESL, 4 = pCM62 vector and 5 = pCMgroESL recombinant plasmid.

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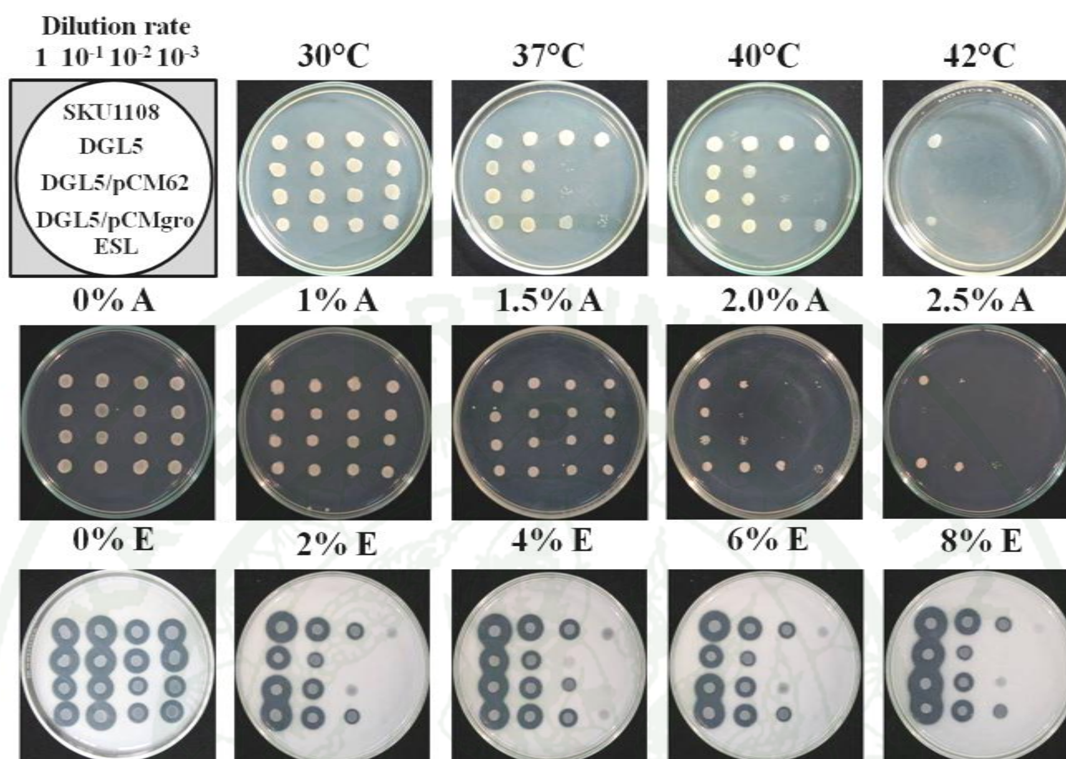

Figure 28 Comparison of growth between *A. pasteurianus* SKU1108 (SKU1108), *groEL* gene disruptant (DGL5), *groEL* gene disruptant harboring pCM62 (DGL5/pCM62) and *groEL* gene disruptant harboring pCMgroESL (DGL5/pCMgroESL). These strains were spot-inoculated on YPGD agar containing 1%, 1.5%, 2% and 2.5% acetic acid; 2%, 4%, 6% and 8% ethanol incubated at 30°C, 37°C, 40°C and 42°C. After incubation for 1-3 d, their growth ability was observed.

1.11 Induction analysis of *groESL* genes

To determine whether the putative σ^{32}- type promoter of *groESL* operon of *A. pasteurianus* SKU1108 was induced by stressors, the expression of the *groESL* gene was observed during different growth phases by monitoring the β-galactosidase activity of *E. coli* DH5α harboring transcriptional *PgroESL-lacZ* fusion plasmid. The 308 bp DNA fragment containing the possible promoter region of *groESL* gene was obtained by PCR amplification (Figure 29) and inserted into promoterless *lacZ* vector pGSA. The *PgroESL* DNA fragment carried 239 nt upstream and continued to 69 nt downstream of ATG start

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site of the *groES* gene. The procedures for construction of transcriptional fusion plasmid was previously described in Materials and Methods, and the recombinant plasmids were transformed into *E. coli* DH5α. Cells were cultured under stress conditions and the β-galactosidase activities were measured (Figure 30). When the strains were cultured at 40°C, with 0.05% acetic acid or 5% ethanol at 37°C comparing with the normal condition, the β-galactosidase activities were exhibited 1.5, 10 and 3 fold, respectively. In case of *E. coli*, acetic acid and ethanol were the stronger inducer than high temperature. Although *E. coli* was unable to grow well in culture media containing slightly concentration of acetic acid or high concentration of ethanol, β-galactosidase activity from these conditions were elevated. Under acetic acid or ethanol exposure, the β-galactosidase activities were significant higher but contrary with growth patterns when compared to standard condition and under high temperature exposure. These results indicated that *A. pasteurianus* SKU1108 *groESL* promoter was able to function in *E. coli* and induced by various stressors. This suggests that promoter from *A. pasteurianus* SKU1108 was recognized in *E. coli*. In *E. coli*, the most effective inducers for the *groESL* promoter were acetic acid, ethanol and high temperature, respectively. The obtained results might be different from *A. pasteurianus* SKU1108 because of its physiology. Recently, Masud *et al.* (2011) reported about ethanol induction and promoter analysis of the NAD^+-*adh* genes from this bacteria by using *lacZ* fusion in *E. coli*. The promoter of the NAD^+-*adh* genes was able to function in *E. coli*. The results obtained from *E. coli* only reflect the physiology of *E. coli* but not *A. pasteurianus*.

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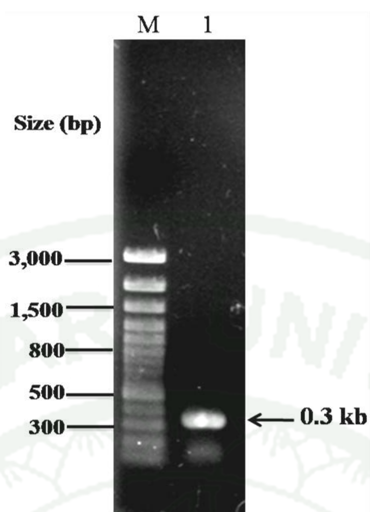

Figure 29 Agarose gel electrophoresis of 0.3 kb PCR products carrying the possible promoter region of *groESL* gene. PCR amplification was carried out by using a pair of specific primers, FgroESL-P and RgroESL-P. Lane M = 100 bp DNA ladder and 1 = *PgroESL.*

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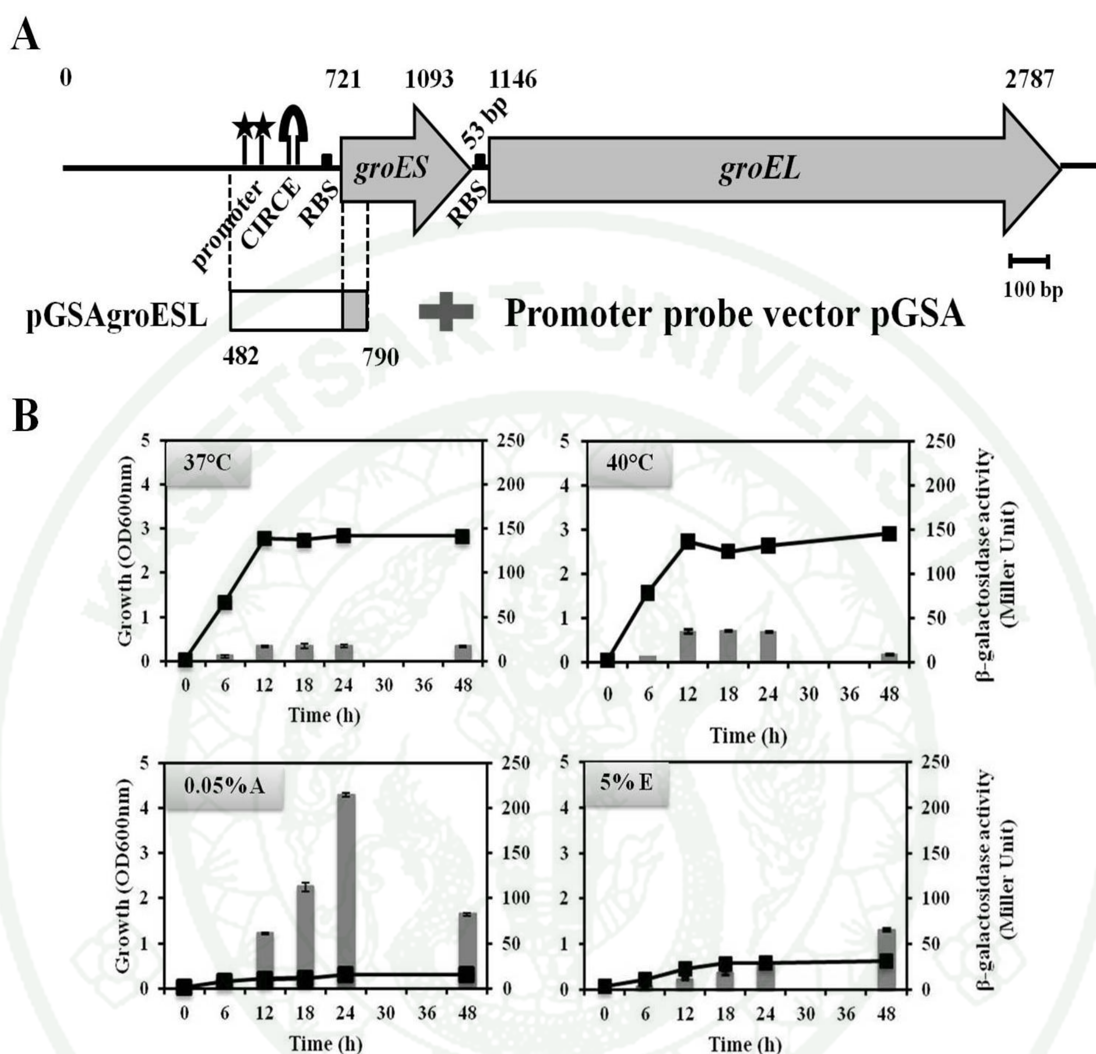

Figure 30 Structure of DNA fragment carrying the promoter region of the *groESL* gene (A) and comparison of growth and β-galactosidase activities of *E. coli* DH5α harboring pGSAPgroESL fusion plasmids (B). These strains were cultured with shaking at 200 rpm in 100 ml of LB medium at 37°C or 40°C in LB medium supplemented with 0.05% acetic acid or 5% ethanol (and 12.5 µg/mg gentamicin) at 37°C. Bacterial growth was determined by using a spectrophotometer at 540 nm. β-galactosidase activity was measured enzymatically as described in Materials and Methods.

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1.12 In silico analysis of CIRCE element in acetic acid bacteria

It has been reported that the sequence highly similar to the CIRCE heat shock element, an inverted repeat relating to regulate *groESL* gene expression, was observed at downstream of promoter in *A. pasteurianus* NBRC 3283 (Okamoto-Kainuma *et al.,* 2002). The sequences putative σ^{32}-dependent promoter and highly conserved regulatory CIRCE element were also found at the upstream region of *groESL* operon in *A. pasteurianus* SKU1108 genome (Figure 31A). We compared CIRCE element detected from all known genome sequences of acetic acid bacteria (4 complete and 16 draft genomes) and observed that these consensus sequences are conserved in all strains (Table 10). To clarify the phylogenetic relationships of these CIRCE elements, we aligned these regions (Figure 31B). Consensus of CIRCE element created by using Weblogo is also shown (Crooks *et al.,* 2004). Results showed that the pattern of CIRCE element was highly conserved in same genera. Phylogenetic analysis revealed that these elements have evolved with the phyletic evolution (Figure 31C). It suggests that the *groESL* operon might play the crucial roles for acetic acid fermentation. The sequence of the downstream of *groESL* promoter of *A. pasteurianus* SKU1108 revealed the presence of a CIRCE element similar to *A. pasteurianus* NBRC 3283. In both strains, *A. pasteurianus* SKU1108 and NBRC 3283 (Okamoto-Kainuma *et al.,* 2002), the *hrcA* gene was detected around *groESL* gene. It can be speculated that the CIRCE element of *A. pasteurianus* SKU1108 is involved in negative regulation at the RNA level by affecting mRNA synthesis and/or stability or at the DNA level by acting as an operator for the repressor protein, HrcA (Zuber and Schumann, 1994; Segal and Ron, 1996a). In this study, we searched and classified the CIRCE elements of all known acetic acid bacterial genome sequences. These sequences may be useful for the determination of the phylogenetic relationships among genera of acetic acid bacteria. Moreover, the presence of a conserved CIRCE in the upstream regulatory region of *groESL* operon of closely related bacteria suggested that their *groESL* operon might be regulated in a conserved way according to their evolutionary relationships (Segal and Ron, 1996b).

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Table 10 List of acetic acid bacteria and the position of the CIRCE element detected in all known genome sequences of acetic acid bacteria used in this study

Strain	DDBJ accession number	strand	initial	end
Gluconacetobacter hansenii ATCC 23769	ADTV01000016	+	33706	33732
Acetobacter pomorum DM001	AEUP01000026	-	63971	63997
Gluconacetobacter sp. SXCC-1	AFCH01000048	+	139727	139753
Gluconobacter morbifer G707	AGQV01000001	+	634565	634591
Gluconobacter oxydans WSH-003	AHKI01000010	+	12293	12319
Acetobacter tropicalis NBRC 101654	BABS01000018	-	4148	4174
Acetobacter aceti NBRC 14818	BABW01001090	-	458	484
Acetobacter pasteurianus NBRC 101655	BACF01000032	+	22024	22050
Acetobacter pasteurianus IFO 3191	BACG01000053	-	3973	3999
Gluconobacter frateurii NBRC 101659	BADZ01000005	+	126224	126250
Acetobacter pasteurianus subsp. *pasteurianus* LMG 1262	CADO01000067	-	11836	11862
Gluconacetobacter europaeus LMG 18890	CADP01000002	+	123320	123346
Acetobacter pasteurianus 3P3	CADQ01000012	-	8229	8255
Gluconacetobacter europaeus LMG 18494	CADR01000004	-	87223	87249
Gluconacetobacter europaeus 5P3	CADS01000002	-	76585	76611
Gluconacetobacter oboediens 174Bp2	CADT01000006	-	11863	11889
Gluconobacter oxydans 621H	NC_006677	+	2082479	2082505
Gluconacetobacter diazotrophicus PAl 5	NC_010125	-	2098382	2098408
Acetobacter pasteurianus IFO 3283-01	NC_013209	+	1929445	1929471
Gluconacetobacter xylinus NBRC 3288	NC_016027	+	2938135	2938161

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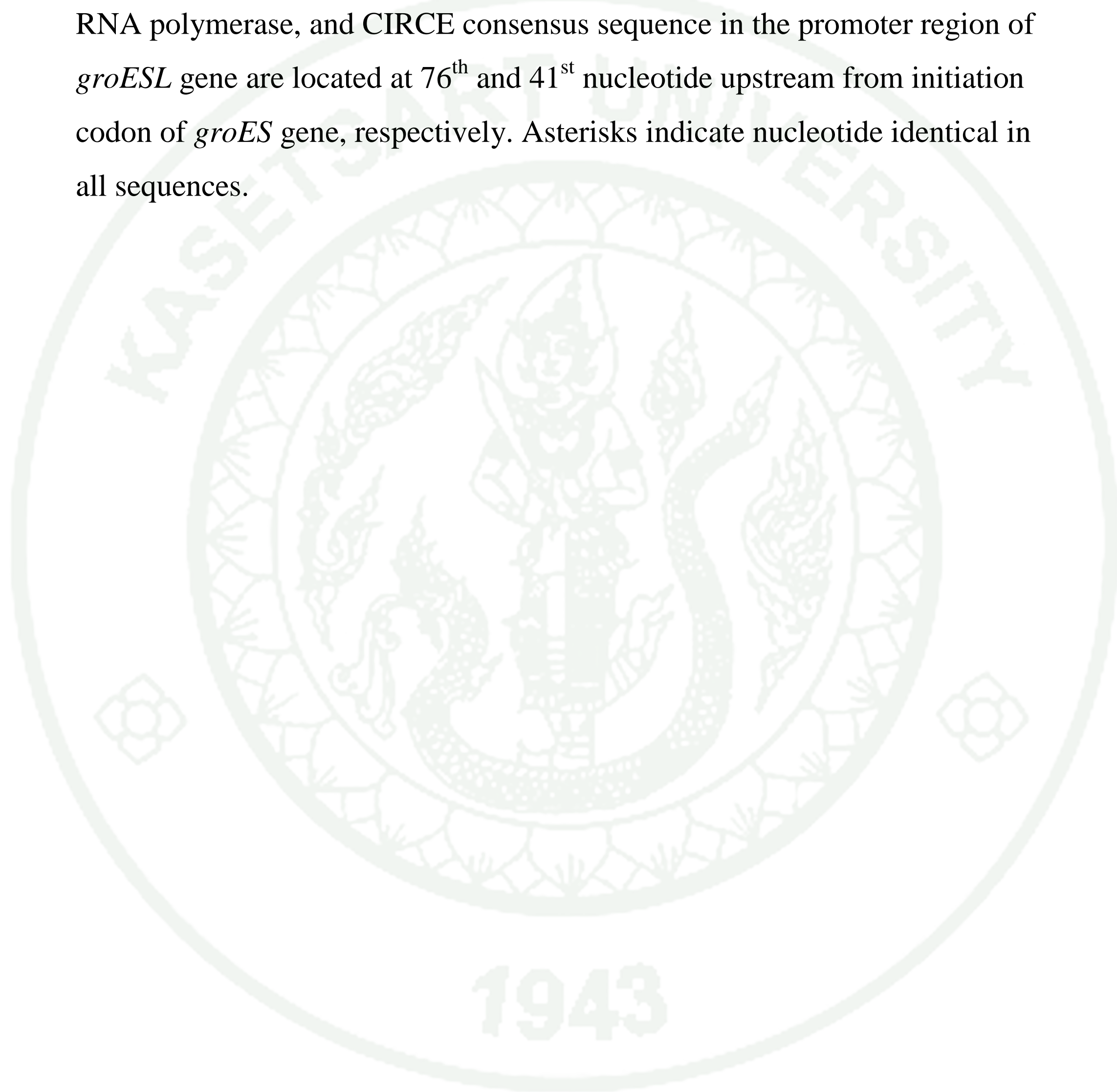
Figure 31 Regulatory region of *groESL* gene in *Acetobacter pasteurianus* SKU1108 (A), alignment of the CIRCE consensus sequences detected from 20 acetic acid bacterial genomes (B), and phylogenetic tree of these consensus sequences (C). The putative -35 and -10 binding sites for *sigma32* (Sig32) subunits of RNA polymerase, and CIRCE consensus sequence in the promoter region of *groESL* gene are located at 76th and 41st nucleotide upstream from initiation codon of *groES* gene, respectively. Asterisks indicate nucleotide identical in all sequences.



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A

0 721 1093 1146 2787

promoter CIRCE RBS groES RBS groEL

(372 bp) (1,641 bp) 100 bp

646 674 681 707

CTTG —— CCTATCT (18 bp.) — 6 bp. — CTGGCACT —— GAGTGCTAA (9 bp.)

CTTG-X_{18}-CYTAT-T CTGGCACTC-X_9-GAGTGCTAA

σ^{32} consensus sequence (putative heat-shock promoter)

CIRCE consensus sequence (Controlling Inverted Repeat of Chaperone Expression)

123 amino acids → GroES ca.13 kDa

546 amino acids → GroEL ca. 58 kDa

B

Gluconacetobacter diazotrophicus PAl 5	CTGGCACTCTCGTGCGGAGAGTGCCAA
Gluconacetobacter hansenii ATCC 23769	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconacetobacter xylinus NBRC 3288	TTAGCACTCTCACGAGGGGAGTGCTAA
Gluconacetobacter sp. SXCC-1	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconacetobacter europaeus LMG 18494	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconacetobacter europaeus 5P3	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconacetobacter europaeus LMG 18890	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconacetobacter oboediens 174Bp2	TTAGCACTCTCATGAGGGGAGTGCTAA
Gluconobacter oxydans WSH-003	TTGGCACTCTCGATACGGGAGTGCTAA
Gluconobacter frateurii NBRC 101659	TTGGCACTCTCGATGTGGGAGTGCTAA
Gluconobacter morbifer G707	TTGGCACTCTCGGTGTGAGAGTGCTAA
*Gluconobacter oxydans*621H	TTGGCACTCTCCATGTGAGAGTGCCAA
Acetobacter pasteurianus IFO 3191	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter pasteurianus subsp. *pasteurianus* LMG 1262	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter pasteurianus NBRC 101655	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter pasteurianus IFO 3283-01	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter pasteurianus 3P3	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter pomorum DM001	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter tropicalis NBRC 101654	CTGGCACTCCCGGGGTGGAGTGCTAA
Acetobacter aceti NBRC 14818	GTGGCACTCCCGGGGTGGAGTGCTAA
	* ****** * * ****** **

C

A. pasteurianus IFO 3191
A. pasteurianus subsp. *pasteurianus* LMG 1262
A. pasteurianus NBRC 101655
A. pasteurianus IFO 3283-01
A. pasteurianus 3P3
A. pomorum DM001
A. tropicalis NBRC 101654

89

A. aceti NBRC 14818

G. morbifer G707

G. frateurii NBRC 101659

G. oxydans WSH-003

*G. oxydans*621H

Ga. diazotrophicus PAl 5

92

63

0.02

Ga. xylinus NBRC 3288

Ga. hansenii ATCC 23769
Ga. xylinus NBRC 3288
Gluconacetobacter sp. SXCC-1
Ga. europaeus LMG 18494
Ga. europaeus 5P3
Ga. europaeus LMG 18890
Ga. oboediens 174Bp2

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2. Investigation of *groESL* Gene Variation in Thermotolerant and Thermosensitive acetic acid bacteria

2.1 Growth characterization of thermotolerant and thermosensitive strains

A total of 129 isolates of acetic acid bacteria (SKU1-129) previously isolated from fruits in Thailand (Theeragool *et al.,* 1996) were selected for thermotolerant and thermosensitive strains. The 3 µl of the inoculum was spot-inoculated on YPGD agar and incubated at 30ºC, 37ºC and 40ºC for 5 d. Comparison of the growth of all isolates was observed as shown in Figure 32. As shown in Figure 32A, the growth of all isolates was similar when incubating at 30ºC. On agar plate incubating at 37ºC, almost isolates could grow well except some isolates as SKU1, 7 and 80 (Figure 32B). Among the 129 isolates of acetic acid bacteria, ten isolates, SKU1, 7, 11, 21, 43, 45, 58, 80, 82 and 92, were the thermosensitive strains (Figure 32C). These strains could not grow well at 40ºC when comparing with other strains. Some thermotolerant and thermosensitive strains were selected for investigation of *groESL* gene variation comparing together.

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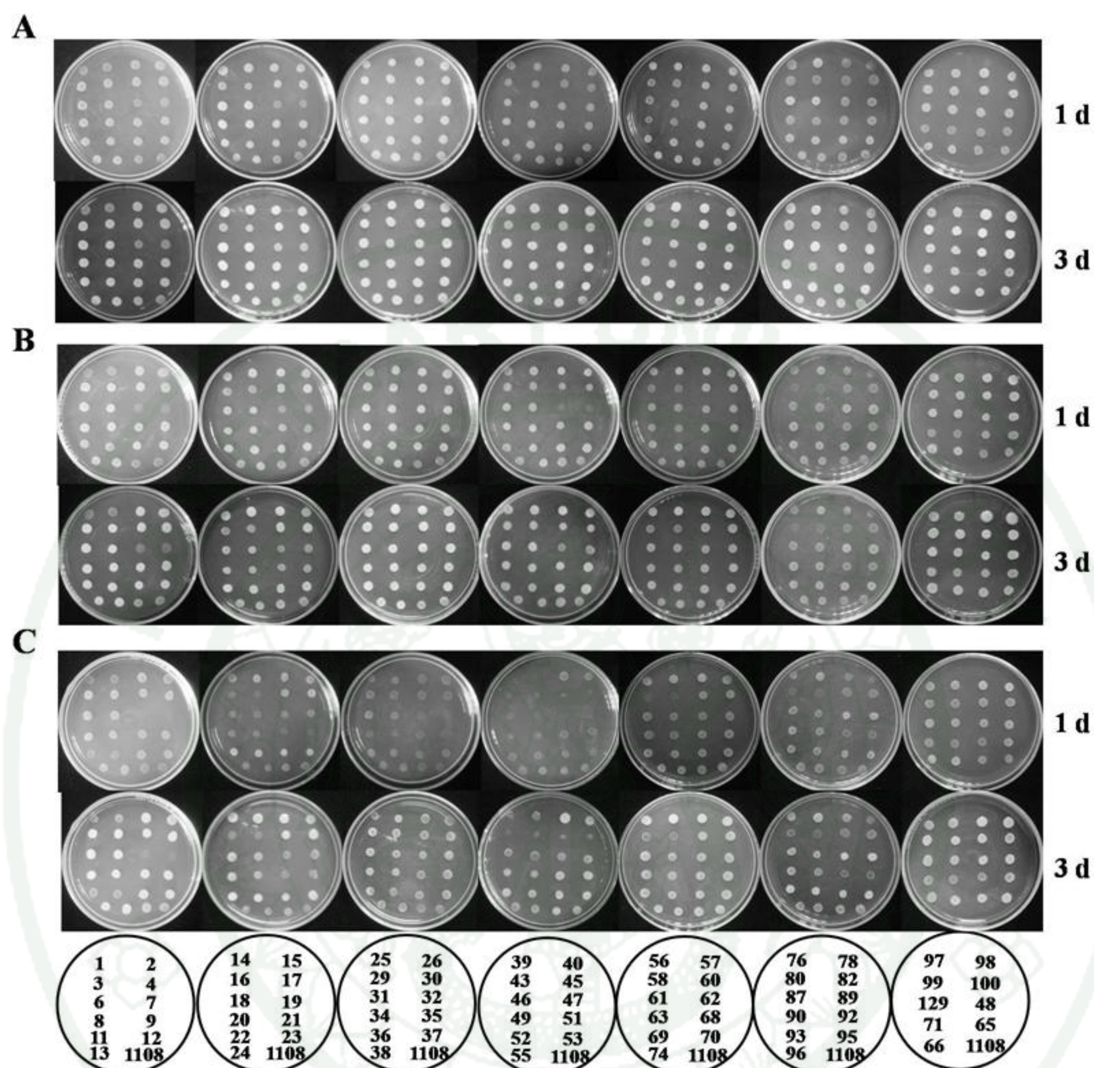

Figure 32 Growth of thermotolerant and thermosensitive acetic acid bacteria, SKU strains. All isolates were spot-inoculated on YPGD agar incubating at 30ºC (A), 37ºC (B) and 40ºC (C) for 3 d.

2.2 PCR amplification of partial *groESL* gene from thermotolerant and thermosensitive strains

Chromosomal DNAs from some thermotolerant and thermosensitive strains were used as template. Thermotolerant acetic acid bacteria used in this study were screened for their growth characteristics at 37°C and 40°C. In contrast, the thermosensitive strains were selected from the isolates exhibited poor growth at high temperature. The partial *groESL*

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genes from thermotolerant and thermosensitive strains were amplified by polymerase chain reaction (PCR) using two specific primers, GF1 (5'-AGG AAA AGC CTA TGG AAG-3') and GR1 (5'-GCT GTG GAA GAA GGC AT-3'). Various patterns of PCR product were amplified from different strains of thermotolerant acetic acid bacteria as SKU1108, SKU19, IFO 3222, IFO 3283, IFO 3298, SKU3, SKU16, SKU46, SKU47, SKU51, SKU76, and some strains of thermosensitive (IFO 3299, SKU1, SKU7, SKU11, SKU80 and SKU82) (Figure 33). However, these primers could not amplify the partial *groESL* gene from some acetic acid bacteria that could not grow at 40°C (SKU21, SKU43, SKU45, SKU58 and SKU92) as shown in Figure 33. To confirm the relationship between pattern of PCR product and strains of thermotolerant and thermosentitive acetic acid bacteria, some of them were selected for preforming 16S rRNA gene analysis. The results showed that the same PCR product patterns were distinguishable from same species but not relating with thermotolerant and themosensitive trait.

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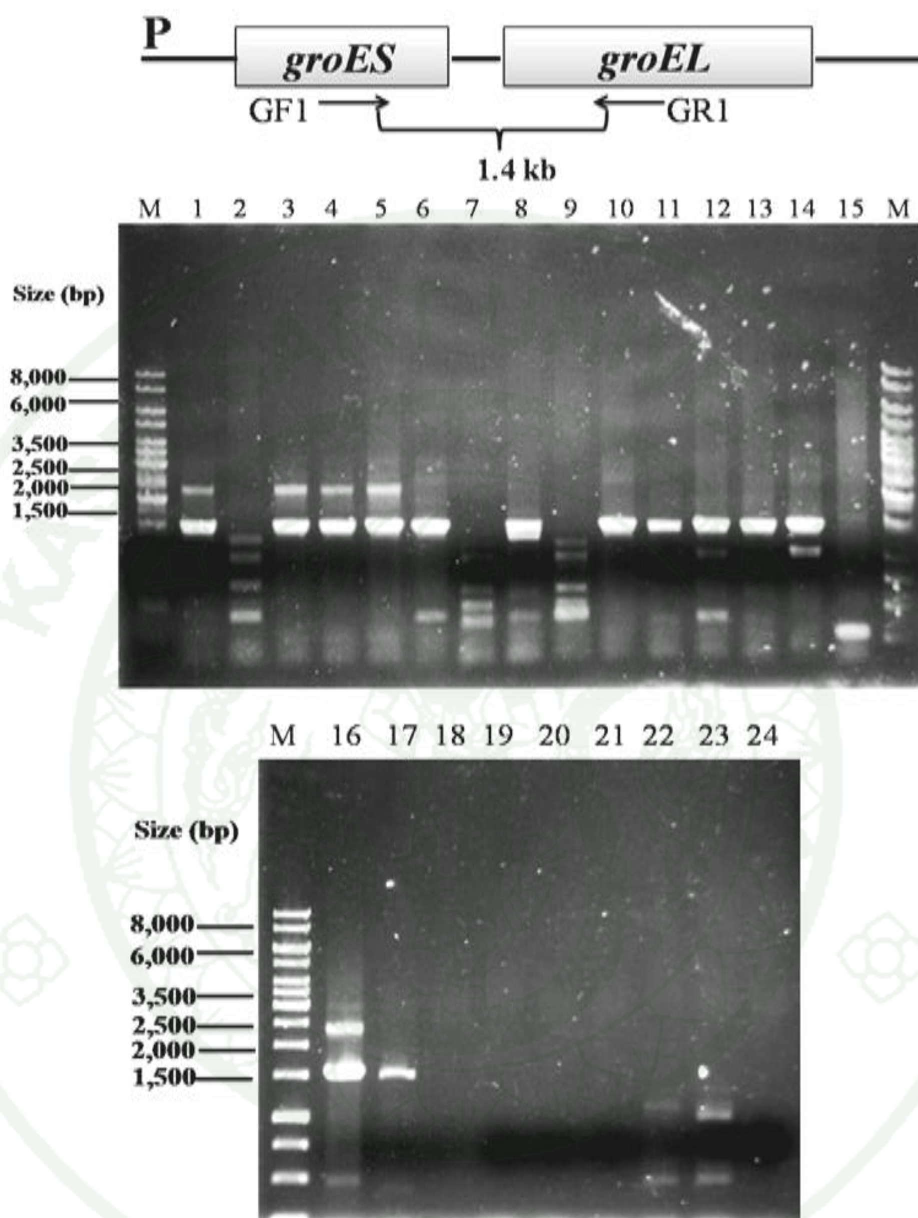

Figure 33 Agarose gel electrophoresis of PCR amplification of partial *groESL* gene from chromosomal DNA of thermotolerant and thermosensitive strains by using primer GF1 and GR1. Lane M = 1 kb DNA Ladder, Lane 1-24 = partial *groESL* gene PCR product from SKU1108, SKU19, IFO 3222, IFO 3283, IFO 3298, IFO 3299, SKU1, SKU3, SKU7, SKU16, SKU46, SKU47, SKU51, SKU76, SKU92, SKU1108, SKU11, SKU21, SKU43, SKU45, SKU58, SKU80, SKU82 and SKU92, respectively.

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2.3 Southern hybridization of *Hind*III-digested genomic DNAs from thermotolerant and thermosensitive strains with partial 1.4 kb *groESL* DNA probe

The 1.4 kb PCR amplified *groESL* gene from *A. pasteurianus* SKU1108 was analyzed in 0.8% agarose gel electrophoresis and recovered from the gel. The purified PCR product was labeled with digoxogenin-11-dUTP (Dig-11-dUTP) by random primed labeling and used as DNA probe for Southern hybridization. The genomic DNAs from some thermotolerant and thermosensitive acetic acid bacteria were completely digested by *Hind*III restiction enzymes. This restriction enzyme was selected due to the *groESL* gene cluster of *A. pasteurianus* SKU1108 that harbor similar restriction map with *A. pasteurianus* NBRC 3283 (Okamoto-Kainuma *et al.,* 2002). These digested DNA were used to hybridize with 1.4 kb *groESL* DNA probe by Southern hybridization. As shown in Figure 34, the positive hybridization signals were detected with several sizes of DNA fragment from thermotolerant and thermosensitive strains. The pattern of signals could not relate with the strains of thermotolerant and thermosensitive acetic acid bacteria. The results indicated that restriction enzyme *Hind*III digested at the different sites on genomic DNA from each acetic acid bacterial strains. The positive signal of each strain was detected depending on the DNA fragment size harboring *groESL* gene. The Southern hybridization results were correlative with the results of partial *groESL* gene amplification from thermotolerant and thermosensitive strains as described previously.

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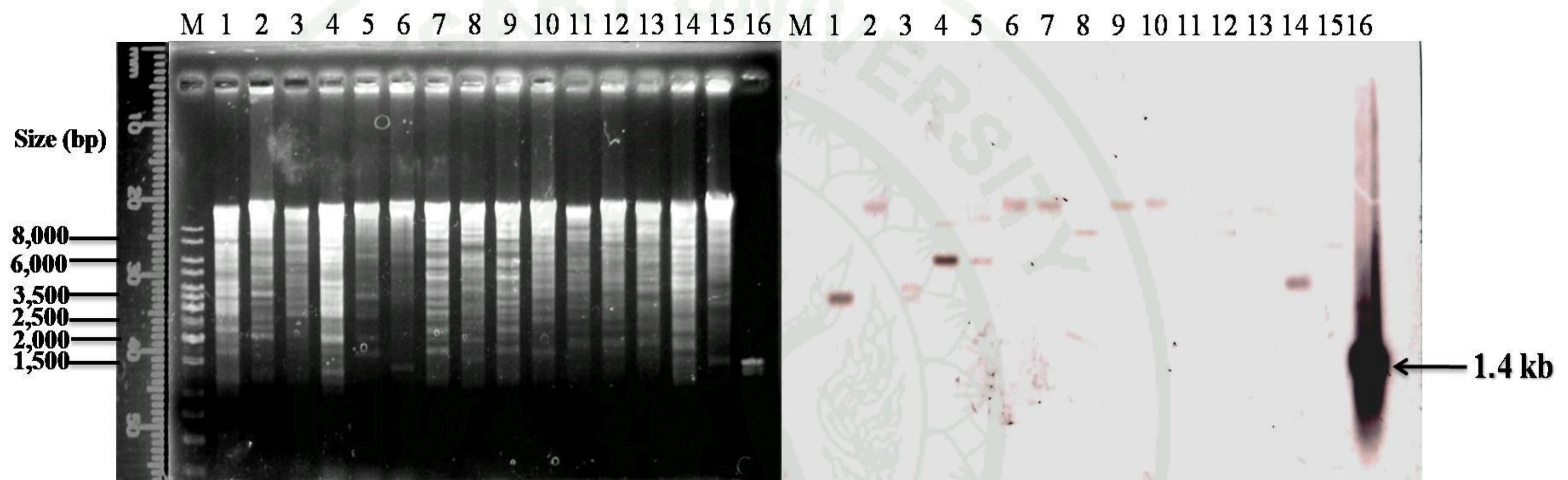

Figure 34 Agarose gel electrophoresis (left) and Southern hybridization pattern (right) of the digested genomic DNA from thermotolerant and thermosensitive acetic acid bacteria. The 1.4 kb *groESL* gene from SKU1108 was used as DNA probe. Lane M = 1kb DNA ladder, 1 = *Hind*III digested genomic DNA of SKU1108, 2 = *Hind*III digested genomic DNA of SKU19, 3 = *Hind*III digested genomic DNA of IFO 3222, 4 = *Hind*III digested genomic DNA of IFO 3283, 5 = *Hind*III digested genomic DNA of IFO 3298, 6 = *Hind*III digested genomic DNA of IFO 3299, 7 = *Hind*III digested genomic DNA of SKU1, 8 = *Hind*III digested genomic DNA of SKU3, 9 = *Hind*III digested genomic DNA of SKU7, 10 = *Hind*III digested genomic DNA of SKU16, 11 = *Hind*III digested genomic DNA of SKU46, 12 = *Hind*III digested genomic DNA of SKU47, 13 = *Hind*III digested genomic DNA of SKU51, 14 = *Hind*III digested genomic DNA of SKU76, 15 = *Hind*III digested genomic DNA of SKU92 and 16 = the 1.4 kb *groESL* probe from *A. pasteurianus* SKU1108.

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From the patterns of partial *groESL* gene PCR product and signals of Southern hybridization, *groESL* gene variations were not found between thermotolerant and thermosensitive acetic acid bacterial strains. However, the same PCR product patterns from partial *groESL* gene amplification were distinguishable from the same species. When comparing with phylogenetic analysis of the 16S rRNA gene sequences revealed that the strains that showed the same patterns of partial *groESL* gene PCR product usually clustered together and belonged to the same species or subspecies. In recent years, some housekeeping proteins such as 60 kDa heat shock protein (HSP60) have been considered as useful phylogenetic markers. They have been successfully used for identification of the genera *Bifidobacterium* (Jian *et al.,* 2001; Ventura *et al.,* 2004), *Enterococcus* (Teng *et al.,* 2001), *Streptococcus* (Teng *et al.,* 2002; Chen *et al.,* 2008; Glazunova *et al.,* 2009), *Rickettsia* (Lee *et al.,* 2003), *Bacillus* (Chang *et al.,* 2003), *Campylobacter* (Kärenlampi *et al.,* 2004), *Lactobacillus* (Blaiotta *et al.,* 2008), *Gluconacetobacter* (Cleenwerck *et al.,* 2010), including *Acetobacter* (Huang *et al.,* 2014). It can be concluded that the acetic acid bacterial *groEL* gene sequences can be used as an alternative of current methods for tracing acetic acid bacterial species, particularly because they allow a high level of discrimination between closely related species of this genus.

3. Identification and Phylogenetic Analysis of Genus *Acetobacter* Based on *groEL* Gene Sequences

3.1 PCR amplification and sequence analysis of 16S rRNA gene

Twenty-four strains were isolated in Thailand by an enrichment culture approach. These strains that were Gram-negative aerobic rods, produced catalase but not oxidase, showed clear zones on glucose/yeast extract/peptone/ethanol/calcium carbonate agar plates, and grew at pH 3.5 were assigned to the genus *Acetobacter*, since they oxidized acetate and lactate to carbon dioxide and water and had Q-9 as the major quinone (Asai *et al.,* 1964; Yamada *et al.,* 1969, 1999). The chromosomal DNA from acetic acid bacteria were used as DNA template for 16S rRNA gene amplification by performing PCR with two primers, 27f primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1525r primer (5'-AAA GGA GGT GAT CCA GCC-3'). The sizes of desired PCR product are 1.5 kb as

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shown in Figure 35. The PCR products of 16S rRNA gene were purified and sent to First Base Laboratory for DNA sequencing analysis. The nucleotide sequences of 16S rRNA gene amplified from chromosomal DNA of these strains were analyzed for sequence identity percentage and the obtained results were summarized in Table 11.

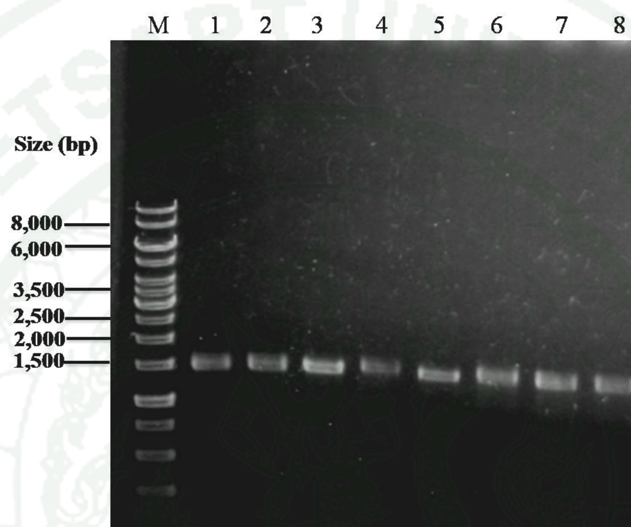

Figure 35 Agarose gel electrophoresis of PCR amplification of 1.5 kb 16S rRNA gene from chromosomal DNA of some *Acetobacter* species by using primer 27f and 1525r. Lane M = 1 kb DNA Ladder, Lane 1-8 = 1.5 kb 16S rRNA gene PCR product from SKU2, SKU9, SKU17, SKU20, SKU60, SKU76, SKU80 and TR5SU1, respectively.

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Table 11 *Acetobacter* strains examined and identified based on 16S rRNA gene and *groEL* gene sequences

Group and strain	Other designation	Isolation source		Identified by (%similarity)	
		Source	Province	16S rRNA gene sequences	*groEL* gene sequences
Group 1		**17% from total**		*A. tropicalis* or *A. senegalensis*	*A. tropicalis*
SKU 2	-	Banana	Bangkok	99.9%	99.7%
SKU 9	-	Banana	Bangkok	99.8%	99.7%
SKU 17	-	Guava	Bangkok	99.9%	99.8%
SKU 60	-	Rambeh	Bangkok	99.1%	99.7%
Group 2		**13% from total**		*A. indonesiensis*	*A. indonesiensis*
BCC 15762	AC27	Fruit	Chiang Mai	99.9%	99.3%
SKU 20	-	Guava	Bangkok	99.0%	99.2%
SKU 21	-	Guava	Bangkok	99.1%	99.1%
Group 3		**9% from total**		*A. persici*	*A. persici*
BCC 15756	AC17	Fruit	Chiang Mai	99.9%	96.9%
BCC 24365	TR5SU1	Fermented rice	Lamphun	99.6%	98.0%
Group 4		**9% from total**		*A.orientalis*	*A.orientalis*
BCC 15812	AC 79	Flower	Kanchanaburi	99.9%	99.5%
BCC 15813	AC 80	Flower	Kanchanaburi	99.9%	99.5%
Group 5		**4% from total**		*A. cibinongensis*	*A. cibinongensis*
BCC 24363	TR5S2	Fermented rice	Lamphun	99.8%	98.3%
Group 6		**13% from total**		*A. pasteurianus*	*A. pasteurianus*
BCC 6290	TISTR102	Fruit	Bangkok	99.0%	98.6%
SKU 76	-	Lychee	Bangkok	99.5%	98.3%
SKU 129	-	Santol	Bangkok	99.1%	98.4%
Group 7		**4% from total**		*A. peroxydans* or *A. papayae*	*A. papayae*
BCC 15884	AD87	Fruit	Kanchanaburi	99.9%	99.2%
Group 8		**9% from total**		*A. fabarum*	*A. fabarum*
BCC 15845	AD38	Mushroom	Kanchanaburi	100.0%	99.2%
BCC 15846	AD39	Mushroom	Kanchanaburi	100.0%	99.2%
Group 9		**4% from total**		*A. okinawensis*	*A. okinawensis*
Strain 15851	AD46	Flower	Kanchanaburi	100.0%	98.6%

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Table 11 (Continued)

Group and strain	Other designation	Isolation source		Identified by (%similarity)	
		Source	Province	16S rRNA gene sequences	*groEL* gene sequences
Group 10		**17% from total**		*A. ghanensis*	*A. ghanensis*
SKU 1	-	Banana	Bangkok	99.9%	99.1%
SKU 7	-	Banana	Bangkok	100.0%	99.1%
SKU 80	-	Orange	Bangkok	99.2%	98.1%
SKU 81	-	Orange	Bangkok	99.3%	99.2%
New species BCC 15839	AD25	Flower	Kanchanaburi	*A. cibinongensis* 98.3%	*A. orientalis* 87.8%

3.2 Phylogenetic analysis based on 16S rRNA gene sequences

In a phylogenetic tree based on 16S rRNA gene sequences, strains isolated and the type strains of all the known *Acetobacter* species were divided into two groups (Figure 36), i.e., the *A. aceti* group and the *A. pasteurianus* group (Yamada and Yukphan, 2008). The topology of the phylogenetic tree was similar to those reported by Lisdiyanti *et al.* (2002), Cleenwerck *et al.* (2008), Tanasupawat *et al.* (2011a) and Iino *et al.* (2012). The twenty-four strains isolated in Thailand were distributed into ten groups phylogenetically except the strain BCC 15839 (Table 10).

In the *A. aceti* group, the strains isolated constituted five groups. Group 1 contained four SKU strains and the type strains of *A. tropicalis* and *A. senegalensis*. Considering the phylogenetic positions observed, the four strains could not exactly be identified as either *A. tropicalis* or *A. senegalensis*. Two SKU strains and one BCC strains of Group 2 were identified as *A. indonesiensis*, two BCC strains of Group 3 were as *A. persici*, two BCC strains of Group 4 were as *A. orientalis*, and one BCC strain of Group 5 was as *A. cibinongensis*. Moreover, one strain, BCC 15839 formed an independent cluster from other type strains in the *A. aceti* group in the 16S rRNA gene based tree. There were no strains isolated that corresponded to *A. cerevisiae*, *A. malorum*, *A. orleanensis*, *A. farinalis*, *A. oeni*, *A. nitrogenifigens*, *A. aceti*, and *A. estunensis*.

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In the *A. pasteurianus* group, the strains isolated constituted five groups. Two SKU strains and one BCC strain of Group 6 were identified as *A. pasteurianus*, one BCC strain of Group 7 was as either *A. peroxydans* or *A. papayae*, two BCC strains of Group 8 were as *A. fabarum*, one BCC strain of Group 9 was as *A. okinawensis*, and four SKU strains of Group 10 were as *A. ghanensis*. There were no strains isolated that corresponded to *A. pomorum*, *A. lovaniensis* and *A. syzygii*.

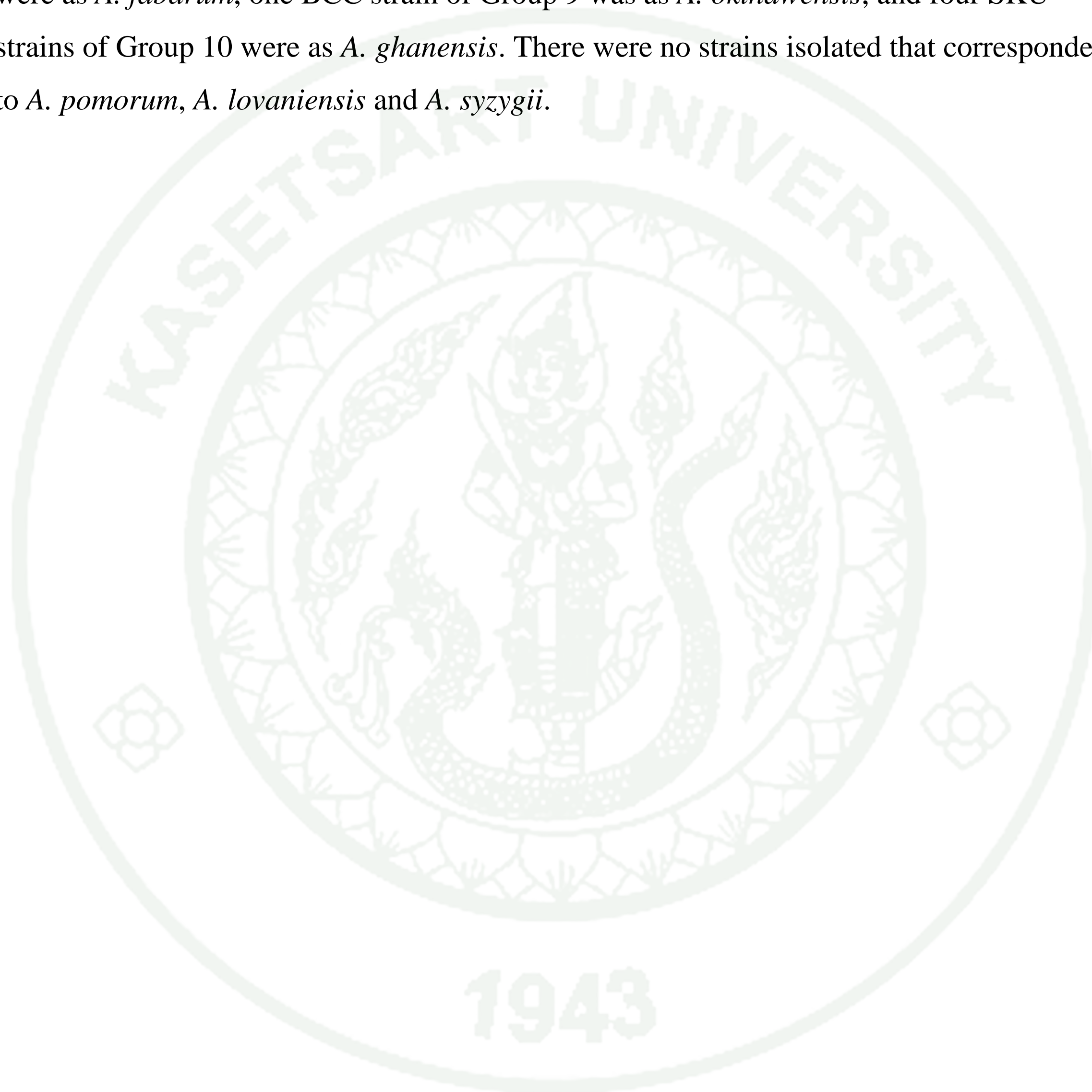

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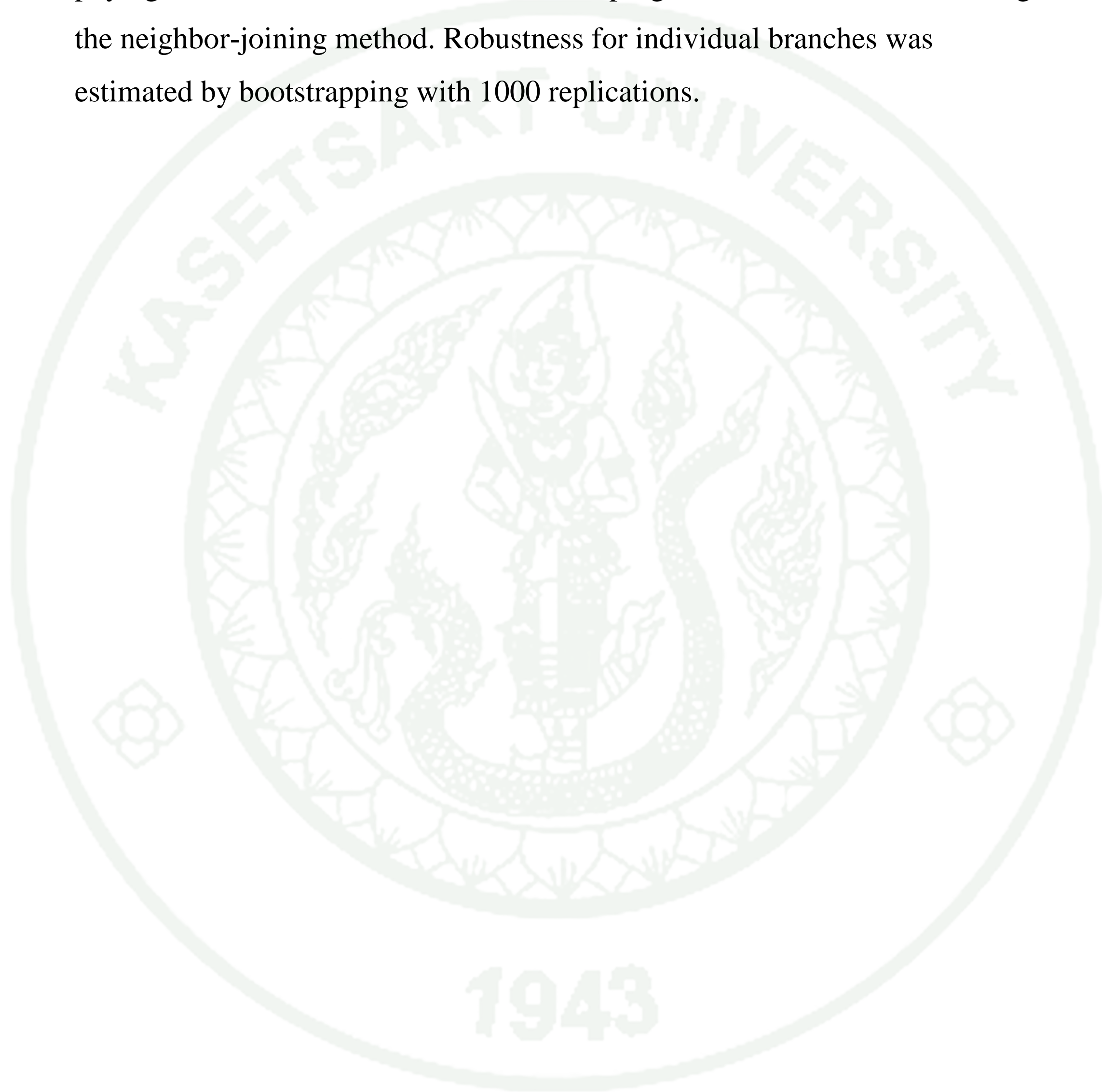
Figure 36 Phylogenetic tree based on 16S rRNA gene sequences of the type strains of all validly described *Acetobacter* species and the strains isolated in Thailand. *Gluconacetobacter liquefaciens* LMG 1381^T was used as outgroup. The phylogenetic tree was constructed with the program MEGA version 5.1 using the neighbor-joining method. Robustness for individual branches was estimated by bootstrapping with 1000 replications.



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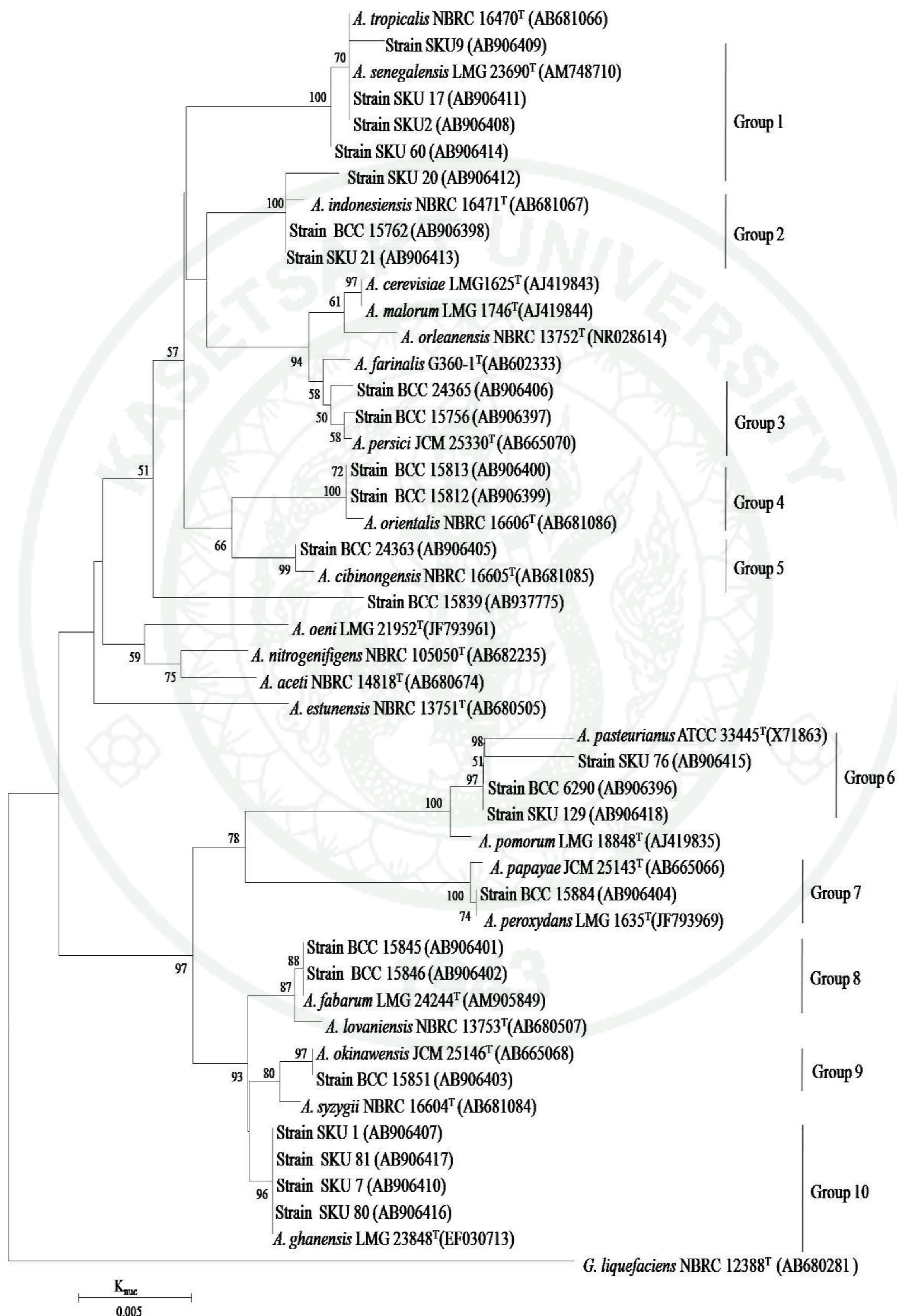

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3.3 PCR amplification and sequence analysis of *groEL* gene

The full length sequences (approximately 1,600 bp) of *groEL* gene were amplified and sequenced by using primer FgroEL and RgroEL from 16 representatives *Acetobacter* species as follows: *A. pasteurianus, A. orleanensis, A. lovaniensis, A. tropicalis, A. indonesiensis, A. syzygii, A. cibinongensis, A. orientalis, A. ghanensis, A. malorum, A. senegalensis, A. fabarum, A. farinalis, A. okinawensis, A. papayae* and *A. persici* (Figure 37). In addition, the nearly full length sequences of *groEL* gene (approximately 1,000-1,400 bp) were amplified and sequenced by using primer FgroELnew and RgroELnew from the type strain as *A. cerevisiae*, *A. peroxydans* and the strain BCC 15839 (Figure 38A). Surprisingly, only one reference strain, *A. pomorum* that was very closely related strain with *A. pasteurianus,* could not amplify *groEL* gene by using primers as described above. The new pair of primer, FgroEL89 and RgroEL89 was designed for *groEL* gene amplification from *A. pomorum*. Finally, the 1200 bp *groEL* gene fragment was amplified from *A. pomorum* (Figure 38A). For the four remaining reference strains as *A. aceti, A. nitrogenifigens*, *A. estunensis* and *A. oeni,* primer groEL-10-F and groEL-11-R (Cleenwerck *et al.,* 2010) were used for 900 bp *groEL* gene amplification and sequencing as shown in Figure 38B. Sequence analysis showed that the identities of *groEL* genes of all the *Acetobacter* species ranged from 80.3% to 99.5%.

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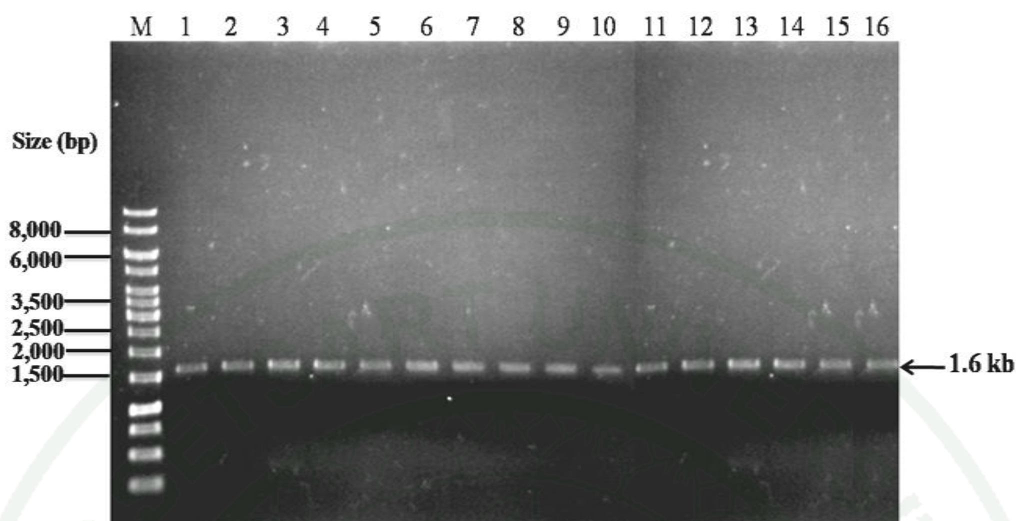

Figure 37 Agarose gel electrophoresis of PCR amplification of 1.6 kb *groEL* gene from chromosomal DNA of *Acetobacter* species by using primer FgroEL and RgroEL. Lane M = 1 kb DNA Ladder, Lane 1-16 = 1.6 kb *groEL* gene PCR product from *A. pasteurianus, A. orleanensis, A. lovaniensis, A. tropicalis, A. indonesiensis, A. syzygii, A. cibinongensis, A. orientalis, A. ghanensis, A. malorum, A. senegalensis, A. fabarum, A. farinalis, A. okinawensis, A. papayae* and *A. persici,* respectively.

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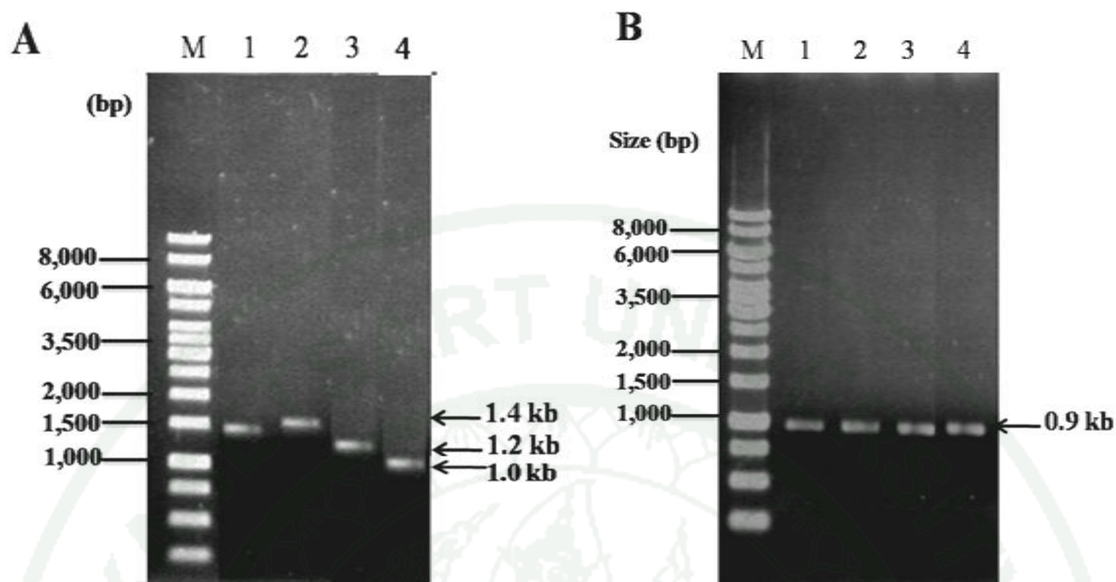

Figure 38 Agarose gel electrophoresis of PCR amplification of *groEL* gene from chromosomal DNA of *Acetobacter* species by using primer FgroELnew /RgroELnew and FgroEL89/ RgroEL89 (A) and groEL-10-F and groEL-11-R (B). A, Lane M = 1 kb DNA Ladder, 1 = 1.4 kb *groEL* gene PCR product from *A. cerevisiae,* 2 = 1.4 kb *groEL* gene PCR product from *A. peroxydans*, 3 = 1.2 kb *groEL* gene PCR product from *A. pomorum* and 4 = 1.0 kb *groEL* gene PCR product from the strain BCC 15839. B, Lane M = 1 kb DNA Ladder, 1-4 = 0.9 kb *groEL* gene PCR product from *A. aceti, A. nitrogenifigens*, *A. estunensis* and *A. oeni,* respectively.

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3.4 Phylogenetic analysis of *groEL* gene sequences

In a phylogenetic tree based on *groEL* gene sequences, strains isolated and the type strains of all the known *Acetobacter* species were not necessarily divided into two groups, i.e., the *A. aceti* group and the *A. pasteurianus* group (Figure 39). For example, the type strain of *A. peroxydans* was located within the members of the *A. aceti* group, which was designated by the 16S rRNA gene sequence analysis. In addition, the type strains of the four species, *A. estunensis*, *A. aceti*, *A. oeni*, and *A. nitrogenifigens* were located outside of the clusters comprised of the members of the *A. pasteurianus* group and the remaining members of the *A. aceti* group.

Group 1 contained four SKU strains and the type strains of *A. tropicalis* and *A. senegalensis*. However, the four strains could be identified as *A. tropicalis*, since the type strain of *A. tropicalis* had a shorter phylogenetic branch than that of *A. senegalensis*. Since Group 7 contained one BCC strain and the type strain of only *A. papayae*, the strain was identified as *A. papayae* but not *A. peroxydans*. The identification of the strains mentioned above was confirmed in Groups 2, 3, 4, 5, 6, 8, 9, and 10 by the *groEL* gene sequence analysis. In case of the strain BCC 15839, it formed an independent cluster among Group 4 (*A. orientalis*) and Group 5 (*A. cibinongensis*) in the *groEL* gene based tree.

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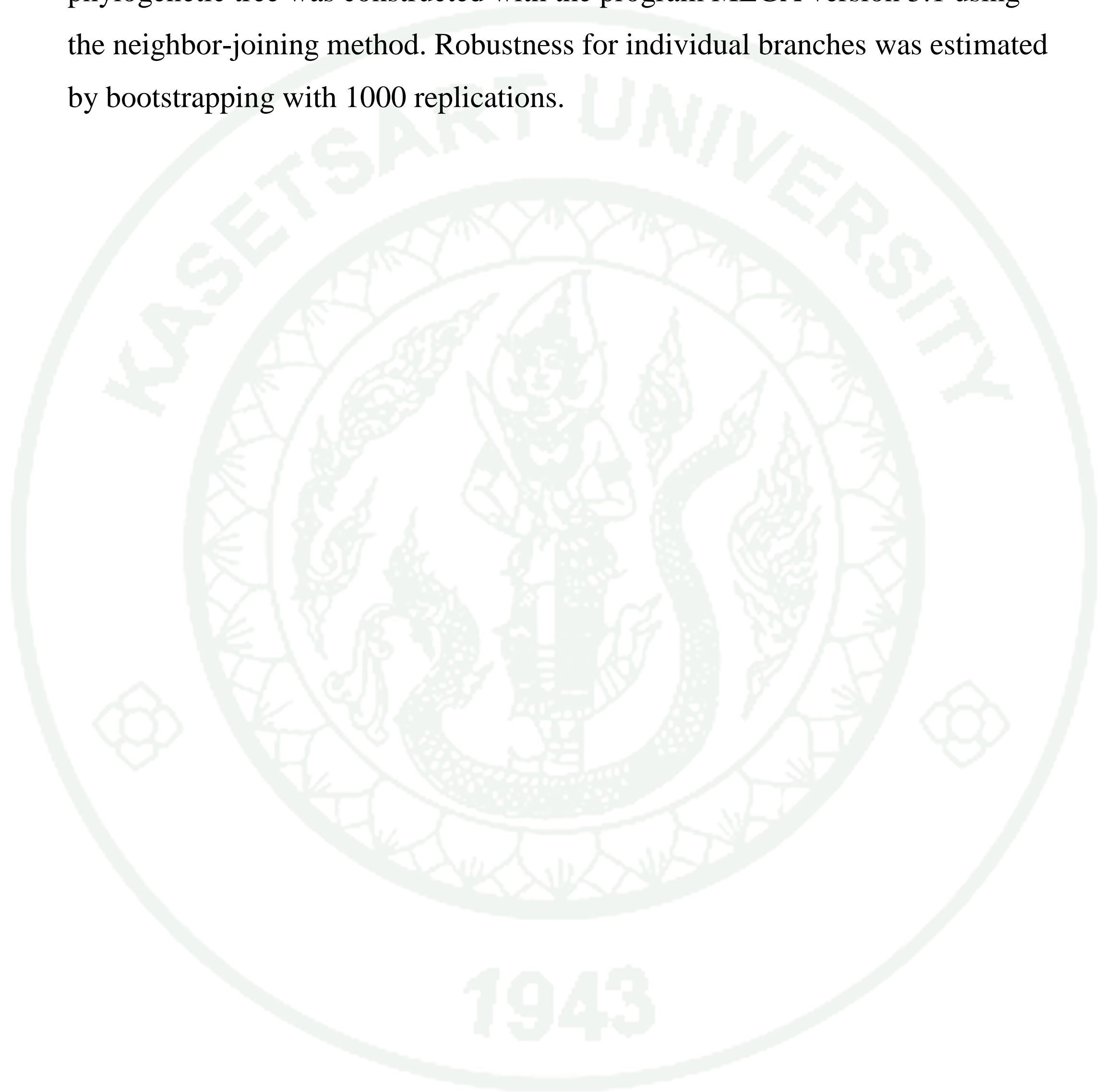
Figure 39 Phylogenetic tree based on *groEL* gene sequences of the type strains of all validly described *Acetobacter* species and the strains isolated in Thailand. *Gluconacetobacter liquefaciens* LMG 1381^T was used as outgroup. The phylogenetic tree was constructed with the program MEGA version 5.1 using the neighbor-joining method. Robustness for individual branches was estimated by bootstrapping with 1000 replications.



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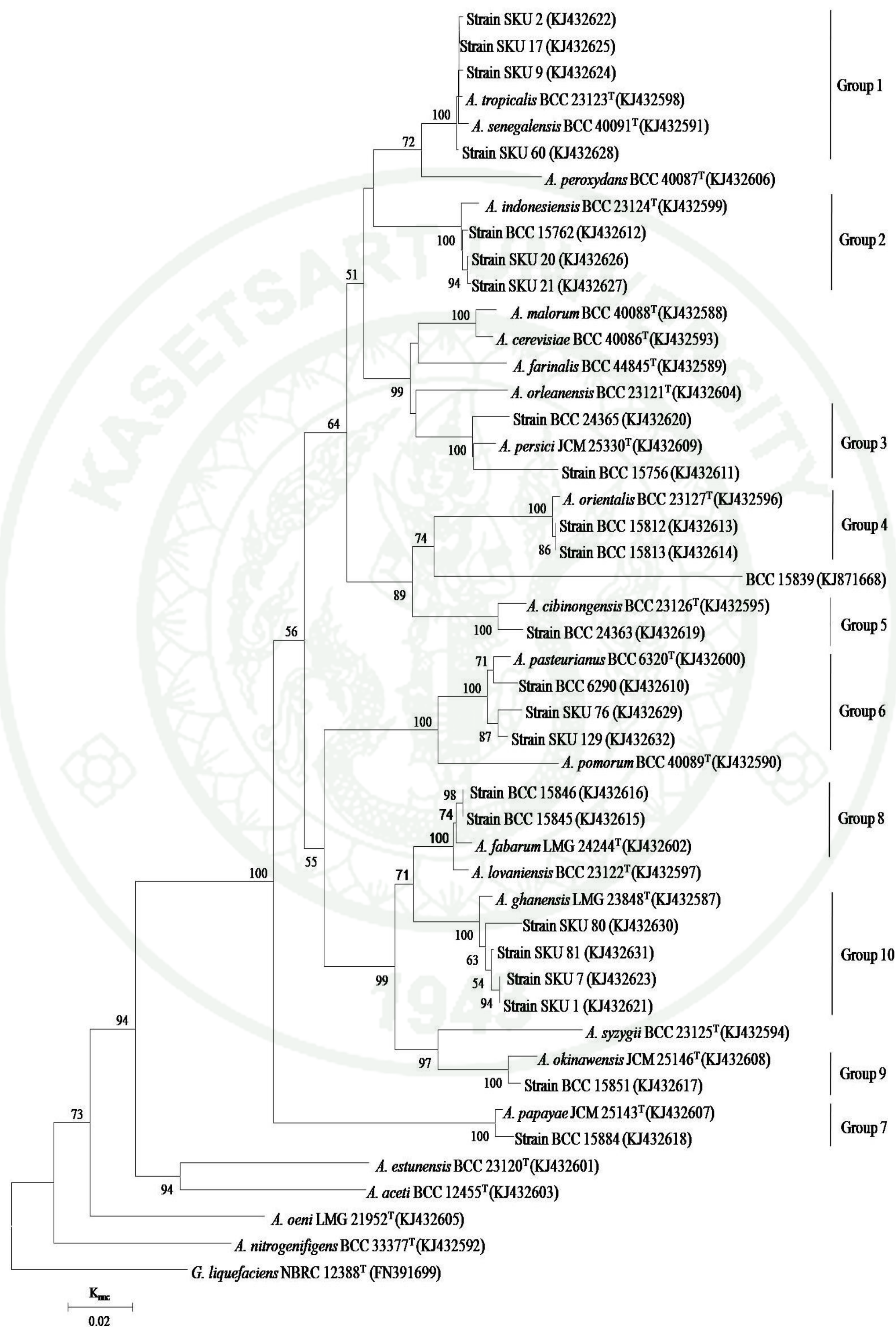

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In acetic acid bacteria, the genus-level identification is very easy by using phenotypic and chemotaxonomic test. The genus *Acetobacter* could be differentiated from others genera by the oxidation of acetate and lactate to CO_2 and H_2O and by ubiquinone analysis. The *Acetobacter* strains have Q9 as the major ubiquinone while all other acetic acid bacteria possess Q10 (Yamada *et al.,* 1997). On the other hand, the taxonomy of acetic acid bacteria at species-level is much more difficult. DNA-DNA hybridizations have been recommended for identify *Acetobacter* strains accurately at species-level (Sokollek *et al.,* 1998; Lisdiyanti *et al.,* 2000, 2001; Cleenwerck *et al.,* 2002). However, DNA-DNA hybridization is a laborious and inconvenient process. Moreover, the results of DNA-DNA hybridization might not be as stable and comparable as those involving nucleotide sequences, because of the different methods and conditions involved.

Because of the inconvenience of DNA-DNA hybridization and the limitations of 16S rRNA gene analysis in the phylogenetic study of closely related bacterial taxa, the sequences of housekeeping genes have been used for resolve this problem (Naser *et al.,* 2005; De Bruyne *et al.,* 2007; Brady *et al.,* 2008; Cleenwerck *et al.,* 2010). In case of acetic acid bacteria, Cleenwerck *et al.* (2010) determined three housekeeping genes; *dnaK* (encoded a DnaK heat-shock protein), *groEL* (encoded a chaperonin protein) and *rpoB* (encoded the β-subunit of bacterial RNA polymerase) for species identification of genus *Gluconacetobacter*. The results revealed that the multilocus of the three housekeeping genes can be useful for species differentiation in the genus *Gluconacetobacter*.

In this study, the partial *groEL* gene sequences analysis was conducted for the *Acetobacter* species identification. The results showed that the partial *groEL* gene sequence similarity among different *Acetobacter* species ranged from 80.3 to 99.5%. By comparison, the 16S rRNA gene sequence similarities among all species ranged from 95.6 to 100% and were exceptionally high for the groups of closely related species. Although the *groEL* gene sequence similarities of the closely related species were higher than 98%, the discriminatory power was still stronger than that of 16S rRNA gene as shown in case of the strains SKU2 and SKU17 (group 1) and BCC 15884 (Group 7). For the strains within the same species (intraspecies) of genus *Acetobacter*, the *groEL* gene sequence similarities were 96.9-99.5%. The results showed that the species tested in the present study displayed

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high sequence similarities (>94%) of *groEL* gene sequences, Thus analysis of *groEL* gene similarity is a powerful tool for the phylogenetic study of *Acetobacter* species.

The topologies of *groEL* and 16S rRNA gene-based trees were comparable in the phylogenetic trees from *Acetobacter* group strains in this study. A phylogram generated from the *groEL* gene sequences of the *Acetobacter* group correlates with the phylogenetic relationships inferred from the 16S rRNA gene sequence and supports the previous data that the genus *Acetobacter* could be organized into two main groups. The topology of phylogenetic tree based on *groEL* gene was basically similar to that of 16S rRNA gene except with the following differences: (1) *A. peroxydans*, which instead of clustering with *A. papayae* same as the 16S rRNA gene phylogenetic tree, existed in an independent lineage from group 1; (2) *A. papayae* was branched independently of the *groEL* tree indicating that the phylogenetic relationship between *A. peroxydans* and *A. papayae* may be more distant than was estimated from 16S rRNA gene; (3) the clustering of *A. aceti, A. estunensis, A. nitrogenifigens* and *A. oeni* species were separated from two major groups but they were grouped together. From the results in this study showed that the phylogenetic analysis based on the partial *groEL* gene sequence similarities would be able to differentiate the strains in genus *Acetobacter* even closely related organisms. The combination of *groEL* gene and 16S rRNA gene analysis is more reliable approach for classification and identification of the genus *Acetobacter.*

Moreover, the comparison study revealed that *groEL* gene showed high variation in *A. peroxydans* and *A. papayae* (Group 7), which had the low variation (99.9-100%) in the 16S rRNA gene. The type strain of *A. peroxydans* (Visser't Hooft, 1925), the *A. pasteurianus* group showed quite different phylogenetic positions in the two phylogenetic trees mentioned above. In the phylogenetic tree based on 16S rRNA gene sequences, it was located within the *A. pasteurianus* group. However, it constituted a cluster along with the type strains of *A. tropicalis* and *A. senegalensis*, the *A. aceti* group in the phylogenetic tree based on *groEL* gene sequences. Such a phenomenon was not found in the type strains of any other species of the genus *Acetobacter.*

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In phylogentic tree based on 16S rRNA and *groEL* gene sequences, one strain, BCC 15839 constituted an independent cluster with long phylogenetic branch. This strain was new species in genus *Acetobacter.* Polyphasic approach should be performed for characterization of this strain. Beside the strain BCC 15839, there were several strains that had long phylogenetic branches in the phylogenetic trees based on 16S rRNA gene as well as based on *groEL* gene sequences. These strains are likely to constitute additional new species in the genus *Acetobacter*. The strains that are candidate of novel species, are SKU 9 and SKU 60 of Group 1, strain SKU 20 of Group 2, strain BCC 24365 of Group 3 and Strain SKU 76 of Group 6 were found in the former phylogenetic tree, and strain BCC 15756 of Group 3 in the latter phylogenetic tree. However, the 16S rRNA gene similarity values of them are more than 99% to the closely related type strains. In the further study, these isolates will be confirmed by performing polyphasic analysis and presented as novel species elsewhere.

Previously, Seearunruangchai *et al.,* (2004) reported that strains of *A. pasteurianus* accounted for 77%, *A. orientalis* did for 8%, and *Gluconacetobacter liquefaciens* did for 15% among the acetic acid bacteria isolated from Thai fruits. Kommanee *et al.* (2008) calculated 55% *A. pasteurianus*, 43% *A. orientalis*, and 2% *A. lovaniensis* among the *Acetobacter* strains that were isolated from fruits, flowers, and other materials. Tanasupawat *et al.* (2009) isolated many *Acetobacter* strains from the same materials mentioned above and found that the Thai *Acetobacter* isolates were identified mostly as the two species, *A. pasteurianus* and *A. orientalis*, along with a small number of *A. ghanensis*, *A. tropicalis*, and *A. syzygii*. In addition, Tanasupawat *et al.* (2011b) used fermented rice products, i.e., ‘khao-khab’ and ‘khao-mak’ in Thai, and starters for sweetened rice for isolation sources and isolated different kinds of *Acetobacter* strains, in which the occurrence of *A. indonesiensis*, *A. lovaniensis*, *A. pasteurianus*, *A. orientalis*, *A. syzygii*, and *A. farinalis* were reported (Tanasupawat *et al.,* 2011a). From the results obtained in the present study, four isolates (17%), four isolates (17%), three isolates (13%), three isolates (13%) and two isolates (9%) were isolated from Thai fruits and flowers, identified as *A. tropicalis*, *A. ghanensis*, *A. pasteurianus*, *A. indonesiensis*, and *A. orientalis*, respectively. The obtained isolates were same as the previous studies described above. Two isolates (9%) identified as *A. fabarum* were isolated from mushroom. Two

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isolates (9%) identified as *A. persici* were isolated from fruit and fermented rice. One isolate was isolated from fermented rice and identified as *A. cibinongensis.* The remaining isolates were isolated from fruit and flower, identified as *A. okinawensis* and *A. papayae.* From the results revealed that the prevalent species as *A. ghanensis*, *A. tropicalis*, *A. pasteurianus*, *A. indonesiensis* and *A. orientalis* were usually found in the isolation sources as flowers or fruits, and in Bangkok. In the present authors' experiment, additionally different kinds of *Acetobacter* species, i.e., *A. cibinongensis*, *A. fabarum*, *A. persici*, *A. okinawensis*, and *A. papayae* including the strain BCC 15839 that was the novel species, were newly reported. The newly species were obtained from other materials such as mushroom, fermented rice, or from fruit and flower in the other part of Thailand but not in Bangkok.

4. Identification and Characterization of the Novel Species in Genus *Acetobacter*

4.1 Phylogenetic analysis based on 16S rRNA, 16S-23S rRNA gene ITS and *groEL* gene sequences

In a phylogenetic tree based on 16S rRNA gene sequences of 1,343 bases derived from the neighbor-joining method, the genus *Acetobacter* was divided into two major phylogenetic groups, i.e., Group I that corresponds to the *A. aceti* group and Group II that corresponds to the *A. pasteurianus* group with a bootstrap value of 100% (Figure 40) (Yamada and Yukphan, 2008). Isolate AD25^T or BCC 15839^T was included in Group I and formed an independent cluster with a bootstrap value below 50% and quite remote from the type strains of any other species of the genus *Acetobacter*. The phylogenetic data obtained suggested that the isolate constitutes a new species within the genus *Acetobacter*.

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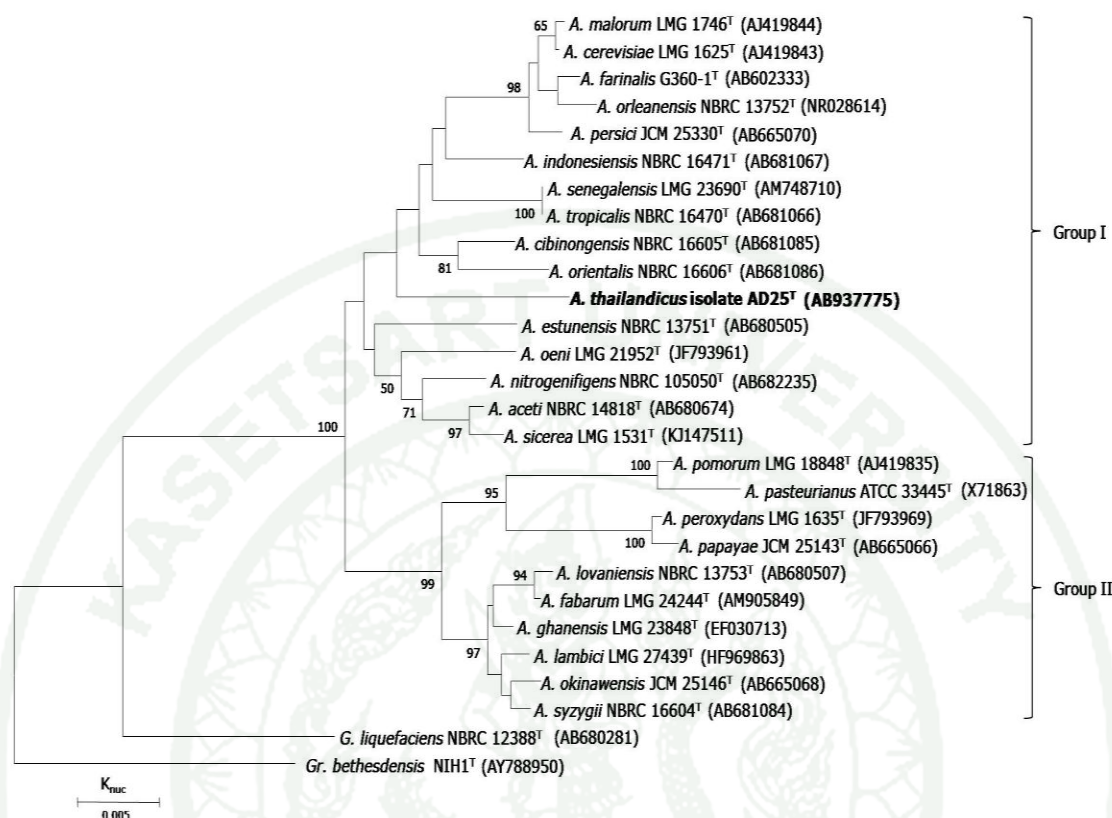

Figure 40 Phylogenetic relationships of *Acetobacter thailandicus* isolate AD25^{T}. The phylogenetic tree based on 16S rRNA gene sequences of 1,343 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. The bootstrap values below 50% were not shown. *Gluconacetobacter liquefaciens* NBRC 12388^{T} and *Granulibacter bethesdensis* NIH1^{T} were used as outgroups.

In a phylogenetic tree based on 16S-23S rRNA gene ITS sequences of 413 bases derived from the neighbor-joining method, the two major phylogenetic groups mentioned above were also found in the genus *Acetobacter* with a bootstrap value of 64% (Figure 41). However, the type strains of the four species, *A. aceti*, *A. nitrogenifigens*, *A. oeni*, and *A. estunensis*, which were once included in Group I, as well as the type strain of *Gluconacetobacter liquefaciens*, which was used as one of outgroups, were not located in the two major groups but in Sub-group I, differing from the two groups in the phylogenetic tree reported by González and Mas (2011) as well as the two groups in the phylogenetic trees of the genus *Gluconobacter* reported by Tanasupawat *et al.* (2004), by Yukphan *et al.*

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(2004) and by Malimas *et al.* (2009). Isolate AD25^T was included in Group I and formed an independent cluster with a bootstrap value of 52%.

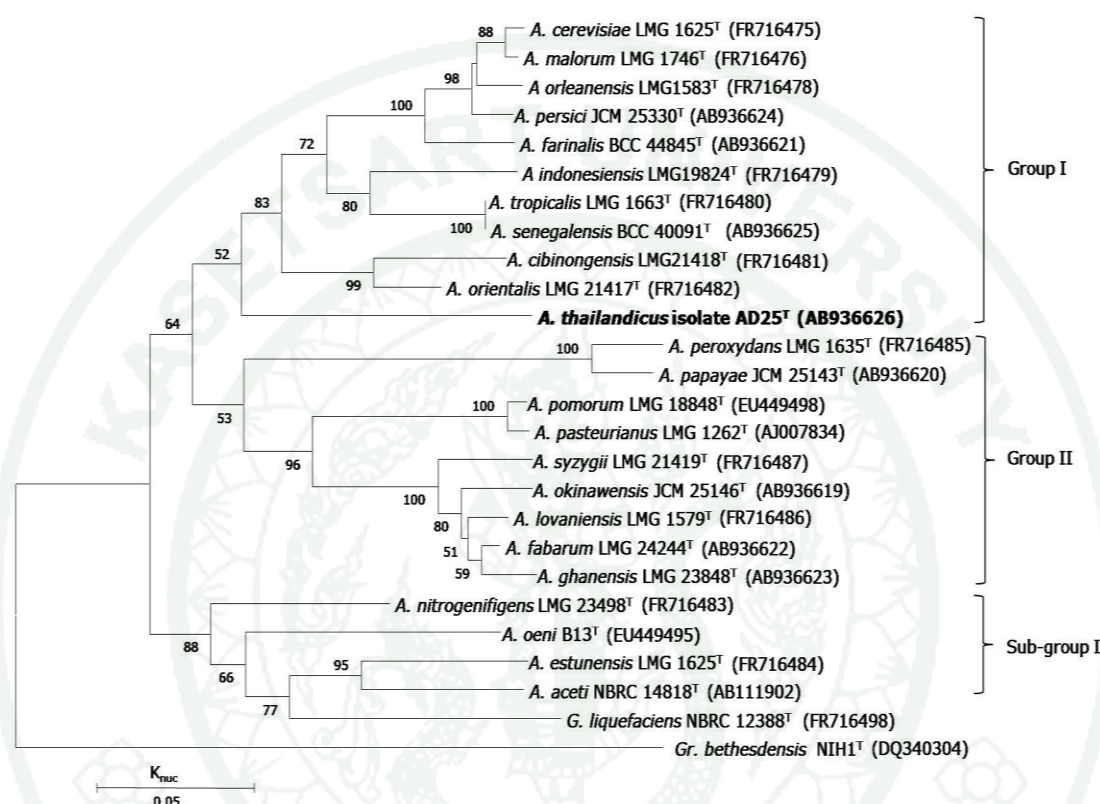

Figure 41 Phylogenetic relationships of *Acetobacter thailandicus* isolate AD25^T. The phylogenetic tree based on 16S-23S rRNA gene ITS sequences of 413 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. The bootstrap values below 50% were not shown. *Gluconacetobacter liquefaciens* NBRC 12388^T and *Granulibacter bethesdensis* NIH1^T were used as outgroups.

In a phylogenetic tree based on *groEL* gene sequences of 866 bases derived from the neighbor-joining method, the resulting two phylogenetic groups were similar to those based on 16S-23S rRNA gene ITS sequences (Figure 42). The type strains of the four species, *A. aceti*, *A. nitrogenifigens*, *A. oeni*, and *A. estunensis* were not located in the two major groups but in Sub-group I. In addition, the two species, *A. peroxydans* and *A. papayae*, which were once included in Group II, were not located in the two major groups

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but in Sub-group II. Isolate AD25^T was located in Group I and formed an independent cluster with a bootstrap value of 70%.

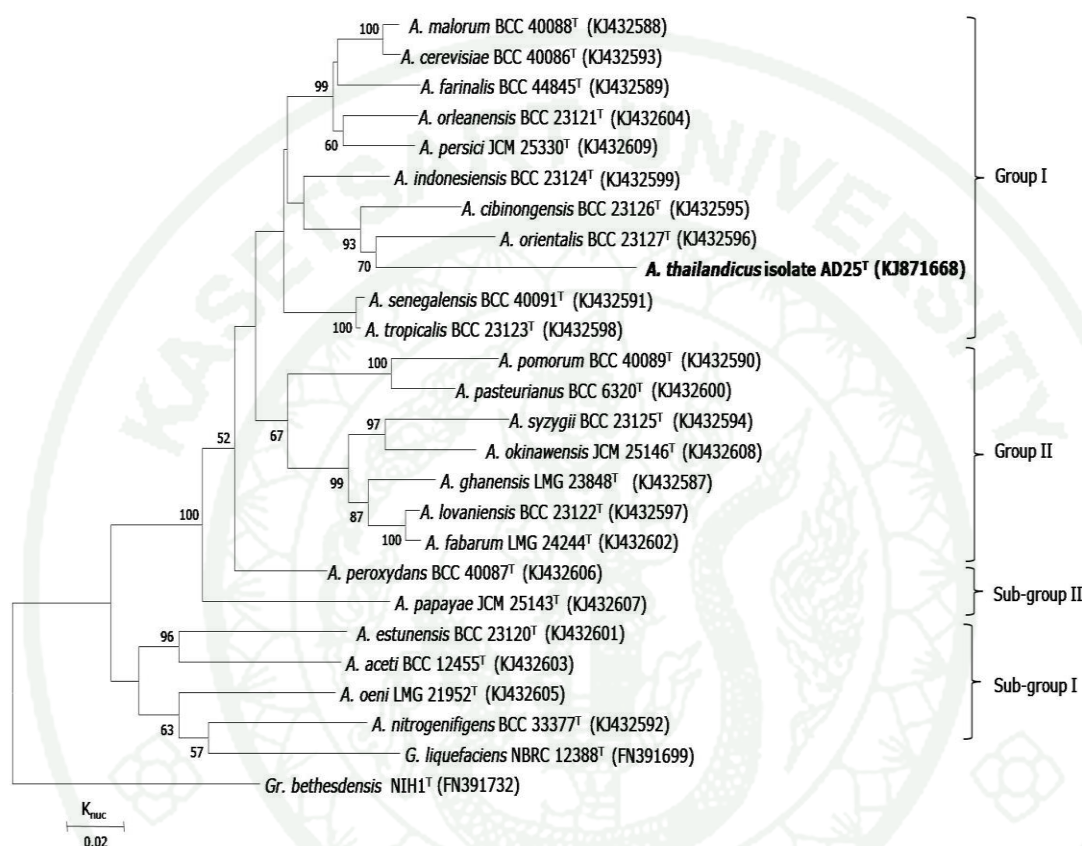

Figure 42 Phylogenetic relationships of *Acetobacter thailandicus* isolate AD25^T. The phylogenetic tree based on *groEL* gene sequences of 866 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. The bootstrap values below 50% were not shown. *Gluconacetobacter liquefaciens* NBRC 12388^T and *Granulibacter bethesdensis* NIH1^T were used as outgroups.

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4.2 16S rRNA gene, 16S-23S rRNA gene ITS and *groEL* gene sequences similarities

The calculated pair-wise 16S rRNA gene sequence similarity values without considering gaps of isolate AD25^T (BCC 15839^T) were 98.3, 98.0, 98.1, 97.8, 97.9, 97.9, 97.6, 98.2, 97.9, 97.9, 97.6, 98.0, 97.5, 97.9, 97.5, 97.5, 97.5, 97.7, 97.4, 97.0, 96.7, 96.9, 96.9, 97.7 and 97.6%, respectively to the type strains of *A. cibinongensis*, *A. indonesiensis*, *A. orientalis*, *A. aceti, A. malorum, A. cerevisiae, A. ghanensis, A. senegalensis, A. tropicalis, A. persici, A. nitrogenifigens, A. farinalis, A. fabarum, A. estunensis, A. syzygii, A. lovaniensis, A. okinawensis, A. orleanensis, A. oeni, A. pomorum, A. papayae, A. peroxydans*, *A. pasteurianus*, *A. sicerae* and *A. limbici.* Interesting is that there was none of similarity values more than 99%. The phylogenetic data obtained indicated that the isolate obviously constitutes a separate species within the genus *Acetobacter*.

In 16S-23S rRNA gene ITS sequences, the calculated pair-wise sequence similarity values without considering gaps of isolate AD25^T (BCC 15839^T) were 83.7, 81.8, 84.0, 77.7, 82.0, 82.0, 83.0, 82.3, 82.3, 81.5, 79.9, 82.8, 83.2, 77.4, 80.8, 83.5, 82.0, 82.3, 76.9, 82.5, 77.7, 78.2 and 82.3% as well, except for *A. sicerae* and *A. lambici*. Similarly, the calculated pair-wise sequence similarity values without considering gaps of the isolate AD25^T(BCC 15839^T) were somewhat high in *groEL* gene sequences, and they were 87.6, 85.6, 87.8, 80.6, 84.5, 84.0, 83.9, 85.5, 85.4, 84.6, 79.4, 83.7, 83.8, 78.9, 82.4, 83.7, 82.4, 84.0, 80.8, 83.7, 81.9, 84.5 and 85.7%, respectively.

4.3 DNA base composition and DNA-DNA hybridization

The DNA base composition of isolate AD25^T (BCC 15839^T) was 51.4 mol% G+C, which was lower in Group I or the *A. aceti* group. When a single -stranded and labeled DNAs from isolate AD25^T (BCC 15839^T) were hybridized with DNAs from test strains, the calculated DNA-DNA relatedness were 100±0.04, 18.1±0.15, 17.6±0.1 and 6.7±0.1%, respectively to isolate AD25^T, *A. orientalis* BCC 23127^T, *A. cibinongensis* BCC 23126^T and *A. tropicalis* BCC 23123^T, which were phylogenetically related. The labeled DNAs from *A. orientalis* BCC 23127^T, *A. cibinongensis* BCC 23126^T and *A. tropicalis*

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BCC 23123^T showed that the DNA-DNA relatedness were 13.2±0.1, 100±0.003, 22.9±0.1 and 5.5±0.1%, 6.8±0.15, 18.7±0.1, 100±0.01 and 5.3±0.1% and 6.8±0.06, 14±0.06, 14.4±0.06 and 100±0.01%, respectively. The genetic data obtained indicated that the isolate constitutes a separate species.

4.4 Phenotypic characteristics

The phenotypic and the chemotaxonomic characteristics were described in the species description of the isolate.

Isolate AD25^T (BCC 15839^T) was quite unique phenotypically (Table 12). In spite of being within Group I or the *A. aceti* group phylogenetically, the isolate was especially distinguished from the type strains of *A. cibinongensis* and *A. orientalis*, which are phylogenetically related, by producing only D-gluconic acid, but not 2-keto-D-gluconic acid, from D-glucose (Table 12). The isolate was also discriminated from them by intense growth on GECA medium with 10% ethanol and by weak growth on Frateur modified Hoyer medium with 3% glucose and by intense acid production from D-mannose, D-galactose and D-xylose. The weak acid production from mellibiose also differentiated the isolate from the type strains of other *Acetobacter* species except for *A. orientalis* and *A. indonesiensis*, which were distinguished from the isolate by the production of 2-keto-D-gluconic acid from D-glucose as well (Table 12).

From the experimental results obtained above, the new species can therefore be introduced in the genus *Acetobacter* with the name, *Acetobacter thailandicus* sp. nov.

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Table 12 Differential characteristics of *Acetobacter thailandicus* isolate AD25^{T} (BCC 15839^{T})

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12[b]
Production from D-glucose												
D-Gluconic acid	+	+	+	+[b]	+[c]	+[b]	+[d]	+[d]	+[b]	+[e]	+[f]	+[b]
2-Keto-D-gluconic acid	-	+	+	+[b]	+[c]	+[b]	+[d]	+[d]	+[b]	+[e]	+[f]	+[b]
5-Keto-D-gluconic acid	-	-	-	-[b]	-[c]	-[b]	-[d]	-[d]	-[b]	-[e]	+[f]	+[b]
2,5-Diketo-D-gluconic acid	-	-	-	-[b]	-[c]	-[b]	-[d]	-[d]	-[b]	-[e]	-[f]	-[b]
Growth in the presence of 10% ethanol v/v (GECA)	+	-	-	-[b]	+[c]	-[b]	-[d]	+[d]	-[b]	-[e]	+[f]	-[b]
Growth on ammoniac nitrogen (Frateur modified Hoyer medium) with D-glucose	w	-	-	-	w	+	-	-	w	+	+	-
Acid Production from												
L-Arabinose	w	-	vw	v(+)[b]	+[c]	v (+)	-[d]	-[d]	v (-)[b]	w[e]	+[f]	+[b]
D-Galactose	+	-	vw	vw	+	vw	+	+	vw	+	+	+
D-Mannose	+	-	w	w	+	+	+	-	w	+	+	+
Mellibiose	vw	-	vw	-	-	vw	-	-	-	-	-	-
D-Ribose	vw	-	-	-	-	-	-	-	-	w	w	w
D-Xylose	+	w	vw	v(+)[b]	-[c]	v(+)[b]	+[d]	+[d]	v(+)[b]	-[e]	+[f]	+[b]
Ubiquinone	Q9	Q9	Q9	Q9[b]	Q9[c]	Q9[b]	Q9[d]	Q9[d]	Q9[b]	Q9[e]	Q9[f]	Q9[b]
DNA base composition (mol%)	51.4	54.5[a]	52.3[a]	55.9[b]	56.0[c]	53.7[b]	57.6[d]	57.2[d]	56.5[b]	56.3[e]	58.7[f]	56.7[b]

Abbreviation: 1, *A. thailandicus* isolate AD25^{T}; 2, *A. cibinongensis* NBRC 16605^{T}; 3, *A. orientalis* NBRC 16606^{T}; 4, *A. tropicalis* NBRC 16470^{T}; 5, *A. senegalensis* LMG 23690^{T}; 6, *A. indonesiensis* NBRC 16471^{T}; 7, *A. cerevisiae* LMG 1625^{T}; 8, *A. malorum* LMG 1746^{T}; 9, *A. orleanensis* NBRC 13752^{T}; 10, *A. farinalis* G390-1^{T}; 11, *A. persici* JCM 25330^{T}; 12, *A. aceti* NBRC 14818^{T}; +, positive; -, negative; v, variable; vw, very weak; w, weak

Cited from [a]Lisdiyanti *et al.* (2001); [b]Lisdiyanti *et al.* (2000); [c]Ndoye *et al.* (2007); [d]Cleenwerck *et al.* (2002); [e]Tanasupawat *et al.* (2011a); [f]Iino *et al.* (2012)

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Description of *Acetobacter thailandicus* sp. nov.

Acetobacter thailandicus (tha.i.lan'di.cus. N. L. masc. adj. *thailandicus* of Thailand, where the type strain was isolated).

Cells are Gram-negative and rod-shaped, measuring 1.0× 1.6-2.6 µm and non motile. Colonies are cream, smooth, glistening, non-pigmented and raised with entire margin on glucose/ethanol/calcium carbonate agar. Grows at pH 3.0 and 3.5 at 30ºC. No growth in the presence of 30% D-glucose (w/v). Grows in the presence of 0.35% acetic acid (v/v). Grows on GECA medium with 10% ethanol and weakly on Frateur modified Hoyer medium with 3% glucose but not on the medium when 3.0% D-glucose (w/v), the carbon source was replaced by 3.0% D-mannitol (w/v) or by 3.0% ethanol (v/v). Ethanol is oxidized to acetic acid. Oxidizes acetate and lactate to carbon dioxide and water. Acetic acid is produced on ethanol/calcium carbonate agar. Catalase is positive, and oxidase is negative. D-Gluconic acid is produced from D-glucose. Unable to produce 2-keto-D-gluconic acid, 5-keto-D-gluconic acid and 2, 5-diketo-D-gluconic acid. No production of dihydroxyacetone from glycerol. Acid is produced from D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose (weakly positive), D-ribose (very weakly positive), mellibiose (very weakly positive), ethanol and 1-butanol, but not from D-fructose, L-sorbose, D-arabinose, L-rhamnose, dulcitol, glycerol, methanol, trehalose, sucrose, raffinose and starch. The major ubiquinone is Q-9. DNA G+C content is 51.4 mol% G+C.

The type strain is isolate AD25^{T} (= BCC 15839^{T} = NBRC 103583^{T}), which was isolated from a flower of blue trumpet vine (*Thunbergia laurifolia*) at Thong Pha Phum, Kanchanaburi, Thailand.

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CONCLUSION AND RECOMMENDATIONS

Conclusion

From this study, a thermotolerant acetic acid bacterium isolated from grape in Thailand, *Acetobacter pasteurianus* SKU1108 was used for characterization of *groESL* operon. The *groES* and *groEL* genes of this bacterium was cloned and sequenced. Sequencing analysis revealed two contiguous open reading frames (ORFs) of 372 nucleotides for *groES* gene and 1,641 nucleotides for *groEL* gene separated by 53 nucleotides spacer. The sequence similar to the putative σ^{32}-dependent promoter and highly regulatory CIRCE element were found at the upstream region of *groESL* operon. GroES and GroEL protein of this strain which show a high degree homology with the same proteins from *A. pasteurianus* NBRC 3283 (formerly assigned as *Acetobacter aceti* IFO 3283), consist of 123 amino acids and 546 amino acids, respectively. Overexpressed the *groESL* gene in *A. pasteurianus* SKU1108 resulted in improved resistance against stressors such as heat, acetic acid or ethanol. The *groESL* gene expression was induced by temperature shift, exposure to acetic acid or ethanol. Furthermore, transcription level of *groESL* steadily increased with elevated temperature, acetic acid and ethanol concentration. Subsequently, the potential role of *groESL* gene was investigated by construction of *groEL* gene disruptant. The growth pattern of *groEL* gene disruptant decreased and exhibited entirely lost toleration activity to stressors comparing with wild type. Complementation of *A. pasteurianus* SKU1108 *groESL* gene in a *groEL* gene disruptant restored significant tolerant activity to stressors. In *E. coli*, 40°C, 0.05% acetic acid and 5% ethanol remarkably induced *groESL* promoter activities about 1.5, 10.0 and 3.0 fold, respectively. In addition, the conserved CIRCE sequences in the upstream regulatory region of the *groESL* operon from all 20 genomes of acetic acid bacteria were analyzed. CIRCE elements were conserved in all acetic acid bacterial genome sequences, and their phylogenetic relationships could be classified by using 27 bp sequences of CIRCE elements. These sequences may be useful for the determination of the phylogenetic relationships among genera of acetic acid bacteria

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The thermotolerant and thermosensitive strains were selected from the total of 129 isolates of acetic acid bacteria. Among all isolates, ten isolates, SKU1, 7, 11, 21, 43, 45, 58, 80, 82 and 92, were the thermosensitive strains. These strains could not grow well at 40ºC when compared with other strains. PCR amplification of the partial *groESL* gene and Southern hybridization with 1.4 kb *groESL* DNA probe were performed for investigation the variation of *groESL* gene between thermotolerant and thermosensitive strains. The partial *groESL* genes from thermotolerant strains were amplified by polymerase chain reaction (PCR) using primers GF1 and GR1 corresponding to positions 97th of the *groES* gene and 1,216th of the *groEL* gene, respectively. However, these primers could not amplify the partial *groESL* gene from some acetic acid bacteria that could not grow at 40°C. Various patterns of partial *groESL* PCR product were amplified from different strains of thermotolerant and some strains of thermosensitive acetic acid bacteria. The results revealed that strains that showed the same patterns of partial *groESL* gene PCR product, are usually clustered together and the results are corresponded well with the phylogenetic analysis based on 16S rRNA gene sequences. The pattern of signals from Southern hybridization could not show the *groESL* gene variation among the strains of thermotolerant and thermosensitive acetic acid bacteria. The positive signal of each strain was detected depending on the DNA fragment size harboring *groESL* gene. The Southern hybridization results were correlative with the results of partial *groESL* gene amplification. The *groESL* gene variation was not found among the thermotolerant and thermosensitive acetic acid bacterial strains.

The *groEL* gene sequences were used as an additional phylogenetic marker for the classification of acetic acid bacteria together with 16S rRNA gene. Twenty-four strains isolated in Thailand by an enrichment culture approach were classified into genus *Acetobacter* by using *groEL* gene and 16S rRNA gene sequences analysis. The topology of *groEL* gene and 16S rRNA gene-based trees was comparable in the phylogenetic trees from *Acetobacter* group strains in this study. A phylogram generated from the *groEL* gene sequences of the *Acetobacter* group correlates with the phylogenetic relationships inferred from the 16S rRNA gene sequence and supports the previous data that the genus *Acetobacter* could be organized into two main groups. On the basis of the 16S rRNA gene sequence and the additional *groEL* gene sequence analyses, the strains isolated were

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divided into ten groups. Group 1 was comprised of four isolates, which were identified as *A. tropicalis*, Group 2 was of three isolates, which were as *A. indonesiensis*, Group 3 was of two isolates, which were as *A. persici*, Group 4 was of two isolates, which were as *A. orientalis*, Group 5 was of one isolate, which was as *A. cibinongensis*, Group 6 was of three isolates, which were as *A. pasteurianus*, Group 7 was of one isolate, which was as *A. papayae*, Group 8 was of two isolates, which were as *A. fabarum*, Group 9 was of one isolate, which was as *A. okinawensis*, and Group 10 was of four isolates, which were as *A. ghanensis*. Beside 10 groups, isolate AD25^{T} (= BCC 15839^{T}) was found to exist at an independent position in phylogenetic trees constructed based on 16S rRNA and *groEL* gene sequences. The topology of both phylogenetic trees showed that the isolate AD25^{T} (= BCC 15839^{T}) was new species, which constituted a cluster separate from the type strains of *A. orientalis* and *A. cibinongensis.*

On the basis of polyphasic taxonomy combining DNA base composition, DNA-DNA relatedness, 16S rRNA, 16S-23S rRNA gene ITS and *groEL* gene sequences analysis including phenotypical and biochemical analysis, the isolate AD25^{T} (= BCC 15839^{T} = NBRC 103583^{T}), which was isolated from a flower of blue trumpet vine, the new species of the genus *Acetobacter* is proposed: *Acetobacter thailandicus* sp. nov.

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Recommendations

Based on those conclusions, there are some points about the *groEL* gene disruptant should be clarified as follows. It is important to prove the GroEL protein expression of the *groEL* gene disruptant under stress condition. Moreover, the global protein expression should be performed for check the protein expression profile of the *groEL* gene disruptant under stress condition. Some gene might be up-regulated or down-regulated. Finally, it is hoped that the results obtained from this study will not only provide basic knowledge in understanding the function of heat shock proteins but also benefit to the basic knowledge implicated to strain improvement for industrial vinegar production.

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LITERATURE CITED

Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita and M. Ameyama. 1978. 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. 2003. New quinoproteins in oxidative fermentation. **Biochem. Biophys. Acta.** 1447: 10-17.

Aiba, H., Adhya, S and de Crombrugghe, B. 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. **J. Biol. Chem**. 256: 11905-11910.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Res.** 25: 3389-3402.

Ameyama, M. and O. Adachi. 1982a. Alcohol dehydrogenase from acetic acid bacteria, membrane-bound. **Methods Enzymol.** 89: 450-457.

________ and ________. 1982b. Aldehyde dehydrogenase from acetic acid bacteria, membrane-bound. **Methods Enzymol.** 89: 450-457.

Andrés-Barrao, C., L. Falquet, S.P. Calderon-Copete, P. Descombes, R. Ortega Pérez and F. Barja. 2011. Genome sequences of the high acetic acid resistant bacteria *Gluconacetobacter europaeus* LMG 18890^T and *G. europaeus* LMG 18494 (reference strains), *G. europaeus* 5P3, and *Gluconacetobacter oboediens* 174Bp2 (isolated from vinegar). **J. Bacteriol.** 193: 2670-2671.

Asai, T. 1934. Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits. A new classification of the oxidative bacteria. **J. Agric. Chem. Soc. Jpn.** 10: 621-629. (In Japanese)

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Asai, T. 1935. Taxonomic studies on acetic acid bacteria and allied oxidative bacteria isolated from fruits. A new classification of the oxidative bacteria. **J. Agric. Chem. Soc. Japan.** 11: 674-708. (In Japanese)

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

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.

Bartowsky, E.J., D. Xia, R.L. Gibson, G.H. Fleet and P.A. Henschke. 2003. Spoilage of bottled red wine by acetic acid bacteria. **Lett. Appl. Microbiol.** 36: 307–314.

Beijerinck, M.W. 1898. **Uber die Arten der Essigbakterian. Zentralblatt fur Bakteriologie, Parasitenkunde. Infektionskrankheiten and hygiene**, 2Abt. 4, 209-216.

Bertalan, M., R. Albano, V. de Pádua, L. Rouws, C. Rojas, A. Hemerly, K. Teixeira, S. Schwab, J. Araujo, A. Oliveira and other 51 authors. 2009. Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. **BMC Genomics.** 10: 450.

Blaiotta, G., V. Fusco, E. Danilo, M. Aponte, O. Pepe and F. Villani. 2008. *Lactobacillus* strain diversity based on partial hsp60 gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. **Appl. Environ. Microbiol.** 74: 208-215.

Copyright by Kasetsart University All rights reserved

Brady, C., I. Cleenwerck, S. Venter, M. Vancanneyt, J. Swings and T. Coutinho. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). **Syst. Appl. Microbiol.** 31: 447-460.

Braig, K. 1998. Chaperonins. **Curr. Opin. Struct. Biol.** 8: 159-165.

Brenner, D.J., G.R. Fanning, A.V. Rake and K.E. Johnson. 1969. Batch procedure for thermal elution of DNA from hydroxyapatite. **Anal. Biochem.** 28: 447-459.

Bukau, B. and A.L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. **Cell.** 92: 351-366.

Chang, L.J., W.H. Chen, F.C. Minion and D. Shiuan**.** 2008**.** Mycoplasmas regulate the expression of heat-shock protein genes through CIRCE–HrcA interactions. **Biochem. Biophys. Res. Commun.** 367(1): 213-218.

Chang Y.H., Y.H. Shangkuan, H.C. Lin and H.W. Liu. 2003. PCR Assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. **Appl. Environ. Microbiol.** 69: 4502-4510.

Chapman, E., G.W. Farr, R. Usaite, K. Furtak, W.A. Fenton, T.K. Chaudhuri, E.R. Hondorp, R.G. Matthews, S.G. Wolf, J.R. Yates, M. Pypaert and A.L. Horwich. 2006. Global aggregation of newly translated proteins in an *Escherichia coli* strain deficient of the chaperonin GroEL. **PNAS.** 103: 15800-15805.

Chaurasia, A.K. and S.K. Apte. 2009. Overexpression of the *groESL* operon enhances the heat and salinity stress tolerance of the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. **Appl. Environ. Microbiol.** 75: 6008-6012.

Copyright by Kasetsart University All rights reserved

Chen H.J., J.C. Tsai, T.C. Chang, W.C. Hung, S.P. Tseng, P.R. Hsueh and L.J. Teng. 2008. PCR-RFLP assay for species and subspecies differentiation of the *Streptococcus bovis* group based on *groESL* sequences. **J. Med. Microbiol.** 57: 432-438.

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

Cleenwerck, I., K. Vandemeulebroecke, D. Janssens and J. Swing. 2002. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. **Int. J. Syst. Evol. Microbiol.** 52: 1551-1558.

______, N. Camu, K. Engelbeen, T. De Winter, K. Vandemeulebroecke, P. De Vos and L. De Vuyst. 2007. *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. **Int. J. Syst. Evol. Microbiol.** 57: 1647-1652.

______, A. Gonzalez, N. Camu, K. Engelbeen, P. De Vos and L. De Vuyst. 2008. *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. **Int. J. Syst. Evol. Microbiol.** 58: 2180-2185.

______ and P. De Vos. 2008. Polyphasic taxonomy of acetic acid bacteria: An overview of the currently applied methodology. **Int. J. Food Microbiol.** 125: 2-14.

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

Copyright by Kasetsart University All rights reserved

Colwell, R.R. 1970. Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus* and related *Vibrio* species. **J. Bacteriol.** 104: 410-433.

Corcoran, B.M., R.P. Ross, G.F. Fitzgerald, P. Dockery and C. Stanton. 2006. Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. **Appl. Environ. Microbiol.** 72: 5104-5107.

Cristiana, C., R.A. Siciliano, L. Muscariello, R. Marasco and M. Sacco. 2006. CcpA affects expression of the *groESL* and *dnaK* operons in *Lactobacillus plantarum*. **Microb. Cell Fact.** 5(35): 1-7.

Crooks, G.E., G. Hon, J.M. Chandonia and S.E. Brenner. 2004. WebLogo: a sequence logo generator. **Genome Res.** 14: 1188-1190.

Crosa, J.H., D.J. Brenner and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and hetero-duplexs. **J. Bacteriol.** 115: 904-911.

De Bruyne, K., U. Schillinger, L. Caroline, B. Boehringer, I. Cleenwerck, M. Vancanneyt, L. De Vuyst, C.M.A.P. Franz and P. Vandamme. 2007. *Leuconostoc holzapfelii* sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of *Leuconostoc* species. **Int. J. syst. Evol. Microbiol.** 57: 2952-2959.

De Ley, J., H. Cattoir and A. Reynaerts. 1970. The quantitative measurement of DNA hybridization from renaturation rates. **Eur. J. Biochem.** 12: 133-142.

______, M. Gillis and J. Swings. 1984a. In: N.R. Krieg, J.G. Holt (Eds.), Family VI. *Acetobacteraceae*, 1th Ed. **Bergey's Manual of Systematic Bacteriology**, Vol. 1. Williams and Wilkins Co, Baltimore, Maryland, pp. 267-278.

Copyright by Kasetsart University All rights reserved

De Ley, J., J. Swing and F. Gossle. 1984b. Key to the genera of the family *Acetobacteriaceae*, pp. 267-278. *In* N. Krieg and J.G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology.** Vol.1. The Williams and Wilkin Co., Baltimore.

Desmond, C., G.F. Fitzgerald, C. Stanton and R.P. Ross. 2004. Improved stress tolerance of GroESL-overproducing *Lactococcus lactis* and probiotic *Lactobacillus paracasei* NFBC 338. **Appl. Environ. Microbiol.** 70: 5929-5936.

Devereux, R. and S.G. Wills. 1995. Amplification of ribosomal RNA sequences. pp. 3.3.1-3.3.2. *In* A.D.L. Akkermans, J.D. van Elsas and F.J. De Bruijn, eds. **Molecular Microbial Ecology Manual.** Academic Publishers, Dordrecht.

Du, X.J., S.R. Jia, Y. Yang and S. Wang. 2011. Genome sequence of *Gluconacetobacter* sp. strain SXCC-1, isolated from Chinese vinegar fermentation starter. **J. Bacteriol.** 193: 3395-3396.

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

Eisen, J.A. 1995. The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. **J. Mol. Evol.** 41:1105-1123.

Ezaka, E., J. Teyssie, S. Ouahrani-Bettache, J.P. Liautard and S. Köhler. 2001. Characterization of *Burucella suis clpB* and *clpAB* mutants and participation of the genes in stress responses. **J. Bacteriol.** 183: 2677-2681.

Ezaki, T., Y. Hashimoto and E. Yabuuchi. 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. **Int. J. Syst. Bacteriol.** 39: 224-229.

Copyright by Kasetsart University All rights reserved

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. **Evolution** 39: 783-791.

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.

Fuentes-Ramírez, L.E., R. Bustillos-Cristales, A. Tapia-Hernandez, T. Jimenez-Salgado, E.T. Wang, E. Martinez-Romero and J. Caballero-Mellado. 2001. Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov., associated with coffee plants. **Int. J. Syst. Evol. Microbiol.** 51: 1305–1314.

Fukaya, M., H. Takemura, H. Okumura, Y. Kawamura, S. Horinouchi and T. Beppu. 1990. Cloning of genes responsible for acetic acid resistance in *Acetobacter aceti*. **J. Bactereriol**. 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.

Georgopoulos, C. and W.J. Welch. 1993. Role of the major heat shock proteins as molecular chaperones. **Annu. Rev. Cell. Biol.** 9: 601-634.

Gevers, D., F.M. Cohan, J.G. Lawrence, B.G. Spratt, T. Coenye, E.J. Feil, E. Stackebrandt, Y. Van de Peer, P. Vandamme, F.L. Thompson and J. Swings. 2005. Re-evaluating prokaryotic species. **Nat. Rev. Microbiol.** 3: 733-739.

Gilbert H.F. 1994. Protein chaperones and protein folding. **Curr. Opin. Biotechnol**. 5: 534–539.

Copyright by Kasetsart University All rights reserved

Glazunova, O.O., D. Raoult and V. Roux. 2009. Partial sequence comparison of the *rpoB*, *sodA*, *groEL* and *gyrB* genes within the genus *Streptococcus*. **Int. J. Syst. Evol. Microbiol.** 59: 2317-2322.

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.

González, A., N. Hierro, M. Poblet, N. Rozes, A. Mas and J.M. Guillamon. 2004. Application of molecular methods for the differentiation of acetic acid bacteria in a red wine fermentation. **J. Appl. Microbiol.** 96: 853–860.

_______, N. Hierro, M. Poblet, A. Mas and J.M. Guillamón. 2005. Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. **Int. J. Food Microbiol.** 102: 295–304.

_______, J.M. Guillamón, A. Mas and M. Poblet. 2006. Application of molecular methods for routine identification of acetic acid bacteria. **Int. J. Food Microbiol.** 108: 141–146.

_______ and A. Mas. 2011. Differentiation of acetic acid bacteria based on sequence analysis of 16S-23S rRNA gene internal transcribed spacer sequences. **Int. J. Food Microbiol.** 147: 217-222.

Gosselé, J., J. Swings and J. De Ley. 1980. A rapid, simple and simultaneous detection of 2-keto, 5-keto- and 2,5-diketogluconic acid by thin layer chromatography in culture media of acetic acid bacteria. **Zbl. Bakt. Hyg. I. Abt. Orig.** C: 178-181.

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

Copyright by Kasetsart University All rights reserved

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

Greenberg, 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.

Gullo, M., C. Caggia, L. De Vero and P. Giudici. 2006. Characterization of acetic acid bacteria in “traditional balsamic vinegar”. **Int. J. Food Microbiol.** 106: 209–212.

_______. and Giudici, P. 2009. Acetic acid bacteria taxonomy from early descriptions to molecular techniques, *In*: Solieri, L. and P. Giudici (Eds.) **Vinegar of the worlds**, Springer, Milan, pp. 41-56.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symp. Ser.** 41: 95–98.

Hartl, F.U. and J. Martin. 1995. Molecular chaperones in cellular protein folding. **Curr. Opin. Struct. Biol.** 5: 92-102.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. **Nature**. 381: 571–580.

Hattori, H., T. Yakushi, M. Matsutani, D. Moonmangmee, H. Toyama, O. Adachi and K. Matsushita. 2012. High-temperature sorbose fermentation with thermotolerant *Gluconobacter frateurii* CHM43 and its mutant strain adapted to higher temperature. **Appl. Microbiol. Biotechnol.** 95: 1531-1540.

Copyright by Kasetsart University All rights reserved

Huang, C.H., M.T. Chang, L. Huang and W.S. Chua. 2014. Molecular discrimination and identification of *Acetobacter* genus based on the partial heat shock protein 60 gene (*hsp60*) sequences. **J. Sci. Food Agric.** 94: 213-218.

Hucker, G.J. and H.J. Conn. 1923. Method of gram staining. **Tech. Bull NY St. Agric. Exp. Stn.** 93: 3-37.

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

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

Ishikawa, M., A. Okamoto-Kainuma, T. Jochi, I. Suzuki, K. Matsui, T. Kaga and Y. Koizumi. 2010a. Cloning and characterization of *grpE* in *Acetobacter pasteurianus* NBRC 3283. **J. Biosci. Bioeng.** 109: 25-31.

_______, _______, K. Matsui, A. Takigishi, T. Kaga and Y. Koizumi. 2010b. Cloning and characterization of *clpB* in *Acetobacter pasteurianus* NBRC 3283. **J. Biosci. Bioeng.** 110: 69-71.

Iyer, P.R., S.M. Geib, J. Catchmark, T.H. Kao and M. Tien. 2010. Genome sequence of a cellulose-producing bacterium, *Gluconacetobacter hansenii* ATCC 23769. **J. Bacteriol.** 192: 4256-4257.

Jian, W., L. Zhu and X. Dong. 2001. New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. **Int. J. Syst. Evol. Microbiol.** 51: 1633-1638.

Copyright by Kasetsart University All rights reserved

Johnson, J. L. 1985. DNA reassociation and RNA hybridization of bacterial nucleic acids. **Methods Microbiol.** 28: 33-74.

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.

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.

Kärenlampi, R.I., T.P. Tolvanen and M-L. Hänninen. 2004. Phylogenetic analysis and PCR-restriction fragment length polymorphism identification of *Campylobacter* species based on partial *groEL* gene sequences. **J. Clin. Microbiol.** 42: 5731-5738.

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.

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

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. **J. Mol. Evol.** 16: 111-120.

Komagata, K. and K. Suzuki. 1987. Lipid and cell wall analysis in bacterial systematic. **Method Microbiol.** 19: 161–206.

Copyright by Kasetsart University All rights reserved

Kommanee, J., A. Akaracharanya, S. Tanasupawat, T. Malimas, P. Yukphan, Y. Nakagawa and Y. Yamada. 2008. Identification of *Acetobacter* strains isolated in Thailand based on 16S-23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses. **Ann. Microbiol.** 58: 319-324.

Kondo, K., T. Beppu and 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.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. Mc William, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. **Bioinformatics.** 23: 2947-2948.

Lasko, 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.

Lee, J.H., H.S. Park, W.J. Jang, S.E. Koh, J.M. Kim, S.K. Shim, M.Y. Park, W.Y. Kim, B.J. Kim, Y.H. Kook, K.H. Park and S.H. Lee. 2003. Differentiation of Rickettsiae by *groEL* gene analysis. **J. Clin. Microbiol.** 41: 2952-2960.

Legault-Démare, J., B. Desseaux, T. Heyman, S. Séror and G.P. Ress. 1967. Studies on hybrid molecules of nucleic acids. I. DNA-DNA hybrids on nitrocellulose filters. **Biochem. Biophys. Res. Commun.** 28: 550-557.

Lemaux, P.G., S.L. Herendeen, P.L. Bloch and F.C. Neidhardt. 1978. Transient rates of synthesis of individual polypeptides in *E. coli* following temperature shifts. **Cell.** 13: 427-434.

Copyright by Kasetsart University All rights reserved

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

Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura and K. Komagata. 2000 Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg, 1996) comb. nov., *Acetobacter lovaniensis* (Frateur, 1950) comb. nov., and *Acetobacter estunensis* (Carr, 1958) comb. nov. **J. Gen. Appl. Microbiol.** 46: 147-165.

_______, _______, _______, _______, _______ and _______. 2001 Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov. **J. Gen. Appl. Microbiol.** 47: 119-131.

______, ______, 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-*Proteobacterium.* **Int. J. Syst. Evol. Microbiol.** 50: 823-829.

______, K. Katsura, W. Potacharoen, R.R. Navarro, Y. Yamada, T. Uchimura and K. Komagata. 2003. Diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines. **Microbiol. Cult. Coll.** 19: 91-98.

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

Copyright by Kasetsart University All rights reserved

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

______, ______, M. Takahashi, Muramatsu M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2009) *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the α-*Proteobacteria*. **Int. J. Syst. Evol. Microbiol.** 59: 466-471.

______, W. Chaipitakchonlatarn, H. Thi Lan Vu, P. Yukphan, Y. Muramaysu, S. Tanasupawat, W. Potacharoen, Y. Nakagawa, M. Tanticharoen and Y. Yamada. 2013. *Swingsia samuiensis* gen. nov., sp. nov., an osmotolerant acetic acid bacterium in the α-*Proteobacteria*. **J. Gen. Appl. Microbiol.** 59: 375-384.

Marmur, J. and P. Doty. 1962. Determination of the base composition of deoxy-ribonucleic acid from its thermal denaturation temperature. **J. Mol. Biol.** 5: 109-118.

Martin, J. 1997. Molecular chaperones and mitochondrial protein folding. **J. Bioenerg. Biomembr.** 29: 35-43.

Martinez-Salazar, J.M., M. Sandoval-Calderon, X. Guo, S. Castillo-Ramirez, A. Reyes, M.G. Loza, J. Rivera, A. Alvarado-Affantranger, F. Sanchez, V. Gonzalez, G. Devila and M.A. Ramirez-Romero. 2009. The *Rhizobium etli* RpoH and RpoH2 sigma factors are involved in different stress responses. **Microbiology.** 155: 386-397.

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

Copyright by Kasetsart University All rights reserved

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.** 138: 39-49.

______, ______, and ______. 2011. Molecular cloning and characterization of two inducible NAD^+-*adh* genes encoding NAD^+-dependent alcohol dehydrogenase from *Acetobacter pasteurianus* SKU1108. **J. Biosci. Bioeng.** 112: 422-431.

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

______, H. Ebisuya, M. Ameyama and O. Adachi. 1992a. Change of the terminal oxidase from cytochrome *a1* 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.

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

______, 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.). **Advanced in microbial Physiology.** Vol. 36, Academic Press, London.

Copyright by Kasetsart University All rights reserved

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

______, ______, H. Toyama, E. Shinagawa and O. Adachi. 1996. Function of multiple heme c moieties in intramolecular electron transport and ubiquinone reduction in the quinohemoprotein alcohol dehydrogenase-cytochrome c complex of *Gluconobacter suboxydans*. **J. Biol. Chem.** 271: 4850-4857.

______, T. Inoue, G. theeragool, J. Trček, H. Toyoma 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(13): 4346–4352.

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

Matsutani, M., H. Hirakawa, M. Nishikura, W. Soemphol, I.A. Ali, T. Yakushi and K. Matsushita. 2011. Increased number of arginine-based salt bridges contributes to the thermotolerance of thermotolerant acetic acid bacteria, *Acetobacter tropicalis* SKU1100. **Biochem. Biophys. Res. Commun.** 409: 120-124.

Copyright by Kasetsart University All rights reserved

Matsutani, M., H. Hirakawa, N. Saichana, W. Soemphol, T. Yakushi and K. Matsushita. 2012. Genome-wide phylogenetic analysis of differences in thermotolerance among closely related *Acetobacter pasteurianus* strains. **Microbiology.** 158: 229-239.

________, M. Nishikura, N. Saichana, T. Hatano, U. Masud-Tippayasak, G. Theeragool, T. Yakushi and K. Matsushita. 2013. Adaptive mutation of *Acetobacter pasreurianus* SKU1108 enhances acetic acid fermentation ability at high temperature. **J. Biotechnol.** 165: 109-119.

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.

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.

Muramatsu, Y., P. Yukphan, M. Takahashi, M. Kaneyasu, T. Malimas, W. Potacharoen, Y. Yamada, Y. Nakagawa, M. Tanticharoen and K. Suzuki. 2009. 16S rRNA gene sequences analysis of acetic acid bacteria isolated from Thailand. **Microbiol. Cult. Coll.** 25: 13-20.

Nakano, S., M. Fukaya and S. Horinouchi. 2004. Enhanced expression of aconitase raises 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: 479-505.

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

Copyright by Kasetsart University All rights reserved

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

Naser, S.M., F.L. Thompson, B. Hoste, D. Gevers, P. Dawyndt, M. Vancanneyt and J. Swings. 2005. Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. **Microbiology.** 151: 2141-2150.

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

Ndoye, B., 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. Tech.** 36: 916 – 923.

_____, I. Cleenwerck, K. Engelbeen, R. Dubois-Dauphin, A.T. Guiro, S. Van Trappen, A. Willems and P. Thonart. 2007. *Acetobacter senegalensis* sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (*Mangifera indica* L.). **Int. J. Syst. Evol. Microbiol.** 57: 1576-1581.

Ogino, H., Y. Azuma, A. Hosoyama, H. Nakazawa, M. Matsutani, A. Hasegawa, K. Otsuyama, K. Matsushita, N. Fujita and M. Shirai. 2011. Complete genome sequence of NBRC 3288, a unique cellulose-nonproducing strain of *Gluconacetobacter xylinus* isolated from vinegar. **J. Bacteriol.** 193: 6997-6998.

Copyright by Kasetsart University All rights reserved

Okamoto-Kainuma, A, Y. Wang, S. Kadono, K. Tayama, Y. Koizumi and F. Yanagida. 2002. Cloning and characterization of *groESL* operon in *Acetobacter aceti*. **J. Biosci. Bioeng.** 94: 140–147.

_____, W. Yan, M. Fukaya, Y. Tukamoto, M. Ishikawa and Y. Koizumi. 2004. Cloning and characterization of the *dnaKJ* operon in *Acetobacter aceti*. **J. Biosci. Bioeng.** 97: 339-342.

_____, Y. Ehata, M. Ikeda, T. Osono, M. Ishikawa, T. Kaga, Y. Koizumi. 2008. Hydrogen peroxide resistance of *Acetobacter pasteurianus* NBRC 3283 and its relationship to acetic acid fermentation. **Biosci. Biotechnol. Biochem.** 72: 2526-2534.

_____, M. Ishikawa, H. Nakamura, S. Fukazawa, N. Tanaka, K. Yamakami and Y. Koizumi. 2011. Characterization of *rpoH* in *Acetobacter pasteurianus* NBRC 3283. **J. Biosci. Bioeng.** 111: 429-432.

Okumura, H., T. Uozumi and T. Beppu. 1985. Construction of plasmid vectors and a genetic transformation system for *Acetobacter aceti*. **Agric. Biol. Chem.** 49: 1011-1017.

Poblet, M., N. Rozès, J.M. Guillamón and A. Mas. 2000. Identification of acetic acid bacteria by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. **Lett. Appl. Microbiol.** 31: 63–67.

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.

Rajaram, H and S.K. Apte. 2003. Heat shock response and its contribution to thermotolerance of the nitrogen fixing cyanobacterium *Anabaena sp.* strain L-31. **Arch. Microbiology.** 179: 423-429.

Copyright by Kasetsart University All rights reserved

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

Roh, S.W., Y.D. Nam, H.W. Chang, K.H. Kim, M.S. Kim, J.H. Ryu, S.H. Kim, W.J. Lee and J.W. Bae. 2008. Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. **Appl. Environ. Microbiol.** 74: 6171 – 6177.

Ruiz, A., M. Poblet, A. Mas and J.M. Guillamon. 2000. Identification of acetic acid bacteria by RFLP of PCR amplified 16S rDNA and 16S-23S rDNA intergenic spacer. **Int. J. Syst. Evol. Microbiol.** l50: 1981-1987.

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

Saito, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. **Mol. Biol. Evol.** 4: 406-425.

Saitou, H. and K. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. **Biochim. Biophys. Acta.** 72: 619-629.

Sakurai, K., H. Arai, M. Ishii and Y. Igarashi. 2011. Transcriptome response to different carbon sources in *Acetobacter aceti*. **Microbiology.** 157: 899-910.

_____, _____, _____ and _____. 2012. Changes in the gene expression profile of *Acetobacter aceti* during growth on ethanol. **J. Biosci. Bioeng.** 113: 343-348.

_____, S. Yamazaki, M. Ishii, Y. Igarashi and H. Arai. 2013. Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. **J. Biosci. Bioeng.** 115: 32-36.

Copyright by Kasetsart University All rights reserved

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

Schleifer, K-H. and E. Stakebrand. 1983. Molecular systematic of prokaryotes. **Annu. Rev. Microbiol.** 37: 143-187.

Schumann, W. 2007. Thermosensors in eubacteria: role and evolution. **J. Biosci.** 32: 549-557.

Seearunruangchai, A., S. Tanasupawat, S. Keeratipibul, C. Thawai, T. Itoh and Y. Yamada. 2004. Identification of acetic acid bacteria isolated from fruits collected in Thailand. **J. Gen. Appl. Microbiol.** 50: 47-53.

Segal, G. and Ron, E.Z. 1995. The *dnaKJ* operon of *Agrobacterium tumefaciens*: transcriptional analysis and evidence for a new heat shock promoter. **J. Bacteriol.** 177: 5952-5958.

_____ and _____.1996a. Heat shock activation of the *groESL* operon of *Agrobacterium tumefaciens* and the regulatory roles of the inverted repeat. **J. Bacteriol.** 178: 3634–3640.

_____ and _____.1996b. Regulation and organization of the *groE* and *dnaK* operons in Eubacteria. **FEMS Microbiol. Lett.** 138: 1-10.

Shin, S.C., S.H. Kim, H. You, B. Kim, A.C. Kim, K.A. Lee, J.H. Yoon, J.H. Ryu, W.J. Lee. 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. **Science.** 334: 670-674.

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.

Copyright by Kasetsart University All rights reserved

Sievers, M., W. Lugwig and M. Teuber. 1994. Phylogenetic positioning of *Acetobacter*, *Gluconobacter*, *Rhodopila* and *Acidiphilium* species as a branch of acidophilic bacteria in the α-subclass of *Proteobacteria* based on 16S ribosomal DNA sequences. **Syst. Appl. Microbiol.** 17: 189-196.

______ and J. Swings. 2005. Family *Acetobacteraceae*. **In Bergey's manual of systematic Bacteriology**, 2nd ed., vol. 2, pp. 41-95. Edited by G.M. Garrity., New York: Springer.

Sigler, P.B., Z. Xu, H.S. Rye, S.G. Burston, W.A. Fenton and A.L. Horwich. 1998. Structure and function in GroEL-mediated protein folding. **Annu. Rev. Biochem.** 67: 581–608.

Silva, L.R., I. Cleenwerck, R. Rivas, J. Swings, M.E. Trujillo, A. Willems and E. Velázquez. 2006. *Acetobacter oeni* sp. nov., isolated from spoiled red wine. **Int. J. Syst. Evol. Microbiol.** 56: 21-24.

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

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

Skerman, V.B.D., V. Mc Gowan, P.H.A. Sneath. 1980. Approved Lists of Bacterial Names. **Int. J. Syst. Bacteriol.** 30: 225-420.

Soemphol, W., A. Deeraksa, M. Matsutani, T. Yakushi, H. Toyama, O. Adachi, M. Yamada and K. Matsushita. 2011. Global analysis of the genes involved in the thermotolerance mechanism of thermotolerant *Acetobacter tropicalis* SKU1100. **Biosci. Biotechnol. Biochem.** 75: 1921-1928.

Copyright by Kasetsart University All rights reserved

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

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

Squires, C.L., S. Pedersen, B.M. Ross and C. Squires. 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. **J. Bacteriol.** 173: 4254-4262.

Stackebrandt, E. and B.M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. **Int. J. Syst. Bacteriol.** 44: 846-849.

______, R.G.E. Murray and H.G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives". **Int. J. Syst. Bacteriol.** 38: 321-325.

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

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

Swings, J. 1992. The genera *Acetobacter* and *Gluconobacter*, In: Balows A., H.G. Trüper, M. Dworkin, W. Harder, H-K. Schleifer (Eds.) **The prokaryotes, A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications**, vol.III, Springer, New York, pp. 2268-2286.

Copyright by Kasetsart University All rights reserved

Takahashi, M, P. Yukphan, Y. Yamada, K. I. Suzuki, T. Sakane and Y. Nakagawa. 2006. Intrageneric structure of the genus *Gluconobacter* analyzed by the 16S rRNA gene and 16S-23S rRNA gene internal transcribed spacer sequences. **J. Gen. Appl. Microbiol.** 52: 187-193.

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

Tamaoka, J., Y. Katayama-Fujimura and H. Kuraishi. 1983. Analysis of bacterial menaquinone mixtures by high-performance liquid chromatography. **J. Appl. Bacteriol.** 54: 31–36.

______. and K. Komagata. 1984. Determination of DNA base composition by reversed-phase high performance liquid chromatography. **FEMS Microbiol. Lett.** 25: 125-128.

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

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

______, J. Kommanee, T. Malimas, P. Yukphan, Y. Nakagawa and Y. Yamada. 2009. Identification of *Acetobacter*, *Gluconobacter*, and *Asaia* strains isolated in Thailand based on 16S-23S rRNA gene internal transcribed spacer restriction and 16S rRNA gene sequence analyses. **Microbs. Environ.** 24: 135-143.

Copyright by Kasetsart University All rights reserved

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

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

Tayama, K., M. Fukaya, H. Okumura, 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.

Teng, J.L., P.R. Hsueh, J.C. Tsai, P.W. Chen, J.C. Hsu, H.C. Lai, C.N. Lee and S.W. Ho. 2002. *groESL* sequence determination, phylogenetic analysis, and species differentiation for Viridans Group Streptococci. **J. Clin. Microbiol.** 40: 3172-3178.

Teng, L.I., P.R. Hsueh, Y.H. Wang, H.M. Lin, K.T. Luh and S.W. Ho. 2001. Determination of *Enterococcus faecalis groESL* full-length sequence and application for species identification. **J. Clin. Microbiol.** 39: 3326-3331.

Teuber, M., M. Sievers and A. Andresen. 1987. Characterization of the microflora of high acid submerged vinegar fermentars by distinct plasmid profiles. **Biotechnol. Lett.** 9: 265–268.

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. p. 23.

Copyright by Kasetsart University All rights reserved

Thi Lan Vu, H., P. Yukphan, W. Chaipitakchonlatarn, T. Malimas, Y. Muramatsu, U. Thi Tu Bui, S. Tanasupawat, K. Cong Duong, Y. Nakagawa, H. Thanh Pham and Y. Yamada. 2013. *Nguyenibacter vanlangensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-*Proteobacteria.* **J. Gen. Appl. Microbiol.** 59: 153-166.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Res.** 22: 4673-4680.

Tomas, C.A., N.E. Welker and E.T. Papoutsakis. 2003. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. **Appl. Environ. Microbiol.** 69: 4951-4965.

Trček, J. and M. Teuber. 2002. Genetic restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. **FEMS Microbiol. Lett.** 19: 69-75.

______. 2005. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S–23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. **Syst. Appl. Microbiol.** 28: 735–745.

______, H. 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.

Copyright by Kasetsart University All rights reserved

Trček, J. and K. Matsushita. 2013. A unique enzyme of acetic acid bacteria, PQQ-dependent alcohol dehydrogenase, is also present in *Frateuria aurantia.* **Appl. Microbiol. Biotechnol.** 97: 7369-7376.

Urakami, T., J. Tamaoka, K. Suzuki and K. Komagata. 1989. *Acidomonas* gen. nov., Incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. **Int. J. Syst. Bacteriol.** 39(1): 50-55.

VanBogelen, R.A., M.A. Acton and F.C. Neidhardt. 1987. Induction of the heat shock regulon does not produce thermotolerance in *Escherichai coli*, **Genes Dev.** 1: 525-531.

Ventura, M., C. Canchaya, R. Zink, G.F. Fitzgerald and D. Van Sinderen. 2004. Characterization of the *groEL* and *groES* loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analyses. **Appl. Environ. Microbiol.** 70: 6197-6209.

Verlander, C.P. 1992. Detection of horseradish peroxidase by colorimetry, *In* Nonisotopic DNA Probe Techniques, ed by Kricka LJ, Academic Press, New York, pp 185-201

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

Wayne, L.G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M.P. Starr and H.G. Trüper. 1987. Report of the Ad Hoc committee on reconciliation of approaches to bacterial systematic. **Int. J. Syst. Bacteriol.** 37: 463–464.

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.

Copyright by Kasetsart University All rights reserved

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

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

______, M. Nunoda, T. Ishikawa and Y. Tahara. 1981. The cellular fatty acid composition in acetic acid bacteria. **J. Gen. Appl. Microbiol.** 45: 7–15.

______, 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 *Gluconacetobacter* to the generic level. **Biosci. Biotechnol. Biochem.** 61(8): 1244-1251.

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

______, K. Katsura, H. Kawasaki, Y. Widyastuti, S. Sano, T. seki, T. Uchimura and K. Komagata. 2000. *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-*Proteobateria.* **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.

______, ______, H.T. Lan Vu, Y. Muramatsu, D. Ochaikul, S. Tanasupawat and Y. Nakagawa. 2012. Description of *Komatagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). **J. Gen. Appl. Microbiol.** 58: 397-404.

Copyright by Kasetsart University All rights reserved

Yamauchi, S., H. Okuyama, E.H. Morita and H. Hayashi. 2003. Gene structure and transcriptional regulation specific to the *groESL* operon from the psychrophilic bacterium *Colwellia maris*. **Arch. Microbiol.** 180: 272–278.

Yoshida, T., Y. Ayabe, M. Yasunaga, Y. Usami, H. Habe, N. 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, M. Takahashi, W. Potacharoen, T. Busabun, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen and Y. Yamada. 2004a. Re-identification of *Gluconobacter* strains based on restriction analysis of 16S–23S rDNA internal transcribed spacer regions. **J. Gen. Appl. Microbiol.** 50: 189–195.

______, W. Potacharoen, S. Tanasupawat, M. Tanticharoen and Y. Yamada. 2004b. *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*. **Int. J. Syst. Evol. Microbiol.** 54: 313-316.

______, W. Potacharoen, Y. Nakagawa, M. Tanticharoen and Y. Yamada. 2004c. Identification of strains assigned to the genus *Gluconobacter* Asai 1935 based on the sequence and the restriction analyses of the 16S–23S rDNA internal transcribed spacer regions. **J. Gen. Appl. Microbiol.** 50: 9–15.

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

______, 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.

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Yukphan, P., T. Malimas, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen and Y. Yamada. 2006. Phylogenetic relationships between the genera *Swaminathania* and *Asaia*, with reference to the genera *Kozakia* and *Neoasaia*, based on 16S rDNA, 16S-23S rDNA ITS, and 23S rDNA sequences. **J. Gen. Appl. Microbiol**. 52: 289-294.

______, ______, 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.I. Suzuki, M. Tanticharoen and Y. Yamada. 2009. *Ameyama chiangmaiensis* gen. nov., sp. nov., an acetic acid bacterium in the α-*Proteobacteria*. **Biosci. Biotechnol. Biochem**. 73: 1-7.

_______, _______, _______, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen and Y. Yamada. 2011. *Neokomagataea* gen. nov., with descriptions of *Neokomagataea thailandicus* sp. nov. and *Neokomagataea tanensis* sp. nov., osmotolerant acetic acid bacteria of the α-*Proteobacteria*. **Biosci. Biotechnol. Biochem.** 75: 419-426.

Zhao, K., M. Liu and R.R Burgess. 2005. The global transcriptional response of *Escherichia coli* to induced σ^{32} protein involve σ^{32} regulon activation followed by inactivation and degradation of σ^{32} in vivo. **J. Biol. Chem.** 280: 17758-17768.

Zolkiewski, M. 1999. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation: a novel multi-chaperone system from *Escherichia coli.* **J. Biol. Chem.** 274: 28083–28086.

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Zuber, U., and W. Schumann. 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis.* **J. Bacteriol.** 176: 1359-1363.

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APPENDICES

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Appendix A

Culture media and antibiotics

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1. Potato medium

Glucose	5	g
Yeast extract	5	g
Polypeptone	10	g
Glycerol	20	ml
Potato extract	15	ml
H_2O	1000	ml

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

3. 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 121ºC for 15 min.

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4. 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 121ºC for 15 min.

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

5. GEY medium

Glucose	0.2	g
Ethanol	5	ml
Yeast extract	1	g
H_2O	95	ml

Sterilization was performed by autoclaving at 121ºC for 15 min. After sterilization, those media were allowed to cool down to 50ºC and then ethanol was added aseptically when indicated.

6. GECA (glucose/ethanol/calcium carbonate/agar) medium

Glucose	2	ml
Ethanol	0.5	ml
Peptone	0.3	g
Yeast extract	0.3	g

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$CaCO_3$	0.7	g
H_2O	99.5	ml

Sterilization was performed by autoclaving at 121ºC for 15 min. For agar, 1.2 g of agar was added to the liquid media before autoclaving. After sterilization, those media were allowed to cool down to 50ºC and then ethanol was added aseptically when indicated.

7. GECA medium containing 10% ethanol

Glucose	2	g
Ethanol	10	ml
Peptone	0.3	g
Yeast extract	0.3	g
$CaCO_3$	0.7	g
H_2O	90	ml

Sterilization was performed by autoclaving at 121ºC for 15 min. For agar, 12 g of agar was added to the liquid media before autoclaving. After sterilization, those media were allowed to cool down to 50ºC and then ethanol was added aseptically when indicated.

8. Basal medium with various carbon compounds

Yeast extract	0.5	g
Bromcresol purple (0.2% in acetone)	small amount	
H_2O	100	ml

All ingredients were mixed and adjusted the pH 6.8. The basal medium (1.9 ml) was distributed into the cotton plugged test tubes (13 x 100 mm) and sterilized at 121ºC for 15 min. After cool down, add 100 µl of 20% sugar which was sterilized by membrane filtation to make the final concentration at 1% sugar (except for ducitol; 1% dulcitol (w/v) was added into the basal medium before sterilization).

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9. Ammoniac nitrogen medium (Frateur modified Hoyer medium)

Glucose	3	g
$(NH_4)_2SO_4$	0.1	g
K_2HPO_4	0.01	g
KH_2PO_4	0.09	g
$MgSO_4.7H_2O$	0.025	g
$FeCl_3.6H_2O$	0.0005	g
H_2O	100	ml

Beside the glucose was used as the sole carbon source, other carbon sources was used by replaced with 3.0 % D-mannitol (w/v) or by 3.0% ethanol (v/v). All ingredients were mixed and adjusted the pH 6.8. Sterilization was performed by autoclaving at 121ºC for 15 min.

10. 0.35% acetic acid in Glucose-ethanol medium

Glucose	1.5	g
Ethanol	0.5	ml
Peptone	0.3	g
Yeast extract	0.3	g
Acetic acid	0.35	ml
H_2O	100	ml

All ingredients were mixed and adjusted the pH 3.5. Sterilization was performed by autoclaving at 121ºC for 15 min.

11. Medium for ketogenesis test

Glycerol	3	g
Yeast extract	0.5	g
Peptone	1	g
H_2O	97	ml

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Sterilization was performed by autoclaving at 121ºC for 15 min.

12. 30% glucose medium

Part I		
Yeast extract	0.5	g
Agar	1.5	g
H_2O	50	ml
Part II		
Glucose	30	g
H_2O	50	ml

The solutions of Part I and Part II were individullly sterilized by autoclaving at 121ºC for 15 min (Part I) and 110ºC for 10 min (PartII). After cool down, the solution of PartI and Part II were mixed before used.

13. Amplicillin (50 mg/ml)

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

14. 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 -20ºC.

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15. 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 -20ºC.

16. 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 -20ºC.

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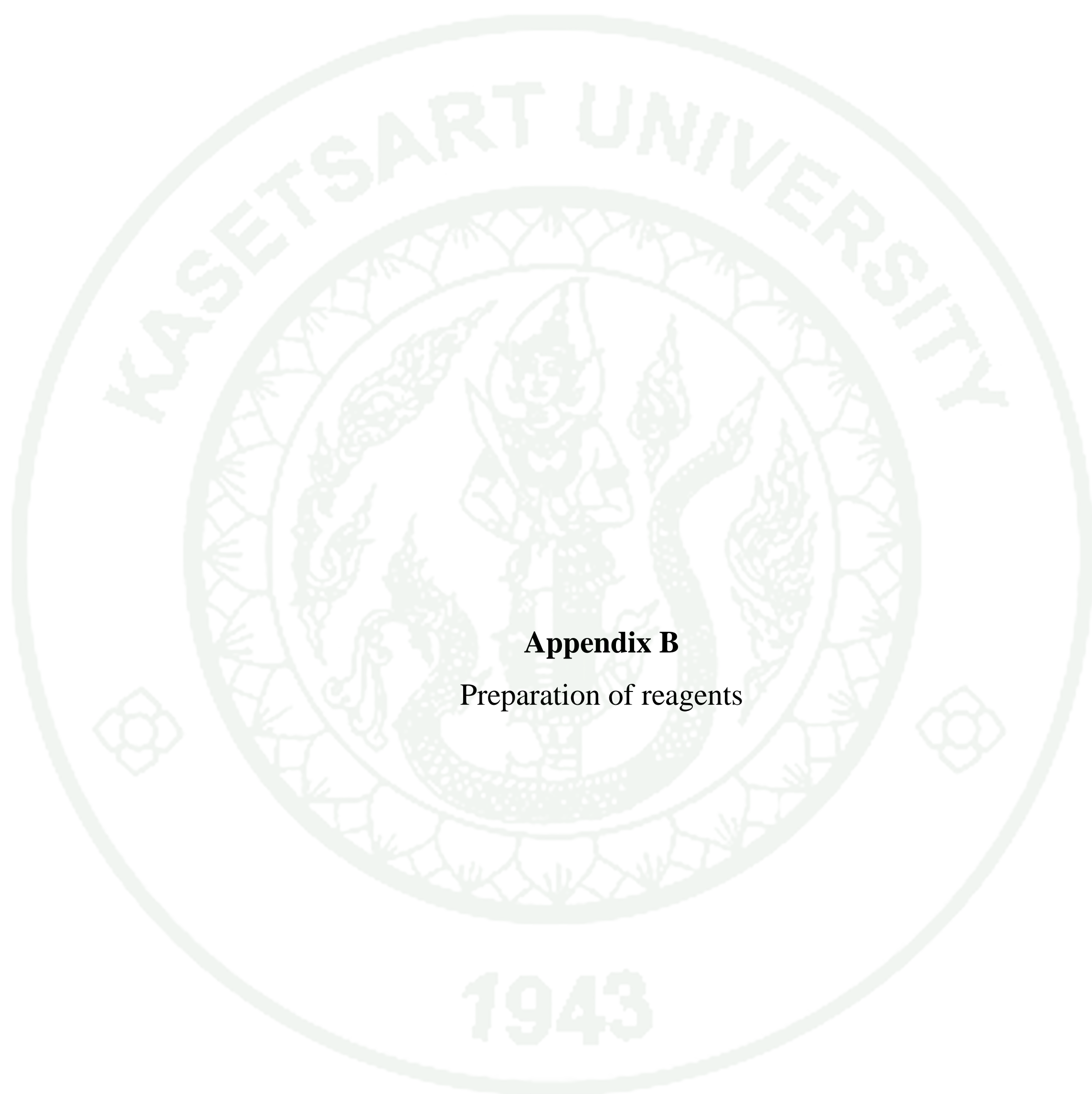

Appendix B

Preparation of reagents

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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 -20ºC. 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.

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1.4 NaOH (0.8N)

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 acetic acid and added double distilled water to adjust volume to 100 ml. Sterilization was performed by autoclaving at 121ºC 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 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 121ºC for 15 min.

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1.9 Sodium acetate (0.5 M, pH 5.5)

A solution was prepared by dissolving 68.02 g of sodium acetate.3H_2O in double distilled water and adjusted pH to 5.5 with glacial acetic acid. Then, the solution was adjusted volume to 1000 ml with double distilled water and sterilized by autoclaving at 121ºC for 15 min.

1.10 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 at 121ºC for 15 min.

1.11 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.

1.12 Saline-EDTA

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

1.13 Phenol: chloroform: isoamyl alcohol

Crystalline phenol was liquided in water bath at 65ºC 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 4ºC.

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1.14 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 sequently heated at 65ºC with stirring. The solution was sterilized by autoclaving at 121ºC for 15 min and stored at room temperature.

1.15 TE buffer

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

1.16 TE buffer with 20 µg/ml RNase A

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

1.17 Gel loading buffer

Bromphenol blue	0.025	g
Glycerol	3	ml

1.18 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

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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)

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

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The solution was adjusted volume to 1000 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 1000 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, autoclaved 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 42ºC for 20 min.

3.6 Buffer I

SSC solution	2	x

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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 RNA extraction

4.1 A solution

Sodium Acetate	20	mM
EDTA pH 8.0	10	mM
SDS	0.5	% (w/v)

4.2 S phenol

Crystalline phenol was liquided in water bath at 65ºC and mixed with chloroform and isoamyl alcohol in the ratio of 25:24:1. The solution was covered with 100 mM Tris-Cl (pH 8.0) and stored in a light-tight bottle at 4ºC.

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5. Reagents for biochemical tests

5.1 Kovac's oxidase reagent

ρ-Dimethylaminobenzaldehyde	3	g
Butanol	75	ml
HCl, concentrated	25	ml

The aldehyde was dissolved in the ethanol at 50-55ºC. After cool down, the acid was added. The solution was kept in dark and stored in refrigerator.

5.2 Fehling solution

Solution A		
$CuSO_4.5H_2O$	16.89	g
H_2O	100	ml
Solution B		
Sodium potassium tartrate	34.6	g
NaOH	10	g
H_2O	100	ml

The solutions A and Solution B were mixed (1:1) before used.

6. Reagents for determination of DNA base composition

6.1 Nuclease P1

0.1 mg of enzyme nuclease P1 was dissolved in 1 ml of 40 mM CH_3COONa and 2 mM $ZnSO_4$ (pH 5.3) solution.

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6.2 Alkaline phosphatase

The 100 units of enzyme bacterial alkaline phosphatase were dissolved in 220 µl of $NH_4H_2PO_4$ solution. The stock of enzyme was stored at 4ºC. The working solution of enzyme was prepared by dilute the stock of enzyme 2.4 unit in 1000 µl of 0.1 M Tris-Cl pH 8.0.

7. Reagents and buffers for DNA-DNA hybridization

7.1 PBSM

$MgCl_2.6H_2O$	0.095	g
1 x PBS	10	ml

7.2 Pre-hybridizaton solution

20 x SSC	1	ml
50 x Denhardt's solution	1	ml
Denatured Salmon DNA (10 mg/ml)	0.1	ml
Formamide	5	ml
H_2O	2.9	ml

Before the solutions were mix, 10 mg/ml of Salmon DNA in TE was boiled in boiling water for 5 min and immediately kept cold on ice bath. This step was prepared before used.

7.3 Hybridization solution

Dextran sulfate	0.25	g
20 x SSC	1	ml
50 x Denhardt's solution	1	ml
Denatured Salmon DNA (10 mg/ml)	0.1	ml

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Formamide	5	ml
H_2O	2.8	ml

Dextran sulfate was dissolved in water before the other reagents were added and mixed. Moreover, 10 mg/ml of Salmon DNA in TE was boiled in boiling water for 5 min and immediately kept cold on ice bath. This step was prepared before used.

7.4 Solution I

BSA	0.25	g
Triton-X-100	50	µl
1 x PBS	50	ml

7.5 Solution II

Streptavidin POD	1	µl
Solution I	4	µl

7.6 Solution III

3, 3’, 5, 5’-Tetramethylbenzidine (TMB)	100	µl
0.3% H_2O_2	100	µl
0.1 M Citric acid	5	ml
(in 10% DMFO and 0.2 M Na_2HPO_4)		

7.7 PBS

Na_2HPO_4	2.30	g
KH_2PO_4	0.4	g
NaCl	16	g
KCl	0.4	g
H_2O	1000	ml

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The solution was adjusted pH to 7.0 and added double distilled water to adjust volume to 1000 ml. Sterilization was performed by autoclaving at 121ºC for 15 min.

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Appendix C

General methods

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1. 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, pH7.9 (Appendix B). Then 10 mg of lyzozyme was added, the cell suspension was incubated at 37ºC 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 50ºC for 10 min without shaking followed by adding 1/10 volume of 10% CTAB/NaCl solution and statically incubated at 65ºC 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 the 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.

2. Isolation of RNA from acetic acid bacteria

Total RNA was prepared by the hot phenol method as described by Aiba *et al.* (1981). The cells were grown in 100 ml of YPGD medium. The cells were harvested and resuspended in 3 ml of a solution of 0.02 M sodium acetate (pH 5.5), 0.5% SDS, and 1 mM EDTA. After addition of 3 ml of redistilled phenol (equilibrated in 0.02 M sodium acetate, pH 5.5), the mixture was incubated at 60ºC for 5 min with gentle shaking. After centrifugation the aqueous phase was re-extracted by phenol. The RNA was precipitated by adding 3 volumes of ethanol to the aqueous phase and chilled at -70ºC for 30 min. The RNA precipitate was collected by centrifugation and redis- solved in 3 ml of the same acetate/SDS buffer. The ethanol precipitation was repeated two more times. The final precipitate was dissolved in 1 ml of distilled water. The RNA concentration was determined by measuring the optical density at 260 nm.

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3. Analysis of plasmid profile of acetic acid bacteria

Plasmid DNA was isolated from wild type and the modified 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 4ºC, 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 37ºC 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 mixture was centrifuged at 12,000 rpm 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 the equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) followed by vigorous shaking. The mixture was centrifuged at 12,000 rpm for 20 min at room temperature. If the sample contained the cellulose, 1/10 volume of CTAB was added to the suspension. Then, it was incubated at 65ºC 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 -80ºC for 30 min was then centrifuged at 4ºC, 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.

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

Alkaline lysis method was described by Sambrook *et al.* (1989) with slightly modification. *E. coli* harboring plasmid was grown in LB broth containing appropriate antibiotic at 37ºC 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 was 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

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on ice 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 -20ºC overnight or -80ºC for 30 min and centrifuged at 12,000 rpm for 30 min at 4ºC. 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 37ºC overnight and stored at -20ºC. Plasmid DNA was analyzed by running in 0.8% agarose gel electrophoresis.

5. 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 transferred to the supplied GeneJET™ spin column by decanting or pipetting, avoid disturbing or transferring the white precipitate and centrifuged for 1 min. 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 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.

6. Purification of DNA fragment by HiYield™ Gel/PCR Fragments Extraction Kit

A gel slice containing DNA fragment was excised with a clean and sharp scalpel or razor blade. The weight up to 300 mg of gel slice was placed in a microcentifuge tube and

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added 500 µl of DF buffer. The gel mixture was incubated at 50-60ºC for 10-15 min or until the gel slice is completely dissolved. The gelmixture was mixed by inversion the tube every few min to facilitate the melting process. After the gel slice has dissolved completely, the solubilized gel solution was transferred up to 800 µl to the DF column and centrifuged for 30 sec. The flow-through was discarded and placed back into the same collection tube. The 600 µl of the Wash buffer was then added to the DF column and centrifuged for 30 sec. The flow-through was discarded and placed back into the same collection tube. The empty DF 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 DF column membrane and allowed standing for 2 min. The purified DNA was eluted by centrifugation for 2 min at maximum speed.

7. Purification of RNA fragments by RNeasy Mini Kit.

The volume of RNA sample was adjusted up to 100 µl with RNase-free water. The 350 µl of the buffer RLT was then added, mixed well. The 250 µl of ethanol (96-100%) was then added to the diluted RNA, mixed well by pipetting. The sample was immediately transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 sec at maximum speed. The flow-through was discarded and placed back into the same collection tube. The 500 µl of the buffer RPE was then added to the RNeasy Mini spin column and centrifuged for 2 min. The column was transferred into a new 2 ml collection tube, and the old collection tube was discarded with the flow-through. The RNeasy Mini spin column was transferred into a new 1.5 ml collection tube and 30 µl of the RNase-free water was added to the center of RNeasy Mini spin column to elute the RNA and centrifuged 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 4ºC. The culture medium was transferred to a centrifuge tube and incubated on ice for 15 min. The bacterial

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cells were harvested by centrifugation at 6,000 rpm for 5 min. The precipitate was washed with cold o.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 10% glycerol. The suspension was dispensed into 65µl aliquots, shocked frozen in liquid nitrogen and stored at -80ºC 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 37ºC 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 0ºC as possible (on ice/in water bath). The culture was transferred to a prechilled sterile tube and centrifuged at 5,000 rpm, 4ºC for 5 min. Remove a much of the supernatant as possible. The pellets were gently resuspended in a total of 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 -80ºC.

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. Then, the cells were heat-shocked at 42ºC 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 37ºC for 30 min with no shaking and further gentle shaking for 30 min. After adaptation, the solution was spreaded on LB agar containing an appropriated antibiotic and incubated overnight at 37ºC.

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11. Conjugation 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 30ºC for overnight and *E. coli* S17-1 was cultured in 5 ml of LB broth at 37ºC for overnight. Exponentially growing donor strain was mixed with an equal volume of recipient cultured in the late exponential phase in an eppendorf tube and 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 inoculated at 30ºC 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 appropriated antibiotic and incubated at 30ºC 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 media was determined by modified method of Saeki *et al.* (1997). 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 below.

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 as follows: the cell pellet was resuspensed 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, 600 µl of Z buffer

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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 28ºC for 2 min. Then, start reaction by adding 200 µl substrate, *o*-nitrophenyl-b-D-galactoside (ONPG; 4 mg/ml), mixed and noted the time of addition precisely. The cell was incubated at 28ºC until a sufficient yellow color has developed. The reaction was stopped by adding 500 µl of 1 M Na_2CO_3, mixed well and recorded the stop time. The reaction mixture was transferred to an eppendorf tube and centrifuged at maximum speed for 5 min to remove cell debris and chloroform. Then, the supernatant was measure the optical density at 420 and 550 nm. The following equation is used to calculate units of β-galactosidase activity.

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

When; T = time of the reaction (min)

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

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CURRICULUM VITAE

NAME : Miss Nittaya Pitiwittayakul

BIRTH DATE : December 28, 1983

BIRTH PLACE : Bangkok, Thailand

EDUCATION : B.S. (Biology) with Second Class Honours
Kasetsart University (2006)

SCHOLARSHIPS/AWARDS

: Awarded a scholarship with the "Strategic Scholarship/Fellowships Frontier Research Network" from the Office of Higher Education Commission, Ministry of Education, Thailand

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