Kasetsart J. (Nat. Sci.) 42 : 701 - 714 (2008)

Molecular Taxonomy of *Acetobacter syzygii* SKU19 and Characterization of Its Acetic Acid Adapted Strains

Wilawan Sintuprapa[1], Gunjana Theeragool[1*], Wichien Yongmanitchai[1], Pattana Srifah-Huehne[2] and Kazunobu Matsushita[3]

ABSTRACT

Acetobacter species are one of the most suitable bacteria for the proteomic analysis of proteins involved in stress response or adaptation. Among 129 isolates of thermotolerant acetic-acid bacteria, two isolates SKU19 and SKU123 were selected as an acetic-acid sensitive and acetic-acid resistant strain, respectively. Molecular taxonomy of these isolates indicated that SKU19 and SKU123 were *Acetobacter syzygii* and *A. pasteurianus*, respectively. To elucidate an adaptive response to acetic acid, acetic-acid-adapted strains were isolated from sequential cultivations of the acetic-acid sensitive strain, *A. syzygii* SKU19, in a medium containing 1% acetic acid. The adapted variants could be divided into two groups based on their growth and ability to further oxidize acetate. The first group consisted of cells with increased overoxidation (rapid acetate oxidizers), while the second group contained cells with increased stability to acetate (slow acetate oxidizers). The membrane-bound quinoprotein alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH) activities of these adapted strains were higher than those of the wild type. The result corresponded well with the increased amount of protein with a molecular mass of 72-80 (subunit I) and 44-54 kDa (subunit II) when the organism was cultivated in a medium containing acetic acid. This study confirmed that acetic-acid resistance in acetic-acid bacteria is conferred by several mechanisms, such as acetate assimilation and PQQ-ADH functions.
Key words: *Acetobacter syzygii*, thermotolerant, acetic acid bacteria, acetic acid, adaptation

INTRODUCTION

Acetic acid is one of the weak organic acids obtained from microbial metabolism and well known as a natural preservative as well as for its cytotoxic effect including the retardation of growth and product formation at concentrations as low as 5 g/l (Lasko *et al.,* 1997). Normally, organic acids affect cell growth in at least two ways; by lowering pHi and by increasing turgor pressure through anion accumulation. These weak acids caused several strong changes in intracellular processes including for example cell division, DNA metabolism and ion transport, so that membrane processes were disrupted and thereby the cell was poisoned (Russell, 1992).

Several species of bacteria are known to be relatively tolerant to weak organic acids, especially acetate. The gram-negative genera, *Acetobacter* and *Gluconobacter*, known as acetic-

1 Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
3 Department of Biological Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan.
* Corresponding author, e-mail: fscignt@ku.ac.th

Received date: 22/04/08 Accepted date: 18/06/08

acid bacteria are the best known examples. These organisms are widely used in vinegar fermentation and are one of the most suitable bacteria for studying the stress response or adaptation to acid, ethanol and thermal stress, when the fermentation process is carried out under high temperature or without a precise cooling system (Saeki *et al.,* 1997). In another study, the microenvironment surrounding the cells during acetic-acid fermentation contained various stressors as follows: (i) high temperature due to the energy generated during fermentation; (ii) ethanol, which is an acetic-acid-fermentation-initiating compound that is always present around the cells; and (iii) acetic acid, which is a product of fermentation and whose level gradually increased as the fermentation proceeded (Okamoto-Kainuma *et al.,* 2002). The previous report on the acetate resistance genes (*aarABC*) of a strain of thermophilic *A. aceti*, was important with regard to acetate resistance on solid media (Fukaya *et al.,* 1990). Additional research identified functions of the *aarA* and *aarC* gene products in citrate synthase (Fukaya *et al.,* 1990) and acetate uptake (Fukaya *et al.*, 1993), respectively. These proteins confer acetic acid resistance in *A. aceti* by acetate assimilation.

More recently, proteome analysis of acetic acid bacteria revealed eight acetate-stress proteins (Asps) that were induced specifically by challenging unadapted *A. aceti* and *G. suboxydans* cultures with 10 g/l acetate (Lakso *et al.,* 1997). Steiner and Sauer (2001) investigated the changes in global protein expression levels during long-term adaptation of *A. aceti* to high acetate concentrations by two-dimensional electrophoresis (2-DE). They reported a complex proteome response with at least 50 proteins that were specifically induced by adaptation to acetate, but not by other stress conditions, such as heat, oxidative or osmotic stress. One of the proteins in cytoplasm whose production was enhanced in response to acetic acid was identified as aconitase (Nakano *et al.,* 2004). In addition, Matsushita *et al*. (2005a) reported that the efflux pump mechanism was responsible for acetic-acid resistance in acetic-acid bacteria. These membrane-associated processes appear to be of major importance for adaptation, because some of the Asps bear N-terminal sequence homology to membrane proteins, for example AatA, a putative ATP-binding cassette (ABC) transporter, which possibly functioned as an exporter of acetic acid (Nakano *et al.,* 2006). Recently, Nakano and Fukaya (2008) reported that their proteomic analysis and those of previous studies indicated that acetic-acid resistance in acetic-acid bacteria is conferred by several mechanisms.

The current study, tried to elucidate an acetic-acid adaptive response in acetic-acid adapted strains isolated from sequential cultivations of the acetic-acid sensitive strain, *Acetobacter syzygii* SKU19. This thermotolerant acetic-acid bacterium was chosen for the preliminary study of acid-stress response due to: its ability to produce acetic acid from ethanol; its low tolerance, but good adaptation to acetic acid; and its high tolerance to ethanol. The adapted strains were characterized by: their genetic background; the relationship between their acetic-acid resistance and PQQ-dependent alcohol dehydrogenase (PQQ-ADH); and their acetate assimilation for acetic-acid adaptation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Thermotolerant acetic-acid bacteria, *Acetobacter* sp. SKU19 (an acetic-acid sensitive strain) and SKU123 (an acetic-acid tolerant strain) isolated from guava and longkon, respectively, were used in this study (Theeragool *et al*., 1996). A potato medium (1 g yeast extract, 1 g polypeptone, 2 g glycerol, 0.5 g glucose, and 20 ml potato extract, filled up to 100 ml with tap water) was used for general cultivation and stock

cultures. A seed culture medium (SCM: 0.5 g yeast extract, 0.5 g polypeptone, 0.5 g glycerol, 0.5 g glucose per 100 ml tap water) was used for the determination of acetic-acid tolerance, bacterial growth, and enzyme activity.

Selection of acetic-acid sensitive and tolerant strains

A total of 129 isolates of thermotolerant acetic acid bacteria (SKU1-129) previously isolated from various fruits in Thailand (Theeragool *et al.,* 1996) were selected for their acetic-acid sensitivity and tolerance. The inoculum was prepared from one loopful of each isolate cultured in potato broth at 30°C, 200 rpm for 18-24 h. For the selection of the acetic-acid sensitive and acetic-acid tolerant strains, 5 µl of the inoculum was dropped on SCM agar containing 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4% (v/v) of acetic acid respectively and incubated at 30°C for five days. The growth of all isolates was observed and the acetic-acid sensitive strains were selected from the isolates that exhibited the poorest growth on the media. In contrast, the acetic-acid tolerant strains were selected from the isolates that exhibited the best growth under the same conditions.

Analysis of 16S rDNA and phylogenetic tree

Chromosomal DNA from the selected acetic-acid bacteria was isolated by the method described by Okumura *et al*. (1985). PCR amplification was performed using a PCR kit (puReTaq Ready-To-Go PCR beads) from Amersham Bioscience (Amersham pharmacia biotech Inc., USA) with the specific primers 27f forward primer, (5′-AGAGTTTGA TCCT GGCTCAG-3′) and 1525r reverse primer, (5′-AAAGGAGGTGATCCAGCC-3′), which were designed from highly-conserved regions of the nucleotide sequence of 16S rDNA of α-Proteobacteria. The purified 1.5 kb PCR product was ligated with pGEM®-T Easy vector system (Promega Corporation, USA) and subcloned into pUC119 before sequencing. The nucleotide sequencing of the cloned 16S rDNA fragment was determined by applying the chain termination method using an ABI PRISM 310 genetic analyzer (Applied Biosystems, USA). The nucleotide sequences obtained were analyzed by homology search using the standard BLAST sequence similarity searching program located at http://www.ncbi.nlm.nih. gov/BLAST/ against available sequences in the Genebank database. The nucleotide sequence of the 16S rDNA was deposited in the DDBJ sequences database with the accession number AB264094. Multiple alignments were carried out with the program CLUSTAL W version 2 (Thompson *et al.,* 1994). Distance matrices for the aligned sequences were determined by using the two-parameter method of Kimura (1980). The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with the program MEGA version 2.1 (Kumar *et al.,* 2001). The robustness of individual branches was estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985).

Isolation of the acetic-acid adapted strains

Isolation of the acetic-acid adapted strains was performed by sequential cultivations of the acetic-acid sensitive strain, *Acetobacter* sp. SKU19, in the SCM broth containing 1% acetic acid. The 5% inoculum of *A. syzygii* SKU19 was inoculated into SCM broth containing 1% acetic acid. The culture was incubated at 30°C, 200 rpm for seven days and 250 µl transferred to five ml of fresh SCM broth containing 1% acetic acid. This was sequentially performed for eight generations and each culture was sampled and checked for its growth on SCM agar containing 1% acetic acid. The culture was incubated at 30°C for five to seven days. The colonies with an adaptation to grow on this medium were selected as the acetic-acid adapted strains.

Determination of growth characteristics and acetate oxidation

All of the acetic-acid adapted strains were precultured in five ml of potato broth containing 1% acetic acid, incubated at 30°C, 200 rpm and their growth was measured by a Klett Summerson photometer until log phase. The 1% inoculum was then inoculated into glucose-free SCM broth containing various concentrations of acetic acid and incubated at 30°C, 200 rpm for 20 days. The growth of all strains was observed by measuring with a Klett Summerson photometer. Acidity of the culture medium was measured by alkaline-titration with 0.8 N NaOH (Saeki *et al.,* 1997). The pH was measured using a pH meter.

To investigate acetate oxidation, the 1% inoculum was inoculated into a glucose-free SCM broth containing 2% ethanol and incubated at 30°C, 200 rpm. The bacterial growth, acidity, residual ethanol, and pH were determined.

Preparation of a crude enzyme solution and enzyme assay

Cells of acetic-acid sensitive, acetic-acid tolerant and acetic-acid adapted strains were grown for two days or until they reached the late-log phase in a glucose-free SCM broth containing 2% ethanol and they were then harvested by centrifugation at 9,000x*g* for 10 min, and washed twice with ice-cold 50 mM KPB (pH 7.5). About one g of wet cells was resuspended in four ml of the same buffer and passed twice through a French pressure cell press (American Instrument, USA.) at 16,000 psi. After centrifugation at 9,000x*g* for 10 min to remove intact cells, the supernatant was centrifuged at 68,000x*g* for 90 min to obtain the membrane and soluble fractions.

The membrane-bound quinoprotein alcohol dehydrogenase, PQQ-ADH (EC 1.1.99.8) and membrane-bound aldehyde dehydrogeanse, ALDH (EC 1.2.99.3) activities were measured colorimetrically with potassium ferricyanide as an electron acceptor by the method of Adachi *et al.* (1978). The rate of reduction of ferricyanide to ferrocyanide gave a quantitative indication of the amount of ethanol oxidized. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of one μmole of substrate per min. All enzyme assays were carried out at 25°C.

Other analytical methods

The ethanol concentration in the culture medium was measured enzymatically (Adachi *et al.,* 1978). The protein content was measured by a modified Lowry method with bovine serum albumin as the standard (Dully and Grieve, 1975). Heme staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12.5% (w/v) acrylamide slab gel with heme-catalyzed peroxidase activity (Thomas *et al.,* 1976). In addition, randomly amplified polymorphic DNA (RAPD) was performed by PCR with the following four random oligonucleotide primers: 70% G+C (5′-AGCGG GCGTA-3′), 80% G+G (5′-CGCGTGCCCA-3′), $(GTG)_{5X}$ (5′-GTGGTGGTGGTGGTG-3′) and AD01 (5′-CAAAGGGCGG-3′)

RESULTS AND DISCUSSION

Molecular taxonomy of *Acetobacter* sp. SKU19 and SKU123

From various kinds of fruits in Thailand, a total of 129 isolates of thermotolerant acetic-acid bacteria previously isolated (Theeragool *et al*. 1996) were selected for their acetic-acid sensitivity and tolerance. Comparison of the growth of all isolates was observed on SCM agar plate containing various concentrations of acetic acid. *Acetobacter* sp. SKU19 could grow well on the SCM agar plate containing 0.25 and 0.5% acetic acid, but it could not grow on the SCM containing 1% acetic acid, whereas *Acetobacter* sp. SKU123 grew well even in the presence of 3.0% acetic acid (data not shown). Therefore,

Acetobacter sp. SKU19 and SKU123 were selected as an acetic-acid sensitive and an acetic-acid tolerant strain, respectively.

The nucleotide sequences of 16S rDNA amplified from chromosomal DNA of *Acetobacter* sp. SKU19 and SKU123 were analyzed for their sequence identity percentage with those of *Acetobacteraceae* type strains (Siever *et al.,* 1994) and the results are summarized in Table 1. The highest identity percentages (99%) were observed when compared with *A. syzygii* and *A. pasteurianus,* respectively. The phylogenetic tree was constructed using the neighbour-joining method (Figure 1). From the result, *Acetobacter* sp. SKU19 and SKU123 were identified as *A. syzygii* SKU19 and *A. pasteurianus* SKU123, respectively.

Isolation and characterization of acetic-acid adapted strains

In order to confirm the acetic-acid sensitivity in *A. syzygii* SKU19 and *A. pasteurianus* SKU123, both strains were cultured in SCM broth containing various concentrations of acetic acid. As shown in Figure 2, *A. syzygii* SKU19 could not grow in SCM broth containing 1.5% acetic acid whereas *A. pasteurianus* SKU123 could grow with a longer lag phase. Both strains exhibited better growth in SCM broth containing 0.5% acetic acid than in SCM broth. In SCM broth containing 1.5% acetic acid, only *A. pasteurianus* SKU123 could grow after five days adaptation. Based on these results, *A. syzygii* SKU19 was selected as an acetic-acid sensitive strain and *A. pasteurianus* SKU123 as an acetic-acid tolerant strain.

To elucidate an adaptive response to acetic acid, the acetic-acid adapted strains were isolated by sequential cultivations of *A. syzygii* SKU19 in SCM broth containing 1% acetic acid for eight generations. Among the 400 colonies, six acetic acid adapted strains (no. 108, 112, 187, 217, 258 and 264) were screened on the basis of their growth on an SCM agar plate containing various concentrations of acetic acid. Interestingly, culture of all adapted strains could grow well in the medium containing 1% acetic acid, whereas *A. syzygii* SKU19 could not (Figure 3). Among six adapted strains, no. 108, 217 and 258 could grow even in the medium containing 2.5% acetic acid, but none of the tested strains could grow in the medium containing 3% acetic acid.

Table 1 Comparison of 16S rDNA sequence identity percentages between *Acetobacter* sp. SKU19 and SKU123 with other acetic-acid bacteria and archaebacteria as the out group.

Strains	%Identity of16S rDNA sequence	
	SKU19	SKU123
A. syzygii (AB052714)	99	97
A. lovaniensis (AJ419837)	99	97
A. pomorum (AJ419835)	97	99
A. pasteurianus (AB086016)	97	99
A. indonesiensis (AJ419841)	97	96
A. aceti (AJ419840)	97	97
A. pasteurianus SKU1108	96	98
Asaia bogorensis (AB025929)	96	95
Kozakia baliensis (AB056319)	95	94
Gluconacetobacter swingsii (AY180960)	95	94
Acidomonas methanolica (AB110714)	94	95
Haloferax volcanii (AB074566)	81	81

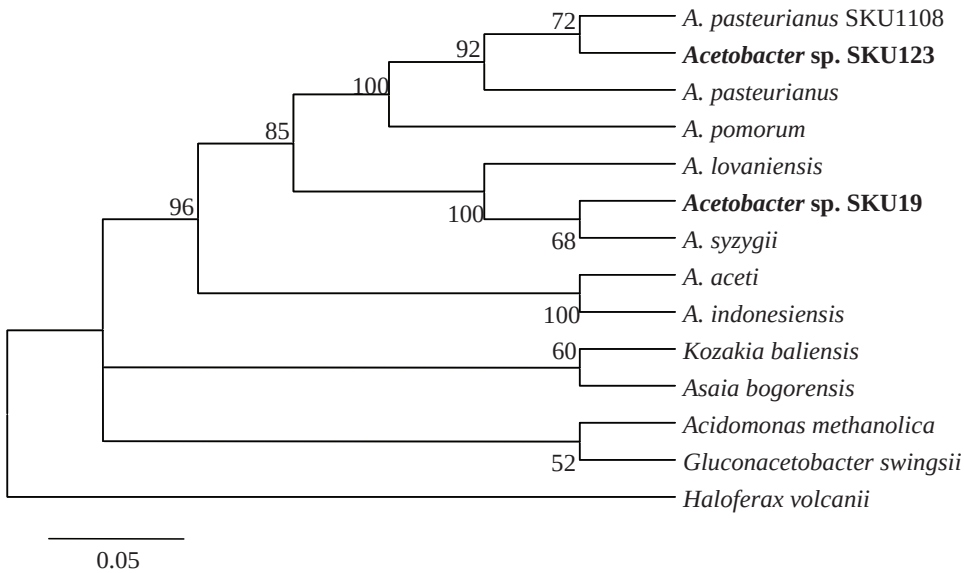

Figure 1 16S rDNA-based tree reflecting the phylogenetic position of *Acetobacter* sp. SKU19, SKU123, related acetic-acid bacteria and archaebacteria. The analysis was done using the following sequence from Genebank: AB025929: *Asaia bogorensis*, AB052714: *A. syzygii,* AB056319: *Kozakia baliensis*, AB086016: *A. pasteurianus*, AB110714: *Acidomonas methanolica*, AJ419835: *A. pomorum*, AJ419837: *A. lovaniensis*, AJ419840: *A. aceti*, AJ419841: *A. indonesiensis*, AY180960: *Gluconacetobacter swingsii, A. pasteurianus* SKU1108, AB074566 *Haloferax volcanii*.

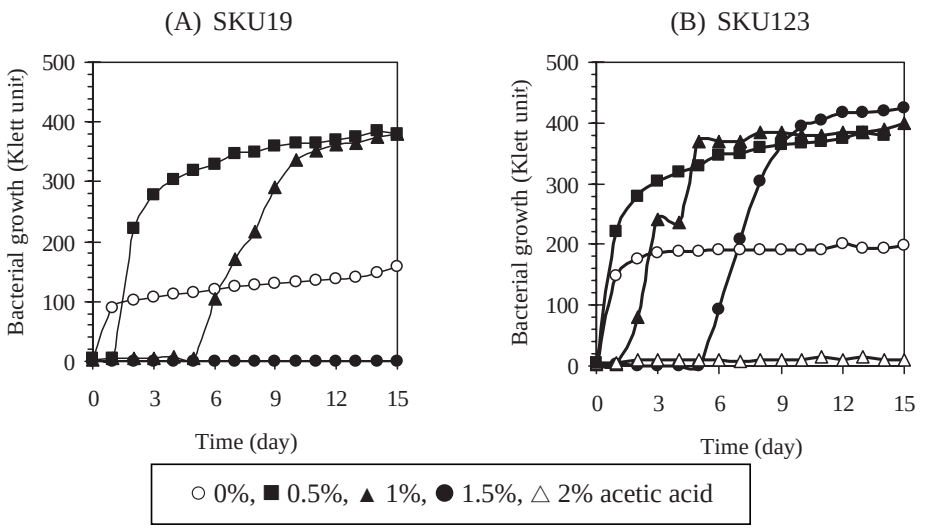

Figure 2 Time-course of growth of (A) acetic-acid sensitive strain, SKU19 and (B) tolerant strain, SKU123 in glucose-free SCM broth containing various concentrations of acetic acid. Both strains were precultured in potato broth at 30°C, 200 rpm for 18-24 h. The 1% inoculum was inoculated into glucose-free SCM broth containing various concentrations of acetic acid, and incubated at 30°C, 200 rpm for 15 days.

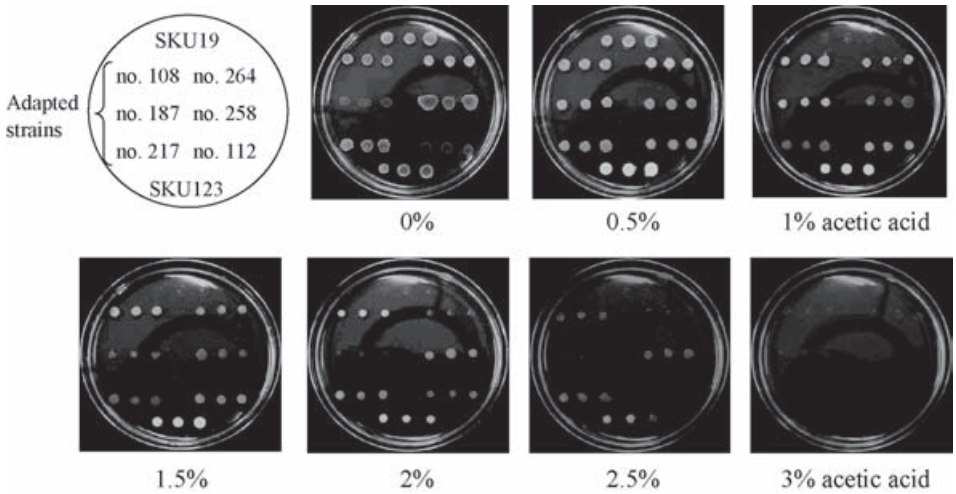

Figure 3 Growth of acetic-acid adapted strains from *A. syzygii* SKU19 on SCM containing various concentrations of acetic acid. All strains were precultured in potato broth containing 1.0% acetic acid and incubated at 30°C, 200 rpm for four days. Five µl of the preculture was inoculated on SCM agar containing 0, 0.5, 1, 1.5, 2, 2.5, and 3% (v/v) of acetic acid and incubated at 30°C for five days.

In order to investigate the adaptive response mechanism in adapted acetic-acid strains, precultured samples were prepared in potato broth containing 1% acetic acid at 30°C for seven days. The culture broth was then spread on SCM agar containing various concentrations of acetic acid. The viable counts of adapted strains were decreased when they were grown on SCM agar containing a higher concentration of acetic acid. However, some adapted strains could grow at a high concentration of acetic acid, so the population of the adapted strain seemed to be a mixed population between sensitive and resistant strains. The resistance to acetic acid and ethanol are crucial factors to determine the fermentation activity of the acetic-acid bacteria, but very little has been learnt about their mechanism. The results showed that the parent strain ceased to grow at 1% acetic acid, while all acetic-acid adapted strains were able to grow at higher than 1% acetic acid. Similarly, all acetic-acid adapted strains were grown in glucose-free SCM broth containing various concentrations of acetic acid (Figure 4). Six acetic-acid adapted strains possessed more acetic-acid resistance than their wild type strain. Thus, the acquired acetic-acid resistance appeared to be an inheritable phenotype acquired by mutation that is more stable than would be expected for a transient physiological adaptation. Steiner and Sauer (2003) showed that the resistance to acetic acid of the adapted strain was probably the result of a transient physiological adaptation and not genetic evolutionary adaptation, which may exclude the possibility of having a mixed population with co-existing resistant and sensitive cells. This was similar to the previously reported short-term conditioning to acetic acid that occurred within three generations (Steiner and Sauer, 2001).

Acetate overoxidation of acetic-acid adapted strains

To determine their ethanol oxidation ability and acetic-acid resistant ability, adapted strains were cultured in glucose-free SCM broth containing 2% ethanol. The growth and ability to oxidize acetate were compared between the

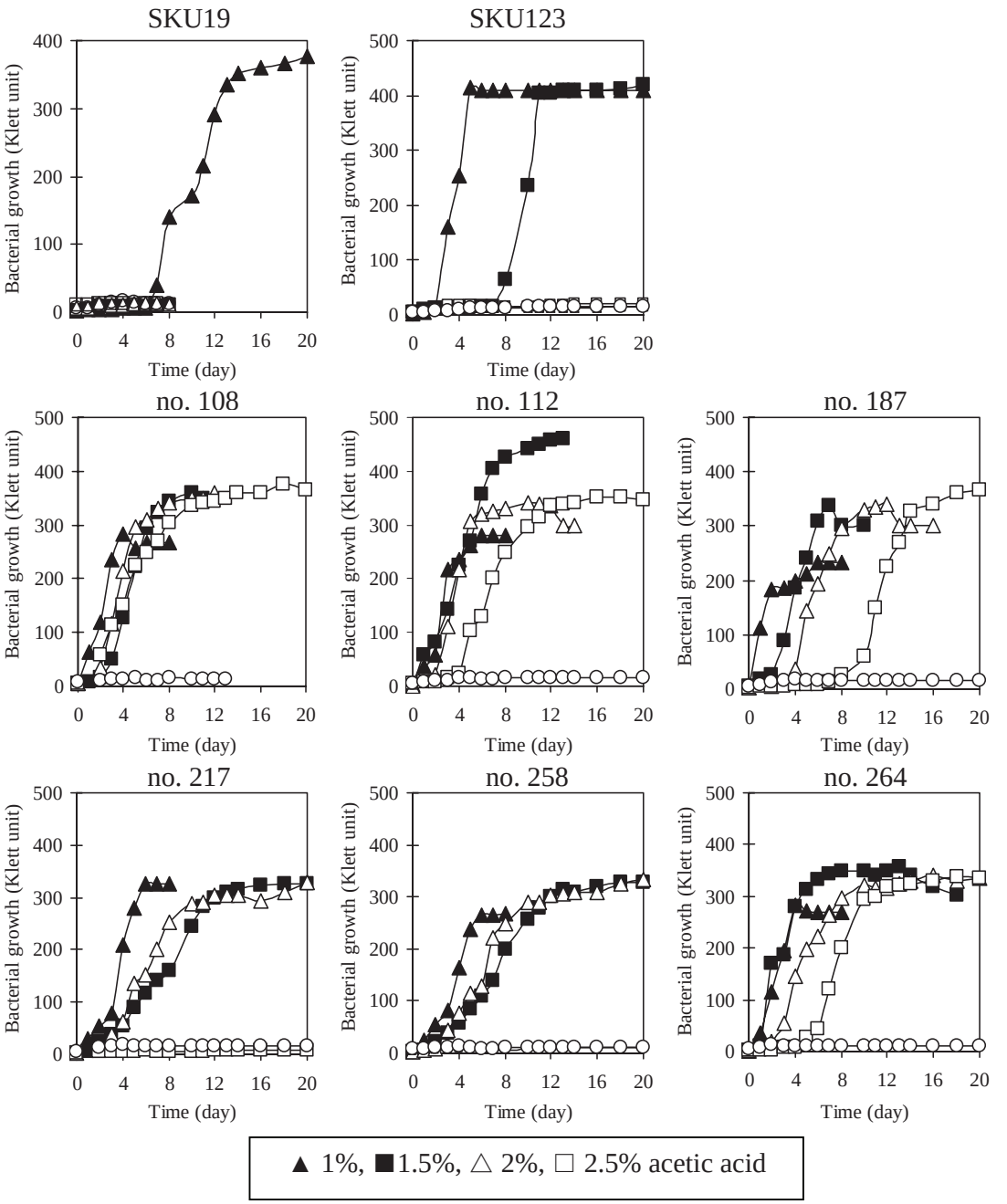

Figure 4 Growth of acetic-acid adapted strains from *A. syzygii* SKU19 in glucose-free SCM broth containing various concentrations of acetic acid. All strains were precultured in potato broth containing 1% acetic acid, incubated at 30°C for four days. 1% of the preculture was inoculated in glucose free-SCM broth containing 1, 1.5, 2, 2.5 and 3% (v/v) of acetic acid and incubated at 30°C, 200 rpm for 20 days.

unadapted *A. syzygii* SKU19 strain and adapted strains and they were categorized into two groups (Figure 5). The first group was the rapid-acetate oxidizer strains consisting of those with increased cells and overoxidation, which included *A. pasteurianus* SKU123 and the adapted strains no. 108 and 187. The second group was the slow-acetate oxidizers consisting of those with increased cells and a stability to acetate, which included the adapted strains no. 112, 217, 258 and 264. These could oxidize ethanol at 2% and also slowly oxidize acetate to carbon dioxide and water similar to the unadapted *A. syzygii* SKU19. The slow-acetate oxidizers showed a phasic growth curve, whereas the rapid-acetate oxidizers showed a typical biphasic growth curve. The first growth phase was accompanied with ethanol oxidation and the second growth phase was accompanied with acetate oxidation, while the slow-acetate oxidizers could not start this second growth phase due to acetate overoxidation. The rapid-acetate oxidizers started overoxidation immediately after conversion of ethanol into acetic acid and thus almost no stationary phase occurred. The results

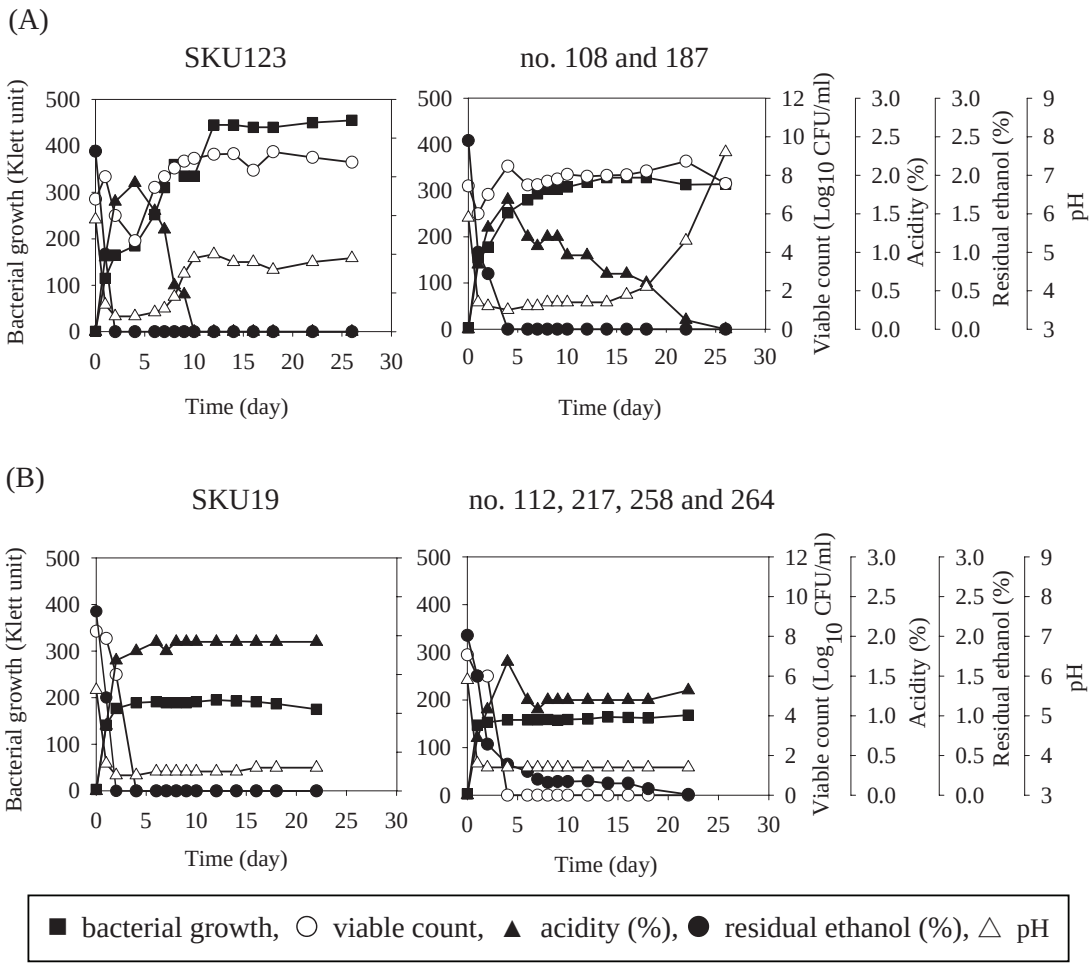

Figure 5 Time-course of the growth and acetate oxidation of wild type and acetic-acid adapted strains in glucose-free SCM broth containing 2% (v/v) ethanol. Growth curves of rapid-acetate oxidizers, *A. pasteurianus* SKU123, no. 108 and 187 are shown in A and slow-acetate oxidizers, *A. syzygii* SKU19, no. 112, 217, 258 and 264 are shown in B.

corresponded well with the study by Matsushita *et al*. (2005b), who reported some variations in the growth phase-pattern which differed from species to species because: 1) some strains do not have an appreciable stationary phase as acetic acid overoxidation started during the ethanol oxidation phase, so they immediately oxidized acetic acid as it was produced; or 2) some strains do not have any appreciable overoxidation and they remain in the stationary phase for a longer period of time; or 3) several species exhibited a stationary phase intermediate between case 1 and case 2.

Enhancement of alcohol and aldehyde dehydrogenase activities and heme staining

To determine the relationship between acetic acid resistance and PQQ-ADH and ALDH activities, acetic-acid adapted strains were cultured in glucose-free SCM broth containing 2% ethanol and harvested in the first stationary phase. The membrane fraction of the adapted strains was prepared and the activity of both enzymes was then measured by the method of Adachi *et al*. (1978). As shown in Figure 6A the specific activities of PQQ-ADH and the ALDH of acetic-acid adapted strains were higher than those of *A. syzygii* SKU19 especially for the adapted strains 112 and 187. This result was confirmed by heme staining which showed a higher intensity of a dehydrogenase subunit (subunit I, 72-80 kDa) and a cytochrome *c* subunit (subunit II, 44-54 kDa) in the acetic-acid-adapted strains and *A. pasteurianus* SKU123 than in *A. syzygii* SKU19 (Figure 6B). The results indicated that the acetic-acid-adapted strains were more resistant to acetic acid than the wild type. The results were also supported by the growth of acetic-acid-adapted strains in glucose-free SCM broth containing various concentrations of acetic acid. It is possible that higher PQQ-ADH activity may produce a larger energy pool available for other membrane-associated processes, such as an acetic-acid export system, which might also explain the capability of all acetic-acid-adapted strains to resist higher concentrations of acetic acid (Chinnawirotpisan *et al*., 2003; Matsushita *et al*., 2005a). Acetic acid is produced by a typical periplasmic oxidation system. All acetic-acid bacteria, except for the genus *Asaia,* contain unique NAD(P)-independent, membrane-bound ADH and ALDH as the primary dehydrogenase responsible for ethanol oxidation. The ethanol oxidation is linked with a respiratory chain reaction in order to generate energy for cell growth (Matsushita *et al*., 2005b). Acetic-acid bacteria can pump acetic acid out by utilizing the proton motive force generated by the ethanol respiration. The most reasonable mechanism responsible for the acetic-acid resistance in acetic-acid bacteria is an efflux pump localized in the cytoplasmic membrane. Therefore, acetic acid production is a closed relationship involving acetic-acid resistance and some functions of PQQ-ADH. Ohmori *et al*. (1982) and Takemura *et al*. (1991) supported this concept since ADH-deficient mutants of *A. aceti* 1031 showed a sensitivity to acetic acid. Moreover, when an ADH gene of *Acetobacter* sp. was introduced to an ADH-deficient mutant of *A. pasteurianus* NP2503, the transformant was resistant to acetic acid. Chinnawirotpisan *et al*. (2003) showed that the PQQ-ADH deficient mutant was obtained from the thermotolerant strain, *A. pasteurianus* SKU1108, by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment and the *adhA* gene disruptant exhibited a complete loss in ethanol oxidation and acetic-acid resistance.

Comparison of the genetic background by randomly amplified polymorphic DNA (RAPD)

To investigate the possible change of the DNA sequence involved in the acetic-acid adaptation in acetic-acid adapted strains, their genomic DNAs were examined for the deficiency of some genetic element(s) using randomly amplified polymorphic DNA (RAPD). The RAPD analysis did not show any obvious difference in genetic background, so the acetic-acid adapted

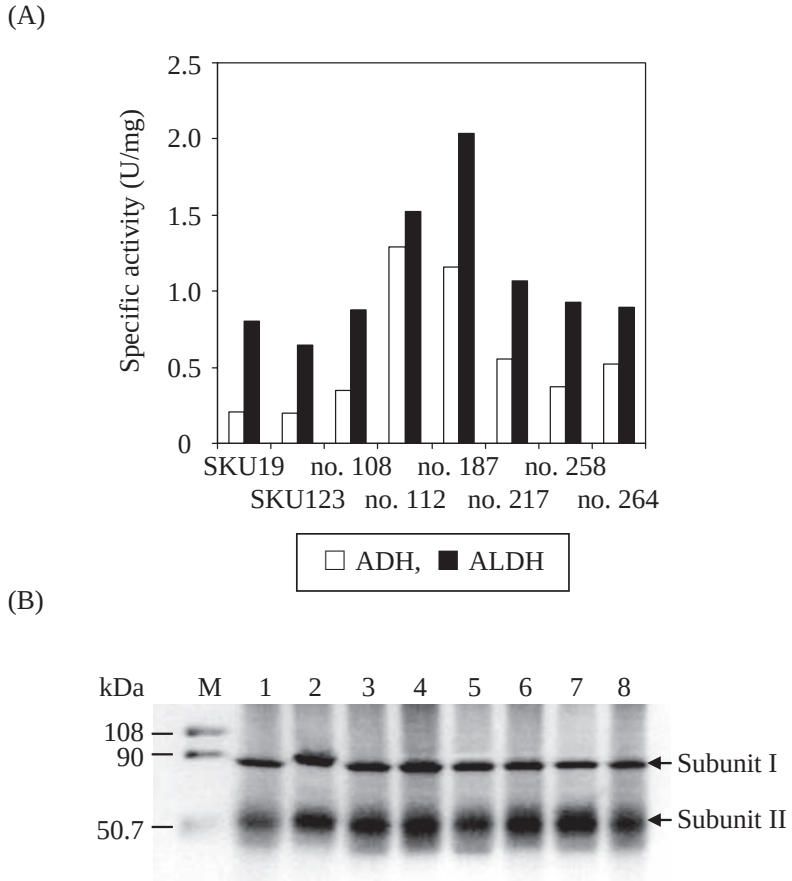

Figure 6 Enzyme activities of (A) ADH and ALDH and (B) heme staining in membrane fraction of acetic-acid-adapted strains from *A. syzygii* SKU19. Lane M, standard protein marker; lane 1, *A. syzygii* SKU19 (acetic-acid-sensitive strain); lane 2, *A. pasteurianus* SKU123; lane 3 to 8, acetic-acid-adapted strains from *A. syzygii* SKU19, no. 108, 112, 187, 217, 258, and 264, respectively. 100 μg of total protein from membrane fraction was loaded in each lane.

strains might not contain any insertion sequences in their genomic DNA (Figure 7).

CONCLUSION

This data indicated that an acetic-acid-sensitive strain can adapt itself to acetic acid. The acetic-acid resistance of the adapted strain probably resulted from a transient physiological adaptation and not a genetic evolutionary adaptation, because the genetic background of the adapted strain and *A. syzygii* SKU19 showed no obvious difference in DNA patterns. Resistance to acetic acid by the adapted strains related to ethanol oxidation and acetate assimilation, which were able to generate a proton motive force. Both the PQQ-ADH and membrane-bound ALDH activities of these adapted strains were higher than for *A. syzygii* SKU19. Previous studies, in addition to proteomic analysis, also described acetate-resistant proteins such as AarABC, aconitase and AatA. Therefore, these studies indicated that resistance to acetic acid in acetic-acid bacteria was conferred by several mechanisms that included an alcohol oxidation-related mechanism and an acetate assimilation-related mechanism.

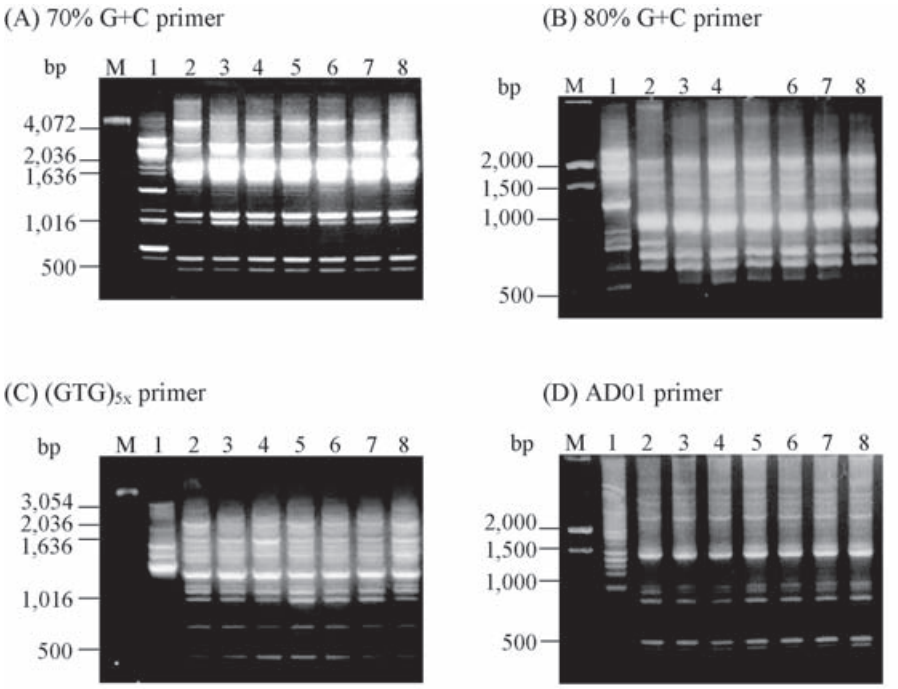

Figure 7 RAPD profiles of genomic DNAs from acetic-acid adapted strains from *A. syzygii* SKU19 with 70%G+C (A), 80% G+C (B), $(GTG)_{5x}$. (C) and AD01 (D) primer. Lane M, 1 kb ladder DNA marker; lane 1, *A. pasteurianus* SKU123; lane 2, *A. syzygii* SKU19; lane 3 to 8, acetic-acid-adapted strains from *A. syzygii* SKU19; no. 108, 112, 187, 217, 258, and 264, respectively.

ACKNOWLEDGEMENTS

The authors would like to express their sincere thanks to the Graduate School, Kasetsart University, the Thailand Research Fund (TRF) and the Association for International Education, Japan (AIEJ) for financial support. Part of this work was carried out under a Core University Program through collaboration between Yamaguchi University and Kasetsart University and was supported by the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Thailand (NRCT).

LITERATURE CITED

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

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

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

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

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. Bacteriol.** 172: 2096-2104.

Fukaya, M., H. Takemura, 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.

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.

Kumar, S., K. Tamura, I.B. Jakobsen and M. Nei. 2001. MEGA 2: molecular evolutionary genetics analysis software. **Bioinformatics** 17: 1244-1245.

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.

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

Matsushita, K., T. Inoue, G. Theeragool, J. Trcek, H. Toyama and O. Adachi. 2005b. 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, India.

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.

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

Nakano, S. and M. Fukaya. 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

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

Okamoto-Kainuma, A., W. Yan, 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.

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.

Russell, J.B. 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. **J. Appl. Bacteriol.** 73: 363-370.

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.

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

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

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

Steiner, P. and U. Sauer. 2003. Long-term continuous evolution of acetate resistant

Acetobacter aceti. **Biotechnol. Bioeng.** 84: 40-44.

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

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.**

Thomas, P.E., D. Ryan and W. Levin. 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome *P*-450 on sodium dodecyl sulfate polyacrylamide gels. **Anal. Biochem.** 75: 168-176.

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.