

Poster Session IV

Physiology and Biochemistry of Acetic Acid Bacteria

PIV-1: Real-time monitoring of ATP generation in *Acetobacter pasteurianus* NBRC3283 using a ^{31}P -NMR method

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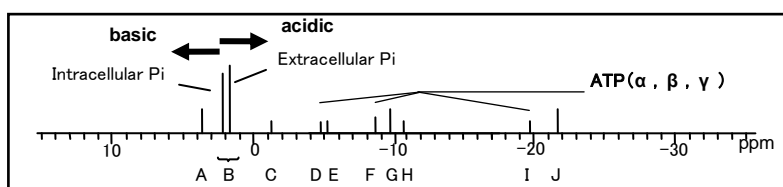
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Acetobacter pasteurianus is a species belonging to the acetic acid bacteria and is known to have two types of energy generating pathway as follows; 1) a pathway via substrate oxidation by enzymes on the cell membrane → electron transfer system → ATP generation; and 2) a pathway via the TCA cycle inside of the cell → electron transfer system → ATP generation. The cells are said to grow by changing between these pathways depending on the circumstances surrounding them; however, there are no reports on the *in situ* observation of this physiology. This study tried to verify this physiological property by real-time monitoring of ATP generation in living cells using a ^{31}P -NMR method.

A. pasteurianus NBRC3283 was cultured to the stationary phase, then collected and resuspended in M9 based minimum medium. The cell suspension was set into an NMR instrument (JEOL ECA600) equipped with a P-probe (Nalorac H-X8FG600) with constant aeration. Measurements were started with the addition of the substrates, and the changes with time in peaks due to ATP molecules were observed. Ethanol, glucose, and glycerol were used as the substrates.

When glucose and ethanol were used as substrates, ATP generation was observed with a decrease in pH of the cell suspension, indicating that substrate oxidation resulted in ATP generation. After oxidizing almost all of the ethanol, ATP generation was also observed with increasing pH in the cell suspension, which indicated the occurrence of peroxidation of acetic acid. Glycerol was shown to generate more ATP compared to ethanol or glucose oxidation in this species.

With these analyses, ATP generation through substrate oxidation at the cell surface was confirmed *in situ*. In addition, the existence of the general aerobic pathway using the TCA cycle, which was responsible for the peroxidation of acetic acid, was strongly indicated. Thus, it was confirmed that the growth of *A. pasteurianus* cells occurred by changing between the two energy generating pathways depending on the circumstances.



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Fig.1 Chemical shift positions of phosphorus containing compounds in the cell
A: sugar monophosphate, B: orthophosphate (Pi), C: phosphoenolpyruvate, D: ATP $^{\gamma}$, E: ADP $^{\beta}$, F: ATP $^{\alpha}$, ADP $^{\alpha}$, G: NAD $^{+}$ /NADH, H: UDPG, I: ATP $^{\beta}$, J: poly phosphate

PIV-2: Proteomic analysis of acetic acid bacteria with several carbon sources

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Acetic acid bacteria can grow with several carbon sources. This property comes from their unique energy generation process by multiple pathways; via substrate oxidation at the cell membrane and via other pathways exist inside of the cell. The cells grow by changing these pathways depending on the circumstances surrounding them. Acetic acid fermentation is one form of these energy acquiring behaviors. However, details of this characteristic property are still obscure, and to understand the physiological characteristics in acetic acid fermentation, it is important to investigate the proteins that are characteristically expressed during the fermentation. For this purpose, proteomic analysis was carried out using *Acetobacter pasteurianus* NBRC3283 and *Gluconobacter oxydans* 621H with three kinds of carbon source including ethanol.

A. pasteurianus NBRC3283 or *G. oxydans* DSM2003 cultured in media containing each carbon source were homogenized in a buffer with several detergents. The extracts were subjected to two-dimensional gel electrophoresis using agarose gel isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension,¹ followed by LC-ESI-IT MS analysis. The proteins were identified using Xcaliber BioWorks software.

In *A. pasteurianus*, the factors related to ethanol oxidation and molecular chaperones, such as GroE, DnaK, and ClpB, increased characteristically when the cells were cultured in ethanol-containing medium. These factors decreased in the peroxidation phase with acetic acid, while factors relating to the TCA cycle and AarC increased in this phase. Thus, the characteristic metabolism of this bacterium with two types of pathway was confirmed by this analysis. The increase in the levels of molecular chaperones was also observed when the cells were cultured in glucose-containing medium but not in glycerol-containing medium. In addition, the same tendency was also observed in *G. oxydans*. Therefore, these chaperones are considered to have important roles in the resistance to acidic conditions during fermentation, and this property seems to be common to acetic acid bacteria.

Comparing the results of the two strains, differences between them were also found. When the cells catabolize the substrates, *A. pasteurianus* increased factors relating to the TCA cycle and AarC, while *G. oxydans* increased factors relating to the glycolytic and pentose-phosphate pathways. These findings were in good agreement with previous reports.

Thus, proteomic analysis is considered to be effective in investigating the characteristic features of the physiology of acetic acid bacteria on a molecular basis.

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PIV-3: Superoxide dismutase and catalase in *Acetobacter pasteurianus* NBRC3283

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Acetobacter pasteurianus is an obligate aerobe that possesses two types of aerobic energy producing pathway. One is via substrate oxidation on the surface of the cells and the other is via the general aerobic pathway using the TCA cycle inside the cells.¹ Both pathways require sufficient amounts of oxygen molecules and the cells change between these pathways depending on the circumstances. When ethanol is a substrate, this bacterium is considered to generate ATP by ethanol oxidation first, and then by the general aerobic pathway using acetic acid produced from ethanol oxidation. The former behavior is called acetic acid fermentation and the latter is called the peroxidation of acetic acid.

In general, aerobic metabolism results in the formation of reactive oxygen species (ROS) in the process of electron transfer, which can cause damage to cells by reacting with many components in the cells. Aerobic cells generally possess mechanisms to resist these ROS, and representative factors involved in the resistance system are superoxide dismutase (SOD) and catalase. SOD catalyzes the reaction of $2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$, and catalase catalyzes the reaction of $\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$. We investigated these enzymes responsible for the detoxification of ROS in *A. pasteurianus* to obtain information about the oxidative stress resistance mechanism in this strain, which is a unique obligate aerobe with two types of aerobic energy metabolism.

Acetobacter pasteurianus NBRC3283 was used in this experiment. SOD and catalase activities were measured by the methods reported by Beauchamp² and Vattanviboon³, respectively. Gene disruption in the chromosomal DNA was carried out by a gene replacement method.

This strain expressed only one type each of SOD and catalase, which were growth dependent. These enzymes were identified as a SodB homologue and a KatE homologue, respectively. There was no isozyme drastically induced by oxidative stressors at log phase (substrate oxidation phase). The disruptant of *katE* showed a delay in growth compared to the parental strain in the peroxidation phase though it was hardly affected in the ethanol oxidation phase. In addition, a growth delay was also observed when the cells were cultured in glycerol or acetic acid containing medium.

From these results, it was suggested that SOD and catalase are involved in the resistance to oxidative stress caused in the general aerobic pathway but not in the substrate oxidation pathway.

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2. Beachamp, C. et al., *Anal. Biochem.*, **44**, 276-287 (1971)
3. Vattanviboon, P. et al., *Gene*, **241**, 259-265 (2000)

PIV-4: NADH peroxidase and quinol peroxidase activities in *A. pasteurianus* NBRC3283

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Acetobacter pasteurianus is an obligate aerobe that performs acetic acid fermentation. This bacterium has two types of energy generating pathway; a pathway via substrate oxidation at the cell membrane and the general aerobic pathway via the TCA cycle. In our study on the resistance mechanism to oxidative stress caused during acetic acid fermentation/oxidation, it was indicated that super oxide dismutase (SOD) and catalase did not perform important roles in the metabolic pathway via substrate oxidation but were important in the general aerobic pathway. This suggests the following two possibilities; 1) the pathway via substrate oxidation does not generate significant levels of reactive oxygen species (ROS), or 2) another oxidative-stress resistance system, which does not use SOD and catalase, exists and functions during metabolism via substrate oxidation. This study attempted to investigate the latter possibility in *A. pasteurianus*.

Attention was paid to the oxidative stress resistance system in anaerobic bacteria like *Lactobacillus*¹ or *Actinobacillus*,² in which the detoxification of hydrogen peroxide by NADH peroxidase or quinol peroxidase has been reported. The detection of these activities was attempted in *A. pasteurianus* NBRC3283.

Catalase activity was measured by monitoring the decrease in hydrogen peroxide.³ NADH peroxidase activity was measured by monitoring the decrease in the absorbance at 340 nm due to NADH, an electron donor, in the presence of hydrogen peroxide.⁴ Quinol peroxidase activity was measured by monitoring the increase in absorbance at 278 nm due to ubiquinol-1, an electron donor, in the presence of hydrogen peroxide.² Both peroxidase activities were analyzed in anaerobic conditions.

Both NADH peroxidase and quinol peroxidase showed stronger activities at log phase than at stationary phase, while catalase showed stronger activity at stationary phase. These activities were detected in the membrane fraction and not in the soluble fraction. These results could indicate that hydrogen peroxide generated during substrate oxidation at the cell membrane might be detoxified by the peroxidases also located on the cell membrane. Thus, the possibility was suggested that a similar system functions in both the anaerobic bacteria and in *A. pasteurianus*, which is an obligate aerobic bacterium.

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PIV-5: Two Component System NtrB/C in *Acetobacter pasteurianus* NBRC3283 Control the Regulations of Membrane-bound Alcohol Dehydrogenase and Aldehyde Dehydrogenase

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Acetic acid bacteria produce acetic acid from ethanol by an oxidation pathway that employs alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) located on the surface of the cell membrane. This behavior is generally called acetic acid fermentation and is applied to vinegar production. During fermentation, the microenvironment surrounding the cells changes as fermentation proceeds; therefore, sensing and adapting to the changing circumstances outside the cells are important to enable the cells to perform fermentation. In general, bacteria are known to perform sensing/adaptation using two-component systems consisting of sensor kinases and response regulators in the cells.¹ This study focused on the NtrB/C two component system,² which is reported to regulate genes controlled by σ^{54} factor, in *A. pasteurianus* NBRC3283 and investigated its relationship to acetic acid fermentation.

The target genes were cloned by a combination of degenerate PCR and inverse PCR methods. It was found that the genes of *nifR3*, *ntrB*, *ntrC*, *ntrY*, and *ntrX* were located in this order on the genome in *A. pasteurianus*, and the *nifR3/ntrB/ntrC* genes and *ntrX/ntrY* genes formed operons, respectively. The *ntrC* deletion mutant showed a remarkable delay in growth in the presence of 1 M ethanol when compared to the parental strain. Such a delay was not observed in the restoration mutant of *ntrC*, which suggested the importance of this factor for growth with ethanol. When the activities of membrane bound type ADH and ALDH were measured in the *ntrC* mutant, lower activities (*ca.* 50%) were detected compared to the parental strain, whereas the activities in the restoration mutant recovered to *ca.* 80%. The same tendency was observed in the mRNA levels of the *adh* and *aldh* genes, indicating that the decrease in the enzyme activities in the *ntrC* mutant occurred at the transcriptional level.

The characteristic sequence of the NtrC binding site and σ^{54} promoter sequence were found upstream of both the ADH and ALDH coding regions in the genome, suggesting that the expression of these enzymes was under control of σ^{54} and NtrC. When gel mobility shift assays were carried out with the upstream regions of the *adh* and *aldh* genes as probes, the NtrC protein bound to them in a dose-dependent manner.

These results indicate that NtrC regulates the expression of ADH and ALDH via σ^{54} -related transcription system and the important relationship of the NtrB/C two component system to acetic acid fermentation.

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PIV-6: Molecular Chaperones in *Acetobacter pasteurianus* NBRC 3283 and Their Relationship to Acetic Acid Fermentation

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In general, acetic acid bacteria produce acetic acid by the oxidation of ethanol on the cell surface for energy generation. Accompanying this process, acetic acid bacteria have considerable resistance to heat generated during fermentation/ethanol/acetic acid, which are stressors for cells in general. Therefore, it is considered to be important to elucidate the mechanisms underlying the resistance of acetic acid bacteria to stressors. From this point of view, the functions of molecular chaperones were examined, which are considered to protect cells against stressors. This study cloned and characterized *dnaKJ*, *grpE*, and *groESL* of *Acetobacter pasteurianus* NBRC 3283, which is a representative strain for vinegar fermentation.

The target genes were cloned by a combination of degenerate PCR and inverse PCR methods. Their expression patterns and regulation were analyzed by northern blot, RT-PCR, and qRT-PCR methods.

The genes of *grpE*, *dnaK*, and *dnaJ* were located in this order on the genome in *A. pasteurianus*.¹ There were two heat shock promoters in the upstream regions of *grpE* and *dnaK*. In addition, a house keeping promoter sequence was observed overlapped with an inverted repeat sequence upstream of the *dnaJ* gene. These genes were found to be transcribed as *grpE-dnaK*, *dnaK*, and *dnaJ* in the ratio of approximately 1:2:0.1. Heat shock (42°C) and ethanol addition (4%) to the cells increased the transcription level of these genes by about 10-fold and 4-fold, respectively. Acetic acid hardly affected their expression levels.

The *groES* and *groEL* genes were found to form an operon with a heat shock promoter upstream of *groES*.² In addition, in the upstream region of the promoter a highly conserved inverted repeat sequence (CIRCE) was observed. In transcription analyses, the *groES* and *groEL* genes were observed to be transcribed as one sequence. These genes were induced by heat shock (42°C) as well as by the addition of ethanol (4%) or acetic acid (3%).

The overexpression of DnaK/J in the cells resulted in improved growth compared to the parental strain under conditions of high temperature (42°C) and in the presence of ethanol (5%). This tendency was also notable in the co-overexpression of GrpE with DnaK/J, indicating that GrpE acts as an effective co-chaperone of DnaK in this strain. The overexpression of GroES/L increased the resistance to acetic acid (1%) as well as to heat (42°C) and ethanol (5%), suggesting that these factors are important in acetic acid fermentation.

These observations indicate that GroES/L, DnaKJ, and GrpE are closely associated with the characteristic nature of *A. pasteurianus* and play important roles in acetic acid fermentation.

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PIV-7: Isolation and characterization of two-component system *chvI/G* genes in *Acetobacter pasteurianus* NBRC3283

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Acetobacter pasteurianus is a type of acetic acid bacteria that is used for vinegar production in Japan and produces acetic acid or gluconic acid by the oxidation of ethanol or glucose, respectively, in oxidative fermentation. The cells become adapted to the acidic conditions as fermentation proceeds. This adaptation enables the cells to grow under acidic condition, which is essential for efficient fermentation. Therefore, the investigation of this adaptation mechanism is important for clarifying the fermentation mechanism. The first step of this adaptation is considered to be the sensing of acidic conditions outside of the cells, and this is reported to be performed by ChvG/ChvI as a global sensor-regulator system for acidic pH in other bacteria.¹ In this study, it was attempted to clone and characterize the *chvG* and *chvI* genes in *Acetobacter pasteurianus* NBRC3283.

The *chvI* and *chvG* genes were cloned from the genome of *A. pasteurianus* by a combination of degenerate PCR and inverse PCR methods. These genes were located tandem in the genome. The ChvI and ChvG proteins possessed all of the features of a response regulator and sensor kinase, respectively.²

Overexpression of these genes did not affect cell growth in media containing ethanol or acetic acid. Disruption of *chvG* caused a delay in growth under the existence of acetic acid; however, the disruptant did not show any different growth behavior in other acidic conditions, such as with gluconic acid, lactic acid, or hydrochloric acid. In addition, ethanol, which is gradually converted to acetic acid during the fermentation process, had no effect on the growth of the disruptant.

In general, the disruption of *chvG* increases the sensitivity to several compounds like detergents or antibiotics³ which affect the cell surface conditions. However, the sensitivity of the *chvG* mutant of *A. pasteurianus* was hardly affected by these compounds.

From these results, it was suggested that ChvG is not a global pH sensor in *A. pasteurianus* although it has some relationship to acetic acid resistance. In addition, the other functions of ChvI/ChvG of this strain seem also to be different from those of other bacteria.

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PIV-8: Expression of Quinoprotein Alcohol Dehydrogenase (PQQ-ADH) and Adaptive Responses to High Concentration of Ethanol and Acetic Acid in Thermotolerant Acetic Acid Bacteria

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PQQ-ADH is a key enzyme involved in ethanol oxidation in acetic acid bacteria. PQQ-ADH deficient mutant of *Acetobacter pasteurianus* SKU1108 (CN6-2) exhibited complete loss in PQQ-ADH activity but increase in NAD-ADH activity when it was grown in the medium containing ethanol. The activities of two NAD-ADHs, ADH I and ADH II, were induced 12 and 20 times, respectively in the medium containing ethanol¹. The results clearly showed that PQQ-ADH involved in ethanol oxidation while NAD-ADHs worked for ethanol assimilation. It has been purposed that mutation in PQQ-ADH led a global metabolic change from ethanol oxidation to ethanol assimilation as an adaptive response to ethanol².

We successfully cloned and determined the nucleotide sequences of *adhA*, *adhB* and *adhS* genes from *A. pasteurianus* SKU1108 (acetic acid resistant strain) and *A. syzygii* SKU19 (acetic acid sensitive strain)³. These three genes coding for PQQ-ADH subunit I (dehydrogenase), subunit II (cytochrome *c*) and subunit III (unknown function), respectively. In *A. pasteurianus* SKU1108; subunit I, II and III consisted of 742, 472 and 205 amino acids with 89, 86 and 98.5% homology, respectively, with the same proteins from *A. syzygii* SKU19 and one additional amino acid was found in subunit I of *A. syzygii* SKU19. Three amino acid alterations, 75Val→75Ala, 134Arg→134Gly and 155Ile→155Thr were also observed in subunit III of *A. syzygii* SKU19. Surprisingly, the molecular mass calculated from relative mobility in SDS-PAGE of purified subunit III protein from *A. pasteurianus* SKU1108 and *A. syzygii* SKU19 was 13 and 10 kDa, respectively, even though both of them consisted of 205 amino acids.

The *adhS* disruptant (DPS) also grew even better than the wild type on the medium containing ethanol even though it completely lost the ability to produce acetic acid and became more sensitive to acetic acid similar to CN6-2. Comparison of PQQ-ADH and NAD-ADH activities in those mutants indicated an induction of NAD-ADH activity in CN6-2 and DPS but not in *adhAB* gene disruptant (DP3). It was also observed that only ethanol but not acetic acid induced *adhS* gene expression. In addition, deletion analysis of upstream region of *adhS* gene suggested that its tentative promoter(s) might be located at around 118-268 bp upstream from an initiation codon. The alterations of several amino acids of subunit III such as 18Leu→18Gln, 36Val→36Ile, 54Val→54Ile, 55Gly→55Asp, 70Val→70Ala, 107Val→107Ala and the mutant lacking 22 amino acid residues at C-terminal did not affect PQQ-ADH activity. However, the change of nucleotide C₁₅ to A₁₅ at a tentative ribosome binding site exhibited low PQQ-ADH activity and complete loss in its activity was obtained in the mutant carrying one amino acid change from 104Thr→104Lys and the mutant lacking 73 amino acid residues at C-terminal.

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PIV-9: Purification and characterization of alcohol dehydrogenases from thermotolerant *Acetobacter pasteurianus* strains

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In this study, we compared the growth properties among thermotolerant and mesophilic *Acetobacter pasteurianus* strains and also the characteristics of membrane-bound alcohol dehydrogenase (ADH) purified from these strains. Thermotolerant *A. pasteurianus* MSU10 and SKU1108 could grow well on potato medium agar plates at 39°C, tolerate to 4% ethanol and 1.5% acetic acid at the same temperature. While a mesophilic *A. pasteurianus* IFO3191 could grow poorly, tolerate to 1% ethanol and 1% acetic acid at the same temperature. From these strains grown with 2% ethanol, ADHs were purified by a DEAE-Toyopearl chromatography. Based on SDS-PAGE, the molecular masses of the three subunits, subunit I, II and III, were almost the same to be 75.8, 56.6 and 15.9 kDa, respectively, in ADHs between MSU10 and SKU1108 strains. While, IFO3191 strain had a little different molecular masses in the three subunits of 73.7, 53.7 and 15.9 kDa, respectively. The optimal temperature of the enzyme activity from MSU10 and SKU1108 strains were found to be 35°C, but 30°C in IFO3191. All the enzymes among these strains were stable up to 50°C for 30 min and retained the activity >50%, which was not much different each other. Effect of pH on enzyme stability was not different among the strains; all the enzymes were stable in a pH rang of 2.3-8.0 for 30 min. The optimum pH of the activity (pH 4.0) was also not different among three enzymes. ADHs of MSU10 and SKU1108 were more resistant to acetic acid than IFO3191 and thus exhibited the resistance >70% even at 8% acetic acid after incubation at 4°C for 30 min. However, at high temperature (30°C), the activity was dramatically decreased to ~30% even with 4% acetic acid.

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PIV-10: High Temperature-Acetic Acid Fermentation in Thermotolerant *Acetobacter pasteurianus* SKU 1108

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Acetic acid bacteria (AAB) are well known to have an ability to oxidize various alcohols or sugar alcohols into their corresponding acids. Vinegar is industrially produced by using the ethanol oxidation ability of AAB, which is carried out by membrane-bound alcohol and aldehyde dehydrogenases (ADH and ALDH) around the optimum temperature between 25°C to 30°C. Many thermotolerant AAB had been isolated from Thailand and *Acetobacter pasteurianus* SKU 1108 was selected as the most suitable strain to do vinegar fermentation at higher temperature.

When acetic acid fermentation of *A. pasteurianus* SKU 1108 was examined at higher temperature, the fermentation in flask culture at 200 rpm was severely impaired at 39°C, though it can grow even at 42°C without ethanol. Thus, to obtain the strain able to do the fermentation at much higher temperature, adaptation of SKU 1108 for the fermentation at higher temperature was carried out by repeated cultivations at 39°C or also by repeated cultivations by increasing the culture temperature gradually from 38 to 39.5°C in the presence of 4% ethanol. Thus, different types of the adapted strains were successfully obtained.

The adapted strain appeared to be advantageous for acetic acid fermentation at higher temperature compared with the original SKU1108 strain. Especially in jar fermentor with high aeration, the adapted strains could perform an effective acetic acid fermentation with 4% ethanol even at 40°C.

In order to understand the underlying mechanism in the thermotolerance of the original thermotolerant strain SKU1108 and also these adapted strains, the enzyme activities involved in alcohol oxidizing system and also the oxidative stress response were examined by comparing these original and adapted strains. One of the adapted strain TI was found to exhibit much higher ADH and somewhat higher ALDH activities when compared with the original strain. Whereas, SOD and catalase activities measured by activity staining were all lower in the adapted strain than in the wild strain. Thus, we are going to present acetate fermentation ability at different temperatures and the physiological properties of these adapted strains, comparing with the original thermotolerant strain SKU 1108.

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PIV-11: Analysis of the gene in high temperature-sensitive mutant of thermotolerant acetic acid bacteria, *Acetobacter tropicalis*

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Acetobacter tropicalis SKU1100 is a thermotolerant acetic acid bacteria (AAB) able to grow even at 42 °C, which is much higher than the temperature that mesophilic AAB strains are able to grow. To elucidate the mechanisms underlying the thermotolerance of *A. tropicalis*, we have attempted to identify genes essential for growth at high temperature by transposon (Tn10) mutagenesis using conjugation method. Among four thousands of the transconjugants obtained, 40 colonies exhibited growth phenotype unable to grow at 39 °C or 41 °C but able to grow well at 30 °C. Using Tail-PCR method, we succeeded to identify the insertion site of Tn10 in chromosomal DNA of the mutant strains. Together with the identified genes and their growth properties at different conditions, the mutant strains were categorized and at least 10 genes seemed to be very important for the growth at high temperature. Different from such mutants exhibiting severe defects in the thermotolerant growth, a mutant strain defective in cytochrome *c* biosynthesis was also obtained, and exhibited high sensitivity to oxidative stress, ethanol, and acetic acid. Reactive oxygen species (ROS) generation were also determined in the mutant cells grown under stress conditions using a reactive fluorescence dye (H₂DCFDA). At high temperature, intracellular ROS was shown to be increased as well as exposing under oxidative stress condition. Interestingly, mutation at the gene for serine protease, which belongs to the group of heat shock protein, decreased the level of ROS after shifting to high temperature, which was different from other mutants such as mutant strain defective in lysyl tRNA synthase generating more ROS compared with the wild strain.

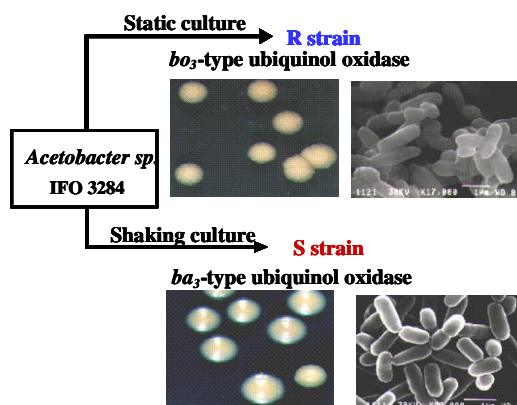
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PIV-12: Heme O and Heme A synthesis genes in *Acetobacter sp.* IFO3284

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Two terminal ubiquinol oxidases, cytochrome *ba*₃ and cytochrome *bo*₃, have been found in *Acetobacter sp.* IFO3284, and shown to be distinctive only in the heme moiety, heme A or heme O, of the O₂ reacting binuclear center. In this strain, two different colonies were isolated from different culture conditions as pellicle-forming R strain and non-forming S strain. S strain, having cytochrome *ba*₃ oxidase, was isolated in shaking culture, and R strain, having cytochrome *bo*₃ oxidase, isolated in static culture. However, the expression of the terminal oxidase was not affected by the culture condition, and both oxidases were shown to occur from the same gene.



It is generally known that heme A and heme O are derivatives of heme B, and are present in many terminal oxidases except for *cbb*₃-type cytochrome *c* oxidase or *bb*₃-type ubiquinol oxidase. Heme O is synthesized by replacement of the vinyl group of heme B with 17-carbon hydroxyethylfarnesyl at position 2 of the porphyrin ring. Heme A is synthesized from heme B via heme O, where the methyl group of heme O at position 8 is replaced with a formyl group. Heme O was first discovered in *E. coli* cytochrome *bo*₃ oxidase. This oxidase consists of four subunits, which are encoded on *cyoABCD* of the *cyoABCDE* operon, and *CyoE* encodes a heme O synthase. The homologue of *E. coli cyoE* has been found as *ctaB* in several other bacteria such as *Bacillus subtilis* and also as *cox10* in yeast. The heme A synthesis gene has also been identified as *ctaA* in *B. subtilis*, because loss of the *ctaA* leads to the deficiency of cytochrome *aa*₃ in the organism.

In this study, heme A and heme O synthesis genes were searched in *Acetobacter sp.* IFO3284 to clarify the heme A synthesis pathway in this bacterium, as they produce a ubiquinol oxidase having heme A as the prosthetic group. As a result, putative *ctaB* and *ctaA* genes were found in the genome of *Acetobacter sp.* IFO3284. *CtaB* exhibit heme O synthesis activity, which was shown both in *in vivo* expression analysis and *in vitro* enzyme assay. *CtaA* was also shown to have heme A synthesis activity by expressing the *ctaA* gene in *E. coli*.

PIV-13: Respiratory chain related to acetic acid fermentation in *Acetobacter pasteurianus* IFO 3283

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Acetic acid bacteria (AAB) are well known for their ability to oxidize various sugars and sugar alcohols into their corresponding acids. Vinegar is industrially produced by using the oxidation ability of AAB from ethanol, and widely used in many food products.

Acetobacter pasteurianus IFO 3283 oxidizes ethanol to acetic acid and further oxidizes acetate after prolonged stationary phase of 5 days, when cultured in 4.0 % ethanol. Thus, the strain exhibits a typical diauxic growth consisting of three phases, ethanol oxidation, acetic acid resistance and acetate overoxidation.¹⁾

It has been shown in the ethanol oxidation phase that acetic acid is produced by alcohol and aldehyde dehydrogenases located on the outer surface of the cytoplasmic membrane, which donate electrons to terminal ubiquinol oxidases, either *ba*₃ or *bo*₃ terminal oxidase, via ubiquinone (UQ). Whereas, in the overoxidation phase, acetate appeared to be oxidized via TCA cycle, which could be operative coupled with the respiratory NADH oxidase system. In general, either NADH dehydrogenase II (NDH II), NADH dehydrogenase I (NDH I), or both works as the primary dehydrogenase of the NADH oxidase system. Genome information from other AAB also suggests the presence of cytochrome *bc*₁ complex or *bd*-type terminal oxidase in the respiratory chain.

Thus, we examined various respiratory chain components of the membranes or vesicles from the ethanol oxidation, acetic acid resistance and acetate overoxidation phases in *A. pasteurianus* IFO 3283 grown on medium containing 4 % ethanol, by comparing the cells grown on glycerol medium, non-acetate fermentation condition. As the results, it was shown that two ubiquinol oxidases, cytochrome *ba*₃ and cytochrome *bd*, are expressed in glycerol medium and in the ethanol oxidation phase, but not detected in the overoxidation phase. It was also shown that NDH II at least, cytochrome *bc*₁ complex and quinol peroxidase work in the respiratory chain.

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PIV-14: Novel Exopolysaccharide from *Kozakia baliensis*

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Acetic acid bacteria are obligate aerobic bacteria and well known as oxidative bacteria. They have strongly oxidizing alcohols, sugars, and sugar alcohols to corresponding oxidation products. Acetic acid bacteria have also been known to produce a relatively large amounts of capsular polysaccharide (CPS) and extracellular polysaccharide (EPS), for example, *Gluconacetobacter xylinus* (*Acetobacter xylinum*) are able to produce thick pellicle consisting of bacterial cellulose¹. Recent works we reported that *A. aceti* IFO 3284 produced novel CPS attached to the outer membrane and shown to be composed of two monosaccharides, glucose and rhamnose, at an approximately equimolar ratio and had molecular mass of higher than 700-kDa². Thermotolerant *A. tropicalis* SKU1100 produced a thick pellicle even at higher temperature such as 37°C and 40°C. The purified CPS was composed of glucose, rhamnose and galactose in an approximately equimolar ratio of 1:1:1 and had molecular mass of 120-kDa³. *A. pasteurianus* produced CPS composed of glucose, rhamnose and xylose⁴.

Our study on diversity of polysaccharide from acetic acid bacteria, we found that *Kozakia baliensis* produced distinct polysaccharide from other acetic acid bacteria. The culture medium used for polysaccharide production comprised 1% glycerol as the carbon source. The polysaccharide was secreted into the culture medium and the character of the culture medium exhibited very high viscosity and then the EPS was harvested after incubation for 5 days. EPS from *K. baliensis* was precipitated from culture medium with alcohol and the yield of dried EPS was 3.72 g/L. EPS of *K. baliensis* was purified by two successive column chromatographies. The purified EPS was recovered at 81.26%. Acid hydrolysis of purified EPS with 2N trifluoroacetic acid at 121°C for 60 min shown to be composed of two monosaccharides, glucose and galactose. The purified EPS was estimated to have an apparent molecular mass of higher than 700-kDa. We investigated free radical scavenging activity of crude and purified EPS using DPPH method. It was shown that both crude and purified EPS had free radical scavenging activity of 28.72 and 18.86%, respectively, compared to that of butyrate hydroxyanisole and butylated hydroxytoluene.

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PIV-15: Differences in cellulose biosynthesis related genes between *Asaia bogorensis* and *Gluconacetobacter xylinus*

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Gluconacetobacter xylinus belonging to acetic acid bacteria (AAB) is one of the well-studied model organisms for cellulose biosynthesis. On the other hand, we found out that *Asaia bogorensis*, which is also classified in AAB showing different energy metabolism from other general AAB, produced bacterial cellulose (BC), too. Intriguingly BC of *A. bogorensis* indicates different characteristic features from those of *G. xylinus*. For instance, the cellulose fibers produced by *A. bogorensis* are much narrower (5-20 nm) than those of *G. xylinus* (40-100 nm), and the crystallinity of cellulose from *A. bogorensis* is also lower.

In this time, we amplified about 13 kbp of DNA fragments containing genes relating cellulose synthesis from total genomic DNA of *A. bogorensis* and determined their nucleotide sequences. The sequenced DNA region contained some open reading frames (ORFs) (Fig.1). The result of homology search based on the deduced amino acid sequence showed that high similarity with cellulose synthase (CesA) and cellulose synthesis related protein (CesB, C) which consist of protein complex called terminal complex (TC) of *G. xylinus*. But CesA and CesB were comprehended one ORF in *A. bogorensis*. At the upstream region of CesAB in *A. bogorensis*, one ORF is homologous to endo- β -1,4-glucanase (CMCase) from *G. xylinus* with 70% similarity, and the other ORF has 49% similarity with a cell morphology protein (yhjQ) of *Azotobacter vinelandii* Av0P. Although two ORFs coding CesD and β -glucosidase are located at the downstream region of CesC in *G. xylinus*, homologous genes of them were not detected in this region in *A. bogorensis*. Alternatively, there were predicted flagellar genes at the downstream region of CesC in *A. bogorensis*. We focused on cellulose-hydrolyzing enzymes related to cellulose-production from the sequence analysis results in the first. CMCase gene shows high homology between *A. bogorensis* and *G. xylinus* as described above. We confirmed that messenger RNA of CMCase was expressed by RT-PCR. In addition, we investigated the activity for CMC, and *A. bogorensis* exhibited highest CMC activity at the cellular membrane fraction. It is reported that *G. xylinus* showed same tendency. On the other hand, β -glucosidase was not detected in the region near the cellulose synthesis operon in *A. bogorensis*, but it indicated β -glucosidase activity at the extracellular, intracellular and cellular membrane fractions, investigating the resolution of pNP- β -D-glucose. And so we are investigating whether it is or not existence of β -glucosidase gene showing similarity with that of *G. xylinus*.

It is suggested that CesD gene not having *A. bogorensis* has significant effect on different characteristic features of BC, as both microorganisms have same cellulose-hydrolyzing enzymes.

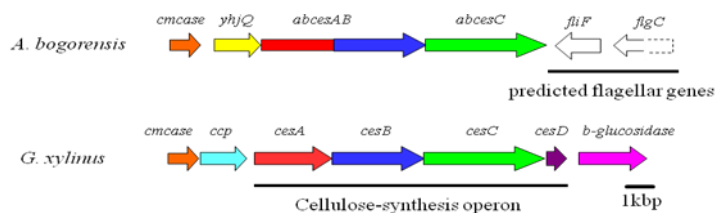


Fig.1
Comparison of
cellulos-synthesis operon of *A. bogorensis* and *G. xylinus*

PIV-16: Flagellum-based motility of *Gluconobacter oxydans*

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In acetic acid fermentation by acetic acid bacteria, ethanol is oxidized into acetic acid by alcohol dehydrogenase and acetaldehyde dehydrogenase located on the periplasmic surface of the cytoplasmic membrane, coupling with reduction of oxygen to water. Such incomplete oxidation is one of key features of acetic acid bacteria. This process can produce energy as the form of proton motive force. Flagella are fiber-form organelles, which require proton motive force in biogenesis and work. This molecular machine spins several hundred revolutions per second and each round consumes several hundred protons (1). Thus, flagellar motility seems to be highly-energy-consuming process in the cells. The genome of *Gluconobacter oxydans* ATCC 621H has one set of flagellar genes (2). Here we examined motility of *G. oxydans*.

G. oxydans spread on soft agar, made of either rich or minimum mineral medium, via an unknown mechanism making "Trails" as well as via flagellum-based swimming. Highest swimming motility of *G. oxydans* was observed at the exponentially growing phase on ethanol-glycerol-glucose or ethanol-glucose medium. There are two *motB* paralogs in the genome of *G. oxydans*, either or both of which function in the rotation of flagella as a part of the proton channel. The BLAST analysis revealed that an *motB* gene (GOX127) adjacent to the *motA* gene (GOX126) was highly homologous to those of several *Pseudomonas* species and several α -proteobacteria as well as to that (GDI1671) of *Gluconacetobacter diazotrophicus* GDI1672. The second *motB* gene (GOX240) adjacent to the gene (GOX239) for an MotA/TolQ/ExbB proton channel protein family was highly homologous to those of several α -proteobacteria as well as to that (GDI503) of *G. diazotrophicus* GDI1672.

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