Acetic Acid Fermentation of \textit{Acetobacter pasteurianus}: Relationship between Acetic Acid Resistance and Pellicle Polysaccharide Formation

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\textbf{Abbreviations}: AAB, acetic acid bacteria; AO-phase, acetate over-oxidation phase; AR-phase, acetate resistance phase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CFU, colony-forming unit; EO-phase, ethanol oxidation phase

\textbf{Acetobacter pasteurianus} strains IFO3283, SKU1108, and MSU10 were grown under acetic acid fermentation conditions, and their growth behavior was examined together with their capacity for acetic acid resistance and pellicle formation. In the fermentation process, the cells became aggregated and covered by amorphous materials in the late-log and stationary phases, but dispersed again in the second growth phase (due to overoxidation). The morphological change in the cells was accompanied by changes in sugar contents, which might be related to pellicle polysaccharide formation. To determine the relationship between pellicle formation and acetic acid resistance, a pellicle-forming \textit{R} strain and a non-forming \textit{S} strain were isolated, and their fermentation ability and acetic acid diffusion activity were compared. The results suggest that pellicle formation is directly related to acetic acid resistance ability, and thus is important to acetic acid fermentation in these \textit{A. pasteurianus} strains.

\textbf{Key words}: acetic acid bacteria; \textit{Acetobacter pasteurianus}; acetic acid resistance; pellicle polysaccharide; acetic acid diffusion

Vinegar is produced industrially with acetic acid bacteria (AAB) and is widely used in many food products. AAB being obligate aerobes are unique microorganisms characterized by their strong ability to oxidize alcohols such as ethanol. In addition, AAB, especially \textit{Acetobacter} and \textit{Gluconacetobacter} species, tolerate high concentrations of acetic acid, and thus both genera are used industrially as vinegar producers. However, in addition to their acetic acid accumulation ability, \textit{Acetobacter} and \textit{Gluconacetobacter} species are also able to oxidize acetic acid. This phenomenon is called acetate overoxidation, and it is a nuisance during vinegar fermentation. The ethanol oxidation (or acetic acid production), tolerance to acetic acid, and over-oxidation of acetic acid are important characteristics of AAB not only for vinegar fermentation, but also for their microbial physiology.\textsuperscript{1}

Acetic acid fermentation is catalyzed by two membrane-bound enzymes, alcohol dehydrogenase and aldehyde dehydrogenase,\textsuperscript{2} which produce large amounts of acetic acid outside the cells. Thus, as well as ethanol oxidation capacity, acetic acid resistance is a crucial factor for AAB to perform fermentation stably. Many studies of the acetic acid resistance of AAB have been reported. The molecular mechanisms conferring acetic acid resistance on \textit{Acetobacter aceti} have been investigated using acetic acid-sensitive mutants, and the aarA gene encoding citrate synthase and the aarC gene were found to be responsible for acetate assimilation.\textsuperscript{3,4} Later, enhanced expression of aconitase was found to lead the acetic acid resistance,\textsuperscript{5} and the acetic acid resistance protein AarC was identified as succinyl-CoA:acetyl-CoA transferase, converting succinyl-CoA and acetate to succinate and acetyl-CoA, which process allows acetic acid to be removed efficiently without substrate-level phosphorylation.\textsuperscript{5,6} The enzymes working in the TCA cycle are related to acetate overoxidation, together with acetyl-CoA synthase and phosphoenolpyruvate carboxylase,\textsuperscript{7,8} but the mechanism responsible for acetic acid resistance cannot be explained only by acetate assimilation, because \textit{Acetobacter} and \textit{Gluconacetobacter} species survive without assimilation of acetate under high concentrations of acetic acid under fermentation conditions.\textsuperscript{9,10} \textit{Acetobacter} species have been found to have proton motive force-dependent and ABC-transporter-like efflux pump systems for acetic acid in \textit{Acetobacter pasteurianus} IFO3283\textsuperscript{10} and \textit{Acetobacter aceti}\textsuperscript{11} respectively. An additional mechanism has also been shown that changes in membrane lipid composition such as phosphatidylcholine and/or a cis-vaccenic acid are related to acetic acid resistance.\textsuperscript{9,12} Hence it has been suggested that acetic acid resistance in AAB is conferred by several different mechanisms.

Bacteria capable of growing under environmental stress can survive by developing a variety of mechanisms of resistance. Biofilm formation is one of such the defense mechanisms. Thus \textit{Pseudomonas aeruginosa} able to produce biofilm are more resistant to heavy metals than planktonic bacteria,\textsuperscript{13} and biofilm cells of \textit{Lactobacillus plantarum} subsp. \textit{plantarum} JCM1149 are more resistant to acetic acid and ethanol than planktonic cells.\textsuperscript{14} \textit{Acetobacter} species, \textit{Acetobacter tropicalis} SKU1100 and \textit{Acetobacter aceti} IFO3284, have been shown to produce capsular polysaccharides as a pellicle...
Materials and Methods

Materials. [1-14C] Sodium acetate (9.25 MBq, 60 mCi/mmol, 200 µCi/ml) was purchased from Amersham Biosciences (renamed GE Healthcare Bio-Science, Tokyo). Hoechst solution and Mounting Fluorescent Solution were from Dojindo (Kumamoto) and Dako (Tokyo) respectively. Yeast extract was kindly provided by Oriental Yeast (Osaka). All other chemicals were commercial products of guaranteed grade.

Bacterial strains, culture media, and culture conditions. A. pasteurianus SKU1108 (NBRC101655; National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan), A. pasteurianus MSU10 (NBRC105870), and A. pasteurianus IFO3283 (Institute for Fermentation, Osaka, IFO, Japan) were used in this study. Each strain of AAB was first cultivated in 5 ml of potato medium at 30°C with rotary shaking at 200 rpm until a klett unit (Klett-Summerson photoelectric colorimeter, Klett New York, NY) of 250 was reached. The seed culture was then transferred to 100 ml of YPG or YPGD medium supplemented with 2% or 4% ethanol in a 500-ml shaking flask. The flask was incubated at 30°C or 37°C with rotary shaking at 200 rpm for 12 d.

Isolation of the S and R strains from A. pasteurianus strains. A. pasteurianus IFO3283, SKU1108, and MSU10 original strains were cultured 30 times by transferring the culture to 5 ml of potato medium every 24 h under shaking or static conditions at 30°C. The culture was diluted and spread onto potato agar plates, from which S-type and R-type colonies were isolated as the S or the R strain.

Transport assay. The A. pasteurianus S or R strains were grown to late exponential phase in YPGD medium without ethanol, washed twice in 50 mM potassium phosphate buffer (pH 6.5), and kept on ice. Before transport assay, cells were suspended in McIlvaine buffer (pH 6.5) or YPGD medium for acetate uptake assay and acetic acid diffusion assay respectively, at a final dry weight of about 0.2 g/ml. The reaction was started by adding acetic acid containing [1-14C] sodium acetate (8 mCi, 6.25 µCi/mmol) to final concentrations of 0.4 mM and 80 mM for uptake or diffusion assay respectively into the cell suspension. Samples of cells, 50 µl in volume, were incubated at 25°C for 10 min for acetate uptake assay and 15 min for acetic acid diffusion assay, and then withdrawn at intervals and filtered through 0.45-µm pore cellulose acetate filters. The filters were quickly washed twice with 3 ml of cold 50 mM potassium phosphate buffer (pH 6.5).

Results

Growth behavior of A. pasteurianus IFO3283 in ethanol culture

In ethanol culture (acetic acid fermentation), as shown in Fig. 1, A. pasteurianus IFO3283 exhibited three clear growth phases, in which acetic acid was produced and remained in the culture medium for a long time before assimilation. In both 4% ethanol culture and 3% ethanol culture, the cell growth of the IFO3283 strain increased concomitantly with the increase in acetic acid, and then reached a plateau after converting all ethanol to acetic acid. We call this stationary phase the acetic acid resistance phase (the AR phase). After remaining on the plateau for about 120 h, cell turbidity increased again, concomitantly with the consumption of accumulated acetic acid, and hence was called the acetate overoxidation phase (AO phase), whereas viable cell numbers, measured as colony-forming units (CFU), started to decrease during the late ethanol oxidation phase (EO phase) and decreased continuously in accordance with acetic acid accumulation. After reaching a threshold (around 10^6 CFU in this case), it increased again before the onset of the AO phase, whereas in the culture containing 1% acetic acid and not any ethanol, the IFO3283 strain grew after a relatively long lag phase, which appeared to be equivalent to the AR phase. In this case, viable cell numbers also decreased, to nearly 10^6 CFU, before commencing utilization of acetic acid.

When we examined the cell situation and morphology during ethanol culture directly by light microscope, the cells appeared to aggregate as ethanol oxidation proceeded. That is, acetate accumulation proceeded in the late EO phase and especially in the AR phase, while such aggregates appeared to vanish during the AO phase (data not shown). When the same cell suspension was observed by fluorescent microscope after staining with fluorescent dyes, the aggregated cell mass appeared to be covered by amorphous materials (Fig. 1B). Although the staining principle is not clear, we think that the amorphous materials might be related to cell pellicles that have been found to be produced at the
cell surface as capsular polysaccharides in Acetobacter species.\textsuperscript{15,16)}

Acetic acid fermentation and pellicle polysaccharide production of thermotolerant A. pasteurianus SKU1108 in ethanol culture

Since growth behavior similar to that of IFO3283 strain, as described above, has been observed in thermotolerant A. pasteurianus SKU1108,\textsuperscript{21)} we examined relationship between acetic acid fermentation and pellicle polysaccharide production in SKU1108 strain. In this study, we assessed acetic acid fermentation with SKU1108 strain, together with morphological changes and with the polysaccharide production, in YPD medium supplemented with 4% ethanol at 37°C (Fig. 2A). We found that SKU1108 strain converted ethanol almost completely to acetic acid for 2 d under these culture conditions and started overoxidation after
Fig. 3. Colony Morphology (A) and Growth Behavior in Static Culture (B) of the S and R Strains Isolated from *A. pasteurianus* SKU1108, IFO3283, and MSU10.

A, The colony shapes of the S and R strains from *A. pasteurianus* SKU1108, IFO3283, and MSU10 was compared. B, These strains were cultivated statically in 5 ml of YPGD medium at 30 °C for 5 d.

5–6 d, earlier than the case of IFO3283 grown at 30 °C. As in the case of IFO3283 strain, the cells appeared to be aggregated and covered by amorphous components, especially in the AR phase (data not shown). Moreover, we collected cells at each phase, the early and late EO phases, the AR phase, and the AO phase, and examined the sugar contents of the cells to estimate polysaccharide production. As shown in Fig. 2B, the sugar contents of the cells increased from the early EO phase to the AR phase, and finally decreased again in the AO phase. Since the sugar contents of the cells have been found to be related to the polysaccharide attached to the cells, which could be detected only in the R strain, as described,14,15) the culture appeared to be rich in cells having pellicle polysaccharides in the late EO phase to the AR phase. This might be related to the acetic acid resistance of the cells. This observation is contrast with the notion that the sugar contents detected in the S strain must have been due to other components such as lipopolysaccharides, not to the pellicle.15) Thus, all the R strains of these species appeared to produce pellicle polysaccharides.

Table 1. Sugar Contents of Cells of *A. pasteurianus* SKU1108, IFO3283, and MSU10 S and R Strains Grown on 4% Ethanol in YPGD Medium for 3 d

<table>
<thead>
<tr>
<th>A. pasteurianus strains</th>
<th>YPGD-4% ethanol medium</th>
<th>Potato medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKU1108 S</td>
<td>0.86 ± 0.04</td>
<td>1.33 ± 0.18</td>
</tr>
<tr>
<td>SKU1108 R</td>
<td>2.03 ± 0.12</td>
<td>3.61 ± 0.49</td>
</tr>
<tr>
<td>IFO3283 S</td>
<td>0.74 ± 0.06</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>IFO3283 R</td>
<td>1.34 ± 0.004</td>
<td>2.88 ± 0.15</td>
</tr>
<tr>
<td>MSU10 S</td>
<td>0.66 ± 0.02</td>
<td>0.86 ± 0.13</td>
</tr>
<tr>
<td>MSU10 R</td>
<td>1.50 ± 0.02</td>
<td>2.55 ± 0.30</td>
</tr>
</tbody>
</table>

*The sugar content is shown as a mean ± SD of three assays.

Isolation of the S and R strains from *A. pasteurianus* and their acetic acid fermentation and acetic acid resistance capacities

Acetobacter species were found to be separated into a pellicle-forming R strain and a non-producing S strain. Therefore, to determine the relationship between the pellicle polysaccharide and acetic acid fermentation capacities, the S and R strains were isolated from the original cultures of *A. pasteurianus* IFO3283 and SKU1108, together with another thermotolerant *A. pasteurianus*, MSU10,23) as described in “Materials and Methods.” As shown in Fig. 3, S colonies obtained from IFO 3283, SKU1108, and MSU10 strains all showed shiny smooth-surfaced colonies (the S type) on agar plates, while the R colonies were rough-surfaced colonies (the R type). All the R strains grew on static culture to form a pellicle on the medium surface, while the S strains grew poorly and did not produce any pellicle. The sugar contents of the S and R strains of these *A. pasteurianus* strains grown on YPGD medium containing 4% ethanol or potato medium were also examined, and were found to be approximately 2-fold and 3-fold respectively higher in the R strain cells than in the S strain cells (Table 1). The difference in sugar contents between the R and S strains appeared to reflect the content of pellicle polysaccharides, since the sugar content detected in the S strain must have been due to other components such as lipopolysaccharides, not to the pellicle.15) Thus, all the R strains of these *Acetobacter* species appeared to produce pellicle polysaccharides.

Acetic acid fermentation with the S and R strains of *A. pasteurianus* strains

Acetic acid fermentation of the S and R strains of IFO3283, SKU1108, and MSU10 strains, were examined in YPGD medium containing 4% ethanol at 37 °C, as shown in Fig. 4. It was found that the SKU1108 and IFO3283 R strains accumulated ∼3.4% acetic acid and...
exhibited the next overoxidation of acetic acid 5 d and 10 d later respectively, although the MSU10 R strain accumulated acetic acid to only 2.4% without over-oxidation, which may have been due to the low acetic acid resistance capacity of the strain. Hence acetic acid fermentation was repeated with the MSU10 R and S strains in YPGD containing 2% ethanol. The MSU10 R strain accumulated 1.9% acetic acid, and then exhibited overoxidation after 5 d. On the other hand, the S strains of the three strains produced only 1.2–1.8% acetic acid, including MSU10 S strain with 2% ethanol, and did not exhibit overoxidation. Thus the S strains appeared not to tolerate the acetic acid produced by themselves.

Based on these results, it is tentatively concluded that the R strains exhibit higher resistance to acetic acid than the S strains, suggesting that the pellicle polysaccharide in the cells gives them high acetic acid resistance capacity.

Acetic acid and acetate transport of the S and R strains of A. pasteurianus strains
Since the pellicle polysaccharides were expected to disturb acetic acid diffusion into the cells, acetic acid uptake and diffusion into the cells were examined with both the R and the S strain. First, using cells of the A. pasteurianus S and R strains, acetic acid (apparently as acetate) uptake was measured with 0.4 mM acetic acid at pH 6.5 (Fig. 5A). All three S strains, IFO 3283, SKU1108, and MSU10, exhibited acetate uptake 3–4 fold higher than the R strains. Uptake was decreased by the addition of an ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP), and of a respiratory inhibitor, NaN₃, the effects of which were different depending on the strain, although the reasons are not clear. In order to show more clearly the effects of the polysaccharides on the diffusion of acetic acid, we measured acetic acid diffusion using a higher concentration of acetic acid (80 mM instead of 0.4 mM for the uptake experiment) under more acidic conditions (pH 3.9 after the addition of 80 mM acetic acid into the YPGD medium). However, since it was very hard to measure the uptake of acetic acid under such a high concentration of cold acetic acid, which disturbs measurements by competing with the uptake of the labeled acetic acid, we carefully repeated the uptake experiments with the high concentration of acetic acid in the presence of CCCP, which can disturb the efflux of acetic acid. As shown in Fig. 5B, acetic acid uptake under these diffusion conditions also showed a higher accumulation of acetic acid in the S strains than in the R strains of at least SKU1108 strain, although no significant difference was seen clearly in IFO3283 or MSU10 strain. These results indicate that acetic acid enters into the S strain more easily than the R strain.

Discussion
AAB generally have a strong capacity to oxidize ethanol to acetic acid and to accumulate it outside the cell, resulting a decrease in the culture pH to less than 4.11 In such a low pH environment, acetate is protonated
to acetic acid ($pK_a = 4.76$), which can penetrate into the cells through a membrane by passive diffusion and decrease the intracellular pH by releasing the proton to kill the cells. Thus, AAB must have some acetic acid resistance mechanisms to grow in a medium with a high concentration of acetic acid. As described in the introduction, AAB have several mechanisms responsible for acetic acid resistance, including acetic acid assimilation (detoxification), acetic acid efflux, and protection against acetic acid diffusion by modification of the lipid compositions of the cytoplasmic membranes.

Acetobacter species have been found to produce pellicle polysaccharides on their cell surface, which might be a kind of biofilm useful for drug resistance, including acetic acid resistance. To examine this idea, we cultured several $A. pasteurianus$ strains on YPGD medium containing 4% ethanol. These strains exhibited three growth phases: they first grow by completely oxidizing ethanol to acetic acid (EO phase), then stop growing and persist for a while in a culture medium filled with a high concentration of acetic acid (AR phase), and finally they start growing again by utilizing the accumulated acetic acid during the AO phase (Figs. 1, 2). During the late EO and AR phases, $A. pasteurianus$ were found to have an amorphous layer surrounding the cells, and also to exhibit higher sugar contents in the cells, which might be due to the production of pellicle polysaccharides, because it has been found that a pellicle-forming R strain produces non-cellulose-hetero-polysaccharides which yield cells exhibiting such high sugar contents. Hence the R strains are expected to become predominant in the late EO and AR phases in ethanol culture. This change in cell type in proportion to the accumulation of acetic acid in the culture medium must be due to an adaptive response for survival under acetic acid stress. A similar adaptive response for acetic acid was seen in $Gluconacetobacter europaeus$ V3, in which the cells changed from short cells to long rods covered with a spongy layer. On the other hand, in the AO phase, the cells became dispersed again and perhaps were reduced in the sugar content, suggesting increases in the S strains. The change from the S to the R strain and vice versa have been shown to be due to the changes in the cell populations, not to conditional expression for the pellicle, because such a S-R exchange has been found to occur by adaptive and spontaneous mutation. The increase in the pellicle polysaccharide surrounding the cells may confer the resistance against acetic acid accumulated during the late EO and AR phases.

In order to determine the relation between acetic acid resistance and the pellicle polysaccharide, we isolated S and R cells from $A. pasteurianus$ IFO3283, SKU1108, and MSU10 and compared acetic acid fermentation.
capacities related to acetic acid resistance between the S and R strains. In all three *A. pasteurianus* strains, the R strains showed clearly high capacity for acetic acid fermentation. The cells produced high acetic acid production of nearly 3.5%, with a typical diauxic growth during the EO, AR, and AO phases, whereas the S strains did not complete fermentation, so as to produce during the EO, AR, and AO phases, whereas the S production of nearly 3.5%, with a typical diauxic growth fermentation. The cells produced high acetic acid strains showed clearly high capacity for acetic acid measured at lower pH, less than the membranes, but real acetic acid diffusion should be to acetic acid or acetate reaching the cytoplasmic which suggests that pellicle polysaccharides are a barrier was found to be lower in the R strain than in the S strain, R strains. Acetate uptake (influx) measured at neutral pH difference in acetic acid diffusion between the S and the ride related to acetic acid resistance, we examined the polysaccharide surrounding the cells is related to acetic acid diffusion into the cells.

In order to determine the function of the polysaccharide related to acetic acid resistance, we examined the difference in acetic acid diffusion between the S and the R strains. Acetate uptake (influx) measured at neutral pH was found to be lower in the R strain than in the S strain, which suggests that pellicle polysaccharides are a barrier to acetic acid or acetate reaching the cytoplasmic membranes, but real acetic acid diffusion should be measured at lower pH, less than the pKₐ value of acetic acids, and also with higher concentrations of acetic acid. Although this experiment was very difficult, as described in “Results,” the R strain had a lower acetic acid accumulation than the S strain in the acetic acid diffusion experiment.

The results obtained in this study suggest that pellicle polysaccharides are involved in the acetic acid resistance of the *A. pasteurianus* strains in that it functions as a biofilm-like barrier to passive diffusion of acetic acid into the cells.

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