Loss of Acetic Acid Resistance and Ethanol Oxidizing Ability in an Acetobacter Strain

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Received May 1, 1981

A thermophilic strain, No. 1023, of Acetobacter aceti was isolated, by which it became possible to carry out submerged vinegar production at 35°C. The thermophilic property of the strain correlated closely with its acetic acid resistance. Strain No. 1023 lost acetic acid resistance at high frequency (more than 55% in isolated strains) and lost ethanol oxidizing ability at lower frequency (about 40%) compared to the above in the stationary growth phase in a liquid medium containing ethanol. The loss of these properties during the stationary phase was confirmed using a genetically marked strain (pro') of No. 1023. Such frequent loss of acetic acid resistance and ethanol oxidizing ability suggested a relationship between these properties and a plasmid. A plasmid, pTA 5001, was found in strain No. 1023, and its molecular weight was determined to be $17 \times 10^6$ daltons by electron microscopy. The plasmid, however, seemed to have no direct connection with acetic acid resistance or ethanol oxidizing ability, because the same plasmid was found in all strains which had no these properties.

There have been many reports that morphological and physiological characteristics of acetic acid bacteria are variable. They include the ability of cellulose formation,1-3) gluconic acid production,4-6) ketogenesis,6) pigmentation7,8) etc. Furthermore the strains which lost their ethanol oxidizing ability, a representative characteristics of acetic acid bacteria, have been reported.5,6) From these results some investigators insist upon the impossibility of differentiation of species in the genus Acetobacter.9,10) But there has been no report of investigation of these mutations of acetic acid bacteria from the view point of genetics.

The authors obtained a thermophilic acetic acid bacterium, identified as Acetobacter aceti No. 1023,11) which had full activity to produce acetic acid in a continuous submerged culture at 35°C and 45% activity even at 38°C. So, it has become possible to carry out submerged acetic acid fermentation at 35°C and to reduce cooling costs to about half of those at 30°C. It seemed that the thermophilic property might depend upon its acetic acid resistance. When Acetobacter aceti No. 1023 was cultured in a liquid medium containing ethanol, it produced strains with reduced acetic acid resistance and ethanol oxidizing ability with a high frequency and produced some strains which completely lost the ethanol oxidizing ability with a lower frequency, after the stationary growth phase. The appearance of revertants from these mutants were less than one out of 10⁹ cells. So it was suggested that the loss of acetic acid resistance and ethanol oxidizing ability would be caused by the omission of a plasmid DNA or the deficiency of some other genetic elements. The present paper deals with the conditions of the appearance of the strains which lost acetic acid resistance or ethanol oxidizing ability, and the isolation of plasmid DNAs from strain No. 1023 and its cured strains.

MATERIALS AND METHODS

Organisms. Acetobacter aceti No. 1023 was employed as a parent strain, which was originally isolated in our laboratory as a thermophilic Acetobacter strain. Strain No. 1023 had strong acetic acid resistance and ethanol
oxidizing ability (Ace^e, Eth^+). Strains d-5 and f-10 were derived from A. aceti No. 1023 during shaking cultures on a medium containing ethanol. Both acetic acid resistance and ethanol oxidizing ability of strain d-5 were reduced (Ace^e, Eth^+). The acetic acid resistance of strain f-10 was much lower and its ethanol oxidizing ability was lost completely (Ace^m, Eth^+). Strain 10-8 (pro^−) was obtained from A. aceti No. 1023 by treatment with N-methyl N’-nitro-N-nitrosoguanidine. The strain retained full activity of acetic acid resistance and ethanol oxidizing ability. The strains used in this study were grown on agar slants containing 1% yeast extract, 0.4% polypepton, 6% glucose, 1% CaCO₃ and 2% agar for 2 days at 30°C and then stocked at 4°C. Transfer to fresh agar slants was repeated bi-monthly.

**Media and culture conditions.** YPG medium (pH 6.5) consisted of 5 g yeast extract (Daigo Eiyo Kagaku), 2 g polypepton (Daigo Eiyo Kagaku) and 30 g glucose in one liter of water. YPG medium was YPG containing 3% ethanol, unless the concentration of ethanol was specified to be otherwise. Solid media were made by adding 2% agar. Cultivation was usually carried out in a 500 ml shaking flask containing 80 ml of the YPGE medium with shaking or in a 2 liter mini-jar fermenter (Iwashiya Model MB-W) containing one liter of the medium under 0.3 v/v per min aeration and 850 rpm agitation at 30°C. The strain was first cultured in a 50 ml test tube containing 5 ml of YPGE medium with shaking overnight at 30°C. One ml of the broth was inoculated into the shaking flask or 10 ml was inoculated into the mini-jar fermenter.

**Counts of viable and total cells.** Viable cells were counted by plating the fermented broth onto YPG agar medium and incubating for 3 days at 30°C. The dilution of broth was carried out with YPG liquid medium. Total cells were counted using a Coulter Counter Model ZC-I (Coulter Electronics, Inc., U.S.A.) with the range of 0.5~∞.

**Isolation of the auxotroph.** Cells of strain No. 1023 in the early logarithmic growth phase in YPGE broth were harvested by centrifugation and washed twice with a phosphate buffer (a mixture of 1/15 M KH₂PO₄ and 1/15 M Na₂HPO₄, pH 6.5). N-methyl N’-nitro-N-nitrosoguanidine was added to the cell suspension to a concentration of 100 μg/ml. The cell suspension was incubated for 20 min at 30°C, and washed twice with the phosphate buffer. During the procedure 45~65% of the cells were killed. After overnight culture in YPGE broth, cells were spread onto a complete agar medium (YPGE with 2% ethanol). The auxotroph was detected by the usual replica plating onto a minimal medium, which consisted of 0.01% K₂HPO₄, 0.05% KH₂PO₄, 0.025% MgSO₄·7H₂O, 0.01% KCl, 0.01% CaCl₂·2H₂O, 0.0005% FeCl₃, 0.4% sodium monoglutamate, 3.0% glucose, 2.0% ethanol and 2.0% agar (pH 6.5).

**Isolation and determination of Ace^e and Eth^- mutants.** The cultures at various growth phases in YPGE broth were diluted and spread over the surface of YPG plates. The plates were incubated for 2~3 days at 30°C. Colonies were randomly picked up onto agar slants containing CaCO₃. The resistances to acetic acid and ethanol were determined by the method described previously. The ethanol oxidizing ability was examined as follows. Each strain was inoculated into 5 ml of YPGE medium in a 50 ml test tube, and cultured with shaking for 72 hr at 30°C. Five ml of each fermented broth was titrated with 1 N NaOH solution. As blank tests, each strain was cultured in the ethanol free medium (YPG). Ethanol oxidizing ability was determined by the production of acid.

**Treatment with curing reagents.** Strain No. 1023 in the early logarithmic growth phase was inoculated into 5 ml of ethanol free medium (YPG) containing various amounts of curing reagents in a 50 ml test tube. The inoculum size was adjusted to 10⁵ cells/ml. The test tube was incubated with shaking at 30°C. Then, cultures which showed growth in the medium containing the highest amount of each curing reagent were plated onto YPG medium, and after incubation individual colonies were isolated.

**Cell lysis and plasmid isolation.** The method described by Guerry et al. was employed with a modification. The cells in one liter of YPGE broth at the late logarithmic growth phase were washed twice with 0.05 M Tris-HCl–0.02 M EDTA–Na₂, pH 8.0, and then suspended in 7 ml of 25% (w/v) sucrose–0.05 M Tris-HCl–0.02 M EDTA–Na₂, pH 8.0. To the cell suspension, 4 ml of lysozyme solution (20 mg/ml in 0.25 M Tris-HCl buffer, pH 8.0) and 4 ml of 0.25 M EDTA–Na₂ solution were added and the mixture was incubated for one hr at 37°C. Three milliliters of 10% sodium dodecyl sulfate solution was added and incubation was continued for 30 min at 30°C. A highly viscous and opaque lysate was obtained. To the lysate, 5 ml NaCl solution was added to a final concentration of 1 M. After standing overnight at 0°C, the mixture was centrifuged for one hr at 14,000 rpm and the supernatant was obtained. To fifteen ml of the supernatant, about 13 g of CsCl was added together with 90 μl of ethidium bromide solution (100 mg/ml in dimethylsulfoxide). After adjustment of the specific gravity to 1.575, the solution was centrifuged to equilibrium for 40 hr at 38,000 rpm in a Hitachi Ultracentrifuge Model 65P using an RPS6T rotor at 20°C. Fractions containing covalently closed circular DNA were pooled, and ethidium bromide was removed by extraction with isoamyl alcohol, followed by dialysis against a buffer solution (0.01 M Tris-HCl, 2 mM EDTA–Na₂ and 0.04 M NaCl, pH 7.5).

**Agarose gel electrophoresis of plasmids.** Electrophoresis was carried out on a horizontal 0.3% agarose gel (length 12.6 cm, width 14.0 cm, thickness 5 mm) at a constant
Acetobacter Mutants

voltage of 15 V for 24 hr. The composition of the electrophoresis buffer was 0.036 M Tris-HCl, 0.032 M KH₂PO₄ and 1.5 mM EDTA·Na₂ (pH 7.5) with addition of 0.5 μg/ml ethidium bromide.

Electron microscopy and determination of molecular weight of plasmids. For visualization of plasmid DNA the method used by Horinouchi et al.¹³ was employed. Pictures were taken with a Hitachi HU-12 electron microscope. The molecular weights of plasmids were determined by comparison of contour length with that of RSF 2124 (7.3 × 10⁶ daltons)¹⁷ used as standard.

RESULTS AND DISCUSSION
Appearance of Ace⁺ and Eth⁻ mutants
The time course of acetic acid production and counts of viable cells and total cells are shown in Fig. 1, when strain No. 1023 was cultured in a mini-jar fermenter aerobically in the YPGE medium containing 3% ethanol. It was found that organisms began to die rapidly after the late logarithmic growth phase, about 20 strains were isolated at various growth phases and their resistances to acetic acid and ethanol, and their ethanol oxidizing abilities were examined. All strains isolated at points A (start) and B (mid logarithmic growth phase) retained full resistance to acetic acid. At point C (late logarithmic growth phase), 20% of the isolated strains could not grow on the YPGE medium with 4% acetic acid at 30°C, and 55% could not grow on the same medium with 3.5% acetic acid at 37°C. At points D and E (stationary growth phase) strains which could not grow on the above acid media increased. All strains isolated at points A, B, C and D exhibited ethanol oxidizing abilities, though the activities were lowered in some strains. But 40% of the strains isolated at point E lost their

![Graph showing the loss of acetic acid resistance and ethanol oxidizing ability of Acetobacter aceti No. 1023.](image-url)

**Fig. 1.** Loss of Acetic Acid Resistance and Ethanol Oxidizing Ability of Acetobacter aceti No. 1023.
Strain No. 1023 was cultured in a mini-jar fermenter at 30°C with YPGE medium containing 3% ethanol according to the culture conditions described in the method. Twenty strains were isolated at various growth phases and their acetic acid resistance, ethanol resistance and ethanol oxidizing ability were examined as described in the method. ○ --- ○, acetic acid produced; ● --- ●, total cells; ○ --- ○, viable cells; △ --- △, acetic acid resistance examined on YPGE plates with 4.0% acetic acid at 30°C; △ --- △, acetic acid resistance examined on YPGE plates with 3.5% acetic acid at 37°C; □ --- □, ethanol oxidizing ability.
ethanol oxidizing abilities completely. On the other hand, the resistance to ethanol was stable all over the cultivation time. The frequency of revertants of these Ace\textsuperscript{e} and Eth\textsuperscript{-} strains was less than one out of 10\textsuperscript{9} cells. On the contrary, when strain No. 1023 was incubated on ethanol free medium (YPG medium), no strains were obtained which had lost both acetic acid resistance and ethanol oxidizing ability (Fig. 2). Thus, it was suggested that the appearance of these mutants would be due to ethanol or its product. The highly frequent loss of acetic acid resistance and ethanol oxidizing ability in the presence of ethanol is reminiscent of the curing of the extrachromosomal genetic character. Thus, we will refer to this phenomenon as "curing" in the following text. Steel et al.\textsuperscript{21} examined the culture conditions for the appearance of celluloseless mutants from the strains of A. acetigenum and A. xylinum which originally produced cellulose, and found that the appearance of celluloseless mutants was promoted by shaking cultures in the presence of ethanol. Thus, we also exam-

![Graph showing genetic changes](image)

**Fig. 2.** Genetic Changes of Strain No. 1023 during Cultivation in YPGE Medium with or without Ethanol. Strain No. 1023 was cultured with shaking at 30°C in YPGE medium with 3.0% ethanol (a) or without ethanol (b). Ten strains were isolated at points A, B, C and D, respectively. Acetic acid resistance, ethanol resistance and ethanol oxidizing ability of each strain were examined by the methods described above. ○—○, acetic acid resistance examined on YPGE plates with 3.0% acetic acid at 30°C; △—△, acetic acid resistance examined on YPGE plates with 2.0% acetic acid at 37°C.
Acetobacter Mutants

Fig. 3. Relation between Ethanol Concentration and Viable Cells.

Strain No. 1023 was cultured with shaking at 30°C in YPGE medium with 2, 3, 4 and 5% ethanol, respectively. Viable cells were measured during the cultivation time. △—△, 2% ethanol; ○—○, 3% ethanol; □—□, 4% ethanol; ●—●, 5% ethanol.

Table I. Relation between Ethanol Concentration and Frequency of Cured Strains

In the cultivation shown in Fig. 3, ten strains were isolated at the late stationary growth phase where viable cells were about 10^4 cells per ml. Their acetic acid resistance and ethanol oxidizing abilities were examined. Figures in the table represent the number of cured strains among the 10 strains.

<table>
<thead>
<tr>
<th>Ethanol in medium (%)</th>
<th>Incubation time (hr)</th>
<th>Viable cells (per ml)</th>
<th>Curing for</th>
<th>Acetic acid resistance*</th>
<th>Ethanol oxidizing ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>70</td>
<td>1.0 × 10^9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>1.3 × 10^9</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>1.6 × 10^4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>1.3 × 10^4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Examination was carried out by incubation on YPGE plates with 3.0% acetic acid at 30°C.

ined the effect of ethanol concentration in the culture medium on the rate of mutation or curing. Strain No. 1023 was cultured with shaking in YPGE medium containing 2, 3, 4 and 5% ethanol, respectively. Ten strains were isolated at the stationary growth phase when the number of viable cells decreased to 10^4 cells/ml, and their acetic acid resistance and ethanol oxidizing abilities were tested (Fig. 3 and Table I). In the culture medium with 2% ethanol, no significant loss of viability was observed in the stationary growth phase, whereas in the media with more than 3% ethanol the viability decreased rapidly in the stationary phase. When strain No. 1023 was incubated on the medium containing 3% ethanol, cured strains for both acetic acid resistance and ethanol oxidizing ability were obtained with
the highest frequency and in this experiment strains which lost acetic acid resistance and ethanol oxidizing ability reached 90% and 60%, respectively. On the contrary, cured strains were rarely found in the cultures with 4 and 5% ethanol. This may be because of rapid death of cured strains in the high acidity attained in the media with 4 or 5% ethanol.

When mutant strain 10-8 (pro⁻) obtained from strain No. 1023 was incubated with shaking in the YPGE medium with 3% ethanol cured strains which had lost acetic acid resistance or ethanol oxidizing ability were also obtained, with unchanged pro⁻ marker. Thus, it was recognized that acetic acid resistance and ethanol oxidizing ability were unstable characteristics compared with the proline requirement.

**Physiological characteristics of No. 1023 and its cured strains**

Physiological characteristics of the three strains, No. 1023 (Ace⁺, Eth⁺), d-5 (Ace⁺, Eth+) and f-10 (Ace⁻, Eth⁻), are summarized in Table II. Marked differences were observed in acetic acid resistance and acetic acid productivity among these strains. The acetic acid resistance of strain d-5 (1.5%) was about one third of that of strain No. 1023 (5.0%), and its maximum acid production (2.4%) was also about one third of that of strain No. 1023 (7.4%). Strain f-10 had lost the ethanol oxidizing ability completely. Furthermore its acetic acid resistance decreased markedly, too (1.0%). The time course of growth and acid production in shaking cultures of strains No. 1023 and d-5 with acid medium were compared (Fig. 4). Strain No. 1023 grew and produced acetic acid rapidly at 37°C and also showed considerable growth and acid production even at 40°C. On the contrary, strain d-5 completely lost its activity at 37°C. Thus, strain d-5 seemed to have lost the thermophilic property in acid circumstances. Except for acetic acid resistance, acetic acid productivity...
Acetobacter Mutants

Fig. 4. Growth and Acetic Acid Production of No. 1023 and Its Cured Strain, d-5.

Strains No. 1023 and d-5 were cultured with shaking in YPGE medium containing 1% acetic acid and 4% ethanol, at 30, 37, 40 and 42°C. Growth and acid production were measured periodically during cultivation. Growth: ●—●, No. 1023; ▲—▲, d-5. Acetic acid produced: ○—○, No. 1023; △—△, d-5.

Table III. Curing of Acetic Acid Resistance and Ethanol Oxidizing Ability of No. 1023 by SDS, SDS-Mitomycin C and Ethidium Bromide

<table>
<thead>
<tr>
<th>Curing reagents</th>
<th>Numbers of strains examined</th>
<th>Numbers of strains sensitive to acetic acid (acetic acid %)</th>
<th>Ethanol oxidizing ability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.0     3.0     4.0</td>
<td>Weak#  No</td>
</tr>
<tr>
<td>Control</td>
<td>52</td>
<td>0       0       0</td>
<td>0     0</td>
</tr>
<tr>
<td>SDS a 0.005%</td>
<td>50</td>
<td>0       8       10</td>
<td>0     0</td>
</tr>
<tr>
<td>SDS 0.005% Mitomycin C 50γ/ml</td>
<td>120</td>
<td>0       4       4</td>
<td>4     0</td>
</tr>
<tr>
<td>Ethidium bromide 100γ/ml</td>
<td>50</td>
<td>0       2       2</td>
<td>2     0</td>
</tr>
</tbody>
</table>

* Acetic acid production less than 2.5%.
# Sodium dodecyl sulfate.

and the thermophilic property, the three strains were identical in many other physiological characteristics. These results indicate that the acetic acid resistance and ethanol oxidizing ability of strain No. 1023 are very variable; whereas other characteristics are fairly stable.
Results of the treatment with curing reagents

The effects of various plasmid-curing reagents on the appearance of Ace⁶ and Eth⁻ mutants were examined. The results of SDS, SDS-mitomycin C, and ethidium bromide treatments are shown in Table III. Ace⁶ strains corresponding to strain d-5 were obtained, but the frequency of appearance was much lower than that with incubation in the YPGE medium.

Plasmid analyses

Plasmid analyses of strains, No. 1023, d-5 and f-10 were carried out to see whether there were any relations between acetic acid resistance or ethanol oxidizing ability and plasmid DNA. And, these three strains were found to have plasmid DNAs by CsCl-ethidium bromide equilibrium centrifugation. Agarose gel electrophoresis and electron microscopy showed that plasmid DNAs isolated from all three strains were identical, having the same molecular weight of $17 \times 10^6$. We named these plasmids, pTA 5001.

The "curing" of acetic acid resistance and ethanol oxidizing ability by cultivation in YPGE medium or, by treatment with curing reagents strongly suggests the presence of a plasmid responsible for these genetic characteristics. But the present plasmid (pTA 5001) seems not to have any relations to these characteristics, because the plasmids isolated from both the parent strain and the "cured" strains, d-5 and f-10, have the same molecular weight.

Fig. 5. Agarose Gel Electrophoresis of Plasmids Isolated from Strains No. 1023, d-5 and f-10 together with Standard Plasmids of Known Molecular Weights.
a, pTA 4 ($2.9 \times 10^6$); b, pHV 14 ($4.6 \times 10^6$); c, pTA 1302 ($5.2 \times 10^6$); d, f-10; e, d-5; f, No. 1023; g, Col El ($4.2 \times 10^6$); h, RSF 2124 ($7.3 \times 10^6$).

Fig. 6. Electron Micrographs of Plasmids Isolated from Strains No. 1023, d-5 and f-10.
①, No. 1023; ②, d-5; ③, f-10.
Acetobacter Mutants

according to agarose gel electrophoresis and electron microscopy. There remains the possibility that another plasmid which is very difficult to detect is really responsible for the acetic acid resistance and ethanol oxidizing ability. In order to find such a hypothetical plasmid, much improvement of the method of detection of plasmids, suitable for this particular strain, will be necessary.

REFERENCES