Hydrogen Peroxide Resistance of Acetobacter pasteurianus NBRC3283 and Its Relationship to Acetic Acid Fermentation

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The bacterium Acetobacter pasteurianus can ferment acetic acid, a process that proceeds at the risk of oxidative stress. To understand the stress response, we investigated catalase and OxyR in A. pasteurianus NBRC3283. This strain expresses only a KatE homolog as catalase, which is monofunctional and growth dependent. Disruption of the oxyR gene increased KatE activity, but both the katE and oxyR mutant strains showed greater sensitivity to hydrogen peroxide as compared to the parental strain. These mutant strains showed growth similar to the parental strain in the ethanol oxidizing phase, but their growth was delayed when cultured in the presence of acetic acid and of glycerol and during the acetic acid peroxidation phase. The results suggest that A. pasteurianus cells show different oxidative stress responses between the metabolism via the membrane oxidizing pathway and that via the general aerobic pathway during acetic acid fermentation.

Key words: Acetobacter pasteurianus; acetic acid fermentation; oxidative stress; catalase; OxyR

Acetobacter pasteurianus is a bacillus belonging to the α-proteobacteria and is an acetic acid bacterium. It expresses alcohol dehydrogenase (ADH) and acetoaldehyde dehydrogenase (ALDH) on the cell membrane and oxidizes ethanol outside of the cell to acetic acid with these enzymes.¹,² The cells acquire ATP by transmitting electrons generated by this oxidative reaction to the electron transport system, which is also on the cell membrane. As a result, acetic acid accumulates on the outside of the cells during growth. This process is called acetic acid fermentation. Due to this property, acetic acid bacteria are used in the production of vinegar and are regarded as industrially important microorganisms.

Energy acquisition by acetic acid bacteria is based on incomplete oxidation of the substrate, and thus the efficiency of ATP generation per ethanol molecule is estimated to be low. Hence acetic acid bacteria are considered to obtain sufficient levels of energy by oxidizing large amounts of substrate. Accordingly, they show obligate aerobic properties and need a large amount of oxygen for growth.³ In general, aerobic metabolism generates reactive oxygen species in cells, since electrons leaked from the electron transport process tend to react with oxygen molecules. Hence cells that rely on aerobic metabolism are thought to be exposed to oxidative stress. Due to their physiological characteristics, acetic acid bacteria are thought to live under constant, intense conditions of oxidative stress during acetic acid fermentation.

In view of this, we investigated the relationship between the oxidative stress resistance ability of this bacterium and acetic acid fermentation. There are several kinds of oxidative stresses caused in aerobic cells, and among them, we deal with hydrogen peroxide resistance ability in this paper. Generally, hydrogen peroxide generated in aerobic cells is removed principally by catalase.⁴ Hence we investigated catalase in this strain and evaluated its effect on the physiology of the cells and on acetic acid fermentation. In connection with this activity we also investigated OxyR, which is generally regarded as a catalase regulating factor. In general, bacterial catalases are roughly categorized into two types: KatG homologs, which are hydrogen peroxide inductive and regulated by OxyR, and KatE homologs, which are growth dependent and regulated by RpoS.⁵ However, recent studies suggest that both catalases might be regulated by both factors.⁶,⁷ This paper is the first report concerning oxidative stress resistance in acetic acid bacteria, industrially-important obligate aerobic bacteria.

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Materials and Methods

Bacterial strains, plasmids, oligonucleotide primers, and culture conditions. The bacterial strains, plasmids, and oligonucleotide primers used in this study are summarized in Table 1. Unless otherwise noted, *Escherichia coli* was cultured in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37 °C with shaking at 120 rpm. *Acetobacter pasteurianus* was cultured in YPD medium (1% yeast extract, 1% polypepton, and 3% glucose) at 30 °C with shaking at 120 rpm. As appropriate, antibiotics were added to a final concentration of 50 μg/ml in LB and to 100 μg/ml in YPD medium.

General molecular biological techniques. General molecular biological techniques, including restriction enzyme digestion, agarose gel electrophoresis, and transformation of *E. coli*, were performed according to standard protocols. A Ligation Kit Ver. 1 (Takara Bio, Tokyo) was used in standard ligations. Plasmids were extracted by the alkali-SDS method, and chromosomal DNA was prepared by the CTAB method. PCR was performed using Ex Taq polymerase (Takara Bio) in a total volume of 100 μl with 10 ng of template DNA and 20 pmol of each primer. The reaction was carried out with 30 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min. Southern hybridization was performed using the DIG Labeling and Detection System (Roche Diagnostics, Tokyo) according to the manufacturer’s instructions at a hybridization temperature of 42 °C. Nucleotide sequences were determined using a CEQ8000 DNA sequencer (Beckman-Coulter, Tokyo). Cycle sequencing for nucleotide sequence analysis was carried out with a Genome Lab DTCS-Quick Start Kit (Beckman-Coulter) according to the manufacturer’s instructions. Sequence data were analyzed using the programs BLASTP (National Center for Biological Information, Bethesda, MD) and Genetyx-Win (Software Development, Tokyo). Competent cells of *A. pasteurianus* were prepared and transformed as described previously.

Enzyme extraction from *A. pasteurianus*. Cells cultured in 100 ml of medium were collected by centrifugation (8,000 rpm × 5 min), and the pelleted cells were suspended in an equal volume of cold 50 mM Tris–HCl buffer, pH 7.8, after two washes with the same buffer. Next, the suspension was transferred to a 1.5-ml microfuge tube (Yasui Kikai, Osaka, Japan), mixed with an equal volume of glass beads (ϕ 0.1 mm, Yasui Kikai), and crushed with a Multi-beads Shocker (Yasui Kikai). The total volume of the cell suspension with beads was

### Table 1. Bacterial Strains, Plasmids, and Primers

<table>
<thead>
<tr>
<th>Strain or reagent</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, ϕ 80lacZΔ M15, ΔlacZ&lt;sub&gt;argF&lt;/sub&gt;U169, deoR, recA1, endA1, hisD18, m&lt;sub&gt;R&lt;/sub&gt;, phoA, supE44, λ&lt;sup&gt;−&lt;/sup&gt;, thi-1, gyrA96, relA1</td>
<td>Toyobo</td>
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<tr>
<td><strong>Acetobacter pasteurianus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBRC3283</td>
<td>wild type</td>
<td>Ref. 9</td>
</tr>
<tr>
<td>3283KE</td>
<td>NBRC3283 ΔkatE::Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>3283OX</td>
<td>NBRC3283 oxyR::Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>Promega</td>
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<td>pBBR122</td>
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<td>Mobitec</td>
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<td>This study</td>
</tr>
<tr>
<td>pMOxyR</td>
<td>pMV24 containing oxyR</td>
<td>This study</td>
</tr>
<tr>
<td>pMKK</td>
<td>pMV24 containing ΔkatE::Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pMOK</td>
<td>pMV24 containing oxyR::Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>km-or</td>
<td>ATGGCCAAGCCGCGTGGGAGAAGGAAAAAAAT</td>
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Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin
adjusted to about half of the microfuge tube. Cell crushing was carried out with three rounds of shaking at 2,500 rpm for 30 s separated by 30-s pauses. This was carried out at 4 °C with circulating coolant in a rotor integrated with the machine. The homogenate was then subjected to centrifugation (15,000 rpm × 15 min), and the resulting supernatant was used as the enzyme solution.

Measurement of catalase activity and protein concentration. Catalase activity was assayed by determining the rate of degradation of hydrogen peroxide, expressed as the change in absorbance at 240 nm, according to the method described by Vattanaviboon et al. This was done using spectrophotometer Model UV-160 equipped with a cell holder which can hold a cuvette at constant temperature (Shimadzu, Kyoto, Japan). The enzyme assay was performed directly in a cuvette with a light pass length of 1 cm and a width of 0.5 cm. The reaction was initiated by the addition of 10 μl of enzyme solution to 1 ml of 15 mM hydrogen peroxide in 50 mM sodium phosphate buffer, pH 7.0. The decrease in absorbance at 240 nm was then monitored continuously for 1 min at 30 °C. The ability to degrade 1 μmol of hydrogen peroxide in 1 min was defined as 1 unit of enzymatic activity. The enzyme assay was carried out in triplicate, and the data were treated statistically.

The protein concentration was determined as described by Bradford using Bio-rad protein assay reagent according to the manufacturer’s instructions. γ-Globulin protein was used as the standard.

Gel electrophoresis and in-gel detection of enzymatic activity. Electrophoresis was performed with 7.5% T native gels. Catalase activity staining was carried out principally by the method of Wayne. After electrophoresis, the gel was immersed in 1 mM hydrogen peroxide solution and shaken gently for 10 min. The gel was washed with MilliQ water, followed by immersion in a solution containing 2% ferric chloride and 2% potassium ferricyanide with gentle shaking. Catalase was observed by the presence of a colorless band on a green background.

Detection of peroxidase activity was performed by the method of Clare. After electrophoresis, the gel was immersed in 1 mM hydrogen peroxide solution and shaken gently for 10 min. The gel was washed with MilliQ water, followed by immersion in a solution containing 1% hydrogen peroxide and 2.3 mM 3,3-diaminobenzidine and shaken gently. Peroxidase activity was observed by the presence of a colorless band on a green background.

Cloning of the katE and oxyR genes from A. pasteurianus. Cloning was carried out by a combination of degenerate PCR and inverse PCR methods, principally as described previously.

Briefly, as a first step, degenerate PCR was carried out to amplify katE or oxyR fragments from A. pasteurianus NBRC3283 chromosomal DNA. The primer sets katE-dF2/katE-dR8 (for katE) and oxyR-dF2/oxyR-dR4 (for oxyR) were used. Based on the sequences of the fragments, new primer sets, katE-iF/katE-iR (for katE) and oxyR-iF/oxyR-iR (for oxyR), were designed and used in inverse PCR with the self-ligated products of HpaI digestion (for katE) and StuI digestion (for oxyR) of chromosomal DNA as templates. The full-length sequence information of these genes was obtained by connecting the sequences of the degenerate and inverse PCR products. The PCR conditions and preparation method for the self-ligation products were as described previously.

Construction of the katE and oxyR mutant strains of A. pasteurianus NBRC3283. Strain construction was carried out by gene replacement using homologous recombination of the corresponding chromosomal DNA sequence. The genes were first amplified by PCR with the primer sets katE-oF/katE-oR (for katE) and oxyR-oF/oxyR-oR (for oxyR). The amplified fragments (2,544 bp for katE and 1,324 bp for oxyR) were then inserted into the KpnI site of the pMV24 vector, and the resulting plasmids were named pMKatE and pMOxyR respectively. Next, a kanamycin resistance gene was inserted between two NotI sites of the katE gene and at the BsuI site of the oxyR gene, generating plasmids pMKK and pMOK respectively. The kanamycin resistance gene was amplified from plasmid pBBR122 (MoBiTec, Goettingen, Germany) by PCR with the primer set km-kF/km-kR (for insertion into katE) and km-oF/km-oR (for insertion into oxyR).

With these plasmids as templates, the katE::km fragment was amplified with the primer pair katE-oF/katE-oR and the oxyR::km fragment with oxyR-oF/oxyR-oR. Subsequently, 1 μg of each fragment was introduced into A. pasteurianus NBRC3283 by electroporation, and the cells were spread on YPD plates containing 100 μg/ml of kanamycin. Colonies recovered on the plates were checked by PCR, and insertion of the kanamycin resistance gene into the target gene was confirmed by sequence analysis and Southern hybridization using chromosomal DNA extracted from the candidate strains.

Hydrogen peroxide sensitivity test. One hundred μl of culture at stationary phase was spread on a YPD-agar plate after adjusting the cell density to OD660 = 1.0 with YPD medium. A paper disc (φ 8 mm, Advantec, Tokyo) containing 10 μl of 1% hydrogen peroxide was placed at the center of the plate. The plate was incubated at 30 °C overnight and the diameter of the inhibition zone was measured. This paper disc assay was carried out in triplicate for each strain and the data were treated statistically.

Growth of strains under various conditions. The cells were cultured in a volume of medium equivalent to 1/5 volume of the conical culture flask at 30 °C with shaking at 121 rpm. YPD2 medium (YP + 2% glucose), YPG medium (YP + 2% glycerol), YPE medium (YP + 1% glucose), YPC medium (YP + 1% potassium acetate), YPC medium (YP + 1% potassium citrate), and YPC medium (YP + 1% potassium succinate) were used. Based on the results of the growth tests, the optimal culture condition for each strain was determined.
ethanol), and YPDA medium (YP + 1% glucose, 1% acetic acid) were used. Growth was checked by measuring the optical density of the culture at 660 nm.

Accession number. The nucleotide sequences determined in this study have been assigned DDBJ Accession nos. AB355642 for katE and AB355643 for oxyR.

Results

Catalase activity in A. pasteurianus cultured in YPD medium

A. pasteurianus NBRC3283 cells were cultured in YPD medium (a glucose-containing medium), and were harvested at the mid-logarithmic, early stationary, and mid-stationary phases. Then the cell-free extracts were assayed for catalase activity and were separated electrophoretically, followed by staining for catalase activity (Fig. 1). Figure 1A shows the catalase activity in the cells at each growth phase. The enzyme activity increased with growth, reaching 13 U/mg protein at the mid-log phase, 65.7 U/mg protein at the early stationary phase, and 109.3 U/mg protein at the mid-stationary phase. Zymogram patterns of catalase activity in the cellular extracts indicated that A. pasteurianus has a single band of catalase activity after gel electrophoresis regardless of the growth phase (Fig. 1B). Based on these results, it appears that under these culture conditions, A. pasteurianus produced only one type of catalase, and that the amount of enzyme increased in tandem with cell growth, showing the strongest activity at the stationary phase.

When the cell free extracts were stained for peroxidase activity after gel electrophoresis, no activity was observed at the position of the catalase activity band, suggesting that catalase in A. pasteurianus is monofunctional (data not shown).

Effect of hydrogen peroxide on catalase expression in A. pasteurianus

A. pasteurianus was cultured in YPD medium at 30 °C, and then hydrogen peroxide was added to the cells to a final concentration of 0, 100, 250, or 500 µM, at mid-log phase or early stationary phase. Subsequently, the cells were cultured for an additional 30 or 60 min, and the changes in catalase activity and zymogram patterns were investigated using cell-free extracts. Notably, the addition of peroxide to a final concentration of up to 100 µM H₂O₂ had little effect on cell growth, a slight effect was observed at 250 µM, and remarkable effects were observed when the concentration was raised to 500 µM.

For log-phase cells, the change in catalase activity was less remarkable, and no additional band of activity was detected after gel electrophoresis regardless of the peroxide concentration or time of incubation. Similar results were obtained from cells at stationary phase, indicating that catalase activity did not change significantly in the presence of H₂O₂.

Therefore, the possibility exists that this strain expresses only one type of catalase and that no isozyme was induced by H₂O₂ in this strain.

Cloning of the gene encoding catalase from A. pasteurianus

Next we attempted to isolate the gene corresponding to the catalase enzyme expressed in the cells. Usually, monofunctional type catalase is encoded by a katE gene. Hence we cloned the katE gene from this strain and then constructed a disruptant according to the procedure described in “Materials and Methods.” When catalase activities in the parental and mutant strains at early stationary phase in YPD medium were measured, almost no activity was detected in the extract from the katE mutant, whereas that from the parental strain showed activity of about 70 U/mg protein (Fig. 2A). When the
same extracts were analyzed by electrophoresis, one activity band was detected in the parental strain at the same position, as shown in Fig. 1, but the band was not present in the mutant strain (Fig. 2B). Therefore, the gene encoding the catalase expressed in A. pasteurianus cells is a KatE homolog.

The putative A. pasteurianus KatE protein sequence showed 51% identity with that of E. coli KatE, 72% with that of Agrobacterium tumefaciens KatE, and 70% with that of Gluconobacter oxydans KatE.

Relationship between KatE and OxyR in A. pasteurianus

In many cases, expression of the katE gene is regulated by RpoS, but many catalases responsible for oxidative stress resistance are regulated by OxyR. The KatE in A. pasteurianus appeared to be the only catalase expressed in this bacterium. Hence the relationship between katE and oxyR was investigated.

For this purpose, the oxyR gene was cloned from A. pasteurianus and a genomic disruption mutant was generated. The putative OxyR protein sequence deduced from the cloned gene showed relatively low identity with E. coli OxyR (35%), but high similarity in amino acid properties was observed even between parts lacking primary sequence identity. In addition, all the features characteristic of OxyR were observed in the A. pasteurianus sequence (Fig. 3).<sup>14,15</sup> Hence we identified this gene as oxyR, and constructed a disruptant of the oxyR gene in A. pasteurianus.

When catalase activities in both the parental and oxyR mutant strains were measured, we detected higher activity in the mutant cells (Fig. 2A) and an activity band at the same position as in the parental strain on a stained electrophoresis gel (Fig. 2B). Thus, the disruption of OxyR in A. pasteurianus resulted in higher KatE activity.

Effects of KatE and OxyR on hydrogen peroxide resistance in A. pasteurianus

To determine the relationship of katE and oxyR to hydrogen peroxide resistance, a hydrogen peroxide paper-disc assay was performed on the parental, katE mutant, and oxyR mutant strains (Fig. 4). As in Fig. 4A, the diameters of the inhibition zones were compared between the parental and katE mutant strains. The inhibition zone of the mutant extended 30% further than that of the parental strain, suggesting that disruption of katE results in an increase in sensitivity to hydrogen peroxide. As shown in Fig. 4B, introduction of a katE-containing plasmid (pMKatE) into the mutant strain reduced the inhibition zone relative to the mutant strain alone. Complementation by pMKatE was reproducible, and the difference was confirmed to be significant by t-test (<0.05).

The hydrogen peroxide sensitivity of the oxyR mutant as compared with that of the parental strain is shown in Fig. 4C. The sensitivity of the mutant was considerably higher than that of the parental strain, with an 80% enlargement of the inhibition zone in the mutant relative to the parental strain. The introduction of pMOxyR, which contains the oxyR gene, caused a recovery to normal sensitivity and significantly reduced the size of the inhibition zone (Fig. 4D).

Based on these results, it appears that both KatE and OxyR are involved in the resistance of A. pasteurianus to hydrogen peroxide.

Growth behavior of the katE and oxyR mutant strains under various culture conditions

To determine the relationship between hydrogen peroxide resistance and acetic acid fermentation, the growth behaviors of the parental and two mutant strains were compared under various culture conditions (Fig. 5). The strains showed similar growth when cultured in YPD2 standard medium (Fig. 5A). However, the growth of the katE and oxyR mutant strains was gradually suppressed from the early stationary phase when the cells were cultured in YPG medium (Fig. 5B), whereas the parental strain grew normally under the same conditions (normal cells showed robust growth, particularly after the early stationary phase). Figure 5C shows the growth of the three strains in YPE medium. Under these conditions, the cells showed two-stage growth patterns similar to other Acetobacter species, as described by Saeki et al.<sup>16</sup> When comparing the behaviors of the three strains, we observed no remarkable difference among them at the log phase, but differences gradually appeared around the stationary phase and became more pronounced with time, ultimately resulting in a marked growth delay for the katE and oxyR mutants. Figure 5D shows the growth of the strains in YPDA...
Fig. 3. Conceptual Translation of the *A. pasteurianus* oxyR Gene.

Above, *Escherichia coli* OxyR. Below, *Acetobacter pasteurianus* NBRC3283 OxyR. Symbols as follows: /, identical amino acid residues; \*, amino acid with similarity; x, general amino acid; h, non-polar amino acid; p, polar amino acid. Two boxed “C”s in the sequences indicate the conserved cysteine residues characteristic of OxyR proteins (C199 and C208 in *E. coli*).

Fig. 4. Hydrogen Peroxide Inhibition Zone Test of Parental and *katE* and *oxyR* Mutant Strains.

Values are based on the diameter of the inhibition zone that formed around a paper disk containing 1% H$_2$O$_2$ on a plate on which 100μl of stationary phase cells were spread. Specifically, the values are the ratio of the zone diameter on the plate for each strain versus the diameter formed by the appropriate control strain (NBRC3283 or NBRC3283[pMV24]). A, Parental strain (NBRC3283) and *katE* mutant strain (3283KE). B, *katE* mutant strain (3283KE[pMV24]) and *katE*-introduced *katE* mutant strain (3283KE[pMKatE]). C, Parental strain (NBRC3283) and *oxyR* mutant strain (3283OX). D, *oxyR* mutant strain (3283OX[pMV24]) and *oxyR*-introduced *oxyR* mutant strain (3283OX[pMOxyR]), n = 3.
medium. In this medium, the growth of the strains was suppressed at the early stages, since the initial pH of the medium was low (pH 3.9) due to the presence of acetic acid. However, the parental strain started to grow 36 h after inoculation and showed relatively robust growth thereafter. Compared with the parental strain, the growth of the katE and oxyR mutant strains was delayed. These growth behaviors were all reproducible.

Judging by these results, hydrogen peroxide resistance abilities due to KatE and to OxyR appeared not to affect growth with membrane oxidation, but did affect growth in glycerol containing and in acetic acid containing media, probably due to a relationship to the catabolism of these compounds.

Discussion

Acetobacter pasteurianus is an industrially useful microorganism classified as an acetic acid bacterium. It is an obligate aerobe with multiple pathways for ATP acquisition in the presence of oxygen. Figure 6 shows the outline of these pathways. The first pathway involves substrate oxidation at the cell membrane, which is explained in the introduction. The cells oxidize ethanol using oxidizing enzymes on the cell membrane, and transmit electrons, which are generated by this oxidation, to the electron transfer system (respiratory chain), also located in the cell membrane. The electron transfer system consists simply of CoQ and a ubiquinol oxidase (UOX), and finally generates ATPs from the proton gradient formed between the inside and the outside of the cell membrane.17,18 Thus acetic acid bacteria can acquire ATP by ethanol oxidation at the cell membrane. The second pathway is a general aerobic pathway that exists in many aerobic cells, consisting of the glycolytic system, TCA cycle, and electron transfer system. NADH molecules generated in the TCA cycle are oxidized by NADH dehydrogenase, causing electrons to be trans-
mitted to the electron transfer system and resulting in ATP generation. By this pathway, the cells can catabolize acetic acid via acetyl-CoA. This property is called the peroxidation of acetic acid. The cells grow via selective use of these pathways, depending on the environment surrounding them. When ethanol is present outside the cells, they grow by oxidizing it on the cell membrane first, and thus produce acetic acid, which is then taken into them to be catabolized by the general aerobic pathway (peroxidation). Due to this characteristic ATP-generating property, this bacterium shows a two-step growth curve when cultured in ethanol-containing medium (Fig. 5C). Thus the strain grows by changing between two types of aerobic energy-generating pathways, depending on the circumstances.

In either case, A. pasteurianus requires a sufficient amount of oxygen for growth, and is expected to be exposed to oxidative stress caused by the electron transfer procedure for ATP generation. Judging by our results, both catalase (KatE) and OxyR are responsible for resistance to hydrogen peroxide, a substance that causes oxidative stress in cells. However, disruption of katE and oxyR affected only the peroxidation phase (after the stationary phase in the growth curve), not the ethanol oxidation phase (the log phase in the growth curve), when the cells were cultured with ethanol as carbon source. The effects of the disruption of katE and oxyR were also shown when the cells were cultured in glycerol and in acetic acid containing media.

Under such culture conditions, which affected the growth of the mutant strains, the carbon sources are thought to be catabolized by the general aerobic pathway via the TCA cycle. In contrast, the cells do not use the TCA cycle during the ethanol-oxidizing phase for ATP generation. Therefore, it might be that the hydrogen peroxide resistance caused by KatE or OxyR is connected to the general aerobic pathway and not to the pathway via oxidation at the cell membrane. The reasons for this difference might be as follows: i) The ethanol oxidation pathway results in lower levels of reactive oxygen species than the general aerobic pathway due to a lack of NADH re-oxidation by NADH dehydrogenase, which has been reported to be one of the main $H_2O_2$-generating points in the electron transfer system. ii) Another oxidative-stress resistance system, which does not use catalase or OxyR, exists in the cells. The resistance systems to oxidative stress reported in anaerobic bacteria such as Lactococcus and Amphotobacillus might provide hints, because these bacteria lack the general aerobic pathway. In any case, it is interesting that the two types of aerobic pathway for ATP generation might cause different responses to oxidative stress.

From the profile shown in Fig. 3, it appears clear that this factor is OxyR. In the oxyR mutant strain, stronger catalase activity was detected than in the parental strain. The following possibilities exist: i) OxyR negatively regulates KatE. ii) KatE is regulated by a factor other than OxyR and is up-regulated in the mutant to compensate for oxyR disruption under conditions of oxidative stress. Case i) is not typical, but control of KatE by OxyR has been reported for several bacteria, including Neisseria, Shinozobium, and Brucella, and as for the two former species, it is possible that OxyR negatively regulates KatE expression. Hence it is possible that the same regulation system works in A. pasteurianus. Regarding case ii), this can occur if KatE is not regulated by OxyR but by some other factor. In many bacteria, expression of KatE is controlled by RpoS, which might also be true for A. pasteurianus. However, no species belonging to the α-proteobacteria, including those for which whole genome sequence data is available, has been reported to have RpoS, suggesting that A. pasteurianus is unlikely to have RpoS either. Indeed, we attempted to clone an rpoS gene from this bacterium by degenerate PCR using primers designed on the conserved regions of the RpoS sequences from Aquifex aeolicus, Borrelia burgdorferi, Escherichia coli, Pseudomonas aeruginosa, Rubrivivax gelatinus, Salmonella enterica, and Yersinia pestis, but we were not successful. Hence some factor other than RpoS might figure in the regulation of KatE in this bacterium. In most cases, OxyRs regulate inductive-type catalases by $H_2O_2$ regardless of positive or negative regulation. Hence case ii) is considered to be more probable, since KatE in A. pasteurianus was not induced by $H_2O_2$, but currently available findings do not make it possible to distinguish between the possibilities described in cases i) and ii), and further study is required.

Although disruption of oxyR caused an increase in KatE activity, not only the katE mutant but also the oxyR mutant became more sensitive to hydrogen peroxide than the parental strain. This appears inconsistent, but it might be explained as follows: OxyR positively controls factors other than KatE that are also responsible for hydrogen peroxide resistance, and in total, the loss of resistance due to these OxyR-regulated factors exceeds the positive effect of increased KatE activity in the oxyR mutant. Such a case was also reported for Shinozobium meliloti, in which the cells became more sensitive to hydrogen peroxide due to disruption of oxyR, although the enzyme activities of KatA and KatB increased in the disruptant. OxyR has been reported to regulate many genes involved in oxidative stress resistance, such as katG, glutathione reductase (gorA), glutaredoxin (grxA), two subunits of alkylhydroperoxidase (ahpCF), Fur repressor (fur), hemH, and saf operon genes in other bacteria. We are working to identify the OxyR-regulated factors responsible for hydrogen peroxide resistance in A. pasteurianus.

In the present study, the parental strain and both mutant strains showed almost the same growth behavior with d-glucose as carbon source. A. pasteurianus grows well with this carbon source (indeed, d-glucose is recommended by the NITE Biological Resource Center, NBRC, of Japan as the standard component of the
Vattanaviboon, P., and Mongkolsak, S., Expression hardly metabolize the gluconic acid produced by this cell membrane, which produces gluconic acid. However, unlike the case of acetic acid, the cells can hardly metabolize the gluconic acid produced by this reaction. Hence a difference in growth behaviors is thought to occur when the cells are grown in YPD versus YPE (Fig. 5A and C).

Here, we investigated the relationship between hydrogen peroxide resistance, which is due to catalase/OxyR, and oxidative fermentation, and obtained results that indicate the possibility of the existence of different oxidative stress circumstances/responses in the cells as between the membrane oxidizing pathway and the general aerobic pathway in *A. pasteurianus*. Although further investigation is required to clarify the whole mechanism, it is interesting that the response to oxidative stress might change depending on the different aerobic pathways operating during acetic acid fermentation.

References