Susceptibility to Hydrogen Peroxide and Catalase Activity of Root Nodule Bacteria

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The root nodule bacteria (free-living cells) tested had higher susceptibility to hydrogen peroxide (H₂O₂) than the other genera of aerobic or facultative anaerobic bacteria tested. The catalase activities tended to have a positive correlation with H₂O₂ resistance among all bacteria tested. Addition of a catalase inhibitor such as 3-amino-1, 2, 4-triazole increased the susceptibility to H₂O₂. These results suggest that the lower catalase activity brings about the higher susceptibility of root nodule bacteria to H₂O₂. Root nodule bacteria seemed to have two or three catalase isozenymes during growth and their catalase activities were higher in log phase than in stationary phase, contrary to other genera of bacteria tested.

Key words: root nodule bacteria; hydrogen peroxide (H₂O₂); catalase

Some toxic forms of oxygen such as hydrogen peroxide (H₂O₂), superoxide radical (O₂·⁻), or hydroxyl radical (OH·), are believed to be naturally generated during the metabolism of cells growing aerobically, and these oxygen species could damage the protein, lipids, and DNA components in organisms.¹ ² Root nodule bacteria form root nodules in legumes and they are present as bacteroids like microsymbiotic organelles in the nodules, and fix nitrogen. Nitrogen fixation is an energy-requiring process and needs large amounts of ATP, which are produced by oxidative phosphorylation. However, the process of nitrogen fixation is oxygen-sensitive and the partial pressure of oxygen inside the nodule is maintained at very low levels, resulting in strongly reducing conditions.³ Thus, these oxygen species are supposed to arise in nodules. Leghemoglobin, which is present in nodules, plays a role in the facilitated diffusion of oxygen within the nodules⁴ and this autocatalysis leads to the production of O₂·⁻ and H₂O₂.⁵ On the other hand, since the host plant could control the nodulation in such a way that the infection during symbiotic interaction is aborted by a hypersensitive reaction, the possibility of the release of oxygen species such as H₂O₂, termed the oxidative burst, is suggested in the early stages of the infection thread formation.⁶ Mehdy found a striking release of oxygen species (oxidative burst) in plant defense against pathogens.⁷ These results suggest that the response of both free-living cells and bacteroids to these toxic forms of oxygen such as H₂O₂ is an important factor for nodulation and the susceptibility to them would exert influence on it. However, little is known about the susceptibility of root nodule bacteria to H₂O₂. To evaluate the susceptibility of root nodule bacteria to H₂O₂, first, we compared the H₂O₂ susceptibility of root nodule bacteria (free-living cells) with that of other genera of aerobic or facultative anaerobic bacteria and report that root nodule bacteria (free-living cells) have higher H₂O₂ susceptibility than other genera of aerobic or facultative anaerobic bacteria, which was mainly caused by the lower level of catalase activity in the cells, although they belong to a group of the aerobic bacteria.

Materials and Methods

Bacterial strains and media. The following strains were used in this study: Rhizobium leguminosarum bv. viciae USDA 2370 and 2443, R. leguminosarum bv. trifolii USDA 2053 and 2145, R. leguminosarum bv. phaseoli USDA 2667 and 2676, Sinorhizobium melliloti USDA 1021 and 1025, Sinorhizobium fredii USDA 191 and 206 were kindly supplied by Dr. van Berkum (Agricultural Research Service, USDA, Beltsville, MD); Bradyrhizobium japonicum S32 was kindly provided from the Tokachi Federation of Agricultural Cooperatives, Obihiro, Hokkaido, Japan, and other strains (Pseudomonas fluorescens AHU1719, Escherichia coli JM109, Proteus vulgaris AHU1144, Serratia marcescens AHU1488, Bacillus subtilis AHU1390, and Micrococcus luteus AHU1427) were from the Laboratory of Culture Collection, Faculty of Agriculture, Hokkaido Univ., Sapporo, Japan. All bacteria used in this study were grown in TY medium⁸ except that B. japonicum was grown in YEM-HM medium.⁹ All media were sterilized by autoclaving at 121°C, 1.2 kg/cm², for 15 min.

A stock solution of hydrogen peroxide (H₂O₂) was filtered (Millipore, pore size 0.45 μm) and then added to the medium described above to prepare an appropriate concentration of this compound. Twenty-four-hour-old cultures (48-hour-old cultures for B. japonicum) (0.1 ml) were transferred to 5 ml of the same medium containing the respective H₂O₂ and incubated at 30°C, aerobically. Growth was monitored by measuring the turbidity of the culture at 660 nm.

Enzyme assays. Cultures in middle log or early station-

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ary phase were centrifuged at 4°C for 10 min at 10,000 × g and soluble extracts were prepared by sonic disruption as described by Clara and Knowles. Protein was estimated by the method of Bradford using bovine serum albumin as a standard. Catalase activity was assayed by measuring the rate of H₂O₂ breakdown at 240 nm. Peroxidase was assayed using NADH, o-dianisidine, or p-phenylene diamine as an electron donor.

Staining of catalase on polyacrylamide gels. Catalase was stained by the method of Clare et al. on 7.5% polyacrylamide gels, i.e., the gels were soaked in horseradish peroxidase (50 µg/ml) in 50 mm phosphate buffer, pH 7.0 for 45 min and then H₂O₂ was added to a final concentration of 5.0 mm followed by soaking for 10 min. The gels were then rapidly rinsed with distilled water two times and placed in 0.5 mg/ml of diaminobenzidine in 50 mm phosphate buffer until the staining was completed.

Results and Discussion

The effects of H₂O₂ on the growth of root nodule bacteria (free-living cells) were compared to those of other genera of aerobic or facultative anaerobic bacteria (Fig. 1). In the presence of 1.5 mm H₂O₂, the growth of root nodule bacteria except for two strains of Rhizobium leguminosarum bv. phaseoli (USDA 2667 and 2676) was severely inhibited and the growth was less than 12% of that in the absence of H₂O₂ (control) (Fig. 1b). Particularly, both strains of Sinorhizobium fredii (USDA 191 and 206) were the most susceptible to H₂O₂ and the growth was decreased to 5% of the control when only 0.6 mm H₂O₂ was added to the culture (Fig. 1a). In the presence of 5.9 mm H₂O₂ the growth of all root nodule bacteria tested was severely inhibited and no strains could grow at least until 7 d after the inoculation. Other genera of bacteria (Pseudomonas fluorescens, Escherichia coli, Proteus vulgaris, Serratia marcescens, Bacillus subtilis, and Micrococcus luteus) showed almost the same growth rate as the control in the presence of 1.5 mm H₂O₂ and all strains tested could still grow to more than 50% of the control even in the presence of 5.9 mm H₂O₂ (Fig. 1c). Specifically, both Bacillus and Micrococcus strains could grow to more than 80% of the control even in the presence of 150 mm H₂O₂ (data not shown). These results indicate that the root nodule bacteria tested tend to have a higher susceptibility to H₂O₂ than the other genera of bacteria.

Catalase and peroxidase activities in the root nodule and other genera of bacteria are shown in the Table. Catalase activities in the former group were in the range from 0.9 to 5.8 (units/mg protein) and the activities in all strains were increased in the range from 2.5 to 11.3 (units/mg protein) by the addition of H₂O₂. The activities in the latter group were from 12.3 to 893.3 (units/mg protein) and the levels of all strains were also H₂O₂-inducible [the levels were increased in the range from 16.1 to 1,460.2 (units/mg protein) by the addition of H₂O₂]. The relationships between H₂O₂ resistance and catalase activities in all bacteria tested are shown in Fig. 2. The results seem to show that there was a positive and mutual correlation between them. For the group of root nodule bacteria (enclosed in a circle), both catalase activities and H₂O₂ resistance were lower than for the others although this bacterial group belongs to the aerobic bacteria. On the other hand, peroxidase activities in the cells with different electron donors, i.e., NADH, o-dianisidine, or p-phenylene diamine, were considerably lower than the catalase activities and did not seem to show a significant difference between root nodule bacteria and others, suggesting that catalase could be mainly responsible for the defense mechanism against H₂O₂ toxicity.

To assess the contribution of catalase to the H₂O₂ resistance of root nodule bacteria, R. leguminosarum bv. phaseoli cells were grown in the presence of both H₂O₂ and the specific inhibitor of catalase, 3-amino-1, 2, 4-triazole (Fig. 3). In the presence of either H₂O₂ (0.6 mm) or the catalase inhibitor (1 mm), the cells could grow more than 90% of the control (without additives) at 24 h after the inoculation. However, when both H₂O₂ and the catalase inhibitor coexisted in the culture, the
### Table: Catalase and Peroxidase Activities in Root Nodule and Other Genera of Bacteria with or without H₂O₂ (0.6 mM)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase</th>
<th>NADPX</th>
<th>ODPX</th>
<th>PDPX</th>
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<tr>
<td></td>
<td>−H₂O₂ + H₂O₂</td>
<td>−H₂O₂ + H₂O₂</td>
<td>−H₂O₂ + H₂O₂</td>
<td>−H₂O₂ + H₂O₂</td>
</tr>
<tr>
<td>A</td>
<td>1.9 8.7</td>
<td><strong>0.03</strong></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>B</td>
<td>2.4 7.5</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>C</td>
<td>2.1 6.4</td>
<td>0.02</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>D</td>
<td>1.2 2.5</td>
<td>0.01</td>
<td>0.03</td>
<td>*</td>
</tr>
<tr>
<td>E</td>
<td>2.6 11.3</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>F</td>
<td>3.3 7.2</td>
<td>0.01</td>
<td>0.04</td>
<td>*</td>
</tr>
<tr>
<td>G</td>
<td>3.8 4.1</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>H</td>
<td>1.8 3.7</td>
<td>0.01</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>I</td>
<td>2.4 3.6</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>J</td>
<td>0.9 2.9</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>K</td>
<td>5.8 8.9</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>L</td>
<td>12.3 16.3</td>
<td>0.02</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>M</td>
<td>33.1 52.7</td>
<td>0.03</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>N</td>
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<td>*</td>
<td>0.01</td>
<td>*</td>
</tr>
<tr>
<td>O</td>
<td>146.6 166.0</td>
<td>0.08</td>
<td>0.08</td>
<td>*</td>
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<tr>
<td>P</td>
<td>265.4 282.3</td>
<td>0.01</td>
<td>0.01</td>
<td>*</td>
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<tr>
<td>Q</td>
<td>893.3 1460.2</td>
<td>0.14</td>
<td>0.20</td>
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</tr>
</tbody>
</table>

Units/mg protein

Each value represents mean of results from at least three trials.


2) expressed as units per milligram of protein

3) Nicotinamide adenine dinucleotide peroxidase

4) o-dianisidine peroxidase

5) p-phenylene diamine peroxidase

6) less than 5 x 10⁻³ units per mg protein

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Cells stopped growing for around 26 h after the inoculation and then started to grow (Fig. 3). The growth repression caused by the addition of the catalase inhibitor in the presence of H₂O₂ was also observed in other root nodule bacteria such as R. leguminosarum bv. trifolii or S. meliloti (data not shown), suggesting that the decrease in H₂O₂ resistance was caused by the inhibition of catalase activity in the cells. These results imply that the catalase in the cells is important for the resistance to H₂O₂ and that lower catalase activity in the cells could be responsible for the higher susceptibility of root nodule bacteria to H₂O₂. As for E. coli, there is a report that catalase-deficient and overproductive mutants of E. coli were more sensitive and resistant to H₂O₂, respectively. ([6,17])

It does not seem to show that there was a strict correlation between H₂O₂ resistance and catalase activities among the root nodule bacteria tested, although catalase could be mainly responsible for the defense against H₂O₂ toxicity. For example, the catalase activity in R. leguminosarum bv. phaseoli USDA2676 was lower than that in B. japonicum strain regardless of the existence of H₂O₂ in the medium (Table). However, in the presence of 1.5 mM H₂O₂, the former strain could grow around 87% of the control, while the growth of the latter strain was inhibited (Fig. 1b). The results of catalase activity staining on gels and the presence of the peroxisome-targeting signal (SKL sequence) in S. meliloti catalase (KatA), which is supposed to be connected with the export into the periplasm, suggest that the catalase in S. meliloti is located in the periplasmic region. ([8]) The location of protecting enzymes against H₂O₂ seems to be important because periplasmic enzymes could be advantageous to the defense against exogenous H₂O₂. Thus, the location of catalase in root nodule bacteria might have an effect upon the mutual correlation between H₂O₂ resistance and catalase activities among them. But, we could not evaluate it.

Figure 4 shows the catalase activities in 6 strains of root nodule bacteria and 3 strains of other genera of bacteria in the middle log and early stationary phases with or without H₂O₂ (0.6 mM). Catalase activities increased in all bacteria tested in the presence of H₂O₂ in both growth phases. However, the levels of catalase in each growth phase had different characteristic features between the root nodule bacteria and others. For the root nodule bacteria, the levels in the log phase were 1.1 to 2.1 times higher than those in the early stationary phase either in the presence or absence of H₂O₂. However, in the case of other genera of bacteria, the levels in the early stationary phase were 1.3 to 13.1 times higher than those in the log phase either in the presence or absence of H₂O₂. These results suggest that the pattern of catalase induction of root nodule bacteria was different from that of the other genera of bacteria, and that the root nodule bacteria had higher levels of catalase in the
Fig. 2. Correlation between Catalase Activities and H2O2 Resistance.
The cells were incubated in TY medium (YEM-HM medium for Bradyrhizobium japonicum) with H2O2 (0.6, 1.5, 5.9, 14.7, 29.4, 58.8, or 147 mM) and the maximum concentration of H2O2 (mM) in which the cells could grow within 24 h (96 h for B. japonicum) by measuring the turbidity of the culture at 660 nm was plotted as ordinate. Catalase activities in the cells in early stationary phase with (crosses) or without (circles) H2O2 (0.6 mM) were plotted as the abscissa. All root nodule bacteria tested are enclosed with a circle. Data points are means of results from at least three trials.

Fig. 3. Effects of 3-Amino-1, 2, 4-triazole on the Growth of Rhizobium leguminosarum bv. phaseoli USDA2676 in TY Medium Containing H2O2.
The cells were grown in TY medium containing H2O2 (0.6 mM) (∆) or 3-amino-1, 2, 4-triazole (1 mM) (○) or both of them (●). The growth of the cells without additives is shown as open circles (○). Data points are means of duplicate from a representative experiment.

Fig. 4. Catalase Activities of Root Nodule and Other Genera of Bacteria in Middle Log and Early Stationary Phases with or without H2O2.
Root nodule (panel a) and other genera of bacteria (panel b) were grown in TY medium (YEM-HM medium for Bradyrhizobium japonicum) with (black bars) or without (white bars) H2O2 (0.6 mM) and catalase activities in the cells in middle log phase (left part) and early stationary phase (right part) were measured as described in Materials and Methods. Each value represents the mean of results from at least three trials. Strains used: B) Rhizobium leguminosarum bv. viciae USDA2443; C) R. leguminosarum bv. trifolii USDA2053; F) R. leguminosarum bv. phaseoli USDA2676; H) Sinorhizobium meliloti USDA1025; I) S. fredii USDA191; K) B. japonicum S32; M) Escherichia coli JM1109; P) Bacillus subtilis AHU1390; Q) Micrococcus luteus AHU1427.

log phase.

The catalase activities of root nodule bacteria were seen by catalase activity staining of polyacrylamide gels using cell extracts from TY cultures sampled at middle log and early stationary phases with or without H2O2, and the following three profiles of zymograms were observed (Fig. 5): First, the catalase-activity-staining gels of R. leguminosarum bv. trifolii showed three catalase isozymes comprising two major bands (labeled 2 and 3) and one minor band (labeled 1) in both growth phases (Fig. 5a). Changes in band pattern were mainly observed in bands 2 and 3, and the inductive response of each isozyme caused by the addition of H2O2 seemed to differ between each phase. That is, in the presence of H2O2, the intensities of band 2 and 3 tended to increase in the log phase and the stationary phase, respectively (Fig. 5a). Catalase-stained gels of other strains such as R. leguminosarum bv. viciae, R. leguminosarum bv. phaseoli, and S. meliloti grown in TY medium also showed two major catalase isozymes in both growth phases, although the relative intensities of each band in the presence or absence of H2O2 differed somewhat with the strains (data not shown); Second, S. fredii also showed two isozymes in each growth phase (Fig. 5b: labeled 1 and 2 in the log phase, labeled 3 and 4 in the stationary phase). However, the Rf values of the bands in each growth phase differed from each other (Fig. 5b);
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Figure 5. Staining of Catalase in Crude Extracts of Root Nodule Bacteria after Electrophoresis on Native Polyacrylamide Gels.

Catalase activities in crude extracts of a) Rhizobium leguminosarum bv. trifolii USDA2053, b) Sinorhizobium fredii USDA191, and c) Bradyrhizobium japonicum S32 in middle log and early stationary phase with (+) or without (-) H₂O₂ (0.6 mM) were detected after electrophoresis on native polyacrylamide gels as described in Materials and Methods. Fifty μg of protein (100 μg of protein for B. japonicum) were put on the gels. Stained bands are shown by the arrows (1 to 4).

Third, B. japonicum also revealed two major isoforms labeled 1 and 2 in both growth phases. However, the relative intensities of each band were clearly dependent on the growth phase, that is, the intensity of band 1 was increased in the stationary phase, but the intensity of band 2 was increased in the log phase whether H₂O₂ existed or not (Fig. 5c).

It seems that the root nodule bacteria have two or three catalase isoforms during growth and the activity of some among them is dependent upon the growth phase or/and the existence of H₂O₂. However, catalase-stained gels of M. luteus showed five distinct isoforms in the stationary phase, but only two isoforms existed in the middle log phase (data not shown). Besides, results obtained from Southern analyses imply that all root nodule bacteria tested have no DNA region similar to kate of E. coli, which is inducible tenfold during growth into stationary phase[20] (data not shown). These results seem to suggest that the differences in the inductive profile of catalase isoforms during growth might be one of the reasons why the patterns of catalase induction in the middle log and stationary phases differ between the root nodule bacteria and others as shown in Fig. 4. It was reported that the kinetics of catalase induction in R. leguminosarum bv. phaseoli was different from that in E. coli, i.e., the catalase activity in R. leguminosarum bv. phaseoli reached a maximum in the early- to mid-exponential phase, but the catalase activity of E. coli reached a maximum in the stationary phase.[20,21] Crockford et al. mentioned that during cell growth, unidentified compound(s) accumulates in the cells and represses catalase activity, but the significance of growth-phase-dependent regulation of catalase activity remains obscure.[20]

This study shows that the root nodule bacteria (free-living cells) have higher susceptibility to H₂O₂ than the other genera of bacteria tested because of the lower catalase activity in the cells. The root nodule bacteria are symbiotic microorganisms and during differentiation into bacteroids they might be able to rely on their host plant for defense against the toxic forms of oxygen such as H₂O₂. It was reported that the production of oxidative protection enzymes by plant cells in nodules was positively correlated with the increase in nitrogenase activity and leghemoglobin content.[22] Thus, higher production of catalase might not be required even if they exist as free-living cells. There is a group of parasitic bacteria that lacks catalase activity because they could depend on the catalase of their host.[23] However, further studies will be needed to explain this phenomenon, i.e., measurement of catalase activity in bacteroids, and then the construction of catalase-deficient mutants and their nodulation efficiency. Results obtained here imply a possibility that the lower catalase activity could be used as a taxonomic index of root nodule bacteria. Besides, since the catalase activity of B. japonicum strain from effective nodules was higher than that in a strain from ineffective root nodules,[24] the improvement of bacterial tolerance against the toxic forms of oxygen like H₂O₂ by increasing the catalase activity in the cells seems to lead to the increase of the nodulation efficiency of the root nodule bacteria.

References