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Effects of H_2O_2 under Low- and High-Aeration-Level Conditions on Growth and Catalase Activity in *Exiguobacterium oxidotolerans* T-2-2^T

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The effects of H_2O_2 under low- and high-aeration-level conditions on growth and catalase activity in *Exiguobacterium oxidotolerans* T-2-2^T were investigated. Continuous addition of 5–200 mM H_2O_2 to the culture medium from the mid-exponential growth phase enhanced the growth of the strain under the low-aeration-level condition, whereas the addition of 5–50 mM H_2O_2 decreased intracellular specific catalase activity and extracellular total catalases activity. The detection of extracellular catalase by the cells and the decrease in intracellular specific catalase activity and extracellular total catalase activity under the high-aeration-level condition account for the stimulation of growth by the introduced H_2O_2 and the decrease in catalase activities induced by O_2 from H_2O_2 in the medium. On the other hand, the addition of H_2O_2 to the medium prior to the initiation of growth inhibited the growth but increased the specific activity of intracellular catalase in the stationary growth phase. Strain T-2-2^T grew when 10 mM H_2O_2 was added to the medium prior to growth. However, the growth was completely inhibited by the catalase inhibitor 3-amino-1,2,4-triazole (3-AT). The continuous addition of H_2O_2 at an appropriate concentration from prior to the initiation of growth to the stationary growth phase under the low-aeration-level condition resulted in higher intracellular specific catalase activity and cell growth rate than single H_2O_2 addition prior to growth.

[**Key words:** catalase, H_2O_2-resistant, *Exiguobacterium oxidotolerans* T-2-2^T]

Aerobic microorganisms metabolize oxygen to sustain their life by various mechanisms such as through their energy production by respiration. During respiration, aerobic microorganisms generate toxic reactive oxygen species (ROSs), such as H_2O_2, as by-products (1–3). The presence of H_2O_2 in cells may result in the generation of even more toxic ROSs, such as hydroxyl radicals, when the molecule interacts with metals (4). Therefore, aerobic microorganisms eliminate H_2O_2 using corresponding enzymes, *e.g.*, catalase (5, 6). On the other hand, parasitic and symbiotic bacteria are attacked by ROSs produced by the external defense system of their host organisms (7–9). It is thought that catalase has an important role in the elimination of H_2O_2 in the extracellular environment in such cases.

Exiguobacterium oxidotolerans strain T-2-2^T has been isolated as an H_2O_2-tolerant bacterium from the drainage of a fish egg processing plant in Hokkaido, Japan, in which H_2O_2 is used as a bleaching agent (10). The strain is able to grow on PYS-2 agar plate containing 10 mM H_2O_2. Furthermore, the specific activity of the catalase of the cell extract of the strain is 558-fold higher than that of *Escherichia coli* IAM 1264. The purified enzyme forms an intermediate of the reaction, compound I, with large-moleculae-size substrates (*e.g.*, peracetic acid) more rapidly than do bovine liver and *Micrococcus luteus* catalases (11). However, the characteristics of growth in the presence of H_2O_2, the mechanisms of H_2O_2 tolerance, and the characteristics of induction of catalase production in this bacterium have not been fully elucidated.

In this study, we investigated the effects of extracellular H_2O_2 and aeration conditions on growth and production of catalase in *E. oxidotolerans* strain T-2-2^T. We discuss the function of catalase in H_2O_2 tolerance and the effect of introduction of H_2O_2 into the culture medium on the induction of the catalase in this microorganism.

MATERIALS AND METHODS

Bacterial strain and growth experiment *E. oxidotolerans* T-2-2^T was cultivated aerobically at 27°C in PYS-3 (peptone-yeast extract-saline medium, version 3; The previous version is described in Ref. 10) broth (pH 7.5) containing (per liter of deionized water) 8.0 g of Polypepton (Nihon Pharmaceutical, Tokyo), 3.0 g of yeast

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extract (Kyokuto, Tokyo), 5.0 g of NaCl, and 5.0 g of sodium succinate. A preculture was prepared using PYS-3 agar medium containing 1.5% agar in PYS broth incubated at 27°C for 15–18 h. A cell suspension prepared in physiological saline from the preculture was adjusted to OD_{650}=0.5, and then 0.5 ml of the cell suspension was inoculated into the main culture medium. The medium of the main culture was prepared as 500 ml of PYS-3 broth in a 2-*l*-volume Erlenmeyer flask. High- and low-aeration-level conditions were generated using baffled and nonbaffled flasks, respectively. Under the low-aeration-level condition, the oxygen concentration was approximately 90% that under the high-aeration-level condition in the absence of an inoculum. The culture was incubated at 27°C with shaking at 120 rpm for 18–20 h. To examine the effect of addition of H_2O_2 to the culture medium of strain T-2-2^T, a one-hundredth volume of H_2O_2 at a concentration 100-fold the desired concentration of H_2O_2 was introduced during cultivation.

Preparation of cell extract Cell extract was obtained as previously described (10). Protein content was determined by the bicinchoninic acid (BCA) method (12) using a BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Enzyme assay conditions Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the disappearance of H_2O_2 using a Unisoku RSP-1000 stopped-flow spectrophotometer, as described previously (11). ε at 240 nm for H_2O_2 was assumed to be 43.6 M^{-1} cm^{-1} (13). The activity of malate dehydrogenase, a cytoplasmic enzyme, was determined by measuring the increase in A_{340} in an assay solution containing 500 μl of a fraction for the estimation of enzyme activity, 5.4 mM β-NAD^+, and 36 mM L-malate in 50 mM Tris–HCl buffer (pH 8.0) in a final total volume of 1.0 ml. The reaction was run at 25°C. The enzyme activity that decomposed 1 μmol of L-malate per min was defined as 1 U of activity.

Electrophoresis and catalase activity staining Native polyacrylamide gel electrophoresis (native PAGE) (7.5%, PAGEL NPU7.5L; Atto, Tokyo) was performed at 4°C. Staining for determining catalase activity was performed as follows (14): the electrophoresed gel was soaked in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mg of 3,3-diamiobenzidine tetrachloride per ml and 10 U of horseradish peroxidase per ml for 45 min in the dark, and then 0.5 ml of 30% H_2O_2 was added to the reaction mixture. SDS–PAGE was performed on a 10–20% gradient gel (PAGEL NPG-1020L; Atto) in accordance with the method of Laemmli (15).

Purification of catalase Intracellular catalase and extracellular catalase from strain T-2-2^T were purified using basically the same procedure, for both, as previously described with slight modifications (11). The cell extract prepared as described above was centrifuged at 131,000×*g* for 60 min to obtain a soluble fraction, which was subjected to the first anion exchange chromatography to purify intracellular catalase. However, for the purification of extracellular catalase, the culture was centrifuged at 10,000×*g* for 15 min and the obtained supernatant was subjected to the first anion exchange chromatography.

RESULTS

Effect of addition of H_2O_2 during culture Five to 200 mM H_2O_2 (final concentration in the medium) was continuously added every 2 h after the culture of strain T-2-2^T showed OD_{650}=0.5 (mid-exponential growth), and growth was monitored turbidimetrically under low-aeration-level condition. The addition of 5–50 mM H_2O_2 stimulated growth, and the strongest effect was observed at a concentration of 25 mM (Fig. 1). Introductions of 50–200 mM H_2O_2 stimulated growth as well as those of 25–50 mM H_2O_2 in the exponential growth phase. However, an earlier decrease in turbidity in the late exponential growth phase was observed in the case of adding 100–200 mM H_2O_2 compared with the case of adding lower H_2O_2 concentrations.

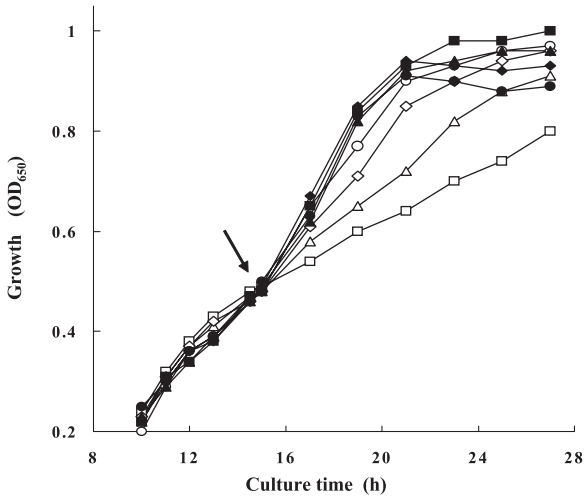

FIG. 1. Effect of continuous addition of H_2O_2 from the mid-exponential growth phase of *Exiguobacterium oxidotolerans* strain T-2-2^T under low-aeration-level condition. The symbols indicate H_2O_2 concentrations (final concentrations in the medium): open squares, 0 mM; open triangles, 5 mM; open diamonds, 12.5 mM; open circles, 15 mM; closed squares, 25 mM; closed triangles, 50 mM; closed diamonds, 100 mM; closed circles, 200 mM. The arrow indicates the time when H_2O_2 was added to the culture medium. H_2O_2 was added to the culture medium every 2 h.

The effects of continuous addition of 5–50 mM H_2O_2 during cultivation on intracellular specific catalase activity and extracellular total catalase activity were investigated, and the results are shown in Fig. 2A and 2B. The intracellular specific catalase activity was almost constant (approximately 33,000 U·mg protein^{-1}) 15–27 h after inoculation, whereas extracellular total catalase activity fluctuated, reaching the maximum (760,000 U in total activity) in the late exponential growth phase when H_2O_2 was not added during cultivation. The presence of extracellular catalase activity accounts for the stimulatory effect of H_2O_2 introduction on growth of the strain. Both the intracellular specific catalase activity and extracellular total catalase activity decreased with the increase in H_2O_2 concentration. A comparison of the mobilities of bands corresponding to proteins exhibiting catalase activity in native PAGE of intracellular and extracellular fractions was performed. The mobilities of the bands corresponding to intracellular and extracellular enzymes were the same (Fig. 3). It was expected that extracellular catalase would be produced as a consequence of disruption of a portion of the cell population during the cultivation. Therefore, we investigated the activity of malate dehydrogenase, an intracellular marker enzyme, in the culture supernatant. Although the activity was detected in the cell extract (50.6 U·mg protein^{-1}), it was not detected in the culture supernatant. This suggests that the existence of extracellular catalase is not due to a physical or an enzymatic disruption of cells.

H_2O_2 (25 mM) was added every 2 h from the 2nd to 16th hour after the initiation of culture under the low aeration

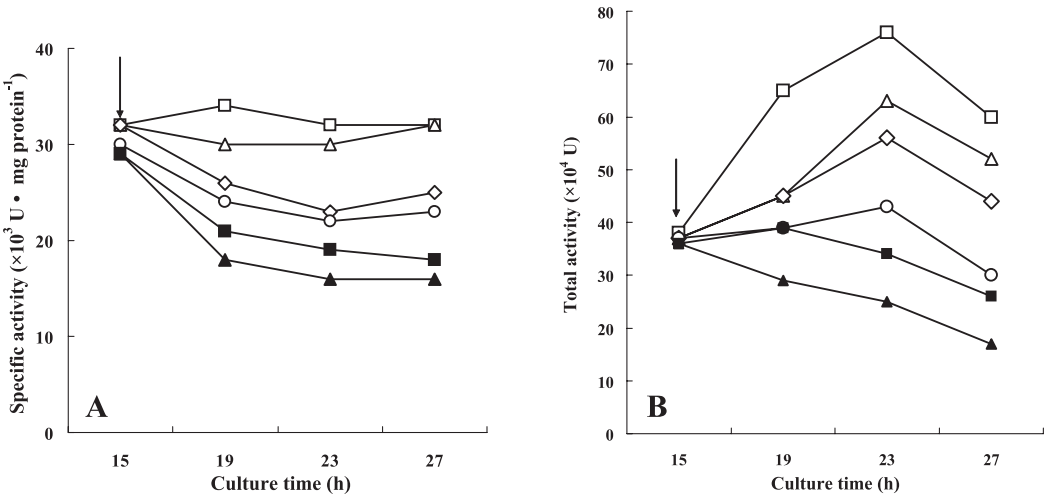

FIG. 2. Specific activity of catalase in cell extract with continuous addition of H_2O_2 from the mid-exponential growth phase of *E. oxidotolerans* strain T-2-2^T (A) and total activities of culture supernatants (B) of *E. oxidotolerans* strain T-2-2^T when H_2O_2 was added in the mid-exponential growth phase under low-aeration-level condition. The symbols indicate H_2O_2 concentrations (final concentrations in the medium): open squares, 0 mM; open triangles, 5 mM; open diamonds, 12.5 mM; open circles, 15 mM; closed squares, 25 mM; closed triangles, 50 mM. The arrow indicates the time when H_2O_2 was added to the culture medium. H_2O_2 was added to the culture medium every 2 h.

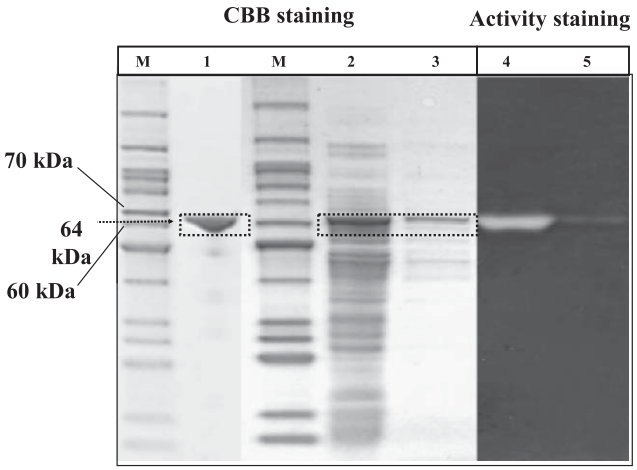

FIG. 3. SDS–PAGE and native PAGE of intracellular and extracellular catalases. Lanes 1–3, SDS–PAGE gel stained by Comassie Brilliant Blue R250; lanes 4 and 5, native PAGE gel stained by catalase activity staining. Lanes: M, molecular weight marker; 1, purified EKTA; 2, cell extract; 3, culture supernatant. Enclosed bands indicate EKTA.

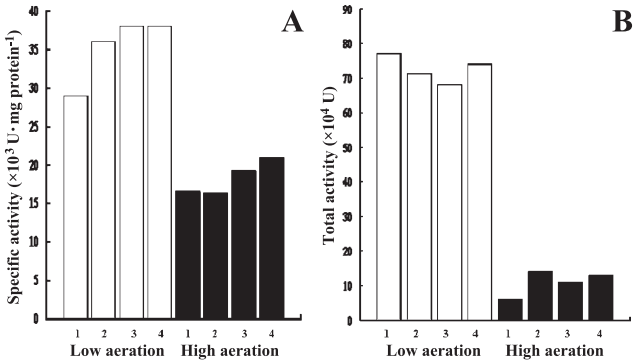

FIG. 4. Specific activities of catalase in cell extract (A) and total catalase activities in culture supernatant (B) of *E. oxidotolerans* strain T-2-2^T when H_2O_2 was added prior to culture initiation under low- and high-aeration-level conditions. The H_2O_2 concentrations corresponding to the numbers are as follows: 1, 0 mM; 2, 1 mM; 3, 5 mM; and 4, 10 mM (final concentrations in the medium).

condition (data not shown). The additions of H_2O_2 at 2, 4 and 6 h after the initiation of culture inhibited growth; the inhibition was complete following the addition of H_2O_2 2 h after the initiation of culture. The effect of the addition of H_2O_2 on growth was strongest 8 h after the initiation of culture. On the other hand, the efficiency for growth inhibition decreased later than the 5th hour after the initiation of culture. A pronounced difference in sensitivity to H_2O_2 was observed in the lag (2–4 h of growth) and early exponential (6 h of growth) phases, suggesting that total activity of catalase produced by the population of cells becomes sufficient for decomposing H_2O_2 introduced into the medium.

Effect of addition of H_2O_2 prior to cultivation The effects of aeration and the introduction of H_2O_2 prior to cultivation were investigated. H_2O_2 at various concentrations (1, 5, 10 and 20 mM) was introduced prior to cultivation, and cells were cultured under high and low aeration conditions (Fig. 4A, B) with 500 ml of medium in a 2-*l*-volume flask shaken at 120 rpm at 27°C. The growth rate and cell density were higher under the high aeration condition than under the low aeration condition. The lag phase of growth became longer with the increase in H_2O_2 concentration added to the medium and the lag phase was longer under the low-aeration-level condition than under the high aeration condition at the same H_2O_2 concentration (data not shown). These results indicate that the cells are much weaker against H_2O_2 at prior to the initiation of growth than those in the mid-exponential growth phase. The production of intracellular catalase increased with H_2O_2 concentration, whereas the production of extracellular catalase was not stimulated by the presence of H_2O_2 under the low-aeration-level condition. In-

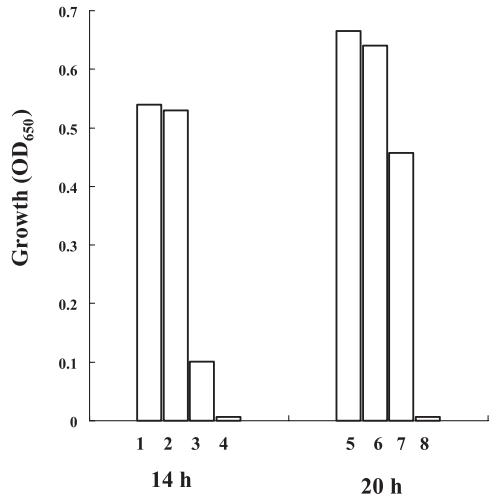

FIG. 5. Effect of a catalase inhibitor, 3-amino-1,2,4-triazole (3AT), on the growth of *E. oxidotolerans* strain T-2-2^T under the low-aeration-level condition. Growth was estimated at 14 h of culture (no. 1–4) and 20 h of culture (nos. 5–8). No. 1, 5: 0 mM H_2O_2; no. 2, 6: 0 mM H_2O_2+ 50 mM 3-AT; no. 3, 7: 10 mM H_2O_2; no. 4, 8: 10 mM H_2O_2+50 mM 3-AT.

tracellular specific catalase activity and extracellular total catalase activity were both higher under the low-aeration-level condition than under the high-aeration-level condition (Fig. 4). These results account for the suppression of catalase production of strain T-2-2^T by O_2 resulting from the continues introduction of H_2O_2 from the mid-exponential growth phase (Fig. 2).

A cell morphological difference was also observed between the lag and exponential growth phases of strain T-2-2^T under the low-aeration-level condition when 10 mM H_2O_2 was added to the medium prior to cell growth (data not shown). Although this morphological difference was observed in the absence of H_2O_2 in the medium, the difference was more easily observed in the presence of H_2O_2 owing to the long period of the lag growth phase. The cells were long rod-shaped in the lag phase but became short rod-shaped in the exponential phase. The cell extract obtained from the lag phase exhibited an activity of 21,000 U·mg protein^{-1}, whereas that obtained from the exponential phase exhibited an activity of 79,000 U·mg protein^{-1}. However, the purified catalases obtained in the lag and exponential phases exhibited activities of 486,000 U·mg protein^{-1} and 384,000 U·mg protein^{-1}, respectively. Although there are a differences in the activities, it is considered that these differences are due to variations in the preparation (approximately 370,000–490,000 U·mg protein^{-1}). The mobilities of the bands corresponding to the two catalases in native PAGE and SDS–PAGE were the same (data not shown).

Effect of inhibitor on H_2O_2 tolerance To determine whether the tolerance of strain T-2-2^T to H_2O_2 is due to the activity of its catalase, the effect of a specific inhibitor of catalase, 3-amino-1,2,4-triazole (3AT), on the strain's tolerance to H_2O_2 added prior to the initiation of growth under the low-aeration-level condition was examined (Fig. 5). Because the inhibitor acts on an intermediate of the reaction (compound I), the sensitivity of the cells to 3AT appeared in the presence of H_2O_2. No inhibition was observed in the presence of only 3AT and partial inhibition was observed in the presence of only H_2O_2 due to its toxicity. The growth recovery (optical density) at 20 h of culture was much higher than that at 14 h of culture after treatment with 10 mM H_2O_2. This difference is probably due to the culture period and the amount of catalase produced in accordance with the culture period. Although it is not clear whether the tolerance is due to intracellular or extracellular catalase, the catalase reaction most likely occurs inside of the cells because extracellular catalase was scarcely observed at the initiation of the growth (data not shown). The above observation demonstrated that catalase has an important role in resistance against H_2O_2 in this bacterium.

Purification of catalase The total catalase activity of the culture supernatant was 880,000 U, whereas that of the cell extract was 6,760,000 U. This indicates that approximately 12% of catalase with respect to total catalase activity in the cells is produced in the extracellular space.

The rate of extracellular catalase recovery was 20%, whereas that of intracellular catalase recovery was more than 30%. Although the reason is not clear, this difference might be due to the insufficient separation of extracellular catalase in the second run anion exchange chromatography. The purified extracellular catalase exhibited the same mobility as the purified intracellular catalase on native PAGE, SDS–PAGE and PAGE for the determination of isoelectric point (p*I*) (data not shown). Furthermore, there is no marked difference in resistance to 2 mM H_2O_2 between the purified intracellular and extracellular catalases (data not shown). However, the specific activity of the purified extracellular catalase was 280,000 U·mg protein^{-1}, which was lower than that of the purified intracellular catalase (approximately 370,000–490,000 U·mg protein^{-1}).

Induction of catalase The above results indicate that the single stimulation with H_2O_2 prior to growth under the low aeration condition increased intracellular specific catalase activity by approximately 1.4- and 1.8–2.3-fold compared with the case of cultivation with no H_2O_2 stimulation and that with H_2O_2 stimulation under the high aeration condition, respectively (Fig. 4A). The effects of the continuous addition of H_2O_2 from prior to the initiation of growth up to stationary growth phase under the low-aeration-level condition on intracellular specific catalase activity and extracellular total catalase activity were examined. The addition of 5 mM H_2O_2 prior to the growth and the continuous addition of H_2O_2 (*i.e.*, 5, 10, 20, 40, 80, 40 and 20 mM) after every 4 h of culture were performed. The intracellular specific catalase activities under the high- and low-aeration-level conditions with continuous H_2O_2 addition from prior to the initiation of growth were about 3.1- and 1.8-fold those without the H_2O_2 addition, respectively (Fig. 6A). The growth stimulatory effect of H_2O_2 was observed in the case of continuous H_2O_2 addition compared with that without the H_2O_2 addition under the low-aeration-level condition (data not shown). Furthermore, the intracellular specific catalase activity (Figs. 4A and 6A) and growth rate (OD_{650}) (data not shown) in the case of continuous H_2O_2 addition was approximately 1.4-fold and 1.2–1.4-fold higher than those in the case of single 5–10 mM H_2O_2 addition prior to the growth under

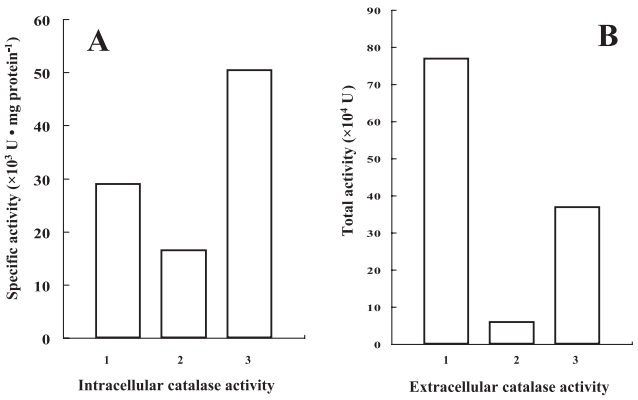

FIG. 6. Intracellular and extracellular catalase activities in the early stationary phase under high- and low-aeration-level conditions with or without addition of H_2O_2. Intracellular and extracellular catalase activities are shown in terms of specific and total activities, respectively. In addition to 5 mM H_2O_2 stimulation prior to the initiation of growth, continuous addition of 5–80 mM H_2O_2 (*i.e.*, 5, 10, 20, 40, 80, 40 and 20 mM; final concentrations in the medium) every 4 h of culture under the low-aeration-level condition was performed. 1, Low-aeration-level condition; 2, high-aeration-level condition; 3, low-aeration-level condition + continuous addition of H_2O_2.

the low-aeration-level condition, respectively. However, the production of extracellular catalase was not enhanced by continuous H_2O_2 addition. It is thought that the introduced H_2O_2 may damage extracellular catalase or that O_2 produced from H_2O_2 decomposition suppressed the production of catalase in strain T-2-2^T.

DISCUSSION

It is thought that the enhanced growth by the introduction of H_2O_2 during the mid-exponential phase of growth of strain T-2-2^T is due to the production of O_2 by the action of extracellular catalase. Furthermore, this production may occur rapidly after the introduction of H_2O_2 into the culture medium owing to the rapidity of catalase reaction and the large amount of extracellular catalase. The introduction of H_2O_2 as an oxygen source for oil-oxidizing microorganisms under underground conditions has been reported (16). However, when the H_2O_2 concentration was 320 $\mu g \cdot ml^{-1}$ (approximately 9.4 mM), no growth occurred. The utilization of H_2O_2 via the action of extracellular catalase may have an important function the resistance of bacteria to H_2O_2 at high concentrations. To the best of our knowledge, this is the first study showing that continuous addition of a high concentration of H_2O_2 (*i.e.*, more than 50 mM) enhances the growth of microorganism.

If a population of cells is exposed to a high concentration of ROSs that exceeds the capacity of the protecting enzyme in that population, oxidative stress will occur in the cells (17). If the H_2O_2 concentration greatly exceeds the capacity of the protecting enzyme of a population of cells, the cells lose viability (18–20). A cells population's capacity to eliminate H_2O_2 is determined by various factors, including growth phase, population density, nutritional status, and external stimulation (21–25). For example, *E. coli* exhibits a higher catalase activity in the stationary phase than in the logarithmic phase (21). In this study, although strain T-2-2^T did not exhibit a strong tolerance to H_2O_2 at the initiation of growth, it exhibited a strong tolerance to H_2O_2 in the mid-exponential phase of growth. It is thought that the H_2O_2 elimination capacity of a cell population is determined by the H_2O_2 elimination capacity of the population (*e.g.*, total catalase activity) corresponding to the growth phase.

As reported for other microorganisms (26, 27), the addition of a low concentration of H_2O_2 prior to culture initiation enhanced the production of catalase. By this stimulation, the cell population becomes able to withstand high H_2O_2 concentrations that would normally be lethal. This phenomenon has been explained by the complex nature of molecular processes of the induction of regulons expressing both protective enzymes and repair systems (26–29). Although the stimulatory effect on intracellular catalase was not observed following the introduction of 5–50 mM H_2O_2 from the mid-exponential phase, induction by continuous addition of 5–80 mM H_2O_2 from prior to the growth produced the stimulatory effect on intracellular catalase (Fig. 6). It is thought that the stimulatory effect of the introduced H_2O_2 on the production of intracellular catalase occurs only in a phase earlier than the mid exponential phase.

Although the production of extracellular catalase by microorganisms has been reported, the secretion mechanism has not yet been clarified (30–33). There is a possibility that extracellular catalase can be detected due to cell disruption concomitant with cell death after the stationary phase of growth. Indeed, extracellular catalase produced by strain T-2-2^T was detected during the logarithmic phase of growth, and catalase activity was detected while there was no intracellular marker enzyme, namely, malate dehydrogenase activity, present in the culture supernatant. These results suggest that the microorganism secretes extracellular catalase into the medium.

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