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Isolation and characterization of *Vitreoscilla* mutant defective in catalase-peroxidase hydroperoxidase I

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Abstract

Mutants of an obligate aerobic bacterium, *Vitreoscilla*, that have deficiency in heat-labile catalase-peroxidase hydroperoxidase I (HPI) were created by EMS treatment. The catalase-peroxidase HPI-deficient mutant showed substantially lower peroxidase activity in exponential and mid-stationary phase compared with the wild type strain. In late stationary phase, the mutant exhibited no peroxidase activity. Peroxidase deficiency in the mutant was revealed by polyacrylamide gels stained for peroxidase activity. Characteristically, catalase levels in the mutant increased about 14- and 8-fold during growth in the exponential and stationary phases, respectively, compared to those in the wild type, suggesting a compensatory effect for protection from H_2O_2 toxicity. The mutant showed differences in physiology from the wild type: retardation in growth rate and decrease in oxygen consumption. Both the wild type and the catalase-peroxidase HPI-deficient mutant of *Vitreoscilla* had lower growth rates in media containing increasing H_2O_2 concentrations. However, the mutant exhibited an additionally decreased growth rate after 6 to 8 h of growth compared to the wild type. The wild type was resistent up to 20 mM H_2O_2, whereas the mutant was very sensitive to high concentrations of exogenous H_2O_2. Although elevated catalase levels would provide protection of the bacteria from the deleterious effect of H_2O_2, it did not appear to be complete. Cell-free extracts of the mutant showed decreased NADH oxidation rates and higher accumulation of H_2O_2 during this oxidation.

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These results may account for the impaired growth and earlier onset of death phase by the catalase-peroxidase HPI-deficient mutant of *Vitreoscilla*.

Key words : Catalase, Catalase-peroxidase, Hydroperoxidase I, *Vitreoscilla*

Introduction

Reactive oxygen species, such as superoxide radical, hydrogen peroxide, and hydroxyl radical, naturally arise during normal metabolism in aerobically growing cells as a result of the incomplete reduction of molecular oxygen. Organisms have evolved sophisticated and efficient enzyme systems to neutralize these potentially injurious reactive oxygen species, including catalases, superoxide dismutases, and peroxidases.

Three classes of heme proteins, commonly designated hydroperoxidases, are involved in the detoxification of hydrogen peroxide: catalases, peroxidases, and catalase-peroxidases. Catalase is usually defined as the enzyme catalyzing the metabolism of H_2O_2 to O_2 and H_2O, whereas peroxidase uses H_2O_2 to oxidize a variety of compounds. Catalase-peroxidase is an enzyme that can catalyze both catalytic and peroxidatic activities [1]. Catalase and peroxidase exist among a large number of aerobic prokaryotes and eukaryotes. In contrast, catalase-peroxidases are identified only from bacteria except for a few species of fungi. Catalase-peroxidaes differ from the typical catalases in some physicochemical properties: pH dependence of the catalytic activity, sen sitivities to temperature, organic solvents and H_2O_2, reduction by sodium dithionite, inhibition by azide and cyanide, and lack of reactivity with 3-amino-2, 2, 4-triazole [1, 2].

Hydroperoxidases, named as HPI and HPII, in *Escherichia coli* have been studied in depth [3-5]. Their synthesis is genetically controlled by at least four noncontiguous loci (*katE, rpoS, katG,* and *oxyR*). HPI, encoded by *katG* [6] and dependent on the oxidative stress response (*oxyR*) regulon [7], is a bifunctional enzyme, having both catalase and peroxidase activities. HPII, encoded by *katE* [8], possesses only catalase activity and is maximally synthesized as cells enter the stationary phase of growth [3] and undergo osmotic changes [9].

The bacterium *Vitreoscilla* sp is an obligate aerobe from the *Beggiatoa* family. It possesses a hemoglobin (VtHb) with a putative function to capture oxygen and feed it to the terminal oxidases under oxygen limiting conditions [10]. *Vitreoscilla* also contains defense enzymes to protect the cells from oxidative damage, superoxide dismutase [11] and a catalase-peroxidase HPI [12]. Whether there is a hydroperoxidase like HPII in *Vitreoscilla* is not known. Catalase-peroxidases would be the primary enzymes protecting the cells from damage by toxic oxygen derivatives resulting from aerobic respiration in the presence of VtHb especially at low oxygen concentration [12]. The precise role of catalase-peroxidase in the resistance of *Vitreoscilla* to hydrogen peroxide could be better investigated if mutants devoid

of one or more peroxidase (*kat*) genes were available. Therefore, the isolation of *Vitreoscilla* mutant defective in a catalase-peroxidase HPI will contribute to our understanding of the role that both catalase and peroxidase play in protecting the cells from toxic oxygen derivatives.

Materials and Methods

Bacterial strain, medium and growth conditions

Vitreoscilla sp strain C1 was cultured in PYA medium at room temperature with shaking at 200 rpm [13]. Cell growth was monitored by measuring absorbance at 540 nm in a Model PU 8600 spectrophotometer with an appropriate dilution. One mℓ culture (1×10^7 cfu/mℓ) of exponential phase was used as the inoculum for 100 mℓ of PYA medium and grown in a shaking incubator (200 rpm) at 20 ℃. At 4-hour intervals, 0.5 mℓ of culture was removed, diluted with 4.5 mℓ of deionized water and the optical density was read at 540 nm.

Isolation of catalase-peroxidase hydroperoxidase I-deficient mutants

(1) EMS mutagenesis

Ethyl ester methane sulfonate (EMS) was used as the mutagen. Cells were grown to early stationary phase in 50 mℓ of PYA medium, and collected by centrifugation at 5,000 × g for 10 min. The pellet was washed in 30 mℓ of sterile 0.1 M potassium phosphate buffer, pH 8.0. The washed cells were centrifuged at 5,000 × g for 10 min, and resuspended in 30 mℓ of the phosphate buffer. The final cell suspension was placed in a 125 mℓ Erlenmeyer flask. Prior to the addition of EMS, a control sample (0.1 mℓ) was removed from the culture, placed in 5 mℓ of 6% sodium thiosulfate, and plated on solid PYA medium after dilutions. EMS was added to yield a final concentration of 3% (*v/v*). After addition of EMS, the flask was incubated at 20 ℃ at 200 rpm, and samples (0.1 mℓ) were removed into 5 mℓ of 6% sodium thiosulfate to inactivate the EMS at 20 min intervals. The treated cells with sodium thiosulfate were diluted and plated on PYA agar. Number of surviving colonies was counted after 3-5 days at 20 ℃.

(2) Isolation procedure of mutants

The colonies that survived in 3% EMS solution for 120 min (10 % survival) were grown in PYA plates, replica plated onto PYA plates and PYA plates containing 1.0 mM hydrogen peroxide, and grown 4 days at room temperature. Colonies that exhibited poor growth in the presence of hydrogen peroxide were selected as presumptive catalase-peroxidase HPI-deficient mutants. These presumptive mutants were further tested for catalase activity by adding a drop of 30 % hydrogen peroxide to the replicates. Mutants exhibited reduced bubbling in the presence of hydrogen peroxide.

Preparation of cell-free extracts

Vitreoscilla cells were harvested by centrifugation at 5,000 × g for 10 min, and washed twice with 50 mM potassium

phosphate, pH 7.2, containing 5 mM EDTA. The harvested cells were then resuspended in the same buffer to one-tenth of the original culture volume and sonicated on ice for 5 cycles of 10 sec on and 30 sec off with a Branson (Danbury, Conn.) model 450 sonifier and microtip at an output setting of 20. The cell debris was removed by centrifugation at 10,000 × g for 45 min at 4 ℃, and the supernatant was recovered and kept on ice.

Enzyme assays

Protein concentration was determined by the method of Lowry et al [14] using bovine serum albumin as the protein standard. Peroxidase activity was assayed spectrophotometrically by monitoring the oxidation of 4-aminoantipyrine at 510 ㎚ at room temperature [15]. The reaction solution was prepared by adding 1.5 ㎖ of 1.7 mM hydrogen peroxide to 1.4 ㎖ of 2.5 mM 4-aminoantipyrine with 170 mM phenol. One ml of this solution was pipetted into a 1.0 ㎝ sample cuvette and 1 ㎖ of distilled water into the reference cuvette. After incubation for 3-4 min, 100 ㎕ of cell extract was added into the sample cuvette, and mixed rapidly. Absorbance was recorded for 4-5 min and the specific activity of the enzyme was calculated as units of activity per ㎎ of protein. Catalase activity was assayed by measuring the decomposition of hydrogen peroxide at 240 ㎚ as described by Aebi [16]. The substrate was prepared by adding 0.16 ㎖ of 30 % hydrogen peroxide to 100 ㎖ of 0.05 M sodium-potassium phosphate, pH 7.0. After 1 ㎖ of the substrate solution was pipetted into a sample cuvette, cell extract was added into the cuvette and mixed rapidly. The absorbance of the samples at 240 ㎚ was measured for 1min. One unit of enzyme was defined as the activity that catalyzed the degradation of 1 μmol H_2O_2 min^{-1}. The specific activity of the enzyme was calculated as units of activity per ㎎ of protein.

Visualization of catalase and peroxidase activities on polyacrylamide gels

Electrophoresis was performed on non-denaturing 8.5% (*w/v*) polyacrylamide gels in a Mini-PROTEAN II Dual Slab Cell (Bio-Rad) at pH 8.8 [17]. Twenty microgram samples of the protein extracts in 10 % glycerol (*v/v*) and 0.02 % bromophenol blue (*w/v*) were applied to the gel. Electrophoresis was for 5 hrs at a constant current of 10 mA. For visualization of peroxidase and catalase activities on the gel, 3,3'-diaminobenzidine (DAB) for peroxidase stain followed by ferricyanide negative stain for catalase was performed as described by Wayne and Diaz [18] with minor modifications for catalase stain : after three washing steps for 15 min in H_2O, the gels were soaked in 0.03 % H_2O_2 for 10 min and rinsed with H_2O for 2 min ; after this, the gels were incubated in a moist chamber for 15 min at room temperature to allow consumption of H_2O_2 by the catalase bands. The gels were then stained in a 50 : 50 mixture of freshly prepared solution of 2 % (*w/v*) ferric chloride and 2 % (*w/v*) potassium ferricyanide for 30 sec with gentle agitation.

Heme staining

Vitreoscilla cell extracts were analyzed by nondenaturing PAGE except that the SDS concentration in the electrophoresis buffer and the gel was reduced to 0.1 %. The gel was pre-electrophoresed overnight at 1 mA to remove excess ammonium persulfate. Immediately before electrophoresis, the sample was mixed with sample buffer ommiting the sulfhydryl reducing agent. After electrophoresis the gel was stained for heme.

Measurement of oxygen consumption

The oxygen concentration was measured with a dissolved oxygen-(DO) sensor in a KFD fermentor. The sensor was standardized to 100% oxygen concentration in oxygen-saturated buffer and submerged in the PYA broth medium within the glass tank of fermentor, and the glass tank was sterilized by autoclaving. After sterilization 10 ml (1×10^7 cfu/ml) of precultured cells was inoculated to 1 liter of medium, and the oxygen concentration was monitored during growth at 20 ℃ at 200 rpm.

Assay for hydrogen peroxide formation during the oxidation of NADH

The concentration of hydrogen peroxide was assayed fluorometrically [19]. For the assay medium, a stock solution of 2',7'-dichlorofluorescin diacetate was made in 0.1 mM in ethanol and activated by hydrolysis with alkali (1 volume of the ethanol solution with 4 volume of 0.01 N sodium hydroxide). The assay medium contained 2 μM 2',7'-dichlorofluorescin diacetate, 2 μg/ml peroxidase, 0.04 mg/ml $ZnSO_4 \cdot 7H_2O$, 0.02 M sodium phosphate, pH 7.1, and 10 μM NADH. The reaction was started by the addition of 100 μl of cell extract to 3 ml of assay medium. The increase in fluorescence at 525 nm was followed with an MK2 spectrophotofluorometer using an excitation wavelength of 503 nm. The standard curve for this assay was obtained using fresh dilutions of 30 % hydrogen peroxide.

The rate of NADH oxidation was determined independently with a PU 8600 spectrophotometer. The assay mixture contained the following: 0.02 M sodium phosphate buffer, pH 7.1 and 10 μM NADH. The reaction was started by the addition of 100 μl of cell extract to 1 ml of assay mixture and was followed by the decrease in 340 nm absorbance. Rates of the NADH oxidation were corrected for the spontaneous breakdown of NADH.

Results

Identification of a catalase-peroxidase HPI-deficient mutant

A number of mutants of *Vitreoscilla* were produced by treatment with EMS for 120 min, which produced 10% survival of the cells. A catalase-peroxidase HPI-deficient mutant was confirmed by assaying peroxidase and catalase activities of crude cell extracts of the isolated mutant (Fig 1). The wild type showed both peroxidase and catalase activities, while the mutant showed catalase activity only. In the wild type, catalase activity increased as

cultures entered stationary phase, while peroxidase activity was highest at mid-stationary growth phase. In the mutant, the peroxidase activity was strongly reduced in exponential and mid-stationary phase compared to that in the wild type. In late stationary phase, the mutant exhibited no peroxidase activity. Interestingly, the mutant had 14- and 8-fold the catalase activity of the wild type at exponential and mid-stationary phases, respectively.

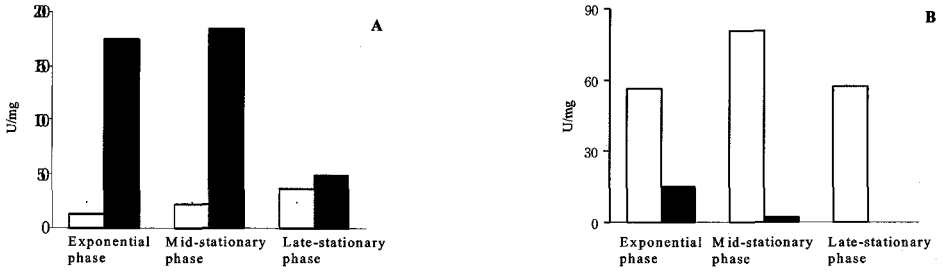

Fig 1. Catalase and peroxidase activities at various growth phases in wild type and in mutant *Vitreoscilla*. (A) Catalase activity. Specific activity is reported as units/mg protein, where one unit of catalase activity is the amount of enzyme that catalyzes the disproportionation of 1 μ mol of H_2O_2/min. (B) Peroxidase activity. Specific activity is reported as units/mg protein, where 1 unit is the amount of enzyme that converts 1 μmol of 4-aminoantipyrine/min under the conditions described in Materials and Methods. White bar : wild type, black bar : mutant.

Peroxidase and catalase activities in the extracts of wild type and catalase-peroxidase HPI-deficient mutant were also analyzed on non-denaturing poly-acrylamide gels (Fig 2). When the gel was visualized with 3,3'-diaminobenzidine, followed by ferricyanide, the wild type showed one band corresponding to HPI. The series of gels stained for either catalase or peroxidase activity shown in Fig 2 confirmed that the mutant did not produce HPI but produced HPII. The results indicate that the high catalase level in the mutant (Fig 1) conferred a selective growth advantage in the presence of H_2O_2. However, this does not necessary mean that high catalase levels would protect bacteria from the killing effect of high doses of H_2O_2. Preparations heated at 68 ℃ for 1 min were used to test the heat stability of the HPI identified in the wild type (Fig 3). The HPI band in lane 4 was disappeared in the heated sample in the lane 3, showing that the *Vitreoscilla* HPI is not a heat stable hydroperoxidase.

Crude extracts of the wild type and the mutant were seperated by non-denaturing PAGE, and then stained for heme (Fig 4). The wild type was showed 3 main bands corresponding heme-stained. The top two bands in wild type are thought to the catalase and catalase-peroxidase, respectively. The band missing in the mutant, probably corresponds to the catalase-peroxidase HPI. The remaining heme-stained polypeptide in the wild type may be the VtHb. The mutant had lesser heme quantity than the wild type including VtHb.

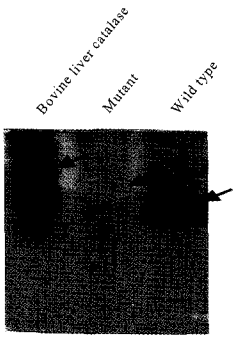

Fig 2. Visualization of peroxidase and catalase in crude extracts of *Vitreoscilla* after electrophoresis on non-denaturing polyacrylamide gel. Gel was stained with 3,3'-diaminobenzidine tetrahydrochloride for peroxidase activity followed by ferricyanide negative stain for catalase activity. Peroxidase activity was visualized as dark band. Catalse activity was appeared as a colorless band. Running conditions and procedures were as described under Materials and Methods. Twenty μg of protein was loaded in each lane.

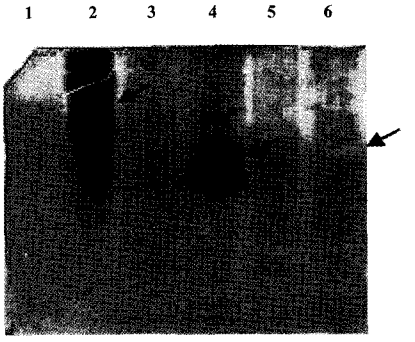

Fig 3. Sensitivity of catalse-peroxidase hydroperoxidase to temperature. Polyacrylamide gel electrophoresis of heated and unheated crude extracts of wild type and mutant of *Vitreoscilla* visualized by sequential DAB and ferricyanide stain. Lanes 1 and 2, Bovine liver catalase, heated and unheated; 3 and 4, *Vitreoscilla* wild type, heated and unheated; 5 and 6, *Vitreoscilla* mutant, heated and unheated. Twenty μg of protein was loaded in each lane.

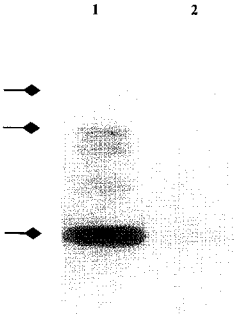

Fig 4. Heme stain of polypeptides from wild type and peroxidase deficient mutant of *Vitreoscilla*. Crude extracts from two strains were separated by non-denaturing PAGE for 90 min at 98 volts on a 8.5 % (*w/v*) polyacrylamide gel and stained for heme as described in Materials and Methods. Twenty μg of protein was loaded in each lane. Lane 1 : wild type, Lane 2 : mutant.

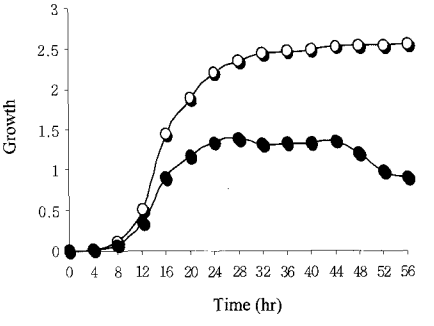

Fig 5. Growth curves of wild type and peroxidase deficient mutant. Cell growth was monitored by measuring absorbance at 540 nm in a spectrophotometer with appropriate dilutions. Growth conditions are described in Materials and Methods. ○, wild type; ●, mutant.

Growth behavior of the catalase-peroxidase HPI-deficient mutant

Cell growth was monitored by measuring absorbance at 540 nm, and growth curves of wild type and mutant are presented in Fig 5. The growth rate of the catalase-peroxidase HPI-deficient mutant in the exponential phase was retarded as compared with the wild type, and the

cell density of the mutant at early stationary phase was roughly one half that of the wild type. Further, the mutant entered a death phase at 40 hrs of cultivation while the wild type remained in stationary phase until the end of the experiment (56 hrs). It was expected that the high catalase level in the mutant might confer a compensatory effect for the peroxidase deficiency in protecting the bacteria from the deleterious effects of H_2O_2. There is a report supporting our result that in other bacteria that there is a correlation between catalase level and level of H_2O_2 resistance [20]. If the elevated catalase did provide protection it did not appear to be complete.

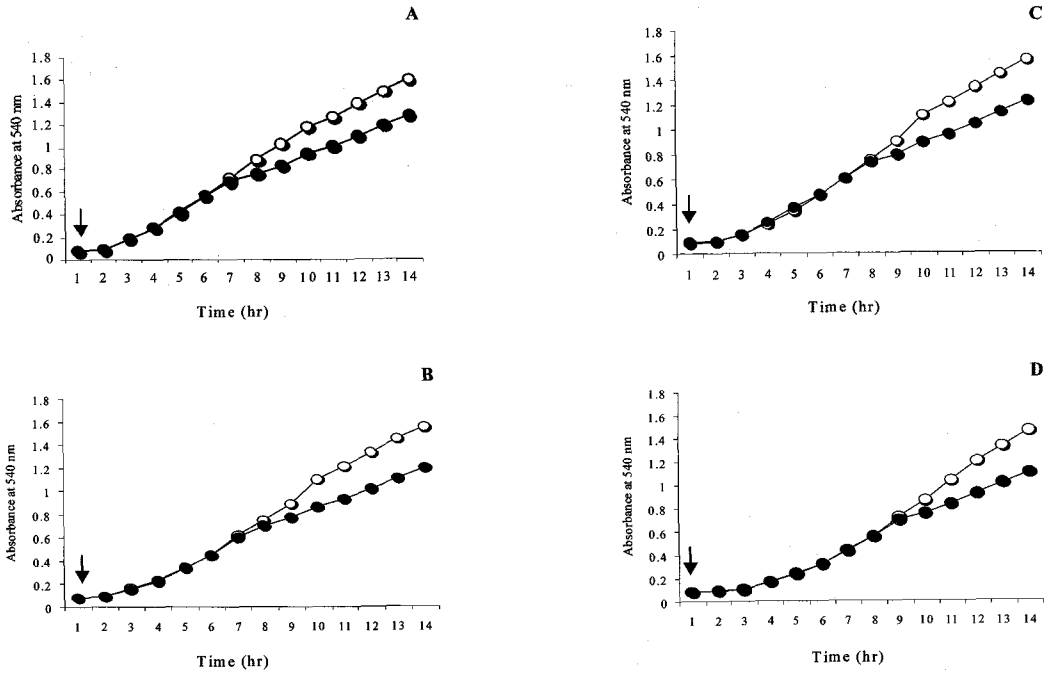

Fig 6. Growth of wild type and peroxidase deficient mutant of *Vitreoscilla* in the presence of H_2O_2. Overnight cultures of each strain were subcultured in PYA medium with a 2 % inoculum and grown for additional hour at 20 ℃. H_2O_2 was added to the cultures and growth was monitored hourly by spectrophotometer at 540 ㎚. Open and closed circles indicate the growths of wild type and mutant, respectively. (A) Growth in the presence of 0.1 mM H_2O_2 concentration. (B) Growth in the presence of 0.2 mM H_2O_2 concentration. (C) Growth in the presence of 1 mM H_2O_2 concentration. (D) Growth in the presence of 2 mM H_2O_2 concentration. Arrow indicates the addition of H_2O_2 to the culture medium. ○, wild type; ●, mutant.

Sensitivity of catalase-peroxidase HPI-deficient mutant to H_2O_2

The effect of concentrations of H_2O_2 on the growth rates of wild type and mutant *Vitreoscilla* was investigated (Fig 6). Both strains were retarded equally in growth rate in the presence of H_2O_2 for the first 6–8 hrs of growth. After that the wild type returned to a growth rate essentially identical to that of the control in exponential phase. In contrast, the

mutant growth rate decreased after the 1st 6-8 hrs. Thus, H_2O_2 inhibits the growth of both strains, but the inhibition of the wild type was only transient and increased to normal after 6-8 hrs whereas that of the mutant decreased at about the same time. An additional lag phase (or decreased gowth rate in exponential phase) and the entering time to the lag phase in the mutant were delayed as H_2O_2 concentration increased. The results indicate that H_2O_2 can arrest in cell division in the mutant.

The viabilities of cells exposed to various high concentrations of exogenous H_2O_2 were evaluated for the wild type and for the mutant. The wild type survival was not affected significantly by H_2O_2 up to 20 mM, *Vitreocilla* were required to a high concentration of H_2O_2 for killing compared with other systems [7, 21]. By contrast, the mutant was very sensitive to H_2O_2 and displays severe survival as 100 % killing at 20 mM H_2O_2 (Fig 7).

Oxygen consumption of the wild type and catalase-peroxidase HPI-deficient mutant

T_{50}, which represents the time after inoculation for the medium oxygen concentration to fall to 50%, was three hours for the wild type and six hours for the mutant (Fig 8). Thus, the respiration of the mutant strain is significantly less than that of the wild type. This may indicate an unknown role for respiration in *Vitreoscilla*. The oxygen concentration dropped to 0% in early-exponential phase for both wild type and the mutant strains. The growth of the mutant was confined under oxygen limiting condition (Fig 5 and Fig 8).

Hydrogen peroxide formation during NADH oxidation

The rate of NADH oxidation was monitored at 340 nm for both strains. The NADH oxidation rate of the catalase-peroxidase HPI-deficient mutant was fifth that of the wild type (Fig 9). This result also suggests that the mutant has some deficiency in its respiratory system. The formation of hydrogen peroxide during the oxidation of NADH by the cell extracts was demonstrated fluorometrically. Although the catalase activity of the mutant was 8 fold higher than that of the wild type, more hydrogen peroxide accumulated in the mutant than in the wild type during the oxidation of NADH (Fig 9).

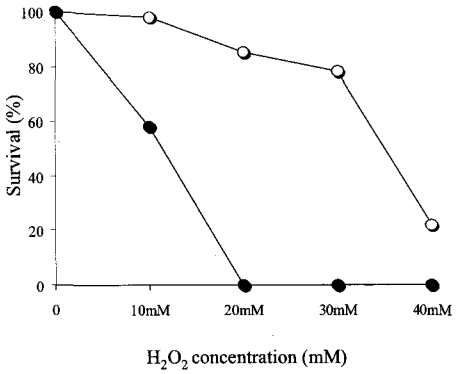

Fig 7. Sensitivity of wild type and peroxidase deficient mutant to high concentration of H_2O_2. Exponentially growing cells were resuspended in 0.1 M potassium phosphate buffered 2 % glucose solution, pH 7.8. H_2O_2 was added to 5 ml cultures with various concentrations for 30 min. 100 μl cells were diluted in 2 % glucose solution and plated onto PYA media. Colonies were counted after 3-5 days at 20 ℃. ○, wild type; ●, mutant.

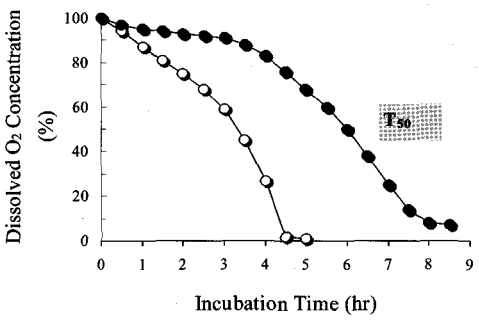

Fig 8. The oxygen consumption of wild type and peroxidase deficient mutant of *Vitreoscilla*. Dissolved oxygen concentration was monitored with a DO sensor during growth in a fermentor. ○, wild type; ●, mutant.

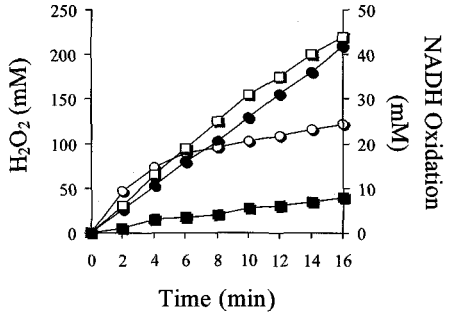

Fig 9. Hydrogen peroxide accumulation during the oxidation of NADH by cell free extracts of wild type and peroxidase-deficient mutant of *Vitreoscilla*. The amount of oxidized NADH in the presence of 100㎕ of cell free extracts was determined spectrophotometrically at 340㎚ as described in Materials and Methods. □, wild type; ■, mutant. Hydrogen peroxide cocncentration was detected fluorometrically by the addition of the cell free extract to 3㎖ of the assay medium and by measuring the increase in fluorescence at 525㎚ using an excitation wavelength of 503㎚ as described in Materials and Methods. ○, wild type; ●, mutant.

Discussion

The increase in peroxidase in *E coli* during growth into stationary phase is the result of induction by H_2O_2 generated by enzymes involved in the metabolism of some carbon sources [3]. Catalase activity in *E coli* is also maximally synthesized as cells enter the stationary phase of growth [3, 4], but the mechanism involved in inducing the synthesis of the enzyme remains unknown. Based on the analyses of the activities of catalase and peroxidase on polyacrylamide gels, the enzyme deficient in the *Vitreoscilla* mutant preliminarily identified as peroxidase part of HPI. However, further biochemical studies are necessary to confirm this preliminary assignment.

The increase of the activities of both peroxidase and catalase in wild type *Vitreoscilla* during the growth cycle might actually be due to the availability of limited oxygen, which would be able to resist *Vitreoscilla* to the increased oxidative stress. It appears to be similar to the heme content of *Vitreoscilla*. It is known that the heme content of *Vitreoscilla* is dependent on the growth phase due primarily to the induction of VtHb synthesis when oxygen becomes limiting [22]. *Vitreoscilla* catalase-peroxidase HPI also increases during this induction such that the ratio of VtHb/catalase stays constant [12]. It was hypothesized that this enzyme protected the cells from H_2O_2 produced by the VtHb under these conditions. The results of this paper, especially the slower growth of the mutant, support this hypothesis.

It has been well known that the rate of exponential growth is influenced by environmental conditions as well as by characteristics of the organism itself. In the stationary phase there is no net

increase or decrease in cell number. However, although no growth occurs in the stationary, many cell functions including energy metabolism and some biosynthetic processes may continue. Thus *Vitreoscilla*, an obligate aerobe, requires continuous supply of oxygen molecule for good growth. VtHb accomplishs this function that is capture oxygen and feed it to the terminal oxidase under an oxygen limiting condition. *Vitreoscilla* hemoglobin enables *Vitreoscilla* to survive under oxygen limiting condition and should be controlled jointly by availability of oxygen. The increased cell density of *Vitreoscilla* increases competition for molecular oxygen and could stimulate VtHb biosynthesis [22]. The decreased oxygen availability and the confined growth of the mutant show that the VtHb content of the mutant would be lower than that of wild type. Consistent with this surmise, the heme quantity of the mutant strain is significantly less than that of the wild type. Practically, the culture media of the mutant show less red than that of wild type at stationary phase. The result is an indirect evidence for a correlation between the synthesis of the HPI and components for the respiratory chain in *Vitreoscilla*.

The cell extract will contain VtHb, NADH-metVtHb reductase, peroxidase, and catalase in addition to respiratory activity (NADH oxidase). The NADH-metVtHb reductase catalyzes the reduction of metVtHb by the oxidation of NADH to keep the hemoglobin in the physiologically active form [23]. Peroxidase is also known to oxidize NADH so both enzymes will contribute to the overall NADH oxidation rate. Therefore, the high NADH oxidation of wild type would be due to the peroxidase activity as well as the high NADH-metVtHb reductase activity. These results suggest that *Vitreoscilla* peroxidase may have a metabolic role as well as an anti-oxidative one, and it may participate in biosynthetic or degradative pathways, as was shown for some peroxidases [2, 24]. This includes lower oxygen uptake, decreased NADH oxidation, and increased accumulation of H_2O_2 during this oxidation by the peroxidase deficient mutant. The latter, but not the former two observations are explainable by the peroxidase deficiency. All of these factors may contribute to the impaired growth of the mutant compared to the wild type. The relatively low NADH oxidation rate in the mutant suggests that the mutant has a decreased metabolic capacity and that the peroxidase-deficiency results in low respiratory activity.

The formation of H_2O_2 generated from NADH cytochrome *o* oxidation in wild type would be more than that of the mutant because the wild type has the higher oxidation rate of NADH and oxygen availability than the mutant. Oxygen derivatives derived from respiration is potentially capable of attacking any of the organic substances including DNA in cells. So, the derivatives must be decomposed to enable cells to survive. Therefore, enzymes that destroy certain oxygen products would be an important factor essential for a growth. The growth of the mutant was also confined initially. We know that the peroxidase activity of wild type has an effect on the oxidation of NADH and the decomposition of the

H_2O_2. Therefore, the accumulated H_2O_2 in the mutant should be caused by the peroxidase deficiency. Interestingly, the catalse-peroxidase HPI deficient mutant shows increased catalase activity compared to the wild type, suggesting a compensatory effect for protection from H_2O_2 toxicity. Also, it is possible that catalase in the mutant is induced by high accumulation of hydrogen peroxide. However, it did not appear to be complete for a defect in peroxidase activity since the mutant has the higher accumulation of endogenous H_2O_2 and was sensitive to killing by H_2O_2. The wild type consumes both endogenous and exogenous H_2O_2 respond to high concentrations and this result contrast well with the mutant. Thus, the catalse-peroxidase HPI, especially peroxidase part, seems like a prevalent enzyme that participate in getting rid of toxic concentrations of H_2O_2 in *Vitreoscilla.* The catalase-peroxidase HPI may be also expressed more constitutively at a high level H_2O_2, enough for H_2O_2 detoxification during respiratory metabolism by VtHb and exogenous challenge.

It is generally accepted that the main function of hydroperoxidases is to scavenge hydrogen peroxide generated under oxidative and other stresses, such as heat shock and nutrient deprivation [25]. However, the molecular mechanisms that regulate the resistance to hydrogen peroxide have not been characterized in *Vitreoscilla.* Thus it is not clear whether the catalase activity of the mutant was revived from another catalase or the factor that relative to DNA damage. However, it is probable that the isolated mutant had been affected in regulatory mechanism involved in induced resistance to hydrogen peroxide. The characterization of this gene of mutant would provide important information as to the nature of the oxidative stress system of this bacterium.

The mutant described here will be a valuable resource in the identification of the mechanisms of regulation of antioxidative enzymes in *Vitreoscilla.* For example, we hope to use the mutant as a means of identifying the roles and interactions of the antioxidative enzymes, catalase, SOD, and peroxidase involved in protection against oxidative stress.

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