A Mechanism of Resistance to Hydrogen Peroxide in *Vibrio rumoiensis* S-1

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A possible mechanism of resistance to hydrogen peroxide (H$_2$O$_2$) in *Vibrio rumoiensis*, isolated from the H$_2$O$_2$-rich drain pool of a fish processing plant, was examined. When *V. rumoiensis* cells were inoculated into medium containing either 5 mM or no H$_2$O$_2$, they grew in similar manners. A spontaneous mutant strain, S-4, derived from *V. rumoiensis* lacking catalase activity did not grow at all in the presence of 5 mM H$_2$O$_2$. These results suggest that catalase is inevitably involved in the resistance and survival of *V. rumoiensis* in the presence of H$_2$O$_2$. Catalase activity was constitutively present in *V. rumoiensis* cells grown in the absence of H$_2$O$_2$, and its occurrence was dependent on the age of the cells, a characteristic which is observed for the HP II-type catalase of *Escherichia coli*. The presence of the HP II-type catalase in *V. rumoiensis* cells was evidenced by partial sequencing of the gene encoding the HP II-type catalase from this organism. A notable difference between *V. rumoiensis* and *E. coli* is that catalase is accumulated at very high levels (~2% of the total soluble proteins) in *V. rumoiensis*, in contrast to the case for *E. coli*. When *V. rumoiensis* cells which had been exposed to 5 mM H$_2$O$_2$ were centrifuged, most intracellular proteins, including catalase, were recovered in the medium. On the other hand, when *V. rumoiensis* cells were grown on plates containing various concentrations of H$_2$O$_2$, individual cells had a colony-forming ability inferior to those of *E. coli*, *Bacillus subtilis*, and *Vibrio parahaemolyticus*. Thus, it is suggested that when *V. rumoiensis* cells are exposed to high concentrations of H$_2$O$_2$, most cells will immediately be broken by H$_2$O$_2$. In addition, the cells which have had little or no damage will start to grow in a medium where almost all H$_2$O$_2$ has been decomposed by the catalase released from broken cells.

There are wide varieties of microorganisms which can live under such unusual conditions as low and high pH, low and high temperatures, high salinity, and high hypodry pressure. These organisms possess specific mechanisms to survive in such environments (11). However, there have been no reports on organisms which inhabit environments with hyperoxidative stress caused by factors such as high concentrations of H$_2$O$_2$.

In the course of normal metabolism of O$_2$ in aerobically growing cells, hydrogen peroxide (H$_2$O$_2$), which is toxic to cells, is mostly generated in the respiratory chain through the incomplete reduction of O$_2$. To counteract the potential hazards of intracellular H$_2$O$_2$, which freely diffuses into cells and harms cell membranes, proteins, or DNAs, organisms possess catalase, a high-molecular-weight heme-containing protein whose primary function is to destroy H$_2$O$_2$, leaving O$_2$ and water as by-products. Challenge by reactive oxygen species occurs from extracellular sources as well as from normal aerobic metabolism. Soil pseudomonads may be exposed to H$_2$O$_2$ as they contact plant roots, and certain soil fungi also produce H$_2$O$_2$ as a mechanism to compete against other organisms. Thus, catalase may be important in overcoming such a challenge.

Recently, Yumoto et al. (27) isolated a bacterium which can grow in the presence of relatively high levels of H$_2$O$_2$ from the drain pool of a fish processing factory. This bacterium is regarded as resistant to such hyperoxidative conditions. The bacterium was gram negative, rod shaped, oxidase positive, facultatively psychrophilic, facultatively anaerobic for both fermentative and respiratory metabolism, and sensitive to the vibriostatic compound O129 (2,4-diamino-6,7-diisopropylpteridine) (27). In the accompanying paper (28), this bacterium was identified as a new species belonging to the genus *Vibrio*. *V. rumoiensis*, from its physiological and biochemical characteristics, analysis of its 16S rRNA sequence, and DNA-DNA relatedness, *V. rumoiensis* S-1 exhibited an extraordinarily high catalase activity compared with other bacteria (27, 28). The catalase activity in cell extracts of this bacterium was orders of magnitude greater than those of *Escherichia coli* and *Bacillus subtilis* (27, 28).

Although the mechanisms of adaptation to high oxidative stress by such microorganisms are still unclear, it is postulated that catalase might be involved in eliminating the toxicity of H$_2$O$_2$. In this study, to determine the possible mechanism of adaptation to hyperoxidative stress in *V. rumoiensis* S-1, the effects of H$_2$O$_2$ on the growth and structure of *V. rumoiensis* S-1 cells were compared with those on a spontaneous mutant of *V. rumoiensis* S-1 lacking catalase activity (strain S-4) and other bacterial strains with normal levels of catalase activity. In order to elucidate the molecular properties of catalase involved in the resistance and survival mechanisms in *V. rumoiensis* S-1, the induction pattern of the catalase was investigated and the partial nucleotide sequence of the catalase gene was determined.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation.** *V. rumoiensis* was isolated from the drain pool of a fish processing factory; this drain pool usually contains several hundred micromolar H$_2$O$_2$. *E. coli* XL1-Blue MRA (P2) was purchased from Stratagene (La Jolla, Calif.). *B. subtilis* IAM 1026 and *Vibrio parahaemolyticus* JCM 2147 were obtained from type culture collections. *V. rumoiensis* cells were cul-
tured in PYS medium (pH 7.5) containing 1% polypeptide, 0.5% yeast extract, and 1% NaCl on a rotary shaker at 200 rpm at 27°C until an absorbance of 2.1 at 600 nm, which is equivalent to 1.97 × 10^8 cells per ml, was obtained. One milliliter of the inoculum was usually transferred to 50 ml of medium containing 5 mM H_2O_2. Strain S-4 was isolated as a spontaneous mutant completely lacking catalase activity during repeated cultivation of *V. rumoiensis* S-1 (29). S-4 cells were cultured as described above: *E. coli*, R. subtilis, and *V. parahaemolyticus* cells were cultured in Luria-Bertani (LB) medium (pH 7.0) containing 0.5% yeast extract, 1% tryptone, and 1% NaCl on a rotary shaker at 200 rpm at 37°C overnight. When *V. rumoiensis* S-1 cells and strain S-4 cells were cultured on a plate containing H_2O_2, both strains were grown in PYS medium on a rotary shaker at 200 rpm at 27°C overnight. Cells harvested at the stationary phase were suspended in sterilized LB medium or PYS medium at a concentration of about 2 × 10^8 cells/ml. Portions (100 μl) of the cell suspensions were spread on plates containing various H_2O_2 concentrations from 0 to 400 μM. Hydrogen peroxide-containing plates were prepared by adding a 1 M H_2O_2 solution to the agar plate (25 ml) so as to give the expected concentration.

Preparation of cell extracts and enzyme assays. Cells of *V. rumoiensis* S-1 and other strains were harvested by centrifugation at 10,000 × g for 10 min at 4°C and washed three times with 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM MgSO_4. The cells, suspended in 1 ml of the same buffer, were sonicated in an ice-water bath for 5 min with a ultrasonic disruptor (type UD-20; Tomy, Tokyo, Japan) at an output setting of 5 and a duty setting of 50. The sonicates were then centrifuged at 10,000 × g for 10 min at 4°C to remove the cell debris and unbroken cells. The supernatants were used as cell extracts.

Catalase activity was assayed in 100 mM potassium phosphate buffer (pH 7.0) containing 30 μl of 1 M TiSO_4 and 0.15 ml of 1 M H_2O_2, and the amount of the enzyme produced in a final volume of 1 ml at 20°C. The reduction of the amounts of H_2O_2 was monitored by measuring the optical density of the reaction mixture at 240 nm with a spectrophotometer (type U-3210; Hitachi, Tokyo, Japan) (27). One unit (23) of catalase was defined as the amount of enzyme capable of catalyzing the reduction of 1 μg of H_2O_2 degraded per min. Protein concentrations were measured by the method of Bradford (3) with the Bio-Rad (Hercules, Calif.) protein assay kit II and bovine serum albumin as a standard.

The concentration of H_2O_2 was measured by the titanium method as follows: 0.8 ml of the sample solution was added to a solution containing 0.25 ml of 20% H_2SO_4 and 0.15 ml of 1 M TiSO_4, and its absorbance at 408 nm was measured.

Cloning of the catalase gene from *V. rumoiensis* S-1. (a) Probe preparation. (a) Detection of the N-terminal amino acid sequence of the *V. rumoiensis* S-1 catalase. *V. rumoiensis* S-1 catalase was purified with a DEAE-Sepharose CL-6B anion-exchange column (Pharmacia, Uppsala, Sweden) and a Sephacryl S-300 gel filtration column (Pharmacia, Uppsala, Sweden) according to the method of Yumoto et al. (29) (details of enzyme purification and characterization will be published elsewhere). To remove minor contaminants, 1.6 mg of the purified *V. rumoiensis* S-1 catalase was subjected to native polyacrylamide gel electrophoresis with a 5 to 20% gradient gel (type NP-20; Altto, Tokyo, Japan) by the standard method of Laemmli (16) and then blotted to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) (25). The membrane was stained with 3% trichloroacetic acid containing 0.1% Ponceau S (Wako, Tokyo, Japan). The catalase band was cut out from the membrane and subjected to analysis with a protein autosequencer (Applied Biosystems 491; Perkin-Elmer, Norwalk, Conn.).

(b) Peptide mapping of *V. rumoiensis* S-1 catalase. Fifty micrograms of the purified *V. rumoiensis* S-1 catalase was digested with 0.5 μg of Lysyl-endopeptidase (Wako) in 1 ml of 10 mM Tris-HCl buffer (pH 9.0) at 37°C overnight. To inactivate the peptidase, 0.6 ml of 10 mM guanidine-HCl was added to the reaction mixture, and the mixture was incubated in a final volume of 5 ml and incubated for more than 30 min at room temperature, and then the mixture was loaded onto a TSK gel ODS-80Ts column (0.46 by 15 cm; Tosoh, Tokyo, Japan) equipped with a reversed-phase high-pressure liquid chromatography system. Peptides were eluted with a linear gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The eluates were monitored by measuring the absorbance at 214 nm. Each peptide fragment was lyophilized, diluted in trifluoroacetic acid, and then loaded onto the protein autosequencer (Applied Biosystems 491).

(c) Genomic PCR amplification. Chromosomal DNA was isolated from *V. rumoiensis* S-1 cells by using the isoPLANT kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Four oligonucleotides, P-1, P-1', P-3, and P-4', were generated according to the N-terminal amino acid sequence and the amino acid sequences of peptide fragments P-1, P-3, and P-4, respectively. Genomic PCR amplification was carried out with these primers and *V. rumoiensis* S-1 genomic DNA as the template. Genomic PCR was carried out as follows. Five microilters of 10% PCR buffer (Takara, Tokyo, Japan), 5 μl of a 2.5 mM deoxyadenosine triphosphate mixture (Takara), 0.7 μg of genomic DNA, 1 pmol of each oligonucleotide primer, 1.25 U of Taq DNA polymerase (Takara), and double-distilled water were mixed in a final volume of 50 μl. The resulting mixture was subjected to PCR amplification: 93°C for 1 min, 72°C for 1 min for one cycle; 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min for 30 cycles, and then 94°C for 1 min, 50°C for 2 min, and 72°C for 5 min for one cycle. The nucleotide sequence of each PCR product was determined (see below). Among the 327 bp fragment, a DNA fragment (gt11A1) of 327 bp was excised out from the gel and subjected to analysis with a protein autosequencer (Applied Biosystems 491).

RESULTS

Effects of H_2O_2 on the growth of *V. rumoiensis* S-1 and strain S-4 cells. To investigate the effects of H_2O_2 on growth, *V. rumoiensis* was cultured either in the presence or in the absence of 5 mM H_2O_2. As shown in Fig. 1A, *V. rumoiensis* S-1 cells grew in the presence of 5 mM H_2O_2, and H_2O_2 concentration at which *E. coli* cells cannot grow (6), and the growth reached the stationary phase 16 h after the inoculation. The growth profile of *V. rumoiensis* S-1 in the absence of H_2O_2 was almost the same as that in the presence of 5 mM H_2O_2 (Fig. 1C). When *V. rumoiensis* S-1 was grown in the presence of 5 mM H_2O_2, the concentration of H_2O_2 in the medium decreased immediately after the inoculation of cells, and H_2O_2 was scarcely detected in the medium 10 min after the inoculation (Fig. 1A). As shown in Fig. 1D, H_2O_2 spontaneously decomposed to half of the original level within 24 h by shaking without inoculation.

Strain S-4 was obtained as a spontaneous mutant of *V. rumoiensis* S-1 lacking catalase activity (29). As shown in Fig. 1B, strain S-4 cells could not grow in medium containing 5 mM H_2O_2, and the H_2O_2 in the medium was decomposed only spontaneously. *V. rumoiensis* S-1 and strain S-4 cells showed almost the same growth profile in the absence of H_2O_2 (Fig. 1C).

Destruction of *V. rumoiensis* S-1 cells and subsequent release of catalase. *V. rumoiensis* S-1 cells accumulate catalase inside the cells, and the catalase is never secreted from the cells (27). To investigate the effects of H_2O_2 on the cell structure of *V. rumoiensis* S-1, cells which had been exposed to 5 mM H_2O_2 were centrifuged, and the localization of the catalase activity was examined. As shown in Fig. 2, most proteins as well as the catalase activity were recovered in the medium after the addition of 5 mM H_2O_2, whereas there was no catalase activity in the medium before the addition of H_2O_2.
inoculum (data not shown), H₂O₂ must be decomposed very quickly by released catalase from disrupted cells.

Effects of the inoculum size of *V. rumoiensis* S-1 on its growth with H₂O₂. When cells of *V. rumoiensis* S-1 were grown in the presence or absence of H₂O₂, their growth profiles were almost the same in some cases (Fig. 1A and 1C), but in other cases a lag time was observed when cells were grown in the presence of H₂O₂ (data not shown). It was likely that a smaller inoculum size resulted in a much longer lag time in the presence of H₂O₂ than in its absence. Since the difference in the length of the lag time of growth is considered to be caused by the size of the inoculum, the growth of *V. rumoiensis* S-1 in 5 mM H₂O₂ was monitored after inoculation with the various inoculum sizes. As shown in Fig. 3A, a smaller inoculum size brought about a longer lag time. When 1,000-μl (1.97 × 10⁸ cells) and 500-μl (9.85 × 10⁷ cells) portions of the preculture were inoculated into 50 ml of medium, the lag times were 4 and 8 h, respectively. However, no growth was observed when an inoculum of less than 1 μl (1.97 × 10⁵ cells) was used. By contrast, cells grew in medium containing no H₂O₂ irrespective of the inoculum size (Fig. 3B).

Effects of H₂O₂ concentration on colony formation by *V. rumoiensis* S-1, strain S-4, and other bacterial strains. It was considered that the resistance of *V. rumoiensis* S-1 to H₂O₂ is primarily due to the endogenously accumulated catalase in cells and/or the released catalase from H₂O₂-disrupted cells when it was grown in a liquid medium. Thus, the colony-forming ability of individual cells of *V. rumoiensis* S-1 was compared with that for strain S-4 and other bacterial strains on agar plates containing various concentrations of H₂O₂. As shown in Table 1, the colony-forming ability of *V. rumoiensis* S-1 was drastically decreased in the presence of H₂O₂. At 10

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**FIG. 1.** Effects of 5 mM H₂O₂ on growth of *V. rumoiensis* S-1 and strain S-4. (A) Growth of *V. rumoiensis* S-1 in 5 mM H₂O₂ (circles) and concentration of H₂O₂ in the medium (triangles). (B) Growth of strain S-4 in 5 mM H₂O₂ (circles) and concentration of H₂O₂ (triangles). (C) Growth of *V. rumoiensis* S-1 (squares) and strain S-4 (circles) in medium containing no H₂O₂. (D) Concentration of H₂O₂ in medium containing 5 mM H₂O₂ with no inoculation.

**FIG. 2.** Changes in the amounts of proteins and catalase activity recovered from cells and culture medium of *V. rumoiensis* S-1 grown in the presence of 5 mM H₂O₂. (A) Recovered protein in precipitates (solid bars) and in medium (open bars). (B) Recovered catalase activity in precipitates (solid bars) and in medium (open bar).
MH_2O_2, the colony-forming ability decreased to 68% of the original in *V. rumoiensis* S-1, whereas 102, 81, and 100% of the original abilities were maintained in *V. parahaemolyticus*, *E. coli*, and *B. subtilis*, respectively. The numbers of colonies on a plate containing 100 μM H_2O_2 were 35.6, 86.6, 86.1, and 98.5% of the original numbers for *V. rumoiensis* S-1, *V. parahaemolyticus*, *E. coli*, and *B. subtilis*, respectively. The colony-forming ability of S-4 was very inferior to that of *V. rumoiensis* S-1. At 100 μM H_2O_2, strain S-4 completely lost its colony-forming ability (Table 1).

**Effects of time of growth on the catalase activity in *V. rumoiensis* S-1.** The catalase activity in cell extracts of *V. rumoiensis* S-1 grown in the absence of H_2O_2 was assayed. As shown in Fig. 4, the catalase activity of 19,655 units/mg of protein at zero time markedly decreased to 7,627 units/mg of protein at 8 h. The catalase activity then increased and reached its maximum at 25 h (Fig. 4).

**Predicted amino acid sequence of the catalase from *V. rumoiensis* S-1.** According to the BLAST homology search, the predicted amino acid sequence deduced from the gt11A1 nucleotide sequence showed high homology with the corresponding sequences of *E. coli* HP II-type catalases and in particular with that of the *H. influenzae* HktE catalase (Fig. 5). Although clone gt11A1 seemed to contain only a partial sequence of the *V. rumoiensis* S-1 catalase gene, the predicted amino acid sequence included the same amino acid sequences determined by partial peptide sequencing.

**DISCUSSION**

Catalases catalyzing the conversion of H_2O_2 to water and O_2 are found in virtually all aerobic organisms and even in anaerobic organisms. Catalases are thought to be involved in detoxifying H_2O_2 that has arisen in the cells and also exogenously added H_2O_2 (1).

As described by Yumoto et al. (27, 28), *V. rumoiensis* S-1 can survive in environments with high levels of H_2O_2, and it accumulates 100 μM H_2O_2, strain S-4 completely lost its colony-forming ability (Table 1).

**TABLE 1. Effects of H_2O_2 concentration on bacterial colony-forming ability**

<table>
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<th>Species or strain</th>
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<th>10</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. rumoiensis</em></td>
<td>315 ± 6 (100)</td>
<td>215 ± 54 (68.3)</td>
<td>112 ± 12 (35.6)</td>
<td>94 ± 5 (29.8)</td>
<td>10 ± 2 (3.2)</td>
</tr>
<tr>
<td>Strain S-4</td>
<td>179 ± 134 (100)</td>
<td>75 ± 57 (41.9)</td>
<td>4 ± 3 (2.2)</td>
<td>0 ± 0 (0)</td>
<td>0 ± 0 (0)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>112 ± 34 (100)</td>
<td>114 ± 0 (101.8)</td>
<td>97 ± 8 (86.6)</td>
<td>21 ± 1 (18.8)</td>
<td>0 ± 0 (0.0)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>230 ± 28 (100)</td>
<td>186 ± 18 (80.9)</td>
<td>198 ± 17 (86.1)</td>
<td>149 ± 6 (64.8)</td>
<td>17 ± 5 (7.4)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>206 ± 5 (100)</td>
<td>206 ± 7 (100)</td>
<td>203 ± 18 (98.5)</td>
<td>184 ± 6 (89.3)</td>
<td>152 ± 5 (73.8)</td>
</tr>
</tbody>
</table>

* Results are means and standard deviations.
mulates a large amount of catalase (levels of 2% of the total soluble proteins inside the cell). As shown in Fig. 1A, when *V. rumoiensis* S-1 was cultured in a medium where 5 mM H$_2$O$_2$ was present, almost all H$_2$O$_2$ in the medium was quickly decomposed after the inoculation of the cells, and they grew quite normally. On the other hand, strain S-4, a spontaneous mutant of *V. rumoiensis* S-1 lacking catalase activity, showed no growth in the presence of 5 mM H$_2$O$_2$, and H$_2$O$_2$ in the medium was not decomposed enzymatically (Fig. 1B), suggesting that in this bacterium catalase could play an essential role in decomposing exogenously added H$_2$O$_2$.

Since H$_2$O$_2$ freely diffuses into cells, it was predicted that exogenously added H$_2$O$_2$ could be decomposed by catalase inside the cells and/or by catalase excreted from cells before it diffused into other cells. Although a very small amount of catalase was detected in the culture medium of *V. rumoiensis* S-1 cells at the stationary phase (28), *V. rumoiensis* S-1 cells were thought not to excrete catalases under conditions without...
as shown in Fig. 2, most proteins and the catalase activity were recovered in the medium 10 min after the addition of H₂O₂, suggesting that V. rumoiensis cells were destroyed by H₂O₂ and catalase was released together with other proteins into the medium. Released catalase could serve to decompose H₂O₂ in the medium (Fig. 1A). Thus, it is suggested that intact cells and/or less-damaged cells of V. rumoiensis remaining after exposure to high concentrations of H₂O₂ would begin to grow in this medium, where no H₂O₂ exists. This scenario implies that individual cells of V. rumoiensis might be rather labile to H₂O₂.

As shown in Fig. 3, a smaller inoculum size brought about a longer lag time in the growth of V. rumoiensis S-1. This is a very usual and expected phenomenon in bacterial growth. However, when volumes of less than 1 μl (1.95 \times 10^5 cells) of the preculture were inoculated into 50 ml of medium containing 5 mM H₂O₂, V. rumoiensis S-1 cells exhibited no growth, while the same number of cells of this bacterium did grow, with a lag time of 8 h, in medium containing no H₂O₂. These results suggest that individual cells of V. rumoiensis S-1 might not have a notable resistance to H₂O₂ and that they could grow only with low H₂O₂ concentrations induced by H₂O₂ decomposition caused by released catalase from cells destroyed by H₂O₂. This speculation was supported by the findings that individual cells of V. rumoiensis S-1 are significantly inferior in their colony-forming ability to other bacterial strains (see below and Table 1), for which levels of catalase activity were 2 orders of magnitude lower than that for V. rumoiensis S-1.

To exclude the involvement of released catalase from destroyed cells in decomposing exogenous H₂O₂, V. rumoiensis S-1 cells were grown on agar plates containing various concentrations of H₂O₂. Thus, the resistance of individual cells to H₂O₂ could be examined. V. rumoiensis S-1 cells were less resistant to H₂O₂ than cells of E. coli, B. subtilis, and V. parahaemolyticus (Table 1). Since intracellular levels of catalase in E. coli, B. subtilis, and V. parahaemolyticus were in the range of 10 to 100 U/mg of protein (27), the colony-forming ability on an agar plate would not be associated with the catalase. However, the occurrence of high levels of intracellular catalase seemed to be involved in the colony-forming ability of V. rumoiensis S-1 compared to that of strain S-4 (Table 1). Although the mechanism of disruption of the cell structure is unknown, the bleb structure of the V. rumoiensis S-1 cell envelope (28), which enlarges the surface area of the cell, might be related to the lability of this organism to H₂O₂. It is likely that V. rumoiensis S-1 and strain S-4 have common structures labile to H₂O₂ and that the lability of strain S-4 to H₂O₂ reflects the inherent lability of the cell structure of V. rumoiensis. Some kind of structure unstable to H₂O₂ and very high-level accumulations of catalase would enable V. rumoiensis S-1 cells to grow in the presence of high concentrations of H₂O₂. This phenomenon might be designated a self-sacrifice strategy of this bacterium for the maintenance of the species.

In E. coli cells, catalase activity fluctuates markedly depending on the phase of growth (11). For example, the HP I-type catalase, which is induced by H₂O₂, is produced during the mid-exponential phase, while the HP II-type catalase, a non-H₂O₂-inducible catalase, is produced during the late exponential to stationary phase (18). As shown in Fig. 4, the total catalase activity dropped at the mid-exponential phase of growth and rapidly increased at the stationary phase in V. rumoiensis S-1 cells grown in the absence of H₂O₂. A similar profile of change in the HP II-type catalase activity was also found in E. coli cells grown in the absence of H₂O₂ (10). In E. coli, katE, which encodes the HP II-type catalase, is expressed at high levels in the stationary phase but not in the exponential phase (17, 19) and is controlled by the stationary-phase sigma factor σ₃, encoded by rpoS (22). Since V. rumoiensis S-1 produced only one catalase species in the absence of H₂O₂ (28), it is suggested that the catalase of V. rumoiensis S-1 could be synthesized in a manner similar to that for the HP II-type catalase of E. coli. This suggestion is supported by the fact that V. rumoiensis S-1 has a catalase gene whose predicted amino acid sequence showed high homology with that of the HP II-type catalase of E. coli and, in particular, with that of HktE of H. influenzae (Fig. 5), although a full-length of the catalase gene has not yet been obtained. Whether an H₂O₂-inducible catalase is present in V. rumoiensis S-1 has never become apparent, because the growth of this bacterium in the presence of H₂O₂ cannot be examined due to the immediate decomposition of H₂O₂ in the medium (Fig. 1A).

As described by Yumoto et al. (27), the catalase activity in cell extracts of V. rumoiensis S-1 was 2 orders of magnitude higher than those in E. coli, B. subtilis, and V. parahaemolyticus. These findings suggested that V. rumoiensis S-1 might have multiple copies of the catalase gene and/or a very specific catalytic motif which would enable the catalase to have a highly specific activity. However, V. rumoiensis S-1 had a single copy of the catalase gene on its chromosomal DNA (12). Thus, it is speculated that V. rumoiensis S-1 would have one or more mechanisms for hyperexpression of the catalase gene.

Despite repeated attempts, we have never cloned the complete catalase gene from V. rumoiensis S-1. Only clones lacking 150 to 190 bp at the 5′ region were obtained (Fig. 5), and in some clones, a recombination might have occurred at the same point in the 5′ region of the catalase gene (12). Such an easy rearrangement of the catalase gene in V. rumoiensis S-1 might be related to the occurrence of mutant lacking catalase, like strain S-4.

In conclusion, V. rumoiensis S-1 produced only one catalase species, belonging to the family of the HP II-type catalase of E. coli. The most remarkable difference between the catalase of V. rumoiensis S-1 and other HP II-type catalases is that the catalase in V. rumoiensis S-1 can be synthesized at quite high levels by unknown mechanisms. When V. rumoiensis S-1 cells are exposed to high concentrations of H₂O₂, cells encountered by H₂O₂ should be broken and, as a result, released catalase should immediately decompose the exogenous H₂O₂. Remaining intact cells and/or less-damaged cells of V. rumoiensis S-1 would begin to grow in this medium, where no H₂O₂ exists. V. rumoiensis S-1 cells apparently are resistant to H₂O₂. However, individual cells of this bacterium are rather labile to H₂O₂. Thus, the resistance of V. rumoiensis S-1 to H₂O₂ and its survival in H₂O₂ are attributable to high-level accumulation of the intracellular catalase and to the H₂O₂-labile cell structure of this bacterium.

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**REFERENCES**

MECHANISM OF *V. RUMOIENSIS* RESISTANCE TO \( \text{H}_2\text{O}_2 \)