Biochemistry (Moscow), Vol. 70, No. 12, 2005, pp. 1369-1376. Translated from Biokhimiya, Vol. 70, No. 12, 2005, pp. 1664-1672.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-154, October 23, 2005.

Antioxidant Status, α-Amylase Production, Growth, and Survival of Hemoglobin Bearing *Escherichia coli* Exposed to Hypochlorous Acid[1]

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Received June 28, 2005
Revision received August 23, 2005

Abstract—In the present work, two matched strains of *E. coli* that bear a recombinant R-amylase gene (MK57) or the R-amylase gene and *vgb* (MK79-hemoglobin expressing strain) were exposed to HOCl. In these cells, glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), α-amylase production, growth and lethality were assessed in the presence and absence of HOCl. It was observed that the hemoglobin makes cells highly susceptible to killing by HOCl. The maximum survival for both strains was with stationary phase cells at any concentration of HOCl. Both strains grown in the presence of 0.0125-0.075 mg/liter HOCl showed a substantial increase in SOD activity and GSH level, with MK79 being the most increased strain in this respect, while the level of CAT activity was decreased in a dose depended manner. Growth of MK57 and MK79 strains decreased as HOCl concentration increased. However, HOCl at concentration above zero enhanced α-amylase production (about 2-fold) in both MK79 and MK57. Furthermore, total amylase production (at all HOCl concentrations) by MK79 was always greater than that by MK57. The results indicate that except for survival, the hemoglobin helps cells to grow better and produces more recombinant products and activates general defense systems more in response to oxidative stress under oxidative condition when compared with the non-hemoglobin-containing counterpart.

Key words: *Escherichia coli*, *Vitreoscilla* hemoglobin, hypochlorous acid, oxidative stress, SOD, catalase, glutathione, α-amylase

The function of hemoglobins in higher organisms as facilitators of oxygen transport and storage are well known, but the biological function(s) of microbial hemoglobins is (are) still not understood precisely. From an evolutionary point of view, microbial hemoglobin (VHb) has been proposed to function in "detoxification", in contrast to a primarily "respiration" function of hemoglobin in higher organisms [1]. Although the aerobic metabolism of bacteria (as well as other organisms) optimally results in the near simultaneous four-electron reduction of O_2 to H_2O, a variable percentage of O_2 reduction occurs initially via either one-electron reduction of O_2 to superoxide ($O_2^{\cdot-}$) or divalent reduction to H_2O_2 [2]. At physiological pH, $O_2^{\cdot-}$ rapidly reacts with itself (dismutates) to form H_2O_2 [2]. To prevent the harmful effects of reactive oxygen species (ROS), cells including *Escherichia coli* contain superoxide dismutase (SOD) and catalase (CAT) as means of eliminating $O_2^{\cdot-}$ and H_2O_2, respectively [3, 4], and general defense systems activated in response to oxidative stress.

Hypochlorous acid (HOCl), the most widely used disinfectant worldwide, is generally considered to be a highly destructive, nonselective oxidant which reacts avidly with a variety of subcellular compounds and affects metabolic processes [5, 6], and mechanisms of its bactericidal activity are still poorly known. Recently, Candeias et al. [7] showed that *in vitro*, hypochlorous acid can also directly generate hydroxyl radicals via a Fenton-type reaction [8]: $HOCl + Fe(II) \rightarrow {}^{\cdot}OH + Cl^- + Fe(III)$, and that in the presence of superoxide anions, hypochlorous acid leads to production of hydroxyl radicals in the reaction $HOCl + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + Cl^- + O_2$ [9], similar to the Haber–Weiss reaction, $H_2O_2 + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + HO^- + O_2$ [10-13].

Vitreoscilla, a filamentous, gliding, gram-negative, strict aerobic bacterium found in oxygen-poor environments [14], produces VHb naturally [15]. The putative function of VHb is to trap oxygen from the air–medium

[1] This work was presented as a poster at the HUPO 4th Annual World Congress, August 28 to September 1, 2005, Munich, Germany.

0006-2979/05/7012-1369

interfaces, facilitating delivery of the gas to cells, and feed it to the membrane terminal oxidases; the kinetic constants for oxygen binding to VHb [15] and nitrite [16] as well as menadione studies [17] reflect this function. The effects of VHb on recombinant cells are supported by related studies, which demonstrate that VHb not only often enhances cell division and recombinant protein, but also stimulates production of metabolite in bacteria and fungi [16-24]. Furthermore, VHb and other unicellular hemoglobins have a greater chemical reactivity than their homologs in multicellular organisms, and they may be associated with protection of cells against oxidative stress [25].

However, there have been no corresponding studies on antioxidant enzyme systems during culture of *Escherichia coli* cells with VHb exposed to HOCl.

Thus, the present study, for the first time, has evaluated not only the overall beneficial effects of VHb on growth/survival and amylase production but has also assessed changes in the activities of enzyme systems involved in oxygen detoxification in *E. coli* MK79 and MK57 strains exposed to HOCl.

MATERIALS AND METHODS

Bacterial strains. All strains used are *E. coli* JM103 derivatives [26]. They were transformed with plasmid pMK57 to yield strain MK57 or plasmid pMK79 to produce strain MK79 [21]. Plasmid pMK57 [21] was created by cloning the 3.0 kb of *B. stearothermophilus* DNA containing the R-amylase gene [27] cloned into pUC8 [26]. Plasmid pMK79 was constructed by inserting a 2.3 kb *Vitreoscilla* DNA fragment containing the 0.6 kb *vgb* gene, as well as the 1.7 kb C-terminal coding region of *uvrA*, into pMK57 [21].

Reagents and hypochlorous acid assay. All chemical used were of analytical grade. N,N-Diethyl-*p*-phenylenediamine, sodium thiosulfate, xanthine oxidase, SOD (from bovine erythrocytes), ferricytochrome *c*, and sodium hypochlorite (NaClO) were from Sigma-Aldrich (USA). Fresh solutions were prepared daily in distilled water and used immediately. NaClO solution in distilled water was stable for several hours. The concentration of hypochlorous acid was determined colorimetrically. The term hypochlorous acid (HOCl) is used throughout the paper for both undissociated acid and hypochlorite ion.

Preparation of inocula for experiments. For each individual experiment, a single colony from a plate was inoculated into 25 ml Luria–Bertani (LB) medium containing 100 μg/ml ampicillin in a 125 ml flask followed by incubation at 37°C overnight with shaking at 175 rpm. The latter was chosen since it has been reported that shaking rate around 150 rpm was favorable for the maintenance of plasmid stability [28]. From each overnight culture, 1 ml was transferred into 24 ml of fresh LB medium (with ampicillin) in a 125 ml flask and incubated under the same conditions for 3 h. Then, from each 3-h culture, 1 ml was transferred in a microfuge tube and centrifuged for 5 min. The supernatant was removed and cell pellet washed twice with ampicillin-free LB medium using 5 min centrifugation. The A_{600} of the washed cells was adjusted with LB medium to 0.50 (unless mentioned otherwise) and 1 ml was inoculated into a 24 ml fresh LB medium in a 125 ml Erlenmeyer flask and incubated under the same conditions for 6 h (log phase cells).

Hypochlorous acid challenge condition assay. At an optical density of 0.6 at 600 nm ($6 \cdot 10^9$ bacterial cells per ml), the cells were spun down by centrifugation at 10,000 rpm for 5 min at 4°C, washed twice with cold 0.05 M phosphate buffer (pH 7), and resuspended in the same volume of phosphate buffer. The number of colony-forming units (CFU) of the culture was not reduced by this washing procedure. The log phase samples were distributed in 5 ml LB medium test tubes, and fresh hypochlorous acid was added at various concentrations (0.0125-0.075 mg/liter). After 22 min incubation at 37°C in the dark with gentle shaking, hypochlorous acid was quenched by the addition of sterile sodium thiosulfate to $5 \cdot 10^{-4}$ M. Culturable bacteria were assayed by plating on LB medium plates after serial dilution in LB medium, and colonies were counted after 36 h incubation at 37°C. Then the survival rate was determined as depletion of cell viability compared to controls receiving no HOCl treatment.

For stationary phase (inocula preparation as described above), the culture was treated after 16 h of growth. After washing, the cells were resuspended in phosphate buffer at a concentration of $6 \cdot 10^9$ cells per ml and treated as described above. Plasmid stability was determined by transferring all colonies from each plate to LB-ampicillin plates.

Growth of MK79 and MK57 in the presence of HOCl. These shake flask experiments were performed in the presence of 0.0125-0.075 mg/liter HOCl at 175 rpm and 37°C in a water bath shaker. Samples were taken for analytical tests at the end of the experiment. These tests included optical density, α-amylase, SOD, and CAT activities, VHb and GSH concentrations, and plasmid stability.

Sample extraction and preparation of cell-free extracts. Antioxidant enzymes of stationary phase (16 h) were determined in growing cells in LB medium containing 100 μg/ml ampicillin and various amounts of HOCl (0.0125-0.075 mg/liter) in a 125 ml shake flask at 37°C and 175 rpm. Cells were harvested by centrifugation (10,000 rpm, 10 min), and the pellet was washed with 50 mM phosphate buffer, pH 8.0. While the cell pellet for catalase was suspended in the same buffer, the cell pellet for SOD was suspended in 50 mM phosphate buffer, pH 8.0, containing 0.1 mM EDTA. The cell pellets for

both enzymes were resuspended in ice cold medium and disrupted with an ultrasound. Extracts were centrifuged (10,000 rpm, 10 min, 4°C). One-milliliter aliquots of the supernatants were transferred to clean microfuge tubes maintained on ice. A portion (50 μl) of each supernatant was analyzed immediately for catalase activity; the remainder was stored at −70°C for subsequent analyses of SOD and catalase.

Super oxide dismutase (SOD) assay. SOD activity was assayed by determining the total amount of enzyme required to produce a 50% inhibition of cytochrome *c* reduction [29]. To a 2 ml quartz cuvette, 1.85 ml of preheated to 25°C solution A (5 ml of xanthine solution mixed with 50 ml of cytochrome *c* solution and 0.1 ml of cell extract supernatant) was added. The reaction was initiated by the addition of 50 μl of solution B containing 0.1 mM EDTA. In all cases, the potassium phosphate buffer consisted of a mixture of KH_2PO_4 and $K_2HPO_4 \cdot 3H_2O$ with the ratio adjusted to give a final pH of 8.0. The rate of reduction of cytochrome *c* was monitored at 550 nm at 25°C. Inhibition of this reduction of cytochrome *c* by SOD was used to assess enzyme activity of cell extract supernatants. One unit of SOD activity was defined as the amount of enzyme that inhibited by 50% the rate of reduction of cytochrome *c*, utilizing a standard curve performed with commercially obtained SOD.

Catalase (CAT) assay. Catalase activity was determined by monitoring the extract-dependent decomposition of H_2O_2 [30]. Eight-hundred-fifty microliters of 50 mM potassium phosphate buffer, pH 8.0, containing 25% (v/v) H_2O_2 and 0.1 ml of cell extract supernatant were made up to 1 ml with potassium phosphate buffer lacking H_2O_2 in a quartz cuvette thermostatically controlled to 25°C. After mixing, the decrease in absorbance at 240 nm was measured immediately over a 1 min period using a Techno spectrophotometer. One unit of activity was expressed as the amount of enzyme catalyzing the degradation of 1 μmol of H_2O_2 per min at 25°C.

Glutathione (GSH) concentration assay. Determination of bacterial GSH concentration was performed with a small modification colorimetrically at 412 nm on the basis of the absorbance of the reaction yield of GSH and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described [31] (a molar absorption coefficient $14{,}200 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ at 412 nm used). Thiol concentrations were expressed as micromoles (or micromolar concentrations) per 10^{10} total cells. Although this technique does not differentiating between GSH and other low-molecular-weight thiol-bearing compounds, GSH constitutes the overwhelming fraction of acid-soluble thiol in *E. coli*. Suspensions of bacteria (1 ml) were centrifuged at 10,000 rpm for 10 min at room temperature. The pellets were washed twice by resuspending them in 0.9% NaCl and centrifuging them at 10,000 rpm for 5 min at room temperature. Precipitation of bacteria was accomplished by the addition of 5 ml of 0.05 M H_2SO_4 with vigorous vortexing and sonication for 5 min in a bath at 4°C. Then, suspensions were incubated for 15 min in an ice bath and centrifuged at 10,000 rpm for 5 min at 4°C. The resulting supernatants were immediately frozen and stored in liquid nitrogen. The frozen samples were rapidly thawed in a water bath at 35°C and diluted with cold 0.1 M HCl containing 1 mM disodium EDTA.

Protein concentration was determined by the Lowry method [32].

VHb concentration of MK79 and α-amylase activity assay. VHb concentration of MK79 was determined as described elsewhere [16, 17]. A typical VHb peak of MK79 (Fig. 1) and base line of MK57 (Fig. 2) is shown.

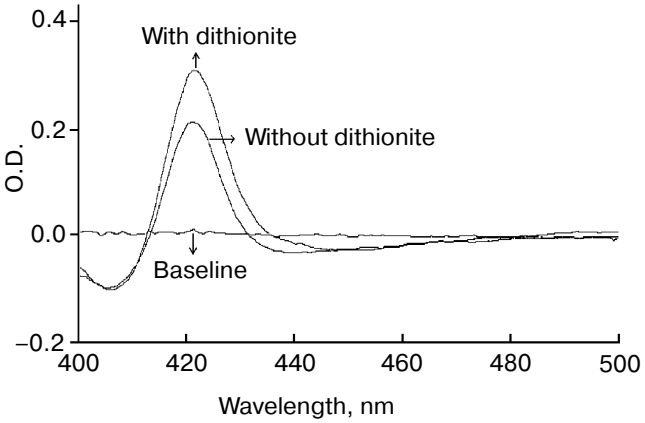

Fig. 1. Absorption spectrum of *E. coli* MK79 strain cells with a typical peak of VHb. Log phase samples were centrifuged and resuspended in 0.1 M potassium phosphate buffer, pH 7.0, to a concentration of 20 mg wet weight per ml. Spectra were taken both before and after reduction of the sample by addition of dithionite. The observed negative absorbance in the range of wavelength 400-415 nm and 440-480 nm is typical for microbial hemoglobin [15].

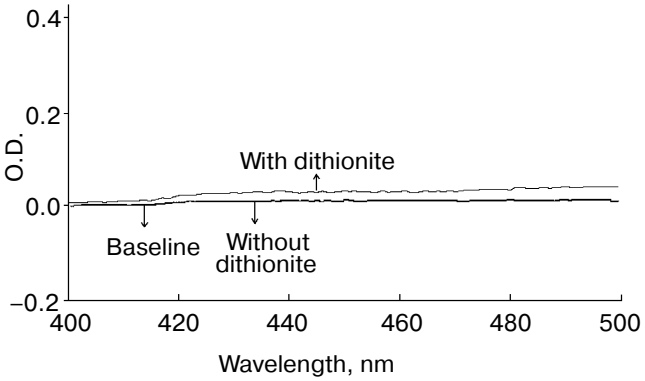

Fig. 2. Absorption spectrum of *E. coli* MK57 strain cells. MK57 showed no hemoglobin peak and was used as a control. Baseline and lines with and without dithionite overlapped. Other details are as in legend to Fig. 1.

Both extracellular and intracellular R-amylase (as described by Liu et al. [33]) was analyzed using a protocol of Miles Laboratories, Inc. (1975) [17, 33].

RESULTS

Previously we have demonstrated that exposure of an *E. coli* strain containing *vgb* to menadione and nitrite leads to the formation of met hemoglobin, but the biological function(s) of microbial hemoglobins is (are) still not understood precisely. Hemoglobin (Hb) acts as a Fenton reagent in biological systems [34, 35]. Several reports have suggested the involvement of *E. coli* antioxidant defenses in protection against HOCl stress. To clarify the influence of VHb on oxidative stress tolerance and growth phase, both logarithmic and late stationary phase cells of MK79 and MK57 were exposed to HOCl. Stationary phase cells were especially examined at this work since our previous studies showed that VHb level increases maximally at this phase in response to oxygen demand by the cells [16, 17]. Winterbourn [36] found that 0.2-3.0 μM (0.0105-0.158 mg/liter) HOCl was a sufficient lethal dose (LD_{50}) against most bacteria. Thus it was examined over a range roughly at this level even though *E. coli* resist HOCl stress up to 76 μM [13].

Cellular SOD, CAT, and GSH activities with and without HOCl. The specific activities of both GSH and SOD increased significantly ($p < 0.05$) [37] following the addition of 0.0125-0.075 mg/liter HOCl to the culture medium (table) when compared with no cells receiving no exogenous HOCl. In general, the level of GSH and SOD elevated gradually with increasing concentrations of HOCl, with MK79 being the most increased strain in this respect. Also, as the concentration of HOCl was increased to a maximum of 0.075 mg/liter HOCl, there was a decrease in catalase. Catalase activity decreased almost 5-fold, nearly linearly, with the addition of 0.0125-0.075 mg/liter HOCl to culture medium, with MK79 being the most affected strain in this respect (table).

Growth comparisons of MK57 and MK79 with and without HOCl. The cell density of MK79 was always higher (both viable and A_{600} bases) than MK57 in the presence and absence of HOCl (table). This result is consistent with our previous studies without HOCl [16, 17]. Also,

Effect of HOCl on hemoglobin concentration, cell growth, α-amylase production, SOD, CAT, and GSH by *E. coli* strains MK57 and MK79

Strain	Parameter	HOCl concentration, mg/liter				
		0	0.0125	0.0250	0.0500	0.0750
MK79	VHb, nmol/g wet weight	66 ± 11* 68 ± 12**	68 ± 17* 66 ± 13**	63 ± 14* 64 ± 12**	67 ± 11* 49 ± 7**	69 ± 16* 39 ± 8**
	Growth, A_{600}	3.32 ± 0.33	3.26 ± 0.46	2.94 ± 0.42	2.80 ± 0.28	2.74 ± 0.26
	α-Amylase***	1.44 ± 0.12	1.78 ± 0.39	2.32 ± 0.58	2.86 ± 0.52	3.12 ± 0.69
	SOD, U/mg	4.1 ± 0.2	5.2 ± 0.1	6.4 ± 0.4	6.8 ± 0.4	7.2 ± 0.6
	CAT, U/mg	32 ± 2.8	18 ± 1.3	12 ± 0.4	9 ± 0.01	6 ± 0.03
	GSH, μmol per 10^{10} cells	11.4 ± 1.2	12.8 ± 1.4	14.8 ± 1.4	17.6 ± 1.6	23.2 ± 2.0
MK57	Growth, A_{600}	3.02 ± 0.28	2.94 ± 0.32	2.86 ± 0.32	2.62 ± 0.24	2.50 ± 0.22
	α-Amylase***	1.22 ± 0.18	1.92 ± 0.36	2.50 ± 0.43	2.68 ± 0.54	2.82 ± 0.56
	SOD, U/mg	3.8 ± 0.2	4.0 ± 0.3	4.6 ± 0.3	4.6 ± 0.2	4.8 ± 0.3
	CAT, U/mg	26 ± 2.6	22 ± 2.1	19 ± 0.7	13 ± 0.03	9 ± 0.02
	GSH, μmol per 10^{10} cells	10.2 ± 1.6	11.2 ± 1.1	13.6 ± 1.2	16.8 ± 1.8	18.2 ± 3.0

Note: The cells were grown at 175 rpm and 37°C for 16 h in shake flasks. All data points are from three individual measurements except for hemoglobin concentration, which is the average of two independent measurements.

* Hemoglobin concentration of MK79 with dithionite addition.

** Hemoglobin concentration of MK79 without dithionite addition.

*** Total α-amylase production is expressed in mg of starch hydrolyzed per min per ml of culture.

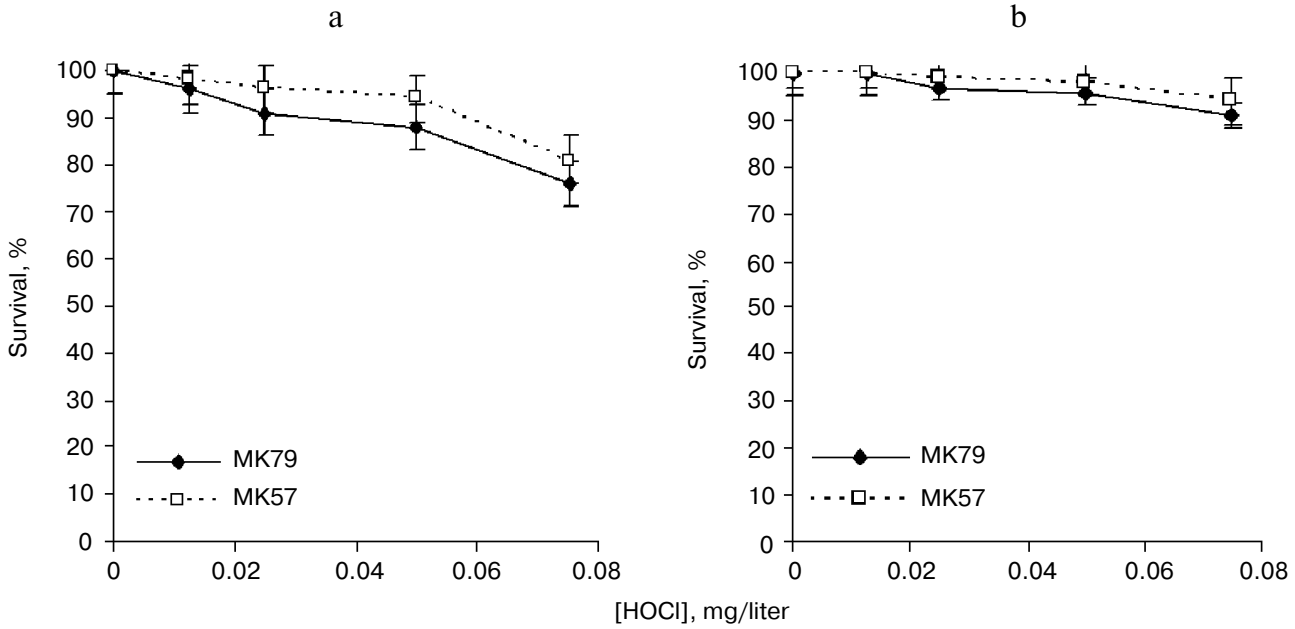

Fig. 3. Survival of exponential (a) and stationary phase (b) of MK79 and MK57 cells exposed to low concentrations of HOCl (0-0.075 mg/liter). Fresh culture (0.1 ml) was inoculated into 5 ml of LB medium in test tubes and grown to $A_{600} = 0.5$ with vigorous shaking at 37°C, and then cultures were exposed to HOCl for 22 min. Survival rate was determined by plating appropriate dilutions of samples and comparing the colony-forming unit versus that of their controls receiving no treatment. The number of surviving bacteria represents the average of three separate experiments.

cell densities for both strains were always greater in the absence of HOCl compared to any HOCl concentration used here. All cultures exhibited low growth rate when grown in the presence of HOCl at concentrations higher than 0.025 mg/liter HOCl (table). Light microscopic examination of cells exposed to HOCl revealed no significant differences in size and overall morphology compared to controls. Both strains showed 100% plasmid stability throughout the experiment.

α-Amylase production of both strains with and without HOCl. Cultures of MK79 and MK57 were grown as for the growth curve experiment and samples were taken at 16 h after inoculation from flasks containing 0-0.075 mg/liter HOCl to be tested for α-amylase. Extracellular α-amylase was greater than intracellular amylase for both strains in the presence and absence of HOCl. Total α-amylase production of MK79 was always greater than MK57 at any concentration of HOCl (table). Interestingly, there is also a large (about 2×) increase in α-amylase production as a result of addition of any HOCl concentration (table). This is true on both per ml of culture and per A_{600} of cells bases.

Lethal effects of HOCl are enhanced by hemoglobin. VHb concentration in MK79 strain was slightly decreased in the presence of HOCl (table). The effect of oxidative stress on cell viability of MK79 and MK57 was investigated by exposing cells to low (0.0125-0.075 mg/liter) and high (1-4 mg/liter) concentrations of HOCl. The survival of exponential and stationary phase cells exposed to various concentration of HOCl is shown in Fig. 3 (low HOCl) and Fig. 4 (high HOCl). The survival of MK57 was significantly greater ($p < 0.05$) [37] than that of the strain expressing VHb (MK79) when challenged in the presence of 0.0125-0.075 mg/liter HOCl regardless of the growth phase (Figs. 3 and 4). The stationary phase cells of both strains indicated a 1.3-to-2-fold higher survival rate than exponentially growing cells in the presence of low concentrations of HOCl. The survival of log-phase MK57 cells was also significantly greater ($p < 0.05$) [37] than that of MK79 cells when challenged with high HOCl concentrations (1-4 mg/liter) (Fig. 4). Overall, MK79 in both phases showed a higher sensitivity to acid challenges. This difference was substantial and dose dependent.

DISCUSSION

Previously we showed that *E. coli* engineered with *Vitreoscilla* hemoglobin (VHb) gene (*vgb*) had higher oxygen uptake than *vgb*$^-$ counterparts. As known, oxygen is vital for most organisms but, paradoxically, damages key biological sites. All aerobic organisms cope with reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, which accumulate in cells as products of the incomplete reduction of

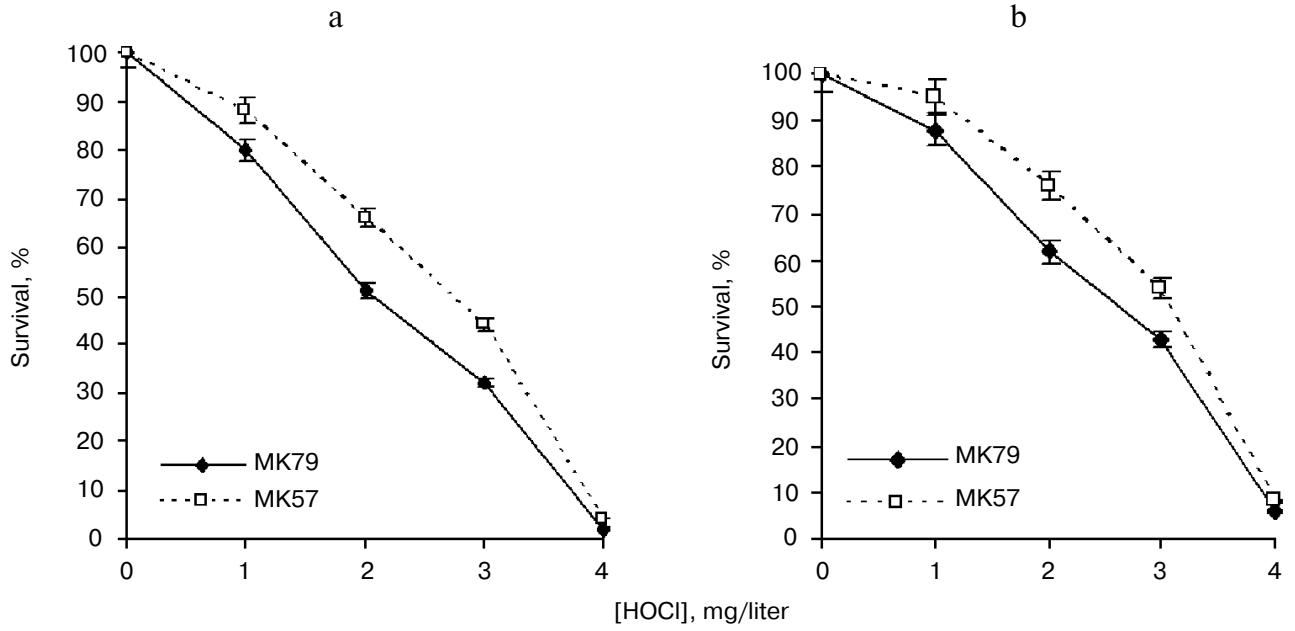

Fig. 4. Survival of exponential (a) and stationary phase (b) MK79 and MK57 cells exposed to high concentrations of HOCl (1-4 mg/liter). Other details are as in legend to Fig. 3.

molecular oxygen [29, 38]. The present study demonstrates for the first time that VHb protein makes cells highly susceptible to killing by HOCl in both exponential and stationary growth phases, as survival of MK79 strain drops almost 2-fold when compared with MK57 strain. This might be a result of strong respiration of the hemoglobin-expressing strain causing endogenous formation of ROS. Many investigators [34, 39, 40] have reported that Hb produces oxidizing species, $^{\cdot}OH$ and/or ferryl ion $[Fe(IV)=O]^{2+}$, in the presence of HOCl through a Fenton and Haber–Weiss-type reaction, thus making cells more susceptible to killing by oxidative stress. Also, the minimum survival for both strains was with exponential phase, since many genes not required for exponential growth are induced in order that cells may survive in the stationary phase, in accordance with results reported for *Streptococcus thermophilus* [41].

At 0-0.075 mg/liter HOCl, growth of MK79 was better than that of its counterpart MK57. This reveals that during growth decreased oxygen in the medium and accumulation of VHb might aid an antioxidant status that ensures the absence of free iron ions via decreased release and increased sequestration. We know that iron is essential for aerobic life and is required for the biosynthesis of a variety of iron bearing proteins and for DNA synthesis. However, when free iron ions are not adequately controlled, it can promote peroxidation of lipid in cell membrane coupled with $^{\cdot}OH$ formation. The oxygen-dependent promoter of *vhb* (P_{vhb}) is maximally induced under microaerobic conditions in both *Vitreoscilla* and *E. coli*, when the dissolved oxygen level is less than 2% of air saturation [42]. In this respect, hemoglobin in MK79 binds oxygen and delivers it to cells at low extracellular oxygen concentration. Thus, VHb did, as expected, eliminated oxygen starvation and *vgb*-bearing cells grow better than their vgb^{-} counterpart. Both oxygen starvation and excess oxygen cause stress in bacteria through the production of harmful ROS, which can damage biomolecules such as proteins, nucleic acids, and cellular membranes, and hypoxia results in a failure to yield sufficient ATP to maintain essential cellular functions.

The importance of oxidative stress and damage in biochemistry is gaining more recognition, as is the significance of antioxidant activity for cellular well-being. Cells are equipped with several defense systems to protect them from the harmful effects of reactive oxygen species [43]. Under normal growth conditions, a balance exists between the amount of radicals produced and the cellular defense mechanisms [44]. In this respect, the "antioxidant" enzymes superoxide dismutase and catalase and glutathione were assayed in a biological model of MK57 and MK79 in the presence and absence of HOCl. It was found that, as the HOCl concentration increased (0.0125-0.075 mg/liter), GSH and SOD activity increased progressively, with the hemoglobin-producing strain having the highest GSH and SOD. The physiological basis of this effect (the highest GSH and SOD in MK79) is not understood but the mechanism behind this buffering characteristic of VHb is thought to prevent the Fenton-type reaction and plays a central role in the overall maintenance of the cellular redox state in *E. coli*. It is

known that GSH is important in maintaining the iron of hemoglobin in the ferrous state (Fe^{2+}). Decrease, increase, or unchanged acid-soluble thiol levels have been also reported in *E. coli* cells. Also, high oxygenation of an *E. coli* strain (like MK79) shifts glutathione homeostasis [45], and a high intracellular GSH level was recently proposed as a potential resistance pathway of *E. coli* cells to HOCl by acting as a scavenger, and also probably by triggering defense systems [46].

Moreover, the highest SOD activity in MK79 perhaps reflects increased oxygen availability promoted by the VHb protein leading, in turn, to intracellular O_2^- production. Because the primary role of SOD is the dismutation of O_2^- to H_2O_2, a linear relationship could be expected between total SOD activity and HOCl concentration. Furthermore, because catalase facilitates the breakdown of H_2O_2, it would be expected that concomitant increases in the activities this enzyme would follow as a greater proportion of total cell protein synthesis is progressively diverted into these enzymatic pathways. However, as the present data indicate, this was not the case. It seems catalase activity, rather than HOCl concentrations, was more directly related to biomass. Moreover, the observed high catalase activity in the *vgb*-bearing strain could be due to the prevented iron-stimulated free radical formation besides, and a buildup in H_2O_2 that might enhance catalase production [46].

In this study, surprisingly, it is also reported that both strains produced more α-amylase as HOCl increased in the range 0-0.075 mg/liter, and MK79 had a bigger advantage over MK57. This is true on both per ml of culture and per A_{600} of cell bases. α-Amylase values of both strains also confirm our previously reported results at zero concentration of HOCl. The growth of both strains was gradually decreased as HOCl increased in the range 0.0125-0.075 mg/liter. So, high α-amylase activity in both strains could be due to HOCl-linked ROS production. This observation is consistent with previous studies, which showed that xanthan gum productivity increased 210% with HOCl-treated cell production [47]. In my lab, HOCl-treated cells were also investigated. Free radical induction may be employed to improve α-amylase productivity. α-Amylase is the most widely used enzyme in starch liquefaction, sizing in textile industries, brewing, paper, and detergent manufacturing processes.

The following conclusions can be made. 1) VHb plays an aggressive role during recovery from HOCl stress. 2) The stationary phase cells of both strains indicated about 1.2-to-2-fold higher survival rate than exponentially growing cells in the presence of any concentration of HOCl. 3) Growth of MK57 and MK79 strains decreased as HOCl increased. 4) MK57 and MK79 strains produced more α-amylase at above zero HOCl concentration (about 2-fold or more). 5) VHb protects the host cell from oxidative stress by triggering defense systems.

This work was done partly at Inonu Universiy Research Center, Malatya, Turkey, and the author wishes to thank this center.

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