Hydrogen peroxide causes *Vibrio vulnificus* bacteriolysis accelerated by sulfonyl fluoride compounds

Michinobu Yoshimura [1, 3], Tetsuro Tamura[1], Ken-ichiro Iida [1], Susumu Shiota [2], Hiroaki Nakayama [1], and Shin-ichi Yoshida [1]

[1] *Department of Bacteriology, Graduate School of Medical Sciences,* [2] *Department of Oral Health, Growth and Development, Division of Oral Infectious Diseases and Immunology, Graduate School of Dental Science, Kyushu University, Fukuoka, Japan,* [3] *Division of Medical Oncology, Hematology and Infectious Disease, Department of Internal Medicine, Fukuoka University, Fukuoka, Japan*

Division of Medical Oncology, Hematology and Infectious Disease, Department of Internal Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan, Fukuoka 814-0180, Japan.

E-mail: myoshimura@fukuoka-u.ac.jp

Tel: (+81) 92 801 1011 Fax: (+81) 92 801 2801

ABSTRACT

Induction of bacteriolysis of *Vibrio vulnificus* cells by 10mM hydrogen peroxide (H_2O_2) was analysed. All *Vibrio* species examined, except for *Vibrio hollisae*, were lysed by 10mM H_2O_2. Bacteriophage induction was not the cause of H_2O_2-induced bacteriolysis. Autolysis is also known to cause bacteriolysis. VvpS protein

is a serine protease of *V. vulnificus* essential for autolysis. *vvpS* mutant underwent H_2O_2-induced bacteriolysis in the same manner as the wild type. Protease inhibitors including serine protease inhibitors did not inhibit H_2O_2-induced bacteriolysis, which means that bacteriolysis is not due to autolysis. Unexpectedly, H_2O_2-induced bacteriolysis was accelerated by adding 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and phenylmethyl sulfonyl fluoride (PMSF) which are serine protease inhibitors. The hydroxyl radical was generated by H_2O_2-AEBSF interaction. It was considered that H_2O_2-induced bacteriolysis was caused by the hydroxyl radical which was generated by Fenton reaction, and possibly mediated by AEBSF. Deferoxamine, an agent chelating ferric ion and Fenton reaction inhibitor, suppressed both H_2O_2-induced bacteriolysis and its acceleration by AEBSF. This suggests that both phenomena were Fenton reaction-dependent, and hydroxyl radical generated by Fenton reaction caused bacteriolysis of *V. vulnificus* though the reason for high susceptibility of *Vibrio* species to hydroxyl radical is not known.

Keywords *Vibrio vulnificus*, Bacteriolysis, Hydrogen peroxide, Hydroxyl radical, Sulfonyl fluoride compounds, Fenton reaction

INTRODUCTION

Vibrio vulnificus is a gram negative halophilic bacterium, found worldwide in normal marine flora (Jones and Oliver 2009, Strom and Paranjpye 2000, Yokochi et al. 2013). This bacterium causes foodborne

disease in humans when consumed in seafood such as raw oysters (Jones and Oliver 2009, Vugia et al. 2013). Furthermore, in compromised hosts such as patients with liver cirrhosis, it invades the blood stream and causes necrotizing wound infection (Jones and Oliver 2009, Neill and Carpenter 2010, Strom and Paranjpye 2000). Patients who have bacteremia and necrotizing wound infection show high fatality, and need combined therapy of surgery and medication including antibiotics (Horseman and Surani 2011).

There are two case reports of successful hyperbaric oxygen (HBO) therapy for advanced *V. vulnificus* infection (Wang et al. 2004, Yagi et al. 2003). In our previous report (Tamura et al. 2012), *V. vulnificus* was shown to have high susceptibility to hydrogen peroxide (H_2O_2) and HBO compared to *Escherichia coli*. Generally, HBO therapy is effective on soft tissue infection such as gas gangrene and Fournier's disease caused by anaerobic clostridial organisms (Bakker 2012, Elliott et al. 1996). *V. vulnificus* is a facultative anaerobic bacteria, just as *E. coli* is, but there is a difference in the HBO and H_2O_2 susceptibility among them (Tamura et al. 2012). The reasons for this difference remain unsolved. When *V. vulnificus* is exposed to H_2O_2, bacteriolysis is observed. Unexpectedly we found that bacteriolysis by H_2O_2 was accelerated by adding sulfonyl fluoride compounds in the bacterial suspension. We found that the compounds induced generation of the hydroxyl radical. The compound accelerated bacteriolysis of vibrio. *V. vulnificus* was thought to be susceptible to both H_2O_2 and the hydroxyl radical.

MATERIALS AND METHODS

Reagents.

Reagents were purchased from companies in parentheses: Hydrogen peroxide (H_2O_2), 4-(2-Aminoethyl) Benzenesulfonyl fluoride hydrochloride (AEBSF), Phenylmethyl sulfonyl fluoride (PMSF), Chloramphenicol (CM), mitomycin C (MMC), Catalase (Sigma-Aldrich, St.Louis, USA), Leupeptin hemisulfate monohydrate, aprotinin, E-64 (Nacalai Tesque, Kyoto, Japan), Deferoxamine mesilate (Novartis Pharma, Tokyo, Japan), Potassium cyanide (KCN), nonfat dry milk (Wako, Osaka, Japan), Laemmli sample buffer (Bio-Rad Laboratories, BioRad, Hercules, CA, USA), Horseradish peroxidase-donkey anti-rabbit IgG conjugate (GE Healthcare UK Ltd, Little Chalfont, UK). *p*-nitrosodimethylaniline (TCI, Tokyo, Japan). Tris-glycine running buffer was prepared (25 mM Tris, 192mM glycine, 0.1% sodium dodecyl sulfate).

Bacterial strains, media and growth conditions.

Vibrio vulnificus strain JCM3725 and *Clostridium perfringens* JCM1290 were obtained by RIKEN Bioresource Center (through the National BioResource Progect of the Ministry of Education, Culture, Sports, Science and Technology, Japan). *Vibrio parahaemolyticus* RIMD2210001 and other *Vibrio* species; *Vibrio fluvialis* RIMD2220001, *Vibrio furnissii* RIMD2223001, *Vibrio harveyi* RIMD2224001, *Vibrio hollisae* RIMD2221001, *Vibrio metschnikovii* RIMD2208013, and *Vibrio mimicus* RIMD2218001 were from BIKEN Research Institute of Microbial Disease, Japan. The following strains used in this study were all our laboratory stock; *Vibrio cholerae* A1552, *Escherichia coli* MG1655, *Escherichia coli* ME9012, and *Staphylococcus aureus* 209P. Organisms of *V. cholerae* and *E. coli*, and *S. aureus* were cultured in yeast extract broth (pH 7.2) containing per liter: 5 g of yeast extract (Difco, Franklin Lakes, NJ, USA), 10g of polypeptone (Wako, Osaka,

Japan), and 5g of NaCl. All strains of vibrios, except for *V. cholera*, were grown in yeast extract broth supplemented with 2.0% (wt/vol) NaCl. We used Brain Heart Infusion medium (Eiken Chemical, Tokyo, Japan) supplemented with 5 μg/ml hemin (Sigma-Aldrich, St. Louis, MO, USA) for *C. perfringens*, and cultured by AnaeroPack Rectangular Jar containing Anaeropack®-Anaero (Nissui Pharmaceutical, Tokyo, Japan). All bacterial strainss were grown at 37 °C.

H_2O_2 induced bacterial cell lysis.

All strains were grown with appropriate media, and cells in the mid-exponential phase were used for assay. Cells were centrifuged (5,800 ×g, 5 min), and washed with PBS, and then centrifuged twice, and suspended in PBS. After H_2O_2 (10 mM) was added, the turbidity was measured at an optical density (OD) difference of 660 nm by spectrophotometer (TAITEC, Hyogo, Japan) at various time points. When necessary, some protease inhibiters and chelating agents were added to reaction solutions, as shown below; leupeptin hemisulfate monohydrate, aprotinin, E-64, AEBSF, PMSF, and deferoxamine mesilate. Catalase was added in reaction solution to eliminate H_2O_2.

Metabolic inhibitors of lysis *of V. vulnificus* by H_2O_2.

V. vulnificus JCM3725 and *E. coli* MG1655 were cultured in appropriate medium, respectively, until OD660 of approximately 0.2. To determine the effect of metabolic conditions, H_2O_2 (10mM) was added to

cultivated medium and OD was measured. To inhibit metabolism such as protein synthesis or ATP synthesis, CM or KCN were added to the culture, and H_2O_2 10mM was added after incubated for 60 min at 37 °C.

Induction of bacteriophage by MMC.

V. vulnificus JCM3725 and lysogenic strain of *E. coli* ME9012 (Liu et al. 1998) and nonlysogenic strain *E. coli* MG1655 (Hayashi et al. 2006) were cultured in appropriate medium respectively until OD660 reached approximately 0.2. Induction of the bacteriophage was performed by adding MMC (0.025-2 μg/ml) to the culture with shaking at 37 °C.

Construction of *vvpS* mutants.

The *vvpS* mutant was constructed by single crossover homologous recombination as described earlier (Elgaml et al. 2014, Funahashi et al. 2002). A 545 bp region of the *vvpS* gene (GenBank accession no. EU232011.1) was amplified by PCR using a forward primer containing the recognition sequence for *Sac*I (5´-GCGAGCTCCCGTAACAGCAACCGCTCTT-3´) and a reverse primer containing the recognition sequence for *Kpn*I (5´-GCGGTACCCGATGCCAAGTTGCGTGATG-3´). The amplicon was inserted into pGEM-T easy vector (Promega, Madison, WI, USA). Competent cells of *E. coli* DH5α were used for transformation through the heat shock method and single colony was isolated from independently cultured plates. Thereafter, sequence was checked and named pYM1007. The *Sac*I-*Kpn*I digested fragment of

pYM1007 was inserted into suicide vector pKTN701 (Nishibuchi et al. 1991). The hybrid plasmid obtained was transformed into *E. coli* SY327λpir, then into *E. coli* SM10λpir. Thereafter, it was transferred to *V. vulnificus* JCM3725 by conjugation, and conjugants were cultivated on a TCBS agar plate (Eiken Chemical, Tokyo, Japan) containing CM 10μg/ml. One suitable *vvpS* mutant named YM0001 was selected after 48 h cultivation at 30 ℃.

Sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting.

The YM0001 and *V. vulnificus* JCM3725 were cultured overnight in yeast extract broth supplemented with 2.0% (wt/vol) NaCl, and was then separated into the supernatant and pellet by centrifugation at 5,800 ×g for 10 min. For SDS-PAGE, this supernatant was dissolved in Laemmli sample buffer with 5% betamercaptoethanol and incubated at 95 ℃ for 5min. SDS-PAGE was performed with 10% acrylamide gels. Electrophoresis was performed by Mini-PROTEAN tetra cell (Bio-Rad Laboratories, BioRad, Hercules, CA, USA) for 120 min at 20 mA in Tris-glycine running buffer. These proteins separated by SDS-PAGE, were transferred to an Immobilon-P transfer membrane (Merck Millipore, Billerica, MA, USA) and blocked with 1% (wt/vol) nonfat dry milk in TBS-0.05% Tween 20 (TBS-T). The membranes were incubated overnight at 4 ℃ with anti-VvpS polyclonal antibodies (1:5000 dilution) gifted from Sang Ho Choi (Lim et al. 2011), followed by incubation for 2 h at room temperature with horseradish peroxidase-donkey anti-rabbit IgG conjugate (1:5000 dilution). The bands were detected with EzWest Lumi

plus (ATTO, Tokyo, Japan) and ImageQuant LAS 4000mini (GE Healthcare UK Ltd, Little Chalfont, UK).

Determination of the hydroxyl radical.

Bleaching of *p*-nitorosodimethylaniline (Bors et al. 1979) was used for determination of the hydroxyl radical. In reaction solutions, *p*-nitrosodimethylaniline (0.05 mM) was added and absorbance (A440) was recorded on a SmartSpec^{TM}3000 (Bio-Rad Laboratories, Hercules, USA).

Determination of peak absorbance of AEBSF.

The absorbance of AEBSF was measured by Flex Station3 (Molecular Devices, California, USA) serially. The catalase (100U/ml) was added to clear the H_2O_2 from PBS. H_2O_2 (10mM) and AEBSF (1mM) were added to PBS, and incubated for 15min. Finally, catalase was added.

RESULTS

H_2O_2 induced bacteriolysis

V. vulnificus and other bacteria were incubated with 10mM of H_2O_2 in PBS. After 20 and 30 min, the OD of *V. vulnificus* and *V. parahaemolyticus* suspensions decreased but other bacteria did not (Fig. 1a). *Vibrio* species other than *V. vulnificus* and *V. parahaemolyticus* were examined to see if bacteriolysis occurs or not under the same condition (Fig. 1b). *V. furnissii*, *V. fluvialis*, and *V. harveyi*, were lysed by 10mM H_2O_2, the

same as *V. vulnificus*. On the other hand, *V. hollisae* was not lysed. *V. metschnikovii* and *V. mimicus* were lysed slower than *V. vulnificus*. *V. vulnificus* was used in the following experiments because it was revealed that the bacterium was sensitive to hyperbaric oxygen (Tamura et al. 2012). *V. vulnificus* was checked for its dose dependency of H2O2-induced bacteriolysis. H_2O_2-induced bacteriolysis occurred quickly in a concentration dependent manner, and bacteriolysis was not observed when 1mM H_2O_2 was added (Fig. 2).

Effect of metabolic inhibitors on H_2O_2 dependent lysis of *V. vulnificus*.

Bacteriolysis by H_2O_2 occurred in growth condition in the media (Fig. 3a) just as in PBS. To study whether bacteriolysis needs metabolism, KCN (respiration inhibitor) or CM (protein synthesis inhibitor) was added to the bacterial suspension. Bacteriolysis by H_2O_2 was not inhibited by KCN (Fig. 3b). Furthermore, KCN alone did not cause bacteriolysis of *V. vulnificus* or *E. coli*. Similary, CM alone neither inhibited nor caused bacteriolysis (Fig. 3c).

Bacteriophage induction.

It is well known that bacteriophage induction by MMC treatment causes bacteriolysis (Ohnishi and Nozu 1986, Otsuji et al. 1959, Pryshliak et al. 2014, Young 2014). We examined whether lysis of *V. vulnificus* due to H_2O_2 was caused by bacteriophage induction or not. When lysogenic strain of *E. coli* ME9012 was incubated with MMC, the OD at 120 minutes first increased, and then decreased (Fig. 4). In addition, λ-phage

was detected by transmission electron microscope in culture media of *E. coli* ME9012 with MMC (data not shown). The decrease of OD was not observed in the nonlysogenic strain of *E. coli* MG1655. The OD of incubation of *V. vulnificus* with MMC (0.25μg/ml-2μg/ml) increased until 30 minutes, but did not change thereafter (Fig. 4). Furthermore, when incubated with lesser MMC (0.025μg/ml) *V. vulnificus* grew, in the same way as that not containing MMC, and the OD did not decrease. Both lysate of *V. vulnificus* by H_2O_2 and supernatant after incubation with MMC were examined by transmission electron microscope, but there were no detectable phages or phage-like particles.

Construction of *vvpS* mutant and bacteriolysis.

It was reported that VvpS is 53 kDa protein, has peptidoglycan (PGN)-hydrolyzing activity, and is essential for autolysis of *V. vulnificus* (Lim et al. 2011). We constructed *vvpS* mutant of *V. vulnificus* and examined whether VvpS is related to lysis by H_2O_2. By Western blotting, the supernatant of *vvpS* mutant YM0001 culture lost the band about 53 kDa although it was detected in wild type (Fig. 5a). The *vvpS* mutant was lysed by 10mM H_2O_2 just as in the wild type. There was no significant difference between wild type and *vvpS* mutant in lysis curves (Fig. 5b).

Effects of protease inhibitors on H_2O_2-induced bacteriolysis of *V. vulnificus*.

Purified VvpS has PGN-hydrolyzing activity on PGN of *V. vulnificus*, and it is inhibited by PMSF, a

serine protease inhibitor (Lim et al. 2011). To confirm VvpS independency of the bacteriolysis, we added serine protease inhibitors PMSF and AEBSF to each reaction solution. Unexpectedly PMSF and AEBSF accelerated the decrease of OD, namely bacteriolysis (Fig. 6a). When Leupeptin (serine-, cysteine- protease inhibitor), aprotinin (serine protease inhibitor), and E-64 (cystein protease inhibitor) were added, OD curves did not show any remarkable change. Because the accelerating effect of AEBSF was especially drastic, we used AEBSF for further analysis of bacteriolysis acceleration. This accelerating effect was examined in other bacteria. This phenomenon was observed in *V. parahaemolyticus* just as in *V. vulnificus*, moderately in *V. cholera*, and to a slight degree in *E. coli* (Fig. 6b). On the other hand, *V. hollisae* did not lyse in the same condition. AEBSF showed its accelerating effect in a concentration-dependent manner (Fig. 6c). When catalase was added to the reaction solution of H_2O_2-induced bacteriolysis, bacteriolysis and AEBSF-acceleration did not occur (Fig. 7), which indicated that H_2O_2 is essential for bacteriolysis.

Detection of hydroxyl radicals.

We hypothesized that the cause of bacteriolysis acceleration by AEBSF was associated with hydroxyl radical production. Thus, to evaluate this hypothesis, we measured OH radical in the reaction solution. The *p*-nitrosodimethylaniline was not bleached by PBS with H_2O_2 or AEBSF alone (Fig. 8a). Fenton reaction generates OH radical from H_2O_2 when interacted with Fe^{2+} (Koppenol 2001). When iron (II) chloride ($FeCl_2$) was added in PBS, a white sediment, perhaps iron (III) phosphate, was generated. Thus we used physiological

saline (0.9%NaCl) instead of PBS in the Fenton reaction assay. When $FeCl_2$ was added to H_2O_2, the *p*-nitrosodimethylaniline was bleached within 1 minute (Fig. 8a). On the other hand, in the case of PBS with both H_2O_2 and AEBSF the absorbance (A 440) gradually decreased for 15 minutes, and thereafter there was no changes. From the bleaching experiment, it was indicated that the OH radical was generated after AEBSF was added.

To evaluate if AEBSF had structural change after this reaction, we measured the peak of UV absorbance. Before adding H_2O_2, the peak was at 265nm (Fig. 8b). After 15 minutes of H_2O_2 addition, the peak was lost (Fig. 8b). It was considered that the reaction of AEBSF with H_2O_2 caused the generation of OH radical accompanied by structural change of AEBSF.

From above results, acceleration of bacteriolysis was considered to be induced by OH radical generation. Hence, we first added AEBSF and H_2O_2 to PBS, and next *V. vulnificus* cells (Fig. 8c). In this condition, OD660 decreased in the same manner as H_2O_2 alone and there was no accelerating effect (Fig. 8c). This data revealed that acceleration was provoked by OH radical.

H_2O_2-induced bacteriolysis in the Fenton reaction model was assayed using physiological saline (0.9%NaCl) to avoid sedimentation. When $FeCl_2$ was added to H_2O_2, it was accompanied by hydroxyl radical generation (Fig. 8a), but there was no accelerating effect on H_2O_2-induced bacteriolysis (Fig. 8d).

Deferoxamine inhibits both H_2O_2 induced bacteriolysis of *V. vulnificus* and the effects of AEBSF.

Deferoxamine is known as an agent chelating ferric ion (Keberle 1964). When deferoxamine was added, the generation of OH radical was inhibited in a concentration dependent manner (Fig. 9a). This result suggested that OH radical generation by interaction between H_2O_2 and AEBSF needs ferric ion. To determine whether bacteriolysis is inhibited by deferoxamine, we added deferoxamine to the H_2O_2 induced bacteriolysis system. When deferoxamine was added, the bacteriolysis was inhibited in a concentration dependent manner of deferoxamine (Fig. 9b). Likewise, deferoxamine delayed the AEBSF-accelerated bacteriolysis (Fig. 9c).

DISCUSSION

This is the first report showing *V. vulnificus* to be highly susceptible to H_2O_2 and OH radical, and lysed by them. H_2O_2-susceptibility seemed to be characteristic to *Vibrio spp*, but there was a species difference among them. *V. cholera*, *V. mimicus* and *V. metschnikovii* were lysed by H_2O_2 more slowly than *V. vulnificus*. Some structural and/or functional differences in membrane may cause the species difference among *V. vulnificus* and other *vibrio* species. Interestingly, H_2O_2 showed no bacteriolytic effect on *V. hollisae*, which is far from other *vibrio* species, genetically based on 16S r RNA gene sequences (Farmer and Janda 2005). Recently, *V. hollisae* was reclassified as the novel genus *Grimontia hollisae*, based on 16SrDNA sequence (Thompson et al. 2003). *Vibrio* species require different concentrations of NaCl in media for good growth. *V. cholera* and *V. mimicus*, however, can grow in media containing no NaCl, and they were lysed more slowly than *V. vulnificus* (Fig. 1a, 1b). It is possible that susceptibility to H_2O_2-induced bacteriolysis may be associated with tolerance of NaCl concentration and the osmotic pressure of their habitats. *G. hollisae* was not

lysed by H_2O_2 though it is a marine bacterium. *V. vulnificus* is a facultative anaerobic, halophilic marine bacteria. *Vibrio* species inhabit the sea, just like *V. vulnificus*. In general, dissolved oxygen in seawater is lower than in the atmosphere and surface water. It is construable that marine bacteria are less encountered by oxygen and reactive oxygen species (ROS) than other bacteria, and have susceptibility to ROS.

The lysis caused by bacteriophage was well investigated (Rice and Bayles 2008, Young 1992, Young 2014). DNA damaging agents, such as MMC and ultraviolet, have been known to cause phage-induced lysis in lysogenic bacterium including *V. vulnificus* (Ohnishi and Nozu 1986, Otsuji et al. 1959, Pryshliak et al. 2014). Under the condition of lysogenic *E. coli* being lysed by phage induction by MMC, *V. vulnificus* JCM 3725 was not lysed. As in MMC, ultraviolet could not cause bacteriolysis of *V. vulnificus* (unpublished observation). Moreover, protein synthesis inhibitors such as CM prevented lysis by phage induction (Otsuji et al. 1959). When KCN or CM was added in culture medium with H_2O_2, the timing of lysis of *V. vulnificus* did not change (Fig. 3c). Moreover, bacteriophage replication needs host cell growth, and some nutrient is necessary for it. But H_2O_2-induced lysis occurs in PBS which does not contain any nutrition. Overall, it is unlikely that bacteriophages are related to H_2O_2-induced lysis of *V. vulnificus*.

Autolysis is also one of the major mechanisms of bacteriolysis (Rice and Bayles 2008). VvpS, a serine protease of *V. vulnificus*, is described as having PGN-hydrolyzing activity and being essential for autolysis (Lim et al. 2011). The *vvpS*-deletion mutant YM0001 had no effect on bacteriolysis by H_2O_2. Despite VvpS being a serine protease as mentioned above, some protease inhibitors, including three kinds of serine protease

inhibitors, were checked for effects on lysis by H_2O_2. Leupeptin and E-64 did not have an effect on bacteriolysis. Interestingly, PMSF and AEBSF accelerated bacteriolysis drastically, but on the other hand, aprotinin had no effect. PMSF, AEBSF and aprotinin are serine protease inhibitors, but they showed different results for lysis by H_2O_2. Overall, it is unlikely that H_2O_2-induced lysis was autolysis.

From our observation where bacteriolysis was accelerated by AEBSF and PMSF, we considered the possibility that sulfonyl fluoride compounds interacted with H_2O_2 and generated some chemical substance. H_2O_2 is a member of a ROS, and generates hydroxyl radical through an interaction with Fe (II) known as the Fenton reaction (Croft et al. 1992, Lloyd et al. 1997). We hypothesized that AEBSF reacted with H_2O_2 and generated hydroxyl radical. In aqueous solution containing AEBSF and H_2O_2 the hydroxyl radical was detected, but not in AEBSF or H_2O_2 alone. In addition to the generation of hydroxyl radical, the peak of UV absorbance (260nm) in AEBSF was lost. From these, it was considered that the hydroxyl radical was generated from the interaction of AEBSF and H_2O_2. Interestingly, hydroxyl radical generation was inhibited by deferoxamine, which is a chelating agent having a high specificity to ferric ion (Keberle 1964). From results of experiments using deferoxamine, it is considered that the ferric ion is necessary for generating hydroxyl radical from H_2O_2 and AEBSF.

To determine if generated hydroxyl radical caused bacteriolysis of *V. vulnificus*, the bacteria were suspended in solution 15 minutes after the reaction of AEBSF and H_2O_2 started (Fig. 8c). The solution that finished generation of hydroxyl radical could not accelerate bacteriolysis. From this relation between

bacteriolysis and hydroxyl radical generation, it was considered that acceleration of bacteriolysis occurred by generated hydroxyl radical. On the other hand, bacteriolysis occurred 20 minutes after cells were suspended in H_2O_2 plus AEBSF solution. In this condition, H_2O_2 was resolved by the reaction with AEBSF, but over 80 % remained (data not shown). It is considered that the remaining H_2O_2 was the effector of bacteriolysis.

In the Fenton reaction ($FeCl_2$ plus H_2O_2), the hydroxyl radical was generated at a greater degree than in the case of AEBSF plus H_2O_2 (Fig. 8a). Despite this fact, there was no accelerating effect for bacteriolysis in the Fenton reaction condition (Fig. 8d). There may be a difference of generation pattern of hydroxyl radical between H_2O_2 plus $FeCl_2$ and H_2O_2 plus AEBSF. In $FeCl_2$ with H_2O_2, hydroxyl radical was generated quickly, taking less than one minute, and stopped. But, it was generated continuously for more than 15 minutes, when AEBSF and H_2O_2 reacted. Furthermore, the Fenton reaction generated a greater amount of hydroxyl radical than that due to AEBSF. The hydroxyl radical had high reactivity and the half-time was very short. It is considered that membranes need to be exposed to the hydroxyl radical continuously for the acceleration of bacteriolysis.

Deferoxamine inhibited bacteriolysis in a dose dependent manner. This phenomenon suggested that ferric ion is necessary for bacteriolysis caused by H_2O_2. It is thought that the hydroxyl radical generated by the Fenton reaction was responsible for bacteriolysis. This result is congruent with a report on the killing of cultured hepatocytes by H_2O_2 (Starke and Farber 1985). In the bacterial cell of *V. vulnificus*, it is considered that hydroxyl radical generated by the Fenton reaction causes lipid peroxidation and membrane injury, finally

leading to bacterial cell lysis.

It is known that patients who have chronic liver disease are susceptible to *V. vulnificus* infection (Jones and Oliver 2009, Neill and Carpenter 2010, Strom and Paranjpye 2000). Patients with liver disease have a defect in the phagocytic system (Blake et al. 1979, Conn 1964). Antiphagocytic activity is important to bacterial virulence (Yoshida et al. 1985). The study compared survival of *V. vulnificus* in blood from healthy individuals, and patients with chronic liver diseases who showed a decrease in neutrophil activity and an increased survival of *V. vulnificus* (Hor et al. 1999). Neutrophils kill bacteria by ROS such as superoxide anion and H_2O_2 generated by oxidative burst (Neill and Carpenter 2010). Our data suggest that *V. vulnificus* has a high susceptibility to ROS, and it emphasizes the importance of neutrophils (phagocytes) to kill this bacteria, for host defense.

ACKNOWLEDGMENTS

We would like to thank Dr. Sang Ho Choi for giving the anti-VvpS polyclonal antibodies. We also would like to thank Shin-ichi Miyoshi for supplying the plasmid vector pKTN701, *E. coli* SY327λpir, and *E. coli* SM10λpir. The original publication is available at springerlink.com.

REFERENCES

Bakker DJ (2012) Clostridial myonecrosis (gas gangrene). Undersea Hyperb Med 39:731-737

Blake PA, Merson MH, Weaver RE, Hollis DG, Heublein PC (1979) Disease caused by a marine Vibrio. Clinical characteristics and epidemiology. N Engl J Med 300:1-5

Bors W, Michel C, Saran M (1979) On the nature of biochemically generated hydroxyl radicals. Studies using the bleaching of p-nitrosodimethylaniline as a direct assay method. Eur J Biochem 95:621-627

Conn HO (1964) Spontaneous Peritonitis and Bacteremia in Laennec's Cirrhosis Caused by Enteric Organisms. a Relatively Common but Rarely Recognized Syndrome. Ann Intern Med 60:568-580

Croft S, Gilbert BC, Smith JR, Whitwood AC (1992) An E.S.R. investigation of the reactive intermediate generated in the reaction between FeII and H2O2 in aqueous solution. Direct evidence for the formation of the hydroxyl radical. Free Radic Res Commun 17:21-39

Elgaml A, Higaki K, Miyoshi S (2014) Effects of temperature, growth phase and luxO-disruption on regulation systems of toxin production in Vibrio vulnificus strain L-180, a human clinical isolate. World J Microbiol Biotechnol 30:681-691

Elliott DC, Kufera JA, Myers RA (1996) Necrotizing soft tissue infections. Risk factors for mortality and strategies for management. Ann Surg 224:672-683

Funahashi T, Moriya K, Uemura S, Miyoshi S, Shinoda S, Narimatsu S, Yamamoto S (2002) Identification and characterization of pvuA, a gene encoding the ferric vibrioferrin receptor protein in Vibrio parahaemolyticus. J Bacteriol 184:936-946

Farmer III JJ, Janda JM (2005) Family I. Vibrionaceae. In: George M. Garrity (ed), Bergey's manual of systematic bacteriology, 2nd edn vol2. Springer Science, New York, pp 491-545

Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H, Horiuchi T (2006) Highly accurate genome sequences of Escherichia coli K-12 strains MG1655 and W3110. Mol Syst Biol 2:2006.0007

Hor LI, Chang TT, Wang ST (1999) Survival of Vibrio vulnificus in whole blood from patients with chronic liver diseases: association with phagocytosis by neutrophils and serum ferritin levels. J Infect Dis 179:275-278

Horseman MA, Surani S (2011) A comprehensive review of Vibrio vulnificus: an important cause of severe sepsis and skin and soft-tissue infection. Int J Infect Dis 15:e157-66

Jones MK, Oliver JD (2009) Vibrio vulnificus: disease and pathogenesis. Infect Immun 77:1723-1733

Keberle H (1964) The Biochemistry of Desferrioxamine and its Relation to Iron Metabolism. Ann N Y Acad Sci 119:758-768

Koppenol WH (2001) The Haber-Weiss cycle--70 years later. Redox Rep 6:229-234

Lim MS, Kim JA, Lim JG, Kim BS, Jeong KC, Lee KH, Choi SH (2011) Identification and characterization of a novel serine protease, VvpS, that contains two functional domains and is essential for autolysis of Vibrio vulnificus. J Bacteriol 193:3722-3732

Liu JQ, Dairi T, Itoh N, Kataoka M, Shimizu S, Yamada H (1998) Gene cloning, biochemical characterization and physiological role of a thermostable low-specificity L-threonine aldolase from Escherichia coli. Eur J Biochem 255:220-226

Lloyd RV, Hanna PM, Mason RP (1997) The origin of the hydroxyl radical oxygen in the Fenton reaction. Free Radic Biol Med 22:885-888

Neill MA, Carpenter CC (2010) Other pathogenic vibrios. In: Mandell GL (ed), Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 7th edn. Churchill Livingstone, Philadelphia, pp 2788-2789

Nishibuchi M, Kumagai K, Kaper JB (1991) Contribution of the tdh1 gene of Kanagawa phenomenon-positive Vibrio parahaemolyticus to production of extracellular thermostable direct hemolysin. Microb Pathog 11:453-460

Ohnishi T, Nozu K (1986) Induction of phage-like particles from a pathogenic strain of Vibrio parahaemolyticus by mitomycin C. Biochem Biophys Res Commun 141:1249-1253

Otsuji N, Sekiguchi M, Iijima T, Takagi Y (1959) Induction of phage formation in the lysogenic Escherichia coli K-12 by mitomycin C. Nature 184(Suppl 14):1079-1080

Pryshliak M, Hammerl JA, Reetz J, Strauch E, Hertwig S (2014) Vibrio vulnificus phage PV94 is closely related to temperate phages of V. cholerae and other Vibrio species. PLoS One 9:e94707

Rice KC, Bayles KW (2008) Molecular control of bacterial death and lysis. Microbiol Mol Biol Rev 72:85-109, table of contents

Starke PE, Farber JL (1985) Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide. Evidence for the participation of hydroxyl radicals formed by an iron-catalyzed Haber-Weiss reaction. J Biol Chem 260:10099-10104

Strom MS, Paranjpye RN (2000) Epidemiology and pathogenesis of Vibrio vulnificus. Microbes Infect 2:177-188

Tamura T, Iida K, Saito M, Shiota S, Nakayama H, Yoshida S (2012) Effect of hyperbaric oxygen on Vibrio vulnificus and murine infection caused by it. Microbiol Immunol 56:673-679

Thompson FL, Hoste B, Vandemeulebroecke K, Swings J (2003) Reclassification of Vibrio hollisae as Grimontia hollisae gen. nov., comb. nov. Int J Syst Evol Microbiol 53:1615-1617

Vugia DJ, Tabnak F, Newton AE, Hernandez M, Griffin PM (2013) Impact of 2003 state regulation on raw oyster-associated Vibrio vulnificus illnesses and deaths, California, USA. Emerg Infect Dis 19:1276-1280

Wang J, Corson K, Mader J (2004) Hyperbaric oxygen as adjunctive therapy in Vibrio vulnificus septicemia and cellulitis. Undersea Hyperb Med 31:179-181

Yagi H, Takechi Y, Hiraki S, Yamaguchi A, Yoshizato M, Furuyama M (2003) A life saving case of Vibrio vulnificus (septic type). Kansenshogaku Zasshi 77:167-173

Yokochi N, Tanaka S, Matsumoto K, Oishi H, Tashiro Y, Yoshikane Y, Nakashima M, Kanda K, Kobayashi G (2013) Distribution of virulence markers among Vibrio vulnificus isolates of clinical and environmental origin and regional characteristics in Japan. PLoS One 8:e55219

Yoshida S, Ogawa M, Mizuguchi Y (1985) Relation of capsular materials and colony opacity to virulence of Vibrio vulnificus. Infect Immun 47:446-451

Young R (2014) Phage lysis: three steps, three choices, one outcome. J Microbiol 52:243-258

Young R (1992) Bacteriophage lysis: mechanism and regulation. Microbiol Rev 56:430-481

Figure Captions

Fig. 1 H_2O_2-induced bacterial cell lysis. All bacterial strains were cultured in appropriate media until exponential phases. Cells were suspended in PBS after being washed once by centrifugation. Turbidity was measured at intervals after hydrogen peroxide (10mM) was added and bacterial suspensions were incubated at 37 °C. (**a**) *V. vulnificus* was compared with other bacteria. *V. vulnificus* (○), *V. parahaemolyticus* (□), *V. cholerae* (Δ), *E. coli* (◊), *S. aureus* (×), *C. perfringens* (▲), *V. vulnificus* without H_2O_2 (●). (**b**) Comparison of

susceptibility of H_2O_2-induced lysis among *Vibrio* species. *V. vulnificus* (○), *V. fluvialis* (□), *V. furnissii* (Δ), *V. harveyi* (◊), *V. hollisae* (×), *V. metschnikovii* (■), *V. mimicus* (▲), *V. vulnificus* without H_2O_2 (●)

Fig. 2 Dose dependency of H_2O_2 on bacteriolysis of *V. vulnificus*. *V. vulnificus* was cultured in yeast extract broth (2%NaCl) at the mid-exponential phase. Cells were suspended in PBS after being washed once by centrifugation. Turbidity was measured at intervals after H_2O_2 (0-100mM) was added and bacterial suspensions were incubated at 37 ℃. H_2O_2 was added at concentration of 0mM (●), 1mM (□), 5mM (Δ), 10mM (○), 20mM (×), 40mM (◊), and 100mM (■)

Fig. 3 Effect of metabolic inhibitors KCN and CM on H_2O_2-induced bacteriolysis of *V. vulnificus*. (**a**) Bacteriolysis assay of *V. vulnificus* and *E. coli* by H_2O_2 (10mM) was performed in the appropriate media. Metabolic inhibitory agents KCN (**b**) or CM (**c**) were added 60 min before H_2O_2 addition in bacteriolysis assay. *V. vulnificus* and *E. coli* MG1655 were cultured in yeast extract broth (2%NaCl) and yeast extract broth (0.5%NaCl) at OD 0.2-0.25. KCN (1mM) or CM (0.1μg/ml) was added to cultured cell, and was incubated at 37 ℃ with shaking for 60min. Thereafter H_2O_2 (10mM) was added and turbidity was measured during incubation at 37 ℃. *V. vulnificus* with H_2O_2 (○), *V. vulnificus* without H_2O_2 (●), *E. coli* with H_2O_2 (□), *E. coli* without H_2O_2 (■)

Fig. 4 Bacteriophage induction by MMC. Bacteriolysis caused by bacteriophage induction was assayed with *V. vulnificus* and lysogenic strain *E. coli* ME9012 and nonlysogenic strain *E. coli* MG1655. All bacterial strains were cultured in an appropriate medium at OD660 0.2-0.25. Various concentrations of MMC were added to bacterial cultures. Thereafter, OD660 was measured during incubation with shaking at 37 °C

Fig. 5 Construction of *vvpS* mutant and its bacteriolysis by H_2O_2. The *vvpS* mutant was constructed by homologous recombination as described in Materials and Methods. (**a**) The wild type *V. vulnificus* JCM3725 and *vvpS* mutant were cultured in yeast extract broth (2%NaCl) overnight at 37 °C, and the VvpS proteins in the supernatants were detected by Western blot analysis. (**b**) The wild type and *vvpS* mutant were compared in bacteriolysis assay with 10mM H_2O_2 in PBS

Fig. 6 Effect of protease inhibitors on bacteriolysis by H_2O_2. (**a**) Serine protease inhibitors (Aprotinin 1μg/ml (□) or PMSF 0.1mM (■), AEBSF 0.1mM (◊)) and cysteine protease inhibitor (E-64 10μM (Δ)), both inhibitors (leupeptin 10μM (×)) were evaluated for their effect on bacteriolysis. *V. vulnificus* was cultured in yeast extract broth (2%NaCl) at exponential phase. Cultured cells were suspended in PBS after being washed once with PBS. Turbidity was measured after hydrogen peroxide (10mM) and any protease inhibitor was added, and incubation was continued at 37 °C. They were compared with no protease inhibitor (H_2O_2 alone (○), without H_2O_2 (●)). (**b**) Effect of AEBSF on H_2O_2-induced bacteriolysis. *Vibrio spp.* and *E. coli* were cultured in

appropriate medium at exponential phase. Cells were suspended in PBS after being washed once with PBS. Turbidity was measured after hydrogen peroxide (10mM) and AEBSF (0.1mM) were added, and incubated at 37 ℃. *V. vulnificus* (○), *V. vulnificus* without H_2O_2 (●), *V. parahaemolyticus* (□), *E. coli* (Δ), *V. hollisae* (×), *V. cholerae* (◊). (**c**) Dose dependency of AEBSF on acceleration of H_2O_2-induced bacteriolysis. Different concentrations of AEBSF in reaction (0.1mM (□), 0.05mM (Δ), 0.025mM (×), 0.0125mM (◊), 0.006mM (■), 0mM (○), no AEBSF and no H_2O_2 (●)) were compared for accelerating effect on *V. vulnificus* H_2O_2 (10mM)-induced bacteriolysis

Fig. 7 Catalase inhibits H_2O_2-induced bacteriolysis. *V. vulnificus* JCM3725 was cultured in yeast extract broth (2%NaCl) at exponential phase. Cells were suspended in PBS after being washed once by centrifugation. Turbidity was measured after H_2O_2 (10mM), AEBSF (0.1mM), and catalase (10U/1μM H_2O_2) were added simultaneously and incubated at 37 ℃

Fig. 8 Detection of hydroxyl radicals after addition of AEBSF. (**a**) Hydroxyl radical was detected by bleaching of *p*-nitrosodimethylaniline. The final concentration of *p*-nitrosodimethylaniline was 0.05mM in PBS or physiological saline (0.9% NaCl). H_2O_2 (10mM) (○) or AEBSF (0.1mM) (□) or H_2O_2 (10mM) plus AEBSF (0.1mM) (Δ) were added to PBS containing *p*-nitrosodimethylaniline. A440 was then measured at intervals. Same as above, H_2O_2 (10mM) and $FeCl_2$ (0.1mM (×), 1mM (●)) were added to physiological saline. (**b**) To

evaluate whether AEBSF had structural change due to reaction with H_2O_2, the change of peak absorbance was measured. Absorbance was measured every 5nm. To clear the effect of H_2O_2 on measurement, catalase was added to the reaction. PBS only (○), catalase (●), H_2O_2 10mM plus catalase (×), AEBSF 1mM plus catalase (Δ), H_2O_2 10mM plus AEBSF 1mM and catalase (□). (**c**) The solution of AEBSF plus H_2O_2 after finishing generation of hydroxyl radicals was examined for bacteriolysis assay. *V. vulnificus* was cultured in medium at exponential phase. Cells grown were washed with PBS once, and separated into supernatant and pellet by centrifugation at 5,800 ×g for 5 min. After centrifugation supernatant was removed. AEBSF (0.1mM) and H_2O_2 (10mM), or H_2O_2 (10mM) only were mixed in PBS and incubated at 37 ℃ for 15min. Then, 15 min after incubation, the pellet of *V. vulnificus* was suspended in each solution. The OD change was measured. (**d**) To evaluate whether the Fenton reaction accelerated H_2O_2-induced bacteriolysis or not, $FeCl_2$ was added in H_2O_2-induced (10mM) bacteriolysis in physiological saline (0.9% NaCl). H_2O_2 (○), without H_2O_2 (●), H_2O_2 plus $FeCl_2$ 1mM (□), H_2O_2 plus $FeCl_2$ 0.1mM (Δ), H_2O_2 plus AEBSF 0.1mM (×)

Fig. 9 Deferoxamine inhibits the H2O2-induced bacterial cell lysis of *V. vulnificus* and hydroxyl radical generation. (**a**) Dose dependency of deferoxamine on hydroxyl radical production, by H_2O_2 plus AEBSF. The final concentration of *p*-nitrosodimethylaniline was 0.05mM in PBS. H_2O_2 (10mM) and AEBSF (0.1mM) and deferoxamine (0mM (◊), 0.5mM (×), 1mM (Δ), 2mM (□), 5mM (○)) were added in PBS containing *p*-nitrosodimethylaniline, then A440 was measured at intervals. (**b**) Dose dependency of deferoxamine on

bacteriolysis by H_2O_2. Bacterial suspensions in PBS OD660nm about 0.2 were incubated with H_2O_2 10mM and various concentrations of deferoxamine (0mM (○), 5mM (□), 10mM (Δ), 20mM (◊), 40mM (×), and without H_2O_2 and deferoxamine (●). (**c**) Dose dependency of deferoxamine on bacteriolysis by H_2O_2 plus AEBSF. Bacterial suspensions in PBS OD660nm about 0.2 were incubated with H_2O_2 10mM and various concentration of deferoxamine (0mM (□), 2mM (Δ), 5mM (◊), 10mM (×), 20mM (■), 40mM (▲)) with AEBSF 0.1mM at 37 ℃, and only H_2O_2 10mM (○)

Fig. 1

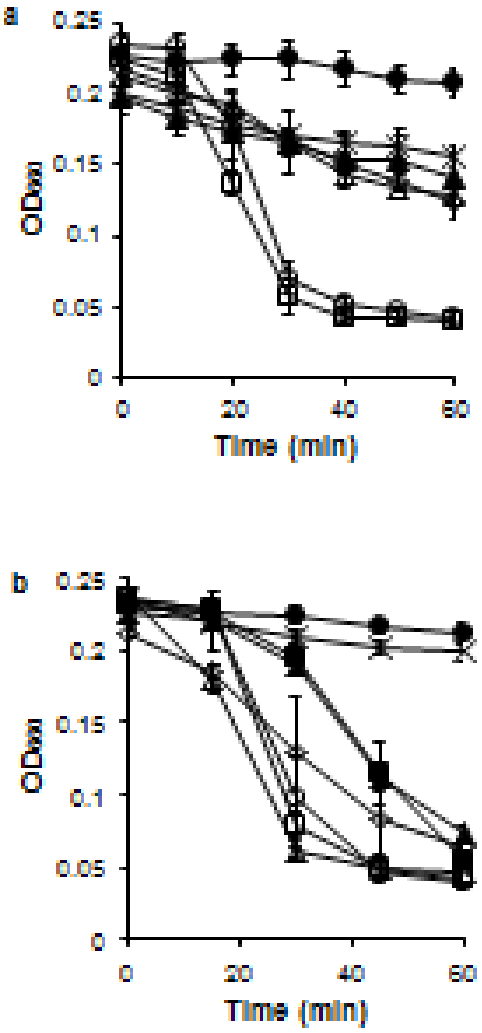
a
0.25
0.2
0.15
0.1
0.05
0
OD600
0
20
40
60
Time (min)
b
0.25
0.2
0.15
0.1
0.05
0
OD600
0
20
40
60
Time (min)

Fig. 2

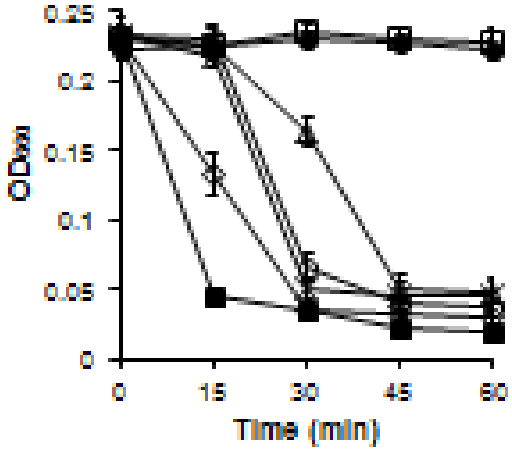

Fig. 3

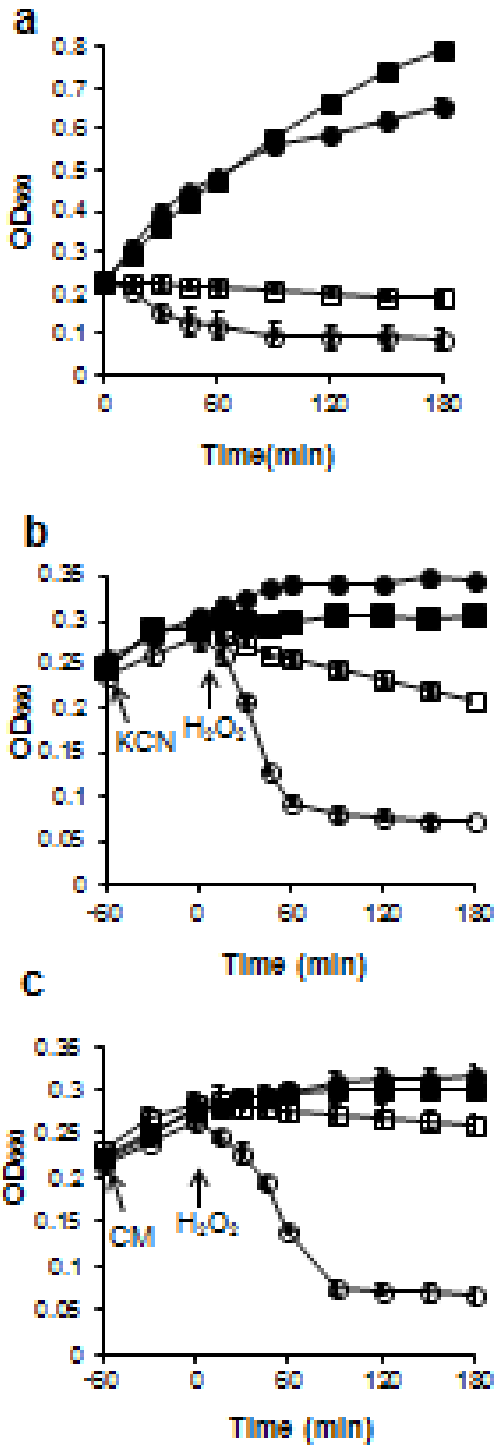
a
OD600
Time(min)
b
OD600
KCN
H2O2
Time (min)
c
OD600
CM
H2O2
Time (min)

Fig. 4

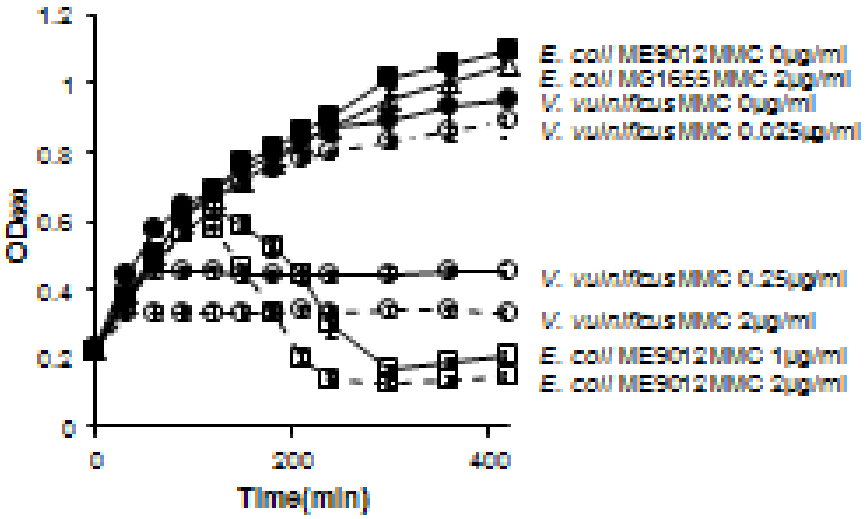
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
200
400
Time(min)
E. coli ME9012MMC 0μg/ml
E. coli MG1655MMC 2μg/ml
V. vulnificus MMC 0μg/ml
V. vulnificus MMC 0.025μg/ml
V. vulnificus MMC 0.25μg/ml
V. vulnificus MMC 2μg/ml
E. coli ME9012MMC 1μg/ml
E. coli ME9012MMC 2μg/ml

Fig. 5

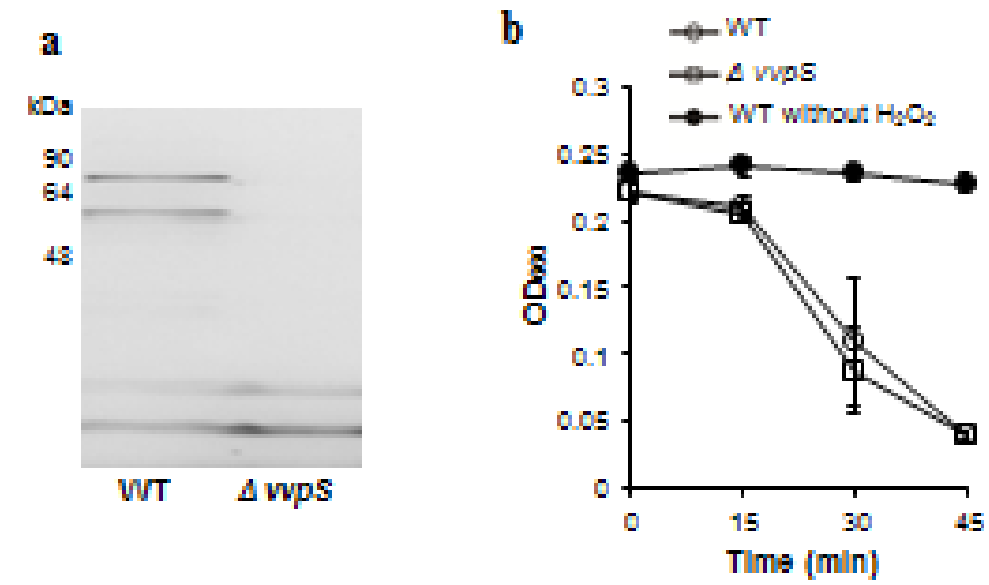
a
kDa
90
64
48
WT
Δ vvpS
b
WT
Δ vvpS
WT without H_2O_2
0.3
0.25
0.2
0.15
0.1
0.05
0
OD600
0
15
30
45
Time (min)

Fig. 6

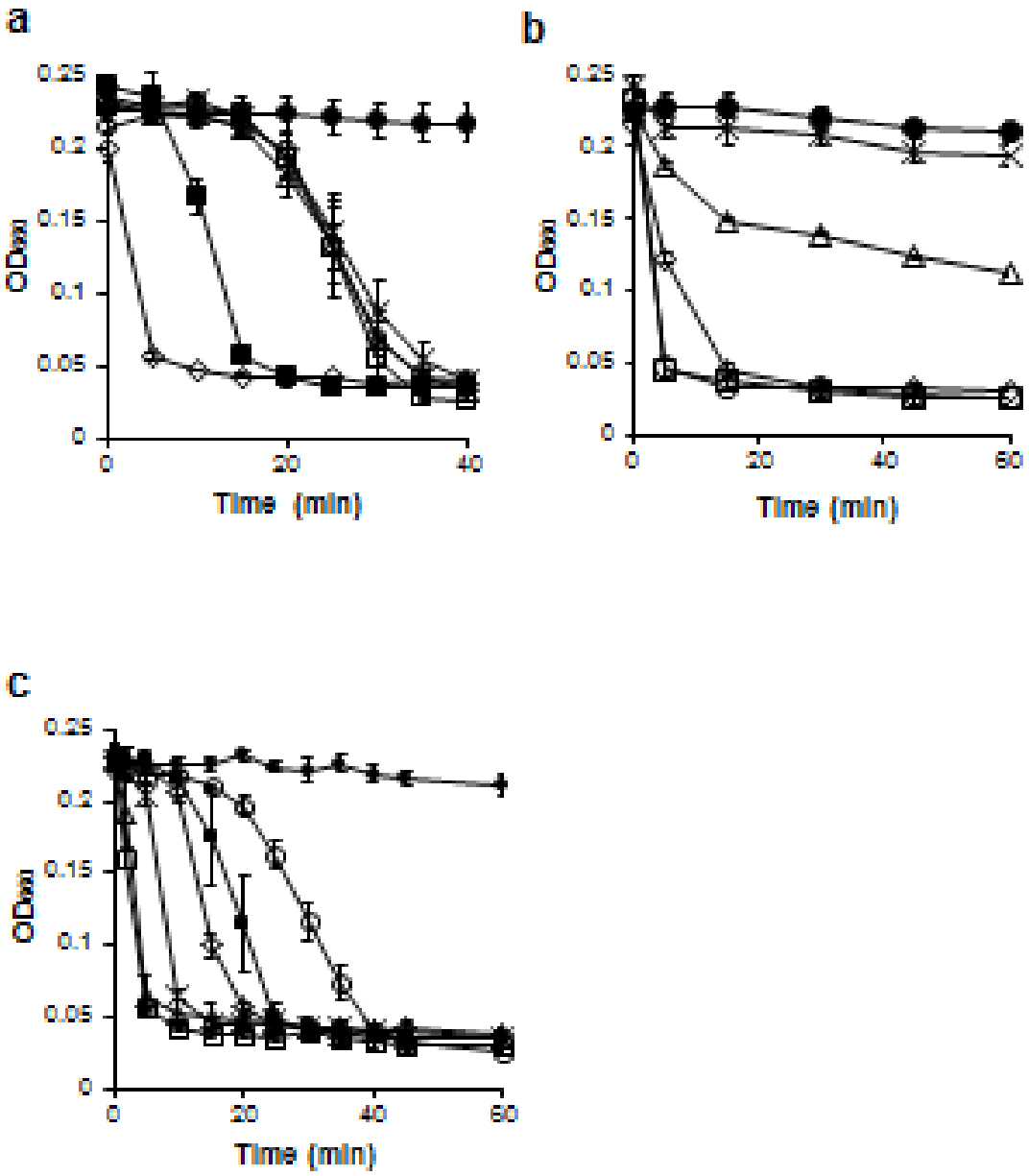

Fig. 7

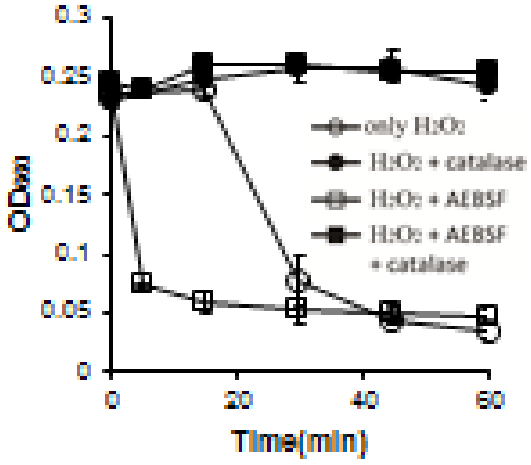
0.3
0.25
0.2
0.15
0.1
0.05
0
OD450
0
20
40
60
Time(min)
only H2O2
H2O2 + catalase
H2O2 + AEBSF
H2O2 + AEBSF
+ catalase

Fig. 8

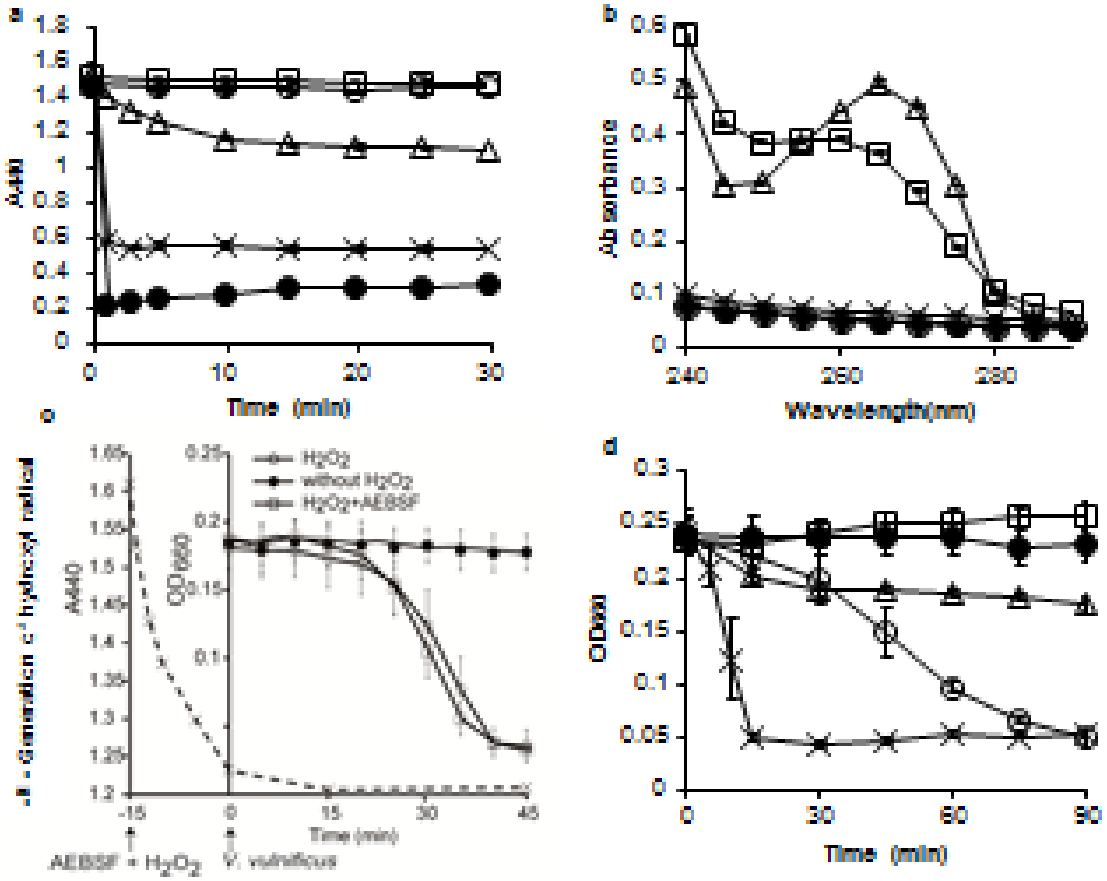
a
A440
Time (min)
b
Absorbance
Wavelength(nm)
c
Generation of hydroxyl radical
A440
OD650
H2O2
without H2O2
H2O2+AEBSF
Time (min)
AEBSF + H2O2
V. vulnificus
d
OD650
Time (min)

Fig. 9