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Note

Presence of Nitric Oxide-Sensing Systems in the Human Pathogen *Vibrio vulnificus*

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Vibrio vulnificus **is a halophilic estuarine bacterium, but this species causes fatal septicemia in humans. *V. vulnificus* may encounter many kinds of stresses either in the natural environment or in the human body. One of the striking stresses is the exposure to the reactive oxygen species including nitric oxide (NO). The present study revealed that NO could participate in the regulation of the *V. vulnificus* community behavior. When the bacterium was cultivated in the presence of sub-lethal doses of an NO donor, the expression of the genes encoding NO-detoxifying enzymes was significantly increased. The NO donor was also found to cause significant increase in production of a metalloprotease, a putative virulence factor, by the bacterium.**

Key words : *Vibrio vulnificus* / Nitric oxide / Oxidative stress / Detoxification.

Vibrio vulnificus is a common Gram-negative halophilic estuarine bacterium; however, this species is an opportunistic pathogen that causes a rapidly progressing and fatal septicemia in human beings, after consumption of raw or undercooked seafood contaminated with the pathogen (Miyoshi, 2006; Jones and Oliver, 2009). This species can control its community behavior through the synchronized regulation of the expression of various genes including those encoding virulence factors and toxins. This regulation is dependent on the bacterial cell density, so that it is called quorum sensing (QS).

The QS cascade is operated by the detection of the small signaling molecule called the autoinducer, and its primary target gene in *V. vulnificus* is *vvp/vvpE* encoding the toxic metalloprotease VVP/VvpE (Kawase et al., 2004; Elgaml et al., 2013, 2014). The purified VVP/VvpE has been documented to enhance vascular permeability due to exocytotic histamine release from mast cells or due to activation of the bradykinin-generating cascade (Miyoshi, S., 2006, 2013). VVP/VvpE also evokes hemorrhagic skin damage through digestion of the vascular basement membrane (Miyoshi, 2006, 2013).

Bacteria are generally exposed to nitric oxide (NO) from environmental or human sources at concentrations ranging from the micromolar to nanomolar levels (Derbyshire and Marletta, 2009; Plate and Marletta, 2013). *V. vulnificus* may be also exposed to different concentrations of NO in the natural reservoirs via UV irradiation of water, as well as in the human body through the oxidative burst of macrophages (Storz and Zheng, 2000; Nowakowska and Oliver, 2013).

Because NO is a potent antibiotic and cytotoxic substance at high concentrations, many bacterial pathogens have evolved the means to sense and detoxify the generated NO (Stevanin et al., 2002; Fang, 2004; Spiro, 2007). For instance, the NnrS protein (the heme-copper membrane protein) and NrfA protein (the cytochrome c nitrite reductase) are known to convert NO to less toxic molecules, such as nitrate, nitrous oxide, and ammonia (Poole and Hughes, 2000; Gardner et al., 2002; Stevanin et al., 2002, 2005; Mills et al., 2008). Consistent with their function in NO clearance, these detoxifying enzymes have sufficient

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TABLE 1. Proteins related to the NO-sensing systems in *V. vulnificus*

Strain	NnrS	NrfA	H-NOX	HqsK	VVP/vvpE
CMCP6	VV1_1781	VV1_3035	VV1_2263	VV1_2264	VV2_0032
YJ016	VV2630	VV1250	VV2082	VV2081	VVA1456

affinity to sense toxic levels of NO. Moreover, the expression of their genes is induced by exposure to NO (Spiro, 2007; Heurlier et al., 2008; Tucker et al., 2010). As shown in TABLE 1, *V. vulnificus* was confirmed to harbor the genes encoding NnrS and NrfA by the results of the genomic project (Chen et al., 2003; Kim et al., 2011). However, no study has been carried out to clarify if NnrS and/or NrfA have roles in NO detoxification.

On the other hand, the recent studies have demonstrated that various bacteria can employ sub-lethal concentrations of NO as a signaling molecule (Carlson et al., 2010; Wang et al., 2010; Henares et al., 2012; Plate et al., 2012). In this system, the heme-nitric oxide/oxygen binding (H-NOX) protein (the protein sharing high sequence homology with the heme-binding domain of the mammalian NO receptors) functions as the NO-sensor (Plate and Marletta, 2013). This H-NOX protein serves as the high-affinity NO sensor in more sophisticated multistep downstream signaling processes, which usually include a histidine kinase (Henares et al., 2012; Plate and Marletta, 2013). The H-NOX-dependent system in *Vibrio harveyi*, the bioluminescent marine bacterium, consists of another protein HqsK, a histidine kinase (H-NOX-associated QS kinase).

Recently, it was found that the H-NOX/HqsK system in *V. harveyi* could activate the QS cascade. Namely, as well as the addition of autoinducer, light production by the bacterium is significantly increased in the presence of the NO donor (Henares et al., 2012). Although the NO-dependence of the QS cascade was not documented, the genomic project evidently showed that *V. vulnificus* has homologues of H-NOX and HqsK (TABLE 1). The degree of homology between *V. vulnificus* proteins and *V. harveyi* proteins is 66% for H-NOX and 67% for HqsK.

In the present study, we studied the effect of NO on the expression of the genes encoding NnrS and NrfA to clarify whether these enzymes participate in NO detoxification in *V. vulnificus*. Moreover, we tested the NO-dependence of the QS cascade of *V. vulnificus*.

To test the effect of NO on the bacterial growth, *V. vulnificus* strain L-180, a clinical isolate, was grown at 37°C in 5 mL of TYE broth (0.5% tryptone, 0.25% yeast extract, 2.0% NaCl, 25 mM K_2HPO_4, pH 7.5) overnight with shaking. Then, an aliquot of the culture was taken and inoculated into fresh TYE broth supplemented with 0 to 200 μM NO donor DPTA NONOate (dipropylenetriamine NONOate) to yield 0 to 300 nM NO as described by Henares et al. (2012). Thereafter, the bacterium was cultivated at 26℃, around the estuarine temperature in the summer season, or 37°C, around the human intestinal temperature, and the growth was monitored by measuring the optical density at 600 nm (OD_{600}).

As shown in FIG. 1, *V. vulnificus* multiplied very slowly in the presence of 200 μM DPTA NONOate, indicating the significant toxic effect of 200 μM DPTA NONOate on *V. vulnificus* cells. However, the bacterium could tolerate 50 or 100 μM DPTA NONOate at either temperature, because the bacterial growth was nearly the same as that in the absence of the NO donor. This finding suggests that the NO-detoxifying enzymes were

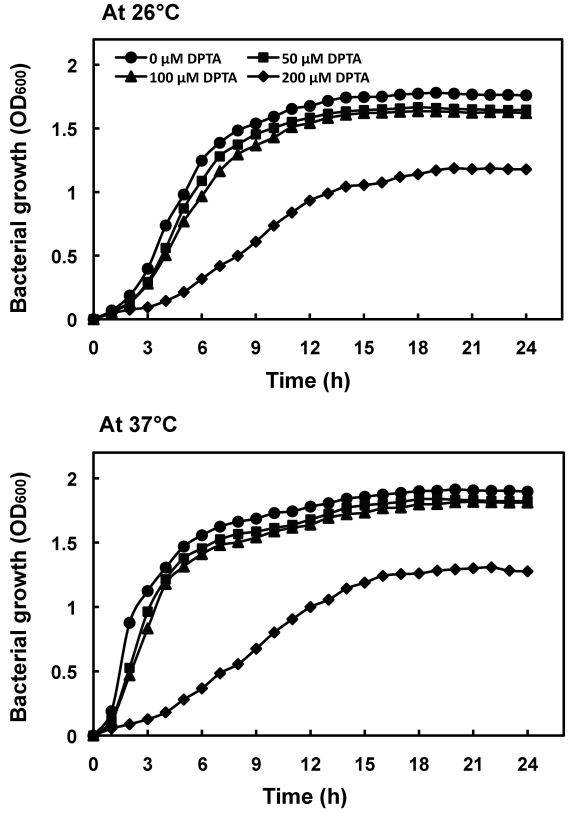

FIG. 1. Growth of *V. vulnificus* strain L-180 in the presence of DPTA NONOate. Strain L-180 was cultivated in TYE broth supplemented with 0, 50, 100 or 200 μM DPTA NONOate at 26℃ or 37℃, and the bacterial growth (OD_{600}) was measured. Data represent the mean of three experiments.

TABLE 2. Oligonucleotide primers used in the study

Gene	Accession No.	Nucleotide sequence (5' ~ 3')		Position
nnrS	CP002469	Forward	GCTAATTTTGCCAGCTACGC	469—488
		Reverse	TAAAGCGACGTGCGGTAAAG	594—613
nrfA	CP002469	Forward	GATGCCTTAGCAGAAGATCC	190—209
		Reverse	GTCTGACATCATCCAATGCG	279—298

induced when *V. vulnificus* was grown in the presence of the sub-lethal doses of NO, as in the case of *Salmonella enterica* and *Vibrio cholerae* (Mills et al., 2008, Stern et al., 2012).

Next, the expression of *nnrS* and *nrfA* genes, which encode enzymes to convert NO to less toxic molecules, were tested by real time reverse transcription PCR (RT-PCR). Strain L180 was cultivated in TYE medium supplemented with or without sub-lethal/non-toxic doses of DPTA NONOate, and the bacterial cells were collected at middle-log or early stationary phase. Then, total RNA was prepared by using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual.

RT-PCR was performed with the MiniOpticon™ real time PCR detection system (Bio-Rad, Berkeley, California, USA) and the iScriptTM one-step RT-PCR kit (Bio-Rad). The reaction mixture consisting of 12.5 μL of SYBR® Green PCR Master Mix, 0.75 μL of the suitable primers (TABLE 2), 0.5 μL of iScriptTM one-step RT-PCR reverse transcriptase, 0.5 μL of the total RNA (100 ng/μL) and 10.75 μL of nuclease free water was incubated at 50℃ for 10 min for the RT reaction. Thereafter, the reverse transcriptase was inactivated by heating at 95℃ for 5 min, and PCR amplification of the target gene was performed for 40 cycles of denaturation at 95℃ for 10 sec, and annealing and extension at an appropriate temperature for 30 sec.

DNA polymerization was conducted in a range of temperatures from 55°C to 95℃ within 20 min to obtain the melting curve for determining the PCR amplification specificity. The RT reaction without the reverse transcriptase was used as a negative control, and 16s rRNA was used as an internal standard. The amount of each mRNA was determined by comparing the C_t value to 16s rRNA. The results indicated that the expression levels of both *nnrS* and *nrfA* genes were significantly increased in the presence of 100 μM DPTA NONOate (FIG. 2). Therefore, two genes encoding NO-detoxifying enzymes may be highly inducible by the addition of NO to growing *V. vulnificus* cells. This finding is in accordance with the studies in other bacteria including *Vibrio cholerae* (Stern et al., 2012).

In *V. vulnificus*, the proteolytic enzyme VVP/VvpE is the best-known extracellular factor regulated positively by the QS cascade (Kawase et al., 2004; Elgaml et al., 2014). Although V. vulnificus may have several genes encoding proteolytic enzymes, VVP/VvpE is the sole extracellular enzyme (Wang et al., 2008). Thus, the effects of the addition of the NO donor on the protease activities in the culture supernatants were tested to clarify whether NO can act as a signaling molecule to activate the QS cascade.

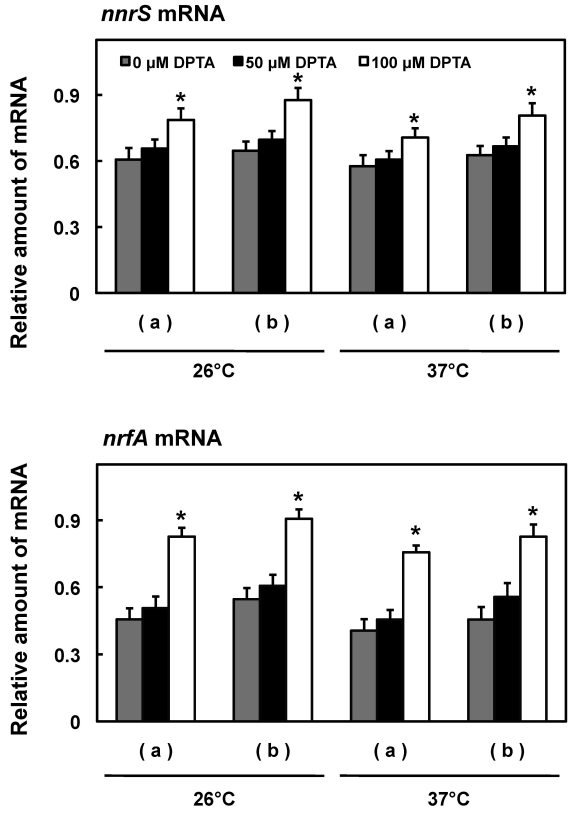

FIG. 2. Effect of DPTA NONOate on the expression of *nnrS* and *nrfA* in *V. vulnificus*. Strain L-180 was cultivated in TYE supplemented with 0, 50 or 100 μM DPTA NONOate at 26℃ or 37℃, and total RNA was extracted at the middle-log (a) or early stationary (b) phase, and the amount of *nnrS* or *nrfA* mRNA was measured by real time RT-PCR. The amount of mRNA was represented using the amount of 16s rRNA as 1.00. The data reflect the mean + S.D. of three experiments. The asterisk indicates significant difference ($P < 0.05$).

Strain L-180 was grown in TYE medium supplemented with or without DPTA NONOate, and at the middle-log or early stationary phase, the cell free culture supernatants were obtained by centrifugation at

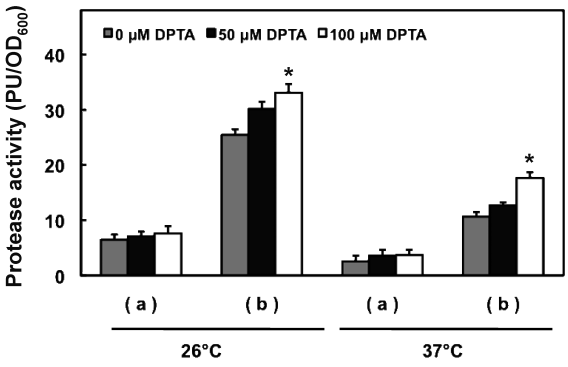

FIG. 3. Effect of DPTA NONOate on the protease production by *V. vulnificus*. The culture supernatants were prepared from the middle-log (a) or early stationary (b) phase of the growth of strain L-180, which was cultivated in TYE broth supplemented with DPTA NONOate (0, 50 or 100 μM) at 26℃ or 37℃. The protease activity (PU/ml) was determined by using azocasein as the substrate, and the specific protease activity (PU/OD_{600}) was calculated. The data reflect the mean + S.D. of three experiments. The asterisk indicates significant difference ($P < 0.05$).

12,000 g for 5 min at 4℃. The protease activity of VVP/VvpE was assayed with azocasein (Sigma-Aldrich, St. Louis, Missouri, USA) as described by Miyoshi et al. (1987). Briefly, the sample was allowed to act at 30℃ for an appropriate time on 1.0 mg of azocasein in a total volume of 0.6 mL of 50 mM Tris-HCl buffer (pH 8.0). The reaction was stopped by the addition of 1.4 ml of 5% trichloroacetic acid. After centrifugation at 5000 g for 5 min, an aliquot of the supernatant was withdrawn and mixed with the same volume of 0.5 M NaOH. Thereafter, the absorbance at 440 nm was measured. One protease unit (PU) was defined as the amount of VVP/VvpE that digests 1 μg of azocasein in 1 min. As shown in FIG. 3, the addition of the NO donor caused significant increase in the protease activity, namely production of VVP/VvpE, at both 26 and 37℃ in a dose dependent manner.

Taken together, our data suggest the presence of the NO-dependent detoxification and regulation systems in *V. vulnificus*. In the former system, the enzymes including NnrS and/or NrfA may contribute to NO detoxification. In the later system, NO may participate in the QS cascade. It may be reasonably conjectured that NO is sensed by the NO sensor H-NOX, then NO/H-NOX blocks the kinase activity of HqsK, which results in the inactivation of the response regulator of the QS cascade and in the activation of the cascade.

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