Neutralization of radical toxicity by temperature-dependent modulation of extracellular SOD activity in coral bleaching pathogen Vibrio shiloi and its role as a virulence factor

Malliga Raman Murali · Subramaniya Bharathi Raja · Sivasitambaram Niranjali Devaraj

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Abstract Vibrio shiloi is the first and well-documented bacterium which causes coral bleaching, particularly, during summer, when seawater temperature is between 26 and 31°C. Coral bleaching is the disruption of the symbiotic association between coral hosts and their photosynthetic microalgae zooxanthellae. This is either due to lowered resistance in corals to infection or increased virulence of the bacterium at the higher sea surface temperature. The concentration of the oxygen and resulting oxygen radicals produced by the zooxanthellae during photosynthesis are highly toxic to bacteria, which also assist corals in resisting the infection. Hence, in this study we examined the effect of different temperatures on the activity of a novel extracellular SOD in V. shiloi. We also partially characterized the SOD and clearly confirmed that the extracellular SOD produced by V. shiloi is Mn–SOD type, as it was not inhibited by H$_2$O$_2$ or KCN. Performing chemical susceptibility killing assay, we confirmed that extracellular SOD may act as first line of defense for the bacteria against the reactive oxygen species. Since, increased activity of novel Mn–SOD at higher temperature, leads to the neutralization of radical toxicity and facilitates the survival of V. shiloi. Hence, the extracellular Mn–SOD may be considered as a virulence factor.

Keywords Vibrio shiloi · Mn–SOD · Reactive oxygen species · Chemical susceptible test

Introduction

Coral reefs are spectacular, massive limestone structures of marine eco system. Corals live in a symbiotic relationship with endosymbiotic algae, zooxanthellae. The zooxanthellae are located within vacuoles in the cells of the host endoderm (Glider and Phipps 1980). The zooxanthellae serve as primary producers and supply their coral host with up to 95% of their photosynthetic products, such as amino acids, carbohydrates and small peptides (Trench 1979). In return, the zooxanthellae receive essential nutrient compounds of ammonia and phosphate from the waste metabolism of the coral (Trench 1979). This tight recycling of nutrients within the association minimizes the loss of nutrients (Muscatine and Porter 1977; Falkowski and Dubinski 1984) and facilitates the high productivity of corals.

Their biological diversity, high productivity, and beauty have made coral reefs one of the most valuable ecosystems on earth. Despite their persistence over millions of years, coral reefs appear to be one of the most vulnerable marine ecosystems. In the past few decades, there has been a large increase in the number of reports on diseases affecting coral reefs. Of particular significance is the coral bleaching disease, which had caused extensive damage to coral reefs throughout the world. Coral bleaching is the disruption in the symbiotic relationship between coral hosts and photosynthetic microalgal endosymbionts, referred to as zooxanthellae (Brown 1997).

Bleaching results in a reduction in zooxanthellae density in the coral’s gastrodermal tissues and/or from decreased...
concentrations of photosynthetic pigment in the algal cells. The loss of zooxanthellae greatly affects the coral host, because these photosynthetic symbionts supply up to 63% of the coral’s nutrients most coral bleaching episodes have been correlated to elevated seawater temperatures (Brown 1997). Even though many causative agents were reported earlier, association of bacteria in bleaching had gained importance after the report on *Oculina patagonica* (coral) bleaching in the Mediterranean Sea due to *Vibrio shiloi* infection (Kushmaro et al. 1996).

*V. shiloi* is a gram-negative, invasive, motile, rod-shaped bacterium (2.4 x 1.6 μM) that has a single polar, sheathed flagellum (Rosenberg and Falkovitz 2004). The infection of corals by *V. shiloi* occurs only at elevated seawater temperature of 30°C. The proposed mechanism of bleaching by *V. shiloi* is that, at elevated sea water temperature, *V. shiloi* adheres to methyl β-d-galactopyranoside receptor of the coral mucus (Toren et al. 1998), penetrates into the host cell (Banin et al. 2000) and produces an extracellular toxin (a proline-rich peptide) which transports ammonia (produced from metabolism of host proteins) into the algal cells (Banin et al. 2001a, b), thereby inhibiting the photosynthesis (Ben-Haim et al. 1999) and lysing the algal cells leading to the bleaching of corals.

Several critical *V. shiloi* virulence factors are produced only at summer seawater temperature (26–31°C) but not at winter seawater temperature (15–20°C). Thus, summer seawater temperatures make *V. shiloi* virulent by causing the expression of genes required for a successful infection (Banin et al. 2000). As Rosenberg and Falkovitz (2004) describe it, the high concentration of oxygen and resulting oxygen radicals produced by the zooxanthellae during photosynthesis is highly toxic to bacteria and is one mechanism by which corals resist infection. Hence, in this study we examined the role of SOD in *V. shiloi* and their modulation by different temperature.

**Materials and methods**

**Bacterial strains and growth conditions**

*Vibrio shiloi* (LMG 19703) was purchased from BCCM™/LMG Bacteria collection, Laboratory for Microbiology, Gent University, Belgium. *V. shiloi* was routinely cultivated in Zobell Marine broth medium from HiMedia™ (1.8% Marine broth and 0.9% NaCl) or, on MB agar (MB medium solidified with 1.8% Bacto agar). The pH of the medium was adjusted to 7.4 and sterilized for 20 min at 15 psi. The plates were incubated at 30°C for 48 h. The cultures were stored for further use at −70°C in 15% glycerol.

**Effect of temperature**

*Vibrio shiloi* were grown at two different temperatures, 23 and 30°C in Marine broth at 150 rpm in an orbital shaker.

**Bacterial SOD activity**

*Vibrio shiloi* cultures grown to exponential phase were pelleted by centrifugation (6,000 x g, 10 min, 4°C). The supernatant was subjected to ammonium sulfate precipitation (40–60% saturation) to obtain secretory protein fraction. Secretory protein was subjected to 12% PAGE under nondenaturing conditions. Prior to the application of the sample, a current was applied to each gel in the Tris (187.5 mM)–EDTA (1 mM) buffer for 30 min to remove radicals such as ammonium persulfate. Protein were then separated by electrophoresis in the presence of Tris (50 mM), glycine (300 mM) and EDTA (1.8 mM) at constant current (40 mA). Wells were loaded with equal concentration of proteins, which were quantified by the method of Lowry et al. (1951). SOD activity was demonstrated as an achromatic zone in a blue background after photoactivation of riboflavin and reaction with nitroblue tetrazolium as described by Sun et al. (1988). Briefly, following electrophoresis, the gel was placed in the dark and soaked in a SOD staining solution containing riboflavin (0.028 mM), nitroblue tetrazolium (0.25 mM), EDTA (1 mM), and TEMED (28 mM) in 50 mM KPi. After 20 min incubation, the staining solution was removed and replaced with 50 mM KPi. The gel was then exposed to light. Areas of SOD activity appeared as achromatic bands in a blue background.

**Determination of SOD type**

To determine the nature of the extracellular SOD, prior to loading the gel, the samples were pretreated for half an hour with a final concentration of 5 mM H2O2 (inactivates Fe–SOD) or 0.1 M KCN (inactivates CuZn–SOD); Mn–SOD is resistant to both treatments (Dunlap and Steinman 1986).

**Electroelution**

The SOD band of *V. shiloi* was excised and electroeluted at 25 V for 20 min at 4°C. 50 mM potassium phosphate buffer (KPi), pH 7.8 was used as both tank and elution buffer. The eluted protein was assessed for its enzyme activity by running on a native PAGE as described above and the molecular weight of the same was assessed by running on a SDS-PAGE according to Laemmli (1970).
Chemical susceptibility assays

Chemical susceptibility killing assays were performed with 0.1 unit/ml xanthine oxidase and varying concentration of hypoxanthine ranging from 50 to 250 μM added to overnight cultures diluted 1:100 in PBS and inoculated to fresh MB medium and incubated for 18 h. Serial dilutions were plated in triplicate at timed intervals for quantitation of colony-forming units.

Results and discussion

Effect of temperature on growth

The growth *Vibrio shiloi* was temperature-dependent. Bacterial isolates grew less well at 23 compared to 30°C (Fig. 1). Bacteria enter into the exponential phase after 6–8 h of growth and the stationary phase was reached after 18–20 h of growth when grown at 30°C. At the lower temperature (23°C), it took a longer period of time for the bacteria to enter into the exponential phase (12 h) and the stationary phase (24 h). This may be due to the fact that the bacteria have to adapt to the cold environment before entering the exponential phase of growth.

Effect of temperature on SOD activity

Figure 2a represents extracellular superoxide dismutase (SOD) profiles and Fig. 2b represents extracellular protein profiles, of *V. shiloi* grown at 23 and 30°C. Only one band of SOD was apparent in *V. shiloi* grown at both the temperatures and showed significant difference in the expression of this antioxidant enzyme, respective to the growth temperature. Earlier, Munn et al. (2008) also reported that, in the Vibrios associated with coral there was significantly increased in activity of extracellular SOD when the cultures were grown at 28°C when compared to cultures were grown to 16°C.

Partial characterization of SOD

Figure 2c shows that the extracellular SOD was not inhibited by pretreatment of the samples with H$_2$O$_2$ and KCN indicating that the SOD produced was not Fe–SOD or CuZn–SOD and may be Mn–SOD.

When the *V. shiloi* SOD was electroeluted and analyzed by non-reducing SDS-PAGE and prior boiling in an SDS sample solubilising buffer that lacked β-mercaptoethanol,
a prominent band appeared in the ~66 kDa region (Banin et al. 2003). When the same sample was run on an SDS-PAGE after reduction and boiling the sample, the band was retained in the same region (~66 kDa) (Fig. 3, lanes 2 and 3) suggesting that the SOD was monomeric in nature.
SOD as virulence factor

To examine role of SOD as virulence factor, we had performed bacterial susceptibility to chemical generated oxygen radical toxicity by reaction of hypoxanthine–xanthine oxidase method. Fig. 4a–f shows viable *V. shiloi* grown at 23 and 30°C in chemically generated oxygen radical toxicity, formed by reaction of various concentration of hypoxanthine ranging from 50 to 250 μM and 0.1 U of xanthine oxidase/ml. *V. shiloi* grown at 30°C is more viable to chemically generated oxygen radical toxicity, formed by reaction of various concentration of hypoxanthine ranging from 50 to 200 μM and 0.1 U of xanthine oxidase/ml (Fig. 4b–e) when compared to viability of *V. shiloi* grown at 23°C. Some amount of susceptibility was observed in *V. shiloi* grown at 30°C to oxygen radical toxicity generated by reaction of hypoxanthine from 250 μM and 0.1 U of xanthine oxidase/ml (Fig. 4f). In external stress (high level of free radical), the viability of *V. shiloi* was greatly reduced at 23°C when compared to viability of *V. shiloi* grown at 30°C. These results can be correlated to increased enzymatic activity of SOD at 30°C when compare to low level SOD expression in *V. shiloi* grown at 23°C.

SOD provides the first line of defense for the bacteria against the toxic effects of reactive oxygen species. It has been already reported that *Vibrio fischeri* (Ruby and McFall-Ngai 1999) and *V. shiloi* must produce SOD for the successful colonization of their host and the *V. shiloi* mutants deficient in SOD production failed to survive in their host environment (Banin et al. 2003). In the present study it was observed that there was change in expression of SOD with respect to variation in growth temperature and hence it may be concluded that *V. shiloi*, when it infects the coral host environment (Banin et al. 2003a,b) provides the first line of defense for the bacteria against the toxic effects of reactive oxygen species during host–pathogen interaction. Hence, it can be considered as a virulence factor.


References


