Virulent Factors of Coral Pathogen *Serratia Marcescens* Associated With White Pox Disease *Acropora Formosa*, Palk Bay, Southeast Coast of India

R.Anithajothi*, G.Umagowsalya, K.Duraikannu, and C.M.Ramakritinan

***Abstract*----**Diverse forms of microorganisms are present in coral holobiont, which not only comprises microbial communities; it also comprises coral animal and endosymbiotic dinoflagellates. Endosymbiotic microbes play a vital role in maintaining coral health. Bacterial communities utilizing the enzyme protease for their colonization on coral host. Colonizing bacteria produced enzymes such as SOD, CAT to overcoming the toxic effects of oxidative stress produced by coral host. In the present study virulent factors of pathogens and endophytic microbes such as protease, SOD, CAT, chemotatic ability and adhesion ability towards coral mucus were studied. Chemotactic ability, adhesion ability and enzyme activities were higher in the pathogen. Upon entry pathogen conferred protection against oxidative stress. We conclude that the enzyme activities such as protease, SOD, CAT, Chemotactic ability and adhesion ability are considered to be major factors in the virulence of *Serratia marcescens* and their main function is to provide protection against oxidative stress.

***Key words*----**Coral pathogen, Oxidative stress, *Serratia marcescens*, White Pox Disease, Virulent factors.

I. Introduction

CHEMOTAXIS is one of the main driving forces for causing infection in the host corals and also important factors of many pathogenic organisms [1], [2] and the migration of pathogenic microorganisms towards suitable host and nutrients indicating that their ability to infect the host involves not only mobility but also chemotaxis [3], [4].

The chemotactic ability of coral pathogenic microorganism that is moving in response to chemical stimuli where the establishment of association with the host [5] and the exploitation of chemical environments may be initially important. Zooxanthellae exudates are the major components in the coral mucus, which act as chemo attractants (specific compound required for adhesion of bacteria) of coral pathogen [3], [6].

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II. Materials And Methods

A. Chemotaxis

Modified Capillary Tube assay was carried out to measure the chemotaxis ability of pathogens [3]. Bacterial cells were grown at 30°C in tryptic soy broth supplemented with 1.2% NaCl and harvested by centrifugation at 5000 x g for 20 minutes. The supernatant was discarded and the cells were washed repeatedly by resuspending in phosphate buffered saline. The cells were pelleted again by centrifugation at 5000 x g for 20 minutes. Finally the pellet was resuspended in an equal volume of sterile distilled water.

Two hundred microlitre of this bacterial suspension was taken in microcentrifuge tubes. The capillary tubes were heated and sealed at one end which containing the substrates i.e., coral mucus and another set of tubes containing 10µl of PBS (control). The tubes were inserted into 1mm below the culture surface and incubated for 60 minutes at room temperature. Then the capillaries were removed and their contents were expelled into 0.3ml of sterile seawater. Bacterial viable counts were calculated using TSA (Tryptic soy agar) agar plate. The chemotatic ability of bacterial isolate was expressed as the ratio of bacterial density in the capillary containing mucus and the bacterial density of the capillary containing 10µl PBS.

B. Adhesion experiment

Adhesion experiment was performed to check the adherence ability of coral pathogen, *Serratia marcescens* to coral. This experiment was conducted simultaneously with infection experiment. Four tanks were used to conduct the adhesion experiment. Two of them were inoculated with pathogen and rests of them were inoculated with non-pathogenic bacteria. Aquarium setup made in such a way that the tank 'PWCF' containing coral fragments challenged with pathogen and 'PWOCF' tank containing without coral fragments inoculated with pathogen, 'CWCF' containing coral fragments challenged with non-pathogenic bacteria and 'CWOCF' tank containing without coral fragments inoculated with non-pathogenic bacteria. The water samples in each tank were collected at 0 h, 6 h, 12 h and 24 h. Viable bacterial counts were performed by plating an aliquot of 100µl water sampled from each tank.

C. Screening for protease activity

Isolated bacterial colonies were plated onto skim milk agar plates (Nutrient agar with 1% skim milk agar powder with 3%

NaCl) and incubated at 37°C for 36 h. A clear zone of skim milk hydrolysis indicated the presence of protease producing organisms. The proteolytic activity was expressed as diameter of clear zones in mm.

D. Effect of temperature on protease activity

The assay was carried out in the reaction mixture by incubating 500µl of 0.5% azocasein in Tris-HCl buffer with 100µl enzyme solution for 60 minutes at 37°C. Reaction was stopped by adding 500µl of 15% Trichloroacetic acid (TCA) solution with shaking [7]. This was left for 15 minutes and centrifuged at 3000rpm at 4°C for 15 minutes. The protein in the supernatant was estimated as described by [8]. One unit of protease activity is defined as the amount of enzyme which liberates one microgram of tyrosine per ml per minute (Tyrosine $ml^{-1}.min^{-1}$) under experimental condition. The protein content in the supernatant was determined at 650nm according to [8].

SOD activity was measured using the method described by Zhang and Qu, (2003). One unit of SOD was defined as the amount of enzyme necessary to produce a 50 % inhibition of the NBT photoreduction. The activity was expressed as Unit per mg protein ($U.mg^{-1}$) [9].

Catalase activity was measured according to the method of Matsumura *et al*., (2002). One unit was defined as mM of H_2O_2 decomposed per minute and the activity referred to milligrams of protein (Units. mg^{-1} protein) [10].

E. Statistical analysis

The data were analyzed using One Way parametric analysis of variance ANOVA for each coral species. Data (means ± SE, n = 3) analyzed by one-way analysis of variance (ANOVA) with the GraphPad Prism 5 software and the least significant differences were compared at $p < 0.05$.

III. Results

A. Chemotaxis

The chemotatic ability of bacteria *Serratia marcescens* towards coral mucus obtained from the healthy coral fragments was checked at two different temperatures such as 20 and 30ºC using Phosphate Bufferd Saline (PBS) as control. At 30°C, the chemotactic ability of *Serratia marcescens* towards PBS control was 0.5×10^4 cells ml^{-1} and in the mucus of *A. formosa*, this was 10×10^4 cells ml^{-1}. At 30ºC, the attraction was 20 times higher than that of PBS control (Figure 1). At 20ºC, the bacteria attracted by coral mucus was 3.5×10^4 cells ml^{-1} while it was 0.3×10^4 cells ml^{-1} in PBS control. The ability of adherence was 11.5 times higher than that of bacteria attracted by the control PBS at 20ºC (Figure 1). The above results states that the chemotactic ability was higher at 30°C.

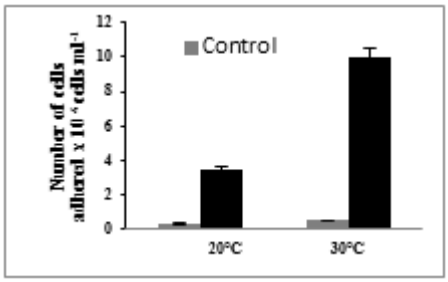

Fig. 1 Chemotatic ability of *Serratia marcescens* towards coral mucus and PBS control at 20 and 30ºC (n= 3)

The findings of the present study reveal that the chemotactic response of S. *marcescens* towards the coral mucus was 20 times higher at 30°C and this was only 11.5 times higher at 20°C when compared with the chemotactic ability of the respective PBS control. This concludes that chemotactic response was higher at elevated temperature. This coincides with the chemotatic response of *Vibrio shiloi* mucosal layer towards *Oculina patagonica* under elevated temperature [11], [12], [13]. The similar chemotactic response has been reported when the coral *Pocillopora damicornis* was infected by *Vibrio coralliilyticus* [14]

B. Adhesion experiment

To check the adhesion ability of the pathogenic bacteria and non-pathogenic bacteria, adhesion experiment was conducted simultaneously as a part of infection experiment. The number of colony farming unit (CFU) calculated for a total period of 24h and presented in Figure 2.

The CFU values in CWCF i.e., inoculated with non-pathogenic bacteria reduced to 6.67% after 24h incubation. At 0^{th} hour, it was 3.0×10^5cells ml^{-1} which declined to 2.7×10^5cells ml^{-1} within 24h incubation. The CFU in PWCF at the 0^{th} h was 2.74×10^5 cells ml^{-1} and it was declined to 0.3×10^5 cells per ml within 24h inoculation. Coral pathogen demonstrated 90% reduction in mean CFUs in seawater samples taken from tank with *Acropora formosa* coral fragments inoculated with pathogenic bacteria with in 24h.

Similarly the mean CFU values observed in PWOCF (Water sample collected from the aquarium inoculated with pathogenic bacteria without coral fragments) and CWOCF tanks were given in Figure 18. The CFU values observed in PWOCF tank was 2.96×10^5cells ml^{-1} at 0^{th} hour and this declined to 2.84×10^5cells ml^{-1} within 24h of inoculation. The percent reduction in CFU was 4.1%. The CFU values observed in CWOCF tanks was 2.84×10^5cells ml^{-1} at 0^{th} hour and it declined to 2.80×10^5cells ml^{-1} within 24h. The reduction in CFU value was about 1.4% (Figure 2). This decline was insignificant. The declining in CFU value of seawater samples collected from the tank containing coral fragments reveals that the pathogenic bacteria was adhered by the coral fragments.

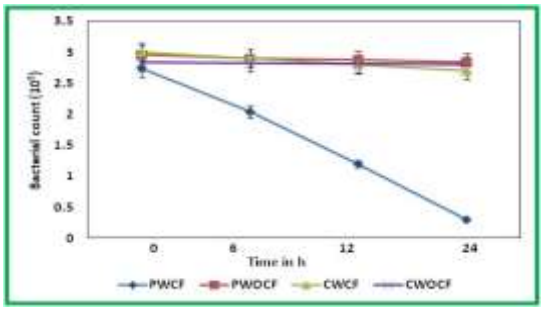

Fig. 2 Adhesion ability of pathogenic bacteria and non pathogenic bacteria with and without coral fragments

- PWCF - Water sample collected from the aquarium containing coral fragments inoculated with pathogenic bacteria.
- PWOCF - Water sample collected from the aquarium inoculated with pathogenic bacteria without coral fragments
- CWCF - Water sample collected from the aquarium containing coral fragments inoculated with non-pathogenic bacteria
- CWOCF - Water sample collected from the aquarium without coral fragments inoculated with non-pathogenic bacteria.

The adhesion experiment conducted in the present study reveals that 90% of coral pathogenic bacteria, *S. marcescens* entered into the coral host within 24 h but the adhesion ability of non-pathogenic bacteria, *H. meridiana* was about 6.7% only. Krediet *et al.,* (2009) have been reported that the population density of *S. marcescens* on mucus was higher (7 x 10^8 cfu.ml^{-1}) than that of *H. meridiana* (8 x 10^7 cfu.ml^{-1}) [15]. Similarly Banin *et al.,* (2001) demonstrated the adhesion ability of *Vibrios* to gastrointestinal tract of fish and coral mucus [11].

In the present study, the coral fragments, *A. formosa* was individually inoculated with the pathogenic bacterial isolates such as *S. marcescens* (SMC) (CP2) and *S. marcescens* (CPI) and a non-pathogenic bacteria *H. meridiana* (CP3). Of these, CP1 and CP3 were isolated from diseased coral *A. formosa* while CP2 *S. marcescens* was obtained commercially available pathogenic bacteria. This study concludes that the infection was higher in *S. marcescens* (CP1) i.e., 75% than that of *S. marcescens* (SMC) (25%) and *H. meridiana* (8.3%). The non-pathogenic bacteria noticed very less infectivity. Further the putative pathogen was able to cause infection followed by inoculation thereby satisfied Hill's criterion 4 [16]. *Serratia marcescens* (pathogen causing WPD in the Caribbean elkhorn coral, *Acropora palmata*), a human pathogen collected from wastewater, found to be a potential pathogen which able to produce disease signs of White Pox Disease within a short span of time i.e., 4-5 days [17] in controlled conditions. The control fragments were unable to perform the transmission of disease, suggesting that motility towards corals and the adhesion may be the factors that involved in pathogenecity of the pathogen [18].

C. Bacterial Protease activity

The proteolytic activity of coral pathogenic bacteria, *Serratia marcescens* exhibited the highest proteolytic activity with a clear zone of 20mm diameter (Figure 3) after 36h incubation. Although, the other bacterial strains isolated from the corals such as *Bacillus* sp., *A. faecalis* and *H.meridiana* exhibited the clear zones of 2.5, 5 and 9mm diameter, respectively (Figure 3).

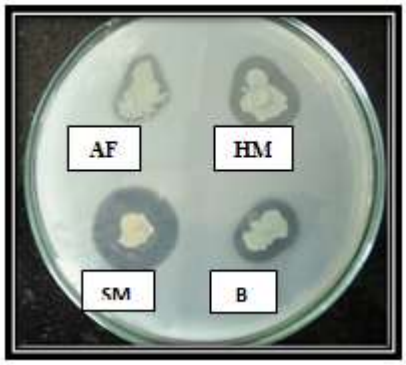

Fig. 3 Bacterial protease activity of pathogenic bacteria isolated from WPD corals.
AF- *Alcaligens faecalis,* HM-*Halomonas meridiana,*
SM - *Serratia marcescens,* B- *Bacillus* sp.

The proteolytic activity of an organism is one of the virulent factors indicating the stress. Proteolytic activity of *S. marcescens* is a probable component of the etiology White Syndrome (WS) through screening of diseased and non-diseased corals [18]. In the present study, it is reported that *S. marcescens* showed highest proteolytic activity than that of *Bacillus* sp., *A. faecalis* and *H. meridiana*. Hence the protease produced by *S. marcescens* serves as a virulent factor [19] and it is reported to cause white pox disease in coral associated with faecal contamination [20]. *S. marcescens* is known to produce both extracellular and cell-associated proteinases [21] which are essential for the breakdown of glycoproteins in the coral mucus because the coral mucus is composed of components like glycoproteins, galactose, fucose, N-acetylgalactosamine, N-glucosamine, sialic acid and mannose [22].

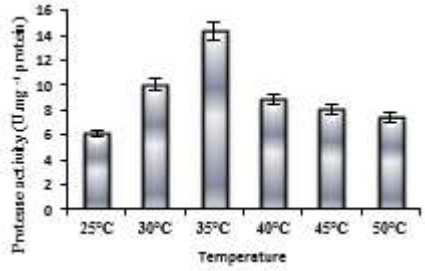

Fig. 4 Effect of temperature on protease activity of pathogenic bacteria *Serratia marcescens*

The effect of temperature on the protease activity of *S. marcescens* was determined by incubating the reaction mixture for 24h between 25 and 50°C at intervals of 5°C (Figure 4). The protease activity was 6.12, 10.2, 14.33, 8.85, 8.03 and 7.36 U.mg $^{-1}$ protein at 25, 30, 35, 40, 45 and 50°C, respectively. Maximum protease activity noticed at 35°C. Below and above these temperatures, there was a significant reduction in the protease activity (Figure 4). The above results

indicate that the optimum temperature for protease activity was 35°C.

Temperature is one of the important factors influencing the enzyme activities. Increasing the temperature of the environment enhances the production of proteolytic enzymes in the animal tissues; while this was maximum at optimum temperature but further increasing or decreasing the temperature inhibit the production of enzymes. The present study shows that the proteolytic activity of pathogenic microorganism increased with increasing the temperature and reached maximum activity at 35°C but further it declined with increasing the temperature [23, 24]. Elevated temperature enhances the production of protease which is one of the important virulent factors of coral pathogen [23].

D. Bacterial Superoxide Dismutase (SOD) activity

The SOD activity of pathogenic and non-pathogenic bacteria isolates such as *Enterococcus* sp., *H. meridiana* and *S. marcescens* was estimated and presented in Figure 5. The coral pathogen, *S. marcescens* showed the highest SOD activity when compared with *Enterococcus* sp. and *Halomonas meridiana* and the activity was 4.4, 4.65 and 6.0 U.mg $^{-1}$ protein, respectively (Figure 5). There was a insignificant variation in SOD activity between *Enterococcus* sp. and *H. meridiana* while *S. marcescens* showed significant increase in SOD activity than that of *Enterococcus* sp. and *H. meridiana* (Figure 5) The increase in activity was 1.4 and 1.3 times higher (ANOVA $p<0.13$) in *H. meridiana* and *S. marcescens* respectively that of *Enterococcus* sp.

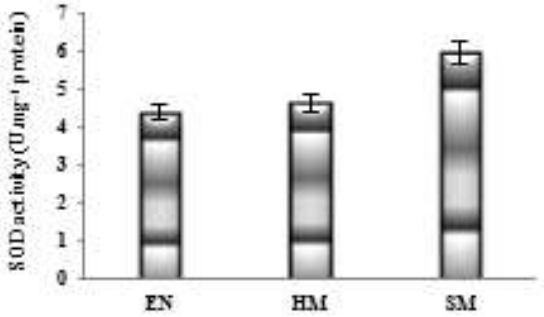

Fig. 5 Superoxide Dismutase activity of *Enterococcus* sp. (EN); *Halomonas meridiana* (HM) and *Serratia marcescens* (SM) isolated from coral *A. formosa.*

E. Bacterial catalase (CAT) activity

Similarly the CAT activity of pathogenic bacteria was presented in Figure 6. *Serratia marcescens* showed maximum CAT activity than that of *Enterococcus* sp. and *Halomonas meridiana* and the activity was 1.5, 0.75 and 0.4units.mg $^{-1}$ protein, respectively. The activity of *Serratia marcescens* and *Halomonas meridiana* was 3.8 and 2 folds higher than that of *Enterococcus* sp. (ANOVA $p<0.08$), respectively. There was a significant variation in CAT activity between all three bacterial isolates.

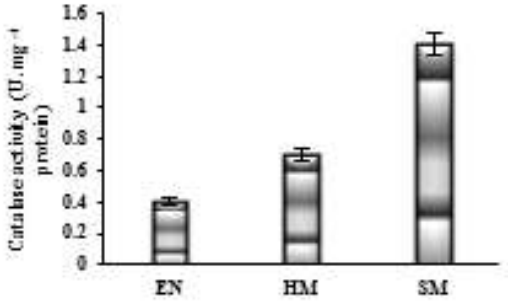

Fig. 6 Catalase activity of *Enterococcus* sp. (EN), *Halomonas meridiana* (HM) and *Serratia marcescens* (SM) isolated from coral *A. formosa.*

The superoxide dismutase activity of bacterial strains such as *Enterococcus* sp., *S. marcescens and H. meridiana* noticed that *S. marcescens* exhibited higher SOD activity than that of other two bacteria i.e., commercial and non-pathogenic organisms. Ben Haim *et al.*, (2003b) have been reported that SOD allows the bacteria to live in oxygen rich environments of corals. The highest SOD activity observed in the pathogenic bacteria *S. marcescens* added the strength to its pathogenic potential [23]. Banin *et al.*, (2003) states that, if the bacteria fail to produce SOD, it cannot able to survive within the host by overcoming the toxic effects of oxidative stress. Similar findings were reported by [25]. The enhancement of SOD and CAT activities in the pathogenic bacteria, *S. marcescens* indicates that a possible role of these enzymes in bacterial survival inside the coral host. Bacterial isolates able to enter into the coral tissue has to produce enzymes such as SOD and CAT in order to overcome the toxic effects of oxidative stress [25].

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