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Characterization of putative virulence factors of *Serratia marcescens* strain SEN for pathogenesis in *Spodoptera litura*

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ABSTRACT

Two *Serratia marcescens* strains, SEN and ICC-4, isolated from diseased insect cadavers were observed to differ considerably in their virulence towards *Spodoptera litura*. The present study was aimed to characterize the possible virulence factors present in the virulent *Serratia marcescens* strain SEN. Both the *S. marcescens* strains were evaluated for the presence of various lytic enzymes such as chitinase, lipase, protease and phospholipase. The virulent *S. marcescens* strain SEN was observed to possess considerably higher activity of chitinase and protease enzymes; activity of phospholipase enzyme was also higher. Although, all the three toxin genes *shlA*, *phlA* and *swr* could be detected in both the *S. marcescens* strains, there was a higher expression of these genes in the virulent strain SEN. *S. marcescens* strain ICC-4 showed greater reduction in overall growth yield in the post-exponential phase in the presence of midgut juice and hemolymph of *S. litura* larvae, as compared to *S. marcescens* strain SEN. Proliferation of the *S. marcescens* strain SEN was also considerably higher in foregut, midgut and hemolymph of *S. litura* larvae, as compared to strain ICC-4. Peritrophic membrane treated with broth culture of the *S. marcescens* strain SEN showed higher damage as compared to strain ICC-4. The peritrophic membrane of larvae fed on diet treated with the virulent strain showed considerable damage while the peritrophic membrane of larvae fed on diet treated with the non-virulent strain showed no damage. This is the first report documenting the fate of ingested *S. marcescens* in *S. litura* gut and the relative expression of toxin genes from two *S. marcescens* strains differing in their virulence towards *S. litura*.

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1. Introduction

Serratia marcescens, a Gram negative bacterium belonging to Enterobacteriaceae, is well known for its role as an insect pathogen (Grimont and Grimont, 1978). *S. marcescens* has been isolated from healthy, moribund and dead insects belonging to Orthoptera, Coleoptera, Hymenoptera, Lepidoptera and Diptera by various workers (Lepesme, 1937; Krieg, 1987; Sikorowski, 1985). It is reported to be a pathogen of several economically important pests as well as beneficial insects (Lauzon et al., 2003). It is usually considered an opportunistic or a facultative pathogen as it is often avirulent to insects when present in the digestive tract and causes lethality, only when it penetrates insect's gut walls and enters hemocoel (Tan et al., 2006). However, reports are available indicating that *S. marcescens* introduced through oral route can cause mortality in insects (Aggarwal et al., 2014; Diaz-Albiter et al., 2012; Nehme et al., 2007).

Two *S. marcescens* strains SEN and ICC-4 differing in virulence against *Spodoptera litura* (Fab.) were isolated from dead insect cadavers. *S. marcescens* strain SEN was found to be highly virulent and caused considerable mortality (98%) in 1st instar larvae of *S. litura* after introduction through oral route. However, in comparison, *S. marcescens* strain ICC-4 was not observed to be very virulent and caused only 10% mortality (unpublished data). The efficacy of a pathogen in causing disease leading to mortality in a host is considerably dependent on its virulence. Many virulence factors are known to promote pathogenicity and some in fact are essential for causing disease in the host. These factors enable the pathogen to enter into the host, evade host defenses and proliferate, cause local damage at the site of infection, disseminate itself or its products and cause lethality (Weiss and Hewlett, 1986). Virulence of bacterial pathogens is known to be influenced by a number of factors such as production of hydrolytic enzymes and toxins.

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S. marcescens is reported to secrete a variety of extracellular hydrolytic enzymes, including chitinases, proteases and nucleases (Hines et al., 1988). It is also reported to produce a number of toxins and factors with hemolytic and cytotoxic activities such as ShlA, PhlA and serrawittin (Swr) which presumably contribute to the virulence and pathogenicity of this opportunistic pathogen (Hertle, 2005; Shimuta et al., 2009; Shanks et al., 2009).

Identification of virulence factors enabling a pathogen to become highly virulent and pathogenic towards insect pest is essential to the development of an effective biopesticide. The present study was aimed to characterize the possible virulence factors present in the virulent *S. marcescens* strain SEN which play an important role in enhancing its pathogenicity and efficacy as a biocontrol agent. To understand the putative virulence factors affecting the pathogenesis of *S. marcescens* strain SEN against *S. litura*, a comparative study of the two *S. marcescens* strains, one highly virulent and the other comparatively non-virulent, has been carried out. The two *S. marcescens* strains have been evaluated for the presence and activity of various hydrolytic enzymes with well known roles in enhancing virulence of a pathogen; production and expression of bacterial toxins and other virulence factors found in pathogenic bacteria involved in causing damage to host tissues and the development of disease has also been investigated. Lethality caused in a host depends to a very large extent on the persistence and proliferation of a pathogen within the host. To determine proliferation and persistence of the pathogen within the host, *S. litura* larvae were fed on diet treated individually with the two *S. marcescens* strains SEN (Genbank Acc. No. KJ443714) and ICC-4 (Genbank Acc. No. KJ443715) and the persistence and/or establishment of these two *S. marcescens* strains in the insect gut and hemoceol of *S. litura* was examined. Although, reports are available on the multiplication of *Serratia entomophila* in the gut of *Costelytra zealandica* larvae (Jackson et al., 2001), to the best of our knowledge this is the first report documenting proliferation and survival of *S. marcescens* in *S. litura* gut; and expression of different toxin genes, presumably playing important role in insect pathogenesis, from the two *S. marcescens* strains differing considerably in virulence.

2. Material and methods

2.1. Growth and maintenance of microorganisms

Two *S. marcescens* strains SEN (Genbank Acc. No. KJ443714) and ICC-4 (Genbank Acc. No. KJ443715) (Supplementary Fig. 1) were grown in nutrient medium (Atlas, 2004) at 30 °C. *S. marcescens* strains SEN and ICC-4 had been isolated earlier from dead insect larvae (Aggarwal et al., 2015). The stock cultures were maintained on slants of NA medium under refrigerated (4 °C) conditions. Subculturing was done as and when required.

2.2. Lytic enzyme activities

S. marcescens (MTCC 8708) was used as standard culture. The bacteria were grown in nutrient broth for 48 h at 30 °C, centrifuged at 8000 rpm for 10 min and supernatant was used as crude enzyme source for carrying out assays of different enzymes.

2.2.1. Chitinase

Chitinolytic activity of the bacterial cultures was quantified by the method of Ohtakara (1988) with slight modification. Instead of glycol chitin, colloidal chitin was used as the substrate to estimate chitinolytic activity. Crude enzyme extract (0.5 ml) was mixed with 0.1 ml of 1% colloidal chitin prepared in 0.1 M sodium phosphate buffer (pH 7) and incubated in a water bath for 10 min at 37 °C. The reaction was stopped by boiling the solution for 4 min. To 0.5 ml of the reaction mixture, an equal volume of distilled water was added. To this 0.5 ml of acetone reagent was added and allowed to boil for 20 min in a water bath. After boiling, 2.5 ml ethanol and 0.5 ml ehrlich reagent (95% Ethanol, 0.8 g p-dimethylaminobenzyladehyde (DMAB), conc HCl-50 ml) were added and the volume was made up to 5 ml. These were incubated at 65–70 °C for 10 min and absorbance was recorded at 530 nm against a standard curve, using a Perkin Elmer spectrophotometer (model Lambda E2201). Three replications per culture were maintained and reaction mixture without crude enzyme extract was used as control. Protein concentrations were estimated according to the method of Lowry et al. (1951). One unit of chitinase activity was defined as the amount of enzyme which produced 1 μmol of amino sugars as glucosamine per min.

2.2.2. Protease

Protease enzyme assay using hemoglobin as a substrate was done according to the method of Anson and Mirsky (1932). The reaction mixture consisting of 250 μl of crude enzyme and 1 ml of substrate (2% hemoglobin in phosphate buffer, pH 7) was incubated at 37 °C for 30 min, after which the reaction was terminated by the addition of 500 μl of 10% TCA. For the control, 500 μl 10% TCA was added to the substrate before the addition of crude enzyme. The precipitated material was removed by centrifugation (1000 rpm for 10 min), 300 μl of the supernatant was mixed with 2.5 ml Cu-alkaline solution (0.5% copper sulphate, 1% sodium potassium tartarate) by vortexing and allowed to stand for 15 min. Thereafter, 200 μl Folin–Ciocalteu reagent (diluted 1:1 with water, v/v) was added to the mixture and allowed to stand for another 20 min. The absorbance was recorded at 660 nm using a spectrophotometer (Lamda EZ 201, Perkin Elmer). Three replications per culture were maintained. Protease activity was expressed as the difference of absorbance between the control sample and the test sample. One unit of protease activity was defined as the amount of enzyme required to release TCA-soluble fraction giving a blue color equivalent to 1 μg of tyrosine per minute. The specific activity of the protease was defined as protease activity per mg protein.

2.2.3. Lipase

Lipase enzyme activity was measured using tributyrin as substrate by the method of Smeltzer et al. (1992). A 0.5% suspension of triglyceride in 100 mM Tris (pH 8.0) was prepared. The suspension was emulsified by sonication (40 W for 3 min). The emulsion was stabilized by adding an equal volume of 0.8% low gelling temperature agarose. Spectrophotometric assays were initiated by adding 1.0 ml of the prewarmed (50 °C) tributyrin emulsion to 100 μl of crude enzyme dispensed into spectrophotometric cuvettes. The reaction was monitored at room temperature by measuring the optical density of the emulsion at 450 nm. Three replications per culture were maintained and reaction mixture without crude enzyme extract was used as control. Enzyme activity was calculated using a lipase standard curve and expressed as U/ml. The specific activity of lipase was defined as lipase activity per mg protein.

2.2.4. Phospholipase

Phospholipase activity of bacterial culture was determined by the method of Durban et al. (2007) using p-Nitrophenylphosphorylcholine (NPPC) as substrate. Ten microliters of a 100-mM solution of NPPC was mixed with 90 μl of the crude enzyme and incubated for 10 min at 30 °C followed by measurement of its absorbance at 410 nm. Three replications per culture were maintained and reaction mixture without crude enzyme extract was used as control. Enzyme activity was expressed as

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