A silkworm infection model to investigate *Vibrio vulnificus* virulence genes

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Abstract. The halophilic marine bacterium, *Vibrio vulnificus*, occasionally causes fatal septicemia in immunocompromised patients. Mice are commonly used as experimental animals to investigate the virulence of *V. vulnificus*, however, a large number of mice are generally required for bioassays. The present study examined whether the invertebrate species, silkworms, can be used instead of mice to investigate *V. vulnificus* virulence. When the silkworms were inoculated with $1.2x10^7$ colony forming units of *V. vulnificus* OPU1-Rf, a virulent strain of *V. vulnificus*, all injected silkworms died within 48 h, however, those injected with culture filtrate or diluent did not. This silkworm infection model was then used to isolate attenuated *V. vulnificus* mutants from 1,016 transposon-inserted mutants. Consequently, a harmless mutant, SW998, was isolated. In this strain, the transposon was inserted into the *rtxA* gene, which is a known *V. vulnificus* virulence gene. In conclusion, the present study demonstrated that silkworms are useful animals for investigating the virulence of *V. vulnificus*.

Introduction

Vibrio vulnificus is a Gram-negative, comma-shaped bacterium found in warm seawater and brackish waters. *V. vulnificus* occasionally causes primary septicemia, gastroenteritis and wound infections in humans. Primary septicemia and gastroenteritis can occur following ingestion of uncooked seafood contaminated with *V. vulnificus* (1), whereas wound infections are often caused by direct contact of an open wound with seawater during marine activities or in the processing of seafood (2). *V. vulnificus* rarely infects healthy individuals, however, immunocompromised patients with an underlying illness, including hepatic disorder, diabetes or immunodeficiency, are susceptible to infection by this bacterium (3). Among the aforementioned clinical states of *V. vulnificus* infection, primary septicemia is the most life-threatening, with a mean mortality rate of >50% worldwide (4). There is no official national system for surveying *V. vulnificus* infections in Japan, however, it was reported that 117 individuals succumbed to mortality between 1975 and 2005 (5), and the annual number of cases of *V. vulnificus*-septicemia has been estimated to be >200 (6). Cases of *V. vulnificus* infection have also been reported annually in other countries. The Centers for Disease Control (Atlanta, GA, USA) reported 32 cases of *V. vulnificus* infection-associated mortality in the US in 2012 (7). In Europe, *V. vulnificus* is a known fish pathogen, particularly in eels, and *V. vulnificus* infection in humans is relatively rare (8). However, due to the global warming, there is concern of an increasing number of cases of *V. vulnificus* infection as this bacterium grows rapidly in warm seawater (9).

Several studies have reported on *V. vulnificus* virulence genes (10-12), however, the mechanism underlying the infection remains to be fully elucidated. Mice have been used as experimental animals to investigate the host-pathogen interactions of *V. vulnificus* infections, however, using a large number of mammals is expensive and raises ethical considerations (13,14).

Previously, invertebrate infection models using nematodes, including *Caenorhabditis elegans* (15), and insects, including *Galleria mellonella* (16), have being applied to investigate bacterial virulence genes (17). In addition, an infection model using silkworms (*Bombyx mori*) for the investigation

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of bacterial pathogenesis has been reported (18). Silkworms are relatively easy to breed in laboratories, and the animal is sufficient large enough for the injection of samples into the hemolymph. The objective of the present study was to use a silkworm model to investigate the virulence of *V. vulnificus*. This model may be aid in the investigation of *V. vulnificus* virulence factors and may assist in the development of effective therapies to protect against *V. vulnificus* infection

Materials and methods

Bacterial strains, plasmids and medium. The *V. vulnificus* OPU1 strain was clinically isolated, and its rifampicin (Rf)-resistant variant, *V. vulnificus* OPU1-Rf, was used in the present study. *Escherichia coli* BW19795 was provided by Dr Barry L. Wanner (Purdue University, West Lafayette, IN, USA (19), which was used as a pUT donor for conjugation. *E. coli* DH10B™-competent cells were purchased from Life Technologies; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The signature-tagged mini-Tn*5*Km2-y67 transposon in the pUT delivery suicide plasmid pool was provided by Dr David W. Holden (Imperial College London, London, UK) (20). Bacterial cells were grown at 37˚C in Luria-Bertani (LB) medium containing 10 g tryptone (BD Biosciences, Tokyo, Japan), 5 g yeast extract (BD Biosciences) and 10 g NaCl/l (21), unless otherwise described. M9 minimal medium without glucose was prepared as previously described (21) and used for bacterial conjugation. The following antibiotics were added to the medium at the indicated concentrations: Rf (100 μg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan), kanamycin (Km; 50 μg/ml; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and ampicillin (Am; 100 μg/ml; Meiji Seika Pharma Co., Ltd.). Bacterial growth was monitored by turbidity using a spectrophotometer (Spectronic 20A; Shimadzu Corporation, Kyoto, Japan).

Silkworm lethality assay. The bacteria were inoculated into silkworms using the protocol described by Hamamoto *et al* (22). The silkworms were raised from fertilized silkworm eggs (Hu·Yo x Tsukuba·Ne) purchased from Ehime Sansyu Co., Ltd. (Yawatahama, Japan). The eggs were incubated in a clean bench (CCV-800E-AG; Hitachi Koki Co., Ltd., Tokyo, Japan) at 25˚C in the dark for 3-5 days, according to the manufacturer's protocol. The hatched larvae were maintained at 27˚C , humidity 60-90% and fed artificial food (Silkmate 2S; Nosan Corporation, Yokohama, Japan) for ~3 weeks. The larvae shed their shells four times, and the fifth-instar larvae were fed antibiotic-free artificial food (Silkmate; Katakura Industries, Tokyo, Japan) for 24-26 h prior to inoculation.

The bacteria were cultivated at 37˚C in 2 ml of LB medium until the optical density at 600 nm (OD_{600}) reached 1.0. The bacterial cultures were diluted with 10 mM phosphate-buffered saline (pH 7.3), containing 0.01% gelatin (PBSG) to cell densities of $2.4x10^7$ and $2.4x10^8$ cfu/ml, and were maintained for up to 2 h at room temperature until they were inoculated into the silkworms. The culture filtrates were prepared by filtering the diluted cultures through a 0.45-μm filter (Merck Millipore, Tokyo, Japan). A 50-μl aliquot of the diluted bacterial cell suspension or culture filtrate was injected into the hemolymph of the silkworms using a 1-ml plastic disposable tuberculin syringe attached to a 27-gauge needle (Terumo Corporation, Tokyo, Japan). The quantities of bacteria injected were estimated by colony forming units (cfu), determined by inoculation of the diluted bacterial culture suspensions onto LB agar and counting the number of colonies grown following incubation for 18 h at 37˚C. The inoculated silkworms were maintained in plastic containers without feeding, and the larval status (dead or alive) was assessed. The silkworms were considered to be dead when they showed no reaction to touch.

Construction of the transposon insertion mutants by conjugation. The transposon insertion mutants were constructed by conjugation, as described previously (23). Briefly, *V. vulnificus* OPU1-Rf was mated with *E. coli* BW19795 harboring the pUTy69 conjugative suicide plasmid, which contained the mini-Tn*5*Km2-y67 Km-resistant transposon. The transposon-inserted mutants were assessed by their growth on Km- and Rf-containing agar medium.

Cloning and sequence analysis of the attenuated mutant. The locations of the transposon-inserted regions in the mutant genome were determined by sequencing of the DNA sequence adjacent to the insertion site, as described previously (23). Briefly, whole genome DNA of attenuated mutants were extracted and digested with the restriction enzyme *Sal*I (Nippon Gene Co., Ltd, Tokyo, Japan). The enzyme digested DNA fragments were ligated into pUC18, followed by transformation into *E. coli* DH10B (Thermo Fisher Scientific, Inc.). The colonies resistant to both Km and Am were selected and the plasmid DNA that ligated with the fragments containing transposon insertion sites of genomic DNA was extracted. The transposon insertion sites were determined by DNA sequencing using the primer P279 (5'-CTAGGTACC TACAACCTC-3') which anneal to the transposon. DNA sequencing was performed with an Applied biosystems DNA sequencing system and the BigDye terminator cycle sequencing kit (Thermo Fisher Scientific, Inc.). Sequence homologies were searched with the BLAST search algorithm at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/)

Results

Lethality of V. vulnificus towards silkworms. To investigate the applicability of silkworms for *in vivo V. vulnificus* infection experiments, the present study first examined the lethality of *V. vulnificus* towards silkworms. The spontaneous Rf-resistant *V. vulnificus* OPU1-Rf was selected for injection into silkworms, as the infectivity and lethality of this strain have been confirmed in mice, and a mutagenesis method using the mini-Tn*5*Km2 transposon was established (23).

Fresh *V. vulnificus* OPU1-Rf cultures with an OD_{600} of 1.0 were diluted with PBSG solution to cell densities of $2.4x10^7$ and $2.4x10^8$ cfu/ml, and were maintained for up to 2 h at room temperature until they were inoculated into the silkworms. A 50-μl aliquot of the diluted culture specimen was injected into the hemolymph of the silkworms from a syringe through the dorsal surface, in a manner similar to that of infection into the human blood stream. The movement of the silkworms reduced

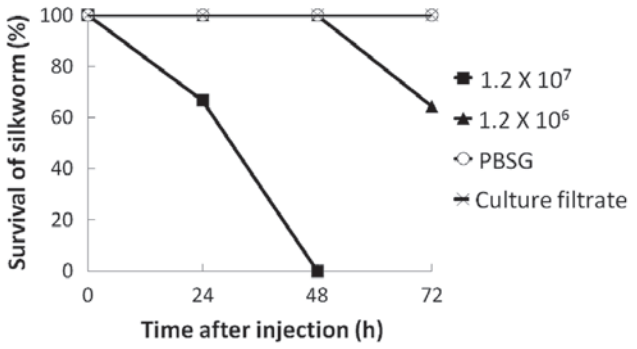

Figure 1. Lethality of *V. vulnificus* to silkworms. *V. vulnificus* OPU1-Rf bacterial cultures ($1.2x10^6$ or $1.2x10^7$ cfu/silkworm), *V. vulnificus* OPU1-Rf culture filtrates or PBSG were injected into the silkworm hemolymph. *V. vulnificus, Vibrio vulnificus*; PBSG, phosphate-buffered saline with 0.01% gelatin.

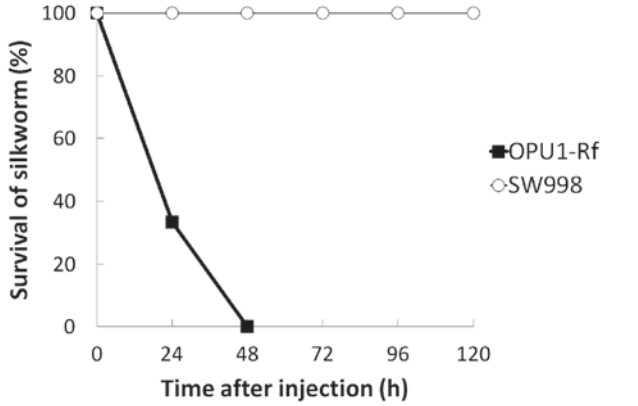

Figure 2. Survival durations of silkworms following being injected with *V. vulnificus* SW998. A total of five silkworms were injected, each with diluted culture media of the *V. vulnificus* OPU1-Rf parent strain and the transposon-inserted mutant SW998. *V. vulnificus, Vibrio vulnificus*; Rf, rifampicin-resistant OPU1 strain.

immediately following the injection, however, the animals behaved normally at ~1 h. This blunting of silkworm behavior was also observed when the animals were injected with the diluent (PBSG) alone. These findings may have been associated with the injection stimulus, an example being a decrease in body temperature caused by the injected solution. As shown in Fig. 1, the silkworms injected with *V. vulnificus* OPU1-Rf began to die at 24 h. By contrast, the silkworms injected with PBSG or culture filtrate remained alive at 72 h. In the deceased silkworms, the points of injection turned black in the initial hours, and these black spots gradually spread throughout the entire body. All silkworms injected with $1.2x10^7$ cfu/silkworm died within 48 h. A reduced dose of $1.2x10^6$ cfu/silkworm extended survival rates, and 60% of the worms were alive at 72 h (Fig. 1). Thus, the virulence of *V. vulnificus* towards silkworms may be dose-dependent at these doses, although only two doses were examined in the present study. These results indicated that *V. vulnificus* is life-threatening to silkworms, and that silkworms can be used for investigating the virulence of *V. vulnificus*. As the injection of $1.2x10^7$ cfu/silkworm led to the death of all silkworms within 48 h, 10^7 cfu/silkworm was used in the subsequent experiments.

Screening of the V. vulnificus-attenuated mutants through the assessment of silkworm lethality. As the silkworms were found to be sensitive to *V. vulnificus* infection, the present study attempted to use a silkworm infection model to identify the pathogenic genes of *V. vulnificus* against silkworms. The attenuated mutants, which had lost pathogenicity to silkworms, were screened from the 1,016 transposon insertion mutants.

The transposon insertion mutants were cultured for 4-6 h at 37˚C with agitation. Culture fluid with an OD_{600} of 1.0 was diluted with PBSG, and a 50-μl aliquot was injected into the hemolymph of silkworms. Subsequently, ~$3.3x10^7 \pm 2.9x10^7$ cfu of the organism were injected into each silkworm, and silkworm status (dead or alive) was monitored for 5 days. In the process of identifying less virulent mutants, which did not lead to silkworm death within 5 days, 78 candidates were obtained (primary screening). To reduce experimental error, a second inoculation of the first candidate mutant was performed. Of the 78 candidates, 16 mutants did not lead to silkworm death within 5 days of injection (secondary screening). To confirm the avirulent properties of the secondary screened candidates, each of the 16 secondary screened candidates was injected into five silkworms. The candidates, in which all five injected silkworms survived for 5 days, were selected as attenuated mutants. Of the 16 mutants, 15 were not selected as an attenuated mutant as the silkworms died during the observation period. The SW998 mutant was found to be avirulent to all five silkworms (Fig. 2).

Transposon insertion sites in the attenuated mutant. As SW998 was avirulent in the silkworms (Fig. 2), the virulence-associated gene was expected to have been disrupted by the transposon insertion in SW998. In order to locate the transposon-inserted gene in the SW998 genome, recombinant plasmid DNA which contained the transposon insertion site was prepared as aforementioned and the DNA sequences adjacent to the transposon insertion sites were determined (Fig. 3). The nucleotide sequences of the cloned *V. vulnificus* DNA fragment adjacent to the transposon insertion site showed a high level of homology to the *V. vulnificus rtxA* gene, which has been reported as a *V. vulnificus* cytotoxic factor (24).

Discussion

Certain *in vivo* experiments designed to investigate the precise mechanisms of microbial infections are difficult to replace using *in vitro* experiments or models. Mice are often used to investigate *V. vulnificus* infection; however, using a larger number of animals may not always be economically or ethically feasible. Random transposon insertion mutagenesis and subsequent screening of attenuated mutants have been applied to identify virulence genes in several bacterial pathogens (25). Thousands of mice are required to isolate attenuated mutants. Animal experiments using mammals are required; however, to obtain satisfactory results, the sacrifice of a large number of animals may be required. *In vivo* infection models using invertebrates instead of mammals have been successfully applied in pathogenic microbe investigations, including *Staphylococcus aureus* virulence genes identified using a silkworm infection model (18). The purpose of the present study

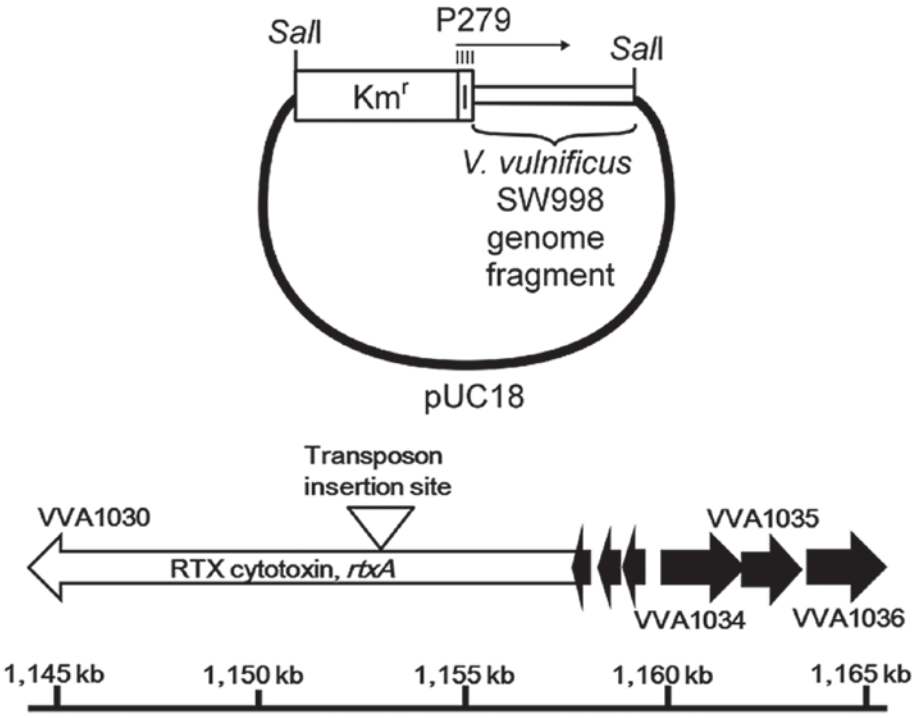

Figure 3. Transposon insertion site of *V. vulnificus* SW998. (A) To identify the transposon-inserted gene, the *V. vulnificus* SW998 transposon insertion region was cloned into the pUC18 plasmid vector, as described previously (18). The *Sa*lI-digested genomic DNA of *V. vulnificus* SW998 was cloned into the pUC18 *Sa*lI site with Km^r as an indicator. The P279 oligonucleotide primer, which was designed to hybridize to the I-ends of the mini-Tn5Km2 transposon, was used to amplify the *V. vulnificus* SW998 transposon insertion site The arrow shows the direction of DNA sequencing. (B) Schematic representation of the *V. vulnificus* chromosomal region around the rtx element and transposon insertion site. Arrows indicate transcriptional directions and coding regions of *V. vulnificus* YJ016 genes (accession no. NC_005140). The line below indicates the gene location. *V. vulnificus, Vibrio vulnificus;* Km^r, kanamycin resistance gene.

was to examine whether the silkworm infection model can be applied to investigate the virulence of *V. vulnificus*.

In the present study, silkworms were demonstrated to be sensitive to the *V. vulnificus*; inoculation of this bacterium into the hemolymph of animals led to death (Fig. 1). Kaito *et al* (26) reported that several bacterial species, including *S. aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *V. cholerae*, which are human pathogens, can also infect and induce death in silkworms when injected into the blood stream of silkworms, whereas non-pathogenic laboratory strains of *E. coli* cannot. Although there is no direct evidence to confirm whether the death of silkworms is caused by the same factors as mammals, silkworms may recognize virulence factors of pathogens as they have the Toll and immune deficiency pathways, which are homologous to the mammalian Toll-like receptor and tumor necrosis factor receptor signaling pathways, respectively (27). Using the silkworm infection model, the SW998 attenuated mutant strain was obtained in the present study, in which the *rtxA* gene was disrupted by a transposon insertion. The *rtxA* gene is a member of the *rtx* gene cluster in the *V. vulnificus* genome. RtxA has been suggested to be a toxin essential for *V. vulnificus* virulence, which has been confirmed by experimental infection of a mouse model and in an *in vitro* tissue culture model (24). In the present study, the injection of culture filtrate did not induce silkworm death (Fig. 1), which suggested that the expression of RtxA increased following interaction of the pathogen with host cells, as reported previously (28). Therefore, the bacteria secreted RtxA in the silkworm *in vivo* and RtxA may be toxic to silkworms. Complete removal of the genes from the genome and lethality assessment are required to determine whether RtxA is actually toxic to silkworms.

In conclusion, the silkworms examined in the present study died when inoculated experimentally with *V. vulnificus* and the *rtxA* gene was identified successfully using this infection model. It is expected this model may be useful for investigating *V. vulnificus* virulence factors and assist in developing effective therapies to protect against *V. vulnificus* infection.

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