Study on the virulence factors of *Vibrio vulnificus* by transposon insertion mutagenesis

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Chapter 1

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 after ingesting 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 when processing seafood (2). Characteristics of this infection are fever, chills, nausea, septic shock, and the formation of secondary lesions on the extremities of patients. Antibiotic treatment improve survival. CDC has announced that doxycycline and a third-generation cephalosporin is generally recommended. However initiation of antibiotic treatment tend to be delayed because *V. vulnificus* can multiply rapidly during infection and there are no pathognomonic symptom of *V. vulnificus* infection except wound infection. *V. vulnificus* rarely infects healthy individuals; however, immunocompromised patients with underlying illness, such as hepatic disorder, diabetes, or immunodeficiency, are susceptible to their infection (3). The mortality rates of *V. vulnificus* infection along with primary septicemia have been reported to be higher than 50% (4, 5).

V. vulnificus infection have been reported in various area. In Japan, no official national system surveys *V. vulnificus infections,* but 117 deaths were reported during 1975–2005 (6) and the annual number of *V. vulnificus* septicemia cases has been estimated at more than 200 (7). *V. vulnificus* infection cases have also frequently been reported every year in other areas of asia such as Korea or Taiwan (8, 9). In the U.S., between 1988 and 2006, the Center for Disease Control and Prevention received reports of more than 900 cases of *V. vulnificus* infection from the Gulf Coast state (10). In Europe, *V. vulnificus* is known as a fish pathogen, particularly of eels, and *V. vulnificus*

infection in humans is relatively rare (11). However, due to the global warming, increasing number of *V. vulnificus* infection cases have been concerned because this bacterium grows rapidly in warm seawater (12). Compared to other infection in the same family such as *V. cholerae* and *V. parahaemolyticus*, *V. vulnificus* infection is a rare, but it is also considered that there are unreported cases. Although there is not so many incidence, *V. vulnificus* infection is the notable infection from the point of view of food sanitation and public health of our country. Because we have food culture to eat raw fish such as sashimi and sushi and *V. vulnificus* infects from eating raw fish or the wound of limbs.

Several possible virulence factors have been reported for *V. vulnificus* infection. Cytolytic hemolysin (VvhA) (13), which resembles *V. cholerae* El Tor hemolysin (14), RTX toxin (15), and proteolytic elastases such as VvpE (16) have been suggested to play a role in the destruction of the host tissue. A polysaccharide capsule is assumed to prevent phagocytosis (17). In addition, *V. vulnificus* induces apoptosis in macrophages (18). Although many virulence factors have been found in this organism, the pathogenic mechanisms of *V. vulnificus* infection remain unknown.

To develop the effective cure and the preventive against infection, identification of an individual etiologic factors of infection is important. Random transposon mutagenesis, one of gene-disruption strategies, has been often used to produce insertion mutants. The insertion of transposon leads to inactivate the gene and if insertion site of transposon related to pathogenesis or attenuation of the bacteria, it expected to obtain to attenuated mutant. Transposon insertion mutants are tested for attenuated virulence *in vivo* or *in vitro*. Infection is complicate process and many genes would be expressed during *in vivo* growth. In the virulence study of *V. vulnificus*, few *in*

vivo test have been carried out. In this study, I attempt to use new two methods to identify *V. vulnificus* virulence factors *in vivo*.

Chapter 2

Signature-tagged Mutagenesis of *Vibrio vulnificus*

To investigate virulence factors of bacterial pathogens, transposon insertion mutagenesis method has been widely used. As described in Chapter 1, when mutant libaries were generated, each indivisual mutant must be assessed for phenotypic defects by using a variety of assay techniques. Since, *V. vulnificus* secretes a variety of toxins, *in vitro* detection methods of the toxins were often used in this organisms. However, expression of pathogenic factors *in vitro* may not always reflect the actual expression of the pathogenic genes in the complicated host environments at the time of infection. Very few studies have been applied *in vivo* screening for *V. vulnificus* infection study. For the study of *V. vulnificus* infection, mice have been used as experimental animals to study the host–pathogen interactions; however, using large number of mammals is costly and may be unethical. Recently, several screening methods for the bacterial *in vivo* virulence genes have been developed. Thus, we attempted to use *in vivo* screening method to investigate the virulence genes of *V. vulnificus*, in which relatively fewer animals are sacrificed. We applied a powerful negative selection transposon insertion method, signature-tagged mutagenesis (STM) (19). In STM, mutations are introduced by random insertion of transposons and each transposon has a different "Tag" DNA sequence that is specifically recognized it. STM allows us to screen attenuated mutants from a pool of large numbers of mutants simultaneously in a single animal model. In this chapter, we attempted to STM to investigate *V. vulnificus* virulence genes that are active *in vivo*. Using STM, we identified several possible virulence genes of *V. vulnificus*.

2.1 MATERIALS AND METHODS

2.1.1 Bacterial strains, plasmids, and growth conditions

V. vulnificus used in this study originated from a clinical isolate, OPU1, and its rifampicin (Rf)-resistant variant, *V. vulnificus* OPU1-Rf. *Escherichia coli* BW19795 was provided by Dr. Barry L. Wanner (20). *E. coli* DH10B^{TM} competent cells were purchased from Life Technologies (Life Technologies, Carlsbad, CA, USA). A signature-tagged mini-Tn*5*Km2 transposon in pUT delivery suicide plasmid pool was provided by Dr. David W. Holden (19). Bacterial strains were grown in a Luria-–Bertani (LB) medium [10 g tryptone (Japan BD, Tokyo, Japan), 5 g yeast extract (Japan BD), and 10 g NaCl/*l*] (21) and incubated at 37 °C unless otherwise stated. Antibiotics were added to the medium at the rate of 100 μg/m*l* for Rf, 50 μg/m*l* for kanamycin (Km), and/or 100 μg/m*l* for ampicillin (Am).

2.1.2 Animal experiments

Five-week-old female ICR mice (SPF/VAF, Crlj;CD1, Charles River Laboratories Japan, Yokohama, Japan) were used for animal experiments. Mice were subcutaneously injected in the back with 250 μg/g body weight of iron dextran 4 hr prior to inoculation to enhance their susceptibility to *V. vulnificus*. All animals used in the present study were cared for in accordance with the guidelines for animal treatment of Kitasato and Okayama Universities, both of which conform to the standard principles of laboratory animal care.

2.1.3 Preparation of digoxigenin (Dig)-labeled DNA probes

Multiplied signature-tagged portions were prepared using PCR. Templates for PCR were heat-denatured extracted DNA from mutant pools. The Dig-labeled oligonucleotides P214 (5′-Dig-TACCTACAACCTCAAGCT-3′) and P295 (5′-Dig-CATGGTACCCATTCTAAC-3′), which recognize common arms adjacent to the 40-bp signature-tagged random sequence regions, were used as PCR primers (Fig. 1). PCR was conducted at 95 °C for 5 min, followed by 25 cycles at 95 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 10 sec, and maintained at 4 °C.

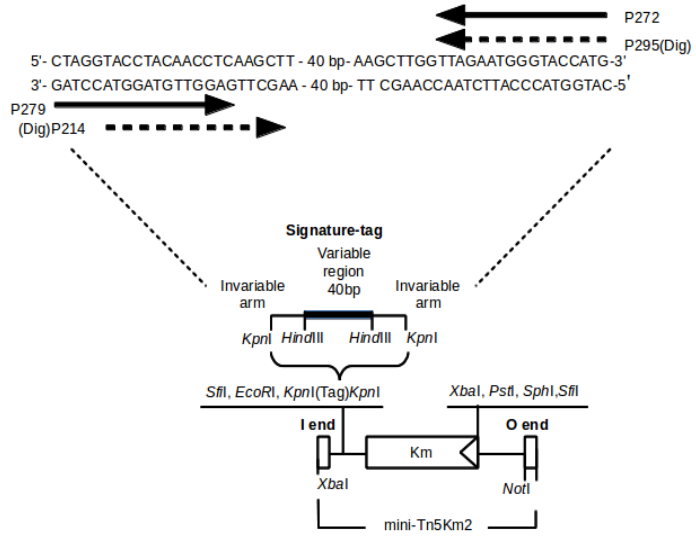

Figure 1. Signature-tagged mini-Tn5Km2. The transposon mini-Tn5Km2 was incorporated into the *V. vulnificus* genome DNA when pUT mini-Tn5Km2 was transferred to *V. vulnificus* by conjugation. Signature tags comprised unique sequences of a 40-bp variable region flanked by invariable arms on either side of the variable region. The mutants with different signature tags were distinguished by hybridization. Invariable arms allowed for amplification of the signature tag by PCR. Primers P272 and P279 were used to prepare unlabeled target DNA on hybridization membranes. DIG-labeled primers P214 and P295 were used to prepare DNA probes labeled at the 5′-end nucleotide.

2.1.4 Dot hybridization

Target DNA for dot hybridization was prepared by PCR using pUT plasmids harboring signature-tagged mini-Tn*5*Km2 as templates and primers P279 (5′-CTAGGTACCTACAACCTC-3′) and P272 (5′-CATGGTACCCATTCTAAC-3′) (Fig.1). The DNA samples were diluted to 50 *n*g/*μl* with 100 *n*g/*μl* of sheared salmon sperm DNA (sssDNA), denatured at 95 °C for 5 min, and chilled on ice. A 1-*μl* aliquot of the denatured target DNA was blotted onto the identical position of each of the 81 sheets of Hybond-N^+ membrane (10 cm × 10 cm, GE Healthcare Japan, Tokyo, Japan), dried, and fixed under UV light. The sheets were maintained at 4 °C until used.

The hybridization processes were performed in a plastic bag using a Dig-labeled hybridization kit (Roche Diagnostics, Mannheim, Germany). The membrane was incubated with prehybridization solution (5× SSPE (21), 3× Denhardt's solution, 1% SDS, 0.1 mg/m*l* denatured sssDNA) without formamide (0.2 m*l*/cm^2 hybridization membrane) for 1 hr at 45 °C. The membrane was transferred into hybridization solution [prehybridization solution containing 50% (v/v) formamide] (0.1 m*l*/cm^2 hybridization membrane). Thereafter, a heat-denatured Dig-labeled DNA probe was added to the hybridization solution at a concentration of 300 *n*g/m*l*, mixed, and incubated for 2 hr at 45 °C in a water bath. The membrane was added to washing solution [2× SSPE, 0.3 M NaCl, 20 mM sodium dihydrogen phosphate, 20 mM EDTA (pH 7.4), and 1% SDS] and shaken to remove nonspecific probe for 5 min (this was discarded). The washing process was repeated twice. The membrane was transferred to preheated washing solution and incubated for 1 hr at 70 °C, followed by incubation in 2× SSPE for 5 min.

The hybridized Dig-labeled probes were detected according to the instruction manual using a Dig detection kit (Roche Diagnostics). The membrane was exposed

overnight to a sheet of X-ray film (Bio Max, Kodak Japan, Tokyo, Japan) and developed.

The intensities of 80 hybridization signals in a sheet of hybridization membrane were scored by the naked eye from 0 to 5 in every film to minimize experimental variations. Hybridization signals with the strongest intensity in the film were given a score of 5 and hybridization signals with no intensity were given a score of 0. The intensities of hybridization signals with INPUT and with OUTPUT probes were compared.

2.1.5 DNA analysis

DNA sequencing was performed with an Applied Biosystems DNA sequencing system (Applied Biosystems, Waltham, MA, USA) and a BigDye terminator cycle sequencing kit (Applied Biosystems). Sequence homologies were searched with the BLAST search algorithm of the National Center for Biotechnology Information. All recombinant DNA experiments in the present study were performed in accordance with the guidelines for recombinant DNA experiments of Okayama Prefectural University, Okayama University, and Kitasato University.

2.2 RESULTS

2.2.1 Construction of transposon insertion mutant library

To identify the pathogenic genes of *V. vulnificus*, we constructed a transposon insertion mutant library. *V. vulnificus* OPU1-Rf was mated with *E. coli* BW19795 harboring pUT mini-Tn*5*Km2 labeled with one of the 80 unique signature tags (T01–T80). This suicide conjugative vector plasmid, pUT, can multiply only in bacteria (such as *E. coli* BW19795) harboring the *pir* gene. When pUT was introduced in *V. vulnificus* by conjugation, the transposon mini-Tn*5*Km2 was transferred from pUT into *V. vulnificus* replicons, allowing *V. vulnificus* to grow on Km-containing agar medium. Conjugation was performed 80 times with each of the respective signature tags (T01–T80). Eighty-one *V. vulnificus* colonies resistant to both Km and Rf were isolated as transposon insertion exconjugants from each conjugation. In total, 6,480 transposon-inserted *V. vulnificus* mutants were isolated (81 exconjugants × 80 signature tags). The 6,480 insertion mutants were divided into 81 set groups (S01–S81) as each set group contained 80 transposon insertion mutants, each having a unique signature-tagged transposon of between T01 and T80. Each set group was used for as a pooled INPUT culture for mouse injection and for template DNA to prepare an INPUT probe.

2.2.2 In vivo passage of INPUT pools

To screen *in vivo* attenuated mutants, the mutants were inoculated into mice by each set group. Eighty mutants in a set group were separately cultured in 96-well flat-bottomed microculture plates containing LB medium for 6 hr at 30 °C. Thereafter, 100-*μl* aliquots of each culture were pooled (S01−S81 INPUT pools). Each INPUT pool containing approximately $3.0 \pm 0.8 \times 10^5$ cfu mutants in phosphate-buffered saline containing 0.01% gelatin (PBSG) was injected into an iron-overloaded mouse

intraperitoneally. This inoculum size of *V. vulnificus* OPU1-Rf allows iron-overloaded mice to survive for at least 7 hr after injection but would kill all mice within 36 hr (data not shown). Mutants that survived and propagated in the mouse were recovered from blood samples obtained by heart puncture 5 hr after the injection of the INPUT pools. Heart blood was inoculated onto LB agar plates containing Km and Rf. Approximately 2–20 × 10^3 colonies resistant to both Km and Rf had grown after overnight culture at 37 °C. All colonies were scraped up and frozen to be stocked as an OUTPUT pool to prepare OUTPUT probes.

2.2.3 Screening of tentative attenuated mutants by hybridization

To estimate attenuated mutants, DNA–DNA hybridization tests were performed and the intensity of hybridization signals with INPUT and OUTPUT probes were compared. If tags were detected with INPUT probes but not with the corresponding OUTPUT probes, those mutants with the tag were expected to lose their ability to survive and multiply in the mouse (Fig. 2). We selected 360 candidate mutants whose hybridization intensities were decreased by more than 2 points as temporarily-attenuated mutants (primary screening). The candidates were subjected to *in vivo* passage and hybridization tests again, as performed in the primary screenings. Thirty mutants whose hybridization intensities differed by more than 3 points were selected as tentative attenuated mutants (secondary screening).

2.2.4 Virulence of tentative attenuated mutants

To confirm the attenuation of the 30 tentative attenuated mutants, lethal doses of the mutants were examined using iron-overloaded mice. Transposon-inserted mutants were cultivated stationarily at 37 °C in 3 m*l* LB broth for 6 hr. A 0.5-m*l* aliquot of 10-fold serially-diluted cultures was inoculated into iron-overloaded mice intraperitoneally and the status (dead or alive) of the mice was checked after 36 hr. The lethal dose of the parent strain, OPU1-Rf, was approximately 10–10^2 cfu/mouse. Unexpectedly, the lethal doses of 19 out of 30 mutants were as low as that of OPU1-Rf. However, the lethal doses of the other 11 mutants were more than 10^3 cfu/mouse (Table 1). Particularly, the lethal doses of S10T79 and S46T31 were as high as 10^6 cfu/mouse, indicating that the virulence of the mutants appeared to have decreased to approximately 1/100,000 compared with the parent strain OPU1-Rf.

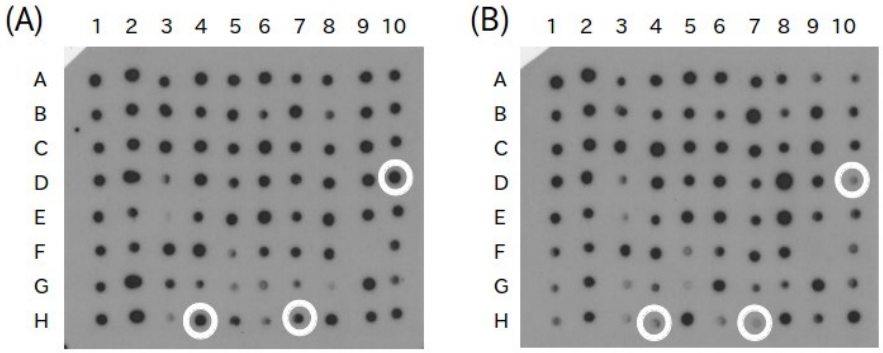

Figure 2. Identification of tentative attenuated mutants *in vivo* by hybridization. Lost mutants of *V. vulnificu*s during infection were identified by DNA–DNA hybridization tests. Two identical membranes dotted with the individually amplified DNA tags (from A1 to H10) were hybridized to the DIG-labeled (A) INPUT and (B) OUTPUT probes. The tags that hybridized to the INPUT probe but not to the OUTPUT probe (such as D10, H4, and H7) were expected to have been lost during infection in the mouse. Therefore, mutants with the tag at D10, H4, and H7 were selected as tentative attenuated mutants.

2.2.5 Insertion sites of mini-Tn5Km2 transposon

To clone transposon insertion sites of attenuated mutants, whole DNA was digested by *Sal*I such that it left intact the I-end of the transposon, the signature tag, and the Km resistance gene of mini-Tn*5*Km2. The digested DNA fragments were cloned into pUC18, followed by transformation into *E. coli* DH10B. Plasmid DNA was extracted from colonies resistant to both Km and Am, in which the transposon insertion sites of genomic DNA were expected to be cloned. DNA sequences adjacent to the transposon were amplified by PCR with the primers P272 or P279, which were annealed to the tag of mini-Tn*5*Km2 (Fig. 1).

The DNA sequences obtained were searched for sequence homologies with *V. vulnificus* genome sequences using the BLAST search algorithm of the National Center for Biotechnology Information. The genes with the highest identification with the database were predicted as transposon-disrupted genes of the attenuated mutants. The putative disrupted genes of *V. vulnificus* identified by STM are summarized in Table 1. The genes obtained in this study may be related to purine metabolism (S10T79, S65T36), sialic acid synthesis (S46T31, S48T58, and S76T76), cell wall biosynthesis (S38T76), membrane transporter (S34T71), and unknown function (S20T62, S37T71). In two mutants (S43T04 and S68T70), transposon-inserted regions showed no homology to any sequence in the GenBank database.

Table 1. Characterization of *V. vulnificus* genes identified by STM

Mutant	Putative product of transposon disrupted gene[a)]	Protein ID[a)]	Putative function	Lethal dose(cfu)[b)]
S10T79	IMP dehydrogenase	AAO08942.2	Purines metabolism	10^{6-7}
S65T36	Phosphoribosylformylglycinamidine cyclo-ligase	AAO10300.1	Purines metabolism	10^{5-6}
S46T31	UDP-N-acetylglucosamine-2-epimerase	AAO09311.1	Sialic acid synthesis	10^{6-7}
S48T58	UDP-N-acetylglucosamine-2-epimerase	AAO09311.1	Sialic acid synthesis	10^{5-6}
S76T76	UDP-N-acetylglucosamine-2-epimerase	AAO09311.1	Sialic acid synthesis	10^{5-6}
S38T76	Aspartokinase	AAO10017.2	Cell wall biosynthesis	10^{4-5}
S34T71	Malate Na (+) symporter	AAO11254	Membrane transporter	10^{3-4}
S20T62	Hypothetical protein	AAO10404.1	Unknown	10^{5-6}
S37T71	Hypothetical protein	AAO10118.1	Unknown	10^{3-4}
S43T04	Unknown		Unknown	10^{5-6}
S68T70	Unknown		Unknown	10^{4-5}

[a)]Gene and protein IDs are from *V. vulnificus* CMCP6 (GenBank accession numbers: AE016795 and AE016796)

[b)]Lethal dose was determined by injection of serially diluted mutant cultures into iron-overloaded mice.

2.2.6 Vaccination with the mutant S10T79 against V. vulnificus infection

To examine whether or not the attenuated mutants had immunogenicity against

V. vulnificus infection, the vaccination of mice was attempted using one of the most attenuated mutants, S10T79. Using 5 iron-overloaded mice in a group, the S10T79 mutant was intraperitoneally injected at a dose of 2.5×10^3, 2.5×10^4, 2.5×10^5 cfu, or mocks, twice with a 2-week interval. One week after the second injection, the mice were challenged by intraperitoneal injection with virulent *V. vulnificus* OPU1, which has a lethality of approximately 10 cfu (data not shown), at a dose of 2.5×10^5 cfu. As shown in Table 2, of the mice injected with PBSG as mocks, all 5 died within 36 hr. On the contrary, all but one of the immunized mice survived more than 36 hr with no symptoms. This result indicates that the mutant S10T79 was effective in preventing *V. vulnificus* infection in experimental vaccination.

Table 2. The effects of vaccination on prevention against *V. vulnificus* infection in mice

Immunized dose with (cfu/mouse)	Dead /Challenged (Numbers of mice)
Mock (PBSG buffer)	5/5
2.5×10^3	1/5
2.5×10^4	0/5
2.5×10^5	0/5

A signature-tagged mini-Tn*5*Km2-inserted mutant *V. vulnificus*, S10T79, was intraperitoneally injected into mice as a vaccine. Vaccinations were given twice with a two-week interval. A week after the secondary vaccination, the mice were challenged with the original strain, *V. vulnificus* OPU1. The status (dead or alive) of mice was judged after 36 hr post the challenge injection.

2.3 DISCUSSION

STM is a negative selection method used to screen transposon insertion mutants that have lost their ability to survive and grow in the host. This method has been applied to screen the virulence factors of many bacterial pathogens (19,22-25). The purpose of this study was to confirm whether STM can be used to identify the virulence genes of *V. vulnificus*. We obtained 11 attenuated mutants whose disrupted genes were suggested to be involved in *in vivo* growth during infection (Table 1).

In two of the mutants, the transposon-inserted genes encoded enzymes involved in purine metabolism, IMP dehydrogenase (*guaB*; S10T79) and phosphoribosylformylglycinamidine cyclo-ligase (*purM* ; S65T36). Many studies have shown that purine nucleotides are required for bacterial growth and purine metabolism plays an important role in bacterial virulence (26-28). Kim et al. (29) reported that in the disruption of *V. vulnificus* nucleotide synthesis genes, AICAR transformylase/IMP cyclohydrolase (*purH*) and UMP kinase (*pyrH*) decreased in virulence. In S10T79 and S65T36, purine nucleotide synthesis *in vivo* may have been reduced, which may have led to the attenuation.

In three of the attenuated mutants (S46T31, S48T58, and S76T76), transposons were inserted into the gene for UDP-N-acetylglucosamine-2-epimerase (*neuC*), which is involved in N-acetylneuraminic acid (Neu5Ac) biosynthesis. Neu5Ac is used for the sialylation of LPS and for capsule formation, which is a significant factor for *V. vulnificus* virulence (30). In *E. coli*, *neuC* mutants express an acapsular phenotype (31) and NeuC is an essential enzyme in the biosynthesis of the capsule in *E. coli*. However,

all 3 *neuC* mutants of *V. vulnificus* represented opaque colonies, indicating capsule formation. Further studies are required to examine the contribution of *neuC* to virulence, particularly to capsule formation.

In the mutant S38T76, the aspartokinase gene (AAO10017.2) was disrupted. In *V. vulnificus*, little attention has been paid toward aspartokinase as a virulence factor, although in other pathogens, the gene has been suggested to be a virulence factor (32,33). The aspartokinase gene (*ask*) of mycobacteria is involved in the synthesis of peptidoglycan, the main function of which is to protect cells against osmotic pressure.

In S34T71, the gene for the malate Na (+) symporter was found at the transposon insertion site. Several transporters are known to play a key role in the homeostasis of intracellular pH, cellular Na^+ content, and cell volumes in bacteria (34).

For the development of the disease, *V. vulnificus* has to multiply in the human body; therefore, its ability to survive in the host (to acquire and metabolize nutrients and to escape from immune systems) could be authentic virulence factors in *V. vulnificus*. Using STM, we obtained 11 attenuated mutants whose disrupted genes were suggested to be involved in *in vivo* growth during infection. Thus, the present study demonstrates the applicability of STM to the search for the virulence factors of *V. vulnificus*. To confirm the attenuation of the tentative mutants, each candidate was solely inoculated into mice. Unexpectedly, 19 of the 30 mutants revealed lethality comparable to that of the parental strain. However, a similar phenomenon has been found in another STM study in *Yersinia pestis* (35). Because the mutants were mixed and inoculated into mice simultaneously during STM, the mutants that succumbed to growth competition would have been chosen as the attenuation candidates. Thus, 19 mutants may have been defeated by competition in multiplying when inoculated into mice simultaneously with others. We may also have

to recognize the possibility that some of the mutants may have reduced growth ability under high free-iron conditions in *in vivo* environments in iron-overloaded mice. Another possibility is that in some mutants, attenuation may have arisen on account of the polar effects of transposon insertion. To negate these possibilities, complete removal of the genes from the genome and lethality tests are required, together with demonstration of the recovery of virulence by trans complementation tests of the genes. Although we need further studies, STM in *V. vulnificus* promises to contribute to the analysis of pathogenesis and to the development of safe and effective vaccines.

Chapter 3

A silkworm infection model to study the *Vibrio vulnificus* virulence genes

In chapter 2, we applied STM to identify virulence factor of *V. vulnificus* and it was shown that STM is a useful method to screen virulence genes *in vivo*. In this study, we found the virulence factors of *V. vulnificus* can be divide into two groups. First, the virulence consists of bacterial factors required for evasion of host defenses and nutrients acquisition within the host. Second, the virulence includes factors involved in cytotoxicity, such as hemolysin. In the STM system, different strains were infected together into the animal and attenuated mutants were detected by loss of their tags. So that means it is unlikely to detect mutations in second group of virulence genes. Because although the gene for the certain toxins was disrupted by transposon insertion, the mutant and tag was still exist. Only first group of virulence genes would be find by STM. It can be a limitation of STM. And more importantly, although STM can reduce number of animal, it still make problem costly and ethically. Recently, invertebrate infection models using nematodes, such as *Caenorhabditis elegans* (36), and insects, such as *Galleria mellonella* (37), are being applied to investigate bacterial virulence genes (38). Moreover, an infection model using silkworms (*Bombyx mori*) to study bacterial pathogenesis has been reported (39). Silkworms are relatively easy to breed in laboratories and is large enough for injection of samples into the hemolymph and intestine. The objective of the current study is to use a silkworm model to study *V. vulnificus* virulence.

3.1 MATERIALS AND METHODS

3.1.1 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 this study. *Escherichia coli* BW19795 was provided by Dr. Barry L. Wanner (20) and used as a pUT donor for conjugation. *E. coli* DH10B^{TM} competent cells were purchased from Life Technologies (Carlsbad, CA, USA). The signature-tagged mini-Tn*5*Km2-y67 transposon in the pUT delivery suicide plasmid pool was provided by Dr. David W. Holden (19). Bacterial cells were grown at 37°C in Luria–Bertani (LB) medium [10 g tryptone (Japan BD, Tokyo, Japan), 5 g yeast extract (Japan BD), and 10 g NaCl/l] (21) unless otherwise described. M9 minimal medium without glucose (21) was used for bacterial conjugation. The following antibiotics were added to the medium at the indicated concentrations: rifampicin (Rf ; 100 μg/ml), kanamycin (Km; 50 μg/ml), and ampicillin (Am; 100 μg/ml). Bacterial growth was monitored by turbidity using a spectrophotometer (Spectronic 20A, Shimadzu Corp., Kyoto, Japan).

3.1.2 Silkworm lethality assay

Bacteria were inoculated into silkworms using the protocol of Hamamoto et al (40). The silkworms were raised from fertilized silkworm eggs (Hu·Yo × Tsukuba·Ne) purchased from Ehime Sansyu Co. (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 instructions*. The hatched larvae were fed artificial food (Silkmate 2S, Nosan Corp., Yokohama, Japan) for approximately 3 weeks. 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 just prior

to inoculation. Bacteria were cultivated at 37°C in 2 ml of LB broth until optical density at 600 nm (OD_{600}) reached 1.0. The bacterial cultures were diluted 1:10 with 10 mM phosphate-buffered saline (pH 7.3), containing 0.01% gelatin (PBSG). Culture filtrates were prepared by filtering the diluted cultures through a 0.45-μm filter (Nihon Millipore K.K., Tokyo, Japan). A 50-μl aliquot of diluted bacterial cell suspension or culture filtrate was injected into the hemolymph of silkworms using a 1-ml plastic disposable tuberculin syringe attached to a 27-gauge needle (Terumo Corp., Tokyo, Japan). The quantities of bacteria injected were estimated by colony forming units (cfu) after inoculating the diluted bacterial culture suspensions on LB agar and counting the number of colonies that grew after 18 h at 37°C. The inoculated silkworms were maintained in plastic containers without feeding, and larval status (dead or alive) was checked. Silkworms were considered dead when they showed no reaction to touch.

3.1.3 Construction of the transposon insertion mutants by conjugation

Transposon insertion mutants were constructed by conjugation, as described previously (41). 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, a Km resistant transposon. The transposon-inserted mutants were judged by growth on Km- and Rf-containing agar medium.

3.1.4 Cloning and sequence analysis of the attenuated mutant

The locations of the transposon-inserted regions in the mutant genome were determined by sequencing the DNA sequence adjacent to the insertion site, as described

previously (41).

3.2 RESULTS

3.2.1 Lethality of V. vulnificus to silkworms

To investigate the applicability of silkworms for *in vivo V. vulnificus* infection experiments, we first examined lethality of *V. vulnificus* to silkworms. The spontaneous Rf-resistant *V. vulnificus* OPU1-Rf was selected for injection into silkworms because the infectivity and lethality of the strain have been confirmed in mice, and we established a mutagenesis method using the mini-Tn*5*Km2 transposon (41).

Fresh *V. vulnificus* OPU1-Rf cultures with OD_{600} of 1.0 were diluted 1:10 with PBSG solution to cell densities of 2.4×10^7 and 2.4×10^8 cfu/ml and were maintained for up to 2 h at room temperature until they were inoculated into silkworms. A 50-μl aliquot of diluted culture specimen was injected into the hemolymph of the silkworms from a syringe through the dorsal surface, in a manner similar to the infection into the human blood stream. The silkworms slowed immediately after the injection but behaved normally after approximately 1 h. This blunting silkworm behavior was also observed when they were injected with diluent (PBSG) alone. Thus, this phenomenon may have been related to the injection stimulus, e.g., possibly the body temperature decrease caused by the injected solution. As shown in Fig. 3, silkworms injected with *V. vulnificus* OPU1-Rf started dying after 24 h. On the other hand, silkworms injected with PBSG or culture filtrate were alive at 72 h. In deceased silkworms, the points of injection turned black within a few hours and these black spots gradually spread

throughout the entire body. All silkworms injected with 1.2×10^7 cfu/silkworm died within 48 h. A reduced dose of 1.2×10^6 cfu/silkworm extended survival and 60% of the worms survived for 72 h (Fig. 3). Thus, *V. vulnificus* virulence to silkworms may be a dose-dependent phenomenon around these doses, although only two doses were examined in this study. These results indicate that *V. vulnificus* is lethal to silkworms, and that silkworms can be used for *V. vulnificus* virulence studies. An injection of 1.2×10^7 cfu/silkworm killed all silkworms within 48 h; thus, we used 10^7 cfu/silkworm in subsequent experiments.

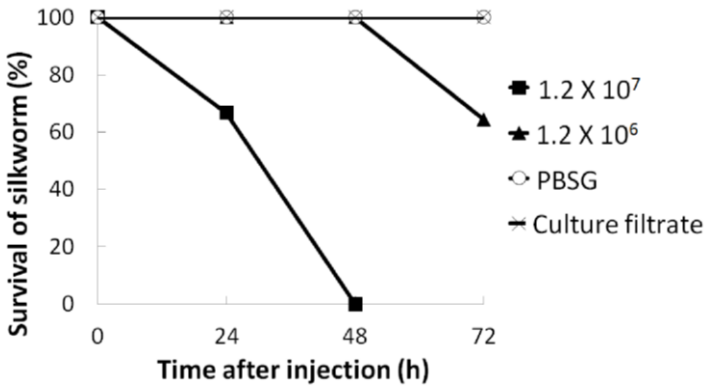

Figure 3. Lethality of *V. vulnificus* to silkworms. *V. vulnificus* OPU1-Rf bacterial cultures (1.2×10^6 or 1.2×10^7 cfu/silkworm), *V. vulnificus* OPU1-Rf culture filtrates, or PBSG were injected into the silkworm hemolymph.

3.2.2 Screening of the V. vulnificus attenuated mutants using the silkworm lethality test

Because the silkworms were sensitive to *V. vulnificus* infection, we attempted to use a silkworm infection test to search for *V. vulnificus* pathogenic genes against

silkworms. The attenuated mutants that 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 shaking. Culture fluid with OD_{600} of 1.0 was diluted 1:10 with PBSG, and a 50-µl aliquot was injected into the hemolymph of silkworms. Approximately $3.3 \times 10^7 \pm 2.9 \times 10^7$ cfu of the organism were injected into each silkworm, and silkworm status (dead or alive) was monitored for 5 days. While attempting to search for less virulent mutants that did not kill silkworms within 5 days, 78 candidates were obtained (primary screening). To reduce experimental error, a second inoculation of the first candidate mutant was carried out. Of the 78 candidates, 16 mutants did not kill the silkworms within 5 days after 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, and the candidates which all five injected silkworms survived for 5 days, were selected as attenuated mutants. Fifteen of 16 mutants were not selected as an attenuated mutant in this study because silkworms died during the observation period. Finally, the mutant SW998 was avirulent to all five silkworms (Fig. 4).

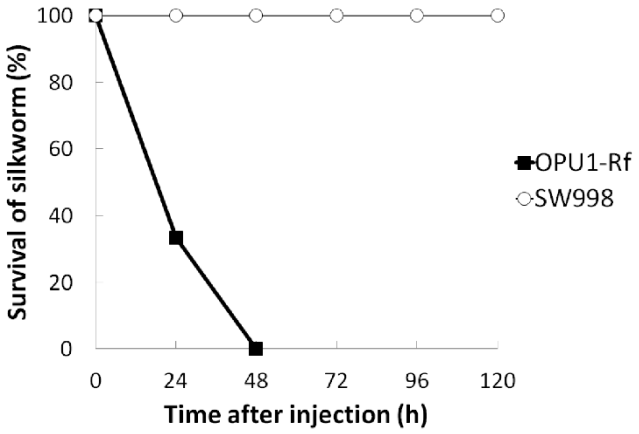

Figure 4. Survival durations of silkworms after receiving an injection of *V. vulnificus* SW998. Five silkworms were injected each with 1:10 diluted culture media of the *V. vulnificus* OPU1-Rf parent strain and the transposon-inserted mutant SW998.

3.2.3 Transposon insertion sites in the attenuated mutant

Because SW998 was avirulent in silkworms (Fig. 4), the virulence-related gene was expected to have been disrupted by the transposon insertion in SW998. The DNA sequences adjacent to the transposon insertion sites were determined to locate the transposon inserted gene in the SW998 genome. The whole SW998 DNA was digested with *Sal*I and ligated into the pUC18 *Sal*I site to clone the transposon insertion site, and several recombinant plasmids containing the Km-resistant gene were cloned (Fig. 5). Because the transposon-inserted sites were expected to be cloned in these clones, the DNA neighboring the transposon was sequenced using the P279 primer oligonucleotide, which was synthesized to anneal to the I-end of the transposon (Fig. 5). The DNA sequences obtained were subjected to a homology search using published *V. vulnificus* genome sequences. The nucleotide sequences of the cloned *V. vulnificus* DNA fragment adjacent to the transposon insertion site were highly homologous to the *V. vulnificus* *rtxA* gene, which has been reported as a *V. vulnificus* cytotoxic factor (42).

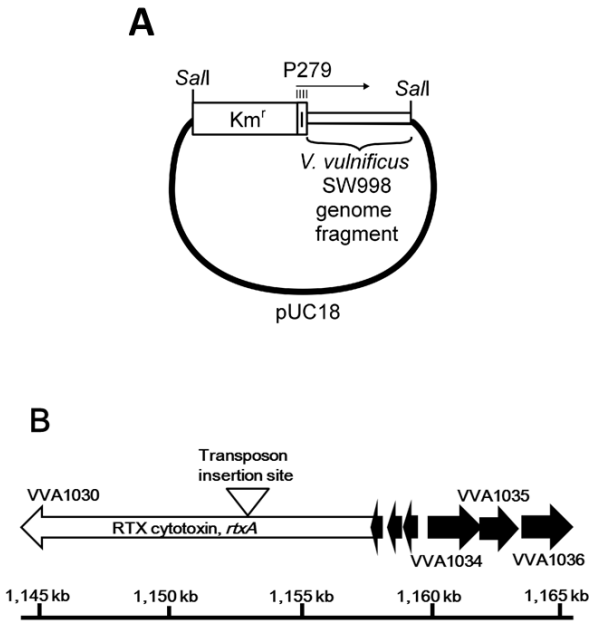

Figure 5. The transposon-inserted site of *V. vulnificus* SW998. (A) To find the transposon-inserted gene, the *V. vulnificus* SW998 transposon insertion region was cloned into the pUC18 plasmid vector, as described previously (18). The *Sal*I-digested genomic DNA of *V. vulnificus* SW998 was cloned into the pUC18 *Sal*I site with kanamycin-resistance as an indicator. The P279 oligonucleotide primer, which was designed to hybridize to the I-ends of the mini-Tn*5*Km2 transposon, was used to amplify the *V. vulnificus* SW998 transposon insertion site. Km^r, kanamycin resistance gene; arrow, direction of DNA sequencing. (B) The *V. vulnificus* chromosomal region around the RTX element and transposon insertion site are presented schematically. Arrows indicate the transcriptional directions and coding regions of the *V. vulnificus* YJ016 genes (accession no. NC_005140). The line under the arrow indicates gene location.

3.3 DISCUSSION

Some *in vivo* experiments designed to investigate the precise mechanisms of microbial infections are difficult to replace by *in vitro* experiments or models. Mice are often used to study *V. vulnificus* infection; however, using many animals may not always be economically and ethically feasible. Random transposon insertion mutagenesis and subsequent screening of the attenuated mutants have been applied to search for virulence genes in many bacterial pathogens. Thousands of mice would be required to isolate attenuated mutants. Animal experiments using mammals are still required; however, to obtain satisfactory results, many animals may need to be sacrificed. *In vivo* infection models using invertebrates instead of mammals have been successfully applied to pathogenic microbe studies, e.g., the *Staphylococcus aureus* virulence genes identified using a silkworm infection model (43). The purpose of this chapter was to examine whether the silkworm infection model could be applied to study *V. vulnificus* virulence.

In this chapter, silkworms were demonstrated to be sensitive to the *V. vulnificus* because they were killed by the inoculation of this bacterium into the hemolymph (Fig. 3). Kaito et al (44) reported that several bacterial species, such as *S. aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *V. cholerae*, which are human pathogens, can also infect and kill silkworms when injected into the blood stream of silkworms, whereas non-pathogenic laboratory strains of *E. coli* cannot. Although we have no direct evidence whether the silkworms died due to the factors same as mammals, silkworms may recognizes virulence factors of pathogens because they have

the Toll and Imd pathways, which are homologous to the mammalian Toll-like receptor and tumor necrosis factor receptor signaling pathways, respectively (45). Using the silkworm infection model, we obtained the SW998 attenuated mutant strain, 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 (42). Injection of culture filtrate did not kill the silkworms (Fig. 3), thus suggesting that RtxA expression increased after the pathogen interacted with host cells, as reported previously (46). That is, the bacteria secreted RtxA in the silkworm *in vivo* and RtxA may be toxic to silkworms, although we would need to completely remove the genes from the genome and conduct a lethality test to demonstrate that RtxA is actually toxic to silkworms.

In conclusion, silkworms died when inoculated experimentally with *V. vulnificus*. Together with this, the *rtxA* gene was identified successfully using this infection model. We are expecting this model to be useful for studying *V. vulnificus* virulence factors and to be helpful for developing effective therapies to protect against *V. vulnificus* infection.

Chapter 4

Conclusion and remarks

V. vulnificus causes highly lethal opportunistic infection. The knowledge of virulence factors would be helpful for developing effective therapies and preventive methods against *V. vulnificus* infection. To find virulence factors of pathogen, random transposon mutagenesis has been often used. After transposon inserted mutants were generated they were tested for attenuated in various screening system. *In vitro* screening have been often used to find attenuated mutant. However, *in vitro* screening have the potential to overlook virulence factors that activated *in vivo*. For *V. vulnificus*, the ability to growth in the human body is the important virulence factor. Very little *in vivo* screening studies of *V. vulnificus* infection have been reported. Although animal study is very important to study pathogens, we always meet the problem costly and ethically. In this thesis, I attempt to use new two methods to identify *V. vulnificus* virulence factors *in vivo*.

In chapter 2, I attempted to apply STM to investigate *V. vulnificus* virulence genes that are active *in vivo*. STM allows us to screen attenuated mutants from a pool of large numbers of mutants simultaneously in a single animal model. We can save the number of animal to be sacrificed by STM. Eleven attenuated mutants whose disrupted genes were suggested to be involved in *in vivo* growth during infection were obtained. Among 11 attenuated mutants, S10T79 which the IMP dehydrogenase gene were disrupted by transposon insertion was most attenuated and it has preventive effect against *V. vulnificus* infection in mouse infection model. IMP dehydrogenase is the enzyme that converts IMP to xanthosine monophosphate (XMP), which is the rate-limiting step in the de novo synthesis of guanine nucleotides. IMP dehydrogenase inhibitors are used as antiviral and immunosuppressive medicines. Purine metabolism plays an important role in various bacterial virulence. Thus IMP dehydrogenase could

be a target for antimicrobial agents against *V. vulnificus* and other bacteria.

In chapter 3, I attempted to use invertebrate infection models using silkworms to study *V. vulnificus* virulence. Silkworms were demonstrated to be sensitive to the *V. vulnificus* and using the silkworm infection model, an attenuated mutant strain, in which rtxA gene was disrupted by a transposon insertion were screend. I expect to the silkworm infection model could be used for various purpose to study *V. vulnificus* infection. For example, it would be useful to search risk factor of host side. High serum iron is one of risk factor of *V. vulnificus* infection. Therefore we have often used iron to make mice to be sensitive to *V. vulnificus*. In this study, a large quantity of *V. vulnificus* were inoculated to silkworm because silkworms did not die with a small number of bacterial injection even iron was used. We may search the factors which make silkworm to be sensitive to *V. vulnificus*. In addition, clinically used antibiotics are effective for silkworm (47). Therefore, novel antimicrobial agents against *V. vulnificus* may be screened using the silkworm infection model.

V. vulnificus infection has gained attention in recent years as an ocean-related disease including both foodborne septicemia and gastroenteritis. At the same time, viral hepatitis (such as hepatitis B and C) kills nearly 1.2 million people annually worldwide (48, 49). Such patients have to be wary of ingesting undercooked seafood because patients with severe hepatic disorders may acquire *V. vulnificus* infection. Therefore, to prevent *V. vulnificus* infection, immunization with the vaccine must be beneficial for these high-risk patients who choose to eat uncooked fresh seafood. In the study of other pathogens, the mutants of the genes for purine nucleotide synthesis had a protective effect as vaccines in animal models (50, 51). In chapter 2, I have demonstrated that even in *V. vulnificus*, the mutant defective in purine nucleotide synthesis (S10T79) was the

effective vaccine, which may be on account of elicited protective antibodies against the pathogen. Safer vaccine would be developed by identification of antigens.

In this thesis I applied two new methods to study virulence factors of *V. vulnificus in vivo*. Two method were demonstrated their applicability to the search for the virulence factors of *V. vulnificus in vivo*. These two methods would be helpful to elucidate the mechanism of a complicated *V. vulnificus* infection.

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