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Signature-tagged mutagenesis of *Vibrio vulnificus*

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ABSTRACT. *Vibrio vulnificus* is the causative agent of primary septicemia, wound infection and gastroenteritis in immunocompromised people. In this study, signature-tagged mutagenesis (STM) was applied to identify the virulence genes of *V. vulnificus*. Using STM, 6,480 mutants in total were constructed and divided into 81 sets (INPUT pools); each mutant in a set was assigned a different tag. Each INPUT pool was intraperitoneally injected into iron-overloaded mice, and *in vivo* surviving mutants were collected from blood samples from the heart (OUTPUT pools). From the genomic DNA of mixed INPUT or OUTPUT pools, digoxigenin-labeled DNA probes against the tagged region were prepared and used for dot hybridization. Thirty tentatively attenuated mutants, which were hybridized clearly with INPUT probes but barely with OUTPUT probes, were negatively selected. Lethal doses of 11 of the 30 mutants were reduced to more than 1/100; of these, the lethal doses of 2 were reduced to as low as 1/100,000. Transposon-inserted genes in the 11 attenuated mutants were those for IMP dehydrogenase, UDP-N-acetylglucosamine-2-epimerase, aspartokinase, phosphoribosylformylglycinamidine cyclo-ligase, malate Na (+) symporter and hypothetical protein. When mice were immunized with an attenuated mutant strain into which IMP dehydrogenase had been inserted with a transposon, they were protected against *V. vulnificus* infection. In this study, we demonstrated that the STM method can be used to search for the virulence genes of *V. vulnificus*.

KEY WORDS: foodborne disease, signature-tagged mutagenesis, vaccine, *Vibrio vulnificus*, virulence

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Vibrio vulnificus is a gram-negative bacterium that lives in sea and brackish water regions. This bacterium enters the human body by ingestion of raw seafood or through the contact of injured skin with sea water [8, 29]. When *V. vulnificus* invades the human body, it may cause severe, life-threatening septicemia. The mortality rates of *V. vulnificus* infection along with primary septicemia have been reported to be higher than 50% [3, 29]. In Japan, the annual number of *V. vulnificus* septicemia cases has been estimated at more than 200 [19, 22]. 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 [4]. *V. vulnificus* infection cases have also frequently been reported every year in other areas, such as Korea or Taiwan [10, 11]. Healthy people are usually not susceptible to *V. vulnificus* infection, except through food-borne gastroenteritis. Individuals with underlying illnesses, such as chronic hepatitis, cirrhosis diabetes or immunodeficiency, are more susceptible to this infection.

Several possible virulence factors have been reported for *V. vulnificus* infection. Cytolytic hemolysin (VvhA) [15], which resembles *V. cholerae* El Tor hemolysin [33], RTX toxin [16] and proteolytic elastases, such as VvpE [20], have been suggested to play a role in the destruction of the host tissue. A polysaccharide capsule is assumed to prevent phagocytosis [25]. In addition, *V. vulnificus* induces apoptosis in macrophages [13]. Although many virulence factors have been found in this organism, the pathogenic mechanisms of *V. vulnificus* infection remain unknown.

Because *in vitro* conditions may not always reflect the complicated *in vivo* host environments, pathogenic genes expressed only in the infection process could be overlooked. Recently, several screening methods for bacterial virulence genes that are active *in vivo* have been developed. In this study, we attempted to apply signature-tagged mutagenesis (STM) [9] to investigate *V. vulnificus* virulence genes that are active *in vivo*. Using STM, we identified several possible virulence genes of *V. vulnificus*.

MATERIALS AND METHODS

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 [18]. *E. coli* DH10B^{TM} competent cells were

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purchased from Life Technologies (Carlsbad, CA, U.S.A.). A signature-tagged mini-Tn*5*Km2 transposon in pUT delivery suicide plasmid pool was provided by Dr. David W. Holden [9]. 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*] [27] 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).

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.

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.

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/μ*l* with 100 *n*g/μ*l* of sheared salmon sperm DNA (sssDNA), denatured at 95°C for 5 min and chilled on ice. A 1-μ*l* 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 [27], 3× Denhardt's solution, 1% SDS and 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

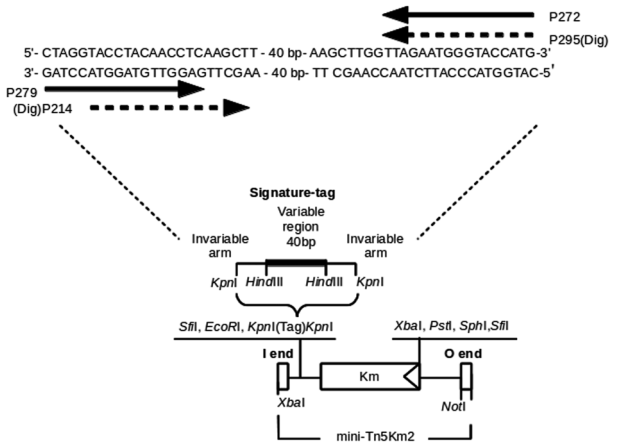

Fig. 1. Signature-tagged mini-Tn*5*Km2. The transposon mini-Tn*5*Km2 was incorporated into the *V. vulnificus* genome DNA when pUT mini-Tn*5*Km2 was transferred to *V. vulnificus* by conjugation. Signature tags comprised unique sequences of a 40-bp variable region flanked by invariable arms on either 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.

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.

DNA analysis: DNA sequencing was performed with an Applied Biosystems DNA sequencing system (Applied Biosystems, Waltham, MA, U.S.A.) 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.

RESULTS

Construction of transposon insertion mutant library: To

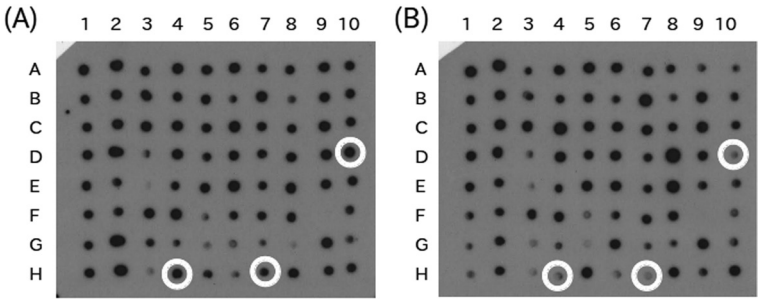

Fig. 2. Identification of tentative attenuated mutants *in vivo* by hybridization. Lost mutants of *V. vulnificu*s during infection were identified by DNA–DNA hybridization tests. Two identical membranes dotted with the individually amplified DNA tags (from A1 to H10) were hybridized to the DIG-labeled (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.

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.

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 $2–20 \times 10^3$ 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.

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 was 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).

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.

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

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.

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.

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 and S65T36), sialic acid synthesis (S46T31, S48T58 and S76T76), cell wall biosynthesis (S38T76), membrane transporter (S34T71) and unknown function (S20T62 and S37T71). In two mutants (S43T04 and S68T70), transposon-inserted regions showed no homology to any sequence in the GenBank database.

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.

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 [5, 6, 9, 17, 26]. 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 2 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 [24, 34, 35]. Kim *et al.* [14] 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 3 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 [32]. In *E. coli*, *neuC* mutants express an acapsular phenotype [1], 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 [2, 23]. 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 [12].

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* [7]. 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.

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 [30, 31]. 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 [21, 28]. We have demonstrated that even in *V. vulnificus*, a mutant defective in purine nucleotide synthesis (S10T79) was an effective vaccine, which may be on account of elicited protective antibodies against the pathogen. Because the transposon may drop out from the mutant and virulence may thereby be recovered, complete deletion of the relevant genes must be required. 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.

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