[SII-5]

Role of TolC in *Vibrio vulnificus* Virulence in Mice

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Abstract

The role of a TolC homologue in the virulence of *Vibrio vulnificus*, a marine bacterium causing serious wound infection and fulminant septicemia in persons with underlying conditions, has been studied. TolC, an outer membrane protein, has been implicated in a variety of bacterial functions including export of diverse molecules ranging from large proteins to antibiotics. A homologue of the *tolC* gene of *V. cholerae*, which has been shown to be required for bile resistance, cytotoxicity and colonization of this organism, was identified in the partially determined genome sequence of *V. vulnificus*. To determine the role of TolC in the virulence of *V. vulnificus*, a TolC-deficient (TD) mutant was isolated by in vivo allelic exchange. Compared with the parent strain, the TD mutant was more sensitive to bile, and much less virulent in mice challenged subcutaneously. This mutant was noncytotoxic to the HEp-2 cells, but its metalloprotease and cytolysin activities in the culture supernatant were comparable to the parent strain. In addition, the resistance of the TD mutant to human serum bactericidal activity as well as its growth in either human or murine blood was not affected. Collectively, our data suggest that TolC may be involved in colonization and/or spread of *V. vulnificus* to the blood stream, probably by secreting a cytotoxin other than the cytolysin.

Vibrio vulnificus, a gram-negative estuarine bacterium, causes severe wound infection and fulminant septicemia in humans, particularly those with underlying conditions such as liver cirrhosis and hemochromatosis (1, 2). Infection is usually acquired via direct contact or ingestion of contaminated seafood; in both cases, skin lesions with hemorrhagic ulcer and edema are commonly seen.

Strains of *V. vulnificus* produce a number of extracellular proteins, including the cytolysin (3), metalloprotease (4) and phospholipase (5), which have been implicated in bacterial invasion into the blood stream where they multiply and result in septicemia. To study the roles of the cytolysin, metalloproteas and phospholipase in the pathogenesis of *V. vulnificus* in mice, we have previously isolated and characterized mutants deficient in one, two, or all of the three factors (6, 7, 8). We found that all the mutants, including the triple-knockout mutant, were as virulent as the parent strain in mice, indicating that the protease, cytolysin and

phospholipase are not major virulence factors. We further found that *V. vulnificus* might produce an as yet unidentified cytotoxin, since the triple-knockout mutant was as cytotoxic as the parent strain to the HEp-2 cells (8). Meanwhile, we isolated a spontaneous noncytotoxic mutant accidentally from a mutant (DD mutant) that was deleted in the cytolysin and phospholipase. This noncytotoxic mutant was four orders less virulent than the wild-type strain or the DD mutant but grew normally in the murine blood. The phenotypes of this noncytotoxic mutant suggest that cytotoxicity might be associated with *V. vulnificus* virulence in mice.

The TolC protein, an outer membrane protein involved in export of diverse substrates ranging from large protein toxins to small toxic compounds (9), has been shown to play a role in bile resistance, cytotoxicity, and intestinal colonization of *V. cholerae* (10). An open-reading frame encoding a polypeptide with 87% similarity in amino acid sequence to the TolC protein of *V. cholerae* was identified in the partially determined genome sequence of *V. vulnificus* strain YJ016 (Fig. 1). To determine whether TolC was involved in the virulence of *V. vulnificus* by exporting cytotoxin(s) other than cytolysin, we isolated a TolC-deficient (TD) mutant by in vivo allelic exchange technique (11). Briefly, a 727-bp deletion (Fig. 1) was introduced to the *tolC* gene carried by a suicide plasmid, and the *tolC* gene with the deletion in the plasmid was later introduced to the *V. vulnificus* chromosome via two sequential homologous recombination events.

Two out of 408 resulting strains were confirmed to contain the deletion in *tolC*. The TD mutant thus obtained grew normally in the LB medium compared with the parent strain. However, unlike its parent strain, the TD mutant was not able to grow in LB medium containing 0.02% bile, suggesting that TolC was required for bile resistance of *V. vulnificus*. The human intestine normally contains 20 mM of bile salt, a concentration much higher than that used in the bile-sensitivity assay, and in this sense, TolC is likely to be important for the survival of *V. vulnificus* in the intestine.

The cytotoxicity of the TD mutant was also investigated. The washed bacterial cells prepared from a 4-h culture of the TD mutant as well as its parent strain were added to the monolayer of HEp-2 cells at an multiplicity of infection (MOI) of 10, and the coculture was incubated for 3 hours. In contrast to the parent strain, which showed almost 100% cytotoxicity, the TD mutant exhibited no cytotoxicity at all. We then examined the cytolysin and protease activities in the supernatant of a 4-h culture of the TD mutant and found that both activities were comparable to the parent strain. These data indicate that TolC may be involved in the export of an unidentified cytotoxin that causes lysis of the HEp-2 cells, but not in the export of the cytolysin. A candidate of the unidentified cytotoxin is an RTX toxin family member reported previously (12). In addition, the cytolysin although is secreted into the culture supernatant when the bacteria are cultured in the LB medium, it is not secreted into the medium under the conditions used in the cytotoxicity assay with the whole bacterial cells.

The virulence of the TD mutant was tested in the C3H/HeNCrj mouse strain. The LD_{50} of the TD mutant challenging subcutaneously into the mouse was 350-fold (3.3×10^7 versus 9.5×10^4) higher than the parent strain, indicating that disruption of *tolC* lowered the virulence of *V. vulnificus* in mice. We then found that the TD

mutant was as resistant as the parent strain to the serum killing effect, and grew normally in either the murine or human blood. Therefore, the reduced virulence of the TD mutant in mice could be due to its defect in colonization and/or invasion from the local infection site into the blood stream, rather than survival in the blood

Based on the phenotypes of the TD mutant (summarized in Table 1) we conclude that TolC of *V. vulnificus* is associated with the virulence of this organism in mice, probably by mediating bile resistance and secretion of a cytotoxin other than cytolysin. We are currently conducting a complementation of the TD mutant with functional TolC expressed from a plasmid to confirm the association of TolC with the phenotypes we observed on the TD mutant.

Acknowledgement

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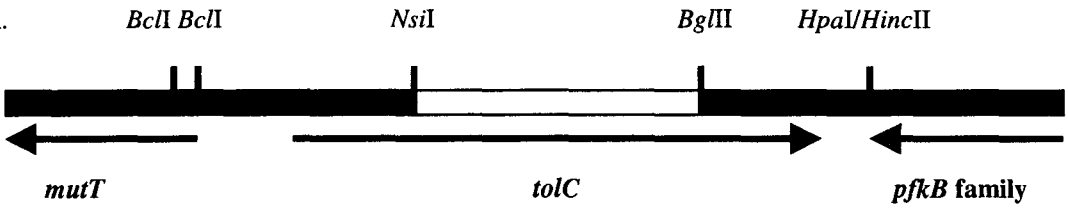
A.



B.

	VC	EC	SE
Similarity (%)	87	62	64

Fig. 1. (A) Restriction map of the *tolC* gene and its flanking regions. The orientation of transcription and the deletion (blank bar) introduced in the TD mutant are indicated. (B) Similarity of *V. vulnificus* TolC to those of *V. cholerae* (EC), E*scherichia coli* (EC), and *Salmonella enteritidis* (SE) in amino acid sequence.

Table 1. Summary of phenotypes of the TD mutant compared with the parent strain

Phenotype	Wild-type	TD mutant
Sensitivity to 0.02% bile	Resistant	Sensitive
Cytotoxicity to HEp-2 cells	Yes	No
Virulence in mice (LD_{50})	9.5×10^4	3.3×10^7
Sensitivity to human serum	Resistant	Resistant
Growth in human and murine blood	Yes	Yes
Secretion of protease and cytolysin	Yes	Yes