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An Improved Procedure for the Pulsed-field Gel Electrophoresis Analysis of *Vibrio vulnificus*

HIN-CHUNG WONG* AND CHENG-JU KUO

Department of Microbiology, Soochow University, Taipei 111, Taiwan, R.O.C.

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ABSTRACT

Vibrio vulnificus is a marine bacterium that causes septicemia with high mortality via wound infection or seafood ingestion. Subspecies typing of *V. vulnificus* can be performed by pulsed-field gel electrophoresis (PFGE). However, this method is hampered by the degradation of chromosomal DNA in about 12% of strains. This study presents a modified PFGE procedure. The outer membrane of *V. vulnificus* cells was lysed utilizing a sucrose-EDTA method and components in the periplasmic space were removed prior to making plugs. Cytoplasmic digestive enzymes were inactivated by an extended proteinase K reaction. Experimental results indicated that five out of eight strains exhibiting smeared DNA in a previous study (Appl. Environ. Microbiol. 70: 5153-5158. 2004) achieved clear banding patterns. This modified PFGE procedure can be applied to improve PFGE typing of untypeable strains of *V. vulnificus*.

Key words: *Vibrio vulnificus*, pulsed-field gel electrophoresis, DNase

INTRODUCTION

Vibrio vulnificus is an autochthonous marine and estuarine bacterium commonly associated with shellfish[1]. This bacterium frequently causes septicemia with high mortality in susceptible persons via wound infection or ingesting contaminated seafood[2]. *V. vulnificus* strains are subgrouped into biotypes 1 and 2. Biotype 1 strains are ubiquitous in marine environments and occasional on human, whereas biotype 2 strains are generally restricted to diseased eels[3].

In addition to biotyping, several molecular techniques have been developed to examine the intraspecific diversity of *V. vulnificus*, including amplified fragment length polymorphism[4], randomly amplified polymorphic DNA[5], pulsed-field gel electrophoresis (PFGE), and ribotyping[6]. PFGE is considered a reliable approach for differentiating strains in many other pathogenic bacteria, including several *Vibrio* species such as *V. cholerae*[7], *V. anguillarum*[8], and *V. parahaemolyticus*[9]. We successfully employed PFGE following *Sfi*I or *Not*I digestion to characterize *V. vulnificus* strains obtained in the United States and Taiwan[10].

The conventional PFGE procedure is limited in that a markedly high percentage (about 12%) of the *V. vulnificus* strains formed smeared DNA and, consequently, were untypeable[10]. Degradation of the chromosomal DNA may result from the presence of highly active digestive enzymes. *V. vulnificus* has several extracellular enzymes, namely, protease[11], phospholipase[12], cytolysin[13], and DNase[14], etc. DNase exhibits multiple functions in bacteria, such as lysing intestinal mucosal linings for the adherence of bacteria, providing carbon and nitrogen sources for bacterial growth, and preventing intrusion of foreign DNA[15]. Degradation of DNA in PFGE is likely due to the presence of Dnase[16]. The nuclease of *V. vulnificus* is a heat-stable alkaline-tolerant periplasmic protein (25 kDa) that can digest RNA and DNA[17]. The aim of this study is to improve PFGE analysis of *V. vulnificus* by removing the periplasmic components.

* Author for correspondence. Tel: +886-2-8819471 ext. 6852; Fax: +886-2-8831193; E-mail: wonghc@scu.edu.tw

MATERIALS AND METHODS

I. *Bacterial Strains and Cultivation*

Eight strains of *V. vulnificus* that exhibited DNA degradation in a previous study were examined[10]. Strain VJ11 was a clinical isolate from Taiwan and strains LSU549 and ORL8074CDC were clinical isolates from the USA Environmental strains CG41, CG54, CG63 and CG65 were from Taiwan, and SS109A-4B2 was an environmental strain from the USA. The clinical strain YJ03 from Taiwan had a clear PFGE pattern and was used as a positive control in this study[10].

Stock cultures were maintained in Tryptic Soy Broth (Difco, Becton-Dickinson Diagnostic Systems, Sparks, NJ, USA) with 10% glycerol at -85°C. *V. vulnificus* was cultured at 37°C in Luria-Bertani Broth (LB, Difco) or Agar (LA, Difco) with a supplement of 1% NaCl.

II. *Removal of Periplasmic Components*

Bacteria on LA-1% NaCl were inoculated to 5 mL LB-1% NaCl, and incubated at 37°C and shaken at 160 rpm for 24 hr. Bacterial cells at the stationary

phase were harvested by centrifugation, resuspended in 2 mL of sucrose-EDTA buffer (100 mM Tris-HCl, 250 mM sucrose, 10 mM EDTA) containing 50 μg/mL lysozyme[16], and incubated at 25°C for 30 min to disrupt the outer membrane. Cells were then harvested and resuspended in buffer containing 10 mM Tris-Base, 10 mM EDTA and 0.83 M NaCl at pH 8.0.

III. *Pulsed-field Gel Electrophoresis*

DNA extraction, DNA digestion and PFGE were performed according to the method previously described[9] with modifications. Agarose plugs were prepared by mixing an equal volume of bacterial suspensions with 1.5% low-melting agarose (FMC Corp., Rockland, ME, USA). Cells in the agarose plugs were lysed with a lysis solution containing 2.5 mg/mL lysozyme, 1% N-sodium lauroyl sarcosine and 0.1 M EDTA, and incubated at 37° C for 24 hr. The plugs were washed in TE buffer (10 mM Tris•HCl, 1 mM EDTA) three times for 20 min each. The cells were then treated with proteinase K (0.5 mg/mL in 0.5 M EDTA and 1% N-sodium lauroyl sarcosine) at 45°C for 72 hr, and washed three times for 30 min each with TE buffer. One plug section (4 × 9 × 1.2 mm) was equilibrated with an enzyme buffer and then placed in 100 μL fresh buffer containing 10 units of SfiI (New England BioLabs, Beverly, MA, USA) and incubated at 37°C for 4 hr.

High molecular-weight restriction fragments were resolved in 1% agarose gel in 0.5% Tris-borate-EDTA buffer using a CHEF apparatus (CHEF-DR II; Bio-Rad Laboratories, Richmond, CA, USA). Running conditions were 200 V for 24 hr at 14°C, with a 1-40 sec pulse time. A lambda ladder PFGE marker (New England Biolabs, Beverly, MA, USA) was used as molecular size markers. Following electrophoresis, gels were stained in ethidium bromide (Sigma Co., St. Louis, MO, USA), destained in distilled water, and photographed over a UV transilluminator V028490 (Vilber Lourmat, Torey, France).

RESULTS AND DISCUSSION

Degradation of DNA, a principal drawback of PFGE, occurs in *V. vulnificus* and many other species including *Campylobacter concisus*[18], *Salmonella enterica*[19] and *V. parahaemolyticus*[20]. Thus, application of this reliable and highly discriminative molecular method is impaired. Several modifications have been applied to minimize DNA degradation. Koort *et al*.[21] altered the running buffer from 0.5× Tris-borate-EDTA to HEPES to decrease DNA degradation during electrophoresis of restricted *Escherichia coli* DNA. As chromosomal DNA of *Clostridium difficile* is highly sensitive to degradation, thiourea was added to the running buffer to minimize the DNA degradation of *C. difficile* during electrophoresis[22]. DNase activity in *C. botulinum* cells was inactivated by a formaldehyde solution[23].

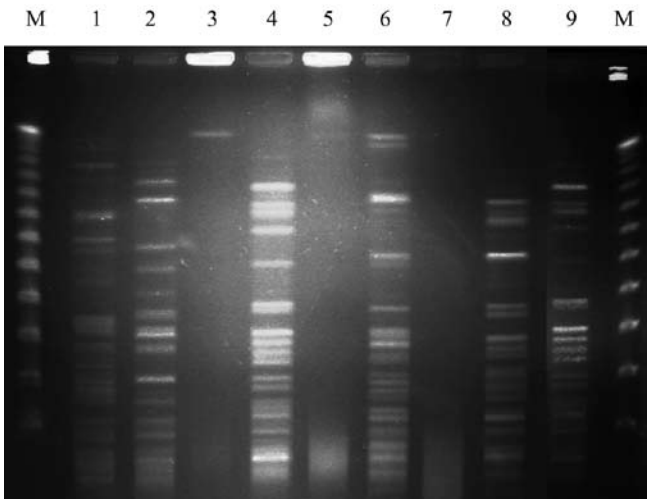

Figure 1. Banding patterns of *Vibrio vulnificus* strains analyzed via a modified PFGE procedure. M, Molecular-sized ladder markers starting at 48.5 kb with increments of 48.5 kb. Lane 1, strain YJ03; 2, strain YJ11; 3, strain SS109A-4B2; 4, strain CG41; 5, strain LSU549; 6, strain CG54; 7, strain CG63; 8, strain CG65; 9, ORL8074CDC. Lane 9 is from another running gel. Except strain YJ03, all other strains exhibited DNA degradation in previous PFGE analysis[10].

In this study, two novel modifications were adopted to improve PFGE for *V. vulnificus*. The sucrose-EDTA method was applied to eliminate periplasmic components, including periplasmic DNase[16]. The incubation time for the proteinase K reaction was increased from 48 to 72 hr to ensure complete inactivation of the degradative enzymes. These modifications likely removed or deactivated nuclease and other digestive enzymes in periplasmic and cytoplasmic fractions. Results in at least triplicate experiments indicated that five of the eight strains that had smeared DNAs, namely, YJ11, CG41, CG54, CG65 and ORL8074CDC, achieved clear banding patterns, on the other hand, SS109A-4B2, LSU549 and CG63 did not show clear patterns (Figure 1). Other problems may hinder the PFGE process. In addition to smeared DNA, most chromosomal DNA of SS109A-4B2 and LSU549 were stuck in the loading wells and did not migrate into the running gel. These two strains formed a thick capsule likely interfered with the lysis of outer membrane, proteinase digestion, restricted enzyme activity or the migration of the restricted DNA fragments. One may also try to use thiourea or formaldehyde to inhibit DNA degradation of strains SS109A-4B2, LSU549 and CG63[23].

In conclusion, this modified PFGE procedure enhances PFGE typing of previously untypeable strains of *V. vulnificus* and other pathogens.

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