GENETIC STUDIES ON VIBRIO FLUVIALIS AND ITS ENTEROTOXINS

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ABSTRACT

Vibrio fluvialis, an estuarine-based halophilic vibrio, causes opportunistic infections in humans resulting in bloody diarrhea. The organism produces an enterotoxin (ET), a cytotoxin (CT), and a cytolysin (CL). We created isogenic non-toxigenic mutants to assess the relative contribution of each toxin to virulence. High level streptomycin (Sm) resistant mutants of the prototype toxigenic strain 807-77 were used as conjugal recipients for TnphoA, which creates gene fusions between target genes and phoA, which encodes the alkaline phosphatase of Escherichia coli. Transposon insertions into genes for secreted or periplasmic proteins were selected on indicator plates. Putative CL- transconjugates (which appear as dark blue colonies) were recovered at a rate of ca. 10^6/recipient cell. Presumptive CL- mutants were screened on blood agar, egg yolk agar, and with a sensitive tube hemolysin assay. Twenty-two CL- mutants were tested vs. red cells from two different species. CL- production was attenuated in 17 and negative in 5 of the mutants. The verified CL- mutants were tested for pathogenicity in infant mice. The CL- mutants were unaltered in their ability to cause fluid accumulation in this model. Various CL- mutants responded differently to increased osmolarity in their growth media as measured by alkaline phosphatase activity of permeabilized cells.

INTRODUCTION

Beginning from the mid-1970's, it became clear that there was a small group of halophilic Vibrios that were serologically heterogeneous and were often confused with members of the genus Aeromonas. The isolates were originally called group F Vibrios and included strains that were both aerogenic and anaerogenic. The aerogenic strains are now a separate species, Vibrio furnissii. Group F Vibrios gained notoriety when the organisms were isolated from patients in Bangladesh presenting with non-cholera diarrhea during outbreaks in 1976-1977. Group F strains were epidemiologically implicated as potential enteropathogens because no other bacterial pathogens were recovered from the patient's stool specimens. Some of these strains were sent to the Centers for Disease Control where they were designated group EF-6. It was concluded in 1980 from DNA-DNA hybridization data, that group F and group EF-6 were identical and that the strains within these groups should be classified as a new species which was named V. fluvialis.
Fluvialis, which means "river" in Latin, is a Gram negative halophilic, curved, rod-shaped bacterium whose natural habitat is stagnant rivers and estuarine waters. The organism causes opportunistic infections in humans resulting in diarrhea, vomiting, abdominal pain, moderate to severe dehydration, and fever. Direct examination of stool samples often reveals leukocytes and erythrocytes and some patients have overt bloody diarrhea.

Viable cells of *V. fluvialis* have been shown to stimulate fluid accumulation in rabbit ileal loops. Different isolates also liberate various combinations of three physically and functionally distinct toxins: i) a cell-bound cholera-like enterotoxin (ET), eliciting diarrhea in animal models, and causing cell elongation in Chinese-Hamster-Ovary (CHO) cells; ii) a non-hemolytic cytotoxin (CT) which results in death and disintegration of CHO cells; iii) a separate hemolytic cytolsin (CL) which evokes morphological changes leading to loss of viability in CHO cells. Oral challenge of infant mice with *V. fluvialis* is followed by fluid accumulation, diarrhea and death. The fluid accumulation ratios recorded in these experiments were similar to those generated by *V. cholerae*.

Because these bacteria elaborate three distinct toxins and incite diarrhea in infant mice, we wanted to determine which toxin or combination of toxins was responsible for diarrhea through the creation of stable mutants defective in toxin production. To achieve this goal we decided to construct insertionally inactivated isogenic mutants using transposon TnphoA recently described by Manoil and Beckwith, which is a fusion between an enzymatically active fragment of the alkaline phosphatase of *E. coli* and Tn5 which codes for kanamycin resistance. TnphoA is delivered by broad host range vectors by conjugation and has been used extensively for the analysis of secreted and periplasmic proteins of Gram negative bacteria.

Here we describe the construction of *V. fluvialis* mutants that no longer produce a functional CL toxin. In addition, we used in vitro and in vivo techniques to verify that mutational events were restricted to the CL gene, tested the virulence of the organism in an animal model, and measured the expression of alkaline phosphatase in 2 of the CL- mutants under a variety of growth conditions.

MATERIALS AND METHODS

**Bacterial and plasmid strains**

*Vibrio fluvialis* strain 807-77 obtained from the Enterobacteriaceae Section of the Centers for Disease Control, Atlanta, Georgia, was a human isolate from an outbreak in Bangladesh. Of the 150 strains tested from our collection, 807-77 produced the highest titers of CL and was thus selected for further study.

*Escherichia coli* strain SM10pir containing plasmid pRT733 (oriR6K, tra, mob*, Ap+, Km+) was used in conjugation experiments. pRT733 is a suicide vector and is a derivative of pJM703.1 which is a pBR325 derivative that has the CoIE1 origin of replication replaced with a oriR6K. The plasmid is only functional in the presence of the pir gene which is supplied by a lambda-pir prophage. This plasmid carries the mob site from RP4. All *E. coli* plasmid bearing strains were kindly donated by Dr. John Mekalanos of Harvard Medical School (Boston, Massachusetts).

Frozen stock suspensions were prepared from all bacterial strains grown for 18 hours at 30°C on Columbia agar (CA; Oxoid, Ltd., England) or in Heart infusion broth (HIB; Difco Laboratories, Detroit, Michigan). Bacteria washed from agar plates with HIB or HIB broth cultures were adjusted to 40%