CONSTRUCTION AND ANALYSIS OF A *mutL* KNOCKOUT STRAIN OF *VIBRIO CHOLEREAE*

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**Abstract**

Most bacterial species contain a DNA mismatch repair system. In *E. coli*, the products of *mutS*, *mutL*, and *mutH*, among others, reduce the rate at which mismatch mutations occur, as well as reduce the incorporation of foreign DNA into the *E. coli* genome (Townsend, 2003). The absence or disruption of these genes increases the background mutation rate (Taddei, 1997). In this study, we describe the construction of a *mutL* deletion mutant of *Vibrio cholerae*. This knockout strain will be used to determine the contribution of methyl-directed DNA mismatch repair in conferring antibiotic resistance in *V. cholerae*. If the knockout of *mutL* produces a hypervariable strain of *V. cholerae*, the strain may also be useful in conducting directed evolution experiments at an accelerated pace. The population of *V. cholerae* mutants obtained in such studies may change the way targets for detection, identification, or even vaccines are chosen.

**Subject Terms**

*Vibrio cholerae*, Dam-mismatch repair, Mutator pathway

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CONSTRUCTION AND ANALYSIS OF A MUTL KNOCKOUT STRAIN OF VIBRIO CHOLERAE

1. INTRODUCTION

Cholera is an ancient disease in the midst of a modern resurgence. With modern medical care, case fatality rates for cholera should be <0.25%; however, in epidemics in remote and medically underserved areas (particularly in Africa), case fatality rates often exceed 10%, and can approach 50%. Due to its ability to cause large epidemics of debilitating disease spread by food and/or water, *Vibrio cholerae* has a significant potential for weaponization. In spite of its potential for causing mass casualties it is a relatively safe organism for laboratory work and therefore is a useful model system for answering basic biological questions applicable to other pathogens.

*V. cholerae* is a diverse environmental species. Seven cholera pandemics have occurred since 1817; in each, cholera has spread rapidly from a focus in Asia to most of the known world (Pollitzer, 1959). In contrast to the diversity seen within *V. cholerae* as a whole, each cholera pandemic appears to be caused by a single, almost clonal strain, with only slight strain differences appearing as the pandemic moves across continents and through time (Momen, 1985; Wachsmuth, 1994). In 1992, a new, non-O1 *V. cholerae* strain (designated *V. cholerae* O139 Bengal), expressing a new O antigen and capsule, appeared in India and spread rapidly across the Indian subcontinent and through much of Asia (ICDDR,B 1993; Nair, 1994), supplanting "native" seventh pandemic *V. cholerae* O1 El Tor strains. These observations highlight the possibility for pandemic spread of "new," emergent strains that carry an appropriate combination of genetic material (including, potentially, novel genes or gene combinations) for a pandemic outbreak.

**Mutator Strains.**

New biological threat agents can be produced by natural mutations, directed evolution, or via more direct molecular engineering techniques. In many cases, the engineered organism is at a disadvantage relative to wild type species in the environment, and this bears directly on its use as a threat agent. The survival of a species is dependent upon a balance between the fidelity of its replication and the increased fitness conferred by its natural mutations. One of the methods for correcting mismatches and mutations that occur during replication is the Dam-dependent mismatch repair system. The main components of this system, which has been found in a number of different species of bacteria, are found in the mutHLS pathway (Aronshtam and Marinus, 1996). When an error is found in replication, the pathway is activated and repairs are performed. Inactivation of the pathway can lead to the development of hypermutator strains that have a significantly increased rate of mutation. Such mutations would be expected to enhance adaptation of bacterial populations by increasing the supply rate of beneficial mutations during periods of strong selection; phrased another way, mutators serve as a "fast track" to generate
adaptive mutations (Taddei, 1997). This concept of “mutator” stains (i.e., strains with a hypermutable phenotype) is well recognized in work with E. coli and Salmonella (LeClerc, 1996). If the right environmental stressors are present, these mutator strains can lead to the production of a mutant with better survival rates or increased pathogenicity under new environmental conditions (Mekalanos, 1979; Townsend, 2003). The hypermutator phenotype is generally lost once the strain has adapted to the new environment, as it is deleterious to survival of the strain under normal conditions. Since an increased rate of mutation could be used to isolate strains with improved biothreat characteristics, the presence of stable hypermutator strains would be a marker for genetically engineered threat agents.

The concepts outlined above have been developed primarily in work with E. coli and Salmonella. Interestingly, in Neisseria meningitidis, mutations in mismatch repair have also been associated with a phenotype demonstrating high-frequency phase variation (Richardson, 2001). In a recent review, Claverys and colleagues (Claverys, 2000) highlighted the variation between the E. coli/Salmonella SOS/mut system and that seen in pneumococci. In contrast to E. coli, pneumococci become competent during exponential growth, accepting any single-stranded DNA, with development of competence dependent on cell density. The pneumococci mismatch repair system Hex can reduce transformation frequencies for point mutations in homologous DNA, but is unable to prevent interspecies transformation. Finally, the system of stress-induced mutagenesis (i.e., the SOS response) seen in E. coli is not present in pneumococci. This results in what Claverys calls “programmed variability,” designed to favor interspecific exchanges in the whole population, rather than the “random,” strain-dependent variability seen in E. coli. This, in turn, leads to the hypothesis that the success of S. pneumoniae as a human pathogen is related to an inherent “genetic plasticity,” which favors adaptation to environmental changes. No studies have been done on mutators in V. cholerae. However, based on the annotation of the V. cholerae N16961 genome sequence, V. cholerae appears to have the intact mut mismatch repair (MMR) system, including mutH, mutL, mutS, mutT, mutU, and mutY (Friedhoff, 2002). The apparent increase in recombination rates within the species raises the possibility that V. cholerae also has elements of “programmed variability,” increasing its ability to adapt to changing environments and its success as a human pathogen.

To understand evolutionary change in cholera, we need to have an understanding of mechanisms involved in this variability, and the contributions they make to the mutation rates of the species as a whole. Therefore, producing a knockout strain of Vibrio cholerae, containing an internal deletion within the mutL gene, would help to determine the effect of the mutHLS pathway on Vibrio evolution.
2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media.

Polymyxin B-resistant *V. cholerae* strain N16961 (wild-type for MMR) was the target strain for mutant construction. Plasmids based on pBluescript (Stratagene, La Jolla, CA) were maintained in *E. coli* strain XL1 Blue (Strategene). Plasmids based on pCVD442 (which requires Pi protein provided in trans for replication) were maintained in *E. coli* strain SM17Apir. Bacteria were stored in LB broth and 10% glycerol at -80°C, and grown in LB broth, on LB agar and LB without NaCl agar. Carbenicillin (100μg/ml), polymyxin B (50U/ml) and sucrose (10%) (SIGMA, St. Louis, MO) were added to the media as required.

Competent cells were made by growing the cultures to mid-logarithmic phase, chilling on ice for 30 min, washing three times with distilled water, and resuspending them in 10% glycerol. Electroporation was performed in chilled, 0.1-cm cuvettes using the *E. coli* Pulser (Bio-Rad, Richmond, CA) at 1.88 kV.

2.2 Molecular Biology.

PCR primers were designed using Primer Express Software (Applied Biosystems, Inc. [ABI], Foster City, CA) and analyzed using the web-based OligoAnalyzer 3.0 software (Integrated DNA Technologies). PCR amplification of gene fragments was performed using an ABI 9600 thermocycler. Agarose gel electrophoresis, restriction enzyme digests, and ligations were performed using standard techniques (Sambrook, 1989). Extractions and purifications were performed according to the manufacturer's instructions in kits purchased from QIAgen (QIAGEN, Inc., Valencia, CA).

3. RESULTS

3.1 Amplification of Genes.

The genome sequence of *Vibrio cholerae* was analyzed for the location, orientation and open reading frame (ORF) of the *mutL* gene. Primers were designed to amplify a truncated version of the *mutL* gene (Δ*mutL*) that did not disrupt the original orientation and ORF (see table). By keeping the ORF intact, any manipulations using the truncated *mutL* gene could attribute phenotypic changes to the disruption of the mutator gene, and not any of the surrounding genes. Using primers 1 and 2, listed in the table, a 602bp fragment was created that contains part of the gene VC0344, which encodes N-acetylmuramoyl-L-alanine amidase, the leading piece of *mutL*, as well as the unique restriction sites for *KpnI*, *Xmal*, and *HindIII* (*mut1*). Using primers 3 and 4, listed in the table, a 596bp fragment was created that contains part of the gene VC0346, which encodes delta(2)-isopentenylpyro-phosphate transferase, the end piece of *mutL*, as well as the unique restriction sites for *XbaI*, *Xmal*, and *HindIII* (*mut2*). (Figure 1A).
These amplified fragments were separated by gel electrophoresis, extracted, and purified.

### Table. Sequences of PCR Primers Used in these Experiments.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Start Site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-TTAGGTACCCCCGGGGGTAGTGGTGATAACC-3'</td>
<td>366523</td>
</tr>
<tr>
<td>2</td>
<td>5'-TTAAAGCTTATTGCTGCCGGATCTGTTATCTTACGC-3'</td>
<td>367043</td>
</tr>
<tr>
<td>3</td>
<td>5'-ACCAAGCTTGTGACTACTGAAGCCGGTCTTTCT-3'</td>
<td>368743</td>
</tr>
<tr>
<td>4</td>
<td>5'-ATTCTAGACCCGGGCGGAGTTAAAGCTCA-3'</td>
<td>369315</td>
</tr>
</tbody>
</table>

*Underlined sequence indicates the unique restriction sites added.*

*Genome sequence from NCBI Genbank Accession NC_002505*

### 3.2 Construction of Intermediates pBNM and pBNMI.

Plasmid pBNM was created by digestion of the purified *mut1* fragment and pBluescript with *KpnI* and *HindIII*, separation by gel electrophoresis, extraction, purification, and joining the two with T4 DNA ligase (Figure 1B). Competent XL1 Blue cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion, and the visualization of a 602bp band after gel electrophoresis.

Similarly, plasmid pBNMI was created by digestion of the purified *mut2* fragment and pBNM with *HindIII* and *XbaI*, separation by gel electrophoresis, extraction, purification, and joining the two with T4 DNA ligase (Figure 1B). Competent XL1 Blue cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion, and the visualization of a 596bp band after gel electrophoresis.
Figure 1. Construction and Cloning of Δ\textit{mutL}, an Allele of \textit{mutL} from \textit{V. cholerae} Strain N16961 Containing an Internal Deletion. A. Physical map of \textit{mutL} and flanking genes. Shaded boxes indicate the genes flanking \textit{mutL}. Solid arrows indicate placement of forward primers. Dotted arrows indicate placement of reverse primers. Numbers correspond to primer sequences located in the table. K: KpnI, H: HindIII, Xm: Xmal, Xb: Xbal. The bar above genome indicates the size of the fragment produced in the intact genome, using primers 1 and 4 from the table. Genes are not drawn to scale. B. Construction of suicide vector pCVD442L, which contains a truncated version of \textit{mutL} from \textit{V. cholerae} N16961. Plasmid pBNM was created by amplifying a fragment of the \textit{V. cholerae} genome containing the 3'end of VC0344 and the 5'end of \textit{mutL}, digesting the fragment and pBluescript with KpnI and HindIII, ligating, and selecting for ampicillin resistant colonies in \textit{E. coli} XL1Blue cells. Similarly, pBNMI was created by amplifying a fragment of the \textit{V. cholerae} genome containing the 3'end of \textit{mutL} and the 5'end of VC0346, digesting the fragment and pBNM with HindIII and Xbal, ligating, and selecting for ampicillin resistant colonies in XL1Blue. The Δ\textit{mutL} fragment was created by cutting pBNMI with Xmal and joining the 1.2-kb fragment with the Xmal-digested plasmid pCVD442, which contains the sac\textit{B} gene of \textit{B. subtilis} and the \textit{bla} gene. The resulting suicide vector was selected by ampicillin resistance in \textit{E. coli} SM17λpir cells. Plasmids are not drawn to scale.
3.3 Construction of pCVD442L.

The suicide vector, pCVD442L, was created by adapting the vector described by Donnenberg and Kaper (1991). Plasmid pCVD442 contains the bla gene, which allows for the selection of antibiotic resistance. This plasmid also contains the sacB gene of B. subtilis, the locus that encodes the enzyme levan sucrase, which is toxic for gram negative organisms only in the presence of sucrose. This conditionally lethal phenotype can lead to the selection of colonies that have undergone an allelic exchange by homologous recombination. Furthermore, this plasmid contains the origin of plasmid R6K and the mob gene, which ensures that its replication is dependent upon π, the pir gene product (Donnenberg, 1991). In this study, the suicide vector pCVD442L was created by the digestion of pBNMI and pCVD442 with Xmal and joining the 1.2kb ΔmutL fragment with the suicide vector using T4 DNA ligase (Figure 1B). Competent SM17λpir cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion and the visualization of a 1.2kb band after gel electrophoresis (Figure 2).

![Figure 2. Agarose Gel of pCVD442L Digested with Xmal. Lane 1: pCVD442 (6,000bp) and ΔmutL (1,198 bp), lane 2: pCVD442, mw, 1 Kb DNA Ladder.](image)

3.4 Construction of Knockout V. cholerae Strain.

Homologous recombination was set up with E. coli SM17λpir containing pCVD442L as the donor strain and V. cholerae N16961 as the recipient strain. Various ratios of the donor and recipient, used to ensure the optimal growth of the bacteria, were pipetted onto a sterile 0.2 μm filter on top of an LB agar plate. These mixed cultures were grown without selection for 6 hr, and the filters were then placed into sterile phosphate buffered saline (PBS). Serial dilutions were spread onto LB plates containing polymyxin B and carbenicillin. Colonies that grew on these plates, under both selections, should be V. cholera that has undergone a single cross-over with the pCVD442L plasmid.

Isolates found to be both polymyxin B and carbenicillin resistant were grown without selection to late logarithmic phase and spread on LB plates without NaCl, but containing 10% sucrose. These same colonies were also split and grown on LB plates containing polymyxin B and carbenicillin for verification of their resistance.
Colonies that grew in both places were then patched onto both sucrose and carbenicillin plates. Colonies that are resistant to carbenicillin would contain a single cross-over event, retaining the resistant plasmid within the genome. Colonies that are unable to grow on the carbenicillin plates, but do grow on the sucrose plates, would have undergone a double cross-over in the *Vibrio* genome, resulting in the Δ*mutL* gene (Figure 3). If the colony had reverted back to the original genome, it would not be resistant to carbenicillin or be able to utilize sucrose well.

Figure 3. Replacement of the Wild-Type *mutL* with Δ*mutL* in the *V. cholerae* N16961 Genome by Homologous Recombination.

3.5 Verification of Double Cross-Over Event.

Twelve colonies were found to be carbenicillin sensitive and able to utilize sucrose. To determine if the colonies contained the Δ*mutL* gene, PCR was performed using primers 1 and 4 from the table. If the *Vibrio* underwent a double cross-over event, those primers would produce an amplicon of approximately 1.2Kb. However, if the *Vibrio* reverted back to the original genome, with another single cross-over, those primers would produce a 2880 bp amplicon. There were nine of the twelve colonies that produced 1.2Kb bands (Δ*mutL*) when run on an agarose gel (Figure 4), the remaining three colonies produced 2.8Kb bands (*mutL*).
Figure 4. Verification of the Replacement of the Wild-Type mutL Allele with ΔmutL.
A. Amplification of mutL DNA from wild-type V. cholerae N16961 and putative mutL deletion strains. Lane 1, V. cholerae N16961; lane 2, 1Kb DNA ladder; lanes 3, 4, and 5, DNA from colonies that were carbenicillin sensitive and sucrose resistant.
B. Physical map of the mutL locus from V. cholerae N16961. Shaded boxes indicate the genes flanking mutL. Solid arrows indicate placement of forward primers. Dotted arrows indicate placement of reverse primers. Numbers correspond to primer sequences located in the table. The bar above genome indicates the size of the fragment produced in the intact genome, using primers 1 and 4 from the table. The bar below genome indicates the size of the fragment produced from the truncated ΔmutL fragment, using primers 1 and 4 from the table. Genes are not drawn to scale.

4. DISCUSSION

We have constructed a polymyxin- and sucrose-resistant strain of V. cholerae that contains an internal deletion in mutL. We confirmed the deletion by PCR, which produced amplicons of the expected size relative to those produced by the wild-type strain. We are sequencing the area spanning the mutation to confirm the PCR analysis and precisely define the deletion. Once we have confirmed the mutL genotype of the mutant, we will determine whether the deletion in mutL affects the background rate of mutation by observing the frequency with which the mutant generates antibiotic-resistant colonies relative to the wild-type strain.

Understanding the mechanisms in V. cholerae that generate genetic variability could lead to a better understanding of how this species adapts to its environment and emerges periodically to cause epidemics. This understanding in turn could enable the discovery of new methods for pathogen detection in somewhat the same way that studies of hypermutator strains of other bacterial pathogens identified
novel approaches to combating antibiotic resistance (Cirz and Romesberg, 2006). Since the human immune system and pathogen detection assays use antibody recognition, an understanding of the origins of strain diversity may improve our ability to develop sensitive detection reagents and contribute to vaccine development. Likewise, the construction of a mutator strain of *V. cholerae* may also enable us to test the hypothesis that hypermutability of loci associated with virulence can contribute to the emergence and survival of strains with increased pathogenicity.
LITERATURE CITED


