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Rapid Detection of Biothreat Agents Based on Cellular Machinery

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Rapid Detection of Biothreat Agents Based on Cellular Machinery

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Abstract

This research addresses rapid and sensitive identification of biological agents in a complex background. We attempted to devise a method by which the specificity of the cellular transcriptional machinery could be used to detect and identify bacterial bio-terror agents in a background of other organisms. Bacterial cells contain RNA polymerases and transcription factors that transcribe genes into mRNA for translation into proteins. RNA polymerases in conjunction with transcription factors recognize regulatory elements (promoters) upstream of the gene. These promoters are, in many cases, recognized by the polymerase and transcription factor combinations of one species only.

We have engineered a plasmid, for *Escherichia coli*, containing the *virA* promoter from the target species *Shigella flexneri*. This promoter was fused to a reporter gene Green Fluorescent Protein (GFP). In theory the indicator strain (carrying the plasmid) is mixed with the target strain and the two are lysed. The cellular machinery from both cells mixes and the GFP is produced. This report details the results of testing this system.

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Contents

Abstract.....	3
Executive summary	7
Introduction	9
Selection of model system.	10
Cloning of <i>mxiE</i> and <i>ipgC</i>	11
Cloning Primers	12
Detection experiments with cloned transcription factors	13
Detection experiments with Congo Red grown <i>Shigella flexneri</i> 90T	13
Conclusions	13
References	15
Distribution List	16

List of Figures

Figure 1. Schematic of bench-top assay.	10
Figure 2. Cloned <i>mxiE</i> and <i>ipgC</i>	11
Figure 3. Typical results from strain mixing assay	14

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Executive summary

The goal of this LDRD was to develop a plasmid based system for the rapid detection and identification of bacterial bioweapons agents. This system was to be designed to detect and identify agents, even if they have undergone genetic modification and the many external and internal characteristics of the organism have been altered. This method utilizes the core genetic machinery of the organism, those mechanisms that are absolutely required for the expression of genes, as a means of detecting and identifying the target organisms. The strength of this system will be that it uses these core processes of the cell that are most difficult to alter, as a basis of identification.

The initial premise was to use the specificity of the cellular transcriptional machinery to detect and identify bacterial bio-terror agents in a background of other organisms. Bacterial cells contain RNA polymerases and transcription factors that transcribe genes into mRNA for translation into proteins. These RNA polymerases in conjunction with restriction factors recognize regulatory elements (promoters) upstream of the gene. It is now recognized that *E. coli* and *Shigella* are closely related with one major difference being the presence of a virulence plasmid in *Shigella* which is largely responsible for the pathogenicity of that species. It became clear that *Shigella* would provide an excellent system for the testing of our detection method.

We have obtained a plasmid (circular self-replicating DNA), for *Escherichia coli*, containing a promoter from the target pathogen *Shigella flexneri* (causative agent of bacillary dysentery) to GFP (Green Fluorescent Protein). This promoter for the gene *virA* is known to be transcriptionally active only in the presence of two transcription factors found only in *Shigella flexneri* called MxiE and IpgC. These transcription factors were cloned into expression vectors and expressed in *Escherichia coli*. The indicator strain (carrying the *virA::gfp* fusion plasmid) was mixed with the target strains (carrying the genes encoding the transcription factors on expression plasmids and mixture of strains are lysed. This allowed the cellular machinery from indicator and target cells to mix. The detection plasmid and assay was then tested against a series of target and non-target strains to determine the specificity and sensitivity of the assay along with the time required for detection. The results from these tests were not satisfactory and indicated that the efficacy of this method of pathogen detection was limited.

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Introduction

Most methods of bacterial species identification are based on three systems. The first is the detection of a set of physiological traits of the organism. The ability to lyse red blood cells, ferment lactose or produce catalase are examples of such traits. These traits can be readily changed by the introduction of mutations that remove a trait or the insertion of foreign genes that add traits. The second system detects specific antigens, usually cell surface proteins that are unique to the target organism. This system can also be defeated by the same genetic mechanisms. The third system in general use is a Polymerase Chain Reaction method that uses the amplification of specific regions of DNA as a means of identifying specific species. Alteration or deletion of the specific DNA sequence recognized by the PCR primers would lead to false negative results.

Bacterial genes are expressed in a two stage process that requires the services of a protein called RNA polymerase and accessory proteins called transcription factors that "transcribes" the DNA sequence into an RNA message that is then "translated" into the protein product by another protein complex, the ribosome.

The research plan was to create a plasmid (an autonomously replicating piece of DNA) which will be designed to contain strong promoters from each target species for genes that are continuously transcribed (called housekeeping genes). In the final form these promoters would be taken from each of the bioweapons species (*Yersinia pestis*, *Bacillus anthracis*, etc), but for this work we will use simulants. Each promoter from a different target bacterium will be placed in front of a gene encoding a different colored fluorescent protein. These proteins are all genetic variants of green fluorescent protein and are available in a number of colors such as red, yellow, and cyan. The resulting plasmid would be called the detection plasmid.

The detection plasmid would be contained in the standard molecular biology species, *Escherichia coli*. Initially the detection assay will consist of mixing the plasmid carrying *E. coli* with the strain to be tested. The mixed bacteria will be gently lysed, thus mixing the cytoplasmic contents of both cells. In this way the detection plasmid will come in contact with the molecular machinery of the test strain. If the RNA polymerase of the test strain recognizes a promoter on the detection plasmid, then the appropriate color fluorescent protein will be produced. The fluorescence emitted at different specific wavelengths will be measured. Because the output is different for each different target strain, it may be possible to simultaneously detect and identify multiple target strains. As the assay is developed, we envision that we will be able to eliminate the need for containing the plasmid in *E. coli*. Instead we will mix the naked plasmid with the target strain after lysis of the strain.

Figure 1. Schematic of bench-top assay.

Target bacterial strain is mixed with detector strain (carrying the detector plasmid) and gently lysed. After a short incubation, the production of fluorescent protein is revealed by the U.V. irradiation of the reaction mixture.

If successful, at the end of the project we would have developed a bench top assay for rapidly detecting and identifying bacterial bioweapons strains. This method is based on biological principals that are unique from those of other detection and identification assays. Our results would serve as both a proof of principal and an assessment of the physical parameters of the assay such as time required and sensitivity achieved. If appropriate the assay could be developed further and adapted to and integrated with other detection platforms and assays.

Selection of model system

An extensive literature search revealed *Shigella flexneri* as an attractive model system. *Shigella* which is known to be very similar to *E. coli* (2) contains a large virulence plasmid that has recently been sequenced (5). Among the many genes on this plasmid which is unique to *Shigella* are *mxiE* and *ipgC* encoding two transcription factors. Recent work has shown that the presence of these two transcription factors is necessary and sufficient for the transcription of a set of virulence genes *in vivo* (1, 3, 4). This has been demonstrated through the use of promoter fusions. The promoters from a number of the virulence genes have been transcriptionally fused to the reporter gene *gfp* which encodes Green Fluorescent Protein. The genes encoding the two transcription factors have been cloned into *E. coli* but attempts to obtain these clone were unsuccessful. *Shigella* virulence appeared to be a perfect model system in that all the molecular machinery that was specific to *Shigella* virulence gene transcription was cloned into and active in *E. coli* so we could use these *E. coli* strains as a non-pathogenic surrogate for *Shigella*. Because we were unable to obtain the *mxiE* and *ipgC* plasmids from their original sources it was necessary to reconstruct the plasmids at Sandia.

Cloning of *mxlE* and *ipgC*

Genes were amplified by PCR with the use of genomic *Shigella* DNA. PCR products were then checked on agarose gels for correct product size. Products were then ligated into pET-101/D-TOPO vectors (5753 bp) using the Champion pET Directional TOPO Expression Kit from Invitrogen. Following ligation, plasmids were transformed into *E. coli* (TOP10 strain). A plasmid preparation was performed on the TOP10 strains. These plasmids were then checked twice for directional and correct size insertion using a T7 5' primer and the appropriate primers listed below.

Following these checks, the plasmids were transformed into BL21STAR(DE3) and pLysE strains of *E. coli*. A plasmid preparation was also performed on these strains, along with a final PCR amplification of the inserted gene with the appropriate primers.

Figure 2. Cloned *mxlE* and *ipgC*

Products were amplified with the appropriate primers from final strains run out on a 2% agarose gel

Cloning Primers

5' *mxiE*

CACCATGTCTTTGAAGCAG

Length	19	Molecular Weight	5774.80
Melting Temperature	59.39	Percent GC	47.37%
Secondary Structure	None	Primer Dimer	No

3' *mxiE* with His-tag tail

GACTATCCCTCGAATTAATTTTAATTTAAC

Length	30	Molecular Weight	9097.00
Melting Temperature	61.99	Percent GC	26.67%
Secondary Structure	Very weak	Primer Dimer	No

3' *mxiE* without His-tag tail

CTCACAATACAATTATATCCCTCG

Length	24	Molecular Weight	7199.80
Melting Temperature	60.12	Percent GC	37.50%
Secondary Structure	None	Primer Dimer	No

5' *ipgC*

CACCATGTCTTTAAATATCACCG

Length	23	Molecular Weight	6927.60
Melting Temperature	61.84	Percent GC	39.13%
Secondary Structure	None	Primer Dimer	No

3' *ipgC* with His-tag tail

ATACTCCTTGATATCCTGAATTG

Length	23	Molecular Weight	6975.60
Melting Temperature	57.63	Percent GC	34.78%
Secondary Structure	None	Primer Dimer	No

3' *ipgC* without His-tag tail

CAAGGAGTAATTATTATGCATAATGTAAGC

Length	30	Molecular Weight	9257.00
Melting Temperature	62.57	Percent GC	30.00%
Secondary Structure	Very weak	Primer Dimer	No

Detection experiments with cloned transcription factors

The two expression strains carrying *mxiE* and *lpgC* without His-tags were grown in LB medium at 37 degrees with appropriate antibiotics until an OD 540 of 0.5 was reached. At this point expression of the cloned transcription factor genes was induced by addition of IPTG to the medium and incubation for a further one hour. At the same time a culture of *E. coli* carrying the reporter plasmid grown under similar conditions. The expression cultures were mixed in equal volumes (1 mL) with the reporter strain culture and lysed by one of a variety of means including sonication, bead beater, and Bug Buster gram negative cell lysis reagent. The cell lysates was then incubated at 37 degrees and the fluorescence of the lysates was measured every 0.5 hours for 6 hours. An *E. coli* strain containing a plasmid encoding *gfp* transcriptionally fused to a *lac* promoter was used as a positive control for fluorescence. Negative controls consisted of each of the expression strains and the reporter strain lysed independently of each other. A number of variations on this basic protocol were tested but unfortunately no increased fluorescence was detected in any of the experimental treatments.

Detection experiments with Congo Red grown *Shigella flexneri* 90T

With the failure of the experiments with the strains containing the cloned *Shigella* transcription factors we decided to attempt detect *Shigella flexneri* that had been grown in medium containing Congo red. Growth under these conditions is known to induce expression of the virulence factors MxiE and lpgC and consequently expression of the downstream virulence genes such as *virA*. *Shigella flexneri* was cultured in LB broth amended with 1mg/L Congo Red to an OD540 of 0.5 (approximately 1x10⁸ cfu/mL) One mL of this culture was mixed with an equal volume of *E. coli* carrying the reporter plasmid grown under the same conditions as above. The mixed culture was chilled on ice and then lysed with either a bead beater or Bug Buster gram negative lysis reagent. The fluorescence signature of the lysates was followed over time as described above. No increased fluorescence was detected during the course of incubation.

Conclusions

Failure to detect either *E. coli* expressing of MxiE and lpgC or *Shigella flexneri* grown on Congo Red is a clear indication that this methodology is unlikely to succeed as a basis for detection. The reason for failure is not clear and it could take significant effort to understand. One possible reason for the failure could be the instability or failure to fold of the reporter molecule GFP. Because the test plasmids needed for these experiments have been constructed, it would be simple to test other reporter systems that may become available

Figure 3. Typical results from strain mixing assay

Results from mixing experiment with Congo Red grown *Shigella flexneri* and an *E. coli* strain containing a *virA::gfp* fusion plasmid. The top panel shows the fluorescence measurements of *Shigella* mixed with the reporter strain taken over the time course of the incubation. No significant change was seen in fluorescence levels. The middle panel shows the no *Shigella* control (reporter plasmid only). As expected no increase in fluorescence was seen during the course of the experiment. A positive control was provided by expressing GFP alone off of a *lac* promoter (bottom panel). This culture was diluted 10 fold before fluorescence was measured. No evidence of GFP production was seen at any point during any of the mixing experiments and the results shown above were typical of all experiments and all timepoints.

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