

# **FY02 CBNP Annual Report: Discovery of DNA Signature of Biothreat Detection Using Suppression Subtractive Hybridization**

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**FY02 CBNP Annual Report: Discovery of DNA Signature of Biothreat Detection  
Using Suppression Subtractive Hybridization**

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## **OBJECTIVES:**

Our goal is to develop robust DNA signatures for rapid and specific DNA-based detection platforms that can be employed by CBNP to detect a wide range of potential agents. Our approach has resulted in highly specific DNA signatures for *Yersina pestis*, *Bacillus anthracis* and *Brucella* species. Furthermore, this approach can be applied to any genome (even uncharacterized ones), which facilitates DNA signature development for detection of newly emerging pathogens.

We are using suppression subtractive hybridization (SSH) as a tool to define large DNA regions specific to multiple biothreat pathogens by comparing them to genomes of the most closely related organisms. This approach has become increasingly accurate as we continue to find new, distinctive strains and ever-closer near-neighbors. With the huge costs incurred by whole genome sequencing, it is not possible to sequence each new bacterial genome. However, it is completely practical to identify genome differences in the laboratory using SSH, and becomes especially useful when comparing new strains to previously sequenced genomes.

## **FY02 HIGHLIGHTS:**

High quality genome sequence data is crucial to the development of robust DNA signatures. Complete genome sequences of numerous pathogens, including those of threat pathogens have been completed within the past year. The DNA sequence information will provide many pathogen signatures. We will undoubtedly see many more genomes completed within the next five years. Within this timeframe, sequencing will still require significant resources, which will only allow for analysis of a couple of different isolates or near neighbors at the most. Given the hundreds, even thousands, of different strains and close relatives that exist in the environment, it is easy to see that approaches relying on whole genome analysis for accurate DNA based detection may be confounded.

SSH provides an experimental approach for the isolation of large DNA regions that are unique to one strain (tester) that are absent from another (driver). DNA is extracted from each genome, and fragmented with restriction endonucleases. A short DNA tag is placed on the end of each of the tester fragments prior to hybridization to distinguish them from the driver fragments. Sequences that are common to both tester and driver will combine with each other, leaving behind tagged unique tester fragments that can be amplified by PCR. These tester-specific fragments are then cloned and sequenced.

### ***Bacillus anthracis.***

Subtractions using *B. anthracis* Ames as tester and closely related non-anthrax *Bacillus* species from the collection of Paul Jackson (LANL) identified twenty-eight DNA signatures that can discriminate *B. anthracis* from these very closely related pathogens. When *B. cereus* ATCC14579 was used as driver, 256 primer candidates yielded 39 *B. anthracis* specific DNA signatures. These signatures were positive for all of the strains in the geographically diverse *B. anthracis* collection of Paul Keim at NAU (i.e. no

false negatives). Furthermore, 6 of them did not cross react with any of the non-anthrax pathogens (i.e. no false positives). Similarly, when *B. thuringiensis* Al Hakam was used as driver, 48 primer candidates yielded 8 signatures that produced neither false positives nor negatives. SSH using *B. cereus* FRI41 as driver yielded 14 *B. anthracis* specific DNA signatures (out of 48 candidates). The twenty-eight *B. anthracis* specific signatures were screened against human, cow, soil and air filter DNA preparations, to ensure no false positives with DNA that might be encountered in environmental samples. These DNA signatures remain robust candidates and will be further validated against a panel of microbial and environmental DNAs. A multiplex reaction consisting of four *B. anthracis* specific signatures provide high confidence detection with as little as 100 picograms of pathogen DNA.

DNA signatures were also generated that rule out the non-anthrax *Bacillus* pathogens from environmental samples. SSH using *B. cereus* FRI41 as tester, and *B. anthracis* Ames as driver, yielded 44 (out of 48) candidates that were tester specific, one of which was specific to FRI41 only. SSH using *B. cereus* FRI13 as tester, and *B. anthracis* Ames as driver, yielded 19 (out of 24) candidates that were tester specific, of which 13 amplified a product from FRI13 only. Clones from subtractions with *B. cereus* FRI42 and *B. cereus* FRI43 as testers are currently being characterized.

### ***Brucella* species.**

DNA signatures that are specific for a single species of *Brucella* remain elusive, indicative of the close genomic similarity of these species. SSH experiments were performed using each of the four pathogenic species (*abortus*, *canis*, *melitensis*, and *suis*) as tester, and one of the other three as driver. SSH was successful in recovering DNA signatures for each species that are present in the tester, but not the driver. We identified 24 out of 1408 candidates for *B. abortus*, 12 out of 555 for *B. canis*, 23 out of 1025 for *B. melitensis* and 26 out of 1010 for *B. suis*. However, when these DNA signatures were screened against all four *Brucella* species, we discovered that most cross-react with at least one other *Brucella* species. Generally, we observed that *B. abortus* and *B. melitensis* DNA signatures cross react with each other, while *B. canis* and *B. suis* signatures cross react. One *B. suis* specific signature derived from SSH appears to react with only *B. suis*. Furthermore, cross\_match analysis using available genome data identified seven potential difference regions specific for *B. melitensis*. One primer set designed against these regions yielded a *B. melitensis* specific primer pair.

Despite the high degree of DNA sequence identity between the four pathogenic *Brucella* species, we were able to devise a multiplex of four primer pairs discriminate the four pathogenic *Brucella* species, based on the profile of the amplified products (Figure 2). Since we were able to find DNA sequences absent from the driver in each of the subtractions, we were able to distinguish each of the species by the different profile of PCR products visualized on agarose gels.

### **Impact:**

SSH is highly efficient at identifying agent specific sequences, from which we can design both species and strain-specific oligonucleotide primers. These primers should produce no false positives or false negatives. Successful candidates are ultimately

submitted to CDC for further development and testing. There are three main considerations for choosing SSH to define these differences. Firstly, SSH protocols can be applied to any genome, even uncharacterized ones, using only a small amount of DNA. Secondly, SSH becomes particularly powerful when sequenced genomes are available, enabling *in silico* comparison (genome sequences are currently available for many biothreat agents including *B. anthracis*, *Y. pestis*, *Brucella melitensis*, and *Francisella tularensis*). Thirdly, SSH provides inexpensive and immediate information about the number and nature of differences in gene content, and can identify strains that differ from reference genomes. While primarily aimed at development of diagnostic tools for CBNP, the signatures will have significant value in the medical and public health communities for early detection and source attribution of epidemics.

#### **Future Outlook:**

Comparative genomics has rapidly become a powerful tool for the study of genetic diversity among bacteria. Since the genome sequence of a single strain within a species does not represent the diversity of genomes within that species, SSH can provide a more cost-effective and rapid approach for the characterization of variable DNA regions. In addition to improved DNA signature development, difference regions can be used to design probes to demonstrate the genetic variation within culture collections. Furthermore, homology searches will identify genes necessary for pathogenicity. Finally, as novel, previously uncharacterized, close relatives are identified as part of “Background Characterization” projects, they can be easily incorporated into SSH screens for relatedness to reference collections. These considerations become increasingly important as more agents are added to the list of biothreat agents, and more near neighbors are isolated from the environment.

#### **Project Report Sidebar:**

SSH facilitates DNA signature development for detection of previously uncharacterized and newly emerging pathogens.

Given the genome diversity within a single bacterial species, approaches that rely on analysis of a single genome for accurate DNA based detection can be confounded.

SSH identifies agent specific sequences that can be used to design both species and strain-specific primers that produce no false positives or false negatives.

#### **Project Citations:**

Radnedge, L., Agron, P.G., Hill, K., Jackson, P.J., Ticknor, L.O., Keim, P. and Andersen, G.L. Genome differences that distinguish *B. anthracis* from closely related non-anthrax *Bacillus* pathogens. *Appl. Env. Microbiol.* (submitted)

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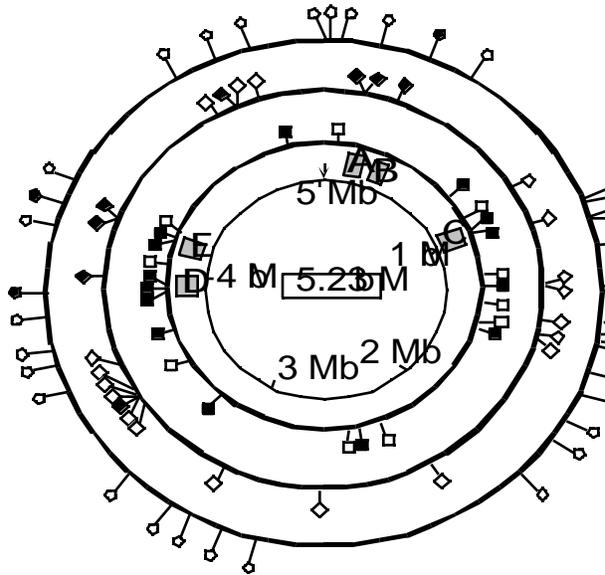


Figure 1: Distribution of *B. anthracis* specific sequ subtractions with *B. cereus* 14579 (circles), *B. th* Al Hakam (diamonds) and *B. cereus* FRI41 (square Sequences that are present in all strains of *B. anti* absent from all of the closest relatives are shown symbols.

Fig 1

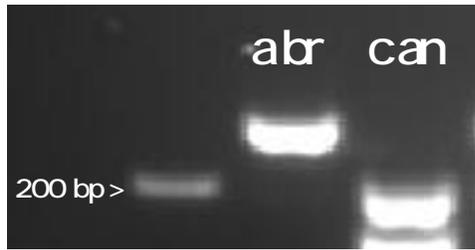


fig 2