

A NOVEL GENE MARKER FOR DIFFERENTIATING AND TRACING ANTHRAX-RELATED MICROBES

Final Report

JPL Task 1062

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A. OBJECTIVES

The objectives of this study are:

1. identification of several of the so-called *B. cereus* strains that have been isolated from various NASA spacecraft-assembly facilities.
2. utilization of a more-rapidly evolving *gyrB* gene marker, whose sequence is more highly differential, while showing analytical sensitivity as high as DNA:DNA hybridization for differentiating and tracing anthrax-related microbes.
3. detailed phenotypic characterization of so-called *B. cereus* serotypes in order to validate this method's accuracy, and thus confidently recommend *gyrB* analysis over DNA:DNA hybridization.
4. evaluation of the potential for deeming this the new "gold standard" method, one that is simple, rapid, and conceivably able to be incorporated into field-deployable instruments.

B. PROGRESS AND RESULTS

1. Scientific Data:

The *gyrB* gene has been found to be much less conserved and more highly differential than the 16S rRNA gene, while at the same time as analytical as DNA:DNA hybridization, suggesting that major changes in the phylogenetic arrangement and nomenclature associated with the *B. cereus*-group may be needed. All studies were performed at the University of Idaho, since proper handling of the strains in a bio-safety level 3 lab was necessitated. The breakdown of various tasks performed during this period is shown below:

Task 1: Identification of capsules in *B. cereus* serotypes to assess virulence: The microscopic examination of all lab- as well as wild-type strains after staining with capsular dye [9] revealed no capsule surrounding the vegetative cells. The positive control, an attenuated strain of *B. anthracis* Sterne, showed clear capsular layer around the cells. Absence of capsular layer in all the examined *B. cereus* and *B. thuringiensis* strains, including spacecraft-assembly-facility isolates, confirmed that these strains are lacking one of the key phenotypic, virulence-associated characteristics of *B. anthracis*.

Task 2: Isolation of plasmids and PCR screening for plasmid-borne genes of *B. anthracis*: Standard alkaline lysis-based plasmid extraction protocols yielded pXO2 plasmid in the *B. anthracis* Sterne strain, but none of the other tested strains yielded any plasmids.

Task 3: Amplification of toxigenic genes associated with *B. anthracis* via chromosomal DNA extraction using PCR primer sets: In the absence of the plasmid, chromosomal DNA was extracted and PCR screening for several toxigenic genes (*cya*, *pag*, and *lef*) was performed for all lab- and wild-type strains. Absence of any fragment for these specified genes in the tested isolates confirmed that these strains do not possess any of the known virulence factors that are normally associated with *B. anthracis*. The positive control strain, an attenuated isolate of *B. anthracis* Sterne, showed 385-bp (*lef*), 993-bp (*lef*), 873-bp (*pag*) amplicons while the Sterne strain did not show any product corresponding to the *cap* gene (Fig 1).

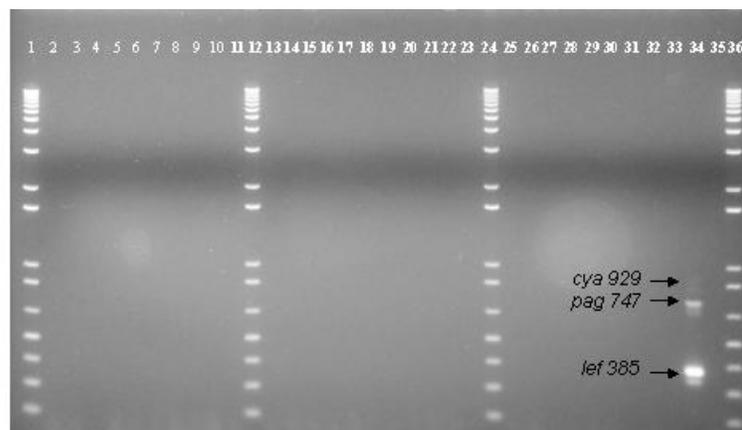


Fig. 1. The PCR amplification of several strains for toxigenic properties associated with *B. anthracis*. *cya*, capsular protein; *pag*, protective antigen protein; *lef*, lethal factor. The PCR amplified product (arrow mark) was noticed only in the positive control, *B. anthracis* Sterne strain. The *cya* gene was not amplified in Sterne strain as expected.

2. Other Results:

In addition to the three main tasks mentioned above, we performed the following studies. The results are summarized:

- a. **16S rDNA.** The bi-directional sequence analysis of the 1.5 kb fragment of the 16S rDNA revealed more than 99% similarity among all 18 *B. cereus* H-serotypes. Similarly, when these sequences were compared to *B. thuringiensis* type strain IAM 12077^T and two sequences derived from *B. anthracis* Pasteur #2H and the complete genome sequence, more than 99% similarity was observed. Not surprisingly, this was also the case when compared to *B. mycoides* type strain ATCC 6462^T. In addition to the *B. cereus* H-serotypes, five serotypes of *B. thuringiensis* were sequenced for 16S rDNA analysis, and revealed a high percentage of similarity (>99%) to all *Bacillus* strains tested. The 16S rDNA-sequence-derived phylogenetic tree (data not shown) showed no grouping whatsoever, and failed to discriminate any one species from another.
- b. ***gyrB* sequence analysis.** The phylogenetic affiliation of various species of the *B. cereus* group based on 1.2 kb *gyrB* sequence analysis is depicted (Fig.2). All sequences were generated in this study except *B. anthracis* genome *gyrB*, which was procured from TIGR. The tree clearly delineates four distinct phylogenetic groupings within this *B. cereus* group. Group one, home to both *B. anthracis* sequences, consisted of ten H-serotypes currently known as *B. cereus*. Group two, housing the *B. cereus* JCM 2152^T type strain, consisted of seven of its own H-serotypes and 1 serotype of *B. thuringiensis* (*aizawai*). Group three, the *B. thuringiensis* group, possessed four of its own serotypes including the type strain. The fourth group was solely represented by the *B. mycoides* type strain, whose sequence similarity was 91% with *B. cereus*, and <90% with both *B. thuringiensis* and *B. anthracis*. As seen in figure 2, two distinct sub-groupings emerge from group 1, one highly homologous to the *B. anthracis* sequences (>99%) and the other only moderately homologous to these sequences (~97%).

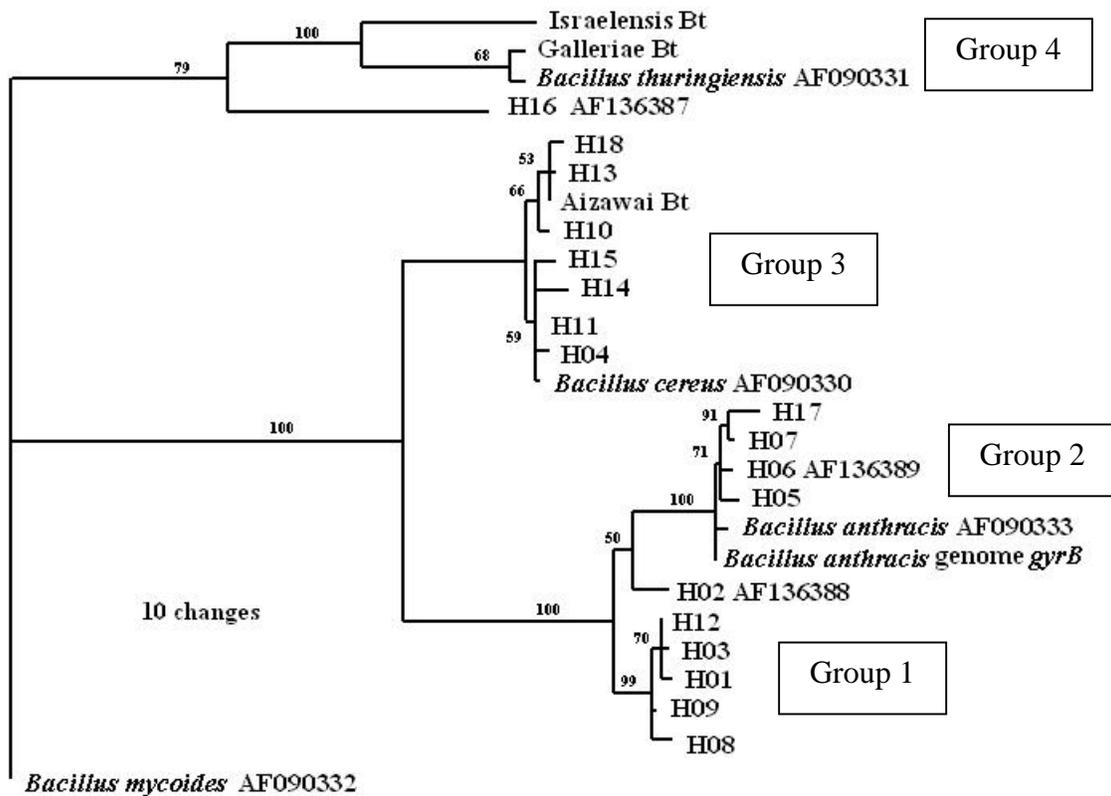


Fig. 2. The phylogenetic tree of several *B. cereus*-group-related strains. There are 4 groups noticed among the strains tested. Of particular interest is the *B. cereus* serotypes grouped into the *B. anthracis* group. Serotypes H05, H06, H07 and H17 showed highest similarities with *B. anthracis* strain whose genome was completely sequenced.

- c. **DNA:DNA hybridization.** Interspecies reassociation values of 10 to 15% were common (Venkateswaran et al., 2002) and also observed between *B. subtilis* and the three species of the *B. cereus* group (data not shown). Amazingly, about 90% of the 900 hybridizations carried out on the 30 strains examined in this study showed reassociation values of more than 50% to one another. As the table and figure show, reassociation values yield the same exact four groups as derived from *gyrB* phylotyping, with the exception of the *B. thuringiensis* group. Group one consisted of the same ten *B. cereus* H-serotypes observed via *gyrB*-based classification, where the attenuated *B. anthracis* genomes hybridized with high affinity. Furthermore, the same two sub-groups were observed among the group one H-serotypes and the same four H-serotypes showed high reassociation values (>75%) with *B. anthracis*. The H16-serotype exhibited 84% reassociation value with *B. anthracis*, the highest among all. The second subgroup of group one consisted of the same six H-serotypes, once again showing moderate similarity to *B. anthracis* (from 66% to 76% hybridization).

Group two also yielded reassociation values mirroring the results of *gyrB* sequence analysis, as the *B. cereus* JCM 2152^T type strain was once again housed in this group, along with six of its H-serotypes; reassociation values ranged from 70 to 88%. Group three, home to the *B. thuringiensis* type strain and serotypes yielded reassociation values ranging from 45% to 54%. Interestingly, *B. thuringiensis* serotypes aizawai and galleriae hybridized to the *B.cereusxxx* type strain at 74 and 66%, respectively. *Bacillus mycoides* was once again the sole member of group four, its highest reassociation value of 58% with *B. thuringiensis* galleriae.

- d. **Storage of *B. cereus*-group related strains.** All these strains were transported with appropriate caution to the University of Idaho and stored in a Bio-safety level-3 lab. We have assurance from the University of Idaho that such strains will be available upon request to us and will not be distributed without prior consent from the Principal Investigator of this study.

C. SIGNIFICANCE OF RESULTS

This task helped to develop a more-accurate, faster, and less-costly method of differentiating anthrax-related microbes. The results indicate that the *gyrB* marker is useful for deciphering and tracing anthrax-related microbes.

D. FINANCIAL STATUS

The total funding for this task was \$30,000, all of which has been expended.

E. PERSONNEL

No other personnel were involved.

F. PUBLICATIONS

- [1] La Duc, M.T., M. Satomi, N. Agata, and K. Venkateswaran, “*gyrB* phylotyping, a rapid alternative to DNA:DNA hybridization for accurately re-evaluating the phylogenetic characterization of the *Bacillus anthracis-cereus-thuringiensis* group”, Appl. Environ. Microbiol. Submitted for publication, 2003.

G. REFERENCES

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- [9] **Duguid, J.** 1951. The demonstration of bacterial capsules and slime. *J. Pathol. Bacteriol.* **63**, 673.
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H. APPENDIX:

Experimental procedures are given below.

Bacterial strains. A total of 18 *Bacillus cereus* strains typed according to H-flagellar antigens [10], five *B. thuringiensis* serovars, one avirulent strain of *B. anthracis*, and *B. mycoides* ATCC 6462^T were procured from the Nagoya Public Health Research Institute or from various culture collections. The source and serotype affiliations of each strain are given in Table 1. The alpha-hemolytic activity was confirmed by streaking all strains onto trypto-soy agar supplemented with 5% defibrinated horse blood, and all plates were incubated at 37°C for 24 hours. Capsular staining and presence of crystal proteins were observed by microscopy per standard protocols [9].

DNA isolation, PCR amplification, cloning, and sequencing. Chromosomal DNA of overnight-grown cultures was extracted by phenol-chloroform solvents and ethanol precipitation (36). The dried DNA was then dissolved in Tris-EDTA (TE) buffer (pH 7.5) and used as the DNA template. The purity of the DNA was checked by agarose gel electrophoresis, and the DNA concentration was measured with a spectrophotometer.

Bacterial small subunit (SSU) rRNA genes were PCR-amplified with B27f and B1512r primers (Pace et al.) under the following conditions: 1 min. 95°C denaturation, 2 min. 55°C annealing, and 3 min. 72°C elongation for thirty-five cycles. Similarly, *gyrB* genes were amplified per the established protocol (Yamada et al.1999). PCR conditions for *gyrB* amplification were as follows: 1 min. 94°C denaturation, 1.5 min. 58°C annealing, and 2.5 min. 72°C elongation for thirty cycles.

Amplification products were purified with a gel excision kit (Qiagen, Chatsworth, CA), after which they were cloned into the pCR-4 TOPO vector by TA cloning (Invitrogen, Carlsbad, CA) per manufacturer's instructions.

Purified plasmids were then sequenced. The identity of the cloned fragment was verified by sequencing from both ends by the dideoxy chain termination method with a Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and with an ABI 373A automatic sequencer as described by the manufacturer (Perkin-Elmer Corp., Foster City, Calif.). DNA sequences were determined from both strands by extension from vector-specific (T7 and M13r primers from pCR-4 TOPO vector) priming sites and by primer walking.

DNA:DNA Hybridization. Cells were suspended in 0.1M EDTA (pH8.0) and digestion of the cell wall was carried out by treating the cells with lysozyme (final concentration, 2mg/mL). The DNA was purified by standard procedures and DNA:DNA hybridization was carried out using microplate methods in microdilution wells. Radioisotopes were used to determine genetic relationships among bacterial strains with photobiotin labeling and colorimetric detection, as previously described (Satomi, et al. 1997).

Virulence gene and plasmid analyses. The presence and/or absence of the virulence-gene-housing plasmids pX01 and pX02 were examined using pre-existing protocols (22,35). Screening for the emetic and enterotoxin genes of *B. cereus* was also carried out (Agata et al).