Species Differentiation of the *Bacillus Cereus* Group using the *pycA* Gene Sequence and an Assessment of its Operational Impact for Pharmaceutical Manufacturers

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1 ABSTRACT

The Bacillus cereus group (BCG) is of high importance in the scientific, industrial, and pharmaceutical communities as members of this group are often found as contaminants in food products and manufacturing facilities. In late 2017, the description of an additional nine novel species was published. As a result, the BCG now includes 17 validly published species. Phylogenetic analysis based on the 16S rDNA sequence has traditionally been used to differentiate between organisms in this group. However, the publication of these additional new BCG species brings a new level of complexity as the 16S sequences for many type species in the group are now identical. Therefore, species within the BCG are too closely related to be accurately differentiated by 16S. Since organisms in the Bacillus cereus group can trigger different levels of concern, rapid and accurate classification and taxonomic separation of the BCG species are critical for investigations in industrial settings. We have evaluated numerous protein-coding gene sequences that have been reported to be phylogenetically discriminatory and concluded that the *pycA* gene is an ideal target for distinguishing all the BCG species under consideration. This method was tested with over 300 diverse samples and a 92% speciation rate was obtained.

2 INTRODUCTION

The members of the *Bacillus cereus* group (BCG) of organisms are very closely related and currently include *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus wiedmannii*, *Bacillus toyonensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus bingmayongensis*†, *Bacillus manliponensis*†, and *Bacillus cytotoxicus*. Late in 2017, we discovered a research article which included the publication of nine new valid species in the BCG[1] (**Table 1**).

The BCG is of great importance as its members are often found as contaminants in many manufacturing facilities and food products. In addition to this, for many years, isolates identifying as *B. cereus* have been one of the most frequently submitted organisms to our facilities.

Phylogenetic analysis based on the 16S rRNA gene sequence has traditionally been used to differentiate between organisms in this group. However, the publication of the additional new BCG species brings a new level of complexity (**Figure 1**). There are many type species in the group with identical 16S rDNA sequences, and overall the species are too closely related to be accurately differentiated. Therefore, 16S rRNA gene sequencing, used by our AccuGENX-ID/BacSeq service, is no longer adequate to differentiate members of this group.

Since organisms in the Bacillus cereus group are known to trigger different levels of concern and corrective action for manufacturers, the classification and taxonomic separation of the BCG species is critical for investigations and environmental monitoring programs.

Since organisms in the Bacillus cereus group are known to trigger different levels of concern and corrective action for manufacturers, the classification and taxonomic separation of the BCG species is critical for investigations and environmental monitoring programs.

In this study, we have carried out extensive research to determine alternative gene targets to the 16S rRNA gene, that would be able to discriminate between the BCG species.

We have evaluated numerous protein-coding gene sequences that have been reported to be phylogenetically discriminatory[13,14,15], and that have shown the best resolution for distinguishing all the BCG species under consideration.

Bacillus cereus group of organisms	References
Bacillus cereus	2
Bacillus thuringiensis	3
Bacillus mycoides	4
Bacillus pseudomycoides	5
Bacillus toyonensis	6
Bacillus wiedmannii	7
Bacillus cytotoxicus	8
Bacillus manliponensis †	9
Bacillus bingmayongensis †	10
Bacillus gaemokensis †	11
Bacillus pacificus	1
Bacillus paranthracis	1
Bacillus tropicus	1
Bacillus luti	1
Bacillus proteolyticus	1
Bacillus mobilis	1
Bacillus nitratireducens	1
Bacillus albus	1
Bacillus paramycoides	1

Table 1. Species in the *Bacillus cereus* group.

† These species have been published in a journal other than the International Journal of Systematic and Evolutionary Microbiology (IJSEM) and are currently not recognized by LPSN as being valid species but are names in common use.

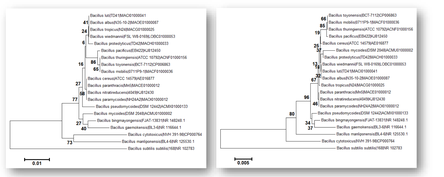

Figure 1. Neighbor-joining phylogenetic trees based on the first 500 bp (left) and nearly full (right) of the 16S rRNA gene sequence for the *Bacillus cereus* group.

3 DEVELOPMENT OF ALTERNATIVE GENE TARGETS

Searched literature; 11 genes were chosen for study (*cpn60, pycA, gyrB, ilvD, purH, gmk, glpF, pta, tpi, rpoB* and *recA*)

Designed the primers for amplifying *cpn60, pycA, gyrB, ilvD, purH,* and *recA* genes

Obtained type strains of all BCG species and amplified the target genes using

Tested a diverse set of samples (n=63) and *selected the pycA gene as an ideal target*

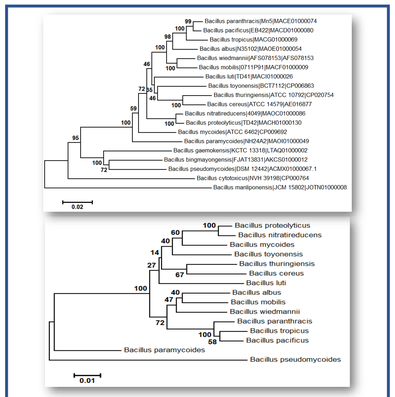

Figure 2. Neighbor-joining phylogenetic tree based on nearly full-length (2890bp) (top) and a partial (532bp) (bottom) sequence of the *pycA* gene for the *Bacillus cereus* group.

4 OPERATIONAL IMPACT

In order to evaluate the operational impact of the newly developed *pycA* assay, we gathered more than 300 diverse samples that would potentially fail to identify to a species with the addition of the nine new BCG species. These samples were sequenced using the partial *pycA* gene fragment and neighbor joining (NJ) trees were constructed to infer phylogenetic relationship. As seen in the example reports below, MALDI-TOF and 16S rRNA gene sequencing methods (**Figures 3A** and **3B, respectively**) were unable to provide a conclusive species-level identification. However, as you can see below (**Figure 3C**), if we use the *pycA* gene sequence as an alternative target, we can obtain better resolution. By analyzing over 300 samples, we were able to achieve a 92% species-level ID rate (**Figure 4).** Despite this high success rate, there were still about 8% of the samples tested that failed to show phylogenetic consistency to make a reliable species call.

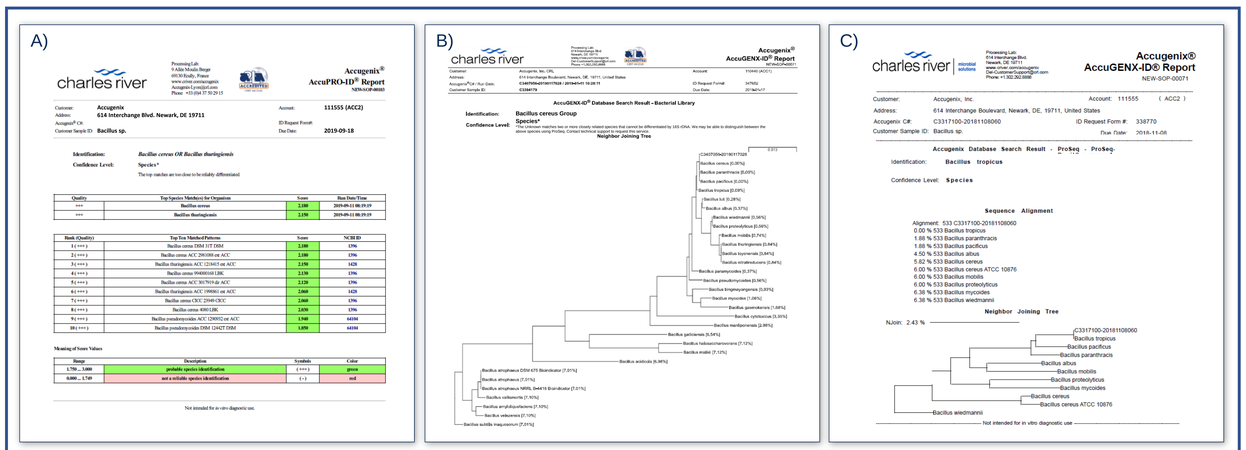

Figure 3. Example Accugenix identification reports for *Bacillus* samples run in house. **A)** MALDI (AccuPRO-ID) report showing two *Bacillus* species that are unable to be distinguished from one another. **B)** A 16S identification report based on the first 500 bp. The NJ tree shown was generated with the top 30 entries and including the nine newest species belonging to the BCG. **C)** NJ tree based on the 532 bp *pycA* gene fragment showing the ability for the target to differentiate between BCG species.

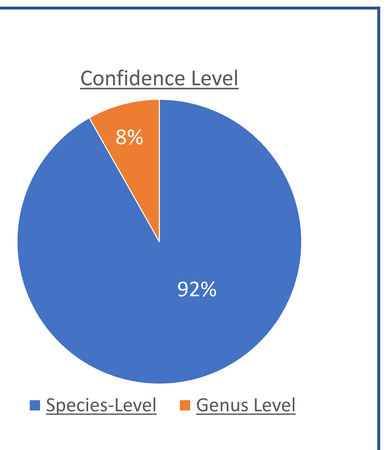

Figure 4. Species-level identification rate based on partial *pycA* gene sequencing for the *Bacillus cereus* group.

5 CONCLUSIONS

- We have conclusive data that show that a portion of the *pycA* gene is an ideal target for speciation among BCG organisms.
- By sequencing the 532 bp *pycA* gene fragment and performing a phylogenetic analysis of the type species in the BCG, we were able to differentiate between the members of this group.
- This method was tested with over 300 genetic diversity samples and a 92% speciation rate was obtained.
- The use of the alternative protein-coding gene target will be beneficial when traditional 16S rDNA sequencing is unable to provide species-level resolution for members of the BCG.

6 REFERENCES

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