Research Article

Identification of pBC218/pBC210 Genes of Bacillus cereus G9241 in Five Florida Soils Using qPCR

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The distribution of the virulent plasmid pBC210 of B. cereus that carries several B. anthracis genes and has been implicated in lethal anthrax-like pulmonary disease is unknown. We screened our collection of 103 B. cereus isolates and 256 soils samples using a quantitative PCR (qPCR) assay that targeted three open reading frames putatively unique to pBC210. When tested with DNA from 2 B. cereus strains carrying pBC210, and 64 Gram-positive and 55 Gram-negative bacterial species, the assay had 100% sensitivity and specificity. None of the DNA from the B. cereus isolates yielded positive amplicons but DNA extracted from five soils collected in Florida gave positive results for all three target sequences of pBC210. While screening confirms that pBC210 is uncommon in B. cereus, this study is the first to report that pBC210 is present in Florida soils. This study improves our knowledge of the distribution of pBC210 in soils and, of public health importance, the potential threat of B. cereus isolates carrying the toxin-carrying plasmid. We demonstrated that sequences of pBC210 can be found in a larger geographical area than previously thought and that finding more B. cereus carrying the virulent plasmid is a possibility in the future.

1. Introduction

The ubiquitous bacterium Bacillus cereus found in soils, fresh and marine waters, dust, and intestinal systems of many insects has usually been linked to food poisoning yet has also caused a variety of serious infections [1–5]. Historically, it has been proposed that B. cereus can acquire one or both B. anthracis megaplasmids (pXO1 and pXO2) and currently it is reported that B. cereus carries similar plasmids (i.e., pBCXO1) and subsequently is able to cause anthrax-like illnesses [1, 6, 7]. The megaplasmid pBCXO1 may have been acquired from B. anthracis or evolved after acquisition from some distant ancestor. Some unusual cases of B. cereus infections were fatal anthrax-like pneumonia in immunocompetent healthy workers [8–10]. An isolate associated with these cases, B. cereus G9241 and related strain 03BB87, carried two large plasmids responsible for their virulence [9, 11, 12]. One plasmid pBCXO1 with >99% similarity to pXO1 carried the genes encoding for toxins, while the second pBC210 (formerly pBC218) appeared to carry the genes that encode for a putative polysaccharide (non-D-glutamyl polypeptide) capsule involved with evasion of host immune responses [9, 11, 13–15]. At 210 kb, the pBC210 is smaller than originally calculated and has been referred to as either pBC218 or pBC210 in the literature [16]. We will use the latter designation. Studies have shown that both plasmids have to be present for G9241 to achieve full virulence [6].

Some researchers debate whether B. cereus G9241 should be considered a frank or opportunistic pathogen [6]. The fact that B. cereus G9241 contains two virulent megaplasmids places it with the pathogenic B. anthracis. However, animal studies have equated the virulence of B. cereus G9241 to that of B. anthracis Sterne as opposed to the highly virulent B. anthracis Ames [6]. Yet healthy individuals have died from infection with B. cereus G9241. Therefore, it is important to determine the distribution of the pBC210 plasmid and any B. cereus strains carrying it. In order to screen our soil sample and Bacillus collection, we developed a rapid quantitative PCR (qPCR) assay to target three open reading frames that have been reported as only in pBC210 and so discriminate B. cereus with pBC210 from closely related bacillus and other bacteria. We screened DNA extracts of 256 soil samples collected from Florida, Texas, and elsewhere in the United States and DNA from 103 B. cereus and 119 other bacterial
strains (64 Gram-positive and 55 Gram-negative) obtained from clinical and environmental (soils, powders, water, and environmental swab) samples held in our bacterial collection.

2. Methods and Materials

2.1. Soil Samples. The Center for Biological Defense (CBD) has a collection of DNA extractions procured from 256 soils collected in Florida, Texas, and elsewhere. Many were collected by CBD in Florida, while other soils were obtained from Dr. Rick Zartman at Texas Tech University, Lubbock, TX, and Dr. Dale Griffin at U.S. Geological Survey, Coastal and Marine Science Center, St. Petersburg, FL. DNA had been extracted from the soils as previously reported and tested for the B. anthracis toxin and capsule genes as reported elsewhere [17–20]. All DNA had been stored at −30°C until used.

2.2. Bacterial Strains. We examined 153 Bacillus strains (one B. cereus G9241, one B. cereus G9241-derived, 13 B. anthracis, one B. badus, 103 B. cereus, five B. marisflavi, five B. megatherium, five B. mycoides, three B. pseudomycoide, five B. pumilus, five B. subtilis, and six B. thuringiensis) (Table 1). In addition, we examined 16 other Gram-positive bacterial strains (four genera) (two Enterococcus faecalis, one Lactobacillus rhamnosus, 12 Staphylococcus aureus, and one Streptococcus pneumoniae) and 55 Gram-negative bacterial strains covering 14 genera (Table 1).

The two B. cereus G9241 strains (CBD 1056 and CBD 1057) were given to CBD by the Hoffmaster laboratory, while the other B. cereus bacterial strains were obtained from the American Type Culture (ATCC, Manassas, VA, USA) or isolated from soils, powders, swab specimens, and marine samples collected in Florida, Texas, and elsewhere. B. anthracis, Burkholderia mallei, and Burkholderia pseudomallei strains were received from either the CDC or the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Bethesda, MD, USA). All other bacteria studied were procured from ATCC, the Florida Department of Health, Bureau of Laboratories, Tampa, FL (FDOH), from Mote Marine Laboratory, Sarasota, FL, or from the University of Washington Medical Center in Seattle, WA.

Manipulations of all bacterial strains except for B. anthracis, B. cereus G9241, B. mallei, and B. pseudomallei were performed in a BSL2 laboratory in a biological safety cabinet following safety practices outlined in the Biosafety in Microbiological and Biomedical Laboratories, 5th Edition (BMBL). All manipulations of cultures of B. anthracis, B. cereus G9241, B. mallei, and B. pseudomallei strains were performed in a biological safety cabinet in a biological safety level 3 (BSL3) laboratory. All safety protocols followed the BMBL practices for the BSL3 environment including the use of protective laboratory clothing and respiratory equipment (US DHHS, 2007). The safety and security requirements by US federal regulation DHHS 42 CFR 73 were strictly adhered to.

2.3. DNA Extraction from Bacterial Isolates. Bacteria were grown on tryptic soy agar supplemented with 5% sheep red blood cells (blood agar (BA)) (Remel, Lenexa, KS, USA). All culture plates were incubated at 35°C overnight (18–24 hours) before performing DNA extractions. Following manufacturers’ instructions, all genomic DNA extractions were performed using the Epicentre (Qiagen Inc., Madison, WI) extraction kits, a MagNaPure Compact automated instrument (Roche, Inc., Indianapolis, IN, USA), or a boil preparation method used by the Tampa FDOH, a reference lab in the Laboratory Response Network, and is described as follows. Bacterial growth from an overnight cultured media plate was removed and placed into 100 μL of sterile water and boiled for 5 minutes, placed onto ice for 2 minutes, and centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was transferred to a 0.1 μM filter tube (Millipore Corporation Billerica, MA) and centrifuged for 2 min at 8,000 ×g. 10 μL of all filtrates and DNA extractions of BSL3 isolates (following the University of South Florida Institutional Biosafety Committee guidelines) were used to inoculate a BA media culture plate and incubated at 35°C for 2 days. Extracts and boil preparation filtrates having no growth were allowed out of the BSL3 environment and made available for molecular work. All DNA were stored at 4°C or −30°C until used.

2.4. Plasmid Extraction of pBC210. Extractions of pBC210 from the two B. cereus G9241 strains (CBD 1056 and 1057) were performed as previously described [21]. The extracts were tested for sterility as the genomic DNA above and stored at 4°C or −30°C until used.

2.5. qPCR Design. Open reading frames (ORFs) that according to other researchers are specific to pBC210, pBC218-0047, and pBC218-0072 (formerly pBC218-0073) were selected as targets for primer design [11]. The whole gene sequence for each ORF was identified on the GenBank sequence for pBC210 (accession number AAEK01000004.1). For the internal amplification control (IAC), the 165 rRNA sequence of B. cereus (GenBank accession number X55060) was used. These sequences were then copied and placed into the online program PrimerQuest by Integrated DNA Technologies (IDT) (Corvallis, IA) to design the real-time PCR primers and probes (Table 2). Parameters necessary for optimal primer and probe design were that the amplicon should be 50–150 bp, the primer melting temperatures close to 50–60°C, and probe melting temperature 10°C higher (68–70°C). Confirmation of pBC210 plasmid in the positive soil DNA extracts was performed using a primer set designed specifically to ORF BC218-0067 that encodes for a transcriptional regulator protein LytR on the pBC210 GenBank sequence (accession number AAEK01000004.1) (Table 2). Later, this primer set was used to test all of the bacterial and soil sample DNA extracts (Table 1).

The qPCR reaction mixture (final volume, 20 μL) consisted of 3 μL of DNA template (ideally 0.5–1.0 ng/μL) and 10 μL TaqMan Fast Universal PCR Master Mix 2X (Applied Biosystems, Foster City, CA). The volumes of the primer and probe working stocks (100 μM) were 0.09 μL of each primer and 0.03 μL of probe, giving final concentrations in the reaction of 450 nM (primer) and 150 nM (probe).
**Table 1: Results of the qPCR assay targeting ORF 0047 and ORF 0072 unique to pBC210 using bacterial and soil DNA extracts as template.**

| DNA source (N)                      | ORF 0047 | PCR assay | ORF 0067 |
|------------------------------------|----------|-----------|----------|
| **Bacteria (244)**                 |          |           |          |
| B. cereus G9241 (2)                | +        | +         | +        |
| **Other Bacillus species (151)**   |          |           |          |
| B. cereus (103)                    | –        | –         | –        |
| B. anthracis (13)                  | –        | –         | –        |
| Bacillus badius (1)                | –        | –         | –        |
| B. marisflavi (5)                  | –        | –         | –        |
| B. megaterium (5)                  | –        | –         | –        |
| B. mycoides (5)                    | –        | –         | –        |
| B. pseudomycoides (3)              | –        | –         | –        |
| B. pumilus (5)                     | –        | –         | –        |
| B. subtilis (5)                    | –        | –         | –        |
| B. thuringiensis (6)               | –        | –         | –        |
| **Other bacteria (71)**            |          |           |          |
| **Gram-positive (16)**             |          |           |          |
| Enterococcus faecalis (2)          | –        | –         | –        |
| Lactobacillus rhamnosus (1)        | –        | –         | –        |
| Staphylococcus aureus (12)         | –        | –         | –        |
| Streptococcus pneumoniae (1)       | –        | –         | –        |
| **Gram-negative (55)**             |          |           |          |
| Achromobacter xylosoxidans (1)     | –        | –         | –        |
| Acinetobacter baumannii (1)        | –        | –         | –        |
| Acinetobacter calcoaceticus (1)    | –        | –         | –        |
| Burkholderia cepacia (15)          | –        | –         | –        |
| Burkholderia mallei (5)            | –        | –         | –        |
| Burkholderia pseudomallei (5)      | –        | –         | –        |
| Burkholderia species (6)           | –        | –         | –        |
| Cedeeea neteri (1)                 | –        | –         | –        |
| Citrobacter freundii (1)           | –        | –         | –        |
| Enterobacter cloacae (1)           | –        | –         | –        |
| Escherichia coli (6)               | –        | –         | –        |
| Klebsiella pneumoniae (1)          | –        | –         | –        |
| Pseudomonas aeruginosa (1)         | –        | –         | –        |
| Salmonella enterica (6)            | –        | –         | –        |
| Serratia marcescens (1)            | –        | –         | –        |
| Shigella flexneri (1)              | –        | –         | –        |
| Stenotrophomonas maltophilia (1)   | –        | –         | –        |
| *Vibrio parahaemolyticus* (1)      | –        | –         | –        |
| **Soils from United States (256)**|          |           |          |
| Florida (69)                       | 5+, 64–  | 5+, 64–   | 5+, 64–  |
| Texas (72)                         | –        | –         | –        |
| Elsewhere (115)                    | –        | –         | –        |

*“+” denotes a positive amplicon that was produced by the assay with a cycle threshold (CT) value of ≤39.99. For ORF 0047, the average CT for the two controls was 16.66 with a range of 15.92–18.73 using plasmid DNA and 29.21 with a range of 20.93–38.25 using genomic DNA. For ORF 0072, the average CT for the two controls was 16.10 with a range of 15.24–18.03 for plasmid DNA and 27.09 with a range of 21.29–33.60 using genomic DNA. All samples positive for the two ORF sequences were also positive for ORF 0067. For ORF 0067, the average CT for the two controls was 15.24 (range of 12.25–15.91) using plasmid DNA and 30.72 (range of 28.59–33.99) using genomic DNA. For the 16S assay, the average CT for the two positive controls was 20.40 with a range of 13.68 to 27.20 for genomic DNA. For all DNA extractions of both bacterial and soil samples, the average CT value was 21.24 and the range was from 12.89 to 39.97. The two B. cereus carrying pBC210 (CBD 1056 and CBD 1057) were tested a minimum of 250 times.*

*“−” denotes a negative result as an amplicon was not detected in the qPCR assay. All samples including the five Florida soils that produced positive results for the target sequences (ORF 0047, ORF 0067, and ORF 0072) were tested in duplicate in multiple runs by two different personnel.*
The remaining 6.79 μL of the reaction mixture consisted of molecular biology grade water (Fisher Scientific, Fair Lawn, NJ). All qPCR was performed on the ABI 7500 Fast Real Time PCR system (ABI, Delray Beach, FL) with the following conditions: initial Taq activation at 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

2.6. Limit of Detection, Sensitivity, and Specificity of qPCR.
To be confident our assay was robust and could detect the pBC210 sequence targets, we determined its limit of detection (LOD), sensitivity and specificity. For the LOD, the concentration (ng/μL) of both genomic and plasmid DNA from the two strains of B. cereus G9241 (CBD 1056 and CBD 1057) was measured using the GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge, England) and then diluted 10-fold from 10^{-1} to 10^{-9} in two separate series and used as template for the qPCR assays as above using the different primer sets. All dilution samples were tested in duplicate in an assay and all assays were performed at least twice. All three target sequence assays were examined. CT values from 30 to 35 were considered positive, 20–30 were strong positives, and 36–39 were weak positives. A dilution that yielded CT values greater than 40 was considered too weak, and the limit of detection would correspond to the concentration of DNA in the preceding dilution.

Although only two isolates of B. cereus G9241 were available, the sensitivity of the assay primer sets was determined by multiple testing (250 tests) of these strains using the optimal concentration (0.75 ± 0.25 ng/μL) of DNA as template. Specificity was determined by testing the assay primer sets against 48 isolates of Bacillus species, including 27 isolates of closely related members of the B. cereus group (B. anthracis, B. mycoides, B. pseudomycoides, and B. thuringiensis), and against both Gram-positive and Gram-negative bacterial isolates listed on Table 1. The presence of pBC210 in B. cereus isolates and in the environment was determined by testing the assay against the 103 B. cereus in our collection (isolated from clinical and environmental sources) and against DNA extracted from 256 soils previously tested for B. anthracis (Table 1).

The qPCR assay targets and identifies the presence of three open reading frames reported by other researchers to be closely related members of the B. cereus group (B. anthracis, B. mycoides, B. pseudomycoides, and B. thuringiensis), and against both Gram-positive and Gram-negative bacterial isolates listed on Table 1. The presence of pBC210 in B. cereus isolates and in the environment was determined by testing the assay against the 103 B. cereus in our collection (isolated from clinical and environmental sources) and against DNA extracted from 256 soils previously tested for B. anthracis (Table 1).

### 3. Results and Discussion

The qPCR assay targets and identifies the presence of three open reading frames reported by other researchers to be exclusively in the pBC210 plasmid [10]. All qPCR assays for the targets consistently gave positive results in over 250 tests with DNA from the two positive control strains (CBD 1056 and 1057) (Table 1). The LOD for the targets in the assay was 0.06471 ng/μL for CBD 1056 and 0.06873 ng/μL for CBD 1057 using genomic DNA and three logs lower for plasmid DNA (Table 3). For simplicity, Table 3 gives the values of only one target sequence (ORF 0047) but all three sequences were examined and yielded essentially the same LOD for both positive control strains. All tests using DNA from other bacteria, including many isolates in the B. cereus group (excluding other B. cereus), produced negative qPCR results. Because the assay had 100% sensitivity and specificity, we were confident that it would detect the pBC210 plasmid in our soil samples. When we tested the DNA extracted from the 256 soils that had been previously tested for B. anthracis plasmids, pXO1 and pXO2, we found five (2%) soil samples that produced positive qPCR amplicons for ORF
set for ORF 0067 for confirmation. All assays were performed in duplicate in multiple runs and by two different personnel in order to rule out errors.

The five soils obtained from Florida yielded positive results for ORF 0047 and ORF 0072. Subsequently, the soils were also tested with the primer and probe

The genomic DNA was extracted using the boil preparation method, while plasmid DNA was extracted as previously described [17]. The starting DNA concentration of CBD 1057 was 38.97 (38.92–40.00) for genomic and plasmid extractions, respectively. The CT averages and ranges for ORF 0067 and ORF 0072 were similar (≥ 40) are regarded as negatives. The limit of detection was then determined to be the prior dilution.

"CT" denotes cycle threshold value.

a "CT" denotes cycle threshold value.
b "Undetected" denotes that no CT value was given for the sample tested.

c "CT" denotes cycle threshold value.

The genomic DNA was extracted using the boil preparation method, while plasmid DNA was extracted as previously described [17]. The starting DNA concentration of CBD 1057 was 38.97 (38.92–40.00) ng/μL and ORF 0047 (Table 1). Upon repeated testing all five soil samples that obtained from genomic DNA template (Table 1 legend). The limits of detection of assay for ORF 0047 using genomic and plasmid DNA extracts from B. cereus G9241 (CBD 1056).

| Genomic DNA concentration (ng/μL) | DNA concentration (μg/mL) | Genomic DNA CT a value (average) range | Plasmid DNA CT a value (average) range |
|----------------------------------|--------------------------|---------------------------------------|---------------------------------------|
| 6.471                            | 1.75                     | 24.68 (24.63–24.73)                   | 14.15 (14.14–14.18)                   |
| 6.471                            | 1.75                     | 24.12 (24.04–24.18)                   | 15.23 (15.12–15.36)                   |
| 0.351                            | 0.175                    | 27.00 (26.95–27.03)                   | 17.24 (17.64–17.89)                   |
| 0.175                            | 1.75 × 10^{-4}           | 30.71 (30.57–31.02)                   | 20.37 (20.37–21.95)                   |
| 6.471 × 10^{-5}                  | 1.75 × 10^{-4}           | 36.35 (35.89–36.58)                   | 24.96 (24.80–25.11)                   |
| 6.471 × 10^{-6}                  | 1.75 × 10^{-5}           | 38.97 (38.92–40.00)                   | 28.41 (28.35–28.54)                   |
| 6.471 × 10^{-7}                  | 1.75 × 10^{-6}           | Undetected                            | 32.63 (31.58–33.77)                   |
| 6.471 × 10^{-8}                  | 1.75 × 10^{-7}           | Undetected                            | 34.87 (34.51–35.29)                   |
| 6.471 × 10^{-9}                  | 1.75 × 10^{-8}           | Undetected                            | 37.03 (36.48–39.57)                   |
| 6.471 × 10^{-10}                 | 1.75 × 10^{-9}           | Undetected                            | Undetected                            |

The genomic DNA was extracted using the boil preparation method, while plasmid DNA was extracted as previously described [17]. The starting DNA concentration of CBD 1057 was 68.73 ng/μL and 36.5 ng/μL for genomic and plasmid extractions, respectively. The CT averages and ranges for ORF 0067 and ORF 0072 were similar (±1.2 and ±1.4, respectively, for genomic DNA) to the values above for both CBD 1056 and 1057. Thus, these were not included in this table. CT values ≥ 40 are regarded as negatives. The limit of detection was then determined to be the prior dilution.

For the five soils obtained from Florida yielded positive results for ORF 0047 and ORF 0072. Subsequently, the soils were also tested with the primer and probe set for ORF 0067 for confirmation. All assays were performed in duplicate in multiple runs and by two different personnel in order to rule out errors. None of these DNA from the 103 B. cereus isolates were unknowns. Therefore, data using these strains were not included in specificity calculations. None of the DNA from the 103 B. cereus isolates produced positive amplicons for the pBC210 targets (Table I) or for pXO1 or pXO2. Most of these isolates came from soils, powders, clinical samples, and marine samples that were collected in various parts of the United States, but primarily from Texas and Florida.

We then screened the DNA from the 103 B. cereus isolates in our collection. These had not been previously examined for pBC210 by other means and so were unknowns. Therefore, data using these strains were not included in specificity calculations. None of the DNA from the 103 B. cereus isolates produced positive amplicons for the pBC210 targets (Table I) or for pXO1 or pXO2. Most of these isolates came from soils, powders, clinical samples, and marine samples that were collected in various parts of the United States, but primarily from Texas and Florida.

This assay appears to identify the B. cereus G9241 from closely related Bacillus spp., and other bacteria with 100% specificity. The assay is based on specific sequences of pBC210 putatively not found on other known Bacillus plasmids [11].
It is theoretically possible that one target or the other could be carried by a currently unknown plasmid but using two putatively unique targets on pBC210 lowers the assay’s potential for false positive results. The possibility of false positives is further reduced by the use of the third DNA target also reportedly unique to pBC210. Yet the possibility still exists that the three sequences are on different plasmids or chromosome of more than one bacterial cell in the soil.

We are the first to find evidence of three DNA sequences reportedly unique to pBC210 in Florida soils. Even though only 256 soils were tested, we still identified five soils that yielded positive qPCR for the three pBC210 targets. This represents 2% of all of the soils tested and 7% of the Florida soils. This work illustrates the need to survey many more soil samples to better understand the prevalence of the targeted genes, the pBC210 plasmid, and virulent B. cereus strains. These five samples are 2% of the total number of soils tested but represent 7% of Florida soils. This higher percentage may indicate that B. cereus or other Bacillus spp. carrying the virulent plasmid are more prevalent in Florida than in Texas or Louisiana. Thus, further work to examine the distribution of pBC210 carrying Bacillus sp. not only in Florida but also in other areas is warranted.

4. Conclusion

This study is the first to report that three sequences reportedly unique to the pBC210 plasmid are present in DNA extractions from Florida soils. We suggest that the distribution of the pBC210 plasmid be expanded from Texas and Louisiana to now include Florida. We were able to identify five soil samples that appear to have the pBC210 plasmid present using qPCR targeting three open reading frames that are putatively only found in pBC210. These five samples are 2% of the total number of soils tested but represent 7% of Florida soils.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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