**Bacillus anthracis**-Like Bacteria and Other *B. cereus* Group Members in a Microbial Community Within the International Space Station: A Challenge for Rapid and Easy Molecular Detection of Virulent *B. anthracis*

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**Abstract**

For some microbial species, such as *Bacillus anthracis*, the etiologic agent of the disease anthrax, correct detection and identification by molecular methods can be problematic. The detection of virulent *B. anthracis* is challenging due to multiple virulence markers that need to be present in order for *B. anthracis* to be virulent and its close relationship to *Bacillus cereus* and other members of the *B. cereus* group. This is especially the case in environments where build-up of *Bacillus* spores can occur and several representatives of the *B. cereus* group may be present, which increases the chance for false-positives. In this study we show the presence of *B. anthracis*-like bacteria and other members of the *B. cereus* group in a microbial community within the human environment of the International Space Station and their preliminary identification by using conventional culturing as well as molecular techniques including 16S rDNA sequencing, PCR and real-time PCR. Our study shows that when monitoring the microbial hygiene in a given human environment, health risk assessment is troublesome in the case of virulent *B. anthracis*, especially if this should be done with rapid, easy to apply and on-site molecular methods.

**Introduction**

Monitoring of microbial hygiene can give an early warning sign for the presence of potential pathogens in an environment so preventive countermeasures can be taken to minimise health risks. To this purpose, microbial hygiene is monitored and controlled at various locations such as hospital wards, potable water distribution systems or air-conditioning systems of modern buildings but also in spacecraft such as the International Space Station (ISS). To secure the health and wellbeing of the crew, characterisation and understanding of the microbial contamination of the ISS is essential [24]. Especially at remote locations such as the ISS, rapid and easy to use methods to monitor microbial hygiene are preferred, which can be used on-site without the need for technical personnel or specialised laboratories. Detection and identification of microbes by conventional culturing is laborious, time-consuming, requires specialist expertise and many species are difficult to culture or uncultivable. In addition, culturing of hazardous microbes such as potential pathogens or so-called technophiles [33] is often undesirable. In this respect, molecular detection methods circumvent many of the disadvantages encountered by conventional culturing techniques [35]. However, for some species, such as *Bacillus anthracis*, the etiologic agent of the disease anthrax, rapid and easy on-site molecular detection can be problematic. The ISS is a complex environment with a regulated atmosphere which forms a unique microbial ecological niche that encompasses several spaceflight related parameters such as microgravity, radiation or the concept of closed environment [14]. The secondly most common identified bacterial species in the ISS environment are spore-formers of the genus *Bacillus* (31.7%) [24]. *Bacillus* species generally are aerobic endospore-forming bacteria that are common in nature. More specifically, the *Bacillus cereus* group comprises six members: *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoideae*, and *Bacillus weihenstephanensis*. *B. cereus*, *B. anthracis*, and *B. thuringiensis* can be considered to be pathovars of a single species and it has been suggested that *B. anthracis* is a monophyletic clone derived from the *B. cereus* and *B. thuringiensis* clade [21,25,28,29]. Whilst *B. cereus* is an opportunistic pathogen that also causes food borne illness, *B. thuringiensis* is an insect pathogen. *B. anthracis* is an obligate pathogenic species that causes the zoonosis anthrax [5,22]. The disease primarily occurs in herbivores, and is usually transmitted to humans by direct or indirect contact with contaminated animals. Two plasmids are essential for the virulence of *B. anthracis*, pXO1 and pXO2 [5,22]. Plasmid pXO1 harbours genes for the anthrax...
Materials and Methods

Sample Collection

Samples were collected from various locations on the interior of the Russian Zvezda Service Module (DOS-8) of the ISS during several spaceflight missions by mainly using a sampling system consisting of a Swab Rinse Kit (927C SRK) tube without medium containing a Dacron applicator (Copan Italia S. p. A. Diagnostics Inc., Brescia, Italy). Swabs were pre-moistened with 55 ml sterile physiological salt solution (B. Braun Melsungen AG, Melsungen, Germany) using DNA-free laboratory techniques. The samples were stored and transported to the microbiology laboratory on Earth at the University Medical Center Groningen. The samples were suspended in Fastidious Broth (Mediaproduts BV, Groningen, The Netherlands) and incubated for 1–2 days at 37°C, after which the suspension was plated on Blood Agar plates and further cultivated overnight on Blood Agar plates at 37°C for 2 days, and further incubated overnight on Blood Agar plates at 37°C. The samples were suspended in Fastidious Broth (Mediaproduts BV, Groningen, The Netherlands) and incubated for 1–2 days at 37°C, after which the suspension was plated on Blood Agar plates and further cultivated overnight on Blood Agar plates at 37°C for 2–3 days.

Preparation of Template DNA for PCR and Sequencing

Bacterial cells were inoculated into 9 ml Brain Heart Infusion (Mediaproduts BV) and grown overnight at 37°C. From this suspension, template DNA was prepared by using either whole bacterial cells without extracting DNA, or extracting DNA based on the method of Boom et al. [3]. Briefly, in the latter case, 0.1 ml bacterial cell suspension was added to 600 ml mixture of L6 and Celleto 545 (Acros Organics, Geel, Belgium), and put at ambient temperature for 10 min. The mixture was centrifuged in an Eppendorf microcentrifuge for 10 s at 13,400 x g. The pellet was washed with 70% ethanol (v/v) and subsequently acetone and centrifuged for 10 s at 13,400 x g after each washing step. The acetone was disposed of after which the pellet was left to dry for 10 min at 56°C. The pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) during 10 min at 56°C.

PCR of gyrB

A Bacillus species-specific chromosomal region was amplified with a PCR assay based on Yamada et al. [34]. Bacterial primers were used to amplify a fragment of the gyrB gene of B. cereus (BC1 and BC2r, 365 bp), B. anthracis (BA1 and BA2r, 245 bp) and B. thuringiensis (BT1 and BT2r, 368 bp) (Table 1). Oligonucleotide primers BC1, BC2r, BA1 and BA2r were obtained from Eurogentec S.A. and BT1 and BT2r from Biolegio. Template DNA was prepared as described above using whole bacterial cells or DNA solution in duplicate (four-fold, in total). All PCR reactions were performed in a total volume of 25 μl containing 1 or 5 μl of template DNA, 0.25 U Super Taq HC (HT Biotechnology Ltd.), PCR Buffer (HT Biotechnology Ltd.), 200 μM of each dNTP (Fermentas Int. Inc., Burlington, Canada) and 100 nM of each primer. Amplification was carried out on an Omegene TR3 CM220 Temperature Cycler (Hybaid Ltd., London, UK) with a profile of 94°C for 5 min, followed by 50 cycles of 94°C for 30 s, 58°C for 75 s and 72°C for 1.5 min and finally one cycle of 72°C for 5 min. To confirm amplification of PCR products of the proper size, electrophoresis was performed through an ethidium bromide stained 1.0% (w/v) agarose gel (Eurogentec S.A., Seraing, Belgium) in TBE buffer (Invirotogen Ltd., Paisley, UK). PCR products were bidirectionally sequenced (BaseClear BV, Leiden, The Netherlands) by using T7, UP and internal primers with proprietary in-house protocols.

Expert Confirmation Analysis

Expert confirmation analysis was conducted on the isolates by the Dutch national veterinary reference laboratory (CVI, WUR, Lelystad, The Netherlands) which encompassed microscopic evaluation of a Gram-stained preparation, evaluation of β-haemolysis, β-phage susceptibility testing and an in-house B. anthracis PCR. β-phage susceptibility is tested for by the capacity of the phage to lyse the culture.

Primers and probes of the PCR specific for B. anthracis were developed with Primer Express 2.0 (Applied Biosystems, Carlsbad, CA, USA) and based on Antwerpen et al. 2008 [1] and Hadjinicolaou et al. 2009 [8] (Table 1). Primers were used to amplify a specific B. anthracis chromosomal fragment with locus tag BA_5345 encoding for a hypothetical protein (BA chrom F and R, 78 bp), the lef gene on plasmid pXO1 (lef F and R, 128 bp) and the capC gene on plasmid pXO2 (CapC F and R, 119 bp). All PCR reactions were performed in a total volume of 20 μl, consisting of 5 μl of template DNA (prepared according to Boom et al. [3]), 14 μl of amplification mixture containing 400 nM of primers (Eurogentec S. A.) and 200 nM of probes (Eurogentec S. A.). PCR reaction buffer (PerfeCTa Multiplex qPCR SuperMix (UNG, Low ROX), Quantast BioSciences Inc., MD, USA) and water and 1 μl of inhibition control (IC). The PerfeCTa Multiplex qPCR Supermix reagents contained uracil-N-glycosylase (UNG)
Table 1. PCR oligonucleotide primers used in this study.

| Target gene | Primer | Sequence (5'-3') | References |
|-------------|--------|------------------|------------|
| gyrB        | BC1    | ATGGTGACCCGATCCAACA | Yamada et al. [34] |
|             | BC2r   | TCACTCGATGTGATTTTTC | Yamada et al. [34] |
| gyrB        | BA1    | ACTGCTTTATAAAACTGACG | Yamada et al. [34] |
|             | BA2r   | CTTCACTACGTGAAATGTG | Yamada et al. [34] |
| gyrB        | BT1    | ATCGTGTACAGATAGACT | Yamada et al. [34] |
|             | BT2r   | CTTCACTACGTGAAATAATTTT | Adapted from Yamada et al. [34] |
| 16S rRNA    | forward | ACCATAAGCCTACTATAAGGAGACTTGTGCCATCTGCTCAG | (R. Rozeboom and P. Terpstra, personal communication); Hiraishi et al. [10] |
| 16S rRNA    | reverse | ATGGTAAACGAGGCCAGTTGGTACCTTGATCGACCTT | (R. Rozeboom and P. Terpstra, personal communication); Hiraishi et al. [10] |
| BA_5345     | BA chrom F | TGGATACATACCCAAAAGGTCACATACCCCAAAGGCTACCTTTGCAACTT | based on Antwerpen et al. [1] |
|             | BA chrom R | TTTGACAAATGACCAAGGTTT | based on Antwerpen et al. [1] |
|             | BA chrom probe | 6-FAM-CCACAGGCGCTAAGAGAGAGCCAGTAAACCC-BHQ-1 | based on Antwerpen et al. [1] |
| lef         | lef F   | TTGAATAATGAAAGGGCTCGATT | based on Hadjinicolaou et al, 2009 [8] |
|             | lef R   | TTCCAGAGCCGATTGCCTGTTGAA | based on Hadjinicolaou et al, 2009 [8] |
|             | lef probe | CY3-AGCGTTGAAAGGAGAAGCTCCACCAATTACACCAAGG-BHQ-2 | based on Hadjinicolaou et al, 2009 [8] |
| capC        | CapC F  | CGATGATTTCAAGGATCTGAT | based on Hadjinicolaou et al, 2009 [8] |
|             | CapC R  | CTCAGGATCCAAAGGATAAACAA | based on Hadjinicolaou et al, 2009 [8] |
|             | CapC probe | CY5-TGCCGCGTAGAAAATTGGCGCAAC-BHQ-2 | based on Hadjinicolaou et al, 2009 [8] |

Bold: T7 and UP extension, respectively.
Abbreviations: FAM, carboxyfluorescein; CY, cyanine; BHQ, Black Hole Quencher.

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Biochemical and Phenotypic Characterisation

Biochemical and phenotypic characterisation was performed on pure cultures by making use of the API system (bioMérieux Inc., Marcy l’Etoile, France) according to the manufacturer’s instructions. The API 20 E tests were used to test for arginine dihydrolase, indole production, acetoin production (VP), gelatinase and nitrate reduction. Inconclusive reactions for acetoin production and nitrate reduction were confirmed with standard culture-based techniques [7]. Anaerobic acid production from D-glucose, glycerol, glycerol, inulin, D-mannitol, salicin and D-trehalose was determined using the API 50 CHB system (bioMérieux Inc.). Supplemental tests were performed by using the VITEK 2 Systems (bioMérieux Inc.) and standard culture-based techniques [7]. Anaerobic growth was determined by cultivation on Brucella Blood Agar (Mediaproducts BV) under a nitrogen atmosphere. Capsule production was tested for by using M’Fadyean polychrome methylene blue and Indian ink stain, on smears of cells that were cultivated on nutrient agar containing 0.8% sodium bicarbonate under a 5% CO₂ atmosphere.

Data Analysis

Nucleotide sequences were aligned by using the BioEdit Sequence Alignment Editor version 7.0.8.0 [9]. Sequence analysis was conducted on determined sequences by searches of the online BLASTN [36] and RDP10 [4] databases.

Results

Eleven B. cereus group isolates were selected based on morphology and Gram stain from a collection of strains isolated from the interior of the Russian Zvezda Service Module of the ISS. These strains were isolated from various sites of the human living quarters, including walls, tables and toilet areas. All S1 isolates were isolated from a first flight and the S2 isolates from a second flight one year later (Table 2). S1-R3O1-FB and S2-R3O1-FB-BA1 were isolated from the same wall panel, but during two consecutive flights one year apart.

16S rDNA Sequence Analysis

16S rDNA sequence analysis results of searches of the online databases are shown in Table 2. Identification to the species level could not be ruled out due to a W at position 1146 of the latter. Isolate S2-R4W1-FB-BA1
Table 2. Overview of isolate characteristics using several methodologies.

| Character                          | B. cereus | B. anthracis | B. thuringiensis | B. mycoides | S1-R1P1-FB | S1-R4D1-FB | S1-R6TC1 | S1-R1O1-FB | S2-R3O1-FB-BA1 | S2-R2W1-FB-BA1 | S2-R4W1-FB-BA1 | S1-R1D2-FB | S1-R2T1-FB | S2-R3J1-FB-BA1 | S1-R5C1-FB | S2-R3J1-FB-BA1 | S2-R4O6S1-FB |
|-----------------------------------|-----------|-------------|-----------------|-------------|------------|------------|-----------|------------|----------------|----------------|----------------|------------|------------|----------------|------------|----------------|--------------|
| 16S rDNA analysis by online database searches |           |             |                 |             |            |            |           |            |                |                |                |            |            |                |            |                |              |
| PCR of gyrB:                      |           |             |                 |             |            |            |           |            |                |                |                |            |            |                |            |                |              |
| B. cereus                        | –         | +           | –               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| B. anthracis                     | –         | +           | –               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| Motility                         | +         | –           | +               | –           | +          | +          | (+)       | (+)        | (+)             | (+)             | (+)             | +          | +          | +               | +          | +               | +             |
| Parasporal crystals              | –         | –           | +               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| Acetoin production               | +         | +           | +               | –           | +          | –          | +         | –          | –               | +               | +               | +          | +          | +               | +          | +               | +             |
| Casein hydrolysis                | +         | +           | +               | –           | –          | –          | –         | –          | +               | +               | +               | +          | +          | +               | +          | +               | +             |
| Penicillin susceptibility R/S     | R         | S/(R)       | R               | R           | R          | R          | R         | R          | R               | R               | R               | R          | R          | R               | R          | R               | R             |
| Capsule production               | –         | +/–         | –               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| Gamma phage susceptibility*      | –         | +           | –               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| Motility                         | +         | –           | +               | –           | (+)        | (+)        | (+)       | (+)        | (+)             | (+)             | (+)             | +          | +          | +               | +          | +               | +             |
| Acid from:                       |           |             |                 |             |            |            |           |            |                |                |                |            |            |                |            |                |              |
| Glycerol                         | +         | –           | +               | –           | –          | –          | –         | –          | –               | –               | –               | –          | –          | –               | –          | –               | –             |
| Salicin                          | +         | –           | (+)             | (+)         | +          | +          | +         | +          | +               | +               | +               | +          | +          | +               | +          | +               | +             |
| VPE                               | +         | +           | +               | +           | +          | +          | +         | +          | (–)             | (–)             | (–)             | +          | +          | +               | +          | +               | +             |
| VITEK 2 Systems BCL card         |           |             |                 |             |            |            |           |            |                |                |                |            |            |                |            |                |              |
| L-aspartate arylamidase          | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | –               | –               | –               | –          | –          | ND              | ND         | ND              | ND            |
| Leucine arylamidase              | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | +               | +               | +               | (+)        | (+)        | (+)              | (+)        | (+)              | (+)           |
| Alanine arylamidase              | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | +               | +               | +               | +          | +          | +               | +          | +               | +             |
| Tyrosine arylamidase             | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | +               | +               | +               | +          | +          | +               | +          | +               | +             |
| Cyclodextrine                    | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | –               | –               | –               | +          | +          | +               | +          | +               | +             |
| Glycine arylamidase              | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | –               | –               | –               | +          | +          | (–)              | (–)        | (–)              | (–)           |
| D-mannose                        | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | –               | –               | –               | +          | +          | +               | +          | +               | +             |
| N-acetyl-D-glucosamine           | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | +               | +               | +               | +          | +          | +               | +          | +               | +             |
| β-glucosidase                    | ND        | ND          | ND              | ND          | ND         | ND         | ND        | ND         | –               | –               | –               | –          | –          | –               | –          | –               | –             |
Table 2. Cont.

| Character         | S1-R5C1-7FB | 2Z-H31-1-FB-BA1 | 51-R4H-1 | 51-R2T-1-FB | 51-R5C1-7FB |
|-------------------|-------------|-----------------|-----------|-------------|-------------|
| gpyruvate         | +           | +               | (−)       | (−)         | (−)         |
| β-glucosidase     | ND          | ND              | ND        | ND          | ND          |
| Phenylalanine     | +           | +               | +         | (−)         | (−)         |
| Phenol red        | ND          | ND              | ND        | ND          | ND          |

Abbreviations: +, positive; v, variable; (−), weak reaction; −, negative; R, resistant; ND, not determined. Symbols for colony morphology indicate similarity and differences. All data were obtained in this study.

The results of the PCR analysis of gyrB are shown in Table 2. The BC isolates were all positive for B. cereus PCR, which is in agreement with 16S rDNA sequence analysis. All BA isolates were positive for B. anthracis PCR. The BW isolate was positive for B. anthracis PCR in two of four reactions.

Expert Confirmation Analysis

All isolates were determined to be negative for B. anthracis by the reference laboratory. Isolate S1-R5C1-FB was slightly inhibited by the phage. The image in a Gram-stained preparation was found to be divergent from B. anthracis for all isolates. PCR results for the B. anthracis chromosomal marker BA_5345 and the lef and capC markers on plasmids pXO1 and pXO2, respectively, were negative for all strains.

Biochemical and Phenotypic Characterisation

All strains were Gram-positive rods with ellipsoidal to cylindrical endospores not swelling the sporangia that lie central to subterminal, which mostly were capable to form chains of cells. All strains were positive for anaerobic growth, starch hydrolysis and lecithinase; all strains were negative for parasporal crystals, growth at 50°C and 65°C, lipase and gas from glucose. In the API 20 E strip, for all strains, nitrate reduction and gelatinase are positive; the indole production test is negative. In the API 50 CHB gallery, acid is produced from glycogen and D-trehalose; acid is not produced from D-arabinose, inulin, and D-mannitol. Of the VITEK 2 Systems BCL card tests, all strains were positive for phenylalanine arylamidase, L-pyrolydonyl-arylamidase, β-N-acetyl-glucosaminidase, D-glucose, D-ribose, maltotriose and esculin hydrolysis; all strains were positive for putrescine assimilation, methyl-A-D-glucopyranoside acidification, D-galactose, D-melezitose, methyl-D-xyloside, myo-inositol, palatinose, L-rhamnose, D-tagatose, Ellman, ala-phe-pro arylamidase, L-lysine-arylamidase, L-proline arylamidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, β-syloxidase. All strains were resistant to polynixin-B and susceptible to oleandomycin. Biochemical and phenotypic characteristics for differentiating the strains from B. cereus, B. anthracis, B. thuringiensis and B. mycoides are presented in Table 2. On the basis of the above characteristics, all strains could be identified as members of the B. cereus group [22,30].

Differentiation by colony morphology was consistent with the results of other methods such as PCR of gyrB and sequence analysis of the 16S rRNA gene, except for isolate S1-R5C1-FB. Of the VITEK 2 Systems BCL card, the BA isolates were mostly distinguishable from the BC and BW isolates with tests for alanine arylamidase and β-glucosidase. None of the strains tested positive for the B. anthracis virulence factor of capsule production.

Discussion

Naturally occurring anthrax caused by B. anthracis is readily controllable, however artificially massive exposures can be created, making it much less controllable and placing it high on the list of potential agents of biological warfare or bioterrorism [5,22]. As a result, B. anthracis has been researched and developed as a biological weapon over many years.
Table 3. 16S rDNA sequence signature comparison of isolates according to the Sacchi et al. [31] type scheme.

| 16S type | Strain | Nucleotide position |
|----------|--------|---------------------|
|          |        | 77  | 90  | 92  | 182 | 189 | 200 | 208 | 1015 | 1036 | 1045 | 1146 | 1462 |
| type 9  B. cereus |        |     |     |     |     |     |     |     |     |     |     |     |     |
|          | S1-R1P1-FB | A   | T   | T   | C   | A   | C   | T   | G   | A   | T   | A   | A   | T   |
|          | S1-R6TC1 | A   | T   | T   | C   | (t) | A   | C   | T   | G   | A   | (c) | T   | A   | A   | T   | (a) |
|          | S1-R3O1-FB | A   | T   | T   | C   | (t) | A   | C   | (t) | T   | G   | A   | (c) | T   | A   | A   | T   | (a) |
|          | S1-R4D1-FB | A   | T   | T   | C   | (t) | A   | C   | (t) | T   | G   | A   | (c) | T   | A   | A   | T   | (a) |
|          | S2-R3O1-FB-BA1 | A   | (g) | T   | T   | C   | A   | C   | (t) | T   | G   | A   | (c) | T   | A   | A   | T   | (a) |
| type 13 B. cereus |        |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |        | A   | T   | T   | C   | A   | C   | T   | G   | C   | T   | A   | T   | T   |
| type 6  B. anthracis |        |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          | S1-R1J2-FB | A   | T   | T   | C   | (t) | A   | C   | T   | G   | C   | T   | A   | T   | W   | T   |
|          | S1-R2T1-FB | A   | T   | T   | C   | (t) | A   | C   | T   | G   | C   | T   | A   | T   | T   |
|          | S1-R4H1-FB | A   | T   | T   | C   | (t) | A   | C   | T   | G   | C   | T   | A   | T   | T   |
|          | S2-R3J1-FB-BA1 | A   | T   | T   | C   | (t) | A   | C   | T   | G   | C   | T   | A   | T   | T   |
|          | S1-R5C1-FB | A   | T   | T   | C   | (t) | A   | C   | T   | G   | C   | C   | T   | A   | T   | T   |
| B. mycoides ATCC 6462 T (AB021192) |        |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          | A   | T   | T   | T   | A   | T   | T   | G   | A   | T   | A   | A   | A   |
| B. weihenstephanensis DSM 11821 (AB021199) |        |     |     |     |     |     |     |     |     |     |     |     |     |
|          | A   | T   | T   | T   | A   | T   | T   | G   | A   | T   | A   | A   | A   |
| S2-R4W1-FB-BA1 |        |     |     |     |     |     |     |     |     |     |     |     |     |
|          | A   | T   | T   | T   | A   | T   | T   | G   | A   | T   | A   | A   | A   |

Abbreviations: R, A or G nucleotide; Y, C or T nucleotide; M, A or C nucleotide; W, A or T nucleotide. Nucleotides between brackets indicate a weak double signal of that nucleotide at that position.

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Due to their resistance to desiccation, heat, radiation and disinfectants, spores of aerobic endospore formers readily survive in soils, dusts, and aerosols, making them troublesome contaminants. *B. anthracis* probably only rarely multiplies in the environment [22,29]. In certain environments however, such as in advanced life support systems like the ISS, build-up of spores of adverse microbial species might occur due to factors such as dust accumulation. In addition, hygiene regimes [27] that make use of cleaning solutions containing disinfectants may clean selectively, favouring microbes and spores that possess features to withstand hostile environments. Indeed, spore-forming bacterial species such as of the genus *Bacillus* have been detected on the ISS in considerable numbers [24].

We isolated strains of members of the *B. cereus* group from the interior of the ISS, of which BC isolates were found to be most similar to *B. cereus* and BW isolates to *B. weihenstephanensis* or *B. mycoides*

The correct identification of virulent *B. anthracis* is a well-documented challenge [16,29]. The BA isolates possessed *B. anthracis*-like biochemical characteristics such as acid from glycerol and salinic reactions. Several of the differentiating characters for *B. anthracis* such as a lack of β-haemolytic activity, lack of motility, penicillin sensitivity, susceptibility to γ-phage lysis or some genetic markers are not truly specific [5,22,25,32], making correct identification challenging and thus leaving these characters inconcusive for the BA isolates. Traits as growth on Phenylethyl Alcohol Blood Agar (PEA) and arginine dihydrolase argued against identification of the BA isolates as *B. anthracis*.

Even though the differentiation of *B. anthracis* from *B. cereus* on the basis of 16S rRNA gene sequencing has been reported to be difficult, it has been proven useful for fast differentiation [2,31]. 16S rRNA gene sequence analysis showed signatures of the BA isolates to be similar to type 15 *B. cereus* and type 6 *B. anthracis* according to the scheme of Sacchi et al. [31]. A conclusive identification of the BA isolates as type 6 *B. anthracis* could however not be made due to a mixed basepair at position 1146 of the latter, which could not be confirmed by the sequencing approach used in our study.

The gyrB gene has been proven to be a useful phylogenetic discriminator for members of the *Bacillus cereus* group [19]. Whilst phenotypic characters and 16S rRNA analysis of the BA isolates pointed towards suspect for *B. anthracis* identification, PCR of chromosomal marker gyrB of the isolates was positive for *B. anthracis*, however, in contrast, PCR of chromosomal marker BA_5345 was negative for *B. anthracis*.

Regarding the phenotypic and genetic characteristics of the BA isolates and the observation that virulence genes of the pXO1 and pXO2 plasmids were not detectable by PCR, the latter which was confirmed by the lack of a detectable capsule, these isolates may best be defined as non-virulent *B. cereus*/*B. anthracis sensu lato* [25].

Identification of virulent *B. anthracis* may be troublesome for several reasons, in particular in determining the presence of the anthrax virulence plasmids [25]. First of all, merely the possession of both plasmids pXO1 and pXO2 is not enough for virulent *B. anthracis* strains, as a result of which they cannot distinguish between the presence of all markers within the same strain or their presence in the microbial community as a whole and as such, hinder the inconclusive determination of whether or not a virulent *B. anthracis* is detected in a sample of mixed species.

For the detection of virulent *B. anthracis* rapid molecular methods are preferred, however in certain situations this can be problematic. We observed the considerable challenge for rapid molecular on-site detection of virulent *B. anthracis* in the situation that multiple species of the *B. cereus* group are present within the same microbial sample. There is an increased chance for this occurrence in the situation that *B. anthracis*-like bacteria are present in the same environment as other members of the *B. cereus* group.

This study describes the preliminary classification of *B. anthracis*-like bacteria which were found to be present on board of the ISS within the same human environment as other members of the *B. cereus* group: *B. cereus*, *B. weihenstephanensis* and *B. mycoides*.

As such this study shows that, besides the known challenges for correctly identifying virulent *B. anthracis* in situations such as of potential biological terrorism, there is also a challenge for monitoring the microbial hygiene in specific human environments. For the monitoring and control of microbial hygiene in human environments at remote locations such as the ISS, it may be difficult to predict certain situations that are critical to human health direct and on-site with a rapid and easy to use molecular methodology, before the emergence of a clinical case. In microbial communities of such environments both *B. anthracis*-like bacteria and other members of the *B. cereus* group may co-exist, as was shown in this study, which is consistent with findings in the natural environment such as soil or aerosols [15,18,23]. As such, the two plasmids pXO1 and pXO2 or their homologues, harbouring the virulence factors for anthrax, might occur simultaneously in separate strains of members of the *B. cereus* group within the microbial population, hampering detection of virulent *B. anthracis* by molecular methods. As a result, the risk assessment of the microbial hygiene situation and whether it is becoming dangerous to human health is difficult to determine for this species by the present methods. Our research shows that not only the primary issue of correct identification of virulent *B. anthracis* is important for future research but that it should also focus on finding a solution for the issue of false positives caused by the need to detect multiple markers within the same sample.

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**Author Contributions**

Conceived and designed the experiments: SPvT HIJR HJMH JED. Performed the experiments: SPvT HIJR. Analyzed the data: SPvT HIJR. Contributed reagents/materials/analysis tools: HIJR HJMH JED. Wrote the paper: SPvT HIJR HJMH JED.
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