Diagnostic challenges within the Bacillus cereus-group: finding the beast without teeth

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Abstract

The Bacillus cereus-group (B. cereus sensu lato) includes common, usually avirulent species, often considered contaminants of patient samples in routine microbiological diagnostics, as well as the highly virulent B. anthracis. Here we describe 16 isolates from 15 patients, identified as B. cereus-group using a MALDI-TOF MS standard database. Whole genome sequencing (WGS) analysis identified five of the isolates as B. anthracis species not carrying the typical virulence plasmids pXO1 and pXO2, four isolates as B. paranthracis, three as B. cereus sensu stricto, two as B. thuringiensis, one as B. mobilis, and one isolate represents a previously undefined species of Bacillus (B. basilensis sp. nov.). More detailed analysis using alternative MALDI-TOF MS databases, biochemical phenotyping, and diagnostic PCRs, gave further conflicting species results. These cases highlight the difficulties in identifying avirulent B. anthracis within the B. cereus-group using standard methods. WGS and alternative MALDI-TOF MS databases offer more accurate species identification, but so far are not routinely applied. We discuss the diagnostic resolution and discrepancies of various identification methods.

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I Introduction

The genus Bacillus is a large heterogeneous group, belonging to the phylum Firmicutes, mainly characterized as aerobic and endospore-forming. Taxonomic misclassifications exist within the genus, leading to proposals to restrict the genus to the two clades of B. subtilis-group and B. cereus-group [1,2]. Bacteria belonging to the B. cereus-group are saprophytic, gram-positive, facultative anaerobic, catalase positive, motile, and spore-forming rods. The B. cereus-group comprises at least eight closely related species: B. cereus sensu stricto (here referred to as B. cereus), B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides, B. cytotoxicus, B. weihenstephanensis, and B. toyensis [1,3]. Isolates of the B. cereus-group are commonly found in soil and food, and few, with the exception of B. anthracis, are considered clinically relevant [4]. B. cereus and B. anthracis are the best-studied representatives within the group, responsible for opportunistic food poisoning and the acute and often fatal anthrax disease, respectively [5]. The
**TABLE 1.** Clinical features and evaluation of clinical relevance of B. cereus-group isolates. Names of isolates identified genomically as B. anthracis are shown in bold. The isolate name identified as B. basiliensis sp. nov. is shown in italics. ID, identification; PICC, peripherally inserted central venous catheter; NA, not available; MRSA, Methicillin-resistant *Staphylococcus aureus*; dd, differential diagnosis; GVHD, graft versus host disease; Age groups: 1, <18 years; 2, 18-50 years; 3, >50 years; Amounts: +, low; ++, moderate; ++++, numerous; CFU, colony-forming unit. Detailed case reports of selected isolates are given in the supplementary material.

| Sample number | Age group | Date of collection | Material | Localization | Culture result (amount) | Infectious disease consultation interpretation | Clinical relevance of B. cereus-group |
|---------------|-----------|--------------------|----------|--------------|-------------------------|-----------------------------------------------|-------------------------------------|
| 128633-19     | 2         | 07/19              | Blood    | Not known    | B. cereus-group         | Bacteremia (1/6 bottles positive), dd          | Relevant                            |
| 607738-19     | 3         | 07/19              | Swab     | Lower leg    | B. cereus-group (2 CFU) | Entercoccus faecalis (+++)                    | Not relevant                        |
| 130277-19     | 2         | 07/19              | Blood    | Venous catheter | Staphylococcus hominis, B. cereus-group | Soft tissue infection after compartment lower leg (E. faecalis) | Relevant |
| 607730-19     | 2         | 07/19              | Biopsy   | Upper leg    | B. cereus-group (+), Staphylococcus pasteurianus (+), *S. aureus* (1 CFU) | Colonization of wound | Not relevant |
| 130415-19     | 3         | 07/19              | Blood    | Venous catheter | B. cereus-group         | Contamination (1/4 bottles positive) | Not relevant |
| 608212-21     | 2         | 08/20              | Swab     | Finger       | Kocuria spp. (1 CFU), B. cereus-group (+) | Soft tissue and bone infection | Relevant |
| 804229-21     | 1         | 09/21              | Swab     | Nose         | B. cereus-group (+)     | Postnatal adaptation disorder, pneumonia, screening for MRSA | Not relevant |
| 607095-21     | 3         | 07/21              | Biopsy   | Lower leg    | E. faecalis (+++), B. cereus-group (+++) | Soft tissue infection after compartment lower leg (E. faecalis, B. cereus-group) | Relevant |
| 203778-21     | 2         | 08/21              | Swab     | Vaginal      | B. cereus-group (1 CFU), vaginal flora (+++), gram labe rods (morphotype gardnareli) (+++) | NA | Not relevant |
| 402914-21     | 1         | 08/21              | Swab     | Skin abdomen | Skin flora (+), B. cereus-group (1 CFU) | Common B-cell acute lymphoblastic leukemia; stem cell transplantation; facial herpes zoster | Not relevant |
| 129606-21     | 3         | 08/21              | Blood    | PICC line    | B. cereus-group         | Contamination (1/4 bottles positive) | Not relevant |
| 607632-21     | 3         | 08/21              | Biopsy   | Lower leg    | E. faecalis (+), B. cereus-group (+) | Soft tissue infection after compartment lower leg (E. faecalis, B. cereus-group) | Relevant |
| 402306-21     | 3         | 08/21              | Swab     | Choledix     | B. cereus-group (+++), Muta calida (+++), *S. aureus* (+++), *S. hominis* ++ | Superficial swab for dermatitis | Not relevant |
| 403291-21     | 2         | 09/21              | Swab     | Abdomen      | B. cereus-group (1 CFU) | Abscess/skin infection with *S. aureus* | Not relevant |
| 403307-21     | 3         | 09/21              | Swab     | Lower arm    | B. cereus-group (+)     | Colonization of wound | Not relevant |
| 135455-21     | 3         | 09/21              | Blood    | PICC line    | B. cereus-group         | Polymicrobial catheter infection/dd enteritis with GVHD | Relevant |

Virulence of *B. anthracis* is enabled by two virulence plasmids, pXO1 and pXO2, carrying genes encoding for the tripartite toxin and the poly-y-D-glutamic acid capsule, respectively [5,6].

In clinical routine diagnostics, the fast and reliable identification of *B. cereus* and *B. anthracis* is crucial because of the pathogenic and biothreat potential of *B. anthracis*. The identification is classically based on morphological features. *B. anthracis* is typically described as non-hemolytic on blood agar with a “Medusa’s head” colony morphology. In contrast, *B. cereus* causes hemolysis and has a grey matt colony morphology [7,8]. Additionally, they can be distinguished by motility, and penicillin and phage y susceptibility, where *B. anthracis* shows susceptibility to both [7–11]. Biochemical reaction profiles can differentiate *B. anthracis* from other members of the *B. cereus*-group [10,12]. However, phenotypic characteristics may vary within and among species resulting in inconsistent species identification [10]. Species distinction by 16S ribosomal RNA gene sequencing has proven to be difficult [3,5,13]. Standard MALDI-TOF mass spectrometry (MALDI-TOF MS) is faster but reliable differentiation within the *B. cereus*-group is difficult using commercial databases [14,15]. Alternative diagnostic approaches include specific PCRs targeting toxins, virulence plasmids and chromosomal markers [16]. In order to investigate the ancestral relatedness of isolates and assign species accordingly, genomics provides the gold standard. Many methods have been used to classify genomes within the *B. cereus*-group: digital DNA:DNA hybridization (dDDH) [3]; the Genome Taxonomy Database (GTDB) [10,17], and average nucleotide identity (ANI), perhaps with a proposed genomospecies cutoff at 92.5% rather than the accepted 95-96% cutoff [18]. Using any of these methods, cases of discrepancy in allocating isolates to species have been described. To add to the confusion, the presence of virulence factors does not always correlate with species identification, as some isolates closely related to *B. anthracis* do not carry pXO1 and pXO2 [3,19,20]. Conversely, these plasmids have been found within the genome of other *Bacillus* species [21].
In this study we analyze 16 clinical \textit{B. cereus}-group isolates from 15 patients, assigned to the \textit{B. cereus}-group by clinical and morphological features and the MALDI-TOF MS Bruker standard database (sDB). We describe the isolates in depth using phenotypic and genomic methods to highlight the diagnostic complexity.

2 Material and methods

2.1 Sample collection and culture

The 16 \textit{B. cereus}-group isolates (Table 1) were collected during July 2019, and between July and September 2021, as well as isolates from interesting clinical cases across this timeframe. Isolates were cultured on Columbia blood agar, supplemented with 5% sheep blood (BD Becton Dickinson, New Jersey, USA) at 37°C under aerobic atmosphere supplemented with 5% CO$_2$ over 24 hours.

2.2 Evaluation of clinical relevance

All clinical cases were evaluated regarding the clinical relevance of the \textit{B. cereus}-group isolates by an infectious diseases specialist.

2.3 Species identification

MALDI-TOF MS Microflex LT (Bruker Daltonics, Billerica, Massachusetts, USA) was used, from single bacterial colonies using a simple smear technique with a 1-μl formic acid overlay and cyano-4-hydroxyinnamic acid (CHCA) matrix solution, compared against the main spectra library Bruker Daltonics DB (BDAL, containing 8468 MSP, revision 9) and subsequently against the security relevant DB (SR DB) MBT SR Taxonomy (2017, containing 200 MSP). Additionally, a full protein extraction protocol was performed [22] and spectra collected on Axima™ Confidence (Shimadzu-Biotech Corp., Kyoto, Japan) were compared against the Mabritec® BCG-Classifier contained in the PAPMID™ database [23], which includes ribosomal marker profiles for \textit{B. anthracis} isolates with and without virulence plasmids. Phenotypic species identification was performed on the VITEK®2 System software version 8.01, (bioMérieux S.A., Marcy-l’Étoile, France) using the VITEK®2 BCL card according to the manufacturer’s protocols. This offers to identify Bacillus species using 46 test substrates (Supplemental material Table 1) [12,24]. We identified four key reactions to distinguish \textit{B. anthracis} from \textit{B. cereus}: L-lysine arylamidase (LysA), maltotriose (MTE), N-acetyl-D-glucosamine (NAG), and growth in 6.5% NaCl (VITEK® representative, personal communication).

Antibiotic susceptibility testing (AST) for penicillin, clindamycin, levofloxacin and vancomycin was performed using the gradient diffusion method (MIC Test Strip, Liofilchem®, Italy) on Müller-Hinton agar (BD Becton Dickinson, New Jersey, USA) resulting in minimum inhibitory concentrations (MIC) in mg/L. Penicillin was interpreted by Clinical and Laboratory Standards Institute (CLSI) breakpoints, and clindamycin, levofloxacin and vancomycin by European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [25,26]. Specific PCR assays for \textit{B. anthracis} targeted the virulence plasmids pXO1 and pXO2 (Supplemental material Table 2), and the chromosomal gyrA gene [27].

2.4 Whole genome sequencing and analysis

DNA was extracted using a Qiagen EZ1 and Qiagen DNA tissue kit, per manufacturer’s instructions. WGS used the Illumina DNA prep kit, and NextSeq500 Illumina platform (PE150). Read data was assembled using Unicycler v0.3.0b [28] and annotated using Prokka v1.13 [29]. Long read sequencing was performed on the Gridion (Oxford Nanopore Technologies) following rapid barcoding. Hybrid assembly was performed using Unicycler v0.4.8 [28], and contigs under 1kb removed.

Read data and assemblies have been submitted under project PRJEB48754. The 16S rRNA gene was extracted from assemblies and compared against the NCBI 16S rRNA database using Blastn. Assemblies were analysed for MLST (https://pubmlst.org/organisms/bacillus-cereus/), rMLST (https://pubmlst.org/species-id), using the TYGS database [30] with additional genomes added for context, GTDB (https://gtdb.ecogenomic.org/), and abricate (https://github.com/tseemann/abricate) using the NCBI [31] and VFDB [32] databases. fastANI v1.32 (https://github.com/ParBLiSS/FastANI) compared putative \textit{B. anthracis} genomes against a comprehensive selection of assembled genomes from NCBI belonging to \textit{B. cereus sensu lato}. Mapping against reference plasmids pXO1 NC_007322, pXO2 NC_007323 and pBC218 AAEK01000004 was performed in CLC genomics Workbench v20.0.2. The core genome MLST (cgMLST) analysis was performed in Ridom SeqSphere + v7.7.5 using the scheme described in [33].

Ethical statement

This retrospective observational case-series has been approved by the local ethical committee following the standards of the Swiss Human Research Act (EKNZ 2021-02112). Whenever possible written informed consent was collected, no patient refused participation.
3 Results

3.1 Clinical case features and routine identification of the isolates

A total of 16 isolates from 15 patients were evaluated. The clinical features of the 15 cases are listed in Table 1. Of the 16 isolates, six isolates were classified as clinically relevant, while 10 were classified as not relevant. Routine analysis using the MALDI-TOF MS sDB assigned 14 isolates to B. cereus and two to B. thuringiensis, with high scores (>2.0) (Table 2).

### TABLE 2. Bacillus species identification results by using VITEK®2 ID, MALDI-TOF MS, and WGS in all 16 isolates. Names of isolates identified genomically as B. anthracis are shown in bold. The isolate name identified as B. basilestis sp. nov. is shown in italics. ID, identification; DB, database; sDB, standard database; SR, security relevant; dDDH, digital DNA:DNA hybridization.

| Sample number | Date of collection | Specimen | VITEK®2 ID Probability MALDI-TOF MS ID standard sDB Score | MALDI-TOF MS ID standard sDB + SR DB Score | MALDI TOF MS Mabrice® PAPMID™ BCG-classifier Species by genome and dDDH (TYGS) |
|---------------|-------------------|----------|----------------------------------------------------------|---------------------------------------------|-----------------------------------------------------------------------------|
| 128633-19     | 15.07.19          | Blood    | B. cereus/ thuringiensis/ mycoides                        | 99% B. cereus 2.44 B. cereus 2.44          | B. cereus B. cereus                                                        |
| 607738-19     | 25.07.19          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 98% B. cereus 2.24 B. anthracis 2.46       | B. anthracis B. anthracis                                                 |
| 130277-19     | 26.07.19          | Blood    | B. cereus/ thuringiensis/ mycoides                        | 99% B. cereus 2.45 B. cereus 2.45          | B. cereus B. cereus                                                        |
| 607730-19     | 26.07.19          | Biopsy   | B. cereus/ thuringiensis/ mycoides                        | 98% B. thuringiensis 2.13 B. thuringiensis | B. thuringiensis B. thuringiensis                                         |
| 130415-19     | 27.07.19          | Blood    | B. cereus/ thuringiensis/ mycoides                        | 98% B. cereus 2.32 B. cereus 2.51          | B. cereus B. cereus                                                        |
| 608121-20     | 21.08.20          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 92% B. cereus 2.20 B. anthracis 2.30       | B. anthracis B. anthracis                                                 |
| 804229-21     | 21.07.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 98% B. cereus 2.27 B. cereus 2.27          | B. paranthracis B. paranthracis                                            |
| 607095-21     | 27.07.21          | Biopsy   | B. cereus/ thuringiensis/ mycoides                        | 95% B. cereus 2.19 B. anthracis 2.36       | B. anthracis B. anthracis                                                 |
| 203778-21     | 02.08.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 95% B. cereus 2.17 B. anthracis 2.22       | B. thuringiensis B. thuringiensis                                         |
| 402914-21     | 05.08.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 90% B. cereus 2.34 B. cereus 2.34          | B. paranthracis B. paranthracis                                            |
| 129606-21     | 10.08.21          | Blood    | B. cereus/ thuringiensis/ mycoides                        | 95% B. cereus 2.28 B. cereus 2.28          | B. paranthracis B. paranthracis                                            |
| 607632-21     | 11.08.21          | Biopsy   | B. cereus/ thuringiensis/ mycoides                        | 99% B. cereus 2.22 B. anthracis 2.40       | B. anthracis B. anthracis                                                 |
| 403206-21     | 26.08.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 96% B. cereus 2.22 B. anthracis 2.37       | B. anthracis B. anthracis                                                 |
| 403291-21     | 01.09.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 93% B. cereus 2.39 B. cereus 2.39          | B. paranthracis B. paranthracis                                            |
| 403507-21     | 16.09.21          | Swab     | B. cereus/ thuringiensis/ mycoides                        | 92% B. cereus 2.22 B. cereus 2.22          | B. genomospecies1.3 B. basilestis sp. nov.                                 |
| 135455-21     | 20.09.21          | Blood    | B. cereus/ thuringiensis/ mycoides                        | 89% B. thuringiensis 2.15 B. thuringiensis | B. mobalis B. mobalis                                                      |

3.1.1 Whole genome sequencing and species identification. All 16 isolates underwent genome sequencing for further characterization (Table 3).

The genome assemblies were used to identify the isolates using several methods (Table 4). GTDB and TYGS give more species-level results, and agree more than the other methods, providing the striking finding that five genomes are defined as belonging to B. anthracis (Fig. 1). This species was not reported at all by 16S rRNA gene identification or rMLST. ANI comparisons for these genomes showed a majority of top hits (96-99.8%) to genomes assigned to B. anthracis (data not shown).
TABLE 3. Genomic features of described Bacillus species. Names of isolates identified genomically as *B. anthracis* are shown in bold. The isolate name identified as *B. basilensis* sp. nov. is shown in italics. Plasmids could only be assigned where ONT sequencing was used, otherwise is given as unknown.

| Isolate     | Illumina mean coverage | ONT mean coverage | Assembly size | #Contigs>1kb | #Circular plasmids | %G + C |
|-------------|------------------------|-------------------|---------------|--------------|--------------------|--------|
| 128633-19   | 69                     | NA                | 5380894       | 38           | unknown            | 35.1   |
| 607738-19   | 54                     | 341               | 5225393       | 1            | 0                  | 35.4   |
| 130277-19   | 60                     | NA                | 5330257       | 48           | unknown            | 35.1   |
| 607730-19   | 57                     | NA                | 5620605       | 42           | unknown            | 34.9   |
| 130415-19   | 186                    | NA                | 6327063       | 228          | unknown            | 34.7   |
| 608121-20   | 67                     | 250               | 5714974       | 11           | 2                  | 35.3   |
| 804229-21   | 101                    | NA                | 5497486       | 71           | unknown            | 35.3   |
| 607095-21   | 102                    | 245               | 5234004       | 1            | 0                  | 35.4   |
| 203778-21   | 160                    | NA                | 5761103       | 57           | unknown            | 34.9   |
| 402914-21   | 30                     | NA                | 5501750       | 8            | 6                  | 35.3   |
| 129606-21   | 91                     | NA                | 5533689       | 72           | unknown            | 35.1   |
| 607632-21   | 119                    | 119               | 5258592       | 2            | 1                  | 35.4   |
| 403206-21   | 204                    | 63                | 5717356       | 107          | unknown            | 35.1   |
| 403291-21   | 62                     | NA                | 5525050       | 91           | unknown            | 34.9   |
| 403072-21   | 84                     | 131               | 5785762       | 3            | 2                  | 35.2   |
| 135455-21   | 59                     | NA                | 5649383       | 46           | unknown            | 35.3   |

However, none of the 16 genomes met the >90% target threshold of the *B. anthracis* cgMLST scheme. One isolate was not assigned to any existing species, being most closely related to genomes GCF_001619425.1 and GCF_002566425.1. We describe this as *B. basilensis* sp. nov.

None of the isolates were found to contain complete plasmids pXO1, pXO2, or alternative capsule-carrying plasmid pBC218, with all isolates mapping to only 5-53% of these references. None were found to contain anthrax toxins nor capsule genes *cya*, *lef*, *pagA* or *capABCDE*. All genomes carry the

TABLE 4. Genomic identification of described Bacillus species. Names of isolates identified genomically as *B. anthracis* are shown in bold. The isolate name identified as *B. basilensis* sp. nov. is shown in italics. MLST was performed at https://pubmlst.org/organisms/bacillus-cereus, and rMLST at https://pubmlst.org/species-id. † Where the 16S rRNA gene was found at 14 copies per genome, with 2-6 SNPs between alleles, a representative version was used for the identification. * GTDB warning: Genome has more than 10% of markers with multiple hits.

| Isolate     | MLST                  | 16S rRNA gene identification: top hit(s) against NCBI rRNA database† | rMLST | GTDB                                  | Species by TYGS, with additional genomes |
|-------------|-----------------------|---------------------------------------------------------------------|-------|---------------------------------------|------------------------------------------|
| 128633-2593 | 19                    | 1534bp: 100% match to Bacillus cereus NR_115714.1                    | B. cereus (50%)/B. thuringiensis (50%) | B. cereus                                | *B. cereus* (100%)/B. anthracis         |
| 607738-75   | 19                    | 1550bp: 99.74% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. amyloidificus                         | *B. cereus* (100%)                      |
| 130277-177  | 19                    | 1515bp: 100% match to Bacillus cereus NR_074540.1                    | B. cereus (100%)                      | B. cereus                               | *B. cereus* (100%)                      |
| 607730-2742 | 19                    | 1550bp: 99.87% match to Bacillus toyonensis NR_121761.1               | B. cereus (50%)/B. thuringiensis (50%)| B. cereus                               | *B. cereus* (100%)                      |
| 130415-8    | 21                    | 1520bp: 99.87% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. barnysepticus                        | *B. cereus* (100%)                      |
| 608121-62   | 20                    | 1550bp: 99.74% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 403299-26   | 21                    | 1349bp: 100% match to Bacillus toyonensis NR_177736.1, Bacillus nitratireducens NR_157730.1, Bacillus thuringiensis B. cereus (100%) | *B. cereus* (100%)/B. anthracis         | *B. cereus* (100%)                      |
| 607095-108  | 21                    | 1550bp: 99.74% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 203778-1    | 21                    | new combination:                                                   | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 402914-144  | 21                    | 1538bp: 99.93% match to Bacillus cereus NR_115714.1                  | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 129606-144  | 21                    | 1302bp: 99.92% match to Bacillus cereus NR_115714.1                  | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 607632-62   | 21                    | 1550bp: 99.74% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 403206-1050 | 21                    | 1550bp: 99.74% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 403291-26   | 21                    | 1550bp: 99.93% match to Bacillus cereus NR_115714.1                  | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 403072-21   | 21                    | 1550bp: 99.81% match to Bacillus fungorum NR_170494.1                | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |
| 135455-784  | 21                    | 1550bp: 99.94% match to Bacillus toyonensis NR_121761.1               | B. cereus (100%)                      | B. anthracis*                           | *B. cereus* (100%)                      |

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class A beta-lactamase gene (A7J11_01054 or A7J11_05168) at >90% identity. With the exception of isolate 403507-21, all isolates carry BcII family beta-lactamase (A7J11_00039, A7J11_05308 or A7J11_05161) at >91% identity, although this gene was disrupted in isolate 608121-20. These genes can lead to penicillin resistance when expression levels are high [34].

These results provide evidence that five isolates are most closely related to B. anthracis, which was not seen from the routine microbiology. Given these discrepant results, and the lack of expert consensus on species identification, further identification methods were performed.

3.2 Identification based on phenotype
All 16 isolates showed the classical morphological characteristics of B. cereus: grey, matt colonies exhibiting large double-zone β-hemolysis. A representative example is shown in Supplemental material Fig. 1. Using the VITEK®2 phenotypic test, all isolates were assigned to B. cereus/thuringiensis/mycoides with an identification probability ranging from 89% to 99%, which is the highest level of resolution provided by VITEK®2 BCL card (manufacturer’s information) (Table 2). For the four key reactions identified, no isolate showed a similar profile to the reference B. anthracis, and all isolates showed a positive tyrosine arylamidase reaction (Supplemental material Table 3 for key reactions and Supplemental material Table 4 for more detailed reaction profiles). AST showed that all isolates were resistant to penicillin, but susceptible to clindamycin and vancomycin, and susceptible to increased exposure with levofloxacin (Supplemental material Table 5).

3.3 Identification based on MALDI-TOF MS and virulence specific real-time PCR
Through re-measuring the MALDI-TOF MS spectra of the isolates and using the SR DB, we assigned six isolates to B. anthracis with high scores (Table 2). Using the Mabritec® PAPMID™ BCG-Classifier database, five isolates were identified as B. anthracis without pXO1, pXO2, and gyrA, this latter likely due
isolates belonging to species Bacillus proteolyticus (Supplementary material Fig. 2).

4Discussion

Isolate identification within the B. cereus-group is challenging. Of the various phenotypic and genotypic approaches used in this study to characterize the 16 isolates, we consider genomic analysis by TYGS with appropriate additional comparators to provide the most accurate species assignment. The TYGS results were in agreement with those from Mabritec® PAPMID™ BCG-Classifier database in all cases, and assigned five isolates to B. anthracis, albeit without virulence plasmids, four isolates to B. paranthracis, three to B. cereus, two to B. thuringiensis, one to B. mobilis, and one isolate was identified as a new species, B. basilevisis sp. nov. This shows the species diversity that can be hidden behind a generic diagnosis of “B. cereus-group”.

Illustrating the lack of species distinction in morphological and phenotypic tests, all our isolates showed morphology and AST patterns typical for B. cereus. The biochemical reaction profile of all our isolates was consistent with B. cereus/thuringiensis/mycoides, showing that the VITEK®2 BCL card offered no additional diagnostic support. Only 5/16 (31.3%) isolates were correctly identified at the species level using routine microbiological methods. Of note, B. paranthracis and B. mobilis cannot be identified by VITEK®2 BCL card.

The original definition of B. anthracis was tightly linked to the presence of the anthrax toxin, making the definition of B. anthracis particularly problematic. Many remain convinced that only tightly related, monomorphic, toxin-carrying strains qualify for the name [18,33]. By dDDH analysis, using the 70% cutoff, the species can be more broadly defined, including non-toxin carrying strains, and those described here [3,19]. B. anthracis is absent from the TYGS database, as no type strain genome sequence exists, which complicates identification. We have shown that isolates belonging to B. anthracis are frequently found in the clinical routine laboratory, albeit non-toxin-carrying isolates representing more diverse members of the species. We find that routine morphological, phenotypic, and standard MALDI-TOF MS methods are insufficient for accurate species identification within the B. cereus-group, and additional methods should be applied in difficult cases.

4.1 Taxonomic description of Bacillus basilevisis sp. nov.

A Gram-positive, rod-shaped bacillus (403507-21) was isolated from human superficial skin swab. On the basis of MALDI-TOF MS identification, the isolate was assigned to the Bacillus cereus-group. 16S rRNA gene sequence (accession number: OY062800) analysis shows over 99.8% nucleotide identity to isolates belonging to species Bacillus proteolyticus, Bacillus
Natural text: wiedmannii, B. cereus sensu stricto, Bacillus fungorum, Bacillus tropicus, Bacillus nitratireducens, Bacillus luti and Bacillus albus. Physiological characterization revealed the following traits for the isolate: growth at 37°C under aerobic conditions. The biochemical characteristics of the isolate as determined by VITEK®2 BCL card revealed the following positive reactions: ellman, L-tyrosinidase, D-glucose, esculin-hydrolyse, N-acetyl-D-glucosamin, pyruvat, D-ribose, alalinidase, phenylalanine-arylamidase, tyrosine-arylamidase, glycin-arylamidase, beta-N-acetyl-glucosaminidase. The results of digital DNA–DNA hybridization with a 70% cutoff, performed on the genome assembly, allowed genotypic differentiation of strain 403507-21 from the validly published Bacillus species and show that it falls within the Bacillus cereus-group. Strain 403507-21 therefore represents a new species, for which the name B. basilensis (Etymology: ba.si.len.sis. L. masc./fem. adj. basilensis, pertaining to Basilea, the Roman name of Basel, Switzerland, where the type strain was isolated) is proposed, with the type strain 403507-21 (DSM 113537, CCUG 75930T).

CRediT authorship contribution statement

Veronica Muigg: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft. Aline Cuénod: Investigation, Data curation, Writing – review & editing. Srinithi Purushothaman: Investigation, Data curation, Writing – review & editing. Martin Siegemund: Investigation, Writing – review & editing. Matthias Wittwer: Investigation, Data curation, Writing – review & editing. Valentin Pfüger: Investigation, Data curation, Writing – review & editing. Kristina M. Schmidt: Investigation, Data curation, Writing – review & editing. Martin Siegemund: Investigation, Writing – review & editing. Nicole Ritz: Investigation, Writing – review & editing. Andreas Widmer: Investigation, Writing – review & editing. Daniel Goldenberger: Data curation, Writing – review & editing. Vladimira Hinic: Data curation, Writing – review & editing. Tim Roloff: Data curation, Writing – review & editing. Kirstine K. Søgaard: Data curation, Writing – review & editing. Adrian Egli: Conceptualization, Supervision, Writing – review & editing. Helena M.B. Seth-Smith: Conceptualization, Supervision, Data curation, Formal analysis, Visualization, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2022.101040.

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