

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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Keywords: *Bacillus anthracis*, *Bacillus cereus*, clinical microbiology, MALDI-TOF MS, Next generation sequencing, Routine Diagnostics, Taxonomy, Whole genome sequencing

Original Submission: 2 December 2021; **Revised Submission:** 18 October 2022; **Accepted:** 18 October 2022

Article published online: 26 October 2022

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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, <18years; 2, 18-50years; 3, >50years; 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 <i>B. cereus</i> -group
128633-19	2	07/19	Blood	Not known	<i>B. cereus</i> -group	Bacteremia (1/6 bottles positive), dd spondylodiscitis	Relevant
607738-19	3	07/19	Swab	Lower leg	<i>B. cereus</i> -group (2 CFU), <i>Enterococcus faecalis</i> (++)	Soft tissue infection after compartment lower leg (<i>E. faecalis</i>)	Not relevant
130277-19	2	07/19	Blood	Venous catheter	<i>Staphylococcus hominis</i> , <i>B. cereus</i> -group	Polymicrobial Hickman Catheter infection (3/4 bottles positive)	Relevant
607730-19	2	07/19	Biopsy	Upper leg	<i>B. cereus</i> -group (+), <i>Streptococcus constellatus</i> (+), <i>S. aureus</i> (1 CFU), <i>Haemophilus parainfluenzae</i> (+), <i>Prevotella denticola</i> (+)	Colonization of wound	Not relevant
130415-19	3	07/19	Blood	Venous peripher Finger	<i>B. cereus</i> -group	Contamination (1/4 bottles positive)	Not relevant
608121-20	2	08/20	Swab	Finger	<i>Kocuria</i> spp. (1 CFU), <i>B. cereus</i> -group (+)	Soft tissue and bone infection	Relevant
804229-21	1	09/21	Swab	Nose	<i>B. cereus</i> -group (+)	Postnatal adaption disorder, pneumonitis, screening for MRSA	Not relevant
607095-21	3	07/21	Biopsy	Lower leg	<i>E. faecalis</i> (+++), <i>B. cereus</i> -group (++)	Soft tissue infection after compartment lower leg (<i>E. faecalis</i> , <i>B. cereus</i> -group)	Relevant
203778-21	2	08/21	Swab	Vaginal	<i>B. cereus</i> -group (1 CFU), vaginal flora (+++), gram labile rods (morphotype <i>gardnarella</i>) (+++)	NA	Not relevant
402914-21	1	08/21	Swab	Skin abdomen	Skin flora (+), <i>B. cereus</i> -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	<i>B. cereus</i> -group	Contamination (1/4 bottles positive)	Not relevant
607632-21	3	08/21	Biopsy	Lower leg	<i>E. faecalis</i> (+), <i>B. cereus</i> -group (+)	Soft tissue infection after compartment lower leg (<i>E. faecalis</i> , <i>B. cereus</i> -group)	Relevant
403206-21	3	08/21	Swab	Cheeks	<i>B. cereus</i> -group (+++), <i>Mixta calida</i> (+++), <i>S. aureus</i> (++), <i>S. hominis</i> (++)	Superficial swab for dermatitis	Not relevant
403291-21	2	09/21	Swab	Abdomen	<i>S. aureus</i> (+), <i>B. cereus</i> -group (1 CFU)	Abscess/skin infection with <i>S. aureus</i>	Not relevant
403507-21	3	09/21	Swab	Lower arm	<i>B. cereus</i> -group (+)	Colonization of wound	Not relevant
135455-21	3	09/21	Blodd	PICC line	<i>B. cereus</i> -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- γ -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 γ 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 genom-species 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 *B. cereus*-group isolates from 15 patients, assigned to the *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 *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% CO₂ over 24 hours.

2.2 Evaluation of clinical relevance

All clinical cases were evaluated regarding the clinical relevance of the *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- μ l 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-Classifer contained in the PAMPID™ database [23], which includes ribosomal marker profiles for *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 *B. anthracis* from *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 *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 Gridlon (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 *B. anthracis* genomes against a comprehensive selection of assembled genomes from NCBI belonging to *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).

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 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. basilensis* 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 Mabritec® PAMPID™ BCG-classifier	Species by genome and dDDH (TYGS)
128633-19	15.07.19	Blood	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	99%	<i>B. cereus</i>	2,44	<i>B. cereus</i>	2,44	<i>B. cereus</i>	<i>B. cereus</i>
607738-19	25.07.19	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	98%	<i>B. cereus</i>	2,24	<i>B. anthracis</i>	2,46	<i>B. anthracis</i>	<i>B. anthracis</i>
130277-19	26.07.19	Blood	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	99%	<i>B. cereus</i>	2,45	<i>B. cereus</i>	2,45	<i>B. cereus</i>	<i>B. cereus</i>
607730-19	26.07.19	Biopsy	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	98%	<i>B. thuringiensis</i>	2,13	<i>B. thuringiensis</i>	2,13	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>
130415-19	27.07.19	Blood	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	98%	<i>B. cereus</i>	2,32	<i>B. cereus</i>	2,51	<i>B. cereus</i>	<i>B. cereus</i>
608121-20	21.08.20	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	92%	<i>B. cereus</i>	2,20	<i>B. anthracis</i>	2,30	<i>B. anthracis</i>	<i>B. anthracis</i>
804229-21	21.07.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	98%	<i>B. cereus</i>	2,27	<i>B. cereus</i>	2,27	<i>B. paranthracis</i>	<i>B. paranthracis</i>
607095-21	27.07.21	Biopsy	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	95%	<i>B. cereus</i>	2,19	<i>B. anthracis</i>	2,36	<i>B. anthracis</i>	<i>B. anthracis</i>
203778-21	02.08.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	95%	<i>B. cereus</i>	2,17	<i>B. anthracis</i>	2,22	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>
402914-21	05.08.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	90%	<i>B. cereus</i>	2,34	<i>B. cereus</i>	2,34	<i>B. paranthracis</i>	<i>B. paranthracis</i>
129606-21	10.08.21	Blood	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	95%	<i>B. cereus</i>	2,28	<i>B. cereus</i>	2,28	<i>B. paranthracis</i>	<i>B. paranthracis</i>
607632-21	11.08.21	Biopsy	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	99%	<i>B. cereus</i>	2,22	<i>B. anthracis</i>	2,40	<i>B. anthracis</i>	<i>B. anthracis</i>
403206-21	26.08.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	96%	<i>B. cereus</i>	2,22	<i>B. anthracis</i>	2,37	<i>B. anthracis</i>	<i>B. anthracis</i>
403291-21	01.09.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	93%	<i>B. cereus</i>	2,39	<i>B. cereus</i>	2,39	<i>B. paranthracis</i>	<i>B. paranthracis</i>
403507-21	16.09.21	Swab	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	92%	<i>B. cereus</i>	2,22	<i>B. cereus</i>	2,22	<i>B. genomospecies</i> 13	<i>B. basilensis</i> sp. nov.
135455-21	20.09.21	Blood	<i>B. cereus</i> / <i>thuringiensis</i> / <i>mycooides</i>	89%	<i>B. thuringiensis</i>	2,15	<i>B. thuringiensis</i>	2,15	<i>B. mobilis</i>	<i>B. mobilis</i>

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. basileusis* 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	5335057	48	unknown	35.1
607730-19	57	NA	5602605	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	5761105	57	unknown	34.9
402914-21	30	NA	5501750	107	unknown	35.1
129606-21	91	NA	5533689	72	unknown	35.1
607632-21	119	401	5258592	2	1	35.4
403206-21	204	63	5717356	8	6	35.3
403291-21	62	NA	5926503	91	unknown	34.9
403507-21	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. basileusis* 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. basileusis* 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
128633-19	2593	1534bp; 100% match to <i>Bacillus cereus</i> NR_115714.1	<i>B. cereus</i> (50%)/ <i>B. thuringiensis</i> (50%)	<i>B. cereus</i>	<i>B. cereus</i>
607738-19	75	1550bp; 99.74% match to <i>Bacillus fungorum</i> NR_170494.1	<i>Bacillus</i> (100%)	<i>B. anthracis</i> *	<i>B. anthracis</i>
130277-19	177	1515bp; 100% match to <i>Bacillus cereus</i> NR_074540.1, <i>Bacillus cereus</i> NR_115714.1	<i>Bacillus</i> (100%)	<i>B. cereus</i>	<i>B. cereus</i>
607730-19	2742	1550bp; 99.87% match to <i>Bacillus toyonensis</i> NR_121761.1	<i>B. cereus</i> (50%)/ <i>B. thuringiensis</i> (50%)	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>
130415-19	8	1520bp; 99.87% match to <i>Bacillus fungorum</i> NR_170494.1	<i>Bacillus</i> (100%)	<i>B. bombysepticus</i>	<i>B. cereus</i>
608121-20	62	1550bp; 99.74% match to <i>Bacillus fungorum</i> NR_170494.1	<i>Bacillus</i> (100%)	<i>B. anthracis</i> *	<i>B. anthracis</i>
804229-21	26	1349bp; 100% match to <i>Bacillus tropicus</i> NR_157736.1, <i>Bacillus nitratireducens</i> NR_157732.1, <i>Bacillus luti</i> NR_157730.1, <i>Bacillus albus</i> NR_157729.1	<i>Bacillus paranthracis</i> (100%)	<i>B. paranthracis</i> *	<i>B. paranthracis</i>
607095-21	108	1550bp; 99.74% match to <i>Bacillus fungorum</i> NR_170494.1	<i>B. cereus</i> (100%)	<i>B. anthracis</i>	<i>B. anthracis</i>
203778-21	new combination: 41,6,12,9,4,114,15	1550bp; 99.94% match to <i>Bacillus toyonensis</i> NR_121761.1	<i>Bacillus</i> (100%)	<i>B. thuringiensis</i>	<i>B. paranthracis</i>
402914-21	144	1538bp; 99.93% match to <i>Bacillus cereus</i> NR_115714.1	<i>B. cereus</i> (100%)	<i>B. paranthracis</i> *	<i>B. paranthracis</i>
129606-21	144	1302bp; 99.92% match to <i>Bacillus cereus</i> NR_115714.1	<i>B. cereus</i> (100%)	<i>B. paranthracis</i> *	<i>B. paranthracis</i>
607632-21	62	1550bp; 99.74% match to <i>Bacillus fungorum</i> NR_170494.1	<i>Bacillus</i> (100%)	<i>B. anthracis</i> *	<i>B. anthracis</i>
403206-21	1050	1550bp; 99.74% match to <i>Bacillus fungorum</i> NR_170494.1	<i>Bacillus</i> (100%)	<i>B. anthracis</i>	<i>B. anthracis</i>
403291-21	26	1509bp; 99.93% match to <i>Bacillus cereus</i> NR_115714.1	<i>Bacillus</i> (100%)	<i>B. paranthracis</i> *	<i>B. paranthracis</i>
403507-21	partial: not all loci found	1550bp; 99.81% match to <i>Bacillus fungorum</i> NR_170494.1	<i>B. cereus</i> (100%)	<i>B. thuringiensis</i>	No matching species (<i>B. basileusis</i> sp. nov.)
135455-21	784	1550bp; 99.94% match to <i>Bacillus toyonensis</i> NR_121761.1	<i>B. mobilis</i> (100%)	<i>B. mobilis</i>	<i>B. mobilis</i>

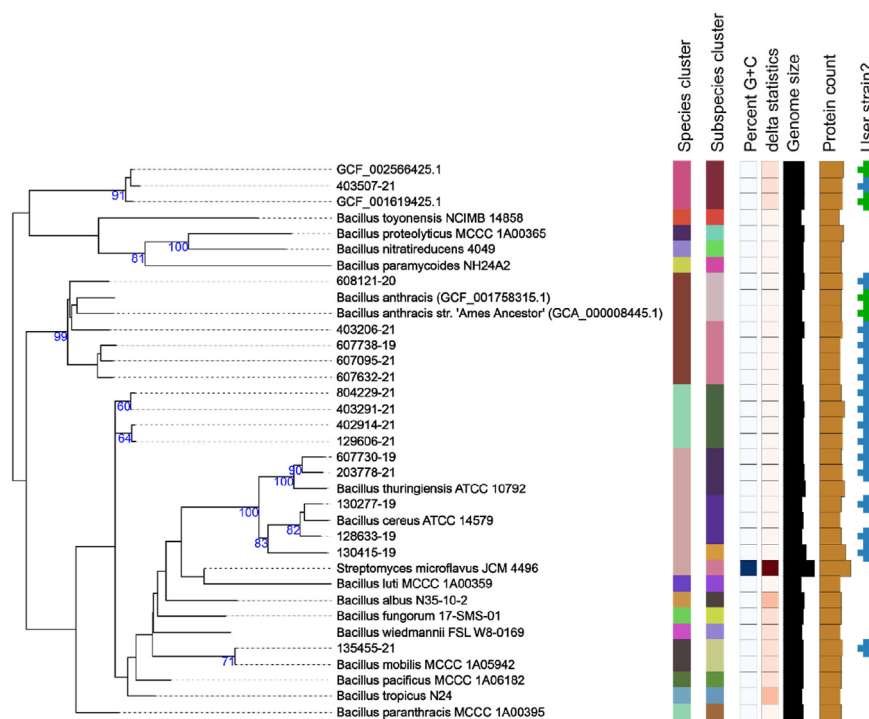


FIG. 1. TYGS GBDP tree showing relationships between isolates and references. Figure was generated by TYGS [30], with genomes sequenced in our hospital (blue + in “User genomes” column), and additional genomes added for context (green +): GCA_000008445.1 and GCF_001758315.1 [19] to reflect *B. anthracis* which is otherwise absent from the TYGS database, GCF_001619425.1 and GCF_002566425.1 to represent members of *B. basilensis* sp. nov.. Species clusters are defined by dDDH with a 70% cutoff. Isolates 607738-19, 608121-20, 607095-21, 607632-21 and 403206-21 can be seen to belong to the species *B. anthracis*, using this standard cut-off (Table 4).

bla1 class A beta-lactamase gene (A7J11_01054 or A7J11_05168) at >90% identity. With the exception of isolate 403507-21 *B. basilensis* sp. nov., all isolates carry *bla2* BclI 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/mycooides* 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® PAMMID™ BCG-Classifer database, five isolates were identified as *B. anthracis* without pXO1 and pXO2, four as *B. paranthracis*, three as *B. cereus*, two as *B. thuringiensis*, one as *B. mobilis* and one as a previously undescribed “genomespecies 13” described here as *B. basilensis* sp. nov. (Table 2). *B. anthracis* specific real-time PCRs were performed on all 16 isolates. All were negative for pXO1, pXO2, and *gyrA*, this latter likely due

to a C-T mutation in the assay probe binding site in the *B. anthracis* isolates (Supplementary material Fig. 2).

4 Discussion

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-Classifer 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. basilensis* 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, and shows that these tools must be used with caution. *B. anthracis* exists in the rMLST database, but the isolates presented here are sufficiently divergent not to be recognized as *B. anthracis* by this method.

Of the isolates, 6/16 were defined as clinically relevant, of which three were identified as *B. anthracis*. Illumina-based WGS offers high resolution identification, but a turn-around time of several days is too slow to generate an impact in clinical management. Using the MALDI-TOF MS sDB, only 4/16 (25%) isolates were correctly identified. This is expected, since *B. anthracis*, *B. paranthracis* and *B. mobilis* are not in the database, and it is known that commercial standard databases do not reliably differentiate species within the *B. cereus*-group [15]. Even using the SR DB, only 9/16 (56,3%) isolates were correctly

identified, including five correctly identified *B. anthracis*, but also one falsely identified as *B. anthracis*. Alternative methods including real-time nanopore sequencing technologies or improved MALDI-TOF MS databases would be valuable additions in these cases. The Mabritec® PAPMID™ database, an online accessible (www.mabriteccentral.com) proprietary database which supports Bruker Biotyper and VITEK® MS bioMérieux data files, is based on in silico predicted ribosomal marker peaks and uses the PAPMID™ algorithm for classification. A sophisticated sample preparation, including the use of sinapinic acid as matrix, was used and all our isolates were correctly identified to species level. The BCG-classifier used is based on deduced reference marker masses from 2204 public *B. cereus*-group genomes, covering 20 accepted species and 21 genomospecies.

Further improvements to MALDI-TOF MS analyses include identifying specific *B. anthracis* biomarker candidates [15], using software such as ClinProTools (Bruker) and Matlab (The Mathworks Inc.), combined with different packages for use in R [14,35], using different matrices [35], and direct detection of anthrax toxin component, the lethal factor [36]. Comprehensive databases can increase the probability of confirmed identification up to 93% [37]. However, these methods need to be evaluated for use in routine diagnostics.

The implications of species mis-identification need to be considered. Defining genomic ancestry does not help in assessment of virulence potential, which is of most clinical and biowarfare agent concern. To address this, suggestions have been made for a new taxonomic nomenclature for the *B. cereus*-group that takes into account phenotypic and genomic characteristics, defining a biovar concept [18] or using BTyper [38]. Anthrax is a notifiable disease: it remains to be clarified whether a non-virulent isolate belonging to *B. anthracis* requires urgent notification.

Using standard genomic methods of species 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 basilensis* 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: OV062800) analysis shows over 99.8% nucleotide identity to isolates belonging to species *Bacillus proteolyticus*, *Bacillus*

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-pyrrolidonyl-arylamidase, D-glucose, esculin-hydrolyse, N-acetyl-D-glucosamin, pyruvat, D-ribose, alanin-arylamidase, 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.sil.en'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

Veronika 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 Pflüger:** Investigation, Data curation, Writing – review & editing. **Kristina M. Schmidt:** Investigation, Data curation, Writing – review & editing. **Maja Weisser:** 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.

Acknowledgements

We thank Prof. Mark Pallen, Quadram Institute, England, for help with species naming. We thank Doris Hohler, Magdalena

Schneider, Clarisse Straub, Elisabeth Schultheiss, Rosa-Maria Vesco, Christine Kiessling, Daniel Gander, Valerie Courtet and Vincent Hartmann (University Hospital Basel) for excellent technical assistance with strain collection and sequencing. We thank Roxanne Mouchet and Samuel Lüdin (Mabritec®) for excellent technical assistance with MALDI-TOF MS. Assemblies and calculations were performed at sciCORE (<http://scicore.unibas.ch>) scientific computing facility at the University of Basel.

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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