Bacillus wiedmannii sp. nov., a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy foods and dairy environments

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A facultatively anaerobic, spore-forming *Bacillus* strain, FSL W8-0169^T, collected from raw milk stored in a silo at a dairy powder processing plant in the north-eastern USA was initially identified as a *Bacillus cereus* group species based on a partial sequence of the *rpoB* gene and 16S rRNA gene sequence. Analysis of core genome single nucleotide polymorphisms clustered this strain separately from known *B. cereus* group species. Pairwise average nucleotide identity BLAST values obtained for FSL W8-0169^T compared to the type strains of existing *B. cereus* group species were <95 % and predicted DNA–DNA hybridization values were <70 %, suggesting that this strain represents a novel *B. cereus* group species. We characterized 10 additional strains with the same or closely related *rpoB* allelic type, by whole genome sequencing and phenotypic analyses. Phenotypic characterization identified a higher content of iso-$C_{16:0}$ fatty acid and the combined inability to ferment sucrose or to hydrolyse arginine as the key characteristics differentiating FSL W8-0169^T from other *B. cereus* group species. FSL W8-0169^T is psychrotolerant, produces haemolysin BL and non-haemolytic enterotoxin, and is cytotoxic in a HeLa cell model. The name *Bacillus wiedmannii* sp. nov. is proposed for the novel species represented by the type strain FSL W8-0169^T (=DSM 102050^T=LMG 29269^T).

The *Bacillus cereus* group, also called *Bacillus cereus sensu lato* (*s.l.*), is a group of closely related micro-organisms of diverse ecotypes, which include pathogenic and non-pathogenic species (Ceuppens *et al.*, 2013). At the time of writing, the *B. cereus* group comprises eight species with validly published names: *B. anthracis* (Logan *et al.*, 1985) *B. cereus* (Smith *et al.*, 1952), *B. cytotoxicus* (Guinebretière *et al.*, 2013), *B. mycoides* (Lechner *et al.*, 1998) *B. pseudomycoides* (Nakamura, 1998), *B. thuringiensis* (Nakamura, 1994), *B. toyonensis* (Jiménez *et al.*, 2013) and *B. weihenstephanensis* (Lechner *et al.*, 1998). In addition to these, three *B. cereus* group strains have been proposed as representing novel species [i.e. '*B. gaemokensis*' (Jung *et al.*, 2010), '*B. manliponensis*' (Jung *et al.*, 2011) and '*B. bingmayongensis*' (Liu *et al.*, 2014)], but have not yet been validly published. *B. cereus* group species are facultatively anaerobic, spore-forming bacteria that are ubiquitously distributed throughout a number of environments (Huck *et al.*, 2007; Ivy *et al.*, 2012; Ceuppens *et al.*, 2013). Depending on virulence gene presence and expression, select strains of these species can cause anthrax or gastrointestinal illness in humans or insects (Kim *et al.*, 2015; Moayeri *et al.*, 2015). Members of the *B. cereus* group are also known food spoilage organisms (Lücking *et al.*, 2013). Therefore, their presence in the food production chain is often monitored (e.g. Miller *et al.*, 2015a).

Strain FSL W8-0169^T was obtained from silo raw milk collected from a dairy powder processing plant in the north-eastern USA in 2012 (Watterson *et al.*, 2014; Miller *et al.*, 2015b). The strain was initially identified as a member of *B. cereus s.l.* based on analysis of a partial sequence of

Abbreviations: ANIb, average nucleotide identity blast; AT, allelic type; DDH, DNA–DNA hybridization; GTR, generalized time-reversible model; HBL, haemolysin BL; MLST, multilocus sequence typing; NHE, non-haemolytic enterotoxin; PI, propidium iodide; SNP, single nucleotide polymorphism; SRA, sequence read archive; WGS, whole genome sequence.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of FSL W8-0169^T is KU198626/SRX1297474. The WGS accession number for FSL W8-0169^T is LOBC00000000, for FSL H7-0353 is LXFL00000000, for FSL H8-0032 is LXFM00000000, for FSL J3-0113 is LXFN00000000, for FSL M7-0044 is LXFO00000000, for FSL M7-0938 is LXFP00000000, for FSL M7-1251 is LXFQ00000000, for FSL P2-0415 is LXFR00000000, for FSL P2-0558 is LXFS00000000, for FSL P4-0569 is LXFT00000000 and for FSL K6-0069 LOBB00000000.

One supplementary figure and three supplementary tables are available with the online Supplementary Material.

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Table 1. Characteristics of the 11 *Bacillus wiedmannii* sp. nov. strains characterized in this study

	FSL W8-0169^T	FSL H7-0353	FSL H8-0032	FSL J3-0113	FSL M7-0044	FSL M7-0938	FSL M7-1251	FSL P2-0415	FSL P2-0558	FSL P4-0569	FSL K6-0069
Source	Raw milk	Ready to eat dairy food product	Dairy food in process	Ready to eat dairy food product	Raw milk	Raw milk	Raw milk	Dairy food in process	Dairy food in process	Dairy food in process	Raw milk
Year of isolation	2012	2005	2005	2012	2011	2011	2011	2009	2009	2010	2012
rpoB AT	61	61	61	417	61	61	61	61	61	61	194
MLST	1081*	1272*	1272*	1094	1271*	1269*	1270*	1268*	644	1266*	1080*
Draft genome length (Mbp)	5.3	5.6	5.6	5.6	5.4	5.4	5.4	5.6	5.6	5.4	5.6
Draft genome G+C content (mol%)	35.3	35.1	35.1	35.1	35.2	35.2	35.2	35.2	35.1	35.2	35.3
No. of contigs	104	75	74	60	73	63	97	93	110	56	158
Draft genome coverage (×)	49	123	121	101	134	142	118	112	114	130	58
Contig N50	189870	248295	247812	542359	233954	301752	206020	203426	196369	329376	77402
BioSample accession no.	SAMN03800026	SAMN04909723	SAMN04909724	SAMN04909725	SAMN04909726	SAMN04909727	SAMN04909728	SAMN04909729	SAMN04909730	SAMN04909731	SAMN03800020
WGS SRA accession no.	SRR2541651	SRR3458441	SRR3458442	SRR3458443	SRR3458444	SRR3458445	SRR3458446	SRR3458447	SRR3458448	SRR3458449	SRR2541606
WGS GenBank accession no.	LOBC00000000	LXFL00000000	LXFM00000000	LXFN00000000	LXFO00000000	LXFP00000000	LXFQ00000000	LXFR00000000	LXFS00000000	LXFT00000000	LOBB00000000

*Novel MLST sequence type.
AT, allelic type; MLST, multi-locus sequence typing; WGS, whole genome sequencing; SRA, Sequence Read Archive.

the *rpoB* gene, which encodes the β-subunit of RNA polymerase. Strain FSL W8-0169^T has *rpoB* allelic type (AT) 61, which was also identified for 12 other *B. cereus* group dairy-associated strains deposited in the Food Microbe Tracker database (http://www.foodmicrobetracker.com). Additionally, two closely related ATs (AT 410 and AT 417) were found in the database, representing 12 and two strains, respectively. Nine strains representing *rpoB* AT 61, one strain representing *rpoB* AT 417 and one strain representing *rpoB* AT 194 were characterized in detail in this study (Table 1). Phenotypic, phylogenetic and whole genome sequence (WGS) data failed to classify these 11 strains into existing *B. cereus* group species. These 11 strains are presented as representing a novel species within the *B. cereus* group, for which the name *Bacillus wiedmannii* sp. nov. is proposed. Strain FSL W8-0169^T is the type strain of *B. wiedmannii* sp. nov.

Phylogenetic analyses

Sequences of the 1471 bp 16S rRNA gene (Fig. 1) and a 632 bp internal fragment of the *rpoB* gene (Fig. S1, available in the online Supplementary Material; Miller *et al.*, 2015a) were aligned using the MUSCLE algorithm; pairwise distance matrices were calculated in MEGA version 6.06 (Tamura *et al.*, 2013). Maximum-likelihood phylogenetic trees were reconstructed using RaxML version 8.2.3 (Stamatakis, 2014) under the Generalized Time Reversible (GTR) model with gamma and invariant site parameters (GTRGAMMAI) and 1000 bootstrap repetitions. The 16S rRNA gene sequence of *B. wiedmannii* sp. nov. strain FSL W8-0169^T was checked for the presence of chimeras using DECIPHER (Wright *et al.*, 2012) and submitted to GenBank (accession number listed in Table S1). The 16S rRNA gene sequence phylogeny (Fig. 1) supports the close relatedness of *B. wiedmannii* sp. nov. with existing members of the *B. cereus* group, as indicated by the $\geq$98.2 % sequence similarity and high bootstrap values. It is known that *B. cereus* group species cannot be delineated based on 16S rRNA gene sequences (Liu *et al.*, 2015), and therefore an *rpoB* gene phylogeny was reconstructed to allow for a more discriminatory analysis.

Based on the *rpoB* gene sequence, the 11 *B. wiedmannii* sp. nov. strains characterized formed a monophyletic, well-supported (bootstrap value of 97) cluster within the *B. cereus* group (Fig. S1). The *rpoB* gene sequences of all 11 *B. wiedmannii* sp. nov. strains were deposited in the Food Microbe Tracker database where they can be found under strain name records.

Genomes of FSL W8-0169^T and 10 other characterized *B. wiedmannii* sp. nov. isolates were sequenced on an Illumina MiSeq or HiSeq platform, respectively. The Nextera XT adapters were trimmed from 250 or 100 bp paired-end reads with Trimmomatic version 0.32, respectively (Bolger *et al.*, 2014). Quality of reads was assessed using FastQC version 0.11.2. Sequences were assembled *de novo* using SPAdes version 3.0.0 or 3.6.2, respectively (Bankevich *et al.*, 2012). Draft genome quality was

verified by QUAST version 3.2 (Gurevich *et al.*, 2013) and sufficient coverage was confirmed using SAMtools version 1.2 (Li *et al.*, 2009). Short reads were submitted to the Sequence Read Archive (SRA) and draft genome assemblies to NCBI GenBank and Prokaryotic Genome Annotation Pipeline (Angiuoli *et al.*, 2008). Assembled draft genomes ranged from 5.3 to 5.6 Mbp (Table 1). The NCBI accession numbers for WGS and draft genome assembly statistics are listed in Table 1.

Whole genome sequences of FSL W8-0169^T and the other 10 strains representative of *B. wiedmannii* sp. nov. were queried against the *B. cereus* PubMLST database (http://pubmlst.org/bcereus/). Two (*gmk* 136, *pta* 234) new ATs and a novel sequence type ST-1081 were identified for FSL W8-0169^T. New ATs were identified also for strains FSL M7-0044 (*ilv* 267, *pur* 236), FSL M7-0044 and FSL H8-0032 (*pyc* 193) and FSL K6-0069 (*glp* 224, *gmk* 135, *ilv* 237, *pta* 233).

Four novel STs were identified as a result of finding a novel AT and four novel STs were identified as a result of new combinations of existing ATs (Table 1). Strains FSL J3-0113 and FSL P2-0558 carried STs that were previously observed in the PubMLST database.

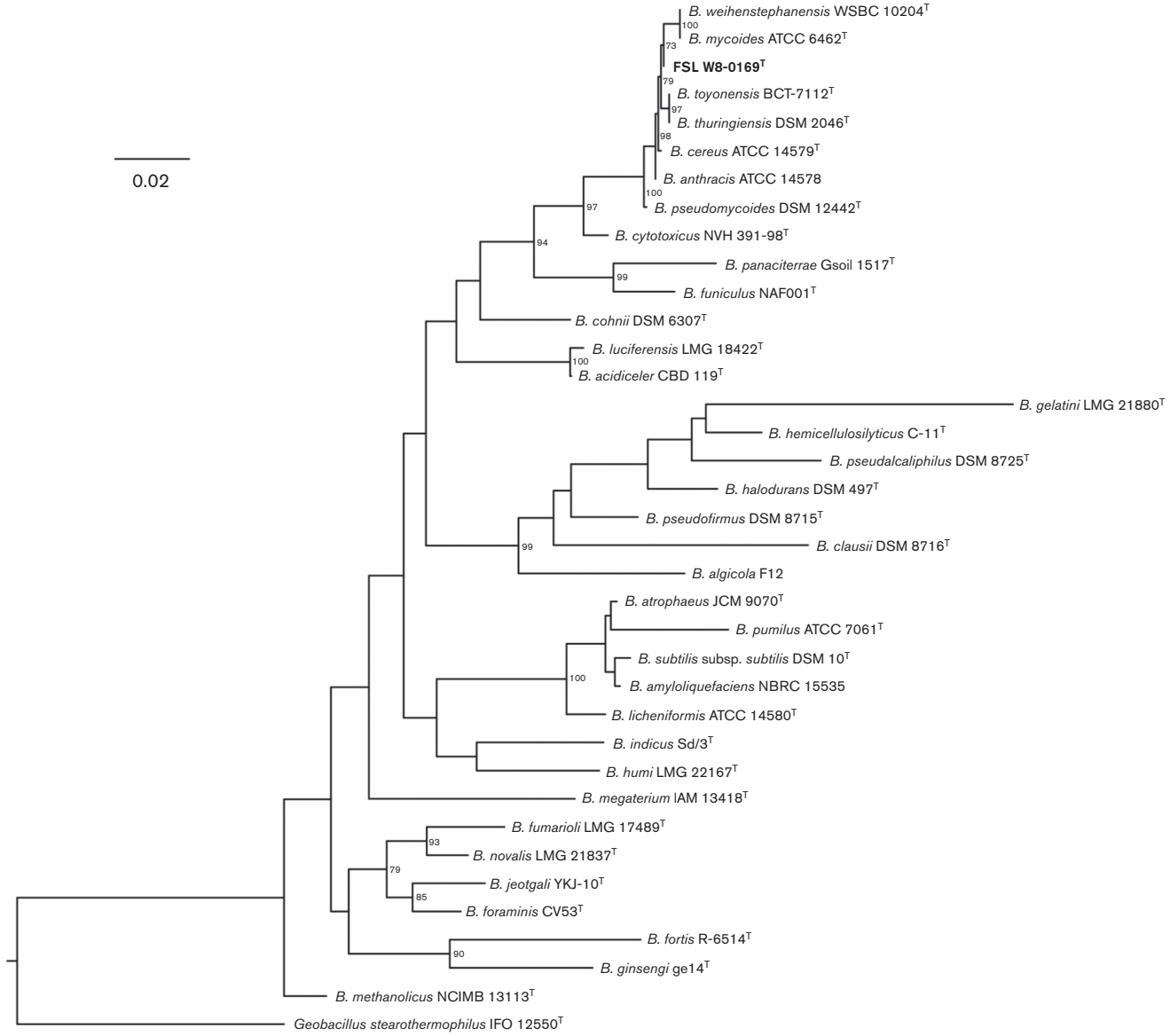

Fig. 1. Maximum-likelihood tree reconstructed in RaxML based on 1471 bp 16S rRNA gene sequences using the generalized time reversible evolutionary model (GTR) with gamma distributed and invariant sites, rooted using *Geobacillus stearothermophilus* IFO 12550^T as an outgroup. Bootstrap values above $\geq$70 are displayed on branches. Bar, 0.02 substitutions per site. The *B. wiedmannii* sp. nov. type strain FSL W8-0169^T is indicated in bold type.

Pairwise average nucleotide BLAST identities (ANIb) between the genomes for the 11 *B. wiedmannii* sp. nov. strains, representative strains of the other eight validly published and three effectively published *B. cereus* group species were calculated (Fig. 2; Richter & Rosselló-Móra, 2009). The representative strains of other *B. cereus* group species were type strains, except in the case of *B. anthracis* where the WGS was not available for the type strain; therefore, the WGS data for the widely used *B. anthracis* strain Ames were used. ANIb was computed using the calculate_ani.py program, which is available on Github (https://github.com/widdow-quinn/scripts/blob/master/bioinformatics/calculate_ani.py). A cladogram (Fig. 2) was built based on the pairwise ANIb similarity matrix using the 'hclust' package in R (R Core Team, 2014). Using a 95 % cut-off for species delineation, these analyses confirmed *B. wiedmannii* sp. nov. as a novel species (Richter & Rosselló-Móra, 2009). The *B. cereus* group species closest to *B. wiedmannii* sp. nov. FSL W8-0169^T is *B. anthracis* (strain Ames with an ANIb of 93.62); the species most distant is *B. cytotoxicus* (strain NVH 391-98^T with an ANIb of 82.73). The minimum pairwise ANIb value between *B. wiedmannii* sp. nov. type strain FSL W8-0169^T and 10 other strains representative of this species was 95.92 %, confidently classifying them in the same species. The ANIb values of the effectively, but not validly, published *B. cereus* group species (i.e. '*B. manliponensis*', '*B. bingmayongensis*', '*B. gaemokensis*') support their recognition as species. Resolving the taxonomic status of these proposed new species would help in minimizing the occurrence of species misclassification of organisms in the *B. cereus* group.

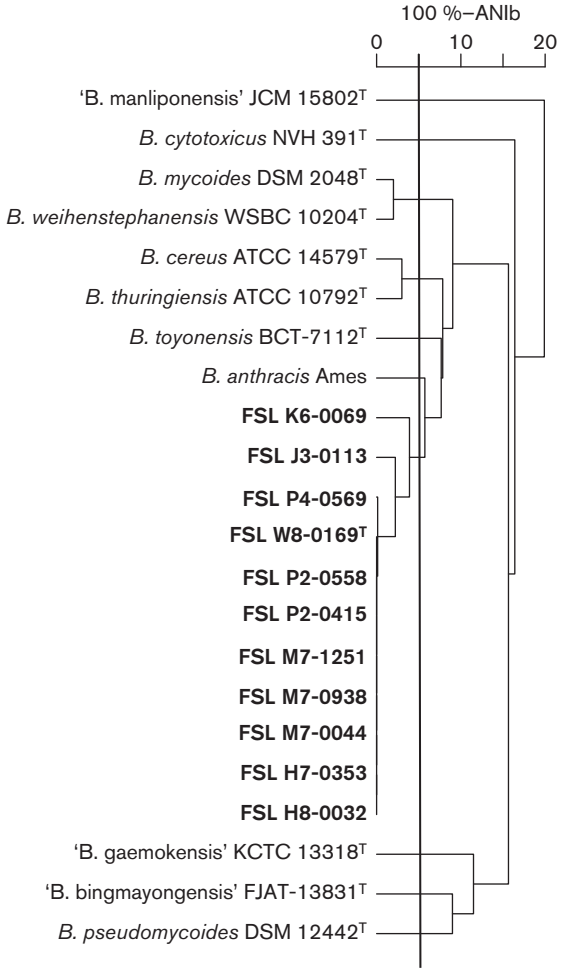

Fig. 2. UPGMA cladogram built based on ANIb values in R. The horizontal rule denotes 100 %−ANIb. The vertical line denotes 100–95 %-ANIb, which correlates to 70 % traditional DNA–DNA hybridization, set as a species cut-off (Chan *et al.*, 2012). The 11 representative strains of *B. wiedmannii* sp. nov. are shown in bold type.

ANIb results were confirmed by *in silico* DNA–DNA hybridization (DDH) conducted using the Genome-to-Genome Distance Calculator (GGDC; http://ggdc.dsmz.de/distcalc2.php; Meier-Kolthoff *et al.*, 2013). Predicted pairwise DDH values between *B. wiedmannii* sp. nov. FSL W8-0169^T and representative strains of other *B. cereus* group species were substantially below the species cut-off of 70 % (Table S2; Richter & Rosselló-Móra, 2009), with the exception of FSL K6-0069, which was classified as a borderline member of *B. wiedmannii* sp. nov., as it had a predicted DDH of 57.9 %, and with a 45.24 % probability of DDH being 70 % or above (Table S2). This strain was deposited with the DSMZ (DSM 102051) and BCCM (LMG 29270).

To further confirm *B. wiedmannii* sp. nov. as a novel taxon, the core genome single nucleotide polymorphisms (SNPs) were identified with kSNP v2 (Gardner & Hall, 2013) using a kmer size of 21 as identified by Kchooser and used to reconstruct a maximum-likelihood phylogenetic tree (Fig. 3). The *B. wiedmannii* sp. nov. type strain FSL W8-0169^T and 10 other representatives of this species formed a monophyletic, robust clade with a bootstrap value of 99. Overall, comparative ANIb and DDH analyses, as well as WGS phylogenetic analysis provide strong evidence in favour of recognizing *B. wiedmannii* sp. nov. as a novel species in the *B. cereus* group.

To examine whether strains belonging to *B. wiedmannii* sp. nov. were found in previous studies, we have classified the 11 *B. wiedmannii* sp. nov. strains into previously defined phylogenetic groups (Guinebretière *et al.*, 2010). We extracted *panC* gene sequences from the WGS and used them for phylogenetic classification with an online tool (Guinebretière *et al.*, 2010; https://www.tools.symprevius.org/bcereus/english.php). All 11 strains were affiliated to phylogenetic group II, which is known for psychrotolerance (Guinebretière *et al.*, 2008) and cytotoxicity (Guinebretière *et al.*, 2010). The combination of these two characteristics sets phylogenetic group II apart from other phylogenetic groups within the *B. cereus* group. This, together with genomic evidence, supports the novel species delineation.

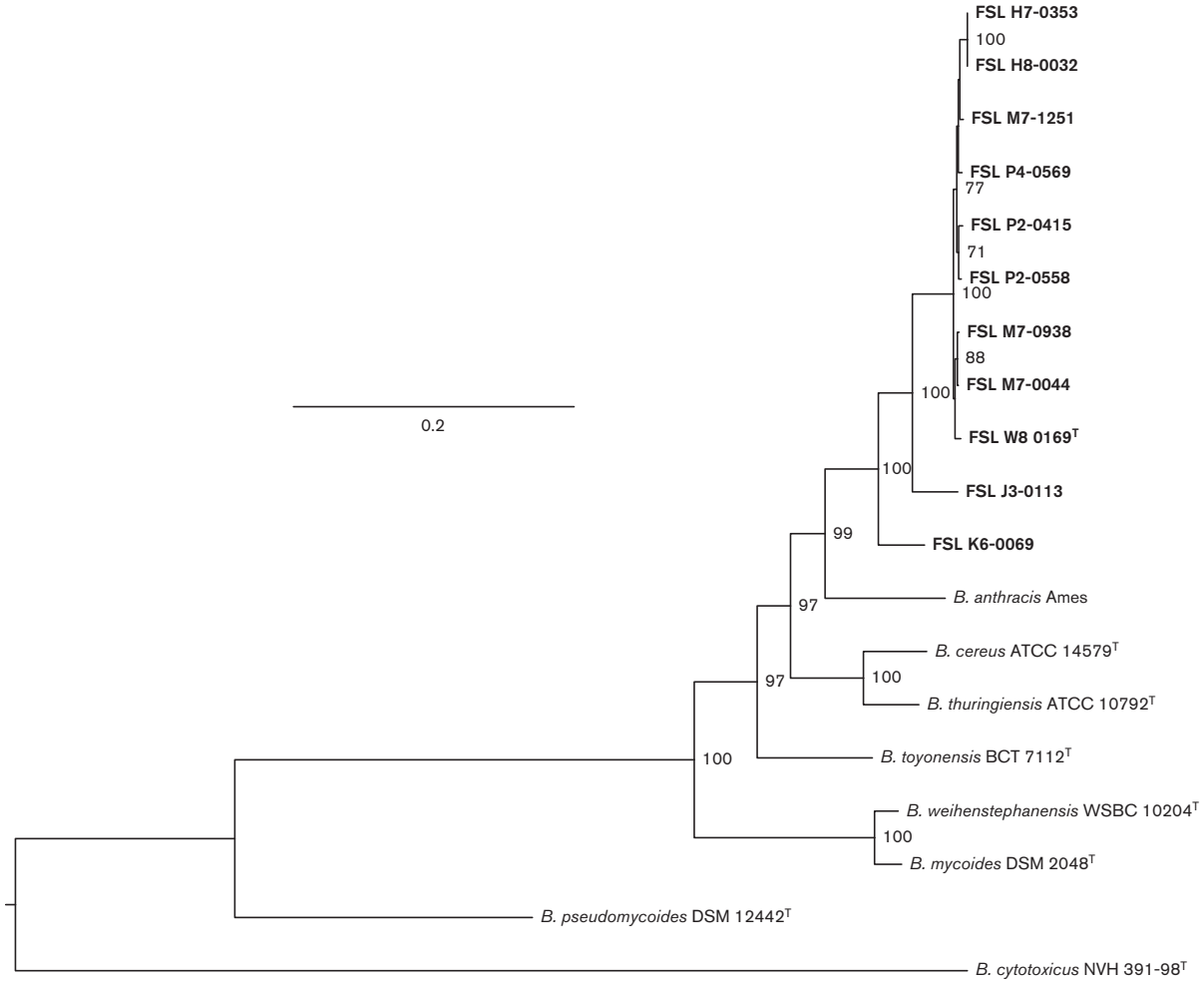

Fig. 3. Maximum-likelihood tree reconstructed in RaxML based on core SNPs using the GTR model with gamma distributed sites and 1000 bootstrap repetitions, rooted by mid-point. Bootstrap values above ≥70 are displayed on branches. Bar, 0.2 substitutions per site. The representative strains of *B. wiedmannii* sp. nov. are shown in bold type.

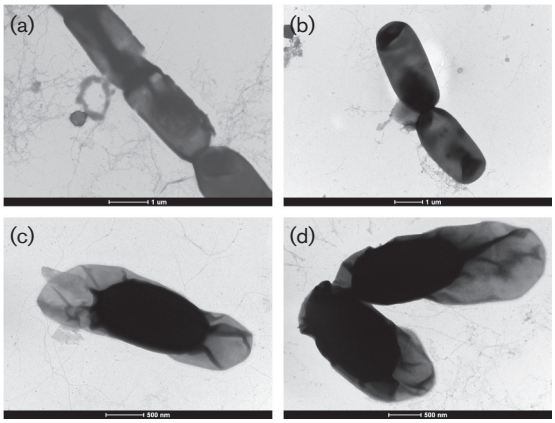

Fig. 4. Transmission electron microscopy (negative staining with 2 % uranyl acetate) images of *B. wiedmannii* sp. nov. FSL W8-0169^T. Panels (a) and (b) show vegetative cells, while (c) and (d) show spores with exosporium. Bars, 1 μm (a, b); 500 nm (c, d).

Phenotypic characteristics

Phenotypic characterizations were performed for *B. wiedmannii* sp. nov. FSL W8-0169^T and the 10 additional *B. wiedmannii* sp. nov. strains (Table S3). Microscopic evaluation of FSL W8-0169^T revealed cells were rod-shaped with an average length of 2.8 μm and average width of 1.2 μm. Transmission electron microscopy (negative staining with 2 % uranyl acetate) images revealed spores in the centre of the vegetative cell (Fig. 4). Colonies grown on brain heart infusion medium (BHI, Becton Dickinson) were large, round and off-white. Colonies grown on Bacara (bioMérieux) plating medium were orange, with an off-white precipitate surrounding colonies, indicative of lecithinase activity. All tested *B. wiedmannii* sp. nov. strains were positive for phosphoinositide phospholipase C based on blue pigmentation on BC/BT agar (R & F Products) plates after 24 h of incubation at 35 °C.

Table 2. Characteristics of the *B. cereus* group strains from this study and previously published studies

Species: 1, *B. wiedmannii* sp. nov. FSL W8-0169^T (*n*=11 strains); 2, *B. cytotoxicus* NVH 391-98^T (*n*=5 strains, data from Guinbretière *et al.*, 2013); 3, *B. pseudomycoides* DSM 12442^T (*n*=7 strains, data from Guinbretière *et al.*, 2013); 4, *B. cereus* ATCC 14579^T (*n*=13 strains, data from Guinbretière *et al.*, 2013 and this study); 5, *B. thuringiensis* CIP 53137^T (*n*=8 strains; data from Guinbretière *et al.*, 2013); 6, *B. weihenstephanensis* WSBC 10204^T (*n*=5 strains, data from Guinbretière *et al.*, 2013); 7, *B. mycoides* CIP 102472^T (*n*=3 strains data from Guinbretière *et al.*, 2013); 8, *B. anthracis* NCTC 10340 (*n*=37 strains; data from Logan *et al.*, 1985 and Guinbretière *et al.*, 2013); 9, *B. toyonensis* BCT-7112^T (data from Jiménez *et al.*, 2013). All species are catalase-positive, Gram-positive rods. All species/strains were positive for acid production from utilization of D-glucose, D-fructose, aesculin and maltose and were negative for acid production from utilization of erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, melibiose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate and 5-ketogluconate. All strains were negative for lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide production, urease, tryptophan deaminase and indole production, as confirmed by API 20E strips. +, Positive result; –, negative result; V, variable trait (i.e. some strains gave a positive reaction while others gave a negative reaction); + W, weak positive result. Data are given for the type strains, with the proportion of positive reactions for all tested strains of the same species in parentheses.

Characteristic	Species								
	1	2	3	4	5	6	7	8	9
Cell diameter >1.0 μm	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+ W	+	+	+	+	+	+	+
Motile	+ (91)	+	+	+	+	+	+	–	+
Rhizoid colony	–	–	+	–	–	–	+	–	–
Starch hydrolysis	+	–	–	+ (67)	+	+	+	+ (97)	+
Egg yolk lecithinase	+	+ W	+ W	+	+	+	+	+	+
Nitrate reduction	+ (73)	+	+	+ (81)	+	+	+	+	+
Arginine dihydrolase	–	+	+ (71)	+ (70)	+ (67)	+ (40)	– (36)	–	+
Citrate	+ (55)	– (33)	– (11)	+ (34)	+ (33)	–	– (60)	–	+
Gelatin	+	+	+ (86)	+	+	+ (80)	+ (67)	+ (70)	+
Voges–Proskauer reaction	+	+ W	+	+	+	+ (60)	+ (67)	+	+
Glycerol	–	–	+ W (17)	– (90)	– (92)	+ W (33)	– (96)	–	–
Ribose	+	+ (67)	+	+	– (81)	+ W	+ W (67)	+	+
Galactose	–	–	–	–	– (38)	–	– (32)	–	–
D-Mannose	–	+	–	– (18)	+ (25)	– (67)	–	–	–
Methyl α-D-glucoside	–	–	–	–	–	– (33)	– (12)	– (3)	+
N-Acetylglucosamine	+	+	+	+	+ (69)	+	+	+	+
Amygdalin	– (36)	+ W (67)	–	+ W (23)	–	+ W (83)	– (50)	–	+ W
Arbutin	+	+	– (33)	+ (82)	+ (63)	+	+ (84)	V (32)	+
Salicin	+	+	+ (67)	+ (64)	+ (75)	+	+ (80)	-	+
Cellobiose	+	+	– (33)	+ (55)	+ (63)	+ w (83)	+ w (75)	–	–
Trehalose	+	–	+	+	+	+	+	+	+
Glycogen	+	–	+	+ (73)	+ (88)	+ (67)	+	+ (92)	+
Maltose	+	+	+	+	+	+	+	+	+
Sucrose	–	–	– (33)	+ (64)	+ (38)	– (33)	+ (50)	+	+
Gentiobiose	–	–	–	– (9)	–	– (33)	–	–	–
Potassium gluconate	– (18)	–	–	– (36)	–	–	– (12)	– (3)	–
Minimum growth temperature (°C)	10 (5–10)	20	10 (8–10)	10 (7–15)	10 (7–15)	5 (5–7)	5 (5–7)	>10	10–45
Maximum growth temperature (°C)	40 (40–43)	50	40 (40–43)	45 (40–45)	45 (40–45)	37 (35–37)	37 (35–37)	<50	45

B. wiedmannii sp. nov. strain FSL W8-0169^T and the 10 additional strains were haemolytic on sheep's blood trypticase soy agar, were Gram-stain-positive and were positive for catalase activity, as determined according to the FDA BAM protocols (U.S. Food and Drug Administration, 2015). All strains were oxidase-negative (BBL Dry Slide Oxidase; Becton Dickinson). All strains hydrolysed starch and casein, as determined by plating on starch agar and skimmed milk agar using standard methods (Logan & De Vos, 2009). All strains were facultative anaerobes, as

Table 3. Cellular fatty acid composition of *B. wiedmannii* sp. nov. FSL W8-0169^T and the type strains of other *B. cereus* group species

Strains: 1, *B. wiedmannii* sp. nov. FSL W8-0169^T; 2, *B. mycoides* KCTC 3453^T; 3, *B. thuringiensis* KCTC 3452^T; 4, *B. weihenstephanensis* KCTC 3975^T; 5, *B. cereus* KCTC 3624^T; 6, *B. pseudomycoides* KCTC 3862^T; 7, *B. cytotoxicus* NVH 391-98^T; 8, *B. toyonensis* BCT-7112^T. Data represent the per cent of the total fatty acids as determined by the Microbial Identification System software. T, Trace amount detected; ND, not detected; NR, not reported. Data for strain 1 are from this study, for strains 2–6 from Jung *et al.* (2011), for strain 7 from Guinebrètiere *et al.* (2013) and for strain 8 from Jiménez *et al.* (2013).

Fatty acid	1	2	3	4	5	6	7	8
iso-$C_{12:0}$	0.8	T	T	1.6	T	6.4	0.4	ND
$C_{12:0}$	0.2	T	T	T	T	3.5	0.6	ND
iso-$C_{13:0}$	6.9	11.8	11.2	16.6	13.9	10.6	7.0	7.1
anteiso-$C_{13:0}$	1.0	T	1.1	1.7	1.7	4.9	1.8	ND
iso-$C_{14:0}$	5.1	2.8	5.1	4.7	4.3	2.4	5.0	2.3
$C_{14:0}$	3.3	3.2	3.6	4.5	3.1	3.4	2.4	3.2
iso-$C_{15:0}$	27.6	33.5	31.3	29.2	33.2	33.2	36.5	38.6
anteiso-$C_{15:0}$	4.0	2.1	3.4	2.7	4	1.9	10.8	3.1
$C_{15:0}$	ND	ND	T	ND	ND	1.1	0.1	ND
$C_{16:1}\omega 7c$ alcohol	1.9	2.1	T	1.3	T	ND	ND	ND
iso-$C_{16:0}$	9.1	5.5	4.5	5.8	4.2	6.8	6.7	5.1
$C_{16:1}\omega 11c$	1.1	2.9	ND	1.2	ND	ND	ND	ND
$C_{16:0}$	7.3	5.3	3.1	7.7	3.1	8.2	10.8	5.6
$C_{15:0}$ 2-OH	0.4	T	T	ND	1.1	ND	NR	ND
iso-$C_{17:1}\omega 10c$	4.7	10.6	2	5.7	2.6	ND	NR	5.8
iso-$C_{17:1}\omega 5c$	2.6	2	4.2	2.1	5.3	ND	NR	4.9
anteiso $C_{17:1}$ A	0.5	ND	ND	ND	T	ND	0.1	ND
iso-$C_{17:0}$	10.1	6.8	13.7	7.6	5.9	8.9	8.2	11.4
anteiso $C_{17:0}$	1.5	T	ND	T	1.5	T	3.4	NR
$C_{18:0}$	0.5	ND	ND	ND	ND	2.6	0.5	NR

determined by the BAM method (U.S. Food and Drug Administration, 2015). All tested strains were motile at 30 °C, with the exception of one strain (FSL H7-0353), as determined by observing growth outside of the inoculation stab on motility agar.

The range of growth temperatures was assessed by plating overnight cultures onto BHI agar (Becton Dickinson) plates and subsequently incubating plates at 5, 10, 15, 20, 25, 30, 40, 45 and 55 °C, for incubation periods as defined by Logan & De Vos (2009). The minimum growth temperature for five out of 11 *B. wiedmannii* sp. nov. strains was 5 °C and all strains grew between 10 and 40 °C. Six out of 11 strains were able to grow at 43 °C. None of the strains grew at 45 °C or above. Sodium tolerance was assessed by inoculating strains into tryptic soy broth (TSB) containing 2, 5, 7, 8, 9 or 10 % (w/v) NaCl, followed by incubation at 30 °C for up to 14 days. Growth of seven out of 11 tested strains was observed at NaCl concentrations up to 5 % (w/v) (for four strains growth was inhibited at concentrations >2 % NaCl), but all strains were inhibited by NaCl concentrations of 7–10 % (w/v). Growth in pH-adjusted TSB incubated at 30 °C for 14 days demonstrated that all strains were capable of growing at pH 5–10.

API CH 50 kits were used to characterize acid production from catabolism of carbohydrates according to the manufacturer's instructions (bioMérieux); incubation was performed at 30 °C for 48 h (Table 2). Of the 11 strains tested, all were able to utilize (acid production from catabolism) D-ribose, D-glucose, D-fructose, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, trehalose, amidon and glycogen. Acid production from D-mannose (positive for one out of 11 strains), amygdalin (positive for three out of 11 strains), potassium gluconate (positive for two out of 11 strains) and *N*-acetylglucosamine (positive for 10 out of 11 strains) was variable. All 11 *B. wiedmannii* sp. nov. strains were also tested with API 20E kits, which were used as per the manufacturer's instructions; incubation was performed at 30 °C for 48 h (see Table 2 for results). Strain FSL W8-0169^T and the 10 additional strains were positive for acetoin production [Voges-Proskauer (VP) reaction] and gelatinase activity. Utilization of citrate as a carbon source was variable (six out of 11 strains were able to utilize citrate). All other reactions included in the API 20E kit were negative (Table 2). Nitrate reduction was determined according to the FDA BAM method (U.S. Food and Drug Administration, 2015) and was variable. Strain FSL W8-0169^T and seven out of the other 10 strains were able to reduce nitrate

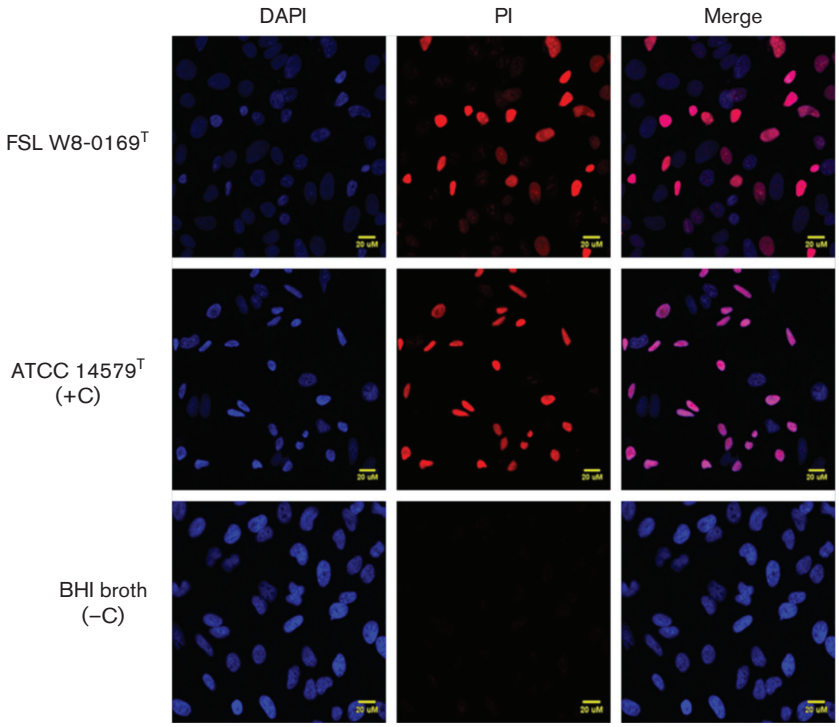

Fig. 5. Cytotoxicity of *B. wiedmannii* sp. nov. FSL W8-0169^T supernatants. HeLa cells grown on coverslips were co-incubated with 5 % (v/v) culture supernatants of FSL W8-0169^T and *B. cereus* ATCC 14579^T (included as a positive control), for 30min with 5 μg PI ml^{-1} (red). After fixation with 4 % PFA, HeLa cells were stained with 1 μg DAPI ml^{-1} (blue). All cells stained blue with DAPI, while only cells with compromised membrane integrity stained red. Incubation with BHI media was included as a negative control. The merged images demonstrate the proportion of cells with damaged membrane due to cytotoxic activity of bacterial supernatant.

to nitrite, while three strains (FSL H8-0032, FSL K6-0069 and FSL H7-0353) were not able to reduce nitrate.

Fatty acid methyl ester extraction and analysis were performed according to the MIDI protocol by MIDI Labs. The content of iso-$C_{13:0}$ in *B. wiedmannii* sp. nov. FSL W8-0169^T was comparable to that in *B. cytotoxicus* and *B. toyonensis*, but was substantially lower than in other *B. cereus* group species (Table 3). The content of iso-$C_{16:0}$, which proved to be the most discriminatory, was higher in *B. wiedmannii* sp. nov. compared to all other validly published *B. cereus* group species. The peptidoglycan type was determined by DSMZ as described previously (Schumman, 2011). *meso*-Diaminopimelic acid was identified as the diagnostic diamino acid for the peptidoglycan type A1γ in *B. wiedmannii* sp. nov. FSL W8-0169^T.

The presence of detectable levels of haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) in cultures grown in BHI for 20 h at 32 °C was evaluated using Duopath Cereus Enterotoxins kits (Merck Millipore). Both HBL and NHE were detected for FSL W8-0169^T and seven additional strains. For three strains (FSL P4-0569, FSL K6-0069 and FSL J3-0113), HBL was not detected, but NHE was. Cytotoxicity was determined by measuring the influx of propidium iodide (PI) into HeLa cells exposed to supernatants (5 %, v/v) of *B. wiedmannii* sp. nov. cultures grown in BHI at 32 °C for 20 h. *B. wiedmannii* sp. nov. supernatants were added to HeLa cells maintained in Eagle's minimal essential medium (EMEM) containing 5 μg PI ml^{-1} and incubated for 30 min at 37 °C with 5 % CO_2. HeLa cells were fixed with 4 % paraformaldehyde (PFA) at room temperature for 10 min. Cells were permeabilized with 1 % Triton X-100 and incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg ml^{-1}) at room temperature for 1–5 min, prior to fixing coverslips and gluing onto microscope slides. Slides were imaged using a Zeiss 710 confocal microscope (Bio-imaging Facility, Cornell University). Images were processed using FIJI software (Schindelin *et al.*, 2012). Bacterial strains were considered cytotoxic if the proportion of PI-positive cells was greater than the average proportion of PI-positive cells for the BHI-negative control. FSL W8-0169^T and all additional strains were cytotoxic (Fig. 5).

Description of *Bacillus wiedmannii* sp. nov.

Bacillus wiedmannii (wied.mann'i.i; N.L. gen. masc. n. *wiedmannii* named in honour of Martin Wiedmann, for his contribution to the understanding of the biology of *Bacillus*).

Cells are Gram-stain-positive rods with an average length of 2.8 μm and average width of 1.2 μm. Endospores are ellipsoidal and are present in the centre of vegetative cells; cells have a non-swollen sporangia. Colonies are positive for egg-yolk lecithinase, with the ability to hydrolyse casein and starch. Facultative anaerobe. Colonies grown on BHI agar at 37 °C for 24 h appear cream-coloured, round and flat, with a rough surface. Growth temperature ranges from 5 and 43 °C, with optimum growth between 20 and 40 °C. Most strains are motile at 30 °C. Citrate utilization and acid production from potassium gluconate and mannose are variable. Can be differentiated from other strains in the *B. cereus* group by the combination of an inability to produce acid from fermentation of sucrose and inability to hydrolyse arginine (arginine dihydrolase-negative). Produces toxins HBL and the non-haemolytic toxin NHE. Cells are cytotoxic in a HeLa cell culture model. Strains can be differentiated from other *B. cereus* group species by *panC* gene sequence comparisons, ANIb and WGS.

The type strain, FSL W8-0169^{T} (=DSM 102050^{T}=LMG 29269^{T}), was isolated from a silo raw milk sample collected from a dairy powder processing plant in the north-eastern USA. The genomic DNA G+C content of the type strain is 35.3 mol%, as determined by genome sequencing.

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