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Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparisons of the species of the group by means of ANI calculations

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ABSTRACT

Strain BCT-7112^T was isolated in 1966 in Japan from a survey designed to obtain naturally occurring microorganisms as pure cultures in the laboratory for use as probiotics in animal nutrition. This strain, which was primarily identified as *Bacillus cereus* var *toyoi*, has been in use for more than 30 years as the active ingredient of the preparation TOYOCERIN®, an additive for use in animal nutrition (e.g. swine, poultry, cattle, rabbits and aquaculture). Despite the fact that the strain was initially classified as *B. cereus*, it showed significant genomic differences from the type strains of the *B. cereus* group that were large enough (ANI values below 92%) to allow it to be considered as a different species within the group. The polyphasic taxonomic study presented here provides sufficient discriminative parameters to classify BCT-7112^T as a new species for which the name *Bacillus toyonensis* sp. nov. is proposed, with BCT-7112^T (=CECT 876^T; =NCIMB 14858^T) being designated as the type strain. In addition, a pairwise comparison between the available genomes of the whole *B. cereus* group by means of average nucleotide identity (ANI) calculations indicated that besides the eight classified species (including *B. toyonensis*), additional genomospecies could be detected, and most of them also had ANI values below 94%. ANI values were on the borderline of a species definition only in the cases of representatives of *B. cereus* versus *B. thuringiensis*, and *B. mycoides* and *B. weihenstephanensis*.

Introduction

The "*Bacillus cereus* group" of Gram-positive, spore-forming bacteria forms a homogeneous independent branch within the *Bacillus* genus and comprises seven closely related species: *B. cereus* [9,35], *B. thuringiensis* [3,35], *B. anthracis* [5,35], *B. mycoides* [7,35], *B. pseudomycoides* [27], *B. weihenstephanensis* [20], and *B. cytotoxicus* [11]. Some of the species of this group (i.e. *B. cereus*, *B. thuringiensis*, *B. anthracis* and *B. mycoides*) were classified about one century ago, much earlier than the discovery of DNA as hereditable material. At that time, the classification of bacteria was generally made on the basis of their habitats, pathogenicity for mammals or insects, and their morphological and physiological characteristics [1,29]. The large extent of synteny among their chromosomes and other genomic similarities led some scientists to propose that these four species could represent a single taxon [29]. The species of the *B. cereus* group are soil-dwelling saprophytes that are of medical and economic importance because of their pathogenicity in humans (e.g. *B. cereus* and *B. anthracis*) or insects (e.g. *B. thuringiensis*, which is used industrially as a biological pesticide and considered harmless to humans [40]). Other species, such as *B. mycoides*, *B. weihenstephanensis* and *B. pseudomycoides*, are considered harmless given

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their hitherto unknown pathogenic nature, despite the fact that some, such as *B. cereus*, may carry similar enterotoxin genes [13,40].

Besides the relevance of members of the genus *Bacillus* as pathogens and for agricultural applications, a large number of strains that have important economic relevance are used as probiotics [14], since these products have been shown to prevent gastrointestinal disorders and infections following extensive use in the swine, bovine, rabbit, poultry and aquaculture industries [14,44]. Among the different spore-forming probiotic strains, *B. cereus* var. *toyoi* (strain BCT-7112^T) has been in use since 1975 when it was officially approved by the Japanese Ministry of Agriculture and Forestry as the commercial preparation TOYOCERIN® (for an extended historical report see the supplementary text). The spores of BCT-7112^T have been used in animal nutrition for swine, poultry, cattle, rabbits and aquaculture for over thirty years in a wide range of countries around the world. In the European Community, TOYOCERIN® was authorized for the first time by the European Commission in 1994 for use in swine, and it became the first microorganism authorized as a feed additive in the European Union, and subsequently it was authorized also for use in poultry, cattle and rabbits. The initial identification studies assigned this strain to the species *B. cereus* [18]. However, given that a series of phenotypic characters can differentiate BCT-7112^T from the main phenotype of the species, it was considered to be a variant and was named *B. cereus* var. *toyoi* [18]. During the years of industrial production, the strain has been subjected to numerous studies in order to prove its non-toxigenic nature (e.g. [41,44]), as well as molecular identification approaches for differentiating BCT-7112^T from food poisoning strains [17,25,28]. Actually, unpublished results on DNA-DNA hybridization have indicated that this strain might not belong to the species *B. cereus* after all, but represents a different species of the group. These results were recently reinforced by means of infrared spectroscopy [25].

The present work was conducted in order to ascertain whether strain BCT-7112^T represented a new species within the *B. cereus* group. The taxonomic study was performed as a polyphasic approach in which the phenotype and genetic traits were extensively studied following the minimal standard recommendations published by Logan et al. [21]. The almost complete genome sequence of the strain was used for studies of pairwise genome comparisons. Altogether, the results indicated that strain BCT-7112^T did represent a new species for which the name *B. toyonensis* sp. nov. is proposed, with the type strain being BCT-7112^T (=CECT 876^T; =NCIMB 14858^T).

Materials and methods

Strains and growth media

Strain BCT-7112^T (=CECT 876^T; =NCIMB 14858^T) was obtained from the collection of Rubinum S.A. (current owner of the strain) and was derived from the original stocks obtained from the Japanese company Toyo Jozo Co. Ltd. (former owner of the strain). For the taxonomic work, the type strains of the following species were used: *B. cereus* (CECT 148^T), *B. thuringiensis* (CECT 197^T), *B. mycoides* (CECT 4128^T), *B. pseudomycoides* (CECT 7065^T), and *B. weihenstephanensis* (LMG 18989^T). For standard growth conditions, in order to obtain biomass for the analyses, the Luria Broth (LB) medium was used (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl).

DNA extraction and pulsed-field gel electrophoresis (PFGE)

The DNA for amplification and whole sequencing purposes was obtained using a modification of the Marmur procedure, as indicated by Urdiain et al. [42]. PFGE was performed as indicated by Klein [17] using the *Sma* I restriction enzyme with the following modifications: lysis buffer was supplemented with 0.5% sarcosyl, 0.2% deoxycholate, and 0.5% triton-X100; the RNAse concentration was 20 mg mL^{-1}; and lysozyme was 100 mg mL^{-1}. In addition, mutanolysine (10 units mL^{-1}) was included. Incubation with the lysis buffer was overnight and incubation with proteinase K was for 48 h.

Sequence analyses

The 16S rRNA gene sequence of strain BCT-7112^T was initially deposited under the GenBank accession number AJ310100 [28], and was identical to that retrieved from the almost complete genome sequence (see supplementary genome fasta file). 16S rRNA gene sequences of the closest reference strains were retrieved either from the latest update of the LTP database as template [45], or directly from the genome sequences of the reference strains given in Fig. 1. Sequences were aligned using the SINA alignment software implemented in the ARB software package [24]. Tree reconstructions using the 16S rRNA gene sequences were performed using either the maximum likelihood algorithm RAxML version 7.0 with the GTRGAMMA model [38], or the neighbor-joining algorithm using the Jukes-Cantor correction in the ARB software package [24] using all homologous positions common to all members of the domain *Bacteria*.

In addition, the housekeeping genes *adk*, *ccpA*, *glpT*, *pyrE*, *recF*, and *sucC*, used for a multilocus typing scheme for the *B. cereus* group [13], and the gene *rpoB* were initially retrieved from the almost complete genome of strain BCT-7112^T (see supplementary genome fasta file). Each gene was blasted against the nucleotide collection (nr/nt) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/) in order to select the closest sequences of the reference genomes listed in Fig. 2 and supplementary Figs. S2–S8. Nucleotide sequences of each gene dataset were aligned using the program ClustalX version 2.1 [19]. Hypervariable and unalignable positions of each alignment were removed by using the program Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with default conditions [4]. The resulting improved alignments were either used for independent tree reconstructions, or they were concatenated in a single stretch (supplementary Table S1). The phylogenetic reconstructions were performed by using either the RAxML algorithm version 7.0 with the GTRGAMMA model [38] and 100 replicates for bootstrap analyses, or by using the neighbor-joining algorithm with the Jukes-Cantor correction as implemented in the ARB software package [24].

Genome sequencing and assembly, and genome comparisons

Shotgun (SG) and paired-end (PE) strategies with 454 technology were performed at Lifesequencing SL (Valencia, Spain) using an FLX+ sequencing version in order to obtain the almost complete genome sequence. A Pacific Bioscience (PacBio) strategy using short (approximately 600 nts) and long (consensus sequences of up to 10 kb) fragments was used to improve the assembly. De novo assembly was performed by the sequencing company using the Newbler assembler v 2.6. Genome annotation was performed using the Lifesequencing annotation pipeline. Nucleotide sequences of the genomes were used to perform genome alignments using progressiveMauve [6]. Genome comparisons between *B. cereus* var. *toyoi* and these genomes were made by BLASTP homology searches between their respective amino acid sequences. In order to assume homology between open reading frames (ORFs), both sequences should have more than 50% identity extending along at least 75% of both sequences. For the assessment of the presence of plasmidic genes and parasporal crystal proteins, the full nucleotide and

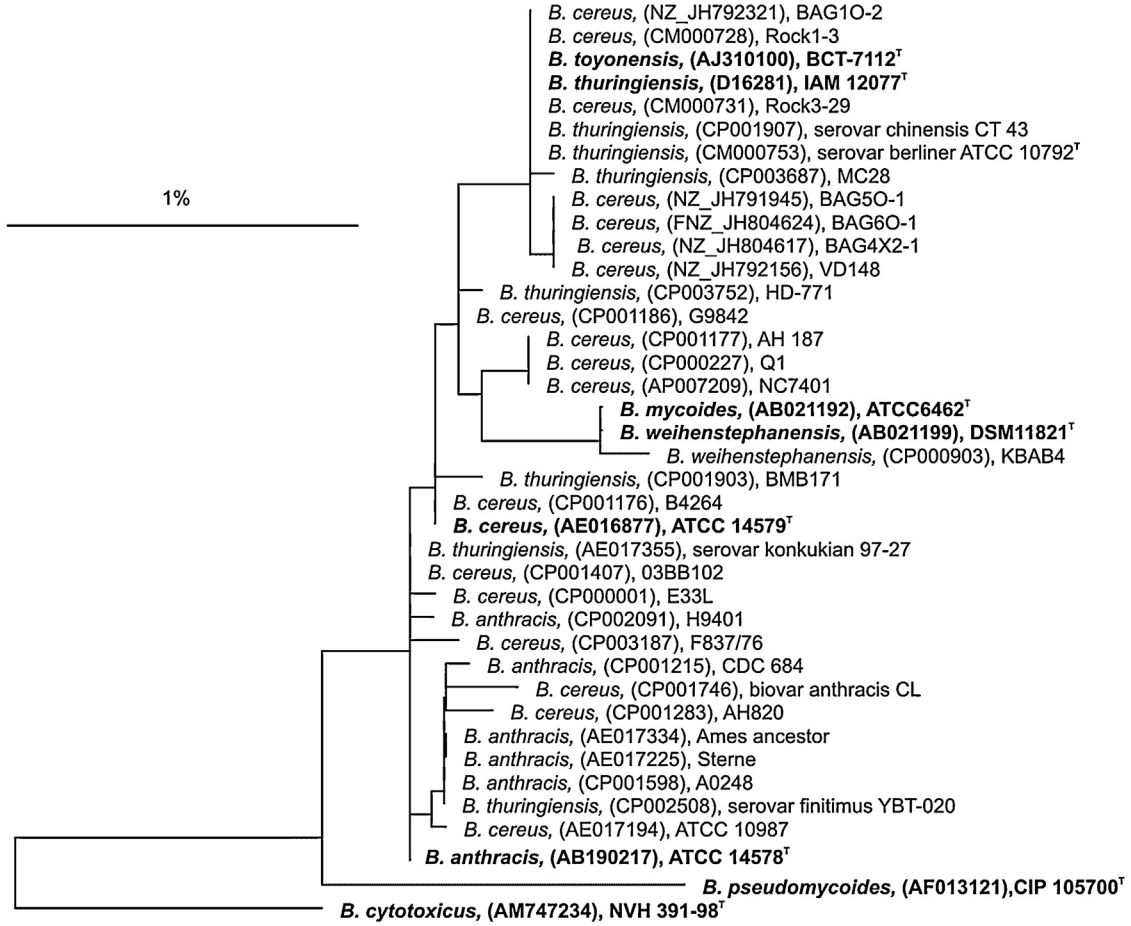

Fig. 1. Phylogenetic reconstruction of the 16S rRNA gene sequences of the *B. cereus* group based on the RAxML algorithm version 7.0 with the GTRGAMMA model [37] and no filter applied. The bar indicates 1% sequence divergence. The type strain sequences are those present in the LTP database [44], and the non-type strain sequences are those retrieved from their respective genome sequences. Accession numbers are indicated in brackets. Bold labeled sequences indicate those that correspond to their type strains obtained from the LTP database.

amino acid sequences for plasmids of *B. thuringiensis* strain MC28 were downloaded (GenBank CP003691.1, CP003692.1, CP003693.1, CP003694.1, CP003689.1, CP003688.1 and CP003690.1).

Average nucleotide identity (ANI) calculations

Reference genomes for comparison purposes were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Sequences were uploaded into the JSpecies software package (http://www.imedea.uib.es/jspecies) in order to perform pairwise genome calculations of the average nucleotide identity (ANI) using the default conditions [30].

Phenotypic analyses

The phenotypic analyses were performed following the minimal standards for describing new taxa of aerobic, endospore-forming bacteria recommended by Logan et al. [21]. The strains were characterized phenotypically using API 20E and API 50CH strips according to Logan and Berkeley [22]. Vegetative cells and spores were harvested at 24 and 48 h, and observed by phase-contrast microscopy. Bacterial cultures were examined by scanning electron microscopy as indicated in the supplementary materials. Catalase, oxidase, starch hydrolysis, DNAse and spore staining [2], as well as parasporal crystal staining [34], were performed as previously indicated. The egg-yolk reaction was performed using Baird Parker agar base covered with a sterile egg-yolk emulsion (Sharlau). For temperature (between 5 and 50 °C, with 5 °C intervals), salinity (between 0 and 7%, with 1% intervals) and pH ranges (between 3 and 11, with 0.5 intervals) cells were cultured in LB, and growth was followed by spectrophotometrically determining values for an optical density of 600 nm.

Chemotaxonomic analysis

The peptidoglycan diamino acid analysis was carried out as described by Schleifer [31], and the extraction of fatty acids and their analysis were performed as described by Kämpfer and Kroppenstedt [16]. Strains were grown under identical conditions (on TS agar at 28 °C for 48 h) and the cells for extractions were taken from colonies of the same size. Fatty acids were identified with Sherlock version 2.11, TSBA40 Rev. 4.1. MALDI-TOF analyses were performed as indicated in the supplementary materials.

Results and discussion

Taxonomic studies on BCT-7112^T

Phylogenetic reconstruction based on 16S rRNA gene sequences

Given the previous studies on the probiotic strain indicating that it could represent a different species within the *B. cereus* group [18], a polyphasic approach was performed in order to clarify its taxonomic status. In the first instance, a phylogenetic reconstruction was generated using the 16S rRNA gene sequence of

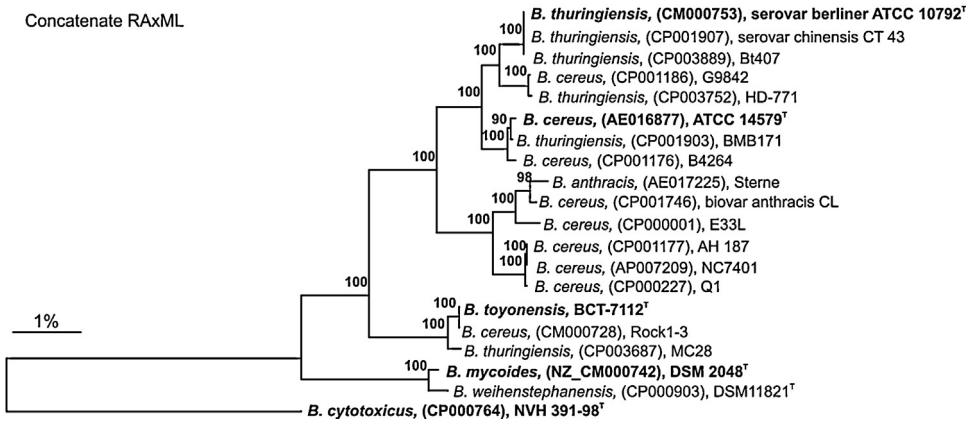

Fig. 2. Tree reconstruction based on a concatenate of the *adk*, *ccpA*, *glpT*, *pyrE*, *recF*, *rpoB* and *sucC* genes previously filtered using the program Gblocks [4] in order to remove hypervariable positions and indels. The reconstruction was based on the RAxML algorithm [37], and the bootstrap values were calculated using 100 replicates. The bar indicates 1% sequence divergence. The position of the type strains with available sequences are highlighted in black.

BCT-7112^T, those of the closest relative type strains of the group, and the closely related strains for which a full or draft genome was publicly available. As seen in Fig. 1, almost all sequences of the *B. cereus* group (except those of the outliers *B. pseudomycoides* and *B. cytotoxicus*) shared identities greater than 99.5%, and the sequence of BCT-7112^T was 99.9% similar to that of the *B. thuringiensis* type strain and other members that also seemed to be closely related. However, it is generally known that the 16S rRNA gene may lack resolution between highly related organisms [8]. At first sight, the reconstruction could have led to the conclusion that BCT-7112^T was in fact a member of the species *B. thuringiensis* rather than *B. cereus* sensu stricto, as originally identified. However, the remaining taxonomic analyses performed (see below) clearly indicated that this strain was not a member of either species of the group. The genome comparisons between strain BCT-7112^T and the closest relative type strains by means of PFGE profiling (Fig. S1) indicated that the fingerprints generated did not match any of the analyzed type strain genomes, and it was clearly different from that of *B. thuringiensis* CECT 197^T.

ANI calculations

In order to shed light on the genomic differences between BCT-7112^T and its closest relatives, the almost complete genome sequence was obtained by pyrosequencing, and it was compared with publicly available genomes. The most relevant comparative parameter for ascertaining the identity of a strain is the calculation of the ANI [30]. In this regard, all genomes present in the database belonging to the *B. cereus* group were compared and the results are shown in Table 1 and supplementary Table S2. Using ANI, the species delimitation borders can be set to about 94–96% identity, which would generally represent approximately 70% DNA–DNA hybridization [30]. As a result, all the genomes analyzed could be grouped into nine ANI groups, which were those represented by type strains of the species *B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. cytotoxicus*, strain KBAB4 of *B. weihenstephanensis* (with high identity to the type strain of the species that is still unsequenced), and four additional groups represented by strains BCT-7112^T, *B. cereus* G9842, *B. cereus* E33L, and *B. cereus* AH187 (the genomes of the latter strains were used as a reference for the comparisons). Despite the fact that the genome sequence of the *B. anthracis* type strain is not available, this species may belong to the group represented by E33L. In all cases, the BCT-7112^T genome shared identities with the reference type strains (and selected strains) of the ANI group of less than 92% (Table 1), which was a value far below the threshold of 94–96% identity that would serve as a boundary for species circumscription [30]. These results indicated that BCT-7112^T represented a different genomospecies from those currently classified within the group. The genome sequence of the remaining species *B. pseudomycoides* is not publicly available. However, given the low 16S rRNA identity in comparison with *B. cereus* and *B. thuringiensis*, its independence was guaranteed. On the other hand, additional genomes were found to have high ANI scores when compared with the genome of BCT-7112^T. In this regard, the genomes of the named strains *B. cereus* Rock1-3, Rock3-28, Rock3-29, BAG1O-2, BAG6O-1, BAG4X2-1, BAG5O-1, VD148, VD115, and *B. thuringiensis* MC28 (Table 1) shared ANI values ranging from 99.9% to 95.8%, which are values that would undoubtedly group them all within the same species. However, none of the listed strains, except MC28, are currently available in the public culture repositories or are published. Only strain *B. thuringiensis* MC28 has some literature available, since it is a strain that was isolated from a virgin forest in China's Sichuan province, and one of its main characteristics is the production of parasporal crystal during the stationary phase [10]. However, no additional information was available for these strains, and efforts to obtain them have to date been unsuccessful, but it would still be interesting to analyze the phenotype of these organisms in the future.

Multilocus sequence analysis (MLSA)

Despite the ANI values undoubtedly indicating that BCT-7112^T represented a new genomospecies within the *B. cereus* group, the phylogenetic reconstruction based on 16S rRNA was of very low resolution. In this context, an MLSA was performed using the gene set *adk*, *ccpA*, *glpT*, *pyrE*, *recF*, and *sucC* previously used for the *B. cereus* group [13], as well as the *rpoB* gene. Once the gene sequences were retrieved from the genomes an MLSA approach was followed, since it has been recommended as an alternative for taxonomic studies [36]. For the analysis, and in order to obtain balanced representatives of each genomic group shown in Table 1, three strains from each group were selected (whenever possible). The genes were analyzed independently (supplementary Figs. S2–S8), or as a single concatenate using either the maximum likelihood (Fig. 2) or neighbor-joining algorithms (Fig. S9). All the trees agreed in their topologies except for that of *pyrE*, which showed a different branching order (Fig. S5). The good congruence between the single-gene reconstructions and the concatenate reinforced the stability of the genealogy observed. The concatenate rendered a sequence stretch of approximately 8208 homologous sites with 1127 informative positions. The bootstrap values shown in Fig. 2 indicated a very stable branching order and mirrored the results observed with the ANI analyses. BCT-7112^T, together with Rock1-3 and MC28, formed an independent branch within the *B. cereus*

Table 1
Genomes used in this study and pairwise ANI values. The genomes are ordered in relation to the different genomic groups observed that could be understood as different genomospecies when ANI ≥ 96%. The results within each frame indicate the genomic groups observed that clearly could be understood as different species. Different genomovars (ANI between 94 and 96%) have been framed within unique species.

Genomes				ANI values (percentage identity) against the target genomes						
Species (names given in the database)	Strain	Accession number	BCT-7112^T	ATCC 10792^T	ATCC 1457 ^{T}	G9842	E33L	AH187	DSM 204 ^{T}	NVH 391–9 ^{T}
B. toyonensis	BCT-7112^T	See supplementary genome fasta file	–	90.35	90.95	90.87	89.61	90.00	89.53	81.1
B. cereus	Rock1-3	CM000728	**99.87**	91.21	91.47	91.45	90.59	90.59	89.51	81.42
B. cereus	BAG1O-2	NZ_JH792321	**99.57**	91.04	91.21	91.22	90.37	90.39	89.46	81.07
B. cereus	BAG6O-1	NZ_JH804624	**99.48**	91.28	91.38	91.49	90.58	90.64	89.36	81.41
B. cereus	Rock3-29	CM000731	**99.36**	91.17	91.4	91.49	90.6	90.6	89.41	81.39
B. cereus	VD148	NZ_JH792156	**99**	90.87	90.99	91.02	90.05	90.2	89.36	80.86
B. cereus	BAG4X2-1	NZ_JH804617	**98.72**	90.97	90.9	91.12	90.15	90.23	89.36	80.98
B. cereus	BAG5O-1	NZ_JH791945	**98.7**	90.78	90.85	91.05	90.08	90.19	89.46	80.87
B. thuringiensis	MC28	CP003687	**98.58**	91.11	91.43	91.33	90.58	90.59	89.56	81.42
B. cereus	Rock3-28	CM000730	**98.45**	91.08	91.29	91.29	90.53	90.6	89.61	81.29
B. cereus	VD115	NZ_JH792165	**95.79**	89.86	89.82	90.03	89.21	89.35	82.69	79.99
B. thuringiensis	Serovar berliner ATCC 10792^T	CM000753	91.73	–	96.68	96.25	91.08	91.31	89.64	81.3
B. thuringiensis	Bt407	CP003889	91.76	**99.67**	96.66	96.35	91.07	91.33	89.97	81.32
B. thuringiensis	Serovar chinensis CT 43	CP001907	91.76	**99.53**	96.65	96.28	91.07	91.3	89.97	81.3
B. cereus	ATCC 14579^T	NC_004722	91.57	96.41	–	95.78	91.2	91.44	89.77	81.33
B. cereus	B4264	CP001176	91.71	96.34	**98.11**	95.88	91.23	91.48	89.77	81.3
B. thuringiensis	BMB171	CP001903	91.71	96.33	**98.96**	95.77	91.26	91.46	89.87	81.38
B. cereus	G9842	CP001186	91.71	96.13	95.81	–	91.03	91.18	90.36	81.29
B. thuringiensis	HD-771	CP003752	91.75	96.05	95.85	**98.77**	91.04	91.21	90.56	81.32
B. cereus	E33L	CP000001	90.88	91	91.46	91.16	–	94.41	86.33	81.36
B. anthracis	Sterne	AE017225	91	91.05	91.53	91.24	**97.15**	94.36	86.14	81.39
B. thuringiensis	Al Hakam	CP000485	90.99	91.13	91.6	91.39	**96.78**	94.31	86.33	81.43
B. cereus	Biovar anthracis CI	CP001746	90.99	91.05	91.6	91.25	**96.89**	94.21	86.14	81.46
B. anthracis	Ames	AE016879	90.99	91.05	91.52	91.25	**97.15**	94.36	86.14	81.39
B. thuringiensis	Serovar konkukian 97 27	AE017355	90.98	91.08	91.55	91.31	**96.92**	94.28	86.14	81.41
B. anthracis	CDC 684	CP001215	90.98	90.94	91.51	91.18	**97.02**	94.26	86.14	81.42
B. cereus	F837/76	CP003187	90.97	91.09	91.56	91.33	**96.73**	94.24	86.33	81.45
B. anthracis	H9401	CP002091	90.97	90.94	91.52	91.18	**97.02**	94.26	85.94	81.42
B. anthracis	Ames Ancestor	AE017334	90.97	90.94	91.51	91.18	**97.02**	94.25	86.14	81.42
B. anthracis	A0248	CP001598	90.97	90.94	91.51	91.18	**97.02**	94.25	86.14	81.42
B. cereus	AH820	CP001283	90.94	91	91.49	91.29	**97.06**	94.33	86.04	81.44
B. cereus	03BB102	CP001407	90.94	90.95	91.48	91.2	**96.54**	94.22	86.23	81.39
B. cereus	AH187 (F4810/72)	CP001177	90.85	90.98	91.65	91.14	94.93	–	85.74	81.43
B. cereus	Q1	CP000227	90.85	91.08	91.71	91.24	94.95	**98.64**	85.74	81.46
B. cereus	NC7401	AP007209	90.81	91.03	91.65	91.17	94.92	**99.94**	85.74	81.45
B. thuringiensis	Serovar finitimus YBT 020	CP002508	90.78	90.84	91.5	91.16	94.71	**96.08**	86.04	81.44
B. cereus	ATCC 10987	AE017194	90.74	90.95	91.32	91.17	94.12	**95.9**	85.45	81.57
B. cereus	FRI-35	CP003747	90.86	91.07	91.52	91.32	94.24	**95.97**	85.45	81.5
B. weihenstephanensis	KBAB4	CP000903	90.45	89.1	89.33	89.39	89.09	89.27	96.96	81.63
B. mycoides	DSM 2048^T	NZ_CM000742	89.53	89.64	89.77	90.36	86.33	85.45	–	74.88
B. cytotoxicus	NVH 391-98^T	CP000764	80.89	80.47	80.92	80.88	80.82	80.98	74.88	–

Figures in bold denote strains belonging to the same genomospecies.

group and was clearly distant from the core species of the group (i.e. *B. cereus*, *B. thuringiensis* and the additional unclassified groups, with that represented by E33L probably being *B. anthracis*). These results reinforced the observation that BCT-7112^T represented a new genomospecies of the group.

Genomic properties

In the present work, sequencing of the whole genome of BCT-7112^T was almost completed (see supplementary fasta file). A total of 540,354 SG reads and 550,423 PE reads were obtained with mean read lengths of 608.75 and 281.53 nts, respectively. Also, 35,766 short-length fragment sequences and 107,202 long-length fragment sequences from PacBio were obtained with mean read lengths of 250 and 2300 nts, respectively. The stretch sequenced could be assembled into a total of 106 long contigs (>500 nts length) and ordered in seven scaffolds, with the longest sequence being 2,895,231 nucleotides and the shortest 194 nucleotides, which resulted in an estimated genome size of 5.8 Mb. The closest full-sequenced published genome by means of ANI values has been that of strain *B. thuringiensis* MC28 [10], which shared an ANI value of 98.5% (Table 1). The complete stretch of MC28 was comprised of 5,414,494 bp, whereas the almost full sequence of BCT-7112^T was 5,033,500 bp. The closure of the BCT-genome is ongoing and the main genome particularities will be published at a later date. However, the stretch sequenced is almost the complete genome of BCT-7112^T, since the hitherto available genomes of the group are in the same range between 5.1 and 5.8 Mb [33]. When aligning both DNA stretches, strain BCT-7112^T aligned with an MC28 homologous stretch of 4,566,281 bp that would correspond to 90.7% MC28 coverage. The alignment of both genomes showed a high degree of

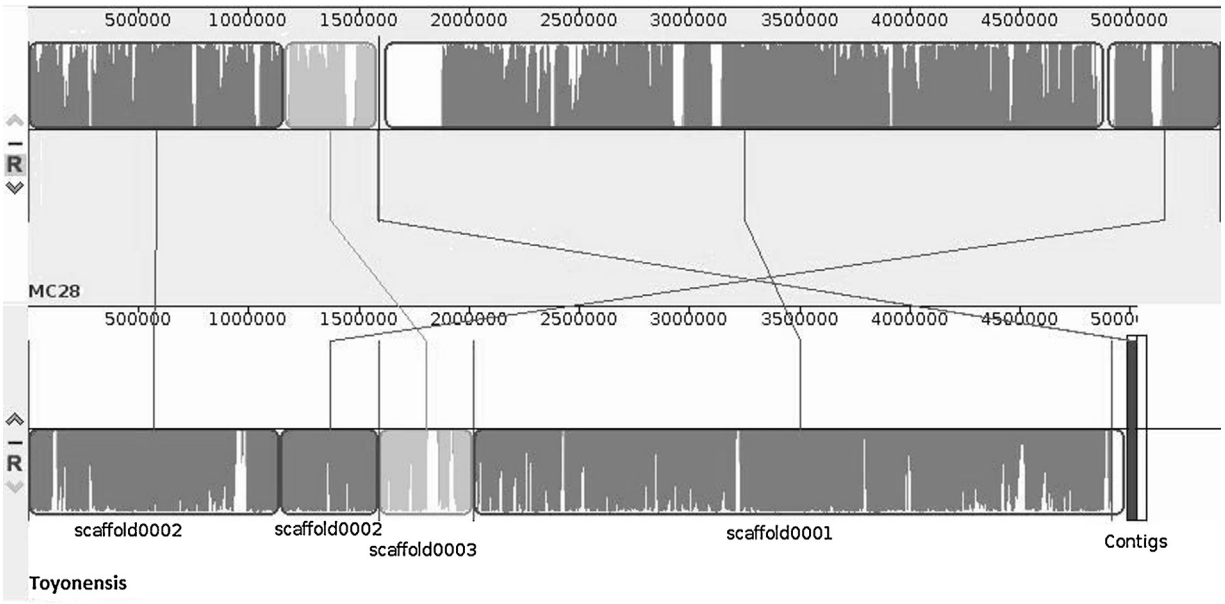

Fig. 3. Genome alignment between the complete genome of strain MC28 and the scaffolds 1, 2 and 3 of BCT-7112^T and MC28, as computed by progressiveMauve software, showing extensive synteny between the two genomes. Filled boxes indicate homology between genomic segments. Blank parts within the boxes show zones with no homology, which are most likely genomic islands.

synteny of 90.2% (Fig. 3). Only two scaffolds (scaffold0004 and scaffold005) could be found that did not show any homology to MC28, and we hypothesize that they may correspond to plasmids since they contained many genes of putative plasmidic origin. Finally, among the relevant taxonomic particularities of such a pairwise comparison, it is worth mentioning that MC28 coded for eleven *cry* genes in three of the plasmids coding for insecticidal crystals [10] that may be responsible for the identification of this strain as *B. thuringiensis*. Strain BCT-7112^T did not show any homologous ORF in the sequenced stretch, which is in agreement with the absence of parasporal crystal observed with the different microscopy staining procedures used.

Phenotypic properties

Cells of BCT-7112^T in the exponential phase showed rod morphology of 3.0–4.4 μm in length and >1 μm in width (Fig. S10). Cells were all endospore-forming, and sporulation occurred in late exponential growth where the spores appeared in a centered or subterminal position within the cell. No parasporal crystal could be observed by any staining procedure or in any electron micrograph, in accordance with the inability to detect any homologous sequence of the *cry* genes observed for the closest relative strain MC28 [10]. Colonies were cereus white but not rhizoidal. Cells were motile, catalase and oxidase positive according to the affiliation with the genus *Bacillus*. Among the metabolic properties analyzed (Table 2), BCT-7112^T could be distinguished from the closest relative type strains by means of several diagnostic properties, such as an optimum growth temperature of 35 °C (despite the growth range between 10 and 45 °C being common to the closest related species), higher salinity tolerance of up to 5% NaCl, and an optimal pH of 6.5. Moreover, diagnostic characters were anaerobic growth, the ability to grow without tryptophan, the presence of arginine dihydrolase, the utilization of citrate, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, D-sucrose, D-trehalose, starch, glycogen and D-turanose, as well as the negative results for glycerol, D-mannose and cellobiose.

Chemotaxonomic properties

In order to understand the chemical differences between the type strains studied, a MALDI-TOF profile analysis of whole cell extracts was initially performed, as previously indicated [26]. The profiles of the three strains BCT-7112^T, *B. cereus* (CECT 148^T) and *B. thuringiensis* (CECT 197^T) were the most closely related in comparison with *B. mycoides* (CECT 4128^T) and *B. pseudomycoides* (CECT 7065^T) (Fig. S11). However, and in accordance with the ANI values observed in Table 1, *B. cereus* (CECT 148^T) and *B. thuringiensis* (CECT 197^T) resembled each other to a much higher degree than BCT-7112^T. The distinct profile patterns observed reinforced the fact that BCT-7112^T could constitute a different species within the *B. cereus* group. The peptidoglycan diamino acid was *meso*-diaminopimelic acid, which has already been described for species of this group, such as *B. cereus*, *B. mycoides* and *B. thuringiensis* [32] and *B. cytotoxicus* [11]. Major fatty acids detected were $C_{15:0}$iso, $C_{17:0}$iso and$C_{13:0}$iso, in addition to other branched fatty acids such as $C_{16:0}$iso, $C_{15:0}$anteiso, as well as unsaturated fatty acids such as $C_{17:1}\omega10c$ and $C_{17:1}\omega5c$. The fatty acid profiles of all type strains of the *B. cereus* group studied were rather similar, as already reported by Kämpfer [15]. However, some additional quantitative (and also minor qualitative) differences were detected (Table 3).

All the results of the polyphasic approach indicated that strain BCT-7112^T represented a new species within the genus *Bacillus*, for which the name *B. toyonensis* sp. nov. is proposed, with strain BCT-7112^T (=CECT 876^T; =NCIMB 14858^T) being designated as the type strain of the species.

Description of Bacillus toyonensis sp. nov.

Bacillus toyonensis (to.yo.nen'sis. N.L. masc. adj. toyonensis, arbitrary epithet formed from the name of a company called Toyo Jozo).

Gram-positive, motile, facultatively anaerobic rods forming ellipsoidal central to subterminal spores in non-swollen sporangia. The length of the rods range between 3 and 4 μm, and cells occur singly, in pairs and occasionally in short chains or filaments. Colonies are generally large, flat with entire to undulate edges, and often form swarming rings around the colony extending through the plate. In general, colonies are matt to granulated, with a whitish to cream color. Growth occurs between 10 and 45 °C, and optimum growth occurs at 35 °C. Like other species of the *B. cereus* group, it is egg-yolk lecithinase positive and mannitol negative. Table 2 shows the different diagnostic phenotypic characters of BCT-7112^T with respect to the closest species of the *B. cereus* group

Table 2
Diagnostic phenotypic characters of strain (1) BCT-7112^T with respect to the closest relative type strains of species, (2) *B. cereus* (CECT 148^T), (3) *B. thuringiensis* (CECT 197^T), (4) *B. anthracis* (data from [11]), (5) *B. mycoides* (CECT 4128^T), (6) *B. weihenstephanensis* (LMG 18989^T), (7) *B. pseudomycoides* (CECT 7065^T), and (8) *B. cytotoxicus* (NVH 391-98^T).

	1	2	3	4*	5	6*	7	8*
Size (μm)	3.0–4.0	3.0–5.0#	3.0–5.0#	nr	3.0–5.0#	nr	3.0–5.0#	nr
Egg-yolk lecithinase	+	+	+	+	+	+	+w	+w
Anaerobic growth	+	+	+	–	+	–	+	+w
Rhizoid colony	–	–	–	–	+	–	+	–
Parasporal crystal	–	–	+	–	–	–	–	–
Growth without tryptophan	+	+	+	+	+	+	+	–
Growth temperature range (°C)	10–45	10–45	10–45	>10–<50	15–40	5–37	10–40	20–50
Optimal growth temperature (°C)	35	30 (37#)	30	nr	30	nr	30	nr
Salinity tolerance range (% NaCl)	0–5	0–4	0–4	nr	0–4	nr	0–2.5	nr
Optimal growth salinity (% NaCl)	0	0	0	nr	1	nr	1	nr
pH tolerance range	5–9.5	5–9.5	5–9.5	nr	5–9.5	nr	5–9.5	nr
Optimal pH	6.5	6	7	nr	8	nr	8	nr
API 20E								
Arginine dihydrolase	+	+	+	+	–	+	+	–
Citrate utilization	+	+	+	–	–	–	–	–
Acetoin production (VP)	+	+	+	+	+	+	+	+w
Gelatinase	+	+	+	d	+	+	+	+
API 50CH								
Glycerol	–	–	–	–	–	+w	+w(–)	–
D-Ribose	+	+	–(+w)	+	+w	+w	+	+
D-Mannose	–	–	+	–	–	–	–	+
Methyl-αD-glucopyranoside	+	–	–	–	–	–	–	–
Amygdalin	+w	+w	–	–	–	+w	–	+w
Arbutin	+	+	+	d	+	+	–	+
Salicin	+	+	+	–	+	+	+	+
Cellobiose	–	+	+	–	+w	+w	–	+
D-Saccharose (sucrose)	+	+	+	+	+	–	–	–
D-Trehalose	+	+	+	+	+	+	+	–
Starch	+	+	+	+	+	+	+	–
Glycogen	+	+	+	+	+	+	+	–
D-Turanose	+	–	–	–	–	–	–	–

In brackets discrepant values of the analyses with the literature; +w, weakly positive; d, 11–89% positive in [11] for *B. anthracis* strains; nr, not reported in the literature. All strains showed a cell diameter > 1 μm. All strains gave positive results for cytochrome oxidase and catalase. All strains gave positive results in the API 20E test for nitrate reduction. All strains gave positive results in the API 50CH test for N-acetylglucosamine, D-glucose, D-fructose, esculin, D-maltose, and negative results for gentiobiose, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, ß-methylxyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-manitol, D-sorbitol, α-methyl-D-mannoside, D-melibiose, inulin, melezitose, D-raffinose, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, gluconate, D-lactose, D-galactose. All strains gave negative results in the API 20E test for lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophan desaminase, indole production and nitrogen production.

Results obtained from [23].

* Results obtained from [11].

Table 3
Cellular fatty acid profiles of strain BCT-7112^T and other species of the *Bacillus cereus* group. Strains: 1, BCT-7112^T; 2, *B. cereus* CECT 148^T; 3, *B. thuringiensis* CECT 197^T; 4, *B. mycoides* CECT 4128^T; 5, *B. pseudomycoides* CECT 7065^T; 6, *B. weihenstephanensis* LMG 18989^T. All data from this study. All strains were cultured on TSA agar at 28 °C for 48 h. Values are percentages of total fatty acids. Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Only fatty acids showing relative amounts >1% are given.

Fatty acid saturated	1	2	3	4	5	6
$C_{14:0}$	3.2	2.9	3.6	3.7	2.6	3.3
$C_{16:0}$	5.6	6.0	5.6	7.1	6.2	10.5
Branched						
iso-$C_{12:0}$					5.2	
iso-$C_{13:0}$	7.1	9.2	8.9	12.3	7.8	12.3
anteiso-$C_{13:0}$		1.2			4.5	
iso-$C_{14:0}$	2.3	4.7	3.9	3.7	5.1	3.9
iso-$C_{15:0}$	38.6	25.4	33.7	15.8	11.2	21.5
anteiso-$C_{15:0}$	3.1	4.5	3.9	3.8	4.7	3.4
iso-$C_{16:0}$	5.1	6.9	5.4	9.6	8.3	6.5
iso-$C_{17:0}$	11.4	10.7	3.6	9.0	11.5	9.8
iso-$C_{17:1}\omega 10c$	5.8	5.6	3.8	16.6		11.7
iso-$C_{17:1}\omega 5c$	4.9	5.7	5.8		6.0	
anteiso-$C_{17:0}$		1.3			2.9	
Hydroxylated						
$C_{15:0}$ 2-OH		1.6	1.0			
Unsaturated						
$C_{16:1}\omega 7$calcohol		1.4		2.9		1.9
$C_{16:1}\omega 11c$				4.7		3.8
Summed feature 2	1.5	2.8	3.0		5.2	
Summed feature 3	11.5	10.2	12.6	10.9	18.8	8.1
Summed feature 4						3.3

Summed feature 2: ($C_{14:0}$ 3-OH/$C_{16:1}$iso I); Summed feature 3: ($C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2OH); Summed feature 4: ($C_{17:1}$ iso I/anteiso B).

(i.e. an optimal growth temperature of 35 °C, a higher salinity tolerance of up to 5% NaCl and an optimal pH of 6.5). In addition, other diagnostic characters are anaerobic growth, the ability to grow without tryptophan, the presence of arginine dihydrolase, the use of methyl-αD-glucopyranoside, D-turanose, citrate, arbutin, salicin, D-sucrose, D-trehalose, starch and glycogen. Glycerol, D-mannose and cellobiose are negative. The major fatty acids are $C_{15:0}$iso, $C_{17:0}$iso and $C_{13:0}$iso, in addition to other branched fatty acids such as $C_{16:0}$iso, $C_{15:0}$anteiso and unsaturated fatty acids, such as $C_{17:1}\omega10c$ and $C_{17:1}\omega5c$. The cell wall peptidoglycan contains meso-diaminopimelic acid. The ANI values calculated with pairwise comparisons between almost full genome sequences to the type strains of the closest relative species are always below 92%. The G + C content is 35.6 mol% for the type strain BCT-7112^T (=CECT 876^T; =NCIMB 14858^T). The 16S rRNA gene entry for the type strain of the species is AJ310100.

Pairwise ANI results between the available genomes of the B. cereus group strains

The assignation of a new strain to one of the different species of the *B. cereus* group classified to date is mainly made based on several simple phenotypic characteristics, such as the synthesis of the parasporal crystal that is generally plasmid coded [20,23]. This fact has led to the situation that differently identified members of the group with distinct species names appear in common genetic clusters (e.g. Table 1, Table S2 and Fig. 2). Such an incoherent distribution of strains in clades that do not match species names has made some authors propose that the species *B. cereus*, *B. thuringiensis* and *B. anthracis* may constitute a single species given their genetic similarities, rather than several paraphyletic taxa [12,33]. This could also be the case for BCT-7112^T and MC28 [10] as *B. toyonensis.* MC28 encodes for the *cry* genes responsible for the synthesis of the parasporal crystal in three of several plasmids, and was originally identified as *B. thuringiensis*. However, BCT-7112^T lacks such genes, and most probably was the reason for its identification as *B. cereus*.

The pairwise genome comparisons (Table 1 and supplementary Table S2) clearly indicated that, among the publicly available genomes, at least nine genomic groups could be observed within the *B. cereus* group. Five of the groups corresponded to already classified species and were represented by the type strains BCT-7112^T (for *B. toyonensis*), ATCC 10792^T (for *B. thuringiensis*), ATCC 14579^T (for *B. cereus*), DSM 2048^T (for *B. mycoides*), and NVH 391-98^T (for *B. cytotoxicus*). A sixth group was represented by strain KBAB4. This genome was used as a reference for *B. weihenstephanensis* given that the genome of the type strain was not available, and both strains appeared to be highly similar [36]. In addition, the results revealed the presence of three additional genomic groups represented by strains G9842, AH187 and E33L (all three were identified as *B. cereus*). The latter would most probably correspond to *B. anthracis* given that the sequenced strains of the species fall within this genomic group, although no type strain genome was available.

Taking into account that ANI values below the boundary of 94–96% correspond to distinct species [30], five major genomospecies could be established (highlighted by a frame in Table 1 and in red in supplementary Table S2): *B. toyonensis* (represented by BCT-7112^T); the *B. cereus–B. thuringiensis* group with three genomovars (represented by ATCC 14579^T, ATCC 10792^T and G9842, respectively, and with inter-genomovar ANI values ranging from 95.8 to 96.7%); the E33L–AH187 group (with inter-genomovar values ranging from 94.1 to 94.9%); the *B. weihenstephanensis–B. mycoides* group (represented by KBAB4 and DSM 2048^T, respectively, with inter-species ANI values of 97%); and *B. cytotoxicus* (represented by NVH 391-98^T). In all cases, the inter-genomospecies values were always below 91.7%. The results obtained here using the program JSpecies gave consistent ANI values, since almost full genome sequences were available in all cases and the amount of aligned stretches for ANI calculation was always above 70%, except for pairwise comparisons with *B. cytotoxicus* that, given the large genetic distance, exhibited aligned stretches of approximately 50–60% (data not shown).

The results here show that the three genomospecies (*B. cereus–B. thuringiensis*; E33L–AH187 and *B. weihenstephanensis–B. mycoides*) harbor genomovars exhibiting ANI values (Table 1 and supplementary Table S2 shadowed in grey) that correspond to the twilight zone where the decision on classifying two different species could be difficult [30]. From the strict genomic point of view, the different genomic groups within each genomospecies could be understood as different genomovars [43]. In the future, should the pairs *B. cereus* and *B. thuringiensis*, and *B. mycoides* and *B. weihenstephanensis* remain independent species, then the classification of the genomovars represented by G9842 and AH187 as new species would be justified from the genomic point of view. However, the decision to combine species into one would depend on the reliability of the phenotypic discriminative traits that give each taxon its own "phenotypic property" [39].

Altogether, the taxonomic structure observed here by means of ANI comparisons and the MLSA with a subset of seven housekeeping genes, appear to be in accordance with previous observations on the phylogenetic divergence based on the overall gene content of the members of the group [46]. In this case, the small clade detected that includes the three strains *B. cereus* Rock1-3, Rock3-28 and Rock3-29, isolated from soil and not associated with human disease [46], would also correspond to the newly classified *B. toyonensis*. Finally, all results point to ancient and stable clade divergences with low putative numbers of genes acquired by horizontal gene transfer given the strong phylogenetic signals observed when comparing the genetic pool of the members of the group [46].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2013.04.008.

References

[1] Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E., Collins, M.D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. 41, 343–346.

[2] Barrow, G.I., Feltham, R.K.A. 1993 Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd ed., Cambridge University Press, Cambridge.

[3] Berliner, E. (1915) Über die Schlaffsucht der Mehlmottenraupe (*Ephestia kühniella* Zell) und ihren Erreger *Bacillus thuringiensis* n sp. Zeitschrift fur angewandte Entomologie Berlin 2, 29–56.

[4] Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.
[5] Cohn, F. (1872) Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen 1, 127–224, 1875 1(Heft2).
[6] Darling, A.E., Mau, B., Perna, N.T. (2010) ProgressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5, e11147.
[7] Flügge, C. 1886 Die Mikroorganismen F.C.W., Vogel, Leipzig.
[8] Fox, G.E., Wisotzkey, J.D., Jurtshuk, P., Jr. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.
[9] Frankland, G.C., Frankland, P.F. (1887) Studies on some new microorganisms obtained from air. Philos. Trans. Roy. Soc. B 178, 257–287.
[10] Guan, P., Ai, P., Dai, X., Zhang, J., Xu, L., Zhu, J., Li, Q., Deng, Q., Li, S., Wang, S., Liu, H., Wang, L., Li, P., Zheng, A. (2012) Complete genome sequence of *Bacillus thuringiensis* serovar *sichuansis* strain MC28. J. Bacteriol. 194, 6975.
[11] Guinebretière, M.-H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M.-L., Lamberret, G., Fagerlund, A., Granum, P.E., Lereclus, D., De Vos, P., Nguyen-The, C., Sorokin, A. (2013) Bacillus cytotoxicus sp nov. is a new thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. Int. J. Syst. Evol. Microbiol. 63, 31–40.
[12] Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolstø, A.B. (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66, 2627–2630.
[13] Helgason, E., Tourasse, N.J., Meisal, R., Caugant, D.A., Kolstø, A.-B. (2004) Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl. Environ. Microbiol. 70, 191–201.
[14] Hong, H.A., Duc, L.H., Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29, 813–835.
[15] Kämpfer, P. (1994) Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* spp. Syst. Appl. Microbiol. 17, 86–98.
[16] Kämpfer, P., Kroppenstedt, R.M. (1996) Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol. 42, 989–1005.
[17] Klein, G. (2011) Molecular characterization of the probiotic strain *Bacillus cereus* var *toyoi* NCIMB 40112 and differentiation from food poisoning strains. J. Food Prot. 74, 1189–1193.
[18] Kozasa, M., Wake, M., Azuma, R. (1977) Taxonomic studies on *Bacillus cereus* T-7112–Biotype. J. Tokyo Vet. Zootechnol. Sci. 25, 38.
[19] Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007) Clustal W and clustal X version 2.0. Bioinformatics 23, 2947–2948.
[20] Lechner, S., Mayr, R., Francis, K.P., Prüss, B.M., Kaplan, T., Wiessner-Gunkel, E., Stewart, G.S.A.B., Scherer, S. (1998) Bacillus weihenstephanensis sp nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Bacteriol. 48, 1373–1382.
[21] Logan, N.A., Berge, O., Bishop, A.H., Busse, H.-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L., Salkinoja-Salonen, M.S., Seldin, L., Ventosa, A. (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. Int. J. Syst. Evol. Microbiol. 59, 2114–2121.
[22] Logan, N.A., Berkeley, R.C.W. (1984) Identification of *Bacillus* strains using the API system. J. Gen. Microbiol. 130, 1871–1882.
[23] Logan, N.A., De Vos, P. (2009) Genus Bacillus Cohn 1872. In: De Vos, P., Garrity, G.M., Jones, D., Frieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, 2nd ed., Springer Dordrecht Heidelberg London, New York, pp. 21–128.
[24] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H. (2004) ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.
[25] Mietke, H., Beer, W., Schleif, J., Schabert, G., Reissbrodt, R. (2010) Differentiation between probiotic and wild-type *Bacillus cereus* isolates by antibiotic susceptibility test and Fourier transform infrared spectroscopy (FT-IR). Int. J. Food Microbiol. 140, 57–60.
[26] Munoz, R., López-López, A., Urdiain, M., Moore, E.R.B., Rosselló-Móra, R. (2011) Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. Syst. Appl. Microbiol. 34, 69–75.
[27] Nakamura, L.K. (1998) Bacillus pseudomycoides sp nov. Int. J. Syst. Bacteriol. 48, 1031–1035.
[28] Pecoraro, S., Bucher, E. (2002) Rapid identification of closely related probiotic *Bacillus cereus* strains and differentiation from wild type bacilli. Arch. Lebensmittelhyg. 53, 49–72.
[29] Rasko, D.A., Altherr, M.R., Han, C.S., Ravel, J. (2005) Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol. Rev. 29, 303–329.
[30] Richter, M., Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U.S.A. 106, 19126–19131.
[31] Schleifer, K.H. (1985) Analysis of the chemical composition and primary structure of murein. Methods Microbiol. 18, 123–156.
[32] Schleifer, K.H., Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36, 407–477.
[33] Schmidt, T.R., Scott II., E.J., Dyer, D.W. (2011) Whole-genome phylogenies of the family *Bacillaceae* and expansion of the sigma factor gene family in the *Bacillus cereus* species-group. BMC Genomics 12, 430.
[34] Sharif, F.A., Alaeddinoglu, N.G. (1988) A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. J. Ind. Microbiol. 3, 227–229.
[35] Skerman, V.B.D., McGowan, V., Sneath, P.H.A. (Eds.), Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30, 225–420.
[36] Sorokin, A., Candelon, B., Guilloux, K., Galleron, N., Wackerow-Kouzova, N., Ehrlich, S.D., Bourget, D., Sanchis, V. (2006) Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and distinct population structure of psychrotrophic strains. Appl. Environ. Microbiol. 72, 1569–1578.
[37] Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden, M.C.J., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A., Whitman, W.B. (2002) Report of the Ad Hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52, 1043–1047.
[38] Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
[39] Tindall, B.J., Rosselló-Móra, R., Busse, H.-J., Ludwig, W., Kämpfer, P. (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int. J. Syst. Evol. Microbiol. 60, 249–266.
[40] Tourasse, N.J., Helgason, E., Økstad, O.A., Hegna, I.K., Kolstø, A.-B. (2006) The *Bacillus cereus* group: novel aspects of population structure and genome dynamics. J. Appl. Microbiol. 101, 579–593.
[41] Trapecar, M., Leouffre, T., Faure, M., Jensen, H.E., Granum, P.E., Cencic, A., Hardy, S.P. (2011) The use of a porcine intestinal cell model system for evaluating the food safety risk of *Bacillus cereus* probiotics and the implications for assessing enterotoxigenicity. APMIS 119, 877–884.
[42] Urdiain, M., López-López, A., Gonzalo, C., Busse, H.-J., Langer, S., Kämpfer, P., Rosselló-Móra, R. (2008) Reclassification of *Rhodobium marinum* and *Rhodobium pfennigii* as *Afifella marina* gen nov. comb. nov. and *Afifella pfennigii* comb. nov., a novel genus of photoheterotrophic alphaproteobacteria and emended descriptions of *Rhodobium*, *Rhodobium orientis* and *Rhodobium gokarnense*. Syst. Appl. Microbiol. 31, 339–351.
[43] Ursing, J., Rosselló-Mora, R., García-Valdés, E., Lalucat, J. (1995) Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. Int. J. Syst. Bacteriol. 45, 604.
[44] Williams, L.D., Burdock, G.A., Jiménez, G., Castillo, M. (2009) Literature review on the safety of Toyocerin®, a non-toxigenic and non-pathogenic *Bacillus cereus* var toyoi preparation. Regul. Toxicol. Pharm. 55, 236–246.
[45] Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.-H., Glöckner, F.O., Rosselló-Móra, R. (2010) Update of the all-species living-tree project based on 16S and 23S rRNA sequence analyses. Syst. Appl. Microbiol. 33, 291–299.
[46] Zwick, M.E., Joseph, S.J., Didelot, X., Chen, P.E., Bishop-Lilly, K.A., Stewart, A.C., Willner, K., Nolan, N., Lentz, S., Thomason, M.K., Shozamannan, S., Mateczun, A.J., Du, L., Read, T.D. (2012) Genomic characterization of the *Bacillus cereus* sensu lato species: Backdrop to the evolution of *Bacillus anthracis*. Gen. Res. 22, 1512–1524.