

1 ***Bacillus clarus* sp. nov. is a new *Bacillus cereus* group species isolated from soil**

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18

19 **Abbreviations:** MLST Multi-Locus Sequence Type; Average Nucleotide Identity BLAST,

20 ANIb; BAM, Bacteriological Analytical Manual; DDH, DNA-DNA hybridization; GTR,

21 generalized time-reversible model; ML, maximum likelihood; MLST, multilocus sequence

22 typing; SNP single nucleotide polymorphism; SRA, Sequence Read Archive; WGS, whole

23 genome sequence.

24

25 GenBank/EMBL/DDBJ accession number for the 16S rRNA of isolate PS00077A^T is

26 MH918154. WGS accession number for the draft genome of isolate PS00077A^T is

27 QVOD000000000. Two supplementary figures and one supplementary table are available with the

28 online version of this paper.

29 **Abstract**

30 *Bacillus cereus* group or *B. cereus* sensu lato (s.l.), is comprised of Gram-positive spore-forming,
31 rod-like bacteria that are widespread in natural environments. Although the species in this group
32 are known to be highly related in terms of phenotypic characteristics, they display different
33 levels of pathogenicity. Biochemical assays are therefore considered to be insufficient for
34 accurate taxonomic classification of *B. cereus* group species. To facilitate accurate taxonomic
35 classification and associated prediction of pathogenic potential, we have conducted comparative
36 genomic analyses of publicly available genome assemblies of *B. cereus* group isolates. Through
37 that, we found that an isolate previously known as *B. mycoides* ATCC 21929 was sufficiently
38 distant from valid and effective type strains to be considered a putative new species. We have
39 conducted biochemical and bioinformatic characterization of strain ATCC 21929 that had been
40 isolated from soil in Papua New Guinea. Strain ATCC 21929 most closely resembles *B.*
41 *paramycoides* NH24A2^T, producing ANIb and DDH values of 86.70% and 34.1%, respectively.
42 Phenotypically, isolate ATCC 21929 does not possess cytochrome c oxidase activity, and is able
43 to grow at a range of temperatures 15°C - 43°C and at a range of pH 6 - 9. With regards to fatty
44 acid composition, this isolate has iso-C17:0 in highest abundance. We propose the strain ATCC
45 21929^T (=PS00077A^T = PS00077B^T = PSU-0922^T = BHP^T) as a new species named *Bacillus*
46 *clarus* sp. nov. to facilitate accurate taxonomic classification of *B. cereus* group isolates.

47 The *Bacillus cereus* group currently comprises 18 valid species, including *B. cereus* sensu stricto
48 (s.s.) [1-3], *B. albus* [4], *B. anthracis* [5], *B. thuringiensis* [6], *B. mycooides* [7], *B. cytotoxicus* [8],
49 *B. luti* [4], *B. mobilis* [4], *B. nitratreducens* [4], *B. pacificus* [4], *B. paramycooides* [4], *B.*
50 *paranthracis* [4], *B.* [4], *B. pseudomycooides* [9], *B. toyonensis* [10], *B. tropicus* [4], *B.*
51 *wiedmannii* [11], and *B. weihenstephanensis* [7]. In addition to these valid species, the group
52 contains effectively published species (i.e., “*B. gaemokensis*” [12], “*B. manliponensis*” [13] and
53 “*B. bingmayongensis*” [14]). Strains from the *B. cereus* group are commonly found as part of the
54 plant and soil microbiome [15]. They can form spores, and they are rod-shaped and facultatively
55 anaerobic [16]. Some *B. cereus* group isolates are important biocontrol agents (e.g., insecticidal
56 *B. thuringiensis* isolates), while others can cause food spoilage or disease in animals or humans
57 (e.g., anthrax, emetic and diarrheal gastrointestinal disease) [1, 15-17]. We have recently
58 developed and published BTyper, a *B. cereus* group whole genome sequence-based
59 comprehensive and rapid genotyping tool [18]. We used BTyper in conjunction with FastANI
60 [19] to investigate genomic diversity of *B. cereus* group isolates for which assembled genomes
61 had been made available through NCBI. We have identified strain BHP with RefSeq assembly
62 accession GCF_000746925.1 as a putative new species based on average nucleotide identity
63 BLAST (ANIb) and *in silico* DNA-DNA hybridization (DDH) values. The strain most closely
64 resembled *B. paramycooides* NH24A2^T, producing ANIb and DDH values of 86.70% and 34.1%,
65 respectively. Strain BHP is also known under alias *B. mycooides* ATCC 21929 and has been
66 reported previously as a producer of an antibiotic compound active against Gram-positive
67 pathogens [20]. For this study, *B. mycooides* ATCC 21929 was obtained from ATCC for whole
68 genome sequencing to verify its identity and for phenotypic characterization. Based on genomic
69 and phenotypic dissimilarities of *B. mycooides* ATCC 21929 compared to valid and effective *B.*

70 *cereus* group species, we propose the strain ATCC 21929^T as a type strain of a new species *B.*
71 *clarus* sp. nov. *B. clarus* type strain is deposited in ATCC (=ATCC 21929^T) and is also known
72 under aliases BHP^T, PS00077A^T, PS00077B^T and PSU-0922^T.

73

74 **Phylogenetic analyses**

75 The genome of the proposed novel *B. cereus* group species *B. clarus* ATCC 21929^T (=BHP^T)
76 was downloaded from NCBI's RefSeq database (RefSeq accession GCF_000746925.1,
77 deposited by Los Alamos National Laboratory in 2014) (Pruitt 2007 Nucleic Acids Res). To
78 confirm the identity of the isolate obtained from ATCC that was phenotypically characterized in
79 this study, the strain was re-sequenced at the Penn State Department of Food Science as part of
80 the FDA GenomeTrakr effort. The re-sequenced genome is accessible under NCBI accession
81 number QVOD00000000. The RefSeq sequence GCF_000746925.1 was used in all further
82 phylogenetic analyses reported in this paper.

83 To confirm that the isolate ATCC 21929^T was a member of the *B. cereus* group, its genome
84 sequence was compared to a database of 16S rRNA sequences of other *B. cereus* group type
85 strains (Table 1) using nucleotide BLAST (blastn) version 2.6.0 [21] and BTyper version 2.2.2
86 [18]. BTyper version 2.2.2 [18] was used to extract 16S rDNA gene sequences from the genome
87 of the proposed novel species *B. clarus* ATCC 21929^T and the 18 published *B. cereus* group
88 species. MUSCLE version 3.8.31 [22, 23] was used to construct an alignment of all 19 16S
89 rDNA genes. RAxML version 8.2.11 [24] was used to construct a maximum likelihood (ML)
90 phylogeny based on the 16S rDNA gene alignment, with the General Time-Reversible (GTR)
91 nucleotide substitution model under the Gamma model of rate heterogeneity and 1,000 bootstrap

92 replicates. FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Graphic for Mac
93 3.1 were used to annotate the resulting phylogenetic tree (Fig. 1).

94 BTyper version 2.2.2 was additionally used to perform *in silico* virulence gene detection,
95 *panC* clade assignment, 7-gene multi-locus sequence typing (MLST), and *rpoB* allelic typing
96 using the assembled genome of *B. clarus* ATCC 21929^T. FastANI [19] was initially used to
97 compute ANI values between the genome of *B. clarus* ATCC 21929^T and the genomes
98 representative of the 18 currently-recognized *B. cereus* group species (Table 1), as well as those
99 of the three effective *B. cereus* group species (Table 1). All ANI values produced by FastANI
100 were < 95, indicating that *B. clarus* ATCC 21929^T meets the genomic criteria for a new species.
101 To confirm this using a blast-based ANI metric (ANIb), pyani version 0.2.7 [25] was used to
102 calculate pairwise ANIb values between proposed novel species *B. clarus* ATCC 21929^T,
103 representative genomes of 18 published *B. cereus* group species, and the type strains of 3
104 unpublished effective *B. cereus* group species. Furthermore, JSpeciesWS [26] was used to
105 confirm that the pairwise ANIb results produced by pyani were below the 95 ANIb threshold for
106 bacterial species (Table 1).

107 Core single nucleotide polymorphisms (SNPs) were identified in 22 *B. cereus* group
108 genomes, including the proposed novel species *B. clarus* ATCC 21929^T, representative genomes
109 of 18 published *B. cereus* group species, and the type strains of three unpublished effective *B.*
110 *cereus* group species (Table 1), using kSNP version 3.1 [27, 28] with the optimal *k*-mer size
111 determined by Kchooser (*k* = 19). A maximum likelihood (ML) phylogeny was constructed
112 using the resulting core SNPs and RAxML version 8.2.4 [24], using the GTR nucleotide
113 substitution model under the Gamma model of rate heterogeneity, a Lewis ascertainment bias
114 correction [29], and 1,000 bootstrap replicates. FigTree version 1.4.3

115 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Graphics for Mac 3.1. were used to annotate the
116 phylogeny (Fig. 2).

117 Novel *B. cereus* group species *B. clarus* ATCC 21929^T could not be assigned to any one
118 of the 7 *panC* phylogenetic clades described by Guinebretiere, et al. [17] based on its *panC*
119 sequence (Table 1). Additionally, it could not be assigned to any known sequence type or allelic
120 type using *in silico* MLST or *rpoB* allelic typing, respectively (Table 1). The strain had MLST
121 allelic types *glp* 97, *ilv* 82, *pta* 83, *pur* 82, *pyc* 72, *tpi* 76, and a new *gmk* allele (*gmk* 179) was
122 defined by submission to the PubMLST database, resulting in a new ST 1834. The isolate has
123 been deposited in PubMLST Isolates database under id 2468. The closest match to the *B. clarus*
124 ATCC 21929^T *rpoB* sequence in the *rpoB* database of Food Microbe Tracker was allelic type
125 342 (AT0342): the two alleles shared 95.1% identity and 99.5% query coverage [30]. The 16S
126 rDNA sequence of *B. clarus* ATCC 21929^T most closely resembled that of the *B. tropicus* type
127 strain (Figure 1), which it matched with 99.8% and 100% nucleotide and coverage, respectively.
128 However, based on both core SNPs detected in all valid and effective *B. cereus* group species, as
129 well as its ANIb metric, the novel species most closely resembled *B. paramycooides* (Figs. 2 and
130 S1). The pairwise ANIb values for *B. clarus* ATCC 21929^T and *B. paramycooides* NH24A2^T were
131 87.33 % and 86.70%, as determined by FastANI and JSpeciesWS (Table 1). The DDH value for
132 these same two strains was 34.1%, with 0.48% probability for DDH being >70% (Table S1),
133 providing strong evidence for a new genomospecies. Genes coding for diarrheal enterotoxin Hbl
134 (*hblABCD*) were detected in the genome of *B. clarus* ATCC 21929^T.

135

136 **Phenotypic characteristics**

137 All phenotypic tests were conducted using *B. cereus* ATCC 14579^T as a control strain. *B. clarus*
138 ATCC 21929^T was confirmed as weakly-positive for production of Hbl and negative for
139 production of Nhe using the Duopath Cereus Enterotoxins kit (Merck) when the culture was
140 grown to the stationary phase at 32°C without shaking. At 37°C, *B. clarus* ATCC 21929^T did not
141 produce Hbl nor Nhe when grown in the same conditions. *B. cereus* s.s. strain ATCC 14579^T,
142 which was used as a control, was positive for production of both toxins when grown at both
143 temperatures without shaking. The cytotoxic potential of *B. clarus* ATCC 21929^T was assessed
144 in a 96-well microtiter plate by incubating 12 replicates of confluent HeLa cells with 5% v/v
145 bacterial supernatant (bacteria grown at 37°C) for 15 min, followed by addition of 10 µl of WST-
146 1 dye solution (Roche) and further 25-min incubation [2]. The final absorbance was determined
147 by subtracting the absorbance values measured at 690 nm from those measured at 450 nm.
148 Percent viability was determined relative to cells treated with 5% v/v Brain Heart Infusion (BHI;
149 negative cytotoxicity control). 0.05% Triton X-100 was used as a positive cytotoxicity control
150 (Figure 3) [2].

151 Gram staining was utilized for the preliminary identification of *B. clarus* ATCC 21929^T.
152 Cells stained Gram-positive and were approximately 3 µm long. Additionally, the morphology of
153 the bacteria grown overnight in BHI broth at 32°C and stained with 2% uranyl acetate negative
154 stain was observed by transmission electron microscopy (Figure S1). *B. clarus* ATCC 21929^T
155 was hemolytic, as confirmed by zones of clearance after streaking a loopful of 24-hour culture
156 suspension onto blood agar and incubating it at 35°C for 24 h. *B. clarus* ATCC 21929^T was
157 oxidase-negative, as confirmed using the Oxidrop reagent (Hardy Diagnostics). It was able to
158 hydrolyze starch and casein at 32°C after 72 hours of incubation, indicating that the strain
159 possesses both amylase and caseinase activity. Assays were conducted following protocols

160 described in Bergey's manual [16]. The ability of *B. clarus* ATCC 21929^T to grow in anaerobic
161 conditions was tested by inoculating anaerobic agar with an overnight culture and incubating it in
162 a jar with anaerobic gas pack at 30°C for 7 days; visible growth was observed after 3 days of
163 incubation. The motility was examined by preparing a Motility Test Medium according to the
164 Bacteriological Analytical Manual (BAM) [31], stab-inoculating overnight culture, and
165 incubating it for 24 h at 32°C. The strain's ability to grow at different temperatures (4, 7, 10, 15,
166 20, 25, 30, 37, 40, 43, 45, and 55°C) was studied by streaking individual well isolated colonies
167 on BHI agar plates, in triplicates, and incubating them up to 3 weeks or until growth was
168 observed (Table 1) [16]. The ability of *B. clarus* ATCC 21929^T to grow at different pH was
169 confirmed by inoculating 10 µl of an overnight culture into BHI broths adjusted to pH 3-11 using
170 appropriate buffers, in triplicate. Citrate buffer was used to supplement BHI adjusted to pH 3, 4,
171 and 5, phosphate buffer was added to BHI adjusted to pH 6,7, and 8, and CAPS buffer was added
172 in BHI adjusted to pH 9,10, and 11. Inoculated BHI tubes were incubated at 30°C for 14 days or
173 until growth was observed based on turbidity. The ability to grow at different concentrations of
174 NaCl was determined by supplementing TSB broth with 0, 0.5, 1, 2, 3, 5, 7, 9, 12, and 15% of
175 NaCl. Tubes were inoculated with 10 µl of an overnight culture, in triplicate, and incubated at
176 32°C for 14 days or until growth was confirmed based on turbidity. Results of the phenotypic
177 tests described above are reported in Table 2. The fatty acid composition was determined by
178 MID Inc. for culture grown at their defined standard conditions, on tryptic soy agar at 28°C. API
179 20E and CH50 biochemical assays (bioMérieux) were performed following the manufacturer's
180 instructions at 32°C (Table 3).

181

182 **Description of *Bacillus clarus* sp.nov.**

183 *Bacillus clarus* (cla'rus. L. masc. adj. *clarus* clear).

184

185 Cells stained as Gram-positive and displayed a long rod-like appearance, 3 µm in length. *B.*
186 *clarus* ATCC 21929^T is highly motile, oxidase negative, hemolytic, possesses amylase and
187 caseinase activity, can reach stationary phase in 16 hours at a growth temperature of 32°C in
188 BHI, and can grow in aerobic and anaerobic conditions. *B. clarus* ATCC 21929^T can grow at pH
189 6-9, temperatures ranging from 15°C- 43°C, and salt concentrations of 0-5%. The optimum
190 conditions of growth are 6-9, 37°C, and 0.5-3% respectively. *B. clarus* ATCC 21929^T is weakly
191 positive hemolysin BL (HBL) production at a temperature of 32°C, as indicated by faint bands in
192 Duopath Enterotoxins test, but does not reduce the metabolic activity of HeLa cells at tested
193 conditions. The fatty acid that was highest in abundance was iso-C15:0. Amongst the least
194 abundant fatty acids were C15:1 ω5c and iso-11:0 3OH. The latter two fatty acids, along with
195 iso-13:0 3OH were not found to be reported for any of the other *B. cereus* group type strains
196 (Table 3). Unique characteristics of *B. clarus* ATCC 21929^T include a higher abundance of iso-
197 C17:0, lower abundance of iso-C16:0 fatty acids, and the ability to grow optimally even at 3%
198 NaCl concentration. *B. clarus* ATCC 21929^T is also oxidase negative, which is a trait shared by
199 only *B. wiedmannii*, *B. gaemokensis*, and *B. manliponensis* of the *B. cereus* group (Table 2).

200

201 **Authors Statements**

202 There are no conflicts of interest and all funding provided to support this study is listed in

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204

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216

217 **Conflicts of interest**

218 The authors have no conflicts of interest.

219 TABLES

220 Table 1. List of *B. cereus* group genomes used in this study.

Species	Strain	Species Status ^a	ResSeq Accession	<i>panC</i> Clade ^b	MLST ST ^c	<i>rpoB</i> AT ^d	FastANI ANI ^e	JSpeciesWS ANIb (% aligned) ^f
<i>B. clarus</i> sp. nov.	ATCC 21929	Novel	GCF_000746925.1	NA	NA	NA	100	100
<i>B. albus</i>	N35-10-2	Published	GCF_001884185.1	II	775	NA	85.8323	84.84 (64.68)
<i>B. anthracis</i>	Ames ATCC 14578	Published	GCF_000007845.1	III	1	NA	86.0863	85.01 (61.66)
<i>B. cereus</i> s.s.	ATCC 14579	Published	GCF_000007825.1	IV	921	158	86.1037	84.84 (63.66)
<i>B. cytotoxicus</i>	NVH 391-98	Published	GCF_000017425.1	VII	NA	NA	83.1993	81.36 (48.48)
<i>B. luti</i>	TD41	Published	GCF_001884105.1	II	764	NA	86.1352	85.09 (60.76)
<i>B. mobilis</i>	0711P9-1	Published	GCF_001884045.1	II	784	194	86.0059	84.81 (61.23)
<i>B. mycoides</i>	DSM 2048	Published	GCF_000003925.1	VI	116	3	86.9787	86.31 (64.88)
<i>B. nitratireducens</i>	4049	Published	GCF_001884135.1	VI	769	NA	86.8859	86.17 (64.12)
<i>B. pacificus</i>	EB422	Published	GCF_001884025.1	II	32	380	85.9859	84.92 (61.75)
<i>B. paramycooides</i>	NH24A2	Published	GCF_001884235.1	VI	780	NA	87.3328	86.70 (64.31)
<i>B. paranthracis</i>	Mn5	Published	GCF_001883995.1	II	761	125	85.9614	84.90 (62.38)
<i>B. proteolyticus</i>	TD42	Published	GCF_001884065.1	VI	765	NA	86.7637	86.23 (65.23)
<i>B. pseudomycooides</i>	DSM 12442	Published	GCF_000161455.1	I	83	148	84.8949	83.90 (59.94)
<i>B. thuringiensis</i>	ATCC 10792	Published	GCF_002119445.1	IV	10	NA	85.9932	84.83 (64.90)

<i>B. toyonensis</i>	BCT-7112	Published	GCF_000496285.1	V	111	129	86.563	85.38 (61.22)
<i>B. tropicus</i>	N24	Published	GCF_001884035.1	II	771	NA	85.9761	85.02 (62.14)
<i>B. weihenstephanensis</i>	WSBC 10204	Published	GCF_000775975.1	VI	196	3	86.9523	86.14 (64.40)
	DMS 11821							
<i>B. wiedmannii</i>	FSL W8-0169	Published	GCF_001583695.1	II	1081	61	86.1474	85.00 (63.07)
<i>B. bingmayongensis</i>	FJAT-1383	Effective	GCF_000299035.1	NA	763	NA	87.7067	83.67 (58.92)
<i>B. gaemokensis</i>	JCM 15801	Effective	GCF_000712615.1	NA	768	NA	84.8374	84.08 (60.40)
	BL3-6							
<i>B. manliponensis</i>	JCM 15802	Effective	GCF_000712595.1	NA	NA	NA	80.4405	77.01 (43.10)
	BL4-6							

221 ^aNovel (proposed in this paper), Published (one of the 18 currently-recognized published and validated *B. cereus* group species), or
222 Effective (previously proposed in a publication as a potential member of the *B. cereus* group, but not validated/published as an official
223 species).

224 ^b*panC* clade assigned *in silico* using BTyper version 2.2.2 using traditional 7-clade scheme proposed by Guinebretiere, et al. [17].

225 ^cMulti-locus sequence typing sequence type; assigned *in silico* with BTyper version 2.2.2 [18].

226 ^d*rpoB* allelic type; assigned *in silico* with BTyper version 2.2.2 [18].

227 ^eAverage nucleotide identity (ANI) calculated using FastANI [19]; proposed novel species *B. mycooides* str. BHP was used as a query
228 and the listed genome was used as a reference.

229 ^fAverage nucleotide identity blast (ANIb) calculated using the JSpecies Web Server (JSpeciesWS [26]), with percent of the genome
230 aligned in parentheses; proposed novel species *B. mycoides* str. BHP was used as a query and the listed genome was used as a
231 reference.

232

233

234 **Table 2.** Phenotypic characteristics of *B. clarus* ATCC 21929^T and other valid and effective *Bacillus cereus* group species type
 235 strains. Species: 1, *B. clarus* ATCC 21929; 2, *B. albus* N35-10-2; 3, *B. anthracis* ATCC 14578; 4, *bingmayogensis* FJAT-13831; 5, *B.*
 236 *cereus* ATCC 14579; 6, *B. cytotoxicus* NVH 391-98; 7, *B. gaemokensis* BL3-6; 8, *B. luti* TD41; 9, *B. manliponensis* BL4-6; 10, *B.*
 237 *mobilis* 0711P9-1; 11, *B. mycoides* DSM 2048; 12, *B. nitratreducens* 4049; 13, *B. pacificus* EB422; 14, *B. paramycoides* NH24A2;
 238 15, *B. paranthracis* Mn5; 16, *B. proteolyticus* TD42; 17, *B. pseudomycoides* DSM 12442; 18, *B. toyonensis* BCT-7112; 19, *B.*
 239 *tropicus* N24; 20, *B. thuringiensis* ATCC 10792; 21, *B. weihenstephanesis* DSM 11821; 22, *B. wiedmannii* FSL W8-0169. The data
 240 for strain ATCC 21929 were produced in this study. All other data were obtained from Liu *et al.*, 2017 [4] unless specified otherwise
 241 in footnotes. In the API 20E tests, all strains were negative for lysine decarboxylase, ornithine decarboxylase, H₂S production, indole
 242 production, mannitol, inositol, sorbitol, rhamnose, melibiose, and arabinose. In the API 50CHB tests, all strains were negative for
 243 erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-
 244 mannitol, D-sorbitol, methyl α -D-mannopyranoside, lactose, melibiose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-lyxose, D-
 245 tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. -, negative; -w,
 246 weakly negative; +, positive; +w, weakly positive; NR, not reported.

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248

249

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
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Oxidase Test	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
Temperature	15-	15-	10-	15-	10-	20-	15-	10-	15-	10-	15-	7-	15-	15-	15-	10-	10-	10-	15-	10-	15-	5-
Range	43	40	50	45	45	50	40	39	40	39	40	39	45	39	45	39	40	45	45	45	40	43
NaCl Range	0-5	0-9	NR	0-4	0-4	NR	0-6	0-7	<7	0-9	0-4	0-9	0-9	0-5	0-9	0-9	0-	0-5	0-9	0-4	NR	0-5
(% w/v)																	2.5					
pH Range	6-9	5-	NR	4-	5-	NR	5-9	5-	5-9	5-9	5-	5-9	5-	5-9	5-	5-	5-	5-	5-9	5-	NR	5-
		10		10	9.5			10			9.5		10		10	10	9.5	9.5		9.5		10
Optimal	37	30	NR	30	30((30-	30	30	NR	30	30	30	30	30	30	30	30	35	30	30	NR	20-
Temperature					37) ^a	37																40
Optimal pH	6-7	7	NR	7	6	NR	7	8	7	7	8	7	6	7	7-8	8	8	6.5	6	7	NR	NR
Optimal NaCl	0.5-3	0.5-	NR	0-2	0	NR	NR	0.5	NR	0	1	0	1	0.5	1-2	0-1	1	0	0-	0	NR	NR
concentration		1																	0.5			
(w/v%)																						
Hemolysis	+	NR	(-) ^b	NR	(+) ^b	NR	NR	NR	NR	NR	(+) ^b	NR	NR	NR	NR	NR	NR	NR	NR	(+) ^c	(+) ^b	NR
Test																						
Casein	+	+	NR	NR	(+) ^a	NR	(-) ^d	+	(-) ^d	+	(+) ^d	+	+	+	+	+	NR	NR	+	(+) ^d	NR	(+) ^a
Hydrolysis																						
Motility	+	-	(-) ^a	(+) ^e	(+) ^a	(+) ^a	NR	+	(+) ^d	+	(-) ^b	-	-	-	-	-	(+) ^a	(+) ^a	+	(+) ^a	(+) ^b	(+) ^a
Anaerobic	+	+	(+) ^a	NR	(+) ^a	(+) ^a	+	+	(+) ^d	+	(+) ^a	+	+	+	+	+	(+) ^a	(+) ^a	+	(+) ^a	(+) ^a	(+) ^a
Growth																						
Starch	+	+	+	(-) ^e	+	-	-	-	-	+	+	-	-	+	-	+	-	+	+	+	+	+

Hydrolysis

Catalase Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryptophan deaminase	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β- Galactosidase	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Arginine Dihydrolase	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	-
Citrate Utilization	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+
Urease	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges– Proskauer reaction	+	+	+	-	+	+w	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	v	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation /oxidation (glucose)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Glycerol	+w	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+(w	+	+	-	+w	-
D-ribose	+	+	+	+	+	+	+	+	+	+	+w	+	+	+	+	+	+	+	+	-	+w	+

(+w

D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
D-galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
D-fructose	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	NR	+	-	-
Methyl α -D-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
<i>N</i> -Acetylglucosamine	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
Amygdalin	+w	-	-	-	+w	+w	-	-	-	-	-	-	-	+	-	-	-	+w	-	-	+w	-
Arbutin	+	+	v	-	+	+	-	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+
Aesculin	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ferric citrate																						
Salicin	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+
Cellobiose	+w	+	-	+	+	+	-	+	-	-	+w	-	-	+	-	+	-	-	+	+	+w	+
Sucrose	+	+	+	+	+	-	+	+	-	-	+	-	+	-	+	-	-	+	-	+	-	-
Trehalose	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Starch	-	+	+	-	+	-	+	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+
Turanose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Potassium gluconate	+w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DNA G+C content	35.2	35	35.	35.	35.	35.	36.	35.	37.	35.	35.	35.	35.	35.	35.	35.	35.	35.	34.	35.	35.
			2	5	3	9	6	5	1	3	2	3	2	2	2	2	4	6	2	8	2
Glycogen	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+	+	+

250

251 ^aData obtained from Miller *et al.*, 2016 [11]

252 ^bData obtained from Klee *et al.*, 2006 [32]

253 ^cData obtained from Ezzell *et al.*, 1990 [33]

254 ^dData obtained from Jung *et al.*, 2011 [13]

255 ^eData obtained from Liu *et al.*, 2014 [14]

256

257 **Table 3:** Fatty acid composition of *B. clarus* ATCC 21929^T and other valid and effective *Bacillus cereus* group species type strains.
 258 Species: 1, *B. clarus* ATCC 21929; 2, *B. albus* N35-10-2; 3, *B. anthracis* ATCC 14578 (no data to show); 4, *bingmayogensis* FJAT-
 259 13831; 5, *B. cereus* ATCC 14579; 6, *B. cytotoxicus* NVH 391-98; 7, *B. gaemokensis* BL3-6; 8, *B. luti* TD41; 9, *B. manliponensis* BL4-
 260 6; 10, *B. mobilis* 0711P9-1; 11, *B. mycoides* DSM 2048; 12, *B. nitratireducens* 4049; 13, *B. pacificus* EB422; 14, *B. paramycoides*
 261 NH24A2; 15, *B. paranthracis* Mn5; 16, *B. proteolyticus* TD42; 17, *B. pseudomycoides* DSM 12442; 18, *B. toyonensis* BCT-7112; 19,
 262 *B. tropicus* N24; 20, *B. thuringiensis* ATCC 10792; 21, *B. weihenstephanensis* DSM 11821; 22, *B. wiedmannii* FSL W8-0169. The
 263 data for strain ATCC 21929 was produced in this study. All other data were obtained from Liu *et al.*, 2017 [4]. NR: not reported; TR:
 264 trace amount.

Fatty acid	1	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
C12:0	0.67	2.7	NR	1.4	TR	2.3	1.3	5.9	1.8	2.7	4.7	1.7	1.6	1.5	3.3	1.4	NR	1	1	2.5	TR
C14:0	3.56	7.9	4.1	4.1	2.4	5	6.9	9	3.6	3.7	7.7	6.8	5.4	3.9	6.5	3.2	3.2	5.5	4.1	3.6	3.3
C15:1 ω5c	0.2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
C16:0	5.43	16.4	9.8	12.5	10.8	12.5	14.8	21	16.7	15.6	30.5	19.9	14.6	33	33.3	9	5.6	12	10.3	18	7.3
C16:1 ω6c	NR	NR	NR	5.9	3.3	NR	NR	NR	NR	6.4	NR	NR	NR	NR	NR	12.3	NR	NR	7.5	3.6	NR
C16:1 ω11c	NR	1.2	NR	TR	NR	NR	1.6	NR	1.3	1.2	2.4	1.7	1	1.2	2.1	NR	NR	1	TR	1.1	1.1
C16:1 ω7c	NR	TR	NR	NR	NR	NR	TR	NR	1.7	NR	TR	TR	TR	TR	TR	NR	NR	1.3	NR	NR	1.9
alcohol																					
C18:0	0.32	6.1	1.7	TR	NR	2.7	2.6	5	3.4	1.6	9.2	2	1.8	5.5	5.3	TR	NR	TR	TR	1.3	TR
C18:1 ω9c	NR	2	NR	TR	TR	1.4	2	1.7	3	TR	1.6	3	2.4	TR	1	TR	TR	TR	TR	TR	NR

iso-C11:0	0.3	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
iso-C12:0	0.66	1.3	TR	1.9	TR	3	1.1	4.9	1.9	2.6	2.2	1.2	1.4	1.5	2.3	8.7	NR	TR	1.5	2.9	TR	
iso-C13:0	14.8	6.9	7.7	20.3	7	7.9	6.8	5	4.9	21.9	8.5	6.4	7	7.9	8.8	12.6	7.1	7.1	18.5	22.3	6.9	
	4																					
iso-C14:0	2.3	4.2	2.9	4.8	5	5.9	4	6.3	5.4	3.4	1.8	3.5	5.5	1.9	2.7	5.5	2.3	5.9	5.2	3.5	5.1	
iso-C15:0	32.0	1	21	20.2	36.5	10.6	14.9	4	10.8	12.5	5.5	13.8	14.7	9	8.3	13.3	38.6	18.9	21.8	12.6	27.6	
	2																					
iso-C15:1 G	NR	1.2	NR	NR	NR	TR	TR	TR	TR	NR	TR	TR	NR	TR	TR	NR	NR	TR	NR	NR	NR	NR
iso-C16:0	2.59	4.6	3.6	3.3	6.7	5.3	5.6	5.7	9.1	2.8	3.2	5.3	7.4	3.6	3.4	8.3	5.1	7.8	3.7	2.9	9.1	
iso-C16:1	NR	NR	NR	TR	TR	NR	NR	NR	NR	TR	NR	NR	NR	NR	NR	2.9	NR	NR	1.3	TR	NR	
ω -5																						
iso-C17:0	12.1	3.8	11.5	6.7	8.2	5.3	6.6	2.7	6	7.5	2.5	5.2	6.5	8.5	4.3	7	11.4	9.6	6.9	6.6	10.1	
	5																					
iso-C17:1	NR	NR	NR	2.7	TR	NR	NR	NR	NR	6.5	NR	NR	NR	NR	NR	TR	NR	NR	3.2	4.2	NR	
ω -11																						
iso-C17:1	NR	NR	NR	1	TR	NR	NR	NR	NR	1	NR	NR	NR	NR	NR	2.3	NR	NR	2.6	TR	NR	
ω -6																						
iso-C17:1	NR	TR	NR	NR	NR	NR	1.8	NR	2.4	NR	1.8	1.7	1.7	1.1	1.6	NR	5.8	3.5	NR	NR	4.7	
ω 10c																						
iso-17:1	5.8	1.3	5.1	NR	NR	2.6	2.7	NR	NR	NR	TR	1	2.1	NR	TR	NR	4.9	2.6	NR	NR	2.6	
ω 5c																						
anteiso-	0.98	1.3	2.2	4	1.8	3.5	1.4	3.3	1.8	3.9	2.4	2	1.7	2.5	2.9	4.9	NR	1	2.8	5.8	1	

C13:0																						
anteiso-	2.16	4.4	7.4	6.5	10.8	5.5	4.6	3.3	6.5	3.8	2.4	6.7	5.6	4.2	3.9	3.6	3.1	4.4	5.3	5.4	4	
C15:0																						
anteiso-	0.6	1.6	2.8	1.5	3.4	2	1.8	1.5	3.2	1.1	1.1	2.7	2.2	2	1.5	1.6	NR	1.7	1.1	1.7	1.5	
C17:0																						
anteiso-	0.55	TR	TR	NR	NR	1.3	TR	TR	1	NR	TR	TR	1	TR	NR	NR	NR	TR	NR	NR	TR	
C17:1 a																						
anteiso-	NR	NR	NR	TR	TR	NR	NR	NR	NR	TR	NR	NR	NR	NR	NR	1.1	NR	NR	TR	TR	NR	
C17:1 ω-6																						
iso-11:0	0.22	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	
3OH																						
iso-13:0	0.46	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	
3OH																						

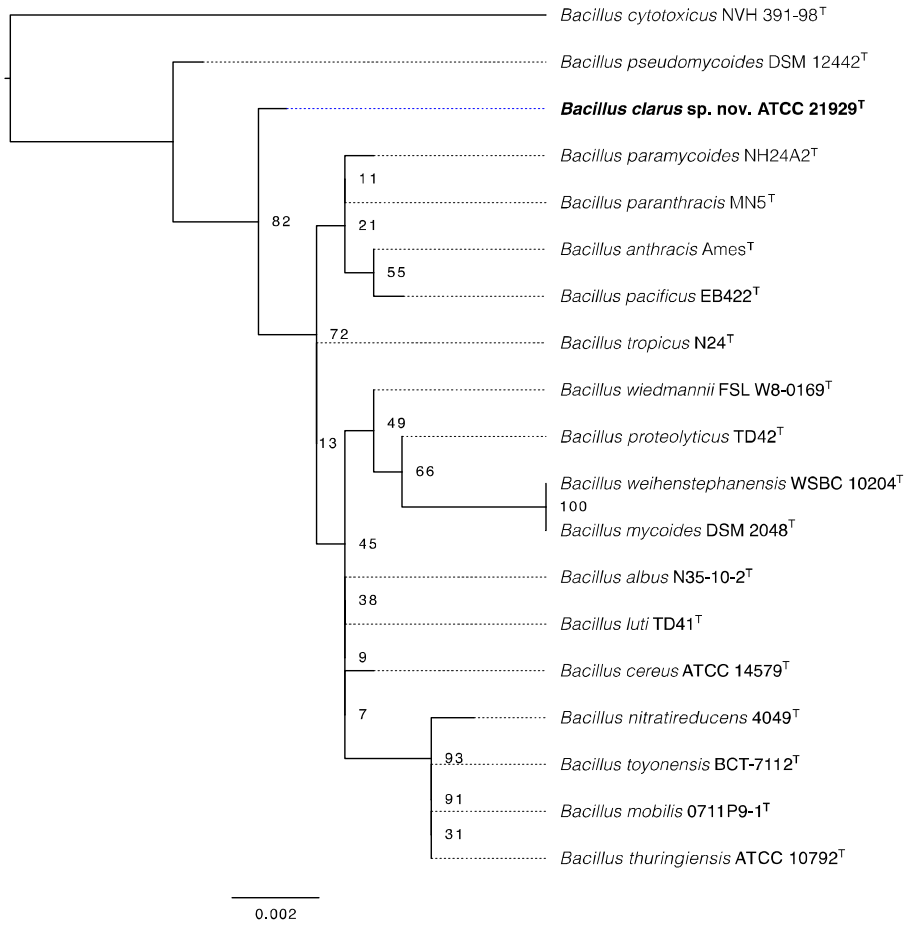
266 **FIGURE LEGENDS**

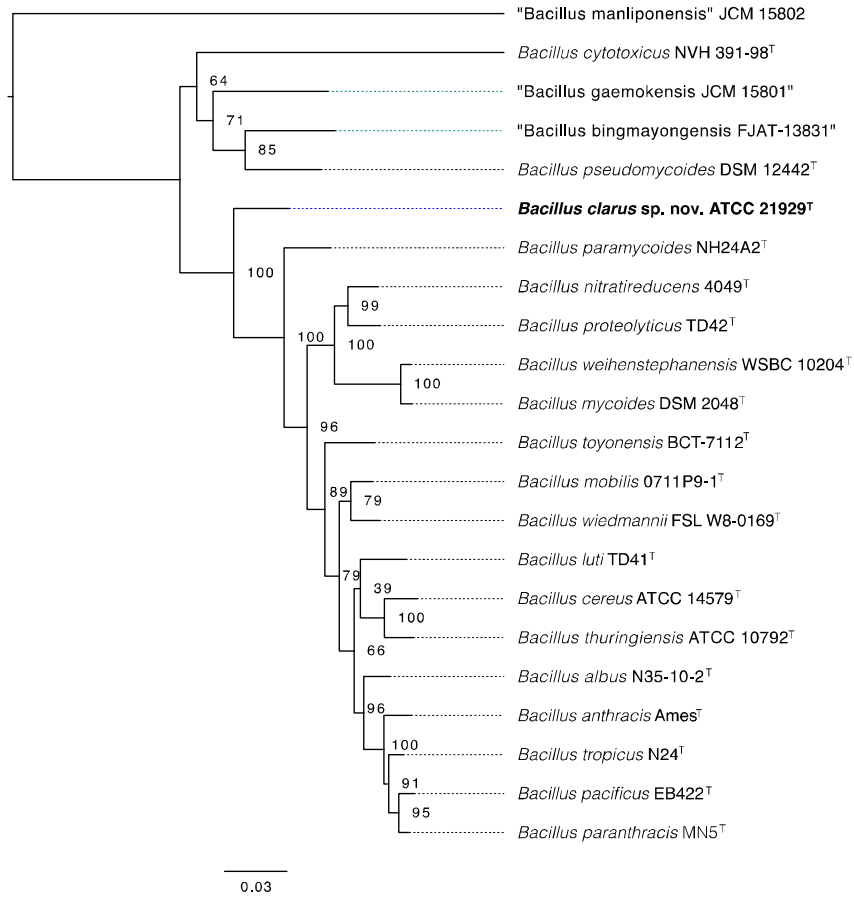
267 **Figure 1.** Maximum likelihood phylogeny constructed using 16S rRNA gene sequences of the 18 currently-recognized *B. cereus*
268 group species and novel *B. cereus* group species *B. clarus* ATCC 21929^T (boldfaced tip label). The tree is rooted at the midpoint, and
269 node labels correspond to bootstrap support percentages using 1,000 replicates.

270
271 **Figure 2.** Maximum likelihood phylogeny constructed using core SNPs identified in 18 currently-recognized *B. cereus* group species,
272 3 proposed effective *B. cereus* group species (tip labels in quotation marks), and novel *B. cereus* group species *B. clarus* ATCC
273 21929^T (boldfaced tip label). The tree is rooted at the midpoint, and node labels correspond to bootstrap support percentages using
274 1,000 replicates.

275
276 **Figure 3.** Cytotoxicity of *B. clarus* ATCC 21929^T and 17 other valid *B. cereus* group species type strains. Percentage viability of
277 HeLa cells when treated with supernatants of *B. clarus* ATCC 21929^T and 17 other valid *B. cereus* group type strains as determined by
278 the WST-1 assay [2]. The columns represent the mean viabilities and the error bars represent standard deviations for 12 technical
279 replicates.

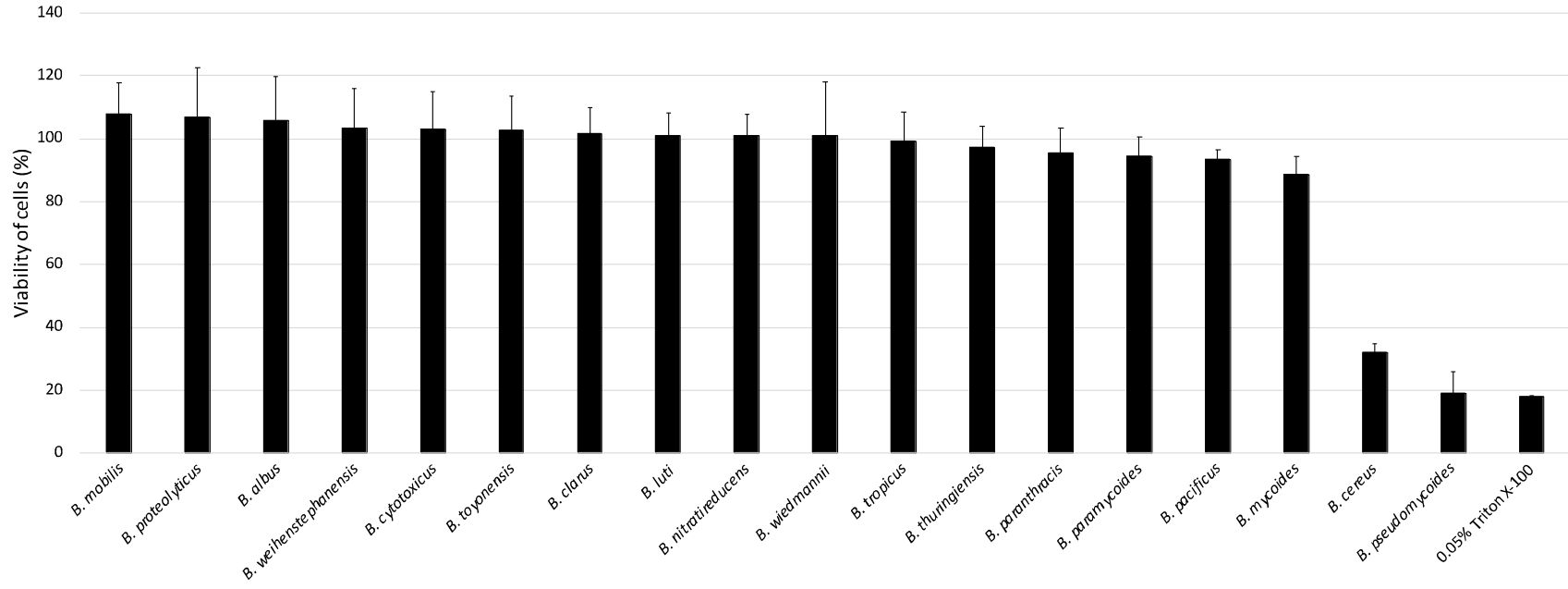
280





285 **Fig. 3**

286



287

288 **References**

289

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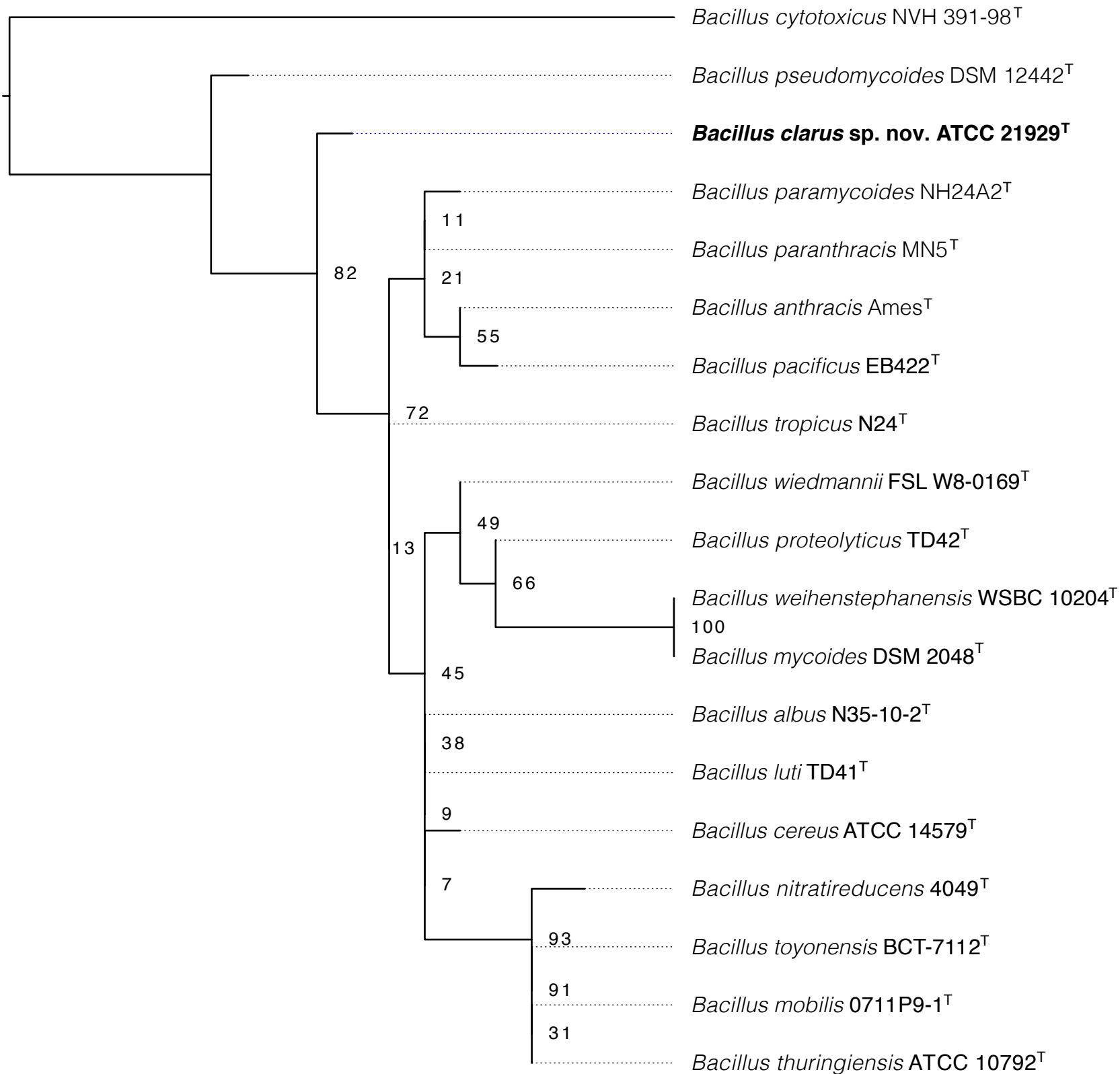
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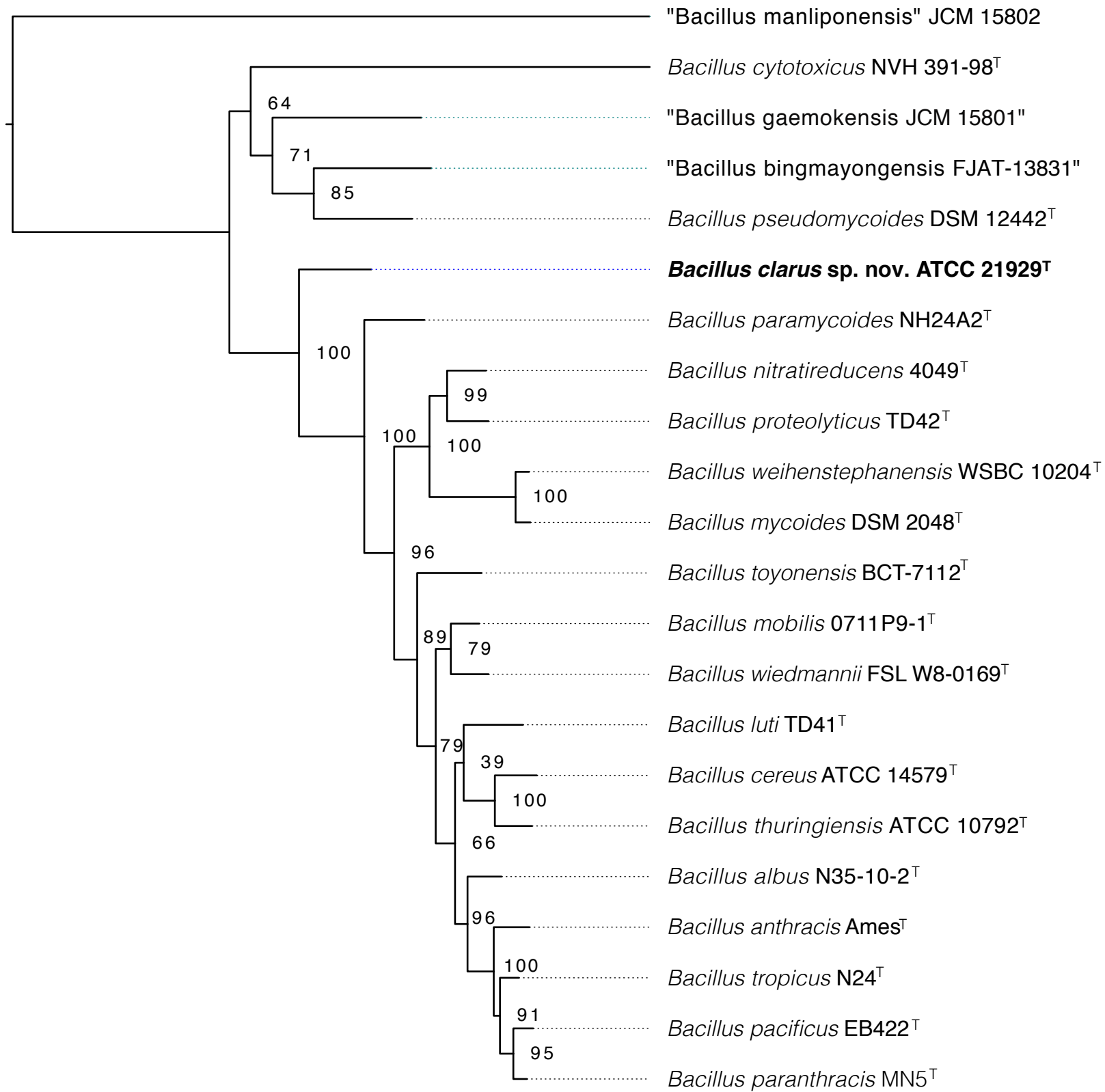
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0.002



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