1 **Genetic distribution of 295 *Bacillus cereus* group members based on *adk***
2 **screening in combination with MLST (Multilocus Sequence Typing) used for**
3 **validating a primer targeting a chromosomal locus in *B. anthracis*.**
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Abstract

The genetic distribution of 295 *Bacillus cereus* group members has been investigated by using a modified multilocus sequence typing method (MLST). By comparing the nucleic acid sequence of the *adk* gene fragment, isolates of *B. cereus* group members most related to *B. anthracis* may be easily identified. The genetic distribution, with focus on the *B. anthracis* close neighbours, was used to evaluate a new primer set for specific identification of *B. anthracis.* This primer set, BA5510-1/2, targeted the putative *B. anthracis* specific gene *BA5510*. Real-time PCR using BA5510–1/2 specifically amplified the target fragment from all *B. anthracis* strains tested and only one (of 288) non-*B. anthracis* strains analysed. This is one of the most thoroughly validated chromosomal *B. anthracis* markers for real-time PCR identification, in which the screened collection contained several very closely related *B. anthracis* strains.

Introduction

The *Bacillus cereus* group consists of six bacterial species; *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*. *B. anthracis* is the causative agent of anthrax, a severe disease of great concern to public health authorities. It is also of military concern due to its potential as a biological weapon agent (Inglesby et al., 2002). *B. cereus,* producing an emetic toxin and enterotoxins, is an opportunistic human pathogen frequently associated with food poisoning (Granum 2007, Schoeni and Wong 2005). This species has also been connected to periodontal-, eye- and wound infections (Drobniewski 1993, Pillai et al., 2006). *B. thuringiensis* is used as an insecticide with great economic advantages based on the production of several insect pathogenic crystal toxins (de Maagd et al., 2001).

52 *B. mycoides* and *B. pseudomycoides* are characteristic for their penchant of making
53 mycelia colonies on agar plates (Di Franco et al., 2002, Nakamura 1998), while *B.*
54 *weihenstephanensis* is a psychrotolerant bacteria often found in dairy products
55 (Anderson Borge et al., 2001). Despite the potential of toxin production in *B.*
56 *weihenstephanensis* (Stenfors et al., 2002), neither this species nor *B. mycoides* have
57 been reported to having caused any human infections. Many of the phenotypical traits
58 that can be used to differentiate species in the *B. cereus* group are plasmid-mediated.
59 The *B. anthracis, B. cereus* and *B. thuringiensis* are closely related due to their high
60 degree of DNA sequence similarity (Helgason et al., 2000, Rasko et al., 2005). Recent
61 publications have reported that *B. cereus* infections may resemble inhalational anthrax
62 (Hoffmaster et al., 2004, Hoffmaster et al., 2006). The *B. cereus* G9241 strain
63 harbours a plasmid with nearly 100% amino acid identity to the *B. anthracis* pXO1
64 plasmid and causes inhalational anthrax-like symptoms. Furthermore, the *B. cereus*
65 strain, E33L, isolated from a zebra carcass in Namibia, probably caused an illness
66 resembling anthrax (Han et al., 2006).
67 Several molecular methods have been used to reveal the genetic relationship
68 of the *B. cereus* group species (Harrell et al., 1995, Helgason et al., 1998, Hill et al.,
69 2004, Sherif et al., 2003, Valjevac et al., 2005). Multilocus sequence typing (MLST)
70 is a molecular method that was first developed for genotyping of *Neisseria*
71 *meningitidis* (Maiden et al., 1998). During recent years this method has been
72 established for several pathogenic agents (Jacobsson et al., 2003, Kotetishvili et al.,
73 2003, Kotetishvili et al., 2005, Manning et al., 2003, Tartofm et al., 2005). To our
74 knowledge, four MLST schemes have been published for the *B. cereus* group
75 members in order to reveal the genetic distribution within the group (Daffonchio et
76 al., 2006, Helgason et al., 2004, Ko et al., 2004, Priest et al., 2004). These protocols

77 use five to seven fragments of various housekeeping genes for MLST analysis.
78 Results from these studies show that *B. anthracis* strains constitute a separate clonal
79 evolutional line among the *B. cereus* group members, without any obvious clustering
80 of other *B. cereus*/*B. thuringiensis* strains. However, selection of markers used in the
81 different MLST methods can influence the exact relation between various strains.
82 The *lef* and *cap* genes, encoding the lethal factor and the poly-D-glutamic acid
83 capsule respectively, located on the pXO1 and pXO2 plasmids respectively, are
84 frequently used as genetic markers for identification of *B. anthracis* (Hoffmaster et
85 al., 2002). It is necessary to possess *B. anthracis* specific chromosomal markers as a
86 supplement to the plasmid-encoded markers due to the fact of plasmid instability. *B.*
87 *anthracis* strains containing only one of the pXO-plasmids are found in nature (Patra
88 et al., 1996, Turnbull et al., 1992), and it cannot be ruled out that bacterial strains may
89 be genetically modified for bioterror actions. Several chromosomally derived PCR-
90 primers for identifying *B. anthracis* have been published, targeted against BA813
91 (Ramisse et al., 1996), *saspB* (Hoffmaster et al., 2002), *rpoB* (Qi et al., 2001), *gyrA*
92 (Hurtle et al., 2004), a fragment crossing a hypothetical protein and a alpha/beta-
93 hydrolase encoding genes (Bode et al., 2004) and *plcR* (Easterday et al., 2005).
94 In this study, the genetic distribution of a collection of 295 *B. cereus* group
95 members has been explored by a modified MLST-screening method using the *adk*
96 gene fragment. The collection has been used to evaluate a novel chromosomal *B.*
97 *anthracis* specific marker for real-time PCR identification.
98
99 **Materials and Methods**
100 ***Bacterial strains***

101 A total of 295 *Bacillus cereus* group isolates were used in this study (Table 1)
102 including 150 *B. cereus* strains, 93 *B. thuringiensis* strains, 11 *B. weihenstephanensis*
103 strains, 6 *B. mycoides* strains, 4 *B. anthracis* strains and 31 *Bacillus* spp. strains,
104 representing a wide variety of clinical, environmental and food isolates. Sequence
105 data for *B. thuringiensis* strain 97-27 subsp. *konkukian* serotype H34 and *B. cereus*
106 strains G9241 and E33L was achieved from GeneBank with accession no/project ID:
107 ID10877 (DOE Joint Genome Institute), ID10788 (TIGR), and NC_006274
108 respectively.
109 ***Bacterial growth***
110 All strains were plated on brain heart infusion agar (BHI) (Acumedia
111 Manufacturers Inc. Baltimore, USA) and grown at 32ºC overnight. In general, cells
112 from one single colony were inoculated in 10 ml BHI-broth and grown at 32ºC
113 overnight.
114 The strains clustering close to *B. anthracis* were streaked on sheep blood
115 agar, and grown at 32 ºC overnight. Characteristic phenotypical features for *B.*
116 *anthracis* were studied by inspecting colonies for lack of hemolysis and cell mobility
117 (microscopy). The penicillin sensitivity of *B. anthracis* was investigated by plating the
118 bacterial cells onto BHI agar plates containing 10.0 µg/ml and 0.5 µg/ml of penicillin
119 G (Farrar and Reboli 1992).
120 ***Extraction of DNA***
121 A 10 ml bacterial culture grown overnight was harvested by centrifugation at
122 2000 x g for 10 min. The pellet was resuspended in 1 ml sterile 10 mM PBS
123 (phosphate buffered saline, pH=7.4) and lysed at 3200 rpm for 1 min using silica
124 beads (500 mg of both 0.1 and 0.5 mm beads) in a Mini-Beadbeater-8 (BioSpec
125 Products, USA). Beads and cell debris were centrifuged at 11000 x g for 1 min and

126 the supernatant, containing a crude DNA extract, was stored at -20 ºC until used as a
127 template in PCR. The DNA was diluted a 100-fold in 10 mM PBS before being used
128 as a template in real-time PCR.

129 ***Real-time PCR***

130 The primers for amplification of the gene fragments for MLST analysis (*adk*
131 (adenylate kinase), *ccpA* (catabolite control protein A), *glpT* (glycerol-3-phosphate
132 permease), *pyrE* (orotate phosphoribosyltransferase), *recF* (DNA replication and
133 repair protein) and *sucC* (succinyl coenzyme A synthetase, beta subunit)) (Helgason
134 et al., 2004), and real-time PCR (*BA5510*, *lef, cap, plcR*) are shown in Table 2. The
135 amplification reactions were performed in 20 µl using 2 µl DNA extract as a template.
136 PCR reactions were performed in a LightCycler® System (Roche Diagnostics,
137 Switzerland) using the Lithos qPCR™ Master Mix (Eurogentec, Belgium) according
138 to recommendations given by the manufacturer of the kit. Optimized annealing
139 temperatures, Mg^{2+}- and primer/probe concentrations are presented in Table 2. The
140 temperature program was as follows: 5 min initial denaturation at 95ºC followed by
141 35 cycles of denaturation at 95ºC for 10 sec, annealing at individual temperatures
142 (Table 2) for 10 sec and primer extension at 72ºC for 25-35 sec. The amplifications
143 were terminated after a final elongation of 5 min at 72 ºC. The PCR fragments were
144 verified by electrophoresis using the Bioanalyzer (Agilent Technologies, USA). PCR
145 products for DNA sequencing were purified using the QIAquick PCR purification kit
146 (Qiagen). The purified PCR products were eluted in 20 µl of sterile water and the
147 DNA concentrations were determined at 260 nm using the NanoDrop® ND-1000
148 Spectrophotometer (NanoDrop Technology, USA).

149 ***DNA Sequencing***

150 DNA sequencing was performed with the ABI prism® 310 Genetic Analyzer
151 (Applied Biosystem, USA) using the BigDye® Terminator v.3.1 Matrix Standard Kit
152 (Applied Biosystem, USA) in accordance with the manufacturer's manual, but with
153 the following exceptions; all reaction volumes were reduced by 50 %, the samples
154 were not vortexed during the washing step and the final ssDNA pellet was air dried
155 for 45 min in room temperature. Both ssDNA strands of the PCR product were
156 sequenced.

157 ***Data analysis***

158 The Staden Package (Staden, 1996) was used for alignment, editation and
159 construction of consensus sequences based on the ABI sequence chromatograms. The
160 *adk* consensus sequences were entered into the Bionumerics v4.50 software (Applied-
161 Maths, Belgium) where a cluster analysis was performed to evaluate the genetic
162 relationship among the strains. Based on a pairwise alignment and a distance matrix, a
163 dendrogram was constructed using the unweighted pair group method with arithmetic
164 mean (UPGMA). In order to achieve higher resolution, the *B. cereus* group strains
165 clustering close to *B. anthracis* in the dendrogram were analyzed by running a MLST
166 scheme according to Helgason et al. (2004). The MLST sequence data were analyzed
167 using computer scripts kindly provided by Erlendur Helgason (Biotechnology Centre,
168 Oslo). Based on allele sequences obtained in this study compared to those reported by
169 Helgason et al. (2004) (data available from GeneBank under accession numbers
170 AY387859 to AY388397), a dendrogram was constructed using the MEGA3.0
171 software package (Kumar et al., 2004) and the neighbour -joining (NJ) method
172 (Saitou and Nei, 1987).

173 ***Nucleotide sequence accession numbers***

174 Nucleotide sequences from the internal fragments from genes sequenced in
175 this study have been submitted to the GeneBank under accession numbers EF553641-
176 EF554074.
177
178 **Results**
179 ***adk-screening and MLST***
180 Sequence comparison of the *adk* gene fragment from 295 isolates of the *B.*
181 *cereus* group members resulted in 54 different sequence types (ST's) (alleles). ST2 to
182 ST8 were clustering close to *B. anthracis* (Fig. 1) and the 24 *B. cereus/B.*
183 *thuringiensis* strains belonging to these ST's turned out to have less than six point
184 mutations in the *adk* gene when compared to *B. anthracis*. Neither of them were
185 identical to the *B. anthracis adk* sequence. However, nine strains showed only one
186 point mutation (ST2). All *B. anthracis* strains tested had identical *adk* sequences.
187 Neither insertions nor deletions were observed, and there was no obvious clustering of
188 the *B. cereus* and *B. thuringiensis* species.
189 To better assess the genetic relationship of the strains belonging to ST2-8 (Fig.
190 1), these strains were compared to 77 other *B. cereus* group members by using a full
191 MLST scheme (Helgason et al., 2004). This MLST scheme compares partial
192 nucleotide sequences of seven housekeeping genes; *adk, ccpA, ftsA, glpT, pyrE, recF*
193 and *sucC*. To achieve a higher discriminating power of the MLST analysis, the scripts
194 used divided each gene fragment into three nearly equal parts, generating allelic
195 profiles consisting of 21 numbers instead of seven (Helgason et al., 2004). These
196 allelic profiles were compared to the previously established profiles of 77 other *B.*
197 *cereus* group members (Helgason et al., 2004), resulting in a dendrogram containing
198 four main clusters; A, B1, B2 and B3 (Fig. 2). Cluster A contains all *B. anthracis*

199 strains analysed in this study as well as 27 *B. cereus* and *B. thuringiensis* strains, in
200 which 17 strains were from this study. Cluster B1, including the *B. cereus* type strain
201 ATCC 10987 and seven *B. cereus*/*B. thuringiensis* strains from this study, contains
202 clinical, food and environmental strains with a predominance of clinical isolates.
203 Cluster B2 and B3 harbour only strains reported by Helgason et al. 2004, with a
204 predominance of environmental strains. In general, there seems to be a weak
205 transition of environmental *Bacillus* isolates dominating cluster B2 and B3, to a more
206 prevalence of clinical or food *Bacillus* isolates clustering closer to *B. anthracis* in
207 cluster A, which is not unexpected. Interestingly, the two environmental *B. cereus*
208 strains DSM318 and DSM336 (originally isolated from soil), located in cluster A, are
209 the closest neighbours to *B. anthracis* in the MLST phylogenetic three (Fig. 2),
210 followed by *B. thuringiensis* BGSC4AJ1, BGSC4AY1 and *B. cereus* NVH0597-99.
211 Strains DSM318 and DSM336 shared three alleles with *B. anthracis* (*ftsA*, *glpT* and
212 *recF*). In *adk*, *ccpA* and *sucC*, only one point mutation was found in each gene
213 fragment, in contrast to ten in the *pyrE* gene fragment. The *B. thuringiensis* strain
214 BGSC4AJ1 displayed an identical allele profile to four *B. cereus* strains isolated from
215 periodontitis patients (AH813, AH816, AH818 and AH820) (Fig. 2). These isolates
216 possessed two identical alleles with *B. anthracis* (*ftsA* and *recF*) and differed in six
217 point mutations. *B. cereus* NVH0597-99 harboured the same six point mutations as
218 found in *B. thuringiensis* BGSC4AJ1, in addition to three other point mutations. *B.*
219 *thuringiensis* BGSC4AY1 displayed identical *recF* allele with *B. anthracis* and had
220 nine point mutations in difference to *B. anthracis*.
221 The *B. cereus* strain NVH246-02 was subject to an MLST analysis, using all
222 seven loci, since its species had not been verified. In this study, NVH246-02 belonged
223 to the MLST cluster B1 (Fig. 1) representing a more distant location from *B.*

224 *anthracis* compared to isolates located in cluster A. Based on these results, we
225 propose that the NVH246-02 strain is not a *B. anthracis* strain. This finding is
226 supported by the observation that no fragments were amplified by real-time PCR
227 using the *B. anthracis* specific BA5510-1/2, BA813-f/r, BAcap-f/r and or BAlef-f/r
228 primers (see section below).

229 ***Novel chromosomal B. anthracis marker***

230 The nucleotide sequence of the *BA5510* gene encoding the techoic acid ABC
231 transporter (ATP-binding protein) was analysed for its potential to be used as a novel
232 *B. anthracis* chromosomal marker, based on bioinformatic analysis of the published
233 *B. cereus* group member genome sequences. A PCR primer set and a hybridising
234 probe, BA5510-1/2 and BA5510-FL/BA5510-640 respectively, targeting this gene
235 was constructed. Only the primer was used for the PCR screening of 292 *B. cereus*
236 group members. The probe was used for those strains resulted in an amplified PCR
237 product. Results showed that a specific amplification of the *B. anthracis* strains A15,
238 A58, A73 and CIP.7700 was obtained, including a fluorescent signal from the
239 hybridising probe. BLASTN analysis showed that the DNA sequence *BA5510* from
240 these four strains was identical to the DNA sequence of the equivalent gene fragment
241 from ten additional *B. anthracis* genomes ('Ames Ancestor', A1055, A2012, Ames,
242 Australia 94, CNEVA-9066, Kruger B, Sterne, Vollum and Western North America
243 USA6153), which have been completely sequenced. However, a similar-sized
244 fragment was also specifically amplified from the genome of *B. cereus* NVH492
245 (including a fluorescent signal from the hybridising probe) isolated from minced meat
246 in Norway. This strain was located in cluster A, close to *B. anthracis* in the MLST
247 dendrogram (Fig. 2), and harboured 58 point mutations compared to *B. anthracis*
248 when using the full MLST scheme. For a comparison, the well-known and frequently

249 used chromosomal marker BA813 (Ramisse et al., 1996) was amplified from 31 of
250 288 non-*B. anthracis* strains (11 %), in which 12 of these strains, B275, BGSC4AJ1,
251 BGSC4AU1, BGSC6E1, BGSC4Y1, BGSC4AS1, BGSC4AW1, BGSC4AY1,
252 NVH1518-99, NVH0500-00, DSM318 and DSM336, were located in cluster A in the
253 MLST dendrogram (Fig. 2). These results showed that the primer set BA813-f/r was
254 not specific for *B. anthracis*, which is consistent with previously published results
255 where four out of 60 non-*B. anthracis* strains were amplified (Ramisse et al., 1999).
256 No amplification was obtained from these 31 strains when using the BA5510-1/2
257 primer set.

258 ***Phenotypic and genetic features***

259 In this study, 24 *B. cereus/B. thuringiensis* isolates were found to be closely
260 related to *B. anthracis* based on the *adk* sequence comparison (Fig. 1). 21 of these
261 strains were analysed for several *B. anthracis* specific phenotypic characteristics.
262 Except for the B283 and B366 strains, all investigated isolates were motile, penicillin
263 resistant and hemolytic when analysed on sheep blood agar (data not shown), which
264 are general characteristics for *B. cereus /B. thuringiensis* strains. Neither motility nor
265 hemolysis was observed when examining the B283 and B366 cells, that were located
266 in cluster B1 in the MLST dendrogram. However, penicillin sensitivity was observed
267 (no growth at 10 µg/ml, 1 colony at 0.5 µg/ml). Also, all 21 strains were used to
268 investigate the presence of the *B. anthracis* specific mutation in the *plcR* gene, a
269 pleiotropic regulator gene of several virulence genes in the *B. cereus* group (Agaisse
270 et al., 1999). This mutation was not found in any of the isolates by nucleotide
271 sequencing of an internal fragment from this gene (data not shown). Thus, *Bacillus*
272 isolates clustering close to *B. anthracis* based on MLST analysis, do not necessarily
273 display phenotypic or other genotypic characters typical for *B. anthracis*.

274 The PCR primers targeting the *lef* and *cap* genes located on the *B. anthracis*
275 plasmids pXO1 and pXO2, respectively (Ramisse et al., 1996) were used for an
276 additional screening of 292 strains in order to elaborate their, if any, presence in these
277 genomes. Amplified products were only obtained from *B. anthracis* A15 (pXO1^{+}) and
278 A73 (pXO2^{+}), with the two BAlef-f*/r and BAcap-1/2* primer sets, respectively (data
279 not shown).

280

281 **Discussion**

282 In this study, a modified MLST method was used to reveal the genetic
283 distribution of 295 strains of the *B. cereus* group members. Strains most similar,
284 genetically, to *B. anthracis* were identified. Studies were performed to elaborate if
285 strains belonging to the *B. anthracis* close neighbours showed any correlation (cross-
286 reaction/talk) with specific real-time PCR analysis for identification of *B. anthracis*.
287 The screening method is based on a sequence comparison of the *adk* gene, which
288 seems to be sufficient in order to reveal a genetic distribution of the group of closely
289 related *B. anthracis* strains similar to that established by a more extended MLST
290 scheme (Helgason et al., 2004). Consequently, *adk* screening approach is labour-
291 saving and less time-consuming, leading to a reduction of manpower in addition to
292 lower costs for screening large strain collections.

293 A DNA sequence comparison of the *adk* gene resulted in 54 different alleles or
294 sequence types, where ST2 to ST8 clustered close to *B. anthracis* (Fig. 1). There was
295 no obvious clustering of the *B. cereus* and *B. thuringiensis* species, which is
296 consistent with previous studies (Daffonchio et al., 2006, Helgason et al., 2004, Ko et
297 al., 2004, Priest et al., 2004). However, if the *adk* gene had been subjected to a higher
298 mutational or recombinational event, these strains would not have been typed by

299 MLST in this study. This would have been the case for the *B. cereus* DSM318 and
300 DSM336 stains if the *pyrE* gene had been used for the initial screening. These two
301 strains have, except for the *pyrE* gene, either common alleles or alleles containing
302 only one point mutation when compared to the similar gene in *B. anthracis*. The *pyrE*
303 gene from DSM318 and DSM336 contained ten point mutations in comparison to *B.*
304 *anthracis*. This indicates that the *pyrE* gene in these strains might have been through a
305 recombination event. Most likely, recombination will occur at all seven loci in the
306 MLST scheme but to a limited extent (Helgason et al., 2004). Theoretically, in this
307 *adk* screening some *B. anthracis* close neighbours might have been left out due to
308 recombinations.

309 An MLST dendrogram including a total of 104 strains generated four main
310 clusters A, B1, B2 and B3, where *B. anthracis* belongs to cluster A (Fig. 2). Eleven
311 strains from this study were clustered closer to *B. anthracis* than the strains G9241,
312 E33L and Bt9727. These strains have previously been reported as close neighbours to
313 *B. anthracis* (Han et al., 2006, Hill et al., 2004, Hoffmaster et al., 2006), containing
314 49, 30, and 19 point mutation, respectively, in difference to *B. anthracis*, and they did
315 not share any alleles with *B. anthracis*. The MLST dendrogram (Fig. 2) obtained in
316 this study showed that the *B. cereus* strains DSM318 and DSM336 are the strains
317 most related to *B. anthracis*. The *B. cereus* strains AH818, DSM318 and DSM336
318 have previously been analysed by a MLST scheme using five genetic loci (16S-23S-
319 rRNA ITS, SG-749, *ywfK*, *plcR* and *cerA*) (Daffonchio et al., 2006), revealing
320 DSM318 as the closest neighbour to *B. anthracis* followed by DSM336 and AH818.
321 However, this finding changed depending on the locus analysed (AH818 was closest
322 using *cerA* and *SG-749*, whereas DSM336 was closest when using *plcR*) (Daffonchio
323 et al., 2006).

324 The *B. thuringiensis* strain BGSC4AJ1 showed identical allele profile to
325 several *B. cereus* isolates causing periodontitis. Previously, the BGSC4AJ1strain was
326 analysed by another MLST scheme (*glpF, gmk, ilvD, pta, pur, pycA,* and *tpi*) (Priest et
327 al., 2004), where four common alleles with *B. anthracis* were found (Kim et al.,
328 2005), placing BGSC4AJ1 nearest to *B. anthracis* among 150 strains of the *B. cereus*
329 group. Our finding of an amplified product using the BA813-f/r primer set genetically
330 connecting DSM318, DSM336 and BGSC4AJ1 to *B. anthracis*, suggested that these
331 strains were all closely related to *B. anthracis*. However, these results showed that the
332 exact relationship will depend on the analytical method applied and the selection of
333 markers used for the MLST method chosen. This has also been addressed by Marston
334 et al. (2006), where discrepant results were obtained when analysing 23 *Bacillus* spp.
335 with traditional microbiological methods and PCR, emphasising the need for MLST
336 and MLVA analyses to achieve a reliable determination of species.
337 The strain NVH246-02, isolated from shrimps imported from Thailand,
338 originally proposed as a *B. anthracis* strain, was analysed using MLST despite a high
339 number of point mutations in the *adk* gene compared to *B. anthracis*. This strain
340 possesses some of the phenotypic characteristics typical for *B. anthracis* (no motility,
341 penicillin sensitivity, no hemolysis) as well as nucleotide sequence identity to the *B.*
342 *anthracis* 16S rRNA gene (Per Einar Granum, personal communication). In this
343 study, NVH246-02 was distantly related to *B. anthracis* based on MLST (Fig. 2) and
344 lack of amplification using the BA5510-1/2, BA813-f/r, BAcap-f/r and BAlef-f/r *B.*
345 *anthracis* specific primer sets. Based on these results, we strongly believe that this
346 strain is not a *B. anthracis* strain.
347 A novel *B. anthracis* specific chromosomal primer set and a probe for real-
348 time PCR identification was constructed targeting the *BA5510* gene. This gene seems

349 to be unique for the *B. anthracis* genome (Dwyer et al., 2004). The BA5510-1/2
350 primer set amplified only the *B. cereus* strain NVH492 among 288 non-*B. anthracis*
351 isolates analysed in this study. BLAST analysis showed that the *BA5510* gene
352 sequence equivalent in the *B. cereus* strains AH820 (only genome shotgun sequence
353 available) and E33L (*tagH* gene, 73% amino acid homology to *BA5510* gene in *B.*
354 *anthracis*) possessed 2 and 386 point mutations, respectively, compared to *B.*
355 *anthracis*. The primer (BA5510-1/2) and probe sequences (BA5510-FL/BA5510-640)
356 showed 100 % identity to the DNA sequence from AH820. However, nine and eight
357 point mutations in primer and probe sequence, respectively, were identified in the
358 *tagH* gene in the E33L strain. These findings strongly suggest that the primer
359 BA5510-1/2 will result in an amplification of the AH820 genome, but this needs to be
360 elaborated regarding the E33L strain. Unfortunately, we were not able perform the
361 necessary real-time PCR analysis to verify these findings, due to not being in
362 possession of these strains.
363 Bode et al. (2004) have reported a real-time PCR assay using a single
364 chromosomal primer set, without the use of probes, resulting in an amplification of a
365 wrong-sized fragment of only one non-*B. anthracis* strain (ATCC21771) (100% *B.*
366 *anthracis* specific if probe was used). However, in that study only 62 non-*B. anthracis*
367 strains were screened. Several other chromosomal markers (BA813, rpoB, gyrA) have
368 been published, but a common feature is their lack of specificity for *B. anthracis*
369 (Ellerbrok et al., 2002, Qi et al., 2001, Ramisse et al., 1999) or their need for a probe-
370 based PCR analysis for single-nucleotide differentiation (Easterday et al., 2005). The
371 chromosomal marker targeting the *saspB* gene (Hoffmaster et al., 2002) has not
372 reported any amplification of non-*B. anthracis* strains, but the primer and probe
373 sequences targeting this gene have not been stated in the publications (Hoffmaster et

374 al., 2002, Marston et al., 2005). This study shows that the constructed BA5510-1/2

375 primer set has a strong potential of being a *B. anthracis* specific marker. The primers

376 possess very high discriminating power. However, use of probes is recommended to

377 avoid possible amplification of non-*B. anthracis* strains, despite there was obtained a

378 fluorescent signal for the NVH492 strain in this study.

379 We here describe an initial *adk*-screening method for identifying closely

380 related *B. anthracis* strains belonging to the *B. cereus* group. Strains closely related to

381 *B. anthracis* were further analysed by using an ordinary MLST analysis, followed by

382 a comparison of the obtained allele profiles to previously published MLST data for *B.*

383 *cereus* group members. This is a convenient method to reveal the genetic distribution

384 of a large collection of *B. cereus* group members. Furthermore, this genetic

385 distribution was used for evaluating a novel *B. anthracis* specific chromosomal

386 marker, targeting the *BA5510* gene. To our knowledge, the BA5510-1/2 primer set

387 seems to be one of the most unique chromosomal marker available, for specific

388 identification of *B. anthracis.* This is based on the evaluation against a total of 288

389 non-*B. anthracis* strains, in which several are closely related to *B. anthracis*. Only one

390 amplified product was obtained among 288 non-*B. anthracis* indicating that a cross-

391 reaction to *B. cereus* group members is exceptionally low.

392

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401 **References**

402 Agaisse, H., Gominet, M., Okstad, O.A., Kolsto, A.B., Lereclus, D., 1999. PlcR is a
403 pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus*
404 *thuringiensis*. Mol. Microbiol. 32, 1043-1053.
405
406 Anderson Borge, G.I., Skeie, M., Sorhaug, T., Langsrud, T., Granum, P.E., 2001.
407 Growth and toxin profiles of *Bacillus cereus* isolated from different food sources. Int.
408 J. Food. Microbiol. 69, 237-246.
409
410 Bode, E., Hurtle, W., Norwood, D., 2004. Real-time PCR assay for a unique
411 chromosomal sequence of *Bacillus anthracis*. J. Clin. Microbiol. 42, 5825-5831.
412
413 Cherif, A., Borin, S., Rizzi, A., Ouzari, H., Boudabous, A., Daffonchio, D., 2003.
414 *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16S-
415 23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. Appl.
416 Environ. Microbiol. 69, 33-40.
417
418 Di Franco, C., Beccari, E., Santini, T., Pisaneschi, G., Tecce, G., 2002. Colony shape
419 as a genetic trait in the pattern-forming *Bacillus mycoides*. BMC Microbiol. 13, 33.
420 Epub.
421

422 Drobniewski, F.A., 1993. *Bacillus cereus* and related species. Clin. Microbiol. Rev. 6,
423 324-338.
424
425 Dwyer, K.G., Lamonica, J.M., Schumacher, J.A., Williams, L.E., Bishara, J.,
426 Lewandowski, A., Redkar, R., Patra, G., DelVecchio, V.G., 2004. Identification of
427 *Bacillus anthracis* specific chromosomal sequences by suppressive subtractive
428 hybridization. BMC Genomics. 5, 15.
429

430 Easterday, W.R., Van Ert, M.N., Simonson, T.S., Wagner, D.M., Kenefic, L.J.,
431 Allender, C.J., Keim, P., 2005. Use of Single Nucleotide Polymorphisms in the *plcR*
432 Gene for Specific Identification of *Bacillus anthracis.* J. Clin. Microbiol. 43, 1995-
433 1997.

434
435 Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B., Pauli, G., 2002. Rapid
436 and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-
437 time PCR. FEMS Microbiol. Lett. 214, 51-59.
438
439 Farrar, W.E., Reboli, A.C., 1992. The Genus *Bacillus*-Medical. In: Balows, A.,
440 Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H., (Eds.). The Prokaryotes: a
441 handbook on the biology of bacteria: ecophysiology, isolation, identification,
442 applications. New York, Springer-Verlag, pp. 1758-1759.
443

444 Granum, P.E., 2007. *Bacillus cereus*. In: Doyle, M.P and Beuchat, L.R., (Eds), Food
445 Microbiology. Fundamentals and Frontiers, 3. edition. ASM Press, Washington DC,
446 pp. 445-455.
447
448 Han, C.S., Xie, G., Challacombe, J.F., Altherr, M.R., Bhotika, S.S., Brown, N., Bruce,
449 D., Campbell, C.S., Campbell, M.L., Chen, J., Chertkov, O., Cleland, C., Dimitrijevic,
450 M., Doggett, N.A., Fawcett, J.J., Glavina, T., Goodwin, L.A., Green, L.D., Hill, K.K.,
451 Hitchcock, P., Jackson, P.J., Keim, P., Kewalramani, A.R., Longmire, J., Lucas, S.,
452 Malfatti, S., McMurry, K., Meincke, L.J., Misra, M., Moseman, B.L., Mundt, M.,
453 Munk, A.C., Okinaka, R.T., Parson-Quintana, B., Reilly, L.P., Richardson, P.,
454 Robinson, D.L., Rubin, E., Saunders, E., Tapia, R., Tesmer, J.G., Thayer, N.,
455 Thompson, L.S., Tice, H., Ticknor, L.O., Wills, P.L., Brettin, T.S., Gilna, P., 2006.
456 Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates
457 closely related to *Bacillus anthracis*. J. Bacteriol. 188, 3382-3390.
458
459 Harrell, L.J., Andersen, G.L., Wilson, K.H., 1995. Genetic variability of *Bacillus*
460 *anthracis* and related species. J. Clin. Microbiol. 33, 1847-1850.
461
462 Helgason, E., Caugant, D.A., Lecadet, M.M., Chen, Y., Mahillon, J., Lovgren, A.,
463 Hegna, I., Kvaloy, K., Kolsto, A.B., 1998. Genetic diversity of *Bacillus cereus/B.*
464 *thuringiensis* isolates from natural sources. Curr. Microbiol. 37, 80-87.
465
466 Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M.,
467 Hegna, I., Kolsto,.A.B., 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus*

468 *thuringiensis*-one species on the basis of genetic evidence. Appl. Environ. Microbiol.
469 66, 2627-2630.
470
471 Helgason, E., Tourasse, N.J., Meisal, R., Caugant, D.A., Kolsto, A.B., 2004.
472 Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl.
473 Environ. Microbiol. 70, 191-201.
474
475 Hernandez, E., Ramisse, F., Ducoureau, J.P., Cruel, T., Cavallo, J.D., 1998. *Bacillus*
476 *thuringiensis* subsp. konkukian (serotype H34) superinfection: case report and
477 experimental evidence of pathogenicity in immunosuppressed mice. J. Clin.
478 Microbiol. 36, 2138-2139.
479
480 Hill, K.K., Ticknor, L.O., Okinaka, R.T., Asay, M., Blair, H., Bliss, K.A., Laker, M.,
481 Pardington, P.E, Richardson, A.P., Tonks, M., Beecher, D.J., Kemp, J.D., Kolsto,
482 A.B., Wong, A.C., Keim, P., Jackson, P.J., 2004. Fluorescent amplified fragment
483 length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus*
484 *thuringiensis* isolates. Appl. Environ. Microbiol. 70, 1068-1080.
485
486 Hoffmaster, A.R., Hill, K.K., Gee, J.E., Marston, C.K., De, B.K., Popovic, T., Sue,
487 D., Wilkins, P.P., Avashia, S.B., Drumgoole, R., Helma, C.H., Ticknor, L.O.,
488 Okinaka, R.T., Jackson, P.J., 2006. Characterization of *Bacillus cereus* isolates
489 associated with fatal pneumonias: strains are closely related to *Bacillus anthracis* and
490 harbor *B. anthracis* virulence genes. J. Clin. Microbiol. 44, 3352-3360.
491

492 Hoffmaster, A.R., Meyer, R.F., Bowen, M.D., Marston, C.K., Weyant, R.S.,
493 Thurman, K., Messenger, S.L., Minor, E.E., Winchell, J.M., Rassmussen, M.V.,
494 Newton, B.R., Parker, J.T., Morrill, W.E., McKinney, N., Barnett, G.A., Sejvar, J.J.,
495 Jernigan, J.A., Perkins, B.A., Popovic, T., 2002. Evaluation and validation of a real-
496 time polymerase chain reaction assay for rapid identification of *Bacillus anthracis*.
497 Emerg. Infect. Dis. 8, 1178-1182.
498

499 Hoffmaster, A.R., Ravel, J., Rasko, D.A., Chapman, G.D., Chute, M.D., Marston,
500 C.K., De, B.K., Sacchi, C.T., Fitzgerald, C., Mayer, L.W., Maiden, M.C., Priest, F.G.,
501 Barker, M., Jiang, L., Cer, R.Z., Rilstone, J., Peterson, S.N., Weyant, R.S., Galloway,
502 D.R., Read, T.D., Popovic, T., Fraser, C.M., 2004. Identification of anthrax toxin
503 genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax.
504 Proc. Natl. Acad. Sci. 101, 8449-8454.
505

506 Hoffmaster, A.R., Ravel, J., Rasko, D.A., Chapman, G.D., Chute, M.D.,
507 Hurtle, W., Bode, E., Kulesh., D.A., Kaplan, R.S., Garrison, J., Bridge, D., House,
508 M., Frye, M.S., Loveless, B., Norwood, D., 2004. Detection of the *Bacillus anthracis*
509 *gyrA* gene by using a minor groove binder probe. J. Clin. Microbiol. 42, 179-185.
510

511 Inglesby, T.V., O'Toole, T., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E.,
512 Friedlander, A.M., Gerberding, J., Hauer, J., Hughes, J., McDade, J., Osterholm,
513 M.T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K.; Working Group on Civilian
514 Biodefense. 2002. Anthrax as a biological weapon, 2002: updated recommendations
515 for management. JAMA. 287, 2236-2252.
516

517 Jacobsson, S., Issa, M., Unemo, M., Backman, A., Molling, P., Sulaiman, N., Olcen,
518 P., 2003. Molecular characterisation of group A *Neisseria meningitidis* isolated in
519 Sudan 1985-2001. APMIS. 111, 1060-1066.
520
521 Kim, K., Seo, J., Wheeler, K., Park, C., Kim, D., Park, S., Kim, W., Chung, S.I.,
522 Leighton, T., 2005. Rapid genotypic detection of *Bacillus anthracis* and the *Bacillus*
523 *cereus* group by multiplex real-time PCR melting curve analysis. FEMS Immunol.
524 Med. Microbiol. 43, 301-310.
525
526 Ko, K.S., Kim, J.W., Kim, J.M., Kim, W., Chung, S.I., Kim, I.J., Kook, Y.H., 2004.
527 Population structure of the *Bacillus cereus* group as determined by sequence analysis
528 of six housekeeping genes and the *plcR* gene. Infect. Immun. 72, 5253-5261.
529
530 Kotetishvili, M., Kreger, A., Wauters, G., Morris, J.G. Jr., Sulakvelidze, A., Stine,
531 O.C., 2005. Multilocus sequence typing for studying genetic relationships among
532 *Yersinia* species. J. Clin. Microbiol. 43, 2674-2684.
533
534 Kotetishvili, M., Stine, O. C., Chen, Y., Kreger, A., Sulakvelidze, A., Sozhamannan,
535 S., Morris, J.G. Jr., 2003. Multilocus sequence typing has better discriminatory ability
536 for typing *Vibrio cholerae* than does pulsed-field gel electrophoresis and provides a
537 measure of phylogenetic relatedness. J. Clin. Microbiol. 41, 2191-2196.
538
539 Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular
540 Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 5, 150-
541 163.

542

543 Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang,
544 Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G.**,**
545 1998. Multilocus sequence typing: a portable approach to the identification of clones
546 within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. 95**,** 3140-
547 3145.

548

549 Manning, G., Dowson, C.G., Bagnall, M.C., Ahmed, I.H., West, M., Newell, D.G.,
550 2003. Multilocus sequence typing for comparison of veterinary and human isolates of
551 *Campylobacter jejuni*. Appl. Environ. Microbiol. 69, 6370-6379.

552

553 Marston, C.K., Gee, J.E., Popovic, T., Hoffmaster, A.R., 2006. Molecular approaches
554 to identify and differentiate *Bacillus anthracis* from phenotypically similar *Bacillus*
555 species isolates. BMC Microbiol. 3, 22.

556

557 Nakamura, L.K., 1998. *Bacillus pseudomycoides* sp. Int. J. Syst. Bacteriol. 48, 1031-
558 1035.

559

560 Pillai, A., Thomas, S., Arora, J., 2006. *Bacillus cereus*: the forgotten pathogen. *Surg.*
561 *Infect. Larchmt.* **7**, 305-308.

562

563 Priest, F.G., Barker, M., Baillie, L.W., Holmes, E.C., Maiden, M.C., 2004. Population
564 structure and evolution of the *Bacillus cereus* group. J. Bacteriol. 186, 7959-7970.

565

566 Qi, Y., Patra, G., Liang, X., Williams, L.E., Rose, S., Redkar, R.J., DelVecchio, V.G.,
567 2001. Utilization of the *rpoB* gene as a specific chromosomal marker for real-time
568 PCR detection of *Bacillus anthracis*. Appl. Environ. Microbiol. 67, 3720-3727.
569
570 Ramisse, V., Patra, G., Garrigue, H., Guesdon, J.L., Mock, M., 1996. Identification
571 and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on
572 plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiol. Lett. 145, 9-16.
573
574 Ramisse, V., Patra, G., Vaissaire, J., Mock, M., 1999. The Ba813 chromosomal DNA
575 sequence effectively traces the whole *Bacillus anthracis* community. J. Appl.
576 Microbiol. 87, 224–228.
577
578 Rasko, D.A., Altherr, M.R., Han, C.S., Ravel. J., 2005. Genomics of the *Bacillus*
579 *cereus* group of organisms. FEMS Microbiol. Rev. 29, 303-329.
580
581 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for
582 reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.
583
584 Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler,
585 D. R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins.
586 Microbiol. Mol. Biol. Rev. 62, 775-806.
587
588 Schoeni, J.L., Wong, A.C., 2005. *Bacillus cereus* food poisoning and its toxins.
589 Review. J. Food. Prot. 68, 636-648.
590

591 Staden, R. 1996. The Staden sequence analysis package. Mol. Biotechnol. 5, 233-241.

592

593 Stenfors, L.P., Mayr, R., Scherer, S., Granum, P.E., 2002. Pathogenic potential of

594 fifty *Bacillus weihenstephanensis* strains. FEMS Microbiol. Lett., 215, 47-51.

595

596 Tartofm, S.Y., Solberg, O.D., Manges, A.R., Riley, L.W., 2005. Analysis of a

597 uropathogenic *Escherichia coli* clonal group by multilocus sequence typing. J. Clin.

598 Microbiol. 43, 5860-5864.

599

600 Valjevac, S., Hilaire, V., Lisanti, O., Ramisse, F., Hernandez, E., Cavallo, J.D.,

601 Pourcel, C., Vergnaud, G., 2005. Comparison of minisatellite polymorphisms in the

602 *Bacillus cereus* complex: a simple assay for large-scale screening and identification of

603 strains most closely related to *Bacillus anthracis*. Appl. Environ. Microbiol. 71, 6613-

604 6623.

605 Table 1

606 *B. cereus* group strains used in this study.

Strain	Species	Strain	Species	Strain	Species	Strain	Species	Strain	Species	Strain	Species
ATCC14579	BC	B349	BC	NVH225	BC	AH652	Bspp.	BGSC4AS1	BT	AH 1144	BW
ATCC10987	BC	B350	BC	NVH237	BC	AH653	Bspp.	BGSC4AT1	BT	AH 1145	BW
AH225	BC	B365	BC	NVH357	BC	AH659	Bspp.	BGSC4AU1	BT	AH 1146	BW
AH228	BC	B366	BC	NVH358	BC	AH663	Bspp.	BGSC4AV1	BT	CCM 4965	BW
AH229	BC	B367	BC	NVH360	BC	AH665	Bspp.	BGSC4AW1	BT	AH 338	BM
AH231	BC	B379	BC	NVH369	BC	AH670	Bspp.	BGSC4AX1	BT	NVH 698	BM
AH232	BC	BGSC6A1	BC	NVH394	BC	AH672	Bspp.	BGSC4AY1	BT	NVH 703	BM
AH233	BC	BGSC6A3	BC	NVH403	BC	AH680	Bspp.	BGSC4AZ1	BT	NVH 710	BM
AH234	BC	BGSC6A4	BC	NVH404	BC	AH690	Bspp.	BGSC4AH1	BT	NVH 720	BM
AH259	BC	BGSC6A6	BC	NVH447	BC	AH691	Bspp.	BGSC4B1	BT	NVH 726	BM
AH1082	BC	BGSC6A7	BC	NVH492	BC	AH694	Bspp.	BGSC4C1	BT	CIP.7700	BA
AH1083	BC	BGSC6A8	BC	NVH519	BC	B285	BT	BGSC4C3	BT	NVH 246-02	BC

AH1084	BC	BGSC6A9	BC	NVH626	BC	B302	BT	BGSC4D1	BT	A15	BA		
AH1085	BC	BGSC6E1	BC	NVH655	BC	B508	BT	BGSC4D11	BT	A58	BA		
AH1086	BC	BGSC6S1	BC	NVH785	BC	B509	BT	BGSC4D22	BT	A73	BA		
AH1087	BC	NVH0391-98	BC	NVH789	BC	B510	BT	BGSC4E1	BT	DSM318	BC		
B275	BC	NVH0075-95	BC	NVH795	BC	B511	BT	BGSC4E5	BT	DSM336	BC		
B276	BC	NVH0139-00	BC	NVH805	BC	B512	BT	BGSC4F1	BT	E33L	BC		
B277	BC	NVH0154-01	BC	NVH838	BC	B513	BT	BGSC4G1	BT	Bt9727	BT		
B278	BC	NVH0165-99	BC	NVH859	BC	B525	BT	BGSC4G2	BT	G9241	BC		
B279	BC	NVH0226-00	BC	NVH862	BC	B526	BT	BGSC4H1	BT				
B280	BC	NVH1125-97	BC	NVH864	BC	B527	BT	BGSC4I1	BT				
B281	BC	NVH1203-97	BC	NVH871	BC	B528	BT	BGSC4J1	BT				
B282	BC	NVH559-97	BC	NVH900	BC	B529	BT	BGSC4L1	BT				
B283	BC	NVH504-96	BC	NVH905	BC	B530	BT	BGSC4M1	BT				
B284	BC	NVH0674-98	BC	NVH906	BC	B531	BT	BGSC4N1	BT				
B3	BC	NVH1230-88	BC	NVH907	BC	B532	BT	BGSC4O1	BT				
B300	BC	NVH0784-00	BC	NVH908	BC	B533	BT	BGSC4P1	BT				
B301	BC	NVH0883-00	BC	NVH910	BC	B534	BT	BGSC4Q1	BT				
B305	BC	NVH0712-01	BC	NVH911	BC	B535	BT	BGSC4Q7	BT				
B306	BC	NVH1411-01	BC	NVH912	BC	B536	BT	BGSC4Q8	BT				
B307	BC	NVH1651-00	BC	NVH914	BC	B537	BT	BGSC4R1	BT				
B308	BC	NVH0230-00	BC	NVH915	BC	B538	BT	BGSC4S2	BT				
B309	BC	NVH1519-00	BC	NVH916	BC	B541	BT	BGSC4T1	BT				
B310	BC	NVH1039-00	BC	IAM1110	BC	B543	BT	BGSC4U1	BT				
B311	BC	NVH0309-98	BC	AH525	Bspp.	BGSC4A1	BT	BGSC4V1	BT				
B312	BC	NVH0861-00	BC	AH528	Bspp.	BGSC4A3	BT	BGSC4W1	BT				
B313	BC	NVH0500-00	BC	AH550	Bspp.	BGSC4A4	BT	BGSC4X1	BT				
B314	BC	NVH1518-99	BC	AH558	Bspp.	BGSC4A5	BT	BGSC4Y1	BT				
B315	BC	NVH1280-99	BC	AH562	Bspp.	BGSC4A7	BT	BGSC4Z1	BT				
B316	BC	NVH1087-99	BC	AH588	Bspp.	BGSC4A8	BT	T01246	BT				
B317	BC	NVH0597-99	BC	AH620	Bspp.	BGSC4A9	BT	T04B001	BT				
B318	BC	NVH1104-98	BC	AH621	Bspp.	BGSC4AC1	BT	T08001	BT				
B319	BC	NVH1105-98	BC	AH623	Bspp.	BGSC4AD1	BT	T26001	BT				
B320	BC	NVH108	BC	AH624	Bspp.	BGSC4AF1	BT	NVH209	BT				
B321	BC	NVH112	BC	AH625	Bspp.	BGSC4AG1	BT	NVH214	BT				
B340	BC	NVH116	BC	AH626	Bspp.	BGSC4AJ1	BT	NVH217	BT				
B341	BC	NVH120	BC	AH629	Bspp.	BGSC4AK1	BT	NVH472	BT				

B342	BC	NVH124	BC	AH630	Bspp.	BGSC4AL1	BT	10204	BW
B343	BC	NVH126	BC	AH631	Bspp.	BGSC4AM1	BT	10381	BW
B344	BC	NVH152	BC	AH635	Bspp.	BGSC4AN1	BT	10387	BW
B345	BC	NVH155	BC	AH638	Bspp.	BGSC4AO1	BT	10396	BW
B346	BC	NVH163	BC	AH642	Bspp.	BGSC4AP1	BT	10403	BW
B347	BC	NVH167	BC	AH645	Bspp.	BGSC4AQ1	BT	10408	BW
B348	BC	NVH199	BC	AH650	Bspp.	BGSC4AR1	BT	10409	BW

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610 Veterinary Science, Oslo, Norway, **CIP**; Department of Analytical Microbiology,
611 CEB, Paris, France, **A**; Dr. Wolfgang Beyer, Hohenheim University, Germany,
612 **ATCC**; American Type Culture Collection, University Boulevard, Manassas, USA,
613 **DSM**; German Collection of Microorganisms and Cell Cultures (DSMZ),
614 Braunschweig, Germany. BC: *B. cereus*, BT: *B. thuringiensis*, BW: *B.*
615 *weihenstephanensis*, BM: *B. mycoides*, BA: *B. anthracis*, Bspp.: *Bacillus* spp.

616

617 Table 2

618 Primer and probe sequences and optimal conditions for real-time PCR analysis.

Primer /Probe	Target genes	Primers (5'→3')	Product size (bp)	Primer-conc. (µM)	Mg^{2+} (mM)	Anneal. temp. (°C)
adk-f/r	*adk*	f: CAGCTATGAAGGCTGAAACTG r: CTAAGCCTCCGATGAGAACA	450[a]	1,0	3,0	57
ccpA-f/r	*ccpA*	f: GTTTAGGATACCGCCCAAATG r: TGTAACTTCTTCGCGCTTCC	418[a]	1,5	2,5	56
ftsA-f/r	*ftsA*	f: TCTTGACATCGGTACATCCA r: GCCTGTAATAAGTGTACCTTCCA	401[a]	1,5	2,0	54
glpT-f/r	*glpT*	f: TGCGGCTGGATGAGTGA r: AAGTAAGAGCAAGGAAGA	330[a]	0,25	2,5	56
pyrE-f/r	*pyrE*	f: TCGCATCGCATTTATTAGAA	404[a]	0,75	2,5	56

		r: CCTGCTTCAAGCTCGTATG				
recF-f/r	*recF*	f: GCGATGGCGAAATCTCATAG	470 [a]	1,5	3,0	56
		r: CAAATCCATTGATTCTGATACATC				
sucC-f/r	*sucC*	f: GGCGGAACAGAAATTGAAGA	504 [a]	0,5	3,5	58
		r: TCACACTTCATAATGCCACCA				
BA813-f/r	*rpoB*	f: TTAATTCACTTGCAACTGATGGG	152	1,0	1,0	58
		r: AACGATAGCTCCTACATTTGGAG				
BAlef-f*/r	*lef*	f*: GCAGATTCCTATTGAGCCAAA [b]	156	1,0	1,0	58
		r: GAATCACGAATATCAATTTGTAGC				
BAcap-1/2*	*cap*	1: ACTCGTTTTTAATCAGCCCG	126	0,2	1,0	58
		2*: GTTGCCGCAAATTTTCTACG [b]				
plcR-f/r	*plcR*	f: CCAATCAATGTCATACTATTAATTTGACAC	103	1,0	1,0	60
		r: ATGCAAAAGCATTATACTTGGACAAT				
BA5510-1/2	*BA5510*	1: CTGCATTGATAGCAATTTCTTCA	162	1,0	1,0	58
		2: CAGGTTGATACATAAACTTTCCA				
BA5510-FL	*BA5510*	GTAATTCCCATCATTAAACCTTTTAATTCGATAT-FL	-	0,2	1,0	58
BA5510-640		CAATCCCTGTTAATTGACCATTAAGCC-640				

619 [a] Fragment size used for MLST analysis. The amplified products contain 30-100

620 additional basepairs.

621 [b] Primers modified in this study.

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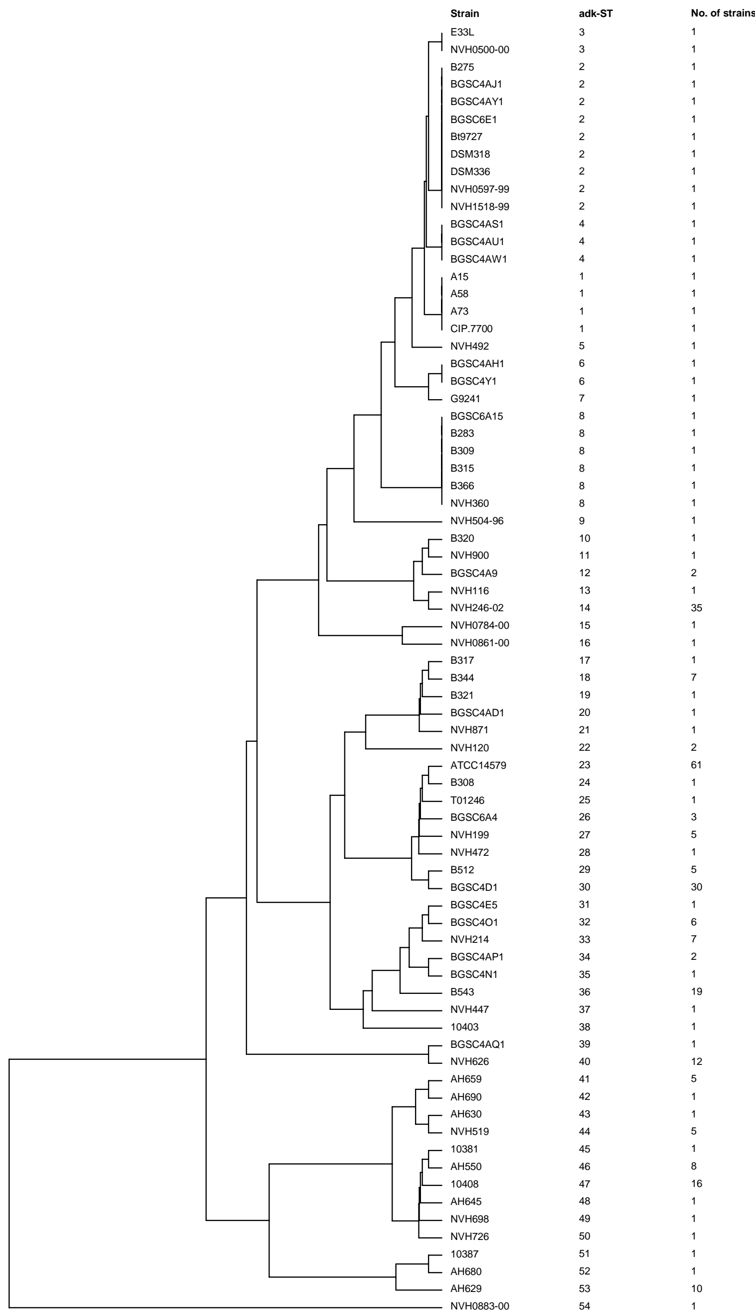
Fig. 1 .Genetic distribution of 295 *B. cereus* group members deduced from *adk*-screening.



642 Fig. 2. Dendrogram deduced from MLST of 104 *B. cereus* group strains.

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