

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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## 27 **Abstract**

28           The genetic distribution of 295 *Bacillus cereus* group members has been  
29 investigated by using a modified multilocus sequence typing method (MLST). By  
30 comparing the nucleic acid sequence of the *adk* gene fragment, isolates of *B. cereus*  
31 group members most related to *B. anthracis* may be easily identified. The genetic  
32 distribution, with focus on the *B. anthracis* close neighbours, was used to evaluate a  
33 new primer set for specific identification of *B. anthracis*. This primer set, BA5510-  
34 1/2, targeted the putative *B. anthracis* specific gene *BA5510*. Real-time PCR using  
35 BA5510–1/2 specifically amplified the target fragment from all *B. anthracis* strains  
36 tested and only one (of 288) non-*B. anthracis* strains analysed. This is one of the most  
37 thoroughly validated chromosomal *B. anthracis* markers for real-time PCR  
38 identification, in which the screened collection contained several very closely related  
39 *B. anthracis* strains.

40

## 41 **Introduction**

42           The *Bacillus cereus* group consists of six bacterial species; *B. anthracis*, *B.*  
43 *cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*.  
44 *B. anthracis* is the causative agent of anthrax, a severe disease of great concern to  
45 public health authorities. It is also of military concern due to its potential as a  
46 biological weapon agent (Inglesby et al., 2002). *B. cereus*, producing an emetic toxin  
47 and enterotoxins, is an opportunistic human pathogen frequently associated with food  
48 poisoning (Granum 2007, Schoeni and Wong 2005). This species has also been  
49 connected to periodontal-, eye- and wound infections (Drobniewski 1993, Pillai et al.,  
50 2006). *B. thuringiensis* is used as an insecticide with great economic advantages based  
51 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 evolutionary 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 32°C overnight. In general, cells  
112 from one single colony were inoculated in 10 ml BHI-broth and grown at 32°C  
113 overnight.

114 The strains clustering close to *B. anthracis* were streaked on sheep blood  
115 agar, and grown at 32 °C 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 °C 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 (*adhk*  
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<sup>2+</sup>- and primer/probe concentrations are presented in Table 2. The  
140 temperature program was as follows: 5 min initial denaturation at 95°C followed by  
141 35 cycles of denaturation at 95°C for 10 sec, annealing at individual temperatures  
142 (Table 2) for 10 sec and primer extension at 72°C for 25-35 sec. The amplifications  
143 were terminated after a final elongation of 5 min at 72 °C. 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 *adh* 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 tree (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 teichoic 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<sup>+</sup>) and  
278 A73 (pXO2<sup>+</sup>), 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 strains 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 BGSC4AJ1 strain 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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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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608 Institute of Microbiology, Munich, Germany, **BGSC**; Bacillus Genetic Stock Centre,  
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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. ( $\mu$ M)	Mg <sup>2+</sup> (mM)	Anneal. temp. (°C)
adk-f/r	<i>adk</i>	f: CAGCTATGAAGGCTGAAACTG r: CTAAGCCTCCGATGAGAACA	450 <sup>a</sup>	1,0	3,0	57
ccpA-f/r	<i>ccpA</i>	f: GTTTAGGATACCGCCCAAATG r: TGTAACCTCTTCGCGCTTCC	418 <sup>a</sup>	1,5	2,5	56
ftsA-f/r	<i>ftsA</i>	f: TCTTGACATCGGTACATCCA r: GCCTGTAATAAGTGACCTTCCA	401 <sup>a</sup>	1,5	2,0	54
glpT-f/r	<i>glpT</i>	f: TGCGGCTGGATGAGTGA r: AAGTAAGAGCAAGGAAGA	330 <sup>a</sup>	0,25	2,5	56
pyrE-f/r	<i>pyrE</i>	f: TCGCATCGCATTTATTAGAA	404 <sup>a</sup>	0,75	2,5	56

		r: CCTGCTCAAGCTCGTATG				
recF-f/r	<i>recF</i>	f: GCGATGGCGAAAATCTCATAG	470 <sup>a</sup>	1,5	3,0	56
		r: CAAATCCATTGATTCTGATACATC				
sucC-f/r	<i>sucC</i>	f: GCGGGAACAGAAATTGAAGA	504 <sup>a</sup>	0,5	3,5	58
		r: TCACACTTCATAATGCCACCA				
BA813-f/r	<i>rpoB</i>	f: TTAATTCACCTTGCAACTGATGGG	152	1,0	1,0	58
		r: AACGATAGCTCCTACATTTGGAG				
BAlef-f*/r	<i>lef</i>	f*: GCAGATTCCTATTGAGCCAAA <sup>b</sup>	156	1,0	1,0	58
		r: GAATCACGAATATCAATTTGTAGC				
BAcap-1/2*	<i>cap</i>	1: ACTCGTTTTTAATCAGCCCG	126	0,2	1,0	58
		2*: GTTGCCGCAAATTTTCTACG <sup>b</sup>				
plcR-f/r	<i>plcR</i>	f: CCAATCAATGTCATACTATTAATTTGACAC	103	1,0	1,0	60
		r: ATGCAAAAGCATTATACTTGGACAAT				
BA5510-1/2	<i>BA5510</i>	1: CTGCATTGATAGCAATTTCTCA	162	1,0	1,0	58
		2: CAGGTTGATACATAAACTTTCCA				
BA5510-FL	<i>BA5510</i>	GTAATCCCATCATTAACCTTTTAATTCGATAT-FL	-	0,2	1,0	58
BA5510-640		CAATCCCTGTTAATTGACCATTAAGCC-640				

619 <sup>a</sup> Fragment size used for MLST analysis. The amplified products contain 30-100

620 additional basepairs.

621 <sup>b</sup> Primers modified in this study.

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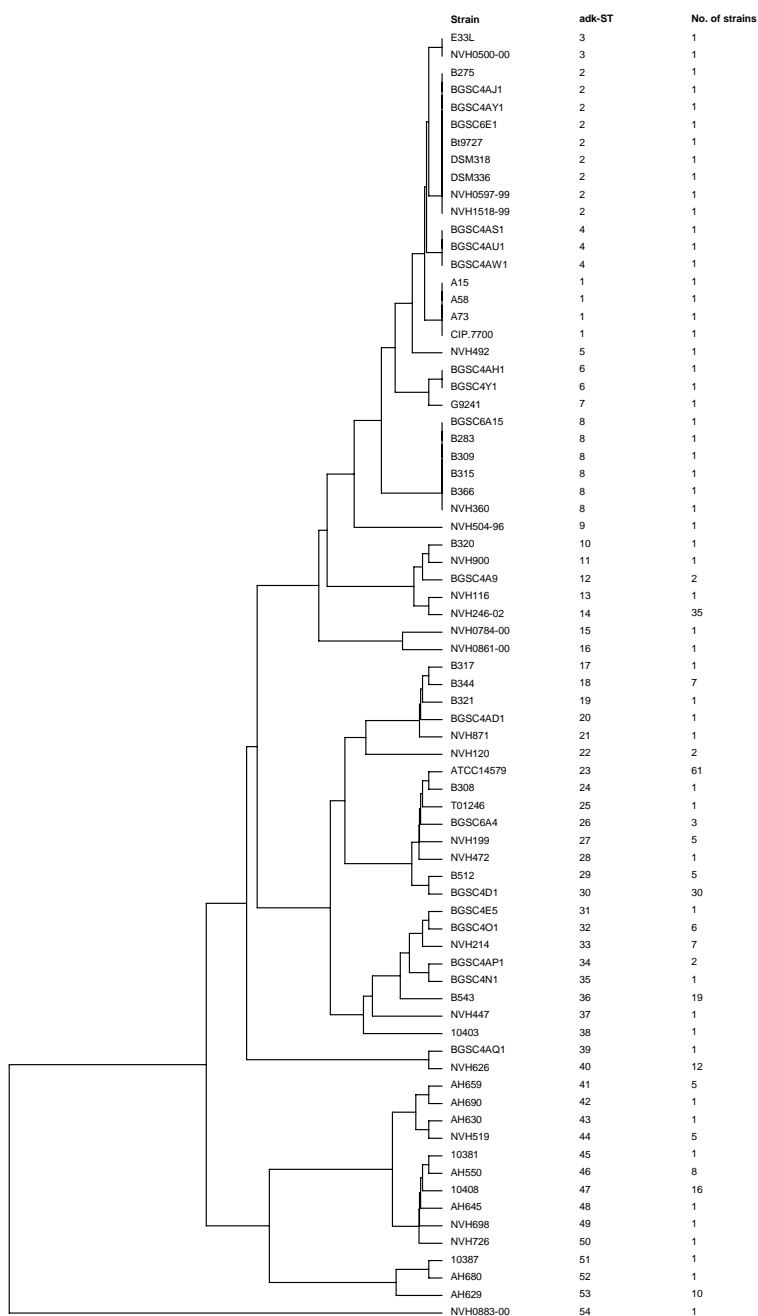
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633 Fig. 1 .Genetic distribution of 295 *B. cereus* group members deduced from *adk*-  
 634 screening.

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

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