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# Comparison of Minisatellite Polymorphisms in the *Bacillus cereus* Complex: a Simple Assay for Large-Scale Screening and Identification of Strains Most Closely Related to *Bacillus anthracis*

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**Polymorphism of five tandem repeats that are monomorphic in *Bacillus anthracis* was investigated in 230 isolates of the *B. cereus* group and in 5 sequenced *B. cereus* genomes in search for markers allowing identification of *B. cereus* and *B. thuringiensis* strains most closely related to *B. anthracis*. Using this multiple-locus variable number of tandem repeat analysis (MLVA), a cluster of 30 strains was selected for further characterization. Eventually, six of these were characterized by multilocus sequence type analysis. One of the strains is only six point mutations (of almost 3,000 bp) away from *B. anthracis* and was also proposed to be closest to *B. anthracis* by MLVA analysis. However, this strain remains separated from *B. anthracis* by a number of significant genetic events observed in *B. anthracis*, including the loss of the hemolysin activity, the presence of four prophages, and the presence of the two virulence plasmids, pXO1 and pXO2. One particular minisatellite marker provides an efficient assay to identify the subset of *B. cereus* and *B. thuringiensis* strains closely related to *B. anthracis*. Based on these results, a very simple assay is proposed that allows the screening of hundreds of strains from the *B. cereus* complex, with modest equipment and at a low cost, to eventually fill the gap with *B. anthracis* and better understand the origin and making of this dangerous pathogen.**

*Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* are spore-forming gram-positive bacteria belonging to the *Bacillus cereus* group. *B. thuringiensis* is an insect pathogen producing plasmid-encoded endotoxins and is widely used as a biopesticide against lepidopteran, dipteran, and coleopteran insect pests (36). *B. cereus* produces an enterotoxin and an emetic toxin that are responsible for diarrhea and emetic syndromes, characteristic of bacillus-associated gastrointestinal illness. *B. cereus* is also responsible for a variety of nongastrointestinal diseases (9). *B. anthracis* causes anthrax, a lethal disease in humans and other mammals. Three types of anthrax infection can occur: inhalational, cutaneous, and gastrointestinal. As a result of its high pathogenicity and the possible scattering of its spores, *B. anthracis* could be used as a biological weapon (19).

*B. anthracis* appears to be genetically extremely close to some members of the *B. cereus-B. thuringiensis* group (14). Previous studies based upon multilocus enzyme electrophoresis (MEE) and sequence analysis (14), sequencing of 16S rRNA (2), and pulsed-field gel electrophoresis/MEE analysis (3) lead to the suggestion that these three bacteria belong to the same species and that some of the key phenotypic properties are conferred usually by plasmids. The loss of these plasmids leads to the inability to differentiate the *B. cereus* and *B. thuringiensis* strains (3, 14). Moreover, *B. cereus* strains can be converted into crystal producers by means of plasmid transfer (12), and some strains of *B. thuringiensis* were also tested for their ability to produce a diarrhea-causing enterotoxin (7).

The presence of the two plasmids pXO1 and pXO2 is essential to the pathogenicity of *B. anthracis*. Detection of these plasmids in association with a chromosomal marker is a common method to distinguish *B. anthracis* from *B. cereus* and *B. thuringiensis* (32, 33). However, distinction between species is sometimes difficult, and an illness resembling anthrax was shown to be caused by a *B. cereus* strain (strain G9241) possessing a plasmid with 99.6% similarity to pXO1 (18). This strain was identified by 16S rRNA analysis and by its phenotype as a *B. cereus* strain, and its virulence was confirmed in A/J mice. Another *B. cereus* strain, ZK, whose genome was recently sequenced, was responsible for an anthrax-like illness in a zebra (GenBank accession number NC_006274). The pathogenicity in immunosuppressed mice of the *B. thuringiensis* 97-27 (subsp. *konkukian* serotype H34), named here CEB97/27 (originally a Centre d'Etudes du Bouchet strain collection name), was also reported by Hernandez et al. (16). This strain was identified as closely related to *B. anthracis* by suppression subtractive hybridization (31) and by amplified fragment length polymorphism (AFLP) (17).

Several molecular methods have been developed to analyze genetic diversity inside the *B. cereus* group and to distinguish *B. anthracis* from the others: AluI restriction of a randomly amplified polymorphic DNA marker specific for the *B. cereus* complex (6), the restriction site insertion-PCR method (5),

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TABLE 1. Reference strains analyzed in this study[a]

| Reference | Species | Source | ID |
|---|---|---|---|
| 02/003 | *B. thuringiensis* | 1 | H1 serovar *thuringiensis* |
| 02/004 | *B. thuringiensis* | 1 | H2 serovar *finitimus* |
| 02/005 | *B. thuringiensis* | 1 | H3aH3c serovar *alesti* |
| 02/006 | *B. thuringiensis* | 1 | H3aH3bH3c serovar *kurstaki* |
| 02/007 | *B. thuringiensis* | 1 | H3aH3d serovar *sumiyoshiensis* |
| 02/008 | *B. thuringiensis* | 1 | H3aH3dH3e serovar *fukuokaensis* |
| 02/009 | *B. thuringiensis* | 1 | H4aH4b serovar *sotto* |
| 02/010 | *B. thuringiensis* | 1 | H4aH4c serovar *kenyae* |
| 02/011 | *B. thuringiensis* | 1 | H5aH5b serovar *galleriae* |
| 02/012 | *B. thuringiensis* | 1 | H5aH5c serovar *canadensis* |
| 02/013 | *B. thuringiensis* | 1 | H6 serovar *entomocidus* |
| 02/014* | *B. thuringiensis* | 1 | H7 serovar *aizawai* |
| 02/015* | *B. thuringiensis* | 1 | H8aH8b serovar *morrisoni* |
| 02/016 | *B. thuringiensis* | 1 | H8aH8c serovar *ostriniae* |
| 02/017 | *B. thuringiensis* | 1 | H8bH8d serovar *nigeriensis* |
| 02/018 | *B. thuringiensis* | 1 | H9 serovar *tolworthi* |
| 02/019 | *B. thuringiensis* | 1 | H10aH10b serovar *darmstadiensis* |
| 02/020 | *B. thuringiensis* | 1 | H10aH10c serovar *londrina* |
| 02/021* | *B. thuringiensis* | 1 | H11aH11b serovar *ioumanoffi* |
| 02/022 | *B. thuringiensis* | 1 | H11aH11c serovar *kyushuensis* |
| 02/023 | *B. thuringiensis* | 1 | H12 serovar *thompsoni* |
| 02/024 | *B. thuringiensis* | 1 | H13 serovar *pakistani* |
| 02/025 | *B. thuringiensis* | 1 | H14 serovar *israelensis* |
| 02/026 | *B. thuringiensis* | 1 | H15 serovar *dakota* |
| 02/027 | *B. thuringiensis* | 1 | H16 serovar *indiana* |
| 02/028 | *B. thuringiensis* | 1 | H17 serovar *tohokuensis* |
| 02/029* | *B. thuringiensis* | 1 | H18aH18b serovar *kumamotoensis* |
| 02/030 | *B. thuringiensis* | 1 | H18aH18c serovar *yosoo* |
| 02/031 | *B. thuringiensis* | 1 | H19 serovar *tochigiensis* |
| 02/032 | *B. thuringiensis* | 1 | H20aH20b serovar *yunnanensis* |
| 02/033 | *B. thuringiensis* | 1 | H20aH20c serovar *pondicheriensis* |
| 02/034 | *B. thuringiensis* | 1 | H21 serovar *colmeri* |
| 02/035 | *B. thuringiensis* | 1 | H22 serovar *shandongiensis* |
| 02/036 | *B. thuringiensis* | 1 | H23 serovar *japonensis* |
| 02/037 | *B. thuringiensis* | 1 | H24aH24b serovar *neoleonensis* |
| 02/038 | *B. thuringiensis* | 1 | H25 serovar *coreanensis* |
| 02/039 | *B. thuringiensis* | 1 | H26 serovar *silo* |
| 02/040 | *B. thuringiensis* | 1 | H27 serovar *mexicanensis* |
| 02/041* | *B. thuringiensis* | 1 | H28aH28b serovar *monterrey* |
| 02/042 | *B. thuringiensis* | 1 | H28aH28c serovar *jegathesan* |
| 02/043* | *B. thuringiensis* | 1 | H29 serovar *amagiensis* |
| 02/044 | *B. thuringiensis* | 1 | H30 serovar *medellin* |
| 02/045 | *B. thuringiensis* | 1 | H31 serovar *toguchini* |
| 02/046 | *B. thuringiensis* | 1 | H32 serovar *cameroun* |
| 02/047 | *B. thuringiensis* | 1 | H33 serovar *leesis* |
| 02/048 | *B. thuringiensis* | 1 | H34 serovar *konkukian* |
| 02/049 | *B. thuringiensis* | 1 | H35 serovar *seoulensis* |
| 02/050 | *B. thuringiensis* | 1 | H36 serovar *malaysiensis* |
| 02/051* | *B. thuringiensis* | 1 | H37 serovar *andaluciensis* |
| 02/052 | *B. thuringiensis* | 1 | H38 serovar *oswaldocruzi* |
| 02/053 | *B. thuringiensis* | 1 | H39 serovar *brasiliensis* |
| 02/054 | *B. thuringiensis* | 1 | H40 serovar *huazhongensis* |
| 02/055 | *B. thuringiensis* | 1 | H41 serovar *sooncheon* |
| 02/056 | *B. thuringiensis* | 1 | H42 serovar *jinghongiensis* |
| 02/057 | *B. thuringiensis* | 1 | H43 serovar *guiyangiensis* |
| 02/058 | *B. thuringiensis* | 1 | H44 serovar *higo* |
| 02/059 | *B. thuringiensis* | 1 | H45 serovar *roskildiensis* |
| 02/060 | *B. thuringiensis* | 1 | H46 serovar *chanpaisis* |
| 02/061 | *B. thuringiensis* | 1 | H47 serovar *wratislaviensis* |
| 02/062 | *B. thuringiensis* | 1 | H48 serovar *balearica* |
| 02/063 | *B. thuringiensis* | 1 | H49 serovar *muju* |
| 02/064 | *B. thuringiensis* | 1 | H50 serovar *navarrensis* |
| 02/065 | *B. thuringiensis* | 1 | H51 serovar *xiaguangiensis* |
| 02/066 | *B. thuringiensis* | 1 | H52 serovar *kim* |
| 02/067 | *B. thuringiensis* | 1 | H53 serovar *asturiensis* |
| 02/068 | *B. thuringiensis* | 1 | H54 serovar *poloniensis* |
| 02/069* | *B. thuringiensis* | 1 | H55 serovar *palmanyolensis* |
| 02/070 | *B. thuringiensis* | 1 | H56 serovar *rongseni* |
| 02/071 | *B. thuringiensis* | 1 | H57 serovar *pirenaica* |
| 02/072 | *B. thuringiensis* | 1 | H58 serovar *argentinensis* |
| 02/073 | *B. thuringiensis* | 1 | H59 serovar *iberica* |
| 02/074 | *B. thuringiensis* | 1 | H60 serovar *pingluonsis* |
| 02/075 | *B. thuringiensis* | 1 | H61 serovar *sylvestriensis* |
| 02/076 | *B. thuringiensis* | 1 | H62 serovar *zhaodongensis* |
| 02/077 | *B. thuringiensis* | 1 | H63 serovar *bolivia* |
| 02/078 | *B. thuringiensis* | 1 | H64 serovar *azorensis* |
| 02/079 | *B. thuringiensis* | 1 | H65 serovar *pulsiensis* |
| 02/080 | *B. thuringiensis* | 1 | H66 serovar *graciosensis* |
| 02/081 | *B. thuringiensis* | 1 | H67 serovar *vazensis* |
| 02/082 | *B. thuringiensis* | 1 | H68 serovar *thailandensis* |
| 02/083 | *B. thuringiensis* | 1 | H69 serovar *pahangi* |
| 02/084 | *B. thuringiensis* | 1 | H70 serovar *sinensis* |
| 02/350 | *B. cereus* | 2 | F4430/73 |
| 02/351 | *B. cereus* | 2 | DSM 4282 |
| 02/352 | *B. cereus* | 2 | 98HMPL63 |
| 02/353 | *B. cereus* | 2 | F2769/77 |
| 02/354 | *B. cereus* | 2 | F284/78 |
| 02/355 | *B. cereus* | 2 | F4815/94 |
| 02/356 | *B. cereus* | 2 | F3003/73 |
| 02/357 | *B. cereus* | 2 | F2081A/98 |
| 02/358 | *B. cereus* | 2 | F2081B/98 |
| 02/359 | *B. cereus* | 2 | F2085/98 |
| 02/360 | *B. cereus* | 2 | DSM 2301 |
| 02/361 | *B. cereus* | 2 | LMG 17605 |
| 02/362 | *B. cereus* | 2 | DSM 4222 |
| 02/363 | *B. cereus* | 2 | DSM 8438 |
| 02/364 | *B. cereus* | 2 | ATCC 4342 |
| 02/367 | *B. cereus* | 2 | LMG 6923T |
| 02/368 | *B. thuringiensis* | 2 | CIP 53.137T |
| 02/369 | *B. thuringiensis* | 2 | T03A001 |
| 02/370 | *B. thuringiensis* | 2 | T14007 |
| 02/371 | *B. thuringiensis* | 2 | T01016 |
| 02/373 | *B. cereus* | 2 | CIP 78.3 |
| 02/374 | *B. cereus* | 2 | CIP 51.27 |
| 02/375 | *B. cereus* | 2 | F4635A/90 |
| 02/376 | *B. cereus* | 2 | F4620/90 |
| 02/379 | *B. cereus* | 2 | CIP 58.32 |
| 02/381 | *B. cereus* | 2 | P1-1 |
| 02/382 | *B. cereus* | 2 | P2-3 |
| 02/383 | *B. cereus* | 2 | P14-1 |
| 02/384 | *B. cereus* | 2 | C413 |
| 02/385 | *B. cereus* | 2 | TC414 |
| 02/386 | *B. cereus* | 2 | Z421 |
| 02/387 | *B. cereus* | 2 | TL811 |
| 02/388 | *B. cereus* | 2 | B2114 |
| 02/389 | *B. cereus* | 2 | L2103 |
| 02/391 | *B. cereus* | 2 | P2103 |
| 02/392 | *B. cereus* | 2 | P15-2 |
| 02/393 | *B. cereus* | 2 | P22-4 |
| 02/394 | *B. cereus* | 2 | P24-1 |
| 02/395 | *B. cereus* | 2 | TZ415 |
| 02/396 | *B. cereus* | 2 | TZ427 |
| 02/397 | *B. cereus* | 2 | C2109 |
| 02/398 | *B. cereus* | 2 | Z4222 |
| 02/400 | *B. cereus* | 2 | K1231 |
| 02/402 | *B. cereus* | 2 | C2104 |
| 96/011 | *B. cereus* | | ATCC 14579 |
| 97/027 | *B. thuringiensis* | 3 | H34 serovar *konkukian* |
| 98/020 | *B. thuringiensis* | 4 | H3aH3b serovar *kurstaki* |
| 98/021 | *B. thuringiensis* | 4 | H14 serovar *israelensis* |
| 99/028 | *B. thuringiensis* | 1 | Bt407 |

[a] Sources of 131 different strains were as follows: 1, Institut Pasteur, Paris, France; 2, INRA, Avignon, France; 3, Hôpital des Armées Bégin, Paris, France; 4, Abbott Laboratories, France. ID, serovar and subspecies; *, eight strains used for the testing of 17 primer pairs.

TABLE 2. *B. cereus* group isolates investigated in this study[a]

| Reference | Species | Additional data/reference | Source | Reference | Species | Additional data/reference | Source |
|---|---|---|---|---|---|---|---|
| 02/216 | *B. cereus* | 8D1 | 1 | 02/561 | *B. cereus* | 94.9.16.344 | 2 |
| 02/217 | *B. cereus* | 4F1 | 1 | 02/562 | *B. cereus* | 94.9.19.398 | 2 |
| 02/219 | *B. cereus* | 18G4 | 1 | 02/563 | *B. cereus* | 95.7.27.304 | 2 |
| 02/220 | *B. cereus* | Italian lactic ferment | 1 | 02/564 | *B. cereus* | 95.5.14.39 | 2 |
| 02/221 | *B. cereus* | Barégine | 1 | 02/565 | *B. cereus* | 95.4.24.34 | 2 |
| 02/222 | *B. cereus* | 406 | 1 | 02/566 | *B. cereus* | 91.3.11.1388 | 2 |
| 02/223 | *B. cereus* | 6C2 | 1 | 02/567 | *B. cereus* | None | 2 |
| 02/224 | *B. cereus* | 7A21 | 1 | 02/569 | *B. cereus* | 92.6.18.420 | 2 |
| 02/225 | *B. cereus* | 9A7 | 1 | 02/570 | *B. cereus* | 92.1.2.183 | 2 |
| 02/226 | *B. cereus* | 10H5 | 1 | 02/571 | *B. cereus* | 92.4.19.242 | 2 |
| 02/227 | *B. cereus* | 2 | 1 | 02/572 | *B. cereus* | 3.53 | 2 |
| 02/228 | *B. cereus* | 19A5 round | 1 | 02/573 | *B. cereus* | 95.3.20.507 | 2 |
| 02/229 | *B. cereus* | 19A5 filamentous | 1 | 02/574 | *B. cereus* | 46 | 2 |
| 02/230 | *B. cereus* | X44 | 1 | 02/575 | *B. cereus* | 95.1.6.117 | 2 |
| 02/231 | *B. cereus* | 109 | 1 | 02/576 | *B. cereus* | 95.7.24.357 | 2 |
| 02/236 | *B. cereus* | W4II | 1 | 02/577 | *B. cereus* | None | 2 |
| 02/481 | *B. cereus* | 7C1 nebulous | 1 | 02/578 | *B. cereus* | 91.3.19.1367 | 2 |
| 02/482 | *B. cereus* | 6A14 | 1 | 02/579 | *B. cereus* | 92.4.12.227 | 2 |
| 02/483 | *B. cereus* | 7A2 | 1 | 02/580 | *B. cereus* | 31.3.19.1292 | 2 |
| 02/484 | *B. cereus* | 7A11 | 1 | 02/582 | *B. cereus* | 92.7.23.208 | 2 |
| 02/485 | *B. cereus* | 7A17 | 1 | 02/583 | *B. cereus* | 95.4.24.253 | 2 |
| 02/486 | *B. cereus* | 6B11 | 1 | 02/584 | *B. cereus* | 95.2.23.398 | 2 |
| 02/487 | *B. cereus* | 7B2 | 1 | 02/585 | *B. cereus* | 92.04 | 2 |
| 02/488 | *B. cereus* | 17B4 | 1 | 02/586 | *B. cereus* | 94.9.9.360 | 2 |
| 02/489 | *B. cereus* | 7C2 nebulous | 1 | 02/622 | *B. cereus* | Biotox 18/12/02 | 3 |
| 02/490 | *B. cereus* | 8C2 nebulous | 1 | 02/623 | *B. cereus* | Biotox 18/12/02 | 3 |
| 02/491 | *B. cereus* | 8C4 pearl | 1 | 02/624 | *B. cereus* | Biotox 18/12/02 | 3 |
| 02/492 | *B. cereus* | 8C12 | 1 | 04/021 | *B. cereus* | 1 | 4 |
| 02/493 | *B. cereus* | 2M4 | 1 | 04/022 | *B. cereus* | 2 | 4 |
| 02/495 | *B. cereus* | 15G9 | 1 | 04/023 | *B. cereus* | 3 | 4 |
| 02/496 | *B. cereus* | 16K3 | 1 | 04/024 | *B. cereus* | 4 | 4 |
| 02/498 | *B. cereus* | Anthracoides horsehair | 1 | 04/025 | *B. cereus* | 8 | 4 |
| 02/499 | *B. cereus* | Anthracoides water | 1 | 04/026 | *B. cereus* | 10 | 4 |
| 02/500 | *B. cereus* | Anthracoides g and j | 1 | 04/027 | *B. cereus* | 26 | 4 |
| 02/501 | *B. cereus* | Anthracoides s | 1 | 04/028 | *B. cereus* | 52 | 4 |
| 02/542 | *B. cereus* | None | 2 | 04/029 | *B. cereus* | Bt001 | 4 |
| 02/543 | *B. cereus* | 94.9.16.344 | 2 | 04/030 | *B. cereus* | Bc1 | 4 |
| 02/544 | *B. cereus* | 92.9.25.319 | 2 | 97/076 | *B. cereus* | TZ-4 | 5 |
| 02/545 | *B. cereus* | 92.9.25.294 | 2 | 97/077 | *B. cereus* | T6-6 | 5 |
| 02/546 | *B. cereus* | 95.7.125.165 | 2 | 97/078 | *B. cereus* | T5-2 | 5 |
| 02/547 | *B. cereus* | 92.1.3.53 | 2 | 97/079 | *B. cereus* | TH-3 | 5 |
| 02/548 | *B. cereus* | 94.9.9.360 | 2 | 97/080 | *B. cereus* | 97/080 | 6 |
| 02/549 | *B. cereus* | 95.4.28.73 | 2 | 97/081 | *B. cereus* | 97/081 | 6 |
| 02/550 | *B. cereus* | 95.4.28.73 | 2 | 97/082 | *B. cereus* | 97/082 | 6 |
| 02/551 | *B. cereus* | 91.3.9.1292 | 2 | 97/083 | *B. cereus* | 97/083 | 6 |
| 02/552 | *B. cereus* | 94.12.16.385 | 2 | 97/084 | *B. cereus* | 97/084 | 6 |
| 02/554 | *B. cereus* | 92.4.19.242 | 2 | 97/085 | *B. cereus* | 97/085 | |
| 02/557 | *B. cereus* | 95.5.12.269 | 2 | 97/091 | *B. cereus* | 97/091 | 7 |
| 02/558 | *B. cereus* | 92.4.24.7342 | 2 | 99/043 | *B. cereus* | 99.819.0254 | 2 |
| 02/560 | *B. cereus* | 92.6.18.420 | 2 | | | | |

[a] The sources of 99 isolates were as follows: 1, soil and dairy, INRA, Jouy-en-Josas, France; 2, clinical isolates, Hôpital des Armées Bégin, Paris, France; 3, soil, Biotox analyses; 4, clinical isolates, Centre Hospitalier, Toulouse, France; 5, CNEVA, France; 6, soil, Institut Pasteur, Paris, France; 7, clinical isolate, Hôpital des Armées Percy, Paris, France.

sequencing of the long 16S-23S rDNA containing tRNA genes (4), real-time PCR analysis of the *rpoB* gene alone (30) or associated with the *cap* gene (24) in a multiplex PCR, characterization of genomic differences that distinguish non-anthrax-causing bacilli from *B. anthracis* Ames by suppression subtractive hybridization (31), *gyrB* sequence analysis (25), and partial sequencing of the *plcR* gene (10, 37).

Multilocus sequence typing (MLST) (15), based on sequencing of 7 essential housekeeping genes, and fluorescent AFLP (17) have proved their efficiency in typing the *B. cereus* group. Both methods show that the genetic diversity is high inside the *B. cereus-B. thuringiensis* group, whereas *B. anthracis* is highly homogeneous and can be considered to be a particularly monomorphic species (all five *B. anthracis* strains investigated have the same MLST type, whereas more than 50 different MLST types are distinguished among 77 strains from the *B. cereus* group). However, these methods are laborious and time-consuming when a large number of strains or isolates must be analyzed. Moreover, the AFLP method requires high-quality standards and strict protocols in order to produce data that can be compared between different laboratories. Such analyses will probably be restricted to the most interesting strains as revealed by simpler, higher-throughput investigations.

TABLE 3. Primers used for PCR amplification of Bcms VNTRs

| Name | Unit length | Primers | | Allele size range (bp) | Expected length (bp)[a] | On *B. cereus* ATCC 14579 genome | | No. of alleles |
|---|---|---|---|---|---|---|---|---|
| | | Orientation[b] | Sequence | | | Tandem repeat location | Corresponding gene | |
| Bcms 08 | 18 | F<br>R | GTGCTGG(W)GCAAACACAGAC<br>TGGTCGCCTGCTTTATAACC | 460–838 | 739 | 797035–797773 | Enterotoxin/cell wall binding protein | 23 |
| Bcms 17 | 16 | F<br>R | ATTGGACAAGAAAAACAAGGTACTG<br>CGCTGATCTTCCATTTGCAT | 215–272 | 215 | 4148084–4148298 | Stage III sporulation protein AH | 4 |
| Bcms 18 | 15 | F<br>R | CCTTGTTTTGCACGCTCAG<br>CTGGTCAACAACCTACTGAAAATGT | 230–283 | 268 | 1273504–1273771 | Hypothetical protein | 7 |
| Bcms 19 | 12 | F<br>R | GGAATAGAAGATGAAGAAGAAGTTACG<br>TTTCG(S)TTTTATTGGTGGTTG | 291–411 | 363 | 4361476–4361838 | SpoVID-dependent spore coat assembly factor SafA | 18 |
| Bcms 20 | 36 | F<br>R | CGCCAAATGTATCGAAAGAA<br>TGCTGATATGGCATTTGATATGG | 412–844 | 451 | 4360634–4361084 | SpoVID-dependent spore coat assembly factor SafA | 20 |

[a] In *B. cereus* ATCC 14579.
[b] F, forward; R, reverse.

Multiple-locus variable number of tandem repeat (VNTR) analysis (MLVA) using a collection of polymorphic markers is currently the method of choice to genotype strains inside the *B. anthracis* species (22, 28). This species is thought to have evolved from a *B. cereus-B. thuringiensis* strain by acquisition of the pXO plasmids. It is thus interesting to identify the closely related strains in order to better understand the evolutionary origin of this pathogen. In the present study, we show that MLVA can be successfully used for this purpose. This is not the traditional use of this technique. We analyzed the polymorphism of selected VNTRs inside a large collection of *B. cereus-B. thuringiensis* strains in an attempt to detect strains closely related to *B. anthracis*. As a result, a two-step assay based on PCR and agarose gel electrophoresis is proposed that represents an interesting approach to rapidly and at a very low cost identify strains of the *B. cereus* complex that are closest to *B. anthracis*.

## MATERIALS AND METHODS

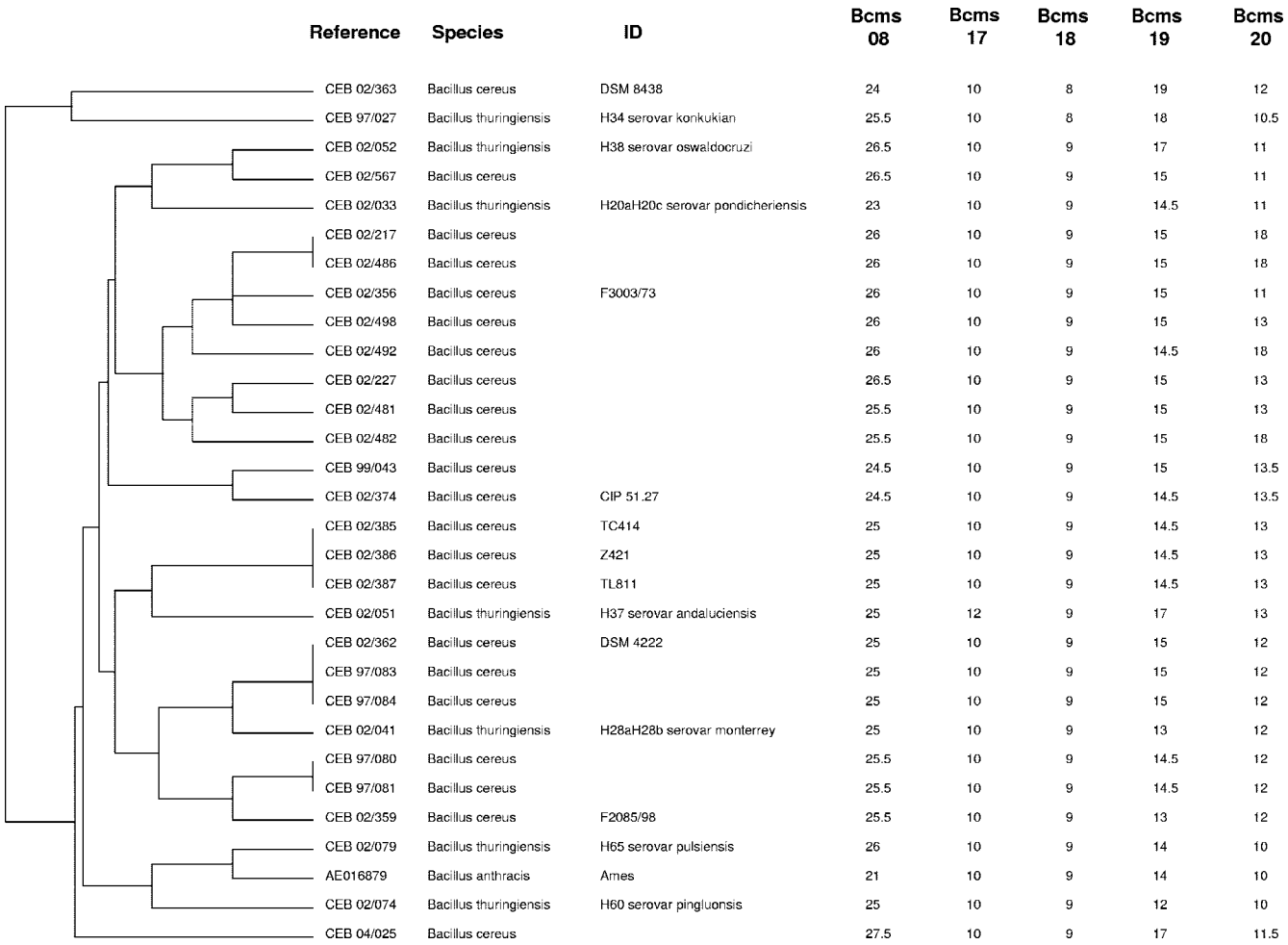
**Bacterial strains.** The microorganisms included in this study are part of the collection maintained by the Centre d'Etudes du Bouchet (CEB). They were obtained from different sources (Tables 1 and 2). A total of 230 strains or isolates were analyzed.

The 131 reference strains (Table 1) originate from well-characterized collections and comprise 90 *B. thuringiensis* and 41 *B. cereus* strains. In particular, the *B. thuringiensis* collection used contains one strain for each *B. thuringiensis* serovar described in reference 26. Similarly, the *B. cereus* strains are a representative panel described in reference 13. The 99 newly investigated isolates (Table 2) were initially identified by the laboratory of origin as being *B. cereus* members of the *B. cereus* group. This was confirmed on the basis of phenotypic criteria (motility and hemolytic capacity) by biochemical criteria analysis with the Vitek system using *Bacillus* Biochemical (BAC) cards (bioMérieux, Marcy l'Etoile, France), by the gram-positive plates and the "dangerous pathogens" database of the Biolog system (AES, Combourg, France) and in some instances by direct microscopic observation (detection of the crystal after Coomassie blue staining).

**DNA purification.** A total of 5 ml of 2YT broth were inoculated by picking a single colony. The culture was incubated overnight at 37°C, and bacteria were harvested by centrifugation at 4,000 × *g* for 20 min. The pellet was suspended in 200 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) with lysozyme (50 mg/μl) and incubated at 37°C for 1 h. The solution was grounded with 0.1-mm glass beads at 2,500 rpm for 90 s in a Mini-Beadbeater (Biospec Products, Bartlesville, Ohio). The supernatant was recovered, and the beads were washed with 250 μl of TE buffer. An RNase solution was added at a final concentration of 500 μg/ml, and the mixture was incubated for 15 min at 50°C. One volume of lysis buffer containing 20 mM Tris (pH 8), 2 mM EDTA, 20 mM NaCl, and 1% sodium dodecyl sulfate was added, along with 100 μg of proteinase K per ml, and the mixture was incubated overnight at 55°C. Chromosomal DNA was extracted by phenol-chloroform by using a Phase Lock gel (Eppendorf, Hamburg, Germany) and precipitated with 2 volumes of ice-cold 95% ethanol. The precipitated DNA was collected by centrifugation for 20 min at 15,000 × *g*. The dried DNA was then dissolved in TE buffer. The quality (average size) of the DNA was checked by 0.7% agarose gel electrophoresis, and the DNA concentration was measured with the DyNA Quant 200 fluorimeter (Hoefer, San Francisco, Calif.).

**VNTR PCR amplification and genotyping.** PCRs were performed in 15 μl containing 2 ng of DNA, 1× PCR buffer, 1 U of *Taq* DNA polymerase, 200 μM concentrations of each deoxynucleoside triphosphate, and 0.3 μM concentrations of each flanking primer. The *Taq* DNA polymerase was obtained from Qbiogen (Illkirch, France) and used as recommended by the manufacturer.

PCRs were run on a MJ Research PTC200 thermocycler (Waltham, MA). An initial denaturation at 96°C for 5 min was followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 1 min, and elongation at 70°C for 1 min, followed by a final extension step of 5 min at 70°C. Then, 5 μl of the PCR products were run on standard 2% agarose gel (Qbiogen) in 0.5× TBE buffer (10× TBE is 890 mM Tris base, 890 mM boric acid, 20 mM EDTA [pH 8.3]) at a voltage of 10 V/cm. Samples were manipulated and distributed (including gel loading) with multichannel electronic pipettes (Biohit, Bonnelles, France) in order to reduce the risk of errors. Gel length of 20 cm were used. Gels were stained with ethidium bromide, visualized under UV light, and photographed. Alleles size was estimated by using a 100-bp ladder (Bio-Rad, Marnes-la-Coquette, France) as a size marker as previously described (27, 28). One reference strain was included for each set of five DNA samples as a control for size assignments. Gel images were analyzed by using the Bionumerics software package version 4.0 (Applied-Maths, Sint-Martens-Latem, Belgium) as previously described (27). The number of repeats in each allele was deduced from the



| Reference | Species | ID | Bcms 08 | Bcms 17 | Bcms 18 | Bcms 19 | Bcms 20 |
|---|---|---|---|---|---|---|---|
| CEB 02/363 | Bacillus cereus | DSM 8438 | 24 | 10 | 8 | 19 | 12 |
| CEB 97/027 | Bacillus thuringiensis | H34 serovar konkukian | 25.5 | 10 | 8 | 18 | 10.5 |
| CEB 02/052 | Bacillus thuringiensis | H38 serovar oswaldocruzi | 26.5 | 10 | 9 | 17 | 11 |
| CEB 02/567 | Bacillus cereus | | 26.5 | 10 | 9 | 15 | 11 |
| CEB 02/033 | Bacillus thuringiensis | H20aH20c serovar pondicheriensis | 23 | 10 | 9 | 14.5 | 11 |
| CEB 02/217 | Bacillus cereus | | 26 | 10 | 9 | 15 | 18 |
| CEB 02/486 | Bacillus cereus | | 26 | 10 | 9 | 15 | 18 |
| CEB 02/356 | Bacillus cereus | F3003/73 | 26 | 10 | 9 | 15 | 11 |
| CEB 02/498 | Bacillus cereus | | 26 | 10 | 9 | 15 | 13 |
| CEB 02/492 | Bacillus cereus | | 26 | 10 | 9 | 14.5 | 18 |
| CEB 02/227 | Bacillus cereus | | 26.5 | 10 | 9 | 15 | 13 |
| CEB 02/481 | Bacillus cereus | | 25.5 | 10 | 9 | 15 | 13 |
| CEB 02/482 | Bacillus cereus | | 25.5 | 10 | 9 | 15 | 18 |
| CEB 99/043 | Bacillus cereus | | 24.5 | 10 | 9 | 15 | 13.5 |
| CEB 02/374 | Bacillus cereus | CIP 51.27 | 24.5 | 10 | 9 | 14.5 | 13.5 |
| CEB 02/385 | Bacillus cereus | TC414 | 25 | 10 | 9 | 14.5 | 13 |
| CEB 02/386 | Bacillus cereus | Z421 | 25 | 10 | 9 | 14.5 | 13 |
| CEB 02/387 | Bacillus cereus | TL811 | 25 | 10 | 9 | 14.5 | 13 |
| CEB 02/051 | Bacillus thuringiensis | H37 serovar andaluciensis | 25 | 12 | 9 | 17 | 13 |
| CEB 02/362 | Bacillus cereus | DSM 4222 | 25 | 10 | 9 | 15 | 12 |
| CEB 97/083 | Bacillus cereus | | 25 | 10 | 9 | 15 | 12 |
| CEB 97/084 | Bacillus cereus | | 25 | 10 | 9 | 15 | 12 |
| CEB 02/041 | Bacillus thuringiensis | H28aH28b serovar monterrey | 25 | 10 | 9 | 13 | 12 |
| CEB 97/080 | Bacillus cereus | | 25.5 | 10 | 9 | 14.5 | 12 |
| CEB 97/081 | Bacillus cereus | | 25.5 | 10 | 9 | 14.5 | 12 |
| CEB 02/359 | Bacillus cereus | F2085/98 | 25.5 | 10 | 9 | 13 | 12 |
| CEB 02/079 | Bacillus thuringiensis | H65 serovar pulsiensis | 26 | 10 | 9 | 14 | 10 |
| AE016879 | Bacillus anthracis | Ames | 21 | 10 | 9 | 14 | 10 |
| CEB 02/074 | Bacillus thuringiensis | H60 serovar pingluonsis | 25 | 10 | 9 | 12 | 10 |
| CEB 04/025 | Bacillus cereus | | 27.5 | 10 | 9 | 17 | 11.5 |

FIG. 1. Cluster A of the dendrogram deduced from VNTR typing of 234 *B. cereus* group strains. The first column indicates the reference of the strains (see Tables 1 and 2). The second column indicates the species. The third column indicates the known identity number. The columns Bcms show the number of motifs obtained for each minisatellite.

amplicon size. The resulting data were analyzed with Bionumerics as a character data set. Clustering analysis was done by using the categorical parameter and the UPGMA (unweighted pair-group method with arithmetic averages) coefficient.

**Sequencing of PCR amplification products and MLST clustering analysis.** PCR was performed in a 60-μl volume. Amplification products of a size greater than 300 bp were purified by polyethylene glycol precipitation as described previously (11). Amplification products shorter than 300 bp were concentrated by ethanol precipitation and treated by ExoSAP-IT from USB Corp. (Cleveland, Ohio) as described by the manufacturer. Sequencing was done by MWG Biotech (Courtaboeuf, France). A portion (10) of the PlcR gene was amplified for sequencing by using 5′-TGGCCATTTTAAGAAGAGTATTGA-3′ and 5′-CAC TCTAGCTTTTCTAGGCATTCA-3′. The primers used for the MLST amplification reactions were as described in reference 15. The MLST sequence data was converted to a character data set by using Bionumerics version 4.0 in order to be able to produce a minimum-spanning tree. The inclusion of hypothetical missing links was allowed in the making of the minimum-spanning tree.

**Search for *B. anthracis* prophages.** The four prophages present in *B. anthracis* and absent in a number of *B. cereus* strains as observed by (35) were investigated by using, respectively 5′-CGGTGACGTGTTAACTGAGC-3′ and 5′-CGTACGTGT TACTCGCCAAA-3′ (prophage 1), AAGTCAATCCTTCCGGGTTT and TCA CCAATCATGGTCAGGAA (prophage 2), CGTTAACCAAACTGGGCAAT and TTATCGTCCTCACGCAGTTG (prophage 3), and TCAGGCATGGGTTAT GTGAA and TCATGATGCTCACGGTTATGA (prophage 4) as primers.

## RESULTS

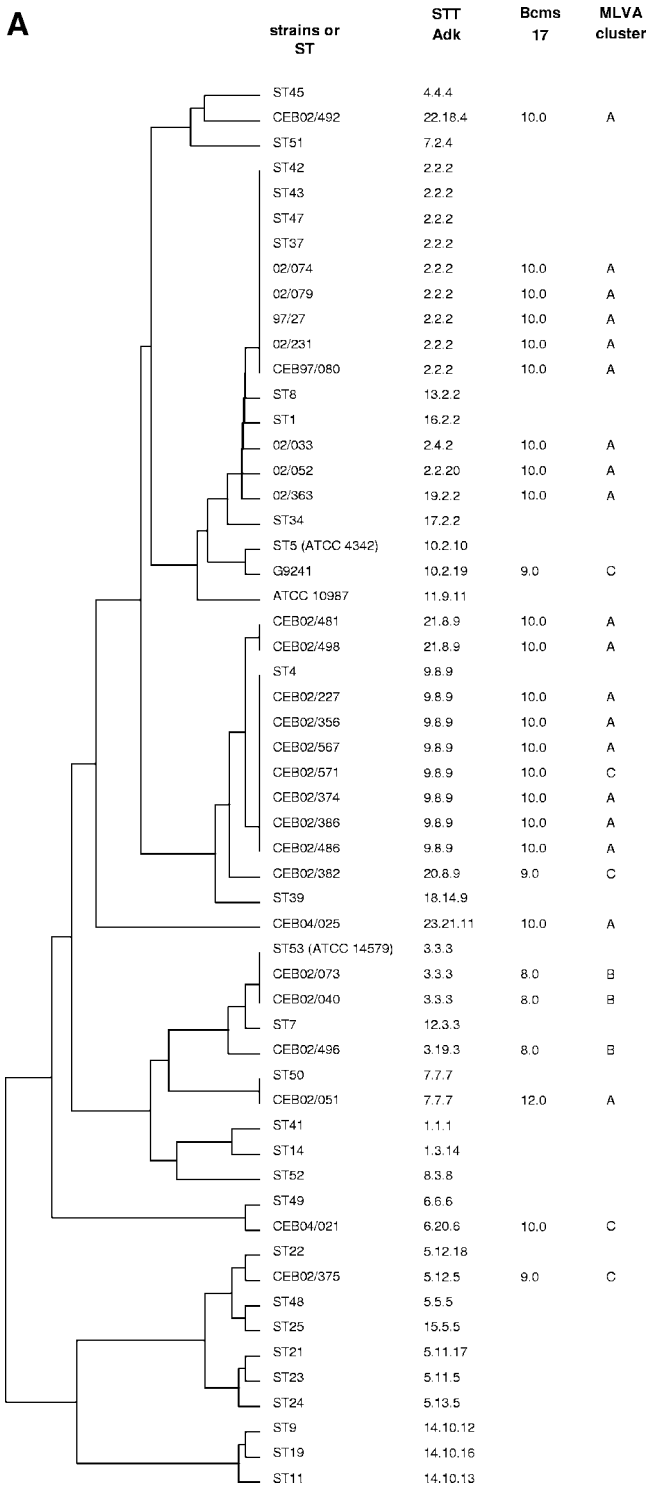
**Analysis of monomorphic tandem repeats.** Le Flèche et al. (28) previously tested numerous tandem repeat loci in order to identify an appropriate set of polymorphic markers for *B. anthracis* strain typing. Seventeen tandem repeats proved to be monomorphic in *B. anthracis*, whereas preliminary investigations demonstrated that at least some were polymorphic in *B. cereus-B. thuringiensis* strains (data not shown). We thus hypothesized that strains of the *B. cereus* group presenting the same allelic combination or at least some common allele sizes with *B. anthracis* for a number of tandem repeats monomorphic within *B. anthracis*, would be more closely related to *B. anthracis*. The flanking sequences of each *B. anthracis* (strain Ames) monomorphic tandem repeat identified by Le Flèche et al. (28) were compared to their homolog in *B. cereus* ATCC 10987 and ATCC 14579 (20, 34). Primers were chosen to match the two sequenced genomes and were given a Bcms suffix. The 17 primer pairs were tested on a subset of eight *B. thuringiensis* strains (indicated by asterisks in Table 1). Only eight primer pairs yielded PCR amplification products in all

eight strains, and polymorphism was observed for five tandem repeats (Table 3). All five minisatellites are part of putative open reading frames, Bcms 19 and Bcms 20 being inside the same gene.

MLVA with these five markers was performed using DNA from 230 strains belonging to the *B. cereus* group including *B. thuringiensis* CEB97/27 strain (17, 31). The allele sizes were converted to repeat unit numbers by using conventions defined earlier (27). A dendrogram was produced that also includes the theoretical alleles of five sequenced genomes: *B. anthracis* Ames, *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* G9241, and *B. cereus* ZK (GenBank accession numbers AE017334, AE016877, AE017194, AAEK00000000, and NC_006274, respectively). The 234 strains were distributed into 199 genotypes, essentially owing to the large number of alleles observed at the Bcms 08 (23 alleles), Bcms 19 (18 alleles), and Bcms 20 (20 alleles) loci (see online data at http://minisatellites.u-psud.fr/).

Three major clusters—A, B, and C—were differentiated by MLVA clustering analysis. Interestingly, the clustering assignment fits very well with the Bcms 17 alleles. Clusters A, B, and C have 10, 8, and 9 motifs, respectively, with rare exceptions. Cluster A, containing *B. anthracis* and 29 other strains, is shown in Fig. 1. Two *B. thuringiensis* strains CEB02/079 (serovar *pulsiensis*) and CEB02/074 (serovar *pingluonsis*) have, respectively four and three alleles of five in common with *B. anthracis* (hence, MLVA scores of 4/5 and 3/5, respectively). Strain CEB97/27 is in cluster A, although only the Bcms 17 allele has the *B. anthracis* size. Cluster B contains 125 strains, including the sequenced strain *B. cereus* ATCC 14579 and the two *B. thuringiensis* strains used as a biopesticide (BtH14 [*israelensis*], listed as CEB98/020 and BtH3a3b [*kurstaki*] listed as CEB98/021); cluster C contains 79 strains, including strains *B. cereus* ATCC 10987, ZK, and G9241 (online data at http://minisatellites.u-psud.fr). *B. cereus* and *B. thuringiensis* strains could not be distinguished by MLVA, a finding in agreement with previous reports using other molecular methods. Apart from the strains closely related to *B. anthracis* cited before, no other strains have a score of 3/5 or more. Thus, the strains belonging to cluster A are *B. anthracis* close-neighbor candidates. The strains derived from patients (Table 2) appear to be equally distributed among all groups.

**Comparison with MLST analysis.** To estimate the validity of the classification observed by VNTR typing, selected strains were analyzed by MLST as developed by Helgason et al. (15). The dendrograms derived from the *adk* sequences alone, or from all seven loci, were previously shown to be in very good agreement, at least in the vicinity of *B. anthracis* (15). For this reason, we first analyzed the *adk* sequence of all strains in cluster A and of some strains from clusters B and C. A dendrogram was produced from the resulting *adk* sequences together with some sequences from Helgason et al. (Fig. 2A) and with the fully sequenced strains. The cluster A strains are separated into two groups, one being very close to *B. anthracis*. Strains CEB02/079 (H65), CEB02/074 (H60), CEB97/27, and CEB97/080 have the same *adk* sequence as the strains of sequence types (STs) 37, 42, 43 and 47, respectively. Strains CEB02/231 and CEB02/571, which were assigned, respectively, to MLVA clusters B and C, in spite of their allele 10 at Bcms 17, have *adk* alleles falling within the cluster A strains. CEB02/051, which was assigned to cluster A, is distant in the *adk* tree. This strain has a Bcms 17 allele 12. Strain CEB04/021, with a Bcms 17 allele 10 and belonging to cluster C, appears distant in the *adk* tree as well.




A complete MLST scheme was applied to the typing of the strains with *adk* alleles closest to *B. anthracis* (Fig. 2B). It confirms that the strains identified by MLVA as being close to

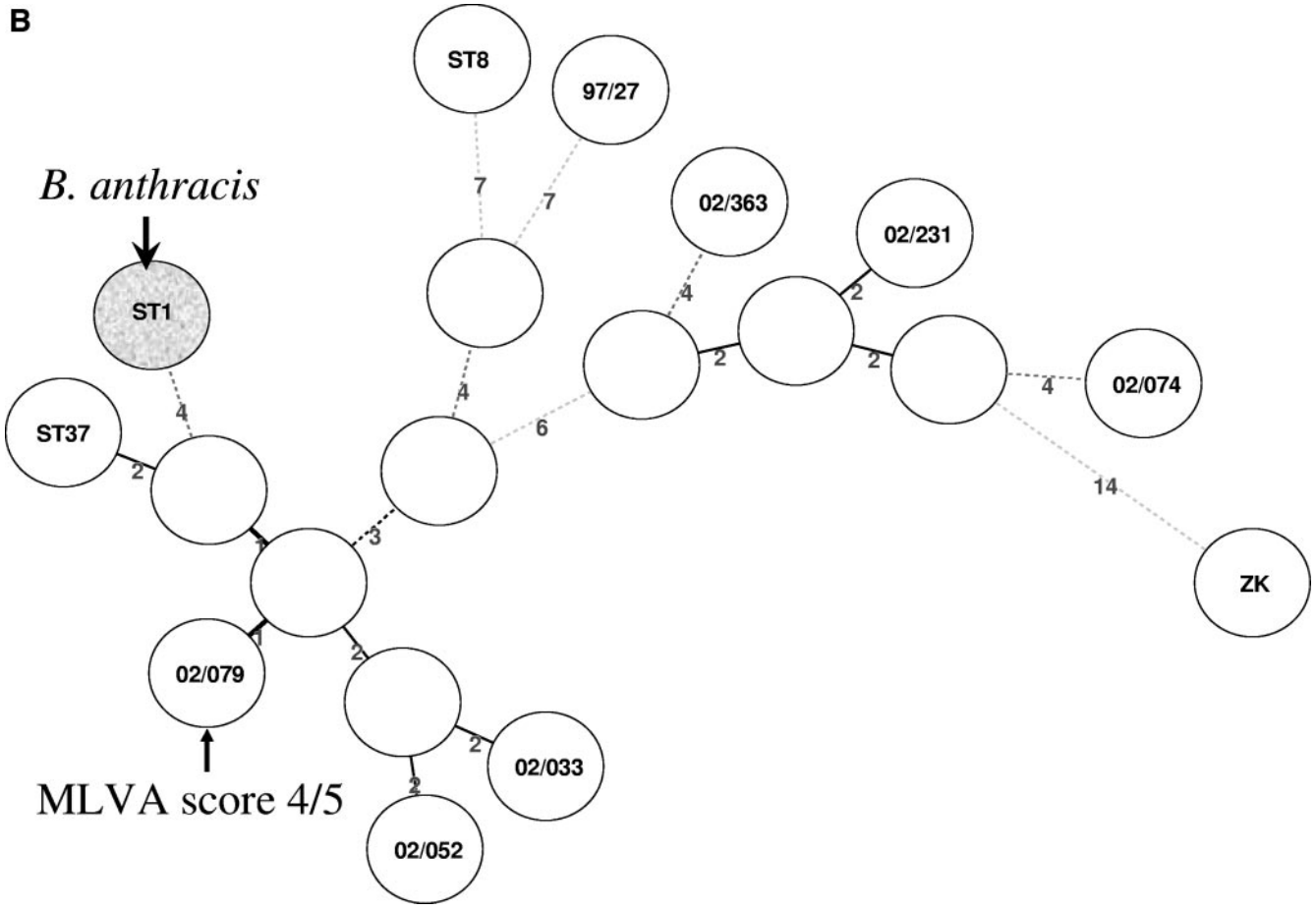



FIG. 2. (A) Genetic relationship among some strains presented here and elsewhere (15) obtained with the *adk* sequence. The dendrogram was constructed by using UPGMA parameters. The first column indicates the reference of the strains (see Tables 1 and 2) or the ST according to (15). The second column indicates the split ST (STT) of *adk* gene sequences as presented previously (15). The two last columns indicate the Bcms 17 allele assignment and the cluster assignment of the strain in the MLVA analysis. (B) Minimum-spanning tree deduced from MLST data among *B. anthracis* closely related strains described here and previously (15). Open circles are hypothetical missing links, which reduce the overall tree length. ST numbers are as presented previously (15). ST1 (*B. anthracis*) is grayed. Strain 02/079 with MLVA score 4/5 is marked with an arrow.

*B. anthracis* have a very similar MLST type. Among them, strain CEB02/079 (H65) seems to be the most closely related to ST1 (*B. anthracis*) (in agreement with the MLVA analysis and the 4/5 score) and to the strains with ST37 in the analysis of Helgason et al. Strains 97/080 and CEB97/27, on the other hand, seem to be closely related, respectively, to ST43 and ST8.

**Sequencing of the Bcms 17 alleles.** Since Bcms 17 allele size alone seemed to efficiently identify a subset of strains closely related to *B. anthracis* (strains with allele 10), we sought to verify whether the sequence of allele 10 was identical in strains of cluster A and different from that of strains with a similarly sized allele in cluster C. We thus further analyzed this locus by sequencing. This minisatellite has a very poor internal motif conservation. Figure 3A shows the manual alignment of part of the locus bearing Bcms 17 in the sequenced *B. anthracis* genome, the three sequenced *B. cereus* (ATCC 14579, ATCC 10987, and G9241), *B. thuringiensis* CEB97/27, and some strains from the present study. In strain CEB04/021, the motif arrangement was clearly different from that of the other alleles of identical size, and many mutations were observed as in *B. cereus* strains of clusters B and C. Similarly, allele 12 of Bcms 17 in strain CEB02/051 showed a very different organization and many mutations compared to alleles of strains closely related to *B. anthracis*. Except for CEB04/021, all of the strains bearing allele 10 had the same motif organization as *B. anthracis*. In the minisatellite region, they were highly similar to *B. anthracis*. CEB97/27, CEB02/079, and CEB02/033 share the identical 10_1 alleles. Allele 10_2 (CEB97/080, CEB97/081, CEB02/363, and CEB02/052) differs by one point mutation. Alleles 10_3 (CEB04/025) and 10_4 (CEB02/074 and CEB02/231) differ by, respectively, one or two distinct additional point mutations (Fig. 3B).

**Prophages and PlcR status.** All strains analyzed in detail and presented in Fig. 2B were hemolytic, as tested by plating on blood agar plates. Sequencing of part of the PlcR gene containing the nonsense mutation responsible for the absence of hemolytic activity in *B. anthracis* shows that strains 02/079, 02/033, 02/052, 02/231, and 02/074 (Fig. 2B) are identical to *B. anthracis* except for the nonsense point mutation. These strains have "allele 3" as described in reference 10. In addition,


FIG. 3. Alignment of Bcms 17 nucleotide sequence from *Bacillus* strains. (A) Alignment of Bcms 17 nucleotide sequence from other *Bacillus* strains. The reference strains are as follows: ATCC 14579, *B. cereus* ATCC 14579; ATCC 10987, *B. cereus* ATCC 10987; G9241, *B. cereus* G9241; Bant, *B. anthracis* strain Ames (allele 10_1). The other strains are identified by their CEB number (see Tables 1 and 2). The flanking sequences are italicized. The residues are grouped according to the Bcms 17 motifs identified in the tandem repeats database (8) for *B. anthracis* strain Ames (35). A dot indicates identity with *B. anthracis* sequence, a dash indicates a gap, and a nucleotide letter indicates positions showing a polymorphism. The numbers in parentheses indicate the theoretical number of motifs according to the MLVA analysis: 08, 215 bp; 09, 227 bp; 10, 242 bp; 12, 262 bp. ∗, Strain CEB 04/021 was assigned a Bcms 17 allele 10, but the actual PCR product size is 245 bp, and this rare allele is indeed quite distinct from the ordinary allele 10. (B) Alignment of different sequences obtained for the 242-bp Bcms 17 allele 10, which had the same motif organization as *B. anthracis*. Flanking sequences are italicized. A dot indicates identity with *B. anthracis* sequence; nucleotide letters indicate positions showing polymorphisms. Allele 10_1 was obtained for *B. anthracis* Ames, CEB97/27, CEB02/079, and CEB02/033; allele 10_2 was obtained for CEB97/80, CEB97/81, CEB02/363, and CEB02/052; allele 10_3 was obtained for CEB04/025; and allele 10_4 was obtained for CEB02/074 and CEB02/231.

all strains in Fig. 3B are missing the four prophages identified by Read et al. (35; data not shown).

## DISCUSSION

In this study, MLVA was used to develop an assay for the identification of strains from the *B. cereus* group most closely related to *B. anthracis*. For this purpose, MLVA markers were selected among loci that were previously shown (28) to be monomorphic in *B. anthracis* but that are polymorphic in the *B. cereus* group. It was then speculated that strains (if they exist) which would show *B. anthracis*-like alleles would be candidate *B. anthracis* closest neighbors. If such an assay could be produced, then it would be easy to run on a large scale because it would require only PCR amplifications followed by agarose gel electrophoresis and allele size measurement. Consequently, it could be run in any laboratory with very basic and standard molecular genetics capacities at a low cost. The approach was tested on a collection of 234 *B. cereus* group strains using five selected markers and proved to be highly successful. On the basis of MLVA polymorphism, strains were distributed in three main clusters A, B, and C. *B. anthracis* strains are included in cluster A (Fig. 1). Incidentally, this MLVA investigation further confirms that the distinction between *B. cereus* and *B. thuringiensis* is not supported by chromosomal polymorphism analysis and only reflects a plasmid content. No strain showed an MLVA profile identical to *B. anthracis*, and only one strain (CEB02/079, cluster A) of 234 was identical to *B. anthracis* strains at four out of five markers. One strain from cluster A has alleles in common with *B. anthracis* for three VNTRs, twenty-five strains from cluster A have alleles in common with *B. anthracis* for two VNTRs, and two strains from cluster A have alleles in common with *B. anthracis* for one VNTR only. CEB97/27 is among these two, although it was shown by MLST to be very close to *B. anthracis* (Fig. 2B). This illustrates the discriminatory power of the MLVA assay in spite of the fact that loci shown to be monomorphic in *B. anthracis* were purposefully used here.

Cluster A strains were further investigated by partial (Fig. 2A) or complete (Fig. 2B) MLST analysis, which dem-

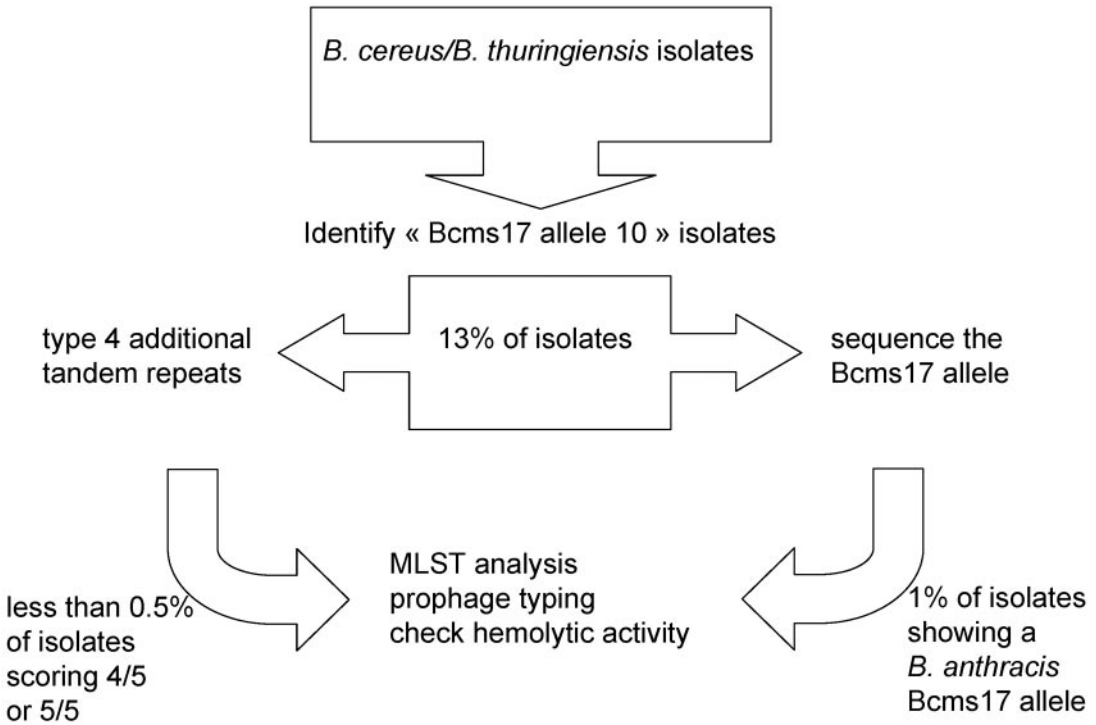



FIG. 4. Typing scheme to screen *B. cereus-B. thuringiensis* for *B. anthracis* close relatives. (First step) PCR amplification at the Bcms 17 locus and identification of "allele 10" strains by agarose gel electrophoresis. (Second step, left) Typing of the four additional markers and identification of strains matching the *B. anthracis* pattern at three or more of these markers. (Second step, right, [alternatively]) Sequencing of the Bcms 17 PCR product. (Third step) MLST analysis of the very rare selected strains, and additional more detailed investigations.

onstrates that indeed CEB02/079 is most closely related to *B. anthracis*. Only six point mutations in the MLST analysis (covering close to 3 kb) separate CEB02/079 (*B. thuringiensis*, H65 serovar *pulsiensis*) from *B. anthracis*, but this is still a significant gap: multiple genetic events separate CEB02/079 from *B. anthracis*, CEB02/079 is hemolytic, and CEB02/079 lacks the four prophages observed in the *B. anthracis* genome (35). One of these prophages contains a *ThyX* (*Thy1*) gene (29). Owing to this prophage acquisition, *B. anthracis* happens to be equipped with two alternate pathways for thymidylate synthesis. Interestingly, among the sequenced *B. cereus* strains, the so-called Zebra killer strain is the only one to share this property (acquired by a seemingly different evolutionary pathway), and ThyX proteins are found in many pathogenic bacteria (29). This suggests that at least some of these prophages may have played a role in the evolution of *B. cereus-B. thuringiensis* toward the highly pathogenic *B. anthracis* and that many additional strains from different geographic origins will need to be analyzed in order to precisely decipher the chronological order of these different events.

Interestingly, it appears that differentiation using only the Bcms 17 allele size is sufficient to identify strains closely related to *B. anthracis* (allele 10). There are four agarose gel size classes of this minisatellite in the complete collection of strains, with allele 12 being present in only one strain. The high degree of internal divergence inside the minisatellite suggests that this is a very old tandem repeat and that no insertion or deletion events occurred in the recent past. This high internal heterogeneity is likely to further prevent the production of new alleles by recombination. A group of five *B. thuringiensis* isolates (CEB02/074 [*B. thuringiensis* subsp. *pingluonsis*], 02/079 [*B. thuringiensis* subsp. *pulsiensis*], 02/041 [*B. thuringiensis* subsp. *monterrey*], 02/033 [*B. thuringiensis* subsp. *pondicheriensis*], and 02/052 [*B. thuringiensis* subsp. *oswaldocruzi*]) with Bcms 17 allele 10 were also grouped by 16S RNA restriction fragment length polymorphism showing that this characteristic is relevant (21). In Fig. 4, we show how this observation can be used to propose a simple typing scheme. In the first step, strains with a Bcms 17 allele 10 are identified by PCR and agarose gel electrophoresis. Only this subset of strains (13% of the population investigated here) needs to be screened for the other four markers (step 2). Strains with at least four loci showing the *B. anthracis* allele are then investigated by MLST analysis (step 3). In the present investigation, only one strain of 234 fulfilled this criteria, and the whole screening process would have required in the present case not more than 350 PCR amplifications and size analyses and the sequencing of seven PCR products. Alternatively (Fig. 4, right side), the Bcms 17 allele of strains showing an "allele 10" could be directly sequenced (step 2). Strains with an allele identical to *B. anthracis* Bcms 17 will be investigated by MLST (three strains in the present collection). This alternate pathway would have required 230 PCR amplification and sizing and sequencing of approximately 50 PCR products. The proportions and figures given are expected to vary according to the population of strains. For instance, a higher proportion of close neighbors is expected to be observed in the geographic area from which *B. anthracis* emerged. The "Bcms 17" assay is very easy to run

since the allele assignment can be derived from PCR amplifications, followed by agarose gel electrophoresis. Kim et al. (23) have used the *vrrA* VNTR marker to determine the genetic relatedness between *B. anthracis* and closely related species. However, *vrrA* is a polymorphic marker for *B. anthracis* strains, showing a divergence between *B. anthracis* strains and a greater one in the *B. cereus* group (1). Moreover, in some strains described here, two amplicons were produced or PCR was weak. In comparison, Bcms 17 was efficiently amplified for the 230 strains, showed only four alleles, and was previously shown to be monomorphic for all *B. anthracis* strains tested.

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