The Genome of a *Bacillus* Isolate Causing Anthrax in Chimpanzees Combines Chromosomal Properties of *B. cereus* with *B. anthracis* Virulence Plasmids

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Abstract

Anthrax is a fatal disease caused by strains of *Bacillus anthracis*. Members of this monophyletic species are non motile and are all characterized by the presence of four prophages and a nonsense mutation in the plcR regulator gene. Here we report the complete genome sequence of a *Bacillus* strain isolated from a chimpanzee that had died with clinical symptoms of anthrax. Unlike classic *B. anthracis*, this strain was motile and lacked the four prophages and the nonsense mutation. Four replicons were identified, a chromosome and three plasmids. Comparative genome analysis revealed that the chromosome resembles those of non-*B. anthracis* members of the *Bacillus cereus* group, whereas two plasmids were identical to the anthrax virulence plasmids pXO1 and pXO2. The function of the newly discovered third plasmid with a length of 14 kbp is unknown. A detailed comparison of genomic loci encoding key features confirmed a higher similarity to *B. thuringiensis* serovar konkukian strain 97-27 and *B. cereus* E33L than to *B. anthracis* strains. For the first time we describe the sequence of an anthrax causing bacterium possessing both anthrax plasmids that apparently does not belong to the monophyletic group of all so far known *B. anthracis* strains and that differs in important diagnostic features. The data suggest that this bacterium has evolved from a *B. cereus* strain independently from the classic *B. anthracis* strains and established a *B. anthracis* lifestyle. Therefore we suggest to designate this isolate as “*B. cereus* variety (var.) anthracis”.

Introduction

The *Bacillus cereus* group comprises six species, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomyoides*. These species are closely related, and the strains of *B. cereus* sensu stricto, *Bacillus thuringiensis*, and *Bacillus anthracis* share highly conserved chromosomes but differ in the virulence encoding plasmids [1]. Whereas *B. thuringiensis* is an insect pathogen [2], *B. cereus* is known mainly as a food poisoning bacterium able to cause diarrhea and vomiting, but is also able to cause more severe infections [3]. *B. anthracis*, the etiological agent of anthrax, is found worldwide and is able to infect virtually all mammals. It is a matter of debate whether these bacteria represent three distinct species or are subspecies of *B. cereus* sensu lato [4,5]. The species-specific phenotype and pathogenicity are often plasmid-encoded [1,6], like the toxins and capsule of *B. anthracis* [7], the insecticidal crystal proteins of *B. thuringiensis* [8], and the cereulide synthesis of emetic *B. cereus* strains [9]. However, other virulence factors like hemolysis, motility, and resistance to antibiotics are encoded on the chromosome [3]. *B. anthracis* is a highly monophyletic clade, and isolates are differentiated by determination of single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs) [10,11]. The pathogen is able to cause edema and cell death by a tripartite toxin consisting of the protective antigen, the edema factor, and the lethal factor [12]. The production of a polyglutamic acid capsule allows the organism to escape the immune system [13]. The virulence factors are encoded on the toxin plasmid, pXO1 [7], and the capsule plasmid, pXO2 [14]. Although sequences of pXO1 and to a lesser extent of pXO2 are widely distributed among strains of the *B. cereus* group [15,16], the presence of plasmids encoding the toxin and capsule genes occurs only rarely.
Here we present the complete genome sequence of a *Bacillus* isolate which induced lethal anthrax in chimpanzee “Léo” in the rainforest of the Tai National Park, Côte d’Ivoire (CI) [17]. The strain belongs to a collection of genetically closely related bacteria, isolated in 2001 and 2002 from deceased wild chimpanzees living in this rain forest area (CI isolates). Pathological and histological examination of “Léo’s” body revealed hemorrhages in nearly all inner organs, particularly in the intestines and lungs, and the lungs were also characterized by edema and emphysema. Microscopic examination revealed Gram-positive, rod-shaped bacteria located intra- and extravascularly in all tissues examined – spleen, liver, lung, lymph nodes, intestines – suggesting an acute bacterial infection as the cause of death [17]. Real time PCR [18] confirmed the presence of *B. anthracis*-like toxin and capsule production [21]. However, the isolates differ from the *B. anthracis* isolate that apparently causes anthrax and possesses both toxin and capsule production [17,19,20]. All these West and Central African strains tentatively grouped as *B. anthracis*-like isolates harbor pXO1- and pXO2-like sequences [17,19] and share plasmid encoded features of the classic *B. anthracis* strains, like toxin and capsule production [21]. However, the isolates differ from *B. anthracis* in important microbiological features, a) they are motile, b) resistant to the γ-phage, and c) some isolates are also resistant to penicillin G [21]. Multilocus sequence typing [21–23] revealed a close relationship with *B. anthracis* and with two atypically virulent isolates of the *B. cereus* group: *B. thuringiensis* serovar konkukian strain 97-27 which was isolated from a case of severe human tissue necrosis and shown to be pathogenic in immunosuppressed mice [1,24,25], and *B. cereus* E33L which was isolated from a dead zebra suspected to have died of anthrax, but it remains unclear if it was the cause of death [26].

For the first time we present the complete genome sequence of a *Bacillus* isolate that apparently causes anthrax and possesses both virulence plasmids of *B. anthracis*, but exhibits a chromosomal background that points to a non-*B. anthracis* member of the *B. cereus* group, e. g. *B. cereus* or *B. thuringiensis*.

### Results and Discussion

#### General genome features

The genome of “*B. cereus* variety anthracis” (*Bc* var. anth.) strain CI consists of four replications, a bacterial chromosome and three plasmids encoding together 5696 protein and 162 RNA genes including 11 tRNA operons, 102 tRNA genes and 30 ncRNA genes (Table 1 and Table S1). According to the typing scheme of Sacchi et al. [27], the CI strain possesses the 16S rRNA gene type 6 like classic *B. anthracis*. The chromosome with its size of 5,488,191 bp is larger than the so far sequenced *B. anthracis* chromosomes. A phylogenetic analysis based on 16S rDNA sequences (Figure 1A) confirmed an almost complete correspondence of all *B. cereus* sensu lato strains (except the cytotoxis NVM strain). Multilocus sequence typing (MLST), however, showed that the *Bc* var. anth. strain CI does not cluster with the classic *B. anthracis* strains but can be grouped between them and *B. thuringiensis* serovar konkukian strain 97-27 (Figure 1B and [21]). The chromosomal background distinguishes the new isolate from typical *B. anthracis* strains and groups it as a new member of the *B. cereus* group. Most importantly, the isolate lacks the four *B. anthracis*-specific prophage regions [19,28] and the nonsense mutation in the gene encoding the regulator PlcR [21,29]. *Bc* var. anth. strain CI harbors the three plasmids pCI-XO1, pCI-XO2 and pCI-14.

The sequences described in this article are available at GenBank under accession numbers CP001746–CP001749.

### Identification of the “*B. cereus* var. anthracis” strain CI core and pan genome

The chromosome sequence of *Bc* var. anth. strain CI shares synteny over the whole length with the chromosomes of all strains of the *B. cereus* sensu lato group including the classic *B. anthracis* strains. The organization of the conserved parts of the chromosomal backbone shows a remarkably conserved structured mosaic (Figure 2A). A genome wide BiBlast comparison of *Bc* var. anth. strain CI with all known *Bacillus* genome sequences available at the time of analysis revealed a set of approximately 4000 (~75% of the genes encoded per genome) orthologous genes shared by all *B. cereus sensu* lato strains with the exception of the untypical small genome of *B. cereus* subspecies cytotoxis NVH 391/98 [30], representing a core genome of the *B. cereus* sensu lato group (Figure S1A and B, Table S2). *Bc* var. anth. strain CI shares most orthologous proteins with *B. cereus* E33L (4229 orthologues) and *B. thuringiensis* serovar konkukian strain 97-27 (4180 orthologues) [1,24,25]. In contrast, only 4114 orthologous proteins are shared with *B. anthracis* strain Ames. If the genomes of the *B. subtilis* group are included in the analysis the number of orthologous proteins decreases to approximately 2300 genes which may represent the core genome of the *Bacillus* ([Figure S1C](#)).

### Genomic islands of “*B. cereus* var. anthracis” strain CI

A selected set of seven strains, four *B. anthracis*, two *B. cereus*, *B. thuringiensis* serovar konkukian and *B. weihenstephanensis* KBAB4 from the BiBlast analysis are depicted in Figure 2A. Several features are apparent. The majority of strain specific genes are located in the regions surrounding the terminus of replication. Twelve genomic regions have been identified in *Bc* var. anth. strain CI which encode genes absent in some or all of the compared strains and which show a clear GC-content deviation as compared to their genomic environment. Six of those regions represent islands of 12 kbp or more in size (Table 2) and are colocalized with genes correlated to mobile genomic elements i. e. integrases, recombinases and transposases. These regions might therefore be considered as strain specific genomic islands probably acquired by horizontal gene transfer [31]. The islands I, II, IV and VI were unique to *Bc* var. anth. strain CI (at the time of analysis). For island V a corresponding region has been found in *B. cereus* AH820, and several ORFs are distributed among the *B. cereus* group. Island III has been assigned as prophage based on the similarities to a prophage of *B. thuringiensis* Al Hakam [32]. The islands II and III are located close to each other and are separated by an insertion which is found in many *B. cereus* sensu lato strains. The majority of genes located within the genomic islands of *Bc* var. anth. strain CI encode proteins of unknown functions. In cases of the islands where an annotation was possible the encoded functions are often found in genomic islands [33] such as phage specific genes, a type I restriction modification system, and a transport system. The finding of defined islands within a highly syntenic chromosomal backbone supports the idea of a conserved genomic mosaic structure as described by Han et al. [26].

The presence of genomic islands I to VI and plasmid pCI-14 in strains of the *B. cereus* group was investigated by PCR analysis (Table 2). For each region, two or three gene fragments were amplified. The analysis included 62 representative strains of *B. anthracis* comprising all six MLVA clusters except B2 [11] and deriving from Europe, Asia, Africa and unknown origins. In addition, 46 non-*B. anthracis* strains of the *B. cereus* group (16 *B. cereus*, 8 *B. thuringiensis*, one *B. mycoides*, one *B. weihenstephanensis*, 20 further strains with unclear species affiliation) were tested which represented all clades and lineages described by Priest et al. [22], including strains acquired from strain collections and all strains...
Table 1. General genome features of bacilli from the B. cereus group.

| Species                                  | replicon            | Size          | G+C content | protein genes | % protein coding | rRNA cluster | tRNA genes | Reference                          |
|------------------------------------------|---------------------|---------------|-------------|---------------|-----------------|--------------|------------|------------------------------------|
| “B. cereus var. anthracis” CI            | chromosome         | 5,488,191     | 35          | 5,353         | 80              | 11           | 102        | this work                          |
| B. cereus var. anthracis                 | pCI-XO1            | 181,907       | 33          | 214           | 77              | -            | -          |                                    |
| B. cereus var. anthracis                 | pCI-XO2            | 94,469        | 33          | 111           | 76              | -            | -          |                                    |
| B. cereus var. anthracis                 | pCI-14             | 14,219        | 38          | 18            | 65              | -            | -          |                                    |
| B. anthracis Ames Ancestor               | chromosome         | 5,227,419     | 35          | 5,309         | 80              | 11           | 95         | [87]                               |
| B. anthracis A2012                       | chromosome         | 5,093,554     | 35          | 5,544         | 81              | n. d.*       | n. d.      | [44]                               |
| B. anthracis str. CDC684                 | chromosome         | 5,230,115     | 35          | 5,579         | 84              | 11           | 98         | Dodson et al., 2009, direct submission, unpublished |
| B. cereus G9241                          | pXO1               | 181,773       | 32          | 206           | 75              | -            | -          |                                    |
| B. cereus G9241                          | pXO2               | 94,875        | 33          | 117           | 76              | -            | -          |                                    |
| B. cereus E33L                           | chromosome         | 5,304,942     | 35          | 6,147         | 80              | n. d.        | n. d.      | [42]; unfinished sequence          |
| B. cereus ATCC 14579                     | pBClin29           | 29,866        | 35          | n. d.         | n. d.           | -            | -          |                                    |
| B. cereus ATCC 10987                     | pBClin15           | 190,861       | 32          | 174           | 58              | -            | -          | [42]; complete sequence            |
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| B. cereus ATCC 10987                     | pBClin15           | 190,861       | 32          | 174           | 58              | -            | -          | [42]; complete sequence            |
| B. thuringiensis serovar konkukian str. 97-27 | chromosome       | 5,237,682     | 35          | 5,117         | 84              | n. d.        | 96         | [26]; JGI finishing team 2004, direct submission, unpublished |
| B. thuringiensis str. Al Hakam           | chromosome         | 5,257,091     | 35          | 4,736         | 82              | 14           | 104        | [32]                               |
| B. weihenstephanensis KBAB4              | chromosome         | 5,602,503     | 35          | 5,332         | 81              | n. d.        | 97         | Lapidus et al., 2006, direct submission, unpublished |

*n. d., no data.

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characterized previously [34]. The sequences derived from island III (putative prophage) were widely distributed, and singular fragments or all three fragments together were detected in a large number of strains. The fragment of BACI_c24450 (putative phage protein) was amplified in almost all B. anthracis strains and in 11 non-B. anthracis strains. The sequence fragment of BACI_c24230 (island II, hypothetical protein) was amplified in 4 non-B. anthracis strains of the B. cereus group. All other sequences tested were specific for Bc var. anth. strain CI. The distribution of the genomic islands within this variety of related strains, which does not follow the dendrograms derived by MLST, supports the hypothesis that the bacteria of the B. cereus group share a common pan genome of which parts can be exchanged by horizontal gene transfer. Especially the encoded prophages are therefore widely distributed within the B. cereus group of strains and might thereby represent a way of horizontal gene transfer.

Island IV is an intervening sequence in the gene for sporulation factor \( \sigma^K \)

In B. subtilis, the \( \sigma^K \) gene encoding the late sporulation factor \( \sigma^K \) is interrupted by a 48 kbp prophage-like element. At an intermediate stage of sporulation, the two \( \sigma^K \) gene fragments are joined in frame by site-specific recombination. The recombination event is reciprocal and the intervening DNA is circularized when it is excised from the chromosome. This event does not need to be reversible because the mother cell and its chromosome are discarded after sporulation [35,36]. The 22 kbp sequence of island IV (Table 2, BACI_c43080-BACI_c43240) is lying in the \( \sigma^K \) gene of the Bc var. anth. strain CI (Figure 3). The insertion site is different from that in B. subtilis and the homology of the encoded proteins does not point to a putative prophage. The function of the majority of proteins is up to now unknown. However, a type I restriction modification system (R subunit: BACI_c43130, S subunit: BACI_c43150, M subunit: BACI_c43160) is encoded that is highly similar to corresponding proteins of Geobacillus kaustophilus and other Gram-positive bacteria but absent from bacteria of the B. cereus group. Type I restriction modification systems were found in B. cereus ATCC 14579 and ATCC 10987, but not in B. anthracis [37], and they occur only rarely in the B. cereus group. A gene for a site-specific recombinase that has 53% similarity to the spoIVCA recombinase gene of the B. subtilis intervening sequence [38] is situated directly downstream and in opposite orientation of the 5’ fragment of the \( \sigma^K \) gene. Since “B. cereus var. anthracis” is able to sporulate efficiently, we assume that the intervening sequence is excised in the mother cell by a reciprocal recombination event similar to that described for B. subtilis [36] and Clostridium difficile [39]. The DNA rearrangement and sporulation kinetics are currently investigated. To our knowledge, this is the first description of an intervening sequence in the \( \sigma^K \) gene of an isolate from the B. cereus group.

Comparative genomics of the plasmids

The different lifestyles of the species of the B. cereus sensu lato group are largely defined by differences in plasmid-encoded features [40]. The pathogenic potential of the species B. anthracis is defined by the two plasmids pXO1 and pXO2, which encode the tripartite toxin and the poly-\( \gamma\)-glutamic acid capsule, respectively. B. thuringiensis isolates harbor plasmids that encode the insecticidal crystal proteins (Bt toxin). The B. cereus sensu stricto plasmid profile is extremely variable. The general features of the Bc var. anth. strain CI plasmids sequenced in the present study and those previously sequenced are outlined in Table 1. The B.
cereus group plasmids range in size from 5 to 466 kb and can be divided into three groups. The first group includes pXO1-like plasmids that share a conserved core region which contains genes that are thought to be involved in plasmid replication and maintenance [40]. This group is comprised of pXO1 (B. anthracis strains), pBCXO1 (B. cereus G9241), pBc10987 (B. cereus ATCC 10987), and pCI-XO1, pCI-XO2 and pCI-14, the sizes of the circles are correlated to relative size of the plasmids. Clockwise transcribed genes are depicted in gold, counter clockwise transcribed genes in green. The inner ring displays the GC-content. Invertible elements A and B in pCI-XO1 are marked in light blue, virulence correlated genes in element B are marked red. Genes for capsule synthesis in pCI-XO2 are depicted in red.

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Table 2. “B. cereus var. anthracis” strain CI regions larger than 12 kbp and plasmid pCI-14.

| Island | I | II | III | IV | V | VI | plasmid pCI-14 |
|--------|---------|---------|---------|---------|---------|---------|---------|
| Genome position | 2076648–2089560 | 2283231–2291389 | 2300576–2312524 | 4061541–4081762 | 4789830–4801917 | 5154639–5165848 | 5154639–5165848 |
| Size | 13 kbp | 12 kbp | 13 kbp | 22 kbp | 12.5 kbp | 12.5 kbp | 14.2 kbp |
| Gene fragments tested in PCR* | BACI_c22180 (638 bp) | BACI_c24230 (535 bp) | BACI_c24450 (300 bp) | BACI_c43090 (745 bp) | BACI_c51040 (327 bp) | BACI_c54520 (604 bp) | BMA_pCI1400090 (448 bp) |
| | BACI_c22220 (677 bp) | BACI_c24340 (619 bp) | BACI_c24500 (438 bp) | BACI_c43150 (748 bp) | BACI_c51070 (354 bp) | BACI_c54560 (756 bp) | BMA_pCI14000190 (445 bp) |
| | BACI_c24550 (445 bp) | BACI_c43220 (426 bp) | | | | | |
| Biological function | unknown (glycogen branching enzyme; camelysin-like protein; hypothetical proteins) | unknown (hypothetical proteins) | putative prophage | type I restriction modification system; hypothetical proteins | transport proteins | unknown (putative ATPase; hypothetical proteins) | unknown (hypothetical proteins) |

*The amplicon size is given in brackets.

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Figure 3. Organization of the sigK locus in “B. cereus var. anthracis” strain CI. The disrupted sigK gene is shown on the top. Shaded rectangles/arrows represent the 5’ and 3’ fragments of the disrupted gene. The intervening sequence is indicated by a dashed line, and the position and orientation of the recombinase gene are indicated by a black arrow. An intact sigK gene and a circularized molecule comprising the excised intervening sequence (bottom) are generated by a proposed reciprocal recombination event.

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B. cereus

10987) and some plasmids derived from periodontal and emetic B. cereus isolates. The second group of plasmids includes pXO2 (B. anthracis strains), pBT927 (B. thuringensis serovar konukian str. 97-27) and pAW63 (B. thuringensis serovar kurstaki str. HD73) [41]. These pXO2-like plasmids share a common backbone including genes involved in replication and putative conjugative functions. The second group also comprises pBC210 (B. cereus G9241), pE33L466 and pE33L54 (B. cereus E33L) which share characteristics with pXO2 [1,40]. Plasmid pBC210 encodes a polysaccharide capsule biosynthesis cluster [42], whereas no virulence-related functions were identified on the two large plasmids of B. cereus E33L [26]. “B. cereus var. anthracis” strain CI harbors three plasmids pCI-XO1 (181,907 bp), pCI-XO2 (94,469 bp) and pCI-14 (14,219 bp) (Figure 2B). The plasmids pCI-XO1 and pCI-XO2 fit perfectly to the groups one and two whereas pCI-14 belongs to the third group of B. cereus plasmids which consists of a series of smaller cryptic plasmids [40].

Comparative sequence analysis revealed that the plasmids pCI-XO1 and pCI-XO2 are highly syntenic and show 99% up to 100% identity to the plasmids pXO1 and pXO2 of B. anthracis. Figure S2A–D shows the results of the comparison using the whole genome alignment tool Mauve [43]. Apart from a small number of SNPs, VNTRs and single nucleotide repeats, no large insertions or deletions have been found, which confirms previous observations on this group of B. cereus plasmids [44]. Differences within the coding regions were not identified. The genetic variability between pCI-XO1 and other pXO1 plasmids of B. anthracis is not larger than the variability between the plasmids of B. anthracis sensu stricto (Figure S3A), and the same is true for pCI-XO2 (Figure S3B and C). The third plasmid pCI-14 was found exclusively in the isolates from chimpanzee “Léo”, not in the other two chimpanzee isolates from Côte d’Ivoire that were analyzed and in none of the isolates from Cameroon. We did not find significant similarity to any known nucleotide or protein sequences in the public sequence databases at the time of analysis, thus the function of the plasmid remains unclear. However, to our best knowledge there are no reports about any B. anthracis isolates harboring a third plasmid in addition to the virulence plasmids. Presence of additional plasmids is a feature thought to be characteristic of non-B. anthracis strains of the B. cereus group [40].

There are other examples of atypically virulent strains causing anthrax-like symptoms with plasmid-encoded virulence factors. B. cereus G9241 harbors a plasmid very similar to pXO1 (pBCXO1) and a second plasmid (pBC210) encoding a polysaccharide capsule [42]. Another strain (B. cereus 03BB102) that was recently sequenced harbors a plasmid (p03BB102_179) that contains both the anthrax toxin and capsule biosynthesis genes [45]. It is a known fact that pXO1- or pXO2-like plasmids or single plasmid-encoded genes can be acquired by horizontal gene transfer [41,46–49], but Bc var. anth. strain CI is the first isolate in which both B. anthracis virulence plasmids are present in a non-B. anthracis chromosomal background.

Plasmid- and chromosome-encoded virulence factors

As expected, the pXO1- and pXO2-encoded toxin components, capsule biosynthesis proteins and regulatory proteins are present in the “B. cereus var. anthracis” strain CI. Under inducing conditions (LB broth with 0.8% bicarbonate in a 5% CO2 atmosphere), protective antigen (PA), lethal factor (LF) and edema factor (EF) were synthesized [21] and immunostaining of bacteria with the monoclonal antibody F26G3 [50] confirmed the production of an anthrax-like capsule (data not shown). Compared to B. anthracis Ames Ancestor, PA, EF and LF contain three, four, and eight amino acid exchanges, respectively. Seven of the eight amino acid exchanges of LF and one of the four exchanges in EF result in related amino acids. The transcriptional regulator AtxA [51] differs by one amino acid from the protein of B. anthracis Ames Ancestor. Interestingly, the CI strain encodes new variants of PA [19,45], EF and the PagR regulator [52] that are also found on the pXO1-like plasmid pBCXO1 of B. cereus G9241. The bslA gene which encodes a putative adhesin [53] contains the same frameshift mutation in pCI-XO1 and in pBCXO1.

The “B. cereus var. anthracis” strain CI possesses several known chromosomally encoded virulence factors of the B. cereus group (Table S3) like hemolysins, non-hemolytic enterotoxins and phospholipases [54]. Like in B. anthracis and B. cereus E33L, the complete 17.7-kbp insertion comprising the grl/hbl operon is lacking in the CI strain [26]. Some plasmid-encoded virulence factors (not shown in the table) like the crystal proteins (β-endotoxins) of B. thuringensis [8] and the emetic toxin of emetic strains of B. cereus [9] were also absent from Bc var. anth. strain CI. Internalin proteins located at the bacterial surface are known to interact with host cells via specific protein receptors [55]. Two putative internalins were detected in the CI strain genome and were found at comparable genome positions as in other B. cereus group chromosomes. BAC1_c13660 exhibits high similarity (more than 90% identity) to proteins from other strains of the B. cereus group, but like in B. anthracis it is truncated at the N-terminus due to a frameshift mutation. BAC1_c05600, however, is only weakly/
The PlcR regulon in "B. cereus var. anthracis" strain CI

Recent analyses showed that the pleiotropic regulator PlcR regulates the expression of 45 genes, including many virulence-related genes, in the reference strain B. cereus ATCC 14579, and a similar result can be expected for other strains of the B. cereus group [56]. In B. anthracis, the regulator is not functional due to a nonsense mutation in the plcR gene [29]. Despite the fact that most of the potential members of the PlcR-regulon as described by Ivanova et al. [37] are present in Bc var. anth. strain CI and that the corresponding transcription units are encoded downstream of plcR boxes our results so far indicate that PlcR is also not functional. The PlcR-regulated phosphatidylinositol-specific phospholipase C protein is inactive in several tests: i) colonies did not exhibit a color change on Cereus Identi agar [21]; ii) no PCR-product was obtained by reverse transcriptase PCR with RNA from Bc var. anth. strain CI; and iii) in western blot, culture supernatants did not react with a phospholipase C specific antibody. In all experiments, the type strain B. cereus DSM 31 (corresponding to ATCC 14579) reacted positive as expected (data not shown). Further reverse transcriptase PCR analyses were conducted to detect the mRNA for PlcR-regulated genes. However, expression of the genes for cereolysin O (cho), phosphatidylcholine specific phospholipase C (phoB) and a serine protease (sgg) (Table S3) was comparable to B. anthracis and either completely abolished or substantially weaker compared to the B. cereus DSM 31 control strain. We assume that PlcR is not active in Bc var. anth. strain CI because its C-terminus that is important for interaction with the PapR cell-cell signaling peptide is altered [57]. A frameshift mutation [insertion of an A-residue] near the stop codon results in a C-terminus of the protein that is slightly altered and four amino acids longer than usual: —SHKKNEMKRT compared to —SHKRMKK in B. thuringiensis serovar konkukian. In addition, the gene for the OppA protein of the OppABCDF transport system that is responsible for reimport of PapR into the cell [50] contains a frameshift mutation in Bc var. anth. CI. Interestingly, identical frameshift mutations in plcR and oppA were detected in all strains from Côte d’Ivoire and Cameroon that were analysed, suggesting that they represent a clonally derived lineage.

Motility of "B. cereus var. anthracis" strain CI

In contrast to B. anthracis, bacteria of the Bc var. anth. strain CI exhibited motility. A detailed comparison of the flagella biosynthesis cluster of strain CI with the corresponding gene clusters of two B. anthracis strains and four B. cereus sensu lato strains revealed a fully functional gene cluster (Figure 4). Ten motility- and chemotaxis-associated genes that contain frameshift mutations in B. anthracis Ames Ancestor are intact in the Bc var. anth. strain CI: motA (BACI_c16760), cheA (BACI_c16790), fliC (BACI_c16880), fliF (BACI_c16950), BA1682 (BACI_c16990), BA1688/BA1689 (BACI_c17050), cheV (BACI_c17060), fliN (BACI_c17120), fliM (BACI_c17130), and fliH (BACI_c17210). Like B. thuringiensis serovar konkukian and B. cereus E33L, the CI strain possesses four flagellin genes fliC1 and fliC2 (BACI_c17090 and BACI_c17100), whereas B. anthracis Ames has only one and B. cereus ATCC 14579 has four flagellin genes. The varying numbers of flagellin genes and the insertion of three different additional sets of genes at the flagellin locus in the B. anthracis strains and B. weihenstephanensis might indicate an evolutionary hotspot.

Older studies suggested that motility genes are also regulated by the PlcR regulon. Expression of flagellin genes was downregulated threefold in a plcR mutant [59], and PlcR boxes were found in the

![Figure 4. Comparison of the flagella gene loci encoding flagella genes in strains of the B. cereus group.](https://example.com/figure4.png)

Figure 4. Comparison of the flagella gene loci encoding flagella genes in strains of the B. cereus group. Motile strains are marked with an asterisk. Nonfunctional genes are depicted in red, corresponding functional genes in green, intact corresponding genes shared by all strains are grey. The essential flagellin genes have been marked purple and inserted gene blocks in blue. "B. cereus var. anthracis" CI contains a fully functional motility locus like strains B. cereus E33L and B. thuringiensis konkukian 97-27. B. cereus ATCC 14579 and B. weihenstephanensis KBA4 contain a duplication of the flagellin genes. The insertion of additional sequences and accordingly the duplication of genes occur in corresponding regions of the motility locus. doi:10.1371/journal.pone.0010986.g004
promoter regions of genes related to motility and chemotaxis [37]. However, in the recent publication by Gohar et al. [56] where a variety of methods was used to determine the genes regulated by PtcR, no motility genes were identified. Therefore, motility of B. var. anth. CI can be explained despite the putative inactivity of PtcR.

Protein secretion systems

The secretion of proteins is crucial for the pathogenic life style within the B. cereus group. “B. cereus var. anthracis” strain CI contains apparently two sec-type secretion systems. One system is fully orthologous to the B. subtilis system for the secretion of unfolded proteins [60]. The second system is orthologous to the so-called secA2 system from B. anthracis and other Gram-positive pathogens. The secA2 secretion system is thought to secrete a specific subset of proteins associated with pathogenicity [60–62]. A comparative genome alignment revealed that Be var. anth. strain CI contains a secA2 locus which is organized exactly like in B. anthracis and closely related B. cereus group strains (Figure 5). Upstream of this locus the CI genome is organized like the B. thuringiensis strains and the majority of B. cereus strains. Interestingly, the strain CI genes are integrated in the corresponding core genome position of their orthologous counterparts in the B. anthracis strains respectively in the genome of B. cereus AH187. A phylogenetic tree of the SecA2 protein sequences revealed a close relationship of the proteins (identities around 99%) except for the B. cereus cytotoxin strain NVH391-98 (identity 96%) and the B. thuringiensis serovar konkukian strain 97-27 (identity 81%) (Figure S4A). Comparison of the secA2 secreted S-layer proteins Sap and EA1 encoded downstream of the secA2 locus indicated that both proteins from Be var. anth. strain CI cluster exclusively with the B. cereus variants and not with the proteins encoded by B. anthracis strains (Figure S4B). Interestingly, B. thuringiensis serovar konkukian does not possess homologs of the S-layer proteins Sap and EA1, but encodes two different S-layer proteins at the corresponding genome position that might have been acquired by horizontal gene transfer.

Evolution of genes

The MLST method is based upon phylogenetic comparison of conserved housekeeping genes and is therefore well suited to follow the path of evolution of a given set of genes by point mutations [63,64]. Following MLST based on the genes classically used for strains of the B. cereus group [22], in which recombination events occur less often than point mutations, the CI strain is a member of clade 1 comprising B. anthracis and mainly B. cereus strains (Figure 1B and [21]). However, it was found that gene acquisition from strains clustering outside the known MLST database is common among clade 1 strains [65]. Consequently the phylogenetic analysis on the S-layer proteins confirmed the intermediate
position of strain CI (Figure S4B) between B. cereus E33L on one side and all classic B. anthracis strains on the other side. These results show the importance of the gene selection for the clustering of a strain by MLST. BiBlast, used for general genome comparison (Figure S1), identified common orthologous proteins within all Bacillus genomes. The knowledge of orthologous genes shared by B. cereus genomes identified the group of genes which evolve by point mutations and are thus suitable for phylogenetic analysis.

**Evolution of genomes and epidemiology of B. anthracis strains**

The genomes of the B. cereus group exhibit a conserved mosaic structure (Figure 2A and [26]). Singhular genes and operons of Bc var. auth. CI encoding diverse virulence factors and antibiotic resistance are differently distributed between strains of the B. cereus group. Some virulence associated operons and their genomic environment are present in all strains, others are restricted to a small number of strains (Table S3 and [66]). Examples are the mersacidin resistance operon that until now was only found in few strains of the B. cereus group and in the CI strain and the secA2 operon described above (Figures 5 and S4). Compared genomic mosaic structures have been found in several organisms of distant phylogenetic groups [67–69]. These structures are usually correlated with the presence of mobile genetic elements like insertion sequence elements, phages, transposases, integrases and recombinases and represent an evidence for strain evolution by horizontal gene transfer. In addition, plasmid transfer within the B. cereus group is well established, and there are numerous mobility genes on pXO1 and conjugative functions on pXO2 [41,48,49]. B. anthracis plasmids are not self-transmissible, but both pXO1 and pXO2 could be transferred from B. anthracis to plasmid-cured B. anthracis or B. cereus recipients with the aid of a mobilizing plasmid [46,47].

In B. anthracis, regulatory mechanisms link chromosomally encoded and plasmid-encoded genes. Some chromosomal genes were shown to be regulated by the plasmid-encoded regulator AtxA [70]. For example, the chromosomal S-layer genes sop and eeg are regulated by AtxA in a way that only eeg is significantly expressed under inducing conditions with CO₂ and bicarbonate [71]. In addition, B. anthracis does not sporulate while growing in the blood of the host but requires the activity of the sporulation initiation pathway and Spo0A to express toxin genes [72]. One of several sporulation sensor kinase genes (BA2636) is inactivated by two different frameshift mutations in B. anthracis and in B. cereus G9241 [73]. It was proposed that acquisition of plasmid pXO1 and pathogenicity may require a dampening of sporulation regulation by mutational selection of sporulation sensor histidine kinase defects. However, no frameshift mutations were detected in the BA2636 homolog of Bc var. anth. CI, and no obvious mutations were found in the other eight potential genes for sporulation sensor histidine kinases. It is possible that regulatory systems of plasmids and chromosome are not linked in a way that is observed in classic B. anthracis, and one reason for that might be that the plasmids were acquired relatively recently and are not yet fully adapted to the chromosome. Further experiments will be performed to assess the linkage between chromosomally and plasmid-encoded genes.

A prerequisite for horizontal gene transfer is the direct contact (conjugation) or indirect contact (transformation or transduction) of donor and recipient strains as vegetative cell. Based on previous results, conjugation is the most probable way of plasmid transfer in the B. cereus group [41,74]. In the past, it was thought that in the environment, B. anthracis strains primarily exist as a dormant, highly stable spore and vegetative cells are limited to the stages inside the host [6]. However, it was shown that some strains of B. anthracis can germinate in the rhizosphere and grow in characteristic long filaments, in which plasmid transfer was documented [75]. B. cereus and B. thuringiensis are ubiquitous soil microorganisms that are able to germinate, grow, and sporulate in the rhizosphere of plants or in soil [76,77]. Genetic exchange resulting in a B. cereus group bacterium possessing the anthrax plasmids is therefore possible both during co-infection in a host or in the soil.

The new B. anthracis isolates have been exclusively detected in CI and CA, but may be present in other regions of Africa where they were eventually misdiagnosed using microbiological methods because they differ from classic anthrax. The ecology of the bacteria is atypical, because they were found in primates in a rain forest area, and classic anthrax is usually a disease of herbivores in the savannah [20]. “B. cereus var. anthracis” strain CI shares more orthologous genes with B. cereus E33L and B. thuringiensis serovar konkukian strain 97-27 than with any B. anthracis strain, contains a chromosomal mutation inactivating the PicR regulon different from all known B. anthracis strains, contains a functional motility operon and harbors pXO1 and pXO2 plasmids in the same range of variability like typical anthrax plasmids. Therefore, one might conclude that strain CI represents a B. anthracis subspecies endemic in rain forests that evolved recently from a motile progenitor similar to B. cereus E33L and B. thuringiensis serovar konkukian strain 97-27.

**Species concept**

B. anthracis was named as the cause of the disease anthrax [1,78]. In the B. cereus group of organisms, virulence and pathogenicity appear to be promiscuous and spread with plasmids [40]. The bacterial chromosomes of this group show a high level of synteny and very high numbers of orthologous genes are shared (Figure S1A-C and Table S2). Such a combination is not observed in any other group of comparably related bacterial genomes. Furthermore, there is evidence for a shared set of core putative virulence factors between different pathogenic and non-pathogenic members of the group (Table S3). Very few chromosomal genes or sets of genes are unique to one species. Subtle changes to regulatory networks may be responsible for the range of phenotypic traits displayed by the B. cereus group members. Based on the classic 16S rDNA phylogeny it is not possible to distinguish members of the B. cereus group [1]. Recently it was suggested to designate strains that appear to reside at the boundary between B. cereus and B. anthracis as B. cereus/B. anthracis sensu lato strains [79]. Based on the finding that the isolate described here represents a bacterium that possesses a chromosomal background of a non-B. anthracis member of the B. cereus group, harbors both the pXO1 and pXO2 virulence plasmids of B. anthracis and apparently causes anthrax, we suggest to designate this and related isolates as “B. cereus var. anthracis” strains CI and CA.

**Methods**

**Genome Sequencing**

DNA from “B. cereus var. anthracis” strain CI was isolated using CTAB treatment and phenol-chloroform extraction as described previously [80]. For preparation of whole shotgun libraries, DNA was fragmented to sizes between 1.5 and 3.0 kbp by appropriate mechanical shearing (Hydroshear, GenEMACHINES, San Carlos CA, USA). DNA fragments were separated by gel electrophoresis after end-repair and cloned using vector pCR4.1-TOPO (TOPO-Cloning Kit for Sequencing; Invitrogen, Karlsruhe, Germany). A total of about 45,600 plasmids were isolated using two BioRobots8000 (Qiagen, Hilden, Germany) and 71,701 sequences
were automatically analyzed on 3730XL (Applied Biosystems, Darmstadt, Germany) and assembled into four replicons. PCR-based techniques on genomic DNA resulted in 3,850 reads which were taken to close remaining gaps and to ensure a minimum quality value of phred 45 on each position within the genome. PCR have been carried out with the BioXact Kit (Qiagen, Hilden, Germany) as described by the manufacturer with product depending variations according the cycling program and the amount of enzyme.

Bioinformatics

Coding sequences (CDS) and open reading frames (ORFs) were predicted with YACOP [81] using therein the ORF-finders Glimmer, Critica and Z-curve. All CDS have been manually curated and were verified by comparison with the publicly available databases SwissProt, GenBank, ProDom, COG, and Prosite using the annotation software ERGO [82]. Complete genome comparisons were done with ACT [83] based on replicon specific nucleotide BLAST [84] and with protein based BiBlast comparisons to all known sequenced bacilli (A. Wollherr, personal communication). Phylogenetic analysis was done with the programs of the PHYLIP software suite [85] and the MEGA4 software using ClustalW multiple sequence alignment for deriving a Neighbor-Joining based tree and bootstrapping with 1000 replicants [86].

Comparative analysis of members of the B. cereus group by PCR screening of selected genomic regions

Standard PCR was performed for the detection of six chromosomal genomic islands and plasmid pCI-14 among a panel of strains from the B. cereus group. Primers (Metabion, Martinsried, Germany) were designed complementary to sequences of the CI strain and used to amplify PCR products in the range from 300 bp to 800 bp (Table 2). The reaction volume was 25 μl with 2.5 μl 10× buffer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 0.6 units of Taq polymerase (Fermentas, St. Leon-Rot, Germany), 0.2 μM of each primer and 10–50 ng of template DNA. The PCR program consisted of one step at 95°C for 5 min, followed by 35 cycles with 95°C for 30 s, 50°C for 30 s and 72°C for 45 s, and a final step at 72°C for 10 min. The primer sequences are available upon request.

Supporting Information

Figure S1 Shared chromosomal genes identified by bidirectional BLAST (BiBlast) of “B. cereus var. anthracis” strain CI and selected chromosomes of bacilli. The colors indicate the number of shared genes with the other strain. Due to strain specific multi copy genes the numbers differ depending on the direction of the BiBlast comparison. (a) Strains “B. cereus var. anthracis” CI, B. anthracis Ames Ancestor and B. cereus E33L, (b) strains “B. cereus var. anthracis” CI, B. anthracis Ames Ancestor and B. weihenstephanensis KBA4 and (c) strains “B. cereus var. anthracis” CI, B. anthracis Ames Ancestor and B. licheniformis DSM13.

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Figure S2 Whole replicon sequence alignments of known pXO1- and pXO2-like plasmids with MAUVE. The colors indicate blocks of high similarity. (a) Sequence alignments of pXO1 plasmids, two invertible regions A and B enveloped by transposases have been identified. Region A represents an IS element and region B represents a 44.5 kb pathogenicity island encoding the anthrax related virulence factors. (b) Sequence alignment of pCI-XO1 and B. cereus G9241 plasmid pBCXO1, a pXO1-like plasmid harboring the pathogenicity island encoding the anthrax toxin. (c) Sequence alignment of pXO2 plasmids. (d) pCI-XO2 versus B. thuringiensis serovar konkukian plasmid pBT9727 lacking the pathogenicity island (PAI). Reference: Darling ACE, Mau B, Blatter F, Perna NT (2004) Mauve: Multiple alignment of conserved genomic sequence with rearrangements. Genome Research 14: 1394–1403.

Found at: doi:10.1371/journal.pone.0010986.s002 (12.32 MB TIF)

Figure S3 Evolutionary relationships of pXO1 and pXO2 plasmids. The evolutionary history was inferred using the Neighbor-Joining method [1]. The bootstrap consensus tree inferred from 500 replicates [2] is shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 188163 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [4]. (a) Phylogenetic tree calculated on full length alignments from pXO1 plasmids of Bc var. anth. CI and different B. anthracis strains. The invertable elements are normalized. (b) Phylogenetic tree calculated on full length alignments from pXO2 plasmids of Bc var. anth. CI and different B. anthracis strains. (c) Phylogenetic tree with pXO2 plasmids from Bc var. anth. CI, different B. anthracis strains and two related plasmids from B. thuringiensis. For plasmids of B. anthracis strains, only the strain designations are indicated. References: 1. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425. 2. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791. 3. Tamura K, Nei M, Kumar S (2004) Prospects for inferring evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [2]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 188163 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [4]. (a) Phylogenetic tree calculated on full length alignments from pXO1 plasmids of Bc var. anth. CI and different B. anthracis strains. (b) Phylogenetic tree calculated on full length alignments from pXO2 plasmids of Bc var. anth. CI and different B. anthracis strains. (c) Phylogenetic tree with pXO2 plasmids from Bc var. anth. CI, different B. anthracis strains and two related plasmids from B. thuringiensis. For plasmids of B. anthracis strains, only the strain designations are indicated. References: 1. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425. 2. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791. 3. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Mol Biol Evol 24: 1596–1599.

Found at: doi:10.1371/journal.pone.0010986.s003 (11.24 MB TIF)

Figure S4 Phylogenetic comparison of SecA2 and S-layer proteins. (a) Rooted phylogenetic tree of SecA2 proteins (b) Phylogenetic tree of S-layer proteins Sap and EA1.

Found at: doi:10.1371/journal.pone.0010986.s004 (12.43 MB TIF)

Table S1 Stable RNAs and Riboswitches

Found at: doi:10.1371/journal.pone.0010986.s005 (0.03 MB DOC)

Table S2 Core and Pan genome of the “B. cereus var. anthracis” strain CI genome and selected Bacillus strains.

Found at: doi:10.1371/journal.pone.0010986.s006 (0.04 MB DOC)

Table S3 Presence or absence of virulence factors and regulatory proteins in “B. cereus var. anthracis” strain CI.
Table S4  Identity of internalin proteins present at comparable genome positions.  Found at: doi:10.1371/journal.pone.0010986.s008  (0.03 MB DOC)

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Author Contributions
Conceived and designed the experiments: SRK HB BA WL CB FHL HE GG RG HL. Performed the experiments: SRK HB BA WL CF DM. Analyzed the data: SRK EBB HB SD AW HL. Contributed reagents/materials/analysis tools: ECH FHL. Wrote the paper: EBB HB GP GG RG HL.

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