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# Characterization of *Bacillus anthracis*-Like Bacteria Isolated from Wild Great Apes from Côte d'Ivoire and Cameroon

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**We present the microbiological and molecular characterization of bacteria isolated from four chimpanzees and one gorilla thought to have died of an anthrax-like disease in Côte d'Ivoire and Cameroon. These isolates differed significantly from classic *Bacillus anthracis* by the following criteria: motility, resistance to the gamma phage, and, for isolates from Cameroon, resistance to penicillin G. A capsule was expressed not only after induction by $CO_2$ and bicarbonate but also under normal growth conditions. Subcultivation resulted in beta-hemolytic activity and gamma phage susceptibility in some subclones, suggesting differences in gene regulation compared to classic *B. anthracis*. The isolates from Côte d'Ivoire and Cameroon showed slight differences in their biochemical characteristics and MICs of different antibiotics but were identical in all molecular features and sequences analyzed. PCR and Southern blot analyses confirmed the presence of both the toxin and the capsule plasmid, with sizes corresponding to the *B. anthracis* virulence plasmids pXO1 and pXO2. Protective antigen was expressed and secreted into the culture supernatant. The isolates possessed variants of the Ba813 marker and the SG-749 fragment differing from that of classic *B. anthracis* strains. Multilocus sequence typing revealed a close relationship of our atypical isolates with both classic *B. anthracis* strains and two uncommonly virulent *Bacillus cereus* and *Bacillus thuringiensis* isolates. We propose that the newly discovered atypical *B. anthracis* strains share a common ancestor with classic *B. anthracis* or that they emerged recently by transfer of the *B. anthracis* plasmids to a strain of the *B. cereus* group.**

*Bacillus anthracis*, the etiological agent of anthrax, is found worldwide and is able to infect virtually all mammals, including humans. The danger of its intentional release is prevalent since the anthrax attacks in the United States in 2001.

Together with *Bacillus cereus* and *Bacillus thuringiensis*, *B. anthracis* is a member of the *Bacillus cereus* group. *B. thuringiensis* is an insect pathogen, and *B. cereus* is known mainly as a food poisoning pathogen characterized by toxin-induced emetic and diarrheagenic syndromes. More severe infections develop mainly in immunocompromised patients or patients with other risk factors (for a review, see reference 12). However, life-threatening and fatal cases of pneumonia and bacteremia resembling infection by *B. anthracis* were also observed in otherwise healthy people (20, 39).

Multilocus sequence typing (MLST) and fluorescent amplified fragment length polymorphism (AFLP) have proved their efficiency in typing members of the *B. cereus* group. Both methods show that genetic diversity is high within the *B. cereus* and *B. thuringiensis* groups, whereas *B. anthracis* is highly homogenous and can therefore be considered a particularly monomorphic species (17, 19, 45). Typing of *B. anthracis* strains and isolates is usually achieved by multiple-locus variable-number tandem repeat analysis (MLVA) (25). However, based on the chromosome genomic comparison reviewed previously by Rasko et al. (48), it is not possible to distinguish members of the *B. cereus* group from one another, and therefore *B. anthracis*, *B. cereus*, and *B. thuringiensis* can be considered one species (16). The differences in pathogenicity among the three species are mainly encoded on plasmids. *B. anthracis* possesses two plasmids, pXO1 and pXO2, both essential for virulence, that carry genes for toxin synthesis (*pag*, *cya*, and *lef*) and capsule synthesis (*capB*, *capC*, *capA*, and *capD*), respectively (37, 41). Regulation of virulence gene expression is different in *B. anthracis* and in other *B. cereus* group members (1, 28, 38, 42, 56).

Fast and reliable diagnosis of *B. anthracis* is of high importance for timely and adequate treatment of a patient. So far, *B. anthracis* strains were easily distinguished from nonanthrax members of the *B. cereus* group by a few microbiological tests that are recommended by the World Health Organization (WHO) and by the Centers for Disease Control and Prevention (CDC); in contrast to *B. cereus* and *B. thuringiensis*, *B. anthracis* is nonmotile, lacks beta-hemolytic activity, and is sensitive to penicillin G and to lysis by the gamma phage. It is able to produce a capsule in vivo and in vitro under appropriate conditions (53). However, not a single diagnostic trait appears to be consistent for all *B. anthracis* isolates (6, 30, 36), and atypical isolates of *B. anthracis* and other members of the *B. cereus* group have been described previously (26). Therefore, the application of molecularly based methods like PCR has become increasingly important for the diagnosis of *B. anthracis* (9, 14, 43, 46).

Anthrax is globally distributed, but the most diverse isolates are found in southern Africa, resulting in speculations that Africa is the origin of *B. anthracis* (24). Herbivorous animals

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TABLE 1. Results of bacteriological examinations of great apes from Côte d'Ivoire and Cameroon

| Ape | Result[d] | | | |
|---|---|---|---|---|
| | Clinical sample | Direct culture | Direct culture, 65°C | Enrichment culture, 65°C |
| Chimpanzee (Léo)[a] | Spleen[c] | +++ | − | − |
| | Lung[c] | +++ | − | − |
| Chimpanzee (Olduvai)[a] | Lung | +++ | − | − |
| | Liver[c] | +++ | − | − |
| Chimpanzee (Dorry)[a] | Spleen | +++ | − | − |
| | Lung[c] | + | + (2 colonies) | + |
| Chimpanzee[b] | Bone Marrow[c] | + (8 colonies) | ND | + |
| | Muscle[c] | + (1 colony) | ND | ND |
| Gorilla[b] | Tooth[c] | + (1 colony) | ND | ND |

[a] From Côte d'Ivoire.
[b] From Cameroon.
[c] DNA isolation from *B. anthracis* colonies derived from these samples.
[d] −, no suspicious colonies detected; +, few suspicious colonies detected; +++, high incidence of suspicious colonies. ND, not done.

are the most susceptible animals, and ungulates of the savannahs are most frequently affected. Cases in primates, except humans, were rarely observed (11). Therefore, it was exceptional to find wild great apes in rainforest regions that had apparently died from anthrax. It was first described in the Taï National Park, Côte d'Ivoire, where at least six wild chimpanzees died of an acute bacterial infection between October 2001 and June 2002. Sequencing of the 16S rRNA gene and real-time PCR using appropriate genome regions indicated the presence of a member of the *B. cereus* group that possessed the plasmid-encoded virulence genes of *B. anthracis* (31). At the end of 2004, more cases of anthrax among great apes were diagnosed: three chimpanzees and one gorilla died at the periphery of the Dja Reserve, Cameroon (32). All great apes were positive for the *B. anthracis*-specific *pag* and *capC* virulence genes. MLVA analyses showed that two different but related strains of *B. anthracis* had infected the great apes from Côte d'Ivoire and Cameroon, respectively. Interestingly, these strains form a highly distinct cluster separate from all other previously described *B. anthracis* strains, and further genetic analyses showed that these strains are significantly different from "classic" *B. anthracis* strains. They lack the four *B. anthracis*-specific prophage regions, regions A, C, D, and E (46), and new alleles of the two toxin genes *pag* and *cya* were identified (34).

In this study, we present data on the isolation and microbiological differentiation of bacteria from chimpanzees and one gorilla. Our results indicate that the isolates from Côte d'Ivoire (termed *B. anthracis* CI) and Cameroon (termed *B. anthracis* CA) are almost identical, with virulence plasmids closely related to those of *B. anthracis* in a chromosomal background of a new member of the *B. cereus* group.

## MATERIALS AND METHODS

**Animal samples and bacterial strains.** Six organ samples collected from three chimpanzees from Côte d'Ivoire stored at $<-70$°C were investigated (31). The samples were collected from intact carcasses of two animals (Léo and Olduvai) and from the partially opened carcass of one animal (Dorry). For the Cameroon apes, a cranium and bones were stored at room temperature, and a muscle sample was stored in RNAlater reagent (QIAGEN, Hilden, Germany) at room temperature. Samples from the muscle and the bone marrow of one chimpanzee and the tooth of a gorilla were studied (Table 1). All isolations were performed under biosafety level 3 conditions. Other bacterial strains from the *B. cereus* group used for MLST were *B. cereus* NCCB 72001 (same as ATCC 10987), *B. cereus* DSM 31 (same as ATCC 14579), *B. cereus* DSM 4312, *B. cereus* DSM 2301, *B. cereus* DSM 609, *B. cereus* DSM 4490 (same as ATCC 11778), *B. thuringiensis* DSM 350, *B. thuringiensis* DSM 2046, *B. thuringiensis* DSM 5815, *B. mycoides* DSM 2048, and *B. weihenstephanensis* DSM 11821. These strains were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) and The Netherlands Culture Collection of Bacteria (Utrecht, The Netherlands) as indicated. The 17 classic *B. anthracis* strains were described recently (26). The sequences of *B. cereus* E33L (formerly termed "Zebra Killer," [ZK]; GenBank accession number NC_006274), *B. cereus* G9241 (accession number NZ_AAEK00000000), *B. cereus* ATCC 4342 (17, 45), and *B. thuringiensis* serovar konkukian strain 97-27 (accession number NC_005957) were derived from the corresponding databases.

**Microbiological studies.** After the addition of sterile distilled water, the organ samples from the chimpanzees from Côte d'Ivoire were homogenized using a mortar. One aliquot was heated at 65°C for 30 min to kill vegetative cells. The untreated and the heat-treated aliquots were plated onto the following agar plates and designated as direct cultures: Columbia agar (Oxoid, Wesel, Germany) with 5% sheep blood, Luria-Bertani (LB) agar, blood-trimethoprim agar (with anthrax supplement [1.6 mg trimethoprim, 6.4 mg sulfamethoxazole, and 20 mg polymyxin B per liter agar medium]), PLET agar (40 g Difco heart infusion agar, 30,000 units polymyxin B, 40 mg lysozyme, 200 mg EDTA, and 40 mg thallous acetate per liter agar medium), Cereus Ident agar (Heipha Diagnostica, Eppelheim, Germany) with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate (44), and Cereus selective agar (Merck, Darmstadt, Germany). In addition, 10 ml of liquid enrichment medium (LB or LB with anthrax supplement) was inoculated with either 0.1 ml of the nontreated or 0.1 ml of the heat-treated aliquot. As only very little material was available from the Cameroon apes, the bone marrow and muscle samples were homogenized as described above, but only the bone marrow sample was heated, and both the heat-treated and the nontreated aliquots were cultivated in enrichment medium. The tooth was incubated in liquid LB medium for a few minutes, and the medium was subsequently streaked onto the different agar plates.

After 24 h of incubation at 37°C, all enrichment cultures were streaked onto the plates indicated above for isolation. The presence of *B. anthracis* in enrichment cultures was tested by real-time PCR as described below. Colony growth was monitored daily. If a high number of colonies suspicious for *B. anthracis* was observed on plates with direct cultures, the corresponding enrichment cultures were not differentiated further. Suspicious colonies were subcultured on the solid media described above.

Tests for susceptibility to penicillin G and the gamma phage test were performed as described in the WHO *Manual for Laboratory Diagnosis of Anthrax* (57). Results of the gamma phage assay were read after 6 to 8 h and after 24 h of incubation at 37°C. Motility of bacteria was observed microscopically by hanging-drop preparation (WHO manual) and by observing growth in tubes with API M motility medium (BioMérieux, Nürtingen, Germany). Gram staining was performed using the microscopy Gram color reagents from Merck. Further bacteriological examinations were performed for selected isolates. Formation of the capsule was tested by cultivation on bicarbonate agar under a 5% $CO_2$ atmosphere. Sensitivity to different antibiotics was analyzed using the Etest (VIVA Diagnostika, Cologne, Germany). The biochemical capacity was tested using the API 50 CHB system (BioMérieux).

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**Electron microscopy.** At least one bacterial isolate from each great ape was studied by electron microscopy. As a control, the classic *B. anthracis* isolate UDIII-7 was used. All bacterial samples (agar or suspension cultures) were first fixed at a biosafety level 3 laboratory in 10% formaldehyde including 1% glutaraldehyde in 0.05 M HEPES buffer (pH 7.2) for at least 2 h. The agar cultures were gently washed with distilled water prior to fixation with 1% $OsO_4$ for scanning electron microscopy (SEM). After stepwise dehydration in graded alcohol, the samples were critical point dried in $CO_2$ (CPD 030; BAL TEC, Vaduz, Liechtenstein), mounted onto the sample stubs, sputter coated with 3 nm Au/Pd (Polaron Sputter Coating Unit E 5100; GaLa Instrumente, Bad Schwalbach, Germany), and examined with a LEO FEG-1530 scanning electron microscope (Carl Zeiss SMT AG, Oberkochen, Germany) at 5 kV.

After a short wash with distilled water, the fixed suspension cultures were first agar block embedded by mixing equal volumes of concentrated bacteria and low-melting-point agar (3% phosphate-buffered saline) and postfixed with $OsO_4$. After block staining with uranyl acetate (2% in distilled water), the samples were dehydrated stepwise in graded alcohol and embedded in LR-White (Science Service, Munich, Germany), which was polymerized at 60°C overnight. The ultrathin sections for transmission electron microscopy (TEM) were prepared with an ultramicrotome (Ultracut S; Leica, Wetzlar, Germany) and placed onto naked 400-mesh grids or onto Pioloform-F (Wacker Chemie, Munich, Germany)-coated 100-mesh grids. The sections were stained with lead citrate and stabilized with carbon evaporation (BAE 250; BAL TEC). The sections were examined using a TEM 902 (Carl Zeiss SMT AG) at 80 kV, and the images were digitized using a slow-scan charge-coupled-device camera (Pro Scan; Scheuring, Germany).

Concentrated bacteria were adsorbed for 1 min on the Pioloform-F-coated, carbon-stabilized, and glow-discharged copper grids and washed three times on droplets of distilled water. After negative staining with 1% uranyl acetate (pH 4 to 4.5), the sample was analyzed by TEM.

**Molecular methods.** Bacterial DNA was isolated according to the protocol for gram-positive bacteria of the DNeasy tissue kit (QIAGEN). For inactivation, colony material of characterized strains of *B. anthracis*, as well as material from *B. anthracis* strains CI and CA, was autoclaved (121°C, 20 min) before DNA isolation.

Real-time PCR was performed with 50-μl volumes using either 1 μl of purified DNA, 3 μl of bacterial culture, or spiked colonies, as described previously by Ellerbrok et al. (14). Conventional PCR for the detection of the Ba813 fragment was performed according to a method described previously by Patra et al. (43). The fragment was sequenced using the ABI PRISM FS BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Darmstadt, Germany). Sequence data were analyzed with the LaserGene software. PCRs for amplification of the SG-749 fragment were performed as described previously (9). Ten microliters of the SG-749 PCR products was restricted with 10 units of AluI (MBI Fermentas, St. Leon-Rot, Germany) and analyzed by agarose gel electrophoresis. In addition, the PCR products were sequenced. MLST was performed by applying the schemes described previously by Helgason et al. (17) and Priest et al. (45). Sequencing was performed as described above. Other conventional PCR and sequencing analyses were performed according to standard procedures using *Taq* polymerase (MBI Fermentas) and the ABI PRISM FS BigDye Terminator Cycle Sequencing Ready Reaction kit.

Plasmids of *B. anthracis* strains were isolated from exponentially growing cultures according to a method described previously by Jensen et al. (23) and separated on a 0.7% agarose gel. Southern analysis was performed by capillary transfer (52), and hybridization with digoxigenin-labeled probes was carried out at 50°C. The same fragments of the *capC* and *pag* genes used for real-time PCR (14) were labeled with PCR DIG labeling mix (Roche, Mannheim, Germany), and the blot was developed using anti-digoxigenin antibodies conjugated to alkaline phosphatase and CDP Star according to the manufacturer's protocol (Roche).

For the detection of protective antigen (PA), bacteria were grown to late log phase in LB broth buffered with 100 mM HEPES (pH 8.0) and 0.8% (wt/vol) sodium bicarbonate in an atmosphere containing 5% $CO_2$ at 37°C. Culture supernatants were analyzed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using standard procedures and subsequently transferred onto an Immobilon P polyvinylidene difluoride membrane (Millipore, Schwalbach, Germany) by semidry blotting. Western blot analysis was performed essentially as described previously (51) by using a monoclonal anti-PA antibody (clone 138; Senova, Jena, Germany) in a concentration of 1.5 μg/ml and a goat anti-mouse antibody conjugated to horseradish peroxidase (Dianova, Hamburg, Germany) in a concentration of 1 μg/ml. Signals were visualized on X-ray film (Hyperfilm ECL; Amersham Biosciences, Freiburg, Germany) by chemiluminescence (ECL substrate; Pierce, Bonn, Germany). As a positive control, 20 ng of a purified recombinant PA preparation kindly provided by W. Beyer, Hohenheim, was used.

Phylogenetic analysis (neighbor-joining tree) was performed using the Phylip algorithm.

## RESULTS

**Microbiological and electron microscopical characterization.** Colonies were assessed to be suspicious for *B. anthracis* if they showed the following features: possession of a so-called "Medusa head" (curl-like projections from the colony edge), no phospholipase C activity (no color change on Cereus Ident agar), no fermentation of mannitol and weak lecithinase activity (pink color and faint precipitation on Cereus selective agar), and distinct growth on blood trimethoprim and on PLET agar. Colonies or suspension cultures were screened for the presence of plasmids as described previously (14). Suspicious colonies could be isolated directly from samples of all five apes examined. However, as indicated in Table 1, the incidence of suspicious colonies differed among the samples, being very high in five of six organ samples from chimpanzees of Côte d'Ivoire (CI) that had been stored at −70°C and being very low (two times one and eight colonies, respectively) in the three samples from apes from Cameroon (CA). The latter samples contained a large number of nonanthrax *Bacillus* species, most likely due to contamination with the soil from the rainforest. After heating of the samples at 65°C, suspicious colonies were detected only in direct and enrichment cultures of one lung sample from Côte d'Ivoire and in the enrichment culture of the bone marrow sample from Cameroon (Table 1). This indicates that the bacteria had already formed spores in these two samples, whereas only vegetative cells were present in the organ samples from intact carcasses.

After 24 h of incubation at 37°C, suspicious colonies from direct or enrichment cultures (primary cultures) of all ape samples were similar and showed the suspicious features described above. On Columbia blood agar, colonies were approximately 5 mm in diameter, rough, and gray-greenish, and some had a mucoid center. The colonies grew with "Medusa head" like classic *B. anthracis* colonies (Fig. 1A to D) and were not beta-hemolytic. After 48 h of incubation, however, growth of the same colonies was atypical for *B. anthracis*. The colonies had a diameter of more than 10 mm with a smooth, shiny, mucoid, yellow-greenish center (diameter, >5 mm) and a dry, gray, weakly jagged edge. Growth on PLET agar was more inhibited than usual for *B. anthracis*, and CI and CA colonies were substantially smaller than typical *B. anthracis* colonies after a 2-day incubation.

The essential characteristics of the *B. anthracis* isolates CI and CA were compared with those of classic *B. anthracis* strains and nonanthrax strains of the *B. cereus* group (Table 2). In contrast to classic *B. anthracis* strains, all isolates from primary cultures from apes were resistant to the gamma phage and motile. In hanging-drop preparations, even short chains of bacteria were clearly motile in some cases. Broth cultures and semisolid motility media were evenly turbid. However, no inverted fir tree effect was observed in tubes with motility medium (data not shown).

Negative staining electron microscopy revealed peritrich flagellation of the *B. anthracis* CI and CA strains, but no

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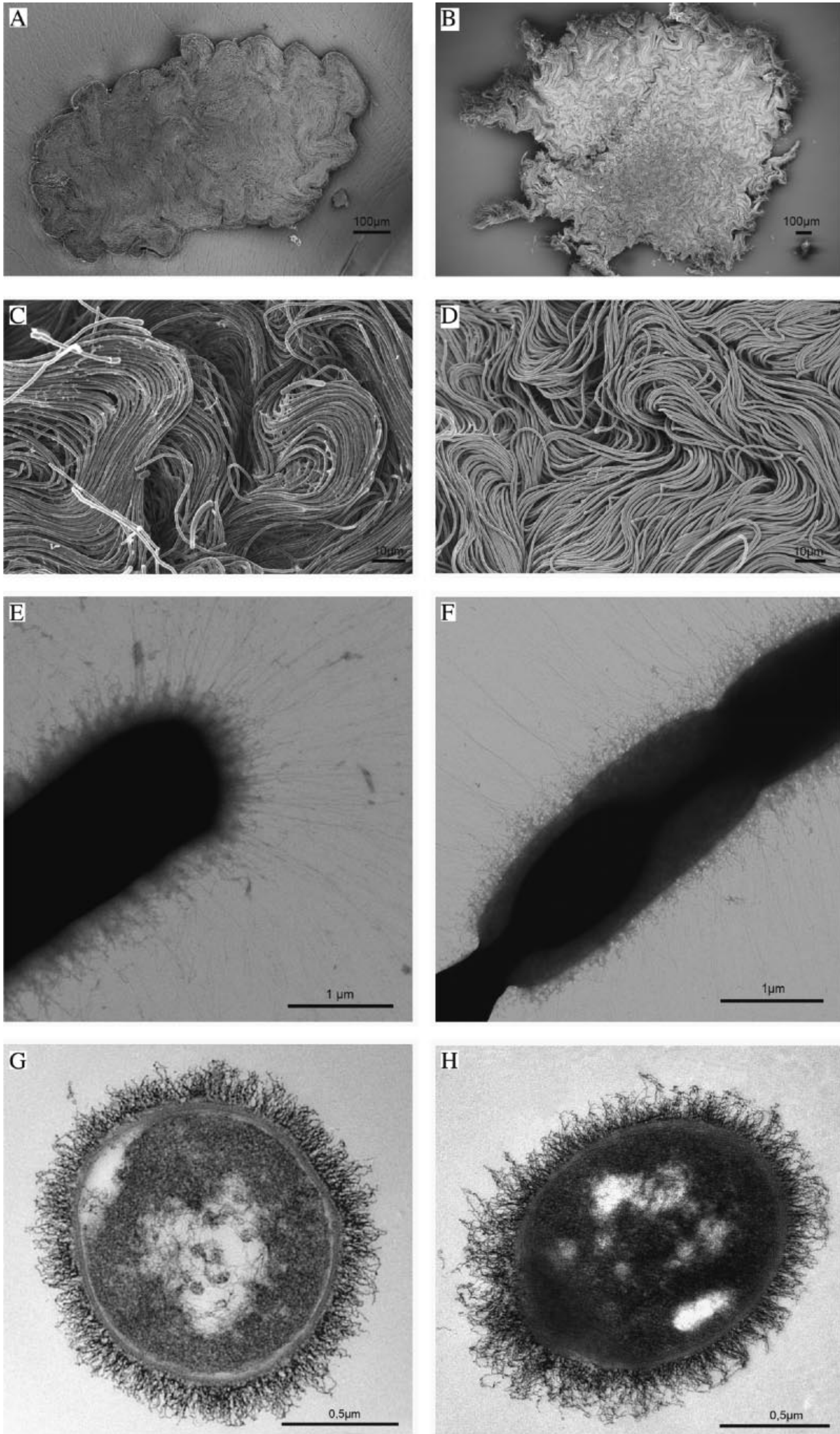


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TABLE 2. Bacteriological discrimination characteristics of atypical *B. anthracis* strains isolated from great apes, classic *B. anthracis* strains, and other strains of the *B. cereus* group

| Microbiological characteristic | Result[a] | | | | | |
|---|---|---|---|---|---|---|
| | *B. anthracis* CI | | *B. anthracis* CA | | *B. anthracis* | *B. cereus* |
| | Primary culture | Subculture | Primary culture | Subculture | | |
| Hemolysis | − | +/− | − | +/− | − | + |
| Motility | + | + | + | + | − | + |
| Susceptibility to gamma phage | − | +/− | − | +/− | + | − |
| Penicillin G | S | S/R | R | R | S | R |
| Capsule | +[b] | +/− | + | +/− | + | Absent in vitro[c] |

[a] S, sensitive; R, resistant; −, negative; +, positive; +/−, some subclones positive, others negative.
[b] Capsule production on bicarbonate agar under a $CO_2$ atmosphere and on blood agar under an ambient atmosphere.
[c] Certain other *Bacillus* spp. can produce a polypeptide capsule but not under normal culture conditions.

flagella were seen on cells of a classic *B. anthracis* strain (Fig. 2A and B). Gram staining of CI and CA isolates showed gram-positive rods and chains that differed in thickness. Spores that did not cause swelling of the cell were formed (data not shown). The difference in cell thickness was also confirmed by scanning electron microscopical studies that revealed capsule-like surface structures on a fraction of the cells (Fig. 2C). These structures were not seen on the surface of cells from a classic *B. anthracis* strain (Fig. 2D). In addition, twisted forms of the bacilli were frequently observed for CI and CA strains and rarely observed for a classic *B. anthracis* strain (Fig. 2E and F), although these atypical cell morphologies were described previously (29).

As expected, a capsule was detected after cultivation of a classic *B. anthracis* strain and the CI and CA strains on bicarbonate agar in a $CO_2$-enriched atmosphere (Fig. 1E to H). Surprisingly, the cells were also encapsulated when the CI and CA strains were grown on common media like Columbia blood or LB agar under ambient atmosphere. This capsule accounts for the structures observed on the surface of bacterial cells and for the mucoid appearance of the colonies.

Susceptibility to penicillin G varied for the *B. anthracis* CI and CA strains (Table 2). Whereas the CI isolates were sensitive, isolates from Cameroon were resistant to penicillin G. However, resistant colonies were also found in subclones of the CI isolate. Susceptibility to a panel of antibiotics was determined using the Etest (Table 3). The MICs determined for the different strains and isolates were comparable for most antibiotics except for tetracycline and amoxicillin-clavulanic acid, in which case the CA strain was less susceptible. For the latter antibiotic, some CA isolates showed intermediate sensitivity. All other MICs indicated sensitivity of the *B. anthracis* CI and CA strains to all antibiotics used in the Etest.

Analysis of the biochemical properties of the CI isolates using the API 50 CHB system showed positive reactions for D-ribose, D-glucose, *N*-acetylglucosamine, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, AmiDon (starch), glycogen, potassium gluconate, gelatin, and nitrate. For the Voges-Proskauer reaction and gluconate or nitrate metabolism, differences between single isolates were observed. CA isolates were positive for D-fructose and nitrate and negative for gluconate and the Voges-Proskauer reaction. All other reactions were identical to those from the CI isolates. Single isolates differed in the metabolism for D-trehalose and gelatin. These reaction patterns point with a high probability towards the presence of *B. anthracis*, but a definitive diagnosis is not possible with these biochemical criteria.

Some bacterioscopic findings were not typical for *B. anthracis*. Due to the different sizes of the bacterial rods and chains (Fig. 2C) and the partially twisted forms (Fig. 2E), the presence of mixed cultures was assumed. To exclude mixed cultures, single colonies were repeatedly subcultured on different agar plates and retested (Table 2). In these subclones, colonies with very different morphologies and characteristics were observed. For example, small smooth colonies were found, which, in contrast to the primary cultures, exhibited strong beta-hemolysis and were sensitive to the gamma phage. All colonies remained negative for phospholipase C activity. Real-time PCR assays revealed the presence of the *pag* gene in all subclones, confirming that all of these subcloned isolates are *B. anthracis*-like. The ability to form a capsule was also variable in the subclones, and could be correlated with the loss of the *capC* marker as indicated by real-time PCR.

**Molecular characterization.** Real-time PCR assays targeting the plasmid markers *pag* (on plasmid pXO1) and *capC* (on plasmid pXO2) as well as the chromosomal marker *rpoB* were performed with initially isolated suspicious colonies, with enrichment cultures, and with DNA preparations (14). Both primary cultures and subcultures of *B. anthracis* strains CI and CA were analyzed. In all cases, fluorescence signals of the *pag* marker appeared early, with cycle threshold ($C_T$) values of 20 to 25, whereas signals of the *rpoB* target were delayed with $C_T$ values above 30. The *capC* gene marker was positive in PCR assays of primary cultures ($C_T$ values of 20 to 25) and in the majority of the subclones. The occurrence of delayed signals of

FIG. 1. Colony morphology and capsule production studied by SEM and TEM. Bacteria isolated from great apes (A, C, E, and G) and classic *B. anthracis* strains (B, D, F, and H) presented the same morphological criteria. The same colony morphology on agar was seen by SEM (A to D). Cells from suspension cultures showed the same capsule and filament (piles) morphology by negative staining (E and F), and a capsule fringe at the outer wall of the bacteria in thin cross-sections by TEM (G and H). Similar results were obtained with all bacterial isolates tested.

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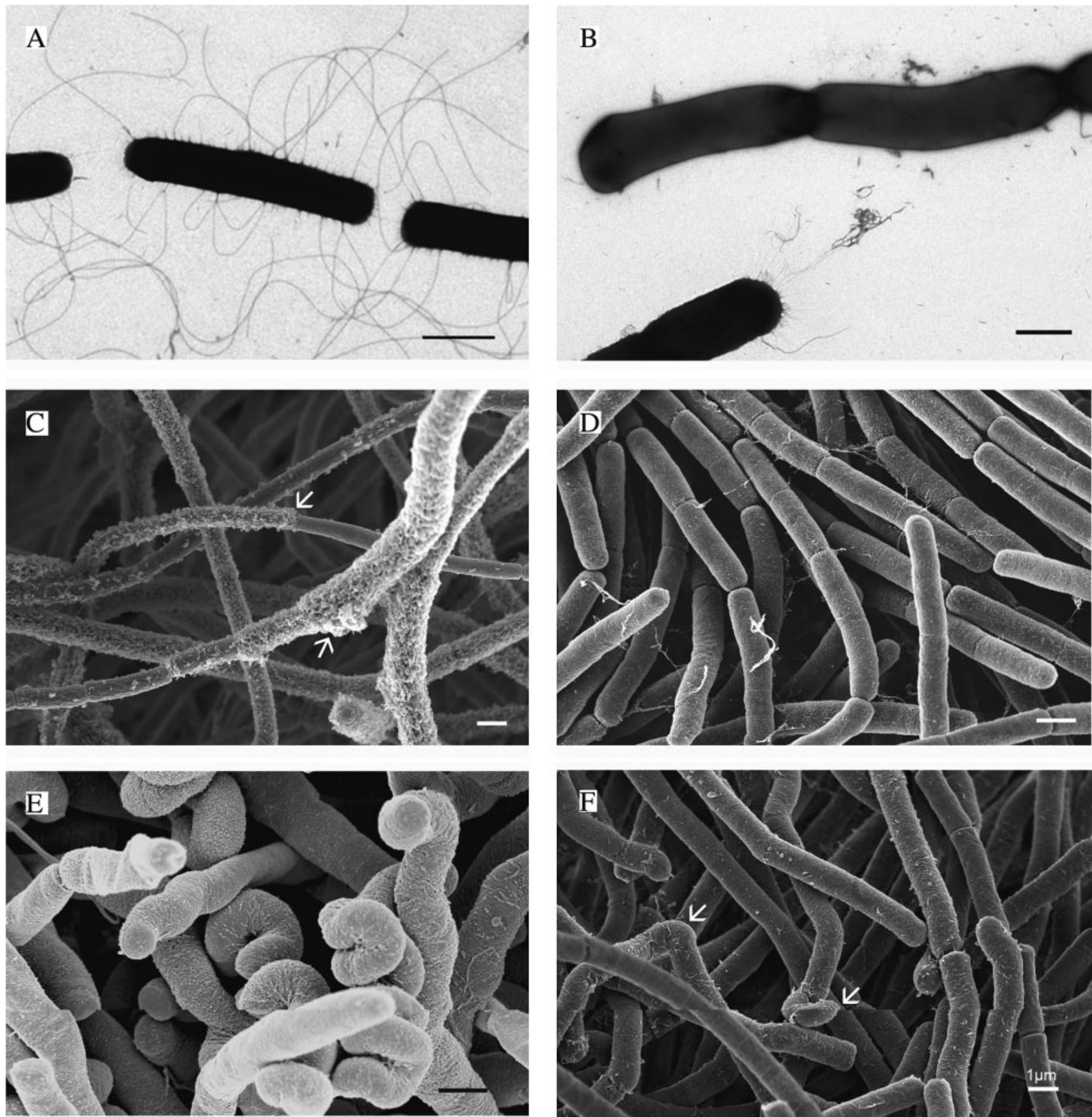


FIG. 2. Cell morphology studied by TEM and SEM. Bacteria isolated from great apes (A, C, and E) and classic *B. anthracis* strains (B, D, and F) presented different morphological criteria. Cells from suspension cultures studied by TEM had flagella (A), in contrast to what was seen in B. Cells from agar cultures studied by SEM showed capsule structures (arrows) on the bacteria, in C in contrast to D, and twisted bacteria (E) in contrast to very rare structures (arrows) shown in F. Similar results were obtained with all bacterial isolates tested. All bars represent 1 μm.

the *rpoB* marker had been observed previously for some strains of the *B. cereus* group (14, 26) and was explained by residual nonspecific priming of the *B. anthracis*-specific primers on the closely related *B. cereus* genomic sequences.

Further analyses were performed with purified DNA from *B. anthracis* CI and CA that was isolated from primary cultures derived from different organ samples (Table 1). The results described below were the same for all isolates tested. The Ba813 gene fragment was amplified from all DNA preparations and sequenced. Compared to the Ba813 sequence found in classic *B. anthracis* strains, the fragment possessed two nucleotide differences (data not shown).

AluI restriction of the SG-749 fragment, a randomly amplified polymorphic DNA marker specific for the *B. cereus* complex, revealed a unique restriction type for all classic *B. anthracis* strains tested (9, 26). However, in *B. anthracis* strains CI and CA, a different restriction pattern was found, which was identical to the patterns found in the environmental isolates *B. cereus* Hohenheim, *Bacillus* sp. strain 2617, and *Bacillus* sp. strain 153 (26). Sequencing of the SG-749 fragment of *B.*

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TABLE 3. Etest results for atypical *B. anthracis* isolates

| Antibiotic | MIC range (μg/ml)[a] | |
|---|---|---|
| | *B. anthracis* CI | *B. anthracis* CA |
| Amoxicillin-clavulanic acid[b] | 0.016–0.023 | 3.0–6.0[c] |
| Ciprofloxacin | 0.038 | 0.047–0.5 |
| Doxycycline | 0.016 | 0.064–0.25 |
| Clindamycin | 0.5–0.75 | 0.75–1.0 |
| Imipenem | 0.047–0.064 | 0.064–0.94 |
| Piperacillin | 0.75–1.5 | 0.75–3.0 |
| Rifampin | 0.19–0.25 | 0.125–0.5 |
| Tetracycline | 0.032–0.47 | 0.25–1.5 |
| Vancomycin | 1.5–3.0 | 2.0–3.0 |

[a] Several isolates were tested for each antibiotic.
[b] Sensitive, MIC $\leq$ 4 μg/ml; resistant, MIC $\geq$ 8 μg/ml.
[c] Some isolates showed intermediate sensitivity.

TABLE 4. Results of multilocus sequence typing

| Allele | Allele no. found in:[a] | | No. of nt differences[b] | Highest homology in BLAST search |
|---|---|---|---|---|
| | *B. anthracis* strains CI and CA | *B. anthracis* strain UDIII-7 | | |
| *adk*[c] | 2 | 25 | 1 | 100%, *B. thuringiensis* serovar konkukian |
| *ccpA*[c] | 35 | 36 | 1 | 100%, *B. thuringiensis* serovar konkukian |
| *ftsA*[c] | 8 | 2 | 2 | 100%, *B. thuringiensis* serovar konkukian |
| *glpT*[c] | (18) | 18 | 1 | 99%, *B. anthracis* Ames |
| *pyrE*[c] | (19) | 19 | 2 | 99%, *B. anthracis* Ames |
| *recF*[c] | 11 | 9 | 1 | 100%, *B. thuringiensis* serovar konkukian |
| *sucC*[c] | (1) | 12 | 3 | 99%, *B. cereus* E33L |
| *glpF*[d] | 34 | 1 | 6 | 98%, *B. thuringiensis* serovar konkukian |
| *gmk*[d] | 1 | 1 | 0 | 100%, *B. anthracis* Ames |
| *ilvD*[d] | (51, 56, 57) | 1 | 6 | 99%, *B. thuringiensis* serovar konkukian |
| *pta*[d] | 1 | 1 | 0 | 100%, *B. anthracis* Ames |
| *pur*[d] | 18 | 1 | 19 | 99%, *B. thuringiensis* serovar konkukian |
| *pycA*[d] | 29 | 1 | 2 | 99%, *B. thuringiensis* serovar konkukian |
| *tpi*[d] | 5 | 1 | 5 | 99%, *B. cereus* E33L |

[a] For new alleles, the number(s) of alleles with highest homology is given in parentheses.
[b] Number of nucleotide (nt) differences between alleles of *B. anthracis* CI or CA and *B. anthracis* UDIII-7.
[c] Alleles belong to the scheme described previously by Helgason et al. (17).
[d] Alleles belong to the scheme described previously by Priest et al. (45).

*anthracis* strains CI and CA revealed six nucleotide differences compared to classic *B. anthracis* strains (data not shown), confirming the restriction pattern through the identification of an additional AluI restriction site, which results in restriction fragments of 496 bp, 166 bp, and 89 bp compared to fragments of 662 bp and 89 bp for classic *B. anthracis* strains.

MLST was performed to assess the phylogenetic relationship of the *B. anthracis* CI and CA strains with classic *B. anthracis* strains and other strains of the *B. cereus* group. Two recently described typing schemes, both based on fragments of seven housekeeping genes, were applied (17, 45). The sequences of all 14 gene fragments were identical for different isolates of *B. anthracis* strains CI and CA. The results are summarized in Table 4. According to the typing scheme described previously by Helgason et al. (17), none of the seven alleles was identical to those found in classic *B. anthracis* strains like the UDIII-7 strain. The alleles from *B. anthracis* strains CI and CA and *B. anthracis* strain UDIII-7 differed by one to three nucleotides. According to the typing scheme described previously by Priest et al. (45), the *gmk* and *pta* alleles of strains CI and CA were identical to the alleles from *B. anthracis* strain UDIII-7; the other five alleles differed by between 2 and 19 nucleotides. The alleles for *glpT*, *pyrE*, *sucC*, and *ilvD* had not been observed previously. Most classic *B. anthracis* strains in our strain collection had sequence type 1 (ST-1) according to both typing schemes. Only strain 5261 had ST-2 (Fig. 3), and strain B19-39 possessed a new *pyrE* allele, which differed by one nucleotide from allele 19 and which was not identical to the corresponding allele from *B. anthracis* strains CI and CA, where two nucleotide differences at other positions were observed (Table 4).

A BLAST search revealed the highest homology for the alleles from *B. anthracis* CI and CA with the corresponding alleles from classic *B. anthracis* strains, from *B. thuringiensis* serovar konkukian strain 97-27, and from *B. cereus* strain E33L. To analyze the phylogenetic relationship of *B. anthracis* CI and CA to classic *B. anthracis* strains and to other *Bacillus* strains, the allele sequences of each typing scheme were concatenated. The concatenated sequences had a length of approximately 3,000 bp for each of the typing schemes. The number of nucleotide differences between *B. anthracis* UDIII-7 and the *B. anthracis* CI or CA strain was 11 for the scheme described previously by Helgason et al. (17) and 38 for the scheme described previously by Priest et al. (45). Based on the concatenated sequences, neighbor-joining phylogenetic trees were constructed (Fig. 3). Both trees confirmed the close relationship between *B. anthracis* CI and CA, classic *B. anthracis* strains, *B. thuringiensis* serovar konkukian strain 97-27, and *B. cereus* strain E33L. The sequence types (45) of the examined strains, if known, are indicated in the legend of Fig. 3.

Virulent strains of *B. anthracis* possess two large virulence plasmids (27), the toxin plasmid pXO1 (182 kb), and the capsule plasmid pXO2 (95 kb). To determine whether the *capC* and *pag* genes of *B. anthracis* strain CI that were detected by real-time PCR were located on plasmids as well, the large plasmids were purified and separated by agarose gel electrophoresis. Southern analysis was performed with probes for the *capC* and *pag* genes. *B. anthracis* strain UDIII-7 was used as a control (Fig. 4). Each of the two plasmids was the same size in both strains, and in Southern analysis, the larger plasmid hybridized with the *pag* probe and the smaller plasmid hybridized with the *capC* probe. Therefore, we conclude the presence of two virulence plasmids related to those of classic *B. anthracis* strains.

The presence of several additional plasmid genes encoding unknown functions or virulence factors was assessed by PCR or by analysis of the unfinished plasmid sequences of the *B. anthracis* CI strain (sequencing is in progress) (H. Liesegang, personal communication). Thus, we confirmed the presence of the transcriptional regulator gene *atxA*, the toxin genes *pag*, *lef*, *cya*, and other genes on the larger plasmid (pXO1-04, pXO1-16, pXO1-45, pXO1-59, pXO1-65, pXO1-78, pXO1-87, pXO1-103, and pXO1-142, according to the sequence reported under GenBank accession number NC_001496) and the presence of the transcriptional regulator genes *acpA* and *acpB*, the capsule biosynthesis genes *capA*, *capB*, *capC*, and *capD*, and other genes on the smaller plasmid (pXO2-04, pXO2-16, pXO2-25,

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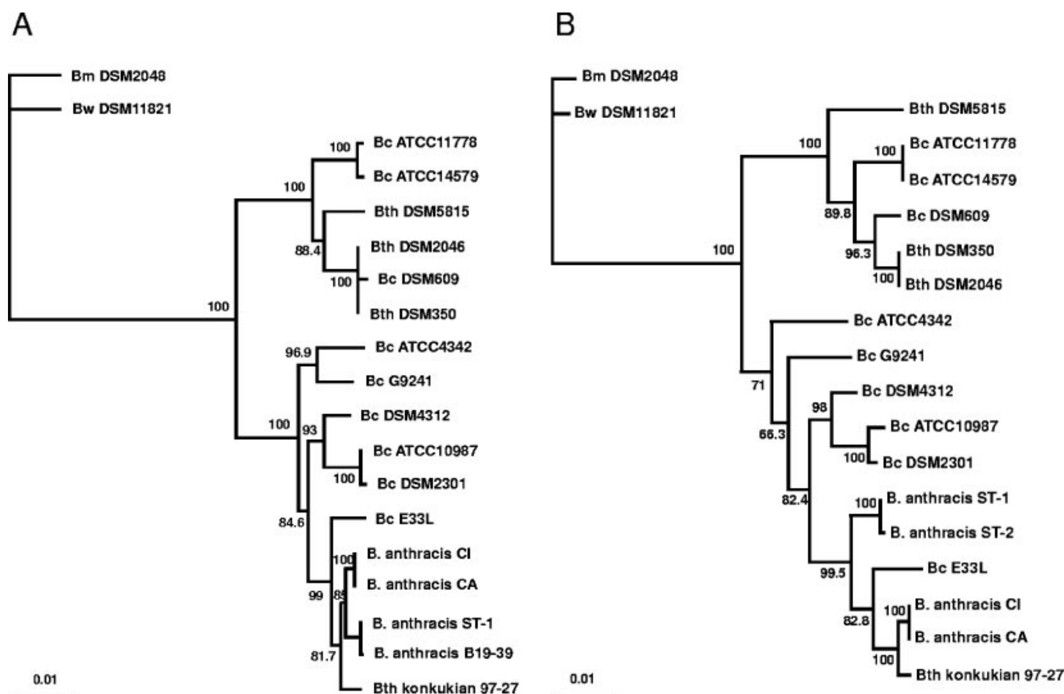


FIG. 3. Neighbor-joining phylogenetic trees for the concatenated allele sequences of different strains of the *B. cereus* (Bc) group. (A) Tree based on the scheme described previously by Helgason et al. (17) comparing 2,977 bp. *B. anthracis* strain B19-39 possessed a new *pyrE* allele, and the other 16 classic *B. anthracis* strains had ST-1. (B) Tree based on the scheme described previously by Priest et al. (45) comparing 2,838 bp. *B. anthracis* strain 5261 was ST-2, and the other 16 *B. anthracis* strains were ST-1. Other STs that could be assigned according to the database at http://pubmlst.org/bcereus/ were as follows: ST-4 (*B. cereus* ATCC 14579), ST-32 (*B. cereus* ATCC 10987), ST-26 (*B. cereus* DSM 4312), ST-34 (*B. cereus* ATCC 11778), ST-10 (*B. thuringiensis* [Bt] DSM 2046 and DSM 350), ST-16 (*B. thuringiensis* DSM 5815), ST-116 (*B. mycoides* [Bm] DSM 2048), ST-38 (*B. cereus* ATCC 4342), and ST-113 (*B. thuringiensis* serovar konkukian strain 97-27). The trees were statistically evaluated with a bootstrap analysis with 1,000 bootstraps. Only relevant bootstrap values above 70% are shown.

pXO2-28, pXO2-37, pXO2-38, pXO2-47, pXO2-66, pXO2-69, pXO2-81, and pXO2-84, according to the sequence reported under GenBank accession number NC_002146). The deduced protein sequences of the transcriptional regulators, the toxins, and the capsule biosynthesis enzymes contained no or only very few amino acid exchanges compared to their homologues in classic *B. anthracis* strains (data not shown).

Western blot analysis was performed to assess the expression of protective antigen by culture supernatants of *B. anthracis* strains CI and UDIII-7 grown in bicarbonate medium under a $CO_2$ atmosphere (Fig. 5). The strains expressed proteins of the appropriate size (83 kDa) that reacted with a monoclonal antibody, confirming the secretion of PA by both *B. anthracis* cultures.

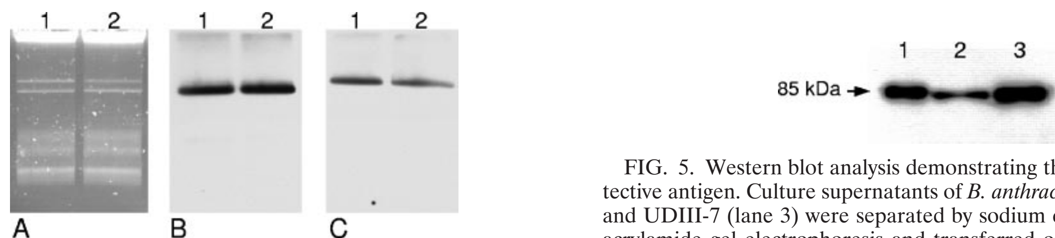


FIG. 4. Detection of large plasmids in *B. anthracis* isolates. Plasmids of *B. anthracis* strains CI (lane 1) and UDIII-7 (lane 2) were detected by agarose gel electrophoresis (A) or by Southern blot analysis with probes for the *capC* gene (B) or the *pag* gene (C).

## DISCUSSION

We were able to isolate *B. anthracis*-like bacteria from all ape necropsy samples that tested positive by PCR for *B. anthracis*. The microbiological features, however, were uncommon because the bacteria were motile and resistant to the gamma phage, and some isolates were also resistant to penicillin G. These microbiological findings would exclude the presence of *B. anthracis* according to currently used differential diagnostic criteria (57). Motile strains were observed only rarely. Liang and Yu (36) previously described numerous motile strains from China with polar flagellation, in contrast to our isolates, which showed peritrich flagellation. Resistance to the gamma phage was seen in 15% of strains tested in a previous study (6). Naturally occurring penicillin G-resistant

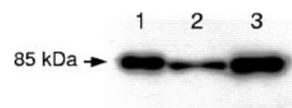


FIG. 5. Western blot analysis demonstrating the expression of protective antigen. Culture supernatants of *B. anthracis* strains CI (lane 2) and UDIII-7 (lane 3) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, and the protein was detected with a monoclonal anti-PA antibody. Purified recombinant PA was included as a control (lane 1). The position of the 85-kDa band of the protein standard is indicated.

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*B. anthracis* isolates have been reported previously (5, 30), and a survey of strains derived from carcasses and soil in South Africa has revealed penicillin G resistance in up to 16% of isolates (40). The fact that one diagnostic trait of an isolate does not meet the typical anthrax criteria would probably not compromise its correct identification. However, in primary cultures of the CI and CA strains, two or three features, respectively, argue against an identification as *B. anthracis*, namely, motility, resistance to the gamma phage, and resistance to penicillin G. Therefore, these isolates would probably be misdiagnosed in routine laboratories if diagnosis is based only on microbiological criteria. Also, testing of the biochemical properties using the API 50 CHB system turned out to be inapplicable for our isolates, because slight discrepancies in the reaction patterns resulted in differing identifications. Although the presence of *B. anthracis* was indicated with the highest probability, no definitive identification was possible. Antibiotic susceptibilities examined by Etest showed MICs in the same range as those described previously by Turnbull et al. (55) for isolates of the *B. cereus* group. However, for amoxicillin-clavulanic acid, ciprofloxacin, and tetracycline, MICs for *B. anthracis* isolates from Cameroon were clearly higher than those described for *B. anthracis* (MICs according to those reported previously by Turnbull et al. are as follows: amoxicillin-clavulanic acid, 0.016 to 0.5 μg/ml; ciprofloxacin, 0.032 to 0.094 μg/ml; tetracycline, 0.016 to 0.094 μg/ml [55]).

As confirmed by Western blot analysis, protective antigen was expressed by the *B. anthracis* CI strain and secreted into the medium when the culture was grown under bicarbonate/$CO_2$ conditions. However, unlike classic *B. anthracis* strains, the CI and CA isolates formed a capsule when cultivated not only on bicarbonate agar in a $CO_2$-enriched atmosphere but also on common agar medium under normal growth conditions. We do not yet know whether the strains express the anthrax-typical poly-γ-D-glutamic acid capsule, but the capsule biosynthesis genes are present and probably functional as suggested by sequencing data (not shown). Subculturing occasionally resulted in a loss of the *capC* marker and consequently in a loss of capsule production. The corresponding isolates will be further analyzed for the presence of the capsule plasmid, because it is known that pXO2 is easily and spontaneously lost (54). In some subclones, beta-hemolysis, sensitivity to the gamma phage, and resistance to penicillin G in previously sensitive CI isolates were observed. The reason for these variations is unclear. One could speculate that regulatory mechanisms that are functional in classic *B. anthracis* strains do not apply or are different in the new isolates. In classic *B. anthracis* strains, the expression of toxin and capsule genes as well as numerous other genes on both plasmids and the chromosome is controlled by the pXO1-encoded pleiotropic regulator AtxA (4, 28, 56). Capsule formation is affected by AtxA via positive control of two pXO2-encoded genes, *acpA* and *acpB*, which are responsible for the $CO_2$-dependent expression of the capsule genes. Low levels of *capB* transcripts were detected during aerobic growth (13), which seemed to be initiated from a promoter not controlled by AcpA or AcpB. This low level of transcription, however, was not high enough to produce a detectable capsule on the cell surface (13). As the *B. anthracis* CI and CA strains form capsules under normal growth conditions, the regulation of capsule generation in these strains differs from that in classic anthrax strains. The reason for these expression differences can not yet be explained, because the *atxA*, *acpA*, and *acpB* genes encoding the transcriptional regulators are present on the pXO1- and pXO2-related plasmids of *B. anthracis* CI, and differences in regulation cannot be easily explained by a lack of one or more of these genes.

PlcR is a pleiotropic transcriptional regulator in nonanthrax strains of the *B. cereus* group that upregulates the expression of more than 100 genes, including those for hemolysis and motility, through binding to an upstream palindromic motif (15, 21, 35). Although these genes are present in classic *B. anthracis* strains, they are usually not expressed due to a nonsense mutation in the *plcR* gene. It was speculated that the acquisition of the pXO1 plasmid induced incompatibility of the regulator AtxA with the chromosomally encoded PlcR (38). Frameshift mutations in four essential genes of the flagellar gene cluster led to the loss of motility in *B. anthracis* (50). However, the data presented in this investigation indicate that flagellar genes are functional in *B. anthracis* strains CI and CA, and beta-hemolytic activity was observed in some subclones. Sequencing of the *plcR* gene of the *B. anthracis* CI strain revealed an insertion of 1 bp near the 3′ end of the gene but no nonsense mutation, resulting in a protein with a slightly altered C terminus and an extension of four amino acids compared to other PlcR proteins (data not shown). Therefore, it is possible that the new *B. anthracis* isolates possess a functional PlcR protein. The two genes for β-lactamases are usually not expressed in *B. anthracis* (7, 8). Their regulation is unclear, because they lack the upstream PlcR binding site, but variations in gene regulation might be the reason for penicillin G resistance in the *B. anthracis* CA isolates and in some CI subcultures.

Recently, a protein (GamR) involved in the bacterial receptor for the gamma phage was identified (10). Sequencing data indicate that a gene with homology to the *gamR* gene is present (data not shown), but the protein and/or other proteins involved in gamma phage propagation are probably not expressed in the primary isolates. The reason for the altered behavior of some subcultures is not known, and no data for the regulation of the phage receptor are available yet. The phenotypic "instability" observed in subclones of the CI and CA strains might result from a relatively recent acquisition of the *B. anthracis* virulence plasmids by a member of the *B. cereus* group. The balanced regulation of different plasmid- and chromosome-encoded genes that is observed in classic *B. anthracis* strains might be the result of a long evolutionary process not yet accomplished in these atypical isolates.

Based on microbiological criteria, the *B. anthracis* CI and CA isolates slightly differed by their antibiotic susceptibilities and biochemical capacities. Previous analyses also revealed different genotypes in MLVA and slightly different *pag* sequences. Phylogenetic tree analyses of the *gyrB* and the *rpoB* genes revealed close relations between the CI and CA strains. However, while *gyrB* clustered with classic *B. anthracis* isolates, the *rpoB* sequences were distinct from those of other *B. anthracis* isolates and, rather, showed homologies to other members of the *B. cereus* group (31, 34). To further characterize these isolates, molecular criteria that are currently used to distinguish *B. anthracis* from other isolates of the *B. cereus* group, like MLST, were applied. These molecular analyses underlined that *B. anthracis* strains CI and CA are closely

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related to each other and clearly showed that they differ from "classic" *B. anthracis* strains.

The presence of the Ba813 marker is not specific for *B. anthracis* strains, as was previously shown for several exceptions (26, 47). AluI restriction of the SG-749 fragment resulted in the same pattern for all classic *B. anthracis* strains tested, but *B. anthracis* strains CI and CA exhibited a pattern that was described previously for some other nonanthrax strains of the *B. cereus* group (26). The only classic *B. anthracis* feature was the presence of two large plasmids with sizes comparable to those of pXO1 and pXO2, possessing the *pag* and *capC* genes, respectively.

MLST confirmed the close relationship of strains CI and CA with classic *B. anthracis* strains but also with two virulent atypical members of the *B. cereus* group (Fig. 3). *B. thuringiensis* is known to be an insect pathogen, but *B. thuringiensis* serovar konkukian strain 97-27 was originally isolated from a case of severe human tissue necrosis and was pathogenic in immunosuppressed mice and thus, in this respect, rather resembled *B. anthracis* (18, 19). The strain possesses the 77-kb plasmid pBT9727 with many open reading frames homologous to sequences of the capsule plasmid pXO2, excluding the sequences necessary for capsule biosynthesis. The second closely related strain, *B. cereus* strain E33L, was originally isolated from the carcass of a dead zebra suspected to have died of anthrax in Namibia. It contains two large and three small plasmids, which do not encode homologues of known virulence factors in *B. anthracis*, *B. cereus*, or *B. thuringiensis* (48). Recently, a *B. cereus* strain was isolated from a patient with inhalation anthrax-like illness. This strain, termed G9241, possesses two plasmids, with one (pBCXO1, 191 kb) having 99.6% similarity to the *B. anthracis* toxin plasmid pXO1 (20). Gene products with similarities to pXO1-encoded gene products were also found on the 218-kb plasmid pBC218. This plasmid encodes gene products for a polysaccharide capsule cluster but not the gene products for the poly-γ-D-glutamic acid found in *B. anthracis*. Interestingly, *B. cereus* G9241 appears to encode fully functional copies of both PlcR and AtxA, and capsule production is not regulated by increased $CO_2$ concentrations. In contrast to the two strains 97-27 and E33L, the relationship of *B. cereus* G9241 with *B. anthracis* is less pronounced when housekeeping genes are analyzed by MLST.

At present, we can only speculate how the atypical *B. anthracis* isolates CI and CA evolved. It can be hypothesized that these strains that were isolated in two regions more than 1,000 miles apart represent an old form that shares a common ancestor with classic *B. anthracis* strains. Another possibility would be a more recent emergence based on the transfer of the *B. anthracis* plasmids to an unknown strain of the *B. cereus* group. Although plasmid transfers were never documented in natural populations, it has been demonstrated that pXO1 and pXO2 could be transferred by conjugative plasmids originating in *B. thuringiensis* (2). Up to now, the CI and CA strains were observed only in rainforests, and their epidemiology is unknown. The source of infection of the great apes remains unclear. Long-term observation of the habituated chimpanzees in the Taï National Park showed that they not only feed on plants or arthropods but also hunt red colobus monkeys (3). However, anthrax infections have not been found in these prey monkeys or other mammals in the areas to date, whereas in general, disease surveillance in such remote regions is challenging and has not been performed systematically (33). It is known that blowflies that feed on infected carcasses are able to disseminate anthrax in their excretions (22). Leaves can be heavily contaminated with these infective excretions, but this source of infection remains speculative. Even contaminated water resources cannot be excluded.

We presented the microbiological and molecular analysis of very atypical isolates of *B. anthracis*, which would probably not have been detected by routine diagnostics. The isolates CI and CA possess virulence plasmids closely related to those of *B. anthracis* but a chromosomal background that is closer to those of atypical *B. cereus* and *B. thuringiensis* strains. In addition, gene regulation was different from that of classic *B. anthracis* strains, and the isolates behaved in an unstable manner upon subcultivation. This might be a hint that the cross talk between chromosome and plasmids is not yet balanced. It was previously suggested that major phenotypic differences between members of the *B. cereus* group might represent alterations in gene expression rather than sequence divergence (21, 49, 50). Sequence analysis of the whole genome of the *B. anthracis* CI isolate will give further information on the relationship of the isolates to other members of the *B. cereus* group. The first evidence for the virulence properties of the CI isolate was shown by its capsule and protective antigen expression, but the virulence of the strains will be further confirmed by in vitro and animal studies.

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## REFERENCES

1. **Agaisse, H., M. Gominet, O. A. Økstad, N. Gilois, V. Sanchis, A. B. Kolstø, and D. Lereclus.** 1999. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. Mol. Microbiol. **32:** 1043–1053.
2. **Battisti, L., B. D. Green, and C. B. Thorne.** 1985. Mating system for transfer of plasmids among *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. J. Bacteriol. **162:**543–550.
3. **Boesch, C., and H. Boesch-Achermann.** 2000. The chimpanzees of the Taï Forest: behavioural ecology and evolution. Oxford University Press, Oxford, United Kingdom.
4. **Bourgogne, A., M. Drysdale, S. G. Hilsenbeck, S. N. Peterson, and T. M. Koehler.** 2003. Global effects of virulence gene regulators in a *Bacillus anthracis* strain with both virulence plasmids. Infect. Immun. **71:**2736–2743.
5. **Bradaric, N., and V. Punda-Polic.** 1992. Cutaneous anthrax due to penicillin-resistant *Bacillus anthracis* transmitted by an insect bite. Lancet **340:**306–307.
6. **Buck, C. A., R. L. Anacker, F. S. Newman, and A. Eisenstark.** 1963. Phage isolated from lysogenic *Bacillus anthracis*. J. Bacteriol. **85:**1423–1430.
7. **Chen, Y., J. Succi, F. C. Tenover, and T. M. Koehler.** 2003. β-Lactamase

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genes of the penicillin-susceptible *Bacillus anthracis* Sterne strain. J. Bacteriol. **185:**823–830.
8. **Chen, Y., F. C. Tenover, and T. M. Koehler.** 2004. β-Lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. Antimicrob. Agents Chemother. **48:**4873–4877.
9. **Daffonchio, D., S. Borin, G. Frova, R. Gallo, E. Mori, R. Fani, and C. Sorlini.** 1999. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. Appl. Environ. Microbiol. **65:**1298–1303.
10. **Davison, S., E. Couture-Tosi, T. Candela, M. Mock, and A. Fouet.** 2005. Identification of the *Bacillus anthracis* γ phage receptor. J. Bacteriol. **187:** 6742–6749.
11. **de Vos, V.** 1994. Anthrax, p. 1262. *In* J. A. W. Coetzer, G. R. Thomson, R. C. Tustin, and N. P. J. Kriek (ed.), Infectious diseases of livestock. Oxford University Press, Oxford, United Kingdom.
12. **Drobniewski, F. A.** 1993. *Bacillus cereus* and related species. Clin. Microbiol. Rev. **6:**324–338.
13. **Drysdale, M., A. Bourgogne, and T. M. Koehler.** 2005. Transcriptional analysis of the *Bacillus anthracis* capsule regulators. J. Bacteriol. **187:**5108–5114.
14. **Ellerbrok, H., H. Nattermann, M. Ozel, L. Beutin, B. Appel, and G. Pauli.** 2002. Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. FEMS Microbiol. Lett. **214:**51–59.
15. **Gohar, M., O. A. Økstad, N. Gilois, V. Sanchis, A. B. Kolstø, and D. Lereclus.** 2002. Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. Proteomics **2:**784–791.
16. **Helgason, E., O. A. Økstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A. B. Kolstø.** 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66:**2627–2630.
17. **Helgason, E., N. J. Tourasse, R. Meisal, D. A. Caugant, and A. B. Kolstø.** 2004. Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl. Environ. Microbiol. **70:**191–201.
18. **Hernandez, E., F. Ramisse, J. P. Ducoureau, T. Cruel, and J. D. Cavallo.** 1998. *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. J. Clin. Microbiol. **36:**2138–2139.
19. **Hill, K. K., L. O. Ticknor, R. T. Okinaka, M. Asay, H. Blair, K. A. Bliss, M. Laker, P. E. Pardington, A. P. Richardson, M. Tonks, D. J. Beecher, J. D. Kemp, A. B. Kolstø, A. C. Wong, P. Keim, and P. J. Jackson.** 2004. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **70:**1068–1080.
20. **Hoffmaster, A. R., J. Ravel, D. A. Rasko, G. D. Chapman, M. D. Chute, C. K. Marston, B. K. De, C. T. Sacchi, C. Fitzgerald, L. W. Mayer, M. C. Maiden, F. G. Priest, M. Barker, L. Jiang, R. Z. Cer, J. Rilstone, S. N. Peterson, R. S. Weyant, D. R. Galloway, T. D. Read, T. Popovic, and C. M. Fraser.** 2004. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc. Natl. Acad. Sci. USA **101:**8449–8454.
21. **Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, E. S. Dusko, R. Overbeek, and N. Kyrpides.** 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature **423:**87–91.
22. **Jensen, G. B., B. M. Hansen, J. Eilenberg, and J. Mahillon.** 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environ. Microbiol. **5:**631–640.
23. **Jensen, G. B., A. Wilcks, S. S. Petersen, J. Damgaard, J. A. Baum, and L. Andrup.** 1995. The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. J. Bacteriol. **177:**2914–2917.
24. **Keim, P., A. Kalif, J. Schupp, K. Hill, S. E. Travis, K. Richmond, D. M. Adair, M. Hugh-Jones, C. R. Kuske, and P. Jackson.** 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. **179:**818–824.
25. **Keim, P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. J. Jackson, and M. E. Hugh-Jones.** 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. **182:**2928–2936.
26. **Klee, S. R., H. Nattermann, S. Becker, M. Urban-Schriefer, T. Franz, D. Jacob, and B. Appel.** 2006. Evaluation of different methods to discriminate *Bacillus anthracis* from other bacteria of the *Bacillus cereus* group. J. Appl. Microbiol. **100:**673–681.
27. **Koehler, T. M.** 2002. *Bacillus anthracis* genetics and virulence gene regulation. Curr. Top. Microbiol. Immunol. **271:**143–164.
28. **Koehler, T. M., Z. Dai, and M. Kaufman-Yarbray.** 1994. Regulation of the *Bacillus anthracis* protective antigen gene: $CO_2$ and a *trans*-acting element activate transcription from one of two promoters. J. Bacteriol. **176:**586–595.
29. **Kolle, W., R. Kraus, and P. Uhlenhuth.** 1931. Handbuch der pathogenen Mikroorganismen. Gustav Fischer und Urban & Schwarzenberg, Jena, Germany.
30. **Lalitha, M. K. and M. K. Thomas.** 1997. Penicillin resistance in *Bacillus anthracis*. Lancet **349:**1522.
31. **Leendertz, F. H., H. Ellerbrok, C. Boesch, E. Couacy-Hymann, K. Matz-Rensing, R. Hakenbeck, C. Bergmann, P. Abaza, S. Junglen, Y. Moebius, L. Vigilant, P. Formenty, and G. Pauli.** 2004. Anthrax kills wild chimpanzees in a tropical rainforest. Nature **430:**451–452.
32. **Leendertz, F. H., F. Lankester, P. Guislain, C. Néel, and O. Drori.** Anthrax in western and central Africa great apes. Am. J. Primatol., in press.
33. **Leendertz, F. H., G. Pauli, K. Maetz-Rensing, W. Boardman, C. Nunn, H. Ellerbrok, S. A. Jensen, S. Junglen, and C. Boesch.** Pathogens as drivers of population declines: the importance of systematic monitoring in great apes and other threatened mammals. Cons. Biol., in press.
34. **Leendertz, F. H., S. Yumlu, G. Pauli, C. Boesch, E. Couacy-Hymann, L. Vigilant, S. Junglen, S. Schenk, and H. Ellerbrok.** 2006. A new *Bacillus anthracis* found in wild chimpanzees and a gorilla from west and central Africa. PLoS Pathog. **2:**e8.
35. **Lereclus, D., H. Agaisse, M. Gominet, S. Salamitou, and V. Sanchis.** 1996. Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. J. Bacteriol. **178:**2749–2756.
36. **Liang, X., and D. Yu.** 1999. Identification of *Bacillus anthracis* strains in China. J. Appl. Microbiol. **87:**200–203.
37. **Makino, S., I. Uchida, N. Terakado, C. Sasakawa, and M. Yoshikawa.** 1989. Molecular characterization and protein analysis of the *cap* region, which is essential for encapsulation in *Bacillus anthracis*. J. Bacteriol. **171:**722–730.
38. **Mignot, T., M. Mock, D. Robichon, A. Landier, D. Lereclus, and A. Fouet.** 2001. The incompatibility between the PlcR- and AtxA-controlled regulons may have selected a nonsense mutation in *Bacillus anthracis*. Mol. Microbiol. **42:**1189–1198.
39. **Miller, J. M., J. G. Hair, M. Hebert, L. Hebert, F. J. Roberts, Jr., and R. S. Weyant.** 1997. Fulminating bacteremia and pneumonia due to *Bacillus cereus*. J. Clin. Microbiol. **35:**504–507.
40. **Odendaal, M. W., P. M. Pieterson, V. De Vos, and A. D. Botha.** 1991. The antibiotic sensitivity patterns of *Bacillus anthracis* isolated from the Kruger National Park. Onderstepoort J. Vet. Res. **58:**17–19.
41. **Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, T. M. Koehler, G. Lamke, S. Kumano, J. Mahillon, D. Manter, Y. Martinez, D. Ricke, R. Svensson, and P. J. Jackson.** 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J. Bacteriol. **181:**6509–6515.
42. **Økstad, O. A., M. Gominet, B. Purnelle, M. Rose, D. Lereclus, and A. B. Kolstø.** 1999. Sequence analysis of three *Bacillus cereus* loci carrying PIcR-regulated genes encoding degradative enzymes and enterotoxin. Microbiology **145:**3129–3138.
43. **Patra, G., P. Sylvestre, V. Ramisse, J. Therasse, and J. L. Guesdon.** 1996. Isolation of a specific chromosomic DNA sequence of *Bacillus anthracis* and its possible use in diagnosis. FEMS Immunol. Med. Microbiol. **15:**223–231.
44. **Peng, H., V. Ford, E. W. Frampton, L. Restaino, L. A. Shelef, and H. Spitz.** 2001. Isolation and enumeration of *Bacillus cereus* from foods on a novel chromogenic plating medium. Food Microbiol. **18:**231–238.
45. **Priest, F. G., M. Barker, L. W. Baillie, E. C. Holmes, and M. C. Maiden.** 2004. Population structure and evolution of the *Bacillus cereus* group. J. Bacteriol. **186:**7959–7970.
46. **Radnedge, L., P. G. Agron, K. K. Hill, P. J. Jackson, L. O. Ticknor, P. Keim, and G. L. Andersen.** 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. Appl. Environ. Microbiol. **69:**2755–2764.
47. **Ramisse, V., G. Patra, J. Vaissaire, and M. Mock.** 1999. The Ba813 chromosomal DNA sequence effectively traces the whole *Bacillus anthracis* community. J. Appl. Microbiol. **87:**224–228.
48. **Rasko, D. A., M. R. Altherr, C. S. Han, and J. Ravel.** 2005. Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol. Rev. **29:**303–329.
49. **Rasko, D. A., J. Ravel, O. A. Økstad, E. Helgason, R. Z. Cer, L. Jiang, K. A. Shores, D. E. Fouts, N. J. Tourasse, S. V. Angiuoli, J. Kolonay, W. C. Nelson, A. B. Kolstø, C. M. Fraser, and T. D. Read.** 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Res. **32:**977–988.
50. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. Økstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. Deboy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A. B. Kolstø, and C. M. Fraser.** 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature **423:**81–86.
51. **Saile, E., and T. M. Koehler.** 2002. Control of anthrax toxin gene expression by the transition state regulator *abrB*. J. Bacteriol. **184:**370–380.
52. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory

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manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
53. **Turnbull, P. C.** 1999. Definitive identification of *Bacillus anthracis*—a review. J. Appl. Microbiol. **87:**237–240.
54. **Turnbull, P. C., R. A. Hutson, M. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Kramer, and J. Melling.** 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. **72:**21–28.
55. **Turnbull, P. C., N. M. Sirianni, C. I. LeBron, M. N. Samaan, F. N. Sutton, A. E. Reyes, and L. F. J. Peruski.** 2004. MICs of selected antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a range of clinical and environmental sources as determined by the Etest. J. Clin. Microbiol. **42:**3626–3634.
56. **Uchida, I., J. M. Hornung, C. B. Thorne, K. R. Klimpel, and S. H. Leppla.** 1993. Cloning and characterization of a gene whose product is a *trans*-activator of anthrax toxin synthesis. J. Bacteriol. **175:**5329–5338.
57. **World Health Organization.** 2003. Manual for laboratory diagnosis of anthrax. World Health Organization, Geneva, Switzerland. [Online.] http://w3.whosea.org/bct/anthrax/.

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