

## Bovine *Bacillus anthracis* in Cameroon<sup>∇†</sup>

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**Bovine *Bacillus anthracis* isolates from Cameroon were genetically characterized. They showed a strong homogeneity, and they belong, together with strains from Chad, to cluster Aβ, which appears to be predominant in western Africa. However, one strain that belongs to a newly defined clade (D) and cluster (D1) is penicillin resistant and shows certain phenotypes typical of *Bacillus cereus*.**

Extensive genetic analysis of a large number of strains of *Bacillus anthracis*, the causative agent of anthrax, led to robust molecular epidemiological data, making this bacterium a model for microbial evolution and the global spread of pathogens and even making it possible for researchers to follow migration or movement of its hosts or host products (5–8, 15). This is possible by virtue of the large data set based on canonical single nucleotide polymorphisms (canSNPs), which classify *B. anthracis* strains into clades (22), and on multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), which groups them further into clusters (6). Although certain geographical regions are extensively sampled, others are poorly studied, which may lead to bias in the analysis of the spread and diversity of strain populations. This, in particular, is the case for western and central African countries, such as Cameroon, where most of Africa's anthrax outbreaks occur (22). A broader genetic diversity of *B. anthracis* strains is expected in that region due to the higher incidence of anthrax there than elsewhere. In order to improve our knowledge regarding the genetic diversity of *B. anthracis*, we analyzed bovine strains from seven sites in Cameroon and compared the results with data from a worldwide strain collection. In addition, we characterized in detail a penicillin-resistant strain that caused bovine anthrax in that area. This strain represents a new clade and a new cluster of *B. anthracis* and shows some genetic traits of *Bacillus cereus*.

Strains of *B. anthracis* isolated from bovines in various areas of Cameroon (Table 1) were identified using standard methods as described previously (17, 23). They all harbored the virulence factor markers *pag* and *cap*, which are specific to *B. anthracis* plasmids pXO1 and pXO2 (19). They were all nonhemolytic and  $\gamma$  phage sensitive (Table 1). One strain, JF3964, showed particular phenotypes that are normally found in *B. cereus*, such as resistance to the *B. anthracis*-specific  $\gamma$  phage and resistance to penicillin. However, in contrast to *B. cereus*, strain JF3964 was non-hemolytic. These phenotypic criteria are used to discriminate *B.*

*anthracis* from *B. cereus*. Additionally, the chromosomal markers *sap* and Ba813 that were used as specific markers for *B. anthracis* (17, 19) were absent in JF3964. However, JF3964 contained the virulence genes which are associated with the two *B. anthracis* plasmids pXO1 and pXO2 (19).

Genetic strain characterization was performed by canSNP analysis, which best roots the phylogenetic relationship of *B. anthracis* strains (22), and MLVA, which allows a finer subtyping of the strains (6). The methods are complementary in studying the genetics of bacterial populations since canSNPs are slowly evolving markers, whereas VNTRs are quickly evolving markers (14). Thirteen canSNPs (A.Br.001, A.Br.002, A.Br.003, A.Br.004, A.Br.006, A.Br.007, A.Br.008, A.Br.009, B.Br.001, B.Br.002, B.Br.003, B.Br.004, and A/B.Br.001) and eight VNTR markers (VrrA, VrrB1, VrrB2, VrrC1, VrrC2, GC3, pXO1, and pXO2) for *B. anthracis* were amplified by PCR, using filtered lysates as template DNA (12), and sequenced. DNA sequencing was carried out with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Rotkreuz, Switzerland) and a BigDye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The canSNP and VNTR sequences were compared to published data (22).

Six characteristic strains of *B. anthracis* from Cameroon (JF3959, JF3060, JF3961, JF3963, JF3965, and JF3966) had the following canSNP characteristics: A.Br.001, T; A.Br.002, G; A.Br.003, A; A.Br.004, T; A.Br.006, A; A.Br.007, T; A.Br.008, T; A.Br.009, A; B.Br.001, T; B.Br.002, G; B.Br.003, G; B.Br.004, T; and A/B.Br.001, A. These characteristics classified them as clade A (Fig. 1) (subclade A.Br.005/006), as defined by Van Ert and colleagues (22). These six strains showed an additional base difference in the canSNP B.Br.004: the nucleotide just downstream of the canSNP is a thymine instead of a cytosine. We confirmed that this mutation is also present in all strains from Chad that were analyzed previously (12). One strain, JF3964, showed a completely different profile that has not yet been described (the characteristics of its canSNPs are A.Br.001, T; A.Br.002, A; A.Br.003, A; A.Br.004, T; A.Br.006, C; A.Br.007, T; A.Br.008, T; A.Br.009, A; B.Br.001, T; B.Br.002, C; B.Br.003, G; B.Br.004, T; and A/B.Br.001, A) and was assigned to a novel clade, designated clade D (Fig. 1), according to the criteria of Van Ert et al. (22).

The sizes of the VNTR amplicons, as determined by sequence analysis, were compared to selected published allele

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TABLE 1. Antibiotic susceptibilities and phage  $\gamma$  sensitivities of *B. anthracis* strains from Cameroon

Strain	Origin, section of Cameroon <sup>a</sup>	$\gamma$ Phage	MIC(s) ( $\mu$ g/ml) of <sup>b</sup> :															
			GEN	AMI	STR	ERY	CLI	Q-D	TET	CHL	ENR	CEP	VAN	LNZ	PEN	AMC	NIT	SXT
JF3959	Makari, NE (GI/D3405)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	1	$\leq 0.5$	1	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	2	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	1, 19
JF3960	Garoua, N (D4863)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	0.5	$\leq 0.5$	1	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	$\leq 1$	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	$\leq 0.5, 9.5$
JF3961	Maloum, N (FED:77)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	0.5	$\leq 0.5$	2	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	2	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	2, 38
JF3963	Padarmé, N (No141)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	0.5	$\leq 0.5$	2	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	2	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	2, 38
JF3964	Koza, NE (No142)	Resistant	$\leq 2$	$\leq 16$	$\leq 4$	0.5	1	2	$\leq 1$	$\leq 4$	$\leq 0.25$	8	2	2	8	$\leq 2, 1$	$\leq 16$	1, 19
JF3965	Boklé, N (D5594)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	1	$\leq 0.5$	1	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	2	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	1, 19
JF3966	Makari, NE (Makari)	Sensitive	$\leq 2$	$\leq 16$	$\leq 4$	1	$\leq 0.5$	1	$\leq 1$	$\leq 4$	$\leq 0.25$	$\leq 2$	2	1	$\leq 0.12$	$\leq 2, 1$	$\leq 16$	2, 38

<sup>a</sup> Geographical origin (original strain number). N, north; E, east; NE, northeast.

<sup>b</sup> AMI, amikacin; AMC, amoxicillin-clavulanic acid (ratio, 2:1); CEP, cephalothin; CHL, chloramphenicol; CLI, clindamycin; ENR, enrofloxacin; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; NIT, nitrofurantoin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole (ratio, 1:19); Q-D, quinupristin-dalfopristin; TET, tetracycline; VAN, vancomycin.

sizes (6), as well as to the *B. anthracis* genome sequences from GenBank (<http://www.ncbi.nlm.nih.gov>). A phylogenetic tree was derived from allelic profile data by the unweighted-pair group method with arithmetic means (UPGMA) with an on-line software (<http://pubmlst.org/>) using the PHYLIP suite of programs and the VNTR markers (4) (Fig. 1). The MLVA profiles matched the clades as defined by the canSNP profiles. Strains JF3959, JF3060, JF3961, JF3963, JF3965, and JF3966 grouped in cluster A $\beta$  (Fig. 1), together with *B. anthracis* strains from Chad, as previously determined (12). The close relationship of *B. anthracis* strains from Cameroon with those from Chad, with both belonging to cluster A $\beta$ , may be explained by the fact that these countries share a border in the northern part of Cameroon, where anthrax is hyperendemic

and where most of the samples were collected. The fact that we observe no particular variations in strains isolated from western and central Africa (Cameroon and Chad) in spite of the high prevalence of the disease underlines the high genetic stability of the species *B. anthracis*. Moreover, strains of cluster A $\beta$  also show a particular phenotype. They all lack anthrose, an unusual sugar that is specific to the exosporium of *B. anthracis* (20, 21). It has to be noted that two *B. anthracis* strains from Cameroon previously analyzed by Lista and colleagues have an MLVA profile identical to that of our strains but were grouped into a new cluster, E (11). However, our analysis showed that this cluster is identical to A $\beta$ , which appears to be a more appropriate designation since A $\beta$  belongs to clade A (6, 22).

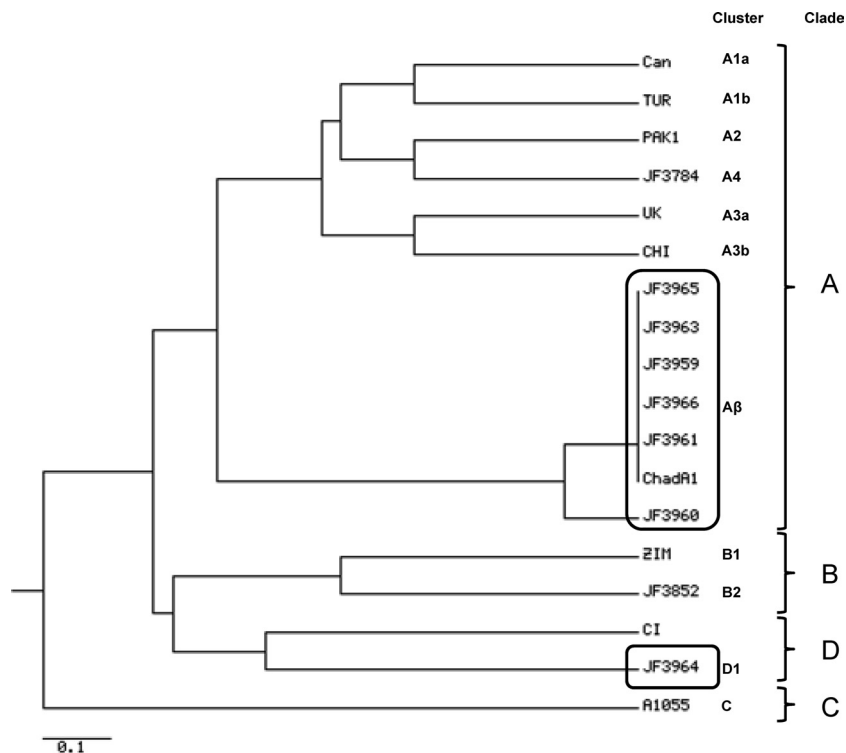


FIG. 1. Dendrogram based on eight VNTR markers of *B. anthracis* strains from Cameroon (this work) and characteristic strains representing all clades and major clusters as reported previously (6, 12, 15). Strains from western and central Africa are boxed. The dendrogram was generated by clustering using the unweighted pair group method with arithmetic means. The scale bar indicates genetic distance. The genetic distance is presented as the absolute number of differences in marker alleles among genotypes.

Strain JF3964, which belongs to the novel clade D of *B. anthracis*, was also placed into a new cluster, designated D1, as determined by MLVA (Fig. 1). The most closely related strain that belongs to the same clade but to a different cluster is *B. cereus* biovar *anthracis* strain CI, which was isolated from a chimpanzee from the Ivory Coast (9, 10) (Fig. 1). Strain CI also shows phenotypic characteristics that are similar to those of strain JF3964, such as being penicillin resistant and  $\gamma$  phage insensitive. It also lacks the specific chromosomal marker *sap* but harbors *cap* and *pag* virulence genes, placing both strains JF3964 and CI between *B. anthracis* and *B. cereus*. Although such particular penicillin-resistant strains of *B. anthracis* were thought to be specific to primates (9, 10), we show that they are also present in cattle with typical symptoms of anthrax. These results reveal these strains to possess a pathogenicity which is very similar to that of classical *B. anthracis* strains.

The presence of both plasmids pXO1 and pXO2 in *B. anthracis* strain JF3964 was confirmed by plasmid extractions from strain JF3964 and the type strain Vollum (as the standard) using the NucleoBond PC 20 plasmid DNA purification kit (Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer's protocol, followed by Southern blotting as previously described (16). Specific probes were constructed by PCR in the presence of digoxigenin-11-dUTP (Roche Diagnostics, Rotkreuz, Switzerland) using the oligonucleotide primer sets pag67/pag68 and cap57/cap58 and standard PCR conditions (16, 17). Both probes targeting plasmids pXO1 and pXO2 hybridized to two different large plasmids in JF3964, with sizes corresponding to those of the strain Vollum<sup>T</sup> (data not shown). Moreover, a real-time PCR assay based on the real-time PCR assay of Coker and colleagues (2) was performed to determine the number of plasmid copies per chromosome. One modification of the protocol was carried out because of the absence of the marker Ba813 in the strain JF3964. The *dnaK* gene was chosen as a chromosomal marker since it is present in a single copy in the genomes of bacteria and it is, like Ba813, not located close to the origin of replication of the chromosome in *B. anthracis*. The primers used for the real-time PCR were dnaK\_F, 5'-GCTTGCTCTTTGCTT GTTCCTAA-3', and dnaK\_R, 5'-TCGATATTGATGCGAA CGGTAT-3', and the probe was dnaK\_P, 5'-FAM-TTCGCA CGTACGTAAAC-TAMRA-3', where FAM and TAMRA are 6-carboxyfluorescein and 6-carboxytetramethylrhodamine, respectively. The copy number per chromosome equivalent to the number of copies of plasmid pXO1 in strain JF3964 was calculated from the ratio of the real-time PCR signal strength for pXO1 (JF3964), normalized to the signal of *dnaK* (JF3964), to the real-time PCR signal strength for pXO1 (Vollum<sup>T</sup>), multiplied by the copy number per chromosome equivalent to that of strain Vollum<sup>T</sup>, which is 11.5 (2). Since the real-time PCR signal is reciprocal to the threshold cycle ( $C_T$ ) value, the corresponding formula is

$$\frac{\text{pXO1}}{\text{chromosome}} = 11.5 \times \frac{C_T \text{ value of } dnaK \text{ in JF3964} \times C_T \text{ value of pXO1 in Vollum}}{C_T \text{ value of pXO1 in JF3964} \times C_T \text{ value of } dnaK \text{ in Vollum}}$$

For the determination of the pXO2 plasmid copy number per chromosome in the strain JF3964, the same formula was ap-

plied using the pXO2 copy number per chromosome in the strain Vollum<sup>T</sup>, which is 1.6 (2).

$$\frac{\text{pXO2}}{\text{chromosome}} = 1.6 \times \frac{C_T \text{ value of } dnaK \text{ in JF3964} \times C_T \text{ value of pXO2 in Vollum}}{C_T \text{ value of pXO2 in JF3964} \times C_T \text{ value of } dnaK \text{ in Vollum}}$$

Four dilutions were tested in triplicate by real-time PCR, and the calculated average copy number ratio for pXO1 in JF3964 was 10.89, with a standard deviation of  $\pm 0.19$ , while for pXO2, it was 1.59, with a standard deviation of  $\pm 0.03$ .

All strains were susceptible to antibiotics commonly used to treat infections caused by Gram-positive bacteria, as determined by the broth dilution method (Table 1) (1, 12), with the exception of strain JF3964. For this strain, the MIC of penicillin was 80 times higher and the MICs of oxacillin and cephalotin were higher than those for other strains of this region and higher than characteristic MIC values for *B. anthracis* (Table 1) (12). However, the strain was sensitive to amoxicillin-clavulanic acid (Table 1), indicating that the penicillin resistance was due to a  $\beta$ -lactamase activity.

The penicillin resistance mechanism of strain JF3964 is likely to be associated with the presence of the  $\beta$ -lactamase genes *bla1* and *bla2* and of a particular *sigP-rsiP* operon which is closely related to that of *B. cereus* but different from that in commonly found *B. anthracis*. Operon *sigP-rsiP* encodes sigma factor P (SigP) and the repressor of sigma factor P (RsiP), the latter of which affects the expression of the  $\beta$ -lactamase genes *bla1* and *bla2* (18). This was recently demonstrated with *B. anthracis* strain 32, which contains a truncated repressor gene, *rsiP*, resulting from one mutation in the *sigP-rsiP* operon. In this strain, the repressor cannot bind to the sigma factor, which enables the latter to interact with RNA polymerase and transcript *bla* genes, leading to a 6,000-times-higher MIC for penicillin (18). The entire *sigP-rsiP* operon was amplified from the penicillin-resistant strain JF3964 by PCR using FIREPol DNA polymerase I (Solis Biodyne) and primers BaSigPXL (5'-GCTTTTTCTATT TTATTATGGCTC-3') and sigmaR (5'-TGCTGCTCTCGTTA CATCAGA-3'). The PCR product obtained was subsequently sequenced using primers BaSigPXL, sigmaL2 (5'-TACGAAAG ATCCACACTTATCA-3'), sigmaL4 (5'-CCCAATGGAACAC CGTAAAG-3'), sigmaL5 (5'-TTGAAACCATAATAAAAGTG AGGGTA-3'), sigmaR, and sigmaR2 (5'-TGTGCTTGCTTTTGTGGTT-3'). DNA sequencing was performed as described above. Comparison of the nucleotide (nt) sequence with sequences in the EMBL/GenBank database using Blastn software revealed the *sigP-rsiP* operon of strain JF3964 to be identical to that of *B. cereus* biovar *anthracis* strain CI (10). Furthermore, the amino acids of SigP are 97.7% identical and 98.3% similar to those of SigP of common *B. anthracis* strains, such as Ames, Sterne, and Vollum<sup>T</sup>. Analogously, the amino acids of RsiP of strains JF3964 and CI are 97% similar and 99% identical to those of RsiP of common *B. anthracis* strains.

The *sigP-rsiP* operon contains -10 and -35 consensus sequences for a putative transcription promoter, followed by a strong ribosome binding site (RBS) upstream from the alternative start codon for SigP, TTG. The open reading frame for gene *rsiP* overlaps the last 11 nucleotides of *sigP* and is preceded by a characteristic RBS 5 nt upstream of the common

start codon for RsiP, ATG. It is worth noting that the start codon for SigP in *B. anthracis* strains, except in JF3964 and CI, is generally ATG, whereas in *B. cereus* and *B. thuringiensis*, it is TTG. We interpret the moderate resistance to penicillin (MIC, 8 µg/ml) in strain JF3964 to be due to a partial repression of SigP by the corresponding RsiP, in contrast to what occurs in common *B. anthracis* strains, which are assumed to exhibit full repression of their SigP by RsiP, and in contrast to *B. anthracis* strain 32, where a truncated RsiP is unable to repress SigP, leading to very high penicillin resistance (18). Although strain JF3964 has additional characteristics specific to *B. cereus*, it harbors the virulence genes which are associated with the virulence plasmids pXO1 and pXO2 in *B. anthracis*. The unusual and penicillin-resistant strains of *B. anthracis*, such as JF3964 and CI, may have evolved from a closely related species of the *B. cereus* group, which, in turn, comprises six closely related members, namely, *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihenstephanensis* (3), since it has been shown that plasmids can be transmitted or transferred spontaneously from one species to another (10, 13).

In conclusion, our analysis of *B. anthracis* strains isolated from cattle in Cameroon revealed that strains isolated from cattle with anthrax in western and central Africa are generally tightly clustered genetically and belong to phylogenetic group Aβ, which has not yet been identified outside this area. Furthermore, a particular subtype of *B. anthracis* belonging to a novel clade (D) with close similarity to *B. cereus* is also present in this region, causing symptoms of anthrax in cattle as well as in primates in western and central Africa. This rarely described type of *B. anthracis* currently contains only two described strains, and both of them belong to clade D but to different clusters (Fig. 1). They deserve particular attention both from the point of view of the microbial evolution of the *B. cereus* group and from a medical and diagnostic point of view.

**Nucleotide sequence accession numbers.** The nucleotide sequence of the *sigP-rsiP* operon is deposited under GenBank/EMBL accession number FR750362. The nucleotide sequences of the VNTRs are deposited under GenBank/EMBL accession numbers FR872834 to FR872889.

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