Identification of an African *Bacillus anthracis* Lineage That Lacks Expression of the Spore Surface-Associated Anthrose-Containing Oligosaccharide[▽]

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The surfaces of *Bacillus anthracis* endospores expose a pentasaccharide containing the monosaccharide anthrose, which has been considered for use as a vaccine or target for specific detection of the spores. In this study *B. anthracis* strains isolated from cattle carcasses in African countries where anthrax is endemic were tested for their cross-reactivity with monoclonal antibodies (MAbs) specific for anthrose-containing oligosaccharides. Unexpectedly, none of the isolates collected in Chad, Cameroon, and Mali were recognized by the MAbs. Sequencing of the four-gene operon encoding anthrose biosynthetic enzymes revealed the presence of premature stop codons in the aminotransferase and glycosyltransferase genes in all isolates from Chad, Cameroon, and Mali. Both immunological and genetic findings suggest that the West African isolates are unable to produce anthrose. The anthrose-deficient strains from West Africa belong to a particular genetic lineage. Immunization of cattle in Chad with a locally produced vaccine based on anthrose-positive spores of the *B. anthracis* strain Sterne elicited an anti-carbohydrate IgG response specific for a synthetic anthrose-containing tetrasaccharide as demonstrated by glycan microarray analysis. Competition immunoblots with synthetic pentasaccharide derivatives suggested an immunodominant role of the anthrose-containing carbohydrate in cattle. In West Africa anthrax is highly endemic. Massive vaccination of livestock in this area has taken place over long periods of time using spores of the anthrose-positive vaccine strain Sterne. The spread of anthrose-deficient strains in this region may represent an escape strategy of *B. anthracis*.

Bacillus anthracis is a sporulating Gram-positive bacillus causing anthrax primarily in herbivores in many countries of southern Europe, South America, Asia, and Africa (32). The majority of anthrax vaccines for cattle are based on live spores prepared from the attenuated, capsule-deficient *B. anthracis* strain Sterne (Weybridge no. 34F2) or similar nonencapsulated strains. The protective effect of a single dose is assumed to last for about 1 year (23), and therefore annual booster vaccinations are recommended for livestock (32). It has been shown that vaccination with strain Sterne does not fully protect mice against challenge with the virulent *B. anthracis* strain Vollum (30). Immune responses against the protective antigen (PA) and spore-associated antigens have been experimentally correlated with protective immunity against challenge with strain Vollum (2). Due to the potential for use of anthrax spores as

an agent of bioterrorism and the persistence of spores in the environment, the development of efficient vaccines and accurate detection methods is needed. The similarity of spore cell surface antigens between the various species of the *Bacillus cereus* group, which comprises *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides*, has made it difficult to create selective antibody-based detection systems for anthrax. However, antibodies capable of species-specific spore detection have been reported (24). The *Bacillus* collagen-like protein of *B. anthracis* (BclA) is a structural component of the outermost surface of the mature endospore. The collagen-like region of BclA is glycosylated with multiple copies of a pentasaccharide (4). This oligosaccharide contains an unusual terminal sugar called anthrose, followed by three rhamnose residues and a protein-bound *N*-acetylgalactosamine. A four-gene operon (designated *antABCD*) that encodes the enzymatic pathway for anthrose biosynthesis was identified (6). These four proteins are annotated as enoyl coenzyme A (enoyl-CoA) hydratase (BAS3322), glycosyltransferase (BAS3321), aminotransferase (BAS3320), and acyltransferase (BAS3319).

Anthrose has been proposed as a potential target for the detection of *B. anthracis* spores or as a target for therapeutic intervention against anthrax (4, 6). After the first chemical synthesis of a truncated version of the pentasaccharide, a tet-

rasaccharide lacking the GalNAc residue (31), several approaches for synthesis of the tetrasaccharide or related fragments have been reported and used in developing immunological tools (1, 3, 8, 13, 19–21). Conjugate vaccines were produced by linking synthetic anthrose-containing saccharides to protein carriers. Immunization of animals with these vaccines allowed the production of monoclonal antibodies (MAbs) and polyclonal antibodies that cross-reacted with native *B. anthracis* spores (5, 11, 26–28), confirming the structural analysis of the oligosaccharide and its expression on the endospore surface. Screening of antitetrasaccharide MAbs and anti-anthrose-rhamnose disaccharide MAbs for cross-reactivity with spores of a broad spectrum of *B. anthracis* strains and related species of the *Bacillus* genus demonstrated a broader presence of anthrose in the *B. cereus* group than initially expected (26, 27). Convergently, genetic evidence showed the presence of the anthrose biosynthetic operon in strains of *B. cereus* and *B. thuringiensis* (6). Additionally, structures similar to that of anthrose were found in the capsular polysaccharide of *Shewanella* sp. strain MR-4 and on flagella of *Pseudomonas syringae* (10). Although they are not strictly specific for *B. anthracis* spores, it has been argued that antibodies against anthrose-containing saccharides may still have potential as immuno-capturing components for a sensitive spore detection system (6, 26, 27). Additionally, antibody responses to the anthrose-containing oligosaccharide may have diagnostic value in confirming aerosol exposure to *B. anthracis* spores (18).

This study demonstrates the absence of the anthrose monosaccharide, as determined by immunological and genetic methods, in *B. anthracis* field isolates collected in West Africa (Chad, Cameroon, and Mali) and an immunodominant role of the anthrose-containing pentasaccharide in a locally produced Chadian Sterne strain-based animal anthrax vaccine in cattle.

MATERIALS AND METHODS

Production of spores from *Bacillus anthracis*. *B. anthracis* strains isolated from cattle carcasses were cultured on tryptone soy agar with 5% sheep blood (Oxoid, Basel, Switzerland) at 37°C for 18 h. All strains used were tested for the presence of the virulence genes *pag*, *led*, *cya* (pX01), and *capB* (pX02) and the *B. anthracis*-specific marker *sap* (surface layer protein) (17) by PCR as well as for the specific phenotypes of sensitivity to phage γ and penicillin and absence of hemolysis. The culture plates were then kept at room temperature for 4 weeks until the colonies appeared to be dry. Subsequently, the colonies were suspended in 1 ml phosphate-buffered saline (PBS) per culture plate and heated at 80°C for 10 min, followed by rapid cooling on ice to avoid germination. Spores were then collected by centrifugation at 4,000 × *g* for 15 min at 4°C and suspended in PBS at 4°C at a titer of 10^9 spores per ml.

SDS-PAGE and immunoblotting. The suspension of *B. anthracis* spores was mixed immediately with an equal amount of 2× loading buffer (0.08 M Tris-HCl [pH 6.8], 20% glycerol, 4.5% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.024% [wt/vol] bromophenol blue) and heated for 10 min at 95°C. This procedure dissolved more than 90% of the spores, while fewer than 1% remained viable. Subsequently the solution was filtered through a 0.22-μm membrane filter before being loaded for 4 to 10% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As a molecular weight marker, See-BluePlus (Invitrogen, Paisley, United Kingdom) was used. Separated proteins were electrophoretically transferred to a nitrocellulose filter by semidry blotting. Blots were blocked with PBS containing 5% milk powder overnight at 4 C. Whole blots or cut strips were incubated with appropriate dilutions of MAbs or immune serum in blocking buffer for 2 h at room temperature. In competition experiments primary antibodies were preincubated for 30 min with synthetic competitors. After several washing steps, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA), with alkaline phosphatase-conjugated goat anti-bovine IgM heavy-chain antibodies (KPL, Guildford, United Kingdom), or with alkaline phosphatase-conjugated

goat anti-bovine IgG heavy-chain antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Blots were finally developed using the ECL system according to the manufacturer's instructions or with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to visualize bands. Anthrose-rhamnose disaccharide (MTD1 to MTD6)- and anthrose-trirhamnose tetrasaccharide (MTA1 to MTA3)-specific MAbs were included in the analysis (14, 26–28).

PCR and DNA sequencing. PCR was performed using DreamTaq (Fermentas, Le Mont-sur-Lausanne, Switzerland) polymerase and synthetic oligonucleotides hybridizing to flanking or internal sequences of the *B. anthracis* anthrose operon genes BAS3319 to BAS3322. Primers employed for PCR amplification are listed in Table 1 and were designed using the genome sequence data for *B. anthracis* Sterne (Weybridge no. 34F2). Thirty-microliter reaction mixtures were used with a GeneAmp 9700 PCR system (Applied Biosystems). Template DNA for PCR was obtained by cell lysis followed by filtration (15) and was denatured at 94°C for 7 min. Thirty-three amplification cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 100 s of extension at 72°C were performed, followed by one cycle of 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis and sequenced by Macrogen (South Korea) using primers listed in Table 1.

Immunization of cattle. The vaccine was produced in Chad using *B. anthracis* strain Sterne (Weybridge no. 34F2) as described elsewhere (33). Four 6-month-old animals were bought from a local farmer in the surroundings of N'Djamena. Before the single subcutaneous injection of the vaccine, the animals received an anthelminthic treatment. Blood samples were collected prior to and at 11, 21, 25, 32, and 183 days after immunization. Aliquots of obtained sera were shipped in dry ice to Switzerland for immunological analysis.

Microarray analysis. Glycan microarray analyses with cattle sera were performed essentially as described previously (14). Briefly, spotted microarray slides were covered with FlexWell-64 (Grace Bio-Labs) layers. Wells were preincubated for 30 min with β-mercaptoethanol (1% [vol/vol] in PBS), blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and washed with PBS. Wells were subsequently incubated with appropriate dilutions of cattle serum in PBS containing 0.5% BSA for 2 h at room temperature. After washing, slides were incubated with Cy3-conjugated affinity-purified goat anti-bovine IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature and then washed. Dried slides were read on a GenePix Personal 4100A (Axon Instruments) microarray scanner at a wavelength of 532 nm. The resulting picture was quantitatively analyzed with GenePix Pro 6 software.

RESULTS AND DISCUSSION

Absence of anthrose in *B. anthracis* field isolates collected in West Africa. Anti-anthrose-trirhamnose tetrasaccharide MAbs and anti-anthrose-rhamnose disaccharide MAbs (14, 26–28) were tested for their reactivities with *B. anthracis* samples isolated from cattle carcasses in four African countries. In immunoblotting experiments with spore lysates, the anti-anthrose-rhamnose disaccharide MAb MTD5 reacted with strain Sterne (Fig. 1C, lane 1), with an internal control strain of *B. anthracis* (strain JF3783, belonging to the globally spread genotype cluster A4 [16]) (Fig. 1C, lane 2), and with an isolate from southern Africa (Zimbabwe) (Fig. 1C, lane 3). The observed staining pattern included both high-molecular-mass (~180/150 kDa) and low-molecular-mass (~60 kDa) bands, as is characteristic for the BclA glycoprotein (25, 27). In contrast, none of the *B. anthracis* isolates collected in Chad (13 isolates), Cameroon (6 isolates), and Mali (1 isolate) were recognized by the disaccharide-specific MAb MTD5 (Fig. 1C, lanes 4 to 6, and data not shown) or by the other antidisaccharide or antitetrasaccharide MAbs tested (data not shown). The BclA glycoprotein, found in *B. anthracis*, *B. cereus*, and *B. thuringiensis*, has highly conserved amino and carboxy termini and is also the immunodominant spore surface antigen in mice (24). Staining of endospore lysate proteins by immunoblotting with an anti-*B. thuringiensis* spore antiserum as the primary antibody yielded multiple-band staining patterns with a prominent high-molec-

TABLE 1. Primers used for PCR amplification and/or sequencing of the *B. anthracis* four-gene anthrose operon (BAS3319 to BAS3322)

Target gene	Primer	Purpose	Sequence (5′→3′)
BAS3319	BAS3319_F	Amplification and sequencing	TGGAATCGCAACGATGAATA
	BAS3319_R	Amplification and sequencing	AAAACGCTAGAAAGAACCTCTG
	BAS3319_S9	Sequencing	CCCCATTAATGCATTTCCAC
	BAS3319_S10	Sequencing	AGGGGAAGTTGGGATTGAAA
BAS3320	BAS3320_F1	Amplification and sequencing	AGCGGTAGTGGTTCCGTATG
	BAS3320_R1	Amplification and sequencing	CCCCATTAATGCATTTCCAC
	BAS3320_F2	Amplification and sequencing	TGGGTGGGGAATTATTCAAG
	BAS3320_R2	Amplification and sequencing	TATTCATCGTTGCGATTCCA
	BAS3320_S7	Sequencing	CGAAATCGTATCTCCGAATG
	BAS3320_S8	Sequencing	GCATGAGCGCATATTTTCCT
BAS3321	BAS3321_F1	Amplification and sequencing	GAACCGACAACGGTTTTGTT
	BAS3321_R1	Amplification and sequencing	GCATGAGCGCATATTTTCCT
	BAS3321_F2	Amplification and sequencing	TTCGCATGGCATTATAGCTG
	BAS3321_R2	Amplification and sequencing	GGAAAATTCCCCAAATCCTT
	BAS3321_F3	Amplification and sequencing	GAGTACCGCGTTTCACCAAT
	BAS3321_R3	Amplification and sequencing	ATGGCGTTTCATTTTTCACA
	BAS3321_S1	Sequencing	CTCGCATTGCTTTTTCATCA
	BAS3321_S2	Sequencing	TGCGTACATTGAAACGGAAA
	BAS3321_S3	Sequencing	GGTTGCCTTCCCCATCTATT
	BAS3321_S4	Sequencing	AGGCAACCTATTGCCAGATG
BAS3322	BAS3322_F2	Amplification and sequencing	TTTCAAAATAATGCGGACGA
	BAS3322_R	Amplification and sequencing	TGATGGATTTCTAAAAAGGGAAA
	BAS3322_S5	Sequencing	CAACCTCCGCCTAGTGCTAA
	BAS3322_S6	Sequencing	TCTCGAGGAGAGGCGTTAAA

ular-mass band, suggesting the presence of the BclA protein moiety in all isolates (Fig. 1B). However, cross-reactivity of the *B. thuringiensis* mouse antiserum with other high-molecular-mass proteins cannot be excluded.

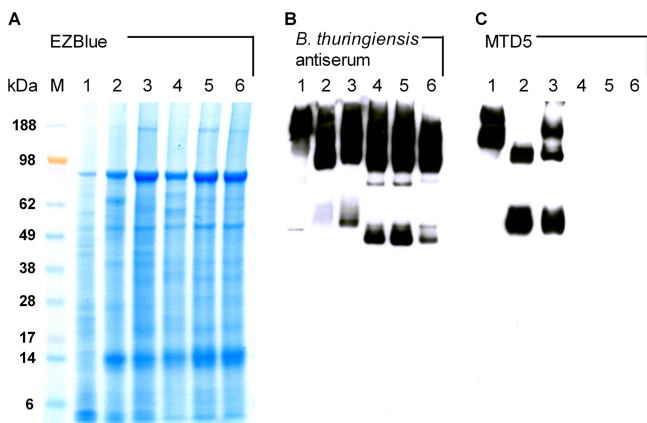Since the anthrose monosaccharide is an essential compo-

FIG. 1. Immunodetection of anthrose-containing tetrasaccharide in *B. anthracis* strains isolated from cattle carcasses in African countries. (A) EZBlue (Sigma) protein staining of *B. anthracis* spore lysates (lane 1, Sterne strain; lane 2, internal reference strain JF3783 from Switzerland; lane 3, isolate from Zimbabwe; lane 4, isolate from Mali; lane 5, one representative isolate from Chad; lane 6, one representative isolate from Cameroon) separated on a 4 to 10% gradient SDS-polyacrylamide gel. (B) Western blot analysis of the reactivity of anti-*B. thuringiensis* antiserum diluted 1:1,000 (27). (C) Cross-reactivity of the anti-anthrose-rhamnose disaccharide MAb MTD5 with the blotted total spore lysates. Similar results were obtained with all other antidisaccharide (MTD1 to MTD4 and MTD6) and antitetrasaccharide (MTA1 to MTA3) MAbs (14, 26–28) tested. MAb MTD5 was used at a concentration of 0.01 μg/ml. The sizes of the molecular mass markers (lane M) are given in kDa.

nent of the epitopes of the tested MAbs (14, 27), the failure of the MAbs to bind to the isolates from Mali, Chad, and Cameroon suggested that these strains might be unable to produce anthrose. To test this hypothesis, the anthrose operons of the African isolates were sequenced. The sequence of the BAS3319 gene, encoding an acyltransferase, was the same for all African strains (data not shown) and identical to that of the sequenced *B. anthracis* strain Sterne (GenBank accession no. AE017225). Sequencing of the BAS3320 gene (encoding an aminotransferase) revealed the presence of three eight-base-pair (AAAAAAAG) tandem repeats at position 230 in all isolates from Chad, Cameroon, and Mali instead of two repeats in all other strains, including the Zimbabwe isolate (Table 2). The observed tandem repeat insertion causes a frameshift mutation, changing the protein sequence and creating a premature stop codon at position 306 (normal termination after base pair 1139). Comparison of the available aminotransferase gene (BAS3320) sequences revealed the presence of a point mutation at position 72 causing an Ile→Met amino acid sequence exchange in the sequenced *B. anthracis* strain A1055 but not in the African isolates. None of the currently published BAS3320 sequences contains three AAAAAAAG tandem repeats (Table 2). In all isolates from western Africa the BAS3321 (glycosyltransferase-encoding) gene carried a single base substitution from C to T at position 892, resulting in a premature a stop codon (normal termination at position 2556) (Table 3). This mutation is not present in any of the currently published BAS3321 gene sequences.

Additionally, in some *B. anthracis* strains three nonsynonymous single-nucleotide polymorphisms (SNPs) compared to the Sterne strain were identified in the BAS3321 gene at positions 1151, 1352, and 1934 (Table 3). Finally, an SNP at position 614 was found in the BAS3322 (enoyl-CoA hydratase-

TABLE 2. Sequence polymorphisms in the BAS3320 gene (1,139 bp, encoding aminotransferase) of the anthrose operon

B. anthracis strain[a]	Polymorphism (amino acid)	
	T→G	Tandem repeat
Sterne	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
A1055	70 AT**G** 72 (M)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
KrugerB	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
A0442	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
A0465	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
CNEVA-9066	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
Zimbabwe	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAG**-------GAA 232
Mali	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAGAAAAAAAG**GAA 240 (frameshift → stop)
Chad (13x)	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAGAAAAAAAG**GAA 240 (frameshift → stop)
Cameroon (6x)	70 AT**T** 72 (I)	211 TTT**AAAAAAAGAAAAAAAGAAAAAAAG**GAA 240 (frameshift → stop)

[a] The *B. anthracis* strains from Zimbabwe, Mali, Chad, and Cameroon were isolated from cattle carcasses. The *B. anthracis* strains Sterne, A1055, KrugerB, A0442, A0465, and CNEVA-9066 are representative hits from sequence comparisons using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

encoding) gene, which has also been reported in other sequenced *B. anthracis* strains (Table 4). At all four positions the strain from Zimbabwe diverged from the strains from Mali, Chad, and Cameroon.

Sequencing of the anthrose four-gene operon revealed the presence of premature stop codons in genes BAS3320 and BAS3321 in all isolates from Chad, Cameroon, and Mali, resulting in shortened open reading frames. It is most likely that these truncated proteins possess no or heavily impaired enzymatic activity. It has been shown previously that experimental inactivation of either gene BAS3321, BAS3320, or BAS3319 results in spores devoid of anthrose (6), indicating that their encoded enzymes are required for anthrose biosynthesis. In contrast, inactivation of gene BAS3322 resulted in spores with about half as much anthrose as found in wild-type spores (6). Furthermore it was reported that inactivation of gene BAS3321 resulted in BclA being substituted with trisaccharides only, suggesting that the enzyme is a dTDP-β-L-rhamnose α-1,3-L-rhamnosy transferase that attaches the fourth residue of the pentasaccharide side chain (7). Inactivation of genes BAS3320 and BAS3319 resulted in the disappearance of BclA pentasaccharides and the appearance of a tetrasaccharide lacking anthrose (7). Thus, the inactivation of enzymes BAS3320 and BAS3321 of the anthrose biosynthetic operon in the strains from Chad, Cameroon, and Mali represents a plausible explanation for the lack of binding of the anti-carbohydrate MAbs.

The observed deficiency in anthrose biosynthesis and the differences in the corresponding biosynthesis genes for the aminotransferase (BAS3320) and the glycosyltransferase (BAS3321), both resulting in premature stop codons, seem to be particular traits of strains from West Africa and are not found elsewhere. This is interesting in that *B. anthracis* strains from West Africa belong to a particular genetic lineage, the genotype cluster Aβ (12; P. Pilo [Bern], unpublished observations), as determined by variable-number tandem repeat (VNTR) analysis according the typing scheme of Keim et al. (9, 29). In this respect, the 8-nucleotide tandem repeat AAAAAAAG that is present in 2 copies in the anthrose biosynthesis aminotransferase gene (BAS3320) in most *B. anthracis* strains worldwide except those from West Africa, where the repeat is present in 3 copies, is proposed as a valuable new VNTR that allows an increase in the resolution of subtyping and is directly associated with the anthrose phenotype.

Antigenicity of the anthrose-containing pentasaccharide of a Chadian veterinary anthrax vaccine. Spore preparations of the attenuated *B. anthracis* vaccine strain Sterne are used as a live veterinary anthrax vaccine. Since many countries manufacture veterinary vaccines on their own, quality control would be important to avoid a resurgence of the disease because of the usage of inadequate vaccines (32). Differences in vaccine quality have been observed but not systematically analyzed (32). In this study, a live animal anthrax vaccine manufactured in Chad from spores of strain Sterne (Weybridge no. 34F2) was

TABLE 3. Sequence polymorphisms in the BAS3321 gene (2,256 bp, encoding glycosyltransferase) of the anthrose operon

B. anthracis strain[a]	Polymorphism (amino acid)			
	C→T	C→T	C→T	A→T
Sterne	892 **C**AA 894 (Q)	1150 AC**G** 1152 (T)	1351 ACC 1353 (T)	1933 AA**A** 1935 (K)
A1055	892 **C**AA 894 (Q)	1150 AC**G** 1152 (T)	1351 ACC 1353 (T)	1933 AA**A** 1935 (K)
KrugerB	892 **C**AA 894 (Q)	1150 AT**G** 1152 (M)	1351 ACC 1353 (T)	1933 AT**A** 1935 (I)
A0442	892 **C**AA 894 (Q)	1150 AT**G** 1152 (M)	1351 ACC 1353 (T)	1933 AT**A** 1935 (I)
A0465	892 **C**AA 894 (Q)	1150 AT**G** 1152 (M)	1351 ACC 1353 (T)	1933 AA**A** 1935 (K)
CNEVA-9066	892 **C**AA 894 (Q)	1150 AT**G** 1152 (M)	1351 ACC 1353 (T)	1933 AA**A** 1935 (K)
Zimbabwe	892 **C**AA 894 (Q)	1150 AT**G** 1152 (M)	1351 ACC 1353 (T)	1933 AT**A** 1935 (I)
Mali	892 **T**AA 894 (stop)	1150 AC**G** 1152 (T)	1351 AT**C** 1353 (I)	1933 AA**A** 1935 (K)
Chad (13x)	892 **T**AA 894 (stop)	1150 AC**G** 1152 (T)	1351 AT**C** 1353 (I)	1933 AA**A** 1935 (K)
Cameroon (6x)	892 **T**AA 894 (stop)	1150 AC**G** 1152 (T)	1351 AT**C** 1353 (I)	1933 AA**A** 1935 (K)

[a] The *B. anthracis* strains from Zimbabwe, Mali, Chad, and Cameroon were isolated from cattle carcasses. The *B. anthracis* strains Sterne, A1055, KrugerB, A0442, A0465, and CNEVA-9066 are representative hits from sequence comparisons using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

TABLE 4. Sequence polymorphisms in the BAS3322 gene (792 bp, encoding enoyl-CoA hydratase) of the anthrose operon

B. anthracis strain[a]	T→C polymorphism (amino acid)
Sterne	613 **TTT** 615 (F)
A1055	613 **TTT** 615 (F)
KrugerB	613 **TCT** 615 (S)
A0442	613 **TCT** 615 (S)
A0465	613 **TCT** 615 (S)
CNEVA-9066	613 **TCT** 615 (S)
Zimbabwe	613 **TCT** 615 (S)
Mali	613 **TTT** 615 (F)
Chad (13x)	613 **TTT** 615 (F)
Cameroon (6x)	613 **TTT** 615 (F)

[a] The *B. anthracis* strains from Zimbabwe, Mali, Chad, and Cameroon were isolated from cattle carcasses. The *B. anthracis* strains Sterne, A1055, KrugerB, A0442, A0465, and CNEVA-9066 are representative hits from sequence comparisons using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

used to immunize cattle. Serum antibody responses were analyzed in immunoblotting experiments with a *B. anthracis* spore lysate (Fig. 2A). These analyses provided evidence for the production of antibody responses against *B. anthracis* proteins. When using IgM-specific secondary antibodies, the preimmune serum revealed a weak and diffuse high-molecular-mass band (>188 kDa) which remained largely constant after immunization. The first traces of a vaccine-induced IgM response were observed at day 21, when at least two distinct protein bands of between 62 and 98 kDa appeared. Already at 25 days after

vaccination staining of these bands decreased, and staining was no longer detectable at day 32. When using IgG-specific secondary antibodies, preimmune serum also stained a high-molecular-mass antigen. Starting at day 21 after immunization, the IgG signal strength of this band increased substantially. Simultaneously, a strong IgG response to two distinct protein bands of between 62 and 98 kDa also emerged. These analyses suggested that immunogenic *B. anthracis* antigens induced an initial production of anthrax antigen cross-reactive IgM and that activated B cells subsequently switched to the expression of Cγ heavy-chain genes. Six months after the single immunization, all animals showed a substantial drop of IgG levels reacting with spore lysates in Western blots, reflecting the relatively short duration of immunity of vaccinated animals. As recommended by WHO (33), this makes annual booster immunizations necessary.

The specificity of the interaction of immune sera with the high-molecular-mass antigen was further investigated by competition Western blot analyses using a set of overlapping synthetic saccharides (Fig. 2D). Binding of serum IgG to the high-molecular-mass band, thought to be the BclA glycoprotein, was reduced greatly by the synthetic tetrasaccharide (Fig. 2B, lane 2) and partially by the anthrose-rhamnose disaccharide (Fig. 2B, lane 3) and anthrose (Fig. 2B, lane 4) but not by trirhamnose (Fig. 2B, lane 5). Competition with the glycan structures tested had no influence on the serological reactions with the lower-molecular-mass antigens that are unrelated to
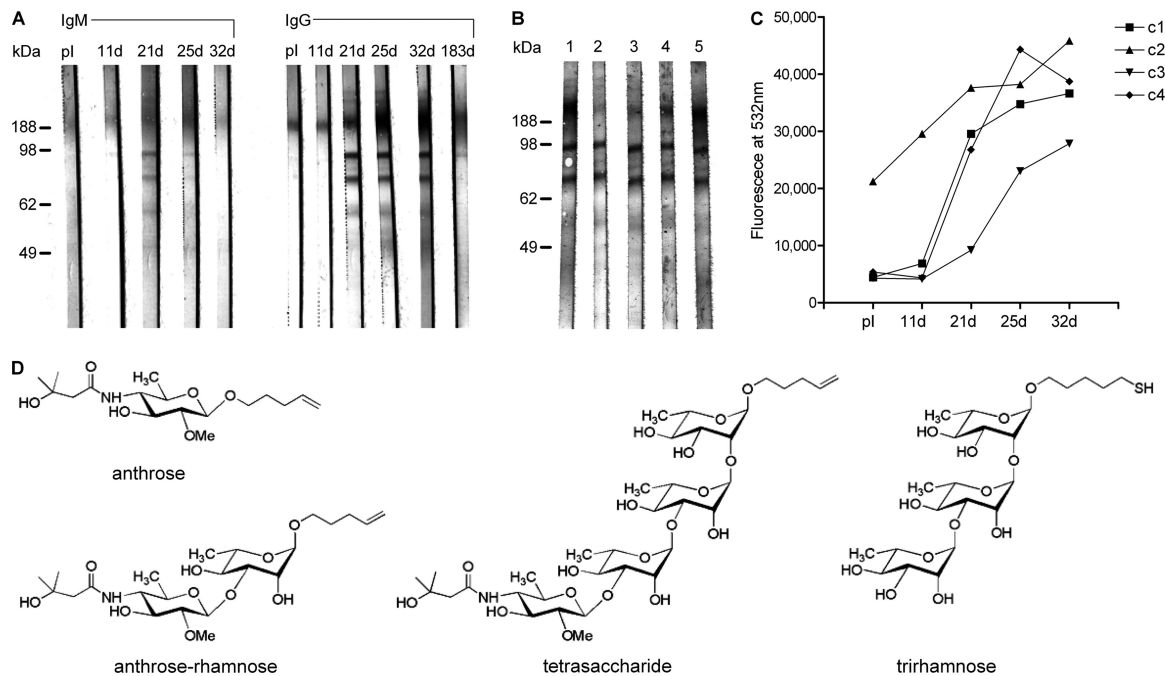

FIG. 2. Antigenicity of the BclA-associated pentasaccharide of the Sterne 34F2 veterinary vaccine in cattle. (A) Development of *B. anthracis* cross-reactive IgM and IgG in Western blotting with total spore lysate. Cattle serum samples taken preimmunity (pI) and at days 11, 21, 25, 32, and 183 after a single subcutaneous vaccination with the Sterne spore vaccine were tested at a dilution of 1:100. IgM and IgG responses of a representative animal are depicted. (B) Competition Western blot experiments with overlapping synthetic carbohydrates were used for epitope mapping. Immune serum of cattle no. 3 taken 32 days after vaccination was preincubated with synthetic competitors and afterwards added to cut strips. The different competitors used were as follows: lane 1, no competitor; lane 2, tetrasaccharide; lane 3, disaccharide; lane 4, anthrose; lane 5, trirhamnose. Immune sera were used at a dilution of 1:100 and synthetic competitors at a concentration of 100 μg/ml. (C) Glycan microarray analysis of the development of antitetrasaccharide IgG responses in the four vaccinated cattle (c1 to c4). Shown are results obtained with individual sera at a dilution of 1:100. (D) Synthetic structures derived from the BclA-associated pentasaccharide.

BclA. These results indicated that the spore vaccine induced a substantial IgG response against the tetrasaccharide, suggesting that the anthrose-containing carbohydrate constituent of the BclA protein is one of the immunodominant antigens in cattle. Thus, the anthrose-containing tetrasaccharide could be used as a target antigen to monitor vaccine-induced antibody responses as a quality control for the livestock vaccines.

Using a previously developed glycan microarray spotted with the tetrasaccharide (14), it was possible to monitor development of this antitetrasaccharide antibody response directly. A single immunization with spores of strain Sterne elicited high levels of specific antitetrasaccharide IgG titers (Fig. 2C) in all four cattle immunized. One of the animals had a substantial preimmunization antitetrasaccharide IgG titer, which was boosted by the vaccination. Since the animals were not reported to have been vaccinated with strain Sterne previously, the observed preimmunization antitetrasaccharide IgG titer in one animal suggests either exposure to an anthrose-expressing *Bacillus* species or the development of cross-reactive antibodies against structurally related antigenic saccharides found on other endemic microorganisms.

West Africa is a region where anthrax is highly endemic and where massive vaccination of livestock has taken place over long periods of time (22). The geographic correlation of massive vaccination with the live vaccine strain Sterne, which produces an antigenic response to anthrose, with the lack of anthrose-positive *B. anthracis* field strains specifically in this area is interesting and leads to the hypothesis that the latter strains could represent escape mutants. This hypothesis could be tested in the future by comparing the protective efficacies of a Sterne vaccine and a similar vaccine derived from an anthrose-deficient *B. anthracis* strain toward anthrose-producing and anthrose-deficient strains.

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