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Peptide-Based Fluorescence Resonance Energy Transfer Protease Substrates for the Detection and Diagnosis of *Bacillus* Species

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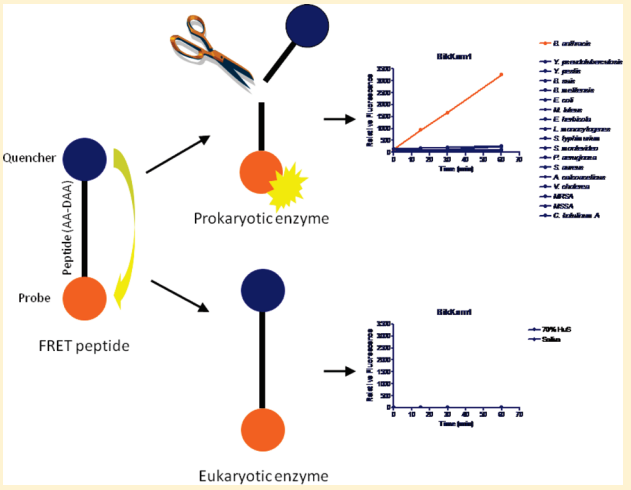
ABSTRACT: We describe the development of a highly specific enzyme-based fluorescence resonance energy transfer (FRET) assay for easy and rapid detection both *in vitro* and *in vivo* of *Bacillus* spp., among which are the members of the *B. cereus* group. Synthetic substrates for *B. anthracis* proteases were designed and exposed to secreted enzymes of a broad spectrum of bacterial species. The rational design of the substrates was based on the fact that the presence of D-amino acids in the target is highly specific for bacterial proteases. The designed D-amino acids containing substrates appeared to be specific for *B. anthracis* but also for several other *Bacillus* spp. and for both vegetative cells and spores. With the use of mass spectrometry (MS), cleavage products of the substrates could be detected in sera of *B. anthracis* infected mice but not in healthy mice. Due to the presence of mirrored amino acids present in the substrate, the substrates showed high species specificity, and enzyme isolation and purification was redundant. The substrate wherein the D-amino acid was replaced by its L-isomer showed a loss of specificity. In conclusion, with the use of these substrates a rapid tool for detection of *B. anthracis* spores and diagnosis of anthrax infection is at hand. We are the first who present fluorogenic substrates for detection of bacterial proteolytic enzymes that can be directly applied in situ by the use of D-oriented amino acids.



The Gram-positive *Bacillus anthracis* bacterium is the causative agent of anthrax. The stability, ease of production, and infectious capacity of the spores confer upon *B. anthracis* a high potential as a biological weapon.[1] During pulmonary anthrax, spores germinate in the lungs ultimately followed by the emergence of vegetative anthrax in the circulation.[2] This systemic infection frequently results in secondary shock and multiple organ failure, which, if untreated, results in death.[3] Therefore, fast point-of-care diagnosis is critical for effective treatment of pulmonary anthrax.

B. anthracis possesses two major virulence components: the pXO1 and pXO2 plasmids.[2] These plasmids encode the biosynthesis pathway for D-glutamic acid and the anthrax toxins lethal factor (LF), edema factor (EF), and protective antigen (PA), respectively.[4−6] Because of their specificity to *B. anthracis* and their absence in the closely related species *B. cereus* and *B. thuringiensis*, to date the pXO1 and pXO2 plasmids are the main targets used in the detection of *B. anthracis* and the diagnosis of anthrax. For instance, numerous antibody-based detection methods for *B. anthracis* target the pXO1 encoded anthrax toxins.[7−9] Other DNA-based techniques used in the detection of *B. anthracis* are (real-time and multiplex) polymerase chain reaction (PCR) targeting pXO1 and pXO2.[10] However, despite their high specificity, these tests do not provide evidence on pathogen viability and disease progression.

Bacterial enzymes, such as proteases, are in theory ideally suited as biomarkers for quick and sensitive identification of microorganisms in clinical samples.[11] Many of these enzymes are released into the surrounding microenvironment and are accessible for detection based on sensitive fluorogenic and/or luminogenic substrates. However, in practice lack of specificity has

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proven to be a large hurdle, which has seriously hampered practical application for diagnosis.[11−13]

Proteases occur abundantly in all organisms, from viruses to men. They are involved in a myriad of processes and functions, from simple digestion of food proteins to highly regulated cascades such as the blood-clotting and the complement cascades.[14] It is therefore obvious that proteolytic activity by itself is not a useful indicator for the presence of bacteria, let alone for a specific pathogen. To overcome this problem, studies have been undertaken to develop substrates with exquisite specificity for protease(s) from a specific pathogen.[15−17] However, the substrates developed thus far still suffer from a lack of specificity, as they can be hydrolyzed by a variety of bacterial and human enzymes.[14] For their use as diagnostic tools, therefore, processing of the sample to isolate the target enzyme under investigation is still needed. This is laborious, time-consuming, and costly and, moreover, prone to yielding erratic results. Multiple enzymatic assays for the detection of *B. anthracis* using the fluorescence resonance energy transfer (FRET) technology have been described by others.[10,17−22] Most of these assays are based on the detection of the anthrax toxins LF and EF.[10,20] Boyer and coworkers studied the LF activity on a 45 amino acid artificial substrate using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).[17,18] When applied to sera of infected monkeys, the assay detected the toxin in the femtomolar range. However, the activity of unrelated proteolytic enzymes will lead to false-positive results, and hence, the detection of LF had to be facilitated by a time-consuming immunoextraction.

In the present study a different approach was developed to enhance the specificity of protease substrates. This approach exploited an intriguing difference between eukaryotic and prokaryotic cells with regard to the use of D-amino acids.

Although L-amino acids are primarily incorporated in natural proteins, the presence of D-amino acids is highly specific for bacterial proteases, in which they are abundantly present as a component of the bacterial cell walls.[23] This led us to hypothesize that bacteria may express proteases which possess a unique property for recognizing and hydrolyzing D-amino acid containing substrates. Such activities can be translated into diagnostic tests.

The aim of the present study was to develop FRET-based substrates for the specific detection of proteolytic enzymes of the *B. cereus* group which includes *B. anthracis*.

MATERIALS AND METHODS

Bacteria. The bacterial isolates used in this study are *B. anthracis* Vollum (ATCC 14578), *B. cereus*, *B. thuringiensis* var. kurstaki aizawai, *B. globigii*, *B. mycoides* (ATCC 14579), *B. subtilis* Marburg (ATCC 6051), *B. megaterium*, (ATCC 15374), *Yersinia pseudotuberculosis* (ATCC 29833), *Y. pestis* HmsF− (ATCC 19428), *Brucella suis* Thomsen, *Br. melitensis* 16M, *Escherichia coli* (ATCC 11775), *Micrococcus luteus*, *Erwinia herbicola* (ATCC 33243), *Listeria monocytogenes* EGDe (ATCC BAA-679), *Salmonella typhimurium*, *S. Montevideo*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staph. aureus* MRSA (ATCC 43300), *Staph. aureus* MSSA (ATCC 25923), *Acetinobacter lwoffii calcoaceticus* Ruh88, *Vibrio cholerae*, and *Clostridium botulinum* A (NCTC 2916). Bacteria were grown overnight in 5 mL of brain heart infusion (BHI) medium (BioTrading, Mijdrecht, The Netherlands) at 35 °C, and at 26 °C for the *Yersiniae*. The next day, the bacteria were pelleted by centrifugation for 10 min at 10 000 rpm. Supernatant, containing secreted enzymes, was sterilized by filtration through a 0.22 μm filter (Millipore, Amsterdam, The Netherlands). The crude samples were used directly or stored at −20 °C for later use.

Table 1. FRET Substrates Designed in This Study

	Sequence
BikKam1	FITC-Leu-D-Leu-LysDbc
BikKam2	FITC-D-Leu-Leu-LysDbc
BikKam3	FITC-Leu-Leu-LysDbc
BikKam4	FITC-D-Leu-D-Leu-LysDbc
BikKam5	FITC-Leu-D-Leu-Leu-LysDbc
BikKam6	FITC-Leu-D-Val-LysDbc
BikKam7	FITC-Gly-D-Leu-LysDbc
BikKam8	FITC-Gly-D-Ala-LysDbc

FRET Assay. FRET substrates were designed by using the MEROPS database[14] and provided by PepScan Presto B.V. (Lelystad, The Netherlands) with a purity of approximately 90%. The identity of the substrates was confirmed by mass spectrometry. The substrates were denoted as "BikKams" (Table 1). Assays were performed in Blackwell, clear bottom 96-well plates (Corning, Lowell, U.S.A.). Enzyme activity in bacterial supernatants was determined by incubating 16 μM substrate with 100 μL of filtered culture supernatant at 37 °C. Filtered BHI medium was used as a negative control. Plates were read with 10 min intervals on a CytoFluor 4000 (Applied Biosystems, Foster City, U.S.A.) with excitation at 485 nm and emission at 530 nm. Relative fluorescence (RF) is the value obtained after correction with the negative control, BHI medium. The measured enzyme activity is defined in RF per minute (RF/min).

Preparation of Spores. *B. anthracis* strain Vollum (ATCC 14578) and *B. subtilis* strain Marburg were cultured by shaking at 35 °C in 250 mL of sporulation broth (SB). At a sporulation efficiency of 99% the suspensions were centrifuged at 4000*g* for 40 min. The pellets were washed with distilled water and resuspended in 1 mL of water. The number of viable spores in the suspensions was determined by plating 10-fold serial dilutions on trypticase soy agar (TSA) plates (BioTrading, Mijdrecht, The Netherlands). Plates were incubated at 35 °C, and spores were enumerated after 1 day of incubation.

Preculture Detection of *B. anthracis* Spores. *B. anthracis* and *B. subtilis* spores were precultured in 1 mL of BHI medium at 35 °C. Samples were taken 0, 1, 2, 3, and 4 h after incubation. After centrifugation the samples were incubated using 16 μM of the BikKam1 substrate. The increase in fluorescence was measured for 1 h with 10 min intervals on a CytoFluor 4000 (Applied Biosystems, Foster City, U.S.A.) with excitation using a 485 nm filter and emission using a 530 nm filter. Relative fluorescence is the value obtained after correction with the negative control, BHI medium. The measured enzyme activity is defined in RF per minute (RF/min).

***In Vivo* Infection Model.** Anesthetized male Balb/C mice (Harlan, Horst, The Netherlands) were intranasally inoculated with 1.25×10^4 *B. anthracis* spores in a 50 μL volume. For each time point 10 mice were used. At 0, 12, and 48 h postinfection, serum was isolated and pooled per time point. Because of the small amount of sera, samples were analyzed using liquid chromatography−electrospray tandem mass spectrometry (LC−ES MS/MS) as described below. During the experiment

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the animals were kept in sterile isolators (UNO, Zevenaar, The Netherlands) in a biohazard animal unit. They were fed irradiated food (Harlan, Horst, The Netherlands) and acidified water ad libitum. The mice were monitored regularly for clinical status and weighed daily. All experimental procedures performed on the animals were approved by the The Ethical Committee on Animal Experimentation of TNO (DEC 2727).

LC−ES MS/MS. For the LC−ES MS/MS analysis of the infected mouse serum, 1:10 in 0.06 M EDTA diluted serum was incubated with 16 μM FRET substrate for 3 h at 37 °C. Assay mixtures were loaded onto an Amicon 10 000 MWCO filter (Millipore, Amsterdam, The Netherlands) that had been preconditioned with 300 μL of 50% acetonitrile and 300 μL of 0.2% (v/v) formic acid in water, respectively. After the sample loading, the filter was washed with 100 μL of 0.2% (v/v) formic acid in water. The collected samples were analyzed using LC−ES MS/MS.

LC−ES MS/MS experiments were conducted on a Q-TOF hybrid instrument (Micromass, Altrincham, U.K.) equipped with a standard Z-spray ES interface (Micromass) and an Alliance type 2690 liquid chromatograph (Waters, Milford, MA, U.S.A.). The chromatographic hardware consisted of a precolumn splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10 or 50 μL injection loop mounted, and a PepMap C_{18} column (15 cm × 1 mm i.d., 3 μm particles; LC Packings, Amsterdam, The Netherlands).

A gradient of eluents A (H_2O with 0.2 v/v % formic acid) and B (acetonitrile with 0.2 vol % formic acid) was used to achieve separation, as follows: 100% A (at time 0 min, 0.6 mL/min flow) to 10% A and 90% B (at 45 min, 0.6 mL/min flow). The flow delivered by the LC equipment was split precolumn to allow a flow of approximately 40 μL/min through the column and into the ES MS interface.

The Q-TOF was operated at a cone voltage of 20−25 V, employing nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 L/h, respectively). MS/MS product ion spectra were recorded using a collision energy of 10−11 eV, with argon as the collision gas (10^{-4} mbar).

RESULTS

Concept and Peptide Design for a FRET-Based Anthrax Detection Method. To design substrates specific for *B. anthracis* we used the MEROPS database to search for known peptidases produced by *B. anthracis.* Peptidases suitable for our purpose should be able to cleave substrates wherein D-oriented amino acids are present to protect the substrate from cleavage by other enzymes. One of the selected enzymes was dipeptidase AC which recognizes substrates with the sequence Leu-D-Leu. Besides in *B. anthracis* this peptidase is also produced by several other bacterial species such as *A. calcoaceticus* and *Brucella* spp.[14,24] To check the specificity of the Leu-D-Leu substrate (BikKam1) the substrate was incubated with culture supernatant of numerous bacterial species. It appeared that BikKam1 was only cleaved by culture supernatants of *Bacillus* spp., whereas no cleavage of BikKam1 by culture supernatants of *A. calcoaceticus* or *Brucella* spp. was observed (Table 2). From these results we hypothesized that probably the cleavage of this substrate is not related to dipeptidase AC. To obtain more insight in the mechanism of action variants on the BikKam1 substrate were designed (Table 1). All substrates designed contained fluorescein isothiocyanate (FITC) as probe and Dabcyl (Dbc) as its quencher. The importance of the placement of D-Leu was investigated by switching the position of Leu and D-Leu in the sequence (BikKam2). The hypothesis of the specificity of the use of D-amino acids was checked by a substrate in which no D-amino acids are present (BikKam3). BikKam4 was designed to see if the specificity could be increased by the replacement of Leu by another D-Leu and in addition to try to increase the cleavage activity another Leu was added to the Leu-D-Leu sequence (BikKam5). The importance of the presence of Leu in the sequence was investigated by the use of substrates in which one of the D-Leu was replaced by another closely related amino acid D-Val (BikKam6). The substrates Gly-D-Leu (BikKam7) and Gly-D-Ala (BikKam8) were designed by Adachi and Tsujimoto[24] and are both cleaved by dipeptidase AC with a higher efficiency than the Leu-D-Leu substrate.

***In Vitro* Evaluation of BikKam Substrates.** To further explore the specificity of all the BikKams (Table 1), the substrates were incubated with culture supernatants, potentially containing secreted proteases deriving from a broad spectrum of bacterial species. In all substrates which contain D-Leu, cleavage of the substrate by bacilli of the *B. cereus* group as well as *B. megaterium* and *B. licheniformis* was observed (Table 2). The only exception was the substrate which consists of two D-oriented amino acids (BikKam4). No cleavage of this substrate by *B. anthracis* or *B. mycoides* was observed. All other bacteria tested did not show activity with any of the D-Leu containing substrates (Table 2) including two bacilli which belong to another part of the *Bacillus* tree: the *B. subtilis* group (Table 2). In case D-Leu was replaced by its L-isomer (BikKam3) a loss in specificity was observed; besides *B. subtilis* and *B. globigii* the substrate was cleaved by culture supernatants of a number of other bacterial species including *P. aeruginosa* and *V. cholerae* (Table 2).

To further substantiate the role of the D-Leu in the BikKam1 substrate, D-Leu was replaced by either D-Val (BikKam 6), which structure is closely related to D-Leu, or D-Ala (BikKam8), where the α-carbon atom is covalently bound to a methyl group. BikKam6 was cleaved by all *Bacillus* spp. though with a lower efficiency. No cleavage of the D-Ala substituted substrate by *Bacillus* spp. was observed (Table 2). Replacement of Leu by Gly had no effect on the cleavage pattern or cleavage efficiency. No cleavage activity by *A. calcoaceticus* or *Brucella* spp. was observed on the Gly-D-Leu and Gly-D-Ala substrates (Table 2).

Identification of the *B. anthracis* Cleavage Sites by LC−ES MS/MS. To identify the cleavage sites, BikKam substrates were incubated with *B. cereus* culture supernatant and analyzed using LC−ES MS/MS. All analyzed substrates appeared to be cleaved directly after the D-amino acid (Table 3). To verify these cleavage sites for *B. anthracis* the experiment was repeated for two substrates using *B. anthracis* culture supernatant. As expected *B. anthracis* cleaved the two substrates at the same position as *B. cereus* did.

Detection of Precultured *B. anthracis* Spores Using BikKam1. To verify the applicability of the BikKam1 substrate to detect anthrax spores, different amounts of *B. anthracis* spores were triggered into vegetative state by precultivating and incubated with the substrate. As a negative control spores of *B. subtilis* were used. After 3 h of preculturing 10^7 *B. anthracis* spores, a significant increase of fluorescence was observed (Figure 1A). In case the spores were incubated for 4 h 10^6 *B. anthracis* spores could be detected. No cleavage was observed when BikKam1 was incubated with precultured *B. subtilis* spores (Figure 1B).

***In Vivo* Diagnosis of Inhalational Anthrax by BikKam1.** To further explore the possibilities for the BikKam1 substrate to

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Table 2. Proteolytic Activity of Bacterial Culture Supernatants against the Designed FRET Substrates[a]

	Leu-D-Leu	D-Leu-Leu	Leu-Leu	D-Leu-D-Leu	Leu-D-Leu-Leu	Leu-D-Val	Gly-D-Leu	Gly-D-Ala
B. megaterium	++	+	-	+	+	+	+	-
B. anthracis (*B. cereus* group)	++	++	-	-	++	+	++	-
B. cereus (*B. cereus* group)	++	++	-	+	+	+	++	-
B. thuringiensis (*B. cereus* group)	+	+	-	+	+	+	+	-
B. mycoides (*B. cereus* group)	+	+	-	-	+	+	+	-
B. licheniformis	++	++	-	++	++	++	+++	-
B. globigii	-	-	+++	-	-	-	-	-
B. subtilis	-	-	++	-	-	-	-	-
A. calcoaceticus	-	-	+	-	-	-	-	-
B. suis	-	-	-	-	-	-	-	-
B. melitensis	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	-	-	-	-	-	-	-	-
Y. pestis	-	-	-	-	-	-	-	-
V. cholerae	-	-	+++	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-
M. luteus	-	-	-	-	-	-	-	-
E. herbicola	-	-	+++	-	-	-	-	-
L. monocytogenes	-	-	-	-	-	-	-	-
S. typhimurium	-	-	-	-	-	-	-	-
S. montevideo	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	+++	-	-	-	-	+
S. aureus (MRSA)	-	-	-	-	-	-	-	-
S. aureus (MSSA)	-	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	-
C. botulinum A	-	-	+++	-	-	-	-	-
Human serum	-	-	-	-	-	-	-	-
Human saliva	-	-	-	-	-	-	-	-

[a] Bacteria were grown at appropriate temperature for 16 h in BHI. The measured enzyme activity is defined in RF/min. RF is the value obtained after correction with the negative control, BHI medium. RF/min: <5 (−), 5−24 (+) less active, 25−124 (++) middle activity, >125 (+++) very active. Phylogenetic tree is adjusted from Kolsto et al. (ref 26).

Table 3. Determination of the *B. cereus* and *B. anthracis* BikKam Cleavage Sites Using MS Analysis[a]

	B. cereus	*B. anthracis*[b]
BikKam1	FITC-Leu-D-Leu ▼ LysDbc	FITC-Leu-D-Leu ▼ LysDbc
BikKam2	FITC-D-Leu ▼ Leu-LysDbc	n.d.
BikKam5	FITC-Leu-D-Leu ▼ Leu-LysDbc	n.d.
BikKam6	FITC-Leu-D-Val ▼ LysDbc	FITC-Leu-D-Val ▼ LysDbc

[a] The cleavage sites of the substrates are denoted with (▼). [b] Not done (n.d.).

detect *B. anthracis*, mice were intranasally infected with 1.25×10^4 spores/mouse. Serum was isolated during the infection at 0 , 12, and 48 h postinfection (p.i.). BikKam1 cleavage products could be detected in the pooled sera of *B. anthracis* infected mice at 48 h p.i. (Figure 2C). At this time point the mice were very ill and showed onset of severe clinical signs. No cleavage products were found in the sera at 0 (Figure 2A) and 12 h p.i. (Figure 2B).

DISCUSSION

In search for a rapid and simple tool for the detection of bacterial protease activity in situ, short and specific substrates containing a D-amino acid were designed. Although L-amino acids represent the vast majority of amino acids found in natural proteins, the presence of D-amino acids is highly specific for bacteria, where D-amino acids are abundantly present as a

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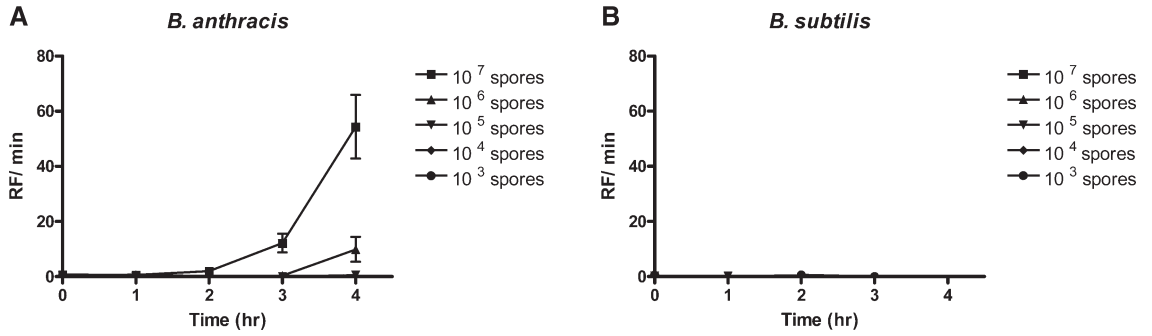

Figure 1. Detection of anthrax spores by BikKam1. Precultivated *B. anthracis* (A) and *B. subtilis* (B) spores were incubated with BikKam1 at 37 °C. After 3 h of preculturing 10^7 *B. anthracis* spores an activity of 11 RF/min, with an increase to 87 RF/min after 4 h, was measured (■). For the detection of 10^6 *B. anthracis* spores, the spores had to be precultured for 4 h (▲). No increase in fluorescence was observed in case *B. subtilis* spores were used (B). Results are expressed as mean ± SEM (n = 3).

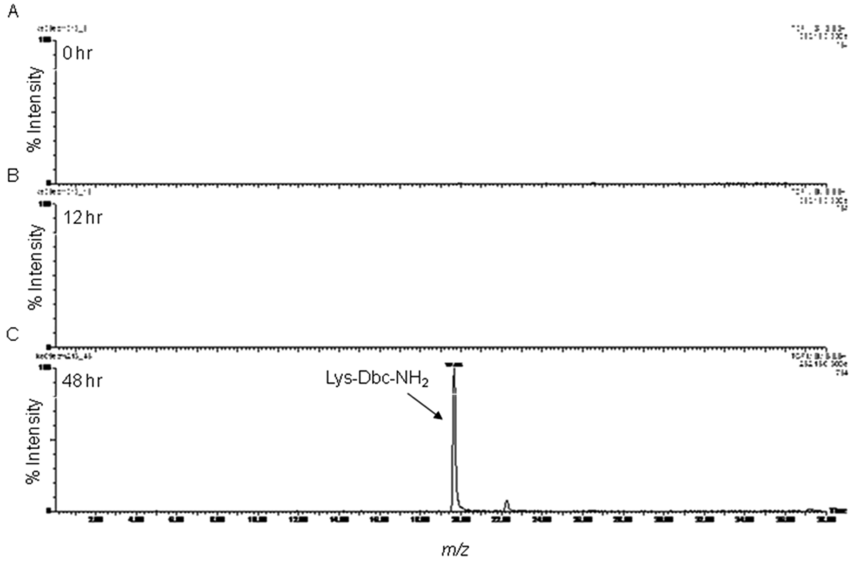

Figure 2. LC−ES MS/MS detection of BikKam1 specific activity in sera of infected mice. Sera taken from *B. anthracis* infected mice were incubated with 16 μM BikKam1 substrate for a 2 h cleavage reaction at 37 °C. At 0 (A) and 12 h (B) postinfection (p.i.) no BikKam1 fragments could be detected. However, at 48 h p.i. a clear peak, identified by MS/MS as the BikKam1 fragment Lys-Dbc-NH_2 (MW 396.2), was observed (C).

component of the bacterial cell walls.[25] The fact that bacteria are able to process D-amino acids led us to hypothesize that bacteria may express proteases which possess a unique property for recognizing and hydrolyzing D-amino acid containing substrates that can be used for detection and diagnostic purposes.

The BikKam1 substrate appeared to be highly specific for the detection of *B. anthracis* and its close relatives (Table 2). No cleavage could be detected in case the substrate was incubated with culture supernatants of *B. subtilis* and *B. globigii* (also known as *B. subtilis* var. niger). These two bacterial species are present in the same branch of the phylogenetic tree of *Bacillus*.[26]

To obtain more insight in the mechanism of action, several BikKam1 analogues were designed. The presence of one or more D-oriented amino acids appeared to be important to maintain the specificity of the substrate. Replacement of the D-Leu by its L-isomer (BikKam3) led to a significant decrease in specificity. The BikKam3 substrate was recognized adequately by *B. subtilis* and *B. globigii* proteases, but it was also cleaved by proteases of a number of other pathogens, including *P. aeruginosa* (data not shown). The position of the D-isomer in the substrate seemed to be of limited importance; BikKam1 and BikKam2 were cleaved with similar efficiency. MS analysis of the cleaved substrates revealed that all analyzed BikKams were cleaved directly after the D-oriented amino acid. Both *B. anthracis* as well as *B. cereus* cleaved the substrates at the same position. Changing the position of the D-isomer (BikKam2) or addition of an extra Leu (BikKam5) had no effect on the cleavage pattern. Also in case the D-Leu was substituted by D-Val (BikKam6) the substrate was still cleaved directly after the D-isomer, though it was with lower efficiency. However, substitution of D-Leu by D-Ala (BikKam8) led to a total loss of cleavage activity. This is probably due to the fact that Ala and Leu differ more in structure than Val and Leu do. Both Leu and Val have two methyl groups in their structure, whereas only one methyl group is present in the structure of Ala. Thus, substitution of Leu by Ala has large effects on the steric design of the substrate.

On the basis of our observations we are tempted to suggest that the BikKam1 substrate might be useful for the detection of *B. anthracis* spores in the so-called "anthrax letters". For this purpose the spores had to be triggered into a vegetative state by three (10^7 spores) or 4 h (10^6 spores) of preculturing to observe a significant increase in fluorescence in time. No increase in

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fluorescence was observed in case *B. subtilis*, flour, washing powder, or talc were used. Currently, culture still is the most common technique to be used in the detection of anthrax.[27,28] The relative ease of this method facilitates a higher throughput compared to immunochemical or PCR methods, which involve multiple and complex sample preparation and assay steps.[10,28,29] One of the methods currently used in the U.S. Postal Offices is the PCR-based technique of Cepheid, with which it is possible to detect 30 anthrax spores in 45 min (http://www.cepheid.com/tests-and-reagents/anthrax/). In contrast, the approach presented in this study is easy to perform, requiring a minimum of experimental steps. Compared to other enzyme-based detection techniques it is fast; yet in 4 h 10^7 *B. anthracis* spores can be detected. Due to the specific character of the BikKam1 substrate there is no need for time-consuming enzyme pre-enrichment or purification. We envisage that by using our FRET assay as rapid prescreening "anthrax letters" can be reliably analyzed within 1 day for the presence of *Bacillus* spp. in the field, without the need of highly trained personnel. To confirm the outcome of FRET-mediated testing eventually a *B. anthracis* specific PCR or culturing can be executed in addition.

To explore the opportunities for the assay to diagnose an anthrax infection, sera of *B. anthracis* infected mice were analyzed using the BikKam1 substrate. Before infection and 12 h p.i. no BikKam1 degradation fragments could be detected. However, at 48 h after infection BikKam1 fragments could be detected by MS analysis. At this time point *B. anthracis* was systemic, and the mice were clearly affected by the disease. To detect inhalational anthrax before the onset of severe clinical signs, the infection needs to be diagnosed before the bacterium is present in the vascular system. However, in order to detect *B. anthracis* secreted enzymes in blood the infection probably has to be systemic. In future experiments bronchoalveolar lavage (BAL) fluid will be used to detect anthrax protease activity at an earlier stage.

Further characterization and identification of the enzyme(s) involved will enhance the possibilities to improve the current methodology. In case the responsible enzyme is known, specific stimulators of the assay can be added to the FRET assay to increase the limit of detection. Moreover, the substrate can be optimized by replacement or addition of amino acids or by the usage of other conjugates.

More likely dipeptidase AC, the enzyme on which the original BikKam1 substrate was based, is not involved in cleavage of the substrates used in this study. Instead of dipeptidase AC we now hypothesize that the cleavage of the substrate is due to a peptidase which plays a role in cell wall metabolism of *Bacillus* spp., where D-amino acids are incorporated in the peptidoglycan (PG).[23] Bacteria release these D-amino acids during their stationary growth phase, probably to synchronize growth inhibition and PG synthesis.[30] Alternatively, we cannot exclude that either FITC or Dabcyl plays a role in enzyme recognition and thereby sterically hinders dipeptidase AC from *A. calcoaceticus* and *Brucella* spp. from cleaving the BikKam substrates.

A candidate enzyme was recently described by Sela-Abramovich et al. who discovered the cysteine peptidase NlpC/p60 (BA1952).[31] This peptidase is present in sera of anthrax-infected mice.

Members of the NlpC/p60 family from the genus of *Bacillus* have shown to be D,L-endopeptidases that hydrolyze the D-γ-glutamyl-*meso*-diaminopimelate linkage in the cell wall peptides.[32] Moreover, BA1952 orthologs are present in the secretomes of *Bacillus* spp. of the *cereus* group.[31] However, the BikKam1 substrate was also cleaved by the more remotely related bacilli such as *B. mycoides*, *B. licheniformis*, and *B. megaterium*. Probably, these bacteria produce other enzymes that possess the same functional capacities as the BA1952 peptidase found in *B. anthracis*.

In conclusion, we report a novel enzyme-based approach for the detection of *B. anthracis*. In this study it is shown that the new test can be applied for the detection of *B. anthracis* spores in anthrax letters. Our *in vivo* study in mice might form the basis for *in vivo* diagnosis of *B. anthracis* infection in humans.

We are the first to use D-amino acid containing substrates that can be used for enzyme-based diagnostic purposes. We feel it tempting to suggest that, besides *B. anthracis*, the described method can be applied in the detection and diagnosis of other pathogens. Specific D-amino acid containing FRET substrates can be designed for the detection and/or identification of other bacterial species.

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