Specific detection of *Bacillus anthracis* using a TaqMan® mismatch amplification mutation assay

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Single nucleotide polymorphisms (SNPs) are increasingly recognized as important diagnostic markers for the detection and differentiation of *Bacillus anthracis*. The use of SNP markers for identifying *B. anthracis* DNA in environmental samples containing genetically similar bacteria requires the ability to amplify and detect DNA with single nucleotide specificity. We designed a TaqMan® mismatch amplification mutation assay (TaqMAMA) around a SNP in the *plcR* gene of *B. anthracis*. The assay permits specific, low-level detection (25 fg DNA) of this *B. anthracis*-specific SNP, even in the presence of environmental DNA extracts containing a 20,000-fold excess of the alternate allele. We anticipate that the ability to selectively amplify and detect low copy number DNAs with single nucleotide specificity will represent a valuable tool in the arena of biodefense and microbial forensics.

INTRODUCTION

The 2001 anthrax letter attacks resulted in five fatalities, cost several billion dollars to the U.S. economy, and illustrated the practical efficacy of this pathogen (*Bacillus anthracis*) as a bioterrorism agent. Routine monitoring and genetic testing of environmental samples for *B. anthracis* nucleic acids is critical for early detection and response in the event of a bioterrorist attack and for subsequent forensic investigations. Yet, molecular detection of *B. anthracis* in the environment is particularly challenging, since samples may contain complex mixtures of DNA signatures, including genetically similar innocuous organisms. In particular, the genetic similarity of *B. anthracis* to other common spore-forming soil bacteria, such as *Bacillus cereus* and *Bacillus thuringiensis*, has presented challenges to identifying *B. anthracis*-specific chromosomal targets (1).

Single nucleotide polymorphisms (SNPs) are valuable markers for the detection and characterization of *B. anthracis* (1–5). For instance, Hurtle et al. (1) demonstrated that a SNP in the *gyrA* gene was a possible *B. anthracis*-specific chromosomal marker. A TaqMan® minor groove-binding assay

designed around the SNP sensitively detected 43 *B. anthracis* strains and did not cross-react with 36 *B. cereus* and 13 *B. thuringiensis* strains. In another example, a single nucleotide nonsense mutation has been identified in the *plcR* gene of *B. anthracis* that is absent in panels of *B. thuringiensis* and *B. cereus* isolates (5–7). Recently, we demonstrated that this nonsense mutation in the *plcR* gene was ubiquitous in 89 globally and genetically diverse *B. anthracis* isolates and is absent from *B. cereus* and *B. thuringiensis* isolates that are known to be genetic near-neighbors of *B. anthracis* (7). Given this, the single nucleotide change in the *plcR* gene represents a promising target for the specific detection of *B. anthracis*.

From a biodefense and bioforensic perspective, using SNP markers for biological threat agent identification should require clear detection of the targeted SNP sequence in the presence of a background of DNA containing the alternate allele. A recent advance in the area of SNP-specific detection was the combination of the fluorogenic 5' nuclease PCR (TaqMan) and the mismatch amplification mutation assay (MAMA) (8,9). Developed by Glaab and Skopek (9), this novel approach, termed TaqMAMA, utilizes

the MAMA to exploit mismatched bases between the PCR primers and the targeted SNP template to support allele-specific amplification, while TaqMan fluorescence allows monitoring of amplification in a real-time, quantitative manner. A powerful attribute of this technique is that it allows for the amplification of low-copy templates with a single nucleotide change in 1000-fold excess of wild-type DNA (9). Here we demonstrate the use of TaqMAMA to selectively amplify and detect *B. anthracis*-specific DNA in the presence of complex mixtures of DNA signatures, including signatures from genetically similar near-neighbors.

MATERIALS AND METHODS

TaqMAMA Primer and Probe Design

Sequences flanking the *plcR* nonsense mutation were obtained either from GenBank® or from sequencing efforts in our laboratory (7) (Figure 1) and were aligned using DNASTAR SeqMan™ II software (DNASTAR, Madison, WI, USA). To promote *B. anthracis* specific amplification, the MAMA primer was designed so that the ultimate 3' base was complementary to the *plcR* nonsense mutation. The penultimate 3' base was designed to mismatch with the shared sequence between *B. anthracis* and the near-neighbors sequences (9). The TaqMan minor groove-binding probe and the reverse primer were designed using Primer Express® software (Applied Biosystems, Foster City, CA, USA) to exploit additional nucleotide differences between *B. anthracis* and near-neighbor sequences that were proximal to the nonsense mutation (Figure 1).

TaqMAMA PCR Protocol

In all experiments, TaqMAMA PCR was conducted in 10-μL reactions that contained 600 nM both forward (5'-GAGTTTGATGTGAAGGT-GAGACATAATC-3') and reverse (5'-TTTGCATGACAAAGCGCTAA-3') primers (see Figure 1 for sequences), 250 nM probe (5'-6FAM-TACTTGGA-CAATCAA-MGB-3'), 1× Platinum®

qPCR SuperMix-UDG (5 mM MgCl₂; Invitrogen, Carlsbad, CA, USA), and 1 μ L template. Thermal cycling was performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) with the following conditions: 50°C for 2 min, 95°C for 2 min, and 50 cycles of 95°C for 15 s, 60°C for 1 min.

Specificity Tests

To initially test the specificity of the assay, 10 pg DNA from the *B. anthracis* Ames strain and 29 genetic near-neighbors (see Figure 1 for strains and sequence information) were used as templates in the assay. We also performed PCR on genomic DNA from *B. cereus* strain 3A and *B. thuringiensis* strain HD1011 at 10 pg, 100 pg, 500 pg, and 1 ng input. We performed 32 replicates of each DNA input level for each strain, with the exception of HD1011 at 500 pg input, which we tested at 64 replicates (288 reactions total).

Sensitivity Tests

Sensitivity was determined by analyzing serial 10-fold dilutions of genomic DNA from the *B. anthracis* Ames strain ranging from 250 pg to 25 fg. To determine assay sensitivity in nontarget DNA backgrounds, we performed analysis on the same *B. anthracis* template ranges in the presence of 50 or 500 pg DNA from the *B. thuringiensis* strain HD1011, air filter DNA extracts from a Biodefense monitoring program, and a combination of air filter DNA extracts and HD1011 DNA (50 or 500 pg).

RESULTS AND DISCUSSION

The TaqMAMA *plcR* assay is designed to amplify this gene fragment in *B. anthracis* but not in closely related isolates of *B. cereus* and *B. thuringiensis* (Figure 1). We found that the assay amplified 10 pg *B. anthracis* DNA but did not amplify any of the

10 pg near-neighbor DNA templates after 50 thermal cycles (Figure 2A). In contrast, the same samples analyzed using a traditional single probe TaqMan minor groove-binding assay (probe recognizes *B. anthracis* SNP allele) showed low-level cross-reactivity with near-neighbors in sequence group 3 [see Figure 1, mean cycle threshold (C_t) = 34.27]. To evaluate if the TaqMAMA would cross-react with higher levels of near-neighbor DNA templates that differed by only the *plcR* nonsense mutation, we performed extensive PCR analysis of genomic DNA from *B. thuringiensis* HD1011 and *B. cereus* 3A. We tested 64 replicates of these near-neighbor templates at 10 pg, 100 pg, 500 pg, and 1 ng per PCR. The assay did not amplify any of the near-neighbor templates at 10 or 100 pg. However, 2 of the 96 replicates at 500 pg input (*B. cereus* 3A) and one at 1.0 ng (*B. cereus* 3A) exhibited weak amplification after 40 amplification cycles (mean C_t = 42.9, C_t range = 41.6–45.0).

Figure 2. Real-time plots indicating the specificity and sensitivity of the *plcR* TaqMan mismatch amplification mutation assay (TaqMAMA). (A) Results of triplicate analysis of 10 pg DNA from *Bacillus anthracis* and 29 near-neighbors. The 29 near-neighbors include the 17 listed in Figure 1 and the following isolates: *Bacillus cereus* strains ATCC 4342, ATCC 14579, F3-27, F3350/87, S2-4; and *Bacillus thuringiensis* strains HD1015, HD681, HD288, HD526, HD974, HD30, and HD50. *B. anthracis* DNA amplified at an average cycle threshold (C_t) of 28.46, whereas the near-neighbor templates did not exhibit amplification. Additional information on strains is presented in Hill et al. (11). (B) Results of TaqMAMA PCR analysis of 10-fold serial dilutions of *B. anthracis* DNA (triplicate samples illustrated). The average C_t values (triplicate analysis) in molecular grade water were as follows: 250 pg, 25.5; 25 pg, 29.0; 2.5 pg, 32.4; 250 fg, 35.7; and 25 fg, 39.0. Insert in the upper left-hand corner is the standard curve plot; R^2 = 0.9995, slope = -3.376.

1. GAGTTTGATGTGAAGGTGAGACATAATCTACTTGGACAATCAAATAGCGCTTTGTCATGGAAA
2. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTTGGACAATCAATACGAAAGAGCGCTTTGTCATGGAAA
3. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTTGGACAATCAATACGAAAGAGCGCTTTGTCATGGAAA
4. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTTGGACAATCAATACGAAAGAGCGCTTTGTCATGGAAA
5. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATCTTTAGACAATCAATACGAAAGAGCGCTTTGTCACGGAAA
6. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATCTCAGATAATCAATACGAAAGAGCGCTTTGTCACGGAAA
7. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACATAGACAATCAATACGAAAGAGCGCTTTGTCACGGAAA
8. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTAGATAATCAATATGAAGAGCGCTTTGTCACAAATAAA
9. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTAGATAATCAATACGAAAGAGCGCTTTGTCACGGAAA
10. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTTAGACAATCAATACGAAAGAGCGCTTTGTCACGGAAA
11. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATACCTTAGACAATCAATACGAAAGAGCGCTTTGTCACGGAAA
12. GAGTTTGATGTGAAGGTGAGACATAATCATGCAAAAGCATTATCTCAGATAATCAATACGAAAGAGCGCTTTGTCACGGAAA

Figure 1. Sequence alignment of *plcR* gene fragments from *Bacillus anthracis* and genetic near-neighbors. The numbered lines indicate the sequences of: (1) primer and probe locations; (2) *B. anthracis*; (3) *Bacillus cereus* 3A, S2-8, and *Bacillus thuringiensis* HD1011; (4) *B. cereus* D5; (5) *B. cereus* AH527; (6) *B. cereus* D17, *B. thuringiensis* HD571, HD44; (7) *B. cereus* F3502/72, R6; (8) *B. cereus* F2-1; (9) *B. cereus* R4, ATCC 33018, *B. thuringiensis* HD1012; (10) *B. thuringiensis* 97-27; (11) *Bacillus* spp. TET 2b-3; and (12) *B. thuringiensis* HD682. Additional information on strains is presented in Hill et al. (11). Colored shading indicates polymorphic sites that are targeted in this assay. Red shading indicates nucleotide differences between near-neighbors and *B. anthracis*. *, *plcR* nonsense mutation; +, penultimate 3' mismatch.

Although these results indicate exceptional specificity of the assay, the rare low-level cross-reactivity with *B. cereus* 3A at 1.0 ng and 500 pg indicates that a weak false positive

result could occur in certain instances. In our estimation, this scenario is unlikely to occur during routine environmental monitoring, in that it requires fairly high concentrations

of a template that differs by only a single nucleotide. *B. cereus* 3A and *B. thuringiensis* HD1011 isolates were selected for specificity testing, since they shared identical sequence in the

Table 1. Average Cycle Threshold Values for Sensitivity Test^a

<i>Bacillus anthracis</i> Template	Experiment ^b	Average C _t (C _t - C _t of control sample)			
		Control Sample (no background)	HD1011 DNA	Air Filter Extract ^c	Air Filter Extract and HD1011 DNA
250 pg	Exp1	24.9	24.4 (-0.5)	26.2 (+1.3)	26.4 (+1.5)
	2	25.7	26.9 (+1.2)	26.8 (+1.1)	27.5 (+2.2)
25 pg	Exp1	28.2	28.2 (0.0)	29.5 (+1.3)	28.9 (+0.7)
	2	28.8	30.0 (+1.2)	29.8 (+1.0)	31.6 (+2.8)
2.5 pg	Exp1	31.0	31.4 (+0.4)	32.8 (+1.8)	33.6 (+2.6)
	2	31.9	32.3 (+0.4)	32.6 (+0.7)	34.1 (+2.2)
250 fg	Exp1	34.6	35.0 (+0.4)	36.4 (+2.2)	35.8 (+1.2)
	2	34.2	35.4 (+1.2)	35.4 (+1.2)	35.3 (+1.1)
25 fg ^d	Exp1	40.4 ^e	40.5 ^f (+0.1)	40.7 ^h (+0.3)	41.1 ^f (+0.7)
	2	40.6 ^f	41.7 ^g (+1.1)	42.3 ^h (+1.7)	40.9 ^f (+0.3)

^aCycle threshold (C_t) values are an average of duplicate reactions unless otherwise noted.

^bExperiment: Exp1 HD1011 DNA background = 50 pg/PCR; Exp2 HD1011 DNA background = 500 pg/PCR.

^c1.0 µL air filter DNA extract spiked into PCR.

^dAll 25 fg samples were replicated 10 times, the C_s from samples that amplified were averaged.

^eOf the 10 replicates, 7 amplified.

^fOf the 10 replicates, 9 amplified.

^gOf the 10 replicates, 10 amplified.

^hOf the 10 replicates, 8 amplified.

plcR gene fragment with *B. anthracis*, with the exception of the single nonsense mutation. In our sequence analysis of the *plcR* gene fragment of near-neighbors, the majority of isolates (26 of 29) had additional mutations in the sequence fragment that is targeted by the assay. We have not observed the *plcR* assay to cross-react with these isolates at 1 ng input levels (data not shown). Therefore, analyzing 500 pg to 1 ng genomic DNA from *B. cereus* 3A and *B. thuringiensis* HD1011, which we estimate corresponds to approximately 100,000–200,000 genome equivalents, represents a rare worse case scenario. Despite this, we suggest that weak positive results in the TaqMAMA could be rapidly investigated by using a dual-probe system (7) that detects the alternate SNP state. This control would differentiate between a very infrequent misamplification of a near-neighbor or the legitimate detection of very low levels of the real target. Additional experiments are underway to investigate factors affecting assay specificity.

The TaqMAMA exhibited quantitative detection of the *B. anthracis* template over a dynamic range (Figure 2B) and permitted sensitive detection in PCRs containing different backgrounds. We compared the sensitivity of the TaqMAMA in PCRs containing: (i)

no background; (ii) 50 or 500 pg of *B. thuringiensis* HD1011 genomic DNA; (iii) air filter DNA extracts; and (iv) a combination 50 or 500 pg of *B. thuringiensis* HD1011 genomic DNA and air filter extracts (Table 1). We used HD1011 genomic DNA in these experiments since we did not observe any cross-reactivity at any concentration (see above) in these templates despite extensive PCR analysis. As illustrated in Table 1, the average C_t values were similar among treatments, although we did consistently measure a slight increase in C_s in samples containing 500 pg HD1011 background and/or air filter extracts, suggesting weak inhibition of the reaction. This inhibition was not alleviated by the addition of 400 ng/µL bovine serum albumin (BSA) to the PCR (Reference 10 and data not shown). Interestingly, this apparent inhibition did not impact the threshold of detection of the assay, which permitted routine detection of the *B. anthracis*-specific SNP at template levels as low as 25 fg, even in the presence of air filter extracts containing a 20,000-fold excess of DNA containing the alternate allele (Table 1). It is important to note that the air filter extracts did not cross-react with the assay or impact the specificity of the assay as illustrated by the lack of amplification in air filter extracts and air filter extracts spiked with 100 or 500 pg

HD1011 template (data not shown).

This *B. anthracis* detection assay represents a significant advance in rapid and specific detection of *B. anthracis* for several reasons. First, the assay is based upon a well-studied chromosomal marker that is present in *B. anthracis* but absent in genetic near-neighbors. The presence of the *plcR* nonsense mutation in globally and genetically diverse *B. anthracis* lineages (7) limits the likelihood of false negative results, whereas the absence of the mutation in *B. cereus* and *B. thuringiensis* strains (5,7) and the specificity of the reaction decreases the probability of false positive results. Second, the sensitivity and specificity of the assay permits low-level detection of the targeted SNP, even in the presence of environmental DNA extracts containing 20,000-fold excess of the alternate allele. Third, the assay is amenable to high-throughput real-time PCR platforms that are currently the mainstay of homeland defense initiatives, such as Biowatch.

In conclusion, the use of the TaqMAMA method to detect the *plcR* mutation in *B. anthracis* permits the specific, low-level detection of this biological threat agent in samples containing environmental extracts and/or genetic near-neighbor DNA. We anticipate that this ability to selectively amplify and detect low copy number biothreat agent DNAs with single nucleotide specificity will represent a valuable tool in the arena of biodefense and bioforensics.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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