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Use of Single Nucleotide Polymorphisms in the *plcR* Gene for Specific Identification of *Bacillus anthracis*

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A TaqMan-minor groove binding assay designed around a nonsense mutation in the *plcR* gene was used to genotype *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* isolates. The assay differentiated *B. anthracis* from these genetic near-neighbors and determined that the nonsense mutation is ubiquitous across 89 globally and genetically diverse *B. anthracis* strains.

The genetic similarities among the pathogenic, spore-forming soil bacteria *Bacillus cereus*, *B. thuringiensis*, and *B. anthracis* have resulted in the suggestion that they be considered members of the same species (3). Interestingly, these bacteria exhibit phenotypic differences and express virulence in diverse ways. *B. cereus* and *B. thuringiensis* are opportunistic pathogens in mammals due to the secretion of nonspecific virulence factors, such as hemolysins, the expression of which is regulated by the transcriptional activator PlcR (8). In *B. anthracis*, PlcR is inactivated due to a nonsense mutation in the *plcR* gene (1), and its virulence in mammals is attributed to the expression of specific toxins under the control of the AtxA regulator (2).

The nonsense mutation in the *plcR* gene of *B. anthracis* may represent an evolutionarily stable, species-specific marker. Research by Mignot et al. (8), in which a functional PlcR was expressed in *B. anthracis*, demonstrated that PlcR- and AtxA-controlled regulons were incompatible, as *plcR* expression interfered with sporulation in *B. anthracis*. Since sporulation is a critical component of the ecology of *B. anthracis*, the authors speculated that a functional PlcR is counterselected in this species. Recent sequence comparisons of the *plcR* genes of two phylogenetically distinct *B. anthracis* lineages revealed the same nonsense mutation in the *plcR* gene (9), providing additional evidence to support the species specificity of this mutation.

To initially test the utility of the nonsense mutation in *plcR* as a species-specific marker for *B. anthracis*, we examined the *plcR* gene fragments that surround the nonsense mutation in several *Bacillus* spp. The strains examined included nine genetically diverse *B. anthracis* strains, nine *B. cereus* strains, six *B. thuringiensis* strains, and one unidentified near-neighbor (TET 2b-3) (4). Sequences obtained either from GenBank or from sequencing efforts in our laboratory were compared using MegAlign (Fig. 1). The nonsense mutation was present in all nine of the *B. anthracis* sequences and was absent in the 16 near-neighbor sequences.

Based upon these sequences, we designed a TaqMan-minor groove binding (MGB) allelic discrimination assay around the nonsense mutation. The TaqMan-MGB probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). One probe was designed to specifically hybridize to the *B. anthracis* sequence (5′-VIC-CAAAGCGCTTATTCG TATT-3′-MGB), and the other was designed to hybridize to the alternate allele (5′-FAM-AAAGCGCTTCTTCGTATT-3′-MGB) (Fig. 1 shows probe locations). Real-time PCRs were conducted in 10.0-μl reaction mixtures that contained 600 nM of both forward (5′-CCAATCAATGTCATACTATTAATTT GACAC-3′) and reverse (5′-ATGCAAAAGCATTATACTT GGACAAT-3′) primers (Fig. 1 shows primer locations), 250 nM of each probe, 1× Invitrogen Platinum qPCR SuperMix-UDG, and 1.0 μl of template. Thermal cycling was performed on an ABI 7900 HT sequence detection system (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

To further evaluate the nonsense mutation in *plcR* as a species-specific marker for *B. anthracis*, we used the assay described above to genotype a collection of *B. anthracis* strains representing 89 unique genetic lineages (6). In addition, we genotyped 29 strains that were identified by amplified fragment length polymorphism analysis as genetic near-neighbors of *B. anthracis* (4) (Table 1 shows strain list). All of the *B. anthracis* isolates supported amplification and were shown to have the *plcR* nonsense mutation genotype (T allele). Not surprisingly, genetic near-neighbors that had mutations in the priming site either failed to exhibit amplification or amplified with lower efficiency relative to the four strains that had complete sequence identity to *B. anthracis* except for the nonsense mutation (Table 1; Fig. 1). Of the 29 near-neighbors, 16 failed to exhibit amplification and the remaining 13 exhibited the G allele genotype (Table 1). The presence of the G allele in 5 of the 16 isolates that failed to amplify in the assay was confirmed via sequencing with flanking primers (Fig. 1).

To test the limit of detection of the assay, we utilized a dilution series generated from DNAs from three diverse *B. anthracis* isolates (Ames [A0462], Kruger B1 [A0442], and Vollum [A0488]). DNA was quantified using a Pico Green assay, and template levels ranging from 100 pg to 10.0 fg were used

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FIG. 1. Sequence alignment of *plcR* gene fragments from *B. anthracis* and genetic near-neighbors. The numbered lines indicate the sequences of (line 1) the primers and probes used in the assay; (line 2) *B. anthracis* strains Ames, Vollum, A2012, A1055, AUS94, CNEVA9066, Kruger, Sterne, and WNA6153; (line 3) *B. cereus* strains 3A (GenBank accession no. AY785766) and S2-8 and *B. thuringensis* strain HD1011; (line 4) *B. thuringensis* strain 97-27 (AY785771); (line 5) near-neighbor strain TET 2b-3; (line 6) *B. cereus* strain AH-527 (AY785767); (line 7) *B. cereus* strain D17 (AY785768) and *B. thuringensis* strains HD682, HD571, and HD44; (line 8) *B. cereus* strains F3502/72 (AY785769) and R6; (line 9) *B. cereus* strain F2-1 (AY785770); and (line 10) *B. cereus* strains R4 and ATCC 33018 and *B. thuringensis* strain HD1012 (AY785772). Light shading indicates areas of polymorphism that are detected in the assay. Darker shading indicates nucleotide differences between near-neighbors and *B. anthracis*. * indicates the nonsense mutation.

in the *plcR* TaqMan assay. The assay reliably detected and genotyped *Bacillus anthracis* DNA template at levels as low as 100 fg, with 10-fg samples exhibiting sporadic amplification (Fig. 2).

TABLE 1. List of *Bacillus* sp. strains examined using the assay developed in this study

Species[a,b]	Strain[b]	*plcR* gene fragment sequence[d]	Avg threshold cycle[e]	TaqMan result (allele)
BA	89 diverse strains[c]	2	26.0[f]	+ (T)
BC	ATCC 4342	NA	No Amp	−
BC	ATCC 14579	NA	No Amp	−
BC	D17	7	No Amp	− (G)[g]
BC	F3-27	NA	No Amp	−
BC	F3502/72	8	27.1	+ (G)
BC	R6	8	27.6	+ (G)
BC	ATCC 33018	10	38.5	+ (G)
BC	D5	NA	28.4	+ (G)
BC	3A	3	24.8	+ (G)
BC	S2–8	3	27.0	+ (G)
BC	F3350/87	NA	No Amp	−
BC	S2–4	NA	34.9	+ (G)
BC	R4	10	34.7	+ (G)
BC	F2-1	9	No Amp	− (G)[g]
BC	AH 527	6	30.0	+ (G)
BT	HD 1015	NA	No Amp	−
BT	HD 681	NA	No Amp	−
BT	HD 288	NA	No Amp	−
BT	HD 526	NA	No Amp	−
BT	97–27	4	33.0	+ (G)
BT	HD 1011	3	26.7	+ (G)
BT	HD 571	7	No Amp	− (G)[g]
BT	HD 682	7	No Amp	− (G)[g]
BT	HD 974	NA	No Amp	−
BT	HD 44	7	No Amp	− (G)[g]
BT	HD 30	NA	No Amp	−
BT	HD 1012	10	33.0	+ (G)
BT	HD 50	NA	No Amp	−
UNK	TET-2B	5	33.3	+ (G)

[a] BA, *B. anthracis;* BC, *B. cereus;* BT, *B. thuringiensis;* UNK, unknown *Bacillus* spp.
[b] Species and strain designations according to reference 4.
[c] The 89 diverse *B. anthracis* strains are described in reference 6.
[d] As represented in Fig 1. NA, strain not sequenced.
[e] Input, 10 pg, average of triplicate cycle threshold values. No Amp, no amplification.
[f] Average of triplicate cycle threshold values from 10-pg input of *B. anthracis* Ames strain.
[g] *plcR* genotypes (Fig. 1) were determined via sequencing using flanking primers.

Our data provide further evidence that the nonsense mutation in the *plcR* gene of *B. anthracis* is an evolutionarily stable, species-specific marker. Although additional genetic changes, such as deletions, could produce a nonfunctional PlcR in *B. anthracis* and potentially cause false-negative results in our assay, this was not observed. The presence of this mutation in the 89 genetically diverse *B. anthracis* lineages examined here, as well as the known genetic homogeneity of the species (5), limits the likelihood of alternate genetic mechanisms for *plcR* inactivation in *B. anthracis*. The recent findings of Slamti et al. (9), which demonstrated that this specific *plcR* nonsense

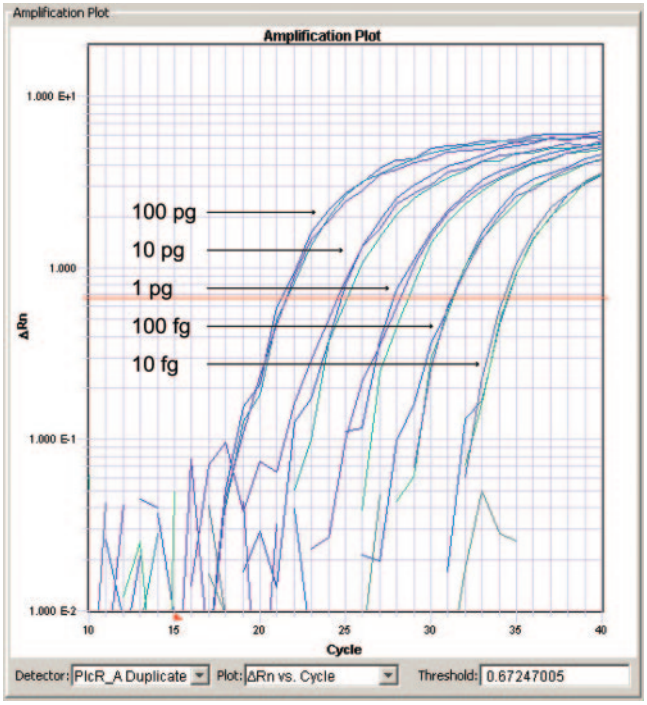

FIG. 2. Results of triplicate analysis of 10-fold serial dilutions of DNA from *B. anthracis* strain A0442. The average cycle threshold values across the three replicates were as follows: 100 pg, 21.4; 10 pg, 24.7; 1 pg, 28.2; 100 fg, 31.2; 10 fg, 34.3. The average cycle threshold values for strains A0488 and A0462 were similar (data not shown), although amplification at the 10-fg level was not consistent.

mutation was not responsible for the nonhemolytic properties of *B. cereus* and *B. thuringiensis* strains, further support the concept that this nonsense mutation is a defining or canonical single nucleotide polymorphism (7) for *B. anthracis*.

The real-time assay presented here represents a potentially valuable diagnostic tool in the event of a future bioterrorist attack. From a biodefense perspective, diagnostic assays allowing rapid and specific identification of *B. anthracis* are critical to initiate appropriate first-response actions, such as remediation measures and prophylactic therapies. As our assay targets a well-characterized, biologically relevant single nucleotide polymorphism, it limits the likelihood of false-negative or -positive results, which can lead to misallocation of resources during an attack scenario. Furthermore, this assay is amenable to high-throughput real-time PCR platforms that are currently used in homeland defense initiatives, such as BioWatch.

In summary, our results indicate that the *plcR* nonsense mutation is ubiquitous in globally and genetically diverse *B. anthracis* isolates and, thereby, represents an excellent target for diagnostic assays. Future studies will involve genotyping more extensive collections of *B. anthracis* and genetic near-neighbors, as well as the optimization and validation of this assay for the specific, low-level detection of *B. anthracis* in complex environmental samples.

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