

Playing Tag with Spores II: Incorporation of an Amino Acid Barcode into the BclA Exosporium Protein of *Bacillus thuringiensis*

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Abstract

Tagging and tracking biological agents and surrogates in the environment requires markers or signatures that are specific to the organism of interest. However, some organisms (e.g. *Bacillus thuringiensis* serovar *kurstaki* (Btk), a potential surrogate for the biological warfare agent *B. anthracis*) are widely distributed in the environment, which can complicate detection of the deliberately released material. Work in other laboratories has utilized fluorescent proteins to track the fate of genetically modified organisms following their release into the environment, but highly expressed artificial markers introduce potential competitive disadvantages to the subject strains.

We report the modification of BclA, an abundant exosporium protein of Btk (BclA^{Btk}) to contain an easily detectable epitope tag. *In silico* models of BclA^{Btk} were constructed based on homology analysis and structural modeling to identify three potential surface-exposed loops that could accommodate a human influenza hemagglutinin (HA) epitope tag. BclA variants containing epitope tags inserted into each of the three loops were expressed in Btk under the control of the native BclA promoter. Strains expressing each variant were allowed to sporulate, and protein extracts from vegetative cells, spores, and from surface protein extracts of spores were probed with anti-HA and anti-BclA antibodies. Tagged BclA variants were observed in spore surface extracts and lysed spores but not in extracts prepared from vegetative cells.

The tagged variants co-migrated with authentic BclA^{Btk}, suggesting that the tagged variants partition to the exosporium fraction in a manner comparable to the native protein. Btk strains expressing the tagged BclA could be visualized by immunofluorescence microscopy using an anti-HA antibody. Btk strains expressing surface-exposed epitope tags, or protein "barcodes," have potential as novel synthetic detection signatures for biothreat agent surrogates, and can serve as unique biological tracers for environmental release studies and/or as training aids for first responders to bioterrorism scenarios.

Background

The exosporium of *B. cereus* group strains (including *B. thuringiensis* and *B. anthracis*) forms a protective barrier around the spore and renders the spore considerably more hydrophobic than *B. subtilis* group strains including *B. atrophaeus* var *globigii* (BG), the historical surrogate for *B. anthracis* [1]. Therefore, *B. thuringiensis* strains including Btk have been investigated as improved surrogates for *B. anthracis* [2]. The exosporium is a multicomponent structure, a dominant feature of which is the collagen-like BclA glycoprotein, which is an immunodominant antigen of *B. anthracis* [3] that is required for efficient entry into mammalian cells [4]. The crystal structure of BclA^{Ba} is known [5], and shows a homotrimeric structure and several potential surface-exposed loops. BclA proteins vary dramatically across *B. cereus* group species, mostly in the length of the highly repetitive collagen-like stalk domain, while the head domain is relatively conserved (Figure 1A, B).

Previously, we had engineered Btk strains containing specific genetic barcodes integrated into a neutral genomic region [6] to facilitate detection in an outdoor setting [7]. We sought to augment our barcoding efforts by developing an immunologic marker for detection. In this study, we introduce a small standard HA epitope tag into the BclA^{Btk} protein and demonstrate its exposure on the spore surface of a Btk strain. The tag is accessible to gentle surface extraction methods and is recognized specifically by anti-HA antibodies, which do not recognize BclA^{Btk} or BclA^{Ba}. Our vision is to create a suite of epitope tagged strains with hand-held immunoassay test tickets specific for each strain. These strains and their cognate assays should be invaluable training aids for potential first responders to bioterrorism and biological warfare attacks and will minimize false-positive results due to the presence of environmental reservoirs of Btk.

Identification of Epitope Insertion Points

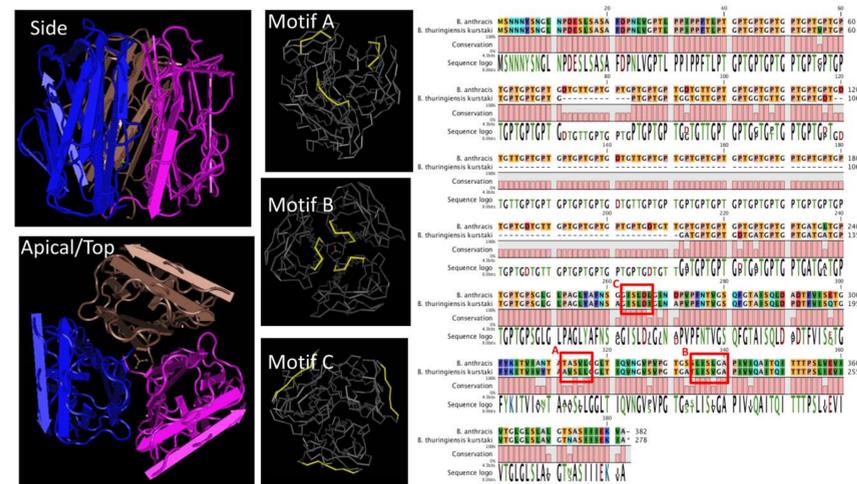


Figure 1: Design and construction of an epitope-tagged BclA protein for spore surface barcoding. A) Left panel: structural analysis of *B. anthracis* BclA protein adapted from (1) showing three potential surface-exposed segments. B) Right panel: alignment of *B. anthracis* and *B. thuringiensis* BclA showing identified segments, designated motifs A-C.

Btk can Express Tagged BclA

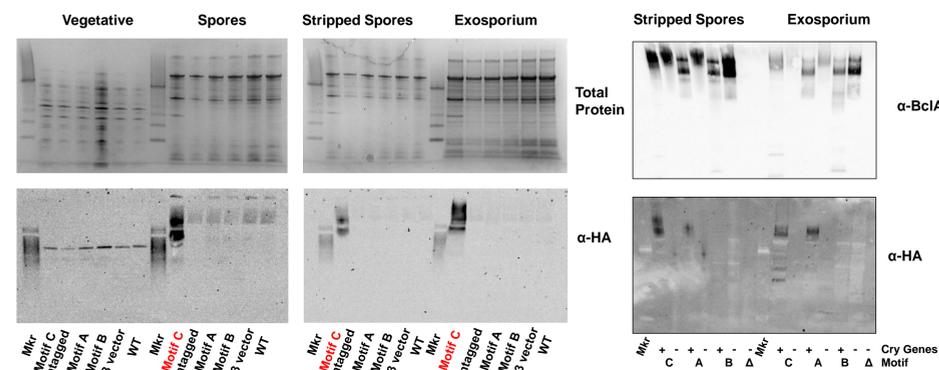


Figure 2: Expression of epitope-tagged BclA in Btk. BclA genes were cloned with epitope tag insertions into pMK3 [8] and transformed into Btk by electroporation. A) Left panel: Total protein and Western Blot analysis of *B. thuringiensis* expressing variants of BclA tagged with the HA epitope at indicated motifs. Only tags inserted at Motif C yield a specific signal by anti-HA Western blot. B) Right panel: Expression of tagged BclA constructs in cry- strains of Btk. Presence of crystal toxin encoding genes and/or plasmids appears to affect BclA expression in Btk. Δ = *B. anthracis* Sterne ΔbclA

BclA_{HA} is Surface-Localized

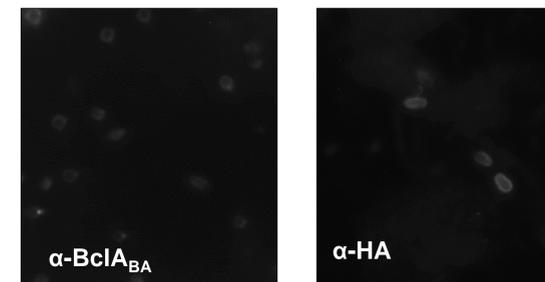


Figure 3: Immunofluorescence analysis of epitope-tagged BclA constructs. Btk strain expressing BclA_{HA} (Motif C) was fixed to a glass slide and stained with anti-BclA or anti-HA antibody. FITC-conjugated secondary antibodies were utilized to detect the primary antibody by fluorescence microscopy.

Allelic Replacement of *bclA* with *bclA*^{HA}

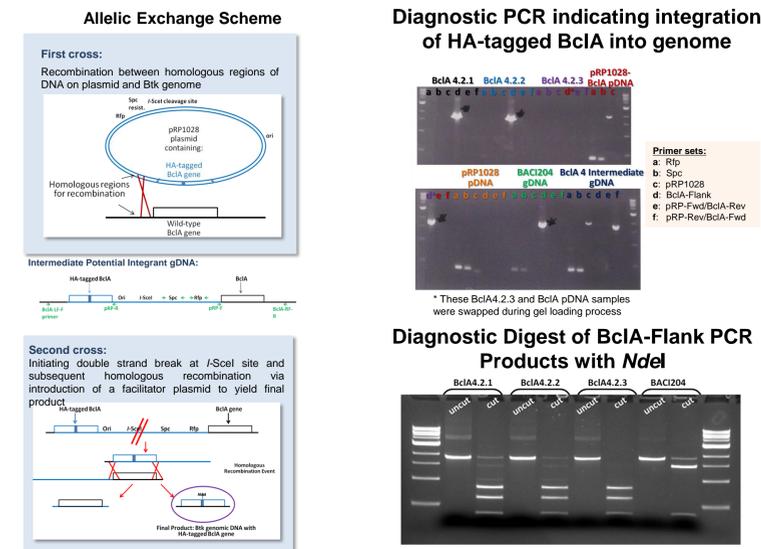


Figure 4: A HA-tagged allele of *bclA* in the temperature-sensitive plasmid pRP1028 was introduced into strain BAC1204 by conjugation, and integrants were selected at 37°C (First Cross). Plasmid pSS4332 expressing I-SceI was then introduced to initiate the second round of homologous recombination, leaving the tagged variant (Second Cross).

Conclusions

- Identified surface-exposed loops of BclA^{Btk} by homology modeling of BclA^{Btk} to BclA^{Ba} crystal structure
- Expression of HA-tagged BclA^{Btk} in Btk yielded detectable HA-reactive bands
- Migration of bands was consistent with authentic BclA^{Ba} and BclA^{Btk} in Western blots
- HA-reactive material was extractable from the spore surface by denaturing wash
- HA-reactive material colocalizes with BclA on the spore surface by immunofluorescence microscopy

Future Work

- ELISA of whole spores and spore extracts to confirm tag accessibility on whole spores
- Development of specific hand-held lateral-flow immunoassay test tickets to detect HA-tagged spores
- Allelic replacement of native *bclA* gene with tagged gene
- Substitution of HA-tag with alternative epitope tags

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