Protein Expression and Purification 80 (2011) 80–90

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

# A *Bacillus anthracis* strain deleted for six proteases serves as an effective host for production of recombinant proteins

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ARTICLE INFO

*Article history:*
Received 28 April 2011
Available online 7 August 2011

*Keywords:*
*Bacillus anthracis*
Protease deficient
Protein overproduction
Edema factor
Gene knockout

ABSTRACT

*Bacillus anthracis* produces a number of extracellular proteases that impact the integrity and yield of other proteins in the *B. anthracis* secretome. In this study we show that anthrolysin O (ALO) and the three anthrax toxin proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), produced from the *B. anthracis* Ames 35 strain (pXO1$^+$, pXO2$^-$), are completely degraded at the onset of stationary phase due to the action of proteases. An improved Cre-*loxP* gene knockout system was used to sequentially delete the genes encoding six proteases (InhA1, InhA2, camelysin, TasA, NprB, and MmpZ). The role of each protease in degradation of the *B. anthracis* toxin components and ALO was demonstrated. Levels of the anthrax toxin components and ALO in the supernatant of the sporulation defective, pXO1$^+$ A35HMS mutant strain deleted for the six proteases were significantly increased and remained stable over 24 h. A pXO1-free variant of this six-protease mutant strain, designated BH460, provides an improved host strain for the preparation of recombinant proteins. As an example, BH460 was used to produce recombinant EF, which previously has been difficult to obtain from *B. anthracis*. The EF protein produced from BH460 had the highest *in vivo* potency of any EF previously purified from *B. anthracis* or *Escherichia coli* hosts. BH460 is recommended as an effective host strain for recombinant protein production, typically yielding greater than 10 mg pure protein per liter of culture.

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## Introduction

The Gram-positive bacterial pathogen *Bacillus anthracis* secretes high levels of the three proteins that are collectively termed anthrax toxin: protective antigen (PA)[1], edema factor (EF), and lethal factor (LF), when grown under conditions thought to mimic those in an infected animal host. PA is a receptor-binding component which acts to deliver LF and EF to the cytosol of eukaryotic cells; EF is a calmodulin-dependent adenylate cyclase and LF is a zinc metalloprotease that cleaves most members of the mitogen-activated protein kinase family for reviews see [1–4]. Because anthrax pathogenesis is highly dependent on the actions of the anthrax toxin proteins, vaccine and therapeutic development efforts have focused on countering toxin action, typically by generating antibodies to PA. The anthrax vaccine currently licensed in the USA, and developed almost 50 years ago [5], consists of a partially purified culture supernatant of a protease-deficient strain (V770-NP1-R). PA is the most abundant protein and the key immunogen in this vaccine. Efforts to produce a recombinant PA vaccine from *B. anthracis* by scale-up of an established process [6] appear to have been hampered by instability of the final product, possibly due to protease contamination.

While the toxin components can be purified as recombinant proteins from *B. anthracis* culture supernatants [6–9], the integrity and yields are limited by the *B. anthracis* proteolytic enzymes that are co-secreted. Two extracellular proteases are reported to be abundant in the *B. anthracis* secretome: NprB (GBAA_0599) – neutral protease B, a thermolysin-like enzyme highly homologous to bacillolysins from other *Bacillus* species, and InhA1 (GBAA_1295) – immune inhibitor A1, a homolog of the immune inhibitors A from other members of the *Bacillus cereus* group [10–12]. These two proteases contain zinc-binding motifs typical for the zincin tribe of metallopeptidases, His-Glu-Xxx-Xxx-His, and belong, respectively, to the M4 and M6 families of metalloproteases according to the MEROPS database (http://merops.sanger.ac.uk). A third metalloproptease, camelysin (GBAA_1290), belonging to the M73 family is found in the secretome of several *B. anthracis* strains. This

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[1] *Abbreviations used:* PA, protective antigen; EF, edema factor; LF, lethal factor; ALO, anthrolysin O; Ap, ampicillin; Em, erythromycin; Sp, spectinomycin; Km, kanamycin.

1046-5928/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.pep.2011.05.016

protease is similar to the camelysin of *B. cereus*, a novel surface metalloprotease [13].

*B. anthracis* also contains a gene encoding the InhA2 metalloprotease (GBAA_0672, M6 family), although it is not known whether this protease is expressed and secreted. This gene is an ortholog of the InhA1 described above (68% amino acid identity). Similarly, the genome of *B. anthracis* also contains genes encoding TasA (GBAA_1288, M73 superfamily), which is an ortholog of camelysin (60% amino acid identity), and MmpZ (GBAA_3159, ZnMc superfamily), which is a putative extracellular zinc-dependent matrix metalloprotease, a member of the metzincin clan of metallopeptidases. This clan is characterized by an extended zinc-binding motif, His-Glue-Xxx-Xxx-His-Xxx-Xxx-Gly/Asn-Xxx-Xxx-His/Asp [14].

We hypothesized that secretion of these and other extracellular proteases might significantly decrease the levels of intact anthrax toxin components in the *B. anthracis* secretome, so that disruption of these protease-encoding genes might result in higher protein yields. We previously inactivated NprB and InhA1 individually and found that NprB deficiency reduced proteolysis of casein [15], while coagulation of human blood by *B. anthracis* required InhA1 for proteolytic activation of prothrombin and factor X [16].

In this report, we describe the adaptation of an improved Cre-*loxP* system for sequentially deleting additional protease-encoding genes of *B. anthracis*. Also, we describe a role of each protease in degradation of *B. anthracis* toxin components and another potential virulence factor, anthrolysin O (ALO) [17]. Our work parallels earlier work to knock out up to eight proteases from *Bacillus subtilis* [18,19] so as to produce an improved expression host. Finally, we suggest that the final *B. anthracis* strain generated, designated BH460, lacking six proteases and being sporulation deficient and free of the virulence plasmids, provides an improved host for production of recombinant proteins. As an example, we show that EF produced from BH460 is highly active, whereas previous *B. anthracis* host strains produced truncated proteins having low potency.

## Materials and methods

### Materials

The proteases, oligonucleotide primers, plasmids, and bacterial strains used and analyzed in this study are listed in Tables 1–4.

### Bacterial growth conditions and phenotypic characterization

*Escherichia coli* strains were grown in Luria–Bertani (LB) broth and used as hosts for cloning. LB agar was used for selection of transformants [20]. *B. anthracis* strains were also grown in LB or FA medium [21]. Antibiotics (Sigma–Aldrich, St. Louis, MO) were added to the medium when appropriate to give the following final concentrations: ampicillin (Ap), 100 μg/ml (only for *E. coli*); erythromycin (Em), 400 μg/ml for *E. coli* and 10 μg/ml for *B. anthracis*; spectinomycin (Sp), 150 μg/ml for both *E. coli* and *B. anthracis*; kanamycin (km), 20 μg/ml (only for *B. anthracis).* SOC medium (Quality Biologicals Inc., Gaithersburg, MD) was used for outgrowth of transformation mixtures prior to plating on selective medium. *B. anthracis* spores were prepared as previously described [22] after growth on NBY minimal agar (nutrient broth, 8 g/l; yeast extract, 3 g/l; $MnSO_4{\cdot}H_2O$, 25 mg/l; agar, 15 g/l) at 30 °C for 5 days. Spores and vegetative cells were visualized with a Nikon Eclipse E600W light microscope (Nikon Instrument Inc., New York).

### DNA isolation and manipulation

Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures [20]. *E. coli* SCS110 competent cells were purchased from Stratagene (La Jolla, CA) and *E. coli* TOP10 competent cells from Invitrogen (Carlsbad, CA). Recombinant plasmid construction was carried out in *E. coli* TOP10. Plasmid DNA from *B. anthracis* was isolated according to the protocol for the purification of plasmid DNA from *B. subtilis* (Qiagen, Valencia, CA). Chromosomal DNA from *B. anthracis* was isolated with the Wizard genomic purification kit (Promega, Madison, WI). *B. anthracis* was electroporated with unmethylated plasmid DNA isolated from *E. coli* SCS110 ($dam^-$ $dcm^-$). Electroporation-competent *B. anthracis* cells were prepared and transformed as previously described [23]. Restriction enzymes, T4 ligase, and Antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). *Taq* polymerase, Platinum PCR SuperMix High Fidelity kit and the TOPO TA cloning kit were from Invitrogen. The pGEM-T Easy Vector system was from Promega. Ready-To-Go PCR Beads were from GE Healthcare Biosciences Corp. (Piscataway, NJ). For routine PCR analysis, a single colony was suspended in 200 μl of TE buffer [20] (pH 8.0), heated to 95 °C for 45 s, and then cooled to room temperature. Cellular debris was removed by centrifugation at 15,000*g* for 10 min. Two microliters of the lysate contained sufficient template to support PCR. The GeneRuler DNA Ladder Mix from MBI Fermentas (Glen Burnie, MD) was used to assess DNA fragment length. All constructs were verified by DNA sequencing and/or restriction enzyme digestion.

### Construction of vectors for protease gene inactivation

*B. anthracis* Ames 35 ($pXO1^+$ $pXO2^-$) (A35) was used for genetic manipulations. The GenBank database (GenBank Accession No. for

**Table 1**
*B. anthracis* Ames ancestor strain genes inactivated or analyzed in this study.

| Protein | Gene | Function/name | Locus tag |
|---|---|---|---|
| NprB | *nprB* | Metallopeptidase | GBAA_0599 |
| InhA2 | *inhA2* | Metallopeptidase | GBAA_0672 |
| TasA | *tasA* | Metallopeptidase | GBAA_1288 |
| Camelysin | *calY* | Metallopeptidase | GBAA_1290 |
| SinI | *sinI* | Regulatory protein | GBAA_1292 |
| SinR | *sinR* | Regulatory protein | GBAA_1293 |
| InhA1 | *inhA1* | Metallopeptidase | GBAA_1295 |
| MmpZ | *mmpZ* | Metallopeptidase | GBAA_3159 |
| ALO | *alo* | Thiol-activated cytolysin (anthrolysin) | GBAA_3355 |
| Spo0A | *spo0A* | Sporulation regulator | GBAA_4394 |
| EF | *cya* | Edema factor | GBAA_pXO1_0142 |
| PA | *pag* | Protective antigen | GBAA_pXO1_0164 |
| LF | *lef* | Lethal factor | GBAA_pXO1_0172 |