Developing a Protein Purification Strategy Using Spore Surface Display of Protein Intein Scaffolding and Self-Cleaving Peptides

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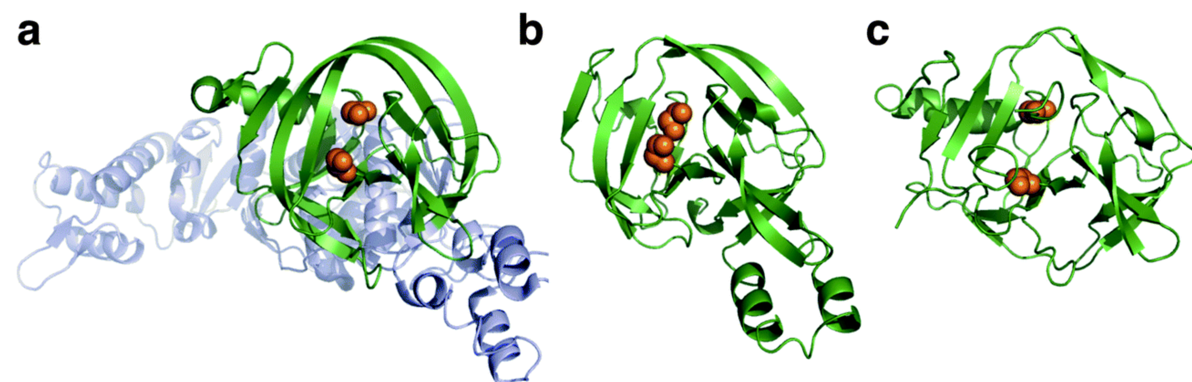
Abstract

This research sets out to develop a methodology of protein purification without using column chromatography. We are attempting to utilize the technology of spore surface display and intervening proteins (inteins) in a way that will lower the costs of production while eliminating the need for affinity tagged proteins. We were able to assemble some of the DNA required for *B. subtilis* transformation and sporulation but were unable to assemble more than two fragments.

Introduction



Comparison of various Hint domain structures. (a) Thermococcus kodakaraensis Pol-2 intein (PDB2CW7). (b) Mycobacterium xenopi GyrA intein (PDB 1AM2). (c) Methanococcus jannaschii KlbA intein (PDB 2JNQ). The Hint domain is shown as green ribbon with the Block A nucleophile and Block G asparagine positions highlighted as orange spheres. In (a), the homing endonuclease domain is shown in blue. [1]

The purification of proteins is central to studying protein structure, function and regulation and purification is often required for industrial use of proteins. Currently the method used to purify recombinant His-tagged proteins is immobilized metal affinity chromatography (IMAC), consisting of chelating resins charged with either nickel or cobalt ions that coordinate with the histidine side chains.

The goal of this research project is to develop an alternative method for purifying proteins.

- Proteins are structurally diverse performing multiple essential functions
- Advent of proteomics and high throughput structural biology created demand for simple methods of purifying (and expressing) recombinant proteins. [3]

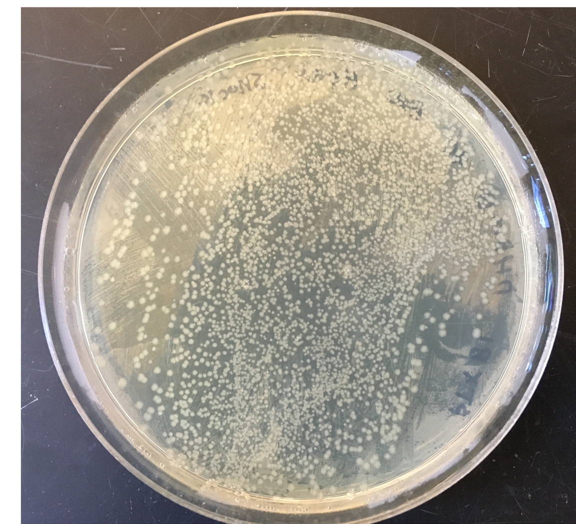
HisPur™ Ni-NTA Spin Column

Problems with Protein Purification

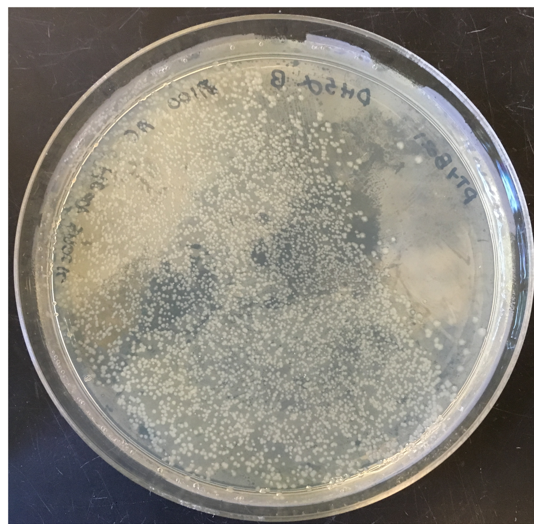
- Chromatography for protein purification is expensive.
- Protease is usually required to cleave targeted protein from affinity tags.

Potential Solutions

- Design new methodology for purifying proteins that is cost effective and simple while providing high purity levels.



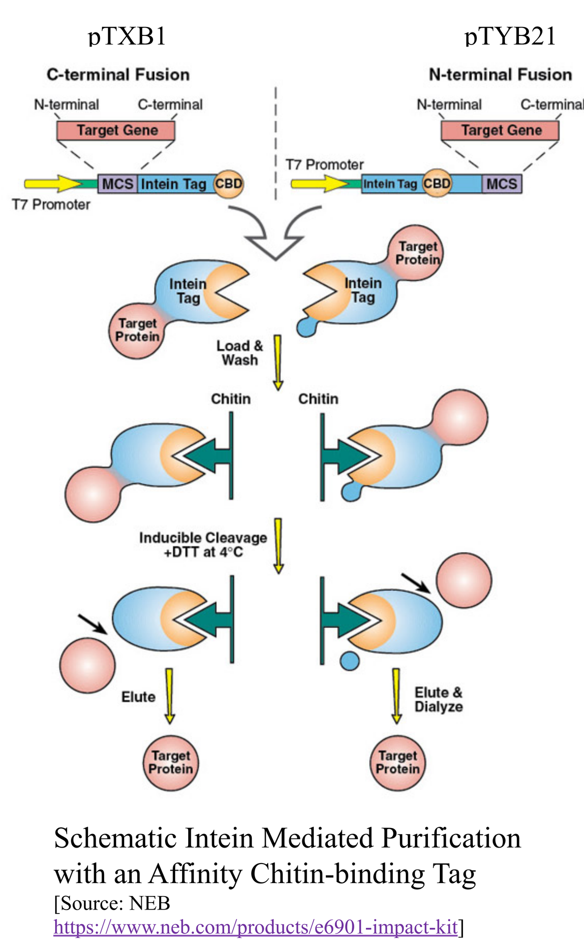
pTXB1 vector (intein) with Dh5αB on A100 plate.



pTYB21 vector (intein) with Dh5αB on A100 plate.

Protein Purification Using Inteins

- Inteins (intervening proteins) are auto-processing domains found in archaea, bacteria, and eubacteria. [7]
- Carry out a process known as protein splicing, which is a spontaneous multi-step biochemical reaction comprised o both the cleavage and formation of peptide bonds. [7]
- Structural recognition between N-intei and C-intein is understood to be highly specific [1, 6, 7, 8] with site specific protein cleavage
- Functional in exogenous context to chemically manipulate polypeptide backbones. [7]
- Engineered self cleaving intein success in bio-separation. [8]
- Through microfiltration, inteins can be used to cleave proteins from ELP tags. [2]



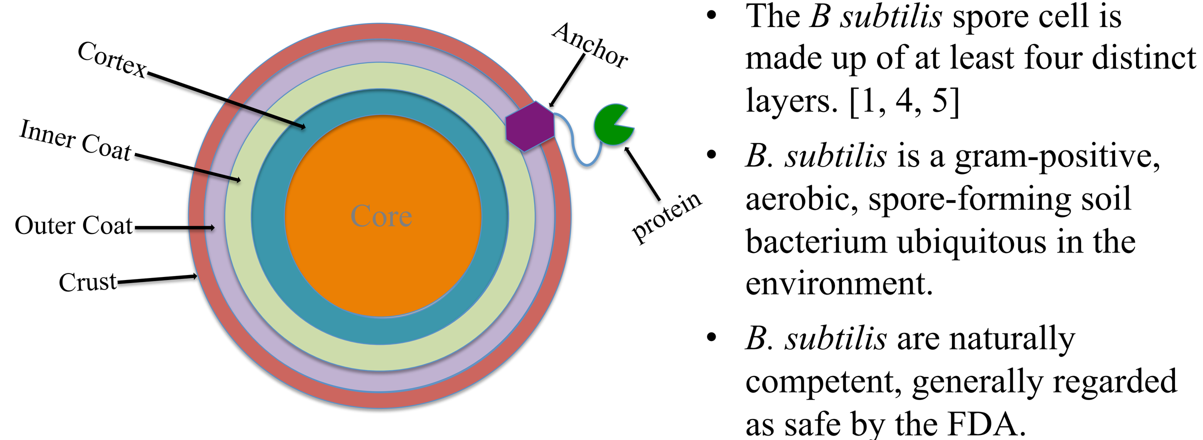
Schematic Intein Mediated Purification with an Affinity Chitin-binding Tag
[Source: NEB https://www.neb.com/products/e6901-impact-kit]

Why use commercial Inteins?*

- Single-column purification without proteases to remove affinity tags.
- Fusion is possible on C-terminus or N-terminus of target protein.

* According to New England BioLabs (NEB) product descriptions.

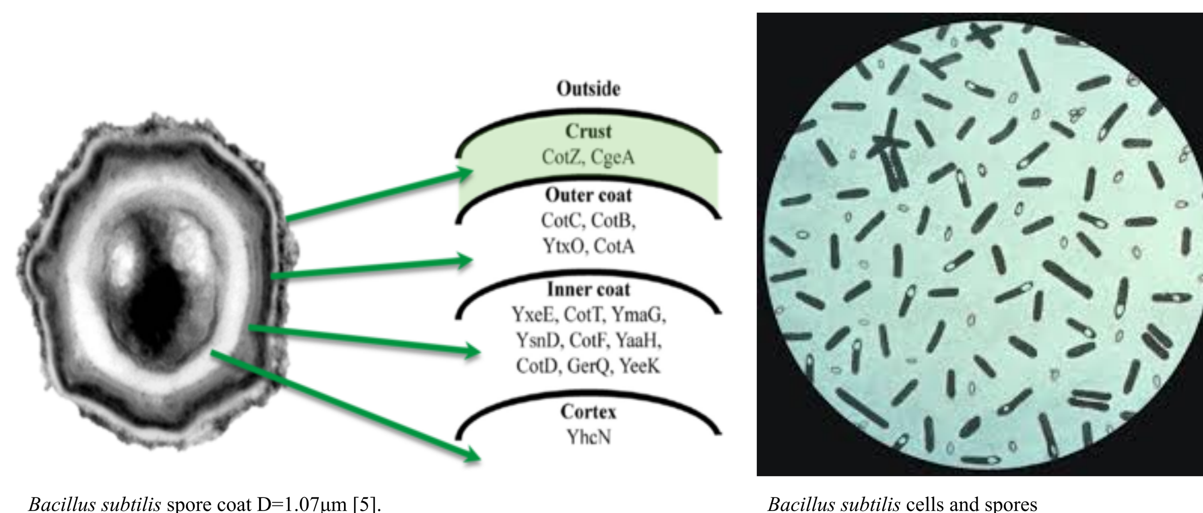
Spore Surface Display



- The *B subtilis* spore cell is made up of at least four distinct layers. [1, 4, 5]
- *B. subtilis* is a gram-positive, aerobic, spore-forming soil bacterium ubiquitous in the environment.
- *B. subtilis* are naturally competent, generally regarded as safe by the FDA.
- Spore surface display is achieved by stably anchoring foreign molecules to endogenous surface protein. [4]

Advantages of spore surface display over other methods

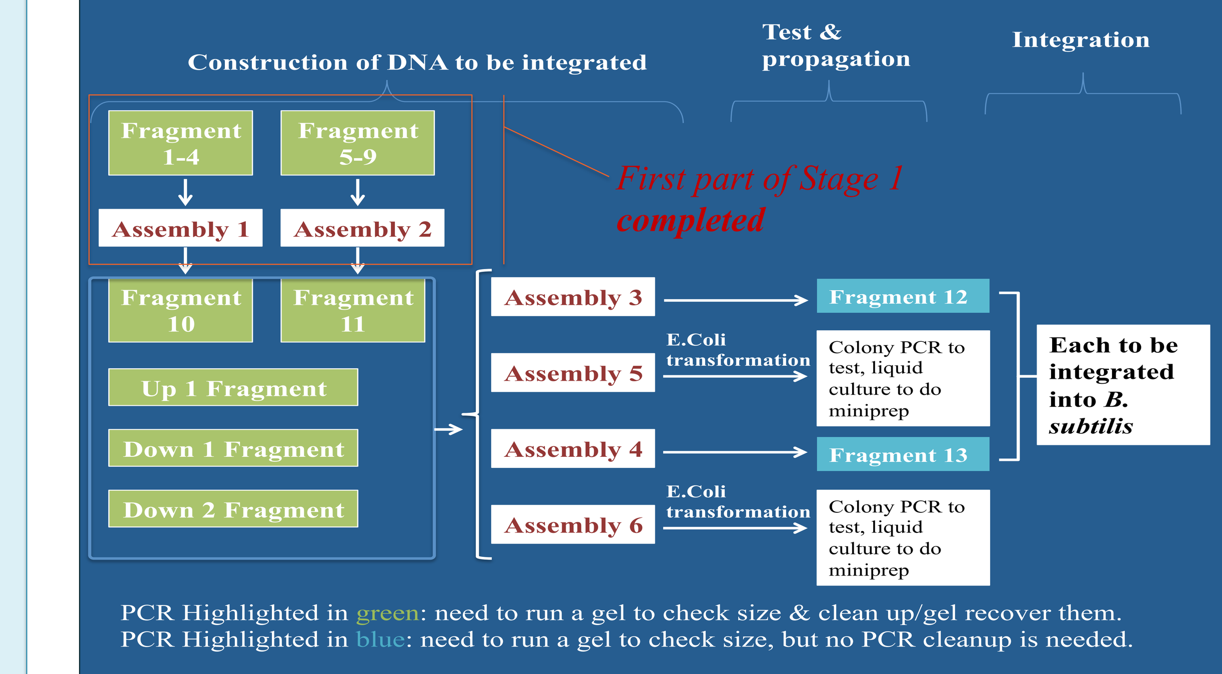
- High stability even after long storage. 4]
- Possibility of displaying large protein with multiple polypeptide chains. [1, 4]



Bacillus subtilis spore coat D=1.07μm [5].

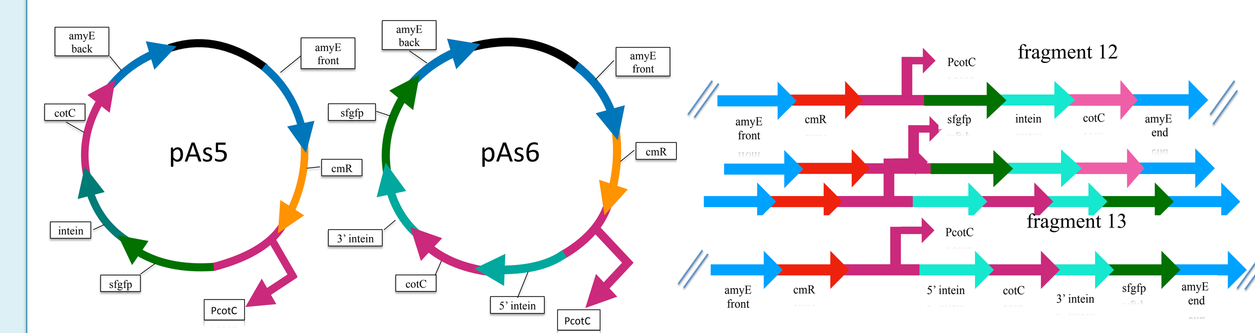
Bacillus subtilis cells and spores

Methods



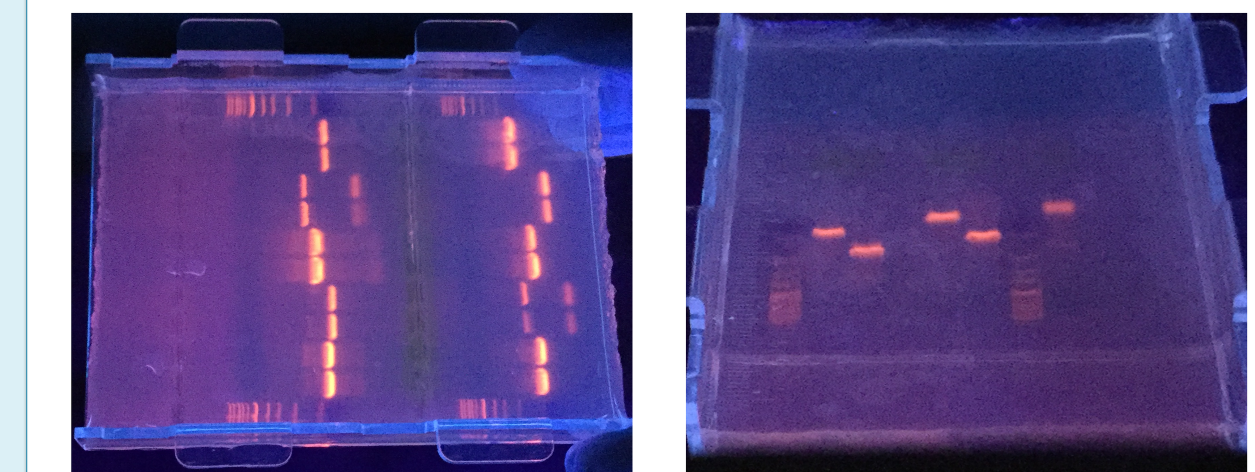
Fragments for Gibson Assembly

Assembly 1 = PcotC+ sfgfp+ intein+ cotC
Assembly 2 = PcotC + 5' intein+ cotC+ 3' intein sfgfp
Assembly 3 = up amyE + cmR + PcotC + sfgfp+ intein+ cotC + dwn amyE
Assembly 4 = up amyE+cmR+PcotC+ 5' intein+ cotC+ 3' intein+ sfgfp + dwn amyE
Assembly 5 = up amyE + cmR + PcotC+ sfgfp+ intein+ cotC + dwn amyE
Assembly 6 = up amyE + cmR+ PcotC+5' intein+ cotC+ 3' intein +sfgfp+dwn amyE

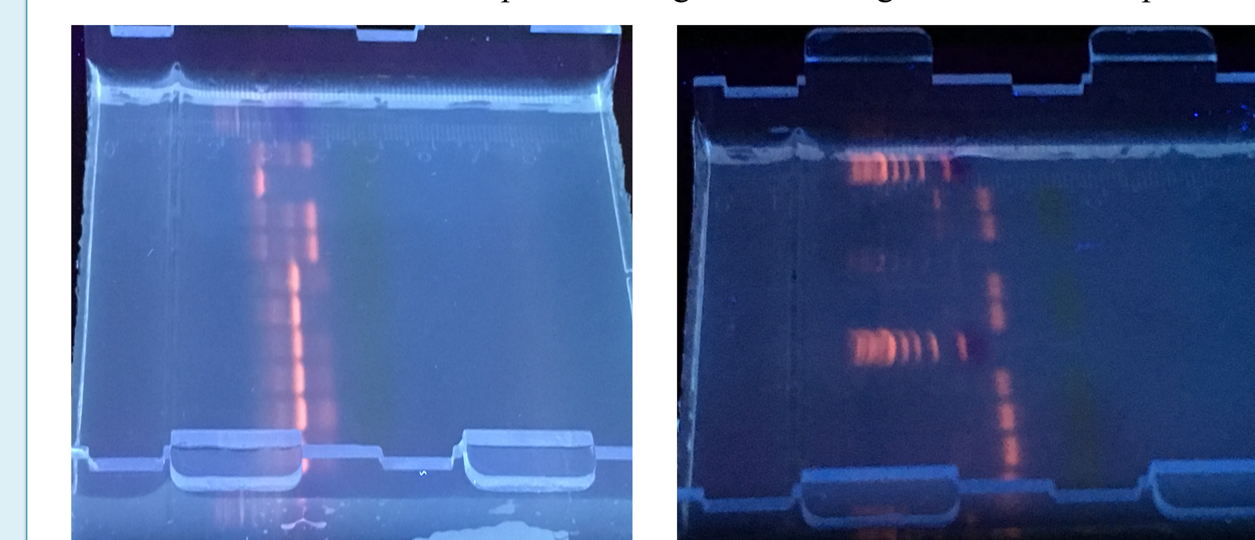


Results

DNA fragments were examined using gel electrophoresis to isolate DNA of specific sizes



Successful *Gel electrophoresis* fragments: in ranges of 240 to 825bp



Failed *Gel electrophoresis* Fragments: incorrect sizes of DNA or RNA.

Discussion

PCR

- Initial experiments to isolate specific DNA fragments by size needed to be completed using three separate protocols with three different polymerases dependent on the size of the fragments.
- PRC failed as evidenced by gel electrophoresis and needed to be completed multiple times to obtain the correct DNA.
- Certain PCR products required gel recovery instead of PRC clean up because there were multiple bands of DNA present.

Gibson Assembly

- Gibson Assembly was not successful at joining multiple fragments of DNA.
- Qbit assays were performed on all fragments to identify the concentration of DNA of each sample in ng/μL.
- Samples were altered to address the differences in DNA concentration for further Gibson Assemblies.
- 2-3 fragments were assembled instead of 4-5 to increase reaction efficiency.

Human Error

- Cross contamination and equipment availability hindered the progress of the experiments.

Future Work

Complete Stage I.

1. Using Gibson Assembly procedure − complete assembly 3-6
   - Products of Assembly 3 and 4 are linear DNA and do not need to be transformed.
   - Assembly 5 and 6 result in circular plasmids, which need to be transformed into *E. coli*.
   - Colony PCR is needed to test whether Assembly 5 and 6 work
2. PCR 12 and 13 with the LongAmp polymerase
   - Run gel to check the size and quality. If it works, it can be directly used for *B. subtilis* transformation

Stage II. Integration into *B. subtilis*
Stage III. Sporulation and test if intein is able to purify sfgfp.

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Acknowledgements

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