# A *CORYNEBACTERIUM GLUTAMICUM* EXPRESSION SYSTEM FOR GLYCOSIDE HYDROLASE ANALYSIS

by

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**A *Corynebacterium glutamicum* expression system for glycoside hydrolase analysis**

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## Abstract

The biological hydrolysis of glycosidic linkages in complex sugars is facilitated by glycoside hydrolases. These enzymes are ubiquitous across all domains of life, playing significant roles in important biological processes like the degradation of cellulosic biomass, viral pathogenesis, antibacterial defense, and normal cellular functions. The potential industrial applications of highly efficient glycoside hydrolases, as well as the fact that a number of lysosomal storage diseases have been attributed to deficiencies in these enzymes [43, 22], merits further study into their structure and activity. For this reason, a handful of novel glycoside hydrolases from *Cellulomonas fimi*, a Gram-positive *Actinobacteria* known for its ability to degrade cellulose [39], will be cloned, expressed and biochemically analyzed.

**Acknowledgements**

I would like to extend my deepest and sincerest gratitude to my supervisor and mentor Dr. Warren W. Wakarchuk. I am indebted to him for all of the opportunities he has afforded me in such a short time and for the chance to grow not only as a scientist, but also as a person. His unfaltering patience and passion for science continue to motivate me and those who are lucky enough to work with him.

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Finally, I would like to thank my friends. If not for them, I would have easily completed my thesis six months in advance.

**Table of Contents**

**List of Figures**

## List of Tables

# Chapter 1

# Introduction

## 1.1 Glycoside hydrolases

Glycoside hydrolases (GHs) are a broad group of enzymes that catalyze the hydrolysis of *O*-glycosidic bonds between two carbohydrate residues or between a carbohydrate and non-carbohydrate moiety [42] (Figure 1.1). These enzymes are ubiquitous in nature, playing significant roles in the degradation of cellulosic biomass for the production of biofuels and various cellular functions [30]. They are primarily utilized in nutrient acquisition among prokaryotes, being found as both intracellular and extracellular enzymes [7]. Of significant historical importance is the bacterial GH β-galactosidase which, in concert with its constitutive genes, has functioned as an invaluable tool for molecular biologists. Among higher organisms GHs can be found within the endoplasmic reticulum and Golgi apparatus where they are involved in the processing of *N*-linked glycoproteins [55], and in the lysosome as enzymes involved in the degradation of carbohydrate structures [18]. Deficiencies of specific lysosomal GHs have been attributed to the development of a range of lysosomal storage (e.g., Tay-Sachs, Hunter, Fabry, Gaucher, and Krabbs) diseases resulting in developmental issues or death [3]. GHs are even involved in processes like viral pathogenesis (eg., viral neuraminidase). Therefore, novel and engineered GHs both have significant industrial and therapeutic potential [65].

### *1.1.1 Originating from soil bacteria*

Cellulose is the most abundant organic polymer on Earth, being composed of β-1, 4-linked glucose monomers (in the form of the repeating disaccharide cellobiose) [32]. The degradation of cellulose by soil microorganisms is a vital step in carbon cycling, releasing $CO_2$ aerobically and various fermentation products anaerobically [65, 5]. Though fungal GHs are preferred for industrial applications, there is still a strong interest in the search for novel bacterial GHs. The diversity of cellulolytic enzymes expressed by

various species of soil bacteria makes them an enticing reservoir of potentially novel GHs [64]. Enhanced thermostability is one of the most desirable properties for a novel GH to possess, since large scale saccharification reactions are commonly carried out at elevated temperatures [56]. Additionally, the synergistic actions of various GHs, which can originate from a wide range of microorganisms, are required for the production of fermentable sugars from cellulosic substrates [5, 56]. For these reasons, novel GHs originating from soil bacteria could be utilized synergistically alongside existing enzyme cocktails to facilitate saccharification of cellulosic substrates.

*1.1.2 CAZy database annotation*

The CAZy (**C**arbohydrate **A**ctive en**zy**mes) database classifies known CAZymes into various families defined by amino acid sequence similarity and fold, with at least one biochemically characterized founding member [8, 26]. Currently, there are only five enzyme classes annotated by CAZy: glycoside hydrolases (GHs) (EC 3.2.1.-), glycosyltransferases (GTs) (2.4.x.y), polysaccharide lyases (PLs) (EC 4.2.2.-), carbohydrate esterases (CEs) (EC 3.1.1.-), and auxiliary activities (AAs) (EC 1.1.3.- and 1.11.1.-). The CAZy database also annotates associated carbohydrate binding modules (CBMs). Currently, there are 135 GH families annotated by CAZy. GH families are systematically updated resulting in the modification, addition, or deletion of entire families. By nature of their classification, members within families will have similar mechanisms, folds, and catalytic residues [37]. GH families can be grouped together in clans where members contain significant tertiary structure similarity, which is more conserved than sequence similarity [23]. The CAZy database allows users to make educated predictions about the fold, mechanism, and substrate of novel CAZymes with only the amino acid sequence.

*1.1.2.1 Mechanism*

Most GHs hydrolyze glycosidic bonds through the use of two catalytic amino acid residues: a general acid (proton donor) and a nucleophile/base [17]. Depending on the positioning of these catalytic residues, hydrolysis occurs via overall retention or overall inversion in configuration around the anomeric carbon (Figure 1.2). The CAZy database indicates both the stereochemical outcome of the reaction as well as the amino acid residues acting as a proton donor and nucleophile (when known) [58]. Though not relevant to this work, a completely unrelated mechanism has been recently demonstrated for two families of glycoside hydrolases utilizing NAD+ as a cofactor [48, 36].

*1.1.2.2 Classes of glycoside hydrolases*

GHs can be classified according to a number of unique schemes in addition to their sequence based annotation in the CAZy database. The utility of these classification schemes depends on the context in which the classification is both made and used. Among these schemes are the Enzyme Commission (EC) number, a mechanistic classification, and the exo/endo classification (Figure 1.3). Exo- refers to the ability of a GH to cleave a substrate at the non-reducing end (most frequently, but sometimes the reducing end as well), while endo- refers to the ability of a GH to cleave a substrate within the chain generating new chain termini [17]. The new chain termini are then accessible for further cleavage by exo-acting GHs.

*1.1.2.2.1 Glycoside hydrolase* family 5

GH family 5 (GH5) is one of the largest CAZy GH families. Previously known as "Cellulase Family A" [25, 20], this group is comprised of enzymes with a generally robust range of known activities including endo-β-1,4-glucanase/cellulase (EC 3.2.1.4), endo-β-1,4-xylanase (EC 3.2.1.8), β-glucosidase (EC 3.2.1.21), β-mannosidase (EC 3.2.1.25), β-glucosylceramidase (EC 3.2.1.45), and glucan β-1,3-glucosidase (EC

3.2.1.58). There are no human enzymes in GH5, though they are widely distributed across archaea, bacteria, and eukaryotes, most notably fungi and plants [37]. GH5 enzymes are retaining enzymes and follow a classical Koshland double-displacement mechanism, utilizing glutamate residues found at the C-terminal ends of β-strands 4 and 7 acting as proton donor and nucleophile, respectively (Table 1.1) [24, 28].

1.1.2.2.2 Glycoside hydrolase family 6

GH family 6 (GH6) was one of the first glycoside hydrolase families classified by hydrophobic cluster analysis, and was previously known as "Cellulase Family B" [25, 20]. The only reported activities for GH6 are endoglucanase (EC 3.2.1.4), and cellobiohydrolase (EC 3.2.1.91) [37]. GH6 enzymes are inverting enzymes with strong evidence supporting an aspartic acid (Table 1.1) playing the role of the proton donor within the enclosed tunnel of the active site [16]. The identification of the nucleophile is far less simple, though there is mounting evidence that it is also an aspartic acid residue located within the active site tunnel [51].

*1.1.3 A brief history of glycoside hydrolases from Cellulomonas*

*Cellulomonas* spp. are Gram-positive coryneform rod-shaped soil bacteria within the *Actinobacteria* phylum that are best known for their ability to degrade complex polysaccharides [1, 10]. *Cellulomonas fimi* ATCC 484 was extensively investigated in the 1980's resulting in the identification of a handful of novel GHs using activity based screening for recombinant enzymes [19]. However, the recent sequencing of the *C. fimi* genome as well as the analysis of the total CAZome (the collection of CAZymes encoded by the genome of an organism) [37] revealed a large number of putative GH genes, many of which have yet to be biochemically characterized [19]. This provided an impetus to revisit this organism in order to identify and characterize these previously overlooked GHs.

*1.1.3.1 Endoglucanases Celf_3184 and Celf_1925*

The first two secreted GH6s isolated and characterized from *C. fimi* ATCC 484, Celf_3184 (CfCel6A) and Celf_1925 (CfCel6B), exemplify how numerous GHs were overlooked due to technical limitations. The identification of these enzymes resulted from the activity based cloning and screening of recombinant libraries as well as purifying enzymes from the host organism [70, 44]. With improved technology giving better sensitivity, a direct proteomic analysis of the secretome of *C. fimi* and *C. flavigena* [66] revealed a number of GHs not detected before in addition to the previously characterized enzymes (Figure 1.4). Many of the newly identified GHs were hypothesized to be novel cellulases or xylanases and a handful of them were identified as being unique to *C. fimi*. Of interest to this study (Figure 1.5) are the GH5, Celf_2403 (which was recently removed from GH5 and currently remains unclassified [37], but for the purposes of this work will be treated as a GH5) and the GH6s, Celf_1230 (CfCel6C), and Celf_0233 (CfCel6D).

*1.1.4 Potential applications of novel glycoside hydrolase family 6s*

The identification and characterization of novel GH6s could serve to improve the efficiency of the saccharification of cellulosic biomass for the production of biofuels. Novel, thermostable GH6s could work synergistically alongside current enzyme cocktails to release more fermentable sugars from various cellulosic substrates. Moreover, understanding the structures and/or amino acid residues responsible for conferring unique traits to particular GHs of interest would greatly facilitate the engineering of these enzymes to improve their efficiency.

**1.2 Problematic expression in *Escherichia coli***

*Escherichia coli* expression systems represent the gold standard in heterologous protein expression, as they offer a cost effective approach to producing heterologous proteins on a large scale. Unfortunately,

the expression of genes originating from *C. fimi* has lead to many complications like irregular folding and aggregation into inclusion bodies (Figure 1.6), resulting in non-functional proteins that are difficult to harvest [33]. The possibility that the difference in GC% content between *E. coli* and *C. fimi* (50.8% and 74.7%, respectively) [46, 11] genes being a contributing factor to expression issues cannot be overlooked, however it is much more probable that there are minor to significant differences in the cellular machinery utilized by these two organisms during the translation, folding, or secretion of proteins. Also, true secretion from the cell is difficult in *E. coli*. The *E. coli* expression issues of secreted, native *C. fimi* GHs could also be linked to the fact that the enzymes don't fold correctly when not destined for secretion. For these reasons, this work seeks to appropriate an alternative expression host in tandem with secretion linked expression.

**1.3 Twin-arginine-translocon dependent secretion**

Protein export from the cytoplasm of a cell to other organelles or out of the cell is a common phenomenon that occurs in both prokaryotes and eukaryotes [54, 49, 47, 67]. In the general secretory (Sec) pathway, pre-protein is transported from the cytoplasm to the extracellular space in an unfolded form and the signal sequence is cut off by a type I signal peptidase (SPase) [14, 15, 60, 45]. The twin-arginine-translocon (TAT) pathway utilizes signal sequences containing two conserved arginine residues "-RR-" in the region near their N-terminus in addition to a type I SPase. TAT translocation is Sec-independent and employs a cytoplasmic chaperone to secrete proteins in a fully folded form (Figure 1.7) [4, 13, 41, 71, 67].

*1.3.1 TorA signal peptide*

The TAT signal peptide of the molybdopterin-containing protein trimethylamine N-oxide (TMAO) reductase (TorA), a protein known to be exported via the TAT pathway in *E. coli*, has been utilized to localize heterologous proteins to the periplasm in *E. coli* as well as for secretion in *Corynebacterium*

*glutamicum* [52, 53]. It has been demonstrated that the fusion of the TorA signal peptide to green fluorescent protein (GFP) simultaneously improves the cytoplasmic folding of heterologous GFP and localizes it to the periplasmic space when expressed in *E. coli* (Figure 1.8) [59]. In *C. glutamicum*, the use of a TorA-GFP fusion resulted in the secretion of active-form GFP into the culture supernatant at quantities approaching 20 mg/L [57].

## 1.4 *Corynebacterium glutamicum*

*C. glutamicum* are Gram positive non-pathogenic, non-sporulating, non-motile rod shaped bacteria that have been widely utilized in industrial applications such as the production of amino acids, nucleotides and enzymes [41, 31, 61]. *C. glutamicum* and *C. fimi* are both members of the *Actinomycetales* Figures 1.9 and 1.10) order [29] and both have genomes that are high in GC% content [69]. Since *C. glutamicum* are widely utilized, fairly well-studied, and bear a genetic resemblance to *C. fimi*, this organism serves as an ideal candidate for a Gram positive protein expression/secretion system.

### *1.4.1 Use as an expression host in tandem with TAT secretion*

The utilization of *C. glutamicum* and TAT dependent secretion is expected to facilitate both the expression and purification of novel GHs originating from *C. fimi*. The employment of an expression host more closely related to *C. fimi* should be able to overcome the aforementioned issues that arise during expression in *E. coli*. Moreover, the secretion of heterologous proteins into the culture supernatant completely eliminates the need for cell lysis during protein purification.

## 1.5 Overall goal

The objective of this project is to generate a stable Gram-positive expression system based in *C. glutamicum* for the expression and subsequent analysis of various novel GHs originated from the Gram-positive soil bacterium, *C. fimi.*

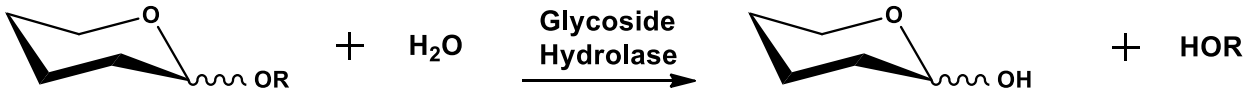



**Figure 1.1: Hydrolysis of carbohydrate polymers by glycoside hydrolases.** Glycoside hydrolases catalyze the hydrolysis of *O*-glycosidic bonds between two carbohydrate residues or between a carbohydrate and non-carbohydrate moiety.

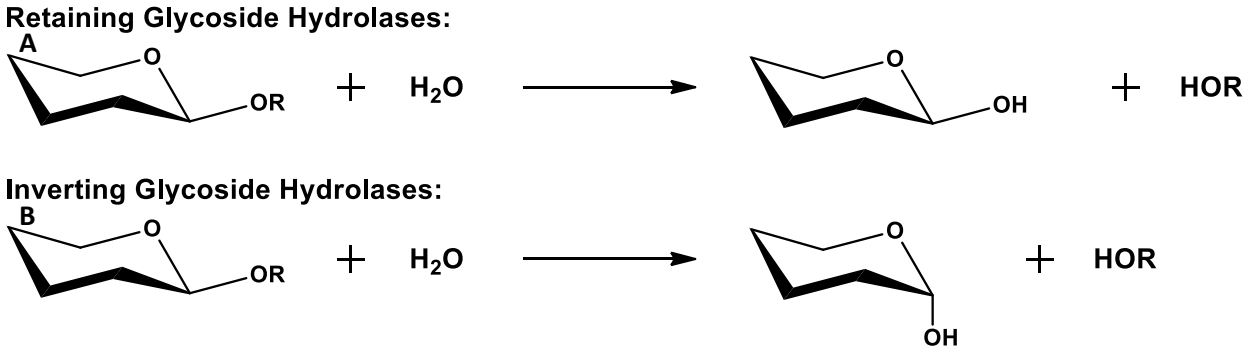



**Figure 1.2: Mechanisms of glycoside hydrolases.** Retaining glycoside hydrolases **(A)** hydrolyze glycosidic linkages via a retaining mechanism that retains the stereochemical orientation around the anomeric carbon, while inverting glycoside hydrolases **(B)** invert the stereochemical orientation around the anomeric carbon.

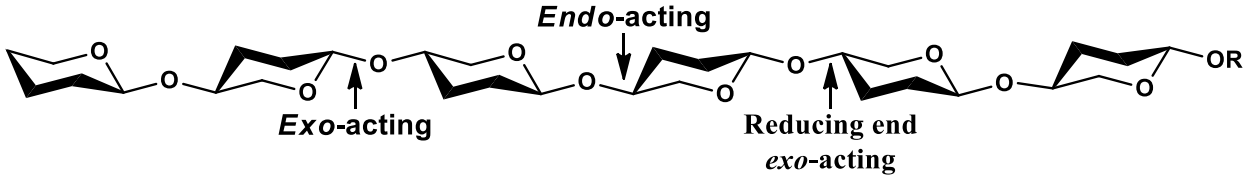



**Figure 1.3: Exo-/endo- classification of glycoside hydrolases.** Exo-acting glycoside hydrolases cleave glycosidic linkages from either the reducing or non-reducing end of a carbohydrate chain. Endo-acting glycoside hydrolases cleave internal sites, generating new chain termini.

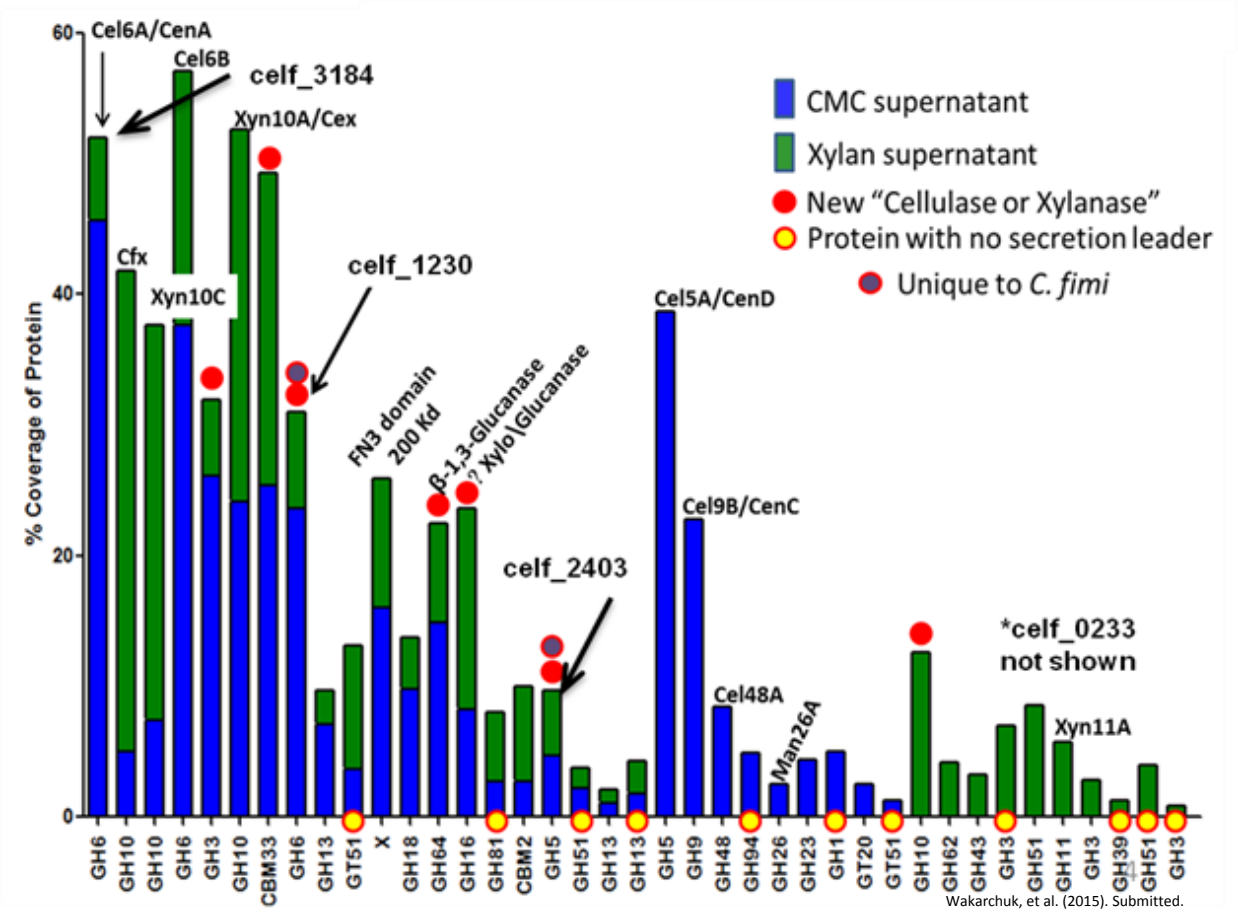



**Figure 1.4: *C. fimi* secretome analysis for cellulases or xylanases by mass-spectrometry.** Proteins with a MASCOT score of less than 50 are not shown. The % coverage of protein is a correlation of the relative amount of protein. Proteins that are unique to *C. fimi* were qualitatively determined by their amino acid sequence. Proteins identified in both supernatant samples are indicated by stacked bars.

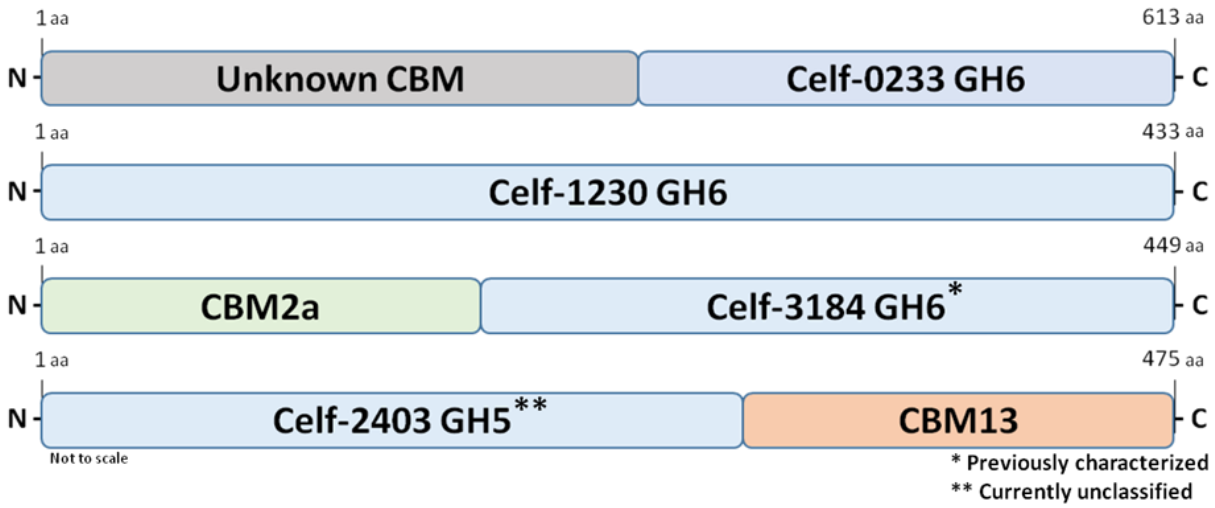



**Figure 1.5: Putative native glycoside hydrolase domain architecture as determined by BLAST.** The GH5, Celf_2403 contains a CBM13. The GH6s, Celf_0233, Celf_1230, and Celf_3184 contain an unknown CBM, no identifiable CBM, and a CBM2a, respectively.

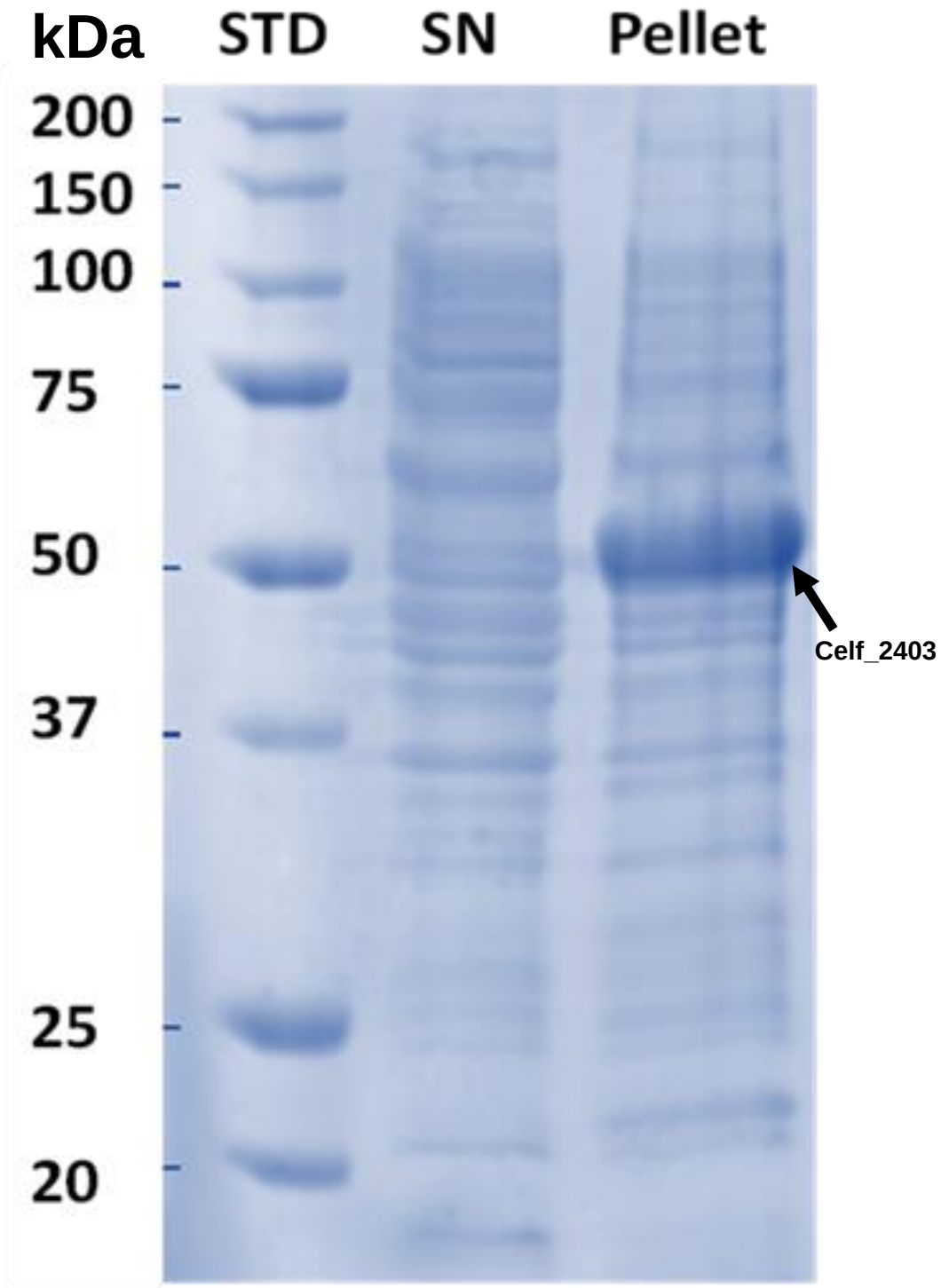



**Figure 1.6: Expression of native *C. fimi* glycoside hydrolase Celf_2403 in *E. coli*.** The native Celf_2403 protein is not expressed in a soluble form in *E. coli*. Any expressed protein forms insoluble inclusion bodies with no discernible activity.

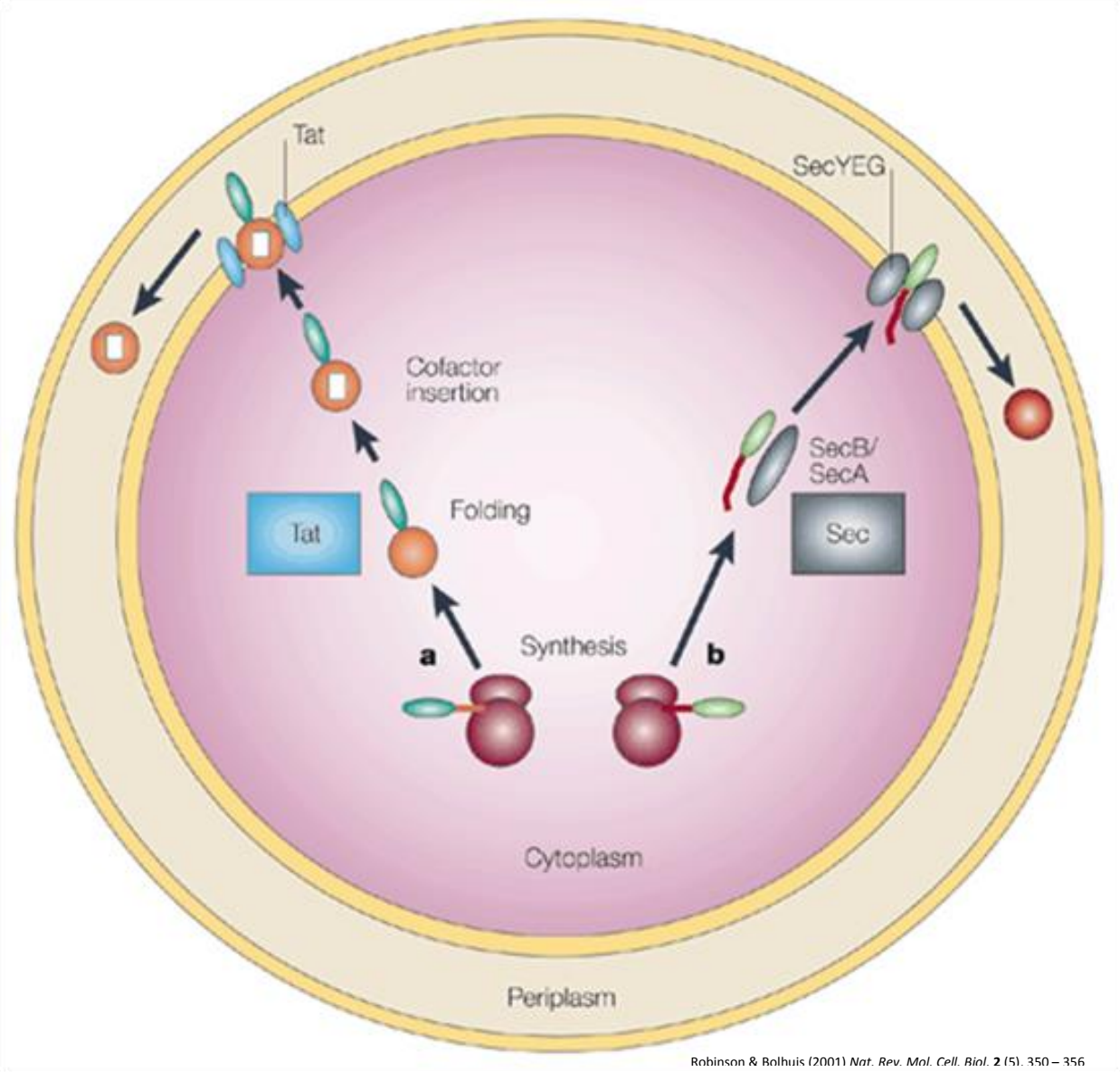
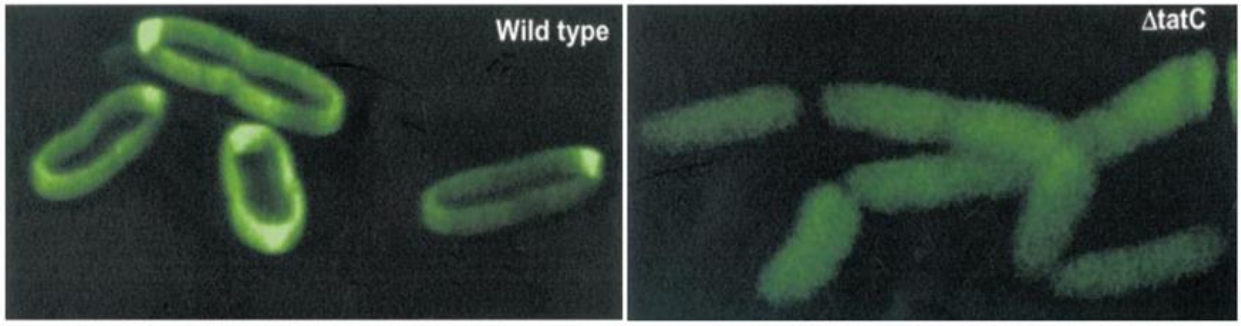
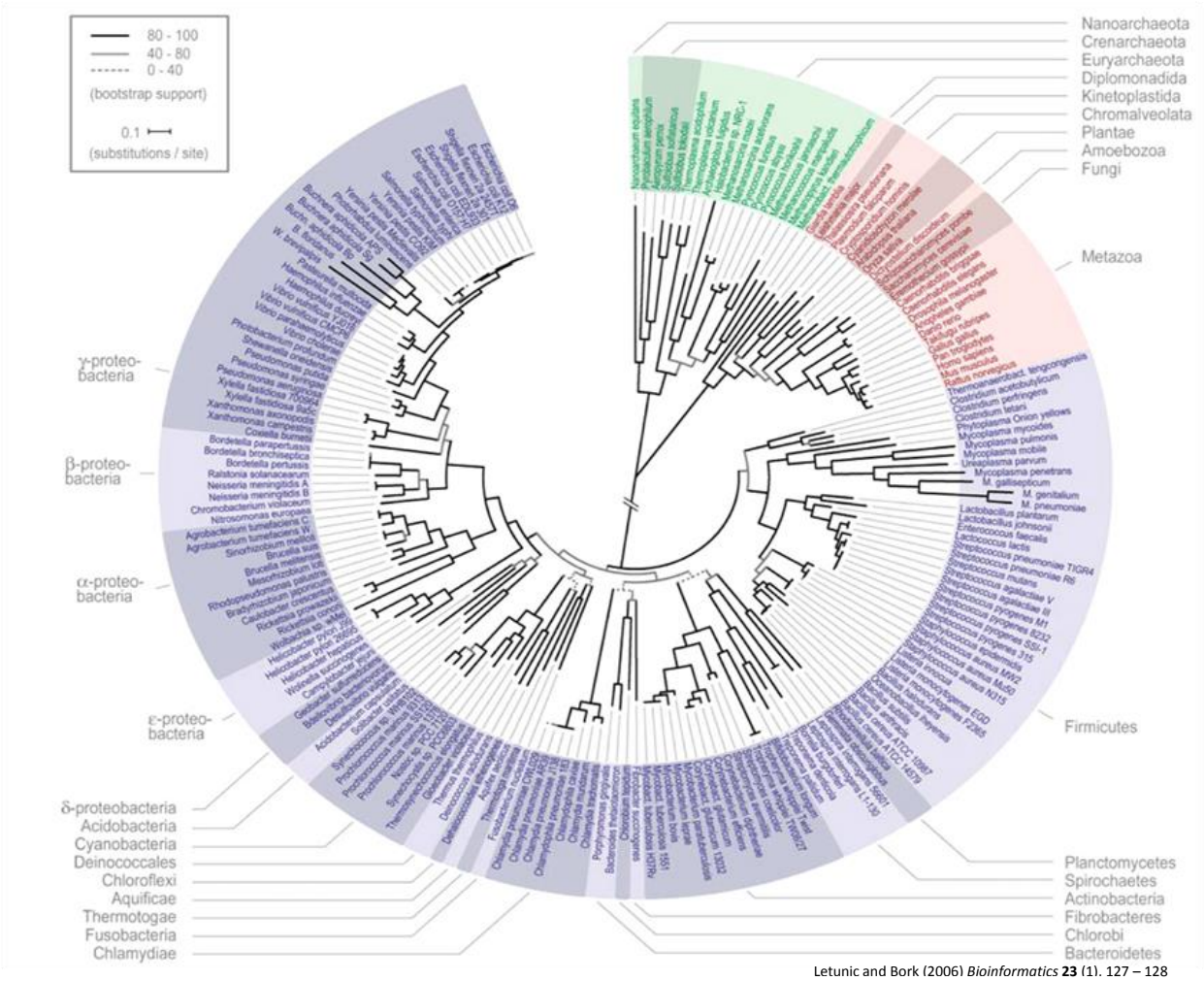



**Figure 1.7: Periplasmic localization via Sec-dependent and Tat-dependent transport.** The Tat pathway **(A)** allows pre-proteins to bind redox cofactors (white square) following synthesis in the cytoplasm. Pre-proteins fold cytoplasmically before being transferred through the Tat apparatus and undergoing processing. The Sec pathway **(B)** associates pre-proteins with the SecB protein (which prevents cytoplasmic folding of the mature domain) before being transferred to SecA. This drives ATP-dependent translocation through the SecYEG complex, threading the pre-protein into the periplasm where it is processed and allowed to refold.



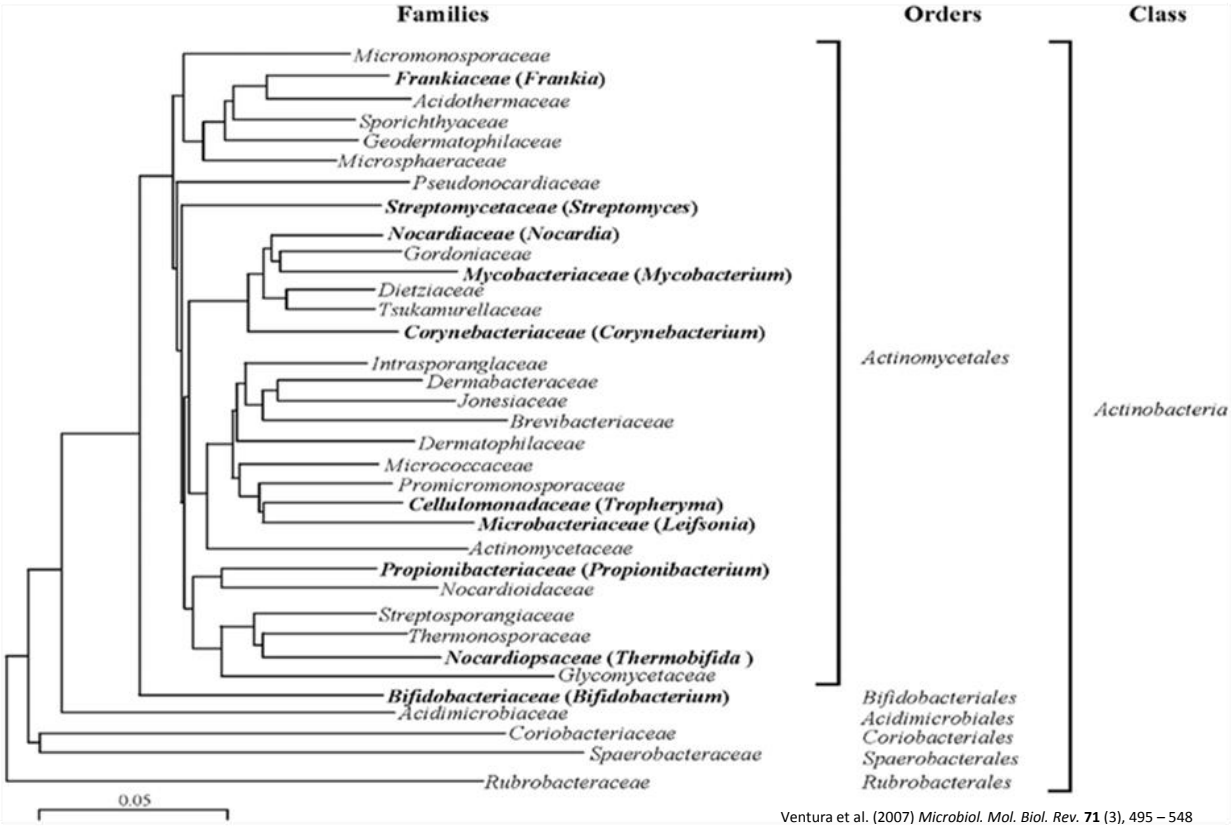

Thomas, et al. (2001) *Molecular Microbiology* **39** (1), 47 – 53

**Figure 1.8: Periplasmic localization of GFP in *E. coli* with TorA signal peptide.** In cells with functional Tat machinery (left panel), TorA localizes GFP to the periplasm. In cells lacking a functional tatC gene (right panel) all localization is lost and mature GFP remains in the cytoplasm.




Letunic and Bork (2006) *Bioinformatics* **23** (1). 127 – 128

**Figure 1.9: Genetic relationship of *E. coli* and *C. fimi*.** A large evolutionary gap exists between *C. fimi* (*Actinobacteria*) and *E. coli* (*γ-proteobacteria*). This distance likely implies a significant difference in the cellular machinery utilized by these two organisms during the translation, folding, or secretion of proteins.




Ventura et al. (2007) *Microbiol. Mol. Biol. Rev.* **71** (3), 495 – 548

**Figure 1.10: Genetic relationship of *C. glutamicum* and *C. fimi*.** *C. glutamicum* and *C. fimi* share both their class (*Actinobacteria*) as well as their order (*Actinomycetales*). This close relationship implies close homology in the cellular machinery utilized during the translation, folding, or secretion of proteins.

**Table 1.1: Characteristics of GH5 and GH6.**

| Family | Mechanism | Proton Donor | Nucleophile or Base | Select Known Activities |
|---|---|---|---|---|
| **GH5** | Retaining | Glutamic acid (experimental) | Glutamic acid (experimental) | endo-β-1,4-glucanase/cellulase (EC 3.2.1.4), endo-β-1,4-xylanase (EC 3.2.1.8), β-glucosidase (EC 3.2.1.21), β-mannosidase (EC 3.2.1.25) |
| **GH6** | Inverting | Aspartic acid (experimental) | Aspartic acid (experimental) | endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) |

# Chapter 2

# Materials and Methods

## 2.1 Strains

Genotypes of bacterial strains can be found in Table 2.7. *C. fimi* ATCC 484 was the source for genomic DNA used during cloning. *C. glutamicum* ATCC 13032 was utilized as an expression host. Both of these strains are type strains for their respective species. ElectroMAX™ DH10B™ (ThermoFisher Scientific) cells were used for routine cloning and the generation of the construct library used in this work. ElectroMAX™ DH10B™ cells are a derivative of the *E. coli* K-12 strain MG1655 optimized for improved transformation efficiency. This strain also contains engineered versions of Endonuclease I (endA1) and RecA (recA1) that are inactive. These modifications help to improve plasmid yield and stability within cells [9]. Shuffle Express™ (New England Biolabs) cells were used for protein expression requiring the formation of disulfide bonds. Shuffle Express™ cells are an *E. coli* BL21 derivative that constitutively expresses cytoplasmic disulfide bond isomerase DsbC, which also acts as a chaperone for protein folding. Additionally, Shuffle Express™ cells contain deletions for gultaredoxin reductase (*Δgor*) and thioredoxin reductase (Δ*trxB*) to prevent the reduction of cysteine residues in the cytoplasm [6]. Strains such as DH5α, BL21, and AD202 were occasionally used for troubleshooting.

## 2.2 Shuttle vector Design

### *2.2.1 Synthetic gene design and synthesis*

Synthetic genes (Table 1) containing the TorA TAT signal sequence were synthesized and supplied by Biobasic Canada (Markham, Ontario). A mutant TorA TAT signal sequence was designed to include a 3' $His_6$ tag which would be present after processing the leader and then used for subsequent protein purification (Figure 2.5). Synthetic genes were designed with a 5' AseI site (VspI/degenerate NdeI) and a 3' NdeI site. Codon usage was optimized to normalize regional GC content.

*2.2.2 Cloning*

Synthetic genes were excised from their parent pUC57 vector in a two-step digestion. First, the pUC57 MCS was excised via digestion with EcoRI and HindIII. The multiple cloning site (MCS) fragment containing the synthetic gene was extracted from the gel and purified using the GeneClean® Turbo Kit (MP Biomedicals) following manufacturer specifications. The purified MCS fragment was further digested with AseI and NdeI to liberate the synthetic gene, which was again purified using the GeneClean® Turbo Kit (MP Biomedicals). Vector DNA (pTGR5) was linearized by digestion with NdeI and gel purified in a similar manner to the synthetic genes. Digestions were allowed to incubate at 37°C for 10 – 30 minutes before heat inactivation at 65° – 85°C. Enzyme concentration in reactions was maintained at 1 µL enzyme (1 U) per 1 µg plasmid DNA and 1/10 total reaction volume according to manufacturer specifications (ThermoFisher Scientific) to minimize star activity. Ligation reactions were prepared with varying molar ratios of insert to vector (3:1 – 10:1) and 10 pmol vector. T4 DNA Ligase was purchased from New England Biolabs. Transformations were carried out via electroporation into the *E. coli* strain DH10B (New England Biolabs) using a 0.2 cm gap electroporation cuvette and a MicroPulser (Bio-Rad) set to deliver a single 2.5 kV pulse. Outgrowth was performed by shaking the cells at 37°C for 1 hour in recovery media (SOC :2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM $MgSO_4$, 20 mM D-glucose)prior to plating onto selective media containing 50 µg/mL kanamycin (BioShop) and overnight incubation at 37°C.

*2.2.3 Verification of TorA modification*

Individual colonies were picked and used to inoculate 2 mL LB (1.0% w/v tryptone 0.5% w/v yeast extract, 1.0% w/v NaCl) containing 50 µg/mL kanamycin (BioShop) and grown overnight with shaking at 37°C. Plasmids were then prepared from cultures following the standard High-Speed Plasmid Mini Prep Kit (FroggaBio) protocol. Clones were screened for inserts with a diagnostic double digestion using

BamHI and NdeI (ThermoFisher Scientific). Clones with the correct insert were further screened for correct orientation with a second diagnostic double digestion using NdeI and EcoRI (ThermoFisher Scientific). Digestions were analysed by agarose gel electrophoresis to confirm their orientation in the vector. Purified plasmids containing correctly oriented inserts were prepared with specific sequencing primers (Table 2.6) and sequenced by The Centre for Applied Genomics (TCAG, Toronto). The shuttle vectors that were generated can be found in Table 2.3 and vector maps can be found in Figures 2.1-2.3.

## 2.3 Generation of glycoside hydrolase construct library

### *2.3.1 Genomic DNA extraction*

*C. fimi* gDNA was harvested from saturated 40 hour cultures grown in low salt LB (1.0% w/v tryptone 0.5% w/v yeast extract, 0.5% w/v NaCl) using the DNeasy Blood & Tissue kit (Qiagen).

### *2.3.2 Primer design and PCR*

Primers (Table 2.2) were designed to include a 5' NdeI site and a 3' EcoRI site and were optimized for GC content, length, and to minimize undesired secondary structures. Primers were synthesized by The Centre for Applied Genomics (TCAG, Toronto). Since primer melting temperatures could not be optimized due to the intrinsically high GC content of the *Cellulomonas* genome, various annealing temperatures were used during PCR amplification (Table 2.2). Cloning was conducted using Q5 High-Fidelity DNA Polymerase and High GC Enhancer buffer (NEB) in the presence of DMSO to aid in the denaturation of high GC content templates.

### *2.3.3 Verification of cloning and sequencing*

Vectors and inserts were digested using NdeI and EcoRI and subsequently purified as described in Section 2.2.2. Clones were prepared as described in Section 2.2.3 and screened for inserts with a

diagnostic double digestion using NdeI and EcoRI. Possible multiple insertion events were ruled out with single digestions using only EcoRI. Digestions were subjected to agarose gel electrophoresis for confirmation. Purified plasmids containing correctly sized inserts were prepared with specific sequencing primers (Table 2.6) and sequenced by The Centre for Applied Genomics (TCAG, Toronto). A list of generated constructs can be found in Table 2.4.

## 2.4 Preparation and transformation of competent cells

### *2.4.1 Chemically competent* E. coli

A 2 mL culture of the *E. coli* strain Shuffle Express was grown in LB media for 16 hours at 30°C with shaking. This was used to inoculate 100 mL LB media at a 1:100 dilution, which was subsequently incubated at 30°C with shaking until early exponential growth was observed, indicated by an $OD_{600}$ measurement of 0.35 – 0.4. The culture was chilled on ice for 10 minutes prior to centrifugation at 3,000 *g* for 15 minutes at 4°C. Cells were gently resuspended in 50 mL ice-cold sterile 100 mM $MgCl_2$. The cells were centrifuged as before and gently resuspended in 25 mL ice-cold sterile 100 mM $CaCl_2$ and incubated on ice for 20 minutes. Following another centrifugation as above, cells were resuspended in 10 mL ice-cold sterile 85 mM $CaCl_2$ supplemented with 15% glycerol. After a final centrifugation, cells are resuspended in 2 mL ice-cold sterile 85 mM $CaCl_2$ and 15% glycerol. Cells were divided into 50 µL portions and snap frozen with a mixture of dry ice and ethanol prior to storage at -80°C. Frozen cells were thawed on ice prior to the addition of 1 µL containing ≈ 100 ng of plasmid DNA. Cells and DNA were incubated on ice for 30 minutes, then heat shocked at 42°C for 30 seconds. Transformations were incubated on ice for 5 minutes before the addition of 950 µL SOC media. Outgrowth was carried out at 30°C for 1 hour with shaking. Transformations were then plated onto selective media containing 50 µg/mL kanamycin (BioShop) and grown overnight at 30°C. Transformants were confirmed via High-Speed Plasmid Mini Prep Kit (FroggaBio) and diagnostic digestion.

*2.4.2 Electrocompetent* E. coli

A 10 mL culture of the *E. coli* strain DH10B was grown in LB media for 16 hours at 37°C with shaking. This was used to inoculate 500 mL LB media at a 1:100 dilution, which was subsequently incubated at 37°C with shaking until early logarithmic growth was observed, indicated by an $OD_{600}$ measurement of 0.35 – 0.4. The culture was chilled on ice for 10 minutes prior to centrifugation at 1,000 *g* for 20 minutes at 4°C. Cells were gently resuspended in 400 mL ice-cold sterile MilliQ $H_2O$ prior to a second centrifugation. Cells were gently resuspended in 200 mL ice-cold sterile MilliQ $H_2O$ prior to a third centrifugation. Cells were then gently resuspended in 40 mL ice-cold sterile 10% glycerol and centrifuged as before. Cells were gently resuspended in 1 mL ice-cold sterile 10% glycerol resulting in a final $OD_{600}$ of 200 – 250. Cells were divided into 50 µL aliquots and snap frozen with a mixture of dry ice and ethanol prior to storage at -80°C. Frozen cells were thawed on ice prior to the addition of 1 µL containing ≈ 10 ng of plasmid DNA. Cells were transformed as detailed in Section 2.2.2. Transformants were confirmed via High-Speed Plasmid Mini Prep Kit (FroggaBio) and diagnostic digestion.

*2.4.3 Electrocompetent* C. glutamicum

A 5 mL culture of *C. glutamicum* was grown overnight in MB media (0.5% w/v yeast extract, 1.5% w/v tryptone, 0.5% w/v soytone, 0.5% w/v NaCl) at 30°C with shaking. This was used to inoculate 200 mL MB media supplemented with 3.5% w/v glycine at a 1:100 dilution and incubated at 30°C with shaking until an $OD_{600}$ measurement of 0.2 – 0.25 was reached. Ampicillin (BioBasic) was then added to a final concentration of 0.5 µg/mL and the culture was further incubated under the same conditions for 1.5 h. The culture was chilled on ice for 10 minutes, then centrifuged at 3,000 *g* for 10 minutes at 4°C. Cells were gently resuspended in 30 mL ice-cold sterile 20 mM HEPES, 5% v/v glycerol (pH 7.2) and centrifuged as before. Resuspension and centrifugation was repeated two more times before the final cell pellet was resuspended in 1 mL ice-cold sterile 5 mM HEPES, 15% v/v glycerol (pH 7.2). Cells were

divided into 100 µL portions and snap frozen with a mixture of dry ice and ethanol prior to storage at -80°C. Frozen cells were thawed on ice prior to the addition of 1 µL containing ≈ 50 ng of plasmid DNA. Cells and DNA were electroporated using a 0.2 mm gap electroporation cuvette and a MicroPulser (Bio-Rad) set to deliver a single 2.5 kV pulse. Outgrowth was carried out with shaking at 30°C for 2 hours in LB media supplemented with 0.5% w/v glucose prior to plating onto selective media containing 25 µg/mL kanamycin (BioShop) and overnight growth at 30°C. Transformants were confirmed via High-Speed Plasmid Mini Prep Kit (FroggaBio), plasmid rescue by DH10B, and diagnostic digestion.

**2.5 TorA-dependant localization of recombinant proteins**

*2.5.1 E. coli periplasmic extraction of recombinant proteins (adapted [2])*

2 mL cultures of Shuffle Express with various constructs (containing GFP, CFI-65, CFI-75, CFI-85 or CFI-93) were grown overnight in LB media with 50 µg/mL kanamycin (BioShop) at 30°C with shaking. This was used to inoculate 50 mL 2YT media (Sigma-Aldrich) with 50 µg/mL kanamycin (BioShop) at a 1:500 dilution and incubated at 30°C with shaking until exponential growth was observed, indicated by an $OD_{600}$ measurement of 0.5 – 0.7. Sterile IPTG (isopropyl β-D-1-thiogalactopyranoside, BioVectra) was added to a final concentration of 0.5 mM and induction was continued overnight at 25°C with shaking. Cells were harvested by centrifugation at 1,100 *g* for 10 minutes at 4°C. The supernatant was decanted and cell pellets were gently resuspended in the residual media and 500 µL of $CHCl_3$ was added. The samples were maintained at room temperature for 15 minutes with occasional mixing, then 5 mL 10 mM Tris-HCl (pH 8.0) was added. Protoplasts were separated from periplasmic contents by centrifugation at 6,000 *g* for 20 minutes at 4°C. The supernatant fraction containing periplasmic proteins was carefully removed and stored at 4°C. Remaining protoplasts were further lysed using CelLytic B™ lysis reagent (Sigma-Aldrich) to analyze both the soluble and pellet fractions. Protoplasts were resuspended in 5 mL of a 0.2X CelLytic B™ solution containing 0.2 mg/mL lysozyme (BioShop), 50 U/mL

Benzonase® (Sigma-Aldrich), and a complete™ Mini EDTA protease inhibitor tablet (Roche) and incubated at room temperature on a rotator for 15 minutes. Extracts were then centrifuged at 16,000 *g* for 10 minutes at 4°C and the supernatant containing soluble proteins was removed and stored at 4°C. Insoluble pellets were resuspended in 5 mL TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and stored at 4°C. Samples were subjected to SDS-PAGE and analysed by Western blotting using anti-$His_6$ peroxidase (Roche).

**2.6 Expression and purification of recombinant glycoside hydrolases**

*2.6.1 Expression and purification in* Escherichia coli

2 mL cultures of Shuffle Express with various constructs (CFI-65, CFI-75, CFI-85, or CFI-93) were grown overnight in LB media with 50 µg/mL kanamycin (BioShop) at 30°C with shaking. This was used to inoculate 200 mL 2YT media (Sigma-Aldrich) with 50 µg/mL kanamycin (BioShop) at a 1:500 dilution and incubated at 30°C with shaking until logarithmic growth was observed, indicated by an $OD_{600}$ measurement of 0.5 – 0.7. Sterile IPTG (BioVectra) was added to a final concentration of 0.5 mM and induction was continued overnight at 25°C with shaking. Cells were harvested by centrifugation at 9,000 *g* for 15 minutes at 4°C and frozen at -20°C until needed. Cell pellets were thawed on ice and mechanically lysed in a mortar and pestle with Celite® (diatomaceous earth, Sigma-Aldrich). Lysed $His_6$ constructs (CFI-65, CFI-75, and CFI-85) were resuspended in 5 mL binding/wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 15 mM imidazole, pH 8.0) containing a cOmplete™ Mini EDTA protease inhibitor tablet (Roche), and clarified via centrifugation at 13,000 *g* for 20 minutes at 4°C. The clarified lysate was added to 1 mL of a 50% Ni-NTA agarose (Qiagen) slurry that had been previously pre-equilibrated in binding buffer and incubated at 4°C for 1 h on a rotator. The lysate-Ni-NTA agarose mixture was loaded onto a disposable column and washed twice with 5 mL binding/wash buffer. Proteins were eluted from the matrix twice in 1 mL elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 300 mM imidazole, pH 8.0). Samples

of each fraction were analyzed via SDS-PAGE, elutions were pooled and buffer exchanged to 50 mM HEPES, 300 mM NaCl (pH7.0). Following lysis, CFI-93 was resuspended in 5 mL preparation buffer (50 mM ammonium bicarbonate, pH9.0) containing a cOmplete™ Mini EDTA protease inhibitor tablet (Roche), and clarified via centrifugation at 13,000 *g* for 20 minutes at 4°C. The clarified lysate was subjected to anion-exchange chromatography on SourceQ (Bio-Rad). Protein fractions were applied to the column using a peristaltic pump at a flow rate of 1 mL/min and eluted with a linear gradient from 0 to 1.0 M NaCl over 30 minutes [12]. Fractions were analyzed by SDS-PAGE and the most pure fractions were pooled and buffer exchanged to 50 mM HEPES, 300 mM NaCl (pH7.0).

*2.6.2 Expression and purification in* C. glutamicum

2 mL cultures of *C. glutamicum* with various constructs (CFI-65, CFI-75, CFI-85, or CFI-93) were grown overnight in LB media with 25 µg/mL kanamycin (BioShop) at 30°C with shaking. This was used to inoculate 50 mL 2YT media (Sigma-Aldrich) with 25 µg/mL kanamycin (BioShop) at a 1:100 dilution and incubated at 30°C with shaking overnight. Sterile IPTG (BioVectra) was added to a final concentration of 0.5 mM and induction was continued over 48 h at 30°C with shaking. Supernatants were clarified via centrifugation at 9,000 *g* for 20 minutes at 4°C. Supernatants were stored at 4°C and cell pellets were frozen at -20°C. A 10X stock solution of binding buffer was diluted with the supernatants of $His_6$ constructs (CFI-65, CFI-75, and CFI-85) to a final concentration of 50 mM $NaH_2PO_4$, 300 mM NaCl, 15 mM imidazole (pH 8.0) and contained a Complete™ Mini EDTA protease inhibitor tablet (Roche). 1 mL of a 50% Ni-NTA agarose (Qiagen) slurry that had been previously pre-equilibrated was added to the supernatant and incubated overnight at 4°C on a rotator. The supernatant-Ni-NTA agarose mixture was loaded onto a disposable column and washed four times with 5 mL binding/wash buffer. Proteins were eluted from the matrix twice in a total of 1 mL elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 300 mM imidazole, pH 8.0). Samples of each fraction were analyzed via SDS-PAGE, elutions were pooled and

buffer exchanged to 50 mM HEPES, 300 mM NaCl (pH7.0). A 10X stock solution of preparation buffer was diluted with the supernatant of CFI-93 to a final concentration of 50 mM ammonium bicarbonate (pH9.0) and contained a cOmplete™ Mini EDTA protease inhibitor tablet (Roche). The supernatant was subjected to anion-exchange chromatography on SourceQ (Bio-Rad). Protein fractions were applied to the column using a peristaltic pump at a flow rate of 1 mL/min and eluted with a linear gradient from 0 to 1.0 M NaCl over 30 minutes [12]. Fractions were analyzed by SDS-PAGE and the most pure fractions were pooled and buffer exchanged to 50 mM HEPES, 300 mM NaCl (pH7.0).

**2.7 Enzyme activity assays**

*2.7.1 PAHBAH*

Increasing concentrations of reducing ends produced by glycoside hydrolases acting on various carbohydrate substrates were monitored using 4-hydroxybenzoic acid hydrazide (PAHBAH) (Sigma-Aldrich). The original protocol by Lever [35] was modified as follows. The PAHBAH reagent (5% w/v PAHBAH, 5% HCl) and the alkali solution (50 mM citrate, 10 mM $CaCl_2$, 0.5M NaOH, pH 8.0) were prepared separately in advance. Prior to use, 1 part PAHBAH reagent was combined with 9 parts alkali solution and the mixture was kept on ice during use. Various concentrations of carbohydrate substrates (generally 0.5% in assay) were combined with 2.5 µg/mL glycoside hydrolase in 50 mM MOPS, 50 mM NaCl (pH 5.5 for celf_3184, pH 6.0 for celf_1230) and allowed to incubate at optimal enzyme temperature. At designated time points 100 µL of the assay were quenched with the addition of 50 µL 0.5 M NaOH. 30 µL of the quenched reaction were placed into a 96-well plate and had 200 µL of the PAHBAH working reagent added to it. After the completion of all time points, 96-well plates were incubated for 10 minutes at 100°C then allowed to slowly cool to room temperature before being read at 410 nm.

*2.7.2 Dye-conjugated substrates*

Azurine-crosslinked-substrates (AZCL, Megazyme) were used to determine the relative activity of purified glycoside hydrolases in a colorimetric assay. AZCL-Hydroxyethylcellulose (AZCL-HEC) and AZCL-Barley-β-Glucan (AZCL- β-Glucan) were utilized to measure cellulase activity. Assays were prepared to a total volume of 1 mL in 50 mM MOPS, 50 mM NaCl (pH 6.0), 5 mg/mL AZCL-substrate, and 100 µg/mL purified glycoside hydrolase. Substrates were preheated and assays were incubated at optimal temperatures of previously characterized enzymes (65°C for CFI-75, 55°C for CFI-93). At designated time points, 150 µL of the assay was removed and centrifuged at 5,000 *g* for 5 minutes to pellet the insoluble material. 100 µL of the chromogenic supernatant was then moved to a 96-well plate and read at 595 nm.

**2.8 Thin-layer chromatography**

The determination of hydrolysis products for celf_1230 was achieved via thin-layer chromatography (TLC). Sufficient separation of standard cellodextrins (G1-G5, 2 mg/mL each) was observed when using a silica gel 60 plate on a plastic backing (Merck). The hydrolysis of cellotetraose (Megazyme) and 1,3:1,4-β-glucotetraose (Megazyme) (Figure 2.4) by celf_1230 was conducted at 65°C (5 mg/mL substrate, 2.5 µg/mL celf_1230, 50 mM MOPS, 50 mM NaCl, pH6.0). Reactions were quenched at various time points by the addition of 0.5 M NaOH before spotting onto the silica plate and being exposed to the liquid phase solvent (ethyl acetate:methanol:$H_2O$:acetic acid in a 4:2:1:0.1 ratio by volume). Carbohydrates were visualized on the TLC plate by spraying with 5% (v/v) $H_2SO_4$ in ethanol and charring over a hot plate.

**2.9 Determination of enzyme concentration**

Enzyme concentrations were determined using $A_{280}$ readings obtained when using a 50 µL microcell and a spectrophotometer. The theoretical molar extinction coefficients ($M^{-1}$ $cm^{-1}$) used for each protein can be found in Table 2.5 along with their molecular weights. Parameters were determined by ExPASy ProtParam [68].

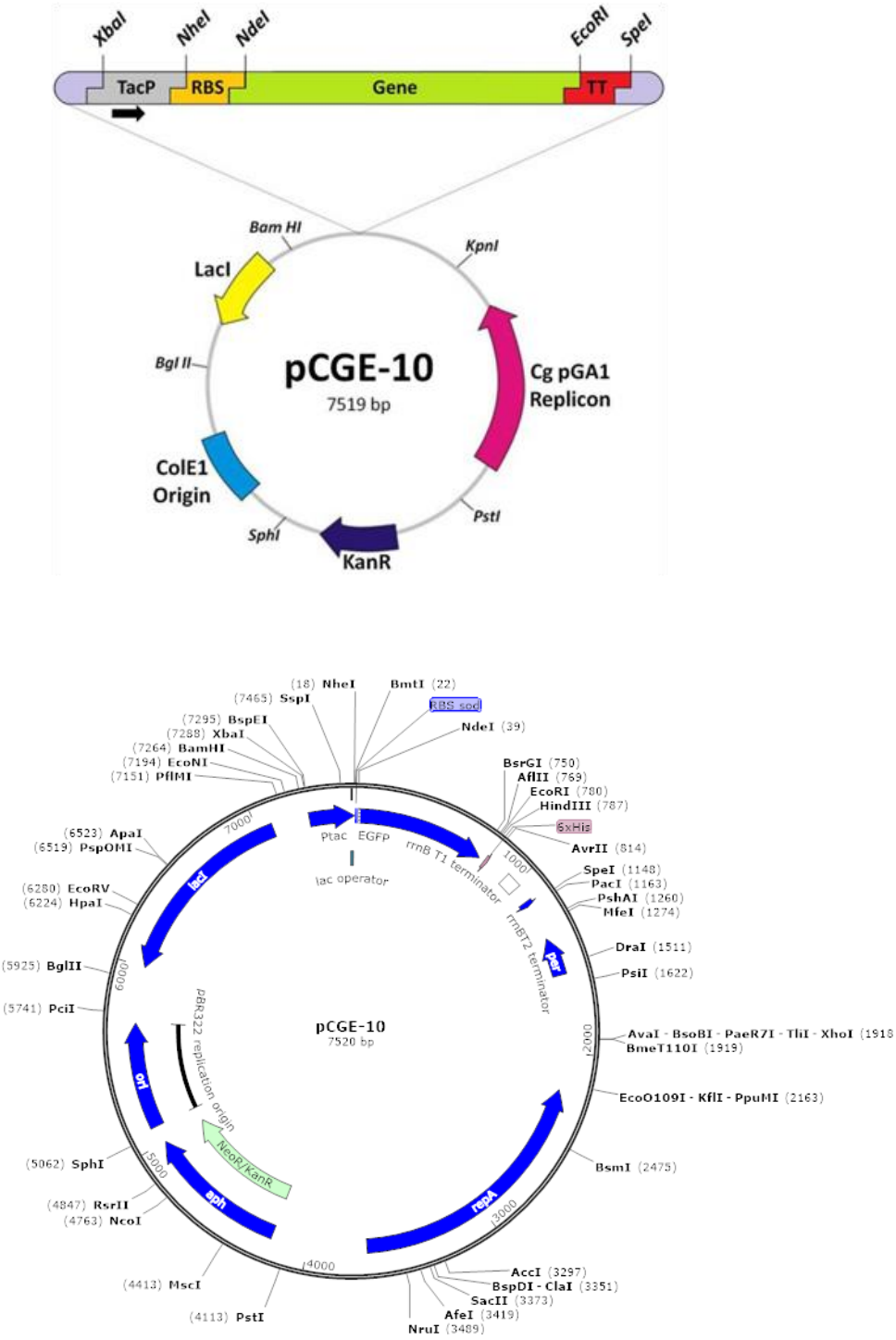
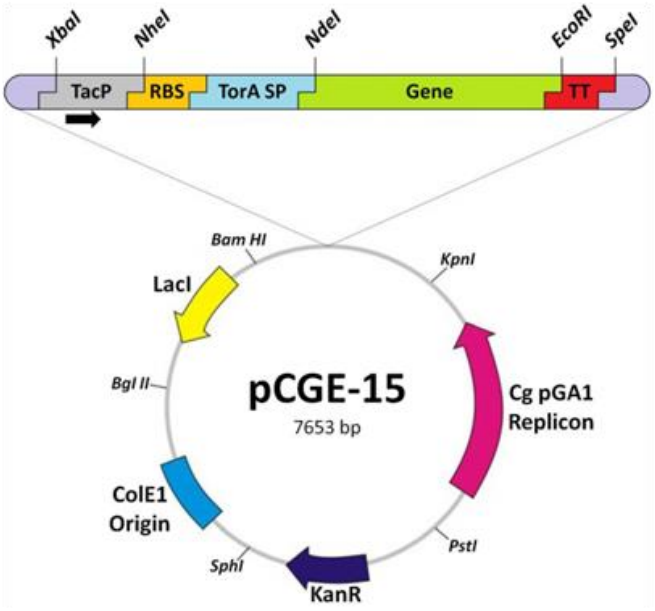



**Figure 2.1: Simplified and detailed vector maps of pCGE-10.**

Vector pCGE-10 features kanamycin resistance (KanR), Lac repressor (LacI) for IPTG induction, ColE1 origin for replication in *E. coli*, and Cg pGA1 replicon for replication in *C. glutamicum*.



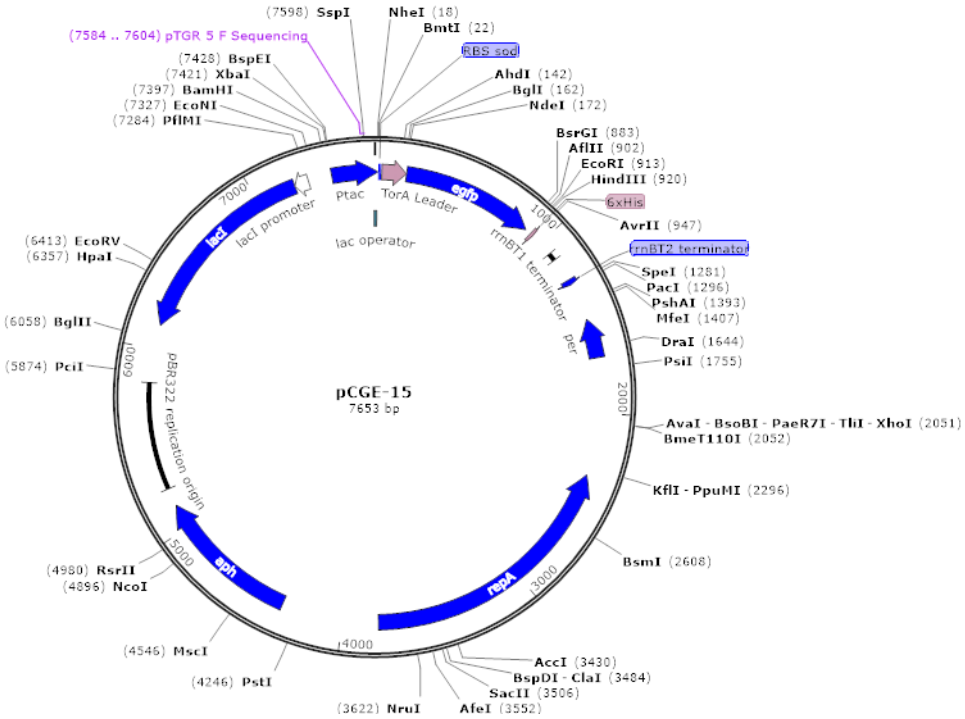




**Figure 2.2: Simplified and detailed vector maps of pCGE-15.**

Vector pCGE-15 features are identical to pCGE-10, including the addition of the TorA TAT signal sequence directly upstream of the MCS.

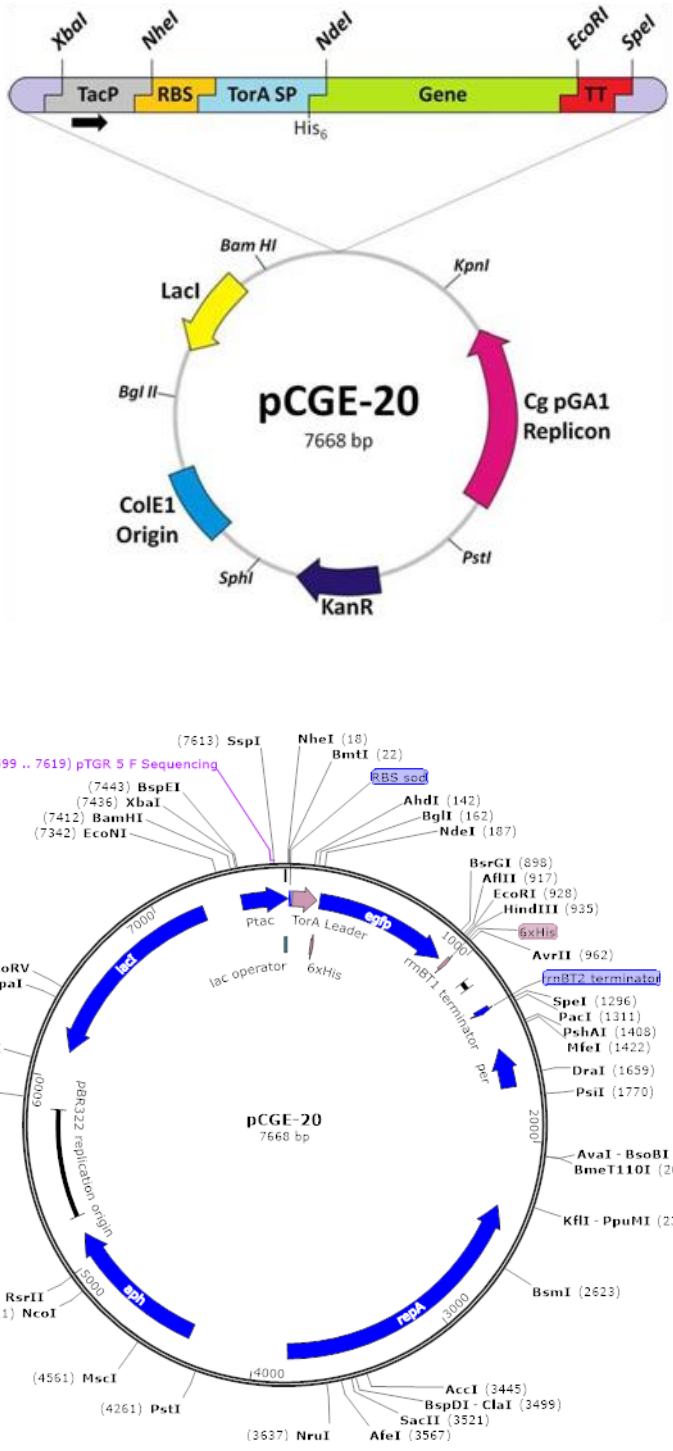



**Figure 2.3: Simplified and detailed vector maps of pCGE-20.**

Vector pCGE-20 features are identical to pCGE-15, including the addition of $His_6$ tag at the C-terminus of the TorA TAT signal sequence.

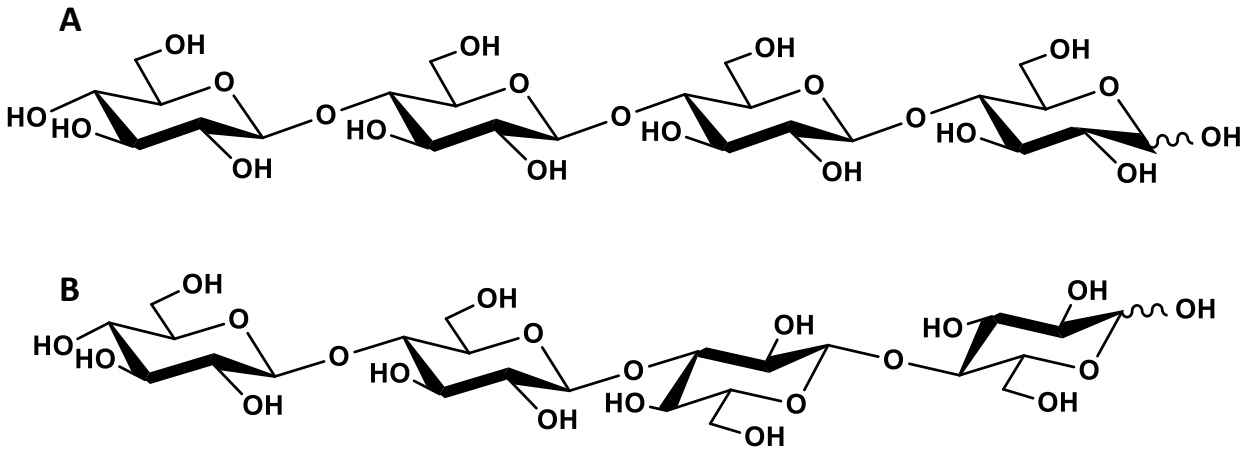



**Figure 2.4: Structural comparison of cellotetraose (A) and 1,3:1,4-β-glucotetraose (B).**

Cellotetraose (A) is composed of 4 glucose β-1,4-linked glucose monomers, while 1,3:1,4-β-glucotetraose (B) is composed of two β-1,4-linkages and a β-1,3-linkage in the middle.

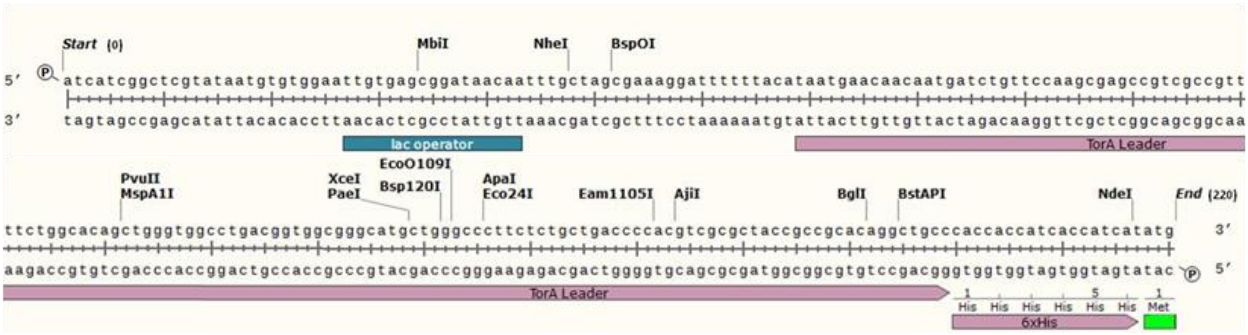



**Figure 2.5 Sequence of TorA signal peptide within expression vector pCGE-20.**

Located between the ribosome binding site and the cloning site of expression vectors pCGE-15 (lacking 6xHIS tag) and pCGE-20, the TorA signal peptide directs expressed proteins to the periplasm in Gram negative bacteria and allows for secretion in Gram positive bacteria.

**Table 2.1: TorA synthetic gene sequence and features.**

| Gene Name | Sequence (5'-3') | Features |
|---|---|---|
| **TorA** | ATTAATGAACAACAATGATCTGTTCCAAGCGAGCCGTCGCC GTTTTCTGGCACAGCTGGGTGGCCTGACGGTGGCGGGCAT GCTGGGCCCTTCTCTGCTGACCCCACGTCGCGCTACCGCCG CACAGGCTGCCCATATG | Encodes TorA TAT signal peptide. Contains 5' AseI (degenerate NdeI) site and 3' NdeI site. |
| **TorA N-His$^6$** | ATTAATGAACAACAATGATCTGTTCCAAGCGAGCCGTCGCC GTTTTCTGGCACAGCTGGGTGGCCTGACGGTGGCGGGCAT GCTGGGCCCTTCTCTGCTGACCCCACGTCGCGCTACCGCCG CACAGGCTGCCCACCACCATCACCATCATATG | Encodes TorA TAT signal peptide. Contains 5' AseI (degenerate NdeI) site, 3' NdeI site, and C-terminal $His_6$ tag. |

**Table 2.2: Glycoside hydrolase cloning primers.**

| Primer Name | Target Gene | Sequence (5'-3') | Tm (°C) | Annealing Temp (°C) | Features |
|---|---|---|---|---|---|
| **1 F** | celf_2403 | GGG GTA TTC **CAT ATG** AGA GCA GCG ACC CGT ACG GCG | 71.42 | 66 | 5' primer with **NdeI** site. |
| **1 FL** | celf_2403 | GGG GTA TTC **CAT ATG** GCC TCC CCC GCG GTC GGT CCC | 78.61 | 74 | 5' primer with **NdeI** site. Lacking native leader. |
| **1 R** | celf_2403 | GGG **GAA TTC** TCA TCA GCC GCG CGT CCA CTG CTG | 74.84 | 70 | 3' primer with **EcoR**I site. |
| **2 F** | celf_1230 | GGG GTA TTC **CAT ATG** GCC CGA CCC TTC CGC ACC CCT CTC GCC | 83.07 | 78 | 5' primer with **NdeI** site. |
| **2 FL** | celf_1230 | GGG GTA TTC **CAT ATG** GGC GGC CCC CCG CAC GGG TAC CCG | 84.75 | 80 | 5' primer with **NdeI** site. Lacking native leader. |
| **2 R** | celf_1230 | GGG **GAA TTC** TCA TCA GCT GCG CGG GCC CCC GAT CGG | 84.13 | 79 | 3' primer with **EcoRI** site. |
| **3 F** | celf_0233 | GGG GTA TTC **CAT ATG** CCC CGT CCC GGT CCG GTC CCG GCC | 89.54 | 85 | 5' primer with **NdeI** site. |
| **3 FL** | celf_0233 | GGG GTA TTC **CAT ATG** ACG ACC GCA CCG GAC CGC GTG CTC | 86.56 | 82 | 5' primer with **NdeI** site. Lacking native leader. |
| **3 R** | celf_0233 | GGG **GAA TTC** TCA TCA CCA GGA CGC GGC CGC GGC CAG | 87.73 | 83 | 3' primer with **EcoRI** site. |
| **4 F** | celf_3184 | GGG GTA TTC **CAT ATG** TCC ACC CGC AGA ACC GCC GCA GCG | 87.09 | 82 | 5' primer with **NdeI** site. |
| **4 R** | celf_3184 | GGG **GAA TTC** TCA TCA CCA CCT GGC GTT GCG CGC CAT C | 85.55 | 81 | 3' primer with **EcoRI** site. |

**Table 2.3: Shuttle vectors.**

| Vector Name | Resistance | Tags | Features |
|---|---|---|---|
| **pCGE-10** | Kanamycin | N/A | N/A |
| **pCGE-15** | Kanamycin | N/A | TorA TAT signal peptide |
| **pCGE-20** | Kanamycin | $His_6$ | TorA TAT signal peptide |

**Table 2.4: Glycoside hydrolase construct library.**

| Construct Name | Gene | Tags | Features |
|---|---|---|---|
| **CFI-63** | celf_0233 | N/A | Amplified from *C. fimi* GH6. |
| **CFI-64** | celf_0233 | N/A | Amplified from *C. fimi* GH6. Lacking native leader. |
| **CFI-65** | celf_0233 | $His_6$ | Amplified from *C. fimi* GH6. Lacking native leader. |
| **CFI-73** | celf_1230 | N/A | Amplified from *C. fimi* GH6. |
| **CFI-74** | celf_1230 | N/A | Amplified from *C. fimi* GH6. Lacking native leader. |
| **CFI-75** | celf_1230 | $His_6$ | Amplified from *C. fimi* GH6. Lacking native leader. |
| **CFI-83** | celf_2403 | N/A | Amplified from *C. fimi* GH5. |
| **CFI-84** | celf_2403 | N/A | Amplified from *C. fimi* GH5. Lacking native leader. |
| **CFI-85** | celf_2403 | $His_6$ | Amplified from *C. fimi* GH5. Lacking native leader. |
| **CFI-93** | celf_3184 | N/A | Amplified from *C. fimi* GH6. |

**Table 2.5: Theoretical molar extinction coefficients of expressed glycoside hydrolases.**

| Gene | Length (AA) | Molecular Weight (kDa) | Extinction Reduced Cys ($M^{-1}$ $cm^{-1}$) | Extinction Non-Reduced Cys ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| **celf_0233** | 613 | 61.78 | 86,400 | 86,650 |
| **celf_1230** | 433 | 46.31 | 107,830 | 108,205 |
| **celf_2403** | 475 | 49.83 | 121,350 | 121,725 |
| **celf_3184** | 449 | 46.70 | 107,370 | 107,745 |

**Table 2.6: Shuttle vector and construct sequencing primers.**

| Primer Name | Target | Sequence (5'- 3') | Tm (°C) | Features |
|---|---|---|---|---|
| **pTGR 7451F** | pCGE-10<br>pCGE-15<br>pCGE-20 | ACGGTTCTGGCAAATATTCTG | 54 | Anneals upstream of *lac* operator and sequences towards MCS. Capable of confirming operator, RBS, and signal peptide sequence (if applicable). |

**Table 2.7: Genotypes of bacterial strains.**

| Strain | Genotype |
|---|---|
| ***Cellulomonas fimi* ATCC 484** | Wild-type |
| ***Escherichia coli* (DH10B)** | F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara*, *leu*)7697 *gal*U *gal*K λ$^-$*rps*L *nup*G |
| ***Escherichia coli* (Shuffle Express)** | *fhuA2 [lon] ompT ahpC gal* λatt::pNEB3-r1-*cDsbC* (SpecR, *lacI$^q$) ΔtrxB sulA11* R(*mcr-73::miniTn10*--Tet$^S$)*2 [dcm] R(zgb-210::Tn10* --Tet$^S$) *endA1 Δgor Δ(mcrC-mrr)114::IS10* |
| ***Corynebacterium glutamicum* ATCC 13032** | Wild-type |

# Chapter 3

# Results

## 3.1 Molecular biology

### *3.1.1 Vector modification*

Synthetic TorA genes ordered from Biobasic Canada (Markham, Ontario) were contained within the MCS of pUC57. Genes were excised using a two-step double digestion. The entire MCS was first excised via digestion with both EcoRI and HindIII, then purified by gel extraction (as described in Section 2.2.2). The MCS fragment (containing the TorA synthetic gene) was then further digested using AseI and NdeI and purified with a secondary gel purification (Appendix Figure 5.1). The TorA synthetic genes were then ligated into the NdeI site of the pCGE-10 plasmid. Since NdeI and AseI sites are compatible, orientation of the TorA gene inserts needed to be determined. Since AseI creates a degenerate site, it was possible to determine insert orientation by digestion with NdeI and another enzyme. To identify the presence of an insert for both pCGE-15 and pCGE-20 (Appendix Figure 5.2 A and 5.3 A, respectively) purified plasmid DNA from clones was digested using BamHI and NdeI. To rule out multiple insertions and confirm orientation purified plasmid DNA from clones was digested using NdeI and EcoRI for both pCGE-15 and pCGE-20 (Appendix Figure 5.2 B and 5.3 B respectively). Vector modifications were confirmed via sequencing as described in Section 2.3.3.

### *3.1.2 Glycoside hydrolase construct library*

Glycoside hydrolase genes were amplified from *C. fimi* genomic DNA using synthetic primers (Table 2.2). Genes were amplified, digested with NdeI and EcoRI, and purified as described in Section 2.2.2 and 2.3.2 (Appendix Figure 5.4 A) prior to cloning into pCGE-15 and pCGE-20. Generated constructs (Table 2.4) were confirmed using diagnostic single and double digestions (to check for multiple insertion events) for

pCGE-10 constructs (Appendix Figure 5.4 B) in addition to pCGE-15 and pCGE-20 constructs (Appendix Figure 5.4 C). Constructs were also confirmed via sequencing as described in Section 2.3.3.

## 3.2 Recombinant protein production

### *3.2.1 Test expression of eGFP*

The *E. coli* strain Shuffle Express was transformed with pCGE-10 and pCGE-15 (unmodified vector, and vector containing the TorA synthetic gene). Both transformed strains were grown, induced to express eGFP, and protein was extracted from various cellular fractions (as described in Section 2.5.1) in order to determine the functionality of the TorA modification. Fractions were first analyzed by visualization with blue light tray to observe differences in fluorescence, then subjected to SDS-PAGE analysis and visualized by UV tray to determine any difference in total eGFP expression. Increased fluorescence was observed in the periplasmic fraction of cells containing pCGE-15, and significantly more fluorescence was also observed in the soluble fraction of these cells (Figure 3.1 A). SDS-PAGE analysis showed that the quantity of eGFP expressed by cells transformed by pCGE-10 and pCGE-15 were equivalent.

### *3.2.2 Expression of glycoside hydrolases in Escherichia coli*

Glycoside hydrolase constructs were transformed into *E. coli* strain Shuffle Express. These cells were grown, induced, and protein was extracted from various cellular fractions as described in Section 2.6.1. Samples were subjected to SDS-PAGE analysis and western blot to identify the production of recombinant proteins (Figure 3.2). As seen previously, CFI-65 and CFI-85 were only produced in an insoluble form in *E. coli*. Both CFI-75 and CFI-93 were produced in a soluble form (CFI-93 does not produce a visible band, so recombinant protein was confirmed via an enzyme activity assay) and CFI-75 was also seen to be exported to the periplasmic fraction (Figure 3.2 B).

Active CFI-75 and CFI-93 were purified from *E. coli* cell lysates (Figure 3.3 A and Figure 3.4 A, respectively) via affinity or anion exchange chromatography. Following the comparison of activity with control proteins, it was determined that 780 µg of CFI-75 and 43 µg of CFI-93 were purified (Figure 3.3 B and Figure 3.4 B, respectively). Celf_1230 [27] and Celf_3184 [12] (used as controls in gels or assays) were produced solely in *E. coli* and originated from previously utilized constructs. Though similar to the methods described in Section 2.6.1, the purification of these proteins is not described here.

*3.2.3 Expression of glycoside hydrolases in Corynebacterium glutamicum*

Glycoside hydrolase constructs were transformed into *C. glutamicum*. These cells were grown, induced, and protein was purified from the culture supernatants as described in Section 2.6.2. Expression and purification of CFI-65 and CFI-85 was not successful with *C. glutamicum* and the data is not shown. Active CFI-75 and CFI-93 were able to be purified from *C. glutamicum* culture supernatants (Figure 3.5 A and Figure 3.6 A, respectively) via affinity or anion exchange chromatography. Following the comparison of activity with control proteins, it was determined that 25 µg of CFI-75 and 350 µg of CFI-93 were purified (Figure 3.5 B and Figure 3.6 B, respectively).

**3.3 Biochemical characterization**

*3.3.1 Control protein activity on cellulose derivatives*

Though the activity of Celf_1230 and Celf_3184 produced in *E. coli* had previously been assayed on CMC [12, 27], their activities on cellulose derivatives remained to be determined. For this reason, the activity of Celf_1230 and Celf_3184 were assayed on wheat arabinoxylan, 1,3:1,4-Barley β-glucan, and tamarind xyloglucan alongside various concentrations of CMC (Figure 3.7). Each glycoside hydrolase was assayed at its optimal temperature and pH (65°C and pH 6.0 for Celf_1230, 50°C and pH 5.5 for Celf_3184) [12, 27] as described in Sections 2.7.1, 2.7.2, and 2.8. Both glycoside hydrolases exhibited significantly improved

activity on 1,3:1,4-Barley β-glucan in comparison to all concentrations of CMC assayed. The hydrolysis products of both Celf_1230 and Celf_3184 on CMC and 1,3:1,4-Barley β-glucan was assayed by TLC as described in Section 2.8 (Figure 3.8) to identify if the β-1,3 glycosidic bond (Figure 2.4) was being cleaved, resulting in the observed increase in activity on the mixed linkage substrate. The result of this assay shows that the β-1,3 linkage is in fact not being cleaved.

The thermostabilities of Celf_1230 and Celf_3184 were assayed on both CMC and 1,3:1,4-Barley β-glucan to observe any trends in activity over time for both glycoside hydrolases and substrates at both their optimal temperatures and +5°C of their optimal temperatures (Figure 3.9). Assays were performed at the optimal pH of each enzyme as described in Section 2.7.2. Both glycoside hydrolases showed improved activity on 1,3:1,4-Barley β-glucan (compared to CMC) over time and across both assayed temperatures. These results show that both glycoside hydrolases are not only more active, but also more thermostable on the 1,3:1,4-Barley β-glucan substrate than on CMC.

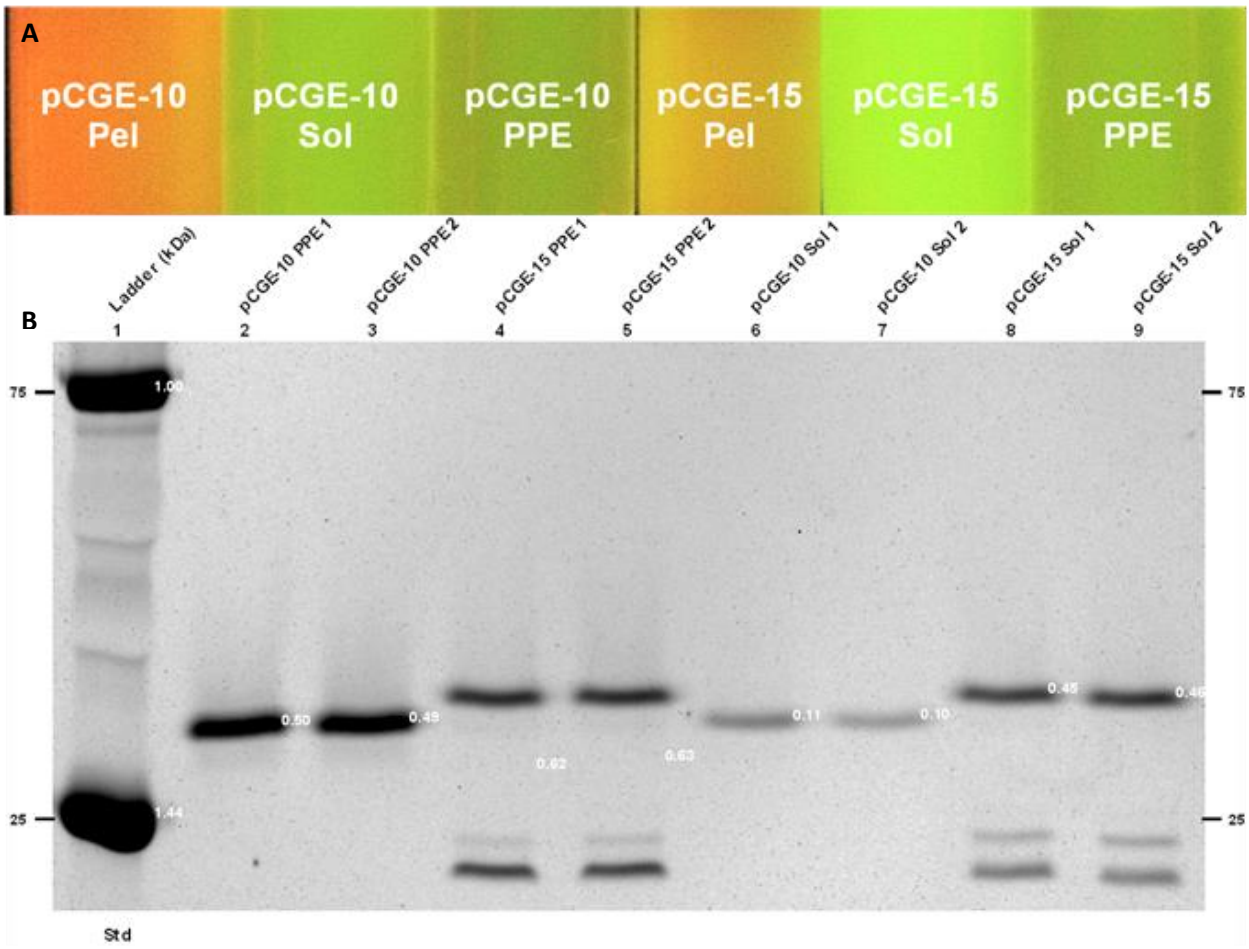



**Figure 3.1: Comparison of GFP activity and localization in expression vectors with and without TorA TAT signal peptide (pCGE-15 and pCGE-10, respectively).** Comparison of GFP fluorescence **(A)** in periplasmic (PPE), soluble (Sol), and pellet (Pel) fractions. GFP expressed with TorA signal peptide shows more fluorescence when visualized with a shortwave blue light box in all fractions, especially the soluble fraction. Increased fluorescence in the soluble fraction of GFP coupled to TorA is the result of a greater proportion of correctly folded and active protein in comparison to GFP without the TAT signal sequence. Fractions were run in side-by-side duplicates on a 12% SDS non-denaturing PAGE to preserve GFP fluorescence and visualized via UV tray **(B)**, relative band intensities are shown. The TorA signal peptide improved GFP activity by a factor of 1.26 in the periplasmic fraction and by a factor of 4.33 in the cytoplasmic fraction.

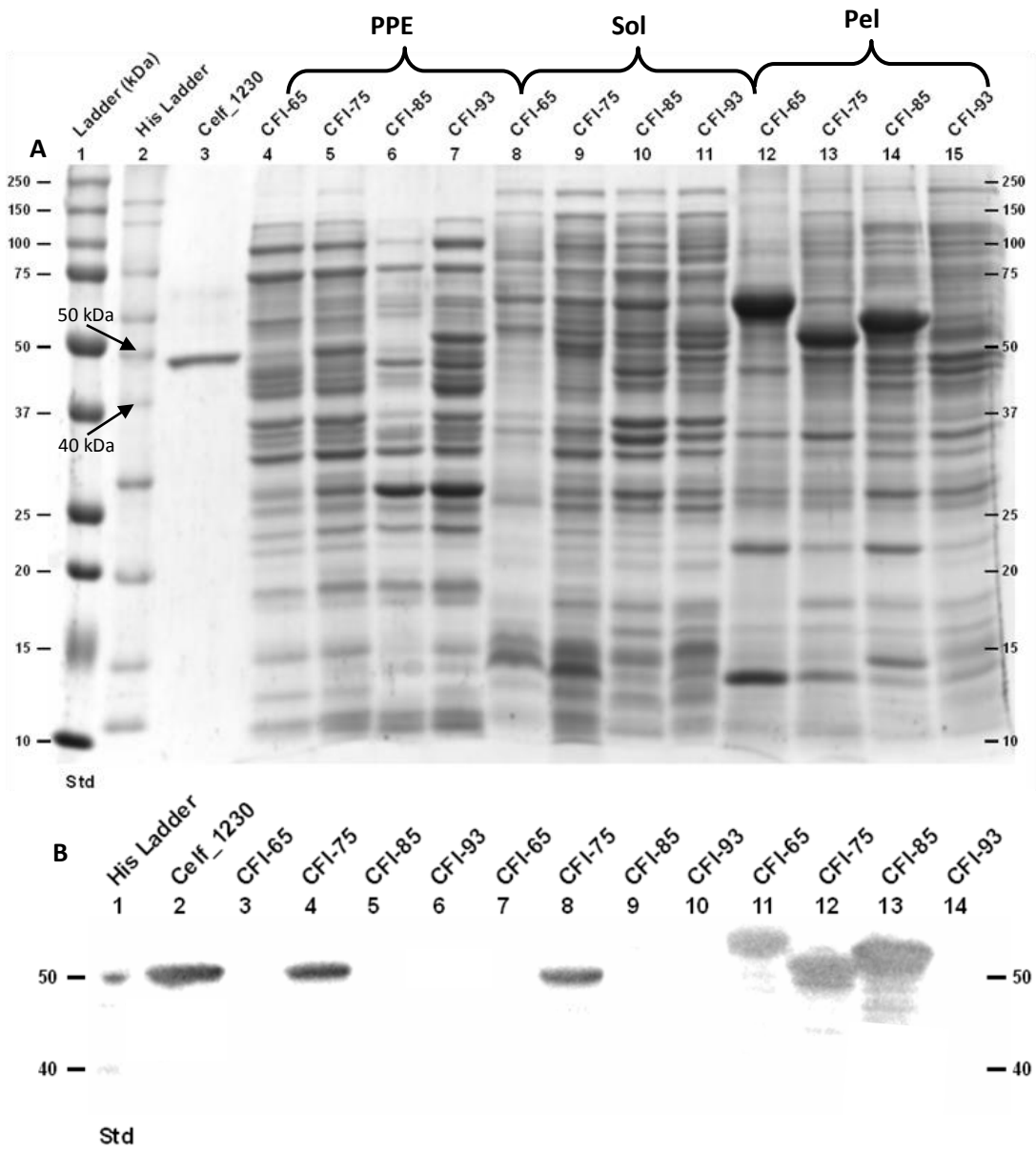



**Figure 3.2: Coomassie stained 12% SDS-PAGE showing periplasmic (PPE), soluble (Sol), and pellet (Pel) fractions of $His_6$ tagged constructs and corresponding Western blot.** In *E. coli*, only CFI-75 and CFI-93 are produced in a soluble form, but at such low levels they are unidentifiable via Coomassie staining **(A)**. The TorA signal peptide is shown to localize soluble CFI-75 into the *E. coli* periplasm **(B)**, while CFI-65 and CFI-85 remain totally insoluble **(B)**. A total of 300 ng of purified 6His tagged Celf_1230 was loaded as a western blot control, while 25 µg of protein from cell lysates was loaded per lane.

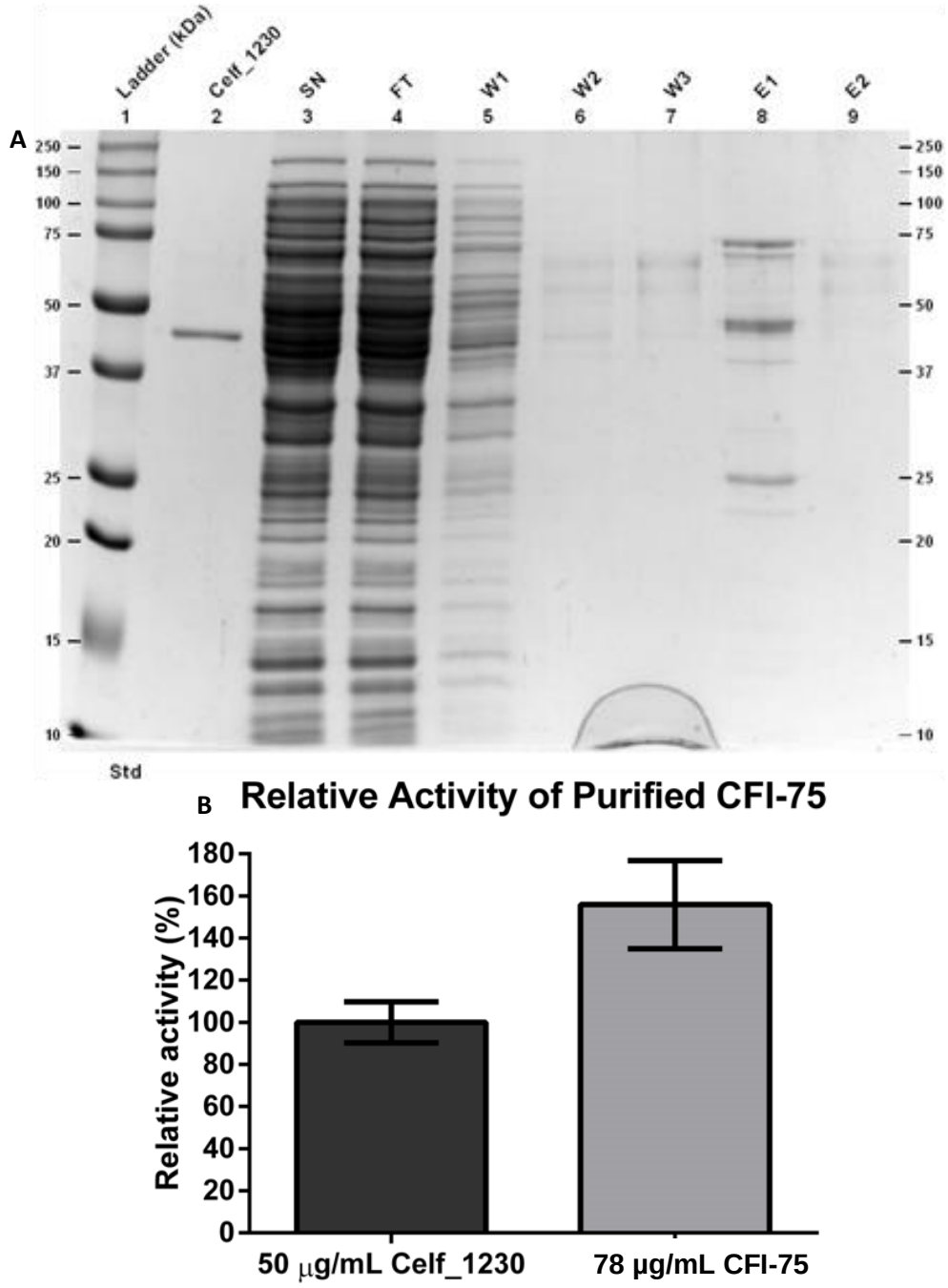



**Figure 3.3: Batch purification of *E. coli* produced CFI-75 with $Ni^{+}$ resin and relative activity on AZCL-Barley β-glucan.** Following purification **(A)** and buffer exchange, protein recovery was determined by testing relative activity against purified Celf_1230 **(B)** with no detectable activity in either the flow-through or wash fractions. In total 780 µg of CFI-75 was purified. SN: lysate supernatant, FT: column flow-through, W1 – W3: wash 1 – wash 3, E1 – E2: elution 1 – elution 2.

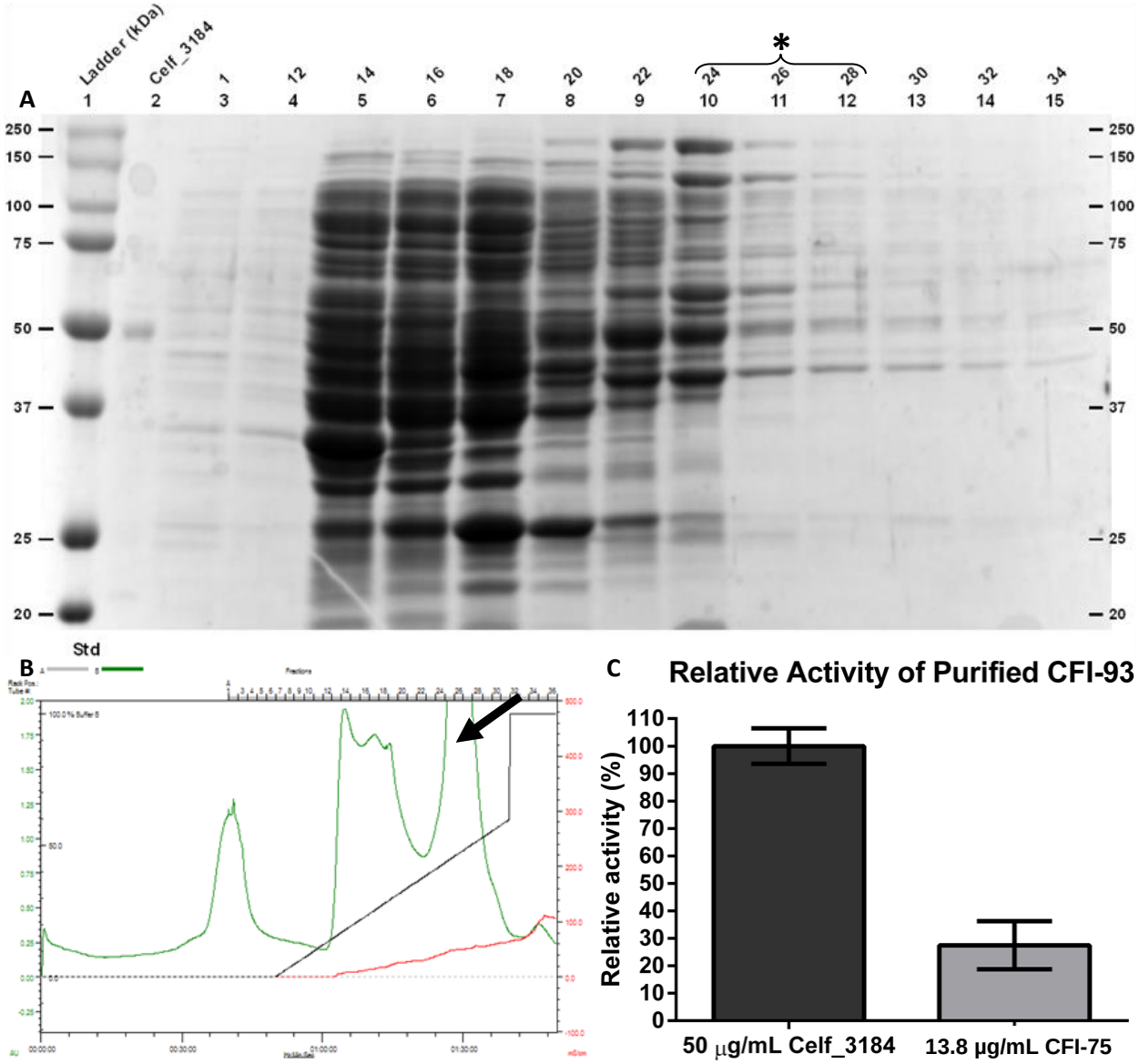



**Figure 3.4: Anion exchange of *E. coli* expressed CFI-93 using 5 mL cartridge of SourceQ resin and its relative activity on AZCL-Barley β-glucan.** Following purification **(A and B)** fractions with activity were pooled and buffer exchanged. Protein recovery was determined by testing relative activity against Celf_3184 **(C)**. In total 43 µg of CFI-93 was purified. Fractions marked by an asterisk contained activity and were subsequently pooled. Black arrow denotes peak on chromatogram containing activity.

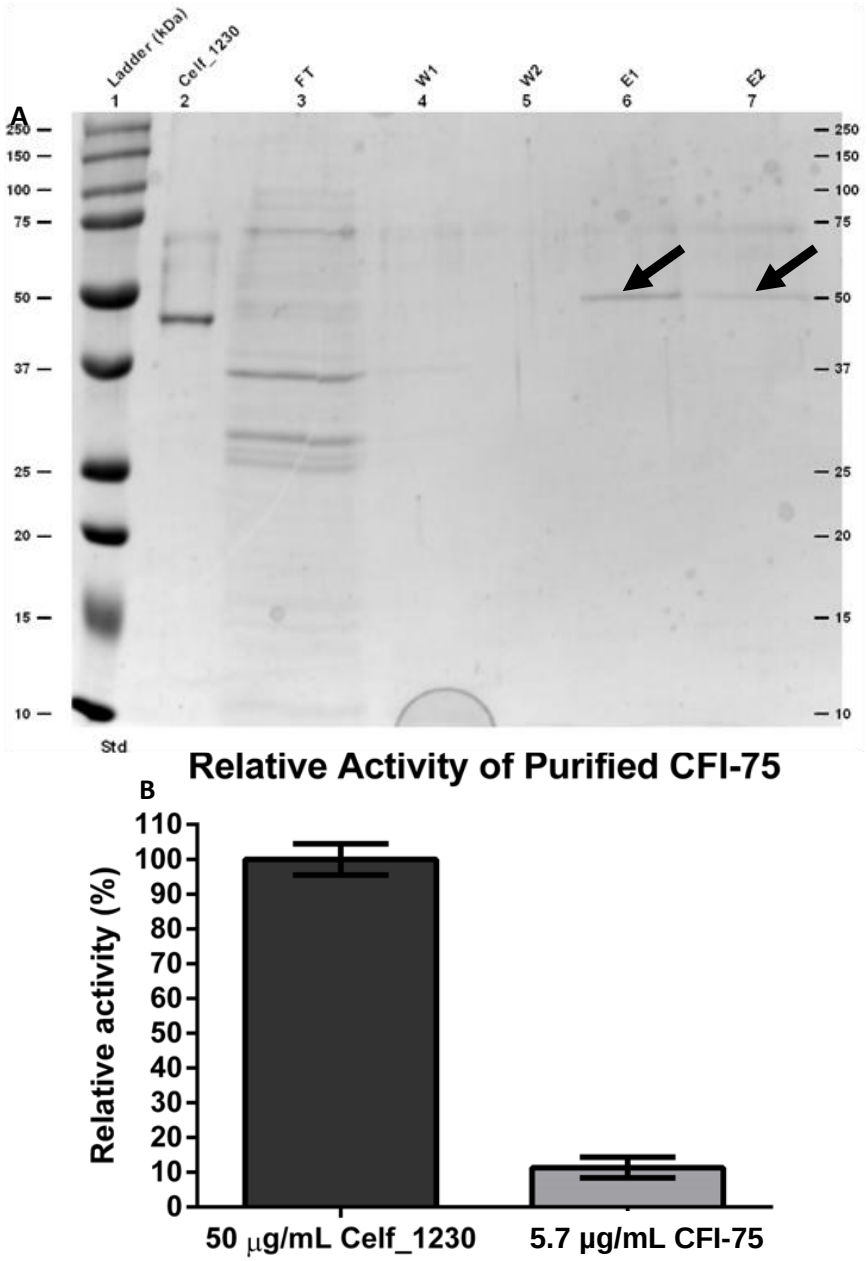



**Figure 3.5: Batch purification of CFI-75 from *C. glutamicum* culture supernatant with $Ni^{+}$ resin and its relative activity on AZCL-Barley β-glucan.** Following purification **(A)** and buffer exchange, protein recovery was determined by testing relative activity against Celf_1230 **(B),** in total 25 µg of CFI-75 was purified. Activity in culture supernatants was only detectable after 32 hour reactions. Black arrows denote purified CFI-75. FT: column flow-through, W1 – W2: wash 1 – wash 2, E1 – E2: elution 1 – elution 2.

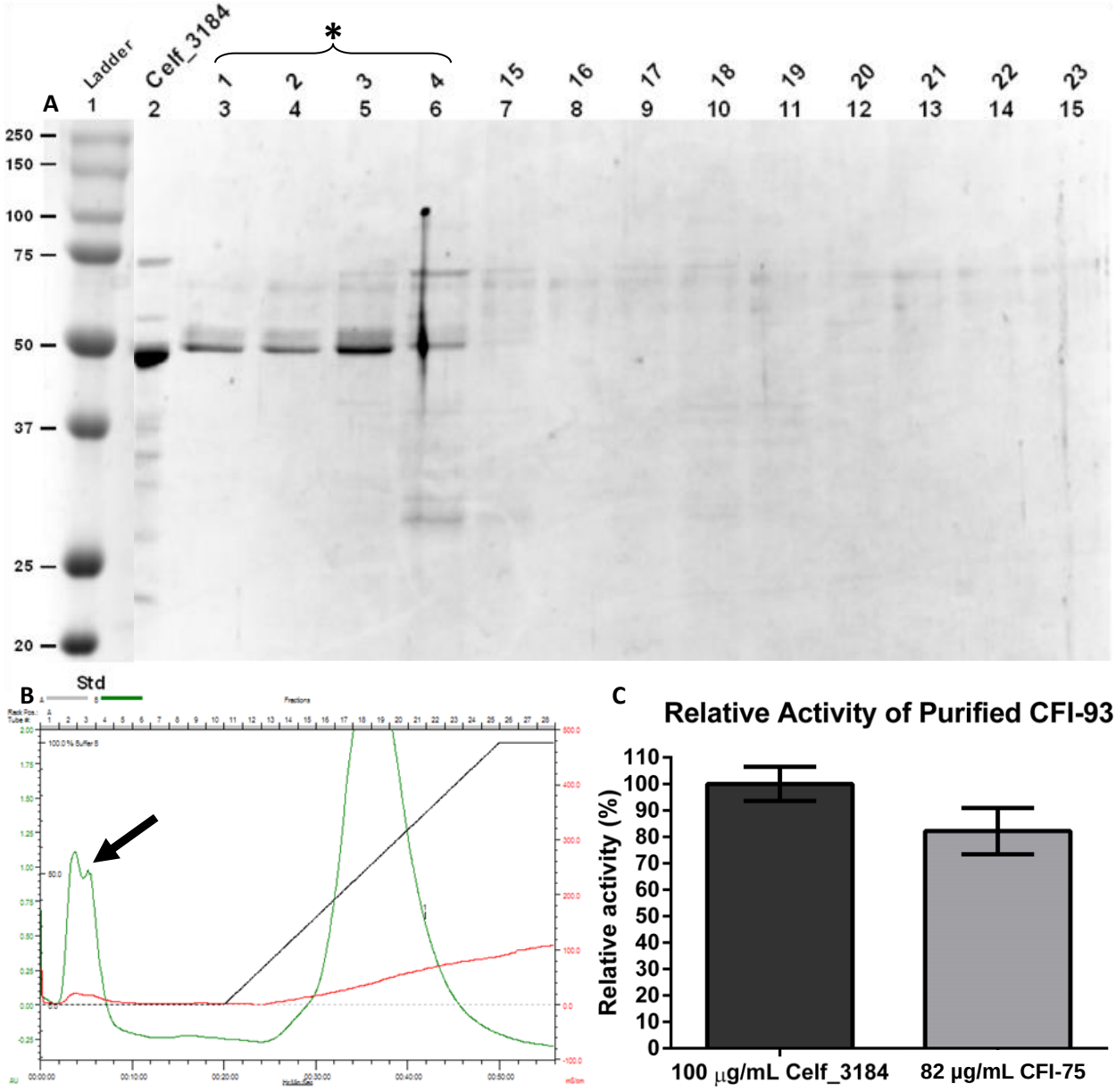



**Figure 3.6: Anion exchange of CFI-93 from *C. glutamicum* culture supernatant using 5 mL cartridge of SourceQ resin and its relative activity on AZCL-Barley β-glucan.** Following purification **(A and B)** fractions with activity were pooled and buffer exchanged. Protein recovery was determined by testing relative activity against Celf_3184 **(C).** In total 350 µg of CFI-93 was purified. Fractions marked by an asterisk contained activity and were subsequently pooled. Black arrow denotes peak on chromatogram containing activity.

A

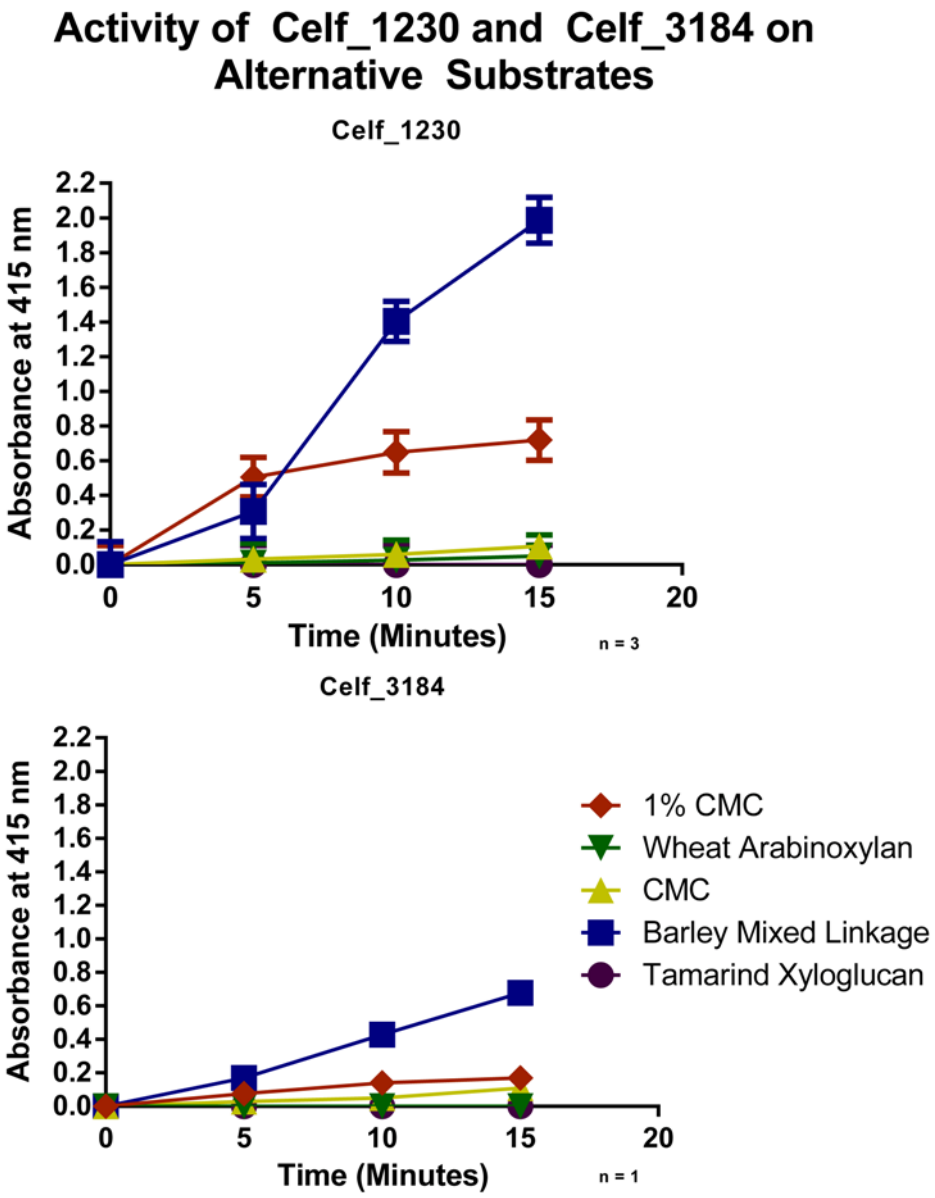



**Figure 3.7: Activity of Celf_1230 and Celf_3184 on alternative substrates.** All substrates assayed at 0.5% unless otherwise specified. Both Celf_1230 **(A)** and Celf_3184 **(B)** display activity on 1,3:1,4-Barley β-glucan that is significantly greater than on the classical glycoside hydrolase substrate CMC. Only one replicate is shown for Celf_3184, but the observed trend was similar across all data sets.

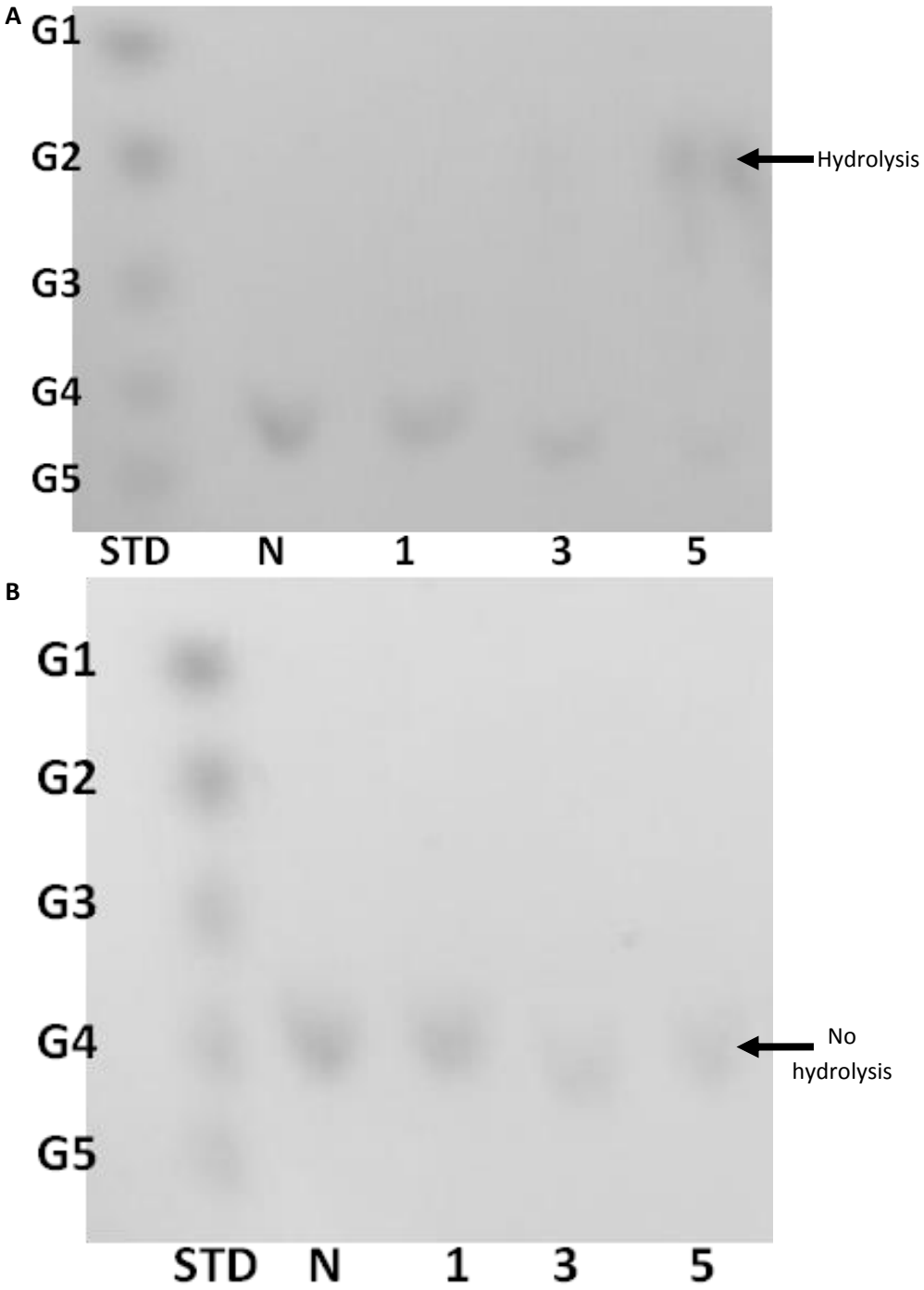



**Figure 3.8: TLC of Celf_1230 hydrolysis products on cellotetraose and 1,3:1,4-β-glucotetraose.**

Celf_1230 hydrolyzes cellotetraose (G4) to cellobiose (G2) rapidly **(A)** but does not hydrolyze 1,3:1,4-β-glucotetraose **(B)**. Time-points in minutes, N: negative reaction, G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, G5: cellopentaose.

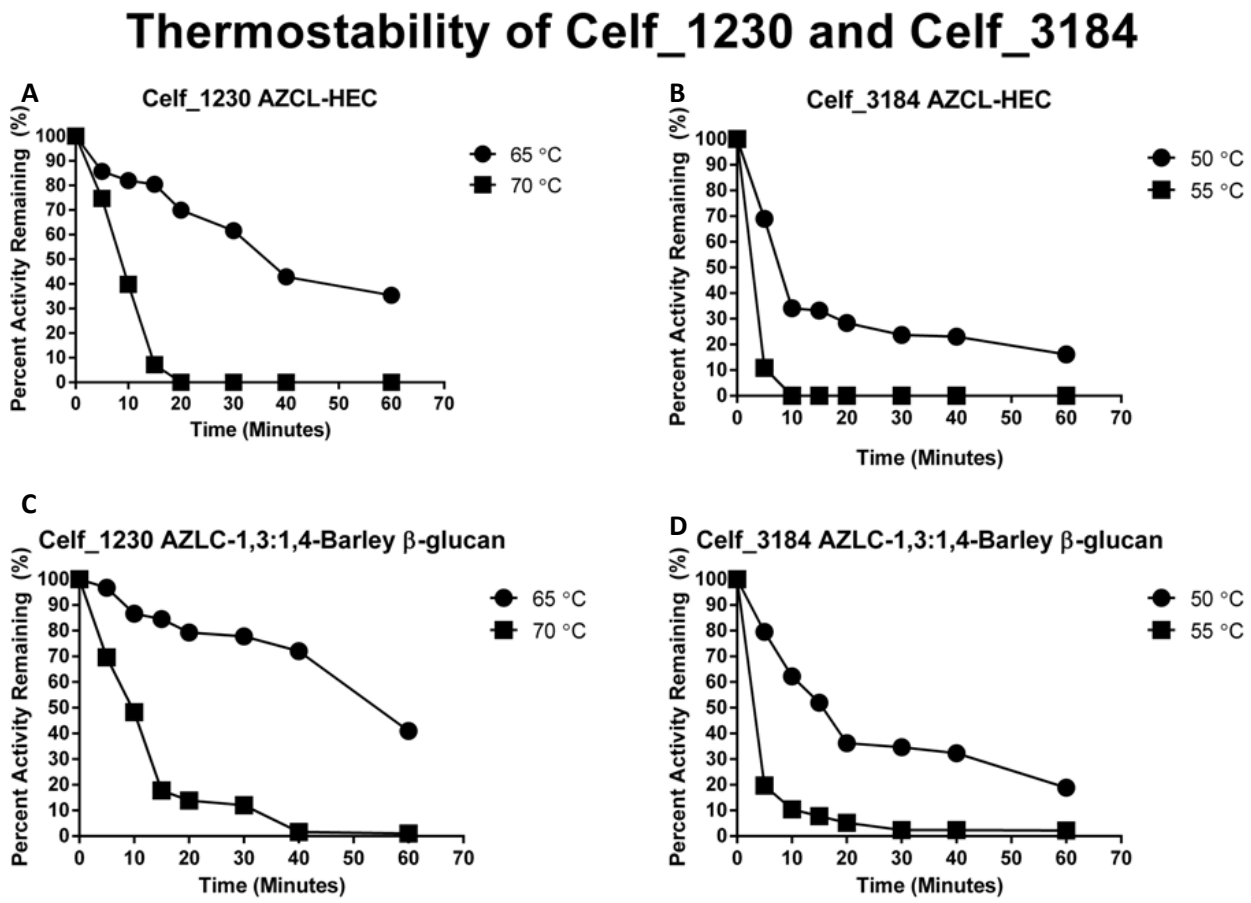



**Figure 3.9: Thermostability of Celf_1230 and Celf_3184 at optimal temperature on CMC and 1,3:1,4-Barley β-glucan.** The thermostabilities of Celf_1230 (left panels) and Celf_3184 (right panels) on both AZCL-HEC (top panels) and AZCL-1,3:1,4-Barley β-glucan (bottom panels). The thermostability profile of Cel6C is unique among characterized glycoside hydrolases originating from *C. fimi*. Displayed data is of a representative data set; however the observed trend is similar across all sets.

# Chapter 4

# Discussion

## 4.1 TAT dependent export

The utilization of a TAT signal peptide during heterologous protein expression in *E. coli* is in itself not novel; however, co-opting this export pathway is an emerging facet of certain Gram-positive expression systems [61, 67, 59, 57]. This work demonstrated two key advantages in using TAT dependent export when expressing heterologous proteins in both *E. coli* and *C. glutamicum*. It is understandable that individuals unfamiliar with TAT dependent export may expect most or all expressed heterologous protein to be located in the periplasm. However, as demonstrated here and in other works [50, 59], that is not the case. In reality, the overall quantity and distribution of expressed protein is nearly identical to the control vector lacking the TAT signal peptide. The primary difference is in the proportion of soluble active protein between vectors with and without the TAT signal peptide. In the case of eGFP expression with the TorA TAT signal peptide, significantly more fluorescence was detected in the cytoplasmic fraction than in the corresponding fraction of the control vector. The TAT export pathway accomplishes this by folding expressed proteins cytoplasmically, where they are able to associate with (predominately) cytoplasmic chaperones and cofactors [50]. The association of cytoplasmic cofactors and chaperones is an important factor in maximizing activity in disulfide bond containing proteins (like eGFP) expressed in *E. coli* strains like Shuffle Express™. This strain has mutations which provide an oxidizing redox potential in the cytoplasm (ΔtrxB and Δgor) in addition to expressing a cytoplasmic disulfide bond isomerase (DsbC) which helps promote the formation of disulfide bonds. Therefore, it is beneficial to have multi-disulfide bond containing proteins fold in the oxidative cytoplasm of similar strains [6]. The improved cytoplasmic fluorescence of expressed eGFP shows the TAT pathway folds proteins cytoplasmically prior to export. Furthermore, the utilization of TAT dependent export in a Gram-positive expression host allows for expressed proteins to be secreted out of the cell (reaching concentrations of 21 mg/L in the case of

GFP), directly into the culture supernatant since Gram-positive organisms lack a traditional periplasmic space [61, 57]. This greatly simplifies the harvest and purification of expressed proteins.

**4.2 Glycoside hydrolase analysis**

*4.2.1 Celf_0233 and Celf_2403*

Neither Celf_0233 nor Celf_2403 were produced in a soluble, active manner in *E. coli*, the entirety of the expressed protein is completely insoluble and inactive. Unfortunately, no heterologous protein production was observed in *C. glutamicum* either. Though more information needs to be gathered in order to accurately determine why these proteins would not express in the Gram-positive expression system, a few hypotheses are currently possible. It was initially thought that the secretion signal differences between the native protein and the TAT sequence utilized by the expression system could account for the shortcomings. It is possible that a protein destined for Sec dependent secretion is not compatible with TAT dependent secretion. However, this is likely not the case since the native Celf_1230 protein has a Sec leader sequence and was produced in detectable quantities in *C. glutamicum* (Figure 3.5). It is much more probable that the expression issues arose from the fact that the *C. glutamicum* expression system still requires a significant amount of optimization. Just like in *E. coli*, growth and induction conditions are intimately related to the stability and structure of individual proteins. Conditions such as optical density at induction, IPTG concentration, induction length and temperature, growth supplements, and using other stains of *C. glutamicum* still need to be checked when attempting to express these proteins before it can be stated that they do not express in the *C. glutamicum* expression system.

*4.2.2 Celf_1230*

The native celf_1230 gene expressed well in both *E. coli* and *C. glutamicum*. Though a significant portion of the *E. coli* produced protein was insoluble, some active protein was still produced. Celf_1230 itself is an intriguing protein, with an optimal temperature of 65°C (Figure 3.9) and originating from a soil bacteria whose optimal growth temperature is only 30°C. This could make Celf_1230 a good candidate for future study and possible engineering for industrial purposes. Mixtures of Celf_1230 and Celf_3184 were previously shown to only have additive activity on cellulose [27] , further study on other types of activity would need to be assayed for synergistic action. Other than the activity of Celf_1230 on 1,3:1,4-Barley β-glucan, its lack of activity on alternate substrates was not surprising as the GH6s only had endoglucanase and cellobiohydrolase activities reported. It was initially thought possible that the significantly improved activity of Celf_1230 on 1,3:1,4-Barley β-glucan compared to the traditional endoglucanase/cellulase substrate, carboxymethylcellulose (CMC) (Figure 3.7), could have been explained by a novel β-1, 3 hydrolysis activity, though this was later rejected after the analysis of Celf_1230 hydrolysis products of 1,3:1,4-β-glucotetraose. This analysis showed that the enzyme only cleaved at the β-1,4 glucose linkage and not at the β-1,3 glucose linkage (Figure 3.8).

*4.2.3 Celf_3184*

The native Celf_3184 protein has been remarkably troublesome to express in our lab in *E. coli*, where it arbitrarily produced active protein. A significantly low induction temperature of 15°C (in tandem with late induction) were previously used [12] to produce soluble protein in *E. coli*. However, with the same construct in *C. glutamicum,* induction during early exponential growth carried out at 30°C was more than sufficient to produce easily detectable quantities of protein (Figure 3.6). This is likely due to the fact that the native Celf_3184 TAT leader sequence is much more compatible with the *C. glutamicum* machinery than in *E. coli*. Regardless, the amount of produced protein in *E. coli* was so insignificant it

was undetectable as a strongly induced protein by SDS-PAGE analysis (Figure 3.2 A). In *C. glutamicum* however, the protein expresses much better even at induction temperatures 15°C higher than that used in *E. coli* (Figure 3.6 A). Activity data for Celf_3184 falls in line with the literature, as this GH has been previously characterized. Celf_3184 exhibited the same characteristic of improved activity on 1,3:1,4-Barley β-glucan compared to CMC.

**4.3 Carboxymethylcellulose is not a preferred substrate**

Cellulose is itself a crystalline water-insoluble polymer, therefore rendering the traditional reducing sugar assay virtually useless for the measurement of cellulase activity from enzymes that do not hydrolyze crystalline cellulose. For this reason, the highly substituted and water-soluble substrate CMC became standard for assaying cellulase activity. The comparison of activity of Celf_1230 and Celf_3184 on both CMC and 1,3:1,4-Barley β-glucan contrast the traditional zeitgeist of CMC being the "ideal" substrate for assaying endoglucanase activity [62]. In fact, there is an emerging trend of moving away from the utilization of CMC due to its high degree of substitution to maximize its solubility. The current observation is that the level of substitution of CMC adversely affects the activity on enzymes acting on it. It is possible that the introduction of alternating β-1,3 and β-1,4 glycosidic linkages alters the stereochemistry of the carbohydrate polymer, making the substrate more accessible to enzymatic cleavage. This "kinked" carbohydrate polymer is much more similar in structure to hemicellulose, which has a random, amorphous structure. Not to be overlooked however, is the possibility that 1,3:1,4-Barley β-glucan is an actual preferred substrate for these particular GH6s from *C. fimi*.

**4.4 Applications of a Gram-positive expression system**

The utilization of this Gram-positive expression system (in concert with a TAT signal peptide) for the production of heterologous proteins has a number of practical applications. Most notably, its use could

decrease the labour and cost of purifying heterologous proteins, since they would be pumped directly into the culture supernatant. From there, the expressed proteins could easily be purified using a variety of chromatography methods including affinity or ion exchange chromatography or even a simple gel filtration. In the case of *C. glutamicum*, there is the added benefit of the organism not producing any secreted proteases, so heterologous proteins would be safe from degradation in the culture supernatant. This expression system also virtually eliminates the need for cell lysis during the harvest of heterologous proteins.

Our lab plans on using this expression system for genes originating from Gram-positive organisms that are troublesome to produce in *E. coli*. Research in the Wakarchuk lab has recently shown that *C. glutamicum* may be an expression platform to understand Gram positive actinobacterial protein O-mannosylation which was first observed with *C. fimi* cellulases in the 1980's (Nakita Buenbrazo, MSc thesis 2016). So far there have not been many other examples of enzymes from Gram-positive bacteria, but examples in other organisms include cellulases from *Clostridium thermocellum*, and *Streptomyces lividans* [38]. Genes from the companion organism *C. flavigena* which is also being examined in our laboratory are also targets for GH and glycoprotein research.

This expression system is also from an organism "generally regarded as safe" or GRAS. This means that proteins produced in this organism are not contaminated with endotoxin that is a serious concern for proteins produced in *E. coli*. Our lab is very interested in the expression of other CAzymes, notably glycosyltransferases, for the modification of therapeutic proteins and mammalian cell surface glycan remodeling. The use of a GRAS expression system could aid with the production of these enzymes in a form which would be better for biomedical applications than the corresponding *E. coli* produced enzymes.

## 4.5 Future directions

### *4.5.1 Optimization of TAT signal sequence*

In order to optimize both the folding and localization of heterologous proteins in both *E. coli* and *C. glutamicum* further study could be directed towards identifying and using a TAT signal sequence that shows reliable functionality in both organisms across a wide variety of protein types. This work brings to light the Celf_3184 TAT leader sequence as a potential avenue for further study in *C. glutamicum* expression and secretion.

### *4.5.2 Activity of glycosylated glycoside hydrolases and other proteins*

While glycosylation of *C. fimi* GHs has not been shown to be required for enzymatic activity, there is evidence for it affecting protein stability [21].Further comparison of production and stability between the glycosylated and non-glycosylated forms of Celf_1230 and Celf_3184 could determine if the modification has any impact on their application to biomass hydrolysis. Furthermore, a significant amount of protein engineering could be directed towards these two GHs to capitalize on their thermostability and possibly even improve their respective activities. Other possibilities are that engineered glycosylation sites might aid in the secretion and stability of other enzymes produced in *C. glutamicum*.

### *4.5.3 Optimization of Corynebacterium glutamicum expression*

A significant amount of work remains to optimize both the growth and expression conditions for *C. glutamicum*. Primarily, identifying the optimal strain for expression and combination of media conditions like buffers to maintain culture pH, amino acids, urea as a nitrogen source, vitamins like biotin and thiamine, glucose as an energy source, and essential anions and cations as well as trace elements like $FeSO_4$ and $MnSO_4$ could greatly increase the levels of heterologous protein production.

This optimization could easily increase protein production many times over as was seen with the addition of $Ca^{+2}$. Calcium sufficient cultures routinely secreted heterologous proteins at levels over 30-fold greater than in comparison to calcium deficient cultures [57]. Furthermore, identifying the optimal induction conditions would also be beneficial. And finally, determining the kinetics of heterologous protein production following induction and how long it maintains its integrity in the culture supernatant (although this is likely protein dependant) are important parameters to consider.

**4.6 Concluding remarks**

This work provides evidence that the expression of heterologous proteins in *E. coli* (in this case for GHs originating from *C. fimi*) does not always produce proteins with the same characteristics as when they are produced in their native organisms (eg., solubility, activity, stability, and expression levels). It supports the thought that it may be time to revisit some previously characterized GHs from other Gram-positive organisms, since they would have all been produced in *E. coli*. Further modifications to the expression vector, such as the utilization of a *C. fimi* TAT sequence and the addition of a tandem promoter could help to produce secreted soluble protein from troublesome genes. In addition, other stains of *C. glutamicum* could also be investigated in an attempt to increase the efficiency of heterologous protein expression. Following optimization, this expression system could serve as an important adjunct to *E. coli* for characterizing new GHs being identified by genome sequencing of environmental or biomedical isolates of Gram-positive bacteria.

**Chapter 5**

**Appendix**

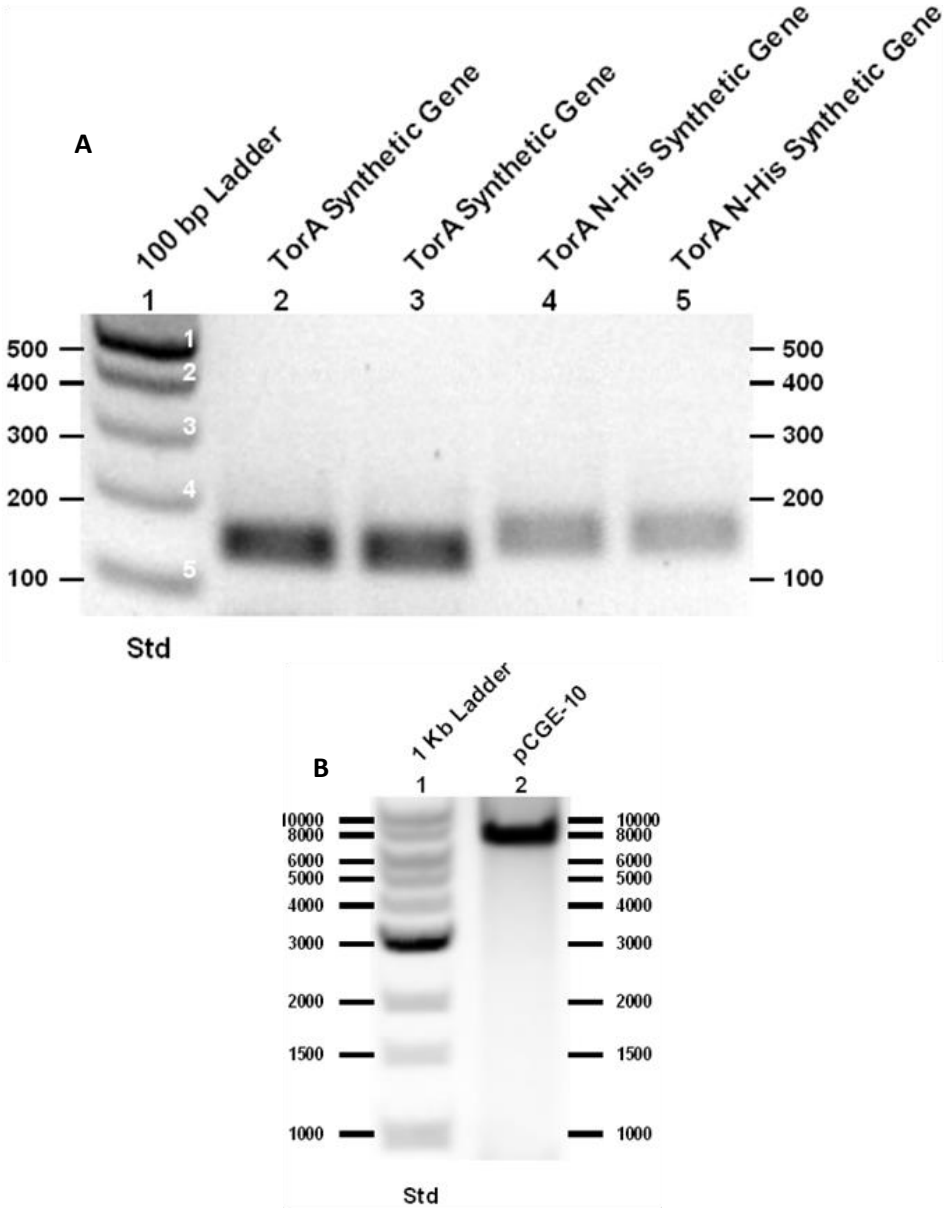



**Figure 5.1: 2.5% agarose gel showing digested TorA synthetic genes (A) and 0.8% agarose gel showing unmodified pCGE-10 (B).** TorA (133 bp) and TorA N-$His_6$ (148 bp) synthetic genes **(A)** that were ligated into the NdeI site of the unmodified pCGE-10 (7519 bp) shuttle vector **(B)**.

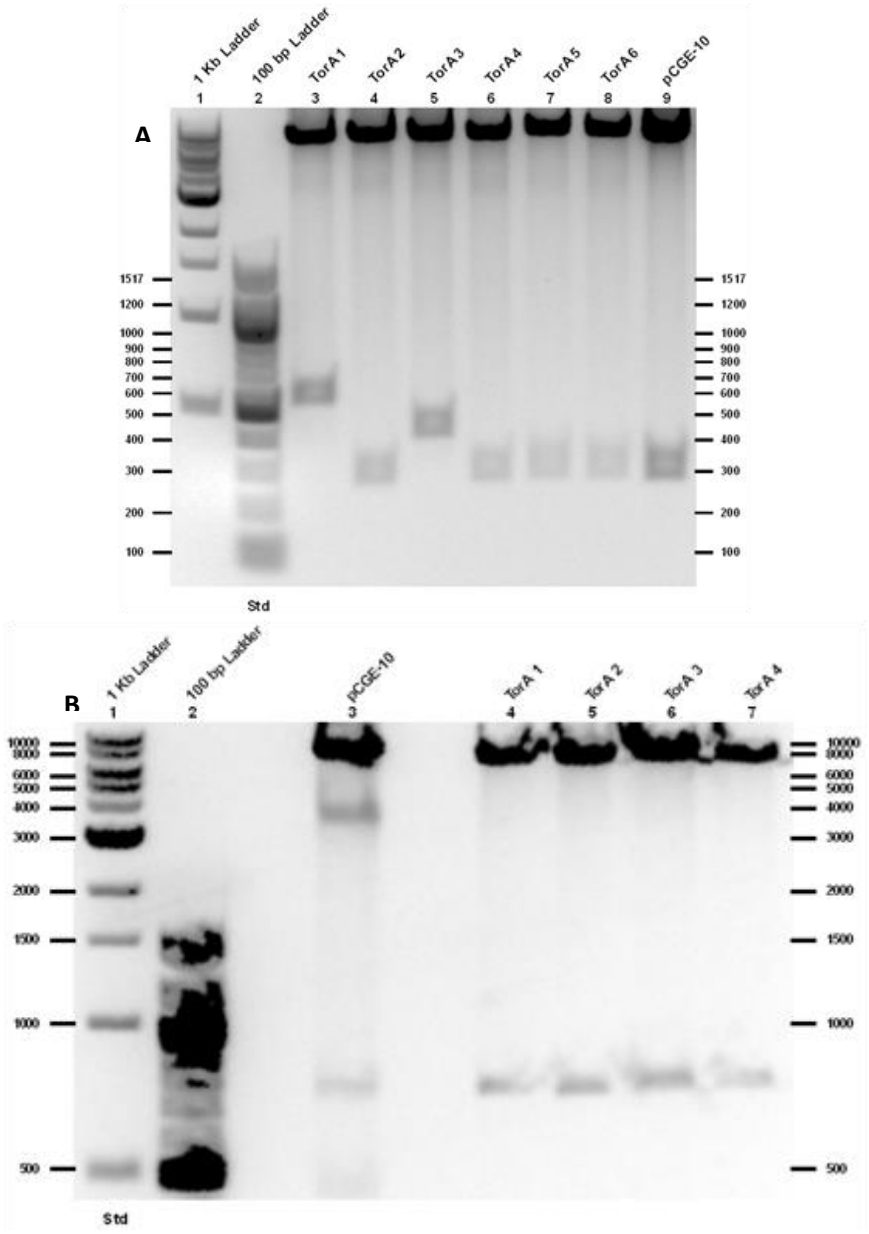



**Figure 5.2: 1.5% agarose gels checking pCGE-15 clones for TorA insertion and synthetic gene orientation.** TorA insertions checked via diagnostic digest with BamHI and NdeI **(A)**. Lane 3 shows a double insertion event and lane 5 shows an insert of the correct size. Orientation of synthetic gene was confirmed by diagnostic digest with NdeI and EcoRI **(B)**. The clone designated "TorA 3" was shown to have the correct insert in the correct orientation and was sequenced for confirmation. Accessory bands in lane 3 are due to incomplete digestion.

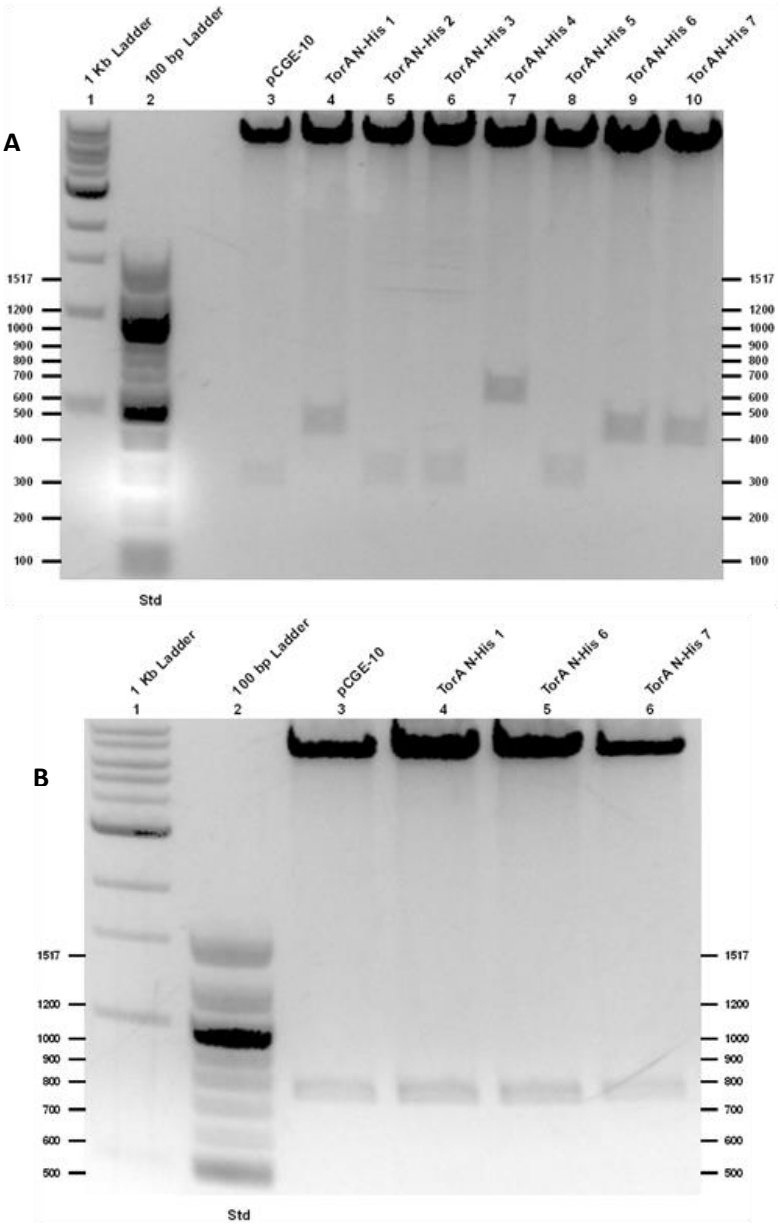



**Figure 5.3: 1.5% agarose gels checking pCGE-20 clones for TorA N-His$_6$ insertion and synthetic gene orientation.** TorA N-His$_6$ insertions checked via diagnostic digest with BamHI and NdeI **(A)**. Lane 7 shows a double insertion event while lanes 4, 9, and 10 show an insert of the correct size. Orientation of synthetic gene was confirmed by diagnostic digest with NdeI and EcoRI **(B)**. The clones designated "TorA N-His$_6$ 1, 6, and 7" were shown to have the correct insert in the correct orientation and were sequenced for confirmation.

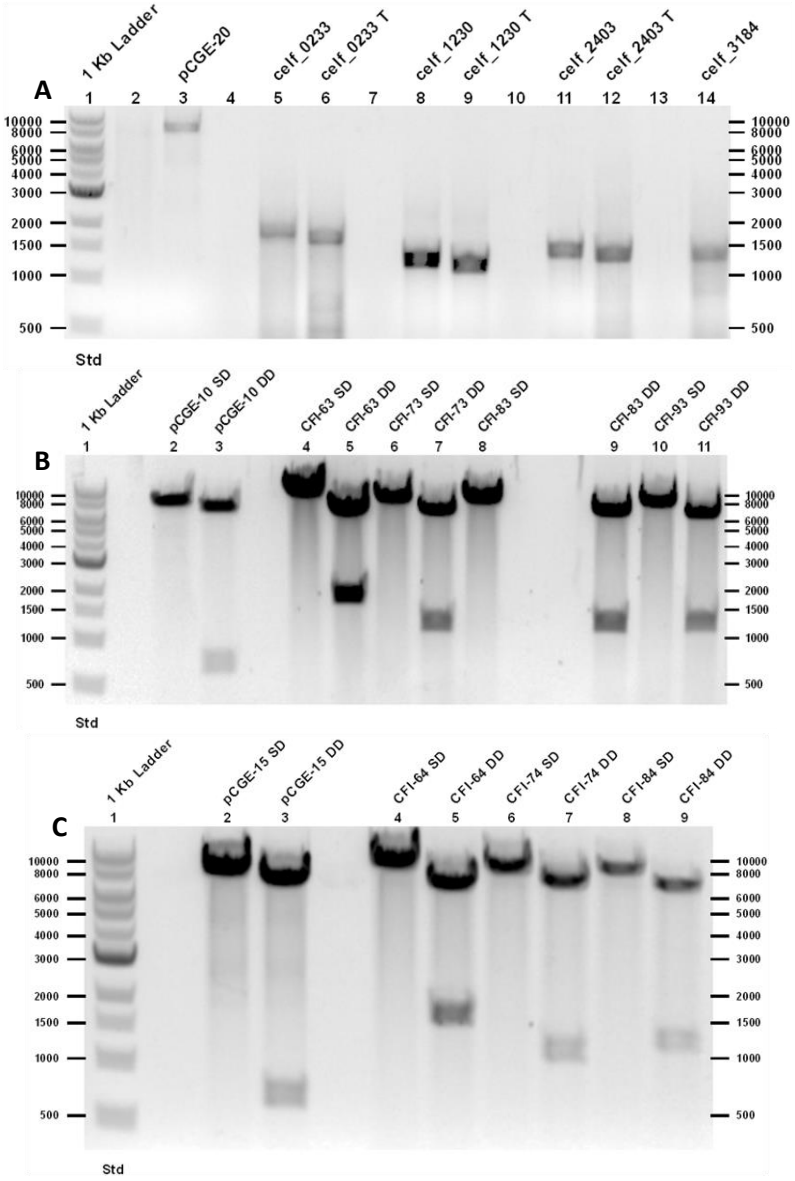



**Figure 5.4: Glycoside hydrolase PCR products and construct library in pCGE-10, pCGE-15 and pCGE-20.**

Ligation reactants **(A)** show amplified native glycoside hydrolases and truncated (T) glycoside hydrolases lacking native leader sequences. Single and double digestions of glycoside hydrolase constructs in pCGE-10 **(B)** with native gene leaders and in pCGE-15 **(C)** lacking native gene leaders. Constructs in pCGE-20 are not shown as they appear identical to pCGE-15 constructs (the only difference being the addition of an N-terminal $His_6$ on inserted gene). The 750 bp band observed in lane 3 of **(A)** and **(B)** is the eGFP stuffer sequence intrinsic to the vector.

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