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Optimization of Chitinase C expression from the pM3CRYY plasmid in *Escherichia coli* BL21(DE3) and determination of secretion status

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SUMMARY Chitin is an essential polysaccharide found in fungal cell walls, crustacean shells, and insect exoskeletons that can be broken down by chitin-degrading enzymes called chitinases. As conventional pesticides contain chemicals associated with a number of severe health risks, chitinase C (ChiC) expressed from the soil microbe *Pseudomonas aeruginosa* has been proposed as a safer and more sustainable alternative to target chitin-containing pests. A previous study generated a ChiC expression plasmid, pM3CRYY, by cloning *chiC* from *P. aeruginosa* PAO1 into a pET28a backbone. Through induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), they demonstrated expression of a 55 kDa protein from pM3CRYY, though the identity of this protein was not confirmed. It is also unknown if the expressed protein is secreted when expressed in *E. coli* BL21. Thus, we aimed to confirm the identity of 6xHis-tag conjugated ChiC off pM3CRYY, and to determine the optimal IPTG induction conditions for ChiC expression. We found that *chiC* in pM3CRYY is conjugated to a 6xHis-tag at the 5' region, and confirmed the presence of His-tagged ChiC in IPTG-induced pM3CRYY *Escherichia coli* BL21(DE3) cultures. Optimal conditions for protein expression were 0.1 mM IPTG for >6 hours at 37ºC. Additionally, lysing cells by bead beating resulted in higher amounts of protein extracted compared to a boiling lysis method. Interestingly, we also detected 6xHis-tagged ChiC in the soluble fractions of induced cultures. However, it was unclear if the protein was being released into the extracellular space because of active secretion or through the action of cells lysing and releasing their intracellular contents. Therefore, we attempted to determine if ChiC was being secreted under induction conditions by probing for the presence of the putative cytosolic chaperone protein, DnaK. Although our results suggest cytosolic proteins and ChiC are released into culture supernatants as a result of cell lysis, this experiment should be repeated before concluding the secretion status of ChiC in this expression system.

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INTRODUCTION

Chemical pesticides often used in agriculture are associated with health risks when humans or animals are exposed through skin contact, inhalation, or ingestion. Negative health outcomes include dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive, and endocrine pathologies. In severe cases, exposure can result in hospitalization or death, warranting an urgent need for alternative pest control measures (1).

Chitin, or β-(1–4)-poly-N-acetyl-D-glucosamine, is a major component of insect and crustacean exoskeletons (2). It is a biodegradable, non-toxic polymer, and an emerging target of insecticides (3). Chitin degradation is catalyzed by chitinase, a hydrolytic enzyme produced by a diverse array of chitinolytic organisms including eukaryotes, prokaryotes,

archaea, and viruses (4). Compared to conventional chemical pesticides, the use of insecticidal microorganisms as biocontrol agents provides a number of advantages, such as minimal consumer health risks and environmental impact. Therefore, chitinase has the potential to be a novel, highly sustainable method of pest management for chitin-containing organisms (2). In particular, chitinase C (ChiC) is a 55 kDa enzyme secreted by chitinolytic soil microbes, including *Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* (5). *P. aeruginosa* is an advantageous strain to use in biotechnological applications because it can inhabit a large range of environments; however, its pathogenicity could limit its suitability to be used on a large scale (6). It is also not an organism optimized for the purification of large amounts of protein in an industrial setting. Therefore, a more practical alternative would be to first optimize an expression protocol in *Escherichia coli* BL21(DE3), and use the purified chitinase enzymes as a direct insecticidal agent.

Previous studies have shown that chitinases originating from *C. violaceum* can be successfully purified from *E. coli* while remaining functionally active against colloidal chitin and synthetic substrates (6-8). A native signal peptide has been identified in *C. violaceum* to direct ChiC's secretion into culture medium (6). Folders et al. found that ChiC expressed by *P. aeruginosa* PAO1 appears to be gradually secreted into culture media (7). They suggest that secretion of this functionally active ChiC in *P. aeruginosa* may rely on cleavage of the N-terminal region of the protein. Sequence analysis of the secreted ChiC in this study also revealed it to be similar to chitinases secreted by other species (7). Two chitinases (I and II) were also purified directly from *P. aeruginosa* K-187 and assayed to show catalytic activity against chitin (8). These studies demonstrate the potential to purify functional chitinase from microbes for use in industrial applications.

More recently, Rocha et al. investigated the expression of *P. aeruginosa* chitinase C in *E. coli* BL21(DE3) by cloning *chiC* into a protein expression vector, pET28a, to create pM3CRYY, a ChiC expression plasmid (6, 9). They confirmed insertion of *chiC* into pET28a using PCR and Sanger sequencing. Using the pM3CRYY plasmid A3 clone, they induced the expression of ChiC using isopropyl β-D-1-thiogalactopyranoside (IPTG) and detected a protein expressed at the expected size of ~55 kDa via SDS-PAGE (9). However, they did not confirm the protein identity. Additionally, the optimal IPTG induction conditions of ChiC expression in *E. coli* BL21(DE3) have yet to be elucidated. Based on findings from previous research, we aim to confirm the identity of ChiC expressed off of pM3CRYY A3, optimize induction conditions, and test whether the protein is secreted from *E. coli* BL21(DE3). Our results demonstrate that the pM3CRYY A3 plasmid does indeed contain the correct ChiC ORF, and that the protein is optimally expressed at 6 hours of 0.1 mM IPTG induction. Comparing ChiC and cytosolic protein DnaK in the supernatants of induced cells also revealed that the enzyme may be released from lysed E. coli BL21(DE3) instead of being secreted.

METHODS AND MATERIALS

Bacterial culture and isolation of pM3CRYY A3 and pET28a. *E. coli* BL21(DE3) was grown on lysogeny broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) agar plates. An overnight culture was obtained by inoculating 5 mL LB with a single colony and incubating at 37ºC while shaking at 250 rpm. Chemically competent *E. coli* BL21(DE3) stocks were prepared (10). *E. coli* BL21(DE3) containing pM3CRYY A3 plasmid and *E. coli* DH5 containing pET28a were obtained from the University of British Columbia's MICB 471 stocks and grown on selective LB agar plates supplemented with kanamycin 25 ug/mL (LB-kan). Plasmids were then isolated from overnight cultures of the strains of interest using the EZ-10 Spin Column Plasmid DNA MiniPrep Kit (BioBasics) in accordance with manufacturer instructions, and resulting preparations were quantified through spectrophotometry. *E. coli* BL21(DE3) containing pET28a plasmid was then obtained by transforming competent *E. coli* BL21 with pET28a plasmid. 50 ng of template was mixed with 100 uL of competent *E. coli* BL21(DE3) briefly prior to incubating for 30 minutes on ice. Following that, samples were heat-shocked in a 42ºC water bath, then put on ice for 3 minutes. 900 uL of LB media was then added and reactions were left shaking at 37ºC for 1 hour before plating on LB-kan plates.

Plasmid identity confirmation using restriction enzyme digest and polymerase chain reaction (PCR) analysis. 500 ng of each isolated plasmid was digested using 1 uL each of Cutsmart EcoRI and HindIII (New England Biolabs, Cat #: R0101S and R0104S) in a 20 uL total reaction volume. Reaction solutions were incubated for 20 minutes at 37ºC. Samples were then run on 1.5% agarose in tris acetate EDTA (TAE) buffer gels prepared with SYBR Safe dye (Invitrogen). For confirmation of plasmid identity using PCR, forward (3chiC F, 5'-TAT CCG AAT TCA TGA TCA GGA TCG ACT T -3') and reverse (3chiC R, 5'- AAT ACA AGC TTT CAG CGC AGC GG -3') primer sequences were obtained from Rocha et al (9). PCR reactions were performed in 50 uL reaction volumes comprising 50 ng of template, 500 nM of each primer, 0.5 uL of Platinum^{TM} SuperFi DNA polymerase (2 U/uL, ThermoFisher), 10 uL of 5x SuperFi buffer, 1 uL 10 mM dNTP mix, and 10 uL SuperFi GC enhancer. The reactions were incubated with 3 minutes initial denaturation at 98ºC, followed by 35 cycles of 15 seconds denaturation at 98ºC, 15 seconds annealing at 55ºC, and 1.5 minutes extension at 72ºC. In the final extension step, the reaction was held at 72ºC for 5 minutes. Reactions were then mixed with 6x Purple Loading Dye (New England BioLabs) before being run on a 1.5% agarose gel. Gels were then imaged using auto-optimal exposure settings.

Sanger sequencing analysis of pM3CRYY A3 and pET28a. pM3CRYY A3 and pET28a plasmids were prepared in 0.2 mL PCR strip tubes in accordance with GENEWIZ (Azenta Life Sciences) Pre-Defined guidelines. Sanger sequencing was performed for each of the two plasmids using GENEWIZ T7 universal primers and *chiC*-specific internal primers (F, 5'-CAAGTACGTGCCTTATCTGCAG-3', R, 5'-GAAGTCTTCTTTCATCGCGTCG-3') obtained from Rocha et al (9). Results were then aligned against the sequence for *P. aeruginosa chiC* CDS (AF279793.1) inserted into pET28a using SnapGene 6.2.

Evaluation of ChiC expression under multiple IPTG concentrations at different time points. Separate cultures of *E. coli* BL21(DE3) transformed with pM3CRYY A3 and pET28a were grown overnight as described previously. Cultures were diluted approximately 1:200 in LB-kan to an optical density (OD_{600}) of 0.04, then incubated at 37ºC while shaking at 250 rpm until an OD_{600} of 0.4–0.6 was reached. The subcultures were aliquoted into separate Erlenmeyer flasks of 30 mL each with the following induction conditions: uninduced, 0.1 mM, 0.5 mM, 1.0 mM, and 5.0 mM of IPTG. Uninduced control and induced cultures were incubated for 24 hours at 37ºC while shaking at approximately 200 rpm. At timepoints of 0, 3, 6, and 24 hours, 1 mL aliquots were removed from each flask and centrifuged at 1000 rcf, 4ºC for 15 minutes. The cell pellets and supernatants were then frozen separately at -20ºC. Samples for bead beating and boiling were taken from the same cultures but OD readings were not normalized prior to preparing the samples for SDS-PAGE.

Evaluation of induction conditions using SDS-PAGE. TGX Stain-Free FastCast 10% acrylamide gels (BioRad) were prepared according to manufacturer protocols. To extract proteins via boiling cell lysis, cell pellets were resuspended in 100 uL of distilled H_2O with 1x protease inhibitor cocktail (1:100, VWR, Cat #: M221), then boiled at 95ºC for 5 minutes. Boiled suspensions were then spun down at 15000 rcf for 10 minutes at 4ºC and the clarified protein-containing supernatants were transferred to a new tube, while the pellet was discarded. To lyse cells via bead beating, the MP Biomedicals FastPrep-24 system was utilized to homogenize supernatants and pellets resuspended in 100 uL dH_2O with 1x protease inhibitor cocktail for 30 seconds at 6.0 m/s (setting for *E. coli* cell lysis). Following homogenization, samples were spun down at 10000 rcf for 1 minute at 4ºC, then the supernatant was quickly transferred to microfuge tubes. Prior to SDS-PAGE, lysates were first spun down to pellet any residual beat beading sand. To prepare samples for SDS-PAGE, lysates were diluted 1:2 in 2x Laemmli buffer (BioRad) with 5% 2-mercaptoethanol added, then boiled at 95ºC for 5 minutes. Briefly, samples were loaded onto 10-well Stain-Free FastCast gels in 25 uL volumes along with 10 uL of PageRuler Unstained Protein Ladder (ThermoFisher, Cat # 26614) and run at 140V for 60 minutes.

Western blot analysis for confirmation of 6xHis-ChiC expression and presence of cytosolic proteins. Stain-Free 10% acrylamide gels were prepared as described above. Separated proteins were transferred to either PVDF or nitrocellulose membranes using Trans-Blot Turbo transfer packs (BioRad, Cat#: 1704156) and the Trans-blot Turbo transfer system. Membranes were blocked while shaking at room temperature for 1 hour with 1x TBS + 1% casein (BioRad, Cat#: 161-0782) prior to blotting using anti-6xHis antibody solution for ChiC or 1x TBS-T + 1% bovine serum albumin (Sigma-Aldrich, Cat#: A7906-500G) for anti-DnaK blots. The membranes were then incubated overnight at 4ºC in 13-15 mL anti-6xHis antibody (Invitrogen, Cat#: MA1-21315) diluted 1:1300 in blocking buffer or anti-*E. coli* DnaK antibody (1:2000, Enzo Life Sciences, Cat#: ADI-SPA-880-J). After performing 3 washes in 1x TBS-T for 5 min each, the membranes were incubated in 15 mL goat-anti-mouse-IgG HRP-conjugated secondary antibody (ThermoFisher, Cat#: 31430) diluted 1:10000 in blocking buffer while shaking at room temperature for 1 hr. Washes were performed using TBS-T as previously described. Visualization of immunoblots was done by incubating membranes in Clarity Western ECL substrate reagents (BioRad, Cat#: 170-5060) for 5 minutes. These steps were repeated using primary antibodies against *E. coli* σ^{70} factor (1:1000 in blocking buffer, Neoclone, WP004) and *E. coli* anti-σ E antibody (1:2000, Neoclone, WP007).

Evaluation of IPTG induction stress via viable cell growth curve analysis. In order to quantify growth rate and the number of viable cells present at each timepoint of the cultures with different IPTG concentrations, induced *E. coli* BL21(DE3) cultures transformed with pM3CRYY A3 or pET28a were diluted in 10-fold serial dilutions and spotted on LB-kanamycin agar plates in triplicate for each IPTG collection time point (0, 3, 6, and 24 hr) and concentration (0.1 mM, 0.5 mM, 1.0 mM). After allowing the spots to dry, plates were incubated overnight at 37°C. Colonies were counted the following day, and CFUs were obtained to generate a growth curve.

RESULTS

The pM3CRYY A3 expression plasmid contains the *chiC* ORF. We first conducted a series of experiments to verify that the plasmid obtained from Rocha et al. was indeed pM3CRYY A3 (Fig. 1A) (9). This was an essential first step since protein expressed from this plasmid would be used for all downstream experiments. A restriction enzyme digest of pM3CRYY A3 using the enzymes EcoRI and HindIII previously used by Rocha et al. to insert the *chiC* open reading frame (ORF) into pET28a revealed the presence of two DNA fragments on an agarose gel. The 6.0 kilobase (kb) fragment corresponds to the pET28a vector backbone, and the 1.5 kb fragment corresponds to the expected size of the inserted *chiC* ORF (Fig. 1B) (9). Digestion of the negative control pET28a with EcoRI and HindIII yielded only a single band of approximately 6.0 kb as expected, since the *chiC* ORF was not present in this vector.

Additionally, a PCR analysis using *chiC*-specific primers found that the reaction performed using pM3CRYY A3 as a template yielded a DNA fragment of approximately 1.5 kb, which was much fainter when using pET28a as a template (Fig. 1C). This unexpected band, presumed to be the *chiC* ORF due to the size being similar to the expected size of the *chiC* ORF, could potentially be due to overflow of DNA from the neighbouring well. To confirm these results, we then performed Sanger sequencing using *chiC* specific primers and primers which bind to the T7 promoter and terminator sequences in the vector backbone. Sequencing results revealed that the ORF found inserted between the EcoRI and HindIII recognition sites corresponded to the known sequence of *P. aeruginosa chiC* with 99.9% sequence identity (Fig. 1D), with only a single silent mutation noted in the ORF.

We also observed the presence of various features in the pM3CRYY A3 expression vector. First, downstream of the predicted ribosomal binding site and upstream of the start codon for *chiC* there were several predicted features, including the sequences for the 6xHis-tag, a thrombin cleavage site, and a T7 tag (Fig. 2A). These features are often found in protein expression plasmids as they can be used for purification of the desired product, and were expected to be present in the original pET28a vector. Additionally, downstream of the *chiC*

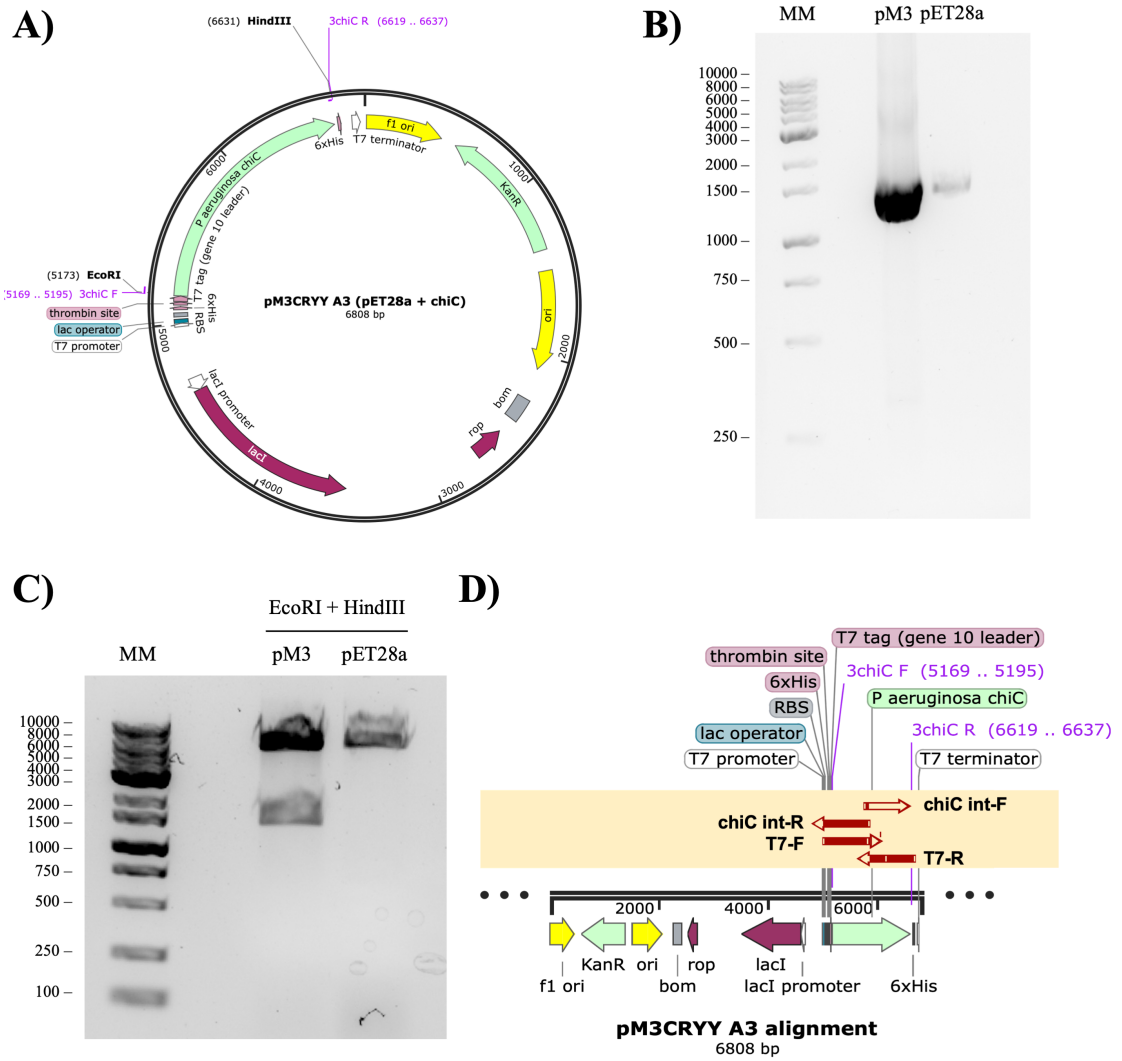

FIG. 1 Identity of pM3CRYY A3 and pET28a was confirmed via restriction enzyme digest, PCR, and Sanger sequencing. (A) Vector map of pM3CRYY A3 generated using SnapGene 6.2. (B) Agarose gel electrophoresis image of EcoRI/HindIII double digest confirmation. (C) Agarose gel electrophoresis image of PCR reaction. MM represents molecular marker. pM3 represents the pM3CRYY A3 vector. (D) Sequencing contig assembly aligned to reference pM3CRYY A3 sequence. Generated using SnapGene® 6.2.

ORF there was an additional ORF for a 6xHis-tag, though this is not expected to be expressed due to the presence of a stop codon at the end of the *chiC* ORF (Fig. 2B).

Bead beating is determined as the optimal cell lysis method for protein extraction. To determine the optimal method for cell lysis and protein extraction, we treated *E. coli* BL21(DE3) transformed with pM3CRYY A3 or pET28a with IPTG at varying concentrations (0, 0.1, 0.5, 1.0 mM) and timepoints (0, 3, 6, 24 hr), then samples taken from the same culture were lysed with either the boiling or beat beating method. The effectiveness of each method was evaluated by running an SDS-PAGE (Fig. 3A-B). It was observed that the bead beating lysis method resulted in greater amounts of protein being extracted from the sample (Fig. 3A) and so subsequent assays utilized this method for cell lysis. In this experiment, we also noted the presence of a protein approximately 55 kDa in size in samples collected from cultures transformed with pM3CRYY A3. Since a similarly sized protein was not observed in the culture transformed with pET28a, this supported that the protein expressed was likely ChiC.

ChiC expression from pM3CRYY A3 is confirmed by western blot using the 6xHis-tag. After observing the presence of a 55 kDa protein in IPTG-induced pM3CRYY A3 cultures, we performed experiments to confirm that the protein was indeed ChiC. We performed a western blot against the 6xHis-tag predicted to be at the N-terminus of ChiC

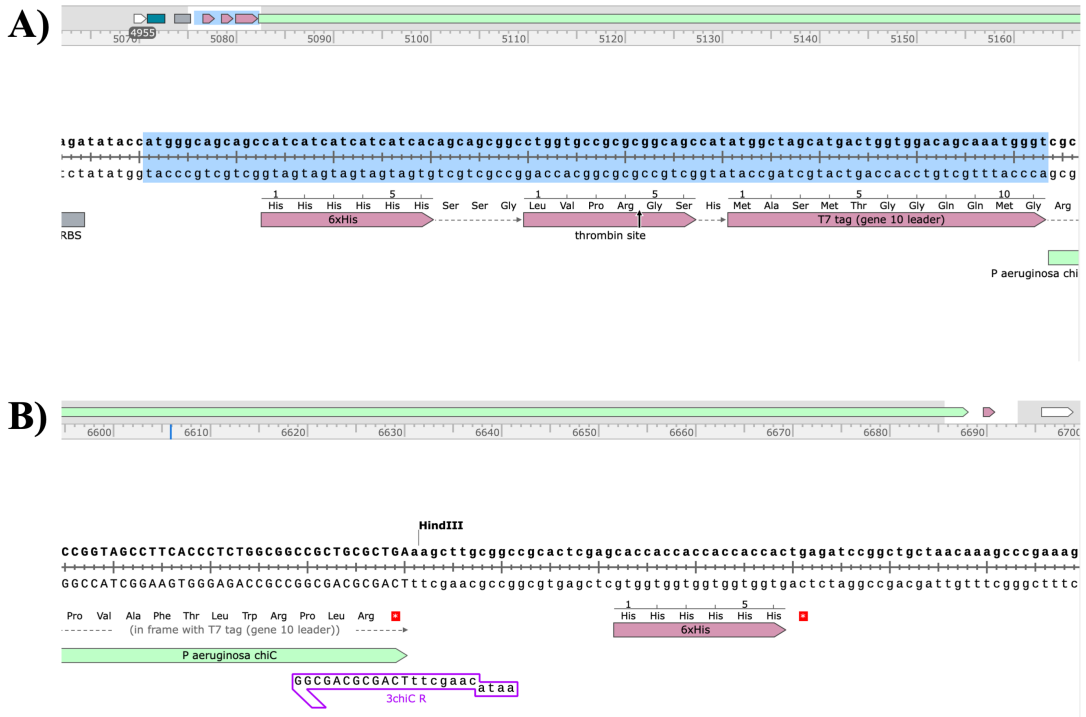

FIG. 2 Annotation of pM3CRYY A3 sequence showing N and C-terminal features. (A) Sequence annotation of the 5' region upstream of pM3CRYY A3. (B) The *chiC* ORF in pM3CRYY A3 and the annotated sequence of the 3' region downstream. Sequence confirmed using Sanger sequencing. Plasmid map generated using SnapGene 6.2.

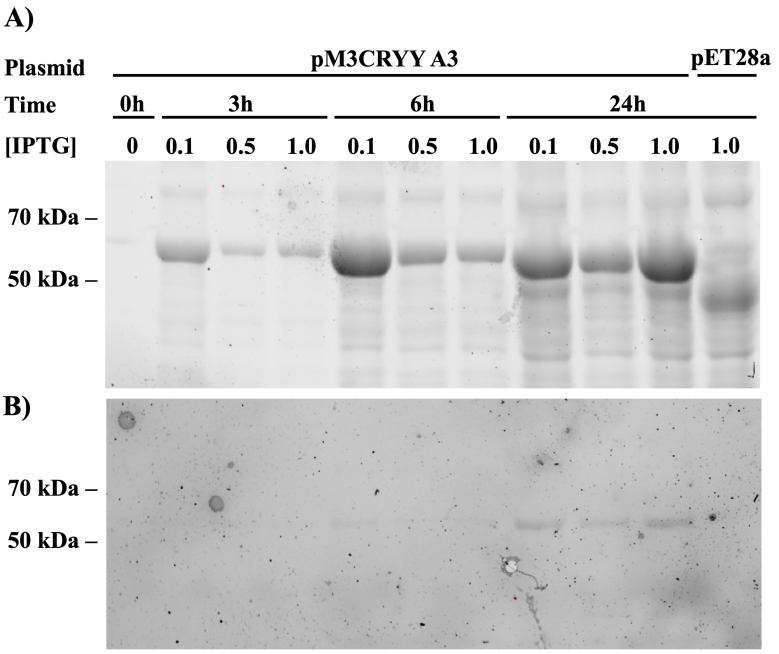

FIG. 3 SDS-PAGE revealed that the bead beating cell lysis method results in greater amount of protein extracted compared to a boiling lysis method. (A) SDS-PAGE image showing proteins extracted from IPTG-induced *E. coli* BL21(DE3) pellets via bead beating cell lysis method. IPTG concentrations and induction times are indicated above. Lane 11 shows an additional negative control corresponding to *E. coli* BL21(DE3) expressing the empty vector pET28a after induction with 1.0 mM IPTG for 24 hours. (B) Boiling method of cell lysis for protein extraction. Conditions as described in (A).

(Fig. 2A), where we observed a specific band at 55 kDa (Fig. 4A) which was absent in the samples from pET28a cultures.

ChiC expression is optimal at 6 hours of induction with 0.1 mM of IPTG and ChiC is present in the supernatants after 24 hours. From our 6xHis-tag western blot (Fig. 4A), we observed increasing ChiC expression in cell lysates between 0 to 6 hours of induction with maximal expression seen at 6 hours, followed by slightly less at 24 hours. Optimal ChiC expression was seen when inducing cultures with 0.1 mM IPTG. Interestingly, when we

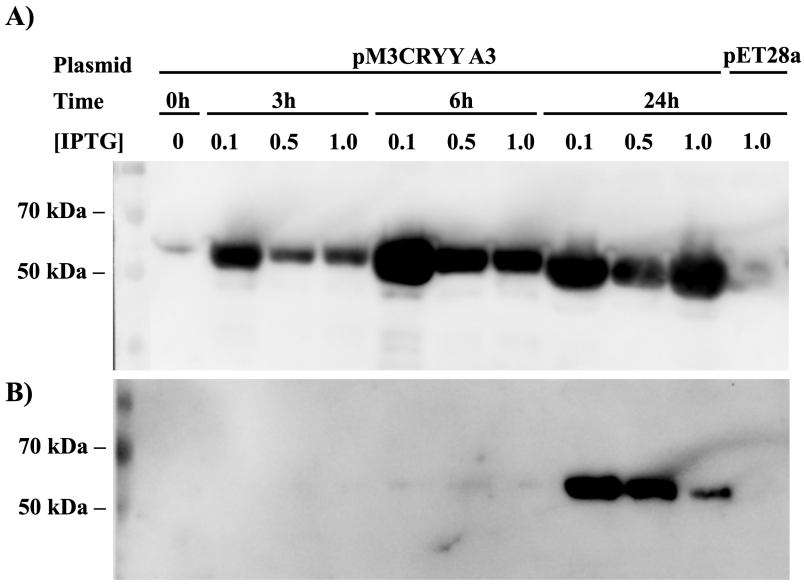

FIG. 4 Maximal expression of ChiC was observed with 0.1 mM IPTG induction for 6 hours and ChiC is present in the supernatants of induced cultures after 24 hours. (A) 6xHis western blot of pellets obtained from *E. coli* BL21(DE3) pellets with bead beating lysis method. IPTG concentrations and induction times are shown above. Lane 11 shows an additional negative control corresponding to *E. coli* BL21(DE3) expressing the empty vector pET28a after induction with 1.0 mM IPTG for 24 hours. (B) Anti-6xHis-Tag western blot against proteins present in culture supernatant. Conditions as described in (A).

performed the same western blot against samples collected from the supernatants of induced cultures, we also observed the presence of a prominent band corresponding to 6xHis-ChiC at 24 hours, though there was a minimal amount of ChiC at 6 hours of induction (Fig. 4B). These supernatant blots are also consistent with the trend seen in cell lysates, where maximal expression of ChiC is seen when inducing with 0.1 mM IPTG.

Detection of cytosolic DnaK in culture supernatant samples via western blot suggests ChiC is released during cell lysis. After observing the presence of 6xHis-ChiC in the supernatant of induced cultures, we sought to determine if this was due to ChiC secretion or due to cell lysis. To investigate this, we performed another western blot on σ^{70} factor and DnaK, which are abundant soluble chaperone proteins that are not secreted by *E. coli*. If DnaK is absent in the supernatant samples whereas ChiC is present, this would support that ChiC is being secreted from *E. coli* via an independent mechanism. Alternatively, if we could detect these proteins in the supernatant samples, this would suggest that both ChiC and these cytosolic proteins are released into the extracellular space by lysed cells. We first attempted to answer this question by probing for σ^{70} factor, where we detected specific bands corresponding to a protein of greater than 100 kDa in the cell lysates but not the supernatant samples (Fig. S1). However, these results were deemed inconclusive since σ^{70} factor is expected to be around 70 kDa. We then re-attempted this experiment with an anti-DnaK blot (Fig. 5). From our western blot, we observed a faint band between 70 and 100 kDa

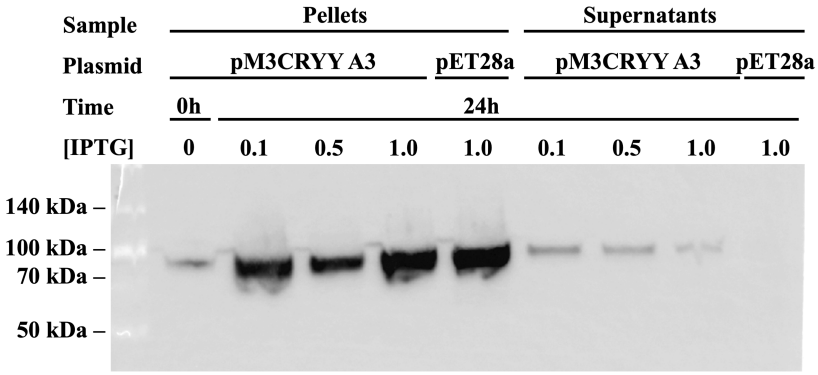

FIG. 5 Cytosolic DnaK is released and detected in the supernatant of induction induced cultures from lysed cells. DnaK western blot against *E. coli* BL21 pM3CRYY A3 cells and supernatants induced with IPTG for 24 hours. Cells were induced for 24 hours with 0, 0.1, 0.5, and 1.0 mM IPTG. Lanes 1–5 are lysates obtained from cell pellets via the bead beating cell lysis method, and lanes 6–9 represent culture supernatant samples.

corresponding to the approximate size of DnaK in the supernatants after 24 hrs of induction with 0.1, 0.5 and 1.0 mM IPTG. The band was most prominent in the 0.1 mM IPTG induction condition (Fig. 5). To assess if this difference in DnaK abundance was due to a decrease in cell viability with increasing IPTG concentration, we also performed a preliminary growth curve assay in an attempt to quantify the number of viable cells in each condition (Fig. S2). From this assay, it seems that IPTG treatment reduces the number of viable cells when comparing uninduced cultures vs. induced cultures. This would suggest that the ChiC detected in the extracellular space was in fact not being secreted by *E. coli* BL21(DE3), but it may be released from dead or dying cells.

DISCUSSION

ChiC from *P. aeruginosa* has been previously cloned into the pET28a protein expression vector by Rocha et al (8). This vector backbone is optimized for protein expression as it contains the universal T7 promoter and terminator, His-tag sequence for purification, thrombin site, and multiple cloning site (MCS) (11). Although the pET28a *chiC* vector pM3CRYY A3 was successfully produced and transformed into *E. coli* BL21(DE3) previously, the identity of the expressed ChiC protein had not been confirmed. Therefore, our first objective in this study was to verify the plasmid sequence and use a western blot to determine whether the protein at the expected size of ~55 kDa is indeed ChiC using an anti-6xHis antibody. After confirming the identity of the protein, we optimized IPTG induction conditions for ChiC expression and sought to determine if it is secreted by *E. coli* BL21(DE3).

Interestingly, the Sanger sequencing results showed that there were two 6xHis sequences in the pM3CRYY A3 plasmid — one at the 5' end of *chiC* and one at the 3' end, but after the *chiC* stop codon (Fig. 1-2). The advantage of having a single tag at the C-terminus rather than the N-terminus is because it can be useful for determining if the protein is in fact being fully translated. However, because we were able to confirm the size of the protein being expressed, we are convinced that ChiC is being fully expressed off of pM3CRYY A3. Between the multiple tags, thrombin cleavage site, and spacers, there are 31 additional amino acids attached to the N-terminus of the ChiC protein. This may pose some potential problems in future experiments because the amount of accessory amino acids could potentially interfere with protein folding. Since proteins are normally folded starting at the N-terminus, the addition of these amino acid residues may disrupt some of the interactions necessary for proper folding. If the protein is misfolded, this could impair the chitinolytic activity of ChiC. This may be why the pET28a vector encodes the thrombin cleavage site upstream of the MCS, which could allow for removal of the 6xHis-tag and some of the extra amino acids at the N-terminus of the protein of interest. However, 14 extra amino acids which include the additional N-terminal T7 tag would remain conjugated to the protein.

Regarding optimal conditions for cell lysis, bead beating was identified as the preferred method due to the increased amount of protein detected via SDS-PAGE (Fig. 3A-B). It was also selected due to its non-protein-denaturing ability to lyse cells, leaving the potential for future functional assays using extracted protein. Boiling as a method for cell lysis resulted in less protein overall, perhaps due to incomplete lysis, or the aggregation of proteins at high temperatures. These protein aggregates can be insoluble and dense enough that they would be removed when centrifuging the samples to remove other impurities such as cell membranes and carbohydrates.

A subsequent western blot against the 6xHis-Tag was performed to determine the conditions required for optimal amount of protein produced mL of bacterial culture. Results suggested that at 37ºC, 0.1 mM IPTG with an induction time of 6 hours yielded the maximum amount of protein (Fig. 4A). IPTG treatment appeared to decrease ChiC expression with increasing concentration and increase the amount of ChiC in a time-dependent manner up to 6 hours. This was as expected because the number of cells in culture – and therefore the amount of protein being produced – should increase with time. However, we observed slightly less ChiC after 24 hours of IPTG induction when compared to 6 hours. We suggest that this may be due to a decrease in cell viability, as IPTG is known to inhibit cell growth by rapidly upscaling the production of proteins under the control of a T7 promoter and diverting cellular resources away from other processes (12). Additionally, we suggest that the two time points

of 6- and 24-hours sample cells at different phases in the growth cycle. *E. coli* BL21 after 6 hours of growth in rich medium should correspond to sampling cells in the exponential growth phase, where cells are rapidly proliferating and producing proteins (13). At 24 hours, the nutrients contained in the growth medium may be depleted, resulting in cells entering the stationary or death phases and a dramatic reduction of the production of proteins. Therefore, our observation that there were decreased amounts of ChiC being expressed at 24 hours compared to 6 hours was not unexpected. For industrial applications, the 6-hour induction time may in fact be preferred for increased chitinase production efficiency.

However, we decided to proceed with probing the samples from 24 hours of IPTG induction. This was due to the fact that we detected ChiC in the supernatants of IPTG-induced cultures most prominently at this time point. Therefore, the following western blots using antibodies against cytosolic proteins σ^{70} factor and DnaK were performed using samples collected after 24 hours of IPTG induction.

In general, various secretion systems exist in different bacteria and can be categorized into classes based on their characteristics such as structure, function, and specificity (14). They can rely on multiple different mechanisms and machinery to transport proteins from where they are translated in the cytosol to the extracellular space. Not all of these secretion systems, however, are conserved across all classes of bacteria – some are specific to the export of only a few proteins in a select number of bacterial species. Folders et al. showed that in *P. aeruginosa*, ChiC is secreted gradually through a novel pathway (7). In the process, they identified an 11-residue N-terminal segment that is cleaved off during the process of secretion (7). However, *P. aeruginosa* is not a typical recombinant protein expression system. *E. coli* BL21(DE3) represents a much more commonly used model, due to it being optimized for the overexpression of proteins and its lack of virulence factors. Although Folders et al. identified that ChiC was not being secreted in *P. aeruginosa* through any of the known protein export systems, we were uncertain if a similar system existed in *E. coli*.

If *E. coli* BL21(DE3) does possess a similar system, based on our sequencing data, we would expect that the N-terminal 6xHis-tag would be cleaved off. However, the ChiC western blot using an anti-6xHis antibody showed that the protein is present at the expected size (Fig. 4), indicating a lack of N-terminal cleavage. This could mean that if *E. coli* BL21(DE3) is secreting ChiC, it does not possess the same machinery as *P. aeruginosa* which cleaves the N-terminal residues during the process of protein export. If the cleavage of the N-terminal segment is necessary for ChiC activity, then the ChiC secreted by *E. coli* BL21(DE3) may not be functionally active.

We next aimed to determine if the proteins detected in the supernatants of the ChiC western blots could be a result of cell lysis rather than secretion. Based on the growth curve generated by spot plating cultures from each induction time point, we saw that treatment with IPTG appeared to decrease the number of viable cells (Suppl. Fig. S2). This is consistent with other studies using *E. coli* BL21, which showed that 1 mM IPTG negatively influenced cell growth compared to 0.1 mM (15). Regardless, this could suggest that ChiC's presence in the supernatant of induced cultures might be due to cell death instead of secretion which would make looking at time points past 24 hours interesting since these might have more cell death, and therefore more ChiC present in the supernatant.

ChiC localization was more robustly determined using a series of western blots. The σ^{70} factor of *E. coli* RNA polymerase is a cytosolic protein that was identified as a potential control, since if it was detected in the supernatant samples, we would know that it was released because of cell lysis (16). However, the σ^{70} antibody detected a protein of 100 kDa rather than the expected 70 kDa size, therefore rendering inconclusive results on the reliability of σ^{70} factor as a determinant of cell lysis or protein secretion (Suppl. Fig. S1). Although we were unable to conclude if the detected protein was in fact σ^{70} factor, it was interesting that this protein was seen only in the insoluble cell lysate fraction and not in the supernatant samples. If this protein were to be the cytosolic σ^{70} factor, this result would show that the ChiC detected in the supernatant was being released as a result of secretion and not via cell lysis.

The cytosolic chaperone protein DnaK was then identified as a candidate for a subsequent western blot (Fig. 5). The observed band at approximately 70 kDa demonstrates that DnaK is present in both the supernatant and pellet samples, though more DnaK is present in the pellets,

as shown by stronger and larger bands. The presence of bands in the supernatant samples could support our hypothesis that cells are being lysed, and DnaK, which is normally an intracellular protein, is being released into the supernatant. Our growth curve data also supports this observation, since it appears that the number of viable cells decreased with addition of IPTG, possibly due to cell death. However, since the supernatant samples were treated with the same lysis method as the pellets without first being spun down to pellet any remaining debris and cells, the proteins present in the supernatant might be from residual cells that were lysed. Therefore, this experiment should be replicated before making any final conclusions.

Limitations This study aimed to determine the optimal conditions for expression of ChiC via Western blotting. We attempted to normalize conditions between experiments by adding IPTG (and therefore beginning induction of ChiC expression) only once cultures had reached a specific OD_{600} absorbance reading. When comparing different lysis methods, we also used samples taken from the same culture in order to ensure that the same amount of bacteria was being treated with each lysis method. However, one additional measure that we could have taken to normalize samples between different conditions and rounds of experimentation was taking a final absorbance reading at each timepoint. This could have helped to account for differences in growth rates between experiments, and would have allowed us to determine if protein expression levels per cell differed between conditions and timepoints. The examination of cell viability and ChiC production at more timepoints before and after 24 hours of culture would have also served to make our study more robust.

Additionally, while we were able to confirm the identity of the expressed ChiC and identify a cytosolic protein useful for determining its secretion status, its localization cannot fully be concluded based on our results. In Figure 5, the band believed to correspond to DnaK seems to be slightly higher than the expected 70 kDa size. Although there is some discrepancy in how proteins migrate in SDS-PAGE and proteins may not always travel at the expected size, this could potentially limit our conclusions on the secretion status of ChiC. In this same blot, there is no band present in the control pET28a supernatant condition, which we would normally expect to see, since the cytosolic DnaK should also be present if the cells are being lysed. Also, performing the beat beating lysis methods on our supernatant samples makes the results shown in Figure 5 not fully conclusive. Though the vast majority of cells should have been removed when separating the cells and supernatant samples from each other, any residual cells in the supernatant samples would have been lysed, releasing their proteins into solution. This may be the reason that proteins are present in the supernatant conditions, making it difficult to distinguish if the proteins present in the supernatants are due to secretion or cell lysis. Future experiments would benefit from first spinning down supernatant samples at a high speed to remove any residual cells before treatment with any lysis method.

Finally, a limitation of our growth curve data (Fig. S2) is that the method used to quantify viable cells was quite labour intensive, limiting the number of replicates that we were able to generate. To confirm our results, it would be beneficial to have repeated the experiment several times.

Conclusions Our study showed that the ChiC expression plasmid pM3CRYY A3 expressed in *E. coli* BL21(DE3) allows for inducible expression of 6xHis-tagged ChiC. We also determined the optimal induction conditions at 37ºC were 0.1 mM IPTG for 6 hours, with a bead beating lysis method extracting greater quantities of protein compared to a boiling lysis method. However, if the protein is in fact being secreted, its presence was detected in the supernatants only after 24 hours of induction. Finally, we attempted to determine if ChiC expressed in *E. coli* BL21(DE3) is secreted, with our results suggesting that it may not be secreted, but future experiments will be needed to confirm these findings.

Future Directions One avenue for other teams to pursue would be to repeat the DnaK Western blot using supernatant samples that were not treated with any lysis method, preventing any residual cells from releasing unwanted proteins. These results would provide stronger evidence as to whether ChiC is secreted by *E. coli* BL21(DE3) or whether it is present in the supernatant because the cells are being lysed. Another area of future exploration

would be to investigate the functionality of extracted ChiC and its ability to degrade chitin using our optimized expression system. This could be done using methods such as the analysis of chitin clearance zones on chitin agar plates. Ultimately, if the ChiC expressed in this pM3CRYY *E. coli* BL21(DE3) system is functionally active, it would have the potential to be used in industrial applications as a biopesticide.

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CONTRIBUTIONS

Laboratory work. JC: Methodology, Investigation, Writing - original draft, review, and editing. **VL:** Conceptualization, Supervision, Methodology, Investigation, Writing - original draft, review, and editing. **CS:** Methodology, Supervision, Investigation, Formal analysis, Visualization, Writing - original draft, review, and editing. **JY:** Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, review, and editing

Manuscript. JC: Introduction, Methods, Discussion, Limitations, References. **VL:** Introduction, Methods, Discussion, Limitations, Future Directions. **CS:** Abstract, Methods, Figures, Results, Conclusions. **JY:** Abstract, Methods, Figures, Results.

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