Expression of *Pseudomonas aeruginosa* PAO1 Chitinase C in *Escherichia coli* BL21 (DE3)

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SUMMARY Chitin is a rigid natural homopolymer found in the exoskeletons of many insects, including agricultural pests. With the importance of chitin in insect metabolism and protection, there have been attempts to identify and develop chitinolytic insecticidal products. Chitinolytic enzymes are naturally produced by bacterial genera such as *Pseudomonas*. Here, we examine chitinase C (ChiC), a secreted 55kDa protein encoded by the *P. aeruginosa* PAO1 gene, *chiC*. ChiC expression in *P. aeruginosa* is controlled by quorum sensing, and its mode of secretion remains poorly understood. We hypothesized that transforming an expression vector containing *chiC* into non-pathogenic *E. coli* BL21 (DE3) would allow for expression of ChiC, and that purified ChiC would retain its previously characterized chitinolytic and insecticidal activity. We cloned *chiC* from an established *chiC*-containing vector (pGKMS21) into pET-28a to create pM3CRYY, a *chiC* expression plasmid. Agarose gel electrophoresis confirmed that we amplified *chiC* from pGKMS21. Next, we ligated *chiC* between the EcoRI and HindIII restriction sites of pET-28a to create pM3CRYY. Upon confirming the identity of the construct via PCR and Sanger Sequencing, we transformed it into *E. coli* BL21 for protein expression. SDS-PAGE analysis of IPTG-induced BL21 pellet lysates and supernatant showed that ChiC was expressed after induction, though secretion of the protein did not occur. Ultimately, our work was able to create a ChiC expression system in *E. coli*, which may be further optimized to study the secretion of ChiC secretion, as well as its intracellular modification and regulation mechanisms.

INTRODUCTION

Chitin is a linear polysaccharide homopolymer of β-1,4-N-acetyl-D-glucosamine and is the second most abundant natural polysaccharide (1). Chitin is an attractive target for agricultural biocontrol, given its crucial role in the survival of many agricultural pests such as phytophagous insects, phytopathogenic fungi, and parasitic nematodes (2). In addition, several insecticides broadly used in agriculture, such as avermectins and carbamates, target the nervous systems of insects, which may have consequences on organisms with similar neurophysiology, including humans (3). Using chitin-degrading enzymes for pest control would minimize non-target effects, thus reducing consumer risks and environmental impact (4).

Several soil bacteria can degrade chitin, including *Pseudomonas*, *Bacillus*, and *Cytophaga*. Chitinolytic microbes in the soil are also notably more efficient at degrading chitin compared to their water- or sediment-associated counterparts (5). Several species of *Pseudomonas,* which can colonize virtually all environments (6), have shown insecticidal activity against agricultural pests. For example, *P. taiwanensis* has shown activity against several pests (e.g. *Plutella xylostella* and *Spodoptera litura)* (7), and *P. fluorescens* has shown insecticidal activity against other insects including aphids and termites (6). Furthermore, a chitinase isolated from *P. fluorescens* strain MP-13 has shown insecticidal activity against *Helopeltis theivora* in vitro. Our study focuses on ChiC, a chitinase isolated from *P. aeruginosa* PAO1 (8). ChiC exhibits insecticidal activity in feeding assays (9), which makes this chitinolytic enzyme a suitable candidate for biocontrol agent studies in a host expression system.

ChiC is a 55kDa extracellular, secreted chitinase enzyme composed of 483 amino acids (8). This enzyme consists of 3 domains: the carbohydrate-binding module, the glycoside

Published Online: September 2022

Citation: Madalena Cunha Rocha, Matthew Ethan Yap, Michael Yoon. 2022. Expression of *Pseudomonas aeruginosa* PAO1 Chitinase C in *Escherichia coli* BL21 (DE3). UJEMI 27:1-10

Editor: Andy An and Gara Dexter, University of British Columbia

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hydrolase family 18 (GH18) domain, and the fibronectin type-III domain, which allow ChiC to degrade chitin. The carbohydrate-binding module allows the binding of chitin (InterPro ID: IPR001223, IPR003610). The catalytic site on the GH18 domain catalyzes random cleavage of β-1,4-bonds within chitin chains (InterPro ID: IPR001579). The fibronectin domain allows for interactions with other proteins (InterPro ID: IPR003961). While the structure of this enzyme has been characterized, little is known about its mode of secretion. ChiC contains an N-terminal sequence of 11 amino acids that is absent in its secreted form, but these residues do not comprise a conventional signal sequence. ChiC is reportedly secreted into the extracellular space through unknown mechanisms that are distinct from type II and type III secretion systems.

Bodykevich *et al.* (2022) (10) sought to explore the biocontrol potential of ChiC and managed to amplify a ~1.6kb DNA fragment containing *chiC* from the genome of *Pseudomonas aeruginosa* strain PAO1 and cloned it into pCR2.1 to create the *chiC*-containing storage vector pGKMS21. However, this construct was not designed to support gene expression, with *chiC* out-of-frame relative to the promoter. Therefore, we hypothesized that cloning *chiC* from pGKMS21 into an expression vector would allow for the expression of *chiC* and secretion of the gene product, ChiC in an *E. coli* host system. In this study, we amplified *chiC* from pGKMS21 and inserted it into pET-28a to create the recombinant expression vector pM3CRYY. The resulting construct was then used to transform *E. coli* strain BL21 (DE3) cells for IPTG-induced expression.

METHODS AND MATERIALS

Preparation of experimental materials, reagents and samples. 50mL of 10mg/mL Kanamycin stock solution was prepared from 0.05g Gibco™ Kanamycin Sulfate (Lot 1913841) and distilled H_2O, and stored at 4°C. 500mL bottles of LB broth or agar were prepared from 5g NaCl, 5g Tryptone, 2.5g yeast extract, distilled water, with or without 7.5g agar. LB + 25μg/mL Kanamycin agar was prepared by adding 1.25mL of Kanamycin stock solution to 500mL of cooled molten LB agar. 1x TAE buffer was prepared by diluting a pre-made stock of 50x TAE. pGKMS21 storage vector containing *P. aeruginosa* PAO1 *chiC* from Bodykevich *et al*. (2022) (10) was isolated from *E. coli* DH5α cells. Extraction of this vector and other plasmids in this project was performed following the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic) protocol. Primers were designed on SnapGene 6.0 and manufactured by Integrated DNA Technologies. 0.1M $CaCl_2$ and 0.1M $CaCl_2$ + 15% glycerol were prepared based on Chang *et al*. (2017)'s UJEMI methods paper on competent cell preparation (11). Liquid culture optical density measurements were read on a Pharmacia Biotech Ultrospec 3000 UV-Vis Spectrophotometer. DNA concentrations were measured using a NanoDrop™2000 spectrophotometer. 10mM isopropyl β-D-1-thio-galactopyranoside (IPTG) stock for induction was prepared from 0.119g IPTG in 50mL of LB + 25 μg/mL Kanamycin broth.

Agarose gel electrophoresis of DNA samples. 1% agarose gels were prepared by dissolving 1g agarose powder (Bio-Rad) in 100mL 1x TAE buffer and 5μL RedSafe™ Nucleic Acid Staining solution. As a result of preliminary protocol testing, all agarose gel electrophoresis procedures in this project were optimised to load ~100ng (10μL of 10ng/μL) of DNA samples with 2μL of 6x Purple Loading Dye (New England BioLabs) into each well. 1 Kb Plus DNA Ladder (Bio-Helix) was loaded onto Lane 1 of each gel. Gels were run at 150V, 500mA then visualized on the transilluminator (Bio-Rad ChemiDoc Imaging System) with Ethidium Bromide stain settings.

PCR amplification of DNA fragments containing *chiC* with restriction sites for EcoRI and HindIII. Amplification of 1.45kb *chiC* with the addition of flanking EcoRI and HindIII restriction sites was conducted using our custom Forward primer: 5' tatccgaattcATGATCAGGATCGACTT 3' and Reverse primer: 5' aatacaagcttTCAGCGCAGCGG 3' ("2γ-3chiC" primers). PCR was performed in 0.2mL Bio-Rad 8-tube strips, each with a reaction volume of 50μL comprised of 0.5μL 2U/μL

Platinum™ SuperFi™ DNA polymerase (Invitrogen), 10µL 5x SuperFi Buffer (Invitrogen), 10µL SuperFi GC Enhancer (Invitrogen), 1µL 10mM dNTPs, 2.5µL 10µM of each primer custom-ordered from IDT, UltraPure™ DNase/RNase-free dH_2O, and 10 ng of template pGKMS21 1:1. PCR tubes were placed in a Bio-Rad T100™ Thermal Cycler set to 105°C lid temperature, 1-min initial denaturation at 98°C followed by 35 cycles of 10-sec denaturation at 98°C, 10-sec annealing at 55°C, 1-min extension at 72°C, ending with 5 mins at 72°C and infinite hold at 4°C. PCR product purification was performed using the GeneJET PCR Purification Kit and standard protocol, with a final elution volume of 20µL. Samples were stored at -20°C until use.

Construction and propagation of pM3CRYY. Purified ~1.5kb PCR amplicon containing *chiC* and restriction sites was digested with EcoRI R0101S and HindIII R0104S (New England BioLabs, exp 2013). 1-hour 20 µL digestion reactions were performed in 0.2mL Bio-Rad PCR tubes, each with 12 µL of UltraPure™ DNase/RNase-free dH_2O, 2 µL rCutSmart™ buffer (New England BioLabs), 1 µL (2U) of each enzyme and 4µL of 144.3 ng/µL purified *chiC*-containing PCR product to obtain *chiC* sticky-end inserts. Expression vector pET-28a isolated from pET-28a(+) *E. coli* DH5α was digested with the same protocol to obtain linearized pET-28a. Double-enzyme digested insert and vector were purified using the GeneJET PCR Purification Kit to remove enzymes, then ligated. 10 µL ligation reactions were performed in 0.2mL Bio-Rad PCR tubes each containing 1µL T4 DNA Ligase and 1µL ligase buffer (Invitrogen), UltraPure™ DNase/RNase-free dH_2O and purified digestion products in 3:1 and 5:1 insert-to-vector ratios. 3µL of ligation products were used to transform 50µL Subcloning Efficiency™ DH5α Competent Cells (Invitrogen) via heat shock at 42°C, followed by 2-mins incubation on ice and 1-hr recovery at 225rpm, 37°C. Transformants were pelleted, resuspended in 100µL 37°C LB, and plated on LB + 25 µg/mL Kanamycin agar. Plates were incubated overnight at 37°C. Candidate pM3CRYY(+) colonies were streaked onto fresh LB-kan agar plates and stored at 4ºC.

Screening of ligation product transformant colonies for the insert. A colony PCR was first performed with the same 2γ-3chiC primers, polymerase and PCR conditions and as the previous procedure used to obtain *chiC* from pGKMS21, but with DH5α transformants as the template. Colony PCR products were visualized and screened for a ~1.5kb insert via agarose gel electrophoresis. 5mL liquid cultures of insert(+) transformants were grown overnight at 225rpm, 37°C in LB+kan broth. Plasmids were then isolated from cultures via EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic) and screened for the insert via PCR and agarose gel electrophoresis using 2γ-3chiC primers, and Platinum™ *Taq* DNA polymerase High Fidelity (Invitrogen), following ThermoFisher Protocol Pub. No. MAN0000948.

Sanger Sequencing of pM3CRYY candidate plasmids. Samples of 4 insert(+) candidate plasmids were prepared in PCR tubes following GeneWiz guidelines and sent for Sanger Sequencing with T7 universal primers and internal primers. 20µl 5µM aliquots of each IDT custom internal primer (Reverse: 5'-gaagtcttctttcatcgcgtcg-3', Forward: 5'-caagtacgtgccttatctgcag-3') were prepared and sent to GeneWiz along with the plasmid samples. Universal T7 primers were provided by GeneWiz. Sequencing results were aligned and compared against a pET-28a + *chiC* reference sequence on SnapGene 6.0. Reads were trimmed to exclude low-quality terminal regions. Resulting sequences covered *chiC* (NP_250990.1) regions from nt5179 to nt5845 and nt5815 to nt6610. Sequences were compared against the *P. aeruginosa* PAO1 complete genome [NC_002516.2] through NCBI BLAST.

Preparation of competent *E. coli* BL21 (DE3) and transformation with pM3CRYY. Competent cell preparation procedures were adapted from Chang et al (2017), UJEMI methods (11). Transformation procedures were likewise adapted from the same methods paper (11), but were optimized based on pilot experiments. Changes to the transformation protocol are described in detail in the supplemental materials (Table 1S).

IPTG induction of gene expression and SDS-PAGE screen of *E. coli* BL21 for 55kDa

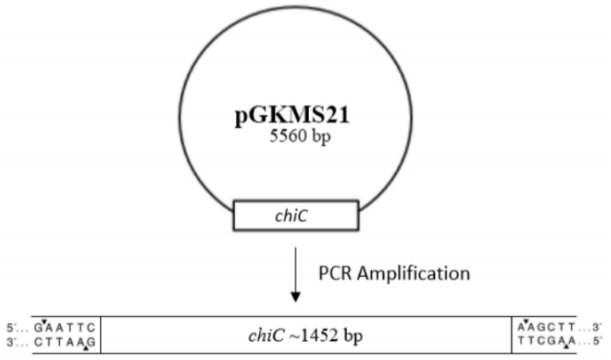
ChiC. Four 5mL liquid cultures of pM3CRYY-transformed BL21 were grown overnight. 2mL aliquots of the overnight cultures were diluted in 48mL of LB+25µg/mL Kanamycin broth in 200mL sterile Erlenmeyer flasks, and incubated at 225rpm, 37°C for 3 hours until OD (A_{600}) of 0.6. Subcultures were then aliquoted into uninduced (control) 1hr-induced and 2hr-induced treatment groups. IPTG induction procedures were adapted from a Fast induction protocol prepared by Biologics International Corp (12). Changes to the adapted protocol are described in the supplemental materials (Table 1S).

10µL of 10-250kDa Precision Plus Protein™ ladder and 15µL of each sample stained with 2x Laemmli dye (Bio-Rad) were then loaded onto two 15-well precast 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad). Gels were run at 300V in 10X SDS-Tris-Glycine buffer. Unpurified BL21 lysates and supernatants from each treatment group were screened for a 55kDa protein band corresponding to ChiC.

RESULTS

A ~1.5kb DNA fragment corresponding to *chiC* was amplified from the pGKMS21 template. PCR was conducted using our 2γ-3chiC forward (5' tatccgaattcatgatcaggatcgactt 3') and reverse (5' aatacaagctttcagcgcagcgg 3') primers designed to add EcoRI and HindIII restriction sites (FIG 1A). To verify that amplification of a DNA fragment containing *chiC* was successful, unpurified PCR product was loaded onto an agarose gel for visualization. The DNA bands were aligned to the 1.5kb marker, corresponding to the size of *chiC* + EcoRI and HindIII sites (FIG 1B). These results indicated that we obtained the correct *chic*-containing DNA fragment.

A)



B)

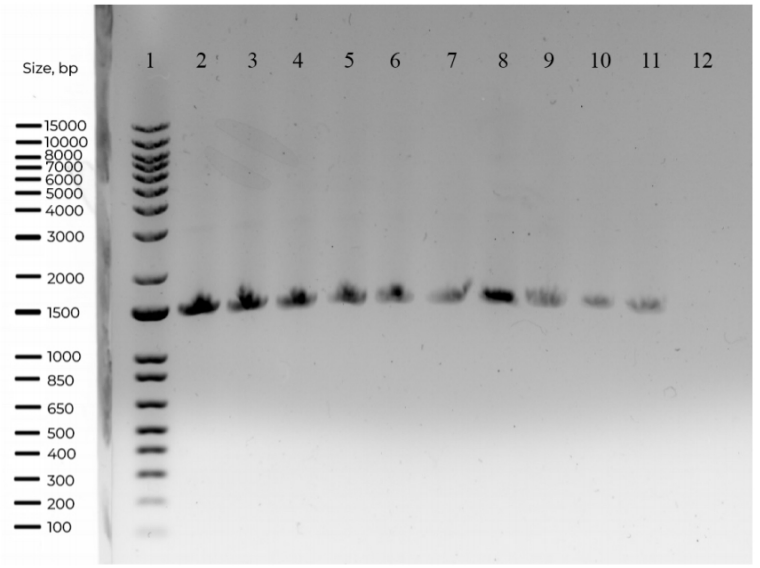


FIG. 1 *Pseudomonas aeruginosa* PAO1 gene *chiC* was amplified from *chiC*-containing template pGKMS21. A) Primers for *chiC* amplification were designed to add restriction sites for EcoRI and HindIII digestion. **B)** Agarose gel electrophoresis of PCR products showed that a ~1.5kb DNA fragment was amplified from pGKMS21. Lane 1 contains 5 µl 1Kb plus DNA Ladder. Lanes 2-9 contain 10 µl of ~10 ng/µl PCR product. Lanes 10-11 contain the positive control (*chiC* amplicon) from a previous PCR run. Lane 12 contains the negative control.

DH5α acquired candidate pM3CRYY constructs from transformation with ligation products. To determine whether digestion and ligation created pM3CRYY, ligation products and controls (no plasmid, double-digested pET-28a without insert, and intact undigested pET-28a) were transformed into DH5α for preliminary screening (FIG. 2A). Only intact pET-28a and ligated insert + vector (pM3CRYY) would propagate and confer resistance to kanamycin. Double-digested empty pET-28a with the addition of ligase without insert resulted in 0 colonies, suggesting that the enzymes were functional, as pET-28a digested with EcoRI and HindIII theoretically would only recircularize if ligated with the insert. Cells transformed with undigested, intact empty pET-28a grew hundreds of Kan^R colonies as expected (FIG 2B; Table 3S). Plated transformants ligation products grew a total of 15 Kan^R colonies, which were later screened for the expected *chiC* insert.

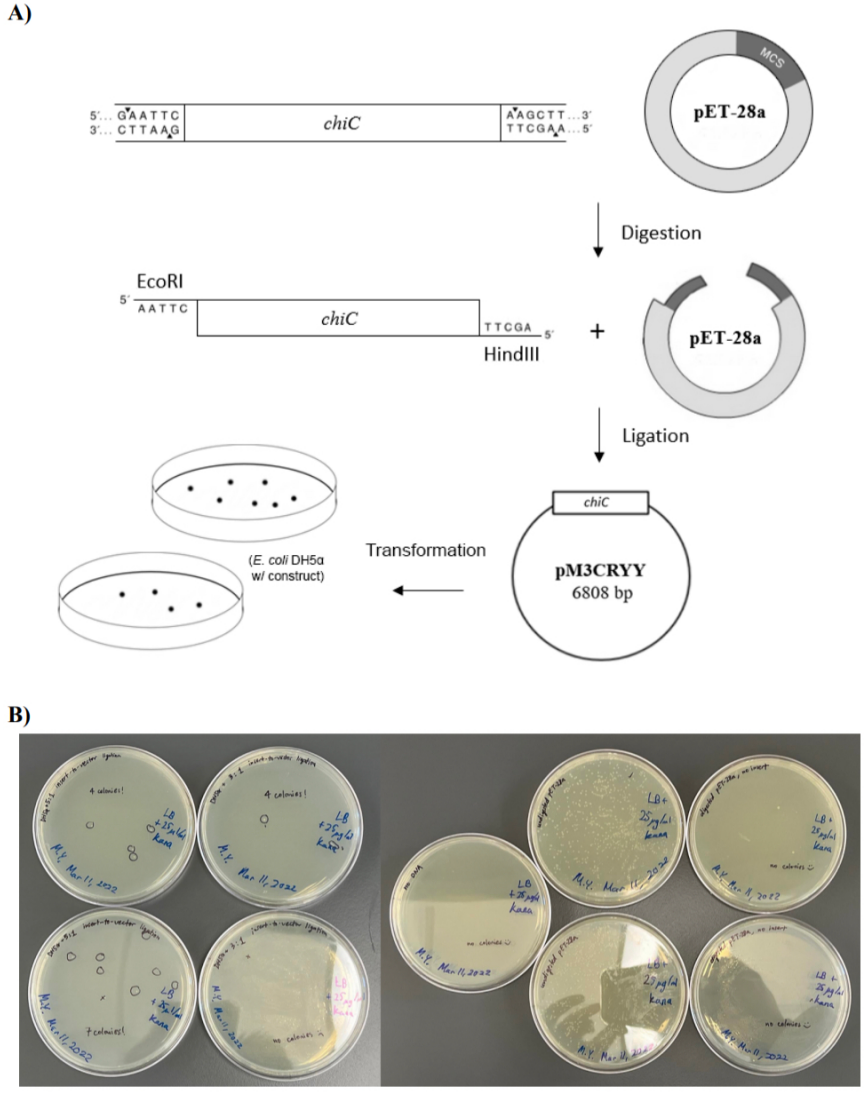


FIG. 2 Purified *chiC*-containing amplicon and pET-28a vector were digested and ligated to create the recombinant *chiC*-expression vector pM3CRYY (pET-28a + *chiC* construct). A) Digestion with EcoRI and HindIII created compatible sticky ends on the insert and vector for unidirectional ligation. **B)** 5:1 and 3:1 insert-to-vector ligation products, H_2O negative control, undigested pET-28a (intact) and double-digested vector (linearized, no insert) were transformed into *E. coli* DH5α which were plated for pM3CRYY propagation and screening.

PCR and Sanger Sequencing of candidate DH5α colony plasmids confirmed the presence of *chiC* and the sequence of pM3CRYY. To ascertain that the vector was correctly constructed, we first performed colony PCR (FIG 1S), then screened plasmids isolated from 9 5:1 ligation product transformant colonies for the insert via PCR. A ~1.5kb band was amplified from all 9 pM3CRYY candidate plasmids and the pGKMS21 positive control. No band was observed from the negative control, indicating true presence of the insert rather than contaminant 1.5kb DNA. (FIG. 3) To verify the identity of the construct and insert, samples

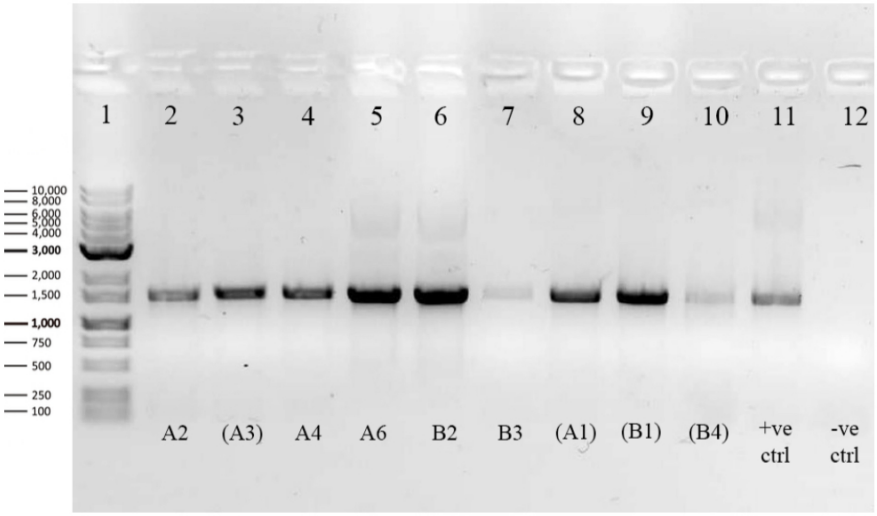


FIG. 3 Plasmids isolated from 5:1 ligation transformant DH5α colonies contained a ~1.5kb insert corresponding to *chiC*. Agarose gel electrophoresis of products from PCR amplification with 2γ-3chiC primers showed that DNA bands of the expected size were seen in all the sample lanes except the negative control. Lane 1 contains the 1Kb Plus ladder. Lanes 2, 3, 4, 5, 8 contain 10µl of 10 ng/µl amplicons from template plasmids of 5:1 plate A and B colonies. Candidate pM3CRYY vectors from transformant colonies A1, A3, B1 and B4 (i.e. pA1, pA3, pB1, pB4) were then arbitrarily selected for transformation into *E. coli* BL21.

of 4 *chiC*(+) candidate plasmids were sent to GeneWiz for Sanger Sequencing. SnapGene alignment showed that the vector backbone was indeed pET-28a (FIG 4). Comparing reads against *P. aeruginosa* PAO1 showed that *chiC* was successfully cloned into the vector with > 99% sequence identity on average (FIG 2S; Table 2S). Of the four candidate plasmids, the sequenced regions of *chiC* from pA3 were identical to *chiC* from the *P. aeruginosa* PAO1 genome (Fig 4). Despite sequencing errors, no overlapping mismatches between the internal and universal primer reads were present. pA1 and pB1 contained regions of low quality, as well as single nucleotide mismatches that were present in reads from both the internal and universal T7 primers. pB4 contained no overlapping mismatches, but had several regions of mismatches on its forward internal primer read.

***E. coli* BL21 (DE3) transformed with sequence-verified pM3CRYY produced a 55kDa protein after induction with IPTG.** To test whether pM3CRYY could function to express the gene in an *E. coli* host, we used IPTG to induce expression through the T7 promoter and screened unpurified *E. coli* BL21 lysates and supernatants for the expected 55kDa protein band via SDS-PAGE. Assorted protein bands from the lysates of BL21 transformed with pM3CRYY included a protein band slightly above the 50kDa marker (FIG 5). Intensities of protein bands of this size showed a noticeable pattern after 2 hours of induction. The protein bands at approximately 55kDa were visibly brighter and thicker after 2 hours of IPTG induction compared to the uninduced controls. In contrast, a faint protein band of this size was observed in only one of the lanes loaded with supernatant samples. As seen in the protein gel, a greater concentration of the 55kDa protein was observed after 2 hours of IPTG induction. However, the protein was primarily found in the lysates, indicating that it was retained inside the *E. coli* BL21 cells.

DISCUSSION

In this study, we aimed to construct a plasmid vector for the expression of *chiC* from *Pseudomonas aeruginosa* PAO1 in *E. coli* BL21 (DE3), a non-pathogenic host. A ~1.5 kb PCR product containing *chiC* was amplified from pGKMS21 and digested with EcoRI and HindIII. The gene fragments were ligated with pET-28a digested with the same enzymes and ligated to create pM3CRYY. The resulting construct was then transformed into *E. coli* BL21 (DE3) for expression. Sanger sequencing confirmed the identity of the construct and showed that pM3CRYY contained a full-length *chiC* that aligned to *chiC* of the *P. aeruginosa* PAO1 reference genome with >99% sequence identity. Despite the overall high sequence alignment to the reference genome, we must acknowledge the point mutations which can alter the amino acid composition of the ChiC protein. Although we did not purify and test the functionality of our ChiC protein, future studies could explore how the enzymatic activity of ChiC may have been altered by such mutations. In addition, mutations in ChiC would also be

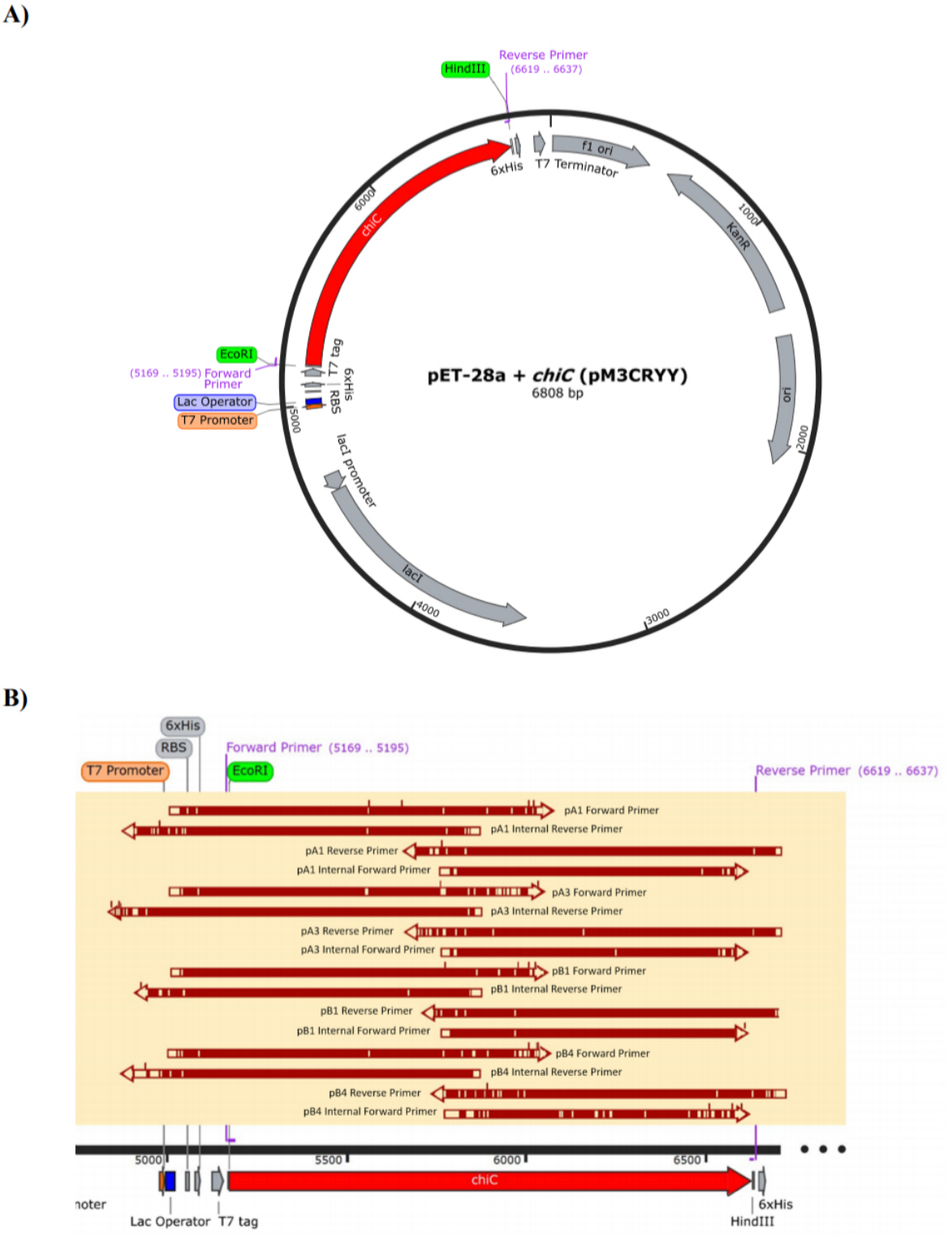


FIG. 4 Alignment of sequence reads to pET-28a + *chiC* reference sequence confirms the identity of the pM3CRYY construct and *chiC* insert. A) Plasmid map of 6.8 kb pM3CRYY, constructed by ligating 1.45 kb *chiC* insert into a pET-28a backbone, shows *chiC* between and in frame with the T7 promoter and terminator. **B)** Sequencing with universal and internal primers shows full gene coverage, with few regions of low base call accuracy and points of likely sequencing error or possible mutation.

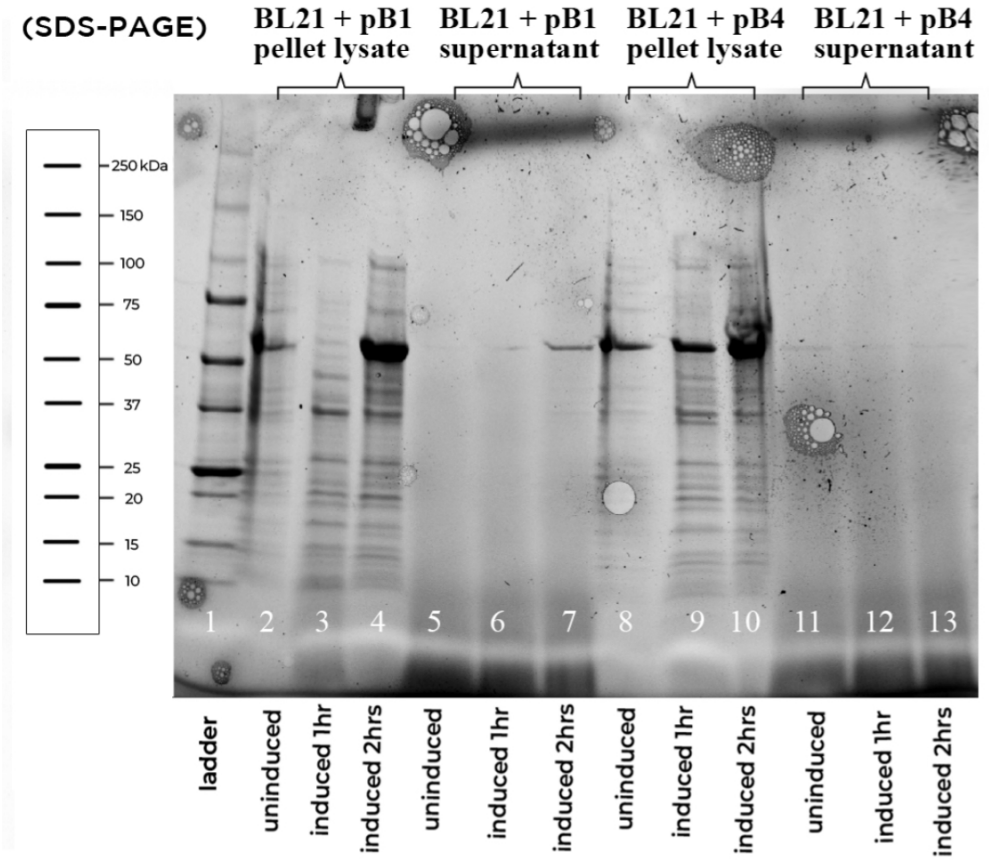


FIG. 5 Lysates of *E. coli* BL21 transformed with verified pM3CRYY (pB1, pB4) contained higher amounts of 55kDa ChiC candidate protein 2 hours after IPTG induction. Lane 1 contains the 10µl of 10-250kDa protein ladder. Lanes 2-4 and 8-10 contain proteins from pellets of BL21 subcultures that were uninduced, induced for 1 hour, and induced for 2 hours respectively. Lanes 5-7 and 11-13 contain proteins from supernatants of BL21 subcultures subject to the same conditions.

instrumental in understanding the interactions with host and bacterial components that allow ChiC secretion.

Based on the NCBI BLAST alignments of the query sequences, we found that pA1, pA3, pB1, and pB4 all had a sequence identity greater than 99% when compared to *chiC* from the reference *P. aeruginosa* PAO1 genome (Table 2S). However, the sequenced reads from pA1, pB1, and pB4 showed several mismatches that may be genuine mutations. In particular, pA1 and pB1 contained overlapping mismatches that were due to either point mutations or consistent sequencing error caused by low base call accuracy. pB4 likewise contained several regions of mismatches, but pB4 reads had notably lower quality scores that make it unreliable until further sequencing confirmation. Therefore, the results suggest that pA1, pB1, and pB4 are not suitable for future research until sequences are reproduced through further sequencing. Instead, we suggest using pA3 for future ChiC expression studies, as it appears to be 100% identical to the *P. aeruginosa* PAO1 *chiC* reference sequences.

We induced expression of *chiC* in pM3CRYY-transformed *E. coli* BL21, and SDS-PAGE of cell lysates identified ~55kDa protein bands corresponding to ChiC, showing that expression of *chiC* was likely successful. However, it is interesting to note that even though ChiC is expected to be secreted into the extracellular space, the 55kDa protein was detected in cell lysates as opposed to the supernatant, suggesting that the enzyme was retained intracellularly rather than secreted. Another possible explanation for this result is that 2-hour incubation period was not enough time for the protein to be secreted. Some studies on ChiC secretion in *P. aeruginosa* observed the initial accumulation of ChiC in the cytosol prior to its gradual secretion over the course of several days (8). As such, the short duration of our IPTG induction protocol might not have allowed for protein secretion.

One other possible explanation is that *E. coli* might not possess the unknown secretion system used by ChiC in its native *Pseudomonas* system (8). In this case, our BL21 expression model would provide a unique opportunity to study ChiC secretion, which could be explored by engineering these pM3CRYY(+) BL21 cells with components of the *P. aeruginosa* PAO1

genome that could be involved in the pathway. This type of experiment could make it possible to identify and test the sufficiency of factors involved in ChiC secretion. The same components could also be deleted from *P. aeruginosa* PAO1 to test for their necessity in ChiC secretion. Although the secretion system used by *P. aeruginosa* PAO1 is unknown, Folders *et al.* (2001) showed that quorum sensing is associated with ChiC expression. Interestingly, quorum sensing in *P. aeruginosa* is often used for the purposes of secreting exoproteins such as elastase (LasB) and it may be possible to identify components of the ChiC secretion pathway through the deletion of genes involved in elastase secretion (8).

Future studies should also aim to investigate the activity of ChiC. The construct in pM3CRYY has the *chiC* gene linked to a 6X Histidine tag, which allows for the purification of ChiC using an Immobilized Metal Affinity Column (IMAC). ChiC could then be tested for insecticidal activity on insect models and further explored as a biocontrol agent in field tests. The insecticidal activity of *Pseudomonas* strains has previously been shown with injection and feeding assays (9), while contact assays are also applicable. Should the insecticidal activity assays produce positive results, options can be explored regarding how to use ChiC as a biocontrol agent in field tests.

Limitations Loading of samples for SDS-PAGE was challenging, as lysate samples were contaminated by DNA that made the samples too viscous to pipet accurately. Even after diluting the lysate samples twice with Laemmli dye followed by centrifugation, the samples continued to adhere to the walls of tubes and tips. With this, volumes of samples loaded onto the gel were inconsistent, and concentrations of the proteins could have been affected. It is possible that sample-loading inaccuracies may be partly responsible for differences in observed protein band intensities. The same issue can also explain the inconclusive results of the first pre-optimized protein gel for IPTG-induced transformants of pM3CRYY candidates pA1 and pA3 (FIG. 3S). Moreover, induction was limited to 2 hours, which might have led to lower levels of detected protein in the supernatant than what could be observed over a longer duration. Nonetheless, it remains clear from our study that the cells transformed with the expression vector pM3CRYY produced a 55kDa protein product.

Conclusions We aimed to determine whether *chiC* of *P. aeruginosa* PAO1 could be expressed in an *E. coli* BL21 (DE3) system. A 1.5kb *chiC*-containing insert was obtained from pGKMS21 and ligated into a pET-28a expression vector to create the *chiC*-expression vector, pM3CRYY. Successful construction of pM3CRYY was confirmed by Sanger sequencing results, which showed more than 99% sequence identity between *chiC* from pM3CRYY and the *P. aeruginosa* PAO1 reference genome. Transformation of pM3CRYY into *E. coli* BL21 and subsequent IPTG induction resulted in the production of a 55kDa ChiC product.

Future Directions Our findings have shown that the expression vector pM3CRYY allows for the expression of *P. aeruginosa* PAO1 *chiC* in an *E. coli* BL21 system. Although it appears that this expression system may not support the secretion of ChiC, future studies may nonetheless use our pM3CRYY construct to express *chiC* in a non-pathogenic host, from which the enzyme could be purified via immobilized metal affinity chromatography. Upon confirming its amino acid sequence and identity, ChiC could then be assayed to test its chitinolytic and insecticidal properties, which could be potentially marketed and commercialized for industrial use as a biocontrol agent. In the context of future agricultural biocontrol applications, ChiC could be tested against an insect model system such as *G. mellonella* and known agricultural pests, such as *Helicoverpa armigera* and *Spodoptera litura* in injection, feeding and contact assays (9). The efficacy of ChiC as an insecticide could also be compared against that of common insecticides currently used in agriculture. Moreover, successful purification of ChiC could facilitate future experiments aimed at optimizing the expression of *chiC* in a host model such as *E. coli* BL21, and provide new insights into unique mechanisms and components that are crucial to understanding ChiC secretion.

ACKNOWLEDGEMENTS

We would like to thank Dr. David Oliver, Jade Muileboom and Gara Dexter of the MICB 401 teaching team for their continued support and guidance in making this project possible. We would also like to acknowledge the previous MICB 401 student team for providing us with pGKMS21 for our initial experiments. Lastly, we would like to thank UBC's Department of Microbiology and Immunology for providing access to the laboratory resources and reagents used in our research.

CONTRIBUTIONS

Laboratory work. Michael took the lead in chiC amplification, digestion and sequence alignment. Madalena took the lead during the ligation of insert and pET-28a to create and propagate our pM3CRYY *chiC*-expression construct. Matthew took the lead in screening for the insert and transforming candidate pM3CRYY vectors into E. coli *BL21*. The team then collaborated on IPTG induction of BL21 transformants and SDS-PAGE of lysate and supernatants.

Manuscript. MCR edited the abstract, drafted the introduction, discussion, conclusion, compiled the references and worked with MEY in drafting the study limitations. MEY worked on the abstract, completed the methods, results, figures, tables, supplemental materials, figure captions and future directions, and edited all sections of the manuscript. MY worked on the abstract, provided the plasmid map, collaborated with MEY to create project flow diagrams and sequencing visuals, and revised the discussion.

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