Recombinant Chitinase C from *Pseudomonas aeruginosa* is expressed and potentially secreted in *Escherichia coli* BL21 (DE3)

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SUMMARY There have been growing efforts to develop natural alternatives to chemical pesticides to mitigate the repercussions of microbiological post-harvest loss on the agricultural industry. Chitin is an attractive target because it is abundant in organisms that are leading causes of post-harvest loss. Many organisms have evolved chitinases which hydrolyze chitin and may serve in the development of a natural biocontrol agent. In this study, we investigated recombinant chitinase C (ChiC) expressed from the previously established pM3CRYY expression vector. We hypothesized that pM3CRYY(+) *E. coli* BL21 (DE3) expresses and secretes recombinant ChiC. Ultimately, we confirmed the retention of chiC in the pM3CRYY expression vector through Sanger Sequencing and evaluated the expression and secretion of ChiC via western blotting. We determined that ChiC is strongly expressed in pM3CRYY(+) *E. coli* and potentially secreted in the extracellular medium. To determine if recombinant ChiC retains its chitinolytic activity, we piloted a functional assay that utilized media clearance on chitin-containing plates as a readout for enzymatic activity. Our findings were inconclusive; however, they serve as an important stepping stone for the improvement of future chitinolytic assays. Furthermore, our study supports the potential of recombinant ChiC expressed in non-pathogenic *E. coli* for the development of a natural biocontrol alternative to synthetic pesticides.

INTRODUCTION

A constant war between phytopathogenic organisms and the agricultural industry has existed as early as the 1930s when synthetic pesticides like DDT emerged to combat postharvest diseases (1). Although effective, chemical pesticides come with a major environmental burden and threaten the health of both animals and humans. Currently, there is a growing effort to develop less toxic biocontrol agents that take advantage of the antagonistic mechanisms that already exist in nature.

Chitin is the second most abundant polysaccharide observed in nature following cellulose (2). From exoskeletons and shells of marine invertebrates to the cell walls of algae and fungi (3-5), this polymer is vital to the architectural reinforcement and self-defense of these organisms (6). Conversely, organisms across archaea, prokaryotes, and eukaryotes produce a host of chitinases and chitin-binding proteins that synergistically function to degrade chitin (3, 6). Specifically, chitinases hydrolyze the beta-1,4 linkages of the beta-1,4-N-acetyl-D-glucosamine (GlcNAc) structure of chitin (3).

Chitinase C (ChiC), which is produced by *Pseudomonas aeruginosa* PAO1, is a 55 kDa protein consisting of three functional domains: a catalytic domain, a fibronectin type III domain, and a chitin-binding domain (Fig. 1A) (7). Since *P. aeruginosa* is known to cause respiratory and blood-borne infections in humans (8), the development of an alternative biocontrol agent to chemical fungicides and insecticides for postharvest disease control in a non-*Pseudomonas* host is an active area of research (5). Furthermore, a non-*Pseudomonas* host would allow for controlled regulation of *chiC* expression and a significantly increased yield of ChiC. However, limited knowledge currently exists regarding the expression and functionality of *P. aeruginosa* ChiC in non-*Pseudomonas* hosts.

Bodykevich *et al*. originally amplified a 1.6 kb DNA fragment containing *chiC* from *P. aeruginosa* PAO1, which they subsequently subcloned into the CR2.1 plasmid to generate

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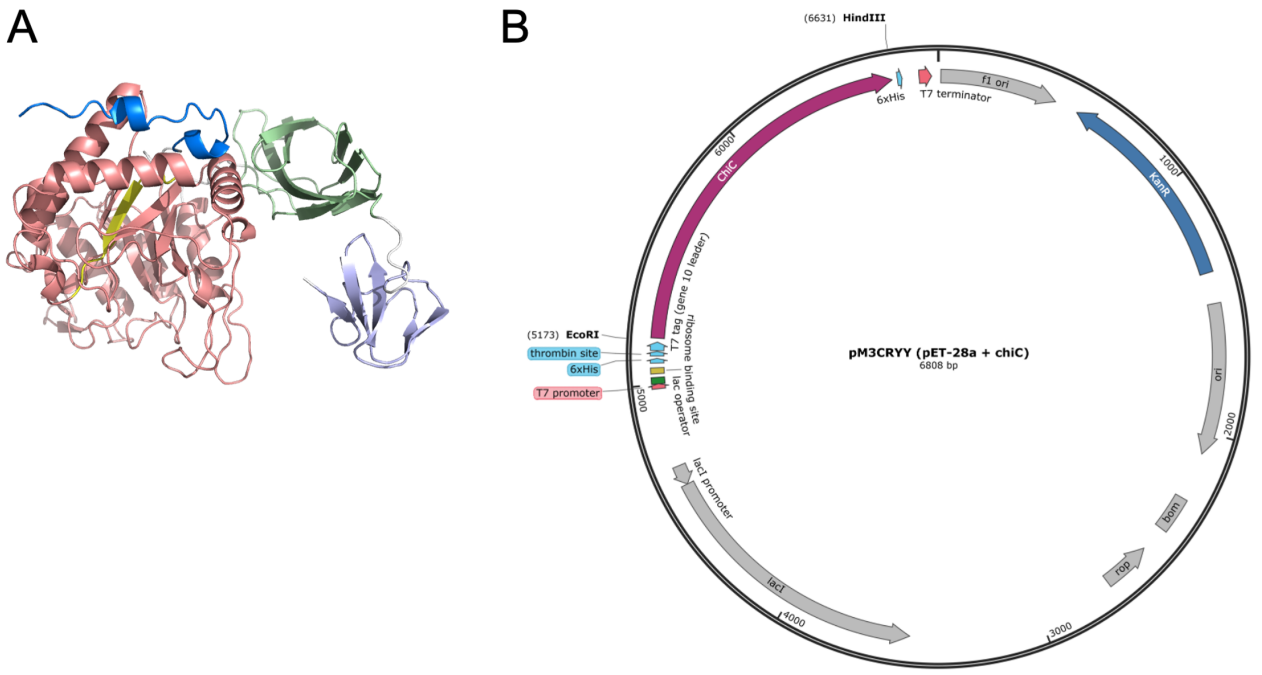

FIG. 1 3D rendering of ChiC and pM3CRYY expression vector construct (A) 3D structural model of ChiC from *P. aeruginosa* generated with ColabFold. Feature annotations include the arbitrarily defined N-terminus (residues 0-20; blue), the glycoside hydrolase family 18 (GH18) domain (pink) containing the ChiC active site (yellow), the fibronectin type-III domain (green), and carbohydrate-binding module family 5/12 (purple). (B) Map of pM3CRYY illustrates the construct generated by Rocha *et al*. (7), which includes the *chiC* insert cloned downstream of a T7 promoter in the pET-28a expression vector.

the pGKMS21 storage vector (9). While they verified that full-length *chiC* was present via Sanger Sequencing, it was not cloned in-frame to a promoter; therefore, the pGKMS21 vector cannot be directly used for expression. Rocha *et al*. (7) continued this research by generating an expression vector to study the expression and secretion of *chiC* in an *Escherichia coli* model system (7). They amplified *chiC* from pGKMS21 and ligated it into a pET-28a backbone vector to create the pM3CRYY expression vector (Fig. 1B). pM3CRYY was transformed into *E. coli* BL21 (DE3) and *chiC* expression was induced with isopropyl β-d-1-thiogalactopyranoside (IPTG) (to induce T7 promoter activity) for 1 and 2 hrs. Following IPTG-induction, protein lysates and supernatant were run on SDS-PAGE to screen for a 55 kDa product.

Although their findings do support the expression of a 55 kDa protein product in pM3CRYY(+) *E. coli*, there was no confirmation of its identity as ChiC. Rocha *et al*. (7) additionally claim that there was no secretion of ChiC. However, we believe their results suggest otherwise as a 55 kDa protein product from their supernatant samples appears in increasing intensity over their 2-hr time course experiment. Rather than a lack of secretion, the faintness of the band relative to the lysate samples may be attributed to the expected dilution of protein in solvent compared to lysates, which concentrates the protein. Furthermore, whether the presumed recombinant ChiC retains its chitinolytic function remains to be investigated through a functional assay.

The overarching objective of our study was to evaluate the expression and secretion of recombinant ChiC in the protein lysates and supernatant, respectively, from pM3CRYY(+) *E. coli* BL21 by immunoblotting. To further investigate the appearance of a supernatant band by 2 hrs (7), we extended the IPTG time course to 4 hrs. We determined that ChiC was highly expressed in protein lysates following 2- and 4-hr IPTG induction with no discernable difference in band intensity between the timepoints. A 55 kDa band of increasing intensity was also observed in the supernatant. Additionally, we aimed to ascertain if recombinant ChiC retains its functionality when expressed in pM3CRYY(+) *E. coli*. To address this question, we performed a functional assay in which zones of clearance around isolated

colonies grown on chitin-containing agar plates was used as a readout for chitinolytic activity. Though our results were inconclusive, they serve as a building block for the development of future functional assays. Overall, our findings support the potential for utilizing the expression of recombinant ChiC in non-pathogenic *E. coli* for development of a natural biocontrol alternative to synthetic pesticides.

METHODS AND MATERIALS

Preparation of experimental materials, reagents, and samples. 25mg/mL Kanamycin (Kan) stock solution was prepared from Gibco^{TM} Kanamycin Sulfate (Lot 1913841) and distilled H_2O by filter sterilizing through a 0.2µm pore and storing at -20ºC. Lysogeny Broth (LB) was prepared from 20g tryptone, 10g yeast extract, 10g NaCl, per liter of distilled H_2O (10g of agar was added for solid media). Kanamycin was added to media at a working concentration of 25µg/mL. 0.1M $CaCl_2$ and 0.1M $CaCl_2$ + 15% glycerol solutions were prepared according to the method outlined by Chang *et al.* (10) on competent cell preparation. 10mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) stock solution was prepared in 25µg/mL LB+Kan broth.

Culturing. Isolated colonies of pM3CRYY(+) *E. coli* BL21 (DE3) from Rocha *et al.* (7) and pET-28a(+) *E. coli* DH5α from the MICB 471 lab grown on 50µg/mL LB+Kan were used to inoculate 5mL of 25µg/mL LB+Kan. Both cultures were grown overnight at 37ºC shaking at 200rpm. The overnight cultures were subcultured in a 1:100 dilution and grown for approximately 3 hrs to achieve an OD_{600} of about 0.4. The pM3CRYY(+) *E. coli* BL21 and pET-28a(+) DH5α *E. coli* cultures were used in downstream experiments and to generate glycerol stocks for storage.

Generation of pET-28a(+) *E. coli* BL21 (negative control). Calcium chloride heat shock transformation procedures were adapted from Chang *et al.* (10) to generate competent *E. coli* BL21 cells and transform them with pET-28a. 25µg/mL LB+Kan plates were used to select for successful transformants, one of which was subsequently used to prepare a starter plate of pET-28a(+) *E. coli* BL21. This starter plate was used to generate liquid cultures for downstream experiments and glycerol stocks for storage.

IPTG induction for ChiC expression. Three 5mL liquid cultures of pM3CRYY(+) and one of pET-28a (+) *E. coli* BL21 were grown overnight at 37°C and 200 rpm in 25µg/mL LB+Kan broth. The resulting cell suspensions were subcultured in a 1:25 dilution and grown for approximately 2 hrs to achieve an OD_{600} of ~0.4. All cultures were normalized to an OD_{600} of 0.3 by diluting as necessary. 100mM of IPTG was added to each culture for a working concentration of 0.1mM and placed on a shaking incubator at 37ºC and 200 rpm for the respective timepoints: pM3CRYY(+) cultures were induced for 0, 2, and 4 hrs, while the negative control, pET-28a(+) culture, was induced for 4 hrs. After each respective time point, the samples were diluted to an OD_{600} of 0.3 before proceeding with the isolation of lysates and supernatants.

Sanger sequencing of pM3CRYY and pET-28a plasmids. The pM3CRYY expression vector was isolated from pM3CRYY(+) *E. coli* BL21 using the EZ-10 Spin Column Plasmid DNA Minipreps Kid (BioBasic, Lot: BS614-N812R0C) according to the manufacturer's instructions. The pET-28a backbone vector was isolated from pET-28a(+) *E. coli* DH5α using the same method. Using the GeneWiz guidelines, four samples of pM3CRYY and one sample of pET-28a were submitted for Sanger Sequencing. The following primers were used for the pM3CRYY samples to obtain full coverage of the *chiC* insert: custom internal forward (IntF, 5'-caagtacgtgccttatctgcag-3'), custom internal reverse (IntR, 5'-gaagtcttctttcatcgcgtcg-3'), universal T7 forward, and universal T7 reverse. The custom internal primers were provided by Rocha *et al.* (7) and the universal primers were provided by GeneWiz. For the pET-28a sample, we used the GeneWiz universal T7 forward primer. For each of the samples, we aliquoted 10µL of isolated plasmid (~30-70ng/µL) and 5µL of 5µM primer into a PCR tube. Sequencing results for pM3CRYY were aligned against the *P. aeruginosa* PAO1 complete

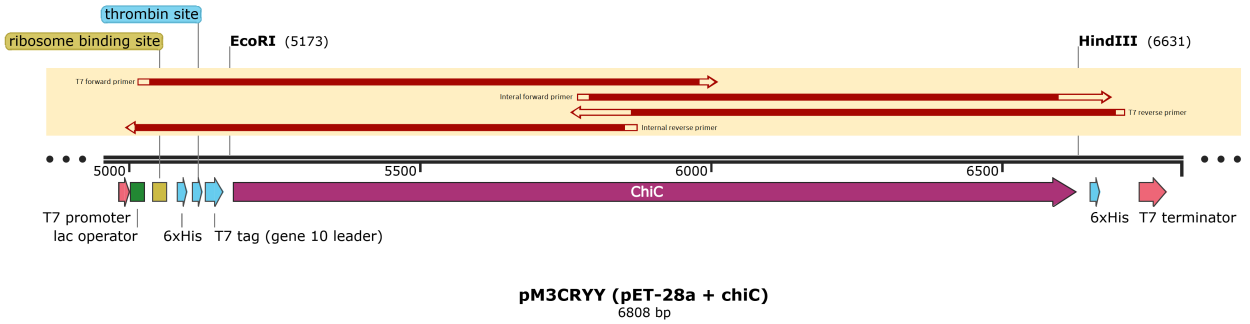

FIG. 2 Sanger sequencing of pM3CRYY confirms pM3CRYY identity and presence of *chiC*. Isolated pM3CRYY from pM3CRYY(+) *E. coli* BL21 prepared by Rocha *et al.* (7) was submitted for Sanger Sequencing using two custom internal primers and two universal primers (T7 promoter and T7 terminator). Sequencing reads were aligned against a pM3CRYY construct on high stringency in SnapGene and demonstrated full coverage of *chiC* and adjacent markers.

genome (NC_002516.2) using NCBI BLAST (Table S1) and against a pM3CRYY reference construct in SnapGene on high stringency, adjusting low accuracy base calls (Fig. 2). The pM3CRYY map was constructed from pET-28a (https://www.addgene.org/browse/sequence_vdb/2565/) and *chiC* (AF279793.1) reference sequences. The pET-28a sequencing read was aligned against a reference pET-28a (https://www.addgene.org/browse/sequence_vdb/2565/) sequence in SnapGene on high stringency (Fig. S1).

Western blotting to determine ChiC expression. Recombinant ChiC expressed by pM3CRYY(+) *E. coli* BL21 was probed for via western blotting. Cell pellets of 0-, 2-, and 4-hr IPTG-induced pM3CRYY(+) *E. coli* BL21 (and 4-hr IPTG-induced pET-28a(+)) were resuspended in 1x protease inhibitor cocktail solution (VWR, Lot: 19F2156957) diluted in dH_2O. Samples were lysed in a homogenizer (MP FastPrep-24 bead beater) with 0.1mm glass beads (BioSpec Products, Cat: 11079101) for 30s at 6.0 m/s then centrifuged at 15000 rpm for 1 min at 4°C to collect protein lysates.

Protein lysates and supernatant samples were loaded for SDS-PAGE onto a 10% TGX Stain-Free FastCast Acrylamide gel (BioRad, Lot: L003997 B) and run at 200V for ~40 mins. Resolved proteins were transferred onto a Nitrocellulose membrane using the Trans-Blot Turbo Transfer Pack (BioRad, Cat: 1704158) and Trans-Blot Turbo Transfer System (BioRad). The membrane was blocked with 5% BSA (Sigma, Lot: 118H0563) diluted in TBS-T, and ChiC was probed for using primary mouse IgG anti-6x-His tag antibodies (Invitrogen, Prod: MA1-21315, Lot: TG267120) diluted 1:1000 in 1% BSA in TBS-T and 1:10000 diluted secondary goat anti-mouse IgG horseradish peroxidase (HRP)-conjugate antibodies (Invitrogen, Lot: RJ240410). The immunolabeled membrane was incubated with a luminol and peroxide detection solution (Clarity Max Western ECL Substrate, Cat: 1705062) and visualized for chemiluminescence using the ChemiDoc imaging system (BioRad).

Preparation of colloidal chitin plates. Nine LB+chitin plates, ten chitin+pH indicator plates, and ten chitin-only minimal medium plates were provided by the MICB 471 lab. Colloidal chitin was prepared by dissolving 10g of crab chitin in 100mL of concentrated HCl. The resulting slurry was kept on ice to reduce excessive heat production and stirred every 5 min for 30 min. Acid slurry was mixed with 2L of chilled, distilled H_2O and resulting white fluffy suspension was stirred for ~20 min. A laboratory coat clothing material (LCCM) filtration apparatus was assembled, in which a piece of LCCM was used as the filtering material in a Buchner vacuum filtration apparatus. Vacuum filtration was used to filter the colloidal chitin-HCl mixture, collecting the colloidal chitin in the LCCM and letting the filtrate drain from the thick slurry. The isolated colloidal chitin was resuspended in 2L of chilled, distilled H_2O and stirred for ~20 min. The filtration process was repeated 15-20 times until the colloidal chitin achieved a near neutral pH. For the chitin+pH indicator plates, basal chitinase medium

was supplemented with colloidal chitin (4.5g/L) and bromocresol purple (0.15g/L), and pH was adjusted to 4.7 (11). Chitin-only minimal medium plates were prepared by adding 8g colloidal chitin, 0.7g K_2HPO_4, 0.3g KH_2PO_4, 0.5g $MgSO_4 \cdot 5H_2O$, 0.01g $FeSO_4 \cdot 7H_2O$, 0.001g $ZnSO_4$, 0.001g $MnCl_2$, 20g agar, and 1L distilled H_2O and adjusting the pH to 8.0 (12). To prepare the LB+chitin plates, colloidal chitin was lyophilized overnight, ground into a fine powder using a mortar and pestle, and added to LB agar plates at 4g/L. All plates were autoclaved at 121°C for 15 min.

Chitinolytic functional assay. pET-28a(+) *E. coli* BL21 and pM3CRYY(+) *E. coli* BL21 were inoculated from 25μg/mL LB+Kan starter plates and grown overnight in the 37ºC and 200 rpm incubator. 1:25 diluted subcultures were grown to achieve an OD_{600} of ~0.4 before they were induced with 0.1mM IPTG for 2 hrs. 2-hr IPTG-induced pET-28a(+) *E. coli* achieved an OD_{600} of 0.810 and pM3CRYY(+) *E. coli* reached an OD_{600} of 1.274, the concentrations of which were directly used in disk soaking and streak plating. Three of each type of chitin plate were soaked with 12μL of each sample in triplicate pipetted onto autoclaved 6 mm Whatman filter paper disks and four of each type of chitin plate were streaked with each sample in duplicate (one less pET-28a(+) *E. coli* for LB+chitin). 2-hr IPTG-induced pET-28a(+) *E. coli* BL21and pM3CRYY(+) *E. coli* BL21 were normalized to an OD_{600} of 0.50 that corresponds to $4x10^8$ cells/mL. Four 1/10 serial dilutions were performed using sterile PBS on a 96-well plate with the normalized starting concentration. 5μL from each concentration was spot plated in triplicate, using three plates from each type of chitin plate. All plates were stored in a box containing a thin layer of sterile water to maintain humidity and incubated at 30°C for five days.

RESULTS

***chiC* is present in the pM3CRYY expression vector isolated from pM3CRYY(+) *E. coli* BL21.** To confirm the identity of pM3CRYY from the pM3CRYY(+) *E. coli* BL21 stock prepared by Rocha *et al.* (7), we isolated and submitted pM3CRYY for Sanger Sequencing (Fig. 2). Four primers were used to achieve full coverage of the *chiC* insert. Alignment of the sequencing reads to a reference pM3CRYY construct on high stringency in SnapGene demonstrated 100% sequence identity; low accuracy terminal regions were excluded and three internal low accuracy base calls were manually adjusted. The sequence alignment also indicated the presence of a 6xHis tag on either terminal end of the *chiC* open reading frame (ORF). Though a stop codon prevents the translation of the C-terminal 6xHis tag, the N-terminal 6xHis tag is still expressed and can therefore be used to probe for ChiC. Thus, our sequencing analysis confirmed the retention of pM3CRYY in our *E. coli* BL21 model, including the presence of *chiC* and adjacent markers such as the N-terminal 6xHis tag.

ChiC from pM3CRYY(+) *E. coli* BL21 is highly expressed following 2- and 4-hr IPTG induction. To evaluate the expression and secretion of recombinant ChiC in pM3CRYY(+) *E. coli* BL21, protein lysates and supernatants were isolated from uninduced, 2- and 4-hr IPTG-induced pM3CRYY(+) *E. coli* and 4-hr IPTG-induced pET-28a(+) negative control for western blotting. A 55kDa band at the expected molecular weight for ChiC indicated its expression and secretion in the pM3CRYY(+) *E. coli* lysates and the supernatants, respectively (Fig. 3). ChiC was not expressed in pET-28a(+) *E. coli* but was constitutively expressed at a basal level in uninduced pM3CRYY(+) *E. coli*. Following a 2- and 4-hr IPTG induction of pM3CRYY(+) *E. coli*, ChiC expression increased significantly compared to the uninduced bacteria. Faint bands in the supernatants increased slightly in intensity over a 2-hr time course, suggesting potential secretion of ChiC. Quality of the SDS-PAGE run is indicated by the stain-free bands. Through immunoblotting, we were able to confirm the expression of ChiC in an *E. coli* model system and lend support to our hypothesis that ChiC is secreted extracellularly.

Piloting a chitin assay to evaluate chitinolytic activity of recombinant ChiC did not validate functionality. To ascertain whether recombinant ChiC is capable of cleaving colloidal chitin, a functional assay utilizing LB+chitin, chitin+pH indicator, and chitin-only minimal medium plates was conducted. pET-28a(+) and pM3CRYY(+) *E. coli* BL21 were induced with IPTG for 2 hrs then plated using disk soaking, streaking, and spot-plating techniques. For LB+chitin plates, the appearance of colonies would indicate pET-28a(+) and

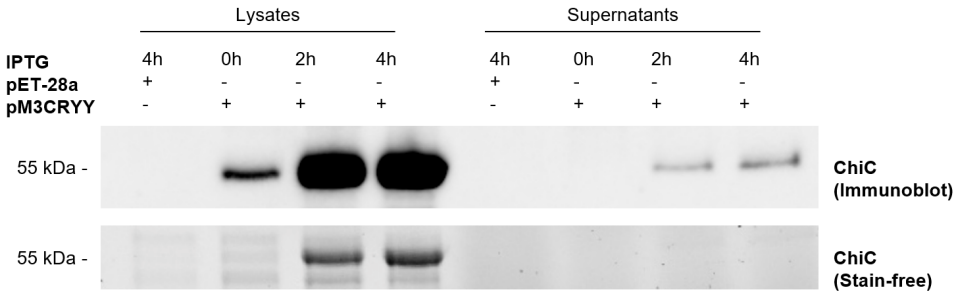

FIG. 3 IPTG induction in pM3CRYY(+) *E. coli* BL21 yields highly expressed ChiC. Lysates and supernatants from negative control pET-28a(+) *E. coli*, uninduced pM3CRYY(+) *E. coli*, and 2-hr and 4-hr 0.1mM IPTG-induced pM3CRYY(+) *E. coli* were loaded for SDS-PAGE and visualized through stain-free imaging (bottom). Western blotting for chemiluminescence imaging was performed using primary mouse IgG anti-6x-His tag antibodies and goat anti-mouse IgG HRP-conjugate secondary antibodies (top). IPTG induction elevated expression of ChiC in the lysates of pM3CRYY(+) *E. coli* and possible secretion in the supernatant.

pM3CRYY(+) *E. coli* BL21 growth using LB, while zones of clearance around colonies in 2-hr IPTG-induced pM3CRYY(+) *E. coli* BL21 would indicate chitinase activity. On chitin+pH indicator plates, the breakdown of chitin into N- acetyl glucosamine would cause a corresponding pH change towards alkalinity and a color change in the pH indicator dye from yellow to purple (11). Additionally, on the chitin-only minimal media plates, the breakdown of chitin would result in zones of clearance around colonies (12). The LB+chitin plates demonstrated growth over two days (Fig. 4). On day one, growth was observed only in pM3CRYY(+) *E. coli* samples as indicated by outward bacterial growth in the disk soaking

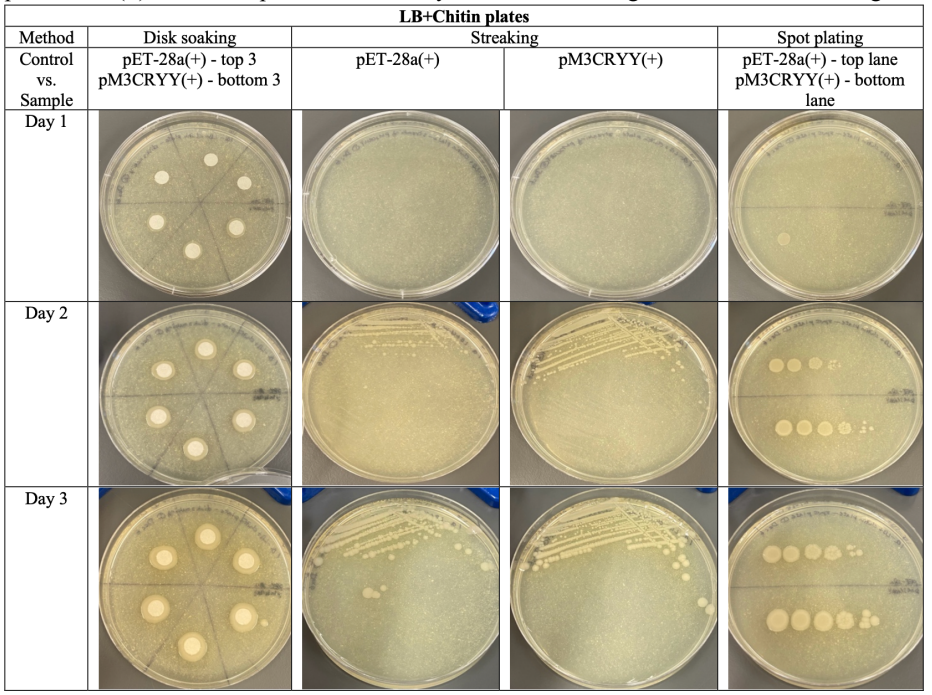

FIG. 4 2-hr IPTG-induced pET-28a(+) and pM3CRYY(+) *E. coli* BL21 demonstrated different growth rates on LB + chitin plates. OD_{600} 0.810 pET-28(+) and OD_{600} 1.274 pM3CRYY(+) *E. coli* BL21 after a 2-hr induction were plated using disk soaking (n=3) and streaking methods (n=2). Normalized to $OD_{600} = 0.5$ ($4x10^8$ cells/mL), four 1/10 serial dilutions were performed using sterile PBS that gave rise to five spot-plated colonies with decreasing concentrations (left to right) (n=3). 2-hr IPTG-induced pET-28a(+) and pM3CRYY(+) *E. coli* BL21 were associated with different growth rates, but the lack of ChiC expression in pET-28(+) *E. coli* BL21 negative control did not deter growth on LB + chitin plates.

method and a single spot for the most concentrated sample ($4x10^8$ cells/mL) in the spot plating technique. No growth was observed in neither pET-28a(+) nor pM3CRYY(+) *E. coli* samples in streaked plates. On days two and three, outward bacterial growth was present around pET-28a(+) *E. coli* disks, similar in size to the pM3CRYY(+) *E. coli* conditions, and five spot-plated colonies were observed in decreasing size consistent with the dilutions. Additionally, growth was seen on the streaked plates for both conditions on day two which increased by day three with more colonies of pM3CRYY(+) *E. coli*. Although ChiC is not expressed in the pET-28a(+) *E. coli* negative control, growth was nonetheless apparent on the LB+chitin plates, albeit at a slower rate than pM3CRYY(+) *E. coli*. Yet, the absence of a color change on chitin + pH plates (Fig. 5) and lack of zones of clearance on chitin-only minimal medium (Fig. 5) and LB + chitin plates (Fig. 4) suggest that pM3CRYY(+) *E. coli* was not capable of cleaving chitin. Based on these findings, we could not validate the retention of chitinolytic activity of recombinant ChiC.

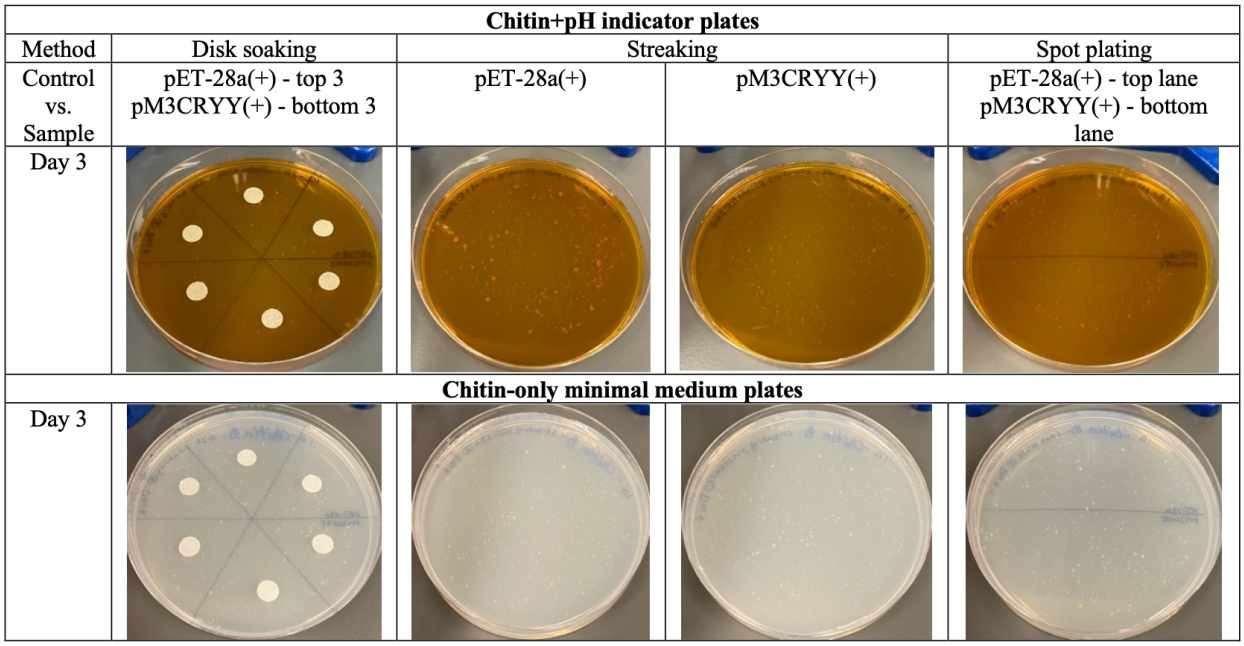

FIG. 5 No growth of 2-hr IPTG-induced pET-28a(+) and pM3CRYY(+) *E. coli* BL21 on chitin+pH indicator and chitin-only minimal medium plates. OD_{600} 0.810 pET-28(+) and OD_{600} 1.274 pM3CRYY(+) *E. coli* BL21 after a 2-hr induction were plated using disk soaking (n=3) and streaking (n=2). Normalized to $OD_{600} = 0.5$ ($4x10^8$ cells/mL), four 1/10 serial dilutions were performed using sterile PBS that gave rise to five spot-plated colonies with decreasing concentrations (left to right) (n=3). No signs of growth were observed on chitin+pH and chitin-only minimal medium plates, indicating that neither pM3CRYY(+) or pET-28a(+) *E. coli* BL21 could utilize chitin as a carbon source for growth.

DISCUSSION

In this study, we investigated the expression, secretion, and functionality of *P. aeruginosa* PAO1 ChiC in *E. coli* BL21 (DE3) transformed with pM3CRYY, the expression vector containing *chiC* (7). To evaluate the expression and secretion of recombinant ChiC, we utilized western blotting to probe for ChiC in the lysates and supernatants of 2- and 4-hr IPTG-induced pM3CRYY(+) *E. coli*. Given that *E. coli* BL21 (DE3) carries the T7 RNA polymerase, which is required for the expression of genes cloned downstream of the T7 promoter on the backbone vector (pET-28a), we hypothesized that *chiC* would be expressed by pM3CRYY(+) *E. coli*. Additionally, though the secretion mechanism of ChiC is not fully understood, the findings by Rocha *et al*. (7) led us to hypothesize that pM3CRYY(+) *E. coli* also secretes ChiC. Thus, we expected to resolve bands at 55 kDa on our immunoblot for both protein lysates and supernatant samples. To address the functionality of recombinant ChiC from pM3CRYY(+) *E. coli*, we aimed to develop a functional assay that would assess whether the recombinant ChiC retained the ability to cleave chitin. Since the entire *chiC* gene (all three

functional domains) is present in the expression vector and because ChiC functionality has been demonstrated in a non-*Pseudomonas* host (3), we hypothesized that the recombinant ChiC produced by pM3CRYY(+) *E. coli* would retain its chitinolytic function. Thus, after incubating 2-hr IPTG-induced pM3CRYY(+) *E. coli* on various chitin-containing plate media, we expected to observe zones of clearance around isolated colonies, indicating chitinolytic activity.

We determined the sequence of the pM3CRYY expression vector in our *E. coli* model to validate our downstream experimental results. Our results confirmed the presence of the *chiC* insert as well as adjacent markers, including 6xHis tags at both termini of the *chiC* ORF. However, examination of the sequence indicated that only the N-terminal 6xHis tag is expressed as a stop codon that prevents the expression of the C-terminal 6xHis tag. The possible implications of having a 6xHis tag at the N-terminal of this recombinant ChiC depend on its location within the 3D structure of the protein; it could interfere with functionality if its position causes protein misfolding or if it blocks a domain interface essential for protein-protein interactions, but it could also have no discernible effect. We generated a replica 3D structural model of ChiC from *P. aeruginosa* PAO1 in the AlphaFold Protein Structure Database using the ColabFold implementation of AlphaFold2 (Fig. 1A) (13, 14). The highest ranked predicted structure generated with default parameters was visualized in pyMOL to annotate relevant protein regions to this study, including the arbitrarily defined N-terminus (residues 0-30), the glycoside hydrolase family 18 (GH18) domain (containing the ChiC active site), the fibronectin type-III domain, and carbohydrate-binding module family 5/12. Based on this model, the N-terminus is within an accessible region; therefore, the likelihood of the 6xHis tag interfering with function is low.

The results of our western blot confirm that recombinant ChiC encoded on pM3CRYY is expressed, corroborating the inference made by Rocha *et al.* (7) regarding the likely identity of the protein band they observed at 55 kDa. Specifically, we determined that ChiC was expressed at low levels in uninduced *E. coli* and significantly increased by 2- and 4-hr IPTG induction with no discernable difference in expression between the timepoints. Although Rocha *et al*. (7) claimed that ChiC is not secreted despite the presence of a 55 kDa protein product by 2 hrs, we ascertained in our extended time course to 4 hrs that ChiC was detected in the supernatant with increasing intensity over the IPTG time course. The intensity of ChiC protein levels from the supernatant are expectedly lower relative to ChiC from protein lysates because proteins in the supernatant are diluted in the medium while lysate proteins are concentrated. However, further studies into ChiC secretion will need to be conducted, as we cannot confirm whether the presence of ChiC in the supernatant is attributed to true secretion or a consequence of bacterial lysis caused by external stresses.

To investigate whether recombinant ChiC produced by pM3CRYY(+) *E. coli* retained its chitinolytic function, we expected to observe the appearance of colonies to indicate bacterial growth and zones of clearance around pM3CRYY(+) *E. coli* colonies to indicate chitinase activity. Although bacterial growth was observed on LB+chitin plates for both pM3CRYY(+) and the negative control pET-28a(+) *E. coli,* with pM3CRYY(+) *E. coli* appearing to grow faster, zones of clearance were never observed (Fig. 4). The difference in growth rates was most likely attributed to the lack of OD normalization (pM3CRYY(+) *E. coli* OD_{600} = 1.274; pET-28a(+) *E. coli* OD_{600} = 0.810) that resulted in faster growing pM3CRYY(+) *E. coli* colonies (Fig. 4). Additionally, abundant growth around the negative control paper disks despite the lack of ChiC expression suggests that LB was preferentially used as a carbon source to promote growth. Based on these findings, we cannot confirm if recombinant ChiC retained chitinolytic activity in *E. coli* BL21.

To further investigate the enzymatic function of recombinant ChiC, we used a color change on pH+chitin plates and zones of clearance on chitin-only minimal medium and on LB+chitin plates as a readout for chitin degradation. Our negative results on these plates (Fig. 4 and Fig. 5) suggest that ChiC may not have retained its chitinolytic activity. However, many other variables could also potentially explain the negative results observed for these plates. For instance, 1) *E. coli* BL21 (DE3) may not encode the chaperon protein required for the correct folding of ChiC, or 2) pM3CRYY might not encode a soluble form of ChiC to allow it to degrade soluble chitin (15). 3) Additionally, even if recombinant ChiC is functional, it only cleaves the beta-1,4 linkages of the beta-1,4-N-acetyl-D-glucosamine structure of chitin,

and *E. coli* BL21 may lack the enzymes needed to further break these products in order to utilize the carbon for growth (3). 4) Alternatively, *E. coli* BL21 may not possess the required exporter to enable sufficient extracellular secretion of ChiC to cause any discernible chitin degradation. 5) Furthermore, although faint bands were observed in the western blot supernatants from 2- and 4-hr IPTG-induced pM3CRYY(+) *E. coli*, it was unclear whether they were indications of ChiC secretion or cell lysis (Fig. 3). Future experiments could address any one of these variables to further investigate the functionality of recombinant ChiC.

For future chitin functional assays, having a positive control, such as ChiC-producing *P. aeruginosa*, would allow us to have more confidence in the methodological approach and preparation of the chitin-containing plates. DNA extractions and PCR with Sanger sequencing can be performed on non-pathogenic *P. aeruginosa* strains to confirm the presence of *chiC*. Alternatively, since the secretion of ChiC in *E. coli* BL21 is still unknown, spotting recombinant chitinase at high concentration could serve as potential positive control. Depending on the control and design of the assay, preparation of colloidal chitin can be adjusted accordingly. Regarding the practicalities of the assay, our plates were incubated at 30°C to ensure optimal enzymatic activity (14) and were placed in a box containing a thin layer of sterile water to prevent the agar from drying out during the five-day incubation period. However, this resulted in heavy condensation that made it difficult to observe growth. A lower temperature of 26°C would slow bacterial growth so as to not occur faster than halo generation while still supporting optimal enzymatic function of ChiC.

Conclusions In this study, we verified that *chiC* is present in the pM3CRYY expression vector and investigated the expression and secretion of recombinant ChiC in pM3CRYY(+) *E. coli* BL21 relative to pET-28a(+) *E. coli* BL21 (negative control). We determined that ChiC was highly expressed in protein lysates following 2- and 4-hr IPTG induction and potentially secreted extracellularly. Additionally, we piloted a chitin functional assay to ascertain if recombinant ChiC retains its chitinolytic functionality when expressed in pM3CRYY(+) *E. coli.* Though our results were inconclusive, they serve as a building block for the development of future functional assays in the hope of utilizing ChiC-secreting non-pathogenic *E. coli* as an alternative biocontrol agent.

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CONTRIBUTIONS

All authors contributed to experimental work in the MICB 471 laboratory. RL was responsible for the Sanger Sequencing method, figure, and results paragraph, discussion, abstract, chitinolytic method, and supplementary material. IL was responsible for the introduction, western blot method and figure, ChiC structural model, discussion, and abstract. SG was responsible for western blot results paragraph, preparation of colloidal chitin plates and chitinolytic functional assay methods, chitinolytic functional assay figures, results paragraph, discussion and conclusion. AB was responsible for preparation of materials, culturing, negative control, and IPTG induction methods, chitinolytic results paragraph, discussion, and abstract.

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