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In vitro screening of *Pseudomonas aeruginosa* PAO1 chitinase C

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SUMMARY Chitin is an abundant aminopolysaccharide constituent of many structural elements in crustaceans, insects, and fungi. They are critical for maintaining structural integrity. As such, chitin is an attractive target for agricultural pest control. Chitinases like chitinase C (*chiC*) from the *Pseudomonas aeruginosa* PAO1 genome are enzymes that exhibit chitinolytic activity. Many chitinase candidates have been screened *in vitro* and *in vivo* to identify key chitinases for downstream biotechnological applications. Previously, Rocha *et al.* cloned *chiC* into a pET-28a expression vector and subsequently confirmed expression in *E. coli* BL21(DE3). However, *chiC* was not purified and its chitinolytic properties remain unknown. Here we aimed to validate previous *chiC* experimental results as well as confirm chitinase activity. We identified a small number of mutations in the pM3CRYY backbone via plasmid sequencing. Following protein expression with IPTG, we purified ChiC and performed *in vitro* colloidal chitin agar assays. We were unable to detect any observable changes (zones of clearing for colloidal chitin plates and color change for colorimetric chitin plates) in our assays. Our findings indicate that currently established chitinolytic assays may need to be further optimized and *chiC* may undergo non-canonical secretion from *P. aeruginosa* which can enhance our understanding of secretion mechanisms.

INTRODUCTION

Chitin is a linear polymer of β-1, 4-N-acetylglucosamine and an essential biomolecule in nature. It is the second most abundant natural polysaccharide after cellulose, and is a crucial structural component in the exoskeleton and gut lining of insects, functioning as a scaffold (1). Chitin also serves as a constituent in various other agriculturally important arthropods, in addition to being a component of vital structures in nematodes and mollusks (2). Given chitin's abundance and significance in a multitude of agricultural pests, it is an appealing target for pesticides and insecticides.

Over the past several decades, extensive pesticidal use has resulted in the rapid evolution of resistance in crop pathogens, weeds, and insects to synthetic compounds (3). Their chemical properties can lead to imbalances in soil, water, and air, causing environmental problems (4). In addition to disruption of ecological balances, there are health implications surrounding misuse of pesticides. For instance, broad spectrum insecticides targeting insect nervous systems can have adverse non-specific activity, impacting humans (5). Such persistent exposure is linked to endocrine suppression, reproductive abnormalities, and cancer (6). As such, organic alternatives to synthetic chemicals have been explored to reduce biomagnification and contamination from pesticide residue.

Promising replacements for chemical pesticides include biopesticides based on microbes and their bioactive compounds (7). These pose a much lower risk to the environment and humans due to their high specificity. However, this limits their target species range, increasing costs and restricting them to niche markets. Thus, recent efforts have focused on the development of products active against multiple pests. Currently, enzyme-based pest controlling products are under investigation such as antifungal chitinases (7).

The recurrence and importance of chitin in insect structure and metabolism is of note when considering pesticidal targets. Chitinase, a hydrolytic enzyme that degrades chitin, has shown promise as a potential biopesticide (8). Chitinases are abundant in chitin-producing organisms, primarily insects, crustaceans, and fungi. The native function of these enzymes in bacterial species allows the use of chitin as a carbon and nitrogen source. They also serve

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alternative functions in various other physiological processes, including tissue remodeling and immune defense.

Pseudomonas fluorescens, a soil bacterium encoding a chitinase, has been shown to have insecticidal activity against aphids, mosquitoes, and termites (10). Notably, a study revealed that a chitinase produced by *P. fluorescens* MP-13 caused *Helopeltis theivora* mortality *in vitro* (11). Our study concerns *chiC*, a chitinase encoded by the *Pseudomonas aeruginosa* PAO1 genome. It is part of a larger glycoside hydrolase 18 family which is further subdivided into three major subfamilies, A, B, and C, based on amino acid homology in the catalytic domain (9). It shares high sequence homology (66-99%) with a number of *Serratia* and *Pseudomonas* chitinases, making it a representative enzyme to study chitinases.

In a previous study, Rocha *et al.* subcloned *chiC* from a storage vector into pET-28a to create pM3CRYY. Following plasmid expression, ChiC was present in cell lysates but not in the supernatant (12). However, the enzymatic activity of purified protein from cell lysates were not assayed (12). In a more recent study, Folders *et al.* observed zones of clearance on colloidal chitin plates after streaking colonies of *E. coli* expressing *chiC* (10). Therefore, we hypothesize that we can detect chitinase activity using ChiC purified from *E. coli* BL21(DE3) on colloidal chitin plates. In this study, we aimed to confirm the *in vitro* chitinolytic activity of *chiC* from both cell lysates and purified protein.

METHODS AND MATERIALS

Preparation of experimental materials, reagents, and samples. 10 mL of 50 mg/mL kanamycin stock solution was prepared from 0.506 g GibcoTM Kanamycin Sulfate and distilled water. The kanamycin stock solution was stored at -20°C. LB broth or agar were prepared from 5 g NaCl, 10 g Tryptone, 5 g yeast extract, distilled water, with or without 1.5% agar. LB with kanamycin agar (LB-KAN) was prepared by adding kanamycin stock to a final concentration of 50 ug/mL.

Plasmid extraction. We picked two pM3CRYY constructs previously confirmed via Sanger sequencing, A1 and A3, to validate via plasmid sequencing. In addition, pET-28a with no gene insert (empty vector) was picked as a protein expression negative control. Glycerol stocks of pM3CRYY A1 and A3 as well as empty vector were streaked onto LB-KAN agar and incubated overnight at 37°C for 16 hours. Per sample, 3 mL of LB-KAN broth was inoculated with an isolated colony and incubated overnight at 37°C for 16 hours. Cells were pelleted at 12,000 RPM for 2 minutes and the remaining supernatant was discarded. Extraction of the plasmids and storage vector was performed following the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic) protocol.

Preparation of chemically competent *E. coli* BL21(DE3) and transformation. We used Chang *et al.*'s calcium competent *Escherichia coli* preparation protocol to prepare chemically competent *E. coli* BL21(DE3) (13). We performed transformation via the canonical heat shock method. Briefly, 100 uL of *E. coli* BL21(DE3) was gently mixed with 1-10 ng of plasmid DNA and subject to the following incubation steps: ice for 30 minutes, 42°C for 30 seconds, then ice for 2 minutes. Heat shocked cells were recovered in 1 mL of antibiotic-free LB broth and incubated with shaking at 37°C for 1 hour. Recovered cells were pelleted at 14,000 RPM for 30 seconds, 950 uL of supernatant was discarded, then the pellet was resuspended in the remaining ~100 uL. These cells were plated on LB-KAN agar and incubated overnight at 37°C for 16 hours.

Plasmid sequencing. Miniprep concentration and purity was assessed on a Nanodrop. A1: 38.1 ng/uL, A260/280 = 2.00, A260/230 =1.48; A3: 38.6 ng/uL, A260/280 = 2.06, A260/230 = 1.28. Miniprep aliquots for A1 and A3 were normalized to 30 ng/uL and shipped to Plasmidsaurus (Oregon, USA) for sequencing. The resulting consensus sequences were mapped to the pM3CRYY plasmid and analyzed for mutations in the plasmid.

Protein expression via Isopropyl β-D-1-thio-galactopyranoside (IPTG) induction. 5 mL of LB-KAN broth was inoculated with an isolated colony of pM3CRYY A1 and grown

overnight on a 37°C shaking incubator for 16 hours. The overnight culture was diluted 1:100 in 500 mL LB-KAN broth and grown for 4 hours in a 30°C shaking incubator. The day culture was split equally for the induced and uninduced portions. IPTG was prepared as a 0.1 M stock solution. 0.1 mM IPTG was added to the induced portion. Both induced and uninduced portions were returned to the 20°C shaking incubator for 24 hours. Per condition, 1 mL was aliquoted in a microcentrifuge tube for SDS-PAGE analysis. Cells were then pelleted at 12,000 RPM for 1 minute. The supernatant and pellet were frozen separately at -80°C.

A pET-28a empty vector was used as a negative control. The IPTG induction was diluted 1:100 in 2 mL LB-KAN broth instead of 500 mL. The IPTG induction was 2 hours instead of 24 hours. The subsequent steps remained the same.

IMAC protein purification. *Preparation of buffers under non-denaturing conditions*. We used the following buffer recipes: lysis/binding buffer (10 mM Tris-Base, 150 mM NaCl and 1 mM EDTA, pH 7.34); wash buffer (20 mM sodium phosphate, 300 mM NaCl and 5 mM imidazole, pH 7.48) elution buffer (20 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 7.40). Lysozyme (1 mg/mL), DNAse I (1 mg/mL), and concentrated protease cocktail (10 uL/mL) were added to the binding buffer prior to cell lysis. *Purification.* The frozen IPTG induced cell pellet was thawed on ice. The cell pellet was then resuspended in 5 mL of lysis buffer and lysed using the FastPrep-24™ Classic bead beating grinder and lysis system for 30 seconds. The sample was then centrifuged at 12,000g for 15 minutes to pellet cellular debris. The resin was equilibrated with 5 mL (5CV) lysis buffer without lysozyme, DNAse I, and protease inhibitor. The supernatant obtained by centrifugation (12,000 x g for 15 min at 4°C) was loaded onto the column. The column was then washed with 10 mL (10CV) of wash buffer. A final volume of 10 mL (10CV) of elution buffer was used.

SDS-PAGE analysis. Protein expression pellets were resuspended in 1 mL of lysis buffer and lysed using the FastPrep-24™ Classic bead beating grinder and lysis system for 30 seconds. Samples were then centrifuged at 12,000g for 15 minutes to pellet cellular debris. Protein expression and protein purification samples were analyzed using SDS-PAGE. Samples were prepared in a 1:1 ratio with 2x Laemmli sample buffer supplemented with β-mercaptoethanol and kept on ice prior to loading. 10 uL of Precision Plus Protein™ Unstained Standards ladder and 15µL of each prepared sample were then loaded onto 15-well gels. 10% gels were prepared according to the TGX Stain-Free™ FastCast™ Acrylamide Starter Kit. Gels were run at 150 V in 10X SDS-Tris-Glycine buffer. Gels were imaged using the BioRad ChemiDoc MP imaging system.

Colloidal chitin agar plates. Colloidal chitin agar plates were prepared by the according to the method by Saima *et al.* (14). Colorimetric agar plates were prepared according to the method by Agrawal and Kotasthane (15). Samples of cell lysates and purified ChiC were inoculated onto eacb plate using two methods: pipetting 20 uL directly onto plate surface and using a microcentrifuge tube bottoms to punch holes in the agar then pipetting 100 uL into the holes.

Protein quantification. Protein concentration in protein elution solution was quantified by performing the Thermo Scientific™ Pierce™ bicinchoninic acid (BCA) assay. Both the bovine serum albumin (BSA) standards and the cell lysates of unknown concentration were run in triplicate. A BSA standard curve was generated by measuring absorbance at 562 nm using the BioRad Microplate Spectrophotometer.

RESULTS

Plasmid sequencing confirms *chiC* insert in pET-28a and low incidence of mutation in the plasmid backbone. Previously, Rocha *et al.* only confirmed the insertion of *chiC* in pET-28a via Sanger sequencing–we sought to sequence the full sequence of pM3CRYY to check for potentially problematic mutations in the plasmid backbone. The expected plasmid size was 6.8 kb containing a 1.5 kb *chiC* insert. Colonies A1 and A3 were sequenced at 296x and 105x coverage, respectively. Read length histograms generated from Plasmidsaurus show the distribution of DNA lengths present in the sample (Fig. 1A). As expected, A1 has a

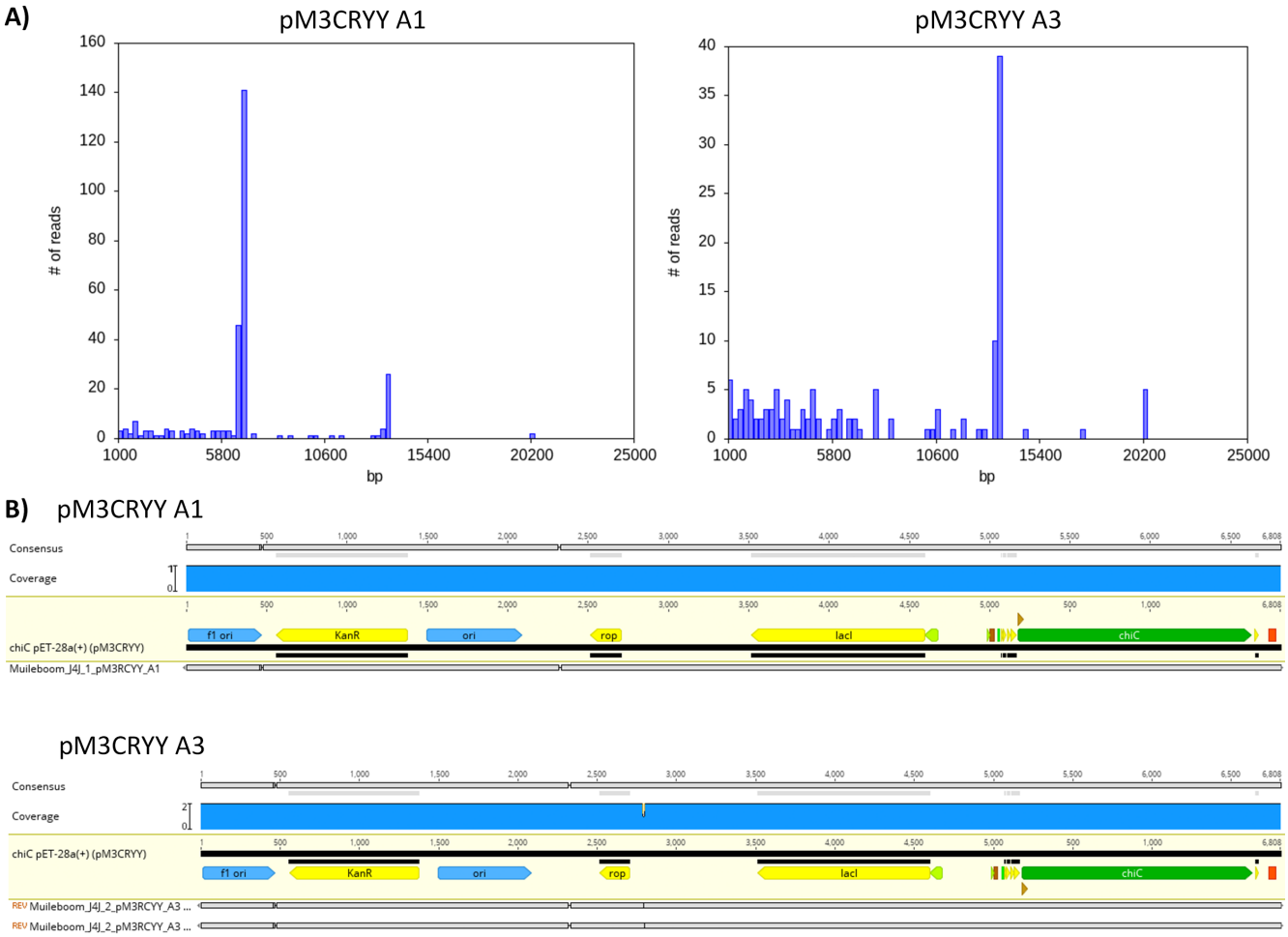

FIG. 1 Full plasmid sequencing of pM3CRYY A1 and A3. Read length histograms of sequenced samples (A). All DNA species identified in the Oxford nanopore run are plotted on a histogram to show any plasmid oligomers or contaminating species. Consensus sequences mapped to an annotated pM3CRYY plasmid map (B). Breaks in the gray consensus sequences below the plasmid map indicate a difference between the reference map and sequence.

dominant peak at ~7 kb. Interestingly, A3 has a dominant peak at ~14 kb, approximately double the expected size, suggesting plasmid dimerization. Mapping the consensus sequences to pM3CRYY supports a monomer and dimer for A1 and A3, respectively (Fig. 1B). We identified the following identical mutations in A1 and A3: T -> C mutation and 1 nucleotide deletion at f1 ori and a 3 nucleotide deletion in a spacer region.

IPTG induces production and intracellular buildup of ChiC in E. coli BL21(DE3) transformed with pM3CRYY A1. To ensure pM3CRYY could function to express *chiC* in an *E. coli* host following -80ºC storage in glycerol, we used IPTG to induce expression through the T7 promoter and screened the *E. coli* BL21(DE3) lysates and supernatants for *chiC* expression. Cell lysates of BL21(DE3) transformed with pM3CRYY produced a thick protein band at 55 kDa (Fig. 2). In contrast, the supernatant of BL21(DE3) transformed with pM3CRYY produced faint protein bands of this size. Interestingly, the uninduced lysates produced a thicker band than the induced cell lysates, potentially indicating leaky expression. There was no protein expression for the pET-28a (control) samples (Fig. 2). ChiC, the 55 kDa protein, was mostly observed in the lystates.

ChiC expressed in *E. coli* BL21(DE3) can be purified using IMAC. To determine whether ChiC could be isolated from *E. coli* BL21(DE3) lysates for downstream chitinolytic activity assays, His-tagged recombinant ChiC purification was performed under non-denaturing conditions using IMAC. SDS-PAGE analysis shows the step-wise process of protein purification (Fig. 3). Subsequent steps of the purification process removed more

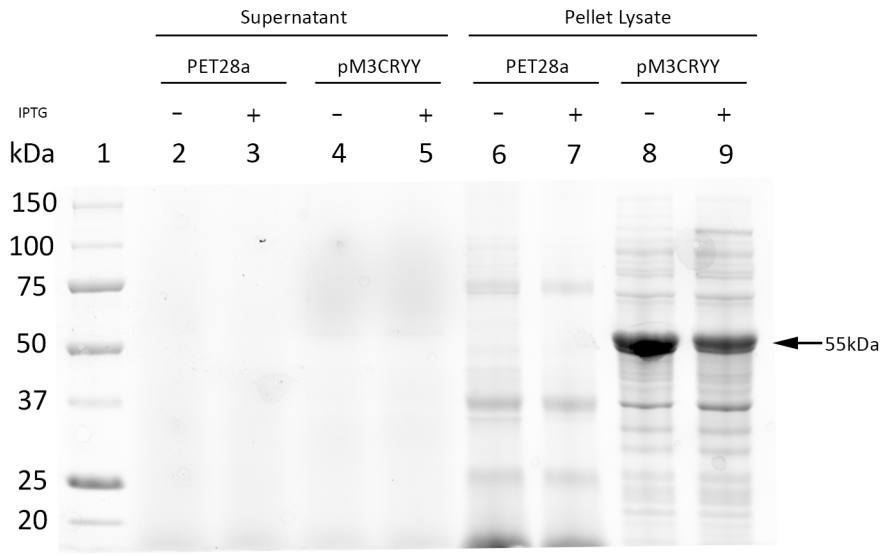

FIG. 2 IPTG induces production and intracellular buildup of ChiC in *E. coli* BL21(DE3) transformed with pM3CRYY A1. Lane 1 contains 10 uL of Precision Plus Protein™ Unstained Standards ladder. Lanes 2-5 contain proteins from supernatants of BL21(DE3) subcultures that were uninduced or induced for 24 hours. Lanes 6-9 contain proteins from pellet lysates of BL21(DE3) subcultures that were uninduced or induced for 24 hours. pET-28a BL21(DE3) subcultures represent the negative controls. IPTG induced the expression of a 55 kDa protein corresponding to the expected molecular weight of ChiC.

contaminating proteins. The band representing eluted protein migrated to ~55 kDa relative to the protein ladder, which matches the IPTG induced lysate protein band (Fig. 3).

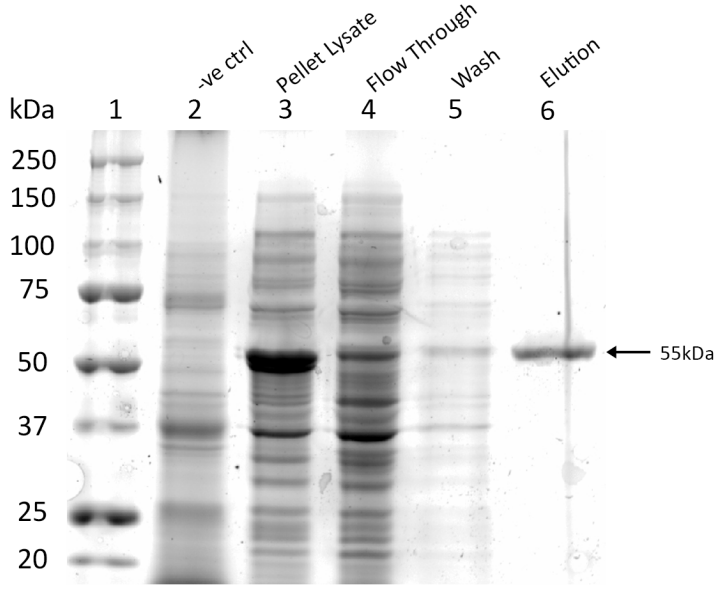

FIG. 3 SDS-PAGE gel of IMAC purification samples. Lane 1 contains 10 uL of Precision Plus Protein™ Unstained Standards ladder. Lane 2 contains uninduced pET-28a cell lysates of BL21(DE3) cultures (negative control). Lane 3 contains cell lysates of induced pM3CRYY A1 BL21(DE3) cultures. Lane 4 containing the binding flow through. Lane 5 contains the wash flow through. Lane 6 contains eluted protein corresponding to the expected molecular weight (55 kDa) of ChiC.

ChiC from cell lysate and purified ChiC fail to create zones of chitin clearing. To test the functionality of ChiC, we inoculated colloidal chitin and colorimetric colloidal chitin agar plates with cell lysates after IPTG induction, purified ChiC, and *E. coli* BL21(DE3) expressing pET-28a (negative control). There were no observable changes within all quadrants of the colloidal chitin agar plates (Fig. 4). All inoculated areas on the colorimetric plates changed color to purple except for the one of the 20 uL negative controls (Fig. 4B). The cell lysates on the colorimetric plates changed to a deeper purple in comparison to the purified protein. In an attempt to quantify the protein concentration in the purified ChiC sample, we ran a BCA assay, which yielded a negative concentration value of -14.00 ug/mL (Supp. Fig. S1). Thus, it is inconclusive whether ChiC expressed in *E. coli* BL21(DE3) is functionally active.

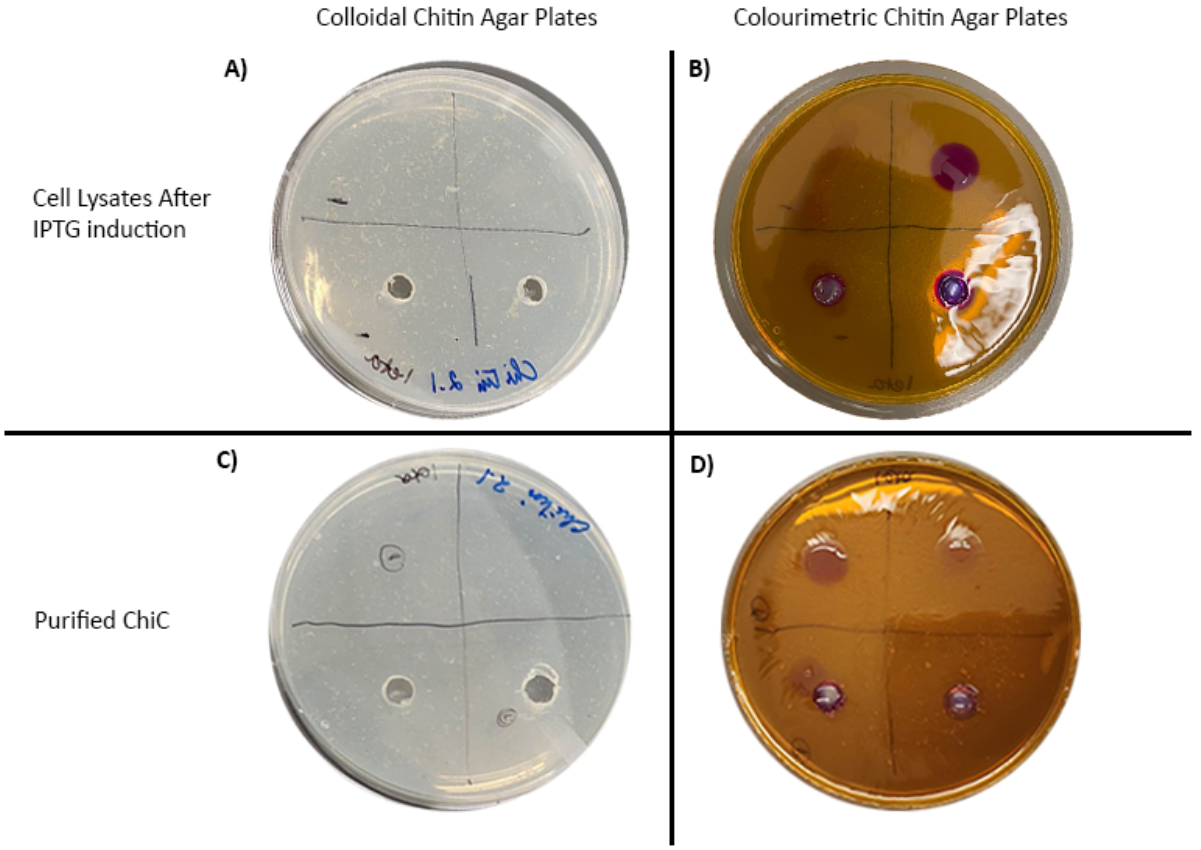

FIG. 4 ChiC plated on colloidal chitin and colorimetric chitin agar plates. Cell lysates of the IPTG induced BL21(DE3) subculture were plated on either colloidal chitin agar (A) or colourimetric chitin agar plates (B). Purified chitin protein in elution buffer was plated on either colloidal chitin agar (C) or colourimetric chitin agar plates (D). The negative control was *E. coli* BL21(DE3) transformed with pET-28a in LB. 20 uL of each sample was directly pipetted onto agar surface and 100 uL was pipetted into the holes.

DISCUSSION

In this study, we aimed to investigate the chitinolytic activity of purified ChiC from *Pseudomonas aeruginosa* PAO1 expressed in a non-pathogenic *E. coli* host. The *chiC* expression plasmid constructed by Rocha *et al.*, pM3CRYY, was transformed into *E. coli* BL21(DE3).

Recent advances in sequencing technologies have reduced the cost of plasmid sequencing to $15 per sample. However, high-throughput sequencing technologies such as nanopore sequencing are more error-prone, requiring several reads of the same DNA sequence to generate a consensus sequence. Efficient multiplexing now allows full plasmid sequencing to be a good alternative to Sanger sequencing–this service can map out the entire plasmid at similar cost without compromising fidelity. Interestingly, we observed potential plasmid dimers for A3. It has been suggested that high copy number plasmids are prone to dimerization via homologous recombination (16, 17). Plasmid dimers are formed by fusing two copies of the plasmid replicons. On the other hand, this could be an artifact of the Nanopore sequencing. While it was possible to verify these two hypotheses by running the Minipreps on an agarose gel and checking for differences in band migration, we proceeded with our experiments using A1, the plasmid monomer, due to time constraints. Identical mutations between A1 and A3 in the plasmid backbone suggest that these mutations existed in pET-28a prior to cloning the *chiC* insert. The exact effects of these mutations are unknown. Perhaps the mutations observed in the origin of replication affect plasmid copy number. Future experiments can test copy number differences by comparing the levels of kanamycin resistance between pM3CRYY and pET-28a.

Following transformation of pM3CRYY into *E. coli* BL21(DE3), we induced overnight expression of *chiC* using IPTG. After running an SDS-PAGE gel of the cell lysates, we confirmed a ~55 kDa band which corresponds to ChiC (10). Chitinases from other bacteria such as *Serratia, Bacillus,* and *Vibrio* (18–20) are known to secrete chitinolytic enzymes and chitin binding proteins into the extracellular space. Similarly, we expected ChiC to be secreted extracellularly. However, in Figure 2, the 55 kDa band appears to be much thicker and darker in the cell lysates compared to the supernatant. Rocha *et al.* also previously had difficulty secreting ChiC (12). This suggests that while ChiC was successfully expressed, the protein accumulated in the cytosol. Folders *et al.* previously demonstrated that *P. aeruginosa* secreted ChiC extracellularly after 4 days of growth (10). It is possible that if we continued to propagate the cells for a longer period after IPTG induction, we could see eventual secretion of ChiC. Folders *et al.* also postulated that *chiC* was not secreted via the type II secretion system typical in *P. aeruginosa*, suggesting that secretion may not occur even after longer propagation if *E. coli* does not possess this novel secretion pathway. A future experiment may attempt to swap this signal peptide (SP) with a known SP targeting a known *E. coli* secretion pathway.

After upscaling the IPTG induction of *chiC* expression, we isolated and purified the His-tagged ChiC protein via IMAC. We confirmed the presence of ChiC in the cell lysate and eluted protein via SDS-PAGE (Fig. 3). After successfully purifying ChiC, we tested its chitinolytic activity. Using colloidal chitin plates prepared by the MICB 471 teaching team, we plated both purified ChiC and cell lysates using two methods: (a) directly spreading the sample onto the agar and (b) punching holes then filling them with sample. We observed no observable changes in chitin clearing for either method, thus we cannot confirm chitinolytic activity. We also tested purified ChiC in a colorimetric chitinolytic assay. This plate is initially dark yellow, turning deep purple when the pH becomes more alkaline at 6.8. Breakdown of chitin by chitinase yields N-acetyl glucosamine, a weak base, and causes this color change on the plate. Both plates with purified ChiC and cell lysates instantaneously turned purple upon plating. This result is also inconclusive as the elution buffer which was used for the purification process had a pH of 7.4, meaning the color change may not have been due to the presence of chitinase. There are a few explanations for these failed experiments. First, it is possible that we plated a very low amount of protein. Via BCA assay, we determined that the concentration of ChiC in the protein elution sample was likely too low to be detected by the assay (Supp. Fig. 1). This suggests that the amount of ChiC spotted on colloidal chitin plates may be under the assay's limit of detection. Indeed, these colloidal chitin assays are known to have low sensitivity (15). In future experiments, ChiC purification likely needs to be repeated to achieve a higher protein yield. Second, the colloidal chitin agar recipe may need further optimization. We acknowledge that we had no true positive control in our experiments–therefore, future experiments should include a known chitinase (likely commercial) to confirm the presence of halos. Our results highlight the need for a simple and quick assay availability for testing chitinolytic activity.

Conclusions In this study, we sought to purify and uncover the chitinolytic activity of *chiC*. From the pM3CRYY A1 glycerol stock, we identified a small number of mutations in the pM3CRYY A1 backbone through plasmid sequencing. Despite these mutations, ChiC was successfully produced and purified as indicated by SDS-PAGE analysis. Unfortunately, we were unable to detect any observable changes for our *in vitro* colloidal chitin agar assays. The *in vitro* and *in vivo* functionality of the purified protein and secretion mechanism of ChiC remains unknown. Thus, future experiments can further investigate these questions. Production and validation of an effective chitinase can be a valuable stepping stone to engineer these enzymes as pest controlling agents.

Future Directions We did not observe zones of chitin clearing in our colloidal chitin assays. It remains unknown whether flaws exist in the assay itself, our technique, or a combination of both. Future works can optimize the colloidal chitin plate recipes or plating methods, focusing on functional assay development for chitinolytic activity. An *in vivo* insecticidal assay on insect larvae of common pests such as *Spodoptera litura* and *Drosophila melanogaster* can offer insight on the pest control potential of *chiC*. A similar experiment is

an antifungal assay against fungi known to be susceptible to chitinase activity. To expand the scope of chitinase experiments, new chitinase candidates can be cloned into a pET vector and screened in a similar manner to this study. Other characterized chitinases have been expressed and purified from BL21(DE3)—these constructs can be used as positive controls. More exploratory work may include searching public sequence databases (*e.g.*, NCBI, Pfam, InterPro) for putative chitinases based on homology to known chitinase sequences. Since we postulate that *chiC* secretion to depend on an unknown secretion pathway not present in *E. coli*, a future experiment may develop a model for visualizing *chiC* trafficking (*e.g.*, GFP-*chiC* fusion) in *E. coli* BL21(DE3) compared to *P. aeruginosa* PAO1. This would involve knocking out the *chiC* encoded by the PAO1 genome.

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CONTRIBUTIONS

Laboratory work. The team collaborated on plasmid purification, transforming BL21(DE3), and IPTG induction of the BL21(DE3) transformants. AT took the lead on protein purification and SDS-PAGE analysis. V.Y. took the lead on the BCA assay.

Manuscript. J.C. completed the introduction. A.T., D.L., and V.Y, collaborated on the methods section. A.T. and D.L. collaborated on the results section. A.T., D.L., J.C., and V.Y collaborated on the discussion section. A.T generated the figures and a majority of their captions. A.T and D.L collaborated on the conclusion. D.L. wrote the abstract, figure 1's caption, and future directions. V.Y. generated the supplemental figure and caption. A.T. and D.L.contributed to the editing of the manuscript.

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