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Assessing the chitinolytic activity of recombinant ChiC from *Escherichia coli* pM3CRYY BL21(DE3)

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SUMMARY Chitin is an abundant polysaccharide that plays a structural role in many organisms, including being present in the cell walls of fungi and exoskeletons of insects. As fungi and insects are common pests of plants that pose a threat in agricultural contexts, chitin is a promising biopesticide target. Chitinases, such as ChiC from *Pseudomonas aeruginosa* PAO1, degrade chitin through hydrolysis, making them a potential biocontrol agent that offers a safer alternative to chemical pesticides. Previously, *chiC* was cloned into the expression vector pET-28a to generate the expression plasmid pM3CRYY. Transforming *Escherichia coli* BL21(DE3) with pM3CRYY produces recombinant ChiC (rChiC), which has recently been shown to retain its chitin-binding activity. However, the chitinolytic activity of rChiC has yet to be demonstrated. Our study aimed to determine if recombinant ChiC from *E. coli* pM3CRYY BL21(DE3) has chitinolytic activity using fungal inhibition, 3,5-dinitrosalicylic acid (DNS), and colloidal chitin-plate based assays. We found that rChiC-expressing pM3CRYY *E. coli* had moderate antifungal effects but that purified rChiC could not degrade colloidal chitin into reducing sugar monomers. We further found that fungal inhibition assays with purified protein and colloidal chitin plates were inadequate for assessing the chitinase activity of rChiC. These results suggest that rChiC likely has chitinolytic activity but that either our extraction and purification methods could not produce sufficient amounts of functional protein for detectable *in vitro* activity or that colloidal chitin is not a suitable substrate for rChiC.

INTRODUCTION

The use of pesticides is common and significant in commercial agriculture but has unfavorable consequences on human health and the environment. Thus, the need for safer alternatives, such as biopesticides, is critical. A possible target for biopesticides is chitin, an insoluble homopolymer of 1,4 β-linked N-acetyl-D-glucosamine (1), which plays a key structural role in the exoskeleton of insects (2) and in the cell wall of fungi (3). As such organisms are common pests of plants, chitinases, enzymes which degrade chitin, may be employed as biocontrol agents in agricultural settings as a non-toxic alternative to chemical pesticides and insecticides (4). This has substantial implications for increasing crop productivity (4).

Although chitin-degrading enzymes can be found in plants and other organisms, microbial chitinases are an encouraging and sustainable option for industrial production (4). Bacteria, such as *P. aeruginosa*, produce chitinolytic enzymes such as ChiC (2). However, the large-scale production of ChiC in *P. aeruginosa* is limited due to its pathogenic nature and the natural yield of chitinase enzymes from their native host is generally low (4), limiting their application as biocontrol agents. This can be improved through the expression of the chitinase gene in a heterologous host, to produce ChiC at a larger scale compared to in its native host (4). Previously, Rocha et al. transformed the frequently used expression strain *E. coli* BL21(DE3), with pM3CRYY, a pET-28a expression vector with *chiC* from *P. aeruginosa* PAO1 inserted (5). Expression of ChiC in an *E. coli* system is an attractive system for studying the characteristics of ChiC.

ChiC from other organisms has previously shown insecticidal activity (2) suggesting its ability to degrade chitin. Chitinases degrade chitin via the random endo-hydrolysis of N-acetyl-D-glucosaminide β-1,4 linkages (4). ChiC contains three domains; a catalytic domain or GH18 domain which catalyzes the cleavage of β-1,4 bonds within chitin, a fibronectin-like

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type III domain which binds and interacts with other proteins, and a chitin-binding domain, which each contribute to chitin degradation (6).

There are several methods used for testing the chitinolytic activity of chitinases, one of which is the DNS assay. This method uses 3,5-dinitrosalicylic acid, which turns red in the presence of reducing molecules and heat, to detect the presence of reducing sugar monomers released from chitin degradation (7). The DNS method has been used to establish chitinase activity of novel bacterial chitinases (3) and to confirm chitinolytic activity in recombinant chitinases expressed in *E. coli* (7).

Antifungal and insecticidal effects of chitinases are also used to characterize chitinase activity. Aktas et al. (3) demonstrated antifungal activity of a purified chitinase from *Stenotrophomonas maltophilia* against the fungus *Fusarium oxysporum* and potato beetle *Leptinotarsa decemlineata*. The chitinase was shown to be effective in reducing the growth of fungus and disrupting the chitin structure of potato beetle. Another study showed that purified chitinase expressed in *E. coli* BL21(DE3) using the pET-28a+ vector exerted broad-spectrum antifungal activity against various pathogenic fungi (7).

Lastly, previous research has shown successful chitinase activity of ChiC using colloidal chitin assays. One study found purified ChiC isolated from *Alteromonas sp.* Strain O-7 to have chitinase activity using colloidal chitin as a substrate (8). Another study showed chitinase activity of *E. coli* BL21 expressed MetaChi18A, also in the GH-18 family, using colloidal chitin (9). Most relevant to this study, however, is that degradation of colloidal chitin media to form halos surrounding bacterial colonies has been observed in *E. coli* DH5α transformed with *P. aeruginosa* PAO1 *chiC* using pUC18 (10). Taken together, these studies provide support for ChiC as an enzyme with chitinase activity that can be detected using various assays. While recent studies have shown that rChiC from *E. coli* pM3CRYY BL21(DE3) retains a functional chitin-binding domain (L. Lin, P.R. Agawin, J. Tong, S. Rai, G. Shoaib; unpublished manuscript), chitinolytic activity has yet to be demonstrated. Here we aimed to assess the chitinolytic activity of rChiC using three separate methods to allow for a greater chance of successful demonstration of chitinase activity. In particular, we aim to demonstrate the chitinase activity of rChiC expressed in *E. coli* pM3CRYY BL21(DE3) using a DNS assay, a fungal assay, and a colloidal chitin assay.

METHODS AND MATERIALS

Sanger sequencing of pM3CRYY to confirm *chiC* sequence. *E. coli* pM3CRYY B4 BL21(DE3) was grown in LB-kanamycin (50 µg/ml) overnight at 37ºC with shaking. Plasmid extraction was performed using the EZ-10 Spin Column Plasmid DNA Minipreps Kit (BioBasic) using the low copy plasmid protocol. Plasmid concentration was determined using NanoDrop™ 2000c (Thermo Scientific). Four samples were then prepared for sequencing according to Genewiz's sample submission guidelines (10) using Genewiz's universal T7 and T7 terminator primers, and custom *chiC* forward and internal primers. Sanger sequencing results were trimmed to remove low-quality reads and aligned using SeqMan Ultra against a pM3CRYY reference construct created with pET-28a and *chiC* sequences using SeqBuilder Pro.

Protein expression. A pM3CRYY B4 BL21(DE3) colony was grown in 5 ml of LB-kanamycin overnight at 37ºC with shaking. 2.5 ml of the overnight culture was diluted 1/100 in LB-kanamycin and incubated again until the OD_{600} reached 0.651. IPTG was added to a final concentration of 0.1 mM and incubated at room temperature with shaking for 20 hours. After induction, cells were pelleted by centrifugation for 10 minutes at 7000 rpm and stored at -70ºC. The same procedure was repeated with a pET-28a colony, with 0.5 ml of overnight culture being diluted 1/100 and then grown to OD_{600} 0.525.

Cell lysis. Cell lysis was performed based on the protocol adapted from L. Lin, P.R. Agawin, J. Tong, S. Rai, G. Shoaib (unpublished manuscript). Lysis buffer was prepared by combining binding buffer (300 mM NaCl, 50 mM Na_sHPO_4, pH 7.5) with 1% Triton X-100 10% glycerol; and 0.01% β-mercaptoethanol (BME), 1 mg/ml lysozyme, and 1X protease inhibitor cocktail. Cell pellets thawed on ice were resuspended in either 10 ml for pM3CRYY or 2 ml

for pET-28a of lysis buffer and incubated on ice for 30 minutes. Lysates were passed through a 23-gauge needle 5 times and then the sample was centrifuged for 12 minutes at 7000 rpm. Soluble fractions were transferred to a separate container and the pellet fraction was stored at -20ºC for downstream SDS-PAGE analysis.

Purification of rChiC by immobilized metal affinity chromatography (IMAC). 2 ml of NEBExpress® Ni Resin (New England Biolabs) was loaded onto a column and the storage buffer was collected as flowthrough and discarded. The resin was first washed with 15 ml of binding buffer before loading 8 ml of the pM3CRYY soluble lysate fraction. The resin was incubated at 4ºC for 30 minutes before flowthrough was collected. The resin was then washed with 20 ml of binding buffer supplemented with 10 mM imidazole. Finally, the protein was eluted using 10 ml of elution buffer (300 mM NaCl, 50 mM Na_sHPO_4, 500 mM imidazole, pH 7.5). Elutions were collected in 1.5 ml fractions and stored at -20ºC. The procedure was repeated for pET-28a lysates using 1/5 the amount of each reagent used for pM3CRYY.

Confirmation of rChiC expression. For both pET-28a and pM3CRYY samples, aliquots of supernatant; insoluble lysate fractions resuspended in binding buffer; soluble lysate fractions; and flowthrough, washes, and elutions from IMAC were added 1:1 to 2X Laemmli Sample Buffer (BioRad) with 10% BME. 10 µl of each sample and of Precision Plus Protein™ Unstained Standards ladder (BioRad) were used for SDS-PAGE analysis. Gels were run at 200 V using 1X SDS Running buffer and imaged using the ChemiDoc™ MP imaging system (BioRad).

Dialysis of rChiC. 6 ml of IMAC elution fractions were pooled and dialyzed using Spectra/Por™ 2 12-14 kD MWCO (Spectrum) dialysis tubing for 24 hours in 2 L of binding buffer. After 3 hours of dialysis, the original buffer was replaced with an equal volume of fresh binding buffer.

Chitin-binding assay. 100 µl of chitin resin (New England Biolabs) was washed three times with 1 ml of dH_2O followed by three times with 1 ml column buffer (20 mM Tris-HCl, pH 8.5; 0.5 M NaCl) by centrifugation at 9000 rpm for 2 minutes. 150 µl of dialyzed protein samples or BSA, as a negative control, was added to the resin and incubated at room temperature for 30 minutes with shaking. Following incubation, samples were spun at 9000 rpm for 2 minutes and the supernatant was collected. The sample was then washed three times, as described above, with 150 µl of column buffer and supernatant was collected each time with a 10 µl aliquot being added to 2X Laemmli Sample Buffer. Beads were then resuspended in 100 µl 2X Laemmli Sample Buffer and heated at 95ºC for 5 minutes to elute the bound protein. The sample was centrifuged and the supernatant was collected. 10 µl of the beads were then resuspended in 10 µl 2X Laemmli Sample Buffer. All samples were then analyzed using SDS-PAGE.

BCA assay for determining protein concentration. The Pierce™ BCA Protein Assay (Thermo Scientific) was used for the quantification of rChiC concentrations via spectrophotometry. The assay was performed according to the manufacturer's instructions. BSA standards were diluted with binding buffer to prepare concentrations of 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml. Undiluted, 1:10, 1:100, and 1:1000 dilutions of rChic were prepared with the same binding buffer. 25 µl of each BSA standard or sample were loaded in wells with 200 µl BCA Working Reagent, prepared from BCA Reagents A and B (50:1). Each standard and sample was run in duplicate. The plate was incubated at 37ºC for 30 minutes and absorbance was measured at 562 nm on the BioTek® Epoch 2 Microplate Spectrophotometer.

Dual culture fungal inhibition assay. Potato dextrose agar (PDA) plates were prepared with 40 µl of 1.0 or 0.1 M IPTG spread on the surface of each plate to obtain a final concentration of 1.0 or 0.1 mM IPTG. *F. oxysporum* cores were grown in the center of the plate at 25ºC overnight. For both induction conditions following incubation, a box was drawn 1 cm away from the edge of the fungus and *E. coli* BL21(DE3) containing either pM3CRYY or pET-28a

was streaked along the perimeter of the box. *P. aeruginosa* was also streaked as a positive control. Plates were incubated at 25ºC and monitored for growth daily.

Disk diffusion assay. 1 cm blocks of an *F. oxysporum* culture were cut with a sterile scalpel, placed in the middle of two PDA plates, and grown to a 2 cm diameter. On one plate, four sterile disks (6 mm) were placed either 0 or 1 cm away from the edge of fungal growth. Subsequently, 20 µl of filter-sterilized purified rChiC (210.8 ng/µl), *E. coli* pM3CRYY BL21(DE3) lysates, binding buffer (negative control), or positive control chitinase from *S. griseus* (Sigma, 1 unit/ml in KH_2PO_4; 50mM, pH 6.0) was loaded onto each disk. To test for the effects of varying concentrations of the positive control *S. griseus* chitinase, the 1 unit/ml stock was diluted to 1:2, 1:4, 1:5, 1:10, and 1:100 using KH_2PO_4 as the diluent. 20 µl of each filter sterilized chitinase dilution was loaded onto a disk 1 cm away from the edge of fungal growth on a separate plate. As a negative control, 20 µl of filter-sterilized KH_2PO_4 was loaded onto a separate disk. Plates were incubated at 25ºC for up to 4 days, and growth was monitored every 24 hours.

Agar well diffusion assay. *F. oxysporum* cores were grown in the center of PDA plates at 25ºC overnight. Once the fungal growth reached a 2 cm diameter, 8 holes each 6 mm in diameter were made 1 cm from the edge of the colony using a sterile wire loop. Then, 80 µl of filter-sterilized purified rChiC (210.8 ng/µl), *E. coli* pM3CRYY BL21(DE3) lysates, binding buffer (negative control) or chitinase from *S. griseus* (positive control) was loaded into the wells. Plates were incubated at 25ºC and growth was monitored daily.

DNS Assay for release of reducing sugars from colloidal chitin. NAG standards ranging from 0 mg/ml to 0.25 mg/ml in 0.05 mg/ml increments were prepared by first diluting 1 mg/ml NAG in sodium acetate buffer (100 mM, pH 4.7) to 1 ml at double the final concentration, and then diluting 1:1 in binding buffer.

The DNS assay was performed using a protocol adapted from Kirubakaran and Sakthivel (7). 1 ml of 1% colloidal chitin in sodium acetate buffer (100 mM, pH 4.7) was added to 1 ml of purified, dialyzed rChiC in duplicate. Reactions were also done with *S. griseus* chitinase (Sigma) resuspended to 1 mg/ml in KH_2PO_4 (50 mM, pH 6.0) as a positive control; and binding buffer and KH_2PO_4 as negative controls. All samples and NAG standards were incubated for 1 hour at 50ºC with reaction mixtures being shaken every 5 minutes. DNS reagent was prepared by dissolving 0.5 g of 3,5-dinitrosalicylic acid in 25 ml water while heating, followed by the addition of 15 g of sodium potassium tartrate and 10 ml NaOH (2 M). Following incubation, 2 ml of DNS reagent was added to each reaction and tubes were placed in boiling water for 5 minutes. 100 µl of each sample, in triplicates, was used to determine absorbance at 540 nm with the BioTek® Epoch 2 Microplate Spectrophotometer. A standard curve was generated using the absorbances of NAG standards and used to interpolate NAG concentrations in samples.

Preparation of colloidal chitin. Colloidal chitin was prepared and provided by the MICB 471 media team, using a protocol adapted from Atkas et al (3). To prepare colloidal chitin, 30 g of crab chitin was mixed with 37% HCI overnight at 4ºC. 400 ml of 95% ethanol was then added to the colloidal chitin-HCl mixture and left to sit overnight at 4ºC. This mixture was then strained using a cotton round. Excess chitin stuck to the cotton round was scraped off and mixed with distilled water for 20 minutes before being strained again through fresh cotton. This process was repeated until the pH of the water-colloidal chitin mixture reached 7. Finally, the water was strained out and the chitin was autoclaved and stored at 4ºC.

Preparation of LB-colloidal chitin plates. To prepare the LB 0.5% colloidal chitin plates, the following components were combined and autoclaved: 500 ml distilled water, 5 g NaCl, 5 g tryptone, 2.5 g yeast extract, 7.5 g select agar, and 2.5 g colloidal chitin. After cooling, 250 µl of filter sterilized 50 mg/ml kanamycin was added to each medium.

Preparation of M9 minimal media colloidal chitin plates. The protocol for M9 minimal media was adapted from the Hancock lab (11). M9 minimal media colloidal chitin agar plates

were made by preparing the following solutions: M9 salts (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, dH_2O to 500ml; autoclaved), 0.5% colloidal chitin agar (250 ml dH_2O, 7.5 g select agar, 1.25 g colloidal chitin; autoclaved), and the supplemental solutions (5 ml 20% glucose; 1 ml 1 M $MgSO_4$; 0.05 ml 1 M $CaCl_2$; 500 µl 50 mg/ml kanamycin; filter sterilized). Following autoclaving, 250 ml M9 salts were aseptically added to 250 ml of the cooled 0.5% colloidal chitin agar medium. Supplemental solutions were filter sterilized and added to the M9 salts plus colloidal chitin agar mediums.

LB-colloidal chitin and M9 minimal media colloidal chitin plate assays. Isolated colonies of pET-28a and pM3CRYY containing *E. coli* BL21(DE3) were subcultured from LB-kanamycin starter plates. Prior to streaking plates with bacteria, 40 µl of 1 M filter-sterilized IPTG was spread-plated on the LB-colloidal chitin and M9 minimal media-colloidal chitin plates. Then, plates were streaked with *E. coli* pET-28a BL21(DE3), *E. coli* pM3CRYY BL21(DE3), or *P. aeruginosa* PAO1 in vertical lines. 3 replicates were produced for each condition. A plain LB plate was also streaked with *P. aeruginosa.* Streaked plates were incubated overnight at 37ºC and growth was observed from days 1-10.

RESULTS

***P. aeruginosa chiC* is preserved in *E. coli* pM3CRYY B4 BL21(DE3).** Prior to examining the activity of ChiC, we first confirmed that *E. coli* pM3CRYY B4 BL21(DE3) contained an unaltered *chiC* through Sanger sequencing. *chiC* from plasmids isolated from an *E. coli* pM3CRYY B4 BL21(DE3) culture were sequenced using T7 and *chiC* internal primers. Though the forward internal primer failed to produce results, the three sequences obtained produced a consensus sequence that covered the entirety of the *chiC* sequence (Figure 1). Aside from a single cytosine residue which was covered by only one sequencing result and read as an unidentified base, the consensus sequence was identical to the *chiC* sequence from *P. aeruginosa* PAO1. This suggests that *chiC* is preserved in *E. coli* pM3CRYY B4 BL21(DE3).

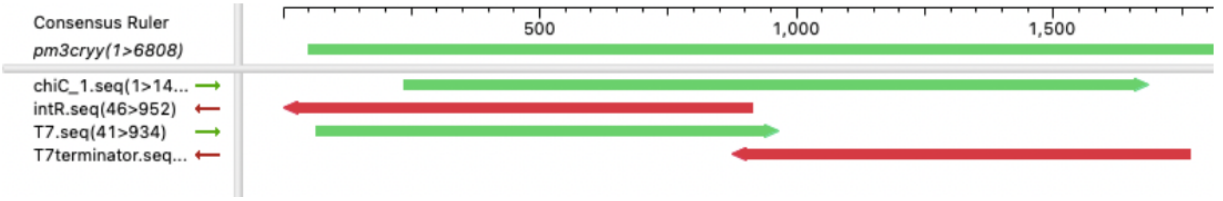

FIG. 1 An unaltered *chiC* sequence is present in pM3CRYY B4. Plasmids isolated from *E. coli* pM3CRYY B4 BL21(DE3) were subject to Sanger sequencing. Sequencing reads using a *chiC* internal primer (intR) and T7 promoter and terminator primers were trimmed for low-quality reads using SeqMan Ultra and aligned to a reference pM3CRYY constructed using SeqBuilder Pro. The position of *P. aeruginosa* PAO1 *chiC* within pM3CRYY is also shown.

***E. coli* pM3CRYY B4 BL21(DE3) has moderate antifungal effects.** To test if *E. coli* pM3CRYY B4 BL21(DE3) has antifungal activity against *F. oxysporum* we observed fungal growth inhibition by *E.coli* using an antifungal plate assay. Since, the cell wall of *F. oxysporum* is composed of chitin, inhibition of fungal growth would indicate chitinolytic activity by *E. coli.* For up to 5 days post-inoculation, we observed inhibition of *F. oxysporum* growth by *E. coli* pM3CRYY B4 BL21(DE3) for both 0.1 mM (n=1) and 1.0 mM (n=1) IPTG treatments (Figure 2). Similarly, the positive control, *P. aeruginosa*, inhibited *F. oxysporum* growth within the box of bacterial growth (n=1). However, *E. coli* pET-28a BL21(DE3), the negative control, did not inhibit *F. oxysporum* for either 0.1 mM (n=1) and 1.0 mM IPTG (n=1) conditions. Based on qualitative observation, *P. aeruginosa* inhibited *F. oxysporum* to a greater extent than *E. coli* pM3CRYY B4 BL21(DE3). This suggests that *P. aeruginosa* has antifungal effects, while rChiC-expressing *E. coli* pM3CRYY B4 BL21(DE3) has moderate antifungal effects.

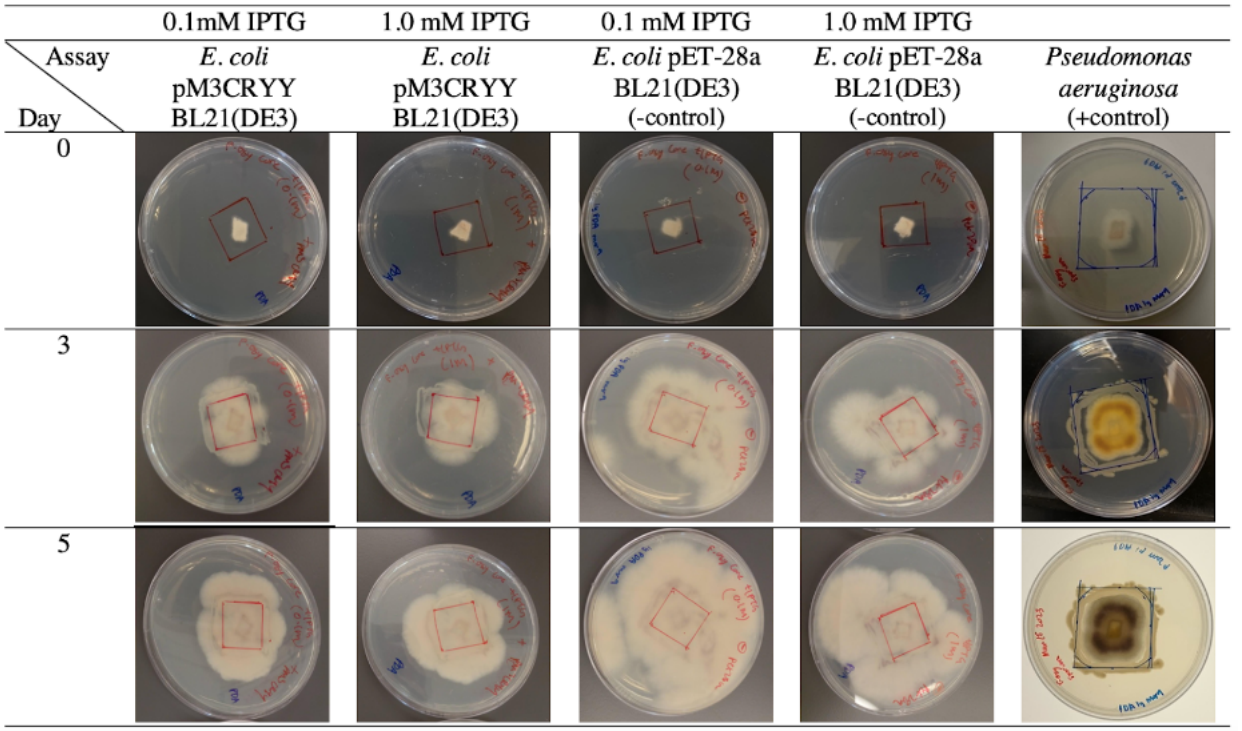

FIG. 2 rChiC-expressing *E. coli* demonstrates moderate antifungal activity. *F. oxysporum* cores were grown on PDA supplemented with 0.1 or 1.0 mM IPTG overnight at 25ºC. *E. coli* colonies containing either pET-28a or pM3CRYY were then streaked in a box surrounding the fungal growth. *chiC* containing *P. aeruginosa* PAO1 was used as a positive control. Fungal growth was observed at 0, 3 and 5 days. One replicate performed for each condition.

Chitin-binding activity is preserved in purified rChiC from *E. coli* pM3CRYY B4 BL21(DE3). As a preliminary test of the functionality of purified rChiC we performed a chitin-binding assay to assess the ability of rChiC to bind to chitin resin beads. Purified and dialyzed protein was incubated with chitin beads followed by multiple washes to remove unbound protein. All wash and elution fractions were then analyzed using SDS-PAGE. The experiment was also done by incubating BSA with chitin beads as a negative control. We found that very little rChiC was present in the washes, with the amount present decreasing with subsequent washes (Figure S2). The darkest bands were seen in the heat elution and resuspended bead fractions. Conversely, for BSA, bands were only seen in the first two washes. These results indicate that purified rChiC retains its chitin-binding activity and can remain bound to chitin beads even after boiling.

Purified rChiC does not have chitinolytic activity using colloidal chitin as a substrate. We then aimed to assess the chitinolytic activity of purified rChiC using a DNS assay to detect reducing sugars released from chitin breakdown (Figure 3). A commercially available chitinase from *S. griseus* was used as a positive control, while the buffers the chitinases were stored in were used as negative controls. While a significant increase in reducing sugar concentrations was observed for the positive control ($p=0.0002$), concentrations from rChiC samples were not significantly different from the negative control. This result indicates that rChiC was unable to release reducing sugars from colloidal chitin under assay conditions.

Fungal inhibition assays are not effective for testing the chitinolytic activity of purified chitinases. To determine if the growth of *F. oxysporum* is inhibited by purified rChiC we observed zones of inhibition using disk diffusion assays. Filter sterilized binding buffer was used as a negative control because it contained no rChiC and would confirm that the buffer itself does not inhibit fungal growth. Commercially available chitinase from *S. griseus* was used as a positive control. No zones of *F. oxysporum* clearance were observed for our purified rChiC, *E. coli* pM3CRYY BL21(DE3) lysates, negative control binding buffer, or the positive control chitinase from *S. griseus* (Figure 4A). Moreover, for our

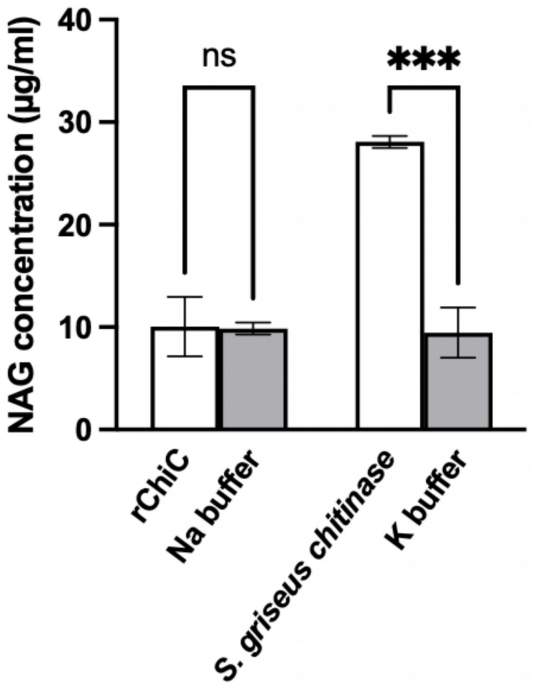

FIG. 3 Purified recombinant ChiC does not display chitinolytic activity. The indicated samples were incubated with 1% colloidal chitin in sodium acetate buffer for 1 hour at 50ºC followed by the addition of DNS reagent and boiling. Absorbance at 540 nm was used to determine reducing sugar concentration based on a set of NAG standards. Data represent the average of 3 technical replicates with error bars indicating standard deviation. p = 0.0002.

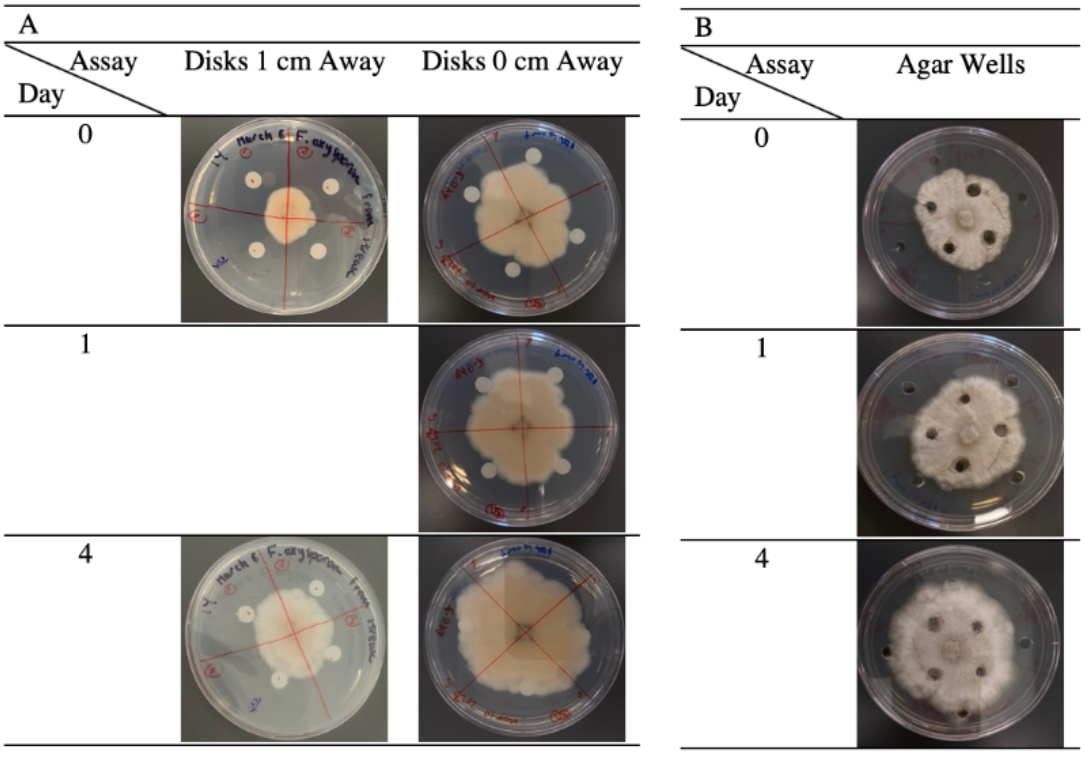

FIG. 4 Disk and well diffusion assays are not effective for testing the antifungal activity of purified chitinases. *F. oxysporum* cores were grown at 25ºC on PDA plates until growth reached a diameter of 2 cm. In the disk diffusion assay (A), sterile filter paper disks were placed 1 cm (left) or 0 cm (right) away from the edge of fungal growth and loaded with 20 µL of filter sterilized rChiC (210.8 ng/µl), cell lysates from IPTG-induced pM3CRYY containing cells, binding buffer, or *S. griseus* chitinase. In the well diffusion assay (B), holes measuring 6 mm in diameter were created in the fungal growth and filled with 80 µL of the same samples as in A. Plates were incubated at 25ºC for up to 4 days, and growth was monitored every 24 hours.

positive control assay to determine which concentration of filter-sterilized chitinase from *S. griseus* would inhibit *F. oxysporum*, we observed no zones of *F. oxysporum* clearance for concentrations of chitinase between 28.9 - 1446.05 µg/ml.

We also used the agar well diffusion method to test if the growth of *F. oxysporum* is inhibited by purified chitinases. Again, no zones of clearance were observed around the wells for our purified rChiC, *E. coli* pM3CRYY BL21(DE3) lysates, negative control binding buffer, or the positive control chitinase (Figure 4B).

Together, these results indicate that fungal inhibition assays were not effective for testing the chitinolytic activity of purified chitinases, including rChiC, at the concentrations used.

LB colloidal chitin plates are not an effective method for assessing rChiC chitinolytic activity. To determine whether *E. coli* pM3CRYY BL21(DE3) rChiC has chitinolytic activity, we performed a LB-colloidal chitin plate assay to detect chitinase activity by the presence of zones of clearing. LB 0.5% colloidal chitin plates with 1 mM IPTG were streaked with *E. coli* pM3CRYY BL21(DE3). *P. aeruginosa* was used as a positive control and *E. coli* pET-28a BL21(DE3) was used as a negative control. Figure 5 shows growth of all three

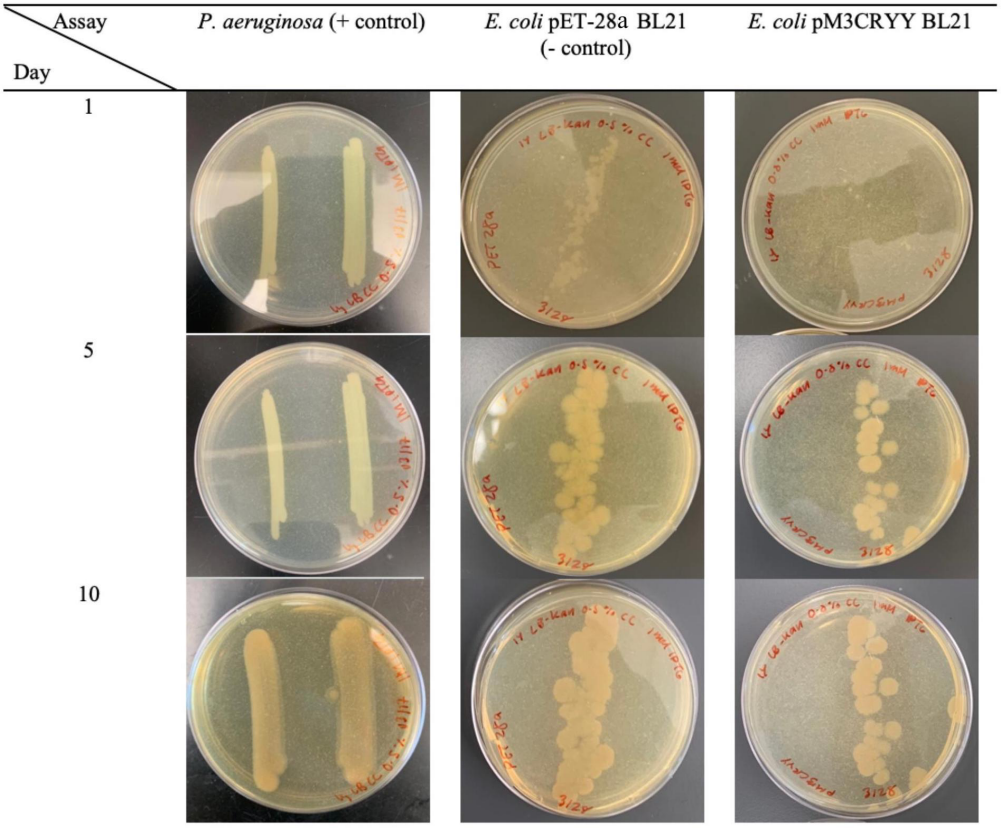

FIG. 5 Chitinolytic activity of ChiC is inconclusive on LB-colloidal chitin plates. *P. aeruginosa* PAO1 (left), *E. coli* pET-28a BL21(DE3) (middle), and *E. coli* pM3CRYY BL21(DE3) (right) were streaked out onto LB 0.5% colloidal chitin plates spread-plated with 40µl of filter sterilized 1mM IPTG. Plates were incubated overnight at 37ºC and growth was observed from days 1 to 10. *P. aeruginosa* PAO1 was used as a positive control. *E. coli* pET-28a BL21(DE3) was used as a negative control. n=3.

bacteria by day 1, indicating the bacteria are viable on this media. By day 5, growth had increased for all three bacteria, but no zones of clearing were visible. By day 10 growth had increased again for all three bacteria, however no zones of clearing were observed in the pET-28a and pM3CRYY *E. coli* plates. On the *P. aeruginosa* plates, there was a fuzzy, translucent line around the perimeter of the inoculated bacteria, however, it was not clear whether this was a sign of chitin degradation or a morphological characteristic of the bacteria growing on LB. To test for this, *P. aeruginosa* was also streaked out onto LB-only plates. *P. aeruginosa* produced the same fuzzy, translucent line around the edge of the inoculum (Figure 6) as seen in the 1 mM IPTG LB 0.5% colloidal chitin plates indicating this was a morphological

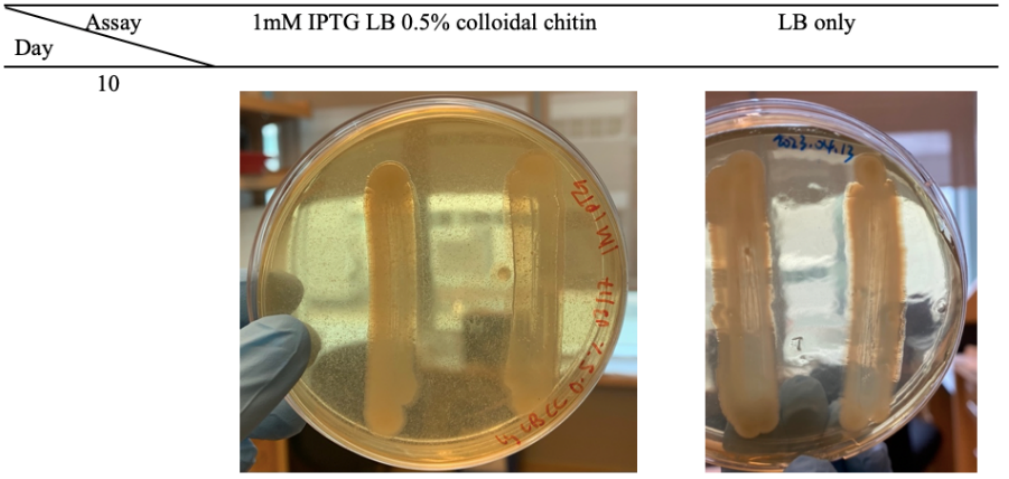

FIG. 6 *P. aeruginosa* morphology on LB plates shows a translucent edge around the bacterial inoculum. *P. aeruginosa* PAO1 growing on 1mM IPTG LB 0.5% colloidal chitin (left) and LB media only (right) on day 10. Plates were streaked with *P. aeruginosa* PAO1 and incubated overnight at 37ºC. *P. aeruginosa* PAO1 produced translucent, halo-like rings around the bacterial inoculum in both conditions.

characteristic of the bacteria growing on LB, not a zone of clearing. As our positive control was unsuccessful, the assay was inconclusive and we were unable to conclude whether rChiC from *E. coli* pM3CRYY retains chitinolytic activity. In the case that *E. coli* pM3CRYY BL21(DE3) was preferentially degrading other more readily available nutrients in the LB over the colloidal chitin, we performed the same colloidal chitin plate assay using M9 minimal media. This was to determine if *E. coli* pM3CRYY BL21(DE3) has chitinolytic activity that is more readily observed in the absence of the other components in LB. Again, plates supplemented with 1.0 mM IPTG were streaked with either *E. coli* pET-28a BL21(DE3) or *E. coli* pM3CRYY BL21(DE3). *P. aeruginosa* PAO1 was not tested on these plates due to time constraints. *E. coli* pET-28a BL21(DE3) was used as a negative control due to its empty vector and absence of the *chiC* gene, to observe what no zones of clearing should look like. Figure 7 shows growth of both pET-28a and pM3CRYY containing *E. coli*

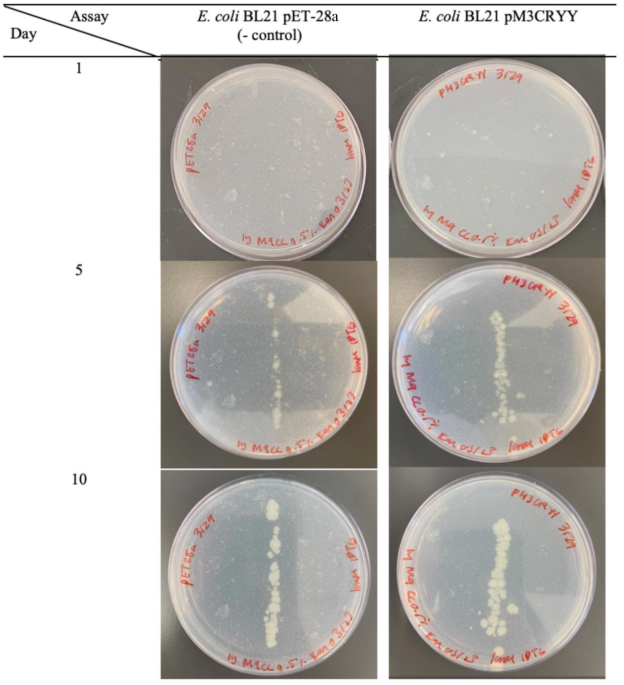

FIG. 7 Chitinolytic activity of ChiC is inconclusive on M9 minimal media colloidal chitin plates. *E. coli* pET-28a BL21(DE3) (left) and *E. coli* pM3CRYY BL21(DE3) (right) were streaked out onto M9 minimal media 0.5% colloidal chitin plates spread-plated with 40µl of filter sterilized 1mM IPTG. Plates were incubated overnight at 37ºC and growth was observed from days 1 to 10. *E. coli* pET-28a BL21(DE3) was used as a negative control. n=3.

by day 1 on the 1 mM M9 minimal media 0.5% colloidal chitin plates, although to a much lower degree than the growth observed for both bacteria on day 1 of the LB 0.5% colloidal chitin plates, indicating the bacteria are viable on this media. By day 5, growth had increased for both bacteria, but no zones of clearing were visible. By day 10 growth had increased again for all three bacteria, and no zones of clearing were visible for *E. coli* pET-28a. However, since the positive control did not work, we cannot conclude if our negative control was successful. No zones of clearing were observed for *E. coli* pM3CRYY either, suggesting it was unable to degrade chitin. However, due to the lack of a positive control, this assay was also inconclusive.

DISCUSSION

The aim of our study was to assess the chitinolytic activity of rChiC from *E. coli* pM3CRYY B4 BL21(DE3) using fungal inhibition, DNS, and colloidal chitin plate-based assays. We first confirmed that the *chiC* sequence was preserved in pM3CRYY extracted from *E. coli* pM3CRYY B4 BL21(DE3) using Sanger sequencing. One of our four sequencing results failed, which may have been caused by a technical error as the primer used had been successfully used previously in Sanger sequencing. Our three sequencing results yielded a consensus sequence that covered all of *chiC*. The consensus sequence exactly matched the *chiC* sequence of *P. aeruginosa* PAO1, except for one cytosine residue in *chiC* which was read as an unidentified base. As this location was only covered by one sequencing read, it suggests that the mismatch was a sequencing artifact, and that the *chiC* sequence was unaltered in pM3CRYY B4.

Next, we tested whether rChiC-expressing *E. coli* pM3CRYY B4 BL21(DE3) had antifungal activity. Since the cell wall of fungi is composed of chitin, antifungal activity against *F. oxysporum* was observed using a fungal inhibition assay. We observed inhibition of *F.oxysporum* growth by *E. coli* pM3CRYY B4 BL21(DE3) and *P. aeruginosa* (positive control), but no inhibition by *E. coli* pET-28a BL21(DE3) (negative control) for both 1.0 mM and 0.1 mM IPTG stimulated plates. This aligns with previous studies that demonstrated that *P. aeruginosa* (PAO25) can inhibit the growth of *F. oxysporum* (2). However, a *P. aeruginosa chiC* knockout control plate should be added to this assay to confirm that the observed *F. oxysporum* inhibition is due the chitinolytic activity of ChiC. Furthermore, the inhibition of *F. oxysporum* by *E. coli* pM3CRYY BL21(DE3), which contains *chiC* and not *E. coli* pET-28a BL21(DE3), which does not contain *chiC*, reveals that rChiC may be responsible for the observed antifungal activity. Notably, the assay results were similar between 1.0 mM and 0.1 mM IPTG plates, but more robust quantification of fungal inhibition and varying concentrations of IPTG need to be tested to determine if there are IPTG concentration-dependent effects on the antifungal activity of *E. coli* pM3CRYY BL21(DE3). This suggests that rChiC-expressing *E. coli* pM3CRYY B4 BL21(DE3) stimulated with 0.1 or 1.0 mM IPTG has moderate antifungal effects. This may have implications for the use of *E. coli* pM3CRYY B4 BL21(DE3) as a biocontrol agent against fungal pathogens in agriculture.

We further aimed to characterize the functional activity of purified rChiC. ChiC expression was induced with IPTG, followed by cell lysis, and purification using nickel affinity chromatography. From SDS-PAGE analysis, we observed that a band corresponding to ChiC was seen in the flowthrough. This could mean that not all the protein was bound to the nickel column, which could be alleviated by increasing incubation time of the protein with the resin. It could also indicate that some of the protein had been degraded from the N terminus and no longer had an intact 6xHis tag, and thus were unable to bind to the nickel column. Our analysis also showed that 10 mM imidazole was sufficient to wash out the majority of the other proteins present in the original lysate. Interestingly, the band corresponding to ChiC seen in all of the samples appeared as a smear (Figure S1). This could be due to degradation leading to variations in ChiC size (12). Since the 6xHis tag that binds to the nickel column is on the N terminus of ChiC, even in the elution fraction, the observed smear could still be attributed to C terminus degradation.

Upon dialysis of the proteins to remove imidazole, which may interfere with protein structure, we observed precipitation in our protein sample. This suggests the formation of protein aggregates, which would decrease the yield of functional protein. This result indicates that the storage conditions of rChiC need to be further optimized. Improvements could

include using glycerol to increase solubility (13) or storing the protein in reducing conditions to prevent disulfide bond formation.

After purifying the protein, we first confirmed that rChiC had chitin-binding activity, as has been previously observed by L. Lin, P.R. Agawin, J. Tong, S. Rai, G. Shoaib (unpublished manuscript). Their study also found that rChiC can bind chitin both with and without the presence of imidazole, suggesting that rChiC may not need to be properly folded to have an active chitin-binding domain. Thus, the presence of chitin-binding activity we observed does not necessarily mean that our protein was fully folded and enzymatically active. Interestingly, while our pre-dialysis rChiC sample showed as a smear upon SDS-PAGE analysis (Figure S1), the fraction that bound chitin showed as a very clean band (Figure S2). As the chitin-binding domain of rChiC is on its C terminus (14), it could be that some of the purified ChiC had been degraded from its C terminus. However, these proteins would have been washed out during the chitin-binding assay, leaving only fully intact ChiC bound to the chitin beads. The presence of these partially degraded proteins may have further decreased the concentration of functional ChiC in our sample and contributed to the negative results observed in downstream chitinase assays.

In contrast to our fungal assay results, we found no evidence of chitinolytic activity with purified rChiC in our DNS assay. There are many reasons why we may have obtained this result. Our BCA assay showed that rChiC concentration was 210.8 ng/µl (Figure S3), which is less than the concentration used for the positive control. Further, we observed precipitated protein in our sample which suggests the formation of disulfide bonds between polypeptides due to an oxidizing environment, which would further decrease the concentration of functional protein. Another possible issue is substrate availability. Colloidal chitin is insoluble and settled at the bottom of tubes during the assay, decreasing the surface area in contact with the protein. The temperature and pH used in the assay may also have impaired chitinase activity. Assay conditions were chosen based on past research which showed that ChiC had optimal chitinolytic activity between pH 4.5 and 5.0 at 50ºC when using carboxymethyl-chitin-Remazol Brilliant Violet as a substrate (2). However, it is possible that optimal conditions for chitinase activity are different using colloidal chitin as a substrate.

Using disk diffusion assays, we observed no zones of clearance for our purified rChiC, *E. coli* pM3CRYY BL21(DE3) lysates, negative control binding buffer, or the positive control chitinase from *S. griseus*. From these results, the lack of inhibition by the binding buffer means that it is an appropriate buffer to suspend rChiC for testing of antifungal activity. The lack of inhibition by *E. coli* pM3CRYY BL21(DE3) lysates suggests that the purification process is not responsible for the lack of activity in our purified rChiC. However, the reason the positive control ChiC from *S. griseus* (2892.1 µg/ml), our purified rChiC (210.8 µg/ml), and the *E. coli* pM3CRYY lysates failed may be due to low loading concentration. Previous literature demonstrated fungal assays with higher chitinase concentrations of 3750 µg/ml (7), hence concentrating rChiC is suggested. There may have also been poor diffusion of the chitinase from the disks, limiting the observable antifungal ability. Further, the stability of the chitinases may have contributed to the negative result, as it is possible that the enzymes had degraded before they could have a substantial inhibitory effect on the fungus. Lastly, the agar well assay did not show any zones of inhibition. This assay can be improved by creating shallow wells so that agar remains at the bottom. Specifically, creating shallow wells would allow us to see inhibition more easily because the fungus would not access the media below a well with inhibitory substances, but would grow into wells without inhibitory substances. Together, these results indicate that fungal inhibition assays were not effective for testing the chitinolytic activity of purified rChiC. This prompts the need for alternative assays to test for chitinolytic activity of rChiC to determine whether it has potential as a biocontrol agent.

To test the chitinolytic activity of *E. coli* pM3CRYY BL21(DE3), we performed colloidal chitin plate assays using LB and M9 minimal media to look for zones of clearing representative of chitin degradation. The LB-colloidal chitin plate assay was inconclusive as our positive result was not successful. This is inconsistent with previous results having demonstrated chitinase activity indicated by zones of clearing on colloidal chitin plates (2). *P. aeruginosa* may not have demonstrated chitinolytic activity due to its preferential use of nutrients in LB as a carbon source over the colloidal chitin (16). Further, it has been

previously reported that expression of the *chiC* gene appears to be regulated by the quorum-sensing system of *P. aeruginosa* (2). An intra- or extracellular signal may be required for the secretion of ChiC (2), and this signal may not have been sufficiently expressed in the LB-colloidal chitin plates. *E. coli* pM3CRYY BL21(DE3) also did not show chitinolytic activity on the LB-colloidal chitin plates. This may also be due to the bacteria preferentially using LB as a carbon source instead of breaking down the colloidal chitin, although this is unlikely, due to the presence of the inducer IPTG. Indeed, *E. coli* pM3CRYY BL21(DE3) also did not produce zones of clearing on the M9 minimal media plates, suggesting other explanations for the absence of chitinolytic activity. This includes issues with the secretion system in *E. coli* pM3CRYY BL21(DE3). The chiC gene is originally from *P. aeruginosa* and is thought to be secreted by a novel system, therefore, *E. coli* transformed with only the *chiC* likely does not contain all the genes necessary for the export of ChiC (2). This explanation is further supported by a previous study suggesting that rChiC is not secreted by *E. coli* pM3CRYY BL21(DE3) (J. Choi, V. Lewis, C. Sie, J. Yap; unpublished manuscript). Additionally, *E. coli* BL21(DE3) may not express the chaperone protein required for the proper folding of ChiC, reducing its functionality. Alternatively, rChiC activity may have been too low to be detected visually, or the preparation of the colloidal chitin plates may have been inadequate. For example, the colloidal chitin did not appear to be fully dissolved in the plates, demonstrated by the small specks of powder in the colloidal chitin plates. This may have affected the final concentration of colloidal chitin in the plates and therefore, the availability of colloidal chitin to the bacteria may have been too low. Similarly, the concentration of IPTG used may not have been ideal for the bacteria. Previous studies have determined soluble rChiC is optimally expressed under induction with 0.1 mM IPTG (H. Kim, D. Li, A. Srinivas, L, Wu; unpublished manuscript), whereas we used a concentration of 1.0 mM IPTG. Based on these findings, we cannot confirm if recombinant ChiC retains chitinolytic activity in *E. coli* BL21 (DE3). However, these preliminary results may have applications for improving the design of future colloidal chitin plate assays.

Limitations Though our preliminary results suggest an antifungal effect from rChiC-expressing *E. coli* and no chitinase activity from purified rChiC, there are several limitations. Foremost, only one replicate was used for each IPTG induction condition in our fungal inhibition assay, and more replicates are needed to confirm that what we observed was biologically based. Additional controls with *P. aeruginosa* chiC knockout and uninhibited *F. oxysporum* are also recommended. Additionally, our DNS assay used different concentrations of positive control chitinase compared to rChiC. This makes it possible that the rChiC concentration was simply too low for any chitinase activity to be detected, and that if the positive control chitinase concentration was decreased to match rChiC, activity would also not be detected. Using heat-denatured chitinases as a negative control would also have been better than using the buffers the proteins were stored in, as was done in our assay, and using the same buffer for both the positive control and rChiC would further improve consistency. Similarly, the concentration of rChiC may be too low for the detection of antifungal effects using disk diffusion assays. Hence, increasing the working protein concentration is suggested.

Conclusions In this study, we aimed to evaluate the chitinolytic activity of rChiC from *E. coli* pM3CRYY B4 BL21(DE3). Through Sanger sequencing, we found that this strain contained an identical copy of *chiC* from *P. aeruginosa* PAO1. Testing the ability of rChiC-expressing *E. coli* to inhibit the growth of *F. oxysporum* showed a moderate antifungal effect. However, our assays for chitinolytic activity using purified rChiC yielded negative results using the DNS method. Antifungal assays using purified rChiC and tests for colloidal chitin degradation by rChiC-expressing *E. coli* were both inconclusive. These findings suggest that *E. coli* pM3CRYY BL21(DE3) has potential applications in biopesticide development, however, more work is needed to confirm and better characterize the chitinolytic activity of the enzyme.

Future Directions As previously mentioned, an immediate first step would be to perform the fungal inhibition assay with more replicates to determine if our preliminary observations of inhibition by rChiC-expressing *E. coli* were significant. Additionally, performing the fungal assay with a ChiC knockout *P. aeruginosa* strain as a negative control would help determine if the fungal inhibition observed in the *P. aeruginosa* PAO1 was specifically due to ChiC. Further testing for *in vitro* chitinolytic activity using more readily available substrates and different pH and temperature conditions is also needed. It would also be beneficial to further optimize the purification process of rChiC to obtain a higher concentration of soluble protein. This could involve concentrating the protein and testing storage buffers with different pH levels to see which one rChiC remains most soluble in, or storing rChiC in reducing conditions to prevent disulfide bond formation. Upon obtaining concentrated protein, it would also be valuable to perform further disk diffusion assays to determine if higher concentrations of purified rChiC have antifungal activity.

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