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Construction of plasmid pRMGS22 for the expression and His-tag purification of recombinant chitinase encoded by the *chiC* gene of *Pseudomonas aeruginosa* PAO1

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SUMMARY The use of chemical pesticides has been the leading approach in agriculture to protect crops from pest and insect-induced damage. However, environmental pollution and human health problems have resulted from excessive chemical pesticide use. As such, there is an urgent need to explore other pesticides that are more ecologically friendly and sustainable. Chitinase, or ChiC, is a naturally occurring enzyme found in *Pseudomonas aeruginosa* capable of degrading chitin, a structural component of the insect exoskeleton. Previous studies have investigated the expression of the *chiC* gene for its potential use as an alternative insecticide. A study by Bodykevich et al. was able to amplify the *chiC* gene from *P. aeruginosa* PAO1 and ligate it into the pCR 2.1 vector, forming the pGKMS21 plasmid. In this study, we sought to subclone the *chiC* gene from the pGKMS21 storage vector into the pET-28a expression vector and transform it into *Escherichia coli* strain BL21-(DE3). The *chiC* gene was amplified from the pGKMS21 vector using gradient PCR and was subsequently ligated into pET-28a, forming the pRMGS22 plasmid. The pRMGS22 plasmid was further propagated in *E. coli* strain DH5α, isolated, and transformed into *E. coli* strain BL21-(DE3). The resulting clones of the pRMGS22 plasmid were analyzed through Sanger Sequencing. The nucleotide sequence and the transcribed protein sequence confirmed the presence of *chiC* from *P. aeruginosa* PAO1 with 100% identity observed using the NCBI BLASTn and BLASTp tools. Future studies will be able to use the pRMGS22 construct to test protein expression and potentially purify the ChiC protein for more in-depth characterization and testing for enzymatic activity. This will allow for further study of the ChiC protein as a potential alternative to chemical pesticides.

INTRODUCTION

The global agricultural industry has historically faced challenges in crop losses due to various species of plant-feeding insect pests (1). This has remained a major issue in spite of significant advancements in agricultural techniques and technology, with an estimated 18% to 20% of crops worldwide lost annually due to arthropods (1). Furthermore, these losses are projected to increase in the future. Increased temperatures due to climate change will result in increased insect pest population growth and metabolic rates, particularly in regions where staple grains are primarily produced (2). Current strategies for mitigating the destruction of crops by pests primarily involve the use of chemical pesticides, with over 3 billion kilograms used annually worldwide (3). These pesticides are composed of a variety of chemical compounds and have been found to be associated with health, environmental, and economic complications (3-7). As pest-induced crop damage increasingly occurs, and as the negative effects of chemical pesticide use continues to intensify, an urgent need for sustainable strategies to protect crops has developed and has led to extensive investigation (4). Natural pesticides produced by various bacteria have emerged as a propitious alternative,

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with considerably fewer risks to both human health and the environment (8). Bacteria, such as those within the *Pseudomonas* genus, produce these compounds naturally, and thus they are known to have specific, biological components as targets, for the purpose of insect pathogenicity (8,9).

Chitin is a naturally occurring polymer that among other functions, serves as a vital structural component in insect exoskeletons (10). As such, chitin is a suitable target for natural pesticides that could potentially be used in the place of synthetic pesticides. A diverse array of bacteria including those within the genera *Bacillus, Pseudomonas,* and *Vibrio* exhibit chitin degradation through the production and secretion of chitinolytic enzymes known broadly as chitinases (10). Chitinases comprise a diverse group of enzymes with varying structure and substrate specificity (11). *chiC* has been identified as a gene that encodes chitinase C (ChiC) which has been shown to contribute to the insecticidal ability in *Pseudomonas aeruginosa* (10). ChiC has been characterized as a 55-kDa secreted protein, with an unknown, and potentially novel, method of secretion that involves the cleavage of the N-terminus (10). Bioinformatic structural analysis of ChiC in previous studies has further identified the multiple protein domains and their biological activity through which chitin is degraded (10,12). A chitin-binding domain facilitates interaction with the chitin compound for degradation by a glycoside hydrolase domain targeting beta-1,4 bonds (10,12). Lastly, a fibronectin type III-like domain is situated between the binding and catalytic domains to maintain optimal spacing for degradation to occur (10).

A study by Bodykevich et al. accomplished the isolation of the *chiC* gene from the strain *Pseudomonas aeruginosa* PAO1 and subsequent amplification of the gene before ligating it into the pCR2.1 vector, resulting in the pGKMS21 plasmid containing the *chiC* insert (12). Results from this study demonstrate the feasibility of cloning the *chiC* gene into desired vectors, and ChiC expression from other species has been achieved in previous studies as well (12,13). The pGKMS21 plasmid serves as a storage vector for *chiC* and does not support protein expression due to the fact that the T7 promotor is on the 3' end of the *chiC* gene in the incorrect orientation (12). As such, further research is required to obtain protein expression in cells that are better suited for propagation of the gene.

We sought to take additional steps towards the goal of ChiC expression. The pET-28a plasmid is a widely used expression vector, as it contains an inducible universal T7 promoter as well as 6xHis-tags on either side of the multiple cloning site that can help facilitate the purification of the final protein product (12,14). As such, the pET-28a vector provides the advantage of easily expressing a recombinant ChiC protein that is more readily purified due to the 6xHis-tags. The *Escherichia coli* strain BL21-(DE3) is widely used for protein production and could be used to improve the efficiency of ChiC expression if transformed with a vector containing the *chiC* insert (12, 15). We hypothesized that the *chiC* gene could be amplified from the pGKMS21 vector, cloned into the pET-28a expression vector, and transformed into *E. coli* strain BL21-(DE3) cells to produce a strain capable of expressing ChiC for future study. In conducting this study, we successfully subcloned the *chiC* gene into the pET-28a expression vector, forming the pRMGS22 plasmid.

METHODS AND MATERIALS

Preparation of experimental materials. Kanamycin stock solution (25mg/mL), 60mM $CaCl_2$ solution (with 10mM PIPES and 15% glycerol), LB broth (tryptone, yeast extract, NaCl, and distilled water), LB agar, and SOC media (tryptone, yeast extract, NaCl, KCl, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, glucose) were prepared following standard protocols. $CaCl_2$ was both autoclaved and filter-sterilized using a 0.2µm filter, as we required more than anticipated. Glucose was added using filter-sterilization through a 0.2µm filter after the SOB media was autoclaved. LB media were autoclaved for sterilization. Most of the media were made selective through the addition of kanamycin at a concentration of 50µg/mL. 1X TAE buffer was made from 50X TAE stock obtained from the teaching team.

Primer design for the pGKMS21 plasmid. Analysis of the *chiC* gene illustrated an overall higher GC-content closer to the 3' end of the gene (69% in the last 300 bp). The subsequent step of the experimental design was to subclone the *chiC* gene isolated from the pGKMS21 plasmid into the pET-28a vector. Custom design primers (forward and reverse: 5'-

ATAGCTAGCATGATCAGGATCGACTT-3', 5'-CTAGAGCTCCAGCGCAGCGG-3') were obtained from Integrated DNA Technologies Inc. to amplify the *chiC* gene from the pGKMS21 vector previously created by Bodykevich et al. (2021). In order to ensure that the 3' 6X Histidine tag on the pET-28a vector would stay in frame of the *chiC* gene after insertion, the last nucleotide of the *chiC* gene was removed from the reverse primer, and replaced with an additional nucleotide on the 5' end. The reverse primer was made shorter in order to reduce the melting temperature to approximately 5ºC of the forward primer. Lastly, the linker regions for the forward and reverse primers contained the restriction site for SacI and NheI respectively to allow for restriction digestion and ligation in later steps.

Plasmid isolation of pGKMS21 and pET-28a vectors. Starter plates containing the pGKMS21 plasmid in OneShot TOP10 *E. coli* cells, created by Bodykevich et al. (2021), and the pET-28a vector in *E. coli* strain DH5α cells obtained from the Haney lab were streaked out onto LB agar plates containing 50µg/mL kanamycin. Overnight cultures of 3mL were made from four colonies of both the OneShot TOP10 cells and four colonies of the *E. coli* strain DH5α cells in glass test tubes placed in a 37ºC shaking incubator at 225 rpm. The Plasmid DNA Minipreps Kit (BioBasic) was used to isolate the pGKMS21 and pET-28a plasmids. The plasmid concentration was then taken using the NanoDrop spectrophotometer. Both plasmids were stored in a -20ºC freezerfor later use.

Creation of chemically competent *E. coli* strain BL21-(DE3) cells. *E. coli* strain BL21-(DE3) cells were obtained from the teaching team and streaked out to obtain isolated colonies for overnight culture. Generation of chemically competent *E. coli* strain BL21-(DE3) cells was done following the American Society for Microbiology protocol (16). Overnight culture of the BL21-(DE3) strain was made in 2mL of LB broth in glass test tubes left in a 37ºC shaking incubator set at 225rpm. 1mL of the overnight culture was then transferred to an Erlenmeyer flask containing 100mL of LB broth and left to incubate for 2 hours in the 37ºC shaking incubator set at 225rpm. An OD_{600} measurement was taken on the Ultrospec 3000 (Biochrom) and determined to be within the targeted range of 0.3-0.4. The *E. coli* strain BL21-(DE3) cell culture was chilled on ice for 10 minutes. 70mL of the culture was transferred into two 50mL Oak Ridge tubes, each containing 37.5mL, and centrifuged using an Avanti™ J-30I centrifuge set at 4000g for 5 minutes at 4ºC. The supernatant was then discarded, and the cells were resuspended in 18.75mL of previously made 60mM $CaCl_2$ solution in each tube and placed in an ice bath for 30 minutes. The suspension was then centrifuged once again using the same parameters, the supernatant was discarded, and the cells were resuspended into 5mL of ice-cold $CaCl_2$ solution before being separated into Eppendorf tubes for long-term storage in -70ºC.

The transformation efficiency of the BL21-(DE3) cells was tested using the previously isolated pET-28a vector. The transformation reaction was set up in a sterile 1.5mL Eppendorf tube containing: 50µL of the competent BL21-(DE3) cells and 1µL pET-28a. The negative control was also set up to include 50µL of the BL21-(DE3) cells, and 1µL of elution buffer from the Plasmid DNA Minipreps Kit (BioBasic). The reactions were incubated on ice for 30 minutes and placed into a 42°C water bath for exactly 2 minutes. The reaction tubes were then immediately removed and placed on ice for 5 minutes. Following this, 1.0mL of prepared SOC medium was added to each reaction tube, and they were incubated at 37ºC for 1 hour in a shaking incubator set at 225rpm. Once completed, 75µL of each reaction was plated on previously prepared LB agar plates containing 50µg/mL kanamycin. The plates were then incubated overnight at 37ºC to allow for growth.

Amplification of *chiC* gene from pGKMS21 vector using SuperFi™ DNA Polymerase. A 1.5kB-sized fragment containing the *chiC* gene, as predicted using SnapGene (version 6.0.2), was amplified from the pGKMS21 vector following the Invitrogen™ Platinum™ SuperFI™ DNA polymerase user guide in 200µL reaction tubes. The reaction components included 10µL of 5X SuperFi Buffer (Invitrogen), 1µL of 10mM dNTP mix (Invitrogen), 2.5µL each of 10µM forward and reverse custom primers, 1µL of 170.1ng/µL isolated pGKMS21 vector, 10µL of 5X SuperFi™ GC Enhancer (Invitrogen), 0.5µL of Platinum™ SuperFi™ DNA polymerase (2U/µL) (Invitrogen), and 22.5µL UltraPure™ Distilled Water

(Invitrogen). The positive control included each of the reaction components, however, the template DNA was replaced with the pUC19 vector (Invitrogen), and their respective forward and reverse primers (5'-GGCGTTACCCAACTTAATCG-3' forward, and 5'-GTGAAATACCGCACAGATGC-3' reverse) obtained from Integrated DNA Technologies Inc. Gradient PCR was performed to optimize the annealing conditions, as the melting temperatures of the forward and reverse primers were 5.6ºC apart at (57.3ºC forward and 62.9ºC reverse). The BioRad T100™ Thermal Cycler was set to the following conditions: initial denaturation of DNA at 95ºC for 5 minutes, 34 cycles of denaturation of DNA at 95ºC for 30 seconds, annealing of DNA set at a gradient (53.1ºC, 54.3ºC, 55.7ºC, 56.8ºC, 57.5ºC, 58.0ºC) for 45 seconds, and elongation at 72ºC for 1 minute and 45 seconds, followed by a final extension period of 72ºC for 5 minutes, and set to hold at 4ºC until gel electrophoresis was performed.

Agarose gel electrophoresis on PCR products for product length confirmation. Agarose gels were made using the 1X TAE buffer, 1 gram of UltraPure™ Agarose (Invitrogen) and 5µL 20 000X RedSafe™ (iNtRON Biotechnology). Upon solidification of the agarose gel within the mold, the electrophoresis container was filled with 1X TAE buffer until the gel was fully submerged. 4µL of each of the six gradient PCR products separately mixed with 4µL of 6X Purple Gel Loading Dye (BioLabs), before being loaded into separate wells. 5µL of the 1Kb DNA ladder RTU (FroggaBio) was added to the left side of the loaded wells. The gel was run at 150V for 30 minutes and visualized using the ChemiDoc™ Imaging System (BioRad). The remaining PCR products were stored at -20ºC until further use.

Digestion of PCR amplified product and pET-28a plasmid with NheI and SacI restriction enzymes. Purification of the PCR amplified product was performed using the PureLink™ Quick PCR Purification Kit (Invitrogen). Concentrations of each of the PCR amplified tubes were then obtained using the NanoDrop spectrophotometer to determine concentrations for the digestion protocol. The three reaction tubes containing the highest concentrations of product, named reactions 2, 5 and 6 (from the annealing conditions from the PCR of 54.3ºC, 57.5ºC, and 58.0ºC respectively) were selected for restriction digestion. The digestion reaction was set up in four 200µL reaction tubes to allow for use of the BioRad T100™ Thermal Cycler. The digestion reaction contained the following components: 2µL of 10X CutSmart™ Buffer (BioLabs), 1µL of NheI (BioLabs), 1µL SacI (BioLabs), either PCR amplified product (varying) or isolated pET-28a vector, and sterilized distilled water (varying). Since the concentrations for the three PCR amplified products and purified pET-28a vector did not have a high enough concentration (1µg/mL), 15.9µL of product 2, 16µL of product 5, 16µL of product 6, and 8µL of the pET-28a vector were used instead. The volume of the UltraPure™ Distilled Water (Invitrogen) was adjusted accordingly to have the reaction volume of each equate to 20µL total. The reaction was set to incubate at 37ºC for 1 hour and was terminated with heat inactivation at 65ºC for 15 minutes, in the BioRadT100™ Thermal Cycler. The thermal cycler was set to 4ºC to store the reaction mixture until ligation could be performed.

Ligation of PCR amplified product into the pET-28a vector to create the pRMGS22 plasmid. The concentration of each of the reaction components from the restriction digest was tested on the NanoDrop spectrophotometer. Three ligation reactions, each containing one of either product 2, 5 or 6, and a positive control were subsequently set up in 1.5mL Eppendorf tubes following a 1:3 vector to insert ratio. The reaction mixture contained the following: 2µL of 5X T4 DNA Ligase Buffer (Invitrogen), 1µL T4 DNA Ligase (Invitrogen), 1µL purified pET-28a vector 3µL of PCR product, and 13µL of autoclaved distilled water. The positive control contained 16µL of autoclaved distilled water and did not contain any PCR product. The ligation reaction was incubated overnight at 16ºC in the BioRadT100™ Thermal Cycler, and then heat-inactivated at 65ºC for 10 minutes the following day. The reaction tubes were then transferred to ice and kept there for the transformation procedure.

Transformation of pRMGS22 into *E. coli* strain DH5α cells. Five 1.5mL Eppendorf tubes were set up for the transformation reaction for each of the ligation products, along with

positive and negative controls. Competent *E. coli* strain DH5α cells were obtained from the teaching team. The transformation reaction was set up in each of the five tubes containing: 50µL of competent *E. coli* strain DH5α cells and 1µL of each ligation product (water in the case of the negative control). The transformation protocol described above to test the transformation efficiency of the *E. coli* strain BL21-(DE3) cells was then followed.

Screening and sequencing of insert in pRMGS22 plasmid. Overnight cultures of 3mL were made from the three ligation reactions (6 colonies of the ligation reaction containing pET-28a and product 2, 6 colonies of pET-28a and product 6, and 2 colonies from pET-28a and product 5) in glass tubes containing LB broth with 50µg/mL kanamycin kept at 37ºC in the shaking incubator set at 225rpm. The isolated colonies were labelled to match their initial PCR reaction and given a number (e.g. 6_1, 6_2, 5_1, 5_2, 2_1, 2_2, etc.). The Plasmid DNA Minipreps Kit (BioBasic) was used to isolate the plasmids from each of the colonies. The concentrations of each of the isolated plasmids were tested using the NanoDrop spectrophotometer. The presence of an insert was confirmed through another restriction digest and running all of the isolated products on an agarose gel.

The restriction digest reaction was set up once again in fourteen 200µL reaction tubes containing the following components: 2µL of 10X CutSmart (BioLabs), 1µL of NheI (BioLabs), 1µL SacI (BioLabs), and varying amounts of the isolated plasmids and sterilized distilled water to total 20µL. Namely, 6_2 had 11.5µL of plasmid, 6_3 had 4.39µL of plasmid, and 5_2 had 4.40µL of plasmid added to the reaction mixture. 16µL of every other plasmid was added to each respective reaction mixture. The reaction parameters were set up in the way previously described on the BioRadT100™ Thermal Cycler.

Two agarose gels were made as previously described, and 4µL of each of the isolated plasmids, combined with 4µL of 6X Purple Gel Loading Dye (BioLabs) and loaded onto the gel, along with 5µL of 1Kb DNA ladder RTU (FroggaBio) on the left and right sides of the wells loaded with the isolated plasmids for ease of comparison.

10µL of two of the plasmids detected to have a band of approximately 1.5kb (namely samples 5_1 and 5_2), and a high enough concentration for Sanger sequencing were sent to an external facility by Genewiz™ (Azenta Life Sciences) to be analyzed and sequenced using the T7 universal promoter and terminator primers. Plasmids were stored at -20ºC until further transformations could be done.

Transformation of the pRMGS22 vector into *E. coli* strain BL21-(DE3) cells. Three 1.5mL Eppendorf tubes were set up for the transformation reaction for the 5_1, 5_2 and 6_4 reaction products due to limitations in materials and time restrictions. The transformation reaction was set up in the three tubes containing: 50µL of previously made competent *E. coli* strain BL21-(DE3) cells and 1µL of the three products. The transformation protocol described above was followed once again. The plates were handed off to the teaching team for long-term storage.

RESULTS

A 1.5 kb product was PCR amplified from the pGKMS21 plasmid. In order to amplify the intended product, two primers were designed to include the initiation and termination sequence of the *chiC* gene on the pGKMS21 plasmid, as well as the 6xHis-tags on the 5' and 3' ends. Due to the 5.6ºC difference in T_m between the primers, a gradient PCR was performed to identify the optimal annealing temperature. The amplified products were then run through gel electrophoresis and visualized under the ChemiDoc™ Imaging System (Figure 1). As shown in the figure, all annealing temperatures tested produced a band around 1.5 kb, which corresponds to the expected size of *chiC*. The positive control lane had a band around 3000 bp. In all of the lanes, there appeared to be a smeared band above the distinct band of interest, especially in the positive control lane. Within the gradient, annealing temperatures of 57.5ºC and 58ºC gave the most intense bands, and all of the PCR products were purified and the plasmid concentrations were measured to determine samples that have the highest plasmid concentrations. These were determined to be products 2, 5, and 6 and so these products were selected to move forward with the restriction enzyme-digestion.

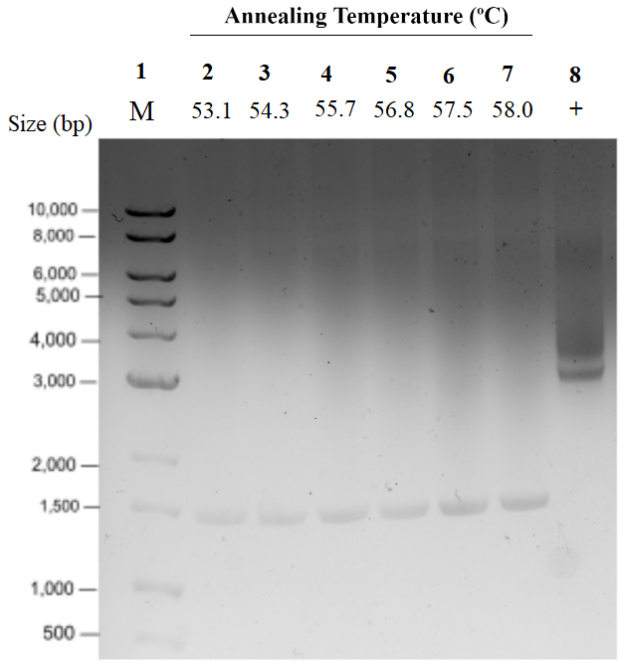

FIG. 1 Agarose gel electrophoresis results indicated the presence of an approximately 1.5 kb PCR product. The *chiC* gene was PCR amplified from the pGKMS21 plasmid using SuperFi polymerase. A gradient PCR was done with annealing temperatures ranging from 53 ºC to 58 ºC, as indicated at the top of each lane. The PCR product was cleaned up and 4ul of each, along with 4ul of 6X Purple Gel Loading Dye, were loaded into each lane of a 1% agarose gel stained with 20,000X REDSafe stain. Lane 1 contains 5ul of a 1kb DNA ladder. Lane 8 contains the amplified pUC19 vector, which acted as the positive control. The gel ran in 1X TAE buffer for 30 minutes at 150 volts. The resulting bands were around 1.5kb (the expected size of the *chiC* gene) and the positive control.

The PCR products were ligated into the pET-28a vector and transformed into *E. coli* strain DH5α and subsequently *E. coli* strain BL21-(DE3) cells. The three PCR products (2, 5, and 6), and the pET-28a vector were restriction digested using NheI and SacI restriction enzymes, and then the PCR product was ligated into pET-28a. The newly formed vector, named pRMGS22, was then transformed into chemically competent *E. coli* strain DH5α cells. Colony screening was then performed to determine if the *chiC* insert was present in the transformed cells. 15 colonies in total from the three plates were selected for plasmid extraction, digestion with SacI and NheI restriction enzymes, and agarose gel electrophoresis analysis. The partial results of the gel electrophoresis can be seen in Figure 2. A band of the expected size of 1.5 kb was only seen in four of the lanes, and in most lanes, this band was very faint, with the notable exception of sample 5_2, which had a very bright band (Figure 2). The new pRMGS22 from samples 5_1, 5_2 and 6_4 were then transformed into chemically competent BL21-DE3 cells.

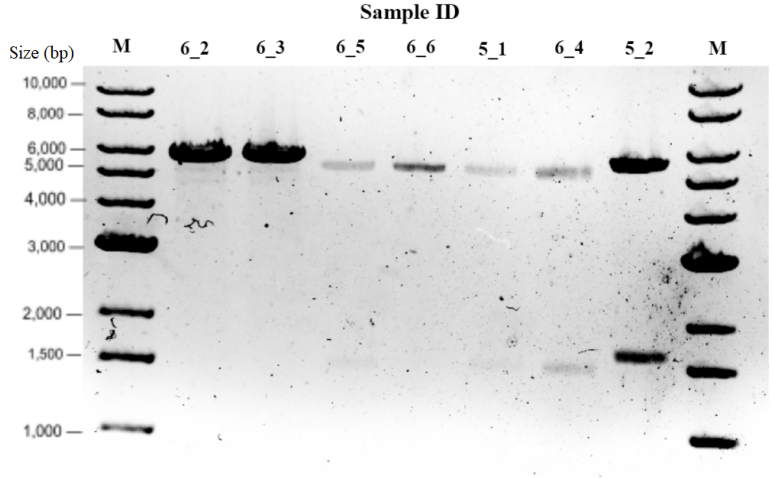

FIG. 2 Agarose gel electrophoresis results of *E. coli* strain DH5α colony screening indicate the presence of a 1.5kb in samples 6_5, 5_1, 6_4, and 5_2. The ligated and transformed pET-28a plasmid was extracted from multiple colonies of *E. coli* strain DH5α cells and was cut with SacI and NheI restriction enzymes. The products were run on a 1% agarose gel stained with 20,000X REDSafe stain for 30 minutes at 150 volts and subsequently visualized under the ChemiDoc™ Imaging System. 15 colonies were screened in total but only the samples that contained an insert of the expected size are shown here.

Two of the isolated pRMGS22 plasmids were analyzed through Sanger sequencing to confirm the presence of the *chiC* insert. pRMGS22 samples 5_1 and 5_2 were screened to contain an insert of 1.5kb and determined to meet the minimum DNA concentration for Sanger sequencing. The plasmids were submitted to Genewiz for Sanger sequencing using the universal primers for the T7 promoter and T7 terminator regions. The 5_2 reverse primer sequence did not prime and so this sample was removed from our analysis. For sample 5_1,

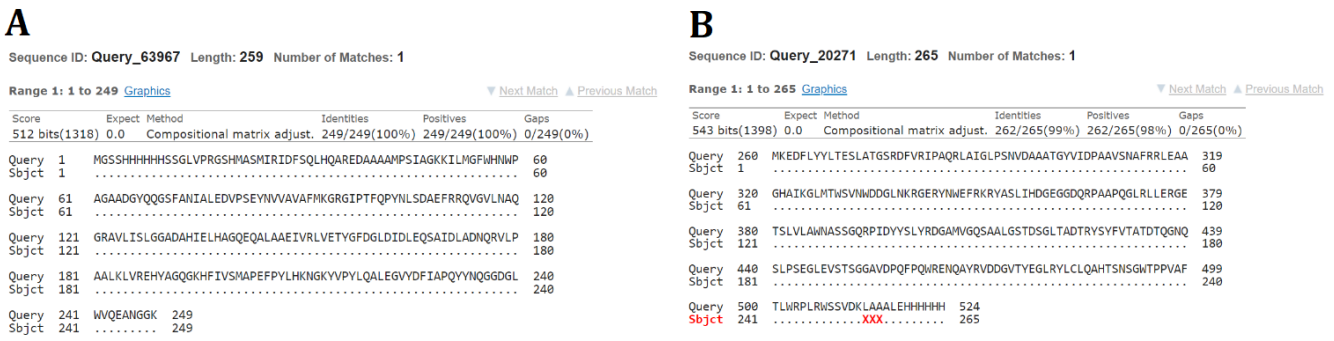

FIG. 3 NCBI BLASTp alignment of the reference chiC amino acid sequence with (A) the 5_1 forward primed amino acid sequence and (B) the 5_1 reverse primed amino acid sequence. The reference *chiC* nucleotide sequence was assembled by using SnapGene to model the ligation of the PCR product into the pET-28a vector, followed by translation into the amino acid sequence using the Expasy Translate tool. This tool was also used to translate the forward and reverse sequencing reads. The amino acid sequences were then aligned using the NCBI BLASTp multiple alignment tool. The reference ChiC sequence is labeled as Query and the forward and reverse sequences are labelled as Subject.

both the forward and reverse sequences were confirmed to align with the *Pseudomonas aeruginosa* PAO1 genome (accession: CP053028.1) through the NCBI BLASTn database. Further alignment of the nucleotide sequence was done using the NCBI BLASTn multiple alignment tool (Supplemental Figure 1). Both the forward and the reverse sequences showed strong alignment with both ends of the reference *chiC* gene, with the forward reaction having 98% identity and the reverse reaction having 97% identity. Most mismatches occurred at the beginning and end of the sequences outside of the gene sequence itself, which was likely the result of poor base call due to primer binding and nucleotide degradation at the end of the sequencing run. The two sequences had a 346 base pair overlap with each other at the middle of the gene, indicating good coverage of the entire *chiC* gene. This alignment also confirmed the presence of the 6xHis-tags on both ends of the insert. An illustration of the coverage of the *chiC* gene provided by both the forward and reverse sequencing reads for sample 5_1 can be seen in Figure 4B.

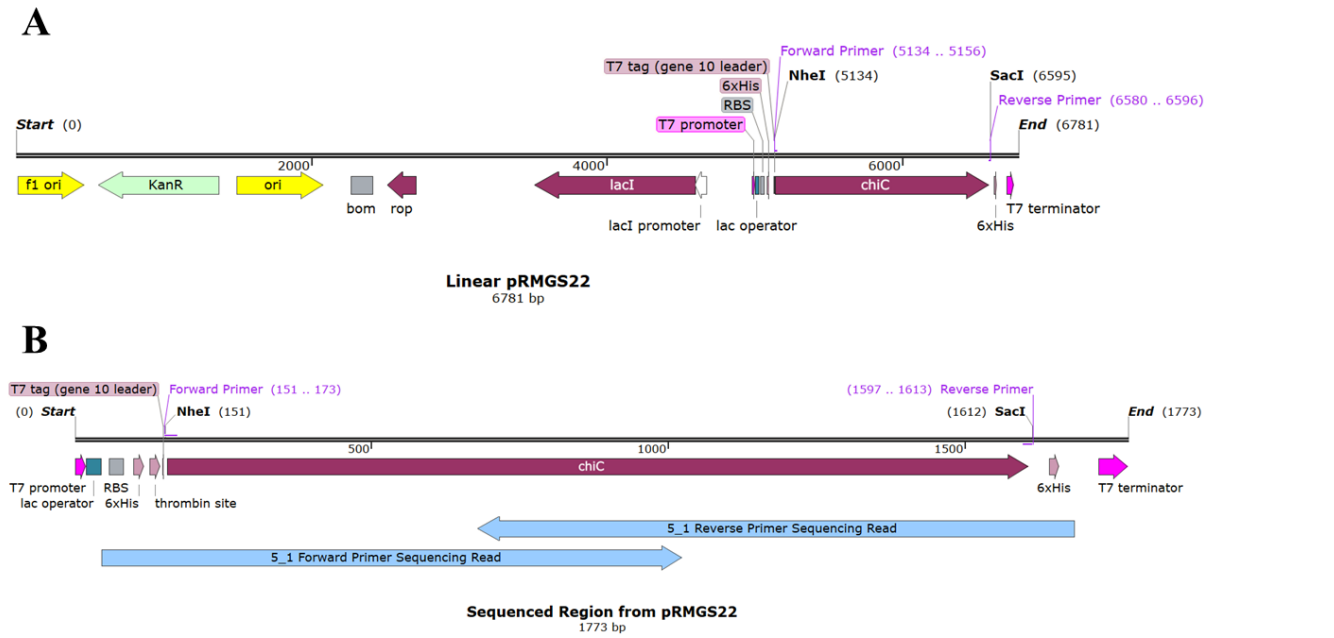

FIG. 4 Annotated amino acid sequence of (A) the forward sequencing read and (B) the reverse sequencing read. Both forward and reverse nucleotide sequences were translated into amino acids using SnapGene. There is no overlap between the ends of the sequences, the end of the forward sequence in Panel A continues on to the beginning of the reverse sequence in Panel B. Using the InterPro domain database, locations of protein domains were identified in the translated sequences, and labeled using SnapGene.

DISCUSSION

The objective of this study was to subclone *chiC* from the previously constructed pGKMS21 plasmid into the pET-28a vector and transform this new plasmid into *E. coli* strain BL21-(DE3) cells for downstream protein expression. We amplified the *chiC* insert from the pGKMS21 plasmid using gradient PCR, digested the PCR product and pET-28a vector using NheI and SacI restriction enzymes to generate sticky ends, and ligated the resulting product into the pET-28a vector, forming the new pRMGS22 plasmid. This new construct was then transformed into *E. coli* strain DH5α cells for propagation, isolated to screen for our insert, and two of the isolated plasmids (samples 5_1 and 5_2) were sent for Sanger sequencing.

Of the two plasmids sent for sequencing, only sample 5_1 contained the entire *chiC* gene. Both the forward and reverse reactions aligned well with the reference *chiC* gene (Supplemental Figure 1), with some mismatches occurring at the beginning of both sequencing reads. However, these mismatches occur outside the *chiC* gene, and thus will not impact the protein sequence when translated. On the other ends of the forward and reverse sequencing reads (Figure 4B), there does appear to be several mismatches in both, however, there was sufficient overlap between the ends of the forward and reverse sequencing reads to provide a comprehensive coverage (Figure 4B). Overall, this strong alignment indicates that the entire *chiC* gene was present in our new pRMGS22 construct for sample 5_1. Following the nucleotide alignment, the two nucleotide sequences were translated into the corresponding amino acid sequence using the Expasy Translate tool (https://web.expasy.org/translate/). Using the NCBI BLASTp multiple alignment tool (Figure 3), these two products were confirmed to have a 100% sequence identity to the reference ChiC from *P. aeruginosa* PA01 (accession: WP_194840396.1, WP_121155046.1). This showed strong alignment, with the forward read having 100% identity and the reverse read having 99% identity. The mismatch in the reverse read occurred between the end of *chiC* and the 3-prime 6xHis-tag, which was likely due to sequencing error and should not impact protein translation. However, this was not able to be confirmed with a chromatogram, and therefore future validation is required.

This work has generated a new plasmid encoding the recombinant ChiC protein that contains 6xHis-tags on both terminals to aid in the protein purification process. As determined by InterPro analysis (https://www.ebi.ac.uk/interpro/), the ChiC protein contains a glycoside hydrolase family 18 domain, a fibronectin 23 type-III domain, and a carbohydrate-binding module family 5/12 (Figure 5). Based on previous research, we predict that the carbohydrate-binding module can bind to the chitin exoskeleton of insects, allowing the glycoside hydrolase domain to enzymatically cleave and degrade chitin polymer (10, 12). Although the function of the fibronectin type-III domain is still unknown, previous studies suggested that since the domain is located between the binding and hydrolase domains, it could potentially serve as a linker region that helps create an optimal orientation for enzymatic cleavage (10). In contrast to the binding and catalytic domains which were evolutionarily more conserved among different chitinase-producing species, previous studies indicated that there was considerable variability in both sequence and length of the fibronectin type-III region among different species, further supporting the notion that it may serve as a linker (10).

By constructing the pRMGS22 plasmid, our study demonstrated that the *chiC* insert can be amplified from the pGKMS21 plasmid and subcloned into the pET-28a vector in frame with and upstream of the T7 promoter. Moreover, this orientation can also allow for protein expression of ChiC when transformed into a new host. This study also determined that a *Pseudomonas* gene can be cloned in a non-*Pseudomonas* host. Thus, pRMGS22 can serve as a storage vector for the *chiC* gene and potentially be used as a *chiC* expression vector. In order to examine the ChiC protein function, future studies may choose to induce ChiC protein expression from the transformed *E. coli* strain BL21-(DE3) cells containing the pRMGS22 plasmid. Moving forward, the ChiC protein can be purified using 6X His-Tag purification methods and tested for enzymatic activity.

Limitations A noted limitation of our analysis is that we do not have access to the Sanger Sequencing trace file, which can allow us to better interpret the effects of sequencing error on our alignment. This is especially important because we were only able to successfully sequence one sample. As a result, future analysis would benefit from confirming our sequencing results. Due to the unknown secretion mechanism of ChiC in *Pseudomonas*

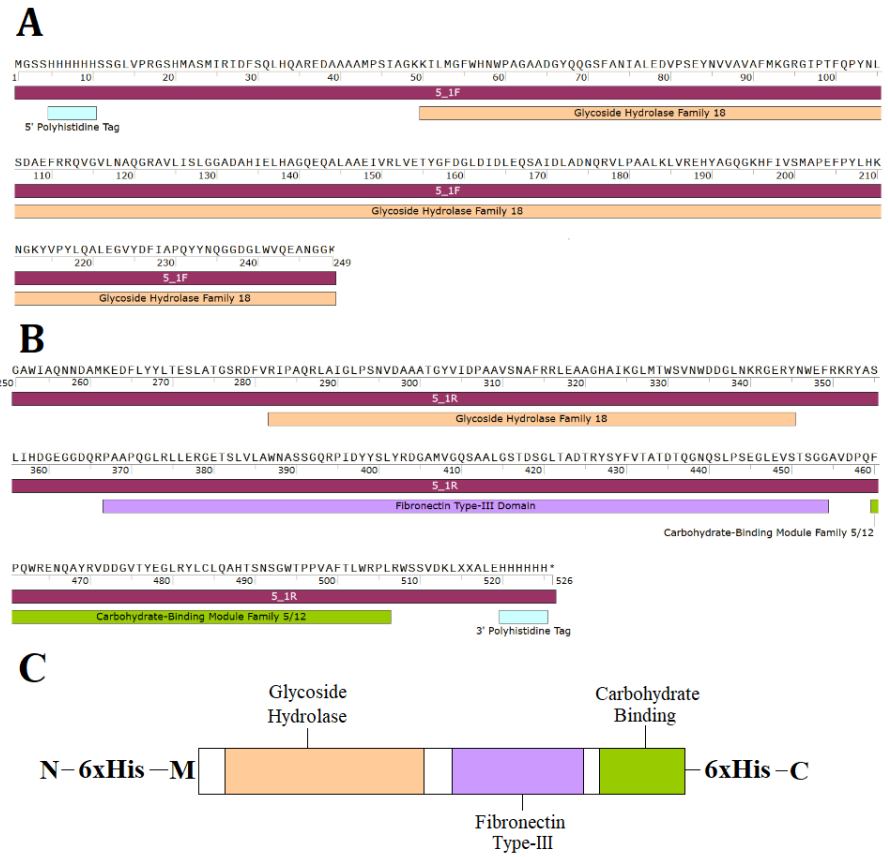

FIG. 5 Annotated amino acid sequence of (**A**) the forward sequencing read and (**B**) the reverse sequencing read. Both forward and reverse nucleotide sequences were translated into amino acids using SnapGene. There is no overlap between the ends of the sequences, the end of the forward sequence in Panel A continues on to the beginning of the reverse sequence in Panel B. Using the InterPro domain database, locations of protein domains were identified in the translated sequences, and labeled using SnapGene. (**C**) A simplified domain map of the full recombinant ChiC construct.

aeruginosa, it is unclear whether our recombinant plasmid would allow for the proper expression and cleavage of ChiC. Previous studies have indicated that the secreted form of ChiC did not contain the N-terminus. Therefore, this may result in the cleavage of the 5-prime 6xHis-tag, which could potentially hinder the protein purification process. Another limitation is the potential codon bias in the *Pseudomonas aeruginosa* PAO1 genome with a higher average GC content in the third codon position (17). Thus, introducing the *chiC* insert into an *E. coli* strain could potentially slow down or decrease the expression levels due to differential preference in codon usage in *E. coli*.

Conclusions We have constructed a recombinant plasmid containing the *chiC of P. aeruginosa* PAO1. Using the pET-28a vector backbone, we cloned *chiC* in frame with the N- and C-terminal 6xHis-tags to facilitate downstream purification steps. We transformed this plasmid into *E. coli* strain BL21-(DE3) cells. Restriction digestion analysis and Sanger sequencing was used to confirm the plasmid. A protein sequence similarity of 100% was found between *chiC* and the reference *chiC* amino acid sequence in *P. aeruginosa* PAO1. The plasmid has been named pRMGS22.

Future Directions Whether the recombinant ChiC protein product has insecticidal enzymatic activity has yet to be determined. In order to address this, future studies could perform purification and testing for the enzymatic activity of ChiC. First, *E. coli* strain BL21-(DE3) cells containing pRMGS22 can be inoculated, grown, and induced for ChiC expression using isopropyl β-D-1-thiogalactoside (IPTG). The overnight culture can then be divided into two portions. The first portion can be lysed and run on an SDS-PAGE gel to visualize the ChiC protein band (~55 kDa). The second portion can be used to perform the M9-chitin agar diffusion test to examine the zones of inhibition on the chitin-containing agar plates (18). Finally, upon confirming the presence of enzymatic activity in ChiC, IPTG induction of protein expression can be performed again, followed by isolation and purification of ChiC through His-Tag purification methods. This step may have higher chances of success due to

the fact that the primers used to amplify *chiC* retain the 6xHis-tags on both the 5-prime and 3-prime ends of the multiple cloning site within the pET-28a vector.

Upon successful isolation and purification of ChiC, further search about its potential applications can be investigated. Since ChiC proteolytic activity only targets chitin, it would be worthwhile to perform an insect bioassay to determine the insecticidal activity of ChiC against insect larvae. As such, these findings could provide an ecologically friendly alternative to the current pesticides in use to potentially mitigate pest-induced crop damage.

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CONTRIBUTIONS

M.P. primarily wrote the Materials and Methods section and contributed to the Introduction, Results, and Discussion sections and conducted the data analysis with S.J. S.J. primarily wrote the Results section and generated the figures, figure captions, and supplemental material as well as contributing to the Discussion section. R.H. primarily wrote the Abstract and Introduction sections and contributed to the Discussion section and assisted in generating figures. G.Y. primarily wrote the Introduction, Discussion, Conclusion and Future Directions sections. All team members were responsible for revising the paper and figures as a whole and determining the final title of the manuscript. Co-authorship credit should therefore be granted equally to all four authors.

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