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Cloning *chiC* from insecticidal *Pseudomonas aeruginosa* PAO1

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SUMMARY Chitinolytic microorganisms display potent insecticidal abilities, which has garnered significant attention in the agricultural industry due to the numerous challenges surrounding pest management. Chitin-degrading bacteria have stood out as a promising and sustainable biocontrol agent, which has motivated research efforts to understand the mechanisms that underlie bacterial insecticidal capabilities. Some bacteria can produce chitinase enzymes, which degrade chitin present in the exoskeletons of insects, ultimately leading to insect lethality. It has been recently discovered that *chiC*, the gene that encodes for the chitinolytic ChiC enzyme, is found in highly insecticidal species, including several pseudomonads. This includes *Pseudomonas aeruginosa* PAO1, which can secrete ChiC into the extracellular environment. In this study, we aimed to bioinformatically analyze ChiC and clone *chiC* from *P. aeruginosa* PAO1 into a plasmid vector. PCR was used to amplify *chiC* from the genome of *P. aeruginosa* PAO1. The PCR product was subcloned into vector pCR2.1, resulting in plasmid pGKMS21. Several clones of pGKMS21 were analyzed by Sanger sequencing and the presence of the full-length *chiC* gene was confirmed. Sequence alignment of the sequenced region from pGKMS21 showed that it had close to 100% identity with the *chiC* (NC_002516.2) from *P. aeruginosa* PAO1. The pGKMS21 construct serves as a foundational tool that can be used in future studies to isolate *chiC* for protein expression and purification studies, allowing for further in-depth characterization of ChiC.

INTRODUCTION

Chitin is the second most abundant polysaccharide found in nature and is known to serve as a primary component in crustacean shells, fungal and algal cell walls, and the exoskeleton of insects (1). Chitin recycling, a critical ecological process that prevents chitin accumulation in the environment, is largely mediated by microorganisms that possess chitin-degrading abilities (1). Bacteria capable of chitin degradation are phylogenetically diverse and include *Bacillus*, *Flavobacterium*, *Vibrio*, and *Pseudomonas* (1, 2). Chitinolytic microorganisms are an active area of research, as some bacteria possess chitin-degrading abilities potent enough to lead to insecticidal activity, bringing promising applicability to the widespread and ongoing issue of pest management in agriculture. Insects cause substantial crop damage and are vectors for disease (3). Conventionally-used chemical insecticides have toxic effects on wildlife, humans, and the environment, which has led scientific and agricultural communities to investigate other strategies for pest control (4). Microorganisms as biocontrol agents have stood out as a promising alternative over others; they present advantages such as nontoxicity to plants and humans, as well as exceptional specificity in their insecticidal activities, such that targeted insects are harmed and beneficial insects are not (3). Of particular interest are species belonging to the *Pseudomonas* genus, which are pathogenic towards insects and can colonize diverse environments (3, 5). As such, much research has attempted to uncover the bacterial mechanisms underlying chitin degradation and insecticidal ability of pseudomonads.

Chitin degradation is an intricate process that involves various proteins, enzymes, components, and regulatory mechanisms (6). Chitinolytic bacteria produce vast amounts of chitinases, which are enzymes central to their ability to degrade chitin (7). Different bacteria possess numerous and differing types of chitinases that act synergistically to catabolize chitin, weakening and degrading the chitin present in the exoskeletons of insects (8, 9). Examples of types of chitinases include ChiA, ChiB, ChiC, and ChiD (1). Given the key role of chitinases

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in bacterial chitin degradation, it may be beneficial to investigate the characteristics and mechanisms of specific types of chitinases.

chiC is a gene found exclusively in species that exhibit high degrees of insecticidal activity (6). *chiC* encodes for chitinase C (ChiC), a secreted chitinolytic enzyme, and is present in the genome of many species belonging to the *Pseudomonas* genus, including *Pseudomonas aeruginosa* strain PAO1 (1, 6). *P. aeruginosa* is a well-studied opportunistic human pathogen, with some strains capable of infecting and killing insects (10, 11). A quorum-sensing system and biofilm formation ability are two virulence traits known to contribute to insecticidal activity in *P. aeruginosa* (3). However, the ChiC enzyme, known to be a significant contributor to the insecticidal ability of other pseudomonads, has not been well-researched in *P. aeruginosa*.

ChiC is a stable secreted multi-domain enzyme that is involved in carbohydrate breakdown. *P. aeruginosa* PAO1 ChiC is a 55-kDa secreted chitinolytic enzyme, composed of 483 amino acid residues, that degrades chitin by randomly cleaving it at internal sites (1, 12). ChiC plays a role in carbohydrate metabolic processes as it has carbohydrate binding and hydrolase activity, hydrolyzing O-glycosyl compounds (UniProtKB: Q9I1H5). The protein is composed of three domains: a glycoside hydrolase family 18 (GH18) domain (amino acids 27-336), a fibronectin type-III domain (amino acids 343-430), and a carbohydrate-binding module family 5/12 (amino acids 436-482) (Fig. 1). The GH18 domain has catalytic properties as this domain is responsible for the hydrolytic cleavage of the beta-1,4-bond within chitin, releasing oligomeric, dimeric, or monomeric products (Interpro: IPR001223). Embedded within this domain is the active site of ChiC (amino acids 135-143) (InterPro: IPR001579). The fibronectin type-III domain has protein binding abilities and is where chitinases, including ChiC, interact with other proteins or enzymes (InterPro: IPR003961). The carbohydrate-binding module family 5/12 domain has carbohydrate-binding and O-glycosyl hydrolase activity, which also allows binding and degradation of glucose-derived chitin (InterPro: IPR003610).

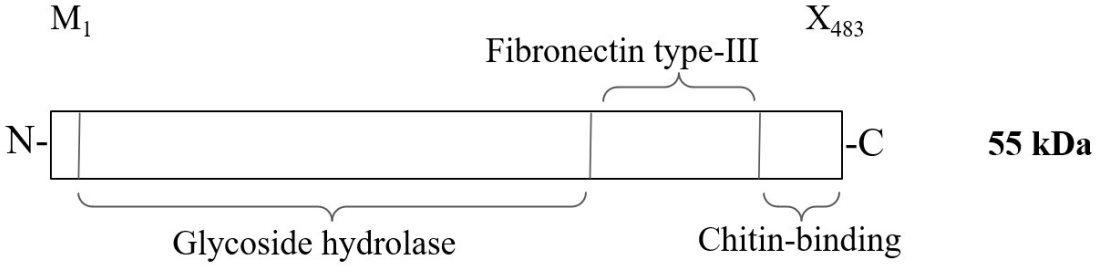

FIG. 1 Protein domain map of ChiC. ChiC consists of three domains: a glycoside hydrolase domain containing the active site for chitin catabolism, a fibronectin type-III domain allowing for ChiC to bind to other proteins, and a chitin-binding domain for binding of glucose-derived chitin.

ChiC is known to accumulate in the cytoplasm of *P. aeruginosa* upon expression, and gradually gets secreted into the extracellular space after long periods of cell growth (1). Extracellular ChiC is shorter than intracellular ChiC by 11 amino acids at the N-terminus, but the residues absent in extracellular ChiC do not resemble any classical signal peptides (1). Additionally, ChiC secretion does not appear to be mediated through type I-III secretion systems (1). Beyond this, comprehensive research on *P. aeruginosa* ChiC and its contributory role in bacterial insecticidal ability is limited.

In this study, we analyzed the *chiC* gene and ChiC protein through the use of bioinformatic tools and constructed a recombinant vector pGKMS21, which contains a pCR2.1 backbone plus a DNA insert containing *chiC*. We hypothesized that *chiC* can be isolated from *P. aeruginosa* PAO1 genomic DNA, amplified, and subsequently cloned into a plasmid vector.

METHODS AND MATERIALS

Bioinformatic analysis of ChiC. All bioinformatic analyses of the *chiC* gene and the predicted ChiC protein were completed using the NCBI reference sequence NC_002516.2 (region c2531840-2530389; gene ID 878587) and NP_250990.1, respectively. Custom primers used for the PCR protocols and Sanger sequencing in this study were designed after analysis of the *P. aeruginosa* PAO1 *chiC* locus using SnapGene (version 6.0). Information related to the protein was obtained through entering the amino acid sequence into the SignalP 5.0 server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) and the ProtParam tool (https://web.expasy.org/protparam/) from Expasy (Swiss Bioinformatics Resource Portal), and browsing databases (e.g. InterPro) linked to the ChiC (ID: Q9I1H5) entry in the UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org/uniprot/Q9I1H5).

Preparation of experimental materials. Stock kanamycin (25 mg/mL), 50 mM $MgSO_4$, 50X TAE, 6X DNA loading dye, LB (tryptone, yeast extract, NaCl, and distilled water) broth and LB agar were prepared following standard protocols. Stock kanamycin and $MgSO_4$ were sterilized by filtering the reagents through 0.2 µm pores. LB media was sterilized by autoclaving. A portion of the media was subsequently used in the preparation of selective media with 50 µg/mL kanamycin only and kanamycin plus X-gal. The following materials were obtained from external sources: *P. aeruginosa* genomic DNA (Hancock Lab at the University of British Columbia) and custom forward (5'-CGCGCCTGTTTTTTCTAAGCG-3') and reverse (5'-AACGCATATGGATATGGCGCCAC-3') primers (Integrated DNA Technologies Inc.) designed to amplify a 1.6 kb *chiC*-containing fragment from *P. aeruginosa* PAO1 genomic DNA by PCR.

Amplification of *chiC*-containing DNA fragments using Pfx DNA Polymerase. Amplification of a 1.6 kb *chiC*-containing fragment from *P. aeruginosa* PAO1 genomic DNA was conducted in 200-µL PCR tubes with a final reaction volume of 50 µL. The reaction mixture included 0.3 mM of each dNTP, 1 mM $MgSO_4$, 0.3 µM of each primer, 1X Pfx Amplification buffer (Invitrogen), 0.2X PCR_x Enhancer Solution (Invitrogen), 1U Platinum Pfx DNA Polymerase (Invitrogen), 45 ng *P. aeruginosa* PAO1 genomic DNA, and autoclaved distilled water. The thermal cycler was set to denature the template DNA for 5 minutes at 94°C before performing 35 cycles of PCR amplification, with denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 100 seconds, and termination at 68°C for 5 minutes. The lid temperature of the thermal cycler was set to 105°C to prevent condensation. PCR products were stored at -20°C until electrophoresis of the samples were performed.

Agarose gel electrophoresis of PCR products. Agarose gels (1%) were prepared using agarose, 1X TAE and 1X SYBR Safe stain to run gel electrophoresis on PCR products. 1X TAE buffer was added to the gel electrophoresis apparatus until the gel was fully submerged. 15 µL of PCR products and 1 µL 1 Kb Plus DNA ladder (Invitrogen) were mixed with 6X DNA loading dye, loaded into separate wells of the gel, and ran at 100 volts. The bands on the gel were visualized using BioRad ChemiDoc Imaging System. When the gel was used to separate and purify PCR products, bands corresponding to the desired DNA fragments were extracted using a sterile scalpel and samples were purified using PureLink™ Quick Gel Extraction Kit (Invitrogen).

Amplification of *chiC*-containing DNA fragments using Taq DNA Polymerase. A second round of PCR was performed to increase the concentration of *chiC*-containing DNA and to add A-overhangs to the 3' ends of the blunt-ended 1.6 kb fragments from the first PCR experiment. The 50 µL reaction mixtures included 0.2 mM of each dNTP, 1.5 mM $MgSO_4$, 0.3 µM of each primer, 1X PCR buffer (Invitrogen), 1% or 5% dimethylsulfoxide (DMSO), 1U Taq DNA Polymerase (Invitrogen), 5 µL of 1.6 kb *chiC*-containing fragment, and autoclaved distilled water. The thermal cycler was set to denature the template DNA for 5 minutes at 94°C before performing 35 cycles of PCR amplification, with denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 100 seconds, and

termination at 72°C for 10 minutes. The lid temperature of the thermal cycler was set to 105°C.

A final round of PCR was conducted, following the same protocol but using the 1.6 kb product with 3'A-overhangs as a template, to increase the concentration of the target 1.6 kb DNA fragment. After confirming the fragment size through electrophoresis, final PCR products were purified using PureLink™ Quick PCR Purification Kit (Invitrogen) prior to plasmid construction.

Construction of pGKMS21. Linearized pCR2.1 was obtained from The Original TA Cloning Kit with pCR2.1 vector (Invitrogen) and 10-µL ligation reactions were performed to construct pGKMS21 (pCR2.1 + 1.6 kb *chiC*-containing fragment) with two purified PCR products (amplified using 1% DMSO or 5% DMSO), at two different vector-to-insert ratios (based on fmol of DNA, 1:1 and 1:3). The reactions included purified 1.6 kb *chiC*-containing fragments (21 ng for 1:1 ratio, 62 ng for 1:3 ratio), 50 ng linear pCR2.1 vector, 1X T4 DNA Ligase Reaction Buffer, 5U ExpressLink T4 DNA Ligase, and sterile water. The reactions were incubated at room temperature for a total of two hours. Ligation products were stored at -20ºC until needed for transformation.

Transformation and screening of One Shot TOP10 competent *E. coli* with pGKMS21. One Shot TOP10 competent *E. coli* cells (50 µL) were incubated with either sterile water (negative control), kanamycin resistance-encoding pET-28a (positive control), or 2 µL of the pGKMS21 ligation mixture on ice for 30 minutes. Following incubation, the cells were heat-shocked for 30 seconds at 42ºC. 250 µL of SOC media was added to each tube and the samples were incubated at 37ºC with shaking for one hour to allow for recovery. 50 µL or 200 µL of the samples were spread plated on LB + kanamycin + X-Gal plates for blue-white screening. Inoculated plates were incubated overnight at 37ºC. The plates were transferred to 4ºC for 2-3 hours before white colonies were selected and streaked onto fresh selective plates. Streaked plates were incubated at 37ºC overnight and stored at 4ºC once sufficient growth was observed.

Isolation and sequence verification of pGKMS21 from transformed One Shot TOP10 cells. Overnight cultures for the selected transformed colonies were prepared in selective media (sterile LB with 50 µg/mL kanamycin) and were incubated in glass test tubes at 37ºC with shaking at 225 rpm. Invitrogen PureLink™ Quick Plasmid MiniPrep Kit was used to isolate the pGKMS21 plasmid from the overnight cultures. The concentrations of isolated plasmids were measured using the NanoDrop spectrophotometer. Each isolated plasmid sample was sequenced using the universal T7 promoter primer, the custom forward primer, and the custom reverse primer. Samples were stored at 4ºC until they were packaged and shipped out for sequencing at the external facility. Sequencing results were aligned to the *P. aeruginosa* PAO1 genome (accession: NC_002516.2) through NCBI BLAST to confirm the presence of the *chiC* insert.

RESULTS

***P. aeruginosa* PAO1 ChiC is a stable protein with an unknown secretion mechanism.** To determine the biochemical properties of ChiC, the amino acid sequence of *P. aeruginosa* PAO1 ChiC was used for analysis in ProtParam. The protein was found to have a theoretical isoelectric point of 5.22 and an amino acid composition dominated by alanine (12.4%), glycine (9.9%), and leucine (9.1%), with the remaining amino acids ranging from 0.0-6.0%. Based on this amino acid composition, ChiC has an extinction coefficient of 96260 M^{-1} cm^{-1}, at 280 nm measured in water. ChiC is classified as a stable protein, carrying an instability index of 36.43. Its half-life was estimated to be 10-20 hours *in vivo* and 30 hours *in vitro*. These findings suggested that ChiC has the potential to be studied *in vitro*, thus cloning *chiC* into a plasmid vector in this study may be a feasible technique to facilitate further research on ChiC.

Additionally, SignalP 5.0 predictions based on the full amino acid sequence showed the lack of a signal peptide in *P. aeruginosa* PAO1 ChiC (Supplemental Fig 1). This suggested that the cleaved N-terminus of ChiC is likely not a known signal peptide that facilitates

transport of the protein out of the cell. Since the protein was not secreted through type I-III secretion systems (1), the mechanism of ChiC secretion remains unknown.

A 1.6 kb DNA fragment was amplified from *P. aeruginosa* genomic DNA. To clone the full 1.4 kb *chiC* gene into a plasmid, custom primers were needed to amplify the gene from the *P. aeruginosa* PAO1 genome using PCR. Since the 5' and 3' termini of the *chiC* gene have different GC content, primers that anneal directly to these regions showed large melting temperature differences (e.g. forward primer 5'-GAAATGATCAGGATCGACTTTTCCC-3' had GC content of 44% and a melting temperature of 58°C; reverse primer 5'-AAGCGCAGCGGCCGCCAGAG-3' had GC content of 75% and melting temperature of 70°C; the melting temperature difference was 12°C) which may result in difficulties in setting an optimal annealing temperature during PCR. To ensure successful amplification of the full *chiC* gene, this study used primers that were designed to anneal at regions flanking the gene, where GC content is similar, at 52% (melting temperatures were at 61°C for the forward primer and 62°C for the reverse primer). The initial PCR experiment that used *P. aeruginosa* PAO1 genomic DNA as template resulted in the amplification of a blunt-ended 1.6 kb fragment which was subsequently used as the template in another PCR experiment for the purpose of increasing the concentration of the fragment and adding A-overhangs on the 3' ends of the DNA (Fig 2). The 1.6 kb fragment with 3' A-overhangs was then used as the template in a final PCR experiment to increase its concentration. The final gel electrophoresis

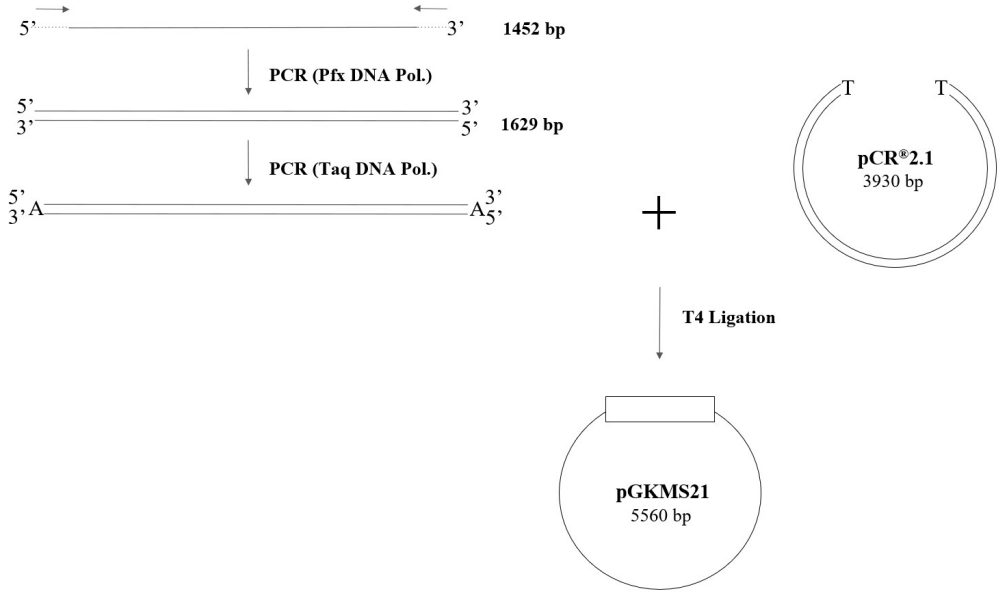

FIG. 2 Illustration of the steps leading to construction of pGKMS21. PCR amplification of *chiC* along with flanking nucleotides upstream and downstream of the gene in *P. aeruginosa* PAO1 genomic DNA resulted in a 1.6 kb fragment, which underwent additional PCR amplification to generate 3' A-overhangs using Taq DNA polymerase. Ligation of the resulting PCR product with linearized pCR2.1 vector yielded pGKMS21.

results showed no visible bands for the negative control reaction, which did not include template DNA (Fig. 3). As expected, a faint 200 bp band was observed, which represented amplification of the pUC19 positive control (Fig. 3). Of the four reactions using the 1.6 kb *chiC*-containing template, two resulted in successful amplification, as indicated by two lanes each containing distinct 1.6 kb bands (Fig. 3). The PCR mixtures used 1% and 5% DMSO, with two replicates (R1 and R2) for each of the DMSO concentrations. Interestingly, although the replicates for each DMSO concentration contained identical PCR components, only the PCR products from the second replicate (R2) of the 1% DMSO reaction and the first replicate (R1) of the 5% DMSO reaction resulted in a visible 1.6 kb band (Fig. 3). Of note, the 1.6 kb band in the 5% DMSO lane was observed to be thicker and of greater intensity than in the 1% DMSO lane. Smeared bands directly below the distinct 1.6 kb band were also apparent in

both the 1% and 5% DMSO lanes, which suggested the presence of contaminants. Nonetheless, the two samples that showed bands corresponding to 1.6 kb were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen) and used for plasmid construction.

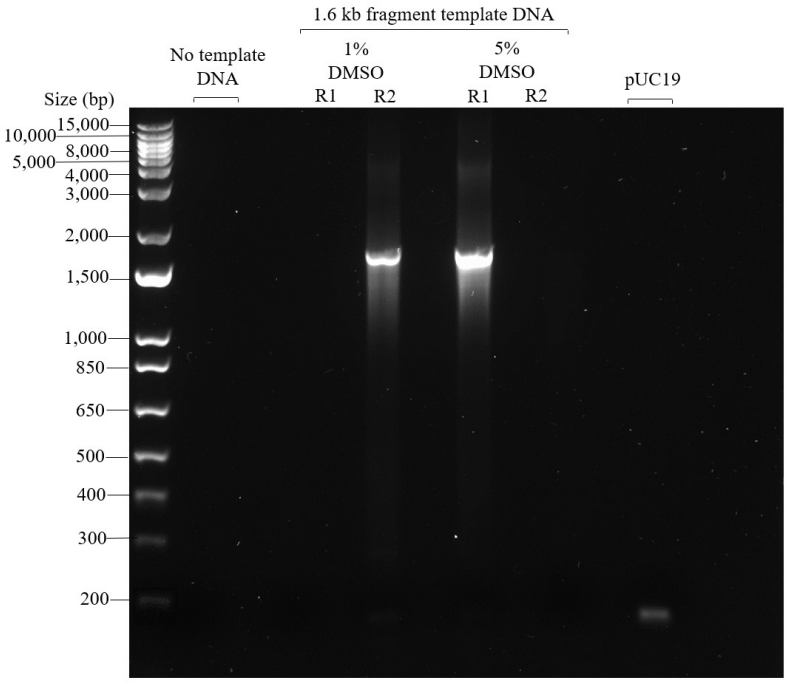

FIG. 3 Agarose gel electrophoresis indicated the presence of a 1.6 kb band fragment containing *chiC* with 3' A-overhangs. The purified 1.6 kb fragment, amplified from *P. aeruginosa* PAO1 genomic DNA, served as the template DNA and was mixed with 6X DNA loading dye and loaded into the wells of a 1% agarose gel stained with 1X SYBR Safe stain. The gel was run in a 1X TAE buffer for 125 minutes at 100 volts. A 1.6 kb band was observed in the lane representing the second replicate (R2) of the 1% DMSO PCR products, as well as in the lane representing the first replicate (R1) of the 5% DMSO PCR products. A faint 200 bp band was visible for the positive control (pUC19 plasmid) and no band was visible for the negative control (no template DNA).

One Shot TOP10 cells acquired the recombinant plasmid pGKMS21 after transformation. To determine whether pGKMS21 plasmid construction was successful, One Shot TOP10 *E. coli* cells were transformed with pGKMS21 ligation products, and the resulting transformants were incubated overnight on blue-white screening media plates. The use of blue-white screening allowed both the identification of colonies of cells that had successfully acquired plasmid and distinguished cells that acquired plasmids with an insert from cells that acquired plasmids without an insert. The negative control plate (cells transformed with water) showed no cell growth and the positive control plate (cells transformed with circularized pET-28a plasmid) was covered with hundreds of white bacterial colonies. Minimal growth (less than three colonies) was observed on plates with 50 µL of transformed cells. Plates with 200 µL of transformed cells showed slightly higher quantities of both blue and white colonies which allowed further analysis. Cells transformed with pGKMS21 ligation mixture with 1% DMSO PCR product showed a higher overall fraction of white colonies, at 5 out of 7 colonies (71% white) for 1:1 ligation and 6 out of 11 colonies (54% white) for 1:3 ligation. Cells transformed with pGKMS21 ligation mixture with 5% DMSO PCR product showed 6 out of 11 colonies (74% white) for 1:3 ligation and 2 out of 11 colonies (18% white) for 1:1 ligation. The presence of colonies indicated growth of cells that successfully acquired either recircularized pCR2.1 or pGKMS21, which both encode kanamycin resistance. Blue colonies corresponded to cells that acquired a recircularized pCR2.1 plasmid without the 1.6 kb insert. White colonies corresponded to cells that most likely acquired the pGKMS21 plasmid with an insert that disrupted the *lacZα* gene (Fig. 4), which prevented the expression of β-galactosidase and metabolism of X-gal to produce blue pigment. The presence of white colonies resulting from transformation using all four ligation mixtures suggested that the competent One Shot TOP10 cells successfully acquired the recombinant pGKMS21 plasmid.

Nucleotide sequence of pGKMS21 indicated the presence of *chiC*. To verify the identity of the plasmid insert and confirm the presence of *chiC*, selected transformed colonies were propagated in selective media and plasmids were isolated and sequenced. Three out of four samples (nine out of the twelve sequencing reactions) returned valid results with acceptable

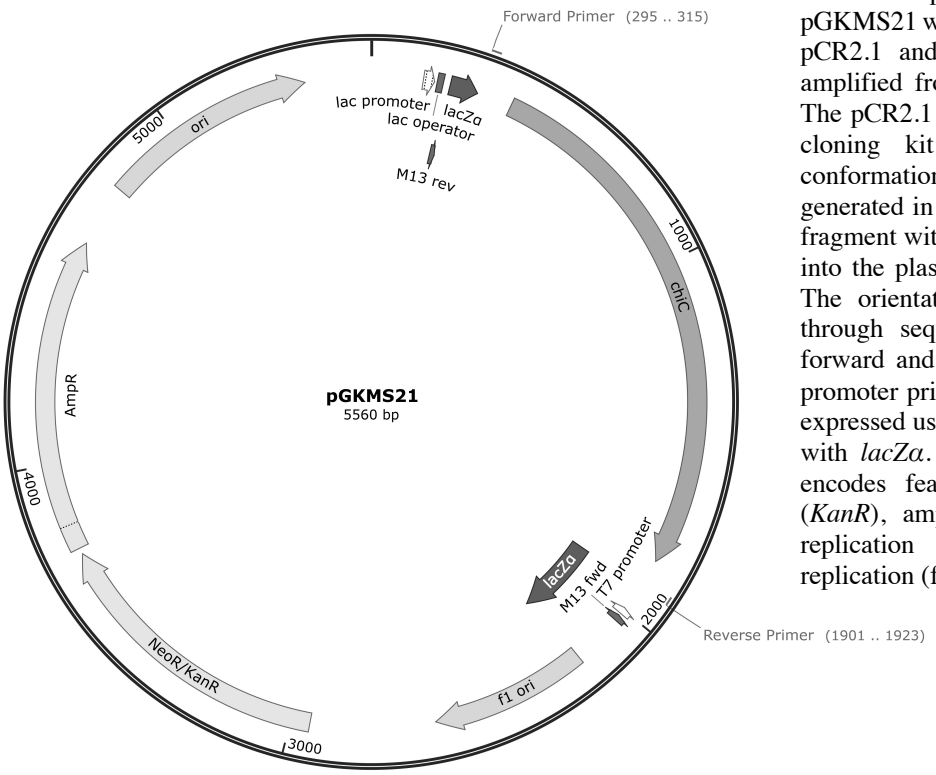

FIG. 4 Map of recombinant pGKMS21 plasmid. pGKMS21 was constructed through ligation of linear pCR2.1 and a 1.6 kb *chiC*-containing fragment amplified from the *P. aeruginosa* PAO1 genome. The pCR2.1 vector was provided as part of the TA cloning kit from Invitrogen in a linearized conformation with the multiple cloning site generated in the *lacZα* gene. Ligation of the 1.6 kb fragment with pCR2.1 allowed the insertion of *chiC* into the plasmid, downstream of the *lac* promoter. The orientation of the gene insert was verified through sequencing the plasmid using indicated forward and reverse primers and the universal T7 promoter primer. The inserted *chiC* gene cannot be expressed using the T7 promoter and is not in frame with *lacZα*. The pCR2.1 backbone of pGKMS21 encodes features such as kanamycin resistance (*KanR*), ampicillin resistance (*AmpR*), origin of replication (*ori*), and phage-derived origin of replication (f1 *ori*).

base qualities when sequenced with custom forward and reverse primers and universal T7 promoter primer. However, the plasmid sample isolated from cells transformed with the 1:3 ligation mixture with 5% DMSO PCR product did not result in priming in all three sequencing experiments. An issue with the isolation of plasmid from the cell was unlikely since all plasmid samples were isolated simultaneously using the same materials and procedure. It was possible that the corresponding selected colony was a contaminant species that had a kanamycin resistance-encoding plasmid which allowed it to grow a white colony on the blue-white screening plate following the spread-plating procedure in the transformation experiment.

The nucleotide sequences from the three sequenced samples were aligned to the *P. aeruginosa* PAO1 genome (accession: NC_002516.2) through NCBI BLASTn. Analysis of the results revealed that the two plasmid samples isolated from cells transformed with ligation mixture with 1% DMSO PCR products had very high sequence homology to published *chiC* (Supplemental Fig. 2). Percentage similarity values ranged from 97.9% to 99.6%, and averaged to 98.8%. Since all of the mismatches occurred within regions near the end of the sequences (Supplemental Fig. 2), the observed base mismatches were most likely the result of poor base calls due to nucleotide degradation at the end of the sequencing run. Alignment of the forward and reverse sequencing results showed alignment to both ends of the *chiC* gene, as well as overlap with one another towards the middle of the gene, which together confirm the presence of *chiC* in full (Supplemental Fig. 2). The plasmid isolated from cells transformed with ligation mixture with 5% DMSO PCR products did not show any alignment with *chiC* or with the *P. aeruginosa* PAO1 genome; BLAST results indicated alignment to an unrelated species, *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) strain sigC, as well as various expression vectors. Since *C. pseudotuberculosis* is a bacterium more commonly found in horses and sheep (13), contamination of transformed colonies with this species in the lab was unlikely. Alignment of the sequences for our sample to the vector sequence database through the VecScreen option in BLAST revealed a 100% match with the pCR2.1-TOPO vector. This may suggest that the plasmid constructed using the 5% DMSO PCR product likely had a small non-*chiC* insertion at the multiple cloning site during the ligation experiment. This small insertion caused disruption of the *lacZα* gene and resulted in

the growth of white colonies that were selected for plasmid isolation. The ligation of a small non-*chiC* fragment with the pCR2.1 vector was possible since the insert fragment was supplied in the ligation reaction in the form of column-purified PCR products. As seen in Fig. 3, final PCR amplification of the 1.6 kb fragment with 3'A-overhangs resulted in bands with smears which indicated possible non-specific amplification or contamination. Since the PCR product was purified using a PCR purification kit that was designed to remove primers, dNTPs, salts, and enzymes, small amounts of PCR products with undesired sizes (e.g. smaller sizes) may have remained in the sample after purification and may have ligated with the pCR2.1 vector by chance to produce a plasmid without a *chiC* insert. These observations suggested that only the two colonies of cells transformed with ligation mixture containing 1% DMSO PCR products acquired *chiC*-containing pGKMS21.

DISCUSSION

In this study, we sought to construct a plasmid vector that contains the full *P. aeruginosa* PAO1 *chiC* gene to allow for further research on the gene and its encoded protein. We amplified a 1.6 kb *chiC*-containing DNA fragment with 3'A-overhangs from *P. aeruginosa* PAO1 genomic DNA. The purified fragments were ligated with pCR2.1, generating pGKMS21 which was propagated in One Shot TOP10 *E. coli* cells following transformation. Plasmids isolated from selected transformants were sequenced and the results confirmed that pGKMS21 contained full-length *chiC* that aligned with *chiC* from *P. aeruginosa* (NC_002516.2) at approximately 99% nucleotide identity.

The pGKMS21 plasmid samples containing *chiC* that showed high sequence similarity to the reference *chiC* in the *P. aeruginosa* PAO1 genome were confirmed to have obtained the full length of the *chiC* gene as indicated by the alignment of all three sequencing reactions which covered both ends of the gene and overlapped in the middle of the gene. Additionally, the potential to produce the full-length ChiC protein by expressing *chiC* from the two pGKMS21 plasmids was simulated using the Expasy Translate tool (https://web.expasy.org/translate/). The amino acid sequence produced from the forward primer sequencing reaction showed that the starting amino acid sequence translated from *chiC* in pGKMS21 was the same as the reference ChiC (MIRID). Furthermore, the amino acid sequence resulting from the reverse primer sequencing reactions showed that the terminating amino acid sequence translated from *chiC* in pGKMS21 was also the same as the reference ChiC (WRPLR). However, it is important to note that there were residue mismatches between the forward and reverse sequences at the center of the amino acid chain. One possible explanation for this observation was that the low fidelity of Taq DNA polymerase, which was estimated 4.4×10^{-5} errors/base/cycle (14), resulted in base-pairing errors during PCR amplification of *chiC*. Another causal factor may be base-call errors during Sanger sequencing as base qualities decreased with the occurrence of indifferentiable peaks towards the end of the sequencing reaction. The latter explanation was more likely in this case since the nucleotide sequences generated from the three sequencing reactions (per sample) showed mismatches with the reference *chiC* gene only near the end of the sequences (center of the gene). Furthermore, the locations of mismatches were different for each sequence despite being in the region of overlap between the forward and reverse sequencing results. For example, within the region of overlap, where there is a nucleotide mismatch between the forward sequence and the reference, the reverse sequence nucleotide at the same location matches with the reference (Supplemental Fig.2).

The construction of *chiC*-containing pGKMS21 in this study demonstrated that *chiC* can be amplified from the *P. aeruginosa* PAO1 genome and inserted into a plasmid vector which allows transfer of genomic material from insecticidal *Pseudomonas* to a non-*Pseudomonas* species. However, the final pGKMS21 plasmid cannot be used for direct expression of ChiC when introduced to a new host. Since the 1.6 kb fragment that contained *chiC* was ligated with linear pCR2.1 through the pairing of compatible TA overhangs during plasmid construction, the DNA insert had the potential to ligate in one of two orientations: *chiC* would either be in the forward or reverse direction relative to the T7 promoter. Comparison of the plasmid sequence alignments showed that the sequencing reactions of the universal T7 promoter primer and the custom reverse primer covered the same region of *chiC* (Supplemental Fig. 2). This suggested that the T7 promoter was downstream of *chiC* and the

gene was inserted in the reverse direction relative to the T7 promoter (Fig. 4). In this orientation, *chiC* cannot be transcribed using the T7 promoter nor can it be transcribed using the *lac* promoter since it is not in frame with *lacZα*. pGKMS21 then cannot be used as a *chiC* expression vector but may serve as a storage and propagation vector for the gene. To investigate the feasibility of producing ChiC through a *chiC* expression vector introduced to a non-*Pseudomonas* host, future studies may amplify the *chiC* gene directly from pGKMS21 instead of *P. aeruginosa* PAO1 genomic DNA for a higher probability of *chiC* amplification and reduced non-specific amplification (Fig. 5).

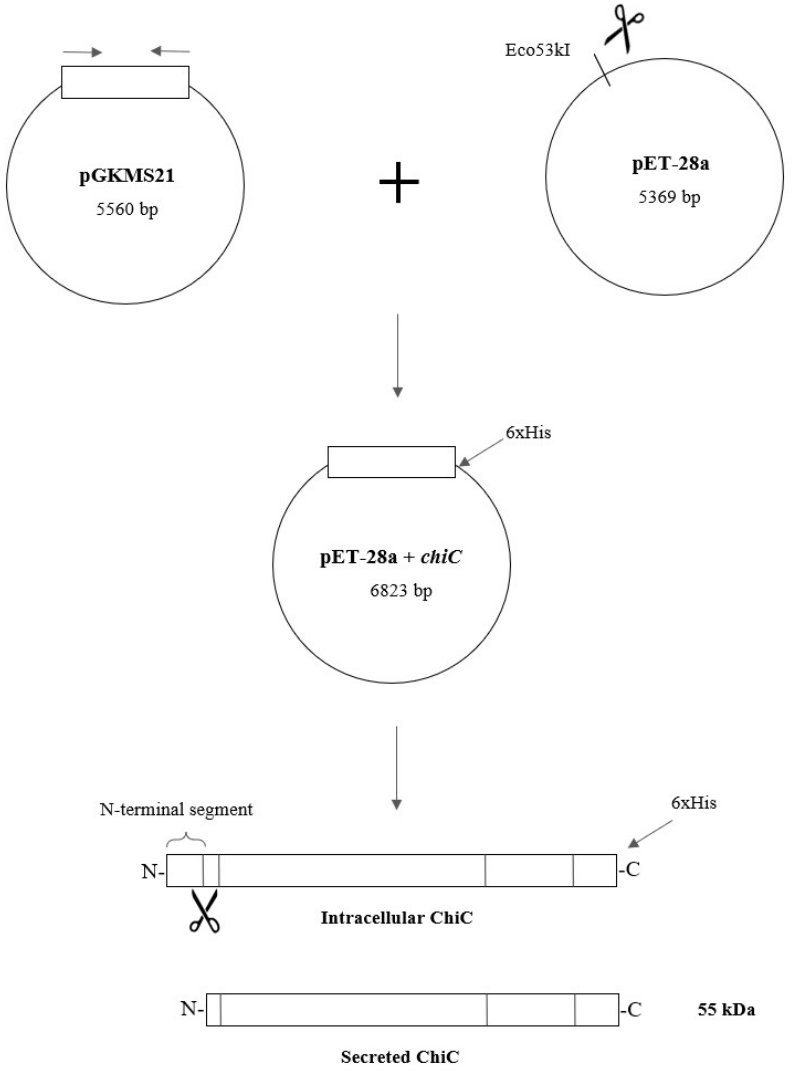

FIG. 5 Proposed approach to construct a ChiC expression vector using pGKMS21. *chiC* isolated from pGKMS21 can be blunt-end cloned into a Eco53kI-digested pET-28a plasmid with T7 promoter and 6xHis tags. The resulting recombinant plasmid can be introduced into *E. coli* BL21 (DE3) to express ChiC.

It should be noted that although previous research has confirmed that *P. aeruginosa* does indeed show insecticidal activities, the use of *P. aeruginosa* in this study does not infer a suggestion that these species be used as a biocontrol agent against insects; rather, *P. aeruginosa* PAO1 serves as a convenient, readily available bacterial model to explore the characteristics of the *chiC* gene and its encoded protein product, ChiC. Successful cloning of *chiC* and construction of pGKMS21 may have downstream applications for future research on ChiC; for instance, *chiC* isolated from pGKMS21 can be cloned into a pET expression vector, and the resulting recombinant plasmid can be introduced into a host expression system. Expressing *chiC* in a host expression system could lead to possible studies into the insecticidal activity of the host expression system.

Conclusions Our study investigated the possibility of constructing a recombinant *chiC*-containing vector using a pCR2.1 plasmid backbone. In parallel with our hypothesis, a 1.6 kb

DNA fragment containing *chiC* was isolated from *P. aeruginosa* PAO1 genomic DNA and ligated with linear pCR2.1 to form recombinant plasmid pGKMS21. Successful cloning of *chiC* into pCR2.1 was demonstrated through transformation of One Shot TOP10 *E. coli* cells with pGKMS21, and further verified through Sanger sequencing of pGKMS21. Sequence similarities close to 100% were noted between *chiC* in pGKMS21 and the previously published *chiC* sequence found in insecticidal *P. aeruginosa* PAO1.

Future Directions Although we were able to successfully clone *chiC* into a plasmid vector, it is unclear whether its protein product would still demonstrate insecticidal potential when extracted after being expressed in a non-*Pseudomonas* host. To address this question, future studies may aim to express ChiC in *E. coli* or other bacterial strains. The pGKMS21 plasmid constructed in this study does not support the direct expression of ChiC as the gene insert was not designed to be in frame with a promoter. Future studies can isolate *chiC* from pGKMS21 and insert the gene into another plasmid, such as pET-28a, in frame with the T7 promoter and 6xHis tags (Fig. 5). The resulting recombinant plasmid can be transformed into a non-insecticidal organism and ChiC can be isolated through His-Tag purification methods (such as immobilized metal affinity chromatography) following overexpression of the protein.

Successful purification of ChiC may facilitate further research on the protein. As indicated previously, the N-terminal segment of ChiC is removed when the protein is secreted extracellularly (1); however, bioinformatic analysis suggested that the N-terminus does not encode an orthodox signal peptide (Supplemental Fig. 1). The mechanism of ChiC secretion and the reason as to why the N-terminal segment is cleaved still remains unclear and, therefore, additional research on the protein can be conducted to address this knowledge gap. Furthermore, insect feeding assays may demonstrate the insecticidal properties of purified ChiC, which can be commercialized as a possible insecticide to control pests and insects.

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