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Construction of inducible expression vector pDNLS-22n containing *P. aeruginosa* PAO1 ChiC

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SUMMARY In response to concerns over traditional pesticides, biocontrol agents have been touted as safer alternatives. This has garnered attention for chitinase as a biopesticide due to its insecticidal activity. Chitinases break down glycosidic linkages in chitin, which is an important constituent of the arthropod exoskeleton. As a result, we sought to characterize the expression of *chiC* which codes for an extracellular chitinolytic enzyme ChiC. We constructed the pDNLS-22n vector to enable inducible expression of *Pseudomonas aeruginosa* PAO1 ChiC in *E. coli*. The *chiC* gene was PCR amplified from the storage vector pGKMS21 and ligated into pET-28a(+) by restriction cloning using the EcoRI and HindIII sites. Sanger sequencing of *chiC* from pDNLS-22n showed complete sequence identity to the reference gene. We thus established an inducible expression vector for ChiC with a leader peptide at the N-terminus that encodes both a 6xHis tag for protein purification and a T7 tag for detection by immunological techniques. Construction of a *chiC* expression vector enables future studies on the structure and function of ChiC and establishes a system for studying the potential implications and challenges associated with creating a viable insecticidal alternative to pesticides.

INTRODUCTION

Approximately 45% of annual food production is lost due to pests such as insects (1). Concurrent with agricultural development, pesticides are a vital tool for plant protection and for enhancing crop yield (1). Extensive use of pesticides is seen to contaminate soil, water, and remains in crops, eventually leading back to humans due to pesticide properties of bioaccumulation and toxicity (1). With chemical insecticides being increasingly linked to various health and environmental issues, a search for effective insecticidal alternatives has sparked increased interest in biocontrol measures such as chitinolytic bacteria. Therefore, developing biological insecticides to replace traditional chemicals can alleviate negative impacts on sensitive ecosystems and provide safer alternatives, while still increasing crop yields and efficiency (2).

A target biomolecule of interest for potential biocontrol measures is chitin. As an insoluble polymer of β-1,4-N-acetyl-d-glucosamine, chitin functions as a structural component in the shells of crustaceans, exoskeletons of insects, in nematodes, and is also located in the cell walls of fungi and algae (3–5).

Chitinases act to degrade chitin through random internal cleavages of glycosidic linkages, producing soluble oligosaccharides (6). Chitinolytic microorganisms partake in recycling of chitin from perished organisms and discarded biological materials whilst displaying potential insecticidal activity (3). Different bacteria, plants, and fungi possess various types of chitinases that act in combination to weaken and degrade chitin (7). Bacterial chitinases are produced to meet nutritional needs by allowing chitin to be used as carbon and nitrogen sources for cell survival (4).

Notably, chitinase C (ChiC) has been associated with several highly insecticidal species from genus *Pseudomonas*, such as *Pseudomonas aeruginosa* PAO1 (8). This gene encodes for a 483 amino acid polypeptide which contains a chitin-binding domain as well as a chitinolytic activity domain, separated by a fibronectin type III domain (3).

In one study, *chiC* from *Streptomyces peucetius* was cloned into plasmid pQEChiC (8). Following pQEChiC transformation into *Escherichia coli* M15 pREP4, the formation of inclusion bodies were observed with minimal secreted levels of ChiC (8). Renatured ChiC

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isolated from the inclusion bodies displayed measurable enzymatic activity (8). Previous groups have expressed a need for further elucidating *P. aeruginosa* ChiC and its relative contribution to bacterial insecticidal ability, due to the limited pool of knowledge available (5, 8). One study created an expression vector for *Pseudomonas* derived chiC to test in a non-*Pseudomonas* host (7). Although Bodykevich *et al.* were successful in cloning *chiC* into a plasmid vector to produce plasmid pGKMS21, it did not support the expression of *chiC* (7). The *chiC* gene was ligated into the pCR2.1 vector in the reverse orientation relative to T7 promoter (7).

In this study, we cloned a functional expression vector of *P. aeruginosa* PAO1 derived *chiC* isolated from pGKMS21 plasmid. We began with amplifying *chiC* from pGKMS21 plasmid using PCR. Purified *chiC* and pET-28a(+) were digested with restriction enzymes and ligated to produce clones of pDNLS-22n. pDNLS-22n was sequenced via Sanger sequencing to confirm the presence and fidelity of *chiC.* We hypothesize that insertion of *chiC* from pGKMS21 into the pET-28a(+) plasmid by restriction cloning will produce a vector with the capacity for expression when transformed into *E.coli* BL21 (DE3).

METHODS AND MATERIALS

Preparation of plasmids and competent *E. coli* cells. TOP10 *E. coli* containing pGKMS21 and *E. coli* DH5α containing pET-28a(+) were grown in overnight cultures of lysogeny broth (LB) with kanamycin (50 μg/mL) at 37°C with shaking. Plasmids were isolated using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic) and quantified using the NanoDrop™ spectrophotometer. *E. coli* DH5α and *E. coli* BL21 (DE3) were made chemically competent following the method described by Chang *et al.* (9). Competent cells were stored in 0.1M $CaCl_2$ with 15% v/v glycerol at -70°C until downstream transformation.

PCR amplification of *chiC* from pGKMS21. 100 ng of pGKMS21 template DNA were used per 50 μL PCR reaction containing 1U Platinum™ SuperFi™ DNA Polymerase, 1X SuperFi™ Buffer, 1X SuperFi™ GC Enhancer, 0.2 mM of each dNTP, 0.5 μM of forward and reverse primers, and nuclease-free water. 2eta-chiC forward and reverse primers contained EcoRI and HindIII restriction sites respectively to produce a 1474 bp *chiC* PCR product for restriction cloning (Integrated DNA Technologies, Inc). A positive control was established using the R1F/R1R primers from Bodykevich *et al.* to produce a 1629 bp product containing *chiC* (7). Nuclease-free water was used in the no template control. The thermal cycler was set as follows for the negative control and pGKMS21 DNA with the 2eta-chiC primers: initial denaturation at 98°C for 30 seconds, 35 cycles of PCR amplification, with denaturation at 98°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 35 seconds, followed by a final extension at 72°C for 5 minutes. For the positive control, the thermal cycler settings were the same except that annealing was conducted at 61°C for 10 seconds. PCR products were analyzed by agarose gel electrophoresis and purified using PureLink™ PCR Purification Kit (Invitrogen™).

Agarose gel electrophoresis of PCR products and restriction digested DNA. 1 % agarose gels were prepared using 1X tris acetate EDTA (TAE) buffer and RedSafe™ nucleic acid staining solution (FroggaBio). 1μL of 100 bp DNA Ladder (Invitrogen™) and 1 Kb Plus DNA Ladder (Invitrogen ™) were mixed with 10X BlueJuice Gel Loading Buffer (Invitrogen ™) to determine the size of DNA fragments. PCR products were also mixed with 10X BlueJuice Gel Loading Buffer, while restriction digested DNA were mixed with 6X Purple Gel Loading Dye (New England BioLabs Inc.) containing sodium dodecyl sulfate (SDS). Equal mass of DNA samples were loaded into sample wells and run at 120V, and visualized on the BioRad ChemiDoc Imaging System.

Restriction digest of *chiC* insert and pET-28a(+) vector. 1 μg of purified *chiC* PCR product and pET-28a(+) plasmid were digested in separate 50 μL reactions containing 1 μL (20 units) of EcoRI-HF ® (New England BioLabs Inc.), 1 μL (20 units) of HindIII-HF® (New England BioLabs Inc.), 1X rCutSmart ™ Buffer (New England BioLabs Inc.), and nuclease-free water for 20 minutes at 37 °C. Undigested and single-digested pET-28a(+) plasmids served as

controls. The restriction enzymes were heat inactivated at 80°C for 20 minutes. The digested DNA was visualized by agarose gel electrophoresis as previously described, and purified using the GeneJET PCR purification kit (Thermo Scientific™).

Ligation and transformation of pDNLS-22n into *E. coli* DH5α. A 3:1 molar ratio of digested *chiC* and pET-28a(+) were ligated in 10 µL reactions containing 1X T4 DNA Ligase Buffer (Thermo Scientific™) , 1 Unit T4 DNA Ligase (Invitrogen™) and nuclease-free water for 1 hour at room temperature. A negative control without the *chiC* insert controlled for background levels of undigested pET-28a(+). The ligation mixtures were transformed into chemically competent *E. coli* DH5α by heat shock as described by Chang *et al.* (9) and grown overnight on LB agar plates with kanamycin (50 µg/mL) at 37°C to select for transformants.

Screening for pDNLS-22n transformants by colony PCR. Colony PCR was performed as previously described using 2eta-chiC primers with the addition of an initial denaturation step at 55°C. Nuclease-free water and *E. coli* DH5α colonies containing empty pET-28a(+) plasmids were used as negative PCR controls. Purified pGKMS21 plasmids were used as positive PCR controls. PCR products were analyzed by agarose gel electrophoresis.

Screening for pDNLS-22n by analytical restriction digests. Positive colonies were grown overnight in liquid LB media with kanamycin (50 µg/mL) at 37°C. Purification of pDNLS-22n was performed using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic). 0.5 µg of purified pDNLS-22n were digested in 25 µL reactions containing 10 units of EcoRI-HF ® (New England BioLabs Inc.), 10 units of HindIII-HF® (New England BioLabs Inc.), 1X rCutSmart ™ Buffer (New England BioLabs Inc.), and nuclease-free water for 20 minutes at 37°C. Purified pGKMS21 and empty pET-28a(+) vectors were also double digested for control. All digested plasmids and undigested pGKMS21 and pET-28a(+) controls were analyzed by agarose gel electrophoresis as previously described.

Sanger sequencing of pDNLS-22n. Purified pDNLS-22n plasmids from three *E. coli* DH5α transformants were sent to GENEWIZ (Azenta Life Sciences) for Sanger sequencing using the universal T7 promoter and T7 terminator primers, as well as custom forward (intF) and reverse (intR) internal primers complementary to *chiC*. Sequencing results were aligned to the pET-28a(+) vector and *P.aeruginosa* PAO1 genome (accession: NC_002516.2) using SnapGene to verify the fidelity and directionality of the *chiC* insert.

RESULTS

PCR amplification of *chiC* from pGKMS21 for restriction cloning. To sub-clone *chiC* from pGKMS21 (Figure 1, step 1), 2eta-chiC primers were used to PCR amplify *chiC* and add compatible restriction sites to the amplicons for directional cloning. EcoRI and HindIII restriction sites were selected for cohesive end cloning on the basis that they are not found within the *chiC* sequence and are unique sites within pET-28a(+), with EcoRI located just 19 bp upstream of HindIII in the polylinker region. The use of these two restriction sites positioned *chiC* in frame for expression with an N-terminus 6xHis tag and T7 tag for downstream protein purification and detection.

The forward and reverse PCR primers were designed to include EcoRI and HindIII restriction sites, respectively, for directional cloning into pET-28a(+). The leader sequence of each primer consists of five random bases at the 5’ end to facilitate restriction enzyme digestion of the *chiC* amplicon. Since the start and end of *chiC* differ greatly in GC content (GC content of the first 20 bases is 40% at the 5’ end and 75% at the 3’ end) , the forward and reverse primer lengths were optimized to yield similar melting temperatures that would result in a more stringent annealing temperature during PCR. As a result, a PCR annealing temperature of 55°C was sufficient to amplify *chiC* from pGKMS21 using our 2eta-chiC primers. A GC Enhancer was also used to improve amplification of *chiC*, which contains a high GC content of 67%.

PCR products were visualized by agarose gel electrophoresis. R1F/R1R primers complementary to the flanking regions of *chiC* were used as positive control for PCR.

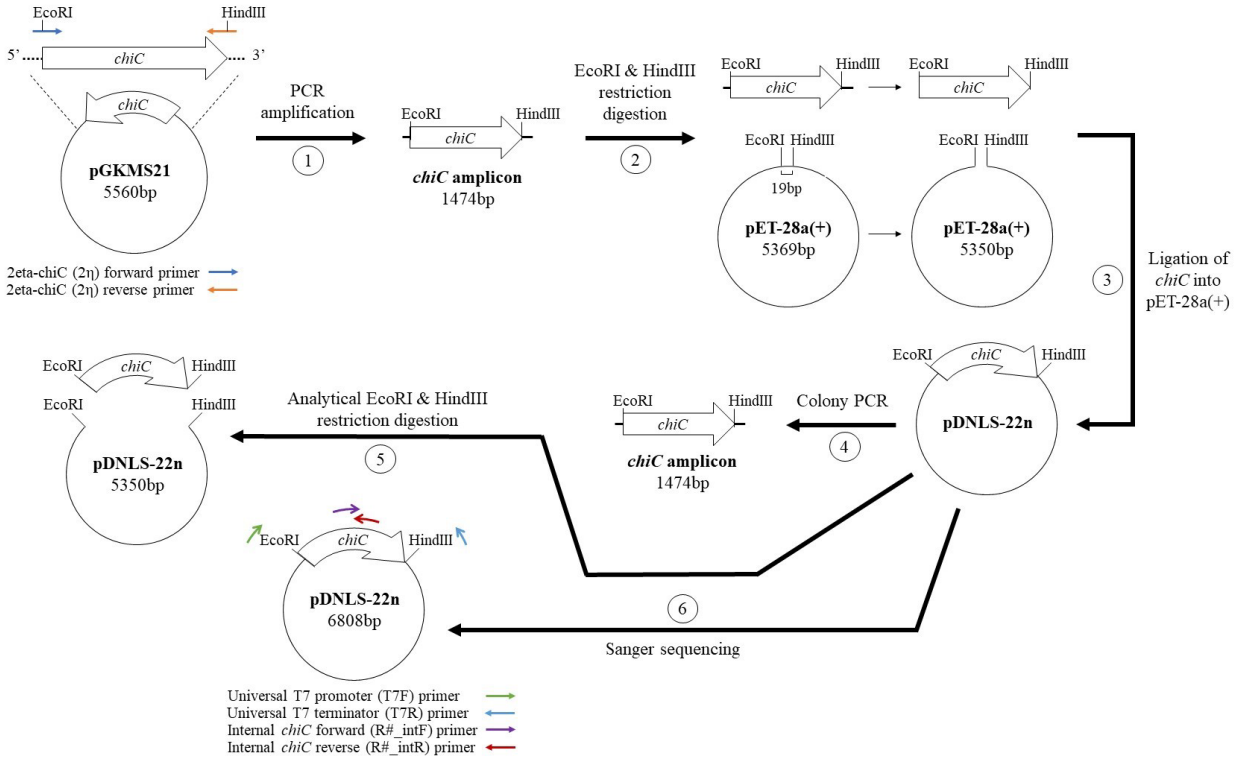

FIG. 1 Workflow for the generation of pDNLS-22n. 1. PCR amplification of pGKMS21 from Bodykevich *et al*. (7) using 2eta-chiC (2η) primers results in a *chiC* amplicon of 1474bp. 2. EcoRI and HindIII double restriction digestion of *chiC* amplicon and pET-28a(+) vector. 3. Ligation of digested *chiC* into digested pET-28a(+) generates the pDNLS-22n plasmid. 4. Colony PCR on pDNLS-22n confirms *chiC* insert presence. 5. Analytical restriction digestion of pDNLS-22n using EcoRI and HindIII confirms the presence of the pET28a(+) backbone and *chiC* gene. 6. Sanger sequencing on pDNLS-22n verifies the sequence and orientation of the *chiC* gene.

Agarose gel electrophoresis showed a DNA band slightly greater than 1500 bp, corresponding to the expected amplicon size of 1629 bp (Figure 2). With the use of 2eta-chiC primers, our expected amplicon size of *chiC* was 1474 bp, which was visualized by a dark band slightly below the 1500 bp DNA marker (Figure 2). Additionally, a faint band for the negative control was observed around 1500 bp (Figure 2). This is indicative of a contaminant present in the no template control (Figure 2). This contamination is most likely resultant from amplification of *chiC* from contaminated pGKMS21 template in the no template control. Overall, these results show that *chiC* was PCR amplified from pGKMS21 using 2eta-chiC primers.

Successful restriction digest of *chiC* and pET-28a(+) with EcoRI and HindIII. To generate compatible sticky ends for ligation, *chiC* inserts and pET-28a(+) vectors were digested with EcoRI and HindIII restriction enzymes (Figure 1, Step 2), and analyzed by agarose gel electrophoresis. Single digested pET-28a(+) plasmids with either EcoRI and HindIII showed singular bands between 5.0 to 6.0 kb, which corresponded to linearized pET-28a(+) expected at 5369 bp (Figure S1). This verified that the EcoRI and HindIII enzymes were active and cleaved only once within the vector to linearize it. In contrast, undigested pET-28a(+) migrated as a single, smeared band between 6.0 to 7.0 kb on the agarose gel (Figure S1). Furthermore, double-digested pET-28a(+) also appeared as a single band between 5.0 to 6.0 kb (Figure S1). Double-digested *chiC* amplicons were approximately 1.5 kb (Figure S1), suggesting that the restriction enzymes did not unexpectedly cleave within the gene. Overall, pET-28a(+) and PCR amplified *chiC* were successfully digested with EcoRI and HindIII.

***E. coli* DH5α transformants acquire pDNLS-22n plasmids.** Following restriction digestion of *chiC* and pET-28a(+), the insert and vector were ligated together (Figure. 1, Step 3). To

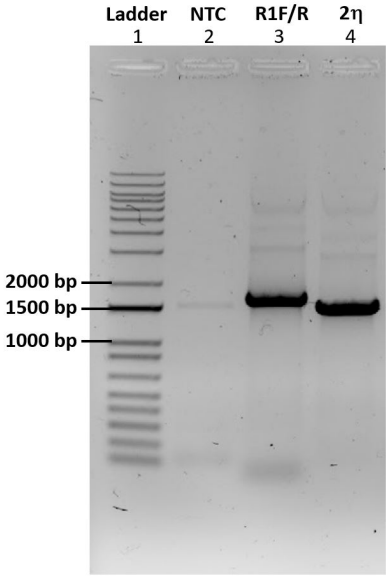

FIG. 2 Agarose gel electrophoresis shows successful PCR amplification of *chiC* from purified pGKMS21. Lane 1: 1 Kb Plus DNA Ladder. Lane 2: no template control (NTC). Faint DNA band indicates PCR contamination with pGKMS21. Lane 3: positive control for *chiC* amplification using R1F/R1R primers from Bodykevich *et al*. (7) produced the expected band size of 1629 bp. Lane 4: *chiC* amplified using 2eta-chiC (2η) primers produced the expected band size of 1474 bp.

determine whether pDNLS-22n was successfully constructed, *E.coli* DH5α transformants were selected on LB agar plates supplemented with kanamycin. The positive control plate of *E. coli* DH5α transformed with empty pET-28a(+) plasmids had colonies when plated on LB-kanamycin, indicating that transformation of chemically competent cells was successful and pET-28a(+) conferred resistance to kanamycin. The use of a ligation control consisting of T4 DNA ligase with digested pET-28a(+) only allowed for the identification of background vectors without inserts; Colony growth may be due to inefficient restriction enzyme activity resulting in undigested or self-ligating pET-28a(+) plasmids conferring resistance to kanamycin. As expected, minimal growth was observed in the no insert ligation control at plating dilutions of 1:10 and 1:100, as well as the resuspended cell pellet from transformation. This indicated low levels of background colonies containing plasmids with the kanamycin resistance marker. In contrast, more colonies were observed on all plating dilutions when transformed with the *chiC* insert and pET-28a(+), indicating the *E. coli* DH5α transformants contain the pDNLS-22n plasmid.

Colony PCR of *E. coli* DH5α transformants indicate the presence of *chiC*. To identify *E. coli* DH5α transformants containing the 1.5 kb *chiC* insert, five clones were screened by colony PCR using 2eta-chiC primers (Figure 1, Step 4). The resultant PCR products were analyzed by agarose gel electrophoresis. While the no template control showed no PCR product as expected, the negative control using empty pET-28a(+) from *E. coli* DH5α hosts showed a faint band at approximately 1.5 kb (Figure 3). It was unclear whether contaminants were introduced during PCR preparation, or sample loading for agarose gel electrophoresis in which spill-over from the positive control lane may have occurred. The positive control, which used purified pGKMS21 as template DNA, showed a light band at 1.5 kb, along with some non-specific PCR products between 1.0 to 1.5 kb (Figure 3). All five *E. coli* DH5α screened by colony PCR contained a band at the expected size of the *chiC* insert, as indicated by the dark DNA bands at 1.5 kb (Figure 3). The difference in band intensity between the positive control and the five screened colonies may be due to variable amounts of template DNA for PCR.

Analytical restriction digests of pDNLS-22n showed *chiC* ligated into pET-28a(+). To verify that *chiC* was ligated into pET-28a(+), purified pGKMS21, pET-28a(+) and pDNLS-22n plasmids were digested using EcoRI and HindIII restriction enzymes (Figure 1, Step 5) and analyzed by agarose gel electrophoresis. Undigested pGKMS21 and pET-28a(+) vectors were intended as controls for restriction digest analysis, but no corresponding bands were present after agarose gel electrophoresis (Figure 4). Double-digested pGKMS21 resulted in a

darker band at approximately 4 kb, and a lighter band between 1.5 to 2.0 kb (Figure 4). Since pGKMS21 was constructed by cloning a 1.6 kb *chiC* fragment in between two EcoRI restriction sites within the pCR2.1 vector (3.9 kb), the larger band on the gel likely

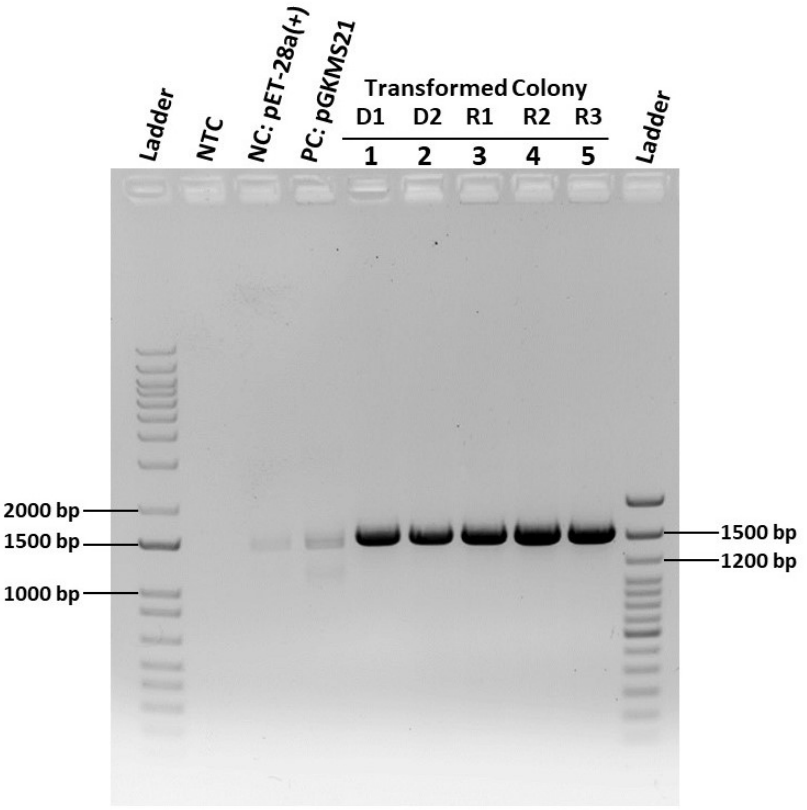

FIG. 3 Agarose gel electrophoresis of colony PCR products, from transformants that potentially contain pDLNS-22n, shows the corresponding bands for the *chiC* insert. Double-digested *chiC* PCR product and pET-28a(+) were ligated at a 3:1 molar ratio, transformed into competent *E. coli* DH5α, and plated at a 1:10 dilution (D1 and D2) and in a resuspended dilution (R1, R2, R3). Transformants were selected on LB agar plates with kanamycin, and screened by colony PCR for *chiC* using 2eta-chiC primers (Figure 1, Step 4). Lane 1 and 10: 1 Kb Plus DNA Ladder and 100 bp DNA Ladder, respectively. Lane 2: no template control (NTC). Lane 3: negative control (NC) using *E. coli* DH5α containing empty pET-28a(+) vectors. Faint band indicates contamination. Lane 4: positive control using purified pGKMS21 contains *chiC*. Lane 5 to 9: all five transformant colonies (D1, D2, R1, R2, and R3) screened contain the expected 1474 bp band corresponding to *chiC*.

corresponded to the pCR2.1 backbone, while the smaller band represented the excised *chiC* insert. The 49 bp fragment was too small to resolve on the agarose gel. Similarly, double-digested pET-28a(+) resulted in a single band between 5.0 and 6.0 kb (Figure 4). Since EcoRI and HindIII restriction sites are only 19 bp away on the 5369 bp pET-28a(+) vector, only the major fragment of the digested plasmid was observed. Double-digested pDNLS-22n from all five *E. coli* DH5α clones resulted in a darker band between 5.0 and 6.0 kb, and a lighter band at approximately 1.5 kb, corresponding to the pET-28a(+) vector and *chiC* insert (Figure 4). These observations support that *chiC* was correctly ligated into pET-28a(+).

pDNLS-22n from transformant colonies R1 and R2 contain the exact pET-28a(+) backbone and *chiC* gene in the appropriate orientation. Purified plasmid from transformant colonies R1, R2, and R3 were sent for Sanger sequencing with universal T7 promoter and terminator (T7F/T7R) and internal forward and reverse (intF/intR) primers to verify the pDNLS-22n sequence (Figure 1, Step 6). Sequences extended from T7F/T7R and intF/intR primers were aligned to the *chiC*-containing pET-28a(+) reference sequence using SnapGene. For transformant R1 and R2, the sequences extended from T7F/T7R and intF/intR primers align to the reference sequence with few gaps, insertions, and mismatches (Figure 5A). Gaps, insertions and mismatches near the 3' end of the primer-extended sequences are likely due to poor quality sequencing, thus, low quality 3' ends were removed for analysis. Analysis of remaining sequencing regions indicate high fidelity of R1 and R2 plasmids to the expected *chiC*-containing pET-28a(+) vector. For transformant R3, there are many gaps, insertions, and mismatches in sequences extended from intR and T7F primers (Figure 5C), indicating lower fidelity to the expected *chiC*-containing pET-28a(+) vector. The high quality sequencing results from R1 and R2 transformants confirm that pDNLS-22n is a 100% match to the expected pET-28a(+) vector containing *chiC* from the *P. aeruginosa* PAO1 genome (accession: NC_002516.2). pDNLS-22n has *chiC* inserted into pET-28a(+) in the correct orientation relative to the T7 promoter and still contains EcoRI and HindIII cut sites. A

plasmid map was generated for pDNLS-22n based on the results from transformants R1 and R2 (Figure 5D).

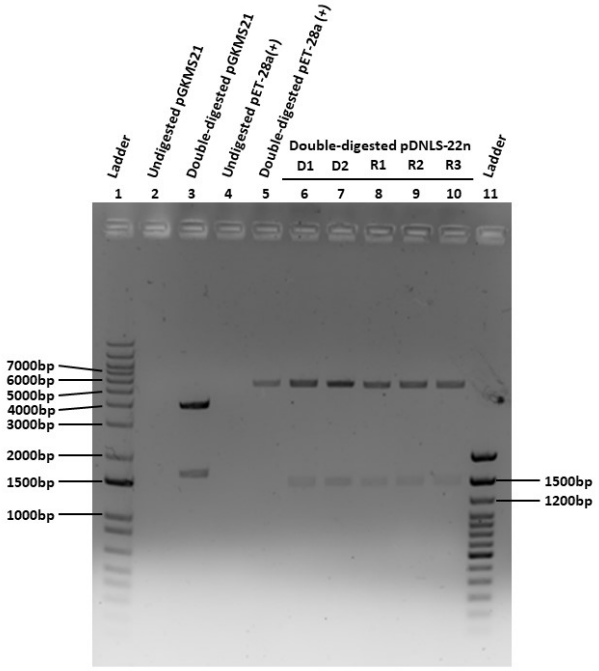

FIG. 4 Agarose gel electrophoresis of restriction digested pDNLS-22n shows *chiC* ligated into pET-28a(+). pDLNS-22n plasmids were purified from all five *E.coli* DH5α transformants (D1, D2, R1, R2, and R3) positive for *chiC* by colony PCR, and double digested using EcoRI and HindIII restriction enzymes (Figure 1, Step 5). Lane 1 and 11: 1 Kb Plus DNA Ladder and 100 bp DNA Ladder, respectively. Lane 2 and 4: undigested pGKMS21 and pET-28a(+), respectively did not appear on gel. Lane 3: double digested pGKMS21 shows expected ~4 kb vector and ~1.6 kb *chiC* band. Lane 5: double digested pET-28a(+) shows expected ~5.4 kb band. Lane 6 to 10: double digested pDNLS-22n from D1, D2, R1, R2, and R3 transformant colonies show bands corresponding to ~5.4 kb vector and ~1.5 kb *chiC* insert.

***E. coli* BL21 (DE3) transformants acquire pDNLS-22n plasmids.** Having acquired the exact sequences of pDNLS-22n that originated from *E. coli* DH5α transformants R1, R2, and R3, purified pDNLS-22n from R1 and R2 were transformed into competent *E. coli* BL21 (DE3) cells and selected for on LB agar plates supplemented with kanamycin. During transformation, the negative control with water showed colony growth when plated on LB agar, but no growth when plated on LB-kanamycin, indicating that *E. coli* BL21 (DE3) cells do not contain a plasmid that confers kanamycin resistance. The LB-kanamycin plates with the transformed *E. coli* BL21 (DE3) cells had several colonies which contain the pDNLS-22n plasmid. *E. coli* BL21 (DE3) and *E. coli* DH5α colonies with pDNLS-22n were then isolated and stored at -80˚C for future applications.

DISCUSSION

In this study, we subcloned the *P. aeruginosa* PAO1 *chiC* from pGKMS21 into pET-28a(+) with the aim of expressing recombinant ChiC in *E. coli* BL21 (DE3). Previously, Bodykevich *et al.* had sought to create an expression vector for *P. aeruginosa* PAO1 *chiC* (7). However, due to the large difference in GC content between the start and end of the 1452 bp *chiC* open reading frame (ORF), Bodykevich *et al.* noted difficulties in designing forward and reverse PCR primers with similar melting temperatures (7). Alternatively, they amplified a 1.6 kb *chiC*-containing fragment using primers that annealed to regions with more similar GC content, and ligated this *chiC*-containing fragment into the pCR2.1 vector by TA cloning to generate pGKMS21. In this study, we were successful in designing 2eta-chiC forward and reverse PCR primers with a predicted melting temperature difference of less than 3°C that amplified the *chiC* ORF only. Without extraneous sequences in the flanking region, *chiC* could be directly cloned into the pET-28a(+) plasmid which contains an innate start codon for expression of the target protein with 6xHis and T7 epitope tags at the N-terminus. Furthermore, we used restriction cloning, instead of TA cloning as described by Bodykevich *et al.* (7), to ligate *chiC* into the recipient plasmid. By generating cohesive ends using EcoRI and HindIII for ligation, this circumvented the issue of generating clones with *chiC* inserted in the reverse orientation relative to the T7 promoter, which did not allow for expression of ChiC from pGKMS21. Thus, PCR amplification of *chiC* from pGKMS21 using a proofreading DNA polymerase yielded a sequence that was 100% identical to the reference

chiC gene (accession: NC_002516.2). Overall, pDNLS-22n contains the ORF of *chiC* in the correct orientation, the EcoRI and HindIII restriction cut sites, and the T7 promoter from which IPTG can induce transcription.

Following the construction of pDNLS-22n, in principle ChiC will be expressed from pDNLS-22n with a N-terminal 6xHis tag and T7 tag. The purpose of the N-terminal 6xHis tag and T7 tag are to facilitate protein purification and detection. After purifying recombinant ChiC by nickel-charged affinity chromatography, the 6xHis tag can be removed at the thrombin cleavage site, leaving behind a T7 tagged ChiC. Since antibodies specific for these epitope tags are commercially available, they are important tools for studying the expression of novel recombinant proteins through immunochemical methods.

Addressing contaminated controls from this study, a no template control (NTC), consisting of water and 2eta-chiC primers, was used for the PCR amplification of *chiC* from purified pGKMS21 experiment. As this negative control does not contain DNA for the 2eta-chiC primers to amplify, no band was expected. However, a faint band was observed (Figure 2), indicating contamination from other samples during gel loading or from pGKMS21 plasmid DNA during preparation of PCR amplification samples. For the colony PCR on transformants that potentially contain pDNLS-22n, a negative control of *E. coli* DH5α containing empty pET-28a(+) vectors was used. As the empty pET-28a(+) vector does not contain the *chiC* gene, there should not be any PCR amplification using the 2eta-chiC primers. Contrary to this expected observation, a faint band corresponding to *chiC* was observed (Figure 3), indicating contamination from other samples during gel loading or from colonies during preparation of PCR amplification samples. In relation to the analytic restriction digest on pDNLS-22n, undigested pGKMS21 and undigested pET-28a(+) plasmids were used as controls to show the contrast of digested pGKMS21 and digested pET-28a(+) plasmids. A 5.6 kb band and 5.4 kb band was expected for the undigested pGKMS21 and pET-28a(+) plasmid, respectively, but no bands were observed (Figure 4). The lack of bands observed may suggest that an insufficient amount of pGKMS21 and pET-28a(+) plasmid was loaded for agarose gel electrophoresis.

E. coli was chosen as the host to express *chiC* due to its fast growth, simple culture procedures, and the availability of genetic tools to control recombinant protein production. However, a potential challenge with expressing *P. aeruginosa* PAO1 genes in a heterologous host such as *E. coli* is that codon usage bias may reduce translational efficiency of recombinant proteins. In contrast to *E. coli* species, which have a GC content of 48-52%, P. *aeruginosa* PAO1 is notable for its high GC content of 67%, which has been shown to dictate codon usage and influence protein expression levels (10, 11). While codon bias can be mitigated by methods such as codon optimization and over-expression of rare tRNAs, this represents an extra step with potential consequences to cell metabolism, protein translational kinetics and function (12). A feasible alternative may be to express *P. aeruginosa* PAO1 *chiC* in a non-pathogenic host with more similarity, such as *Pseudomonas putida.* However, these strains must also contain the T7 expression system in order to induce expression of *chiC* from pDNLS-22n (13).

Limitations Although sequencing results show that *chiC* is inserted into the pET-28a(+) vector in the correct orientation for transformants R1 and R2, we were unable to confirm the sequences of some features necessary for *chiC* expression in pET-28a(+). *chiC* expression should be induced from the T7 promoter and terminated at the T7 terminator. R1 sequencing results from the intR primer indicated two poorly sequenced bases within the T7 promoter. The T7F primer did not sequence the T7 promoter, thus, we were unable to confirm the validity of the intR sequencing results from transformant R1. Neither intF and T7R primers sequencing covered the T7 terminator, so the sequence of the T7 terminator in pDNLS-22n from transformant R1 is unknown. R2 T7F/intR and T7R/intF primers also did not support sequencing of the R2 T7 promoter and terminator, respectively. Without T7 promoter and terminator sequences, we do not know if there are any mutations that may affect *chiC* expression from the T7 promoter or termination at the T7 terminator of pDNLS-22n from transformants R1 and R2.

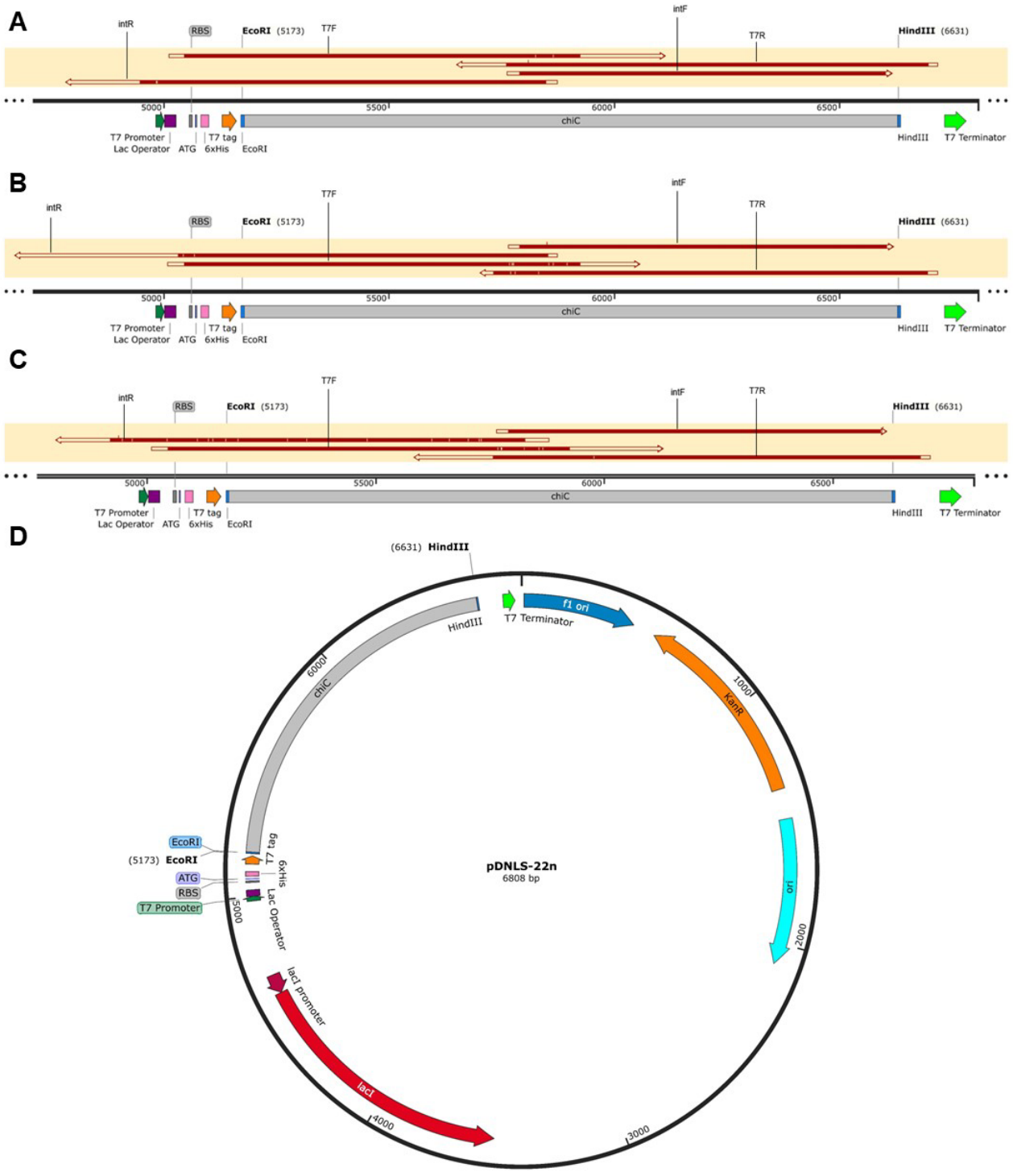

FIG. 5 Sanger sequencing results of the transformant plasmids indicate that pDNLS-22n from transformant colonies R1 and R2 contains the exact pGKMS21-originated *chiC* gene in the proper orientation. pDNLS-22n plasmids were purified from three *E. coli* DH5α transformants (R1, R2, R3). Purified plasmids, universal T7 promoter and terminator primers (T7F/T7R), and internal *chiC* forward and reverse primers (intF/intR) were sent to GENEWIZ for Sanger sequencing (Figure 1, Step 6). (A-C) Using SnapGene, sequences extended from T7F/T7R and intF/intR primers were aligned to the expected pET-28a(+) vector with the ligated *chiC* gene. Gaps, insertions, and mismatches are observed in primer arrows (red) aligned against R1 (A), R2 (B), and R3 (C) transformant plasmids. (D) The pDNLS-22n plasmid map shows that pDNLS-22n includes the pET-28a(+) backbone and pGMKS21-originated *chiC* gene.

Given the shortened timeline of our project, we were unable to proceed with inducing the expression of *chiC* using IPTG in *E. coli* BL21 (DE3) cells. Resultantly, we were unable to purify ChiC using nickel-charged affinity column chromatography in order to determine its protein structure and chitinolytic activity. As such, we are unable to conclude whether pDNLS-22 can express *chiC* in its native form and whether the expressed protein product is still chitinolytic. In addition, previous studies have mentioned the formation of inclusion bodies during the expression of *chiC* when transformed into *E. coli* M15pREP4 with low levels of exogenous secretion of ChiC (8). It has been noted that the expression of *chiC* in *P. aeruginosa* PAO1 is performed in an unprecedented manner, independent from conventional Type I and III secretion systems. Since only *chiC* was ligated into pET-28a(+) backbone for the construction of pDNLS-22n, pDNLS-22n does not encode the appropriate cellular machinery required for exogenous secretion of *chiC.* Therefore, we are limited in concluding whether transformation with pDNLS-22n will result in successful expression and subsequent secretion of *chiC.*

Conclusions In our experiment, we demonstrated the successful amplification of *P. aeruginosa* PAO1 derived *chiC* from pGKM21 plasmid, where *chiC* was successfully cloned into pET-28a(+) vector by restriction digest cloning. Verification of the construct fidelity through Sanger sequencing demonstrated that the plasmid consisted of a 100% match to the *P. aeruginosa* PAO1 *chiC* reference gene. Therefore, our results provide an expression vector for *chiC*, termed pDNLS-22n. Importantly, we improved upon previous work by ensuring the gene was ligated in the correct orientation for appropriate expression. This lays the groundwork for future investigations of *chiC* in a non-chitinolytic host.

Future Directions Having pDNLS-22n constructed offers a means to study *chiC* in a safer, non-pathogenic host with fast replication times and inexpensive media. Studying *chiC* in a host with a lower GC content offers reduced barriers in sequencing and genetic manipulation for proceeding experiments. Subsequent experiments may scaffold on our work for further examination of *E. coli* as a host expression system. This allows for further characterization of how ChiC functions in a wildtype host and can build towards testing the synergistic effect of ChiC when expressed with other chitinases.

Previous researchers have demonstrated that multiple chitinases are able to degrade chitin individually, but also in conjunction with other chitinases to produce a synergistic effect of chitin degradation (6). Determining possible synergistic effects of pDNLS-22n derived ChiC chitinolytic activity is limited by the lack of other *P. aeruginosa* PAO1 derived chitinases in *E. coli*. Nonetheless, chitinolytic assays with purified ChiC may be supplemented with other chitinases to characterize any synergistic activity.

To test the functionality of a chitinolytic ChiC product derived from *P. aeruginosa* PAO1, the chitinase enzyme activity assay from Folders *et al.* can be employed (3). *E. coli* containing pDNLS-22n can be streaked onto LB plates with colloidal chitin (3). If ChiC is expressed from pDNLS-22n, chitin degradation is expected to occur, which would result in the formation of a halo around *E. coli* colonies on the LB-colloidal chitin plates (3). pDNLS-22n was constructed to express ChiC with a 6xHis tag and T7 tag, which may affect enzymatic function. Although the 6xHis tag and T7 tag are relatively small compared to ChiC, their effects on chitinolytic activity and stability will need to be empirically determined.

To determine the three-dimensional structure of ChiC, the recombinant protein can also be purified from induced *E. coli* BL21 (DE3) cells, and imaged by X-ray crystallography. Determining the three-dimensional structure of ChiC allows for visualization of the active site and the functional domains of the protein, which will be crucial in understanding its potential to degrade chitin.

As stated previously, ChiC operates in conjunction with secretion systems in the natural environment. Given our current understanding, we are limited in our ability to predict the degree of efficacy with which ChiC will be secreted in a host which does not encode for these systems. Future studies may look at whether ChiC is secreted or cytoplasmic when expressed in *E. coli* BL21 (DE3). If such an issue were to arise, it may be pertinent to explore the suitability of other hosts that have secretion systems similar to *P. aeruginosa*. Indeed, if such assays prove that ChiC remains functional and secretion can be reasonably achieved,

this prompts further research into the feasibility of chitinases as commercial biocontrol agents.

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CONTRIBUTIONS

All individuals of the team were integral to the completion of this draft manuscript and participated in the planning, data collection, and analysis for this study. S.P. was responsible for the introduction, abstract, results, and discussion. Both N.C., and L.X. were responsible for the abstract, materials and methods, results, discussion, and future directions. D.Y. was responsible for the future directions and conclusion. All members were involved in the editing of this manuscript. Co-authorship should be considered equal for all 4 authors.

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