

# Attempted Construction of Recombinant Vectors Designed to Study the Solubility of Overexpressed Proteinase Inhibitor 2 when Co-expressed with Thioredoxin

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**Overexpression of recombinant proteins in *Escherichia coli* can result in biologically inactive aggregates of misfolded proteins known as inclusion bodies. The fusion of thioredoxin A (TrxA) to target proteins has been used in commercial plasmids to increase the solubility of overexpressed protein which can reduce the formation of inclusion bodies. The objective of this study was to create recombinant plasmid vectors that would overexpress TrxA fused to proteinase inhibitor II (PI2), a protein domain containing 8 disulphide bonds known that is known to form inclusion bodies. We hypothesized that the co-expression of TrxA in either *cis* or *trans* with PI2 would increase levels of soluble PI2 when overexpressed in *E. coli*. We designed oligonucleotide primers to amplify the PI2 gene from plasmid pE32PI2 using polymerase chain reaction. We found that the addition of dimethyl sulfoxide at concentrations between 0.25% and 1.0 % was required for PCR amplification of the PI2 gene from pE32PI2. In this study the PI2 gene was successfully amplified by PCR using DMSO as an additive. The expected DNA band sizes were confirmed by gel electrophoresis.**

*Escherichia coli* overexpression vectors have been instrumental in the biotechnology and pharmaceutical industry in the mass production of protein. One complication that can arise with high-level protein expression is the production of misfolded protein (2). Protein misfolding can result in the formation of protein aggregates known as inclusion bodies. Misfolded protein shows a reduction in biological activity (2). Purification and refolding of misfolded protein can be attempted but these steps are complex and time-consuming, reduce the overall purification yield, and are often unsuccessful (3). Finding conditions that result in expression of soluble, active protein is often most desirable.

Thioredoxin (trx) is a biological disulfide reductase, which has been used as a recombinant fusion partner to increase the solubility of overexpressed proteins in *E. coli* (4,5). Although the precise mechanism is unclear, thioredoxin is proposed to affect the redox state of the cell environment. PI2 originates from eukaryotic cells, which are known to have different redox states compared to prokaryotes (10). This difference in redox state causes the formation of abnormal disulfide linkages in overexpressed proteins within *E. coli* (10). Coproduction of thioredoxin enhances protein solubility by altering the redox state of *E. coli*, through the reversible oxidation of cysteine thiol groups (11).

Proteinase inhibitor 2 (PI2) is an endo-acting peptide bond hydrolase, found in a variety of tomato and potato plants (6,7). PI2 has been shown to form inclusion bodies when overexpressed in *E. coli*. PI2 is composed of 2 domains which contain a total of 8 disulfide bonds (8). Thus, it was chosen to investigate the influence trxA would have on a protein containing a high number of disulfide bonds relative to the size of its tertiary structure. Previous work has attempted to generate various PI2 constructs to investigate the effects of trxA on the

solubility of PI2, but our analysis indicated that PI2 was absent in these putative clones (1, 2).

The aim of our study was to generate a pET32a(+)-PI2 plasmid construct and a pET32a(+)-PI2/ $\Delta$ trxA plasmid construct to evaluate the effects of Trx on the solubility of PI2. Primers were designed with linkers encoding restriction sites to enable directional cloning of PI2 from pE32PI2 into pET32a(+). Putative clones were isolated and subjected to colony PCR, which revealed fragments of similar size to PI2, however, subsequent analysis did not confirm PI2 nucleotide sequence.

## MATERIALS AND METHODS

**Strains and Plasmids.** The pET32a(+), obtained from Novagen, is the template vector used in this study. JP061, JP062, JP063, JP064, CD111, CD112, CD113, and CD114 plasmid constructs (TABLE 1) were preserved in *E. coli* DH5 $\alpha$  strains as part of the Ramey strain collection (Department of Microbiology and Immunology, University of British Columbia). Cells containing each of the plasmids were grown overnight at 37°C at 180 RPM in Luria-Bertani (LB) liquid medium (pH 7.0) containing tryptone (1.0%), yeast extract (0.5%), and NaCl (1.0%).

**Plasmid Isolation.** Plasmid DNA was isolated using the Invitrogen PureLink HQ Mini Plasmid DNA Purification Kit (Cat# K2100-01). DNA concentration and purity of the plasmid products were assessed by measuring the absorbance at 260 nm and 280 nm using a Beckman Coulter DU530 spectrophotometer.

**Restriction Digestion of Plasmids.** The identity of pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, pCD114, and pE32PI2 plasmids were confirmed using: restriction digestion, PCR, and Sanger sequencing. To confirm the identity of the clones, each of the plasmid isolates from JP061, JP062, JP063, and JP064 were digested with 20 units of New England BioLabs EcoRI (Cat# R0101S) and 10 units of New England BioLabs NcoI (Cat# B0101S) with 5  $\mu$ l of 10x NEBuffer EcoRI (Cat# B0101S). Plasmids CD111, CD112, CD113, and CD114 were digested with 15 units of New England BioLabs EcoRI (Cat# R0501S) and New England BioLabs RsrII (Cat# B7004S) with 5  $\mu$ l of 10x NEBuffer 4 (Cat# B7004S). The pE32PI2

**TABLE 1 Strains and plasmids used in this study.**

Name	Description	Source
<b>Strains</b>		
DH5 $\alpha$	Bacterial host for plasmid storage.	UBC
BL21 (DE3)	Bacterial host for protein overexpression.	UBC
<b>Plasmids</b>		
pET32a	Expression vector with thioredoxin and ampicillin resistance.	Novagen
pJP061/062	pET32a/PI+PI2 gene with fused thioredoxin gene.	Park, UBC.
pJP063/064	pET32a/PI+ $\Delta$ trx PI2 gene with a deleted thioredoxin gene.	Park, UBC.
pCD111/112/113/114	pET32a/PI+ $\Delta$ trx/trx+PI2 gene with a non-fused thioredoxin gene.	Duronio, UBC.
pE32PI2	Contains original PI2.	Tai Man Louie.

plasmid isolates were digested using a series of single and double restriction enzyme reactions. For each of the single digests, 3  $\mu$ l of pE32PI2 plasmid was mixed with 0.5  $\mu$ l of the enzyme (NcoI, Hind III, EcoRI, XbaI), 1  $\mu$ l of 10x NEBuffer 4 (Cat# B7004S), and 5.5  $\mu$ l of sterile distilled water. For each of the double digests, 3  $\mu$ l of pE32PI2 plasmid was mixed with 0.5  $\mu$ l of each enzyme (NcoI+HindIII, NcoI+EcoRI, XbaI+EcoRI, HindIII+EcoRI), 1  $\mu$ l of 10x NEBuffer 4 (Cat# B7004S), and 5  $\mu$ l of sterile distilled water. Each of the reactions were performed in a final volume of 10  $\mu$ l for 90 minutes at 37°C.

**PCR Amplification of *PI2*.** Attempts were made to amplify a 590 bp fragment encoding the *PI2* gene from the putative clones pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, pCD114, and pE32PI2. Shown in TABLE 2 are the oligonucleotide primers that were designed to amplify the *PI2* gene. The *PI2* forward primer contains four extra nucleotides in order to engineer an NcoI restriction site, while the *PI2* reverse primer introduces an EcoRI restriction site. All PCR reactions were carried out in volumes of 50  $\mu$ l. Each reaction was set up to contain final concentrations of 1x PCR buffer, 1 mM MgSO<sub>4</sub>, 0.2 mM dNTPs, and 0.5 mM of both forward and reverse primers. Next, 100 ng of template plasmid was added to each reaction along with 0.5  $\mu$ l of Taq Polymerase (Invitrogen, Cat# 10342053). PCR cycle conditions used are as follows: an initial denaturation step of 5 min at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes. All PCR products were subsequently resolved on 1% agarose gel and visualized by exposure to UV light. In addition, varying amounts of dimethyl sulfoxide (DMSO) were also added to reaction tubes during PCR optimization experiments.

**TABLE 2 Oligonucleotide primers used to amplify *PI2*.** Restriction cut sites for NcoI (CCATGG) and EcoRI (GAATTC) are indicated in bold, while start and stop codons are underlined.

Name	Sequence (5' - 3')	T <sub>m</sub> (°C)
PI2 Forward	TAC <b>CCATGG</b> CTGTTCA <b>CAAGG</b> AA GTT	58.6
PI2 Reverse	CC <b>GAATTC</b> ACATTGCAGGGTAC ATAT	57.3

**DNA Sequencing of plasmids.** Sequencing reactions of JP061, JP062, JP063, JP064, CD111, CD112, CD113, CD114, and pE32PI2 were prepared according to instructions from GeneWiz DNA Sequencing. For each sequencing reaction, the plasmid isolate was eluted using 300  $\mu$ l sterile distilled water instead of TE buffer. The concentration of each plasmid isolate was measured using a Beckman Coulter DU350 spectrophotometer to ensure a minimum concentration of 50 ng/ $\mu$ l for use as the template. 10  $\mu$ l of template DNA was mixed with 5  $\mu$ l of 5 pmol/ $\mu$ l diluted primer and the samples were loaded into PCR strip tubes. The sequencing reactions were then submitted for sequencing to GeneWiz DNA Sequencing.

**CaCl<sub>2</sub> Transformation.** Competent DH5 $\alpha$  and BL21 (DE3) *E. coli* cells were prepared by CaCl<sub>2</sub> treatment (9). A 0.1M CaCl<sub>2</sub> stock solution was prepared by mixing 2.94 g of CaCl<sub>2</sub>·2H<sub>2</sub>O with 200 ml of distilled water. Competent cells were divided into aliquots of 0.1 ml and stored in -80°C. For each transformation, 10  $\mu$ l of DNA were added to 0.1 ml of competent cells. Cells were subject to heat shock at 42°C for 45 seconds and returned to ice for 1 minute. About 1.2 ml of LB liquid medium was added and each mixture was incubated at 37°C for one hour. The transformed cells were spread plated onto LB agar containing ampicillin (100 $\mu$ l/mg).

**Isolation of *PI2* PCR Product.** *PI2* was amplified from pE32PI2 plasmid. The amplified products were subsequently loaded onto a 1.0% agarose gel and products were resolved at 100V for 1 hour. The distinct band found at approximately 590 bp was excised and purified using the Invitrogen PureLink Quick Gel Extraction Kit (Cat# K2100-12) according to the manufacturer's instructions. The product of the gel extraction was used as the template for subsequent PCR reactions.

**Construction of Functional *PI2* Vectors.** To construct overexpression vectors containing *PI2*, plasmid isolates of pET32a, JP061, JP064, and CD111 were prepared. These plasmids along with the purified putative *PI2* fragments were subject to overnight restriction digestion, generating suitable sticky ends for DNA ligation. These putative *PI2* insert DNA fragments were then ligated using each of the cleaved vector DNA (pET32a(+), JP061, JP064, and CD111). For each reaction, 50 ng of vector DNA, 150 ng of insert DNA, 1  $\mu$ l of 10x T4 DNA ligase buffer (Cat# B69), 1.5  $\mu$ l of ATP, 2  $\mu$ l of 50% polyethyleneglycol (PEG) and 5  $\mu$ l of T4 DNA ligase (Cat# EL0014) were mixed and added to a final reaction volume of 50  $\mu$ l using sterile distilled water. Each ligation reaction was incubated at 20°C overnight. Products from each ligation were transformed into BL21 (DE3) *E. coli*.

## RESULTS

**Confirmation of identities of putative clones.** Our initial goal of this study was to confirm the identities of plasmids pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114 (1, 2). We carried out restriction enzyme double digests of plasmids pJP061, pJP062, pJP063, and pJP064 using NcoI and EcoRI. Double digests were also performed on plasmids pCD111, pCD112, pCD113, and pCD114 using RsrII and EcoRI. Based on the previous study by Duronio (2), both pJP061 and pJP063 were expected to generate band sizes of 5900 bp and 590 bp, the latter of which corresponds to the size of the *PI2* gene. pJP062 and pJP064 bear a deletion of the 300 base pair *trx* gene and were expected to generate bands of 5600 bp and 590 bp. pCD111, pCD112, pCD113,

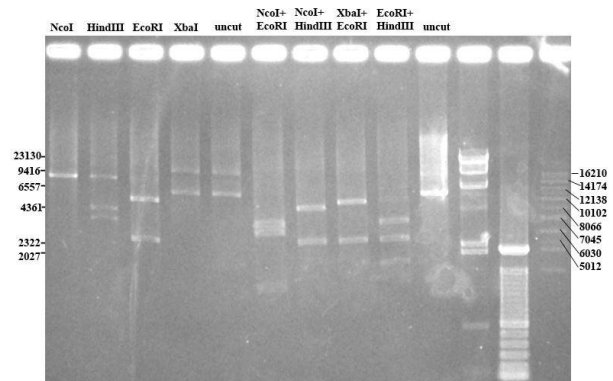


**FIG 1 Restriction digests of putative clones** 1.0% agarose gel showing the products of double digests of plasmids isolated from putative clones. pJP061, pJP062, pJP063, and pJP064 were digested with NcoI and EcoRI, while pCD111, pCD112, pCD113, and pCD114 were digested with RsrII and EcoRI. Lane 5 was loaded with 0.5  $\mu$ g of high molecular weight mass ladder and marker sizes are indicated on the left. Lane 6 was loaded with 0.5  $\mu$ g of low molecular weight mass ladder with marker sizes indicated on the right.

pCD114, which contain the *trx* gene, were expected to generate similar band sizes to JP061 and JP063. While all of the clones generated the correct larger band sizes of either 5900 bp or 5600 bp, a band size of 590bp corresponding to *PI2* was not found in any of the plasmids (Figure 1). To further investigate the identities of the clones, PCR was used to screen for the putative *PI2* inserts using primers designed to flank the *PI2* insert site. A band consistent with the expected *PI2* size was not observed suggesting that the *PI2* inserts may not have been present (data not shown). Each plasmid was successfully sequenced as suggested by the even distribution of peaks representing bases in the raw sequencing data (data not shown). Additionally, by using BLAST analysis, the sequenced region contained an unknown sequence that did not show any homology to *PI2*, which was expected to be present. Therefore, these data suggest that *PI2* was not present in pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114.

**Restriction enzyme digest and characterization of pE32PI2 construct.** Since little is known about the original pE32PI2 construct, a restriction enzyme digest was performed for characterization purposes. In Figure 2, the lane with uncut pE32PI2 shows two bands, suggesting that pE32PI2 may have two conformations, circular and supercoiled. Based on the single cut NcoI, the pE32PI2 plasmid appears to be 6600bp. The pE32PI2 plasmid was later sequenced using the *PI2* reverse primer where it was determined that the plasmid contained the *PI2* gene sequence (data not shown).

**PCR Amplification of *PI2* from pE32PI2.** PCR was used to amplify *PI2* for subsequent cloning steps. Using primers designed by Park (2006), a PCR was carried out to amplify the *PI2* gene from the pE32PI2 plasmid. Primers contained linkers with restriction sites for downstream cloning steps. Initial PCR attempts yielded no *PI2* product



**FIG 2 Restriction digests of pE32PI2** Isolated pE32PI2 plasmids were digested overnight with various restriction enzymes in 10 $\mu$ L reactions and fragments were resolved on a 1.0% agarose gel. Lanes 1 to 4 are products of single digests with the indicated restriction enzyme. Lanes 5 and 10 contain uncut pE32PI2 plasmid; two bands corresponding to either the supercoiled (lower) or the circular conformation of the plasmid are apparent. Lanes 6 to 9 contain the products of double digests of pE32PI2 with the indicated enzyme pairs. DNA marker sizes of a Lambda DNA/HindIII DNA marker (lane 11) and an Invitrogen supercoiled DNA ladder (lane 13) are indicated to the right and left of the figure, respectively. An additional GeneRuler 100 bp ladder was ran in lane 12. All DNA marker lanes were loaded with 0.5  $\mu$ g of the DNA ladder.

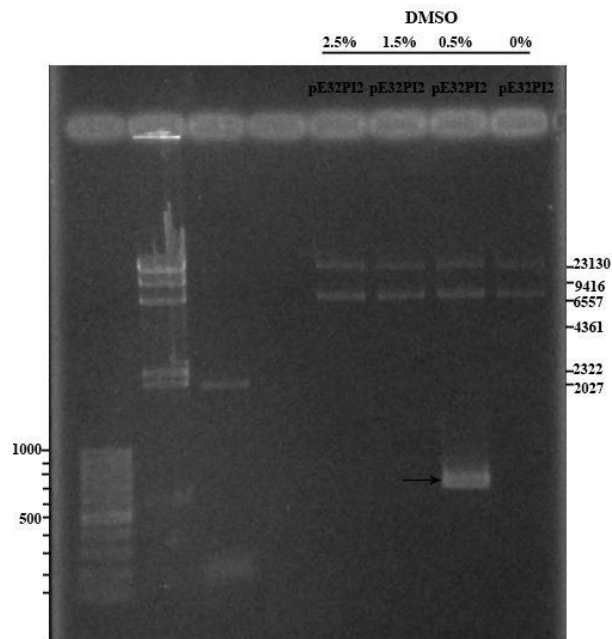
(data not shown). PCR was conducted in which DMSO was added in varying concentrations as a PCR enhancing agent (Figure 3) (12). A DMSO concentration of 0.5% was found to be the only concentration to generate the expected band size of 590 base pairs. Bands corresponding to the sizes of the two conformations of the pE32PI2 plasmid are also apparent, which may be indicative of excessive template DNA in the PCR (Figure 3). An additional PCR was done in which DMSO concentrations of 0.25% to 1% successfully generated *PI2* amplicons via PCR (Figure S1).

**Colony PCR and DNA Sequencing of Transformants Containing the Putative *PI2* insert.** After the attempted ligation of the putative *PI2* sequence into the pET32a(+), JP061, JP064, and CD111 plasmids constructs were introduced into competent BL21 (DE3) *E. coli* cells using CaCl<sub>2</sub> heat shock transformation. Vector DNA that is incompletely digested will give rise to high background so therefore ligations of pET32a(+) without insert DNA were performed as controls. Transformants were obtained for all conditions, except for the condition in which ligation into pJP061 was attempted. Colony PCR amplified a product of 590 base pairs derived from the modified pET32a(+) and pJP064 amplified an approximately 590 bp product resembling *PI2* (Figure 4A). Colony PCR of the pCD111 transformants amplified a product of approximately 200 base pairs, which was not expected (Figure 4B). For both gels, a positive control from Invitrogen containing template and primers known to amplify, and a negative control using sterile dH<sub>2</sub>O as template, indicated that the PCR reaction was successful. This data suggested that our modified pET32a(+) and JP064 constructs may have contained the correct *PI2* sequence.

## DISCUSSION

To investigate the increase in solubility of overexpressed protein observed with fusion to thioredoxin, constructs were generated with expression of TrxA in *cis* or *trans* with PI2. Expression of constructs were to be induced and inclusion body formation assessed to further explore the role of TrxA in the reduction of aggregates. Confirmation of previously constructed plasmids was required before expression could be induced. A double restriction digest with NcoI and EcoRI was prepared to identify the putative plasmids. A band of 590 bp (*PI2*) was absent suggesting that *PI2* was not present. Band sizes of 5900 bp (pET32a) in lanes 1&3 and 5600 bp (pET32a- $\Delta$ trx) in lanes 2&4 of Figure 1 demonstrated that *trx* was present and absent in the respective constructs. PCR using primers designed to flank *PI2* revealed that *PI2* was absent in all of the constructs. The putative constructs were then sequenced for *PI2*. We found that the incorrect nucleotide sequences in plasmids pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114 (1, 2).

Using the nucleotide sequence of *PI2* a primer pair flanking the gene was designed for targeted amplification. It was found that 0.25-1% DMSO was necessary for the amplification of *PI2* PCR products. DMSO facilitates strand separation by base pair disruption, and could reduce possible secondary structure in the *PI2* nucleotide sequence. The products of PCR were resolved on a gel to determine if products of the expected size of *PI2* were present. Fragments of desired size were observed, however there were also non-specific products on the gel. Due to the low DNA concentration of previous gel extractions, PCR products were directly digested with restriction enzymes and ligation into the target vector was attempted. Ligation products were transformed into *E.coli* and screened via colony PCR for insertion of *PI2*. Colonies were observed bearing PCR products of the correct size, however, sequencing results showed that incorrect sequences were present. A significant number of inserts were of 200-300 bp in size, smaller in comparison to the *PI2* sequence. Ligation or amplification of the smaller non-specific PCR products may have been favored in the reaction. In addition, these plasmids containing the smaller PCR product insert may also have higher transformation efficiency again due to its smaller size. Another explanation that may explain the lack of transformants with the desired *PI2* insert is due to *PI2*'s toxicity when expressed in the *E. coli* strain BL21. Basal level of the T7 RNA polymerase transcribes the target protein enough to hinder growth or cause cell death. Transformants with the *PI2* recombinant vector would not develop into colonies and therefore be absent in colony screening. This would



**FIG 3. PCR amplification and DMSO optimization of PI2**

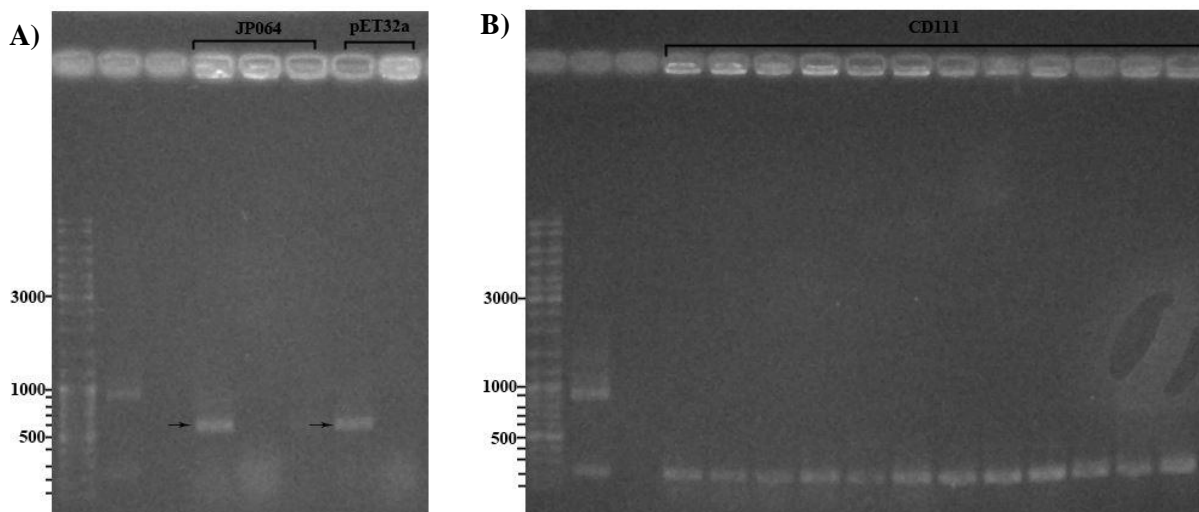
Lanes 5 to 8 contain PCR amplification products of pE32PI2 with varying concentrations of DMSO as indicated above the figure. A positive PCR control from Invitrogen was loaded onto lane 3, and a negative control using sterile dH<sub>2</sub>O as template was loaded onto lane 4. Lanes 1 and 2 were loaded with 0.5  $\mu$ g of GeneRuler 100 bp and Lambda DNA/HindIII ladders respectively. Band sizes are as indicated.

explain why previous studies came to the same conclusion, as only colonies without *PI2* recombinant vectors would be generated. This could also explain why a large concentration of 590bp PCR products is not sufficient to produce transformants with the correct insert.

## FUTURE DIRECTIONS

The amplification and isolation of *PI2* to generate a pET32a(+)-*PI2* construct proved to be challenging. To eliminate the problem of amplifying non-*PI2* products in the PCR of pE32PI2 it would be helpful to sequence pE32PI2 to analyze potential non-specific primer binding. Primers should be redesigned with higher specificity to reduce non-specific products and allow for accurate sequencing. PCR amplification of *PI2* from pE32PI2 and sequencing of the subsequent PCR products with newly designed primers should be performed to confirm *PI2* isolation.

To solve the possible problem of *PI2* transformants not generating colonies, basal T7 RNA polymerase should be inhibited. This could be solved by co-transformation of competent cells with pLys S/R. pLys S/R is a small plasmid that encodes a T7 lysozyme. T7 lysozyme binds onto the C-terminal domain of T7 RNA polymerase forming a complex and conformational change that causes T7 RNA polymerase to fall off the T7 promoter. This



**FIG 4. Colony PCR to screen for PI2 inserts.** Attempted isolation and ligation of PI2 into vectors pET32a(+), JP064, and CD111 was performed. Transformants were subjected to colony PCR to screen for the insert. (A) Lanes 4 to 6 show colony PCR products from the modified JP064 and Lanes 7 and 8 show colony PCR products from the modified pET32a(+). Lane 1 was loaded with 0.5  $\mu$ g of O<sup>+</sup>GeneRuler 100-10,000 bp ladder, and band sizes are indicated to the left of the figure. Lanes 2 and 3 served as positive and negative controls, respectively. (B) Lanes 4 to 15 show colony PCR products from the modified CD111. Lane 1 was loaded with 0.5  $\mu$ g of O<sup>+</sup>GeneRuler 100-10,000 bp ladder, and band sizes are as indicated. Lanes 2 and 3 served as positive and negative controls, respectively.

inhibits basal level transcription of the toxic *PI2* gene of interest. However, this is only effective on low or basal levels of T7 RNA polymerase. Therefore when IPTG is added and large amounts of T7 RNA polymerase is generated, T7 lysozyme is no longer active and transcription continues unhindered. Another strategy would be to transform the plasmid into an *E. coli* strain that lacks T7 polymerase (e.g. DH5 $\alpha$ ) as an intermediate cloning step. This may allow propagation and sequencing of the plasmid before testing expression in another *E. coli* host, such as strain BL21.

The difficulty in sequencing *PI2* may be from secondary structures that form when strands are separated. Future experiments could combat this problem with the addition of DMSO, similar to the PCR protocol outlined.

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