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Generation of Recombinant Plasmid Constructs to Assess the Ability of NADH:Flavin Oxidoreductase to Solubilize Proteinase Inhibitor 2 in Bacterial Protein Overexpression Systems

JEI EUNG PARK
Department of Microbiology and Immunology, UBC

A possible solution to the problem of conversion of overexpressed proteins into insoluble aggregates in bacterial protein expression systems is to express the target protein fused to a reductase enzyme. One such example is *Escherichia coli* thiol reductase (Trx), currently used in the Novagen pET expression system marketed by EMD Biosciences. In the present study, an alternative redox protein, the highly soluble *E.coli* NADH:flavin oxidoreductase (Fre) was chosen to investigate whether it would solubilize a protein called proteinase inhibitor 2 (PI2), which is relatively insoluble when expressed in high concentration. In order to compare the properties and characteristics of thiol reductase and flavin oxidoreductase, attempts were made to generate fusion constructs of *trx*:PI2 and *fre*:PI2, as well as a control construct with PI2 by itself, using the Novagen pET32a vector plasmid. Due to time constraints and a problem with amplification of *fre*, the pET32aΔ*trx*/*fre*$^+$/PI2$^+$ construct was not generated. However, potential clones of the pET32a/PI2$^+$ and pET32aΔ*trx*/PI2$^+$ recombinant constructs were generated and isolated. Further study is necessary to confirm the identity of these constructs, to generate the pET32aΔ*trx*/*fre*$^+$/PI2$^+$ construct, and to express these constructs under appropriate conditions to assess and compare the effects of flavin oxidoreductase and thiol reductase on solubility of PI2.

When studying a protein of prokaryotic origin, an obvious choice is to use *Escherichia coli* expression system. The method is quick (i.e. *E.coli* grows rapidly) and cheap, and *E.coli* possesses all the machinery necessary for protein folding and post-translational modifications. For expression of eukaryotic proteins, as well, the first expression system of choice is normally *E.coli*, for the same rationale. However, many eukaryotic proteins do not fold properly in *E.coli* and form insoluble aggregates (inclusion bodies). Indeed, a common problem encountered in many bacterial protein expression systems, including *E.coli*, is the aggregation of the overexpressed protein, which renders the protein product non-functional or inactive.

Strategies exist that can generate active proteins through refolding of bacterial inclusion body proteins (7). Alternatively, protein solubility and stability may be improved by expressing the protein at a lower temperature, but wide applicability of this approach has not been vigorously investigated (4). An effective approach to overcoming this problem is to express the target protein fused to a reductase enzyme such as thiol reductase (Trx, also called thioredoxin). Thioredoxin is a protein that carries out a variety of cellular functions, such as the reduction of disulfide linkages in proteins, metabolism of sulphate, and participating as a cofactor for phage T7 DNA polymerase (B. Novy, J. Berg, Yaeger, K., and R. Mierendorf, 1995. inNovations. 3:4 http://www.emdbiosciences.com/docs/NDIS/inno03-003.pdf). LaVallie and colleagues reported in 1993 that when a variety of mammalian cytokines and growth factors were produced as thioredoxin fusion proteins, the corresponding solubility in the cytoplasm of *E.coli* had dramatically increased (2). Furthermore, thioredoxin has inherent thermal stability, and is localized on the cytoplasmic face of the adhesion zones between the inner and outer cell membranes; both properties aid in rapid purification of thioredoxin fusion proteins (2). The mechanism by which thioredoxin as a gene fusion partner increases the solubility of proteins has not exactly been determined, but the finding that the solubility of foreign proteins can be increased by overproduction of Trx strongly indicates that the redox state affects the solubility of foreign proteins (9).

Perhaps, other redox proteins, such as the highly soluble *Escherichia coli* NADH:flavin oxidoreductase (Fre), may function as novel alternatives to thioredoxin in increasing the solubility of overexpressed proteins in bacterial expression systems. In the present study, a relatively less soluble protein called proteinase inhibitor 2 (PI2) was chosen to study its solubility under different expression conditions – by itself, or fused to thioredoxin or flavin oxidoreductase. Using the Novagen pET32a plasmid from EMD Biosciences as the starting point, which allows the cloned gene of interest to be fused to thioredoxin for overexpression with enhanced solubility, attempts were made to create three different constructs – one with PI2 expressed as a fusion of Trx, one with PI2 expressed as a fusion of Fre,

TABLE 1. Bacterial strains and plasmids.

Escherichia coli strains		Description	Source
DH5α		Bacterial host for plasmid amplification	UBC
DH5α: SW057		pET32aΔtrx, Amp^r_{100} [a]	Wong (8)
DH5α: JP061 & JP063		pET32a/PI2$^+$, Amp^r_{100}	This study
DH5α: JP062 & JP064		pET32aΔ*trx*/PI2$^+$, Amp^r_{100}	This study
Plasmids			
pES1		pET30 plasmid with *fre* fragment, Kan^r_{50} [b]	Tai Man Louie
pE32PI2		Plasmid containing PI2 gene, Amp^r_{100}	Tai Man Louie
pET32a		*trxA*$^+$, Amp^r_{100}	Novagen
pET32a/PI2$^+$		pET32a with PI2 inserted	This study
pET32aΔ*trx*		pET32a with *trxA* deleted	Wong (8)
pET32aΔtrx/PI2$^+$		pET32a with *trxA* deleted and PI2 inserted	This study

[a] Amp^r_{100}: resistance to ampicilin at 100 ug/ml; [b] Kan^r_{50}: resistance to kanamycin at 50 ug/ml

and one with PI2 expressed by itself. By doing so, the relative effectiveness of Trx and Fre in increasing the solubility of overexpressed PI2 could be determined and compared.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Table 1 summarizes the bacterial strains and plasmids used in this study. *E.coli* DH5α culture was grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or on LB agar plates (LB broth with 15g/L agar added), supplemented with the appropriate antibiotics, at 37°C with or without shaking at 150 rpm. Purified plasmids were prepared using the Invitrogen PureLink HQ Mini Plasmid Purification Kit (Cat# K2100-01) or PureLink HiPure Maxiprep Plasmid Kit (Cat# K2100-07).

Confirming the Identity of *E.coli* DH5α:SW057, a Potential pET32aΔ*trx* Clone. In a previous study by Wong (8), a potential pET32aΔ*trx* clone, *E.coli* DH5α: SW057, was obtained. To confirm the identity of the clone, the plasmid was isolated along with the wild-type pET32a plasmid and mapped after digestion with the restriction endonucleases RsrII and SacI. Two hundred nanograms of plasmid DNA (pET32a and SW057) was digested with 4 units of RsrII (New England Biolabs, Cat# 5015) and 10 units of SacI (NEB, Cat# R0156S) in NEBuffer 4 (NEB, Cat# B7004S). Reactions were performed in 15 ul total volume, at 37°C for 3.5 h. At the end of the digestion, samples were added with 6x Gel Loading Buffer (5) and resolved on a 1% agarose gel (Agarose 3:1, Amresco, Cat# E776-250) in 1x Tris-Borate-EDTA (TBE) at 150 V.

Amplification of *fre* from pES1. Attempts were made to amplify the 790 bp *fre* sequence from the plasmid pES1, using the forward and reverse primers designed by Kazem (1). The forward primer has the sequence 5'-TAGGGGAATTGTGAGCGGATAAC A-3', and is complementary to the *fre* sequence upstream of the translational start site. The reverse primer has the sequence 5'-GGGTTTTTTCATATGTCCGATAAATGC-3', and contains three mutation sites to introduce an NdeI restriction enzyme site and to change the *fre* stop codon to a glycine residue for generation of a fusion protein with PI2. Refer to the study by Kazem for the details of the primer designing process (1). 30 ul reactions were set up, each at the final concentration of 1x PCR buffer, 3.33 mM MgCl2, 0.25 mM each of the four deoxynucleotide triphosphates, and 0.66 uM of each primer (forward and reverse). One hundred nanograms of uncut pES1 plasmid were used as the template DNA, and 0.5 ul of the Invitrogen Taq polymerase was added (Cat# 18038-042). Gradient PCR was performed in the Biometra T Gradient Apparatus (Whatman, Florham Park, NJ) thermocycler with an initial denaturation period of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45.3°C, 48.0°C, 51.6°C, 55.2°C, or 58.6°C for 1 min, and extension at 72°C for 90 sec. Cycles ended with a final extension period of 7 min at 72°C. The PCR amplified *fre* product was then resolved on a 1% low melting point agarose gel (Amresco, Cat# 0815-25G), in 1x TBE. The DNA band at the 790 bp position was subsequently excised. In order to obtain *fre* product without any trace amounts of the template pES1 plasmid DNA, a new PCR reaction was set up with the same final reagent concentrations as above. The excised agarose gel fragment (790 bp) was stabbed with a needle to pick up trace amounts of the amplified *fre* DNA, and the needle was swirled inside the new PCR reaction tube to transfer the DNA into the mixture to serve as template DNA. Amplification was performed with the same set of temperature and cycle parameters as above, but with the annealing temperature of only 55.2°C. Alternatively, the excised agarose was gel purified, using the Fermentas DNA Extraction Kit (Fermentas, Cat# K0513), and the extracted DNA was used as the template for subsequent PCR re-amplification of *fre* fragment.

Amplification of PI2 from pE32PI2. Primers were designed for amplification of proteinase inhibitor 2 gene from pE32PI2 plasmid. As illustrated in Figure 1, the forward primer contains four nucleotides to the front of the sequence complementary to 5' end of PI2 in order to generate an NcoI restriction endonuclease site, while the reverse primer contains five nucleotides after the sequence complementary to the 3' end of PI2 so that an EcoR1 site is introduced to the amplified PCR product. The NcoI and EcoR1 restriction sites would allow subsequent cloning of the amplified PI2 fragment into the multiple cloning site of pET32a plasmid with directional specificity. The primers were synthesized by Integrated DNA Technologies (Coralville, IA), and reconstituted to stock concentrations of 20 uM. 30 ul reactions were set up, each at the final concentration of 1x PCR buffer, 3.33 mM MgCl2, 0.2 5mM each of the four deoxynucleotide triphosphates, and 0.5 uM of each primer (forward and reverse). One hundred nanograms of uncut pE32PI2 plasmid were used as the template DNA, and 0.5 ul of the Invitrogen Taq polymerase was added. Gradient PCR was performed with an initial denaturation period of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48.0°C, 51.6°C, 53.4°C, 55.2°C, 56.9°C, or 58.6°C for 1 min, and extension at 72°C for 90 sec. Cycles ended with a final extension period of 7 min at 72°C. The PCR amplified PI2 product was then resolved on a 1% low melting point agarose gel (Amresco, Cat# 0815-25G), in 1x TBE. The DNA band at 590 bp position was subsequently excised and gel purified using the Fermentas DNA Extraction Kit (Fermentas, Cat# K0513). The extracted DNA was then used as the template for subsequent PCR re-amplification of PI2 fragment to obtain products clean of initial pE32PI2 template plasmid DNA. Re-amplification

PI2 gene (GenBank Accession Number: **AY517498**)

```
  (start)
1   atggctgttc acaaggaagt taatttcgtt gcttacctac taattgttct tggtaagatt
61  ttcctttact ccttttttttt aaaaaaaata aaaaataaaa aatcttgctt tatatatata
121 tacacaagta gttttatatt tttcctttat attatatttg tttgtaggat tattggtact
181 tgtaagcgcg atggagcatg ttgatgcgaa ggcttgcact ttagaatgtg gtaatcttgg
241 gtttgggata tgcccacgtt cagaaggaag cccggaaaat cgcatatgca ccaactgttg
301 tgcaggttat aaaggttgca attattatag tgcaaatggg gctttcattt gcgaaggaga
361 atctgaccca aaaaacccaa atgtttgccc ccgaaattgt gatacaaata ttgcctattc
421 aaagtgtccc cgttcagaag gaaaatcgct aatttatccc accggatgta ccacatgctg
481 cacagggtac aagggttgct actatttcgg taaaaatggc aagtttgtat gtgaaggaga
541 gagtgatgag cccaaggcaa atatgtaccc tgcaatgtga
                                        (stop)
```

< **Forward Primer Design** >

- Insert four nucleotides to the front of the sequence complementary to 5'end of PI2, in order to engineer an NcoI site (in the right reading frame for expression in pET32a).

[NcoI]
5' – A G C C A T G G C T G T T C A C A A G G A A G T T – 3'
(start)

  - Length: 25; Tm: 62°C; %GC: 48; MW: 7700 g/mol

< **Reverse Primer Design** >

- Insert five nucleotides following the sequence complementary to 3' end of PI2, in order to engineer an EcoR1 site.

[EcoR1]
5' – A A T A T G T A C C T G C A A T G T G A **A T T C G** – 3'

- Since this is the reverse primer, the actual sequence to be ordered is as following:

5' – C G A A T **T C A** C A T T G C A G G G T A C A T A T T – 3'
(stop)

  - Length: 26; Tm: 59°C; %GC: 38; MW: 7700 g/mol

1) Forward primer: 5'-AGCCATGGCTGTTCACAAGGAAGTT-3'
2) Reverse primer: 5'-CGAATTCACATTGCAGGGTACATATT-3'

FIG 1. Sequence of PI2 gene (GenBank Accession: AY517498) and designing of primers for amplification of PI2 from pE32PI2 plasmid and subsequent cloning into the multiple cloning site of pET32a plasmid using NcoI and EcoR1 restriction endonucleases. A PCR product of roughly 590 bp is expected using these primers and the pE32PI2 template DNA.

was performed using the same reagent concentrations and temperature and cycle parameters, but with only one annealing temperature of 55.2°C.

Cloning of PI2 into pET32a and pET32aΔtrx. Re-amplified 590 bp PI2 fragment was directly purified using the Fermentas DNA Extraction Kit (Fermentas, Cat# K0513), without having to be resolved through an agarose gel. Purified PI2 DNA was then digested with NcoI and EcoR1 restriction endonucleases. In a 50 ul reaction, 1 ug of PI2 DNA was digested overnight with 20 units of EcoR1 (NEB, Cat# R0101S) and 20 units of NcoI (NEB, Cat# R0193S) in NEBuffer 3 (Cat# B70035) at 37°C. In separate reactions, vector plasmids – pET32a and pET32aΔ*trx* – were also digested with NcoI and EcoR1. For vector plasmids, 1 ug of DNA was digested with 10 units of EcoR1 and 5 units of NcoI in 20 ul reactions for the same period of time (O/N) at 37°C. The next day, digested products were checked by agarose gel electrophoresis, and the enzymes were inactivated by heating at 65°C for 20 min. Digested and linearized vector plasmids were subsequently treated with Antarctic phosphatase (NEB, Cat# M0289S) to dephosphorylate the cleaved sticky ends of the plasmids to prevent autoligation during the ligation step with the PI2 insert fragment. The use of the Antarctic phosphatase instead of the regular calf thymus alkaline phosphatase was so that the phosphatase enzyme could be heat inactivated more readily prior to the vector-insert ligation process. 20 ul of plasmid digests (1 ug of plasmid DNA) were combined with 0.5 ul of enzyme (5 units/ul), 2.5 ul 10x Reaction Buffer (NEB, Cat# B0289S), and 2 ul water, and were incubated at 37°C for 1 h. The Antarctic phosphatase enzyme was then inactivated by heating at 65°C for 10 min. Ligation reactions were set up as the following. 360 ng of the insert (digested PI2) and 360 ng of digested and phosphatase treated pET32a or pET32aΔtrx were mixed together with 1 ul of ligase enzyme (T4 DNA ligase, Invitrogen, Cat# 15224-025) and 8 ul of 5x ligation buffer (Invitrogen, Cat# Y90001), for a total volume of 40 ul. Reactions were incubated at 14°C overnight.

Transformation of *E.coli* DH5α Cells with Ligation Reaction Mixes. Chemically competent *E.coli* DH5α cells were prepared with calcium chloride treatment (5). One hundred microlitre isolates of competent cells were thawed on ice, and 10 ul (about 180 ng of DNA)

of the 40 ul ligation mix was added to each tube and gently mixed by swirling the piepette tip around. To another 100 ul of thawed competent cells, 200 ng of uncut pET32a plasmid DNA was added, to serve as a positive transformation control. The cells were incubated on ice for 30 min and heat shocked at precisely 42°C for exactly 90 sec, followed by another 10 min on ice. Two hundred and fifty microlitres of LB broth without any antibiotics was added to each tube and the transformed cells were incubated at 37°C for 3 h with shaking. After the outgrowth period, everything from each tube was plated onto an LB agar plate supplemented with 100 ug/ml of ampicilin, and incubated overnight at 37°C.

RESULTS

Confirming the Identity of *E.coli* DH5α:SW057, a Potential pET32aΔ*trx* Clone. Restriction mapping of pET32a and SW057 with the RsrII and SacI restriction endonucleases is illustrated by Figure 2. Since the RsrII restriction endonuclease site occurs only once in the pET32a plasmid – within the *trxA* region – and the SacI site occurs once outside of the *trxA* region, digesting pET32a with the two enzymes would yield two DNA bands while pET32aΔ*trx* digested with RsrII and SacI would only give a single DNA band. As expected, digest of wild-type pET32a plasmid (Lane 2) contains two distinct DNA bands, one between the 5 kb and 7 kb bands of the molecular weight marker, and the other at further down the gel, around 400 bp position. Treatment of the potential pET32Δ*trx* plasmid from *E.coli* DH5α: SW057 clone with the same two enzymes (RsrII and SacI; Lane 3), also as anticipated, yielded a single DNA band at approximately the same position as the large band in Lane 2.

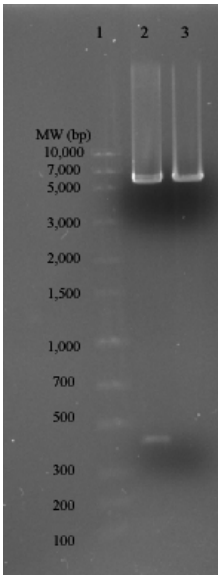

FIG. 2. RsrII and SacI digest of pET32a and SW057 (potential pET32aΔ*trx*) plasmids. Lane 1 contains 4 ul of DNA Ladder Mix (MassRuler, Fermentas, Cat# SM1282). Lane 2 and 3 are digests of pET32a and SW057, respectively. Both lanes (2 and 3) have a band at approximately 5.5 kb, but lane 2 contains a second band at about 400 bp position.

Amplification of *fre* from pES1. Despite repeated attempts with varied cycles and temperature conditions and reagent concentrations, amplification of *fre* from pES1 failed to yield a single specific product at 790 bp, the expected size of amplified *fre* fragment. As can be seen in Figure 3, multiple DNA bands are found in close proximity of one another at all annealing temperatures tested. All lanes contain a distinct DNA band along the 700 bp position. Lanes 3 to 6 show a band at a position expected of the 790 bp *fre* fragment, with the highest signal intensity being observed at the annealing temperature of 55.2°C (Lane 5). Smaller DNA fragments are also found. Lanes 2 to 4 possess a band slightly below the 500 bp molecular weight position. Faint traces of a DNA band can be found in lanes 3, 4, and 6 between 200 and 300 bp molecular weight markers. Lane 4 has yet another DNA band with the size of between 100 and 200 bp. Accumulation of unused deoxynucleotide triphosphate molecules is very apparent in all five lanes, indicated by the presence of strong intensity bands at a position below the 100 bp molecular weight marker. Overall, no apparent relationship seemed to exist between the annealing temperature and the signal intensity or the number of DNA bands produced.

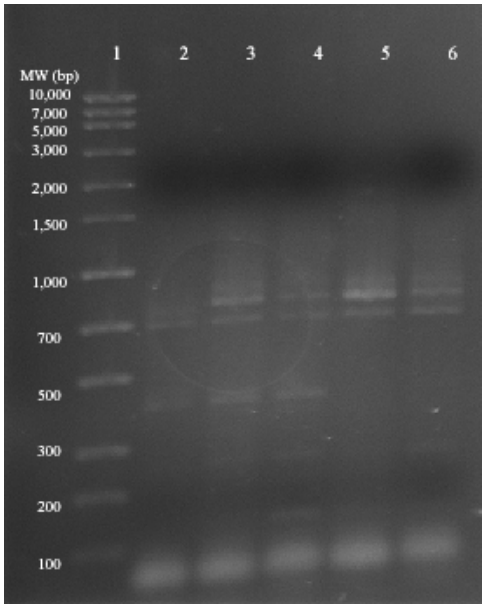

FIG 3. Amplification of *fre* from pES1 plasmid via gradient PCR. Lane 1 contains 5 ul of the Fermentas MassRuler DNA Ladder Mix. Lanes 2 to 6 represent a gradient of five different annealing temperatures between 45°C and 60°C – 45.3°C, 48°C, 51.6°C, 55.2°C, and 58.6°C, respectively. All reactions were done with 100 ng of pES1 plasmid as template DNA, with reagent concentrations and temperature and cycle parameters outlined in Materials and Methods section. All five lanes show the presence of multiple DNA bands at various positions, with high concentrations of unused deoxynucleotide triphosphates accumulated at the bottom of the gel.

Amplification of PI2 from pE32PI2. Amplification of PI2 from pE32PI2 plasmid was successful at all annealing temperatures tested, with a 590 bp DNA band indicative of a single PI2 amplification product uniformly observed in all six lanes (Fig.4a). When the gel picture was taken immediately after the samples had been resolved, the bands seen at the 590 bp position were completely blocked by the dye front and could not be visualized (data not shown). Therefore, the gel was left in distilled water overnight, and a new picture was taken the next morning (Fig.4a). No dyes were observed that could interfere with band visualization, but the DNA molecules themselves, especially the small DNA fragments, seemed to be diffusing out of agarose, thereby resulting in blurry dull bands seen in Fig 4a. This fuzziness made relative comparison between samples in different lanes impossible, but by adjusting the exposure level, it could be estimated that highest level of amplification was achieved in the sample in lane 6, with the annealing temperature of 55.2°C. All samples seen in Fig 4a contain not only the amplified PI2 DNA, but also the pE32PI2 template plasmid DNA (seen at above the 10,000 bp molecular weight marker; slow migration down the gel due to coiling and/or supercoiling), which may interfere with subsequent applications of the amplified PCR product. Therefore, these samples were purified directly using the Fermentas DNA Extraction Kit, without being resolved through an agarose gel, and then used as template DNA in subsequent amplification of PI2. Figure 4b illustrates the result of this re-amplification experiment, which shows a single amplification product at the same 590 bp position, without any trace amounts of the original pE32PI2 template plasmid DNA.

Construction of pET32a/PI2$^+$ and pET32aΔ*trx*/PI2$^+$. Re-amplified PI2 DNA was purified (with Fermentas DNA Extraction Kit) for subsequent reaction with restriction endonucleases EcoR1 and NcoI. Two vector plasmids, pET32a and pET32aΔ*trx* were also digested with the same restriction endonucleases, and were treated with phosphatase enzyme to prevent autoligation. Digested insert DNA, PI2, and vector DNA, pET32a or pET32aΔ*trx* were then ligated overnight at 14°C. Same amount of insert and vector DNA were mixed together (360 ng each). Competent *E.coli* DH5α cells were transformed with a portion of the ligation mix (total of 180 ng DNA), and grown on LB agar plates containing ampicilin overnight. The positive transformation control plate (cells transformed with uncut pET32a plasmid) resulted in numerous colonies throughout the plate. Very few colonies were observed on plates of ligation mix-transfored cells. Two potential clones with the pET32a/PI2$^+$ recombinant plasmid were isolated and named "JP061" and "JP063". Two potential clones with the pET32aΔ*trx*/PI2$^+$ recombinant plasmid were also isolated and named "JP062" and "JP064".

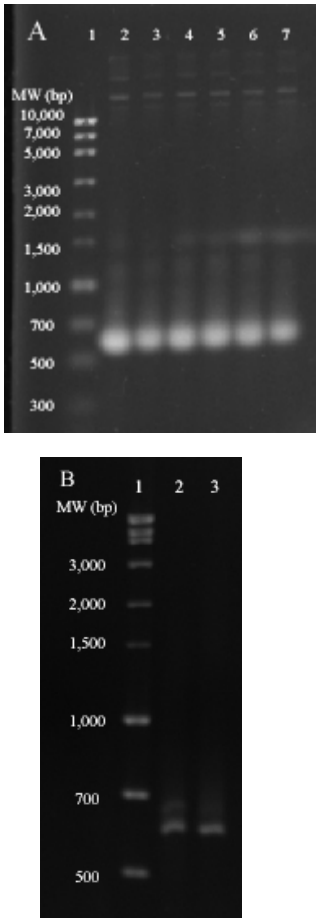

FIG 4. A. Amplification of PI2 from pE32PI2 plasmid via gradient PCR. Lane 1 contains 5ul of Fermentas MassRuler DNA Ladder Mix. Lanes 2 to 7 represent a gradient of six different annealing temperatures between 45°C and 60°C – 48°C, 51.6°C, 53.4°C, 55.2°C, 56.9°C and 58.6°C, respectively. All lanes show a single positive amplification product at the expected size of approximately 590 bp. Various forms of the pE32PI2 template plasmid are also observed in all lanes (2 to 7). All reactions were done with 100 ng of pE32PI2 plasmid as template DNA, with reagent concentrations and temperature and cycle parameters outlined in Materials and Methods section. **B.** Amplification of PI2 using a purified PI2 PCR product (using the Fermentas DNA Extraction Kit; direct purification without gel electrophoresis) as the template DNA. All reaction conditions and temperature and cycle parameters remained the same, with annealing temperature of 55.2°C. Lane 1 contains 5 ul of Fermantas MassRuler DNA Ladder Mix. Lanes 2 and 3 contain duplicate PCR reaction samples. Both lanes show a single DNA band at the expected position of approximately 590 bp. This time, no signs of the pE32PI2 plasmid DNA are observed.

DISCUSSION

Wild type pET32a plasmid is 5900 bp long. The *trxA* region constitutes 330 bp, so pET32Δ*trx* plasmid would be that much smaller, at about 5570 bp. The two

restriction endonucleases, RsrII and SacI, were chosen because the two plasmids would produce different band patterns when digested with the two given enzymes. More specifically, as the RsrII restriction endonuclease site only occurs within the *trxA* sequence (at position 589 on vector map; refer to Novagen (3)), only the wild type pET32a with the *trxA* sequence intact would be subject to cleavage by RsrII. SacI would also cut the plasmid only once, but its restriction site occurs outside of the *trxA* sequence (at position 190 on vector map), thereby making both pET32a and pET32aΔ*trx* subject to cleavage. As such, when pET32a plasmid is digested with RsrII and SacI, two DNA fragments would result, one approximately 400 bp long and the other 5500 bp. On the other hand, pET32aΔ*trx* is only cleaved by SacI, so it would simply be linearized and migrate to its appropriate size position, approximately 5570 bp. These are the exact banding patterns observed in Figure 2. Sample in lane 2, digested pET32a, show two DNA bands, one between 5 kb and 7 kb size markers, and the other between 300 bp and 500 bp markers. In lane 3, the digest of SW057 plasmid shows a single large DNA band between 5 kb and 7 kb size markers. These results indicate and confirm the probably absence of the *trxA* sequence in the plasmid in strain SW057.

Attempts to amplify *fre* from pES1 failed repeatedly throughout the study. Amplification of multiple DNA fragments indicates low reaction specificity, which is usually observed at low annealing temperatures as primers bind to non-specific regions on template DNA. In this case however, because multiple bands are observed at all annealing temperatures tested (up to 58.6°C), the temperature by itself does not explain why non-specific amplification may have occurred. In addition, there is no apparent relationship between DNA fragment signal intensity and annealing temperature. Looking at the DNA fragment slightly above the 700 bp position, which is suspected to be the fragment of interest (790 bp), high signal intensity is observed at annealing temperature of 48°C, but the intensity decreases significantly at 51.6°C, comes back strong at a higher temperature of 55.2°C, and dies down again at 58.6°C. These observations are very strange, especially when compared to previous results obtained by Kazem (1), Wong (8), or Shah (6), all of whom reported successful amplification of *fre* from pES1 using the same set of primers and nearly identical experimental conditions and parameters. Any concerns with PCR reagents can be disregarded as they produced a positive amplification product for PI2 (Fig.4). Regardless, a few explanations may be suggested for the results observed in this study. First of all, the forward and reverse primers may have been degraded or contaminated over extended period of usage and storage. Since the specificity and success of PCR depends critically on the specificity and composition of primers, any problem with the primers may directly result in amplification failure or amplification of multiple DNA products. For future attempts, a new batch of forward and reverse *fre* primers should be ordered and used. Another speculation was that there may be certain structural features of the pES1 template plasmid DNA that led to poor specificity and efficiency (as suggested by accumulation of unused dNTPs) in primer binding, such as an internal hairpin loop structure within the plasmid. However, the validity of this speculation is questionable, as such observations (poor primer binding specificity and efficiency) were never made in previous studies where *fre* amplification was attempted.

Contrary to *fre*, PI2 was amplified successfully to yield a single specific product of expected size at 590 bp. Figure 4 illustrates very clearly the specificity and efficiency of PI2 amplification, which was confirmed over and over again in repeated experiments. Newly synthesized forward and reverse primers were designed so that restriction endonuclease recognition sites – NcoI and EcoR1 – could be engineered at the ends of the sequence for insertion into the vector plasmid in the right reading frame. The number of nucleotide sequences not complementary to the target sequence was minimized, with only a four nucleotide overhang in the forward primer and a five in the reverse primer. It should be noted that the forward primer has two nucleotides to the front of the NcoI recognition sequence, and the reverse primer has one nucleotide following the EcoR1 recognition sequence. The presence of these nucleotides before and after the respective enzyme recognition sites would ensure efficient cleavage of the amplified PI2 fragment by the two restriction enzymes. In other words, if the amplified PI2 fragment was to start and end with an NcoI and EcoR1 enzyme sites, respectively, the restriction enzyme cleavage efficiency would be reduced to 50% or less, and subsequent ligation reaction efficiency would also be affected.

With the success of PI2 amplification and re-amplification following DNA purification, the PI2 fragment was ready to be cloned into the two vector plasmids available, pET32a and pET32aΔ*trx*. Both the insert DNA (PI2) and the vector plasmids were digested with NcoI and EcoR1, and the efficiency of enzyme cleavage was checked by gel electrophoresis (data not shown). Following Antarctic phosphatase treatment of the digested vector plasmids, ligation reactions were set up as outlined in Materials and Methods. Based on the results from transformed *E.coli* cells, it seems that ideal ligation conditions were met when the reaction was incubated overnight at 14°C, in 40 ul total volume, with 1 ul of T4 DNA ligase from Invitrogen and the same amount (in ng, not the molar

ratio) of insert and vector DNA. As two unique enzyme sites with sticky ends were utilized, any insert-vector ligation would occur with directional specificity (i.e. PI2 would insert in only one direction). Transformation of *E.coli* DH5α cells were done chemically, but very few colonies (less than 5) were found on agar plates. Even the positive control plate, plated with cells transformed with uncut pET32a vector plasmid, did not have nearly as many colonies as would be normally expected. Such poor transformation efficiency might be due to growth of transformed *E.coli* cells in microfuge tubes during the outgrowth period following the final incubation step on ice. Cells should have been transferred to appropriate glass or plastic culture tubes to provide sufficient oxygenation for recovery and growth. With narrow, pointy tip at the bottom, microfuge tubes do not provide optimal surroundings for bacterial growth. Cells may have simply sunk to the bottom without much room for growth, which may explain why so few colonies appeared on agar plates. Nevertheless, potential clones were identified and isolated, ready for screening. Due to time limitation, screening of the four clones, JP061~JP064 was not performed, but that would be the logical step to be followed.

FUTURE EXPERIMENTS

Potential pET32a/PI2$^+$ and pET32aΔ*trx*/PI2$^+$ clones, JP061 and JP063, and JP062 and JP064, respectively, must be checked to confirm their identities. To do so, the clones' plasmids should be prepared, digested with NcoI and EcoR1, and resolved through an agarose gel. Digested pET32a/PI2$^+$ plasmid would show two DNA bands on gel, one at 5900 bp and the other at 590 bp. Digested pET32aΔ*trx*/PI2$^+$ plasmid would also show the band at 590 bp, but the other larger fragment would be at 5600 bp, as it lacks the *trxA* sequence. Next step would be to generate the missing construct, the pET32a/*fre*$^+$/PI2$^+$ plasmid. As noted in this study, however, PCR amplification of *fre* is the first thing that must be accomplished. Whether the problem is with the primers, the template DNA, or whatever else, *fre* must be amplified and inserted into the pET32aΔ*trx* plasmid via restriction digest with NdeI enzyme. It should be paid attention, though, that the process of cloning amplified *fre* into pET32aΔ*trx* using NdeI digestion has been the most troublesome and technically challenging step in most, if not all, previous studies on this topic (1, 6, 8). Also, because the PI2 DNA sequence contains an NdeI restriction site, cloning of *fre* into pET32aΔ*trx* must occur prior to insertion of the PI2 fragment. Having said that, once *fre* is correctly inserted into the Δ*trx* plasmid, cloning of PI2 into the recombinant plasmid should pose no technical hurdles. Once all constructs are generated and their identities confirmed, they should then be transformed into *E.coli* BL21 cells for protein expression studies. At that point, assays can be established to measure and directly compare the solubilities of PI2 expressed different conditions – by itself, fused to Trx, or fused to Fre.

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