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Cloning EDTA monooxygenase as a Model Protein to Characterize the Effects of Flavin oxidoreductase on Solubility of Proteins in Protein Overexpression Systems

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***Escherichia coli* thiol reductase has been utilized by Novagen to create an expression system for insoluble and poorly soluble proteins which normally form aggregates when overexpressed and become inactivated. Believing that the redox state maybe responsible for the solubilizing mechanism, we conducted the following study to assess the role of *E. coli* flavin oxidoreductase in increasing solubility of such proteins. Primers were designed for amplification of *E. coli* flavin oxidoreductase. We attempted replacing the thiol reductase sequence in the Novagen vector with the amplified sequence. Due to time limitations, we were only able to construct a control vector with an insoluble protein sequence, EDTA monooxygenase A. Construction of other vectors were attempted but failed due to specific properties of restriction enzymes used. Further work is required to complete the constructs necessary to fully examine the effects of flavin oxidoreductase on protein solubility.**

Aggregation of over-expressed proteins is a problem in protein overexpression systems that contributes to inactivation of the protein (3). Thiol reductase has been shown to prevent formation of aggregates in protein overexpression systems (5). In a study done by Yasukawa et al., several mammalian proteins including mouse c-Myb, cAMP response element-binding protein 1 (CRE-BP1), the p53 tumor suppressor gene product, ser/thr kinases, and *Xenopus mos* proto-oncogene product (Mos) were assessed in terms of their solubility in presence and absence of Thiol reductase (Trx) or *E.coli* chaperon GroESL (5). These proteins formed insoluble aggregates when produced in the absence of either protein. Nevertheless, Yasukawa et al. report that four out of eight proteins examined had an increase in solubility after being co-expressed with the GroESL or the Trx (5). The two proteins that increased in solubility the most when co-expressed with Trx were c-Myb and p53 tumor suppressor gene product (5). The exact mechanism of resolving aggregates is not understood but it appears that the redox state may be responsible for the state of solubility. Based on these results and the incorporation of Trx in a Novagen vector which also increases solubility, we hypothesized that perhaps flavin oxidoreductase (Fre) may also demonstrate this effect when co-expressed with insoluble proteins. However, we were uncertain whether the potential benefit needed the enzyme expressed separately or needed the enzyme to be expressed as a fusion protein with our model insoluble protein.

To test these possibilities, we intended to generate two sets of constructs; one with the protein being expressed as a fusion of Fre and one with the protein being independently but co-expressed with the Fre. To generate these constructs, we designed sense and anti sense primers (Fig. 1) and amplified the *fre* gene out of pES1 plasmid, kindly provided by Tai Man Louie. An Nde1 restriction site and an EcoR1 restriction site flank the *fre* sequence. We then obtained the *trx* containing Novagen plasmid, pET32a (Fig. 2). We intended to remove the *trx* from the pET32a and replace it with the *fre*. Two Nde1 sites flank the *trx* in the plasmid hence we designed primers to change the EcoR1 restriction site of the *fre* to an Nde1 site (2).

Upon removal of the *trx*, we intended to make three constructs: one construct with the *fre* and our insoluble protein, EDTA monooxygenase A (*emoA*) as a fusion protein; one construct containing the *fre* only and one construct with the *emoA* alone. We intended to express these constructs in BL21(DE3) (4) and assess the amount of expressed protein solubilized in the broth and compare it with the amounts in the insoluble pellet with each of the constructs. We expect to see more EmoA in the insoluble fraction of the culture with the EmoA alone in pET32aΔTrx compared to that of pET32aΔTrx/Fre-EmoA (pETFre/EmoA). We expect to see no EmoA in the insoluble fraction of pET32aΔTrx/Fre (pETFre). We started by making the constructs but due to time limitation we were only able to make pET32aΔTrx-EmoA.

FIG. 1 Flavin oxidoreductase (*fre*) sequence in the plasmid pES1 and primer designs. The yellow highlighted region is the primer-binding site of the Fre region, giving rise to a PCR fragment of approximately 790 bp. The forward primer is complementary to the *fre* sequence while the reverse primer contains three mutation sites to change the *fre* stop codon to a glycine for generation of a fusion protein with EmoA and to create an Nde1 restriction site.

MATERIALS AND METHODS

Preparation of competent DH5α cells.500 mls of DH5α cells were grown in LB broth (containing 10g of tryptone, 5g of yeast extract, and 10g of NaCl) overnight. The culture was harvested then washed with 25 mls of cold 100mM $MgCl_2$ two times. The culture was then washed with 25 mls of 100mM $CaCl_2$. After this final wash the culture was re suspended in 20 mls of 100mM $CaCl_2$ containing 10% glycerol and aliquoted into 200µl aliquots, then stored at -70°C. This method was adopted from Shierley Chelliah, Technician, working at the Biomedical Research Centre at UBC.

Amplification of the *fre* fragment from pES1. Primers were designed using Lasergene software (by DNASTAR Inc.) and synthesized by Nucleic Acid Protein Service (NAPS) for amplification of the *fre* fragment from the pES1 plasmid. Figure 1 shows the binding site and the sequence of the forward and reverse primers. The reverse primer contains three mutations to create an Nde1 site and to change the stop codon at the end of the *fre* sequence into a glycine to allow generation of a fusion protein. Primers were hydrated to 20µM concentrations. 3 µl, 4 µl or 5 µl of uncut pES1 was added to the PCR Master Mix (Table 1). The *fre* was amplified over 40 cycles in the thermocycler. During each cycle the DNA was denatured at 94°C, annealed to primers at 54°C, and was extended at 72°C (Table 1).

Cloning of *fre* fragment into pET32aΔTrx. 2 µg of pET32a plasmid was digested with Nde1 which flanks the *trx* coding region of the plasmid. The digest was left overnight at 37°C. The digest was cleaned using Qiagen Reaction Cleanup Kit. The amplified *fre* fragment from pES1 was purified using Qiaquick PCR Purification Kit. Several ligation reactions were set up as outlined in table 2 to assess which conditions result in highest ligation efficiency. 2µl of the ligation reaction was used to transform chemically competent

TABLE 1. PCR conditions for *fre* amplification.

Master Mix		Temperature Parameters	
Reagents	Vol/rxn	Temp.	Duration
20µM Forward Primer	1 µl	1) 94°C	3 min
20µM Reverse Primer	1 µl	2) 94°C	30 sec
50 mM $MgCl_2$	2 µl	3) 54°C	1 min
10X PCR Buffer	3 µl	4) 72°C	90 sec
10mM dNTP	3 µl	5) Return to 2	40 cycles
Taq polymerase	0.25 µl	6) 72°C	10 min
dH_2O	14.75 µl	7) 4°C	Until end
Total volume	30 µl		

DH5α cells. Later, the same experimental conditions were tested except the purified pET32a digest and the purified Fre PCR product digest were heated to 95°C and cooled to 25°C at a very slow rate.

Transformation of DH5α cells. 2µl of ligation reaction was added to 100µl of chemically competent cells. The mixture was incubated on ice for 10 minutes followed by a heat shock at 42°C for 90 seconds. The mixture was then incubated for an additional 10 minutes on ice. 1 ml of SOC high nutrient broth, without any antibiotics, was then added and the cells were incubated in a 37°C water bath for 2-3 hours. After incubation at 37°C, 200µl of the mixture was plated on an agar plate with 50µg/ml concentration of ampicillin. The plates were then incubated overnight in a 37°C incubator.

Cloning of *emoA* fragment into pET32aΔTrx. 1 µg of pET32aΔTrx plasmid, kindly provided by Aileen Kartono, was digested with EcoR1 and incubated overnight at 37°C. The digest was cleaned using Qiagen Reaction Cleanup Kit. 3 µg of pEmoA plasmid was also digested with EcoR1 overnight at 37°C. The digest was run on 0.9% Tris-Boric acid-EDTA (TBE) agarose gel and the 2.4 kb EmoA fragment was gel purified using Qiagen Gel Purification Kit. Several ligation reactions with different insert to vector ratios were set up as outlined in table 3 to assess which conditions result in highest ligation efficiency. 2µl of each ligation reaction was used to transform chemically competent DH5α cells.

Screening of potential positive clones. 39 colonies were picked from plates transformed by ligation reaction 1a (1-23) and ligation reaction 1b (A-P). The colonies were grown in 1ml of LB broth with 50µg/ml Ampicillin overnight. The next morning, 700 µl of the culture was used for a plasmid prep using Gibco CONCERT Miniprep system. To minimize the overall size of the screening, 700µl of the cultures were pooled together in groups of 4. The isolated plasmids were then digested with Hind III. They were incubated overnight at 37°C and resolved on a 0.8% TBE agarose gel.

RESULTS

Amplification of *fre* fragment from pES1. Following the PCR and thermocycling conditions outlined in table 1, we were able to successfully amplify *fre* from the plasmid. The reactions that contained at least 4 µl of the template had a product (Fig. 3). When the product was purified with Qiagen PCR Purification Kit and run on a gel, the streaking and smearing effect, observed in Figure 3, was removed (data not shown).

Cloning of *fre* fragment into pET32aΔTrx. None of the ligation conditions tested resulted in appearance of any colonies after the reaction was used to transform chemically competent cells. The control used was transformation of DH5α cells with an uncut plasmid (containing the Amp resistance sequence). The control plate had numerous colonies. The results were consistent for every reaction with the control plate positive and the ligation-mix transformed plate negative.

Cloning of *emoA* fragment into pET32aΔTrx. The gel purified *emoA* fragment was successfully ligated into pET32aΔTrx. The three sets of reactions contained a 5:1, 10:1, and 1:1 insert of vector ratio, respectively. All three sets of transformations produced colonies on 50µg/ml Ampicillin plates. The ligation reactions incubated at 4°C yielded higher number of colonies compared with their counterparts, incubated at 25°C.

Screening of potential positive colonies. The ligation set with a 5:1 insert to vector ratio was chosen to be screened for presence of *emoA* positive colonies. The expected fragment sizes after digestion with HindIII were a 6.6-7.8 kb and a 260-1460 bp fragment if one copy of the *emoA* was ligated into pET32aΔTrx in the correct direction (Fig. 4A and 4B). The range is due to a 1.2 kb size difference between the coding sequence of *emoA* (1.2kb) and the size of the EcoR1 pEmoA fragment containing the *emoA* (2.4 kb). This range accounts for the extreme case scenario where the extra 1.2 kb of the 2.4 kb *emoA* is located entirely 5' or 3' to the HindIII cut site. When the pooled digests were resolved on a 0.8% TBE agarose gel, we identified two sets that exhibited the proper band pattern (Fig. 5). We did not expect to be able to see the lower molecular weight bands as they would run off of the gel. Pooled colonies 21-23 and I-L contained bands that were approximately 6.6 to 7.8 kb in size. The remainder of the individual cultures from colonies 21-23 and I-L were not tested and plasmid isolation needs to be done individually for each culture to determine which cultures contain the construct. Since many bands of the

TABLE 2. Ligation reactions of *fre* and gel purified ΔTrx pET32a. Transformation of each reaction was coupled with a control transformation of an uncut plasmid (pES1) to assess the viability of the cells.

Reagents	Reaction 1	Reaction 2	Reaction 3	Reaction 4	Reaction 5
Insert (*fre*)	3 µl	6 µl	9 µl	9 µl	9 µl
Vector (ΔTrx pET32a)	1 µl	2 µl	3 µl	3 µl	3 µl
Buffer	4 µl	4 µl	4 µl	4 µl	4 µl
Ligase	2 µl	2 µl	2 µl	1 µl	1 µl
dH_2O	10 µl	6 µl	2 µl	3 µl	3 µl
Total volume	20 µl	20 µl	20 µl	20 µl	20 µl
Ligation temperature and duration	15°C 24h	15°C 24h	15°C 24h	25°C 20 min	25°C 16h

TABLE 3. Ligation reaction of *emoA* and ΔTrx pET32a. A control transformation of pEmoA was included to assess viability of the cells and the number of colonies expected in presence of actively expressed EmoA.

Reagents	Reaction 1a	Reaction 1b	Reaction 2a	Reaction 2b	Reaction 3a	Reaction 3b
Vector (ΔTrx pET32a)	3 µl	3 µl	4 µl	4 µl	2 µl	2 µl
Insert (*emoA*)	9 µl	9 µl	20 µl	20 µl	2 µl	2 µl
Buffer	4 µl	4 µl	8 µl	8 µl	4 µl	4 µl
Ligase	1 µl	1 µl	2 µl	2 µl	1 µl	1 µl
dH_2O	3 µl	3 µl	10 µl	10 µl	11 µl	11 µl
Total volume	20 µl	20 µl	40 µl	40 µl	20 µl	20 µl
Ligation temperature and duration	4°C 72h	25°C 72h	4°C 72h	25°C 72h	4°C 72h	25°C 72h

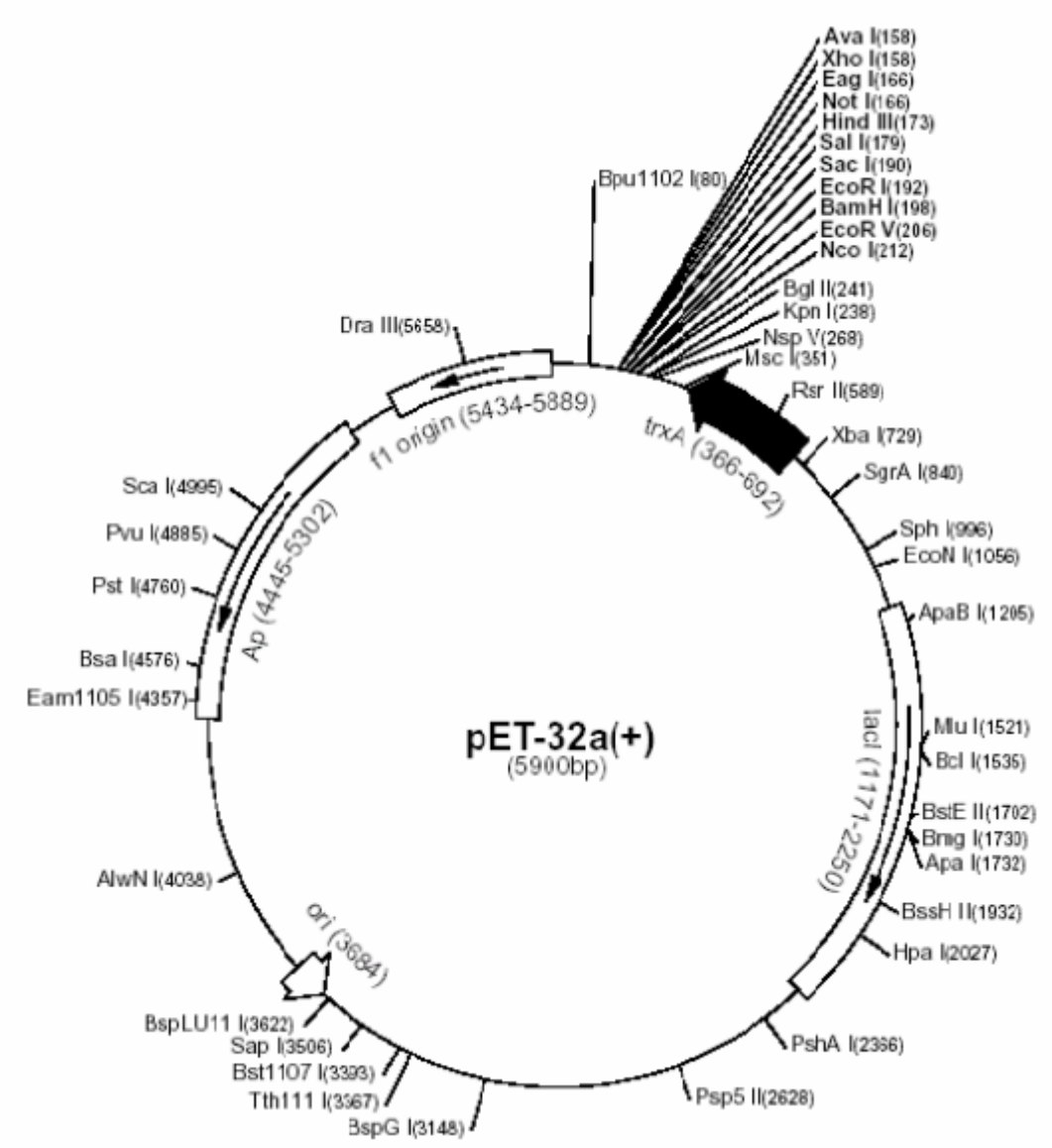

FIG. 2 Novagen pET32a vector (2). The *trxA* sequence is flanked by two Nde1 restriction sites, which was utilized for replacing the *trx* with the *fre* sequence.

1 kb plus molecular marker did not resolve properly on the gel, the exact band sizes could not be determined.

DISCUSSION

Amplification of the *fre* fragment from pES1. The designed forward primer targeted the starting sequence of the *fre* in pES1 plasmid and was of optimal length and melting temperature and near optimal GC content thus as we expected it did bind to the sequence. The reverse primer targeted the end sequence of the *fre* as well as some residual pES1 sequence. This design was carried out in order to enable us to induce mutations in the amplified fragment. The mutations were induced to change the stop codon at the end of the *fre* sequence into a glycine codon. This would enable us to create a fusion protein once the *fre* and the *emoA* sequences were cloned into pET32aΔTrx. The other mutation sites were created to change an EcoR1 site to an Nde1 site at the end of the *fre* sequence to simplify cloning of the fragment into the plasmid. The reverse primer was of optimal length and melting temperature while the GC content was low. We attempted several other sequences with higher GC contents however, they

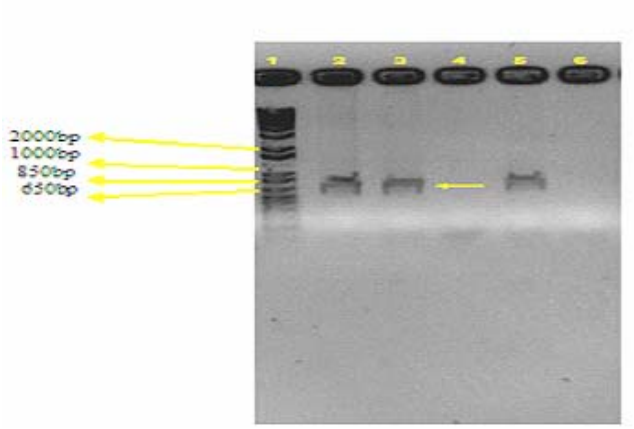

FIG. 3 Amplification of the *fre* fragment from pES1. Lane 1 contains 2 µg of Invitrogen's 1kb plus molecular marker. Lanes 2-6 are the different PCR reactions. Lane 2 contains the reaction with 5µl of the template, lanes 3 and 5 contain the reactions with 4µl of the template, and lanes 4 and 6 contain the reactions with 3µl of the template. No product was observed in lanes 4 and 6. The arrow points to the amplified band in lane 3 which was sized and determined to be approximately 788 bp (Appendix 1).

were not expected to bind to the sequence. Using these primers and the PCR conditions outlined in table 1, we

were able to generate a fragment of 788bp (Fig. 3) which we suspect to be *fre*. However, since our molecular weight standard did not resolve very well on the gel we cannot be sure of the real size of the fragment and it may or may not be *fre*. A reliable confirmation method would have been sequencing this fragment after cloning it into a TOPO vector. We were unable to confirm by sequencing due to shortage of time.

Cloning of *fre* fragment into pET32aΔTrx. The amplified *fre* fragment contained two Nde1 sites, one at each end. We intended to use these sites to create a sticky end in the vector and in the fragments and ease the ligation of the two. We initially attempted the ligation by digesting pET32a with Nde1 and gel purifying it. Since the *trx* sequence in the pET32a is also flanked by two Nde1 sites (which are the only two Nde1 sites in the plasmid) we anticipated that the large gel purified fragment to be pET32aΔTrx. We attempted to ligate this fragment with the Nde1 digested *fre* PCR fragment however the two pieces probably never ligated as no colonies grew on the plates after being transformed by the ligation reaction. We tried a variety of conditions, changing ligation temperatures, ligation times, and even the ligase and its buffer to assess if working conditions could be achieved. After failure of all the tested conditions, we suspected the role of the enzyme since the same enzyme was used to generate the digested PCR fragment and the pET32a. We hypothesized that a part of the enzyme may be interacting with our sequences and preventing the ligation. To address this, we heated the purified PCR and pET32aΔTrx fragments to 95°C in order to disrupt any DNA/protein interactions. The fragments were then allowed to cool to 25°C at a slow rate to allow re-annealing of their DNA since at such high temperature the fragments would be denatured as well. However, despite this modification to our protocol, we still were not able to ligate the Nde1 digested *fre* into the Nde1 digested pET32aΔTrx. Perhaps an alternate method to address our concern with the enzyme would have been using an Isoschizomer of Nde1 such as BfuC1. If this construct was successfully made and expressed we would have expected to obtain no EmoA in either the soluble fraction or the insoluble fraction of our expression system.

Cloning of *emoA* fragment into pET32aΔTrx. This ligation was completed successfully as all of the ligation reactions with different conditions yielded colonies upon transformation into DH5α cells. Our only concern was the directionality of the insert since the *emoA* fragment was flanked by EcoR1 restriction sites and could have ligated into pET32aΔTrx in either direction. To address this concern, we chose a restriction enzyme that created an asymmetric cut in the plasmid and in the *emoA* sequence to tell us its direction.

Screening of potential positive colonies. These colonies were chosen from the DH5α plate which was transformed by the ligation reaction with 5:1 insert to vector ratio. We deliberately chose this plate since we anticipated ligation of more than one fragment into the vector. The plate with the 1:1 ratio would have potentially contained many colonies with circularized pET32aΔTrx without any *emoA* being ligated into the plasmid. The coding sequence of *emoA* is 1.2 kb while the *emoA* fragment cloned into pEmoA is 2.4 kb (1). We were unable to determine the location of the extra 1.2 kb in the sequence hence, we included it in our calculations of the expected fragment size of the right construct. On a 0.8% TBE agarose gel, we expected to observe a band of 6.607 to 7.807 kb in size for the plasmid that contained 1 copy of *emoA* inserted in the right direction. If the direction was reverse, on the same gel, we expected two bands in the size ranges of 1.033 to 2.233 kb and 5.834-7.034 kb. Since the band sizes in the plasmid pools for clones 21-23 and I-L were greater than 2.0 kb, we considered these to be potentially positive colonies. Due to poor separation of the bands in the samples and the molecular weight marker we could not size these bands to determine if they were truly within our expected range. An alternative to this method would have been sending each individual construct of the seven possible positives for sequencing.

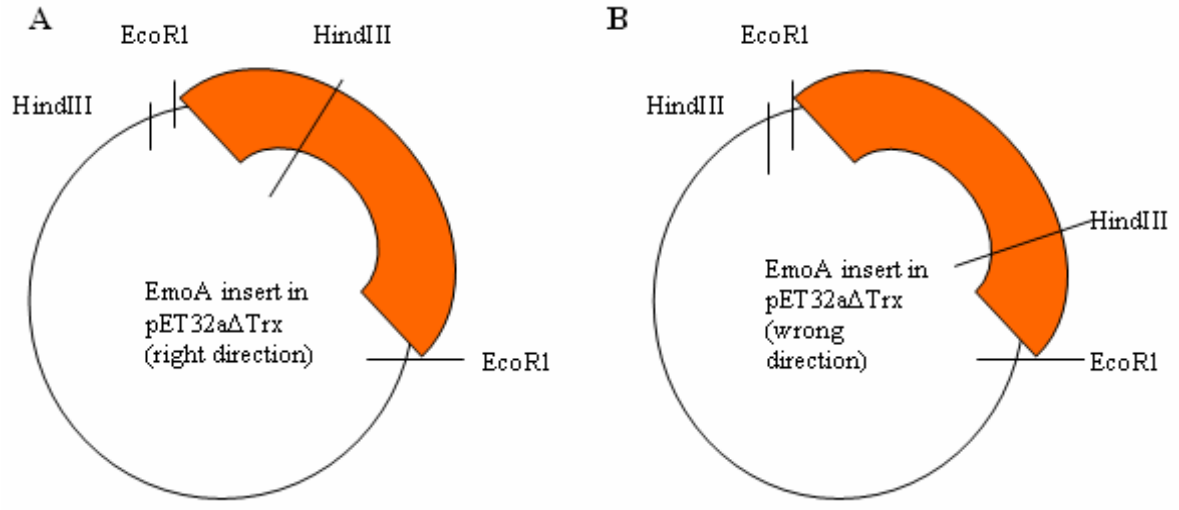

FIG. 4 Map of pET32aΔTrx/EmoA. Due to uncertainty in the potential size of the clone, one fragment would be 0.26 kb to 1.46 kb and the other would be 6.607 to 7.807 kb in size when digested with HindIII, if *emoA* is inserted in the proper direction (A). If the *emoA* fragment is inserted in the opposite direction (B) the resulting fragments from a HindIII digestion are expected to be 1.033 to 2.233 kb and 5.834 to 7.034 kb. The shaded region represents the *emoA* sequence.

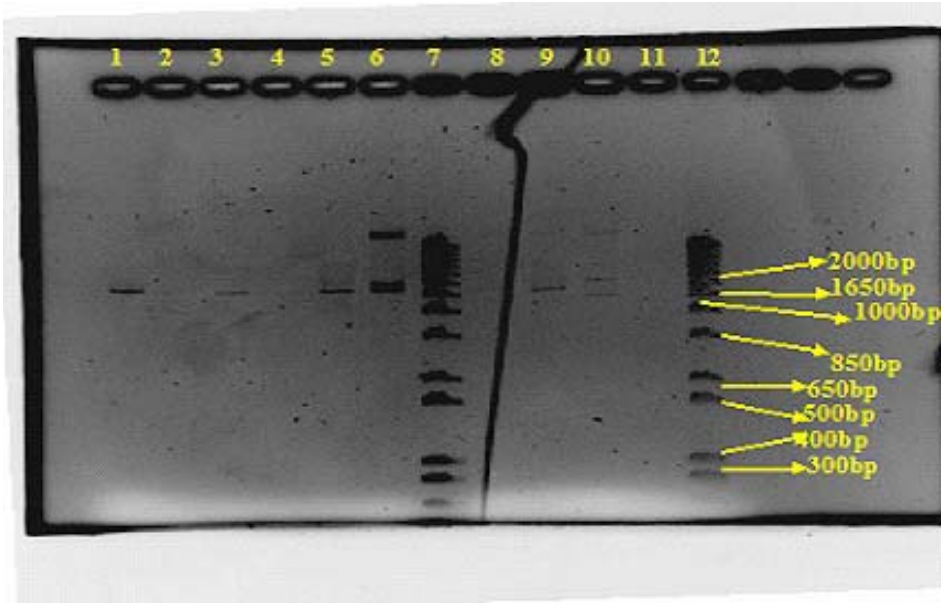

FIG. 5 HindIII digest of potential positive colonies. Lanes 7 and 12 contain 2 µg of 1kb plus DNA ladder. Lane one contains plasmid from clones 1-4, lane two is plasmids from clones 5-8, lane three is plasmids from clones 9-12, lane four is plasmids from clones 13-16, lane five is plasmids from clones 17-20, lane six is plasmids from clones 21-23, lane eight is plasmids from clones A-D, lane nine is plasmids from clones E-H, lane ten is plasmids from clones I-L, and lane eleven is plasmids from clones M-P. The intensity of the bands in lane 6 indicates that there may be more than one positive colony when compared with the intensity of lane 10.

FUTURE EXPERIMENTS

One can prepare a plasmid prep of the individual colonies that may potentially be positive for pET32aΔTrx/EmoA and determine the exact band sizes. Some other future experiments with this project include making the remaining constructs. The construction of pFre can be attempted again by heating the digested, amplified *fre* and heating the Nde1 digested pET32aΔTrx and allowing them to re-anneal for at least 6 hours. If an Nde1 associated protein is

responsible for blocking the ligation site of the insert and the vector, the heating may resolve the problem.
Once this construct is made, one has to make the pFre/EmoA construct. These constructs should then be transformed into BL21 (DE3). Protein expression assays need to be set up to measure the amount of protein in the soluble and the insoluble fractions of each set of cultures.

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Appendix 1

Calibration Graph of 1 Kb Plus Ladder

MW	log MW	Distance
2000	3.30103	1.9
1650	3.217484	2.2
1000	3	3
850	2.929419	3.3
650	2.812913	3.8
500	2.69897	4.15
400	2.60206	4.55
300	2.477121	5
200	2.30103	5.55
100	2	6.2

slope	-0.28757
intrcpt	3.874237

Observed *fre* band: 788 bp