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Attempted Construction of a pET32a(+) Vector Containing EDTA Monooxygenase A

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Protein overexpression vectors, such as Novagen's pET vectors are very efficient cloning and expression systems for recombinant proteins in *Escherichia coli*. It has been shown that overexpression of heterologous proteins can lead to their aggregation within the cell known as inclusion bodies. Novagen's pET32a(+) vector reduces the formation of inclusion bodies by increasing the solubility of proteins when fused directly to highly soluble thioredoxin A (TrxA). In this study, we designed PCR primers that allowed for cloning of a translational fusion between TrxA and EDTA monooxygenase (EmoA). We successfully amplified *emoA* from the pEmoA plasmid in an effort to subclone the gene into pET32a(+). We confirmed the necessity of DMSO as a PCR enhancer and demonstrated that concentrations ranging from 5 – 9% were adequate to amplify *emoA*. Putative constructs were screened by colony PCR and restriction digests in an effort to identify positive clones. This analysis established that *emoA* had not been inserted into pET32a(+) and further attempts to clone and identify the construct were futile.

The use of overexpression systems to produce large amounts of heterologous proteins in *Escherichia coli* is a cornerstone of the biotechnology and pharmaceutical industries. A major problem with high level expression of these proteins is the formation of insoluble protein aggregates in the cytoplasm, known as inclusion bodies, which affects the native folding and reduces activity of the expressed protein (7). Protein recovery subsequently involves lengthy, complicated and costly solubilization and refolding protocols (7). To circumvent the problem of inclusion bodies, expression vectors like the Novagen pET32a(+) vector have been used to express the desired target as a fusion protein with a native soluble *E. coli* protein, thioredoxin (TrxA), which has been shown in increase the solubility of a number of insoluble heterologous proteins (5, 10).

Ethylenediaminetetraacetate (EDTA) monooxygenase (EmoA) has been characterized as a flavin mononucleotide-utilizing monooxygenase isolated from several *Agrobacterium sp.* strains, including BNC1, which facilitates the degradation of EDTA to EDDA (2). It is a protein that has been shown to form inclusion bodies upon expression in *E. coli* (6), and previous work has attempted to test the effect of the Trx fusion partner on EmoA solubility (4, 9, 11). Thus far, both the PCR amplification of *emoA* from the pEmoA and the insertion of the PCR product into pET32a(+) have proven difficult.

The aim of this study was to create a stable, verified pET32a(+)-*emoA* plasmid construct in an effort to evaluate the effect of Trx on the solubility of EmoA. We aimed to create the construct using newly designed PCR primers and restriction digestion of the product and vector that would ensure directional insertion of the *emoA* into the pET32a(+). Once formed, this construct was to be used to express Trx-EmoA as a fusion protein whose solubility could be compared to expressed EmoA alone. Furthermore, the construct was to be used to test new fusion partners for EmoA by deletion of *trx* and insertion of a putative solubility aid such as *fre* (flavin oxidoreductase). A number of putative clones were screened using PCR and restriction digestion; however, our analyses failed to identify positive pET32a(+)-*emoA* clones.

MATERIALS AND METHODS

Strains and Plasmids. The pEmoA and pET32a(+) plasmids (Table 1) were maintained in *E. coli* DH5α strains as part of the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). For pEmoA and pET32a(+) plasmid isolation, DH5α cells were grown in Luria-Bertani (LB) liquid medium (pH 7.0) containing tryptone (1%, BD Science), yeast extract (0.5%, BD Science), and NaCl (1%) overnight at 37 °C under aeration and agitation (200rpm). For colony PCR, DH5α cells were streaked for isolation onto LB agar (LB liquid medium with 1.5% agar, BD Science) and incubated overnight at 37 °C.

Plasmid Isolation. Plasmid DNA was isolated from DH5α cells growing in LB liquid media as per the manufacturer's instructions of the PureLink HQ Mini Plasmid DNA Purification Kit (Invitrogen, Cat. No. K2100-01). DNA was eluted with sterile distilled and stored at -20°C. DNA concentration and purity were determined using a Beckman Coulter DU530 spectrophotometer set to read absorbance at 260 nm and 280 nm.

TABLE 1. Strains and plasmids used in this study.

Name	Features	Source
Strains		
DH5a	*sup*E44 ? lacU169 (f 80lacZ? M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A1	Hanahan
Plasmids		
pET32a(+)	Novagen expression vector with *trxA* and *bla* resistance	MICB 421 Culture Collection
pEmoA	pTrc99A plasmid containing *emoA* and *bla* resistance	MICB 421 Culture Collection

TABLE 2. Oligonucleotide primers used in this study.

Name	Sequence[a] (5'-3')	T_m (°C)
SCGWemoA-F	acacac**GGATTC**ccaggaggcctcttaatgc	55
SCGWemoA-R	acacac**CTCGAG**gcttggtcaacctagcgg	56

[a] Start and stop codons are underlined. Restriction cut sites are indicated in bold capitals. *Bam*HI recognition site: GGATTC, *Xho*I recognition site: CTCGAG.

Primers and PCR amplification of *emoA*. A 1326 bp fragment encoding *emoA* was amplified from the pEmoA plasmid by colony PCR. The SCGWemoA-F forward primer (Table 2) is complementary to the sequence upstream of *emoA* and contains a *Bam*HI site and the SCGWemoA-R reverse primer introduces a *Xho*I restriction site. Introduction of *Bam*HI and *Xho*I restriction sites allowed for cloning of *emoA* into the pET32a(+) expression vector (FIG. 1). All reactions for the purpose of *emoA* amplification were carried out in 50 µl total volume. Each reaction contained 5% v/v DMSO (Fisher, Cat. No. PI-20684), 200 µM dNTP's, 1.5 mM of $MgCl_2$, 1X Taq Buffer, 2.5 U of Taq Polymerase (Invitrogen, Cat. No. 10342053) and a final primer concentration of 0.5 µM. Template cells were added to each reaction by toothpick from an isolated colony grown on LB agar overnight. To amplify *emoA* PCR cycle conditions were 30 seconds at 98°C, 30 seconds at 52°C, 1 minute at 72°C, as well as 2 minutes at 98°C for the initial denaturation and 5 minutes at 72°C for the final extension. A positive control PCR reaction containing TOPO positive PCR control primers and TOPO positive control DNA was included. All PCR reactions were cleaned using the PureLink PCR Purification Kit (Invitrogen, Cat. No. K3100-01) and stored at -20°C.

Construction of pET32a(+)-*emoA*. Two *Bam*HI/*Xho*I double digest reactions were carried out simultaneously; one for the *emoA* PCR product and the other for the pET32a(+) vector. Both reactions contained; 1X BSA, 5 µl NEBuffer 4 (New England Biolabs), 0.5 µl of each *Bam*HI and *Xho*I, as well as 1.6 µg of *emoA* or 2.3 µg of pET32a(+) in a total volume of 50 µl. Reactions were incubated overnight at 37°C. The vector was then Antarctic Phosphatase (New England Biolabs, Cat. No. M0289S) treated by adding to the 50 µl volume, 6 µl of the 10X Antarctic Phosphatase Reaction Buffer, 5 U of Antarctic Phosphatase and 3 µl of sterile distilled water to bring the reaction to a final volume of 60 µl. The reaction was then incubated at 37°C for 30 minutes. The digestion reaction was not cleaned prior to Antarctic Phosphatase treatment as per the manufacturer's guarantee that the enzyme was fully functional in NEBuffers when supplemented with the 10X Antarctic Phosphatase Reaction Buffer. The Antarctic Phosphatase was heat inactivated at 65°C for 5 minutes and both the treated pET32a(+) vector and untreated *emoA* insert digestion reactions were cleaned using the PureLink PCR Purification Kit (Invitrogen, Cat. No. K3100-01). An overnight ligation reaction was then set up with 0.27 µg pET32a(+) vector, 0.20 µg *emoA* insert, 1.5 µl 10X T4 DNA ligase buffer, 1 µl of T4 DNA ligase and 2.5 µl of sterile distilled water, for a total reaction volume of 15 µl. Vector control and insert control ligation reactions were also set up with sterile distilled water replacing the *emoA* insert and pET32a(+) vector, respectively. Following the overnight incubation at 16°C, the T4 ligase was heat inactivated at 65°C for 10 minutes and desalted on 800 µl of a Sephadex G-50 DNA Grade F column (Cat. No. 17-0573-02, GE Healthcare) by centrifugation at 0.8 rcf for 2 minutes.

Bacterial transformation. Methods involving electroporation and chemical transformation were employed in the study. Chemically competent DH5α cells were transformed by heath shock as described by the manufacturer (Invitrogen, Cat. No. 18265-017). Electro-competent DH5α cells were graciously donated by Dr. Lindsay Eltis (Department of Microbiology and Immunology, University of British Columbia). For each transformation, construct and vector control, 4 µl of construct was added to a sterile, chilled 0.2 cm electroporation cuvette (Biorad) to which 40 µl of DH5α cells was added. The cell/construct mixture was then put through a MicroPulser (Biorad) set at the Ec2 program, rinsed with 500 µl of nonselective LB liquid medium, collected in a sterile culture tube and incubated for 1.5 hours at 37°C under aeration and agitation (200 rpm). Following transformation, either 20 µl or 200 µl of each pET32a(+)-*emoA* and vector control outgrowth cultures were then respectively plated on LB agar plates containing 100 µg/ml ampicillin.

PCR and restriction digest screening of potential clones. In an effort to reduce the amounts of reagents used, the total volume of the colony PCR reactions were scaled down to 10 µl. Each reaction contained the same proportions of reagents as described above for colony PCR, but the amounts were all scaled down $1/5^{th}$. Twelve colonies growing on LB agar plates containing 100 µg/ml ampicillin, following transformation, were selected for the screen. The PCR program used was the same as described above. The reactions were then run on a 1% agarose gel with a double digested *emoA* as a band size control for the PCR screen. Alternatively, potential clones were screened via double digestion of the construct with *Xba*I and *Hind*III, which was expected to produce two bands of approximately 5.6 kb and 1.6 kb in size. As with the PCR based screen, the double digestions were also scaled down to a final volume of 10 µl. In an effort to avoid inaccuracy when working such small volumes, a master mix was prepared, an aliquot was delivered to each digestion reaction, to which 2 U of each the *Xba*I and *Hind*III were added.

Mfold analysis of the *emoA* sequence. Mfold is a collection of related software applications available online for the prediction of the secondary structure of single stranded nucleic acids. A putative sequence incorporating the *emoA* sequence with 50 bp of pET32a(+) both upstream and downstream was analyzed for secondary structure

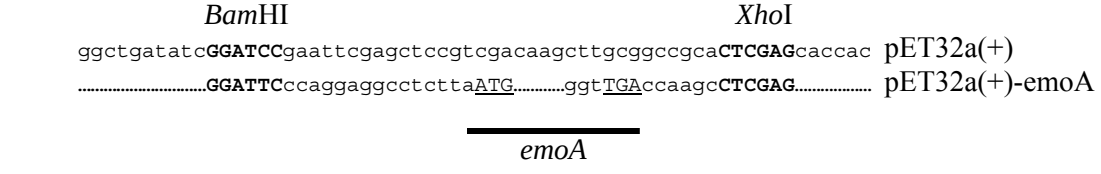

FIG. 1. Design of the *trxA-emoA* fusion in the putative pET32a(+)-*emoA* construct. The first 3 codons in the *Bam*HI restriction site are in frame with *trxA* transcription, meaning that there are 7 amino acids fusing TrxA and EmoA. The start and stop codons for *emoA* are underlined and the restriction cut sites used are indicated in bold.

at PCR extension temperatures of 72 °C. When temperature is altered from standard conditions, the free energy, enthalpy, entropy and an estimated T_m is computed using a simple 2-state model. The results are displayed graphically as a dot plot and the structure of sequences is shown graphically (Fig. 3). Detailed procedures for the correct use of Mfold are detailed by Zucker *et al.* (12).

RESULTS

PCR amplification of *emoA* from pEmoA requires the addition of 5% or greater DMSO. Using the information obtained previously by Honeyman *et al*. (3), where the *emoA* sequence was successfully amplified with the addition of DMSO to enhance the PCR reaction, we tested our redesigned primer set (Table 2). We found that amplification was not successful in reactions containing either no DMSO or supplemented with 1% DMSO. As reported by Honeyman *et al*. (3), a concentration of 5% DMSO in the reaction mixture enhanced the PCR process and by applying this method we obtained the expected *emoA* PCR product. The amplified *emoA* sequence included non-coding regions and the *Bam*HI and *Xho*I restriction sites 5' and 3' respectively. The length of this sequence was 1326 bp, which was clearly evidenced by agarose gel electrophoresis (Fig. 2A and 2B). We further investigated the effectiveness of DMSO on enhancement of our PCR reaction by testing higher concentrations of it and found that within a range of 5 – 9% DMSO, *emoA* is amplified equally as well.

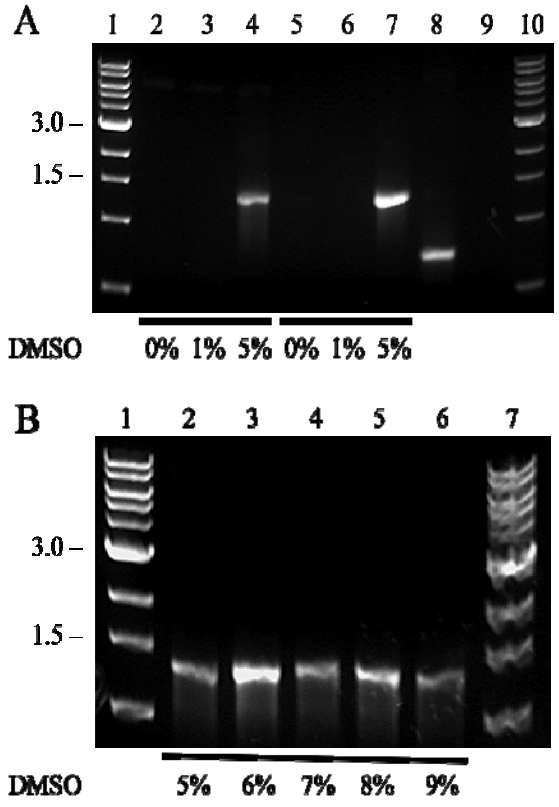

FIG. 2. PCR amplification of *emoA* (1326 bp) from pEmoA with DMSO treatment. (A) Comparison of amplification from plasmid preparations in lanes 2 – 4 and amplification from pEmoA colonial material, lanes 5 – 7. A positive 750 bp TOPO PCR control (Invitrogen) was run in lane 8 and a negative control in lane 9. Lanes 1 and 10 are 1 kb molecular mass ladders. (B) Effect of increasing DMSO concentrations on PCR efficiency in lanes 2 – 6. Both 1% TAE-agarose gels are flanked by 1 kb molecular mass ladders (New England Biolabs) with units in kb.

Transformation by electroporation or chemically competent methods yields ampicillin-resistant colonies. The pET32a(+) protein expression vector encodes *bla*, the gene conferring ampicillin resistance. Our strategy was to digest the vector with *Bam*HI and *Xho*I, remove the 5' phosphate groups with phosphatase treatment and ligate this to a similarly-digested *emoA* amplicon. Following ligation we transformed the construct into *E. coli* DH5α by two methods: electroporation and chemical competence. Both methods were successful in producing colonies that grew on ampicillin-selective media. Electroporation of electrocompetent cells produced more colonies than transformed chemically-competent cells, but re-circularization appeared to be an issue with DH5α cells electroporated with the vector-only ligation controls. The transformation of the chemically competent cells produced fewer ampicillin-colonies but was more discriminating in that no colonies were observed on vector-only controls. Adequate ampicillin-sensitive controls were set up to ensure the selectivity of the antibiotic and no growth was observed on these plates.

Colony PCR screening of transformants indicates that *emoA* was not inserted into the pET32a(+) expression vector. Following the transformation of both the electro- and chemically-competent cells we used colony PCR (5% DMSO) to detect the *emoA* sequence from twelve randomly selected transformants. Neither transformation method yielded amplified product nor emoA transformants even though a positive control was included and was successful (data not shown). To determine the nature of the failed PCR reactions we then carried out extensive Mfold sequence analyses and restriction digests to map our putative constructs.

Mfold analysis detected extensive secondary structure in the putative pET32a(+)-*emoA* sequence at PCR extension temperatures. As previously reported by Honeyman *et al*. (3), Mfold analysis (12) indicated the formation of secondary structure within the reverse primer binding region (5' to the *emoA* coding sequence). Honeyman *et al*. (3) suggested that at a PCR extension temperature of 67.5°C there is a hairpin loop of ΔG = -5.6 kcal/mol; however, hairpin structures are generally tolerated at -6 kcal/mol (8). Therefore, we analyzed 100 bp segments of the putative pET32a(+)-emoA sequence that corresponded to both

the forward and reverse primer annealing sites. We found no evidence of secondary structure lower than –1.2 kcal/mol (data not shown). However, we hypothesized that the difficulty in amplifying *emoA* was a direct result of the secondary structure present within the coding sequence of the gene itself. The entire 1326 bp *emoA* sequence, including 50 bp of pET32a(+) both upstream and downstream of the gene was analyzed by Mfold at 72°C extension temperature. We found that *emoA* has extensive secondary structure that is 5-fold more stable than a randomly selected gene from *E. coli* called *yebQ*, similar in GC content to *emoA* and, at 1374 bp, is similar in size. The ΔG of the most stable *emoA* structure at 72°C is -130.4 kcal/mol (FIG. 3).

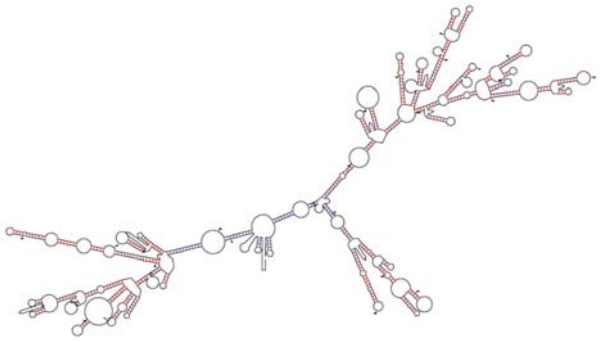

FIG. 3. Extensive secondary structure within the *emoA* sequence and 50 bp of flanking pET32a(+) as determined by Mfold. The structure above has ΔG of -130.4 kcal/mol at PCR extension temperatures of 72°C and exhibits hair-pin loop structures throughout the majority of its sequence.

Chemically competent transformants yielded stable plasmids lacking the *emoA* insert. We randomly selected four transformants from both the electro- and chemically competent transformations and then isolated plasmids from overnight plates containing ampicillin. The plasmids were subjected to double digestion with *Hin*dIII and *Xba*I. If the plasmid contained the *emoA* insert we would have expected a fragment of 1585 bp to appear on an electrophoretic gel. Unfortunately, our restriction analysis clearly demonstrated that none of the clones contained *emoA* because no bands of approximately 1600 bp were observed, while bands of approximately 500 bp were present (Fig. 4A). These low molecular weight bands correspond to the 556 fragment expected if pET32a(+) were to be digested with both *Hin*dIII and *Xba*I restriction enzymes (FIG. 5). Of significant interest is that we were unable to isolate plasmids from electroporated cells (FIG. 4B). The plasmid preparation was carried out exactly the same as that for the chemically-competent transformed cells, yet only large, faint smears were detected by electrophoresis. An additional 50 electroporated transformants were screened by double digestion with *Hin*dIII and *Xba*I ; however, this analysis did not yield plasmid (data not shown).

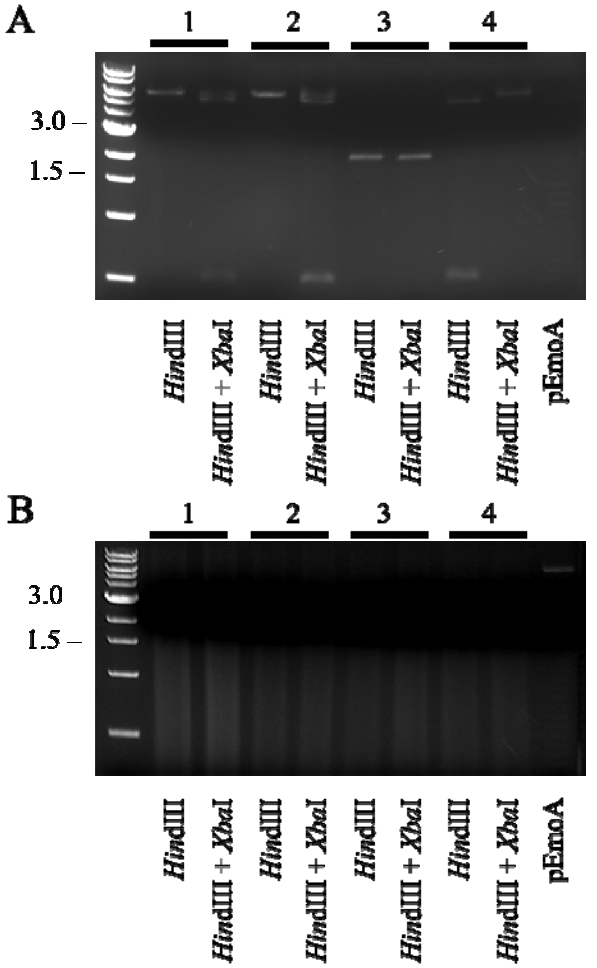

FIG. 4. Restriction enzyme digestion analysis of transformed DH5α cells. (A) Plasmid isolations from four colonies of transformed chemically-competent *E. coli* DH5α were subjected to *Hin*dIII digestion or *Hin*dIII and *Xba*I digestion. No cells containing the putative construct were isolated. (B) Digested plasmid isolations from four randomly selected electroporated transformants. No bands were observed. Both 1% TAE-agarose gels are flanked by a 1 kb molecular mass ladders (New England Biolabs) with units in kb and an uncut pEmoA control.

DISCUSSION

Transformation of DH5α with a putative pET32a(+)-*emoA* construct produced ampicillin resistant colonies for both chemically and electro-competent cells. The electroporated transformants displayed a 10% vector background (data not shown); however, the chemically competent cells showed no growth on the vector-only control plates. According to product information obtained from New England Biolabs, the 10%

background growth on the electroporated vector-only control plates is 8% higher than the approximate2% background expected for Antarctic Phosphatase treated DNA and much higher than the 0.1% background

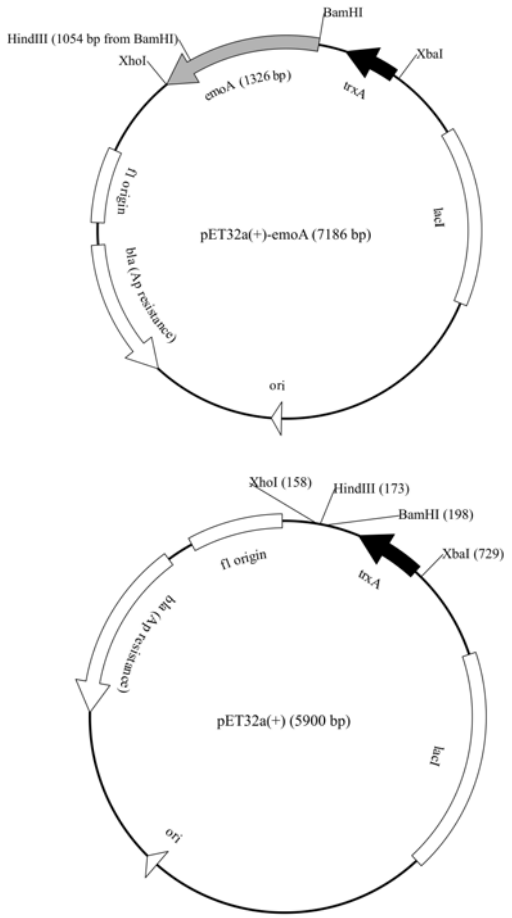

FIG. 5. Plasmid maps of pET32a(+) and the putative pET32a(+)-emoA construct. The native pET32a(+) vector contains a *Hin*dIII restriction site within its multiple cloning site. This *Hin*dIII is lost when *emoA* is inserted between *Bam*HI and *Xho*I; the *emoA* sequence, however, contains another *Hin*dIII site.

expected from the *Bam*HI-*Xho*I double digestion suggesting problems with these two procedures. Since there was no growth on the ampicillin-sensitive control plates, it can be concluded all of the ampicillin-resistant colonies were the result of vector uptake during the transformation procedures.

Unfortunately, the colony PCR screening failed to confirm the presence of *emoA* inserted into pET32a(+) in the transformants. This result suggested two things: either the PCR failed due to high degrees of secondary structure within the putative pET32a(+)-emoA sequence and primer annealing sites or the *emoA* sequence was absent. The Mfold analysis showed significant, stable secondary structure in *emoA* and it is possible the 5% v/v DMSO was insufficient in relaxing this high degree of secondary structure enough to perform PCR. Furthermore, the secondary structure may have proved a hindrance to successful ligation of the insert to the vector.

To determine the presence of *emoA* in the transformed DH5α cells a double digestion of plasmid preparations was done with *Hin*dIII and *Xba*I. If the insert was present the double digest would result in DNA fragments of 1525 bp and 5601 bp with *Hin*dIII cleaving the DNA inside the *emoA* coding region and *Xba*I cutting in the vector sequence (FIG. 5). Instead, we obtained bands of approximately 500 bp and 5300 bp (FIG. 4A), which is consistent with the digestion pattern expected if the native *Hin*dIII site were still present in the vector and no *emoA* was inserted (FIG. 5). This finding was helpful because it showed that digestion with *Bam*HI and *Xho*I was not removing pET32a(+)'s endogenous *Hin*dIII site, suggesting that the digestion with one or both of these enzymes was incomplete. Both enzymes are 100% active in the buffer used in the digestion.

It seems likely that the high degree of secondary structure inherent in the *emoA* sequence is both a factor in the PCR amplification of this gene and in the restriction digestion of any amplified sequences. Despite the fact that the restriction cut sites are located on the termini of the *emoA* amplified sequence, the secondary structure may be interfering allosterically with the digestion enzymes by hiding the restriction cut sites. Identification of putative constructs was also challenging because of these factors and the lack of alternate suitable methods to screen for positive clones was time consuming and expensive. Further experiments need to devise ways to account for these difficulties.

FUTURE EXPERIMENTS

The construction of pET32a(+)-*emoA* proved to be challenging. Moreover, it was difficult to determine if the vector had *emoA* inserted; therefore it would be beneficial to have a second marker such as *lacZ* (blue-white colony screening) to make screening of transformed colonies faster and easier. This could be done either by cloning *lacZ* into the pET system or by cloning the *emoA* into a different vector containing *lacZ* intersected by a multiple cloning site. Disrupted *lacZ* produces white colonies, which would then be selected so that *trx* could be cloned in last (since *lacZ* disruption only works for one cloning event).

Digestion conditions also appeared to be a problem, specifically the double digestion with *Bam*HI and *Xho*I. Therefore, a sequential digest may prove more effective than a double digest and would elucidate any inefficiency in enzyme cleavage. Furthermore, once

the construct is made, expression assays can be carried out in pET32a(+) host cells to evaluate the effect of thioredoxin A on the solubility of EDTA monooxygenase A.

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