Journal of Experimental Microbiology and Immunology (JEMI) Vol. 6:72-76

Attempted Cloning of *Escherichia coli csg* Into Flavin Reductase or Thioredoxin In The pET32a (+) Vector To Assess Disulfide Bond Effects on Amyloid Deposits Formation

WINNIE KWOK
Department of Microbiology and Immunology, UBC

The decreased levels of thioredoxin activity within amyloid plaques in Alzheimer's patients support the idea that the disease is initiated by reactive oxygen species. One suggestion for therapy is to create a reducing environment near the amyloid plaques, such as in the temporal or frontal lobe of the brain. The goal of this experiment was to determine if amyloid-like proteins called curli in *Escherichia coli* will express soluble subunits rather than amyloid-like fibrous tangles when fused with thioredoxin, a ubiquitous reducing enzyme. Fusing thioredoxin to a gene that tends to form inclusion bodies when overexpressed in *Escherichia coli* expression vectors produces viable, soluble protein with native conformation. An attempt was made to clone the curli gene, *csg*, into pET32a(+), a commercially available vector containing thioredoxin to determine if amyloid-like plaque formation is affected by disulfide linkages. A second objective was to determine if the NADPH:flavin oxidoreducatse can similarly produce soluble proteins. The project failed to yield any successful ligation of *csg* to either thioredoxin or NADPH:flavin oxidoreducatse.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposits of amyloid plaques in the temporal and frontal lobes of the brain thereby affecting learning and memory processes. AD is a truly devastating disorder that robs the patients of their identity and history. With the aging baby boomer generation, AD is predicted to increase threefold in the next 50 years (10). However, much remains unknown about the formation of the amyloid plaques and the mechanism by which atrophy of neurons arises. Research indicates that oxidative stress is one of the early triggers of AD through the generation of hydroxyl radicals as indicated by increase levels of lipid peroxidation (9). Specifically, thioredoxin (Trx) and thioredoxin reductase, appear to guard against reactive oxygen species damage since Trx levels are decreased in AD patients as determined by quantitative reverse transcriptase polymerase chain reaction (8). Trx is a 12kDa protein found in all eukaryotes. It acts on sulfide-sulfide bonds in proteins and functions as a scavenger for reactive oxygen species (7). In addition, Trx is hypothesized to be involved in protein folding and regulation, reduction of dehydroascorbate, repair of oxidatively damaged proteins, and sulphur metabolism (11).

Trx and other redox proteins may prove to be an AD therapy since the nucleation process where amyloid fibers are formed from precursor subunits may require disulfide bond linkages (6). The reduction of disulfide bonds between resides 2 and 7 of amyloids reduce fiber formation as precursors cannot link to from polymers (6).

In the pET32a (+) expression vector system by Novagen, *trx* is fused in frame with a target protein to create a fusion protein that greatly enhances solubility. Without this fusion system, the overexpression of foreign protein within an *E.coli* system leads to protein aggregates in the cytoplasm, or inclusion bodies. Although inclusion bodies are readily separated from other cell debris, the difficulty lies in obtaining the native, active protein with the proper conformation. One objective of this study was to determine if NAD(P)H:flavin oxidoreductase (flavin reductase or Fre) has similar potential to *trx* to allow the expression of soluble target proteins. *Fre* catalyzes the reduction of FAD, FMN and riboflavin with NADH as a reductant to create $FADH_2$ (13).

To study the effects of *fre* or *trx* on amyloid fiber formation, *E.coli* curli proteins were chosen for analysis. *E.coli* produces extracellular fibers that resemble beta-amyloid proteins of AD based on biochemical, biophysical and imaging comparisons (1). Curli binds specially to Congo Red, one of the standard dyes to determine if a patient suffered from AD, and curli demonstrates similar aggregation cluster morphology under electron microscope micrographs. Biologically, these proteins are used by *E.coli* and other infectious bacteria such as the *Salmonella* species to attach themselves on to epithelial surfaces to colonize the host (1). Although it has yet to be investigated whether curli proteins rely on disulfide bonds in their secondary structure or whether these bonds are involved in the formation of polymers, preliminary analysis using disulfide bond prediction computer

programs such as the Genomic Disulfide Analysis Program suggests curli proteins are capable for disulfide bond formation.

MATERIALS AND METHOD

Bacterial Strain and culture condition: *E.coli* K12 MG1665 was used to obtain genomic DNA for PCR amplification of *csg*. Cultures were streaked on Luria-Bertani (LB) agar plates from freezer stocks, and a single colony was inoculated in 50 mL LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) with 0.1 mg/mL ampicillin. The culture was incubated on a shaker incubator at 37°C and 150 rpm overnight.

E.coli DH5α was made competent using the calcium chloride method. Briefly, *E.coli* was grown to a density of about 0.15 to 0.2 at OD_{650}. Cultures were centrifuged, supernatant was removed, pellet was resuspended with 0.1 M cold calcium chloride and chilled on ice for 20 minutes. The culture was centrifuged again and the pellet resuspended in 0.1 M calcium chloride with 80% glycerol, and stored aliquots at –80°C until use.

Genomic DNA Isolation 1 mL of overnight MG1665 culture was placed into a microcentrifuge tube and spun for 1 minute at 8,000 rpm. DNA was isolated according to Proteinase K protocol for bacterial cultures in QIAamp DNA Mini kit from Qiagen.

Gene cloning and expression. To overproduce *csg* in *E.coli*, PCR primers were designed to clone *csg* into pET32a(+) vector (Novagen). The forward primer (5'-AGT-AGC-GGA-TCC-TGC-AGC-AAT-CGT-ATT-CTC-CGG-T-3' binds to *csg* at position 15-48 in which an Bam HI restriction site (underlined) was introduced. The reverse primer 5'TGA TGA AAG CTT GCG TTG TTA CCA AAG CCA ACC TGA 3' binds to *csg* at position 403 to 449 (GenBank U00096) in which a Hind III restriction site (underlined) was introduced. The restriction site sequences were inputted into National Centre for Biotechnology Information BLAST program and the enzymes does not cut within the *csg*. PCR primers were synthesized from the Nucleic Acid Protein Services unit on the University of B.C. Campus.

The gene was amplified with the Csg forward and reverse primers (20 pmol) and the genomic DNA from MG1665 was amplified using initial denaturation at 95°C for 3 minutes, then 30 PCR cycles set with the following thermal profile: denaturation at 95°C for 1 minute, annealing at 60°C for 30 seconds, extension at 72°C for 42 seconds. PCR reagents were obtained from the Invitrogen DNA Taq polymerase kit using 5 ul of 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]; 1.5 ul of 50 mM $MgCl_2$, 1 ul of 10 mM dNTP, 0.25 ul of 5 U/ul DNA Taq polymearase, and 32.5 ul of sterile water in 40 ul of master mix per reaction. The PCR product was resolved on a 0.8% TBE agarose gel and the appropriate sized 434 bp band was excised and the DNA was purified using the MiniElute Gel Extraction kit from Qiagen. The PCR product was cut with the restriction endonuclease Bam HI at 37°C for 2 hours, then reaction buffer and excess enzymes were removed using MiniElute Reaction Clean-up kit from Qiagen. The digested DNA was eluted in 20 ul of water and a second digestion using the restriction endonuclease Hind III was performed at 37°C for 2 hours. The digested PCR product (50 ng) was then ligated using T4 ligase from New England Biolabs into pET32a (100 ng) from Novagen that was previously digested with Bam HI and Hind III, gel-purified and designated as pETBHTrx.

A second ligation was performed using pET32a that had been digested with Nde I to remove the *trx* to produce pETN2. The pETN2 was then treated with 5 units of Antarctic Alkaline Phosphatase (New England Biolab) and the phosphatase was inactivated by heating at 65°C for 5 minutes. The *csg* PCR product was also ligated into pETN2 to produce pETBH. 100 ng pETBH and 100 ng pETBHTrx was mixed into 100 ul of chemically competent DH5α and placed on ice for 30 minutes. A control using 200 ng of pES1 was performed simultaneously. The cells were heat shocked for 90 seconds in a 42 °C heating block filled with water and then iced for 10 minutes. SOC media (2.0 g tryptone, 0.5 grams yeast extract, 1.0 mL of 1 M NaCl, and 0.25 mL of 1M KCl for one liter of SOC media) was added to the cultures and placed in a 37°C shaker bath for 1.5 hours after which the bacterial cells were plated onto 0.1 mg/mL ampicillin agar plates and incubated overnight at 37°C.

To clone *fre* into pET32a , pES1 was a generous gift from Dr. Tai Man Louie. pES1 contains *fre* cloned into a LIC vector from Novagen using Nde I and Eco RI. The forward primer (5'-TAG-GGG-AAT-TGT-GAG-CGG-ATA-ACA-3') binds at –72 to -44 bp from the *fre* start codon (GenBank M61185). The reverse primer (5'-GGG TTT TTT CAT ATG TCC GAT AAA TGC-3' overlaps the *fre* at position 690-702. The expected product size was 790 base pairs. The introduced Nde I restriction site is underlined in the reverse primer.

For both *csg* and *fre*, the vector:insert ration varied from 1:3, 1:1, 1:5, and 1:10. The T4 DNA ligation was performed at either room temperature for two hours or at 16 °C overnight.

RESULTS

***Csg* Amplification.** *Csg* was PCR amplified from the genomic *E.coli* K12 MG1665 DNA and a single band of approximately 450 bp was amplified and resolved on a 0.8% agarose gel (Fig. 1)

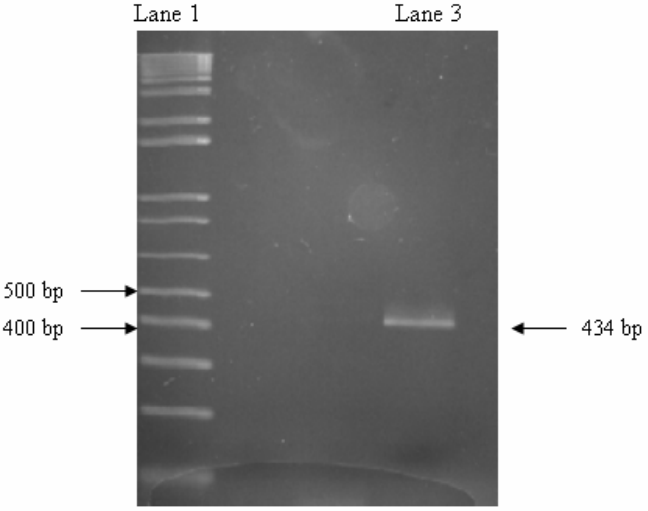

FIG. 1 PCR amplification of *csg*. Lane 1. 1 kb Plus DNA ladder (Invitrogen). Lane 3. PCR product of *csg* from genomic MG1665 DNA. The expected product size is 434 base pairs.

The transformation of pETBH and pETBHTrx into DH5α yielded 4 colonies for the pETBH and 3 colonies for the pETBHTrx while the pES1 control produced more than 200 colonies per plate. A digestion of the resulting pETBH and pETBHTrx colonies with Bam HI and Hind III to confirm if *csg* was successfully ligated showed that the colonies were false positives (Fig 2).

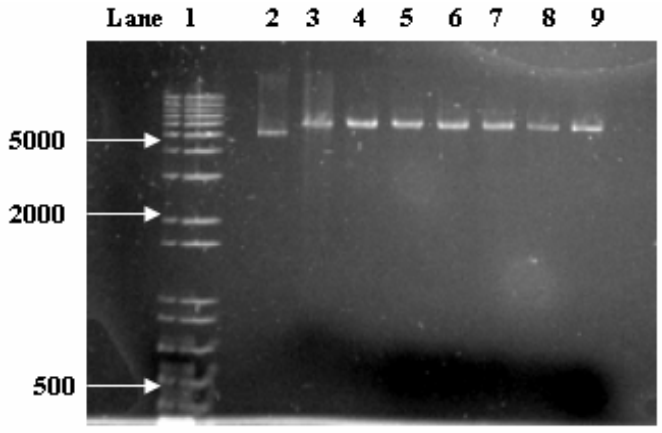

FIG. 2 Restriction enzyme digestion with Bam HI and Hind III to determine if *csg* was inserted into the pETBHTrx or pETBH. The ligated vector was transformed into DH5α and colonies that grew overnight were clonally grown in LB broth. Lane 1: 1 Kb Plus DNA ladder from Invitrogen. Lane 2: Bam HI nicked pET32a. Lane 3-6: pET32a with *csg* inserted into MCS and digested with Bam HI and Hind III. Lane 7-9: pETBH with *csg* inserted in MCS and digested with Bam HI and Hind III.

DISCUSSION

Although both *csg* and *fre* PCR products appeared as single bands of the expected sizes when analyzed on an agarose gel, no transformants containing the insert was isolated. Lanes 3 to 6 contained transformed plasmids of what should be PCR-amplified *csg* inserted into the MCS of pET32a. The digestion of the transformed plasmids with BamHI and HindIII was expected to yield two bands if the ligation of *csg* was successful: a 434 bp representing the *csg* insert and an approximately 5.9 kb band for the pET32a vector. Likewise, two bands were expected from the BamHI and HindIII digestion of the pETBH in lanes 7-9 with a 434 bp band for *csg* and a second band at 5.5 bp for the pET32a vector with *trx* removed. However, in both the pETBHTrx and pETBH transformed plasmids, only single bands were observed after the double digestion (Fig 2) suggesting that the colonies were false positives. False positives are entirely possible since the transformation frequency of pETBHTrx and pETBH was incomparable to the frequency in the control. The bacteria colonies on the control were too many to count for 200 ng of DNA whereas transformation with pETBHTrx and pETBH generated less 5 colonies.

However, pETBHTrx and pETBH were a larger size on the agarose gel than the BamHI nicked pET32a suggesting that the *csg* was inserted. The pETBH size on the agarose gel was the correct predicted size at 6 kb, assuming that the *trx* was indeed cleaved out from pET32a and *csg* was inserted. To confirm if *trx* in pETBH was digested from pET32a, a PCR could be conducted using *trx* primers based on the nucleotide sequences provided by Novagen. For the pETBHTrx, the expected size was 6.3 kb if *csg* was inserted but from figure 2, there was no size difference between pETBHTrx and pETBH.

Two plausible explanations for pETBH may be that the insert was ligated but the agarose gel was too insensitive to detect the insertion. It is possible that the digested pETBH may have had *csg* but because of the loading volume, the insert may not have yielded a sufficiently strong intensity band to be visualized even with maximal exposure times on the GelDoc. The band intensities of the vector were not exceptionally bright even though the vector was 14 times larger than the insert. One future modification is to overload the digested pETBHTrx and pETBH beyond 1 ug in hopes that if there was a small fraction of these plasmids with the insert present, it would be more visible on the gel.

Second, the csg insert may be present but the restriction enzymes failed to cleave it out from the transformants. To check if either the BamHI or the HindIII restriction sites inserted into the primers for the amplification of *csg* can still be recognized by the respective enzymes, one could cut the pETBH with a single enzyme, e.g. with BamHI only, and run the digested product with the non-linearized pET32a. It is expected that the non-linearized pET32a would adopt a more coiled form and thus will run faster relative to linearlized DNA of the same molecular weight. Therefore, if the singly cut pETBH contains the BamHI restriction site that is still recognized by the enzyme, this band will run slower than the uncut pET32a. The gel should be repeated with a HindIII single digestion of the pETBH to determine if all the inserted restriction sites were reconstructed.

To definitively conclude if the *csg* was inserted into pETBHTrx and pETBH, a PCR could be performed using the forward and reverse *csg* primers originally used to clone the *csg* from the genomic MG1665 but using the transformants as a template. However, a BLAST search should be performed to confirm that the primers will not amplify any regions within the pET32a vector.

Since the experiment was hindered at the cloning stage, the subsequent discussion is focused on probable sources of error and suggests possible solutions for future experiments.

A source of error for the experiment may be with the ligation procedure leading to low transformation percentages. One test to access the whether the ligation kit was functional for this system would be to ligate a lineralized pET32a to determine if the ligated plasmid could transform the DH5α. If no transformants were obtained, it suggests modifications to the ligation procedure should be made. For example, although different vector to insert ratios were tested, the insert and vector may simply fail to bind. If the insert is

added at a molecular concentration that is significantly greater than the vector (typically 4 mg/mL), linear concatamers of the insert forms. A method to improve ligation efficiency without altering the vector:insert ratio beyond the typically 3-5 fold insert excess is to use up to 5 % (w/v) polyethylene glycol (PEG). More than 5% PEG (w/v) decreases the transformation frequency.

Because *trx* was flanked by Nde I restriction sites, it is possible for the digested vector or the *fre* insert to self-ligate. Although some the Nde I nicked vector was treated with alkaline phosphatase to dephosphorylate the 5' end, there was negligible differences between the number of transformants in the treated and untreated vectors. A limitation to alkaline phosphatase is that it is applied to either the vector or the insert but not both; therefore, the untreated digested *fre* remains capable of self-ligation. In addition to alkaline phosphatase of the pET32a, the 2'-deoxyribose at the 3'-end of the insert can be replaced with a 2',3'-dideoxyribose. Self-ligation is prevented at the replaced 3'-end, while the 5'-phosphate permits ligation with the 3'-hydroxyl end of the dephosphorylated DNA strand (12). Briefly, to produce the replaced 3' end, the insert is treated with T4 DNA polymerase and ddNTP and dNTP. The ends are then filled in completely using Taq DNA polymerase (12). The ligation efficiency for the 3'ddNTP is 1.4 x 10^4 colony/5 fmol DNA versus 2 x 10^3 for the 3'OH insert at a 0.5 fmol/µl concentration of vector and insert in the reaction mixture (12).

For the *fre* ligation into the pET32a vector, it may be worthwhile to consider using a ligation independent cloning vector as in the Xun et al. publication (13). The restriction sites inserted into the forward and reverse primers were flanked by sequences complementary to the target to enhance binding. However, it has been suggested that these short base sequence near the terminii may be insufficient for stable association with and cutting by some restriction endonucleases (4). One solution is to form concatamers of the PCR amplified insert using T4 ligase so that the restriction enzyme sequences lie within an internal site that is more readily recognized (4). PCR fragments containing terminal restriction enzyme sites that were ligated prior to digestion had a 50% increase in cloning efficiency over the control (4).

Once the *csg* is cloned into the pET32a, the orientation of the insert should be verified by PCR amplifying the transformants using a vector specific primer and an insert specific primer. To express the curli gene, the pET32a with the *csg* insert must be transformed into an expression host that contains a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control and grown with the inducer IPTG.

To determine if curli is expressed, cultures should be grown on YESCA plates (10 g/L cosamino acid, 1g/L yeast, 20 g/L Bacto agar, 20 mg/L Congo red and 10 mg/L Comassie brilliant blue G) at 26 °C (1). Curli binds to the Congo Red thereby, a greenish halo will appear around the circumference of the colony whereas the agar surfaces without any bacterial growth remain red. The bacterial colonies with and without the greenish halo should be isolated and grown clonally in LB broth.

The bacteria should be lysed and the supernatant analyzed on a 12% SDS-polyacrylamide gel. To see the curli subunits, the proteins are first treated with 90% formic acid to separate the polymers into a 17.5 kD and a 32 kD band corresponding to monomers and polymers, respectively (1).

The *trx* should be cleaved out of the pET32a containing the *csg* using Nde I and the vector re-circularized. A second set of transformation using the expression host should be performed and then plated on YESCA plates to determine if the *trx* had any effect on the curli production. If curli production is indeed affected by disulfide-disulfide bond formation, the *fre* should be cloned to determine if it has the same effect on curli formation as *trx*.

ACKNOWLEDGEMENTS

The author would like to thank Dr. William Ramey for assistance on project design and consultation; Jennifer Sibley for technical assistance; and Dr. Tai Man Louie at
Kemin Consumer Care for the pES1. The author would also like to thank May Kazem and Aileen Kartono for the *fre* primers and suggestions.

REFERENCES

1. **Chapman M., L. Robinson, J. Pinkner, R. Roth, J. Heuser, M. Hammar, S. Normark, S. Hultgren.** 2002. Role of E. coli curli operons in directing amyloid fiber formation. Science **295**: 851-855.

2. **Hayashi K., M. Nakazawa, Y. Ishizaki, N. Hiraoka and A.Obayashi.** 1986.
Regulation of inter- and intramolecular ligation with T4 DNA ligase in the presence of polyethylene glycol. Nucleic Acids Res. **14**: 7617–7631.

3**. Honda, K., G. Casadesus, R. Petersen, G. Perry, M. Smith. 2004.** Oxidative stress and redox-active iron in Alzheimer's Disease. Ann. N.Y. Acad. Sci. **1012:** 179–182 .

4. **Jung, V., S.Pestka and S.Pestka**. 1990. Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites. Nucleic Acids Res. **18**: 1656.

5. **Kang, C., V. Jayasinha, and P. Martin. 2003.** Identification of peptides that specifically bind $A\beta_{1-40}$ Amyloid in vitro and amyloid plaques in Alzheimer's Disease brain using phage display. Neurobiol Dis.**14:** 146-56.

6. **Koo, B., and A. Miranker.** 2005. Contribution of the intrinsic disulfide to the assembly mechanism of islet amyloid. Protein Sci. **14**:231-9.

7. **Lippoldt, A., C.A. Padilla, H. Gerst, B. Andbjer, E. Richter, A. Holmgren and K. Fuxe.** 1995. Localization of thioredoxin in the rat brain and functional implications. J. Neurosci. **15**: 6747–6756.
8. **Lovell, M., W. Ehmann, S.Butler and W. Markesbery**. 1995. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's Disease. Neurology **45:** 1594–1601.
9. **Lovell, M., C. Xie, S. Gabbitaand, and W. Markesbery**. 2000**.** Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's Disease brain. Free Radic Biol Med. **28**: 418-427.
10. **Mattson, M.** 2004. Pathways towards and away from Alzheimer 's disease. Nature **430:** 631-639.
11. **Trotter, E.W., and & C. M. Grant**. 2003**.** Non-reciprocal regulation of the redox state of the glutathione–glutaredoxin and thioredoxin systems. Nature. **4**:184-188.
12. **Ukai, H., M. Ukai-Tadenuma, T. Ogiu, and H. Tsuji**. 2002. A new technique to prevent self-ligation of DNA. J Biotechnol. **97:** 233–242.
13. **Xun, L., and E. Sandvik**. 2000. Characterization of 4-hydroxypheylacetate 3-hydroxylase (hpab) of E. coli as a reduced flavin adenine dinucleotide-utilizing monooxygenase. Appl.Environ.Microbiol. **66**:481-6.