Attempts to construct an Amyloid fiber-plasmid model to study the effect of Thioredoxin and Flavin reductase on overexpression of the Curli Protein in *Escherichia coli*.

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Alzheimer’s disease is a debilitating neurodegenerative disease characterized by the formation of insoluble amyloid plaques in the brain. *Escherichia coli* curli, encoded by the *csg* gene, is the prokaryotic equivalent of amyloid protein. To study the effect of a reducing protein on the solubility of curli, attempts were made to fuse curli to thioredoxin and flavin reductase. Both proteins are believed to enhance protein solubility by breaking disulfide bridges among thiol groups. Thioredoxin is featured in the Novagen pET32a(+) plasmid as a fusion protein that enhances solubility upon overexpression with a foreign protein. Three constructs were designed to study the curli-amyloid model: curli/thioredoxin, curli/flavin reductase, and curli alone. Plasmids from five potential pET32a(+) constructs, SW052 to SW056, were isolated by alkaline lysis method and analyzed by restriction digest with *HincII*, *pFlMI*, and *BglI*. No appropriate restriction endonuclease fragments were generated from the tested constructs, but a comparison of the linearized plasmids revealed the absence of an appropriate 790bp *fre* fragment. Another 67 clones, including SA067 to SA069 were generated for curli/thioredoxin, and 6 clones, SA061 to SA066 were generated for curli alone. Neither of the two clones gave appropriate bands in a simple restriction digest and gel electrophoresis experiment. More sequencing experiments and restriction digests must be done to assess the potential of the SA061 to SA069 constructs.

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Alzheimer’s disease (AD) is a neurodegenerative condition characterised by chronic deterioration and loss of intellectual and cognitive skills caused by the accumulation of amyloid β (Aβ) plaques in the brain (10). Limited knowledge of AD pathogenesis coupled with a growing senior population, necessitates the need to study Aβ plaque pathogenesis. This can be achieved with the creation of an amyloid fiber-plasmid model. Akterin et al. postulated the origin of AD to be Aβ peptide deposits in the brain that oxidize the redox protein, thioredoxin (Trx), to liberate an arrested apoptotic signal, thereby generating cell death (1). AD pathogenesis has also been attributed, in terms of plaque formation, to the nucleation of Aβ precursors via disulfide bond formation (9). Trx is an active reducing agent of disulfide bonds (17). It specifically reduces structural disulfide linkages that enable Aβ polymerization, thereby restricting Aβ to their subunit phenotype (9). This Trx-mediated protective effect is supported by consistently low levels of Trx and Trx isoforms in AD regions of the brain (1,11).

*Escherichia coli* (*E. coli*) curli proteins share a resilient, non-branching, β-sheet-enriched morphology with eukaryotic amyloids (2). Plaque formation that is based on nucleation events is analogous in both systems, making curli an ideal candidate for the amyloid fiber-plasmid model (4, 9).

The effect of Trx on the polymerization of amyloid fibers was studied as a function of its effect on the solubility properties of curli. The Novagen pET32a(+) expression vector was used for its ability to facilitate the expression of the target curli protein as a fusion protein with Trx. The reducing property of Trx was expected to prevent curli aggregate formation by facilitating the generation of soluble subunits. This would be enhanced by fusion protein-mediated inhibition of irreversible conformational changes and product aggregation that is normally observed in the expression of isolated proteins. Flavin reductase (Fre) is another redox protein which acts on substrates such as FAD and FMN, reducing them to FADH₂. It shares potentially similar properties to Trx in decreasing aggregate formation of fused insoluble protein targets (9). To assess whether their reducing effects are similar and to investigate Fre’s viability as an alternative to Trx, constructs of *fre*, *trx*, and the gene for curli, *csg*, were used to create the amyloid fiber-plasmid model. A total of three constructs were designed for the curli model: curli alone, curli fused with Trx, and curli fused with Fre. This would be achieved by ligating *csg* into pET32a(+) to generate pET32a(+)*csg*, the curli-Trx positive control for curli solubility. The negative control, curli protein alone, would be made by excising the *trx* gene from pET32a(+) with restriction enzymes, and the test
clone, pET32a(+/ΔtrxA/csg) that encodes curli-Fre would be generated by ligating csg into the construct expressing the negative control.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions** The following bacterial strains and plasmids were used in the study as shown in Table 1.

All the strains were grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or LB plates (LB broth, 15 g/L agar) supplemented with antibiotics as appropriate from table 1, and incubated overnight at 37°C and 250-400 rpm in a shaker incubator.

To make *E. coli* DH5α competent, a starter culture of 25 mL was prepared by inoculating LB broth the night before, and then used to inoculate 500 mL of LB broth. The culture was incubated as indicated above until an OD₅₅₀ of approximately 0.6 was reached. Two-hundred and fifty millilitres of culture was then harvested in a sterile oakridge tube by centrifuging at 5,000 rpm for 10 minutes at 4°C with a JA-14 rotor in a Beckmann J21 centrifuge. Cells were washed twice with 25 mL ice cold 100 mM magnesium chloride solution, followed by a third wash with 25 mL ice cold 100 mM calcium chloride. Finally, the cells were gently resuspended in 20 mL of 100 mM calcium chloride containing 10% glycerol. The cell suspension was distributed among 100 pre-chilled Eppendorf tubes in 200 uL aliquots, and immediately frozen on dry ice. Competent cells were stored at -70°C until further use.

**PCR Amplification of csg from K12 MG1665** To compensate for poor growth observed in liquid *E. coli* K12 MG1665 cultures, a colony from the streaked plate was used to isolate genomic DNA. The Qiagen QIAamp DNA minikit (Cat # 51304) was used. Following the Proteinase K protocol (12), a colony of *E. coli* DH5α was resuspended in 180 uL of lysis buffer (ATL), mixed with 20 uL Proteinase K (>600 mAU/mL), and incubated at 56°C and 200 rpm for 2-3 hours. The remaining steps were carried out as directed by the Proteinase K protocol. Eluates were combined and quantified in an Ultraspec 3000 UV/Visible Spectrophotometer by reading the absorbance at 260nm and 280nm.

A 430 bp csg fragment was amplified from 1 ng of isolated K12 MG1665 DNA by PCR using 20 pmol forward and reverse primers designed by Winnie Kwok (9). The forward primer, 5'-AGT-AGC-GGA-TCC-TGC-AGC-AAT-CGT-ATT-CTC-CGG-3', introduced a BamHI restriction site as underlined at position 15-48, while the reverse primer, 5'-TGA-TGA-AAG-GTTGCG-TTG-TTA-CCA-AAC-ACC-ACC-TGA-3' introduced a HindIII site at position 403-449. The PCR mastermix was completed with 5 uL of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 uL of 50 mM MgCl₂, 0.2 uL of 50 mM dNTP mix, 0.25 uL of 5 U/uL Taq polymerase, and sterile water to fill up a 50 uL reaction volume. Initial denaturation of template DNA was carried out at 95°C for 3 minutes, followed by denaturation at 95°C for 1 minute, 30 seconds of annealing under a gradient of 52°C to 70°C, extension at 72°C for 42 seconds, for a total of 32 cycles, and a final extension at the same temperature for an extra minute. The PCR product was then resolved on a 1% TBE gel, and the remaining reactions were stored at 4°C until further use.

**Confirmation of pET32a(+/ΔtrxA/fre+)** clones The SW051 to SW056 strains are potentially positive pET32a(+/ΔtrxA/fre+) *E. coli* DH5α clones (16). The strains were grown and the plasmids were isolated using the following alkaline lysis method. Ten millilitres of LB broth containing 100 μg/mL ampicillin was inoculated with each clone, and allowed to incubate overnight at 37°C and 200 rpm. Cells were harvested the next day in a 2147 rotor of a Biofuge 17R centrifuge at 4,000 rpm for 10 minutes and 4°C. The supernatant was discarded, and the cells were washed with 500 uL of STE buffer (0.1 M sodium chloride, 10 mM Tris-Cl pH 8, 1 mM EDTA). After a second 10 minute-spin at 4,000rpm, the pellet was resuspended in 200 uL of 50 mM glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA and vortexed. Four-hundred microlitres of 0.2 N sodium hydroxide and 1% SDS was added, inverted five times, and stored on ice. Three-hundred microlitres of a cold solution of 5 M potassium acetate (60 mL), glacial acetic acid (11.5 mL), and water (28.5 mL) at pH 4.8 was added, inverted to maximize precipitation, and stored on ice for up to 5 minutes. The supernatant was discarded, and the cells were washed with 500 uL of STE buffer (0.1 M sodium chloride, 10 mM Tris-Cl pH 8, 1 mM EDTA). After a second 10 minute-spin at 4,000rpm, the pellet was resuspended in 200 uL of 50 mM glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA and vortexed. Four-hundred microlitres of 0.2 N sodium hydroxide and 1% SDS was added, inverted five times, and stored on ice. Three-hundred microlitres of a cold solution of 5 M potassium acetate (60 mL), glacial acetic acid (11.5 mL), and water (28.5 mL) at pH 4.8 was added, inverted to maximize precipitation, and stored on ice for up to 5 minutes. The supernatant was transferred to a fresh Eppendorf tube and an equal volume of phenol:chloroform (1:1) was added to isolate the nucleic acids from the proteins. The mixture was vortexed, and 600 uL of the upper aqueous layer containing the nucleic acids was transferred to a new
Table 2. The ligation reaction conditions for csg, pET32a, and pET32a(+)/ΔtrxA, using Fermentas’ 10X buffer (B0202S) and T4 DNA ligase (M0202S).

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* no pET22a (transformation control)
** phosphatase background control
*** ligase activity control
** including one for pET32a(+)/ΔtrxA

Results

Transformation

Prepared competent cells were thawed at room temperature for 10 minutes. One-hundred microlitres was aliquoted into sterile, pre-chilled Eppendorf tubes. Ten microlitres of ligation reaction was added, and incubated on ice for 50 minutes. The cells were heat-shocked at 42ºC for 90 seconds exactly, and transferred to ice for 10 minutes. Two-hundred and fifty microlitres of prewarmed SOC media (2 g tryptone, 0.5 g yeast extract, 1 mL/L 1M sodium chloride, 0.25 mL/L 1M potassium chloride; or Invitrogen 15454-034) was added, and the cells were incubated horizontally at 37ºC and 190 rpm in the walk-in incubator for 1 hour. Approximately 200 µL of each reaction was plated on LB agar supplemented with 50 µg/mL ampicillin. The cultures were incubated overnight at 37ºC, and stored at 4ºC.

Selected colonies of transformed cells were grown in 10 mL LB broth supplemented with 100 µg/mL ampicillin, and incubated overnight at 37ºC. The plasmids were isolated by the alkaline lysis method outlined for SW051-SW056 clones. Five micrograms or 10 µg of plasmid was digested with HindIII and BamHI for 1 hour at 37ºC, and resolved on a 1% TBE gel.

Csg amplification

Eleven micrograms of csg was amplified from E. coli K12 MG1665. The product was resolved on a 1% TBE gel and a 430 bp fragment was observed (Figure 1). The cleanest band was produced under 68.1ºC annealing temperature of 70ºC. The highest concentration of product was produced under 68.1ºC annealing temperature, and was thus used for cloning.

Confirmation of pET32a(+)/ΔtrxA/Δfre clones

Following the alkaline lysis isolation of plasmids from strains SW052 to SW056, at least 100 µL of plasmid solution was obtained at 23 µg/mL for SW052, 815 µg/mL and 641 µg/mL for SW053, 447 µg/mL for SW054, 181 µg/mL for SW055, and 477 µg/mL for SW056. SW051 was lost during the phenol:chloroform-based nucleic acid-isolation step when isopropanol was accidentally added before the isolation. Despite attempts to precipitate the DNA by diluting the 100% alcohol with water, SW051 could not be salvaged. A purity check was performed to analyse...
the dialysis-based purification of each plasmid solution on a 1% TBE gel. Figure 2 shows the results for SW053 (A) and SW052, SW054, SW055, and SW056 (B). In figure 2a, a single band was observed at ~6000 bp. The band intensity decreased from lanes 3 to 5, and 6 to 8, corresponding to 3.5 ug or 5 ug, 2 ug, and 1 ug of plasmid loaded into the wells of the respective lanes. The absence of other bands indicated a highly pure plasmid solution. Figure 2b shows a single band of the same size for SW054 and SW056, but an empty lane for SW052 and SW055.

FIG. 1. Csg was PCR-amplified under annealing temperatures of 61.2ºC (lanes 1 and 2), 68.1ºC (lane 3), and 70ºC (lane 4). Each condition produced a 430 bp fragment. The Mass Ruler Express DNA ladder from Fermentas (SM1283) was used to evaluate the fragment’s size.

FIG. 2. A. The HindIII digestion of SW053 produced a single band 5,994 bp (~6000bp) in size. Lanes 3-5: represent 3.5 ug, 2 ug, and 1 ug of 815 ug/mL plasmid DNA solution. Lanes 6-8: 5 ug, 2 ug, and 1 ug of 641 ug/mL plasmid DNA solution. Lanes 1-2: 10 uL and 5 uL of Mass Ruler Express DNA ladder from Fermentas to determine the loading volume for optimum band resolution. B. The HindIII digestion of clones SW052, SW054, SW055, and SW056 generated a 6,000 bp fragment for SW054 and SW056. No bands were observed for SW052 and SW055. Between 500 ng to 1 ug of DNA was loaded into the wells.

FIG. 3. Restriction digest analysis of the composition of clones SW052-SW056 with BglII, PfII, and HindIII. A. Lanes 2-7: triple restriction digest of SW052 (lane 2), SW053 (lane 3), SW054 (lane 4), SW055 (lane 5), SW056 (lane 6), and pET32a (lane 7) generated 4,729 bp, 2,081 bp and 1,963 bp fragments from pET32a only. Lanes 8 to 13 represent BglII digests of SW052, SW053, SW054, SW055, SW056, and pET32a respectively. 5,979 bp, 4,206 bp and 2,207 bp fragments were generated from pET32a. The PfII digest of SW052 in lane 14 failed to produce any bands. B. Lanes 2-6: PfII digest of
SW052, SW053, SW054, SW055, SW056, and pET32a. pET32a was nicked into 17,176 bp and 9,557 bp fragments. Lanes 7 to 12: *Hin* _cII_ digest of SW052, SW053, SW054, SW055, SW056, and pET32a. Digest generated 8,500 bp and 3,741 bp fragments from pET32a. Smears in lanes 13 and 14 belong to λ/HindIII (Invitrogen 15612-013) and ligated λ/HindIII (reaction #9, Table 2) respectively.

*Fre* was PCR-amplified from 1 ng of pES1 plasmid. Eight microlitres of the product was resolved on a 1% TBE gel and the results are shown in Figure 4. Faint bands of the expected 890 bp size were seen in lanes 4 to 7 corresponding to annealing temperatures of 56.4ºC, 63.6ºC, 68.1ºC, and 61.2ºC respectively. A bright row of bands was observed along the gel front, representing a high proportion of unused dNTPs and primers.

**FIG. 4.** PCR amplification of *fre* from pES1 plasmid generated an 890 bp fragment under an annealing temperature of 56.4ºC (lane 4), 63.6ºC (lane 5), 68.1ºC (lane 6), and 61.2ºC (lane 7). 51.9ºC (lane 1), 54.0ºC (lane 2), and the negative control (lane 8) failed to produce any bands. Unused dNTPs and primers accumulated at the bottom of the gel, accounting for the relatively low PCR yield. Eight microlitres from 50 ul. of PCR reaction volume was run in each lane. The gel was stained for 53 minutes in 0.2 ug/mL ethidium bromide solution.

**CONSTRUCTION OF pET32a(+)*trxA/csg**

One microgram of *csg* was ligated into 1 ug of pET32a(+)*trxA* vector in a set of reactions. Twenty-four hours of incubation with competent *E. coli* DH5α cells at 37ºC produced 67 colonies under the conditions for reaction #2 (Table 2). None of the remaining conditions produced colonies, including the controls.

Three colonies, SA067, SA068 and SA069, were randomly selected and their plasmids were isolated by alkaline lysis, producing a yield of 57 ug, 34 ug, and 70 ug respectively. 10 ug (SA067, SA069) or 5 ug (SA068) of plasmid underwent a double digest with *Hind*III and *Bam*H1. The digests were resolved on a 1% TBE gel. SA068 and SA069 produced the expected 5,590 bp fragment while SA067 generated the expected 5,960 bp fragment (Figure 5).

**CONSTRUCTION OF pET32a(+)*trxA/csg***

For pET32a(+)*trxA/csg*, both reactions (#1, #6) produced 3 colonies each – SA061, SA062 and SA063 (for reaction #1), and SA064, SA065, and SA066 (for reaction #2). The plasmids from each clone were isolated by alkaline lysis, and digested with *Bam*H1 and *Hind*III. The products were resolved on a 1% TBE gel as shown in Figure 5. An identical band in the expected 5,590 bp size was observed for all 6 clones.

**DISCUSSION**

Curli is encoded by *csg*, a 430 bp gene that was successfully amplified by PCR (Figure 1). An attempt to clone *csg* into pET32a(+)*trxA* and pET32a(+)*trxA/csg* successfully generated 67 clones of potential pET32a(+)*trxA/csg* clones and 6 colonies of potential pET32a(+)*trxA/csg* clones in *E. coli* DH5α respectively. To ensure directional cloning and eliminate non-specific recombination, *Bam*H1 and *Hind*III were selected to create unique ends for ligation. Restriction digest with the same enzymes on pET32a(+)*trxA/csg* clones SA061 to SA069 yielded an identical 5,900 bp fragment with the exception of a slightly larger fragment (5,960 bp) for SA067 (lane 10). The digest containing the pET32a control produced an unusual smear that is concentrated around 5,900bp, an indication that the clones are unlikely to be pET32a(+)*trxA/csg*. Lanes 4-13: SA061, SA062, SA063, SA064, SA065, SA066, SA067, SA068, SA069, and pET32a. Lane 2 contains the Fermentas Mass Ruler Express DNA ladder.

**FIG. 5.** Double restriction digest of clones SA061 to SA069 yielded an identical 5,900 bp fragment with the exception of a slightly larger fragment (5,960 bp) for SA067 (lane 10). The digest containing the pET32a control produced an unusual smear that is concentrated around 5,900bp, an indication that the clones are unlikely to be pET32a(+)*trxA/csg*. Lanes 4-13: SA061, SA062, SA063, SA064, SA065, SA066, SA067, SA068, SA069, and pET32a. Lane 2 contains the Fermentas Mass Ruler Express DNA ladder.

**DISCUSSION**

Curli is encoded by *csg*, a 430 bp gene that was successfully amplified by PCR (Figure 1). An attempt to clone *csg* into pET32a(+)*trxA* and pET32a(+)*trxA/csg* successfully generated 67 clones of potential pET32a(+)*trxA/csg* clones and 6 colonies of potential pET32a(+)*trxA/csg* clones in *E. coli* DH5α respectively. To ensure directional cloning and eliminate non-specific recombination, *Bam*H1 and *Hind*III were selected to create unique ends for ligation. Restriction digest with the same enzymes on pET32a(+)*trxA/csg* clones SA067 to SA069 generated a 5,590 bp fragment from pSA068 and pSA069, and a 5,960 bp fragment from SA067. Since *csg* does not contain restriction sites for either enzyme, fragment sizes of 25 bp and 6,305 bp (*trxA/csg*) or 5,875 bp (*trxA* alone) were expected. Both 5,590 bp and 5,960 bp are relatively close to 5,875 bp. Assuming that the fragments were linear, these fragment sizes indicate
that the ligation and transformation reactions did not work. However, the resolution of the gel and band sizes is insufficient to distinguish between the various plasmids with certainty. As such these results are still ambiguous and more experiments are necessary. Twenty-five base-pair fragments were likely undetected because they were too small to be accurately resolved on a 1% gel with the Fermentas Mass Ruler Express DNA ladder. Also, the band may have melted under the voltage used, or may have run off the gel completely. The transformation reactions for pET32a(+)ΔtrxA/csg also failed as the 5,590 bp fragment lacked an extra 430 bp fragment indicative of csg insertion. This may be explained by the large plasmid size that decreases the efficiency of transformation (11). The 5,590 bp comes very close to the 5,575 bp cleaved pET32a(+)ΔtrxA fragment expected from HindIII-BamHI double-digestion, if the vector was indeed pET32a(+)ΔtrxA.

To address the ligation issues, isoschizomers could be used to generate sticky ends. Unfortunately isoschizomers do not exist for some enzymes or are not commercially available. An isoschizomer does not exist for HindIII but BstI exists for BamHI. Isoschizomers have limitations in that they don’t always generate the same ends. They are also sometimes difficult to digest after ligation. Both HindIII and BamHI are sensitive to methylation. With isoschizomers, methylation often prevents re-digestion at the restriction site. To prevent methylation, wild-type sources could be avoided, and PCR-amplified DNA products sought instead. Another source of non-recombinant clones is the presence of ambiguous compatible ends that have been cut by different enzymes. Although HindIII lacks compatible ends, BamHI is compatible with BclI, BglII, MboI, and XhoI. Also during ligation, lower concentration of DNA template increases the chances of finding compatible sticky ends. The optimal insert:vector ratio is 2:1, close to the 2:1 and 3:1 ratios that successfully transformed E. coli DH5α cells – a positive reinforcement of the ligation conditions used.

Isolating pET32a(+)ΔtrxA/cre clones, SW051 to SW056, with the Pure Link HQ Mini Plasmid Purification kit from Invitrogen (K2100-01) failed to produce enough yield, so the alkaline lysis method was used instead. SW051 was lost during the phenol:chloroform step if some proteins in the interface layer were transferred along with the aqueous layer for alcohol precipitation. Other plausible reasons could be that the volume loaded into the wells was too low, or that the fragment sizes were too dispersed that no similar sized fragments could accumulate to form a band.

Wong (14) had initially reported SW051, SW054, and SW055 as pET32a(+)+ΔtrxA/csg positives, observing a 5,500 bp pET32a(+)+ΔtrxA fragment and at least one other fragment in each (15). In this study however, HindIII-nicked SW053, SW054 and SW056 produced a 6,000 bp corresponding to pET32a(+)+trxA, but lacking any csg insertion. This discrepancy raises doubt on the initial characterization of the clones. It is also possible that the restriction digest did not go to completion, a problem that could be avoided by using enzymes with buffers of lower salt concentration (13). A triple digest with BglII, PflMI, and HincII, and separate digests with each enzyme was performed on the clones. No bands were observed for any of the clones. The possibility of malfunctioning enzymes was eliminated by the multiple bands observed in pET32a(+) digests. This reinforced the inconsistencies between the plasmid concentrations derived by spectrophotometry and that required by the agarose gel standards.

The tactic to use pre-made pET32a(+)+ΔtrxA/csg clones was abandoned. Fre was PCR-amplified from pES1 plasmid under a gradient of annealing temperatures, albeit inefficiently as indicated by a high proportion of unused dNTPs and primers. The reaction may have been compromised by the presence of EDTA or phosphates that would have affected the integrity of the DNA. Despite the low yield, an annealing temperature of 63.6°C was able to produce a relatively intense band. However, due to the lack of time the cloning process had to be halted at this point.

Despite the attempts to clone csg, it is necessary to remember that the sequences used to design the experiments were just references from the NCBI Genbank database. The strain may have still mutated along the way, and may no longer have been identical to that in the database. This may or may not have adversely affected the progress of this study. To keep track of these changes in the future, more elaborate restriction enzyme digests should be used.

The curli-based amyloid fiber model was designed to study the pathogenesis of eukaryotic amyloid plaques in Alzheimer disease (AD) patients. The model was to be composed of thioredoxin-curli and flavin reductase-curli fusion proteins, as well as free curli
proteins, to compare the effects of reducing thioredoxin (TrxA) and flavin reductase (Fre) on the solubility of curli. Since none of the proteins were successfully generated in this study, further experiments have to be designed to circumvent the problems with ligation and transformation encountered thus far. The results will provide hope for a cure to this debilitating, neurodegenerative disease, and a better future for a population that isn’t getting any younger.

FUTURE EXPERIMENTS

To start, isolate the plasmids from SA061 to SA069 and digest them with a different combination of restriction enzymes to create a unique restriction map for each. Alternatively, the clones could be sent for sequencing to reliably characterize the composition of the plasmids. Similarly, the PCR products of fre and csg could be sent for sequencing to identify any mutations. To address the ligation problems, the efficiency of T4 DNA ligase could be investigated using different manufacturers and conditions.

Once the correct clones have been created, the recombinant plasmids should be transformed into a competent expression host like E.coli BL21. The cells that successfully grow in ampicillin and IPTG-fortified LB broth should be harvested and inoculated on YESCA plates. Positive clones will be identified by green colonies. The protein solubility can then be analyzed by SDS-PAGE using 90% formic acid treatment and protein polymerization studies.

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