Co-expression of PDI and *P. falciparum* vaccine candidate AMA1 in *Pichia pastoris*

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← **Malaria, caused by the *Plasmodium* parasite, claims the lives of 1.5 to 3 million victims each year. Vaccine development against malaria remains a challenge due to the complexity of the *Plasmodium* parasitic life cycle. This experiment, formed through an internship at NIAID's Malaria Vaccine Development Branch (MVDB), may potentially contribute to the development of a malaria vaccine. In this project, two genes were co-expressed in *Pichia pastoris* yeast: a protein disulfide isomerase (*PDI*) gene and a gene for a malaria antigen vaccine candidate, Apical Membrane Antigen I (*AMA1*). PDI is a chaperone protein shown to increase the expression of other proteins by facilitating protein folding within the *Pichia*'s endoplasmic reticulum. AMA1 is a *Plasmodium* antigen that is currently MVDB's lead vaccine candidate. In this experiment, the genes for *AMA1* and *PDI* were cloned into a plasmid, which was transformed into the *Pichia pastoris* protein expression system. It was found that co-expressing these genes within a vector increased the levels of AMA1 vaccine candidate production. These results could potentially save MVDB significant funds by increasing the production yields of AMA1 proteins for use in future vaccines.**

INTRODUCTION

The millions of malaria infections occurring yearly pose a remarkable threat to global health. The annual devastation of malaria is currently beyond comprehension: 1.5 to 3 million people die each year from malaria infections, and ninety percent of fatalities occur amongst sub-Saharan African children, less than five years of age [4].

The malaria disease is caused by *Plasmodia*, a group of species from the Sporozoa protozoan parasites. Each stage of the *Plasmodium* parasitic life cycle has a role in the transmission, disease, and latency periods of the malaria infection [3]. Four different types of malaria are specific to human infection, and these are caused by four related *Plasmodium* species: *falciparum, vivax, malariae* and *ovale* [3]. Of the four *Plasmodium* species, *Plasmodium falciparum* is the most serious, leading to severe morbidity and mortality around the world [1]. As such, the majority of vaccine development efforts target the *Plasmodium falciparum* species [8].

Vaccine development against malaria remains a challenge due to the complexity of *Plasmodium*; the parasite has an extensive life cycle and multiple antigenic variants [8]. Antigen from *Plasmodium* are stage-specific, where antigen from the parasite's erythrocytic stage differ highly from those produced during its hepatic stages or mosquito stages [8].

Global impact of malaria is not only medical, but social and economic as well, and the production of an effective vaccine remains a crucial and pressing issue worldwide [8]. We can only hope that by gaining a better understanding of the transmission, life cycle, and drug resistance of *Plasmodium,* we will eventually develop vaccine options that will lead to the eradication of this global health concern.

Plasmodium **Vaccine Development**

While there are no vaccines in standard use against the *Plasmodium* parasite, the development of an effective vaccine could lower the increasing costs of drug treatment and save millions of lives [8]. Malaria is becoming more of a global threat as *Plasmodia* develop increasingly significant drug resistance. Factors such as the high prevalence of disease and continuous drug pressure have led to drug-resistant strains of *Plasmodium*. And similarly to the drug treatment guidelines set for HIV treatment, the World Health Organization now has guidelines to increase the combination therapy of malaria in order to slow the development of resistance. High rates of gametocyte recombination in *Plasmodium* may provoke the development of highly-resistant strains [3].

The prevalence of drug-resistant *Plasmodium* parasites and the enormous economic costs of treatment in tropical and sub-tropical countries make an effective vaccine an urgent necessity [8]. The complicated life cycle of malaria poses serious challenges in vaccine development; the malaria parasite life cycle has a variety of protective immune responses, and usually different, often polymorphic, antigens [8].

AMA1 Vaccine Candidate

Apical membrane antigen (AMA1) is currently the lead vaccine candidate of the Malaria Vaccine Development Branch (MVDB) [6]. The AMA1 protein is found on the apical end of the *P. falciparum* species*,* when the parasite is in its erythrocytic stage in the human bloodstream. Because of its location on the outside of the parasite as well as its role in the invasion of the red blood cells, the AMA1 antigen is an excellent target for vaccine development [6]. It has been shown that vaccination with AMA1 in rodents and primate models can elicit an effective antibody response against the *P. falciparum* parasite [6]. With promising clinical trials underway, the large-scale production of recombinant AMA1 proteins is required for development of the AMA1 vaccine.

Pichia Pastoris **Protein Expression System**

Pichia pastoris is a species of methylotrophic yeast commonly used for the production of recombinant proteins such as AMA1 [1]. This yeast has several advantages as

a protein expression system, one being that it is single-celled and easy to manipulate and culture [1]. In addition, *P. pastoris* is faster and less expensive than other expression systems derived from higher eukaryotes and typically give higher expression levels[1]. As a eukaryotic species, *P. pastoris* can perform many of the post-translational modifications seen in higher eukaryotes, such as proteolytic processing, folding, disulfide bond formation and glycosylation [1]. This makes *P. pastoris* well equipped for the production of therapeutic proteins which require post-transcriptional modification [5].

Protein Disulfide Isomerase (PDI)

Protein Disulfide Isomerase (PDI) is a 57 kDa protein found in the endoplasmic reticulum of *P. pastoris* [5]. Here, it serves the critical role of rearranging proteins that have been formed with incorrect disulfide pairings [5]. The disulfide bonds of misfolded proteins are broken and reformed by PDI [5]. This protein works efficiently and without radically changing the normal folding pathway. It ensures that secreted proteins are correctly folded [5].

This quality correction mechanism is crucial to high-production protein expression systems such as that found in the methylotrophic yeast, *P. pastoris*. When the *P. pastoris* expression system is used to produce high levels of therapeutic proteins, there is an increased risk for error. Overexpressing proteins in a nonoptimal, nonnatural environment can lead to deleterious problems and saturation of the secretory pathway due to the buildup of misfolded proteins [5]. Therefore, it has been examined whether the over-expression of PDI can improve the secretion of recombinant proteins in yeast by decreasing levels of misfolded proteins [5].

In a study by Inan et al., it was discovered that the expression of a potential vaccine candidate, *Necator americanus* secretory protein (Na-ASP1) increased with an increase in the PDI copy number. Inan et al. increased the PDI copy number by amplifying the *PDI* gene into a PDI expression plasmid and transforming the plasmid into *P. pastoris* cells, which contained plasmids with the gene for the *Necator americanus* secretory protein. These results demonstrate the importance of PDI's refolding capacity in the endoplasmic reticulum in order to facilitate the secretion of recombinant proteins containing disulfide bonds [5]. Furthermore, it is believed that these results could be applicable for other proteins and may facilitate the understanding of *P. pastoris* secretory pathways [5].

Folding and processing in the endoplasmic reticulum are some of the most critical obstacles for maximum expression of recombinant proteins in *Pichia pastoris* [5]. By

increasing the copies of PDI expressed in *P. pastoris*, it may be seen that the levels of recombinant vaccine candidate proteins such as AMA1 may be increased as well.

In this experiment, we attempted to develop a co-expression vector for *P. pastoris*, which expressed the genes for both *PDI* and *AMA1*. It was our hope that the overexpression of PDI could facilitate protein folding in the *P. pastoris* secretory pathway, leading to higher levels of AMA1 protein expression, potentially for use in vaccine development.

MATERIALS AND METHODS

Cloning of the *AOX1* promoter

To generate our desired vector we needed to clone the *AOX1* promoter and gene into our expression vector, pPICZaA. We used PCR ligation to create the *AOX1* promoter. PCR amplifications were performed with the DNA polymerase *Phusion*, 5mM dNTP mix, primers and a Stratagene Robocycler. Two PCR reactions, in attempts to form two products, the 5' end of *AOX1* and the 3'end of *AOX1*, were performed. 1 cycle at 98ºC (30 s), 5 cycles at 98ºC (10s), 55ºC(30s),72ºC(60s), respectively, followed by 15 cycles at 98ºC (10s), 58ºC(30s), 72ºC(60s), respectively, and 1 cycle at 72ºC(10 min) were employed using genomic DNA as template. Two products of lengths 295 bp (3' *AOX1*) and 827 bp (5' *AOX1*) were obtained and prepared for a PCR ligation. The second round of PCR, to create the full fragment, used the two products from the first amplification (3' *AOX1* and 5' *AOX1*) as a template. Again, PCR amplifications were performed as before. The cycles were employed as follows: 1 cycle at 98ºC, 20 cycles at 98ºC (10 s), 60ºC (30 s), 72ºC(60 s), respectively, and 1 cycle 72ºC(10 min). The expected product of 1078 bp (*AOX1*) was obtained and prepared for insertion into the pPICZaA vector.

The *AOX1* PCR product and the pPICzaA vector were both digested with restriction enzymes BamHI and BglII in preparation for ligation. The pPICzaA vectors were dephosphorylated, using a phosphatase. DNA ligase was used to ligate a 5:1 fmol ratio of insert (*AOX1*) to vector (pPICzaA). Ligated products were transformed into Invitrogen's chemically competent TOP10 *E. coli*., the tranformants were selected on LB agar plates containing 50 ng/µl Zeocin and incubated at 37ºC overnight.

Colonies showing Zeocin resistance were picked from plates and grown up separately in LB media with 50µg/ml Zeocin. Cultures were incubated 24 hours, 37º at 250 rpm. Plasmids were isolated from *E.coli*,, and were sequenced by a commercial provider. Final plasmid products were identified as either pPICzaA AOX-R or pPICzaA AOX-F, depending on the orientation of their *AOX1* gene.

Cloning of the *PDI* gene

Cloning of the *PDI* gene into pPICzaA AOX-R and pPICzaA AOX-F did not require PCR, as the *PDI* gene could be excised from a separate vector using the BamHI restriction enzyme. Similarly, the pPICzaA AOX-R and pPICzaA AOX-F vectors were cut with BamHI and then dephosphorylated with a phosphatase in preparation for ligation. DNA ligase was used to ligate a 5:1 fmol ratio of *PDI* insert to either of the vectors (pPICzaA AOX-R or pPICzaA AOX-F). Ligated plasmids, pPICZaA AOX-R/PDI and pPICZaA AOX-F/PDI, were transformed into *E. coli* as before.

Colonies showing Zeocin resistance were selected and DNA was isolated and sequenced as before. The two plasmid products were renamed Pzr-PDI-RE and Pzf-PDI-F5, depending on the orientation of the *PDI* gene.

Cloning of the *AMA1* gene

Similar methods were used to clone the *AMA1* gene into the newly created expression vectors: Pzr-PDI-RE and Pzf-PDI-F5. XbaI and XhoI restriction enzymes were used to isolate the *AMA1* gene from a separate vector. Both the *AMA1* gene and the Pzr-PDI-RE and Pzf-PDI-F5 vectors were then cut with the XbaI and XhoI restriction enzymes. The vectors were desphosphorylated with a phosphatase. Cut *AMA1* inserts and vectors were ligated in a 5:1 fmol ratio, and the ligated products were transformed into Invitrogen's chemically competent TOP10 *E. coli*. Transformed *E. coli* cells were selected on 50 ng/µl Zeocin LB agar plates incubated at 37ºC overnight.

Colonies showing Zeocin resistance were picked from plates and grown up separately in tubes containing media broth with 50µg/ml Zeocin. Tubes were incubated 24 hours, 37º at 250 rpm. Plasmids were isolated from two of the *E.coli* colonies, and DNA was sequenced by a commercial provider. The two plasmid products were renamed Pzr-PDI/synAMA-Ala/c and Pzf-PDI/synAMA-Ala/2, depending on the orientation of their *AMA1* gene.

Transformation of vectors into *Pichia pastoris*

Five plasmids were chosen for transformation into the X33 strain of *Pichia pastoris*: Pzr-PDI/synAMA-Ala/c and Pzf-PDI/synAMA-Ala/2, as well as 3 controls, including Pzr-PDI-RE and Pzf-PDI-F5 and pPICzaA/AMALI-32. These five plasmids were digested with SacI to linearize the plasmids for *P. pastoris* transformation.

P. pastoris cells were transformed by electroporation with 10ng of each plasmid. Transformants were selected for on YPD plates containing 1M Sorbitol and .1mg/mL Zeocin. Plates were incubated at 30C for 3 days.

Characterization of Proteins

Transformants were initially screened through colony blots to assess *P. pastoris* secretion. Colonies were grown on a low protein-binding cellulose acetate membrane. The membrane was transferred to methanol induction media plates overlaid with a nitrocellulose membrane. Plates were incubated 24 hr at 30ºC. The membrane was developed as a western blot, using Anti-AMA1 antibodies.

Colonies showing significant expression on the colony blot as well as an additional clone of transformed *Pichia* containing the AMA1 Production Cell Bank plasmid were selected for further analysis through shake flask test inductions. Clones were grown in methanol induction media to induce protein expression. Culture supernatants containing secreted proteins were collected at 0, 24, 48, 72 and 96-hour time-points and stored at -80ºC.

A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the shake flask test induction supernatants. Secreted proteins were visualized with Coomassie Brilliant Blue dye.

A time-course western blot was performed on the AMA1 production cell bank clone as well as our best expressing clone, Pzf-PDI/synAMA-Ala/2 F2. Samples were run on an acrylamide gel for 100 minutes at 25 mA. The gel was transferred to a nitrocellulose membrane and run at 30V for 75 minutes. The membrane was blocked and then incubated overnight in anti-AMA1 antibody solution. The rinsed membrane was developed in phosphate substrate for several minutes.

RESULTS

In this experiment, three genes were cloned into a pPICzaA vector: an *AOX1* promoter, a *PDI* gene, and an *AMA1* gene. The cloning map of the final vector Pzf-PDI/synAMA-Ala/2 is shown (Figure 1). It should be noted that vector Pzr-PDI/synAMA-Ala/c, containing the reverse orientation of the three genes, is the mirror image of Pzf-PDI/synAMA-Ala/2 (Figure 1).

In order to initially characterize the transformed *P. pastoris* cells, a colony blot for AMA1 secretion was performed (Data Not Shown). However, results from the colony blot were inconclusive, and colonies that seemed to have better expression,

includingPzf-PDI/synAMA-Ala/2 colonies 1,2,5,6, the L32 colony 1, and the pPICzaA-AMA1 colonies 9 and 10, were selected and analyzed further by shake flask test inductions.

Supernatant containing secreted proteins from the shake flask test inductions were collected at 0, 24, 48, 72 and 96 hour time-points. An SDS-PAGE showing protein secretion in supernatant collected at the 96 hour time-point was stained with Coomassie blue dye (Figure 2). Coomassie Brilliant Blue is a non-specific dye , which shows all protein expression, including that of PDI. Figure 2 shows bands at approximately 55 kDA, indicating the presence of PDI as well as bands approximately at 66 kDA, indicating AMA1 expression.

Each column contained supernatants from one colony expressing different vectors. The far left column contained supernatant from the background strain of *P. pastoris* X33, which was not transformed with any vectors (Figure 2). As expected, no bands that indicated PDI or AMA1 expression appeared in this column. The column on the far right contained a vector with *PDI* and no *AMA1* gene (Pzf-PDI-P11), and it was seen that a band for PDI and not AMA1 was present as expected (Figure 2). The 2 columns labeled in white had a vector containing the *AMA1* gene and no *PDI* gene (pPICZaA-AMA1-A9 and A10); these samples contained a band for AMA1 expression and no band for PDI expression (Figure 2). Similarly, the column labeled in green contained the current clone being used for AMA1 production by the MVDB; this clone also contained a vector with an *AMA1* gene but no *PDI g*ene (Figure 2). Again, no band was present to indicate PDI expression, but a band was visible that indicated AMA1 expression. The columns labeled in yellow were from colonies with the co-expression vector (Pzf-PDI/synAMA-Ala/2 F1, F2, F5, F6), and they contained bands for both PDI and AMA1 (Figure 2).

On the Coomassie stained SDS-PAGE, darker bands indicated increased protein expression. Here, it was seen that the darkest AMA1 bands corresponded to the colonies with the co-expression vector, and that these AMA1 bands were dramatically darker than those from vectors without the *PDI* gene(Figure 2). The best total expression was seen in one of the co-expression vectors (Pzf-PDI/synAMA-Ala/2-F2). As such, this colony was selected for further analysis through a time-course western blot.

← Figure 3 shows a time-course western that was performed on co-expression vector Pzf-PDI/synAMA-Ala/2-F2 as compared to the AMA1 production cell bank clone. This clone contains the vector currently being used by MVDB for AMA1 production. The left side of the western blot showed the time-course of AMA1 expression for the co-expression vector Pzf-PDI/synAMA-Ala/2-F2, starting at 0 hours to 96 hours (T0 to T96) (Figure 3). The bands indicated AMA1 expression, and again, darker bands indicated higher levels of AMA1 expression. It could be seen by the progressively darker bands from 0 hours to 96 hours, that AMA1 protein expression increased over time. The right side of the western blot showed the AMA1 production cell bank clone, and again, it could be seen that protein expression increased slightly from 0 hours to 96 hours (Figure 3). When comparing the two clones, there was a dramatic difference in the darkness of bands, showing that the co-expression vector had significantly more AMA1 protein expression (Figure 3).

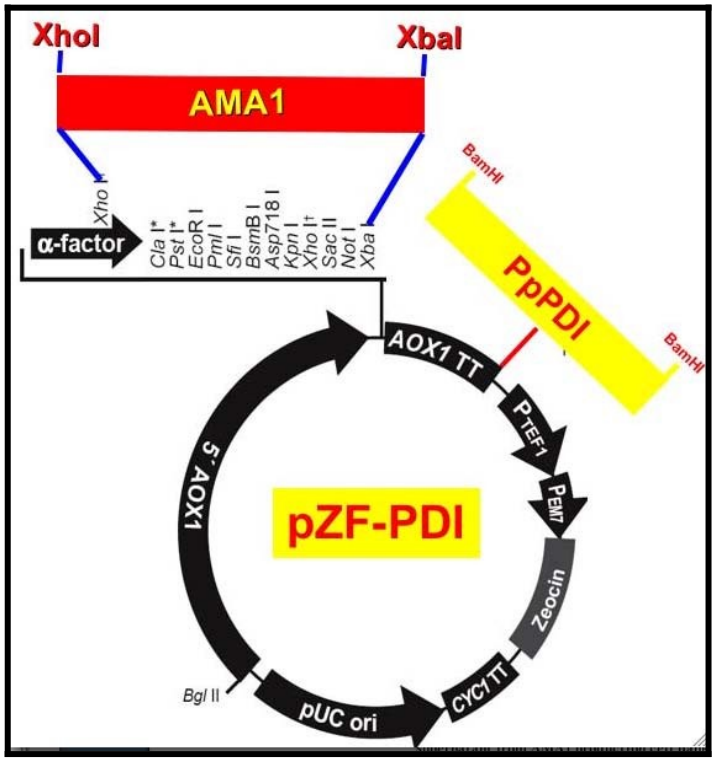

Figure 1. Cloning map of the co-expression vector: contains *AMA1* and *PDI*.

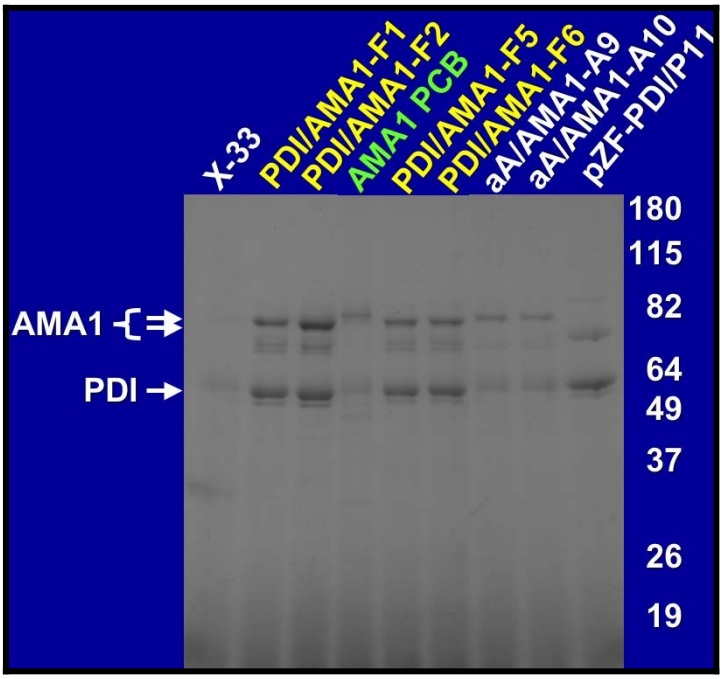

Figure 2. Coomassie stained gel of shake flask test induction transformants at 96 hour time-point: lane 1, X-33 background strain (no transformation); lane 2,3, clones with co-expression vectors Pzf-PDI/synAMA-Ala/2-F1, F2, respectively; lane 4, AMA1 production cell bank clone; lane 5,6, co-expression vectors PDI/synAMA-Ala/2-F5, F6, respectively; lane 7,8, clones with AMA1 expression vector and no *PDI* (pPICZaA-AMA1-A9 and A10); lane 9, clone with PDI expression vector and no AMA1 (Pzf-PDI-P11).

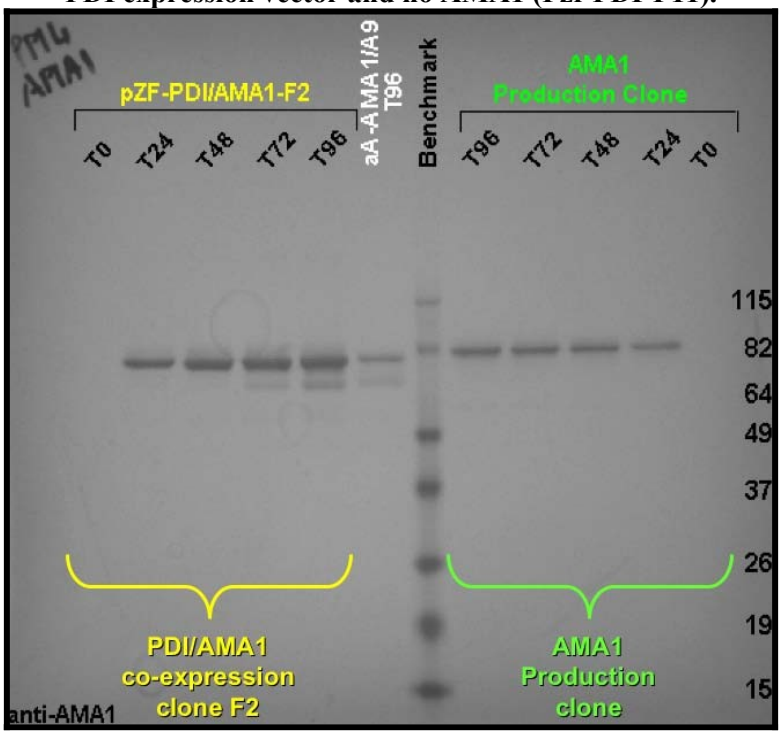

Figure 3. Time-course western blot from shake flask test inductions: lanes 1-5, T0-T96 supernatant from co-expression vector clone Pzf-PDI/synAMA-Ala/2-F2; lane 6, T96 supernatant from pPICzaA-AMA1-A9; lane 7, molecular weight marker; lanes 8-12, T96-T0 supernatant from AMA1 production cell bank clone.

DISCUSSION

Through this experiment, it was our hope to accomplish two goals: 1) to successfully produce a PDI co-expression vector, and 2) to determine whether the co-expression vector could allow for increased AMA1 expression.

Sequencing by a commercial provider showed that the DNA sequence of the co-expression vector was as expected, demonstrating that cloning techniques used to insert the *AOX1* promoter, *PDI*, and AMA1 were carried out successfully. One challenge in the development of this vector was the production of an *AOX1* promoter that did not include a BamHI restriction site within the sequence.

P. pastoris is unique in that it uses a tightly regulated alcohol oxidase I promoter (*AOX1*). The yeast metabolizes methanol with the enzyme alcohol oxidase [1]. The *AOX1* gene that codes for the enzyme induces enzyme expression with increased methanol levels [1]. The *AOX1* gene has been isolated and its promoter is used to drive the expression of the gene of interest [1]. *P. pastoris* vectors have DNA sequences for the *AOX1* promoter, followed by unique restriction sites for insertion of a foreign gene [1].

Typically, the *AOX1* promoter contains a BamHI restriction site within the middle of the sequence, as well as one near the end. Since the BamHI restriction enzyme was needed to cleave the end of the *AOX1* promoter in preparation for insertion into the pPICzaA vector, the additional BamHI restriction would have resulted in an unwanted cleavage within the sequence. In order to prevent the presence of this additional BamHI site, we performed a PCR ligation which allowed us to amplify both the 5' end and the 3' end of the promoter separately without the extra BamHI site. The amplified end pieces could be combined in a PCR reaction and amplified together in order to form a complete *AOX1* promoter with a synonymous change that removed the extra BamHI site. This process was carried out successfully, as it was found that the final *AOX1* promoter was not cut into multiple pieces when exposed to the BamHI restriction enzyme, and thus did not contain the unnecessary original BamHI site.

The second goal of this experiment was to determine whether the co-expression vector could allow for increased levels of AMA1 expression, when compared to vectors which only contained genes for *AMA1* and no *PDI.* Our results from both the Coomassie stained gel as well as the time-course western lead us to believe that AMA1 expression levels increased with the co-expression of PDI and AMA1. The Coomassie stained gel was able to detect for expression of both PDI and AMA1, and it was found that the co-expression

vectors showed high levels of expression for both PDI and AMA1, demonstrating the presence of functional *PDI* and *AMA1* genes within the co-expression vector (Figure 2). When comparing the levels of AMA1 expression between the co-expression vectors and the vectors containing only *AMA1* and no *PDI*, there was a significant difference in the darkness of the AMA1 bands (Figure 2). The AMA1 production cell bank clone and the other two clones containing genes for *AMA1* and no *PDI*, had dramatically lighter AMA1 bands than the co-expression vectors, indicating higher levels of AMA1 expression from the co-expression vectors (Figure 2). Again, it should be noted that the AMA1 production cell bank clone is currently the clone being used by the MVDB to produce AMA1 for vaccine use.

In the time-course western blot of the AMA1 production cell bank clone and the co-expression vector clone (Pzf-PDI/synAMA-Ala/2-F2), a significant difference could be seen between the bands of the co-expression vector clone and the production cell bank clone (Figure 3). Since Anti-AMA1 antibodies were used to detect AMA1 secretion, this western blot was specific for AMA1 expression levels, where darker bands indicated higher levels of AMA1 expression. It was seen for both clones that from 0 hours to 96 hours, AMA1 expression bands darkened, indicating that AMA1 expression increased over time (Figure 3). When comparing the darkness of bands for the co-expression vector clone and the AMA1 production cell bank clone, it was seen that bands for the co-expression vector were significantly darker, which again indicates that the co-expression vector had higher levels of AMA1 expression (Figure 3).

The results from the colony blot , which was the initial test used to detect AMA1 secretion, were inconclusive (Data Not Shown). Dots indicating AMA1 expression were hardly visible, and control samples, which were known expressers of AMA1, did not have any dots. However, it was found that a sticky, clear substance seemed to be growing between the plate and the nitrocellulose membrane, leading us to believe that plate contamination most likely affected the results of our colony blot. However, the presence of slightly visible dots allowed us to select a few of the colonies for further analysis through the shake flask test inductions.

Overall, the results from the Coomassie stained gel and the time-course western demonstrate that PDI over-expression can indeed be beneficial for the production of AMA1. While the mechanisms for this phenomenon were not explored in this experiment, past studies have speculated that PDI and other chaperone proteins can prevent cell stress associated with the unfolded protein response induced by the accumulation of misfolded

proteins [5]. By rearranging the misfolded disulfide bonds of proteins in the endoplasmic reticulum, PDI can prevent misfolded proteins from saturating the cell's secretory pathway [5]. Since folding and processing in the ER are some of the most critical obstacles for maximum expression of proteins in *P. pastoris*, PDI can have a significant impact on the efficiency of the secretory pathway.

In order to further confirm the success of this PDI co-expression vector, it may be important to analyze the amino acid sequence of the AMA1 and PDI proteins. The western blot and Coomassie stained gel indicated that the secreted AMA1 and PDI proteins were generally the correct size, and the recognition of AMA1 with anti-AMA1 antibodies indicated that the secreted AMA1 was generally correct in its conformation. However, sequencing the AMA1 and PDI amino sequences could further confirm that no minor abnormalities exist within the proteins' configuration.

The main accomplishment of this project was the production of a successful PDI co-expression vector. Although this co-expression vector was only tested with the AMA1 gene, it may also be that this co-expression vector could increase the protein expression of other vaccine candidates. A potential next step in this experiment would be to test this PDI co-expression vector with other therapeutic recombinant proteins to further determine its capacity as a tool for recombinant protein production. Similarly, another future experiment would be to create a co-expression vector which included genes for other ER chaperone proteins, such as *BiP,* in order to determine if the over-expression of many chaperone proteins affects recombinant protein expression.

The effects of the PDI co-expression vector developed in this experiment are important for production labs such as the MVDB. Currently, the MVDB's Good Manufacturing Practice (GMP) production runs for generating AMA1 proteins cost over $280,000 each. Increases in AMA1 expression yields in each run through the use of a PDI co-expression vector could result in significant savings.

In the future, it may be seen that the AMA1 protein becomes the target for an effective *P. falciparum* vaccine in humans. Through this experiment, it was shown that PDI has a profound effect on the levels of AMA1 protein expression in *P. pastoris*. Potentially in the future, *P. pastoris* expression systems containing an over-expression of PDI may someday be key to the development of vaccines utilizing large amounts of these therapeutic proteins. As research efforts towards the malaria vaccine progress, it may be that the use of

PDI in a co-expression vector for therapeutic protein expression will help to achieve this long-awaited goal of effective *P. falciparum* vaccine development.

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