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M. McWilliams, G. Chan, C. Radford, K. Tokaryk, M. Russell, D. Mah
Canada West Biosciences Inc.

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M. McWilliams, G. Chan, C. Radford, K. Tokaryk, M. Russell, D. Mah
Canada West Biosciences

D. Mah, Project Manager
Canada West Biosciences
113-339 50th Ave S.E.
Calgary, AB T2G 2B3
1-403-255-3788

B.J. Berger, Contract Scientific Authority
Defence R&D Canada – Suffield
1-403-544-4621

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Michael McWilliams

Gene Chan, Cynthia Radford, Kara Tokaryk, Michelle Russell, and David Mah

Canada West Biosciences Inc.
113-339 50th Ave. SE
Calgary AB T2G 2B3
Contact: D.C.W. Mah, 1-403-255-3788

Contract Technical Authority: Dr. Bradley J. Berger
Chemical and Biological Defence Section, DRDC Suffield
Phone No. 1-403-544-4621

DRDC-Suffield CR 2005-110

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Author

Michael McWilliams

Approved by

[Enter name here]
[Enter title here]

Approved for release by

[Enter name here]
[Enter title here]

Abstract

Antibiotic resistance is of increasing medical concern. As the old therapies become ineffective, new drug targets must be identified. Metabolic pathways offer attractive chemotherapeutic drug targets which have until recently been laborious to discover. The advent of large scale genome sequencing projects has revealed the information required to begin the identification and analysis of new target enzymes involved in many metabolic pathways in some of the medically important pathogens such as *P. falciparum*, the causative agent of malaira, and *B. anthracis* which causes anthrax.

Results: We characterized enzymes from the following organisms: the protozoan *Plasmodium falciparum,* the bacilli *Bacillus cereus, Bacillus anthracis,* and *Bacillus Subtilis,* and the mycobacteria *Mycobacterium tuberculosis, Mycobacterium marinum,* and *Mycobacterium smegmatis.*

To this end, we cloned the chosen enzymes into various *E. coli* expression vectors and induced their expression in small-scale culture. After confirmation of expression, we purified the enzymes using affinity FPLC and performed preliminary enzyme assays to determine the activity of the expressed protein. We found some of the enzymes could not be expressed at all, while others were expressed only as inclusion bodies. We performed kinetic studies on those enzymes that were expressed, and found to be active.

The *P. falciparum* ASAT was demonstrated to have a V_{max} of 15.36 nMol/min/mg and a K_m of 2.07 mM when glutamate and KMTB were used as substrate. The *B. anthracis* amino transferase was shown to have similar preference for isoleucine, leucine, and valine as amino donors, with KMTB and KG as amino acceptors with K_m values between 0.41-1.75 mM and V_{max} values between 0.13-0.45 µmol/min/mg. The *P. falciparum* cysteine desulfurase enzyme was successfully produced and analyzed for specific activity in E. coli BL21 Rosetta cells. We found that the induction conditions made a large difference in enzyme activity; the maximum specific activity obtained was 40.34 µmol Alanine/min/mg using the pET41b vector. The *M. tuberculosis* MTAN enzyme was demonstrated to have a V_{max} of 189.47 nmol/min/mg and a K_m of 0.49mM using MTA substrate. Substrate level inhibition with a K_{si} of 0.879 was noted with this enzyme. The *M. smegmatis* MTAN enzyme was demonstrated to have a V_{max} of 2.466 nmol/min/mg, and a K_m of 0.09 mM. The *M. smegmatis* MTAP enzyme was demonstrated to have a V_{max} of 3128.742 nmol/min/mg and a K_m of 3.185 mM.

We also examined the utility of a commercially available xylose inducible *Bacillus megaterium* expression system. We modified pWH1520 parent expression vector to include 10x histidine affinity tags, and sequences which signal for secretion to the growth media. Preliminary expression results with the *B. megaterium* system were encouraging.

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Executive summary

Introduction: The research being pursued is a study of enzyme characterization. However, the enzymes of interest come from pathogenic organisms such as *P. falciparum* which is the causative agent of malaria and the *Mycobacteria* which cause diseases such as tuberculosis and leprosy. Certain pathogenic organisms are difficult or even impossible to grow under standard laboratory conditions. This makes the study of metabolic pathways and associated potential drug targets in these organisms more complicated. By isolating the genes coding the enzymes in the pathways, and expressing the genes in bacterial expression systems which are easier to manipulate, the relevant proteins may then become available to study.

Bacterial protein expression systems offer several positive attributes: relative ease for incorporation of new protein in an existing system; high protein production; well developed purification methods; and scaling up production. This report describes our efforts to clone and characterize enzymes from pathogens using commercially available protein expressions systems.

Initially we used the *E.coli* based expression systems BL21 codon+ and rosetta based on reports of success by others. Eventually, we began to explore the use of a *B. megaterium* expression system as an alternative for bacillus derived proteins. *B. megaterium* is known for lacking proteases common to other bacilli, and there is a commercially available expression vector based on the xylose operon for ease of cloned gene expression.

Results: We generated an array of completed expression clones of various enzymes related to metabolic pathways for both the *E. coli* and *B. megaterium* expression systems. In addition we modified a commercial vector for *B. megaterium* to take advantage of protein secretion pathways and affinity tags for easing downstream purification.

Some of the enzymes that were produced in these expression systems demonstrated detectable activity. We performed kinetic assays on these enzymes during their characterization.

Conclusions: We were able to express most of the identified target enzymes in two bacterial expression systems. However only a few of those enzymes demonstrated detectable enzyme activity. Other expression systems may improve on this result.

Future directions: Follow up research to this work demonstrated successful inhibition of the *B. anthracis* amino transferase enzyme with amino-oxy compounds. We expect that similar studies will be done on the enzymes that were produced during the course of this project.

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Introduction

Microbial organisms represent one of the greatest medical challenges. There are numerous species of different lineages which are destructive agents. Malaria and tuberculosis are significant diseases in developing countries. Anthrax is one of many bacterial bioterror agents. As resistant strains arise it is with earnest that countermeasures should be developed. For example, the rate of resistance amongst anthrax isolates to penicillin has increased to 11.5% by some estimates [1], and resistance to ciprofloxacin can be induced *in vitro* after a few selective trials.[2]

Targeting the components of prokaryotic protein expression has been a successful method of developing antibiotics in the past, and will continue to yield results in the future because the components of protein expression while not identical are shared by all of life with the exception of viruses.

Though we tried to examine a number of enzymes from distinct metabolic pathways, we had the most success with enzymes involved in the regeneration of methionine consumed during polyamine biosynthesis.

The *de novo* synthesis of methionine is energetically expensive, requiring aspartate, ATP, NADPH, succinyl-coenzyme A or acetyl-coenzyme A, cysteine or H_2S and 5-methyltetrahydrofolate. [3] Most organisms have evolved an enzymatic pathway to regenerate methionine, making this pathway an excellent drug target. Methionine is an important amino acid because this amino acid represents a critical bottleneck in protein production: if no methionine is available, protein synthesis does not begin and growth is arrested. This is also an important pathway because it represents a common target amongst important protozoan and bacterial pathogens. This report summarizes the effort to produce and characterize these critical enzymes.

An overview of the methionine recycling pathway is shown in Figure 1 illustrating the enzymatic steps involved, with the targets whose kinetics were successfully examined in this study highlighted. Following Figure 1 is Table 1 outlining which enzymes were successfully examined during the course of this work including enzymes unrelated to methionine regeneration.

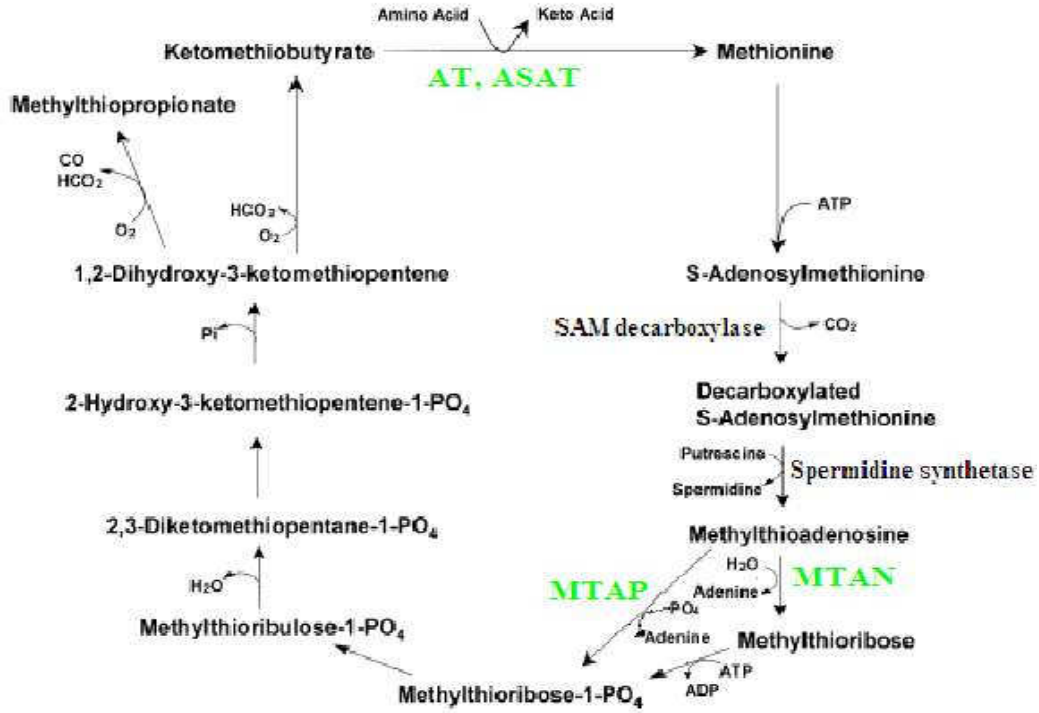

Figure 1: Enzymatic pathway for methionine regeneration* highlighting enzymes whose kinetics were successfully analyzed

*diagram adapted from[3]

Table 1: Enzymes whose Kinetics were Examined

Organism	*Enzyme/Gene name*	*Abbreviation*
B. anthracis	amino transferase	AT
P. falciparum	aspartate amino transferase	ASAT
P. falciparum	cysteine desulfurase	NIFS
M. smegmatis	methylthioadenosyl transferase	MTAN
M. smegmatis	methylthioadenosyl phosphorylase	MTAP
M. tuberculosis	methylthioadenosyl transferase	MTAN

These genes were inserted into inducible bacterial expression vectors. Expression of the gene was induced and the resulting protein was purified by affinity FPLC, and analyzed for enzymatic activity. Some of this work has been previously published [3], [4], [5].

For our expression studies, we initially used *E.coli* expression lines (BL21 derivatives Codon+, Rosetta and Origami) based on the reports of success by others. We also produced a number of active enzymes using these systems. Later we switched to a *Bacillus megaterium* expression system. *B. megaterium* was chosen for a variety of technical reasons: safe handling, rapid growth, ease of transformation, and lack of proteases which could degrade the protein of interest. In addition, *B. megaterium* is thought to have two protein secretion pathways called “sec” and “tat”. The sec secretion

pathway was shown to be present in *B. megaterium* by the contract scientific authority in unrelated research by producing *B. anthracis* protective antigen [DRDC internal publication]. The existence of the tat pathway in *B. megaterium* has yet to be established. By taking advantage of these secretion pathways, proteins of interest can be more easily purified as they are secreted into the growth medium where there are fewer contaminants. To this end, some changes were made to an existing commercial xylose inducible expression vector, called pWH1520. The features we added were the sec and tat signal peptide sequences to take advantage of the protein secretion systems, and 10x histidine affinity tags to allow for one step purification by immobilized metal affinity chromatography (IMAC) of expressed proteins. We were able to express a few enzymes successfully using this system, but as yet have not characterized their enzymatic activity.

Materials and Methods

Materials and methods:

1. Cells and Reagents:

For primary cloning of genes, *E. coli* XL-10 (Stratagene: La Jolla, CA) or TOP10 (Invitrogen: Carlsbad, CA) strains were chosen because they are recombinase deficient. These cells were routinely cultured on selective LB media at 37°C. Following sequencing of the primary clones, secondary (expression) clones were made using the following strains of *E. coli* BL21: codon+, rosetta, and origami (Stratagene). Some expression clones were also made using *B. megaterium* (MoBiTec: Goettigen, Germany).
All expression cells were also cultured routinely in LB broth at 37°C. For induction, other media and parameters were often used, and these are indicated in the Tables 3, 5, and 6 when available. All *E. coli* expression systems used were inducible with IPTG, and where no specific induction method is listed, this can be assumed. The *B. megaterium* system was xylose inducible, and where no induction information is given, this can also be assumed.

Specific recipes for various reagents used in this work may be found in Annex A.

2. Cloning of target Genes:

In some cases, genomic DNA from pathogenic species was obtained by growing a small culture, and then using phenol-chloroform extraction, and ethanol precipitation to obtain DNA. In other cases gDNA was already available from previous research performed in this laboratory. gDNA from *P. falciparum*, *B. anthracis* Ames strain (USAMRIID: Fort Detrick, MD), *B. cereus* 14579 (American Type Culture Collection: Manassas, VA), *B. subtilis* 168 (ATCC 23857), *M. tuberculosis* H37Rv, *M. marinum* and *M. smegmatis* (NCTC-8159: London, UK) were used as templates for PCR amplification of genes of interest.

Genome data was obtained from public databases, and from specific groups (Integrated genomics: Chicago, IL and TIGR: Rockville, MD) when appropriate. Sequences were then analyzed using the BLAST program within Bio-Edit (Ibis Therapeutics, Carlsbad CA). Primers were designed depending on the ligation scheme used. In the case of ligation independent cloning (LIC), a special 12 base LIC sequence flanking the complementary sequence was required. In the case of double digestion, the primers had restriction sites incorporated flanking the complementary sequence. Specific PCR primer sequences are listed in Table 2. PCR amplification of target genes was performed as in the protocols section (Annex B).

Plasmid strains used included pCALnEK (Stratagene), pCALnFLAG (Stratagene), the pET family(Novagen, San Diego, CA), and pWH1520 (MoBiTec). In some cases LIC was performed, though in other cases, restriction endonucleases (Promega: Madison, WI) were used to create compatible sticky ends for directional insertion using T4 ligase. The recombinant plasmids were transformed into the target primary clone species through chemically induced competence of host cells.

3. Functional expression:

The expression clones were induced by the appropriate method. This generally involved growing cultures in liquid media until the cell density reached an A_{650nm} of 0.6-0.8, followed by induction with either IPTG for the *E. coli* types or xylose for *B. megaterium*. Specific induction conditions are listed in Tables 3, 5, and 6, which summarize the specific details of protein expression.

Most expressed enzymes had affinity tags attached, and these were used to purify and assay fractions for activity by FPLC. The Tags used include: calmodulin binding, maltose binding, cellulose binding, GST, and 10x histidine. Each of these proteins was purified with the appropriate affinity column by FPLC, and used appropriate running and elution buffers. The composition of these buffers appear in Annex A when available. Analysis of FPLC fractions by SDS-PAGE was performed to confirm the presence and molecular weight of the purified enzyme. Eluted enzymes were often concentrated using molecular weight cut off centrifugal filters (Pall filtron: East Hills, NY), and stored at 4° C for immediate use, or in 20% v/v glycerol for long term storage.

4. Kinetic Studies:

When soluble, active enzyme was obtained in sufficient quantities, enzyme assays were performed to determine kinetic properties. As a number of different enzymes were analyzed, each assay had its own parameters. In general, an enzyme was paired with its substrate in varying concentrations in the presence of applicable cofactors. Assays were incubated at 37°C for 30 minutes followed by quenching and storage at -20°C until analysis by HPLC. Substrate and/or product peak areas were used to determine kinetic properties of the enzyme. The reaction contents are recorded in Annex A when possible. Kinetics of enzymes are summarized in the Tables 4, 5, and 6. The K_m and V_{max} values were obtained using the Scientist software program (Micromax, Salt Lake City UT)

5. Plasmid Engineering:

Initial modification of pWH1520 was by a summer student whose work was unrelated to this project. The initial change made was to include a histidine affinity tag , and the plasmid created was named pWH1520b (Fig 2). In an earlier experiment unrelated to this work, the contract scientific authority produced *B. anthracis* protective antigen using the *B. megaterium* system and pWH1520. The result was that PA was secreted into the growth media. Analysis of the PA sequence showed a secretory signal peptide that is recognized by *B. megaterium* as a functional secretory sequence and allows secretion of the protein into the surrounding medium. The sec sequence was amplified from *B. anthracis* PA, and inserted in pWH1520 to create pWH1520s (Fig 2). An additional change was made to combine the histidine affinity tag from pWH1520b, resulting in pWH1520sb (Fig 2).

Three more custom plasmids were created: pWH1520HS (Fig 2), pWH1520HSH (Fig 2) and pWH1520HTH (Fig 3). H refers to a 10x histidine affinity tag, and S and T refer to the sec and tat protein secretion signals respectively. Each of the secretion signals had an enterokinase cleavage site incorporated, which if processed correctly by the cell

would allow removal of the signal peptide and subsequent protein secretion into the growth media. In the case of HSH and HTH, removal of the signal peptide would leave a 10x histidine tag intact for affinity purification of secreted protein.

Results and Discussion

Results:

6. Protein expression and enzyme production:

Qualitative analysis of FPLC fractions for enzyme activity in HPLC indicated that there was successful expression of *B. cereus* MTAN, *P. falciparum* ASAT, NIFS and UridP, *B. anthracis* AT, *M. tuberculosis* BCAT, MTAP, and MTAN, and *M. marinum* MTAN and MTAP. These results are summarized in Tables 3-6. Enzymes that were not successfully expressed would either form misfolded inclusion bodies, or were undetectable by SDS-PAGE.

Kinetic information was obtained for *P. falciparum* ASAT and NIFS2, *B. anthracis* AT, *M. tuberculosis* MTAN, and *M. marinum* MTAN and MTAP. These results are summarized in Tables 4, 5, and 6. In the case of *M. tuberculosis* MTAN, substrate level inhibition was observed and the resulting K_{si} recorded (Table 6).

Detailed substrate studies were performed with *B. anthracis* AT. This enzyme was show to have similar preference for leucine, isoleucine, and valine as amino donors when KMTB was the acceptor(V_{max} = 0.43-0.45 µmol/min/mg); a significant drop in reaction velocity when KG was the acceptor (V_{max} = 0.25-0.33 µmol/min/mg). The *P. falciparum* NIFS enzyme showed highly variable activity (specific activity = 0.16-40.34 µmol Ala/min/mg) depending on the induction conditions .

7. Optimized pWH1520 Plasmid Derivative Construction:

In this work pWH1520b was tested with pilot induction of *M. tuberculosis* BCAT, MTAN, and MTAP with some success, and pWH1520sb was used for pilot induction of *M. tuberculosis* MTAP and *B. subtilis* YKRV with no active enzyme produced. The remaining plasmids pWH1520s, pWH1520hs, pWH1520hsh and pWH1520hth were not tested for expression in the course of this project. During the construction of pWH1520hs there was a problem with PCR fidelity which required site-directed mutagenesis to correct the error. pWH1520hsh and pWH1520hth were then derived using the correctly mutagenized pWH1520hs. Despite the lack of expression, all plasmids were sequenced to ensure that the additional sequences added were in frame with the XylA' leader. In the case of pWH1520hth, a primary clone of *B. subtilis* ykrv in pWH1520hth was prepared. Maps of the changes to the vector are included in Figures 2, 3, and 4. The potential of the *B. megaterium* expression system for was not sufficiently explored in this work. With the expression plasmids developed during the course of this work, studies of the protein secretion mechanisms would be easy to accomplish.

Conclusions and further directions:

The expression results indicated that bacterial proteins were expressed more reliably in bacterial expression systems than the corresponding protozoan proteins. Only 6 of 40 large scale inductions of protozoan proteins (*P. falciparum* enzymes) resulted in active enzymes as confirmed by FPLC analysis. In Contrast 10 of 28 large scale

inductions of bacterial enzymes resulted in active enzymes confirmed by FPLC analysis. It is not surprising that protozoan proteins would have problems with expression considering that bacterial systems are unable to perform post-translational modifications which contribute to stability, and in the case of enzymes, activity.

Of the expression plasmids that we screened, the pCALnEK showed the most reliable production with 8 out of 13 large scale inductions resulting in active enzyme. In contrast the pET family did not perform well, with only 3 of 28 large scale inductions resulting in active enzyme.

Some follow-up work to this research has already been completed and published exploring the effect of known inhibitors [4], the effect of different growth media on enzyme activity and production [3], and phylogenetic analysis to determine relatedness of the enzymes between species [5].

One long term direction which stems from this work would be to fully characterize different metabolic pathways which are suitable drug targets for each of the pathogenic organisms studied here. As of now, it is not known for certain whether these pathways are fully functional, as the existence of genomic sequences for the intermediate enzymes does not guarantee that they are produced or active. Nevertheless, since some of the pathogens are intracellular parasites, they may scavenge intermediates from their host's active pathways, and may still be important targets for new antibiotic development.

References

[1] Turnbull PC, Sirianni NM, LeBron CI, Samaan MN, Sutton FN, Reyes AE, Peruski LF: MICs of Selected Antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest J Clin Microbiol. 2004 August; 42(8): 3626–3634.

[2] Athamna A, Athamna M, Abu-Rashed N, Medlej B, Bast DJ, Rubinstein E: Selection of *Bacillus anthracis* isolates resistant to antibiotics. *J. Antimicrob. Chemother.*.2004; 54: 424-428.

[3] Berger BJ, English S, Chan G, Knodel MH: Methionine regeneration and aminotransferases in *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus anthracis*. J Bacteriol 2003, 185:2418-31.

[4] Berger BJ, Radford CL, Knodel MH, Venos ES: Branched-chain amino acid aminotransferase and methionine formation in *Mycobacterium tuberculosis* BMC Microbiology 2004, 4:39

[5] Berger BJ, Knodel MH: Characterization of methionine adenosyltransferase from *Mycobacterium smegmatis* and *M.tuberculosis*. BMC Microbiol 2003, 3:12

Tables

Table 2: Genomic and Vector PCR Primers

Genomic Primers	
Primer Name	***Primer Sequence***
B. anthracis-PAG2	Not listed in lab manual
B. anthracis-PAG3	Not listed in lab manual
B. cereus-Mtan1	5'-GACGACGACAAGATGTAGAATTGCTGAATTGGAG
B. cereus-Mtan2	5'GGAACAAGACCCGTTTAACTCTTCTAACACTTTTA
B. subtilis-YKRV-F	5'- ATCGATGCGGCCGCAATGAAATTTGAACAGTC-3'
B. subtilis-YKRV-R	5'-ATCGATTCGCGATTATAAGGTCTTGTCAATG-3'
B. subtilis-ykRV HS-1	5'-ATCgATggTACCATATgAAATTTgAACAgTC-3'
B. subtilis-ykRV HS-2	5'-ATCgATTCgCgATTATAAggTCTTgTCAATg-3'
B. subtilis-ykRV HSH-1	5'-ATCgATTgATCAATgAAATTTgAACAgTC-3'
B. subtilis phoD 1	5'ACGTAGGCGGCCGCA GATGGCATACGACAGTCG
B. subtilis phoD2	5'ACGTAGGGATCCTCATCGGATTGCTTCACCCCG
B. subtilis phoD tat 1	5'-GAGACTGGGCCCGATGGCATACGACAG
B. subtilis phoD tat 3	5'-AGACTGGTACCCGTTAGGCGCAGCA
M. marinum-MTANw1-pET	5'-ATCGATCATATGATGACGATAGGTGTTATCTG-3'
M. marinum-MTANw2-pET	5'-ATCGATGGATCCTCAGTCCACCACCGACAGCAG-3'
M. marinum-MTANw1-pCAL	5'-ATCGATGGATCCATGACGATAGGTGTTATCTG-3'
M. marinum-MTANw2-pCAL	5'-ATCGATCCGCTTTCAGTCCACCACCGACAGCAG-3'
M. marinum-MTAPw1-pET	5'-ATCGATCATATGATGCACAACAATGAGCCGATG-3'
M. marinum-MTAPw2-pET	5'-ATCGATGGATCCTCATGGCAACTCGAACGG-3'
M. marinum-MTAPw1-pCAL	5'-ATCGATGGATCCATGCACAACAATGAGCCGATG-3'
M. marinum-MTAPw2-pCAL	5'-ATCGATAAGCTTTCATGGCAACTCGAACGG-3'
M. tuberculosis-BCAT W1	5'- CGATGCGGCCGCGAATGACCAGCGGCTCCCTTCAA-3' (NotI)
M. tuberculosis-BCAT W2	5'- ATCGATGGTACCCTACCCCAGCCGCGCCATCCAGC-3' (KpnI)
M. tuberculosis-MTAN W1	5'-ATCGAGGCGGCCGCGAATGGCGGTGACGGTCGGCG-3' (NotI)
M. tuberculosis-MTAN W2	5'-ATCGAGGGTACCTCAACCGGCAGCAAGCGCAG-3' (KpnI)
M. tuberculosis-MTAP W1	5'-ATCGACGGTACCTAATGCACAACAATGGGCGCATG-3' (KpnI)
M. tuberculosis-MTAP W2	5'-ATCGATGGATCCTCATGGCAGCTCGAACGGCAA-3' (BamHI)
M. tuberculosis-MTAP-F	5'-ATCGATCATATGATGCACAACAATGGGCGCATG-3'
M. tuberculosis-MTAP-R	5'-ATCGATGGTACCTCATGGCAGCTCGAACGGC-3'
P. falciparum-Arg1	5'-GACGACGACAAGATGTTGGATACTATAGAA AGTTA
P. falciparum-Arg2	5'GGAACAAGACCCGTTTACACTATATCGTATCCTAA
P. falciparum-ASAT ConA-F	5'-ATCGATGCGGCCGCAATGGATAAGTTATTAAGC-3'
P. falciparum-ASAT ConA-R	5' ATCGATTCGCGATCATATTTGACTTAACGAAAGAC-3'
P. falciparum-ASAT1	Not listed in Lab manual
P. falciparum-ASAT2	Not listed in Lab manual
P. falciparum-ASAT3	5'-ATATGATTGAATCAAGGGGA

Genomic Primers	
P. falciparum-ASAT4	5'-TTTTCTTCAAACTTTCG
P. falciparum-ASAT5	5'-CAATGTATTGGAGGTACTGGTGCTA
P. falciparum-ASAT6	5'GGATTGACATACCGAAAAGGCGA
pCAL-LIC1	Not listed in lab manual
pCAL-LIC2	Not listed in lab manual
Plasmid Primers	
pWH1520hs-1 (5' Ba PAG sec + ApaI)	5'-AGGATCGGGCCCGATGAAAAAAGCAAAAGTGTTAATA-3'
pWH1520hs-2 (5' Ba PAG sec + KpnI)	5'-AGGATCGGTACCGAACTTCTGCCTGAATCACCTCTAA-3'
pWH1520hsh-1	5'-AGCTGGTACCAGAGTGGCCATCATCATCAT-3'
pWH1520hsh-2	5'-AGCTGGATCCCTTGTCGTCGTCGAT-3'
pWH1520HS-mut1	5'-CGACGACAAGCATATGGGCCCGATGAAAAAAC-3'
pWH1520HS-mut2	5'-GTTTTTTCATCGGGCCCATATGCTTGTCGTCG-3'

Table 3: Protein Expression

Sample	Expected Total Size KDa	Pilot	Large Scale Time OD600	Induction Conditions	FPLC
B. anthracis PA in BL21 pLysS and BL21 codon+ (plasmid identity is not available)	83	-	NA	NA	NA
M. tuberculosis SAM synthase in BL21 codon+ (plasmid identity is not available)	43	included	NA	NA	NA
B. cereus MTAN in pCALnEK in BL21 codon +	30.6	included	1st run: 3.25h 0.610 2nd run: 2.25h 0.586	1st run: 28°C, 250 RPM for 3h 2nd run: 28°C, 225 RPM for 2h	+ -
P. falciparum ASAT parental in pCALnEK in BL21codon+	~52	NA	3h 0.6	RT, 250 RPM for 3 hours	+
P.falciparum ASAT mutant A in pCALnEK in BL21 codon+	~52	-	3h 0.6	RT, 250 RPM for 3 hours	+
P. falciparum ASAT mutant A in pCALnEK in rosetta pLysS	~52	NA	3h 0.6	RT, 250 RPM, 3 hours	+
P. falciparum ASAT parental in pCALnEK in rosetta pLysS	~52	NA	3h 0.6	RT, 250 RPM, 3 hours	+
B. cereus MTAN in pET19b in BL21 pLysS	~28	-	NA	NA	NA
B.cereus MTAN in pET19b in BL21 codon+	~28	+	2h 10 min 0.645	28°C, 225 RPM, 3 hours	+
B. anthracis AT in BL21 codon+ (plasmid identity unavailable)	33.1	+	2h 0.769	28°C, 250 RPM for 3 hours	+
P. falciparum NifS in pET19b in BL21 Codon+	55.5	-	2.7h 0.62	28°C 1mM 3hr	-
P. falciparum NifS in pET19b in Rosetta	55.5	Included	6.0h 1.486	24°C 0.5mM o/n	+
P. falciparum NifS in pET19b in BL21 Codon+	55.5	NA	NA	Time Course Self Induction	-
P. falciparum NifS in pET19b in BL21 Codon+	55.5	NA	NA	Induced Time course 1mM 2Hr	Included band in 6hr sample only
P. falciparum NifS in pET19b in Origami	55.5	-	NA	NA	NA
P. falciparum BCKAD 1α in pET19b in BL21 Codon+	52.7	75%Included 25% Soluble	2.6h 0.618	28°C 1mM 3Hr	Included
P. falciparum BCKAD 1β in pET19b in BL21 Codon+	45.8	Included	2.6h 0.749	28°C 1mM 3Hr	Included
P. falciparum BCKAD #2 in pET19b in BL21 Codon+	53.7	50% Soluble 50% Included	2.6h 0.916	28°C 1mM 3Hr	+

P. falciparum BCKAD #3 in pET19b in BL21 Codon+	74.3	50% Soluble 50% Included	2.6h 1.025	28°C 1mM 3Hr	NA
P. falciparum Dipthine synthase in pCAL in BL21 Codon+	36.7	Included	2.8h 0.624	28°C 1mM 3Hr	NA
P. falciparum FAD synth in pCAL in BL21 Codon+	44.4	90% Included 10% Soluble	2.7h 0.613	28°C 1mM 3Hr	NA
P. falciparum UridP in pCAL in BL21 Codon+	31.9	50% Included 50% Soluble	3.8h 0.718 6.0h 0.531	28°C 1mM 3Hr 22°C 1mM o/n	Included
P. falciparum Hethiazole in pCAL in BL21 Codon+	38.9	50% Included 50% Soluble	2.8hh 0.631	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET19b in BL21 Codon+	66	Small Included	2.7h 0.636	28°C 1mM 3Hr	-
P. falciparum NifS2 in pET19b In Rosetta	63.6	Faint band	3.0h 0.547	28°C 1mM 3Hr 20°C 0.1mM o/n	- -
P. falciparum NifS2 in pCAL in BL21 Codon+	68.2	75%Included 25% Soluble	2.8h 0.648	28°C 1mM 3Hr	-
P. falciparum NifS2 in pCAL in Rosetta	68.2	50% included 50% Soluble	3.4h 0.723	28°C 1mM 3Hr	-
P. falciparum NifS2 in pET28b in BL21 Codon+ Rosetta	66.4	- Small amount included	3.0h 0.585	28°C 1mM 3Hr 28°C 1mM 3Hr	-
P. falciparum NifS2 in pET30b in BL21 Codon+	68.4	-	NA	NA	NA
P. falciparum NifS2 in pET 30b in Rosetta	68.4	Included	3.0h 0.705	28°C 1mM 3Hr	-
P. falciparum NifS2 in pET32a in BL21 Codon+	80.7	Included	NA	NA	NA
P. falciparum NifS2 in pET32a in Rosetta	80.7	Included	3.0h 0.559	28°C 1mM 3Hr	Included
P. falciparum NifS2 in pET34b in BL21 Codon+	85.3	-	NA	NA	NA
P. falciparum NifS2 in pET34b in Rosetta	85.3	Included		28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	Wispy band - -
P. falciparum NifS2 in pET39b in BL21 Codon+	92.7	-	NA	NA	NA
P. falciparum NifS2 in pET39b in Rosetta	92.7	Included	3.0h 0.609	28°C 1mM 3Hr	Iincluded
P. falciparum NifS2 in pET40b in BL21 Codon+	95.2	-	NA	NA	NA
P. falciparum NifS2 in pET40b in Rosetta	95.2	Included	3.0h 0.625	28°C 1mM 3Hr	-
P. falciparum NifS2 in pET41b in BL21 Codon+	95.2	NA	NA	NA	NA

P. falciparum NifS2 in pET41b in Rosetta	95.2	Slight included Some soluble		28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	Small included band Ox Glut. Failed Wispy band
P. falciparum NifS2 in pMALc in BL21 Codon+	106.2	2/3 included 1/3 soluble	NA	NA	NA
P. falciparum NifS2 in pMALc in Rosetta	106.2	90% included 10% soluble	3.4h 0.737	28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	Large included band Wispy band Wispy band
P. falciparum NifS2 in pMALp in BL21 Codon+	108.8	-	NA	NA	NA
P. falciparum NifS2 in pET44b in BL21 Codon+	125.0	Two bands Smaller band 1/3 included, 2/3 soluble Correct band 2/3 included, 1/3 soluble	NA	NA	NA
P. falciparum NifS2 in pET44b in Rosetta	125.0	Slight included, some soluble	3.4h 0.664	28°C 1mM 3Hr	Included
M. tuberculosis-SS in pWH1520 in *B.megaterium*	43.0	-	NA	37°C 0.5%Xyl 3Hr	NA
M. tuberculosis BCAT in pWH1520b-1 in *B. megaterium*	46.5	-	4.5h 0.246	37°C, 250 RPM for 2.6 hours	+
M. tuberculosis MTAN in pWH1520b-4 in *B. megaterium*	34.2	-	4.5h 0.245	37°C, 250 RPM for 2.6 hours	-
M. tuberculosis MTAP in pWH1520b-6 in *B. megaterium*	34.8	-	4.5h 0.331	37°C, 250 RPM for 2.6 hours	+
M. tuberculosis MTAP in pWH1520b-6 in *B. megaterium*	34.8	-	0.27	37°C, 250 RPM for 18 hours	-
M. tuberculosis MTAN in pCAL in BL21 codon+	32	-	6.3h 1.536	20°C, 250 rpm, 16.9 hours	- high enzyme activity in HPLC could be host
M. tuberculosis MTAN in pCAL in BL21 codon+	32	+	6.3h 1.536	37°C, 250 RPM for 16.3 hours	+ High enzyme activity in HPLC
M. tuberculosis MTAN in pCAL in BL21 codon+	32	+	autoinduction	37 °C, 250 RPM for 15.9 hours	+
M tuberculosis MTAP in pCAL in BL21 codon+	31.9	NA	autoinduction	37°C, 250 RPM for 16.6 hours	+

M. tuberculosis DAAT2 in pET19b in BL21 codon+	~35	included	NA	NA	NA
M. tuberculosis MTAN in pWH1520b in *B. megaterium* with variable tetracycline concentration	34.2	NA	3h 0.365	37°C, 250 RPM for 3 hours	+
M. tuberculosis MTAP in pET19b in BL21 codon+	~30.5	+	autoinduction	37°C, 250 RPM for 15.5 hours	+ low activity in HPLC
M tuberculosis MTAP in pET19b in RosettaGami	~30.5	included	NA	NA	NA
M. marinum MTAN in pET19b in BL21 codon+	~34	+	conditions not recorded	37°C, 250 RPM for 15.5 hours	- low activity noted in HPLC
M. marinum MTAP in pET19b in BL21 codon+	~30	band	conditions not recorded	37 °C, 250RPM for 15.5 hours	- low activity noted in HPLC
M. tuberculosis MTAP in pWH1520sb in *B. megaterium*	~34.8	-	NA	NA	NA
B. subtilis YKRV in pWH1520sb in *B. megaterium*	44	-	NA	NA	NA
B. subtilis YKRV in pWH1520sb in *B. megaterium*	44	included	4.6h 0.425	37°C, 250 RPM for 2.5 hours	NA
B. subtilis YKRV in pWH1520sb in *B. megaterium*	44	-	NA	NA	NA
B. subtilis YKRV in pWH1520sb in *B. megaterium*	44	-	NA	NA	NA
M. tuberculosis -MTAP in pWH1520sb in *B. megaterium*	27.9	NA	4.6h 0.504	37°C, 250 RPM for 2.5 hours	-
B. subtilis PHOD in pWH1520b in *B. megaterium* cultured in LB	~67	75% soluble, 25% insoluble	NA	NA	NA
B. subtilis PHOD in pWH1520b in *B. megaterium* cultured in LPDM	~67	Included	NA	NA	NA

Notes: Included = The protein was insoluble and inactive when expressed.
NA = not applicable, this means the procedure was not performed.
+ = positive result
- = negative result

autoinduction = Self induction system which is designed to automatically induce when growth density reaches a certain range

Table 4: Gene Chan's Enzyme Studies

B. anthracis amino transferase (AT)		
Amino acid donor and substrate	***V_{max} (µmol/min/mg)***	***K_m (mM)***
Isoleucine + KMTB	0.451 ± 46.0	1.66 ± 0.52
Leucine + KMTB	0.444 ± 60.7	1.75 ± 0.58
Valine + KMTB	0.434 ± 58.3	3.23 ± 1.18
KMTB + Leucine	0.415 ± 24.0	0.95 ± 0.20
Isoleucine + KG	0.331 ± 0.038	0.67 ± 0.28
Leucine + KG	0.253 ± 0.009	0.41 ± 0.06
Valine + KG	0.335 ± 0.044	0.71 ± 0.33
KG + Leucine	0.127 ± 0.009	0.83 ± 0.22
P. falciparum mutant A aspartate amino transferase (ASAT)		
Substrate	***V_{max} (nmol/min/mg)***	***K_m (mM)***
glutamate	15.36	2.07

For the *B. anthracis* AT The concentration of the amino acid donor was varied from 10mM to 0.1mM and the concentration of the acceptor was constant at 10mM. KMTB is ketomethylthiobutyrate, and KG is ketoglutarate.

Table 5: Cynthia Radford's enzyme studies

Sample	Procedure	Results	Induction Conditions	Specific Activity µmol Ala/min/mg
P. falciparum NifS in pET19b in BL21 Codon+	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS in pET19b in BL21 Codon+ self Induced Time course	AA-HPLC	NA	Sample over 22 Hrs	NA
P. falciparum NifS in pET19b in Rosetta Stationary Phase Induction o/n	AA-HPLC Colorimetric	NA NA	24°C 0.5mM o/n 24°C 0.5mM o/n	NA
P. falciparum NifS in pET19b in Codon+ Matrix refolding	AA-HPLC	NA	28°C 1mM 3Hr	NA
M. tuberculosis-SS in pWH1520 in *B. megaterium*	Sam-Syn HPLC	NA	NA	NA
P. falciparum NifS2 in pET19b in BL21 Codon+ Concentrated Eluate	AA-HPLC AA-HPLC Colorimetric	NA NA NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET19b in Rosetta o/n	HPLC Colorimetric	NA NA	24°C O.5mM 24°C O.5mM	NA NA
P. falciparum UridP in pCAL in Bl21 Codon+	AA-HPLC	Activity in flow through, could be E.coli	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET19b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2in pET28b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pCAL in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET30b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2in pET32a in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET34b in Rosetta	AA-HPLC	Positive ~150mAU ala peak	28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	20.86 +/- 1..33 NA NA
P. falciparum NifS2 in pET39b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET40b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA
P. falciparum NifS2 in pET41b in Rosetta	AA-HPLC	Positive ~150mAu ala peak	28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	40..34 +/- 3.13 NA 0.31 +/- 0.01

P. falciparum NifS2 in pMALc in Rosetta	AA-HPLC	Positive ~ 115mAu ala peak	28°C 1mM 3Hr 20°C 0.1mM o/n 37°C 1mM 3Hr	0.19 +/- 0.02 0.16 +/- 0.01 0.17+/- 0.02
P. falciparum NifS2 in pET44b in Rosetta	AA-HPLC	NA	28°C 1mM 3Hr	NA

Notes:
AA-HPLC = autoanalysis HPLC, this is an HPLC protocol
NA = not applicable, the assay was not performed, or no results are available

Table 6: Kara Tokaryk's Enzyme studies

Sample	*Km*	*Vmax (nmol/min/mg)*	*Ksi*
M. tuberculosis-MTAN in pCAL in BL21 codon+ autoinduced at 37°C for 15.9 hours MTA Assay #4	65.62 ± 17.55 uM	54.22 ± 3.36	NA
M. tuberculosis-MTAN in pCAL in BL21 codon+ autoinduced at 37°C for 15.9 hours SAH Assay #1	70.2 ± 3.0 uM	77.69 ± 3.24	1.058 ± 0.060
M. tuberculosis-MTAN in pCAL in BL21 codon+ autoinduced at 37°C for 15.9 hours MTA Assay #5	0.499 ± 0.067 mM	189.47 ± 6.30	0.879 ± 0.45
M. smegmatis-MTAN in pCAL in BL21 codon+ autoinduced at 37°C for 16 hours SAH Assay	0.0906 ± 0.0073 mM	2.466 ± 0.051	NA
M. smegmatis-MTAP in pCAL in BL21 codon+ autoinduced at 37°C for 16 hours SAH Assay	0.4094 ± 0.0999 mM	7.021 ± 0.681	NA
M. smegmatis-MTAP in pCAL in BL21 codon+ autoinduced at 37°C for 16 hours MTA Assay #2	3.185 ± 0.314 mM	3128.742 ± 174.407	NA

Notes:
MTA = methylthioadenine
SAH = s-adenosylhomocysteine

Figures

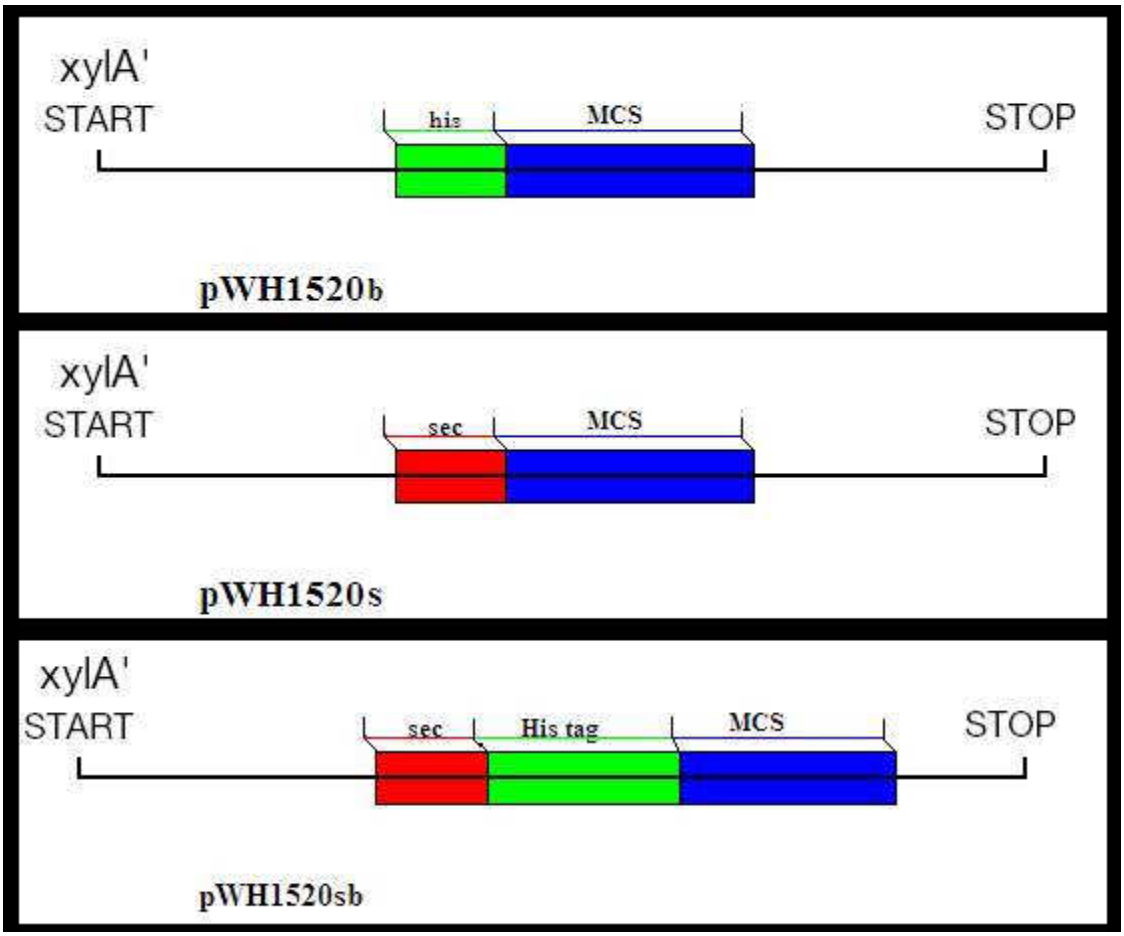

Figure 2: Map of modifications made to pWH1520. pWH1520b was not constructed by people working on this project, but it is included here for completeness. Subsequent modifications can be traced to pWH1520b. pWH1520s includes the sec signal sequence to allow expressed protein to be secreted to the growth media. pWH1520sb includes a histidine tag to allow for affinity purification.

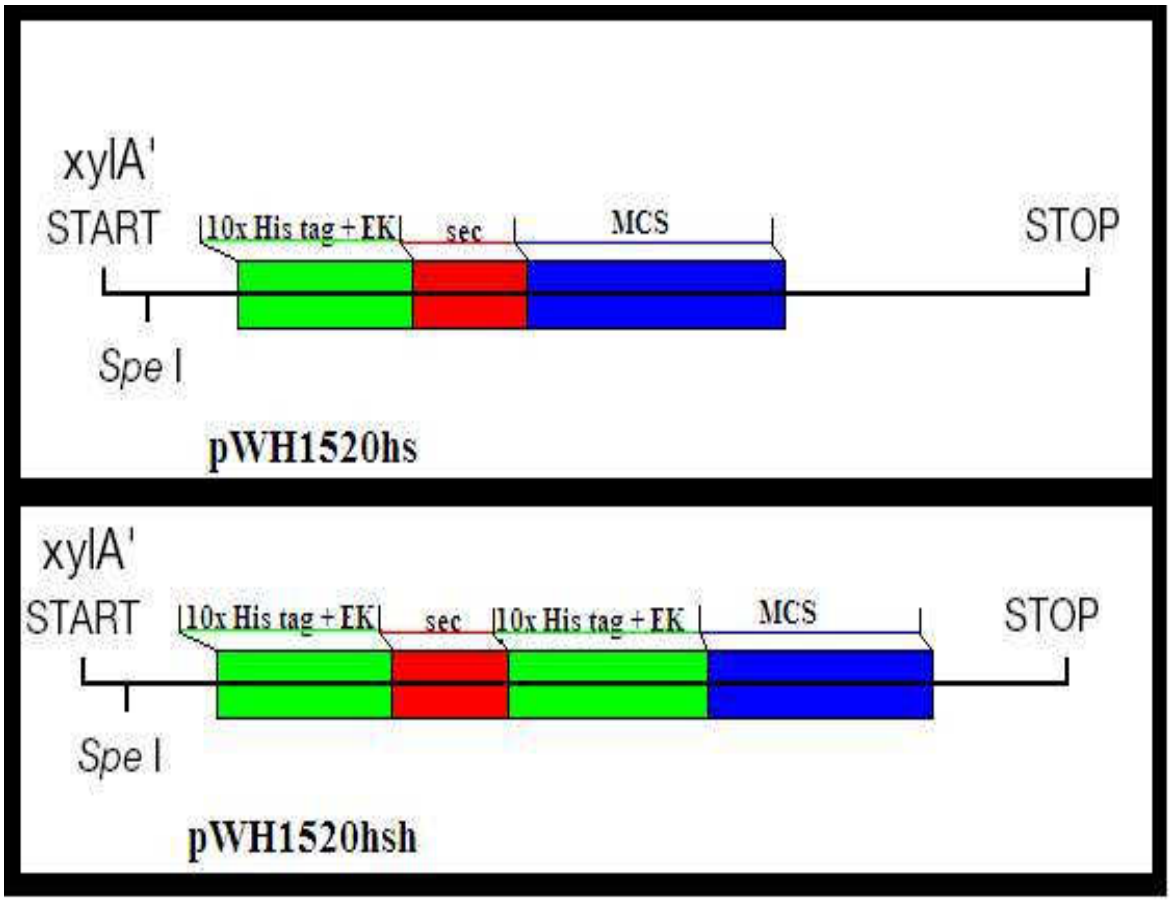

Figure 3: Map of modifications made to pWH1520. In hs, an affinity tag plus the sec sequence (protein secretion pathway sequence from *B. anthracis*) were added before the multiple cloning site, while in hsh, two affinity tags were added. The sec sequence contains a cleavage site, and the second 10x histidine tag allows for purification of secreted protein.

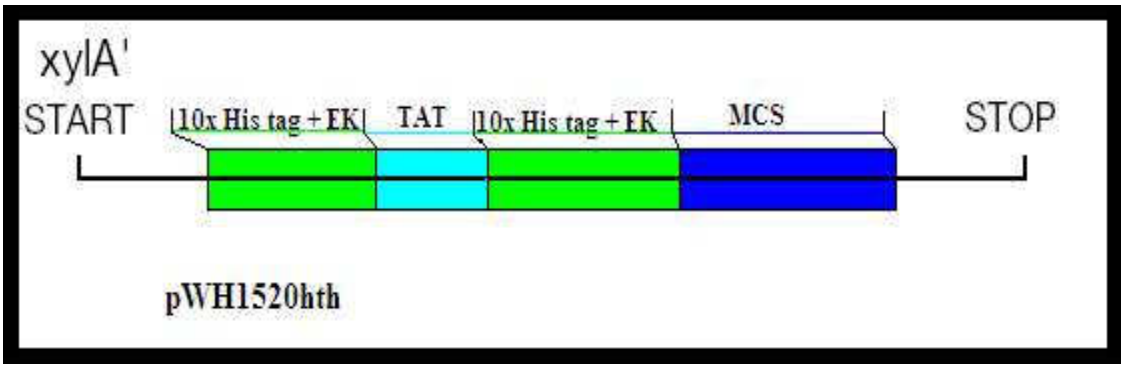

Figure 4: Map of modifications made to pWH1520hs. In hth two affinity tags and the TAT sequence (protein secretion pathway sequence from *B. subtilis*) were incorporated. The TAT sequence contains a cleavage site for a membrane bound endoprotease, and the second 10x histidine tag allows for purification of secreted protein.

Annexes

ANNEX A: Solutions

Gene Chan's Solutions:

Malarial Culture Media:

7.95g RPMI 1640 + HEPES + Glutamine
0.5g Sodium Bicarbonate
1.0g Glucose
2.5g Albomax II
500ml dH2O
13.6mg hypoxanthine
200µl gentamycin (50mg/ml)
pH to 7.4
Filter sterilized and stored at 4°C

Malarial Stabilating Buffer:

0.324g Sodium Chloride
1.512g Sorbitol
36ml dH2O
14ml Glycerol (28% final concentration)
Filter sterilize and store at 4°C

Saponin Solution:

1g saponin
50ml 1X Phosphate buffered saline

Phosphate buffered saline (PBS):

2.7mM potassium chloride [0.201g]
1.2mM magnesium chloride hexahydrate [0.245g]
138mM sodium chloride [8.065g]
8.1mM disodium hydrogen phosphate [0.972g]
1000ml dH2O
pH to 7.4, autoclave, and store at 4°C.

Cynthia Radford's Solutions:

Competence solutions:

Solution 1
10mM Rubidium Chloride
50mM Manganese Chloride.4H2O
30mM Potassium oxaloacetate

10mM Calcium Chloride
10% w/v glycerol
pH to 5.0 with 0.2M acetic acid

Solution 2
10mM Rubidium Chloride
10 mM MOPS
75mM Calcium Chloride
15% w/v glycerol
pH to 6.8 with Sodium Hydroxide

FPLC buffers:

IMAC

Buffer A (pH 7.4)
50mM HEPES
750mM Sodium Chloride

Buffer B (pH 7.4)
50mM HEPES
750mM Sodium Chloride
800mM Imidazole

Calmodulin Column

Buffer A (pH 7.8)

10mM HEPES
150mM Sodium Chloride
1mM Imidazole
1mM Magnesium acetate (tetrahydrate)
1mM Dithiothreitol
2mM Calcium Chloride (dihydrate)

Buffer B (pH 7.8)
10mM HEPES
1.2M Sodium Chloride
1mM Dithiothreitol
3mM EGTA

Maltose Binding Protein buffers

Buffer A (pH 7.4)

20mM TRIS
200mM Sodium Chloride

1mM EDTA
1mM Dithiothreitol

Buffer B (pH 7.4)
20mM TRIS
200mM Sodium Chloride
1mM EDTA
1mM Dithiothreitol
25mM Maltose

Cellulose Binding Domain (CBD) buffers

10X Buffer A (pH 7.5)
200mM TRIS
800mM Sodium Chloride

1X Buffer A (pH 7.5)
20mM TRIS
800mM Sodium Chloride

Buffer B
50% Glycerol

Glutathione S Transferase buffers:

Buffer A (pH 7.3)
140mM Sodium Chloride
2.7mM Potassium Chloride
10mM Potassium Phosphate
1.8mM Sodium dihydrogen phosphate

Buffer B (pH 8.0)
50mM TRIS-HCl
40mM Reduced glutathione

HPLC buffers:

Amino Acid Buffer A
1.36g Sodium Acetate into 500 ml dH2O
90 µl triethylamine
pH to 7.2 using 2% acetic acid
1.5ml tetrahydrofuran
Filter with 0.2 um filter

Amino Acid Buffer B
1.36g sodium acetate into 100m dH2O
pH to 7.2 with 2% acetic acid
200ml acetonitrile
200ml methanol

Derivitization Reagent for Amino Acid Assays
10mg ortho-phtalaldehyde
1.0ml 0.4M Borate pH 10.5
12µl 3-mercaptoproprionic acid

Isocratic BufferA
100mM Sodium dihydrogen phosphate
8mM Heptane Sulfonate
pH 2.65 with Phosphoric Acid

Substrate for UridP HPLC Assay pH 7.4
1mM Uridine
1mM Thymidine
in 200mM phosphate buffer

Substrate for Colorimetric Assay PfNifS pH 8.0
50mM TRIS
50mM Potassium Chloride
10mM Magnesium Chloride
5mM Dithiothreitol
2mM l-cysteine
Trace pyridoxal phosphate

P. falciparum NifS reaction substrate
50mM HEPES
2mM l-cysteine
1mM TCEP Tris(carboxyethyl)phosphine OR 5mM Mercaptopropionate

Sam-Syn Buffer A
0.1M Sodium dihydrogen phosphate
8mM Heptane Sulfonate
in 470mL dH2O
pH 2.65 with concentrated Phosphoric Acid
Add 2% Acetonitrile (10mL)
Make up to final volume 500mL

Sam-Syn Buffer B
0.15M Sodium dihydrogen phosphate
8mM Heptane Sulfonate
in 350mL dH2O
pH to 3.25 with concentrated Phosphoric acid
Add 26% Acetonitrile (10mL)

Make up to a final volume of 500mL

Column Refolding buffers:

Buffer 1:Solubilization/Denaturation Buffer
20mM TRIS-HCl pH 8.0
500mM Sodium Chloride
10% Glycerol
8M Urea
Trace Pyridoxal phosphate

Buffer 2:Wash Buffer
20mM TRIS-HCl pH 8.0
500mM Sodium Chloride
10% Glycerol
8M Urea
Trace pyridoxal phosphate
50mM Imidazole

Buffer 3: Refolding Buffer
20mM TRIS
500mM Sodium Chloride
10% Glycerol
8M Urea
Trace pyridoxal phosphate
50mM Imidazole
5mM Reduced Glutathione
0.5mM Oxidized Glutathione

Buffer 4: Refolding Buffer

20mM TRIS pH 8.0
500mM Sodium Chloride
10% glycerol
50mM Imidazole
Trace pyridoxal phosphate
5mM reduced glutathione
0.5 mM oxidized glutathione

Buffer 5: Elution Buffer

20mM TRIS pH 8.0
500 mM Sodium Chloride
10% glycerol
800 mM Imidazole
trace pyridoxal phosphate
5mM reduced glutathione

0.5mM oxidized glutathione

Miscellaneous buffers:

10X TAE

24.2g TRIS Base
5.72ml Acetic Acid
2.26 g EDTA-4Na+
Bring to 500ml dH2O

Coomassie R250

0.025% Coomassie Brilliant Blue R250
40% methanol
7% Acetic Acid

Destain

200ml Methanol
35ml Acetic Acid
265ml dH2O
Final Volume 500ml

Terrific Broth

To 900 ml dH2O add;
12g bacto-tryptone
24g bacto-yeast
4ml glycerol
Autoclave, cool to 60°C

To 90 ml dH2O add:
2.31g Potassium dihydrogen phosphate
12.54g Potassium hydrogen phosphate
Bring to 100ml final volume
Autoclave.
Add together.

ABI 5X Sequencing Dilution Buffer pH 9.0

400 mM TRIS-HCl
10mM Magnesium Chloride

5X Blue Dye for Agarose gel Loading

0.25% Bromophenol Blue
0.25% Xylene cyanol
30% Glycerol

Carbenicillin Stock 50mgs/ml

0.5g of Carbenicillin into 10ml dH2O
Filter Sterilize
Use at 50µg/ml

Chloramphenicol Stock 34mgs/ml

0.34g of Chloramphenicol into 10mLs ETOH
Use 50µg/ml

Kanamycin Stock 30mgs/ml

Add 0.3g of Kanamycin to 10mls dH2O in 15ml Falcon tube
Filter Sterilize.

Use at 30µg/ml

Low phosphate defined media:

50mM TRIS
3.03mM Ammonium Sulphate
6.8 mM Trisodium Citrate
3.04mM Iron Chloride
1.0mM Manganese chloride
3.5mM Magnesium Sulphate
0.01mM Zinc Chloride
0.5% glucose
0.05% casamino acids
10mM L-arginine
0.065 mM Potassium dihydrogen phosphate
pH 7.1
Autoclave

Since the Zinc Chloride and Potassium dihydrogen phosphate had such low concentrations, they were made up as 100X and 10X solutions respectively. The Zinc Chloride would not readily dissolve, but a few drops of concentrated HCl fixed this. Furthermore the addition of acid to the Zinc solution was not a problem because the whole media solution had to be brought to pH 7.1 with concentrated HCl, and the Tris started the whole solution off at a basic pH.

ANNEX B: Methods

Preparing Primers:

1. Dissolve the primer in equal amount of µg of product into µl of dH2O.
2. Vortex
3. Store at -70°C.
3. To make a working stock primer, add together 100µl of reconstituted primer with 100 µl dH2O. Store at -20°C.
4. Sequencing Primers are diluted to 3.2pmol/µl

PCR amplification:

(This is a general procedure, often a factor will need to be adjusted to get results; an example is given)

1. Add dH2O to bring reaction to 50µl
2. Add 5µl 10X PCR buffer (this usually contains Magnesium at an appropriate concentration)
3. Add 1µl dNTP
4. Add 1µl Primer A (100pmol/µl)
5. Add 1µl Primer B (100pmol/µl)
6. Add 2µl Target DNA
7. Add 0.5µl Taq:Pfu 5:1
8. Pipette mix

Then follow this program with the PCR thermocycler

1. 95°C 1.5 min
2. 95°C 1min---\
3. 55°C 1min----30 cycles The annealing temperature is the most often changed
4. 72°C 1min---/ parameter, this depends on primer sequence.
5. 72°C 10 min final extension
6. Test the product by gel electrophoresis

DNA Gel extraction:

(using the Qiaex II gel extraction kit)

1. Run an extraction gel at 40V, spaces between loaded lanes.
2. Turn Heating block on to 50° C. Weigh microfuge tubes.
3. Excise band from gel over limited UV. Take photo.
4. Weigh gel slice. Add 3 volumes Qxl.
5. Resuspend buffer QXll by vortexing for 30 sec.
6. Add 20 µl QXll. Vortex.
7. Add 10 µl 3M NaOAc. Vortex.
8. Incubate 50° for 10 min. Vortex every 2 min.

9. Spin 13,000 rpm 30 sec. Remove and discard Supernatant.
10. Resuspend pellet in 500 µl Buffer QXl. Vortex.
11. Spin 13,000 rpm for 30 sec. Discard Supernatant.
12. Resuspend pellet in 500 µl P.E. Vortex.
13. Spin 30 sec. Discard Supernatant.
14. Resuspend pellet in 500 µl P.E. Vortex.
15. Spin 30 sec. Discard Supernatant.
16. Air dry pellet for 20-25 min in small incubator
17. Add 20µl dH2O. Vortex. Incubate at RT 5 Min.
18. Spin 30 sec. Carefully transfer supernatant to a clean microfuge tube.
19. Add 20 µl dH2O. Vortex. Incubate at RT 5 Min.
20. Spin 30 sec. Carefully transfer supernatant to the clean tube.
21. Store purified DNA @ -20° C.

Restriction Digestion

(these reactions can vary depending on the requirement. As such, An example will be given)

1. Add 6µl dH2O
2. Add 1µl 10X 'multicore' (this is different for the each enzyme check instructions for optimal concentrations)
3. Add 2µl miniprep plasmid DNA (this could be purified PCR product)
4. Add 0.5µl of each enzyme (eg. NdeI and BamHI)
5. Incubate at 37°C for 1 hour
6. Some enzymes can be heat inactivated by a few minutes at high temperature, if not, perform a gel purification on digested products.

Primary Cloning (Transformation of primary line):

1. Combine
   - 100µl XL-10 or Top10 competent *E. coli* cells
   - 2-5µl miniprep of plasmid plus insert
2. Incubate on Ice for 30 minuntes.
3. Heat Shock at 42°C for 30 seconds
4. Incubate on ice for 10 min
   - (If Kanamycin resistance need 2 min on ice, add 80µl SOC medium, 30 min 37°C 250 rpm incubation for recovery time)
5. Plate all on LB plus antibiotic

Miniprep to recover plasmid DNA:

(This is performed using the Qiagen Qiaquick spin miniprep kit. A variation on the standard kit is required for gram positive organisms because the peptidoglycan layer prevents efficient lysis with the standard procedure. When the pellets are initially resuspended in buffer P1, lysozyme is added to a concentration of 10mg/ml, followed by incubation at 37°C for 1 hour. It is often useful to calculate the total amount of P1

required and add the lysozyme first in a master mix, as the larger volume allows for more accurate measurement of the lysozyme weight, and avoids having to maintain a concentrated lysozyme solution.)

1. Save 250µl for possible stabilating.
2. Centrifuge culture in 15ml Falcon tubes, 4500 rpm, 15min, 4°C.
1. Resuspend pellet in 250µl Pl
2. Transfer to microfuge tube.
3. Add 250µl P2. Invert 4-6X.
4. Add 350µl N3. Invert 4-6X immediately.
5. Spin 13,000 rpm 10min.
6. Transfer supernatant to a spin column.
7. Spin 30-60 sec. Discard flow through.
8. Add 500µl PB. Spin 13,000 RPM 30-60 sec. Discard flow through
9. Add 750µl PE. Spin 13,000 RPM 30-60 sec. Discard flow through
10. Spin 13,000 RPM an additional 1 min.
11. Place spin column in a clean 1.5 ml utube.
12. Add 50µl dH2O to center of column. Let stand 1 min.
13. Spin 13,000 RPM 1 min. Reserve flow through.

Stabilate creation (Bacterial Glycerol Stock)

1. Take 500µl from a 5ml overnight culture grown at 37°C
2. add 500µl of 20% glycerol + appropriate antibiotic at stame concentration used for plating made up in glycerol.
3. freeze at -70°C

Competent Cell Prepreparation:

1. Culture cells at 37°C, 250 rpm in LB overnight (no selection).
2. Dilute 1/100 into fresh LB.
3. Grow at 37°C, 250 rpm until OD_{600}=0.4.
4. Pellet cells at 3650 rcf for 10 min at 15°C.
5. Pour off supernatant.
6. Resuspend cells in 50ml solution 1. (See Annex A)
7. Incubate on ice for 1 Hour.
8. Pellet cells 3650rcf for 10 min at 4°C.
9. Resuspend pellet in 8ml solution 2. (See Annex A)
10. Aliquot into chilled microfuge tubes.
11. Store at -70°C.

Secondary cloning (Transformation of expression line):

1. Add 1µl of miniprep into 10µl Competent Expression cell Line
2. Incubate on ice for 30 min.

3. Heat Shock 42°C for exactly 30 seconds
4. Incubate on ice for 10 min
    - (If strain is Kanamycin resistant, incubate for 2 min on ice, add 80µl SOC medium, Incubate for 30 min 37°C 250 to allow for kanamycin resistance proteins to be expressed).
5. Plate all on LB plus appropriate antibiotics

PCR Sequencing:

1. Add 8µl of terminator ready reaction mix
2. Add 10µl target DNA (often dilutions are made of target, and all are sequenced because the reaction can be sensitive to template concentration, but a 1/100 or 1/1000 dilution will catch the sensitivity)
3. Add 1µl sequencing primer (3.2pmol/µl) (3' and 5' in separate reactions)
4. Add 1µl dH2O

Then follow this program with the PCR thermocycler
1. 96°C 30 seconds
2. 50°C 15 seconds
3. 60°C 4 minutes
4. 30 cycles from 1.

Then the reaction has to be 'cleaned' to remove excess fluorescent dyes
(This uses the Princeton Separations Centrisep spin column)
1. Tap column to make gel settle to the bottom
2. add 800µl dH2O and shake column gently
3. let column equilibrate for at least 30 minutes
4. Remove bubbles by gently tapping column
5. Allow 10 minutes for gel to settle again
6. remove upper and lower caps and allow excess dH2O to drain by gravity. Start with a pasteur pipette tip if required. Discard flow through
7. Centrifuge at 750X g for 2 minutes; discard wash tube containing flow through
8. Place column in new 1.5ml microfuge tube, apply sequencing reaction to the EXACT center of the column without cracking the column (it is fragile, and if it cracks you have to start over).
9. Centrifuge at 750X g for 2 minutes, discard the column, and reserve the cleaned reaction.
10. Vacuum dry for roughly 2 hours.
11. The lyophilizate is capped, and can be stored at -20°C until reconstitution and being run in the sequencer.

Malarial Stabilate Creation:
1. Resuspend malarial cultures in 15ml tubes

2. Centrifuge at 1500 RPM, 4°C for 5 minutes
3. Remove supernatant with sterile technique
4. Resuspend the cell pellet in an equal volume of malarial stabilating buffer (Gene Chans Solutions)
5. Store at -70°C

Malarial gDNA isolation:

1. Resuspend malarial cultures, combine in a 50ml tube if convenient.
2. Add 1/10 volume of saponin solution (Gene Chans Solutions)
3. Incubate at 37°C for 10 minutes
4. centrifuge the lysed cultures at 3500 RPM, 4°C for 10 minutes
5. Discard supernatant, resuspend pellet in 1X PBS (Gene Chans Solutions)
6. Centrifugation and resuspension are continued until the solution is mostly free of hemoglobin (by visual inspection) discard final supernatant
7. Pellet can be stored at -20°C at this point
8. Add one volume of buffer saturated phenol and one volume chloroform/isoamyl alcohol (24:1) and vortex
9. Centrifuge at 15,000 RPM for 1 minute, collect the upper (aqueous) phase in a new 1.5ml microfuge tube
10. repeat the above step, and combine the upper aqueous phase
11. add one volume of chloroform/isoamyl alcohol, vortex and centrifuge at 15,000 RPM for 1 minute
12. collect the upper aqueous phase, add 2 volumes of cold absolute ethanol and 1/10 volume of 3M sodium acetate
13. Store at -20°C overnight to allow the DNA to precipitate
14. Centrifuge at 14,000 RPM for 15 minutes at 4°C; decant the absolute ethanol
15. Add 1ml 70% ethanol and centrifuge at 14,000 RPM for 15 minutes at 4°C;decant the ethanol
16. Air dry the pellet at 37°C
17. Add 200µl dH2O and incubate at RT overnight
18. Store frozen.

Inserting a PCR amplified gene in the LIC protein expression system:

Vector Prep:

1. dH2O to 10µl
2. 1-2µl digested vector
3. 1.0µl 10X buffer
4. 1.0µl 10mM dTTP
5. 1U (0.5µl) Pfu or T4 polymerase

Insert Prep:

1. dH2O to 10µl

2. **2**.0µl insert
3. 1.0µl 10X buffer
4. 1.0µl dATP 10mM
5. 0.5µl Pfu or T4 polymerase
6. 72°C for 10 min
7. cool to RT (10-15 min)

Ligation/Transformation are combined in one step:
1. Add 1.0µl of prepped vector to the insert
2. Pipette mix
3. Incubate over 1Hr at RT
4. Transform 5µl of rxn mix into 100µl competent cells
5. Plate all on LB plus appropriate antibiotic.
6. Incubate at RT overnight

Pilot induction with BL21 pLysS and BL21 codon+ strains:
1. Grow picked colonies in 5ml of selective LB at 37°C, 250 RPM overnight
2. Centrifuge cultures at 3500 RPM, 4 °C for 15 minutes
3. resuspend pellet in 5ml fresh selective LB
4. 100µl of suspension is used to seed new 5ml cultures of selective LB
5. cultures are incubated at 37°C, 250 RPM
6. Absorbance readings are gathered at 600nm till desired density is reached.
7. Cultures are centrifuged at 3500 RPM, 4 °C for 15 minutes, the supernatant decanted, and the pellet resuspended in 5mls of fresh selective LB
8. 50µl of 100mM IPTG is added to induce, and cultures are further incubated at 28°C, 250 RPM for 3.25 hours.
9. Cultures are centrifuged at 3500 RPM, 4°C for 15 minutes
10. pellets are resuspended in 200µl dH2O and stored at -20°C

SDS-PAGE:

(Different resolving and stacking gel concentrations are used, so an example of a 7.5% resolving and 4% stacking gel will be used)

Resolving gel:
1. Add 3.04ml of dH2O
2. Add 725µl 3M TRIS
3. Add 940µl 40% acrylamide
4. add 250µl 2% Sodium dodecyl sulphate
5. add 5µl TEMED
6. Set up the glass plates, and electrophoresis apparatus
7. add 50µl of 10% ammonium persulphate (APS)
8. quickly pour resolving gel to a little over ¾ of possible height.

9. layer a little water above the resolving gel to ensure a uniform interface
10. shake apparatus to remove water after the gel has fully polymerized

Stacking gel:
1. add 1.85ml dH2O
2. add 300µl 3M Tris
3. add 250µl 40% acrylamide
4. add 125µl 2% SDS
5. add 2.5µl TEMED
6. add 50µl 10% APS
7. quickly pour gel above the resolving gel
8. leave enough room for the comb teeth to displace the gel to the top of the apparatus. This is something to get a feel for. If you overestimate on the gel amount, the wells become deformed and hard to load evenly. If you underestimate, you probably won't get your whole sample in.

Direct Insert Ligation:
(This is a multi-step procedure, and an example will be given here)

Vector Preparation for Direct Insert Ligation
1. Vectors are usually grown up in broth + appropriate antibiotic if necessary then miniprepped and digested. Digestion is performed to completion.
2. Combine the following
   - 20µl pCALnFlag
   - 2.5µl 10X Buffer E (supplied with LIC kit)
   - 1.0µl BamHl
   - 1.0µl Hindlll
3. Incubate at 37°C for 1Hr in a water bath.
4. Add 0.5µl BamHl and 0.5µl Hindlll
5. Incubate at 37°C for 1 Hour in a water bath.
6. Add 1µl Alkaline Phosphatase
7. Incubate at 37°C for 30min in a water bath.
8. Heat treat enzymes at 80°C for 20min.

Insert Preparation for Direct Insert Ligation
1. Insert is PCR amplified, and purified.
2. Combine the following:
   - 10µl purified PCR
   - 1.4µl 10X Buffer E
   - 1.0µl BamHl
   - 1.0µl Hindlll
3. Incubate at 37°C for 1 Hour in a water bath
4. Add 0.5µl BamHl and 0.5µl Hindlll
5. Incubate at 37°C for 30min in a water bath

6. Heat treat at 80°C for 20min.

Direct Insert Ligation

1. Combine the following:
   - 11µl dH2O
   - 4µl 5X DNA ligation buffer
   - 2µl digested pcr product
   - 2µl digested purified AP treated vector
   - 1µl T4 ligase
2. Incubate at RT for 10-30min
3. Transform into competent cells

Pilot Induction of BL21 codon+ and rosetta cells using non-standard induction parameters:

1. Scratch in stabilated cell culture or pick colony in 5 mls broth and appropriate antibiotic.
2. Incubate overnight at 37°C, 250rpm.
3. Next AM, save cuvette of broth + antibiotic for spec calibration.
4. Add 100 µl of cell culture in 5mls broth and antibiotic X2 (one uninduced, one induced)
   - Stabilate o/n culture if needed
5. Incubate at 37°C, 250rpm
6. continue till OD600 of 0.6-0.8
7. Cool to 28°C
8. Add IPTG to 1mM final concentration
9. Induce for 2-3 hrs, 28°C, 250 rpm
10. Transfer to 15ml Falcons
11. Spin 4500rpm 4°C 15min
12. Discard supernatant
13. Resuspend pellet in 200µl dH2O
14. Freeze at -70°C for 10min or -20°C o/n
15. Sonicate 2X or more on ice. Sonicate uninduced samples first.
16. Spin 4500 rpm 20min 4°C (except uninduced samples)
17. Separate sup and pellet of induced samples
18. Add 200µl dH2O to pellet. Vortex
19. Prepare samples for SDS-PAGE

Large Scale Induction for BL21 rosetta cells:

1. Scratch in stabilated cell culture into 10ml broth plus appropriate antibiotic
2. Incubate overnight(16.5h) at 37°C, 250rpm
3. Next morning add all of o/n culture to 500ml broth plus appropriate antibiotic
4. Incubate at 37°C, 250rpm until OD600 = 0.6-0.8

5. Induction parameters can differ, but generally this lab begins with
   - 28°C, 3Hr, 1mM IPTG
   - 20°C, o/n, 0.1mM IPTG
   - 37°C, 3Hr, 1mM IPTG
6. Harvest cells by centrifuging 20 min at 4500 rpm, 4°C
7. Resuspend cell pellet in 5-10ml of Buffer A (supplied with kit)appropriate for the purification procedure.
8. Freeze at -20°C until ready for purification.

FPLC Column Elution Parameters:

1. IMAC
   - 0%B
   - 10%B
   - 100%B

2. Calmodulin, CBD, Amylose-Agarose
   - 0%B
   - 100%B

3. GST
   - 0%B
   - 100%B

General flow parameters:

0% Flow 0.2ml/min, Chart Speed 0.2mm/min, Fractions 35min/7ml
100% Flow 1ml/min, Chart Speed 1mm/min, Fractions 7.5 min/7.5 ml

Stabilate concentrated enzymes:

1. Use 20% glycerol for storage at -20°C.
2. Cut end of pipette tip off to deliver 500µl to 2mls of concentrated enzyme.
3. Note: Many enzymes stabilated by this method have lost activity over time (order of weeks to months). This is a crude method.

Protocol for Column Refolding:

(This method attempts to recover misfolded protein from inclusion bodies to an active form)

1. Start a seed culture by scratching in stabilated culture into 5mLs broth with appropriate antibiotic. Incubate 3°C 250rpm o/n (16.5h)
2. Next morning seed 10mL broth with antibiotic, incubate 37°C 250 rpm until OD600 is between 0.6 and 0.8. Induce to create inclusion bodies. Harvest cells by centrifuging at 4500 rpm for 15min at 4°C. Pour off supernatant and freeze cell pellet at -20°C.

Sonicate cell pellet, and spin to obtain supernatant.

3. Equilibrate column with 5ml buffer 1. (Annex A) When using pharmacia pump p-3500 pump at 0.5ml/min, pressure limit upper 02, lower 00. Load column with 1ml of supernatant, allow 2Hr room temperature incubation to allow protein to bind to the column.
4. Wash column with 5ml of solubilization buffer. Wash with 5ml Buffer 2 (Annex A). Wash with 5ml Buffer 3 (Annex A).
   Wash with 5ml of Step Gradients

	8M	7M	6M	5M	4M	3M	2M	1M	0M
Buffer 3 mL	10.0	8.75	7.5	6.25	5.0	3.75	2.5	1.25	0
Buffer 4 mL	0	1.25	2.5	3.75	5.0	6.25	7.5	8.75	10.0

5. Wash with elution buffer 5mL.
6. Visualize by SDS-PAGE

P. falciparum NifS Enzyme reaction for HPLC:

1. Pipette mix:
   - 95µl substrate
   - 5µl enzyme
2. Incubate at 37°C for 30 min in a water bath
3. Add 10µl of 10mM Iodoacetic acid
4. Incubate 10 min in the dark at room temperature
5. Store at -20°C until HPLC analysis

HPLC AA method Sample preparation:

1. Pipette mix the following:
   - 50µl Borate 0.4M pH10
   - 10µl Sample
   - 10µl derivitization reagent
2. Load into the HPLC

Colorimetric Assay PfNifS pH 8.0:

Sample prep for Colorimetric Assay

1. Mix the following:
   - 1ml Substrate
   - 10µl sample
2. Seal the microfuge tubes with parafilm.
3. Incubate at 37°C for 30 min
4. Add 100µl each of 20mM dimethylphenylenediamine (0.4182g in 6.28ml Hcl, 3.72ml dH2O) 30mM FeCl3 (0.08109 in 1ml HCl, 9ml dH2O)

5. Using a 20G1 needle, inject 200µl of the liquid through the lid. Cover the lid with parafilm.
6. Incubate for 20 min at room temperature (in the dark)
7. Read the absorbance at 650nm on the spectrophotometer.
8. A Standard curve can be created using Na2S as the analyte.

UridP HPLC Assay pH 7.4:

Sample Prep

1. Pipette mix the following:
   - 100µl substrate
   - 10µl sample
2. Incubate at 37°C for 30 minutes in a water bath
3. Store Frozen at –20°C

HPLC Sample Prep

1. Add 100µl of Isocratic A Buffer to each of the prepared samples.
2. Run with SamSyn method.

ANNEX C: Personnel Time-line:

Gene Chan worked on this project from August 2001 – March 2002 for Canada West Biosciences Inc.

Cynthia Radford worked on this project from July 2002 – August 2003 as an employee of Canada West Biosciences Inc. Her continued work to January 2004 is included.

Kara Tokaryk worked on this project from November 2003 – January 2004 for Canada West Biosciences Inc.

Michelle Russell worked on this project from September 2004 - November 2004 for Canada West Biosciences Inc. Her continued work to December 2004 is included.

Michael McWilliams worked on this project from February 2005 to the end of March 2005 for Canada West Biosciences Inc. The contract ended March 31, 2005.

List of symbols/abbreviations/acronyms/initialisms

AA (HPLC)	auto analyser
ASAT	aspartate aminotransferase
APS	ammonium persulphate
AT	amino transferase
ATCC	American type culture collection
BCAT	branched chain aminotransferase
BCKAD	branched chain alpha keto acid dehydrogenase
BL21	a strain of *E.Coli* optimized for protein expression
CBD	calmodulin binding domain
CBDS	Chemical-Biological Defense Section
DAAT2	d-aspartate aminotransferase
dATP	deoxyadenosinetriphosphate
DNA	deoxyribonucleic acid
DND	Department of National Defence
dNTP	deoxyribonucleotidetriphosphates
dTTP	deoxythymidinetriphosphate
DRDC	Defence Research and Development Canada
EDTA	ethylenediaminetetraacetic acid
EK	enterokinase
FAD	flavin adenine dinucleotide
FPLC	fast protein liquid chromatography
gDNA	genomic DNA
GST	glutathione S-transferase
HPLC	high pressure liquid chromatography
IMAC	immobilized metal-ion affinity chromatography
IPTG	isopropyl thiogalactopyranoside
kDa	kilo dalton
KG	ketoglutarate
KMTB	ketomethylthiobutyrate

K_{si}	enzyme kinetics term describing the effect of substrate level inhibition
LB	Luria-Bertani
LIC	ligation independent cloning
MCS	multiple cloning site
MTA	methylthioadenosine
MTAN	methylthioadenosine nucleoside
MTAP	methylthioadenosine phosphorylase
NCTC	National culture type collection (United Kingdom)
NIFS	cysteine desulfurase
OD	optical density
PA	*B. anthracis* protective antigen
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiousi*
PHOD	phosphodiesterase D
rcf	relative centrifugal force
RPM	revolutions per minute
RT	room temperature
SAMSYN	s-adenosylmethionine synthase
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SEC	A protein sec retion pathway in B. subtilis
SOC	a type of growth media for bacteria
SS	s-adenosylmethionine synthase
TAT	Twin Arginine Translocase, a protein translocation pathway in B. subtilis
T4	a bacteriophage
Taq	*Thermus aquaticus*
TEMED	tetramethylethylenediamine
TOP10	a strain of *E.coli* used for plasmid production
URIDP	uridine phosphorylase
USAMRIID	United States Army Medical Research Institute for infectious diseases.
UV	ultraviolet

V_{max}	enzyme kinetics term referring to the maximum rate of catalysis
XL-10	a strain of *E.coli* used for plasmid production
XylA'	xylose leader region
YKRV	enzyme similar to aspartate aminotransferase

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Antibiotic resistance is of increasing medical concern. As the old therapies become ineffective, new drug targets must be identified. Metabolic pathways offer attractive chemotherapeutic drug targets which have until recently been laborious to discover. The advent of large scale genome sequencing projects has revealed the information required to begin the identification and analysis of new target enzymes involved in many metabolic pathways in some of the medically important pathogens such as *P. falciparum*, the causative agent of malaira, and *B. anthracis* which causes anthrax.

Results: We characterized enzymes from the following organisms: the protozoan *Plasmodium falciparum,* the bacilli *Bacillus cereus, Bacillus anthracis,* and *Bacillus Subtilis,* and the mycobacteria *Mycobacterium tuberculosis, Mycobacterium marinum,* and *Mycobacterium smegmatis.*

To this end, we cloned the chosen enzymes into various *E. coli* expression vectors and induced their expression in small-scale culture. After confirmation of expression, we purified the enzymes using affinity FPLC and performed preliminary enzyme assays to determine the activity of the expressed protein. We found some of the enzymes could not be expressed at all, while others were expressed only as inclusion bodies. We performed kinetic studies on those enzymes that were expressed, and found to be active.

The *P. falciparum* ASAT was demonstrated to have a V_{max} of 15.36 nMol/min/mg and a K_m of 2.07 mM when glutamate and KMTB were used as substrate. The *B. anthracis* amino transferase was shown to have similar preference for isoleucine, leucine, and valine as amino donors, with KMTB and KG as amino acceptors with K_m values between 0.41-1.75 mM and V_{max} values between 0.13-0.45 µmol/min/mg. The *P. falciparum* cysteine desulfurase enzyme was successfully produced and analyzed for specific activity in *E. coli* BL21 Rosetta cells. We found that the induction conditions made a large difference in enzyme activity; the maximum specific activity obtained was 40.34 µmol Alanine/min/mg using the pET41b vector. The *M. tuberculosis* MTAN enzyme was demonstrated to have a V_{max} of 189.47 nmol/min/mg and a K_m of 0.49mM using MTA substrate. Substrate level inhibition with a K_{si} of 0.879 was noted with this enzyme. The *M. smegmatis* MTAN enzyme was demonstrated to have a V_{max} of 2.466 nmol/min/mg, and a K_m of 0.09 mM. The *M. smegmatis* MTAP enzyme was demonstrated to have a V_{max} of 3128.742 nmol/min/mg and a K_m of 3.185 mM.

We also examined the utility of a commercially available xylose inducible *Bacillus megaterium* expression system. We modified pWH1520 parent expression vector to include 10x histidine affinity tags, and sequences which signal for secretion to the growth media. Preliminary expression results with the *B. megaterium* system were encouraging.

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Bacillus anthracis, *Bacillus megaterium*, Mycobacterium tuberculosis, *Mycobacterium smegmatis*, *Plasmodium falciparum*, methylthioadenosine, phosphorylase, nucleosidase, cysteine, desulfurase, expression vectors