CHAPTER 4

Cloning of CYP6P9 (resistant and susceptible) *An. funestus* for functional expression in *Escherichia coli* to examine their pyrethroid (permethrin) metabolising potential

4.1 Introduction

P450s are well known to metabolize pyrethroids or organophosphates in other insect species such as houseflies (Amichot *et al.*, 1997; Hardstone *et al.*, 2007). Müller *et al.* (2008) recently reported that field-caught permethrin-resistant *An. gambiae* was over-expressing CYP6P3 and that this P450 was able to metabolise both alpha-cyano and non-alpha-cyano pyrethroids. CYP6P3 in *An. gambiae* is the ortholog to CYP6P9 in *An. funestus* (Amenya *et al.*, 2005; Matambo *et al.*, 2010). Over-expression of a P450 does not mean that the P450 gene is involved in detoxification of an insecticide. It is only after it is clearly demonstrated that the specific P450 gene metabolizes the pyrethroid, can one stipulate that the gene is involved in resistance. The rate of insecticide metabolism by P450 enzymes helps determine the survival of an organism. Determining the enzymatic activity helps establish the efficiency of catalysis of substrates and any metabolites that may be produced, supports the role of the enzyme in the detoxification process.

Commonly used protein expression systems include those derived from bacteria (such as *E. coli* and *B. subtilis*) (Baneyx, 1999), yeast (such as *S. cerevisiae*) (Cregg *et al.*, 2000), cultured baculovirus/insect cells (Kost *et al.*, 2005), and mammalian cells (Rosser *et al.*, 2005; Lackner *et al.*, 2008). Cytochrome P450s have been expressed in a variety of these heterologous expression systems however; the bacterial system has proven to be the least demanding and provides large amounts of catalytically active P450s for metabolic and structural studies (Pritchard *et al.*, 2006). The reproducibility of the bacterial expression...
system is far superior to those of the former two cellular models and is also lower in cost. However, the expressed proteins often become overproduced and aggregate into inclusion bodies causing them to fold incorrectly and making them biologically inactive (Baneyx, 1999).

Various methods have been tested to get high-level heterologous expression of unmodified recombinant cytochrome P450 enzymes. However, this has only been achieved by altering the 5’-end of the native cDNA, resulting in amino acid changes within the P450 protein chain. Such methods include making NH2-terminal translational fusions to bacterial leader sequences or signal peptides, such as ompA and pelB (Guengerich et al., 1997; Matambo, 2008). The NH2-terminal segment of P450 is very hydrophobic and is considered to be the membrane-binding domain. The other more conventional way has been to replace the natural P450 NH2-terminus with a sequence (MALLLAVF) derived from the bovine steroid 17α-hydroxylase. CYP17A was the first P450 to be expressed in *E. coli* (Waterman et al., 1995; Barnes et al., 1991). Heterologous expression of a functional P450 monooxygenase system in *E. coli* requires the co-expression of P450s together with cytochrome P450 reductase (CPR), which supplies P450s with electrons from NADPH, as well as cytochrome b5 (b5), glutathione, detergent and a particular phospholipid composition to be present for optimal measurements of activity (Blake et al., 1996).

Isolating individual P450 enzymes has proven to be challenging due to the instability of insect P450 and the presence of interfering substances in microsomes (Scott, 1999). Expression plasmids such as the highly regulated tac-promoter expression plasmid pCWori+, have been used to express many eukaryotic and prokaryotic P450s (Barnes et al., 1991; Helvig et al., 2004; Matambo, 2008). This vector has been used successfully in
expressing other insect P450s in *Escherichia coli* (*E.coli*) such as cockroach CYP15A1 (Helvig *et al.*, 2004).

Previous strategies to express *An.gambiae* CYP6P3 (Müller *et al.*, 2008) and CYP6Z2 (McLaughlin *et al.*, 2008) using the *E. coli* signal peptide (*ompA*) were shown to be unsuccessful. This too was unsuccessful for CYP6P9 in *An. funestus* (Matambo, 2008). Replacing the natural P450 amino-terminus with a sequence (MALLLAVF) derived from the bovine steroid 17α-hydroxylase is a common strategy for P450 expression (Barnes *et al.*, 1991). Matambo (2008) was the first to provide evidence of permethrin (pyrethroid) detoxification by microsomal P450 from the resistant *An. funestus* FUMOZ-R strain and was also the first to isolate and characterize full-length CYP6P9 and express the recombinant CYP6P9 protein in *E. coli*.

This was only successful when the expression vector was changed from the insect pIX4.0 plasmid to pCWori+ plasmid and when eight of the first amino acid sequences were replaced by the 17α-hydroxylase (MALLLAVF) amino acid sequence. P450 induction with IPTG and ALA was however slow compared to the normal one to two days (Pritchard *et al.*, 2006). Matambo (2008) performed permethrin metabolism using FUMOZ-R and FANG total microsomal P450. Chloroform extraction was used to extract permethrin metabolites from the mosquitoes tested, however the HPLC used was not sensitive enough to detect distinctive metabolites or permethrin peaks and therefore could not give clear evidence of permethrin metabolism within FUMOZ-R. *Anopheles funestus* b5 and *An. gambiae* CPR were also expressed in *E. coli* (Matambo, 2008). *Anopheles gambiae* CPR was used instead of *An. funestus* due to its high sequence similarity in this gene between the two mosquito species (Matambo, 2008). Matambo *et al.* (2010) showed that there are
only four amino acid differences between two distinctive CYP6P9-coding regions in resistant and susceptible An. funestus strains. Although their functional significance is not yet known, these variations may possibly be associated with differences in insecticide metabolism (Matambo et al., 2010).

Based on the above findings, successful experimental procedures and the fact that overexpression of CYP6P9 has been associated with resistance (Amenya et al., 2008; Wondji et al., 2009), the aim here was to express the CYP6P9 FUMOZ-R (An. funestus) and An. gambiae CPR constructs in E. coli, previously used by Matambo (2008), and to confirm its permethrin metabolising potential because of its strong association between gene expression and the resistance phenotype. The specific activity of the enzyme was also to be characterized. Cloning and expression of CYP6P9 isolated from the pyrethroid susceptible strain (FANG) was to be carried out in conjunction with FUMOZ-R, in order to compare metabolic activity between resistant and susceptible strains. As the CYP6P9 gene from the FANG strain has never been cloned successfully for functional expression in E. coli, the N-terminal modification (as performed for FUMOZ-R) had to be performed prior to expression analysis in this strain. The changes incorporated only alter the membrane anchor and therefore this N-terminal modification should not be problematic and should allow for functional expression in E. coli. However, such changes could alter the efficiency of expression. Permethrin metabolism was analysed by reverse phase High Performance Liquid Chromatography (HPLC) at the Liverpool School of Tropical Medicine (LSTM), United Kingdom, due to lack of equipment and facilities at the NICD, Johannesburg.
4.2 **Materials and methods**

4.2.1 *Preparing CYP6P9 (FANG) for biochemical characterisation:*

*Amplification of CYP6P9 (FANG)*

Based on the principle using the MALLLAVF sequence, derived from the bovine steroid 17α-hydroxylase, as described by Barnes *et al.* (1991), the CYP6P9 gene was amplified from plasmid pA092 (pCWompA2::CYP6P9-FANG; 2µg in 8 µl prepared by miniprep) with the forward primer ECG145 (5’-

TTTCATATGGCTCTGTATTAGCAGTTTTTGCCGCGTTCATCTTCGTAGTG-3’), to introduce an NdeI restriction site (underlined) and to introduce some changes to the N-terminal to make it similar to the 17α leader sequence (four of the first eight amino acid residues are altered and a change at nucleotide 41) to that observed in CYP6P9 FUMOZ-R (GeneBank accession number: AY729661). The reverse primer ECG226 (5’-

TTTTCTAGACTACAACCTTTCCACCTCAAGTAATTACCC-3) incorporated an XbaI restriction site (underlined) and added a stop codon and changed a mutation present in pA092. The CYP6P9 sequence in pA092 would now match the FANG (GeneBank accession number: EU450763) submission perfectly, except for the MALLLAVF leader and a change at nucleotide 1523 that changes the lysine codon in FANG to an arginine. This change is reverted back by using the ECG226 reverse primer. The PCR protocol (B.L. Spillings, personal communication, NICD) and cycling conditions are given in Tables 4.1 and 4.2 respectively.

*Ligation and transformation*

The resulting 1.5Kb 17α-CYP6P9-FANG (pA092) product was gel extracted (QIAquick gel extraction kit, Qiagen, cat no: 28704, South Africa) and then either, a) digested (via
restriction enzymes NdeI and XbaI) and ligated directly into the expression plasmid pCWori+ (5Kb) (kindly donated by Dr Michael Waterman, Vanderbilt University, Department of Biochemistry, Nashville, Tennessee, U.S.A) to create the construct pCW::17α-CYP6P9 (FANG), or b) cloned into the holding vector pGEM-T easy (Promega, U.S.A, cat no: A1360), transformed into competent cells (Section 4.2.2), mini-prepped, digested and then gel purified the gene for ligation into pCWori+. Subcloning of the gene was performed to ensure that there was no pA092 carry over from the amplification as this could lead to contamination of the final transformation. The pCW::17α-CYP6P9 (FANG) construct would then be expressed in E. coli to perform metabolic studies on this strain. All constructs were verified by DNA sequencing.

Ligations (10 µl) (Table 4.3) were performed in both 8:1 and 10:1 ratios, calculated using the formula (Promega technical manual: pGEM®-T and pGEM®-T Easy Vector Systems, www.promega.com):

\[
\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}
\]

| Table 4.1: PCR protocol to create 17α-CYP6P9-FANG (pA092) |
|----------------------------------|------------------|
| **Reagents**                     | **Volume (µl)**  |
| 10X buffer (NH₄)                 | 2.5              |
| MgCl₂ (50mM)                     | 1                |
| dNTPs (0.2mM)                    | 2.5              |
| ECG145 (10µM)                    | 0.5              |
| ECG226 (10µM)                    | 0.5              |
| Taq (Bioline)                    | 0.2              |
| dH₂O                             | 16.8             |
| pA092 (30ng µl⁻¹)                | 1                |
| **Total volume**                 | **25**           |
### Table 4.2: PCR cycling conditions to create 17α-CYP6P9-FANG (pA092)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
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<td>95</td>
<td>1 min</td>
</tr>
<tr>
<td>Denaturation</td>
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<td>95</td>
<td>30 sec</td>
</tr>
<tr>
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<td></td>
<td>45</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72</td>
<td>5 min</td>
</tr>
</tbody>
</table>

### Table 4.3: pGEM-T Easy vector ligation setup

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl) 8:1</th>
<th>Volume (µl) 10:1</th>
<th>Control insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x rapid ligation buffer, T4 DNA ligase</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>pGEM-T Easy vector (50ng µl⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PCR product (200 – 250ng µl⁻¹)</td>
<td>3.0</td>
<td>3.9</td>
<td>-</td>
</tr>
<tr>
<td>T4 DNA ligase (3 Weiss units µl⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dH₂O</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Control insert DNA (4ng µl⁻¹)</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10.0</strong></td>
<td><strong>10.9</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

### 4.2.2 Transformation of CYP6P9 (FANG)-pGEM-T into JM109 competent cells

The *E. coli* competent cells (JM109 competent cells, Promega, cat no: L2004, South Africa) were removed from -70°C and placed on ice for 5 minutes to thaw. JM109 cells (Yanisch-Perron *et al.*, 1985) are an ideal host for many molecular biology applications and are convenient for transformation and subcloning. Whilst thawing, the ligase (in all ligations) was inactivated by heating to 70°C for 15 minutes. Once thawed, competent cells were gently mixed and 100µl transferred to each of the chilled culture tubes.

Ligations were added to the JM109 competent cells (100µl) and left on ice for 30 minutes. Cells were then heat-shocked in a 42°C waterbath for 50 seconds and then immediately placed on ice for 2 minutes. Tube contents were then added to 1ml LB-glucose medium and incubated for 60 minutes at 37°C with shaking (225rpm). Each transformation
reaction was diluted 1:10 and 1:100 and 100µl of each dilution streaked onto LB agar antibiotic plates (100µg/ml Ampicillin) each containing 20µl 5-bromo-4-chloro-3-indolyl-β-galactosidase (Xgal: 40µg/ml) and 100µl Isopropyl-β-D-thiogalactopyranoside (IPTG: 0.5mM). Plates were incubated at 37°C for 12-24 hours. Controls included a ligation control and transformation control. Transformation efficiency was assessed as a ratio of recombinant (white colonies) to non-recombinant (blue colonies). When the total recombinants were greater than 80% the cells were considered competent. Transformants were screened via PCR.

4.2.3 Screening of inserts using PCR

Transformants were screened by PCR using ECG152 and ECG226 primers (Section 4.2.1), as well as SP6 (5’-TAC GAT TTA GGT GAC ACT ATA G-3’) and T7 (5’-GTA ATA CGA CTC ACT ATA GGG-3’) primers. 10µl PCR reaction mixture containing 1µl 1X buffer (NH₄), MgCl₂ (1.5mM), dNTPs (0.2mM), 6pmol of each primer, and 2.5 units of Bioline Taq™ was prepared. The PCR cycling conditions (B.L. Spillings, personal communication, NICD) involved an initial denaturation step of 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 56.5°C for 30 sec and 72°C for 50 sec. A final extension of 72°C for 2 min followed. PCR products were electrophoresed on a 1.5% (w/v) agarose gel in 1X TAE buffer. Inserts for CYP6P9 showing the required band at 1.5Kb were used for further experiments for cloning into the expression plasmid pCWori+.

4.2.4 Plasmid purification (mini-prep) by SDS/alkaline lysis

Plasmid purification was carried out according to manufacturer’s instructions using the NucleoSpin® Plasmid kit (Macherey-Nagel, cat no: 740588.50, Germany), whereby plasmid DNA is liberated from the E. coli host cells by SDS/alkaline lysis. Following
screening of inserts, selected clones were inoculated into 5ml of LB medium and incubated overnight at 37°C with shaking (200rpm). Following incubation, bacterial cells were harvested by centrifugation (11,000 x g, 30 sec) and the cell pellet resuspended in 250µl Buffer A1. Plasmid DNA was liberated from the E. coli host cells by SDS/alkaline lysis using 250µl buffer A2, inverted 6-8 times and incubated at room temperature for 5 min or until lysate appeared clear. The mixture was neutralized by adding 300µl Buffer A3, mixed by inverting 6-8 times and centrifuged at room temperature (11,000 x g, 5 min). The resulting supernatant was loaded onto the NucleoSpin® plasmid column and centrifuged (11,000 x g, 1 min). All flow-through was discarded. The silica membrane was then washed by adding 500µl buffer AW (prewarmed to 50°C) and centrifuged (11,000 x g, 1 min) before proceeding with the addition of 600µl ethanolic Buffer A4 and centrifuged (11,000 x g, 1 min). Flow-through was discarded and the NucleoSpin® plasmid column was placed into an empty collection tube. The silica membrane was then dried by centrifuging (2 min at 11,000 x g) and the collection tube discarded. The NucleoSpin® plasmid column was placed into a 1.5ml microcentrifuge tube and pure plasmid DNA eluted under low ionic conditions, following incubation for 2 min at 70°C with 50µl slightly alkaline buffer AE (prewarmed to 70°C) and centrifuged (11,000 x g, 1 min).

4.2.5 Preparing E. coli membranes for functional CYP6P9 (FUMOZ-R)

Flasks containing 0.2L Terrific Broth (with 50mg/L ampicillin and 34mg/L chloramphenicol) were inoculated with the CYP6P9/CPR-expressing strain (from glycerol stock A100), previously transformed by Matambo (2008). The flasks were shaken at 150rpm and 37°C with 150rpm until the culture reached an optical density at 595nm of 0.8-1.0 units. The culture was then cooled to 25°C and supplemented with 1mM isopropyl β-
D-1-thiogalactopyranoside (IPTG) and 0.5mM 5-aminolevulinic acid (ALA), to induce expression and act as a heme precursor respectively. This culture was left to shake at 25°C and 150rpm orbital shaking for 2-3 days to achieve optimal P450 expression, monitored every 12 hours using CO-difference spectroscopy (Cary Spectrophotometer) by the method of Omura and Sato (1964). The cells were harvested and bacterial membranes prepared as described previously (Pritchard et al., 2006; McLaughlin et al., 2008). Total protein concentration was determined by Bradford assay with bovine serum albumin (BSA) standards. CPR activity was estimated by monitoring the reduction of cytochrome C (Pritchard et al., 2006), and cytochrome b5 (b5) concentration was determined by reduced versus oxidised difference spectroscopy (Omura and Sato, 1962). CYP6P9 was expressed at 97–225nmol of P450 L⁻¹ of culture. The isolated bacterial membranes contained 27mg ml⁻¹ CYP6P9 protein and the specific activity of CPR, calculated using a predicted pathlength of 0.85cm, = 0.0552*slope/protein concentration [where slope = 70.835 (n =3); protein concentration = 27mg ml⁻¹ ] contained 86.88nmol cytochrome c reduced min⁻¹mg⁻¹ protein.

4.2.6 Storage of plasmids and membrane preparations

Transformed cells containing CYP6P9 insert, as well as membrane preparations for CYP6P9 (FUMOZ-R) were stored as glycerol stocks as described by Sambrook et al. (1989). To the overnight culture (500µl), 90% glycerol (200µl) was added and stored at -70°C. When the plasmid or membrane preparation was needed, the surface of the frozen culture was scraped using a sterile pipette and inoculated into 5ml of LB broth (for transformed cells with insert) or 200ml Terrific Broth (for membrane preparations) respectively. These were then incubated overnight at 37°C with shaking (150rpm) and
then purified as described in Section 4.2.4 or prepared for functional expression as described in Section 4.2.5.

4.2.7 In-vitro permethrin insecticide metabolism analysis

The insecticide was first prepared at a stock concentration of 1-5mM. Permethrin metabolism reactions (adapted from Müller et al., 2008) were performed in 1.5ml tubes at 30°C with 1200rpm shaking following the composition: 0.2M Tris.HCl (pH 7.4), 1mM glucose-6-phosphate (G6P), 0.25mM MgCl₂, 0.1mM beta-nicotinamide adenine dinucleotide phosphate (oxidised; NADP), 1U/ml G6P dehydrogenase (G6PDH), 10µM permethrin (mixture of isomers), and 2% (v/v) ethanol (from permethrin stock solution). Reactions were performed with 0.2µM CYP6P9 and 0.8µM b5 (An. gambiae). NADP and G6PDH were excluded from reactions for negative controls (NADPH absent). Reactions were incubated at 30°C for 0, 5, 10, 20 and 40 minutes, and quenched by addition of an equal volume of acetonitrile and incubated for an additional 20min at 30°C with 1200rpm shaking to ensure all permethrin dissolved. After 5min centrifugation at 20,000 x g, the sample supernatant was transferred to glass HPLC vials. 100µl of the supernatant was loaded onto a mobile phase of 92% methanol and 8% water, with a flow-rate of 1ml min⁻¹ and 23°C for separation on a 250mm C18 column (Acclaim 120, Dionex, UK). Pyrethroid elution was monitored by absorption at 232nm and quantified by peak integration (Chromeleon, Dionex, UK). Permethrin quantity was estimated by integrating the trans-permethrin peak at 10.8 min and the cis-permethrin peak at 12.75min. Varying concentrations of substrate (0.5 -16µM) were used for permethrin kinetics, determined over a 10min period. Rates of permethrin turnover from three independent reactions were plotted versus permethrin substrate concentration. \( K_m \) and \( V_{max} \) were determined using
SigmaPlot 11 (Systat software, Inc) by fitting to the Michaelis-Menton equation using non-linear regression.

4.2.8 Cytochrome b5 preparation

The *E. coli* strain A183 was used to express a His-tagged *An. gambiae* cytochrome b5 (Dr. M.J.I Paine, LSTM, UK) that is subsequently solubilized from whole-cell lysate and purified by Ni-affinity chromatography. The culture was grown as described for CYP6P9, except the culture was only grown for 14 hours at 30°C before harvesting by centrifugation. The cell pellet had a red-brown colour and was then processed according to the method outlined by Holmans et al. (1994) except without the 50°C incubation step following the cell lysis. Inclusion of this step results in the degradation of most of the functional b5. The resulting supernatant which now contained the solubilised, full-length, His-tagged b5 appeared clear and red. The supernatant was purified by Ni-affinity chromatography by loading onto a gravity-fed 1ml Ni-NTA column and purified with varying buffers, eventually eluting a red coloured solution. The b5 sample was then concentrated using VivaSpin centrifugation columns and stored in aliquots at -20°C. Total protein content was measured by Bradford assay and the b5 concentration determined by spectroscopy, using the original extinction coefficient from Omura and Sato (1964): reduced b5/vs oxidized b5 $A_{424} - A_{409} = 185$/mM/cm; b5 measured 66.76µM L⁻¹.
4.3 Results and discussion

4.3.1 CYP6P9 (FANG) cloning and preparation of E. coli membranes for expression studies

P450 expression studies in An. gambiae using the E. coli ompA signal peptide were shown to be unsuccessful (Müller et al., 2008; McLaughlin et al., 2008). The incorporation of Histidine tags is also known to be problematic for cloning of An. funestus P450s (Matambo, 2008). For successful cloning as demonstrated by Müller et al. (2008) for CYP6P3 in An. gambiae, the P450 amino-terminus is replaced with a sequence (MALLLAVF) derived from the bovine steroid 17α-hydroxylase (Barnes et al., 1991).

pA092 (CYP6P9-FANG) is from the plasmid pCWompA-2, which is a modified version of pCWori+ for fusing P450 genes to the sequence for E. coli’s outer membrane protein A signal peptide. OmpA signal peptides have not worked well for expression of CYP6P9 which is why CYP6P9 (FANG) needed to be extracted from this construct, modified with a 17α sequence (MALLLAVF) and then sub-cloned into pCWori+.

Initially, CYP6P9 (FANG) was amplified from pA092 with primers ECG145 and ECG152 [designed by Matambo (2008) for CYP6P9-FUMOZ] and directly ligated into pCWori+.

These primer sequences were as follows:

ECG145

(TTTCATATGGCTCTGTATTAGCAGTTTTGCCGCTTATCTTCTCGTAGTG) - forward primer with NdeI site (underlined) and 17alpha modification. The shaded regions anneal to pA092 with a Tm of approximately 55 °C.

ECG152 (CGCTCTAGACTACAACTTTTCCACCTTC) - reverse primer with XbaI site (underlined). The shaded regions anneal to pA092 with a Tm of approximately 43 °C.
Direct ligation and transformations were problematic in that there were no white colonies present. Minipreps of these colonies were made for screening and sequencing purposes, using primers designed by Matambo (2008). Digests on the minipreps were also performed using NdeI and XbaI to determine if three fragments were present to confirm the existence of the insert, plasmid, and plasmid plus insert. Screening of the transformants revealed no insert was present at 1.5Kb, only non-specifics above 2Kb, and digests were not successful as multiple bands were being observed at incorrect sizes. These CYP6P9 (FANG) minipreps were then sent for sequencing to clarify results.

Sequencing revealed that the sequence alignment did not match pCWori+, although the insert was the correct CYP6P9 (FANG) sequence. The entire construct matched up with pA092 but there was a 5’ Histidine tag and an error at the 5’ end, whereby an NdeI site at the 5’ end was present instead of an XbaI site. Due to two XbaI sites being present, this would explain why it would not ligate into pCWori+ as NdeI was required, and this construct [pGEM-T::ompA-6P9 (FANG)His] could therefore not be used in expression studies.

For this reason a new 5’ end primer was designed, namely ECG226 (Section 4.2.1.1). ECG145 and ECG226 were used to create the modified 17α-CYP6P9-FANG construct (Figure 4.1) with no Histidine tag. The fragments were gel extracted and their concentrations measured on the Nanodrop® spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Values ranged from 40.2ng/µl -64.7ng/µl per fragment. Sequencing verified the construct was the correct size and had the 17α modifications with NdeI and XbaI restriction sites available for cloning into the expression plasmid pCWori+ (sequencing analysis performed by Bradley Stevenson, LSTM, UK).
When pCWori+ is treated with NdeI and XbaI, it releases a 500bp fragment, leaving a 5Kb linear plasmid for ligation with the 17α-CYP6P9-FANG via NdeI/XbaI. Digests were carried out in a 50μl total volume comprising: 10X Tango buffer (10μl), plasmid with insert (0.5 -1μg/μl), enzyme 1 (NdeI) (0.2 units), enzyme 2 (XbaI) (0.2 units) and dH2O. Digests occurred for 3-12 hours. The digest of pCWori+ was successful and plasmid was gel extracted (Figure 4.2).

Figure 4.1: Amplification from pA092 using primers ECG145 and ECG226 resulting in a 1.5Kb fragment, namely 17α-CYP6P9 (FANG) with no Histidine tag. Lanes 1-3: using 1μl of 30ng/μl pA092; Lanes 4-6: using 0.5μl of 30ng/μl pA092. These amplicons were gel extracted and either a) directly ligated into the expression plasmid pCWori+ (5Kb) to create the construct pCW::17α-CYP6P9 (FANG), or b) subcloned into the holding vector pGEM-T easy.

Figure 4.2: Digestion of pCWori+ showing plasmid at 5Kb and a 500bp fragment. The 5Kb plasmid was gel extracted for use in creating the expression construct pCW::17α-CYP6P9 (FANG).
The 17α-CYP6P9 (FANG) sequence was then subjected to direct ligation into the expression plasmid pCWori+. This was not successful, despite multiple attempts with different ligations (5:1; 8:1; 10:1) and restriction digests. Various other bacterial expression plasmids such as pA023 (pb13::ompA-CYP6Z2), pA123 (pCW::17alpha-CYP6P3), pA004 (pCWompA-2), pA082 [pCWmod1::ompA(AP)CYP6M2] and pA094 [pCWori::17alpha-CYP6P9 (FUMOZ)] were digested and gel extracted to see if they would ligate with the 17α-CYP6P9 (FANG) sequence, however there was no success with any of these bacterial plasmids. The digest for pA004 was unsuccessful; no insert was incorporated into the plasmid. The digests for constructs pA023 and pA123 were successful (as indicated in Figure 4.3 and Figure 4.4 respectively) and the plasmids were extracted successfully, however ligations and transformations were not as successful. The main problem seemed to arise at the transformation step. An established yeast or insect cell system was not available at the NICD or the LSTM, UK where expression studies were performed so the use of these expression systems with the FANG construct could not be investigated. Investigating the use of other plasmids may provide more successful results for this construct in future.

![Image](image_url)

**Figure 4.3:** Digests of bacterial plasmid pA023 for use for ligation with the 17α-CYP6P9 (FANG) sequence. Lanes 1-6: 1) No DNA, 2) and 4) A023 digested with NdeI and XbaI resulting in a 6 Kb plasmid, 3) no sample loaded, 5) pA123 with XbaI only, 6) pA123 with NdeI only.
Figure 4.4: 4.9Kb insert was gel extracted from plasmid pA123 (Lanes 1 – 7) following digestion with XbaI and NdeI for use in cloning of 17α-CYP6P9 (FANG).

Due to direct ligation being difficult and with no success, the route of subcloning into a holding vector such as pGEM®-T easy was the next available option. This construct was successfully cloned into pGEM-T easy and was then transformed into DH5α competent cells, miniprepped and then digested and gel extracted for ligation into pCWori+. The digest of 17α-CYP6P9 (FANG) in pGEM-T easy vector shows everything is in tact (Figure 4.5) and was confirmed via sequencing. A glycerol stock of the sequenced 17α-CYP6P9 (FANG) in pGEM-T easy vector was made and named pA259 [pGEM-T easy::17α-CYP6P9 (FANG), with no his tag] (Appendix A3). This pGEM-T-CYP6P9 (FANG) construct was transformed into DH5α cells and PCR confirmed the insert was present (Figure 4.6). In conjunction to pCWori+, the success of using pA082 in the final transformation was also examined to see if it would ligate with the insert.
Figure 4.5: Lanes 1-4: digest of 17α-CYP6P9 (FANG) in pGEM-T easy vector (FANG minipreps), using restriction enzymes NdeI and XbaI. Insert is at 1.5Kb, pGEM-T at 3Kb and pGEM-T plus insert at 4.5Kb. 
Lane 5: A digest of plasmid pA082. Insert was attempted for ligation with plasmid pA082 (4Kb).

Figure 4.6: pGEM-T easy/CYP6P9 (FANG) clones screened for insert following transformation into DH5α competent cells. The insert is present at 1.5Kb. MW = Molecular weight marker, HYP MW = Hyperladder MW.

The transformation of 17α-CYP6P9 (FANG)/pA082 plasmid into DH5α cells produced white clones. White clones were screened via PCR to check for insert, and selected clones were cultured overnight in LB-ampicillin so that minipreps could be made of them and these minipreps were then also screened via PCR. Screening of the white clones and minipreps revealed no insert. These minipreps were digested with NdeI and XbaI restriction enzymes to determine if it would remove the 17α-CYP6P9 (FANG) sequence.
from the pA082 plasmid, however fragments for the 17α-CYP6P9 (FANG) insert were not being detected at the correct size of 1.5Kb (Figure 4.7). What was observed was a band at 2.0Kb. This 2.0Kb fragment was not gel extracted and sequenced as it was considered as non-specific. The white clones selected were considered to be false positives. JM109 competent cells were also used as DH5α cells were not showing success, however this did not affect the outcome. Cloning into pCWori+ was also unsuccessful despite subcloning.

Due to time constraints at the Liverpool School of Tropical Medicine, no further work could be conducted on trying to transform the 17α-CYP6P9 (FANG) construct. This work was not continued at the NICD as no facilities or equipment were close at hand to perform the cloning and expression studies (in different and established bacterial, yeast or insect cell systems) and it was not feasible due to project time constraints. No metabolism analysis could then be performed between CYP6P9 susceptible and resistant strains. Metabolism analysis of CYP6P9 (FUMOZ-R) was then conducted as a standalone study.

![Image of gel electrophoresis](image.png)

**Figure 4.7: Digest of 17alpha-CYP6P9 (FANG)/pA082** – Lanes 5 and 6 shows a digest of this construct; however the insert should be at around 1.5Kb and not 2Kb.
4.3.2 **CYP6P9 (FUMOZ-R) expression in E.coli and permethrin metabolism studies**

CYP6P9 (FUMOZ-R) was co-expressed with *An. gambiae* cytochrome P450 reductase (CPR) in *E. coli* to produce a functional monooxygenase complex, as described previously (Pritchard *et al.*, 2006; McLaughlin *et al.*, 2008). This expression vector contained cytochrome P450 as detected by the characteristic reduced CO/reduced difference spectrum with an absorbance maximum at 450nm (Figure 4.8). Cells were harvested on day two, as the level of cytochrome P450 expression was 225nmol of P450 L⁻¹ of culture (stock concentration of 0.2µM) with a small amount of (inactive) cytochrome P420. The isolated bacterial membrane preparation contained 27mg ml⁻¹ CYP6P9 protein and the specific activity of CPR was 86.88nmol cytochrome c reduced min⁻¹mg⁻¹ protein.

Cytochrome b5 was prepared and its expression measured by spectrometry (Figure 4.9). This functional enzyme system was used to study the metabolism of permethrin substrate. Previous range-finding experiments (B.J. Stevenson, personal communication, Liverpool School of Tropical Medicine) determined that 0.1µM P450 (CYP6P9/CPR) with 0.8µM b5 and 10µM permethrin were ideal for determining the kinetics of substrate depletion. However, as 0.1µM showed low activity levels (data not shown) reactions were performed with 0.2µM CYP6P9 to try and increase activity.

CYP6P9 permethrin metabolism was evaluated over a time period of 40 minutes, using a 10µM mixture of four isomers. HPLC analysis showed that permethrin eluted with R and S trans-isomers at 10.6 min and R and S cis-isomers at 12.7 min (Figure 4.10). Elution for *An. funestus* CYP6P9 was at an earlier time point than that of *An. gambiae* CYP6P3, which eluted with R and S trans-isomers at 16.1 min and R and S cis-isomers at 17.4 min (Müller *et al.*, 2008).
Figure 4.8: Typical Fe$^{3+}$ vs Fe$^{2+}$-CO difference spectrum used for the quantitation of (*E. coli*) recombinant P450 CYP6P9, measured over three days. The main absorbance is at 450nm, whilst the absorption at 420nm varies from preparation to preparation and is most likely caused by other sources containing heme or partially degraded P450’s. The peak at 420nm is usually much reduced in membrane samples. The size of the peak at 450nm was measured (using the absorbance at 490nm as reference), and quantified using the extinction coefficient: $e_{\text{cytP450}} = 0.091\mu\text{M}^{-1}\text{cm}^{-1}$. Key: Day one = blue, Day 2 = pink, Day 3 = orange.

Figure 4.9: Cytochrome b5 (b5) spectrum from 380nm to 580nm. The size of the peak at 420nm was measured using the original extinction coefficient from Omura and Sato (1964): reduced b5/vs oxidised b5 $A_{424} - A_{409} = 185/\text{mM/cm}$. Total protein concentration was measured by Bradford Assay and b5 concentration determined by spectroscopy. Cytochrome b5 measured 66.76µM L$^{-1}$. 

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NADPH was needed for permethrin metabolism, as no significant change in permethrin concentration was observed over the time period for elimination, not even after the full 40 min (Figure 4.11). However in the presence of NADPH, 27% of the total permethrin was eliminated in 40 min and metabolites were visible in the presence of NADP+. Over time permethrin showed a steady rate of decrease (Figure 4.12).

Substantial rates of metabolism of permethrin were observed by the heterologously expressed CYP6P9. Analysis of initial metabolic rate in response to permethrin concentration revealed Michaelis-Menten kinetics (Table 4.4 and with reference to Appendix A4): the average Vmax was 2.45±0.18 min\(^{-1}\) and the average Km was 10.04 ± 0.98 µM (± S.E.M; n = 3). Substrate turnover values (k\(_{\text{cat}}\) values) were in the range of 11.4–14 min\(^{-1}\) (average k\(_{\text{cat}}\) value = 12.28 min\(^{-1}\); n = 3), which were relatively quick compared to rates observed for the in-vitro P450 metabolism of pyrethroids reported from other species such as lepidopteran CYP6B8, which had a Vmax of α-cypermethrin of 13 min\(^{-1}\) (Li et al., 2004). Kasai et al. (1998) demonstrated via in-vitro metabolism using larval microsomes, that the JPal-per strain, from the mosquito Culex quinquefasciatus, had 5- and 20-fold higher permethrin metabolism than the susceptible strain. One of the kinetics analyses and Michaelis-Menten plots are shown in Figure 4.13. Raw data and calculations are further explained in Appendix A4. Being able to identify such differences in An. funestus resistant and susceptible strains would be interesting to compare with C. quinquefasciatus and would provide insight into the metabolic efficiencies of these two strains for permethrin metabolism.
Figure 4.10: HPLC analysis showing that in the presence of NADPH, permethrin is eluted with R and S\textit{trans}-isomers at 10.6 min and R and S\textit{cis}-isomers at 12.7 min. The presence of putative NADPH-dependent metabolites is indicated by arrows. Green lines indicate 0 min (start of reaction); black line indicates 40 min (end of reaction).

Figure 4.11: HPLC analysis showing that when NADPH is absent no permethrin metabolism occurs and no metabolites are produced.
CYP6P9 in FUMOZ-R is roughly a 7-fold faster metabolizer of pyrethroids (permethrin) than An. gambiae CYP6P3, which had turnover values in the range 0.5–2min⁻¹ (Müller et al., 2008). This could be due to the fact that cytochrome b5 was incorporated into our reaction system, whereas b5 was absent with CYP6P3, leading to the enhanced activity of this cytochrome P450 (Feyereisen, 2005, Müller et al., 2008). $K_{cat}/K_m$ values ranged from $5.15 \times 10^7$ M⁻¹ s⁻¹ to $9.93 \times 10^7$ M⁻¹ s⁻¹ (average = $8.33 \times 10^7$ M⁻¹ s⁻¹; n = 3), which relates to a very high catalytic efficiency in this species. This value however does not make it a ‘perfect enzyme’ as enzymes that have a $K_{cat}/K_m$ ratio of $10^5$ are regarded as perfect because this ratio approaches the diffusion limit (Benner, 1989). It will also not be perfect here as this experiment was performed in-vitro and not under ‘physiological’ conditions. It was also postulated by Benner (1989) that enzymes in vitro that have $K_{cat}/K_m$ values higher than $10^7$ M⁻¹ s⁻¹ are expected to occur as multi-enzyme aggregates. This could be possible for CYP6P9. This fast metabolism may account for the increased transcription of CYP6P9 following pyrethroid exposure as observed in the previous chapter.

![Figure 4.12: Decrease of permethrin (Area MAU*min) by CYP6P9 (FUMOZ-R) over time, showing a steady decline of permethrin in the presence of NADPH (+VE) and very little decline, if any without NADPH (-VE).](image-url)
Table 4.4 Parameters determined by substrate saturation kinetic analysis

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
<th>(K_{\text{cat}}) (min(^{-1}))</th>
<th>(K_{\text{cat}}/K_m) (M(^{-1}) s(^{-1}))</th>
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<tbody>
<tr>
<td>1</td>
<td>(V_{\text{max}})</td>
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<td>14.03 min(^{-1})</td>
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<td>(K_m)</td>
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</tr>
<tr>
<td>3</td>
<td>(V_{\text{max}})</td>
<td>2.28</td>
<td>0.24</td>
<td>11.41 min(^{-1})</td>
<td>9.93 (\times 10^7)</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>6.89</td>
<td>1.29</td>
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<tr>
<td><strong>Averages</strong></td>
<td><strong>(V_{\text{max}})</strong></td>
<td><strong>2.45</strong></td>
<td><strong>10.04</strong></td>
<td><strong>K_{\text{cat}})</strong> (min(^{-1}))</td>
<td>**K_{\text{cat}}/K_m) (M(^{-1}) s(^{-1}))</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td><strong>12.28</strong></td>
<td><strong>8.33 (\times 10^7)</strong></td>
</tr>
</tbody>
</table>

Note: \(K_{\text{cat}} = V_{\text{max}}/\left[E\right]_T\); where enzyme concentration = 0.2µM

\(K_{\text{cat}}/K_m = K_{\text{cat}}/K_m \times 10^6\) M x 60 sec

Figure 4.13: A kinetics analysis of CYP6P9 activity – showing Michaelis Menton plot and derivative Lineweaver-Burke plot. \(V_{\text{max}} = 2.28\) (StdErr =0.25), \(K_m = 6.90\) (StdErr = 1.30); calculated using SigmaPlot 11 (Systat software, Inc).
This result correlates well with the fact that FUMOZ-R is the resistant phenotype and it was expected to have a high catalytic efficiency due to its success at survival once exposed to pyrethroids, both in the laboratory and in the field.

Some HPLC runs did not produce such high yields of metabolites as seen in Figure 4.10 (mA232 range: 1.0 - 8.0) and the reason as to why this may have occurred was investigated. It was thought that the percentage of ethanol may have played a role in permethrin stabilisation. The reason as to why ethanol was chosen in comparison to using other solvents such as DMSO or methanol was based on a paper by Chauret et al. (1997) which compared different solvents with P450s. It was found that DMSO was a poor choice since it could mimic O₂ and interact with the Fe²⁺ at the heart of the P450 and it could inhibit the activity of several P450s even at low levels (0.2%) (Chauret et al., 1997). This is consistent with the fact that DMSO is a P4502E1 inhibitor (Yoo et al., 1987). It was also found by experimentation that when either methanol or ethanol was used for solvent; pyrethroids that consisted of one stereoisomer (e.g. deltamethrin) racemised at a slower rate in ethanol (Bradley Stevenson, personal communication). For this reason ethanol was chosen for this experiment. It is also of interest to note that most proteins are fine with up to 5% ethanol (Stevenson et al., 2011). The ethanol percentage was varied using 6.66%, 10%, 14.2% and 20% ethanol. It was found that 20% ethanol nearly resembled the negative control, whereas 6.66% provided for production of permethrin metabolites (data not shown). It was concluded that increasing the ethanol percentage in this reaction did not stabilize permethrin and yielded fewer, if any, metabolites, and was therefore not what may have led to our distinct metabolite peaks previously observed.
Future development of the FANG construct needs to be looked at to determine any differences between these two strains with regard to pyrethroid metabolism. The fact that FANG and FUMOZ-R differ by four amino acids (Matambo et al., 2010) is a vital aspect, as the catalytic power of an enzyme, the kinetic order, Michaelis constants and regulatory properties of an enzyme can be altered simply by changing a small number of amino acids (Benner, 1989). Identifying which amino acid change may be causing a difference between these two strains, using site-directed mutagenesis techniques, would be an important finding to understanding detoxification mechanisms between these strains.
4.4 Conclusion

Prior studies by Matambo (2008) allowed for the functional expression of CYP6P9 (FUMOZ-R) in *E. coli* along with its cognate redox partner CPR and this allowed for functional enzyme characterisation and permethrin metabolism studies. However, this was not possible for CYP6P9 (FANG) as cloning into the final expression plasmid was problematic despite multiple attempts in various bacterial plasmids. CYP6P9 has been shown to play a major role in the detoxification of pyrethroids and in this study CYP6P9 was found to metabolise permethrin in an NADPH-dependent manner. There are four isomeric forms of permethrin; (R) cis, (R) trans, (S) cis and (S) trans, which differ in the spatial arrangement of the atoms. The cis-isomer has been noted to be more toxic as it is not as easily hydrolysed as the trans-isomer (Müller *et al.*, 2008). HPLC chromatography cannot separate the cis and trans forms into the (R) and (S) forms, however metabolism of the active form occurs as both (1RS) cis and (1RS) trans isomers are eliminated from enzyme reactions (Müller *et al.*, 2008). Permethrin metabolism by CYP6P9 (FUMOZ-R) revealed Michaelis-Menten kinetics; substrate turnover values (*k*<sub>cat</sub>) were in the range of 11.4–14 min<sup>−1</sup>; *K*<sub>cat</sub>/K<sub>m</sub> values (5.15 x 10<sup>7</sup>M<sup>−1</sup>s<sup>−1</sup> to 9.93 x 10<sup>7</sup>M<sup>−1</sup>s<sup>−1</sup>) suggested *An. funestus* to be catalytically efficient; and CYP6P9 in FUMOZ-R was found to be approximately a 7-fold faster metabolizer of pyrethroids (permethrin) than *An. gambiae* CYP6P3. Although permethrin metabolism in *An. funestus* has been elucidated, the protein structure needs to be viewed in order to understand whether this P450 is metabolizing this insecticide at single or multiple sites and whether it can metabolise other xenobiotics too. The causative mutations responsible for the overexpression of this P450 gene are still unknown. Site directed mutagenesis studies may help determine this, however determining the underlying protein structure would be most advantageous to understand its physiological interactions.