Chapter 2: An efficient method for the deletion of parasite-specific inserts in malarial genes

2.1 Introduction

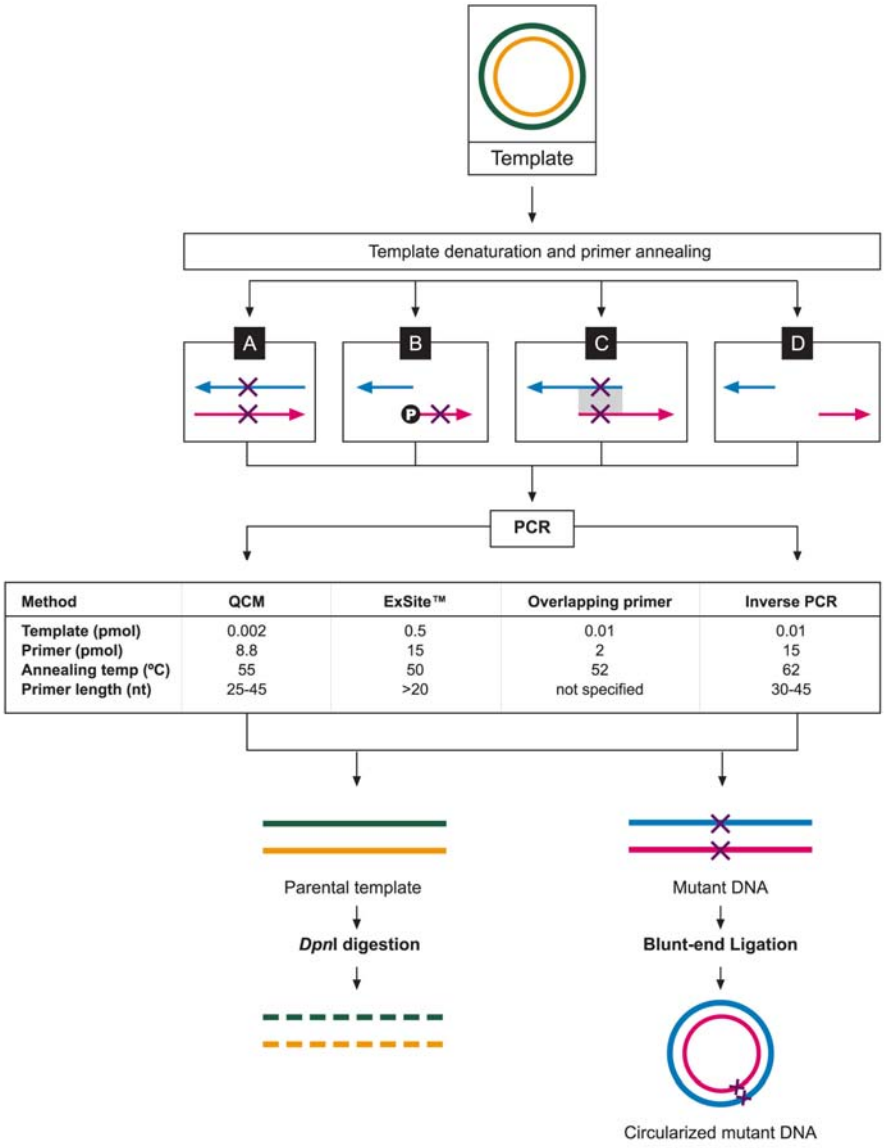
Specialised organisms like *P. falciparum* have unique adaptations that include generally larger protein sizes compared to orthologues due to the bifunctional arrangement of proteins and the presence of parasite-specific inserts (Mehlin *et al.*, 2006). In general, these inserts are species-specific, rapidly diverging, non-globular regions containing low-complexity areas consisting of mainly Lys and Asn residues that form flexible prion-like domains extending from the protein core (Pizzi and Frontali, 2000; Pizzi and Frontali, 2001). Up to 90% of *P. falciparum* proteins contain at least one low-complexity region that may co-localise with parasite-specific inserts. These proteins are also up to 50% longer compared to their yeast counterparts (Gardner *et al.*, 2002; DePristo *et al.*, 2006). The exact evolutionary origin and functional advantages of these inserts remain elusive. It has, however, been proposed by Karlin *et al*. that these inserted regions are adaptive as they appear to promote protein-protein interactions and mRNA stability (Karlin *et al.*, 2002). For example, in *P. falciparum* it has been demonstrated that stabilisation of interdomain interactions of the bifunctional malarial drug target, DHFR/TS, is mediated via an essential parasite-specific insert (Shallom *et al.*, 1999; Yuvaniyama *et al.*, 2003). DHFR/TS also regulates its own translation by binding to cognate mRNA (Zhang and Rathod, 2002). Some parasite-specific inserts have been implicated in malaria pathogenesis due to an increase in the antigen diversity and resultant incomplete immune response of the human host to *P. falciparum* (Gardner *et al.*, 2002).

Due to the ever-increasing resistance of malarial parasites to commercially available drugs, it is of extreme importance to identify novel drug targets. The bifunctional *P. falciparum* AdoMetDC/ODC (PfAdoMetDC/ODC) regulates the synthesis of polyamines, essential molecules for DNA and RNA stabilisation (Assaraf *et al.*, 1987). In addition to its unique bifunctional nature, the protein contains six parasite-specific inserts of up to 180 residues (Müller *et al.*, 2000; Birkholtz *et al.*, 2004; Wells *et al.*, 2006). Previous analysis of the structure-activity relationships indicated that these inserts are important for activity of the respective decarboxylase domains and act as mediators of protein-protein interactions in the bifunctional protein complex (Birkholtz *et al.*, 2004).

Site-directed mutagenesis is an important technique used in studying protein structure-activity relationships. Non-PCR based deletion mutagenesis methods mostly use sequence-

specific exonuclease-based enzymatic procedures but has the disadvantage that a single-stranded template is required (Braman, 2002). Since the development of PCR, oligonucleotide-mediated site-directed deletion mutagenesis has become a technically straightforward and efficient endeavour [reviewed in (Ishii *et al.*, 1998; Braman, 2002)]. Widely used PCR-based mutagenesis methods include the QuickChange™ site-directed mutagenesis (QCM) and ExSite™ methods (Stratagene) that are effective for the deletions of areas of up to 12 bp (Figure 2.1 A and B, respectively) (Papworth *et al.*, 1996). Several modifications to the QCM have been reported to be successful for deletions in large genes. This includes a partial overlapping primer design method allowing seven bp deletions (Figure 2.1 C) (Zheng *et al.*, 2004) and inverse PCR methods with a maximal deletion of 102 bp (Figure 2.1 D) (Wang and Wilkinson, 2001). However, none of these methods have been reported to be consistent in removing areas >100 bp in genes (Makarova *et al.*, 2000).

In this study, a restriction enzyme (RE)-mediated inverse PCR is described that successfully removes large areas in abnormally large genes (gene size ~4300 bp). This method was specifically designed for use on large parasite-specific inserts of the large PfAdoMetDC/ODC protein. The deletion mutagenesis efficiency of this RE-mediated inverse PCR method was compared to the existing methods described above by deleting a 411 bp parasite-specific insert in the PfAdoMetDC domain of the bifunctional PfAdoMetDC/ODC protein. In addition, its application to delete an insert in another malarial gene was also investigated to validate the method.



Method	QCM	ExSite™	Overlapping primer	Inverse PCR
Template (pmol)	0.002	0.5	0.01	0.01
Primer (pmol)	8.8	15	2	15
Annealing temp (°C)	55	50	52	62
Primer length (nt)	25-45	>20	not specified	30-45

Figure 2.1: Overview of four currently used deletion mutagenesis PCR methods.
(A) QuickChange™ site-directed mutagenesis method, (B) ExSite™ site-directed mutagenesis method, (C) Overlapping primer method, (D) Inverse PCR method. The template plasmid (green and yellow, top panel) is denatured during the first step of the PCR reaction to allow the primers containing the desired mutation to anneal to the specific target sites. In the QCM method the primers completely overlap and the mutations are incorporated into both of the primers (A). The ExSite primers do not overlap at all; instead only one of the primers contains the mutation. One or both of the primers must also be phosphorylated (B). Partial overlapping at the 3'-ends is characteristic of the overlapping primers. The mutation is present in both of the primers within the overlapping region (C). And finally, primers for the inverse PCR method do not overlap, but simply start at the opposite ends of the desired area to be deleted (D). The pink and blue arrows are the sense and antisense primers. Crosses on the primers represent the mutation sites and P is the single phosphorylated ExSite™ primer in (B). The primers are extended during which the desired mutations are incorporated and subsequently amplified. Two different linear PCR products are created during the PCR reactions, parental template and mutated DNA. The parental DNA is degraded by a *Dpn*I digestion step while the mutant DNA is circularised by blunt-end ligation. The newly formed mutant plasmids can subsequently be transformed into competent cells.

2.2 Methods

2.2.1 Deletion mutagenesis PCR

2.2.1.1 Cloning of genes into pASK-IBA3

All five methods described below (QCM, ExSite™, overlapping primer, inverse PCR and RE-mediated inverse PCR) used the *P. falciparum AdoMetDC/ODC* gene (gene size ~4300 bp) cloned into a pASK-IBA3 vector [vector size ~3100 bp (Institut für Bioanalytik, Germany)] as template (total template size ~7400 bp and called pA/Owt henceforth) (Müller *et al.*, 2000; Birkholtz *et al.*, 2004; Wells *et al.*, 2006). All methods were used to delete the 411 bp A3 insert in the PfAdoMetDC domain. The *P. falciparum* pyridoxal kinase (*PfPdxK*, gene size ~1500 bp) was also cloned into the same pASK-IBA3 vector (total template size ~4600 bp) (Wrenger *et al.*, 2005) to delete a 618 bp parasite-specific insert (A.O. Tastan Bishop, personal communication). The mutagenesis primers that were used in each of the deletion mutagenesis methods are listed in Table 2.1 below.

Table 2.1: Primers used for the various deletion mutagenesis protocols

Primer Pair	Primer	Length (bp)	Tm* (°C)	Primer Sequence (5' to 3')	Mutagenesis method
P1	A3consF	43	78	gctttatgatagtagtgatgctgataatt ataataaggaaagc	QuickChange™ site-directed method
	A3consR	43	78	gctttccttattataattatcagcatcact actatcataaagc	
P2	A3overF	49	79	gatagtagtgatgctgat↓aattataat aaggagagctttttatataatg	Overlapping primer method
	A3overR	55	80	gctttccttattataatt↓atcagcatca ctactatcataaagctttaaattatcc	
P3	A3reF	27	73(62)	CGCggatccaattataataaggaa agc	ExSite™, Inverse (Wang and Wilkinson, 2001) and RE-mediated inverse PCR methods
	A3reR	34	79(69)	CGCggatccatcagcatcactacta tcataaagc	
P4	PdxkF	34	78(67)	CGCggatccaatctaaattttctttgg gtatgtg	RE-mediated inverse PCR method
	PdxkR	38	79(67)	CGCggatcctttccttcttaattcaagt atatttttgg	

The *Bam*HI restriction sites for primer pairs P3 and P4 are underlined. Capital letters show the 5'-terminal overhangs of the P3 and P4 primer pairs The P3 primer pair was used with and without *Bam*HI restriction digestion for the RE-mediated inverse PCR and inverse PCR methods, respectively. * The Tm's were calculated according to the Stratagene formula: 81.5 + 0.41(%GC) - 675/N) or for primer pairs P3 and P4 with the Rychlik *et al*. formula: 69.3 + 0.41(%GC) - (650/N) (Rychlik *et al.*, 1990) as indicated in parentheses. ↓ indicates where the deletions were made with the overlapping primer method.

2.2.1.2 QuickChange™ site-directed mutagenesis (QCM)

According to the manufacturers' recommendations, a 50 µl reaction contained 10 ng template (0.002 pmol for the 7400 bp template used here), 125 ng of each of the primers (8.8 pmol

each of A3consF and R), 1 × *Pfu* reaction buffer, 200 µM of each dNTP and 2.5 U *Pfu* DNA polymerase (Fermentas, Canada). The temperature cycles were as follows: incubation at 95°C for 30 sec, followed by 30 cycles of 95°C for 30 sec, 55/60°C for 1 min, 68°C for 2 min/kb and a final extension at 68°C for 2 min/kb.

2.2.1.3 ExSite™ PCR-based site-directed mutagenesis

The PCR reaction with a final volume of 25 µl was set up as follows: 0.5 pmol template, 15 pmol of each primer (A3reF and R), 1 × *Pfu* reaction buffer, 200 µM of each dNTP and 2.5 U *Pfu* DNA polymerase (Fermentas, Canada). The temperature cycles were as follows: incubation at 94°C for 4 min, 50°C for 2 min, 68°C for 2 min/kb of template, followed by 18 cycles of 94°C for 1 min, 56°C for 2 min and 68°C for 1 min/kb, followed by a final incubation at 68°C for 5 min.

2.2.1.4 Overlapping primer method

A typical deletion mutagenesis reaction with a final volume of 50 µl, contained 50 ng template (0.01 pmol for the 7400 bp template), 2 pmol of each primer (A3overF and R), 1 × *Pfu* reaction buffer, 200 µM of each dNTP and 2 U of *Pfu* DNA polymerase (Fermentas, Canada). The cycling parameters were 94°C for 3 min, 16 cycles of 94°C for 1 min, 52°C for 1 min and 2 min/kb at 68°C with a final extension for 1 hr at 68°C (Zheng *et al.*, 2004).

2.2.1.5 Inverse PCR method

The PCR reaction set up followed the protocol as indicated by Wang *et al*. A typical deletion mutagenesis reaction with a 50 µl final volume contained 1 ng template (0.15 fmol for the 7400 bp template), 150 ng of both primers (15 pmol of A3reF and R), 1 × *Pfu* reaction buffer, 200 µM of each dNTP and 2.5 U *Pfu* DNA polymerase (Fermentas, Canada). The temperature cycles were as follows: 95°C for 3 min, 18 cycles of 95°C for 45 sec, 62°C for 1 min and 68°C for 2 min/kb, with a final extension at 68°C for 2 min/kb. The same reaction was also repeated with the addition of 5% dimethyl sulfoxide (DMSO) (Wang and Wilkinson, 2001).

2.2.1.6 RE-mediated inverse PCR method

For the RE-mediated inverse PCR method described here a typical deletion mutagenesis reaction with a 50 µl final volume contained 50 ng template (0.01 pmol for the 7400 bp template), 150 ng of both primers (15 pmol each of A3reF and R), 1 × *Pfu* reaction buffer, 200 µM of each dNTP and 2.5 U ExTaq DNA polymerase (TaKaRa Biomedicals, Japan). The temperature cycles were typically as follows: 95°C for 3 min, 18 cycles of 95°C for 45 sec, 56°C (Table 2.1) or 62°C for 1 min and 68°C for 2 min/kb, with a final extension at 68°C for 2 min/kb (Wang and Wilkinson, 2001). However, follow-up experiments have shown a 10^5

molar excess primer:template ratio to be optimal (where the best results were obtained with 0.15 fmol template and 15 pmol primer).

The effectiveness of this method in deleting a parasite-specific insert in another A+T rich *P. falciparum* gene was also tested. The reaction conditions and temperature cycles for the removal of the insert from *PfPdxK* were identical as given above.

All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems, USA).

2.2.1.7 Analysis of mutagenesis products

PCR products were visualised with 1% w/v agarose (Whitehead Scientific, SA) electrophoresis in TAE (40 mM Tris-OAc and 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). The electrophoresis runs were performed in Minnie Submarine HE33 Gel units [Hoeffer Scientific systems, (Amersham Biosciences, UK)] at a speed of 8 V/cm. DNA bands were visualised at a wavelength of 312 nm on a Spectroline TC-312A UV transilluminator (Spectronics Corporation, Germany) and gel images were captured digitally with a 4.0 mega pixel Canon Powershot G2 camera using the IC Capture Software.

Correctly sized PCR products were subsequently treated with 10 U of *Dpn*I for 3 hrs at 37°C to remove parental templates. For the RE-mediated inverse PCR method, 10 U *Bam*HI (Fermentas, Canada) was additionally added to the *Dpn*I digestion in a dual compatibility buffer Tango™ (33 mM Tris-OAc, 10 mM Mg-OAc, 66 mM K-OAc, 0.1 mg/ml BSA, pH 7.9). Products were purified with the High-Pure PCR product purification kit (Roche Diagnostics, Germany) and ligated for 16 hrs with 3 U of T4 DNA Ligase (Promega, USA) at 22°C for blunt-end ligation and 4°C for sticky-end ligation (RE-mediated inverse PCR product). The resulting circular plasmids were transformed into electrocompetent DH5α cells (Gibco BRL, Life Technologies, USA).

2.2.1.8 Electroporation into DH5α cells

DH5α *E. coli* (Gibco BRL, Life Technologies) cells were inoculated in 10 ml Luria Bertani (LB) liquid medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7.5) and grown overnight at 37°C with agitation. The overnight culture was diluted 1:100 in 500 ml preheated LB-medium. The inoculations were grown at 37°C with agitation (250 rpm) until the optical density at 600 nm reached 0.5. The cultures were placed on ice for 20 min. The following steps were performed on ice. The cells were harvested at 1000 x g for 10 min at 4°C. Pellets were resuspended in 10 ml cold dddH_2O with swirling motions. Ice-cold dddH_2O (240 ml) was added to the cell suspensions and gently mixed after which the cells were once again

collected at 1000 x g at 4°C for 10 min. The supernatants were then discarded and the pellets were resuspended in the remaining fluid. These wash steps were repeated once again. The pellets were resuspended in 10 ml 10% v/v sterile glycerol and transferred to sterile 50 ml centrifuge tubes. These were incubated on ice for 1 hr. The cells were pelleted at 1000 x g for 10 min at 4°C and the supernatants were immediately discarded. The pellets were resuspended in 1 ml 10% v/v glycerol, aliquoted into ice old Eppendorf tubes and immediately frozen at -70°C.

The plasmids were precipitated in the presence of 1 mg/ml tRNA and 0.3 M NaOAc (pH 5), followed by the addition of three volumes of absolute ethanol. The mixtures were centrifuged at maximum speed for 40 min at 4°C. Supernatants were discarded and the pellets were washed with 100 µl 70% ethanol and centrifuged for 10 min at the same speed. The pellets were dried *in vacuo* and subsequently dissolved in 5 µl dddH_2O.

The electrocompetent DH5α cells (100 µl) were thawed on ice to which the concentrated plasmids were added. These mixtures were placed into ice old electroporation cuvettes (Eppendorf, Germany) and immediately electroporated at 2000 V. Preheated LB-glucose (20 mM glucose in LB-medium) was immediately added to the shocked cells and incubated at 37°C for 30 min in a shaking incubator (250 rpm). Cells containing the transformed plasmids were plated onto LB-amp agar plates (1% agar w/v in LB-medium with 100 µg/ml ampicillin) and incubated overnight at 37°C (250 rpm).

2.2.1.9 Plasmid isolation

The plasmids from five clones for each of the three different mutagenesis methods that produced PCR products were isolated. Five colonies of each were inoculated overnight in 5 ml LB-amp (LB-medium with 50 µg/ml ampicillin) at 37°C in a shaking incubator set at 250 rpm. The cells were collected by centrifugation for 10 min at 13000 x g in a Heraeus Sepatech Medifuge. The High Pure Plasmid Isolation kit (Roche diagnostics, Germany) was used for plasmid isolation and the instructions were followed according to the manufacturer's protocol. Briefly, the pellets were suspended in 250 µl Suspension buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8) followed by the addition of 250 µl Lysis buffer (0.2 M NaOH, 1% w/v SDS). The suspensions were incubated at room temperature for 5 min. A volume of 350 µl pre-cooled Binding buffer (4 M guanidine hydrochloride, 0.5 M KOAc, pH 4.2) was added to each suspension followed by an incubation period on ice for 5 min. The suspensions were centrifuged at 13 000 x g for 10 min after which the supernatants were transferred to separate filter-tubes and once again centrifuged for 1 min at the same speed. The filters were washed with Wash buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) and the DNA was eluted in 100 µl Elution buffer (1 mM Tris-HCl, pH 8.5), after which the concentrations of the

purified plasmids were determined spectrophotometrically on a GeneQuantpro spectrophotometer (Amersham Biosciences, UK).

The concentrations were measured at a wavelength of 260 nm and the purities were analysed by their A260/280 ratio. A pure double-stranded plasmid should have a A260/280 ratio between 1.7 and 1.9 (Sambrook *et al.*, 1989).

2.2.1.10 Restriction enzyme mapping

Five clones for each of the three different mutagenesis methods that produced PCR products were analysed with *Hind*III restriction mapping. In the wild type pA/Owt plasmid, this enzyme should cut three times resulting in bands of ~3900, ~3100 and ~450 bp. However, in the ~400 bp deletion mutants, one of the sites is removed resulting in only two bands sized ~3900 and ~3100 bp. Application of the RE-mediated inverse PCR method on the deletion of ~600 bp from the *PfPdxK* gene also resulted in a PCR product. Five clones were analysed with *Eco*RI restriction mapping. The deletion removes an *Eco*RI site resulting in the linearisation of only the wild type non-mutated DNA (~4600 bp template).

2.2.1.11 Nucleotide sequencing

These same clones were thereafter analysed with nucleotide sequencing to confirm the mutagenesis results. The K215A1 (5'-gcttctacgtttgcattctgttcgg-3') sequencing primer was used as it binds 46 residues upstream of this area in *PfAdoMetDC/ODC*. Nucleotide sequencing of the *PfPdxK* mutants was not performed as mutagenesis could be confirmed by both the decrease in PCR product size (~4000 bp) and the removal of the *Eco*RI digestion site upon the deletion of the 618 bp parasite-specific insert.

The reactions were made up to a final volume of 20 µl and contained ~750 ng template, 5 pmol of the sequencing primer, 1 x Big Dye Sequencing buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0) and 2 µl of Big Dye Ready Reaction mix version 3.1 (Applied Biosystems, USA). The sequencing cycles were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems, USA) with an initial denaturation step at 96°C for 1 min followed by 25 cycles of denaturation for 10 sec at 96°C, annealing at 50°C for 5 sec and extension at 60°C for 1 min. The labelled extension products were purified with ethanol precipitation. Briefly, the products were mixed with 2 µl of 3 M NaOAc (pH 4.6) and 50 µl ice-cold absolute ethanol. These were then centrifuged at 11 000 x g for 30 min at 4°C. The supernatants were discarded and the DNA washed with 250 µl 70% ethanol and subsequently collected by centrifugation for 10 min at the same speed. The pellets were dried *in vacuo* and stored at 4°C in the dark.

The products were analysed with an ABI PRISM 3100 capillary sequencer (PE Applied Biosystems). Sequences obtained were evaluated with the BioEdit version 5 (Hall, 1999).

2.3 Results and Discussion

2.3.1 Analysis of mutagenesis products

The wild type malarial *PfAdoMetDC/ODC* gene cloned into pASK-IBA3 was used as template for a comparative study of all the deletion mutagenesis methods described here (Figure 2.2 A and C, lane 1). Subsequent gel electrophoresis analysis of the PCR products obtained by the different methods showed that only the overlapping primer method, inverse PCR method and RE-mediated inverse PCR method yielded products ~7000 bp in size (Figure 2.2 B and C, lanes 2, 3, 4). However, no PCR products could be visualised for the QCM or ExSite™ methods.

The products obtained by the overlapping primer method, inverse PCR and RE-mediated inverse PCR method were further analysed for mutagenesis efficiency with *Hind*III restriction enzyme screening and occurrence of the expected ~3900 and ~3100 bp bands.

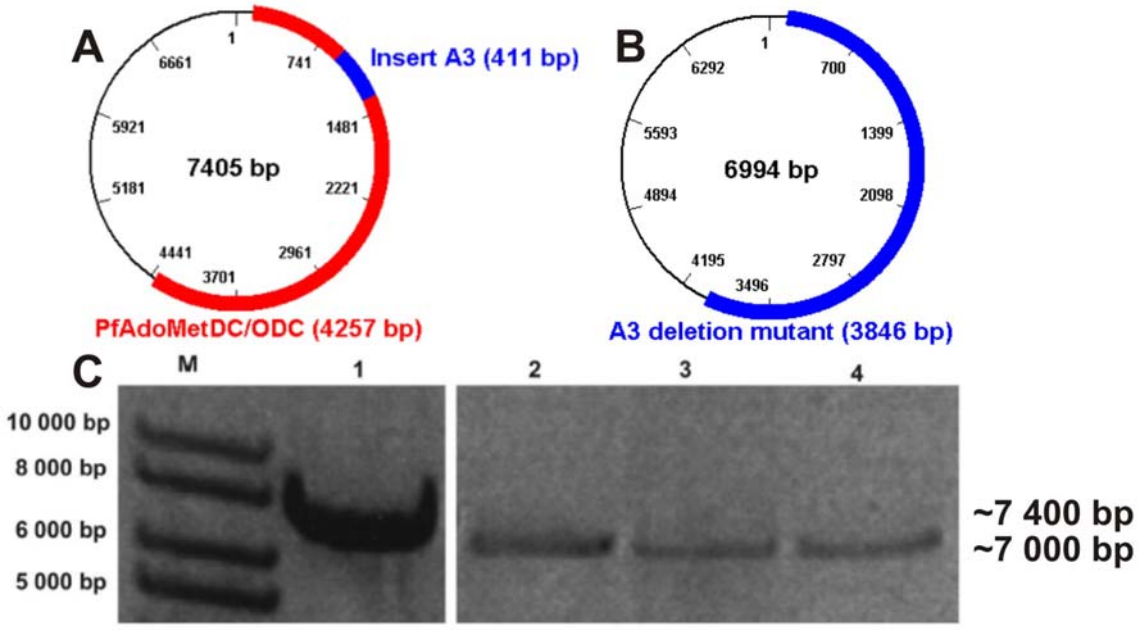


Figure 2.2: Agarose gel electrophoresis of the PCR products obtained with the various deletion mutagenesis PCR methods.
Schematic representations of the wild type (A) (gene size 4257 bp) and the 411 bp deletion mutant (B) (gene size 3846 bp) *PfAdoMetDC/ODC* genes inserted into a pASK-IBA3 vector (vector size ~3100 bp). (A) Wild type *PfAdoMetDC/ODC* is shown in red and the insert region in blue (B). The agarose electrophoresis gel of the deletion mutagenesis PCR products is given in (C) indicating the wild type PCR product (~7400 bp) and the deletion mutants (~7000 bp) produced by the different deletion mutagenesis methods. (M) 1 kb DNA marker, (1) A/Owt, (2) Overlapping primer, (3) Inverse and (4) RE-mediated inverse PCR products.

Two out of five clones obtained with the overlapping method were mutant and did not contain the 411 bp insert. No transformation-competent mutated genes were obtained for the inverse PCR method. The RE-mediated inverse PCR method described here resulted in four mutated out of five clones analysed. Nucleotide sequencing verified the 40% and 80% mutagenesis efficiencies obtained for the overlapping primer and RE-mediated inverse PCR methods, respectively (Table 2.2).

Table 2.2: Deletion mutagenesis efficiency results for the different protocols used

Primer pair	Mutagenesis method	PCR product size	RE mapping with *Hind*III	Deletion efficiency after nucleotide sequencing (%)
P1	QuickChange™ site-directed	No product	NA	0
P2	Overlapping primer	7000 bp	~3900 bp ~3100 bp	40
P3	ExSite™	No product	NA	0
	Inverse PCR	7000 bp	~3900 bp ~3100 bp	0
	RE-mediated inverse PCR	7000 bp	~3900 bp ~3100 bp	80

Five clones were analysed for each of the different PCR-based mutagenesis methods based on duplicate PCR products.

Figure 2.3 shows representative nucleotide sequences of the deletion products after the A3 insert was removed from *PfAdoMetDC/ODC* with the overlapping primer (A) and RE-mediated inverse PCR (B) methods. With the latter method, the *Bam*HI recognition sites of the forward and reverse primers are inserted in place of the deleted insert.

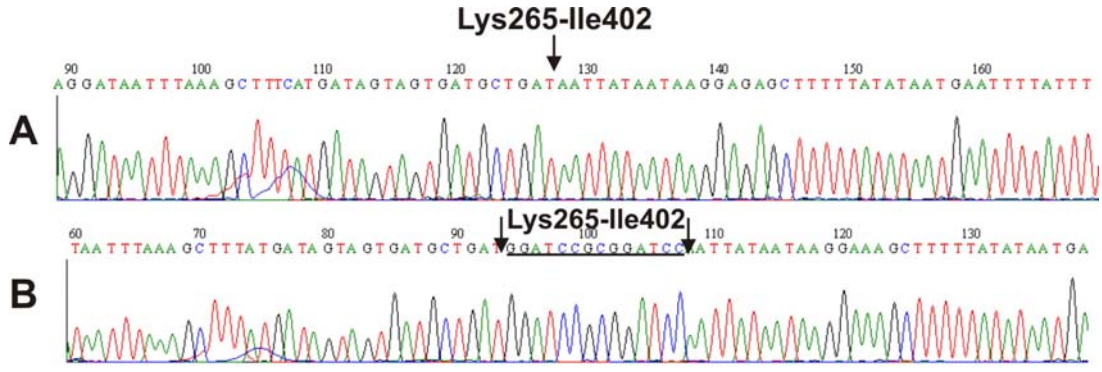


Figure 2.3: Nucleotide sequencing chromatograms of the largest insert deletion products (A/OΔA3) obtained with the overlapping primer and RE-mediated inverse PCR methods.
The arrow in (A) indicates the position where the overlapping primer method removed the A3 insert. The black bar in (B) shows the nucleotides corresponding to the *Bam*HI recognition sites that were inserted in place of the insert during the RE-mediated inverse PCR method.

The QuickChange™ site-directed mutagenesis method (QCM) requires that both of the mutagenic primers contain the desired mutation and anneal to the same sequence on

opposite strands of the plasmid. The method is limited to primers of 25 to 45 nt in length with melting temperatures approximately 10°C above the extension temperature of 68°C. The mutation should preferably be in the centre of the primer flanked by at least ten bases on either side. The GC content must also be at least 40%, and the primers must terminate in a G or a C base, which is challenging when working with the A+T rich genome of *P. falciparum*. The QCM method claims 80% efficiency for point mutagenesis but was unsuccessful in this deletion mutagenesis study (Table 2.2). This also explains previous inconsistent results produced in this laboratory by application of the QCM deletion mutagenesis method for the deletion of significantly sized areas in various other malaria genes (Birkholtz *et al.*, 2004).

The ExSite™ PCR-based site-directed mutagenesis method uses higher template concentrations and reduced PCR cycles to minimise potential second-site mutations. Primers for this method must be greater than 20 bases in length. The mismatched portions of the primers should be at or near the 5'-terminus of one or both of the primers with 15 or more of the matching sequence at the 3'-terminus. One or both of the primers must be 5'-phosphorylated. In order to make a specific mutation, the alteration must be included within the primers and their 5'-termini must meet but not overlap. Any bases between the 5'-termini will subsequently be deleted in the final product. The application of the ExSite™ method did not result in any product (Table 2.2).

Zheng and his co-workers modified the QCM protocol by using primers with partial overlaps at the 5'-termini to prevent self-extension [overlapping primer method, (Zheng *et al.*, 2004)]. This method was applied to vectors of up to 12 000 bp in length and yielded significantly improved PCR mutagenesis results. The modified primers were proposed to overcome the limitation of the melting temperature of primer design dictated by QCM. At least eight non-overlapping bases must be present on the 3'-termini of the primers, and the mutations may be as close as four nt away from the 5'-terminus. The primers must also terminate in a G or a C residue. The overlapping primer method was reported to delete up to seven bp (Zheng *et al.*, 2004) and had a 40% mutagenesis efficiency in deleting 411 bp from the ~7400 bp template in this study (Table 2.2).

Inverse PCR employs two inverted tail-to-tail primers to amplify an entire gene/vector except for the region that needs to be deleted. This method has been successful in deleting up to 102 bp in large plasmids (12 000 bp) (Wang and Wilkinson, 2001). According to Wang *et al*., for this method to be effective the primers must be similar in size, between 30 and 45 nt in length and have a melting temperature of at least 78°C with an applied annealing temperature of 68°C (Wang and Wilkinson, 2001). The number of PCR cycles must also be preferably less than 20. The application of this method produced the expected 7000 bp

deletion product as judged by gel electrophoresis only in the absence of DMSO (Figure 2.2 C, lane 3). However, the correct deletion product was not present in any of the five clones screened (Table 2.2). The primary causes for such background after PCR-based mutagenesis techniques could include the mis-priming and subsequent generation of incorrect, transformation-competent PCR products or the self-annealing of the 5'-overhanging CGC ends of the primers that could prevent subsequent blunt-ended ligation (Chiu *et al.*, 2004). The results presented here support other examples, which suggests that a maximum of only 12 bp can be removed with the inverse PCR method (Makarova *et al.*, 2000).

The inverse PCR method was subsequently modified to incorporate unique restriction enzyme sites at the 5'-ends of both the inverted tail-to-tail primers (RE-mediated inverse PCR, Figure 2.4). The primers of the RE-mediated inverse PCR method designed here included 5'-terminal overhangs (CGC in this instance) to improve the efficiency of the restriction digestions. This is followed by unique restriction enzyme sites that generate sticky-ends to improve the ligation efficiency (Sambrook *et al.*, 1989). This would additionally increase the number of deletion products by eliminating any primary product still containing the inserted region. The designed primers were not dependent on a similar length due to the requirement to terminate in one or more G or C bases at the 3'-end to increase the specificity of the PCR reaction. This feature is particularly important in the application of PCR on A+T rich *P. falciparum* genes. This method produced four out of five correct mutated products for a large area (411 bp) in a large gene (*PfAdoMetDC/ODC*, gene size 4257 bp) (Table 2.2).

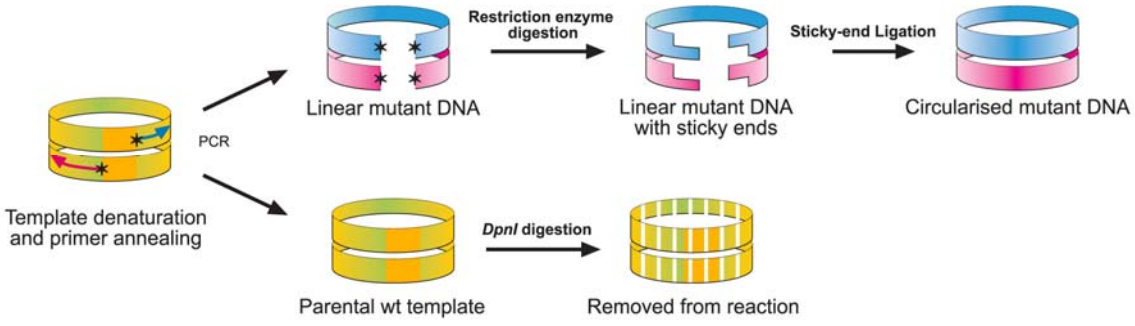


Figure 2.4: Schematic representation of the RE-mediated inverse PCR method.
The primers are designed in such a way that they contain unique restriction enzyme sites (represented by stars) and anneal to the opposite ends of the desired region to be deleted. The PCR reaction results in the synthesis of both parental, wild type template DNA (yellow and green in the bottom panel), which is subsequently removed during a *Dpn*I digestion step, as well as linear mutated DNA (pink and blue in the top panel). Digestion with the unique restriction enzyme creates linear DNA with sticky-ends, which improves the ligation efficiency and subsequent circularisation of the PCR product containing the deletion mutation.

In addition, the RE-mediated inverse PCR method was verified with a second *P. falciparum* gene as template to prove that this method is efficient in deleting large areas from smaller A+T rich genes.

2.3.2 Verification of the RE-mediated inverse PCR method

The subsequent application of the RE-mediated inverse PCR method on a second A+T rich malarial gene (*PfPdxK*, gene size 1536 bp) resulted in a PCR product that greatly differed in size compared to that of the wild type (Figure 2.5).

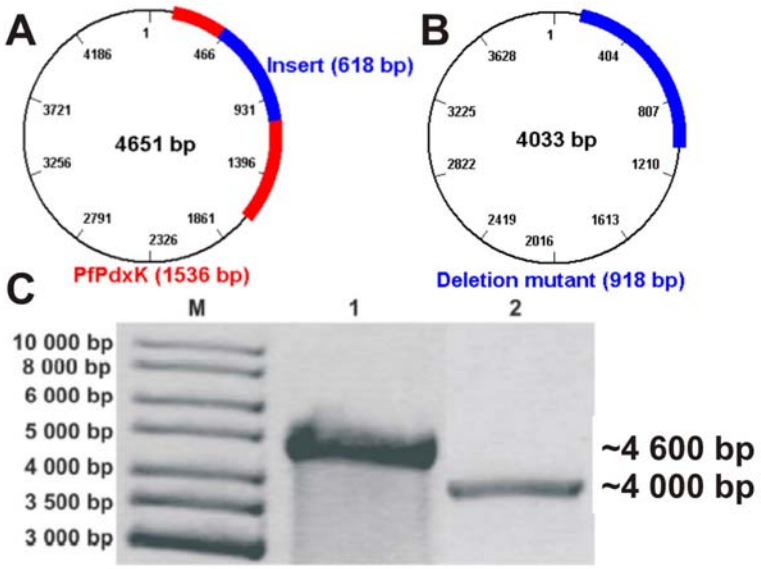


Figure 2.5: Agarose gel electrophoresis of the PfPdxK insert-deleted PCR product after RE-mediated inverse PCR.
Schematic representations of the wild type (A) (gene size 1536 bp) and the 618 bp deletion mutant (B) (gene size 918 bp) *PfPdxK* genes inserted into pASK-IBA3 vectors (vector size ~3100 bp). Wild type *PfPdxK* (A) is shown in red while the deletion mutant is in blue (B). The gel of the deletion mutagenesis PCR products is given in (C) showing the wild type product of ~4600 bp and the deletion mutant at ~4000 bp produced by the RE-mediated inverse PCR method. (M) 1 kb DNA marker, (1) wild type PfPdxK, (2) RE-mediated inverse PCR product.

Five clones were analysed with *Eco*RI restriction mapping. The deletion of the insert in *PfPdxK* removes an *Eco*RI site resulting in the linearisation of only the wild type plasmid (~4600 bp) (Figure 2.6).

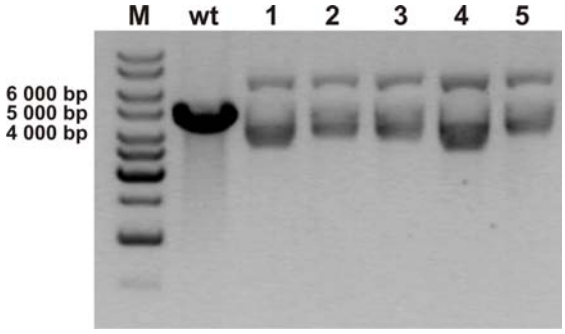


Figure 2.6: *Eco*RI restriction enzyme mapping of the wild type and insert-deleted PfPdxK plasmids obtained with the RE-mediated inverse PCR method.
The agarose electrophoresis gel of the restriction enzyme digested products shows the linear wild type *Eco*RI digested product ~4600 bp (wt) and the circular deletion mutant plasmids of ~4000 bp (Lanes 1 to 5). (M) 1 kb DNA marker.

Restriction enzyme mapping showed that the insert was deleted in all five of the plasmids that were analysed giving a 100% mutagenesis efficiency. The RE-mediated inverse PCR method is therefore highly efficient in deleting up to 618 bp (roughly one third of the entire gene) in a smaller malarial gene. The efficacy of this method may therefore be influenced by the template size, with smaller templates resulting in marginally higher efficacy. However, this is not dependent on the particular gene sequence.

The RE-mediated inverse PCR method is a straightforward method in which the primers do not require either 5'-phosphorylation or purification by polyacrylamide gel electrophoresis or HPLC as specified by the general inverse PCR protocol (Wang and Wilkinson, 2001). Large deletions can be made without increasing the length of the primers as the desired mutation is not incorporated into the primer sequence but is simply deleted by extending the plasmid during the PCR reaction. A further advantage of this method is that PCR temperature cycles of less than 20 are needed, which decreases the incidence of DNA polymerase error rates. The method was not dependent on the addition of 5% DMSO (results not shown) as is often needed by the inverse PCR method for the prevention of secondary structure formation in both primers and template. Additionally, there is no requirement for a high primer GC content as with the QCM method, which is useful with the A+T rich *P. falciparum* genes. Primer options for QCM and inverse PCR are furthermore limited by the requirement that the primer melting temperature must be >78°C, which was not the case in the RE-mediated inverse method. In the absence of unique restriction sites, alternative methods including DiSec/TriSec (Dietmaier *et al.*, 1993), which allows the generation of specified sticky-ends, may be used or a restriction-independent method like the overlapping primer method should suffice.

In this chapter, the inability in deleting a reasonably large (>100 bp) DNA region with existing oligonucleotide-based deletion mutagenesis methods led to the application of a highly

efficient RE-mediated inverse PCR method for the deletion of large areas in abnormally large *P. falciparum* genes. In the next chapter, the use of this method in the deletion of inserts in the PfAdoMetDC domain of PfAdoMetDC/ODC will be described.