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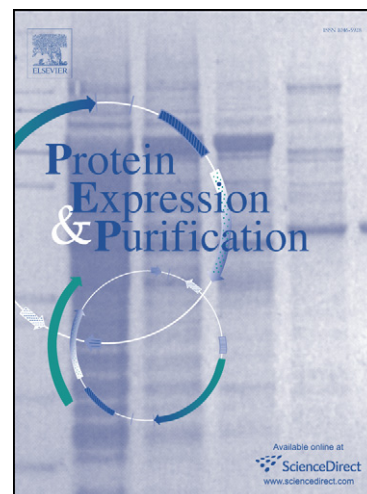
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Overexpression, purification and assessment of cyclosporin binding of a family of cyclophilins and cyclophilin-like proteins of the human malarial parasite *Plasmodium falciparum*

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Key words: Plasmodium falciparum, malaria, cyclophilin, cyclosporin, hexahistidine tag, metal-chelate affinity chromatography, thermal melt.

Abstract

Malaria represents a global health, economic and social burden of enormous magnitude. Chemotherapy is at the moment a largely effective weapon against the disease, but the appearance of drug-resistant parasites is reducing the effectiveness of most drugs. Finding new drug-target candidates is one approach to the development of new drugs. The family of cyclophilins may represent a group of potential targets. They are involved in protein folding and regulation due to their peptidyl-prolyl *cis-trans* isomerase and/or chaperone activities. They also mediate the action of the immunosuppressive drug cyclosporin A, which additionally has strong antimalarial activity.

In the genome database of the most lethal human malarial parasite *Plasmodium falciparum*, 11 genes apparently encoding cyclophilin or cyclophilin-like proteins were found, but most of these have not yet been characterized. Previously a pET vector conferring a C-terminal His₆ tag was used for recombinant expression and purification of one member of the *P. falciparum* cyclophilin family in *Escherichia coli*. The approach here was to use an identical method to produce all of the other members of this family and thereby allow the most consistent functional comparisons. We were successful in generating all but three of the family, plus a single amino-acid mutant, in the same recombinant form as either full-length proteins or isolated cyclophilin-like domains. The recombinant proteins were assessed by thermal melt assay for correct folding and cyclosporin A binding.

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According to the latest report of the World Health Organisation [1], about 3.3 billion people are at risk of malaria, leading to about 250 million cases a year and nearly one million deaths. In Africa alone it contributes to the death of about 10,000 pregnant women and 700,000 children each year. By far the most significant species is *Plasmodium falciparum*, which causes severe infections and death, and enjoys widespread geographic distribution [2]. Chemotherapy plays a major role in malaria control but the antimalarial drug armoury must constantly be renewed because drugs are continually falling prey to resistance [3]. One approach to new drug development lies in the identification of novel drug targets in the parasite, guided by analysis of genome sequence data [4]. Due to the almost overwhelming obstacles to purifying all but the most abundant *P. falciparum* proteins directly from the parasites, potential target proteins are typically produced in recombinant form for structural analysis and measurement of binding and/or inhibition of activity by small molecules [5].

P. falciparum is well known for having genes that can be particularly resistant to heterologous expression in bacterial and yeast hosts. A variety of factors are thought to account for this: the genome is 80% AT and has a codon bias well removed from that of *Escherichia coli*; glycosylation patterns unique to the parasite are utilized [6]; and *P. falciparum* proteins are generally larger than homologues in other species, for example as much as 50% larger on average than homologues in *Saccharomyces cerevisiae* [7]. In addition, it is not uncommon for *P. falciparum* genes to contain cryptic start sites for *E. coli* that might produce mRNAs with altered protein-coding sequences or translation efficiencies resulting in multiple, truncated products when overexpressed in these bacteria [8]. Despite the difficulties, there are many examples of *P. falciparum* proteins expressed in *E. coli* and with the simplicity and speed of the T7 expression vector system, *E. coli* is the host of choice for most of the groups studying structural genomics [9]. The vector used often adds an oligohistidine affinity tag [10] that is relatively small, allows purification by a relatively simple protocol using immobilized metal affinity chromatography (IMAC¹) [11], and rarely affects the functional characteristics or solubility of the tagged protein [12].

The immunosuppressive drug cyclosporin A (CsA) was shown in the early 1980s to have anti-malarial activity in cultured blood-stage parasites and in vivo, and even to reduce the severity of malaria in experimental infections of owl monkeys [13, 14, 14a]. The

¹ Abbreviations used: CLD, cyclophilin-like domain; CsA, cyclosporin A; FKBP, FK506-binding protein; IMAC, immobilized metal affinity chromatography; PPIase, peptidyl-prolyl *cis-trans* isomerase; RT, reverse transcriptase.

immunosuppressive effects of cyclosporins have limited their development as antimicrobial agents, but the appearance of non-immunosuppressive derivatives of CsA with pronounced antimalarial activity emphasized the importance of further investigating the action of these drugs upon the parasite [14, 14a, 15]. In humans, by inhibiting signalling through the phosphatase calcineurin to a combination of transcription factors, CsA suppresses the transcriptional activation of the interleukin-2 gene [16]. Of these transcription factors, the nuclear factor of activated T-cells appears to be the most sensitive to the drug and is the only pathway for which the molecular mechanism of CsA-immunosuppression is well characterised. CsA exerts its action as part of a complex with its receptor, a member of the family of proteins called cyclophilins [16, 17]. The mechanism(s) of antimalarial action of CsA and other cyclosporins is/are unknown but two major cyclosporin receptors have been identified, namely the parasite cyclophilins PfCYP19A and PfCYP19B [18, 19]. The precise role of these two proteins in the antimalarial action of these compounds is not yet clear [19]. Moreover, ten other cyclophilin-related gene sequences are present in *P. falciparum* genomes, and some of them are expressed in blood-stage parasites, but their contribution if any to cyclosporin action is unknown. The chemically unrelated immunosuppressant FK506, which acts analogously to CsA following binding to its FK506-binding protein (FKBP) receptor, also has antimalarial activity [15].

Regardless of their origin, the structural conservation of cyclophilins throughout evolution and the presence of peptidyl-prolyl *cis-trans* isomerase (PPIase) activity in most members, and molecular-chaperone activity in some, underline their importance and suggest that cyclophilins may play an important role in malarial parasite development [17, 19]. So far, two recombinant *P. falciparum* cyclophilins PfCYP19B and PfCYP19A [20, 21] (see [19] for nomenclature) have been successfully purified and biochemically characterised. Functionally active PfCYP19B (minus its secretory signal peptide) was produced in milligram quantities in *E. coli* using the pET22b+ vector, which generated a protein with a C-terminal His₆ tag [20]. Similar approaches have been used in high-throughput expression of various *P. falciparum* genes [9]. Since our intention was to compare the functional properties and cyclosporin-binding capacity of all of the cyclophilin/cyclophilin-like family members, we wished to obtain recombinant proteins under conditions as close to identical as possible. We therefore adopted the pET system with a C-terminal His₆-tag for ‘medium-throughput’ production of this family of proteins.

Materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated.

Routine culture of P. falciparum

The *P. falciparum* 3D7 strain was maintained in continuous culture in human erythrocytes according to the method of Trager and Jensen [22]. Parasites were cultured routinely in culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, 0.01% [w/v] gentamicin, 0.18% [w/v] sodium bicarbonate, hypoxanthine (50 µg/ml), 10% albumax® [a serum substitute: Invitrogen, Dun Laoghaire, Ireland]) and washed erythrocytes (at a haematocrit of 2.5% or 5% [v/v]). Parasites were maintained in Petri dishes at 37°C in a candle jar. Culture medium was replaced depending on the parasitaemia, which was monitored by microscopic examination of Giemsa-stained smears.

Harvesting of parasites

Free parasites were released from infected erythrocytes according to the saponin method of Zuckerman et al. [23]. Freed parasites were sedimented by centrifugation at 975 x g at 4°C for 15 min and washed twice with cold saline sodium citrate buffer. Pellets were either used immediately or resuspended in freezing solution (10% [v/v] glycerol, 2 mM phenylmethylsulphonyl fluoride, pepstatin A (1 µg/ml), leupeptin (20 µg/ml), prepared in phosphate-buffered saline) for storage at -80°C.

Cloning of P. falciparum cyclophilin and cyclophilin-like genes: amplification by PCR

Of the cyclophilin or cyclophilin-like open-reading frames discussed below (Results), six were found not to have any introns, thus genomic DNA could be used as template. Primers displayed in **Table 1** were used to amplify their coding sequences, with *NdeI* and *XhoI* sites at the 5' and 3' ends, respectively, to facilitate subsequent cloning into the pET22b+ expression vector. Note that, where indicated, due to the presence of other domains within the gene and difficulties in amplifying large AT-rich regions, only the

cyclophilin-like domain (CLD) was amplified. DNA and RNA were isolated from harvested parasites as described elsewhere [24].

PCR was performed using ~1 µg *P. falciparum* 3D7 genomic DNA, 0.5 µM primers, 2 U *Pfu* turbo® DNA polymerase (Stratagene, La Jolla, California, USA) and 0.5 mM each of dATP, dTTP, dGTP and dCTP (Stratagene) in a TC-3000 thermocycler (Techne, Staffordshire, UK) (95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 45°C for 1 min, 72°C for 3 min; and a last step of 72°C for 10 min).

Amplification by reverse transcriptase PCR (RT-PCR)

The other cyclophilin/cyclophilin-like genes showed at least one predicted intron in their sequences, although one of these (*pfccyp19B*) had already been cloned [20]. Therefore a two-stage RT-PCR was performed on total RNA isolated from *P. falciparum* 3D7. Firstly, cDNA synthesis was performed on ~2 µg RNA in the presence of a specific primer, pre-incubated at 70°C for 10 min. Thus, *NdeI* (or *NcoI*) and *XhoI* sites were incorporated. Then 0.5 µM of each dNTP, 10 mM dithiothreitol (New England Biolabs, Ipswich, MA, USA) and 30 U of avian myeloblastosis virus reverse transcriptase (USB Products, Stauffen, Germany) were added. The samples were incubated at 42°C for 30-60 min, followed by 10 min at 70°C to inactivate the enzyme. Amplification of cDNA was performed as described before, except with the template being cDNA rather than genomic DNA. In all cases PCR products were purified using the quick protocol of a wizard SV® gel and PCR clean-up system (Promega, Wisconsin, USA).

Cloning of putative P. falciparum cyclophilin genes into pET22b+ or pET21d+

pET22b+ and pET21d+ (Novagen, Darmstadt, Germany) were isolated from *E. coli* XL-1 Blue cells (Stratagene) using a wizard *plus* SV® miniprep DNA purification system (Promega). Purified plasmids and the corresponding PCR-amplified cyclophilin/cyclophilin-like coding sequences were digested with either *NdeI* (pET22b+) or *NcoI* (pET21d+) (according to the PCR primers) and *XhoI* (Roche). pET21d+ was used where the presence of an *NdeI* site within the gene precluded the easy use of pET22b+. Briefly, 30-µl reactions were set up in microfuge tubes using 0.2–0.5 µg DNA, 10 U of *NdeI* or *NcoI* and 10 U of *XhoI* in the appropriate buffer. Tubes were incubated for 3 h in a 37°C water bath. DNA was purified and ligated together using 1 unit of T4 DNA ligase (Roche) at room temperature

overnight. Competent *E. coli* XL-1 Blue cells were transformed using the heat-shock method at 41°C and plated on to L-agar (10% [w/v] tryptone, 5% [w/v] yeast extract, 5% [w/v] NaCl, 1.5% agar) supplemented with ampicillin (Amp; Roche) at 100 µg/ml and incubated overnight at 37°C. Resulting colonies were screened for the presence of the desired construct. In all cases, the integration of the fragment into the vector was confirmed by a slower migration pattern in agarose gels after digestion with a one-cutter restriction enzyme due to their larger size, and the release of the gene fragment after double digestion of the resulting plasmids.

Site-directed mutagenesis

Plasmid pETPfcYP19A was used as a template. In order to change the tryptophan in position 128 for phenylalanine (W128F) in the *pfycyp19A* gene, the following overlapping primers were designed: 5' CCTTAGTACCTTGCCCATCTTAGATGGTAAACATGTTG 3' and 5' CAACATGTTTACCATCTAAGAATGGGCAAGGTACTAAGG 3'. PCR was performed using ~1 µg pETPfcYP19A and the concentrations of primers, dNTPs and polymerase indicated above. The PCR protocol was 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 50°C for 1 min, 72°C for 10 min; and a last step of 72°C for 10 min. The PCR product was transformed into competent *E. coli* BL21 cells and the DNA of the few successful transformants was sequenced (GATC Biotech, Germany) to confirm that the constructs included the correct mutation.

Harvesting and lysis of E. coli cells

Recombinant proteins were produced by inoculating L-broth, supplemented with Amp (100 µg/ml), with overnight cultures of *E. coli* BL21 (or Rosetta in case of PfcYP32) harbouring the desired plasmid, and growing at 30–37°C (depending on the particular recombinant protein) with agitation at 200 rpm to an OD₆₀₀ of 0.6–0.7. Protein expression was induced by the addition of 0.5–2 mM IPTG and the culture incubated for 2–5 hours. Cells were harvested by centrifugation at 6,000 x g for 10 minutes at 4°C in a Sorvall RC50 plus centrifuge using a SLA-3000 rotor. Pellets were frozen at -20°C. Pellets were prepared for lysis by resuspending in buffer A (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4) supplemented with complete mini protease inhibitor (EDTA-free) tablets (Roche), lysozyme (1 mg/ml) and 0.3% (v/v) Triton X-100. The cells were broken by

passage twice through a french press, and clarified by spinning at 35,000 x g in a Sorvall RC50 plus centrifuge using an SS-34 rotor for 45 min at 4°C.

Nickel-chelate affinity chromatography

A 5-ml histrap® HP column (GE Healthcare, Buckinghamshire, UK) was equilibrated with 10 column volumes of buffer A prepared without imidazole. Clarified lysate (prepared in buffer A) was applied to the column at a flow rate of 2–3 ml/min using a peristaltic pump. The resin was washed with buffer A supplemented with 5 column volumes of increasing concentrations of imidazole (10–120 mM in 10-mM increments) followed by 10 column volumes of buffer A + 500 mM imidazole. Ten-ml eluted portions were collected from each concentration and analysed by SDS-15% PAGE [25]. The desired amount of protein sample (maximum volume of 20 µl) was loaded onto gel. Proteins separated by this technique were visualized by staining with Coomassie blue reagent (0.15% [w/v] Coomassie brilliant blue R-250, 45% [w/v] methanol, 10% [v/v] acetic acid, filtered to remove particulate material). Gels were subsequently destained in a solution of 20% (v/v) methanol, 7.5% (v/v) acetic acid. When gels were sufficiently destained, they were exposed to white light using an AlphaImager 2200 gel imaging system and photographed. The purest fractions were pulled together and concentrated, if needed, by ultrafiltration through Amicon ultra 10 concentrators (Millipore, Cork, Ireland).

Thermal melt assay

The thermal melt curves of the different recombinant proteins were obtained by a slight modification of the original method described elsewhere [26]. The recombinant proteins (1 µM) were mixed with sypro orange® (Invitrogen) in 50 µl. Then, the mixture was incubated in a quantitative PCR machine (RG-3000 Corbette Research) at increasing temperatures, from 30°C to 95°C with 1°C/min increments and the fluorescence of the sample was measured every 0.2°C. The effects of CsA on the thermal melt curves were assessed as described in the Results section.

Results

Data mining, alignments and annotated data

First, it should be stated that the nomenclature used for this cyclophilin family is similar to the convention reported by Galat [27] in which cyclophilin is abbreviated to CYP and a species-specific prefix, and a suffix representing the approximate molecular mass in kiloDaltons of the mature protein, are added, e.g. PfCYP19A (one of three *P. falciparum* 19-kDa cyclophilins) [19]. After an exhaustive online search in the latest version of two main gene sequence databases PlasmoDB [28] and the National Center for Biotechnology Information [29], 12 nucleotide sequences were found to be annotated as or to have sequence similarity to cyclophilins (**Fig. 1** and **Table 2**). Only two gene products had been previously characterized biochemically: PfCYP19B [20] and PfCYP19A [21]. In addition, over-expression of the gene of PlasmoDB ID PF08_0121 (named PfCYP25 here, PfCYP24 previously [19]) in *E. coli* had been described but its product was not purified [30]. The accession numbers of the genes are listed in Table 2. The smallest of these open-reading frames contains 125 and the largest 747 amino acids. The grade of sequence identity with the archetypal, cytosolic human cyclophilin hCYP18 (hCYPA) was calculated using the PHYRE server [31], and varied from 61% for PfCYP19A to 1% for PfCYP14. This latter open-reading frame, even if it has been annotated as a RNA-binding protein containing a 85-residue cyclophilin-like domain (CLD), is probably too short and the sequence too distant to hCYP18 (1%) for it to be a *bona fide* cyclophilin. For example ScCYP17 contains the shortest cyclophilin domain described in *S. cerevisiae* and is composed of 162 residues.

All of the genes shown in Fig. 1 contain a cyclophilin-like domain but four of them also show other motifs, e.g. PfCYP19B a secretory signal peptide at the N-terminus, PfCYP32 a putative mitochondrial targeting sequence, and PfCYP87 a WD40 repeat, which might serve as a platform for assembly of protein complexes. All of them have been found to have orthologues in most of the annotated *Plasmodium* species (data not shown), which is an indicative of the high level of conservation of this family of proteins. PfCYP19B has already been shown to be located primarily in the cytosol of the parasite, despite its apparent signal sequence [18], but was also exported to the membrane of the infected erythrocyte [32]. The putative mitochondrial signal of PfCYP32 was further investigated since mitochondrial proteins of *P. falciparum* are very different from human mitochondrial proteins, making them attractive potential drug targets [33]. It was scanned for mitochondrial sequences with MitoProt [34], PlasMit [35] and PFM Pred [36]. All algorithms predicted PfCYP32 to be a mitochondrial protein. Therefore we attempted to produce PfCYP32 with and without its putative mitochondrial transit peptide as described below. Surprisingly, when scanning the

other proteins studied here, PfCYP25 was also predicted to be mitochondrial with PFM Pred but not the two other algorithms, while PfCYP81 was so with PlasMit only. None of the sequences was predicted to possess an apicoplast (plastid) targeting signal using PlasmO AP [37] or a PEXEL motif [38], an indicator of export to the erythrocyte [39].

The phylogram in **Fig. 2** displays inferred historical relationships among these cyclophilins. The distance of one group from the other groups indicates the degree of relationship; that is, closely related amino acid sequences are located on branches close to one another. PfCYP14 was set apart from the others as the most remotely related to any of the other sequences. Furthermore, the cyclophilin-like domains of PfCYP72 and PfCYP81 were distantly connected with the others confirming what is apparent in Fig. 1. However, with an approximated likelihood above 60% the other sequences are gathered together, although the cyclophilin-like domains of the larger sequences, such as PfCYP87CLD and PfCYP52CLD, still fall into a sub-tree apart from the others. There is also a probability above 60% that the other well-known cyclophilins – PfCYP19A and PfCYP19B – are part of the same evolutionary family.

Due to the low sequence identity with hCYP18 for PfCYP14 (1%) and PfCYP72 (8%), it was investigated in the literature what was the lowest identity of a known cyclophilin to this archetypal one. To our knowledge, the lowest sequence identity to hCYP18 of a known cyclophilin is 7% for a *Drosophila melanogaster* protein (DmCYP20) [27]. Thus, PfCYP72 was included in this study. On the contrary, based on the alignment, overall sequence similarity and size (see above), it was concluded that PF13_0122 (called “PfCYP14” in this study for convenience) is not a cyclophilin and has been mis-annotated. It was not investigated further here.

PCR amplification and cloning into a pET vector

All of the cyclophilin and cyclophilin-like genes or the corresponding cyclophilin-like domains were amplified (**Fig. 3**), either by standard PCR or RT-PCR depending whether gDNA or mRNA, respectively, was used as a template. The difficulty of amplifying long *P. falciparum* sequences due to their high AT content is well known. Frequently, the desired activity is supported by a discrete domain, and thus it is often not necessary to express the full-length protein to address a particular biological question. Moreover, the probability of successfully expressing a soluble protein decreases considerably at molecular weights above ~60 kDa [40]. Therefore, when the full length of the gene could not be amplified, as was the

case of the four largest genes, we focussed only in the CLD. Both the *pfryp32cld* and the *pfryp32* (full-length including putative mitochondrial signal) genes were amplified successfully. In some cases it was necessary to optimize the PCR conditions to remove incorrectly-sized bands. In a few cases (*pfryp23*, *pfryp25*, *pfryp81cld* and *pfryp87cld*), mainly due to annealing temperatures as low as 45 °C and the long annealing times required, those bands remained visible. Only the correctly sized bands were cut from the gel, purified and subsequently used to clone into the corresponding vector.

The presence of an *NdeI* restriction site in the sequence of *pfryp72cld* impeded the use of pET22b+, and pET21d+ was used instead. Both plasmids provide a C-terminal His₆-tag. However, despite several attempts *pfryp72cld* remained refractory to cloning. All of the other PCR products except *pfryp87cld* were successfully cloned into pET22b+. All the putative clones were corroborated by sequencing. Note that *pfryp19B* had previously been cloned into pET22b+ and transformed into *E. coli* BL21 [20], therefore PfCYP19B was directly produced and purified from this pre-existing clone (our ‘prototype’ for cyclophilin family overproduction) in this study. As the amplification of full-length *pfryp72* and *pfryp87* was not possible and the cloning of their respective cyclophilin-like domains was not successful, the investigation of these two sequences was discontinued.

Induction and purification of recombinant proteins

In most cases, pET22b+, transformed into *E. coli* BL21, provided a highly convenient tool for the expression of the proteins of interest, since 11 recombinant proteins (including two versions each of PfCYP19A and PfCYP32) were produced and 9 of these were successfully purified using IMAC, as shown in **Fig. 4** and **Table 3**. In a first small-scale induction several concentrations of IPTG and induction times were assessed, as well as different temperatures. This allowed us to optimize the conditions to produce each protein in the most efficient manner. In most cases lower concentrations of IPTG (0.5 mM) and longer incubation times (5 h) at 30 °C provided satisfactory yields. For PfCYP19C, PfCYP19A, PfCYP19A W128F, PfCYP19B, PfCYP23 and PfCYP25, less intense bands were visible as well as the major bands but these were likely to be minor degradation products. The altered form of PfCYP19A (W128F) was purified as for wild-type PfCYP19A but a lower final yield was obtained. Soluble production of PfCYP32 (full-length) and PfCYP81CLD proved unfeasible even when the Rosetta strain, which bears plasmids encoding tRNA genes for all of the ‘problematic’ rarely-used codons encoding Arg, Ile, Gly, Leu and Pro (except for Arg

CGA/CGG), was employed. There was an unexpectedly large variation in the concentration of imidazole required to elute the different proteins, in spite of their sequence similarities and broadly similar numbers of histidine residues (Table 3).

Assessment of protein stability and CsA binding by thermal melt assay

In order rapidly to characterise the correct folding and likely functionality of the proteins we used the thermal melt assay. It has been suggested that a good melting curve is predictive of catalytic activity in recombinant enzymes [41]. The fluorescence approach measures fluorescence from a dye, such as sypro orange®, whose emission properties changed upon interaction with unfolded protein [42]. The melting temperature (T_m) was defined as the temperature where the first derivative of that fluorescence curve vs. temperature was maximal [26].

Since the melting properties of each independent protein may vary depending on the buffer used in this assay, the first step performed was the optimization of the melting curves of the purified proteins using four different buffers: standard buffer (100 mM HEPES, 150 mM NaCl, pH 7.5), potassium phosphate (pH 7.0), sodium phosphate (pH 7.5) and sodium citrate (pH 5.5). For most of the proteins, when dissolved in standard buffer, the resulting melting curve was optimal (see PfCYP19B curve in **Fig. 5**). Also, it could be observed that thermally induced unfolding was an irreversible process with a sharp transition between the folded and unfolded states. PfCYP23 and PfCYP26 showed better curves with the sodium phosphate buffer while PfCYP25 seemed to be more stable in the potassium phosphate. Under the conditions of the assay, only one of the protein constructs, PfCYP32CLD, did not display a melting curve that allowed derivation of the T_m . This parameter ranged between 42 and 56.5 °C for the other proteins examined here, with the exception of the PfCYP26 T_m which was exceptionally low (32.5 °C) (**Fig. 6**).

This assay was adapted to measure whether or not the recombinant cyclophilins were capable of binding to CsA. In previous reports, it has been suggested that shifts in ΔT_m larger than 2°C upon addition of the ligand being tested indicate significant binding [41].

PfCYP19A and PfCYP19B were the only CsA-binding proteins (**Fig. 6**), giving a T_m shift greater than 2°C when incubated at concentrations as low as 0.5 μ M. The T_m varied less than 1°C for all the other proteins meaning that if they bound to CsA it would be only at concentrations higher than 25 μ M. This includes the single-point mutant of PfCYP19A in which the Trp128 that has been previously demonstrated to be crucial to CsA binding in

cyclophilins of other species [43] was changed to a Phe. In that case, the CsA-binding affinity decreased more than 200-fold, since no significant change in the T_m was observed in the presence of CsA concentrations as high as 100 μ M (**Fig. 6**). This validates the method as an accurate way to assess CsA binding.

Discussion

The members of the cyclophilin family are not only important for proper protein folding, as *cis-trans* isomerization of peptidyl-prolyl bonds is a necessary and rate-limiting step for the folding of some proteins, but cyclophilins also tightly bind to the immunosuppressive drug CsA [18]. Although well described in T-lymphocytes, the mechanism of antimalarial action of this drug remains to be elucidated. Cyclophilins are widely distributed and have been implicated in several important cellular functions such as signal transduction, regulation of multi-subunit complexes and the stress response, but they are missing for instance in a number of fungal genomes suggesting that the function of this subfamily might not be essential in all organisms [44]. It was therefore intriguing that *P. falciparum* possesses no fewer than 11 open-reading frames with substantial sequence similarity to known cyclophilins, while having only one convincing member of the FKBP family, PfFKBP35 [45, 46]. Among the 11 cyclophilin-related sequences, there is considerable diversity in gene architecture, presence of additional domains and similarity to cyclophilins of other species (discussed in more detail in [47]).

Many large projects are devoted to developing methods to generate large numbers of purified proteins. However, the task has proved challenging: on average, for proteins from bacteria, only 50–70% of soluble proteins and 30% of membrane proteins can be readily expressed in recombinant form, and only 30–50% of these expressed proteins can be purified to homogeneity [48, 49]. The coding regions of the *P. falciparum* genome contain 76.5% AT. Together with its codon bias that is divergent from that of *E. coli* [50], it seemed likely that the genetic make-up of the parasite contributes significantly to the failure rate in protein expression. In a previous report, differences in the % AT did not in themselves appear to affect expression or solubility, and it was suggested that the stability and secondary structure of the encoded RNA could be more of an issue than the codons themselves [51]. As part of a structural genomics initiative, 1000 open reading frames from *P. falciparum* were tested in an *E. coli* protein expression system. Three hundred and thirty-seven of the targets were expressed in *E. coli* [9]. For every soluble protein obtained, more than four were observed to

express insolubly and more than 10 did not express at all. In that study, it was concluded that the one physical protein quality which was associated with both expression and solubility was predicted isoelectric point (pI). The tendency towards insolubility with higher pI was remarkable: of the 288 targets with a pI above 10 only one was soluble. At very low pI values, there appeared to be a tendency towards a lack of expression which was compensated for by greater level of solubility among those that did express. Another published study of recombinant expression of a large number of *P. falciparum* proteins obtained five soluble proteins from 95 single-exon constructs fused to maltose binding protein or glutathione-S-transferase [52].

To the best of our knowledge, this is the first time that the same host/vector system has been used to produce and purify an entire (or almost entire) family of proteins in *P. falciparum*. In view of the statistics mentioned above, the successful overexpression of 9/11 of these recombinant proteins in *E. coli* and the further purification of all these proteins but one is a satisfactory achievement. It may be significant that the only two that have not been purified after having been cloned into the vector, PfCYP32 and PfCYP81cld, had the highest predicted pI's of all this group of proteins, 9.8 and 9.76 respectively. Expression of the former was induced using the Rosetta strain, but the product was completely insoluble, while the latter was not induced in any of the strains used in this study. The high success rate in this study may be related to the similarity between the proteins being produced, meaning that (in accord with our initial hypothesis) a method that worked for one cyclophilin was perhaps more likely to work for others.

To give an initial assessment of the likely functionality of the novel cyclophilins produced here, we used a thermal-melt assay as an indication of folded three-dimensional structure. This assay was successfully used to assess a variety of *P. falciparum* proteins [53] including a putative cyclophilin that we call PfCYP23 which yielded approximately the same T_m as in our study (52.1 °C). It has also been stated that melting curves are a reasonable indicator of whether a protein is denatured or natively folded. It was shown that enzymes with poor melting curves are less likely to be active, suggesting that a poor melting curve is often a result of poor folding. However, it was evident that in many instances a treated enzyme can suffer substantial deterioration of its melting curve but still retain significant activity [40]. This case may need to be considered for PfCYP26 since it gave a T_m that was exceptionally low (32.5 °C). A limitation of this method is that it cannot distinguish between proteins that are properly folded and proteins that are misfolded in a way that shields their hydrophobic regions from solvent. In a recent report on 61 proteins, including some from *P.*

falciparum, 25% did not display a melting curve that allowed derivation of the melting temperature [54]. In our case, just with a simple change of the buffer, with no more additives (e.g. glycerol), a melting curve was obtained for eight out of nine recombinant proteins. If indeed these proteins are properly folded, our expression system will facilitate further characterization. This assay has also been used in several studies to assess target binding of a large number of novel antimalarial compounds [55]. We used it to establish whether or not our recombinant proteins were capable of ligating CsA. Only PfCYP19A and PfCYP19B showed the ability to bind CsA, both of them at concentrations as low as 0.5 μ M. This confirms the previous finding that PPIase activity of these two proteins was inhibited by CsA [19] and further suggests that it is unlikely that any of the novel cyclophilins described here is involved in mediating the antimalarial action of this drug. Other, non-immunosuppressive cyclosporins [15] and unrelated, non-immunosuppressive cyclophilin ligands (our unpublished data) have however been found to have antimalarial activity and we have not ruled out a role of one or more of the novel cyclophilins in the actions of these compounds.

Outstanding questions about the *P. falciparum* cyclophilin family include whether all of its members can (like PfCYP19A and PfCYP19B) display PPIase activity and whether any demonstrate chaperone activity, as shown for some immunophilins [56] including PfFKBP35 [45]. Cyclophilins have the potential to be important in the export of *P. falciparum* virulence determinants to the host erythrocyte [19] and/or the response of the parasite to stresses such as the heat shock it encounters upon transfer from the mosquito vector to the human host [57]. Moreover, reports of potent and selective antiparasitic activity of non-immunosuppressive derivatives of CsA have led to much interest in immunophilins as possible drug targets [19]. This concept is supported by the fact that there are cyclophilin types that are absent from their mammalian hosts, such as PfCYP72 [47]. Therefore, understanding the roles of cyclophilins in the mechanisms of action of cyclosporins might contribute to the development of non-cyclosporin agents that act in a similar way.

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Figure legends

Fig. 1. Domain architectures of all the cyclophilin and cyclophilin-like proteins annotated in PlasmoDB. RRM, RNA recognition motif; SP, signal peptide; put Mito, putative mitochondrial transit signal; Ring/U-box, domain with a zinc finger-type domain and a ubiquitin binding domain; SYF2, splicing factor 2; WD40, 40-amino acid domain, Trp/Asp-containing β -propeller repeat. The red boxes correspond to the cyclophilin or cyclophilin-like domain and show the % amino acid sequence identity shared with the archetypal hCYP18. Above the diagrams the first/last residue number of the corresponding domain are indicated. NB: “PfCYP14” is not considered a *bona fide* cyclophilin (see text). *, Cyclophilin previously described in the literature (see text).

Fig. 2. Evolutionary relationship of the cyclophilin and cyclophilin-like proteins in *P. falciparum*. The evolutionary history was inferred using the Neighbour-Joining method [58]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Statistical support for branches is given as approximate likelihood ratios at the nodes. Phylogenetic analyses were conducted in MEGA4 [59].

Fig. 3. PCR amplification of the cyclophilin and cyclophilin-like domains. Lane 1, molecular size marker; lane 2, *pfryp19c* (504 bp), lane 3, *pfryp19a* (516 bp); lane 4, *pfryp23* (615 bp); lane 5, *pfryp25* (654 bp); lane 6, *pfryp26* (681 bp); lane 7, *pfryp32* (843 bp); lane 8, *pfryp32cld* (567 bp); lane 9, *pfryp52cld* (597 bp); lane 10, *pfryp72cld* (597 bp); lane 11, *pfryp81cld* (657 bp); lane 12, *pfryp87cld* (486 bp). Expected sizes are indicated in brackets.

Fig. 4. SDS-15% polyacrylamide gels showing expression and purification of His₆-tagged cyclophilins and cyclophilin domains. A) PfCYP19C; B) PfCYP19A; C) PfCYP19 AW128F; D) PfCYP19B; E) PfCYP23; F) PfCYP25; G) PfCYP26; H) PfCYP32CLD; I) PfCYP52CLD. Lanes 1, extracts of uninduced *E. coli*; lanes 2, extracts of *E. coli* induced with IPTG; lanes 3, soluble fractions of IPTG-induced *E. coli* extract; lanes 4, insoluble fractions; lanes 5, samples of diluted soluble fractions applied to the column; lanes 6, flow-through (unbound) fractions; lanes 7–17, fractions eluted with 20–120 mM imidazole (10-mM increments); lanes 18, fractions eluted with 500 mM imidazole. Running positions of

molecular mass standards are indicated to the left. Boxed areas show the fractions chosen for further analysis with the apparent molecular masses of the major bands.

Fig. 5. Thermal melt assay of PfCYP19B. A) Thermal melt curve of PfCYP19B; B) First derivative of the curve in (A). The protein/dye mixture was incubated at increasing temperatures from 30°C to 95°C with 1°C/min increments and the fluorescence of the sample was measured every 0.2°C. The concentration of the recombinant protein was 1 μ M.

Fig. 6. Thermal melt study of CsA binding and importance of the W128 residue. First derivatives of curves for PfCYP19C (A), PfCYP19A (B), PfCYP19B (C), PfCYP19A W128F (D), PfCYP23 (E), PfCYP25 (F), PfCYP26 (G) and PfCYP52CLD (H). See Fig. 5 legend for details. The concentrations of CsA are indicated in the labels.

Fig. 1

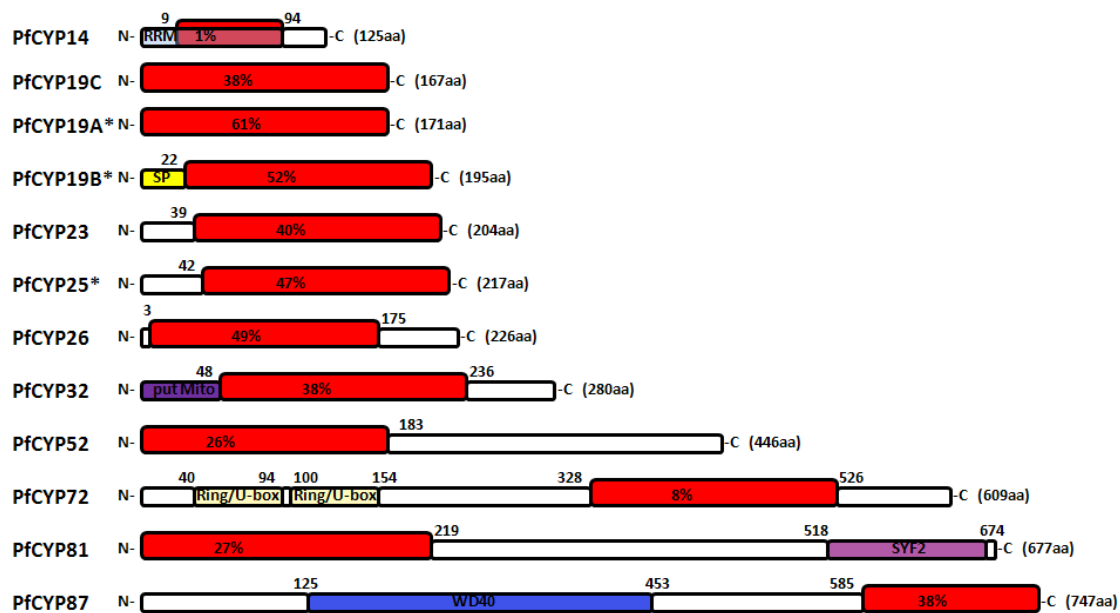


Fig. 2

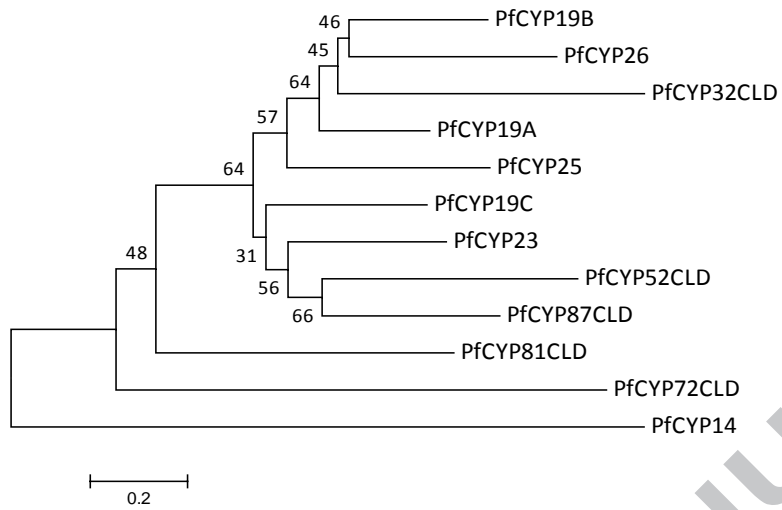
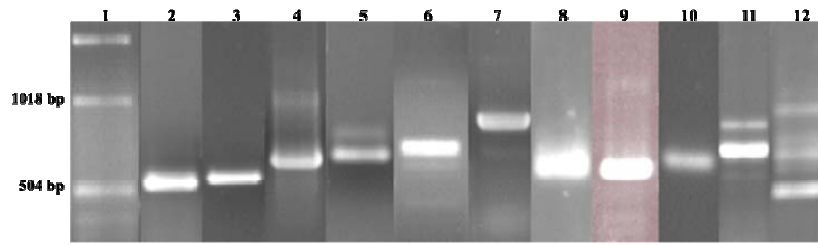


Fig. 3



ACCEPTED MANUSCRIPT

Fig. 4

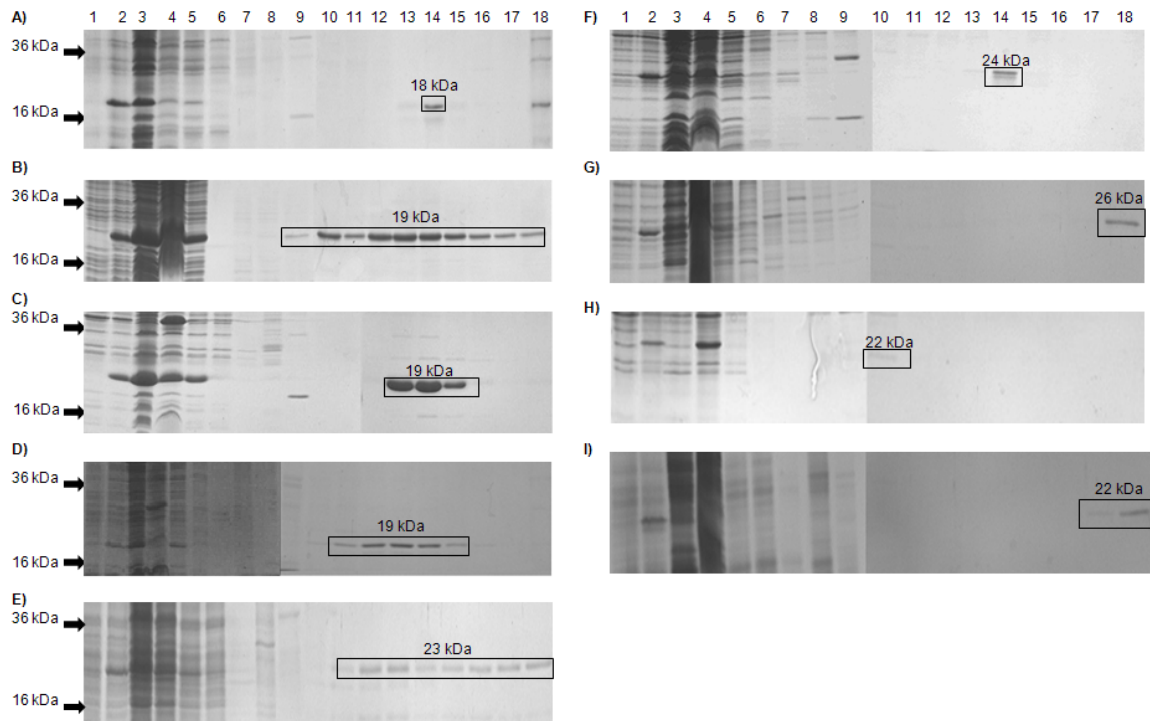


Fig. 5

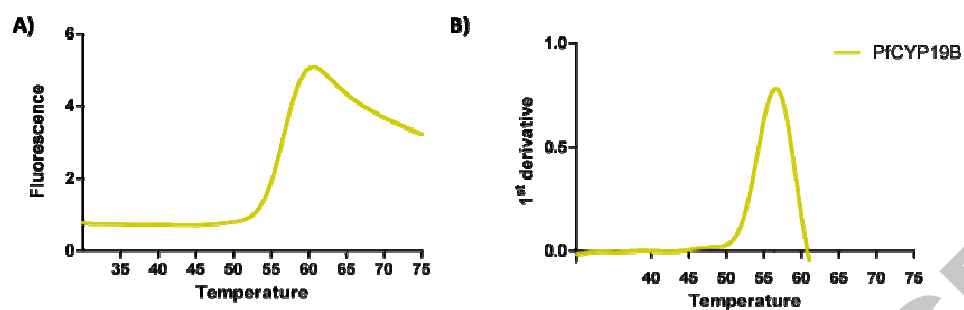


Fig. 6

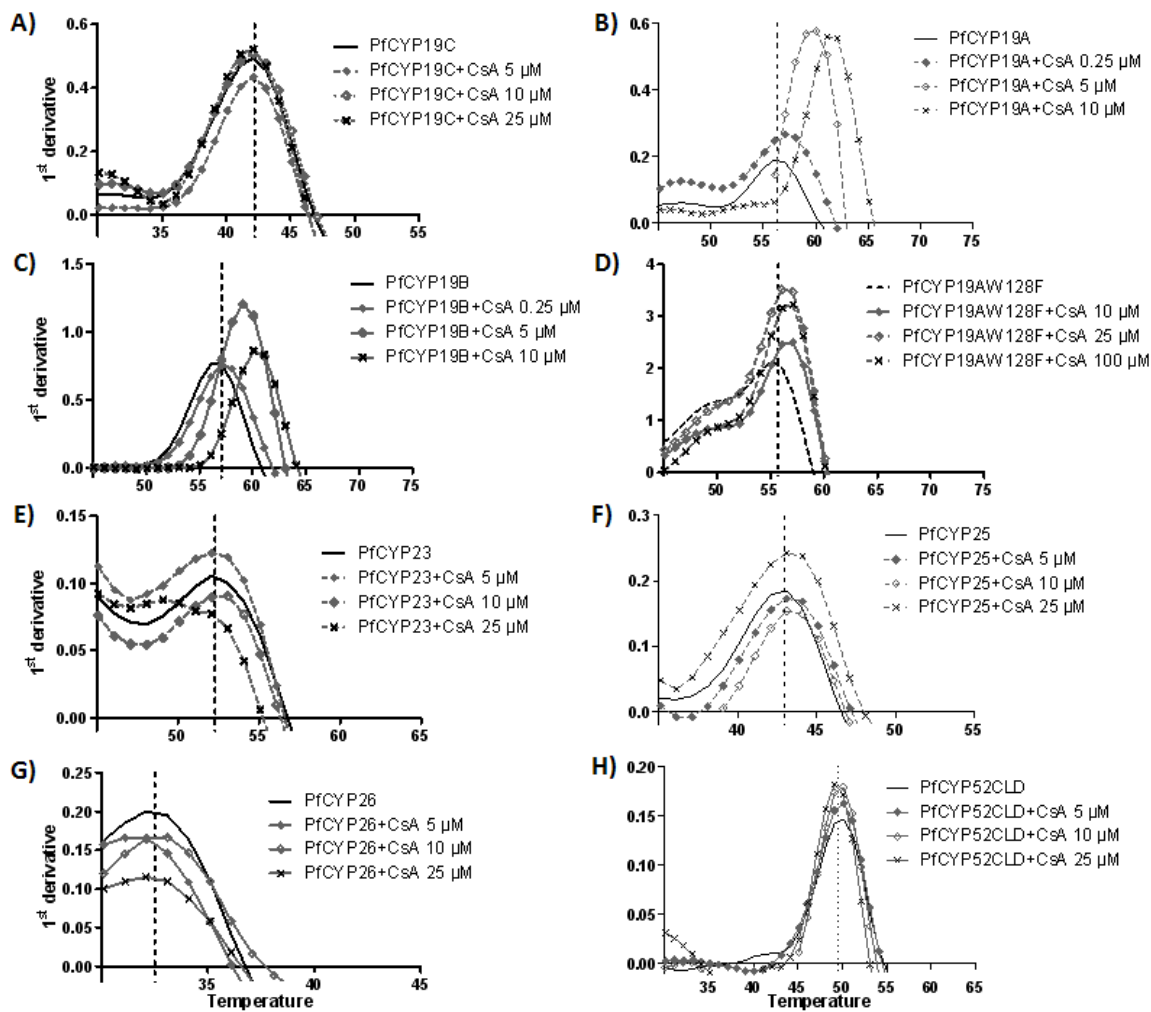


Table 1. Putative cyclophilin genes and primers used to amplify them.

PlasmoDB ID ^a	Gene name ^b	Forward primer ^c	Reverse primer ^c
PF11_0170	<i>pfcyp19C</i>	5' ggaatt ccat atgcttttgagtatacatatacaaat 3'	5' ccgctc gag attatccaactgattctctc 3'
PFC0975c	<i>pfcyp19A</i>	5' ggaatt ccat atgagtaagaggagtaaag 3'	5' ccgctc gag caattcaccacaatcagt3'
PFE1430c	<i>pfcyp23</i>	5' ggaatt ccat atgttcacctatgcaaag 3'	5' ccgctc gag atcgacattgaccgctg 3'
PF08_0121	<i>pfcyp24</i>	5' ggaatt ccat atgaaaaattgaatcagaat 3'	5' ccgctc gag taattetccgattctac 3'
PFL0120c	<i>pfcyp26</i>	5' ggaatt ccat atgaaatctctaatctctgtg 3'	5' ccgctc gag aaaaatgtttattatattatacatatt 3'
PFL0735w	<i>pfcyp32</i>	5' ggaatt ccat atgctttataaaagtgtgaaa 3'	5' ccgctc gag taagctgttttttttctt 3'
PFL0735w	<i>pfcyp32cld</i>	5' ggaatt ccat atggcaaaagaaaaacaatattt 3'	5' ccgctc gag taagctgttttttttctt 3'
PF14_0223	<i>pfcyp52cld</i>	5' ggaatt ccat atgicagaagtgtatagc 3'	5' ccgctc gag atctttatgtactctctgtttata 3'
PFI1490c	<i>pfcyp72cld</i>	5' catg ccat ggcatatataagactaattaactgat 3'	5' ccgctc gag ccaacatgaaaagtccatttta 3'
PF08_0128	<i>pfcyp81cld</i>	5' ggaatt ccat atgaaaaagaaaaagtatgtttatc 3'	5' ccgctc gag tgatgggtttcaagttgtttc 3'
PFE0505w	<i>pfcyp87cld</i>	5' ggaatt ccat atgaccattcataaaaaatactcc 3'	5' ccgctc gag attattaatttttatattaatatt 3'

^a Accession numbers shown as those used in www.plasmodb.org.

^b The suffix 'CLD' indicates that only the cyclophilin domain was amplified.

^c Both the *Nde*I (or *Nco*I) site and *Xho*I sites are bolded in the forward and reverse primers, respectively.

Table 2. Properties of cyclophilin and cyclophilin-like genes and their products.

Protein name ^a	PlasmoDB ID ^b	Coding sequence (bp) (CLD ^c)	Amino acids (CLD)	Predicted pI (CLD)	Motifs/Domains ^d	Location
“PfcYP14”	PF13_0122	378	125 (92)	4.6	RRM, CLD	ND, ^e probably nuclear
PfcYP19C	PF11_0170	504	167 (167)	6.35	CYP	ND
PfcYP19A*	PFC0975c	516	171 (171)	8.2	CYP	Cytosolic ^f
PfcYP19B*	PF11_0164	588	195 (173)	7.73	SP, CYP	Mainly cytosolic but has cleavable signal peptide ^f , iRBC ^g
PfcYP23	PFE1430c	615	204 (160)	5.31	CYP	ND
PfcYP25*	PF08_0121	654	217 (175)	6.78	CYP	ND
PfcYP26	PFL0120c	681	226 (172)	8.54	CYP	ND
PfcYP32	PFL0735w	843 (567)	280 (189)	9.8 (7.17)	Mito, CYP	ND, probably mitochondrial
PfcYP52	PF14_0223	1341 (549)	440 (183)	7 (7.71)	CYP	ND
PfcYP72	PF11490c	1830 (597)	609 (199)	9.48 (9.21)	CYP	ND
PfcYP81	PF08_0128	2034 (657)	677 (219)	9.76 (9.3)	CLD, SYF2	ND, probably nuclear
PfcYP87	PFE0505w	2244 (486)	747 (162)	7.34 (7.69)	WD40, CLD	ND

^a Cyclophilins previously described in the literature are marked * (see text).

^b Accession numbers from www.plasmodb.org.

^c CLD, cyclophilin-like domain.

^d See **Fig. 1** for abbreviations.

^e ND, Not determined.

^f Ref. [18].

^g iRBC, infected erythrocyte ghosts [32].

ACCEPTED MANUSCRIPT

Table 3. *P. falciparum* cyclophilins or cyclophilin-like proteins solubly produced in *E. coli*.

Protein name	Number of histidine residues in the sequence^a	Concentrations of imidazole required to elute (10 mM increments)	Yield (mg/l)
PfCYP19C	5	90 mM	2.6
PfCYP19A	4	50–120 and 500 mM	19.8
PfCYP19 AW128F	4	80–100 mM	3.2
PfCYP19B	8	70–110 mM	8.3
PfCYP23	6	80–120 and 500 mM	2.2
PfCYP25	6	90 mM	1
PfCYP26	6	500 mM	2.2
PfCYP32CLD	5	500 mM	0.7
PfCYP52CLD	9	120 and 500 mM	1

^a Not including His₆ tag.