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## Selection of Yeast Strains With Enhanced Expression of *Plasmodium falciparum* Proteins

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### Abstract

The poor expression of *Plasmodium falciparum* proteins in heterologous systems and the difficulty in obtaining sufficient material directly from the parasite have limited the experimental characterization of many of the approximately 5200 proteins encoded by this organism. To improve the expression of *P. falciparum* proteins in the yeast *Saccharomyces cerevisiae*, we selected yeast *ura3* mutants that acquired the ability to utilize the *P. falciparum* orthologue (*PfOMPDC*) of *URA3* to grow on media lacking uracil. Two of these mutant strains, BY#29 and PJ#17, expressed up to 100-fold more of four *P. falciparum* proteins as a result of mutations in either *HRP1* or *KAP104*, respectively. These mutations, as well as a temperature-sensitive *rna15* mutation, likely decrease the efficiency of mRNA 3' end formation and produce longer mRNAs of *P. falciparum* genes. These yeast strains may be useful for the analysis and purification of *P. falciparum* proteins.

### Keywords

*Plasmodium falciparum*; protein expression; yeast; *Saccharomyces cerevisiae*

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*Plasmodium falciparum* causes the most severe form of human malaria and is responsible for more than one million deaths each year [1]. Because no effective vaccine for malaria exists and resistance to currently used drugs is increasing, there is an urgent need for the identification of new vaccine and drug targets. Although the availability of the *P. falciparum* genome sequence [2] has been invaluable for malaria research, the majority of the parasite's genes have insufficient homology to known proteins to predict their functions. At the experimental level, few genes have been characterized. A small number of *P. falciparum* proteins have been expressed and purified, but this approach has been hampered by the difficulties in expressing *P. falciparum* proteins in heterologous organisms or *in vitro* systems. These difficulties likely stem from unique features of *P. falciparum* genes, which are AT-rich (74% on average in protein coding regions), and which encode significantly longer proteins than do orthologous genes due to the presence of low complexity inserts consisting primarily of amino acids encoded by AU-rich codons. For *E. coli* expression, the overabundance of these AU-rich

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codons prompted the development of the pRIG plasmid, which boosts the levels of tRNAs corresponding to codons that are common in *P. falciparum* but rare in *E. coli* [3]. Although some *P. falciparum* proteins express well by this approach, many are still poorly expressed or insoluble [4,5].

Alternative approaches to express *P. falciparum* proteins in heterologous systems have been investigated, with limited success, including the use of orthologous genes from other *Plasmodium* species or from *Cryptosporidium parva* [5]; expression in baculoviruses [4], the slime mold *Dictyostelium discoideum* [6], and wheat germ extracts [7]; and whole gene synthesis, in which the sequence of the gene is altered to match the AT content and codon usage of the heterologous host [4]. Another host cell that has been used to express several *P. falciparum* genes is the yeast *Saccharomyces cerevisiae* (for example, [8]). Despite some successes, however, many proteins fail to express in *S. cerevisiae*, primarily due to the high AT content of *P. falciparum* genes. In this study, we describe an unbiased selection of *S. cerevisiae* mutant strains that express the *P. falciparum* orotidine 5'-monophosphate decarboxylase (PfOMPDC, PF10\_0225) protein.

Expression of *P. falciparum* genes in *S. cerevisiae* can result in mRNAs that are truncated after AT-rich sequences [8]. These AT-rich sequences resemble *S. cerevisiae* sequences that encode the positioning and efficiency elements in the 3' untranslated regions of yeast RNAs that are responsible for specifying the site of polyadenylation [8]. Thus, mRNAs from *P. falciparum* genes expressed in yeast are often prematurely truncated and unable to synthesize full-length proteins [8]. Since the truncated mRNAs would encode partial open reading frames with no stop codons, we suspected that the yeast mRNA surveillance pathway specifically degrades these RNAs, preventing the accumulation of the encoded proteins [9]. If this interpretation is correct, then mutations in yeast genes required for degradation of aberrant RNAs would be predicted to increase production of truncated *P. falciparum* proteins. One such gene is *SKI2*, which encodes an RNA helicase that functions in conjunction with the exosome to degrade aberrant yeast mRNAs [9,10]. We transformed *ski2Δ* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ski2::KANMX4*) and *SKI+* yeast strains with *P. falciparum* protein expression constructs encoding the *PfCRK1*, *PfMRK1* and *PfPK6* genes as fusions to the bacterial *lexA* gene. Western blot analysis (Fig. 1A) demonstrated that *P. falciparum* fusion proteins were detectable in the *ski2Δ* mutant, but not in the *SKI+* parent, and that the sizes of the proteins detected on the blot were much smaller than the predicted coding capacity of the genes (observed sizes of ~37, 34, and 30 kDa versus predicted sizes of 100, 58, and 59 kDa for the LexA-PfCRK1, LexA-PfMRK1, and LexA-PfPK6 fusion proteins, respectively), indicating that truncated proteins were produced in the *ski2Δ* strain. This result provides additional support for the hypothesis that premature transcription termination of *P. falciparum* mRNAs is the primary problem associated with expression of *P. falciparum* proteins in yeast [8].

In an effort to obtain mutant yeast strains with an increased capacity to express full-length *P. falciparum* proteins, we began by transforming yeast strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) [11] and PJ69-4a (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) [12] with a high copy number yeast expression construct based on the plasmid p425GPD [13] in which the strong yeast *GPD1* promoter drives transcription of *PfOMPDC* (Fig. 1B). Both BY4741 and PJ69-4a contain a mutated *URA3* gene, which renders them unable to grow on media lacking uracil unless a functional *URA3* gene is supplied on a plasmid. PfOMPDC catalyzes the same reaction as *S. cerevisiae* Ura3, and is encoded by a 969 base pair ORF that is 76% AT, approximately the same as the average *P. falciparum* gene. Although the PJ69-4a parental strain carrying either the *LEU2*-containing vector or the *PfOMPDC* expression construct could grow on media lacking leucine, it could not use the *PfOMPDC* gene to grow on media lacking uracil (Fig. 1C); the same features were true for the BY4741 strain (data not shown). However, by plating a

large number of yeast cells, we identified more than 50 spontaneously occurring mutants from each parental strain that grew on media lacking uracil. In general, these strains grew slowly, even on rich media, compared to the parental strains, with doubling times at least twice as long.

A subset of the mutant strains with the fastest growth rates was selected for further analysis. To confirm that the growth on media lacking uracil was due to mutations in the yeast genome and not to changes in the PfOMPDC expression plasmid, we cured the mutant strains of their plasmids by counterselection on 5'-fluoroorotic acid (5-FOA), which kills strains that have Ura3 activity. The resulting strains were re-transformed with empty expression plasmid or plasmid containing the *PfOMPDC* gene and replica-plated onto media lacking leucine or uracil. For 33 of 38 mutants tested, growth on media lacking uracil required the *PfOMPDC* expression plasmid (Fig. 1C, showing a representative subset of 8 strains). This result demonstrated that the yeast strains surviving the selection protocol had acquired the ability to transcribe full-length *PfOMPDC* mRNA and to translate that mRNA into a functional PfOMPDC protein capable of rescuing yeast growth on media lacking uracil.

To identify strains among these yeast mutants that were able to synthesize other *P. falciparum* proteins in addition to PfOMPDC, we transformed the two parental and 28 mutant strains with yeast expression plasmids for the *P. falciparum* proteins PfPPP and PfPK6, each protein constructed as a fusion to the *E. coli* LexA protein. Immunoblotting with an antibody against LexA showed that neither parental strain expressed LexA-PfPPP or LexA-PfPK6, whereas 20 mutant strains expressed full-length LexA-PfPPP, 4 expressed full-length LexA-PfPK6, and 3 expressed both (Fig. 2A and data not shown). We further evaluated the two fastest growing of these strains, BY#29 and PJ#17, for their ability to express two additional *P. falciparum* proteins, PfMRK1 and PfMAP1, which could not be detected in parental strains, and found that both strains produced full-length LexA-PfMAP1, but only BY#29 synthesized detectable levels of LexA-PfMRK1 (Fig. 2A). Reprobing each blot with an antibody against *S. cerevisiae* glucose 6-phosphate dehydrogenase protein indicated equivalent or slight overloading of samples from the parental strains. By serial diluting total cell lysates from BY#29 and PJ#17, we used the blots to estimate that the mutant strains express at least 64- to 128-fold more LexA-PfPPP than BY4741 and PJ6-4a, respectively (Fig. 2B). These experiments demonstrated that BY#29 and PJ#17 carry mutations that improve the expression of *P. falciparum* proteins in the absence of selection for the biochemical activity of these proteins.

To identify the mutations responsible for the improved expression of *P. falciparum* genes, we rescued the slow-growth phenotype of strains BY#29 and PJ#17 by transforming them with a yeast genomic DNA library [14]. By testing individual genes encoded on inserts of plasmids that restored wildtype growth, we determined that the mutated gene responsible for the slow growth phenotype and the ability to use the *PfOMPDC* gene to grow on media lacking uracil was *HRP1* in strain BY#29, and *KAP104* in strain PJ#17 (Fig. 3A and data not shown). Sequencing of the 1602 bp ORF of the *HRP1* gene from BY#29 revealed a C to A mutation at position 1592 that converts the Pro at position 531 to Glu. Analysis of the 2754 bp *KAP104* ORF from PJ#17 revealed a G to T mutation at position 1777 that results in a stop codon at position 593. We also identified a 114 base pair deletion of nucleotides 1042–1155 in *KAP104* in another mutant strain (PJ#27) that generates an in-frame deletion of amino acids 348 to 385.

The Hrp1 and Kap104 proteins are both involved in mRNA 3' end processing and transport of polyadenylated RNA out of the nucleus. Hrp1 is both an RNA-binding protein that binds to and shuttles polyA+ RNA out of the nucleus [15] and a component of cleavage stimulation factor 1 (CSF1), a complex that binds to immature mRNAs and specifies the cleavage site at which the polyA tail will be added [16]. Mutations in *HRP1* result in defects in mRNA 3' end

formation and accumulation of polyA<sup>+</sup> RNA in the nucleus. Kap104 is a nuclear importin  $\beta$  subunit protein whose role is to shuttle polyA<sup>+</sup> RNA-binding proteins, including Hrp1, into the nucleus [17]. Mutations in *KAP104* disrupt nuclear export of polyA<sup>+</sup> RNA by blocking re-import of polyA<sup>+</sup> shuttling proteins into the nucleus, which leads to defects in 3' end formation that result in aberrantly long mRNAs [17,18]. Both *KAP104* and *HRP1* are essential for yeast viability, which likely accounts for the slow growth of the mutant strains.

To determine if other strains with mutation in genes required for 3' end formation also improved expression of *P. falciparum* genes in yeast, we assayed a strain (CMHy58.1A, *MATa ura3-52 leu2 $\Delta$ 1 his3 $\Delta$ 200 rna15-58*) with a temperature-sensitive mutation in *RNA15* [18]. Rna15 is also a component of CSF1 [19], but does not shuttle in and out of the nucleus and has no known role in RNA transport [18]. CMHy58.1A cells have severe defects in 3' end formation at both 37° and 42° C [18]. Immunoblot analysis of the expression of three LexA-*P. falciparum* fusion proteins in CMHy58.1A revealed that expression of LexA-PfFRAN was not affected at the nonpermissive temperature, whereas the levels of full-length LexA-PfPPJ and LexA-PfPK6 were significantly increased (Fig. 3B). LexA-PfPPJ was also detected in CMHy58.1A at 30° C (Fig. 3B), suggesting that 3' end formation is also affected at temperatures that are permissive for growth. Consistent with this observation, CMHy58.1A transformed with the PfOMPDC expression plasmid was able to grow slowly on media lacking uracil. These results suggest that defects in 3' end formation, as opposed to the ability to shuttle in and out of the nucleus, are primarily responsible for the improved expression of *P. falciparum* genes in BY#29 and PJ#17.

In summary, we used the inability of *S. cerevisiae ura3* strains to express the *P. falciparum* orthologue of the yeast *URA3* gene and to grow in the absence of uracil as a selection for yeast mutants with increased expression of the parasite's proteins. Yeast *hrp1* and *kap104* mutants produced sufficient PfOMPDC to survive the selection, and also showed increased expression of four other *P. falciparum* proteins for which no selection was imposed. In addition, an existing temperature-sensitive *rna15* mutant with defects in 3' end processing activity expressed full-length *P. falciparum* proteins, with the additional feature that it can be grown and transformed at the permissive temperature and shifted to the restrictive temperature when *P. falciparum* protein production is required.

These data provide additional support for the hypothesis that misinterpretation of AU-rich *P. falciparum* sequences as mRNA 3' end processing signals is the primary impediment to expression of *P. falciparum* genes in *S. cerevisiae* [8]. In addition, these strains should prove valuable as a heterologous system for *P. falciparum* protein expression. First, the parasite's proteins may be produced for purification and biochemical analysis or crystallization. Second, other yeast mutations can be placed into these backgrounds for complementation by *P. falciparum* genes, enabling subsequent analysis of the encoded proteins. These studies can take advantage of the extensive array of yeast functional genomics tools, as has been done for bacterial proteins (for example, see ref. [20]). Third, yeast can be an effective platform for small molecule screens. For example, pairs of yeast strains expressing a *Plasmodium* gene and its human orthologue can be screened for small molecules that preferentially inhibit the *Plasmodium* protein. Finally, PJ#17 is a yeast two-hybrid strain and should improve the results of yeast two-hybrid screens with *P. falciparum* proteins by enabling the expression of larger fragments from a wider variety of genes.

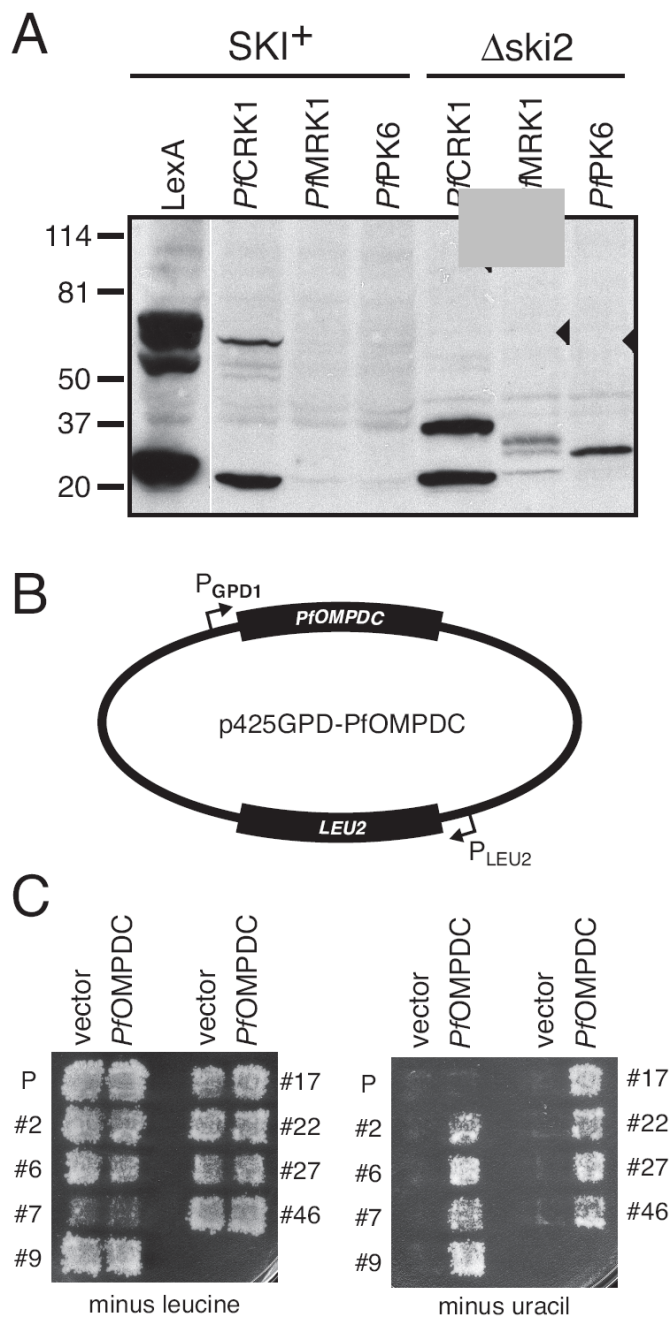
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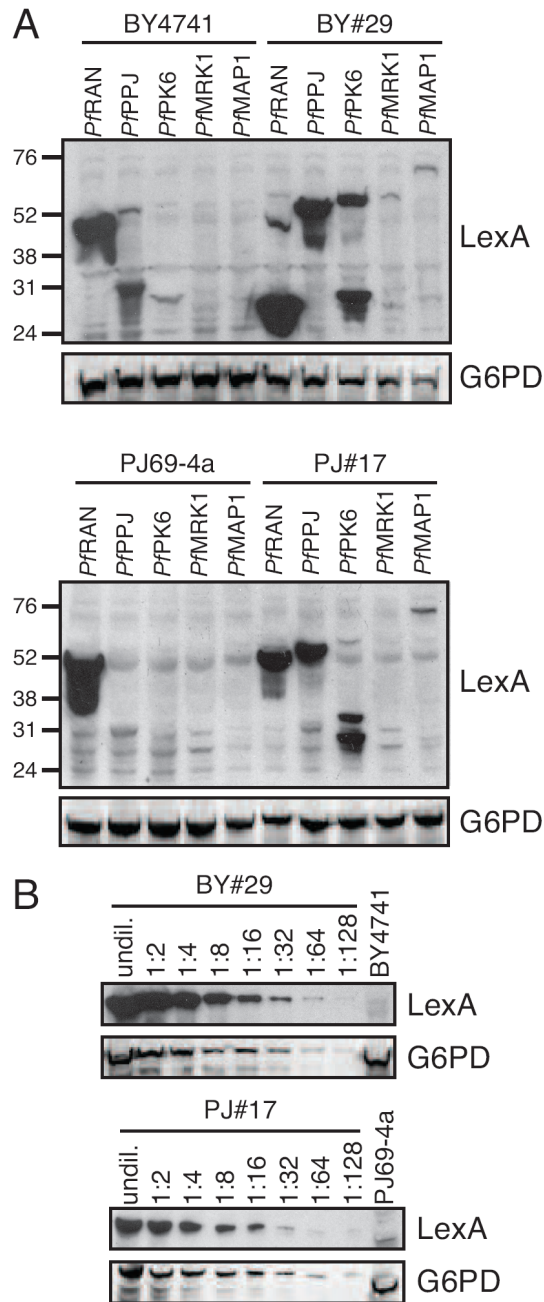
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**Fig. 1.** Expression of *P. falciparum* proteins in mutant yeast strains. **A.** An *S. cerevisiae* strain that lacks *SKI2* expresses truncated *P. falciparum* proteins. Plasmids encoding the *PfCRK1*, *PfMRK1*, and *PfPK6* genes in-frame with the *E. coli lexA* gene were transformed into BY4741 (*SKI*<sup>+</sup>) and *ski2*Δ yeast strains. Total protein lysates were separated by SDS-PAGE and subjected to immunoblot analysis with an anti-LexA antibody. Molecular weight markers are indicated at left. The predicted sizes of the LexA-PfCRK1, LexA-PfMRK1, and LexA-PfPK6 fusion proteins are 100, 58, and 59 kDa, respectively (indicated by black triangles). The ~26 kDa protein detected in lanes 1 and 4 is the same size as LexA protein produced by the parental LexA expression plasmid (not shown). **B.** Plasmid p425GPD-PfOMPDC, which encodes the

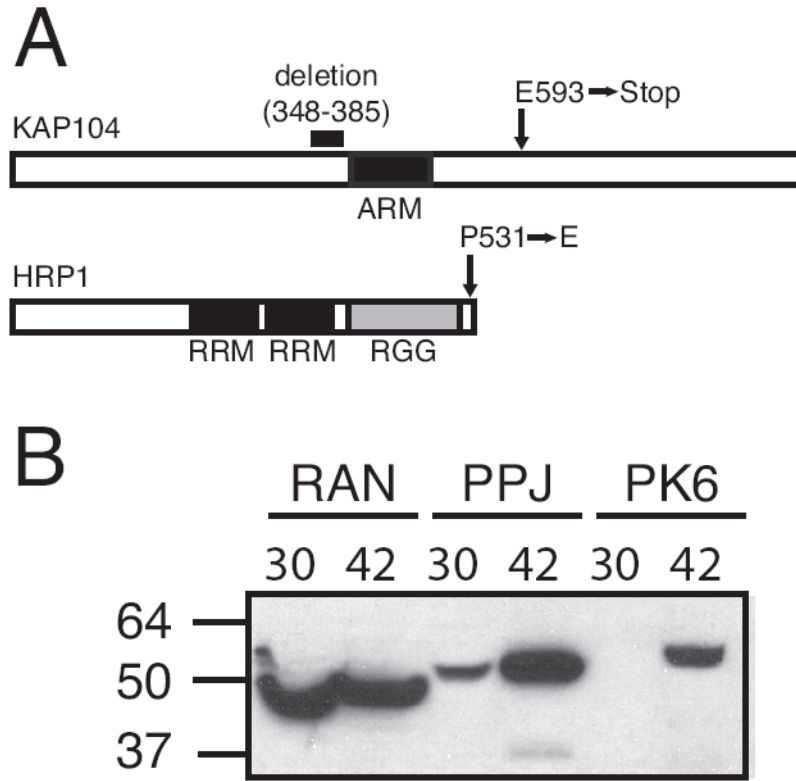
*P. falciparum* orthologue of the *S. cerevisiae* *URA3* gene. C. Selection of yeast strains that express functional PfOMPDC. The *S. cerevisiae* strains PJ69-4a [12] and BY4741 [11] were transformed with parental plasmid p425GPD or plasmid p425GPD-PfOMPDC. Yeast harboring either plasmid grew on media lacking leucine (due to expression of the *S. cerevisiae* *LEU2* gene encoded by the plasmids), but did not grow on media lacking uracil. Spontaneous yeast mutants that acquired the ability to express PfOMPDC and grow on media lacking uracil were selected, cured of their plasmids by growth on 5-fluoroorotic acid (5-FOA), and transformed with either p425GPD or p425GPD-PfOMPDC. Growth of a subset of eight mutant strains on media lacking leucine or uracil is shown. “P” indicates the parental yeast strain PJ69-4a.

**Fig. 2.**

A. Expression of *P. falciparum* proteins in mutant yeast strains that express PfOMPDC. Total protein lysates were prepared from the parental yeast strains BY4741 and PJ69-4a, and from PfOMPDC-expressing strains BY#29 and PJ#17, transformed with the *lexA* expression plasmid or plasmids encoding the *P. falciparum* genes *PfRAN*, *PfPPJ*, *PfPK6*, *PfMRK1*, and *PfMAP1* as fusions with the *lexA* gene. Lysates were separated by SDS-PAGE and subjected to immunoblot analysis with an anti-LexA antibody. The blots were then reprobed with an antibody against the yeast glucose-6-phosphate dehydrogenase as a loading control. Molecular weight markers are indicated at left. The predicted sizes of the LexA-PfRan, LexA-PfPK6, LexA-PfPPJ, LexA-PfMRK1, and LexA-PfMap1 fusion proteins are 46, 58, 59, 59, and 114



kDa, respectively. B. Estimate of the fold increase in LexA-PfPPJ expression in BY#29 and PJ#17 compared to parental strains. Undiluted and two-fold serial dilutions of total protein lysates from BY#29 and PJ#17 and undiluted total lysate from BY4741 and PJ69-4a expressing PfPPJ were subjected to SDS-PAGE and probed with anti-LexA antibody. Blots were reprobed with an antibody against the yeast glucose-6-phosphate dehydrogenase (G6PD) as a loading control.

**Fig. 3.**

A. *S. cerevisiae* strains that express functional PfOMPDC have mutations in Hrp1 or Kap104 proteins. To identify the genes responsible for the increased expression of PfOMPDC, BY#29 and PJ#17, which grow slowly, were transformed with a high copy number yeast genomic library [14] and plated on media lacking uracil. Plasmids from large, fast-growing colonies were isolated and sequenced from both ends, which narrowed the chromosomal region harboring the mutated gene to approximately 10 kb on chromosome 15 of BY#29 and to approximately 5.2 kb on chromosome 2 of PJ#17. Candidate genes encoded on the genomic DNA library plasmids were PCR-amplified, cloned into plasmid p424GPD, and transformed into the mutant strains harboring p425GPD-PfOMPDC to determine if expression of the candidate gene restored wildtype growth rate and reversed the ability of the mutant strains to express PfOMPDC. This analysis identified *HRP1* and *KAP104* as the genes that were mutated in BY#29 and PJ#17, respectively. This figure represents the Kap104 (918 amino acids) and Hrp1 (534 amino acids) proteins as white bars. Black and gray regions indicate protein domains. The positions of mutations in these proteins that confer enhanced expression of *P. falciparum* genes are indicated by arrows (point mutations) or a horizontal bar (deletion). B. A yeast strain with a temperature-sensitive mutation in *RNA15* expresses *P. falciparum* proteins. The yeast strain CMHy58.1A, which encodes a temperature-sensitive mutation in *RNA15* [18], was transformed with parental *lexA* expression plasmid or plasmids encoding the *P. falciparum* genes *PfRAN*, *PfPPJ*, or *PfPK6* as fusions with the *LexA* gene. Yeast were grown at 25° C to an OD600 of 1.0, and shifted to either 30° or 42° C. Total protein lysates were prepared after 4 h at the elevated temperatures, separated by SDS-PAGE and subjected to immunoblot analysis with an anti-LexA antibody. Molecular weight markers are indicated at left.