J. Parasitol., 103(1), 2017, pp. 118–122

Transient Expression of *Plasmodium berghei MSP8* and *HAP2* in the Marine Protozoan Parasite *Perkinsus marinus*

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ABSTRACT: *Perkinsus marinus* is a protozoan parasite of molluscs that can be propagated in vitro in a defined culture medium, in the absence of host cells. We previously reported that *P. marinus* trophozoites can be transfected with high efficiency by electroporation using a plasmid based on *MOE*, a highly expressed gene, and proposed its potential use as a "pseudoparasite." This is a novel gene expression platform for parasites of medical relevance for which the choice of the surrogate organism is based on phylogenetic affinity to the parasite of interest, while taking advantage of the whole engineered surrogate organism as a vaccination adjuvant. Here we improved the original transfection plasmid by incorporating a multicloning site, an enterokinase recognition sequence upstream of GFP, and a His-tag and demonstrate its potential suitability for the heterologous expression of *Plasmodium* sp. genes relevant to the development of anti-malarial vaccines. *Plasmodium berghei HAP2* and *MSP8*, currently considered candidate genes for a malaria vaccine, were cloned into *p*[MOE]:GFP, and the constructs were used to transfect *P. marinus* trophozoites. Within 48 hr of transfection we observed fluorescent cells indicating that the *P. berghei* genes fused to GFP were expressed. The expression appeared to be transient for both *P. berghei* genes, as florescence of the transfectants diminished gradually over time. Although this heterologous expression system will require optimization for integration and constitutive expression of *Plasmodium* genes, our results represent attainment of proof for the "pseudoparasite" concept we previously proposed, as we show that the engineered *P. marinus* system has the potential to become a surrogate system suitable for expression of *Plasmodium* spp. genes of interest, which could eventually be used as a malaria vaccine delivery platform. The aim of the present study was to test the ability of marine protozoan parasite *P. marinus* to express genes of *P. berghei*.

According to the World Malaria Report in 2015, an estimated 188 million cases of malaria and 395 thousand malaria deaths occurred worldwide (WHO, 2015). In sub-Saharan Africa alone, malaria causes 10% of children's death under the age of 5, making malaria the fourth highest cause of death. Although insecticides have been used to control mosquito populations with some success, in recent years malaria mosquitoes appear to have acquired increased resistance to the insecticides used in both insecticide-treated bed nets and indoor residual spraying. For example, pyrethroid resistance was detected in all major malaria vectors in 2014. Additionally, resistance to anti-malarial drugs has been increasing dramatically in both *Plasmodium vivax* and *Plasmodium falciparum* (WHO, 2015). Therefore, it is widely recognized that a preventive vaccine continues to be the strategy of choice in the fight against malaria. However, malaria is caused by any of 5 different species of *Plasmodium* parasites of distinct virulence and overlapping geographic distribution, which, together with their complex life cycles with multiple stages in both the mosquito vector and the human host, has made the development of efficient vaccines extremely difficult (Ouattara and Laurens, 2015). Approaches for development of a malaria vaccine have aimed mostly at *P. falciparum* pre-erythrocytic stages and include a recombinant protein with adjuvant (e.g., RTS,S/AS01) or the whole sporozoite (e.g., PfSPZ and PfSPZ-CVac) (Ouattara and Laurens, 2015). Other approaches include asexual erythrocytic stages (e.g., Pfs25) (Ouattara and Laurens, 2015). Most efforts, however, have been hindered by multiple challenges in the vaccine manufacture, preservation, storage, and delivery (Ouattara and Laurens, 2015). In this regard, efficacy and longevity metrics for the RTS,S/AS01 vaccine still are below the benchmark values, providing approximately 50% efficacy in preventing infection and 47% efficacy in protection from severe disease (Rts et al., 2012; Olotu et al., 2013). Therefore, a global strategy for anti-malaria vaccines should include not only the optimization of the magnitude and durability of protective efficacy (Hoffman et al., 2015) but also the search for alternative platforms that will improve their efficient delivery (Fernández Robledo and Vasta, 2010).

Perkinsus marinus was described in the early 1950s as the causative agent of 'Dermo' disease in eastern oysters (*Crassostrea virginica*) (Perkins, 1996), and since then several more species have been described world wide (Fernández Robledo et al., 2014). Unique to the genus *Perkinsus* is that the species in this group are one of the few protozoan parasites that can be propagated in vitro as clonal cultures in defined media and in the absence of host cells, reaching cell densities of 10^7 cells/ml (Gauthier and Vasta, 1993; La Peyre et al., 1993). The availability of culturing methodologies for *Perkinsus* spp. enabled a variety of studies addressing diverse aspects of *Perkinsus* biology (Fernández Robledo et al., 2014) and the development of a transfection system (Fernández Robledo et al., 2008; Cold et al., 2016). On the other hand, the Perkinsozoa is one of the earliest diverging groups in the lineage leading to dinoflagellates, branching off close to the ancestor from which the Ciliata, Dinozoa, Chromera, and Apicomplexa originated (Leander and Keeling, 2003; Saldarriaga et al., 2003; Gile et al., 2006; Moore et al., 2008). The Perkinsozoa is exclusively comprised of marine protozoan parasites of molluscan bivalves or microeukaryotes (Leander and Keeling, 2003; Saldarriaga et al., 2003; Gile et al., 2006; Moore et al., 2008). Based on the close phylogenetic affinity with the apicomplexan group, also exclu-

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DOI: 10.1645/16-88

sively comprised of protozoan parasites of medical and veterinarian relevance (Morrison, 2009), we introduced the 'pseudoparasite' concept, an expression platform for which the choice of the surrogate organism is based on its phylogenetic affinity to the target parasite, while taking advantage of the whole engineered organism as a vaccination adjuvant (Fernández Robledo and Vasta, 2010). Indeed, we have already shown that upon the feeding of live *P. marinus* to DR4.AE0 mice, while the gastrointestinal tract and lungs remain unaltered, strong humoral and cellular responses to the parasite were elicited, supporting the rationale for using genetically engineered *P. marinus* as a new oral vaccine platform to induce systemic immunity against infectious agents (Wijayalath et al., 2014).

In this study, we improved the original plasmid vector used to develop the transfection system in *Perkinsus* spp. (Fernández Robledo et al., 2008; Cold et al., 2016) to include a multiple cloning site (MCS) and the sequences coding for enterokinase and a His-tag. Two partial *Plasmodium berghei* genes, which are currently recognized as good candidates for a malaria vaccine, were cloned into new plasmid vector and used to transfect *P. marinus* trophozoites. Both genes were expressed in the transfectant cells, although transiently, supporting the "pseudoparasite" concept.

Cultures of the wild-type *P. marinus* CB5D4 (ATCC No. PRA-240) (Shridhar et al., 2013) were maintained in DME:Ham's F12 (1:2) supplemented with 5% fetal bovine serum in 25 cm^2 (5–8 ml) polystyrene canted neck cell culture flasks with vent caps (Corning®, Corning, New York) at 26–28 C in a microbiology incubator as reported elsewhere (Gauthier and Vasta, 1995).

The transfection vector *p*[MOE]:GFP (green fluorescent protein) was derived from *p*MOE[MOE]:GFP (former *p*PmMOE-GFP; Fernández Robledo et al., 2008). The MCS (underlined sequences) and His-tag (×6, lowercase) were built using the forward primer 5′ CCGCTGTTCAACGCAATCATGGACGTCGGTACCATGCATGAGCTCCACC 3′ and the reverse primer 5′ CAACTAGATGTGTTGTCTTAatgatgatgatgatgGTGGAGCTCATGCATG 3′ to include *Aat* II, *Kpn* I, *Nsi* I, and *Sac* I restriction sites upstream of the sequence coding for GFP to produce *p*[MOE]:MCS-HIS. Using the forward primer 5′ CCGCCGAGCTC*gacgacgacgac*AAGATGGTGAGCAAGGGCGAGGAGC 3′ and the reverse primer 5′ CGTAGGGAGCTCCTTGTACAGCTCGTCCATGCCG 3′ an enterokinase site (lowercase *italic*) was introduced upstream of the GFP using the *Sac* I restriction site (underlined sequences) to produce *p*[MOE]:GFP (Fig. 1A). The database PlasmoDB was mined for *P. berghei* merozoite surface protein 8 (*MSP8*) and hapless protein 2 (male gamete fusion factor) (*HAP2*) (Aurrecoechea et al., 2013); *MSP8* (PBANKA_1102200) full gene (1,230 bp) was amplified from *P. berghei* gDNA using the forward primer 5′ GGTACCAAAAGGAGTTCACAAATAATAATTT 3′ and the reverse primer 5′ TTCAATAGTATGCATATATATAATGGGTACC 3′ to include *Kpn* I site and cloned into *p*[MOE]:GFP to produce *p*[MOE]:MSP8-GFP (Fig. 1B). Similarly, *HAP2* gene (PBANKA_1212600) exon 2 (2,235 bp) was amplified using the forward primer 5′ GGTACCAGAAAAGGAGGAAAAGGGTATTTCC 3′ and the reverse primer 5′ GGTAAAAGTAAAATACCCCCATTGCGAGGTACC 3′ to include a *Kpn* I site and cloned into *p*[MOE]:GFP to produce *p*[MOE]:HAP2-GFP (Fig. 1B). All constructs were sequenced to verify orientation and sequence. The sequences corresponding to *p*[MOE]:MCS-HIS, *p*[MOE]:MSP8-GFP, and *p*[MOE]:HAP2-GFP have been deposited in GenBank™ database (accession numbers: KX423758–60).

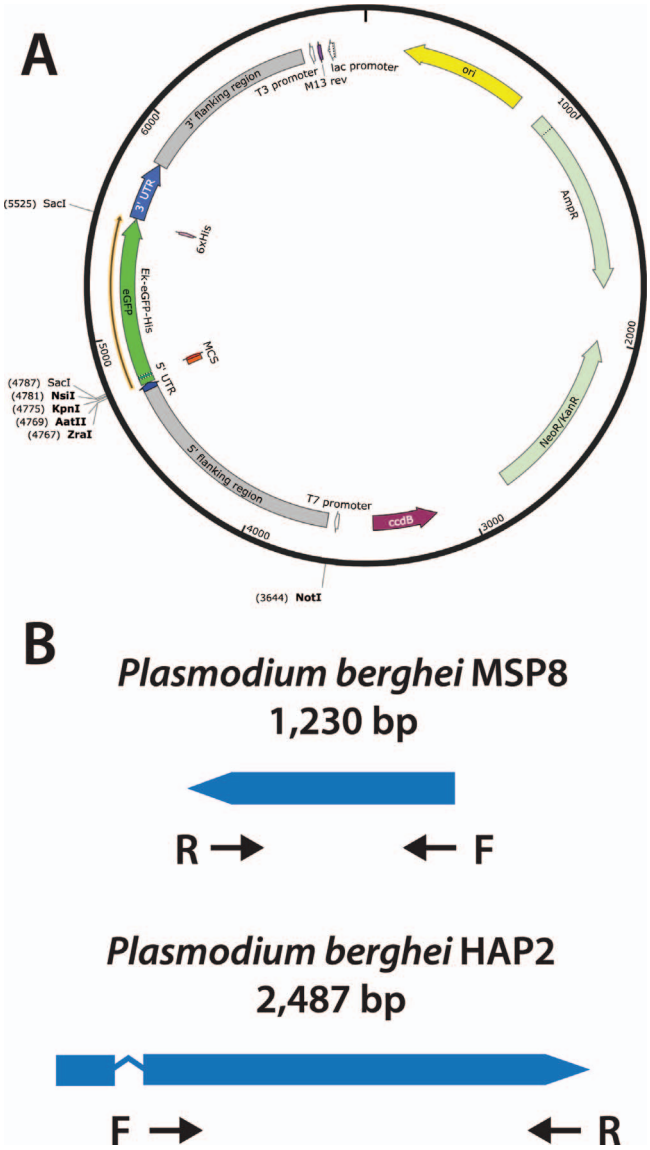

FIGURE 1. *Perkinsus marinus* transfection plasmid map and *Plasmodium berghei* gene maps. (**A**) Detail of the plasmid (*p*[MOE]:GFP) developed for cloning and transfection of *Perkinsus* spp. (**B**) *Plasmodium berghei MSP8* and *HAP2* genes, arrows indicate the forward (F) and reverse (R) primers used to amplify and clone the genes into *p*[MOE]:GFP.

Perkinsus marinus trophozoites in the log phase (5×10^7 cells) were resuspended in 100 µl of Lonza's solution V containing 5–10 µg of plasmid *p*[MOE]:HAP2-GFP or *p*[MOE]:MSP8-GFP (1:1, supercoiled:*Not* I linearized) and electroporated using the D-023 program in a Nucleofector™ II (Lonza, Walkersville, Maryland) (Fernández Robledo et al., 2008). After transfection, the cells were recovered in fresh culture medium and maintained as above. Seven and 2 biological replicates were performed for *p*[MOE]:MSP8-GFP and *p*[MOE]:HAP2-GFP, respectively. Transfected cultures were monitored over time for green fluorescence using standard excitation/emission filters (488/507

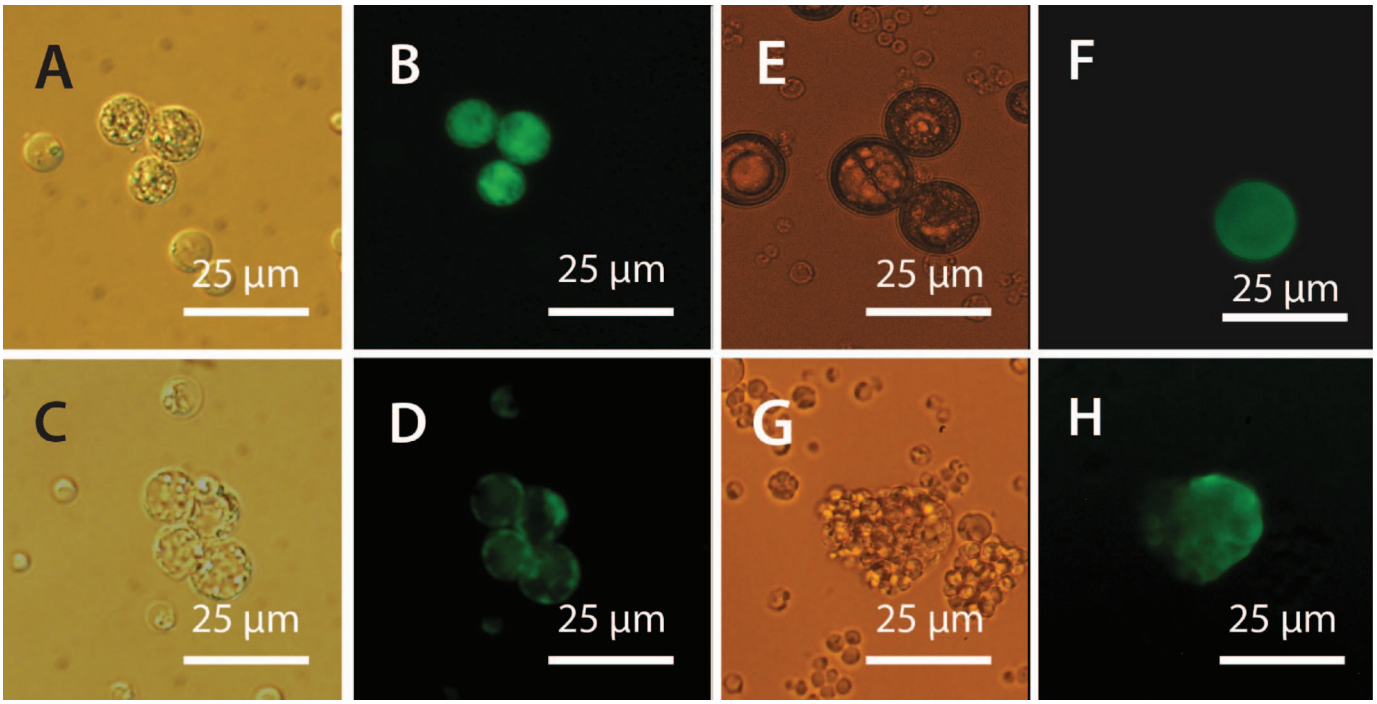

FIGURE 2. *Perkinsus marinus* transfectants expressing *Plasmodium berghei MSP8* and *HAP2*. (**A–D**) *Perkinsus marinus* transfectants expressing *P. berghei* MSP8. (**E–H**) *Perkinsus marinus* transfectants expressing *P. berghei* HAP2. Trophozoites expressing *p*[MOE]:HAP2-GFP appear to be propagating (G–H). (A, C, E, G, bright field; B, D, F, H, blue light excitation).

nm) under an Olympus IX-70 transmitted-light fluorescence microscope.

Here we report the transient expression of 2 apicomplexan genes in *P. marinus,* a protozoan parasite of marine and estuarine bivalves (Norén et al., 1999). The vector used to clone *P. berghei HAP2* and *MSP8* genes was derived from the original plasmid used to develop the transfection methodology for *Perkinsus* spp. (Fernández Robledo et al., 2008; Cold et al., 2016). The newly improved plasmid *p*[MOE]:GFP uses the genome sequences and untranslated regions (UTRs) of *MOE*, a highly expressed gene in *P. marinus* trophozoites with no homologues so far in GenBank™ other than *Perkinsus* spp. (Fernández Robledo et al., 2008). Compared to the original plasmid (*p*MOE[MOE]:GFP), where GFP was cloned fused to MOE using a *Pci* I restriction site (Fernández Robledo et al., 2008), the new developed vector offers a MCS for directional cloning. Additional features added to the plasmid vector include a His-tag to facilitate future purification of recombinant proteins and an enterokinase site at the cleavage of GFP.

HAP2 is a male-gamete-specific protein conserved across vast evolutionary distances (Cole et al., 2014; Speijer et al., 2015). The fertilization of *Plasmodium* spp. is a complex process that takes place in the gut of female mosquitos and begins upon the uptake of the parasite gametes included in the blood meal (CDC, 2016). Transmission-blocking the sexual stages of *Plasmodium* spp. is a good candidate for an anti-malarial vaccine to further prevent the development of *Plasmodium* gamete fusion (Gonçalves and Hunziker, 2016). Alternatively, vaccines using MSP have been used to inhibit erythrocyte invasion (Ouattara and Laurens, 2015). For proof of concept, we targeted both HAP2 and MSP8 in *P. berghei*, the *Plasmodium* species used in the mouse model for malaria. The rationale for the choice of both genes was based on the ease for their amplification directly from the *P. berghei* genome: *P. berghei MSP8* is an intron-less gene and *P. berghei HAP2* has a short intron close to the transcription starting site (Aurrecoechea et al., 2013). The results show that we were able to amplify and clone most of the *MSP8* and *HAP2* coding sequences (CDSs) into *p*[MOE]:GFP; transfected trophozoites showing fluorescence were observed 48 hr after transfection (Fig. 2A–H); compared to the transfection with the original plasmid (*p*MOE[-MOE]:GFP), the fluorescent cells were observed later (19 hr vs. 48 hr) after *P. marinus* started propagating (doubling time 24 hr; Gauthier and Vasta, 1995) and only few cells per transfection experiment showed green fluorescence, indicating a much lower transfection efficiency (<1%) than previously reported (Fernández Robledo et al., 2008). The fluorescence patterns observed in trophozoites transfected with either *p*[MOE]:MSP8-GFP or *p*[MOE]:HAP2-GFP were similar (Fig. 2). These patterns consisted in patches of fluorescence in most of the cell; in some trophozoites we also observed a concentration of fluorescence in the cytoplasm, which was also observed in *P. marinus MOE* (ATCC No. 393) and was attributed to the expressed fusion proteins (HAP2-GFP, MSP8-GFP) remaining in the trophozoite cytoplasm. This was consistent with the predicted expression outcome, since as compared to *P. marinus* MOE, both *HAP2* and *MSP8* lack a signal sequence for targeting the gene product to the membrane or cell-wall (Fernández Robledo et al., 2008). Interestingly, in the case of *p*[MOE]:MSP8-GFP we also observed some trophozoites with green fluorescence in the vacuole, an organelle of unknown function that occupies most of the cytoplasm (Perkins, 1996). Follow-up of the cultures transfected with either *p*[MOE]:MSP8-GFP or *p*[MOE]:HAP2-GFP revealed

no clear division of those cells expressing the fused proteins. An exception to this observation was a single trophozoite transfected with *p*[MOE]:HAP2-GFP that appeared to actively divide by schizoogony (Fig. 2G–H). However, in later stages of the cultures containing transfectants no fluorescent cells were observed, indicating that either the plasmid was not inherited during the cellular division or the cells expressing the fusion proteins were not viable. In our previous studies with the original *p*MOE[-MOE]:GFP, its integration into the genome of the sub-cloned transfectants occurred as a single event, and by non-homologous recombination (Fernández Robledo et al., 2008). In our present study an alternative explanation for the loss of fluorescence is that integration may have happened by either homologous or non-homologous recombination, but either causing a disruption in the regulation of an essential cell function or resulting in a truncated protein. Unfortunately we were unable to pursue sub-cloning of the transfectants because we lacked sufficient fluorescent cells. Future options to explore to improve both the efficiency of expression and to retain the plasmid by the transfected cells are to synthetize the genes of interest using the *P. marinus* codon use table and to incorporate into the transfection vector a resistance cassette for positive selection (see below).

Over the years multiple gene expression platforms, from bacteria to in vitro cell-free systems, have been proposed and tested to express genes from apicomplexan parasites (reviewed in Fernández Robledo and Vasta, 2010). Ultimately, the choice of the platform has been determined by the primary objectives pursued, from production of adjuvant for vaccines to expression of recombinant proteins for testing drug activity or ultrastructural studies, among others, but all exhibit multiple advantages and disadvantages (reviewed in Wijayalath et al., 2014).

The oyster disease ("Dermo") caused by *P. marinus* is highly prevalent in oyster populations in the United States (Marquis et al., 2015), and most shellfish consumers have been enterically exposed to this and related parasites. Furthermore, we have shown that feeding DR4.EA0 mice with live *P. marinus* results in IFNγ secretion and significantly increased titers of specific IgM and IgG antibodies, indicating live *P. marinus* stimulated both humoral and cellular response to the parasite, with no pathological effects observed (Wijayalath et al., 2014). Thus, it was concluded that *P. marinus* could be used as an effective oral vaccine delivery platform (Wijayalath et al., 2014). At the present time, the main challenges to achieve continued protein expression in heterologous systems is the lack of reliable methods to achieve stable integration of the transfected gene or to achieve the construction of inducible expression vectors that provide a robust expression of heterologous genes or, alternatively, to achieve transfection vectors incorporating a drug resistance cassette that facilitates the selection of transfectants. The results from the present study represent a significant advance in addressing these problems for the use of the parasite *P. marinus* as a gene expression platform for proteins of biomedical interest, and based on our previously reported *p*MOE[MOE]:GFP, a drug selection cassette for *P. marinus* has been developed using *Sh-ble* fused to mCherry, which confers resistance to the glycopeptide antibiotic bleomycin (Sakamoto et al., 2016). The selection of transfectants with the new plasmid containing the resistance cassette will also benefit from the recently developed methodology for plating *Perkinsus* spp. in solid medium (Fernández Robledo et al., 2008; Cold et al., 2016). The difference in GC content between *P. marinus* and *P. berghei* genome (47.4% vs. 23.7%) (Nikbakht et al., 2014) did not hinder the expression of the selected *P. berghei* genes in *P. marinus*. Nevertheless, gene synthesis of genes is currently advantageous due to low cost and speed (Gibson et al., 2008; Hutchison et al., 2016), and future attempts to express *Plasmodium* spp. genes in *P. marinus* will benefit from codon optimization by synthesis of the genes of interest.

In summary, we have shown that *P. marinus* has the ability to express genes of *P. berghei* and that in the absence of a drug resistance cassette the expression appears to be transient. Developing transfection vectors for genome integration and/or incorporating a resistance cassette into the vector would accelerate the design and construction of 'pseudo-*Perkinsus*' carrying and expressing gene candidates for malaria vaccine (Fernández Robledo and Vasta, 2010), which can be tested in the malaria mouse model (Wijayalath et al., 2014).

We thank Isabelle Coppens (Johns Hopkins Bloomberg School of Public Health) for kindly providing us with *P. berghei g*DNA. This study was supported by the National Institutes of Health (grant 1R21AI076797-01A2); the National Science Foundation, Research Experiences for Undergraduates (grant 1460861); and the Gordon and Betty Moore Foundation, Palo Alto, California (grant 4962).

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