using only the officially reported area by the main foreign variety (100,000 hectares) and main Chinese variety (120,000 hectares) and using the variety-specific yields (3440 kg/ha for the foreign variety; 3500 kg/ha for the main Chinese variety) and the variety-specific cost savings per kilogram (0.8 yuan or $0.416 for the foreign variety; 0.9 yuan or $0.468 for the main Chinese variety). In PPP terms, the benefit of the Chinese variety is 120,000 ha × 3500 kg/ha × 0.468 $/kg = $197 million. If the total estimated cotton area (700,000 hectares) or the average savings per kilogram [1.18 yuan/kg or 0.62 $/kg (Table 4)] were used, estimated benefits would be higher. Some benefits are due to labor savings, which may not be immediately of value to the farmer. In the longer run labor savings is important for increasing productivity.

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# Complete Development of Mosquito Phases of the Malaria Parasite in Vitro

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**Methods for reproducible in vitro development of the mosquito stages of malaria parasites to produce infective sporozoites have been elusive for over 40 years. We have cultured gametocytes of *Plasmodium berghei* through to infectious sporozoites with efficiencies similar to those recorded in vivo and without the need for salivary gland invasion. Oocysts developed extracellularly in a system whose essential elements include co-cultured *Drosophila* S2 cells, basement membrane matrix, and insect tissue culture medium. Sporozoite production required the presence of para-aminobenzoic acid. The entire life cycle of *P. berghei*, a useful model malaria parasite, can now be achieved in vitro.**

For over a century, a major objective of malaria control programs has been to block parasite transmission by mosquitoes. Such approaches would clearly benefit from a better understanding of parasite development within the vector, initiated when gametocytes are taken up in a blood meal. Fertilization of macrogametes within the mosquito midgut produces zygotes that transform into motile and invasive ookinetes. These penetrate and traverse the midgut epithelium and become sessile vegetative oocysts lying beneath the midgut basement lamina, each potentially producing 2 to 8000 sporozoites. Knowledge of the mosquito-related factors regulating these processes is improving (*1–3*), but it is difficult to determine the specific and separate effects of these factors in vivo. Early events associated with midgut invasion have recently been studied in vitro with the use of midgut preparations (*4–6*) or co-cultured mosquito cells (*7*), but these systems do not sustain long-term development or simulate oocyst interaction with the basal lamina and do not permit investigation of sporozoite differentiation.

Fertilization and ookinete development can be achieved in vitro for many malaria parasite species, including *Plasmodium berghei*, a parasite of rodents (*8*, *9*). These culture systems have facilitated the study of ookinete molecules that may be targeted by antibodies induced by transmission-blocking vaccines or drugs (*10*, *11*). After many pioneering attempts (*12*, *13*), it is only recently that in vitro transformation of *Plasmodium gallinaceum* and *Plasmodium falciparum* ookinetes into oocysts and sporozoites has been achieved, but the numbers of oocysts produced are low and, more importantly, the infectivity of these sporozoites has not been demonstrated (*14*, *15*). Here we confirm the need for a basement membrane–like substrate such as Matrigel, which may mimic the basal lamina of the mosquito midgut epithelium. In addition, co-culture with *Drosophila melanogaster* S2 cells is necessary for development, although the role of these insect cells is unclear.

We have based our work on the previously described *P. gallinaceum* culture system (*15*) and, where appropriate, substituted conditions that more nearly mimicked the mosquito environment or provided factors known to enhance oocyst growth in vivo. Thus, a culture system has been developed that consistently supports the transformation of large numbers of *P. berghei* ookinetes to extracellular oocysts and the production of infective sporozoites with efficiencies approaching those seen in vivo.

*Plasmodium berghei* ANKA (clone 2.34) ookinetes were produced in vitro (*8*, *9*) and cultured to produce oocysts in eight-chamber slides (*16*). Previously, cultures of other malaria species used supplemented RPMI 1640 (*15*), a mammalian medium traditionally used to culture ookinetes. A comparison of oocysts growing extracellularly in RPMI 1640 and Schneider's medium (*17*), whose composition mirrors the high aminoacidaemia of mosquito hemolymph (*18*), demonstrated that Schneider's medium significantly improved oocyst yield [multiple analysis of variance (MANOVA) over time: $F_{3,66} = 3.06$, $P = 0.03$ (*19*)]. Therefore, a classic insect medium, Schneider's medium, was used in all subsequent investigations. Nutrition of oocysts may be better supported by this medium, or Schneider's medium may be more suitable for the co-cultured insect cells because growth of *Drosophila* S2 cells is retarded in RPMI (*20*).

Extracellular oocyst development did not occur if chambers were not initially coated with Matrigel. Many ookinetes burrowed into the Matrigel matrix within hours and, within 1 to 2 days, transformed into oocysts within and on the surface of the matrix. Parasites not firmly attached to the matrix were probably removed during the repeated medium changes, which may account, in part, for the decline over time in oocyst number recovered from each chamber (*19*). We have previously observed that *P. berghei* ookinetes attach to plastic wells coated with the basal lamina components laminin, collagen IV, or fibronectin. Some ookinete-oocyst

**Table 1.** Summary of optimum culture conditions for sporogonic stages of *P. berghei*. *Drosophila melanogaster* S2 cells were incubated at 19° to 20°C in air on a layer of Matrigel in a ratio of 10:1 with ookinetes (*36*).

| Oocyst culture medium (pH 7) | Per 100 ml |
|---|---|
| Schneider's medium | 83.48 ml |
| Fetal bovine serum, heat-inactivated | 15 ml |
| $NaHCO_3$ | 23.8 mM |
| Hypoxanthine | 36.7 mM |
| Lipoprotein and cholesterol | 200 μl |
| PABA | 44 nM |
| Penicillin | 10,000 U |
| Streptomycin | 10 mg |
| Gentamicin | 20 mg |

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transformation occurs when bound to a laminin-collagen mixture (*21*). Ookinete proteins that may act as ligands for laminin (*22*) or collagen IV include the surface protein Pbs21 (*23*), Pgs28 (*24*), and a 215-kD protein, possibly PbCTRP (*21*, *25*).

The culture of erythrocytic schizonts requires microaerobic conditions. Therefore, we compared the effect of air with low-oxygen gas phase on oocyst development (*9*). No significant effects on parasite development were observed due to gas phase when analyzed by days of interaction (MANOVA: $F_{3,42} = 0.5417$, $P = 0.66$) (*19*). All subsequent incubations were performed in air. The hypothesis that fluctuations in midgut lumen pH normally associated with blood meal management (*26*) influence oocyst development was not upheld (*9*, *19*), and oocyst differentiation in vitro was more efficient at a pH close to that of the hemocoele (pH 6.81 ± 0.08) (*27*) than the range of pH found in the gut during blood meal digestion (pH 7.68 to 8.03 in *Anopheles stephensi*) (*26*).

Pilot studies established that extracellular oocyst growth was markedly improved by the presence of *Drosophila* S2 cells (*19*). We then asked whether oocyst development could be improved by co-culturing with cell lines from susceptible (*Anopheles gambiae* line, Sua 4.0, kindly supplied by H.-M. Müller, European Molecular Biology Laboratory, Heidelberg, Germany) or nonsusceptible mosquitoes [Mos20, from *Aedes aegypti* (*28*)]. All cells were added at a cell:ookinete ratio of 10:1. Cell type significantly affected the number of oocysts recovered throughout the culture period (MANOVA: $F_{3,28} = 33.51$, $P < 0.0001$) (*19*). However, the *Drosophila* cell line supported significantly more oocysts than either mosquito cell line (Tukey's post hoc pairwise comparisons, $P < 0.001$ for all comparisons). In the absence of Matrigel, *P. berghei* ookinetes transform into early oocysts intracellularly in *Aedes albopictus* cells (*29*) and in *A. gambiae* 4a-3A cells (*7*). The latter system supports intracellular oocyst development, including nuclear division and expression of circumsporozoite protein but not sporozoite formation (*7*). The role of co-cultured "feeder" cells in the sustained development of oocysts is unknown, but they could provide either soluble nutrients or unidentified intercellular matrix molecules (*24*) that may be involved in host-parasite signaling.

The development of *P. berghei* oocysts in the presence of S2 cells was very similar to that seen in the mosquito. Mosquitoes were fed on a *P. berghei*–infected mouse (*8*, *9*), and groups of five female mosquitoes were dissected at various times post-infection. Comparisons of oocyst diameters in vivo and in vitro showed that growth proceeded at a similar rate in both conditions (*19*) and that maximum diameters of ~40 μm were achieved by day 15. In contrast to reports of cultured *P. gallinaceum* oocysts (*15*), all cultured *P. berghei* oocysts were spherical and had a clearly defined capsule (Fig. 1D). Oocysts expressed Pbs21 until day 6 (Fig. 1B) and circumsporozoite protein was first detected 7 days after culture, as has been reported in vivo (*30*) (Fig. 1, E and H). Staining with 4′,6′-diamidino-2-phenylindole (DAPI) revealed several foci of nuclear material (Fig. 1, C, F, and I), indicative of the expected endomitosis.

Para-aminobenzoic acid (PABA), previously shown to support enhanced oocyst development in mosquitoes (*31*), was found to be essential for sporozoite differentiation in vivo. When added to Schneider's medium at a final concentration of 11 to 44 nM, oocysts containing sporozoites were visible by day 15. By day 20, the first sporozoites were naturally released from a small percentage of oocysts and were found adhering to Matrigel (Fig. 1, J and K). Parallel observations on parasite development in vivo showed that salivary gland invasion by sporozoites (and, therefore, prior oocyst rupture) was also first detected on day 20. Recovery of sporozoites adherent to the Matrigel increased with rising PABA concentrations to an optimum at 44 nM (*19*). In eight replicate experiments, culture medium removed from all wells every 2 to 3 days from day 20 onward contained large numbers of sporozoites.

Sporozoites were collected from 25- or 28-day-old cultures, washed, and resuspended in phosphate-buffered saline (PBS) and inoculated intravenously into Charles River Derived (C.D.) mice at doses of 4000 (25 day), 7000 (28 day), or 14,000 (28 day). Four of ten, one of three, and three of three mice became infected, respectively, demonstrating a patent parasitaemia by day 7 post-inoculation, a time consistent with the 6.3-day pre-patent period described after inoculation of salivary gland sporozoites (*32*, *33*) (Fig. 1L). This clearly demonstrates that a period of residence in the mosquito salivary glands is not essential to the onset of sporozoite infectivity, as suggested previously (*33*). One of these mice was then used as a blood meal source for *A. stephensi* mosquitoes, and 8 of 12 of the fully engorged mosquitoes developed oocyst infections on their midguts by day 10.

As described previously (*34*), ~40% of the initial ookinete inoculum undergoes apoptosis and ~68% of the survivors differentiated into oocysts by day 15. These efficiencies compare with 10 to 30% for cultured *P. gallinaceum* (*15*) and 77% for *Plasmodium yoelii* in vivo in *A. stephensi* (*35*). The ability to contrast parasite development in this culture system with the events of natural infection may enhance our ability to understand the regulation of parasite development in both susceptible and refractory mosquitoes.

We have described the successful culture of all the sporogonic stages of *P. berghei* in vitro. However, the partial success previously achieved with the avian parasite *P. gallinaceum* and the human parasite *P. falciparum* with the use of a comparable but less developed culture system (*14*, *15*) gives every reason to expect our protocol to work with other strains and species of *Plasmodium*. Whilst recognizing the potential applicability of this technique to a wide range of malaria species, we also appreciate that the pos-

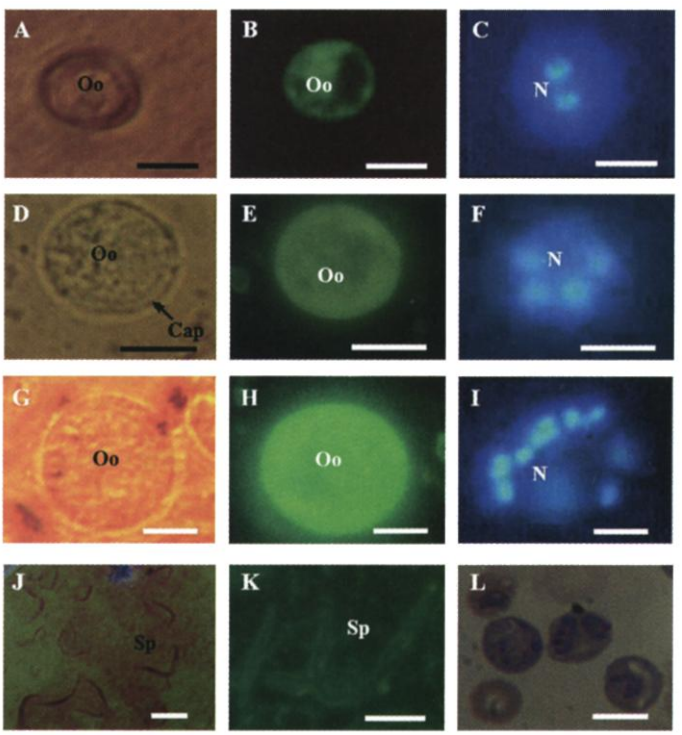


**Fig. 1.** In vitro sporogonic development of *P. berghei*. (**A** through **C**) Day 3. Bars, 5 μm. (**D** through **F**) Day 9. Bar, 10 μm. (**G** through **I**) Day 15. Bars, 10 μm. (**J** and **K**) Day 25. Bars are as follows: (J), 4 μm, (K and **L**), 5 μm. (A, D, and G), light microscopy; (B, E, H, and K), fluorescence microscopy. Cell labeling was performed with reagents as follows: (B), Oocyst surface labeled with antibody 13.1 (antibody to Pbs21); (E, H, and K), parasite labeled with antibody 3D11 [antibody to circumsporozoite protein (CSP)] oocyst wall; (C, F, and I), oocyst nuclei (marked "N") labeled with DAPI; (J), Giemsa-stained sporozoites (marked "Sp") free on the Matrigel; (L), mouse erythrocytes infected with *P. berghei* 7 days after inoculation of sporozoites produced in culture. Oo, oocyst; Cap, oocyst wall.

sibility of culturing each and every stage of the life cycle of the rodent parasite *P. berghei* immediately opens up important new areas of investigation in this useful model species.


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36. Cold Matrigel (90 μl) was pipetted into each culture well (*16*) and gelled at 37°C for 30 min. Ookinetes were co-cultured with *D. melanogaster* S2 cells (*17*) kindly supplied by J.-L. Imler, CNRS, Strasbourg, France. Ookinetes and S2 cells were suspended in either RPMI 1640 medium, supplemented as in (*15*, *19*), or Schneider's medium (Sigma), supplemented as in Table 1. Ookinete ($10^4$ per chamber)–S2 cell suspension was pipetted onto solidified Matrigel in the chamber plate and incubated at 19° ± 1°C. Most of the medium and S2 cells were changed every 48 to 72 hours.


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REPORTS

# A Bacterial Guanine Nucleotide Exchange Factor Activates ARF on *Legionella* Phagosomes


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**The intracellular pathogen *Legionella pneumophila* subverts vesicle traffic in eukaryotic host cells to create a vacuole that supports replication. The *dot/icm* genes encode a protein secretion apparatus that *L. pneumophila* require for biogenesis of this vacuole. Here we show that *L. pneumophila* produce a protein called RalF that functions as an exchange factor for the ADP ribosylation factor (ARF) family of guanosine triphosphatases (GTPases). The RalF protein is required for the localization of ARF on phagosomes containing *L. pneumophila*. Translocation of RalF protein through the phagosomal membrane is a *dot/icm*-dependent process. Thus, RalF is a substrate of the Dot/Icm secretion apparatus.**


*Legionella pneumophila* are aquatic bacteria that infect and grow within protozoan hosts in most freshwater ecosystems (*1*). When these bacteria are inhaled by humans, *L. pneumophila* will replicate in alveolar macrophages, resulting in a severe pneumonia known as Legionnaires' disease (*2*, *3*). *Legionella pneumophila* replicate within phagocytes by first creating a specialized vacuole that is similar morphologically to the endoplasmic reticulum (ER) of its host (*4*, *5*). Biogenesis of this replicative vacuole requires the Dot/Icm transporter (*6*), which is a type IV protein secretion apparatus (*7*, *8*). Pathogens such as *Agrobacterium tumefaciens* and *Helicobacter pylori* use type IV transporters to inject bacterial proteins directly into the cytosol of eukaryotic host cells (*9*–*11*). It is thought that the Dot/Icm transporter is used by *L. pneumophila* to inject proteins into host cells in order to control the biogenesis of a replicative organelle by modulating the activity of host factors involved in vesicle traffic. However, genetic screens that have been successful in isolating virulence determinants required for growth of *L. pneumophila* in host cells, including the genes encoding the Dot/Icm secretion apparatus, have not revealed any injected proteins (*7*, *8*, *12*, *13*).

The host protein ADP ribosylation factor–1 (ARF1) is found on phagosomes containing wild-type *L. pneumophila* but is not localized to phagosomes containing *L. pneumophila dot/icm* mutants (*14*). ARF1 is a highly conserved small GTP-binding protein that acts as a key regulator of vesicle traffic from the ER and Golgi [reviewed in (*15*)]. Because ARF1 localization on phagosomes containing *L. pneumophila* requires the Dot/Icm transporter, an injected bacterial protein may be required for ARF1 recruitment. To find proteins that are injected into host cells by the Dot/Icm transporter, we focused on bacterial gene products that may play a direct role in localization of ARF1 to phagosomes containing *L. pneumophila*. Because the association of cytosolic ARF onto vesicle membranes is coincident with GTP activation, we searched the genome of *L. pneumophila* for proteins that had homology to ARF-specific guanine nucleotide exchange factors (GEFs) (*16*).

We identified a *L. pneumophila* gene that encodes a 374 amino acid protein with a Sec7-homology domain (Fig. 1A) (*17*). Sec7-homology domains are found in a diverse family of eukaryotic ARF-GEFs and are sufficient to stimulate the exchange of GDP for GTP [reviewed in (*18*)]. This *L. pneumophila* gene was called *ralF*. A gene from the intracellular pathogen *Rickettsia prowazekii* (*19*) that is predicted to encode a protein that has 42% identity to the full-length RalF protein was identified using the RalF protein sequence as a BLASTP query (Fig. 1A). Currently, the RalF protein and this *R. prowazekii* protein are the only two prokaryotic gene products known to contain a Sec7-homology domain.

The RalF protein was detected (*20*) in wild-type *L. pneumophila* (Fig. 1B, Lp01) and in an isogenic mutant that has a defective Dot/Icm transporter (Fig. 1B, Lp01 Δ*dotA*) but was not detected after the *ralF* gene was deleted from the *L. pneumophila* chromosome (Fig. 1B, Lp01 Δ*ralF*). The RalF protein was also produced by *L. pneumophila* Philadelphia-1 (Fig. 1B, Lp philadelphia-1), which is a clinical isolate obtained from the first documented *L. pneumophila* disease outbreak (*3*). These data demonstrate that a protein containing a Sec7-homology domain is produced by *L. pneumophila*.


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