Rodent and nonrodent malaria parasites differ in their phospholipid metabolic pathways[S]

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Abstract **Malaria, a disease affecting humans and other animals, is caused by a protist of the genus *Plasmodium*. At the intraerythrocytic stage, the parasite synthesizes a high amount of phospholipids through a bewildering number of pathways. In the human *Plasmodium falciparum* species, a plant-like pathway that relies on serine decarboxylase and phosphoethanolamine N-methyltransferase activities diverts host serine to provide additional phosphatidylcholine and phosphatidylethanolamine to the parasite. This feature of parasitic dependence toward its host was investigated in other *Plasmodium* species. In silico analyses led to the identification of phosphoethanolamine N-methyltransferase gene orthologs in primate and bird parasite genomes. However, the gene was not detected in the rodent *P. berghei*, *P. yoelii*, and *P. chabaudi* species. Biochemical experiments with labeled choline, ethanolamine, and serine showed marked differences in biosynthetic pathways when comparing rodent *P. berghei* and *P. vinckei*, and human *P. falciparum* species. Notably, in both rodent parasites, ethanolamine and serine were not significantly incorporated into phosphatidylcholine, indicating the absence of phosphoethanolamine N-methyltransferase activity. To our knowledge, this is the first study to highlight a crucial difference in phospholipid metabolism between *Plasmodium* species. The findings should facilitate efforts to develop more rational approaches to identify and evaluate new targets for antimalarial therapy.—**Déchamps, S., M. Maynadier, S. Wein, L. Gannoun-Zaki, E. Maréchal, and H. J. Vial. **Rodent and nonrodent malaria parasites differ in their phospholipid metabolic pathways. *J. Lipid Res.* 2010.** 51: **81–96.**

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Malaria is a disease caused by an intraerythrocytic protozoan parasite of the genus *Plasmodium*, which is transmitted by dipterans and affects vertebrates such as reptiles, birds, and mammals, including humans (see supplementary Table I). By contrast with other Apicomplexa parasite species that can infect a broad range of metazoans, *Plasmodium* species have a narrow specificity range regarding insect and vertebrate hosts (1). *Plasmodium falciparum* is, thus, responsible for the most severe form of malaria in humans only. Other species, such as *P. vivax*, two *P. ovale* subspecies (2), *P. malariae*, and, according to recent reports, *P. knowlesi* (3) cause less complicated forms of human malaria. The host specificity of *P. knowlesi* is not restricted to humans because it also infects monkeys.

The evolutionary history of *Plasmodium* species has been highly debated, especially the position of *P. falciparum*, either grouped with avian parasites (4, 5) or placed as a sister species to other mammalian parasites including rodent parasites. The findings of most recent analyses using three classes of rare genomic changes and mitochondrial RNA genes unambiguously support a mammalian clade and no

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Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; CDP-Cho, cytidine-diphospho-choline; CDP-DAG, cytidine-diphospho-diacylglycerol; CDP-Etn, cytidine-diphospho-ethanolamine; CEPT, choline/ethanolamine-phosphotransferase; Cho, choline; EST, expressed sequence tag; Etn, ethanolamine; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; P-Cho, phosphocholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; P-Etn, phosphoethanolamine; PL, phospholipid; PMT, phosphoethanolamine N-methyltransferase; PS, phosphatidylserine; SD, serine decarboxylase; SDPM pathway, serine decarboxylase - phosphoethanolamine methyltransferase pathway; Ser, serine.

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grouping of *P. falciparum* with avian parasites. Phylogenetic studies revealing that *P. falciparum* could be grouped with bird parasites likely reflects an artifact of long-branch attraction and problems associated with extreme base compositions (6). Molecular phylogeny data would, therefore, corroborate the related host specificities and parasite's biology in mammalian parasites and should be consistent with marked differences between mammalian and avian parasite clades.

Laboratory animal models are necessary for the study of human malaria but none of the human malaria agents could infect mice or rats. Although simian malaria parasites such as *P. knowlesi* or *P. cynomolgi* can be considered as the most relevant models, rodent malaria parasites are actually the most practical ones. Well-known *Plasmodium* species infecting rodents include *P. berghei*, *P. chabaudi*, *P. vinckei*, and *P. yoelii* (see supplementary Table I). By comparing the complete genome of *P. falciparum* with concatenated genomic shotguns of rodent malaria parasites, Kooij et al. (7) observed a high level of conservation between ~85% of the genes from *P. falciparum*, *P. berghei*, *P. chabaudi*, and *P. yoelii* in terms of content and order in so-called syntenic regions. Concerns about the accuracy of rodent malaria models for drug or vaccine development are focused on the extent of genomic divergences, which might be responsible for substantial differences in human and rodent pathogeneses. Whenever a gene representing a possible target for malaria chemotherapy is identified in *P. falciparum*, it is, thus, crucial to gain insight into both the similarities and differences in the genomics and biology in closely related species.

Because genomic information is now available for numerous *Plasmodium* species, the stable inheritance of potential drug and vaccine targets can be analyzed. Whole genome synteny maps are particularly interesting to detect intrasyntenic indels, which can be foci for species-specific genes that play roles in host-parasite relationships (7).

Phospholipid synthetic pathways can provide key information to trace and understand the evolution of eukaryotes. Indeed, few phospholipid pathways occur in eukaryotes, which can be parallel redundant routes. The conservation or withdrawal of these routes, throughout evolution, also provide clues to understand membrane lipid dynamics in relation to cell development and, possibly, in the case of infectious pathogens, to introduce therapeutical treatments. Consequently, some genes, like those studied here, are markers of complete pathways.

The biosynthesis of phosphatidylcholine (PC), the most abundant membrane phospholipid (PL) in *P. falciparum*, is a remarkable example of the level of parasitic adaptation and dependence toward its host and represents a promising target for novel chemotherapies (8, 9).

In *P. falciparum*, PC can be synthesized de novo by two major routes using choline (Cho) or ethanolamine (Etn) as precursors (8, 10, 11). In the so-called Kennedy pathway, Cho diverted from the host is phosphorylated into phosphocholine (P-Cho) and subsequently coupled to cytidine triphosphate (CTP), thus, generating cytidine diphosphate (CDP) -choline (CDP-Cho) that serves as a direct substrate for PC synthesis. De novo synthesis of PC following the Kennedy pathway can be summarized as:

$$\text{Cho} \rightarrow \text{P}-\text{Cho} \rightarrow \text{CDP}-\text{Cho} \rightarrow \text{PC} \qquad \text{(pathway } \mathbf{II}\text{)}$$

A similar de novo pathway does exist for *Plasmodium* synthesis of phosphatidylethanolamine (PE) from ethanolamine.

Besides, some eukaryotic cells, particularly *Saccharomyces cerevisiae* and human liver cells (12, 13), are able to methylate PE to PC (pathway **II**). The corresponding genes coding for PE N-methyltransferases (PEMT) have not yet been found in any *Plasmodium* species. Nevertheless, the high capacity of *P. knowlesi*-infected erythrocyte to intensively methylate PE (introduced by phospholipid transfer proteins) into PC clearly indicates the presence of PEMT activity (14). Moreover, there is strong evidence that this PE N-methylation pathway also occurs in *P. falciparum* based on similar labeling from radioactive ethanolamine and serine (Ser) (10).

Alternatively, Ser, which is diverted from the host or from hemoglobin degradation in the food vacuole, was found to be decarboxylated into Etn, which is subsequently phosphorylated into phosphoethanolamine (P-Etn). P-Etn can be methylated into P-Cho, which serves as a substrate to form PC. This alternative pathway in *P. falciparum* can be summarized as:

$$\text{Ser} \rightarrow \text{Etn} \rightarrow \text{P-Etn} \rightarrow \text{P-Cho} \rightarrow \text{CDP-Cho} \rightarrow \text{PC}$$

(pathway **III**)

In this pathway, serine decarboxylase (SD) enzymatic activity was first described by our group in *P. knowlesi* and *P. falciparum* (10). The gene and the related catalytic activity was subsequently identified in plants (15), whereas the corresponding plasmodial gene has not yet been identified. Etn can then be phosphorylated, giving rise to PE.

Surprisingly, P-Etn can also be converted into P-Cho by S-adenosyl methionine (SAM)-dependent triple methylation carried out by a plant-like phosphoethanolamine N-methyltransferase (PMT or PEAMT) (EC 2.1.1.103). *P. falciparum* PMT (PfPMT), which was revealed by the *P. falciparum* genome sequencing program (16), is encoded by a single gene (*PfPMT*) of chromosome XIII (PlasmoDB accession number: MAL13P1.214). P-Cho is then integrated into the CDP-choline pathway for the synthesis of PC.

The PfPMT enzyme was characterized in vitro and also in a surrogate system, as after introduction of the gene into a mutant yeast, the exogenous enzyme was shown to methylate P-Etn (11, 17). Orthologous *PMT* genes have been previously detected in plants such as *Arabidopsis thaliana* (Genbank AAG41121) (18) and then in *Caenorhabditis elegans* (Genbank AAO38584.1 and AAB04824) (19, 20), but not in humans (11).

Therefore, *P. falciparum*, which does not possess a significant amount of cholesterol, uses a bewildering variety of metabolic pathways for the synthesis of PLs, which are its main membrane lipids. Because SD and PfPMT provides *P. falciparum* with a novel pathway to use host Ser as a precursor for its major membrane PL, this plant-like pathway (III) seems to be a specific feature of the host-parasite relationship.

Therefore, we investigated its occurrence in *Plasmodium* species infecting a broad range of hosts. Both syntenic and biochemical studies have revealed that PL biosynthetic pathways differ between plasmodia invading rodent and nonrodent hosts. Thus, by contrast with the consensus view that mammalian *Plasmodium* species are grouped separately from avian parasites and, particularly, that *P. falciparum* is related to rodent parasites on an evolutionary scale, we report here an important lineage specificity that differentiates *P. berghei*, *P. yoelii*, and *P. chabaudi* from nonrodent parasites. This discovery is one of the few examples of different metabolic features between *Plasmodium* species and has a major impact with respect to the animal models.

MATERIALS AND METHODS

Chemicals

[1-^{3}H]ethan-1-ol-2-amine, [methyl-^{14}C]choline, L-[U-^{14}C]serine, phosphoryl-[methyl-^{14}C]choline, and CDP-[methyl-^{14}C]choline were purchased from GE Healthcare (UK), and CDP-[1,2-^{14}C]ethan-1-ol-2-amine from ARC International (St. Louis, MO). Cho, P-Cho, CDP-Cho, Etn, P-Etn, cytidine-diphospho-ethanolamine (CDP-Etn), Ser, lysophosphatidylcholine (LysoPC), PC, PE, and phosphatidylserine (PS) (from bovine brain) were from Sigma (St. Louis, MO). Albumax I and RPMI 1640 were obtained from Gibco (France). TLC silica gel 60 plates with a concentrating zone were from Merck (Darmstadt, Germany). Cellulose powder (CF 11) was obtained from Whatman Laboratory Division (Maidstone, UK).

Biological materials

The animal study was performed at the Centre d'Elevage et de Conditionnement Experimental des Modèles Animaux, Montpellier (France), under permission no. A34370 and approved by the local animal research committee, in compliance with European Regulations and French legislation.

P. falciparum parasites (3D7 strain) were cultured in human O+ erythrocytes (Blood Bank of Montpellier, France), as previously described (21), under 5%O2/5%CO2/90%N2 in a gas chamber using RPMI 1640 medium supplemented with 25 mM HEPES buffer (pH 7.4) and 0.5% Albumax I.

Female Swiss albino mice weighing 30–40 g were obtained from Charles River Laboratories France (France). *P. vinckei petteri* (279BY) and P. *berghei* ANKA strains were provided by Dr. I. Landau (Paris, France) and Dr. O. Billker (London, UK), respectively. Mice were inoculated intraperitoneally with 2×10^{8} cryopreserved *P. berghei*- and *P. vinckei*-infected erythrocytes suspended in 0.2 ml saline medium. Parasitemia was routinely monitored on Giemsa stained thin blood smears.

Total blood from infected (10–20% parasitemia) and uninfected mice was harvested by cardiac puncture. After centrifugation (700 *g* for 10 min), cells were suspended in Ringer's buffer (122.5 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM D-glucose, 1 mM K_2HPO_4, 10 mM HEPES, pH 7.4) and then passed through a cellulose powder column (CF 11) to remove white blood cells (22), as monitored by microscopic examination of Giemsa stained smears (less than 1‰ of white blood cells).

In silico studies

Search for the phosphoethanolamine N-methyltransferase gene in human, simian, rodent, and bird Plasmodium genomic resources. PMT gene sequences were sought via available genomic resources: mainly PlasmoDB (23); GeneDB; the J. Craig Venter Institute, particularly for *P. yoelii* genomic data; the National Center for Biotechnology Information (24); and data from ongoing sequencing projects of *P. reichenowi* and *P. gallinaceum* genomes at the Sanger Institute, kindly provided by A. Pain, C. Newbold, and M. Turner. The *PfPMT* gene was previously characterized at position 1,696,925-1,698,144 on chromosome XIII (PlasmoDB accession number: MAL13P1.214). The *PfPMT* sequence was used as a probe to retrieve orthologous genes in annotated genomic sequences of *P. vivax* (*PvPMT*, PlasmoDB accession number Pv083045) and *P. knowlesi* (*PkPMT*, PlasmoDB accession number PKH_121150) by conventional BLAST searches (using BLOSUM62 matrix and an E-value cutoff at $1e^{-10}$). Orthologous *PMT* genes were sought in unassembled *Plasmodium* genomes (Sanger Institute sequencing project): *PMT* genes could be identified in *P. reichenowi* (*PrPMT*, concatenation of previously unassembled sequences) and *P. gallinaceum* (*PgPMT*, concatenation of previously unassembled sequences and the genomic contig 226551.c000128434). No gene with significant sequence similarity was detected in any available genomic data of *P. berghei*, *P. yoelii*, or *P. chabaudi*.

Determination of syntenic regions in available bird, human, simian, and rodent genomic data corresponding to the phosphoethanolamine N-methyltransferase gene context in P. falciparum. Conservation of the genomic context of *PfPMT* on *P. falciparum* chromosome XIII (including PF13_0234, PF13_0235, *PfPMT*/MAL13P1.214, MAL13P1.215, PF13_0236, and PF13_0237 ordered genes) was sought in *P. reichenowi*, *P. vivax*, *P. knowlesi*, *P. gallinaceum*, *P. berghei*, *P. yoelii*, and *P. chabaudi* based on the precomputed genomic comparisons available via the PlasmoDB genome browser, manually validated and revised using additional information obtained from available genomic shotguns. Gene models were corrected on the basis of genomic sequences overlaps and available expressed sequence tags (ESTs).

Molecular phylogenies. Four phylogenetic methods including maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ) and the Bayesian method (BM) were used to infer the gene tree for each set of orthologous genes. ML, NJ, and BM inferences were performed using PHYML (25), NEIGHBOR (26), and MrBayes (27), as implemented in the Phylogeny.fr platform (28). In all cases, multiple alignments were computed using the MUSCLE method (29) and ungapped alignments were selected using GBlocks (30) for tree computation. MP trees were constructed using PROTPARS in the PHYLIP package (26). The level of bootstrap support was inferred by 100 resamplings of the alignment. Trees were also constructed using the TULIP method, which is compositional bias insensitive, using the Smith Waterman alignment method, the BLOSUM62 matrix, and n = 1,000 sequence randomizations (31). The topology of phylogenetic trees constructed in this study was confirmed by all methods. Because *P. gallinaceum* is a taxon responsible for artifacts of long-branch attraction and problems associated with extreme base compositions (6), the *P. gallinaceum* sequence was set as an outgroup for the tree constructions. For each gene, molecular phylogeny representations were then first computed using the ML method, and the resulting tree topology was assessed by comparing bootstrap values with those obtained with other methods.

Study of plasmodial phospholipid biosynthesis

Labeling assays. Erythrocyte suspensions, either infected or not, were washed several times in Ringer's buffer. The erythrocytes were incubated at 20% hematocrit in 500 µl of Ringer's buffer containing 50 µM [^{14}C]choline (0.0469–0.056 Ci/mmol), or 10 µM [^{3}H]ethanolamine (2.4 Ci/mmol) or 38.7 µM [^{14}C]serine (0.0568–0.16 Ci/mmol). Suspensions were incubated at 37°C for

4 h *(P. falciparum)* or 3 h (*P. berghei* and *P. vinckei*). Reactions were stopped at 4°C and cell suspensions were washed twice with 0.9% cold NaCl.

The cellular lipids were extracted according to the procedure of Folch (32) as modified by Rock (33). The organic and aqueous phases of Folch's extract were evaporated and the dried materials were dissolved in 100 µl chloroform/methanol (2:1) and 100 µl ethanol/water (1:1), respectively.

The organic phase was fractionated by silica gel TLC developed with chloroform/methanol/acetic acid/water 65/43/1/3 (Retention Factors LysoPC:0.03, PC:0.13, PS:0.28, and PE:0.7) (34).

Water-soluble metabolites were obtained from the aqueous supernatant and were fractionated by silica gel TLC developed with MetOH/0.5%NaCl/30%NH_4OH 50/50/1 (Retention Factors Cho:0.07, Etn:0.16, P-Cho:0.32, P-Etn:0.57, CDP-Cho:0.71, Ser:0.78, and CDP-Etn:0.84) (35).

Radioactivity was located by autoradiography or by counting one strip of the plate. Kodak BioMax MS Films were obtained from Perkin Elmer (France). In addition, intensifying screens (Kodak BioMax TranScreen LE, Perkin Elmer) were used for TLC of [1-^{3}H]ethanolamine-labeled metabolites. Radioactive spots were scraped off directly into scintillation vials and radioactivity was determined by liquid scintillation counting (Ultimagold, Perkin Elmer) and counted in a Beckman LS 6500 spectrometer. Spots were identified by migration and revelation of the appropriate standards with iodine vapor or ninhydrin reagent. In some cases, appropriate radioactive standards (Cho, P-Cho, CDP-Cho, Etn, CDP-Etn, and Ser) were also located by autoradiography.

Data analysis. In all radioactive determinations, incorporation into a metabolite was considered as significant only when a radioactive spot for the metabolite was detected on the TLC autoradiogram. Otherwise, incorporation was considered as not significant and the threshold of incorporation, defined as twice the radioactive background of the TLC plate, is indicated. The amounts of labeled precursors incorporated into water-soluble metabolites and lipids were computed on the basis of the incorporated radioactivity and specific activity of precursors in the incubation medium. When [U-^{14}C]serine was incorporated into Etn, P-Etn, PE, P-Cho, or PC, radioactivity was corrected for the loss of one radioactive carbon unit.

The results are expressed as nmol/10^{10} cells/h and are means of at least two independent experiments with independent batches of erythrocytes and solutions conducted in triplicate. In the case of parasitized cells, the values were corrected for the activity of unparasitized cells present in each preparation. This subtraction was not valid when the part detected in infected erythrocytes was not overriding relative to the part in uninfected erythrocytes.

RESULTS

Phosphoethanolamine N-methyltransferase gene is identified in human, simian and bird *Plasmodium* species but not in rodent malaria parasites

Orthologous genes of *PfPMT* were sought in sequenced *Plasmodium* genomes using conventional sequence comparison methods (BLAST comparison, using BLOSUM62, with an e-value cutoff of $1e^{-10}$) and were only detected in *P. vivax*, *P. knowlesi*, *P. reichenowi*, and *P. gallinaceum*, i.e., human, simian, and bird *Plasmodium* species. We did not detect any similar sequences in genome shotguns of *P. berghei*, *P. yoelii*, or *P. chabaudi*, i.e., rodent malaria parasites.

In *P. vivax* and *P. knowlesi*, *PMT* genes were previously annotated, i.e., Pv083045 (PlasmoDB) and PKH_121150 (GeneDB), respectively, based on automatic gene predictions. The predicted sequence for *PvPMT* cDNA was confirmed by alignment with two EST (expressed sequence tag) sequences (EST939228, EST921231) obtained from the dbEST NCBI database (36), supporting the functional expression of *PMT* in *P. vivax*. At the date of writing, no EST corresponding to *PkPMT* has been identified. *P. reichenowi* and *P. gallinaceum* genomes have only been partially sequenced (genome shotgun) and not yet annotated (data from the Sanger Institute sequencing projects). In both species, we detected *PMT* genes based on conventional BLAST searches. We reconstructed the *P. reichenowi* gene based on overlapping genomic fragments. *PrPMT* and *PfPMT* DNA sequences were almost identical, thus allowing us to propose a gene model with a spliced sequence for *PrPMT*. Likewise, the *PgPMT* gene model was reconstructed based on the assembly of overlapping genomic fragments and contig sequences, leading to a similar gene structure. In both *PrPMT* and *PgPMT*, the assembly of overlapping fragments allowed us to correct minor sequencing inaccuracies (e.g., frame shifts) at the ends of read or contig sequences. Protein sequences encoded by *PrPMT* and *PgPMT* exhibit a high level of sequence similarity with the PfPMT sequence (97 and 71% identity, respectively). These five deduced PMT proteins have almost the same length (263 to 266 amino acids) and share over 62% sequence identity (**Fig. 1A**). The positions of all exon boundaries are conserved among protein sequences, thus supporting the ORF predictions of four exons. Like PfPMT, the four other PMT have a single SAM-binding domain consisting of four conserved catalytic motifs (I, postI, II, III) (11, 37). The molecular phylogeny of PMT genes was inferred by an ML method, using the PHYML program (25) (Fig. 1B). Confidence in the resulting tree topology was assessed by bootstrap values, compared with those obtained with other methods, i.e., MP, NJ, and BM inferences (Fig. 1B).

To our knowledge, no *PMT* gene has ever been annotated in any rodent malaria parasite. We scanned all available genomic sequences from rodent *Plasmodium* species using the genomic, cDNA or protein sequence of the full-length *PfPMT* or of the catalytic domain. No homologous sequence was identified, based on BLAST searches, in any contig, unassembled genome, EST, or protein sequence database. Rodent malaria genomes have been partially sequenced and some gaps remain between adjacent contigs (38, 39). It is, therefore, possible that still unsequenced *PMT* genes have been missed in rodent *Plasmodium* species.

Syntenic regions corresponding to the genomic context of phosphoethanolamine N-methyltransferase gene in *P. falciparum* indicate an intra-syntenic deletion in rodent malaria parasites

To determine whether the *PMT* gene is really absent from rodent *Plasmodium* genomes, we analyzed the correspond-

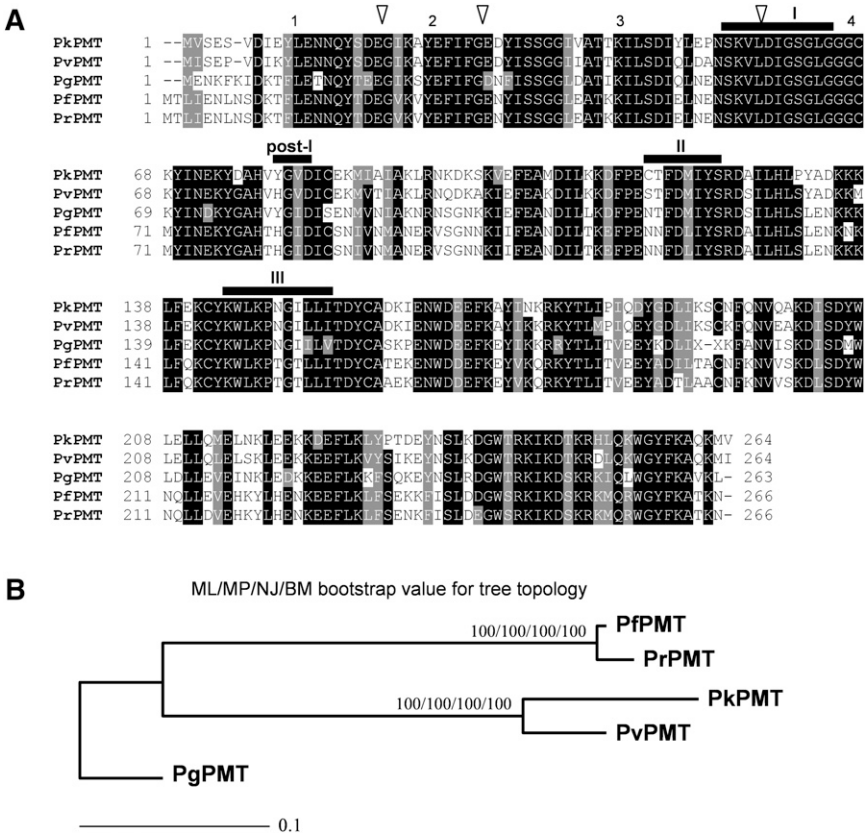

Fig. 1. Alignment and phylogenetic trees of phosphoethanolamine N-methyltransferases from nonrodent *Plasmodium* species. A: Multiple alignment of amino acid sequences deduced from annotated genes (*PfPMT*, *PvPMT*, and *PkPMT*) or gene models reconstructed from this study (*PgPMT* and *PrPMT*). Alignment of the translated sequences was performed using ClustalW and BoxShade. Identical residues are shaded in black and similar residues are shaded in gray. The length (in amino acids) is indicated. The arrowheads mark the conserved exon boundaries and the number of each exon is given above the alignment. The horizontal bars correspond to the four conserved motifs (I, postI, II, III) of the SAM-binding domain (11). B: Four phylogenetic methods, including maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ) and Bayesian method (BM), were used. ML, MP, NJ, and BM inferences were performed using PHYML, PROTPARS in the PHYLIP package, NEIGHBOR, and MrBayes, respectively. The level of bootstrap support was inferred by 100 resamplings of the alignment. As *P. gallinaceum* is a taxon responsible for artifacts of long-branch attraction and problems associated with extreme base compositions (6), the PgPMT sequence was set as an outgroup for the tree constructions. The PMT phylogenetic tree was then first computed using the ML method, and the resulting tree topology was assessed by bootstrap values compared with those obtained with MP, NJ, and BM methods.

ing syntenic regions. Syntenic regions are genomic segments from different species in which the gene content and order have been conserved throughout evolution. Thus, we compared the genomic context in *P. falciparum* chromosome XIII with syntenic regions recovered from available sequenced genomes (*P. vivax*, *P. knowlesi*, *P. chabaudi*) or from contigs and sequence fragment assemblies (**Fig. 2**). The *PMT* genomic position observed in *P. falciparum*, downstream of PF13_0235 and upstream of MAL13P1.215 (two genes of unknown function), is strikingly conserved in nonrodent plasmodia. The PF13_0235 and MAL13P1.215 genes are also conserved in rodent *Plasmodium*, but the homologous genes are strictly contiguous with no trace of any gene (or pseudogene) separating them (Fig. 2).

We compared the molecular phylogenies of *PMT* flanking genes. Confidence in the tree topology resulting from the ML inferences was assessed by bootstrap values compared with those obtained with other methods, i.e., MP, NJ, and BM inferences. **Figure 3** shows the molecular phylogenies of *PMT* genes and the upstream PF13_0235 and downstream MAL13P1.215 sequences reconstructed using available *Plasmodium* information. The tree topology considering nonrodent sequences is conserved, e.g., the sister species *P. knowlesi* and *P. vivax*, and is consistent with the current view of the *Plasmodium* genus phylogeny (6). Considering PF13_0235 and MAL13P1.215, and including homologs from *P. berghei*, *P. yoelii*, and *P. chabaudi*, the obtained tree topology is also conserved with a group corresponding to rodent sequences (framed in light gray in Fig. 3). These local phylogenomic analysis findings provided further support in favor of a shared evolution for this genomic region.

Thus, the *PMT* gene was not detected in any unassembled genomic sequence of any rodent parasite nor in the genomic context conserved between mammalian *Plasmo-*

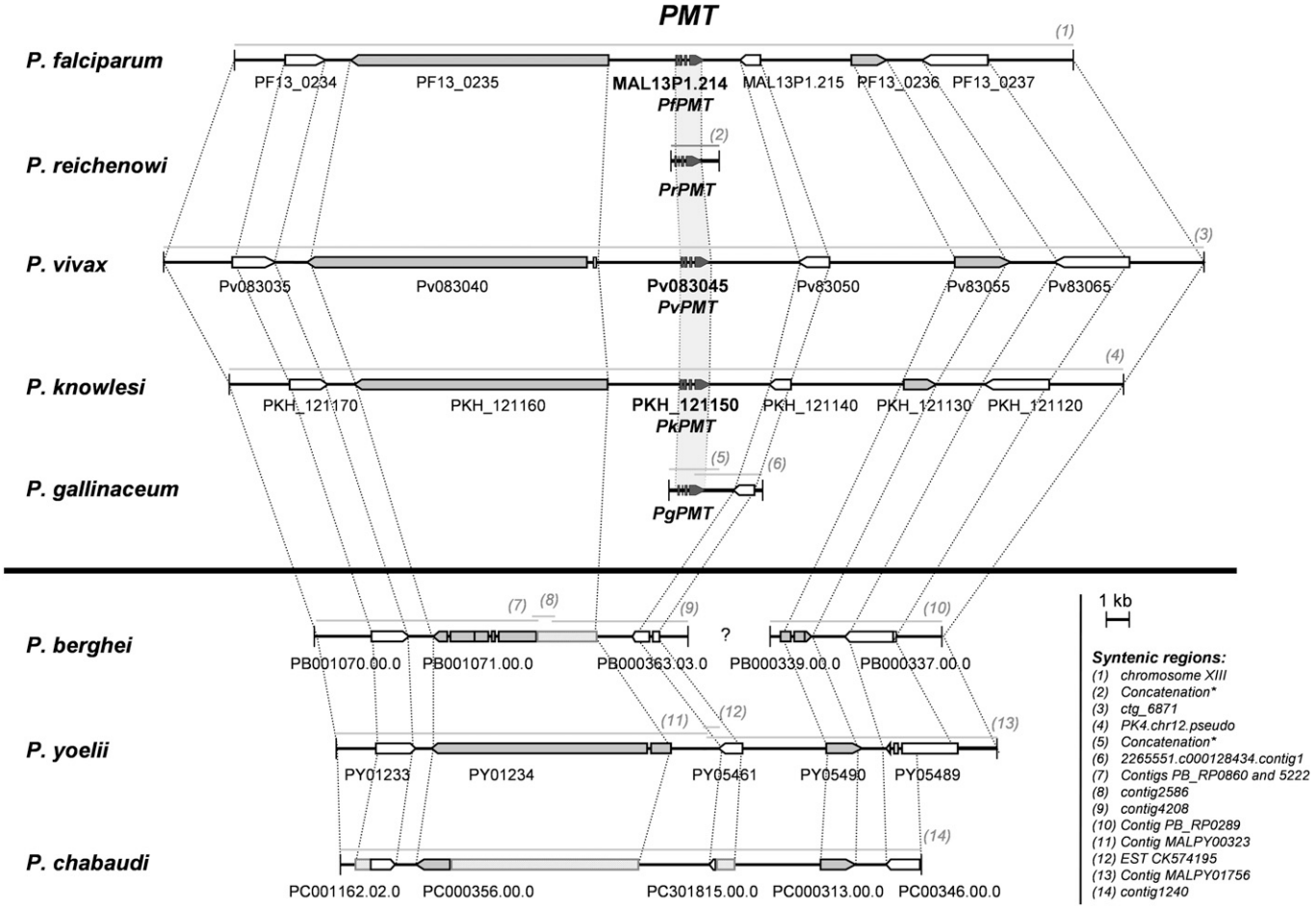

Fig. 2. Syntenic regions corresponding to the genomic context of the phosphoethanolamine N-methyltransferase gene in *P. falciparum* in nonrodent and rodent malaria parasites. Conservation of the genomic context of *PfPMT* on *P. falciparum* chromosome XIII (including PF13_0234, PF13_0235, *PfPMT*/MAL13P1.214, MAL13P1.215, PF13_0236, and PF13_0237 ordered genes) was sought in *P. reichenowi*, *P. vivax*, *P. knowlesi*, *P. gallinaceum*, *P. berghei*, *P. yoelii*, and *P. chabaudi* based on the precomputed genomic comparisons available via the PlasmoDB genome browser, manually validated and revised using additional sequence fragment assembly information. Gene models were corrected based on genomic sequence overlaps and available ESTs. * Concatenation of shotgun sequence reads: *(2)* reich168f02.q1k, reich361b12.p1k, reich361b12.q1k, reich239b04.p1k and reich246a01.q1k;*(5)* Pgal1168b04.q1k, Pgal0396b09.p1k, Pgal1168b04.p1k and Pgal0848d12.p1k.

dium species and in which the *PMT* gene is identified in nonrodent parasites.

Labeling experiments reveal that the phosphoethanolamine N-methyltransferase pathway is lacking in *P. berghei* and *P. vinckei* rodent malaria parasites

In silico data strongly suggested that the orthologous gene *PMT* was lacking in the different sequenced rodent malaria parasites. However, we might have been unable to detect the gene, or an unrelated gene coding for phosphoethanolamine N-methyltransferase activity could exist in these genomes. Biochemical experiments were carried out in an attempt to confirm the findings of the syntenic studies dealing with the *PfPMT* gene. Metabolic studies were performed with human *P. falciparum* and rodent *P. berghei* and *P. vinckei* malarial parasites. Using radioactive ethanolamine as precursor was the most relevant way to reveal P-Etn methylation through P-Cho formation. The labeling experiments were also performed with two other radioactive precursors, i.e., Cho and Ser, to allow us to shed light on the poorly known PL synthesis pathways in these two rodent malaria species (40, 41).

Radioactive labeling assays were carried out with uninfected erythrocytes and mature stages of *P. falciparum*-, *P. berghei*-, or *P. vinckei*-infected erythrocytes, as described in Materials and Methods. The erythrocytes were incubated with radioactive precursors, i.e., [^{14}C]choline, [^{3}H]ethanolamine, or [^{14}C]serine, for 4 h (*P. falciparum*, 48 h cycle) or 3 h (*P. berghei* and *P. vinckei*, 24 h cycle) in Ringer's buffer. The concentrations of [^{14}C]choline, [^{3}H]ethanolamine and [^{14}C]serine in the suspensions (50 μM, 10 μM, and 38.7 μM) were in the same range as the physiological levels, i.e., 10–40 μM, 2.5 μM, and 107 μM, respectively (42–44). The cellular lipid and nonlipid metabolites were extracted and analyzed by silica gel TLC. The metabolites were identified using appropriate standards and quantified as described in Materials and Methods.

The results (**Fig. 4**; supplementary Tables II–IV) highlighted the ability of the three nitrogenous polar heads to

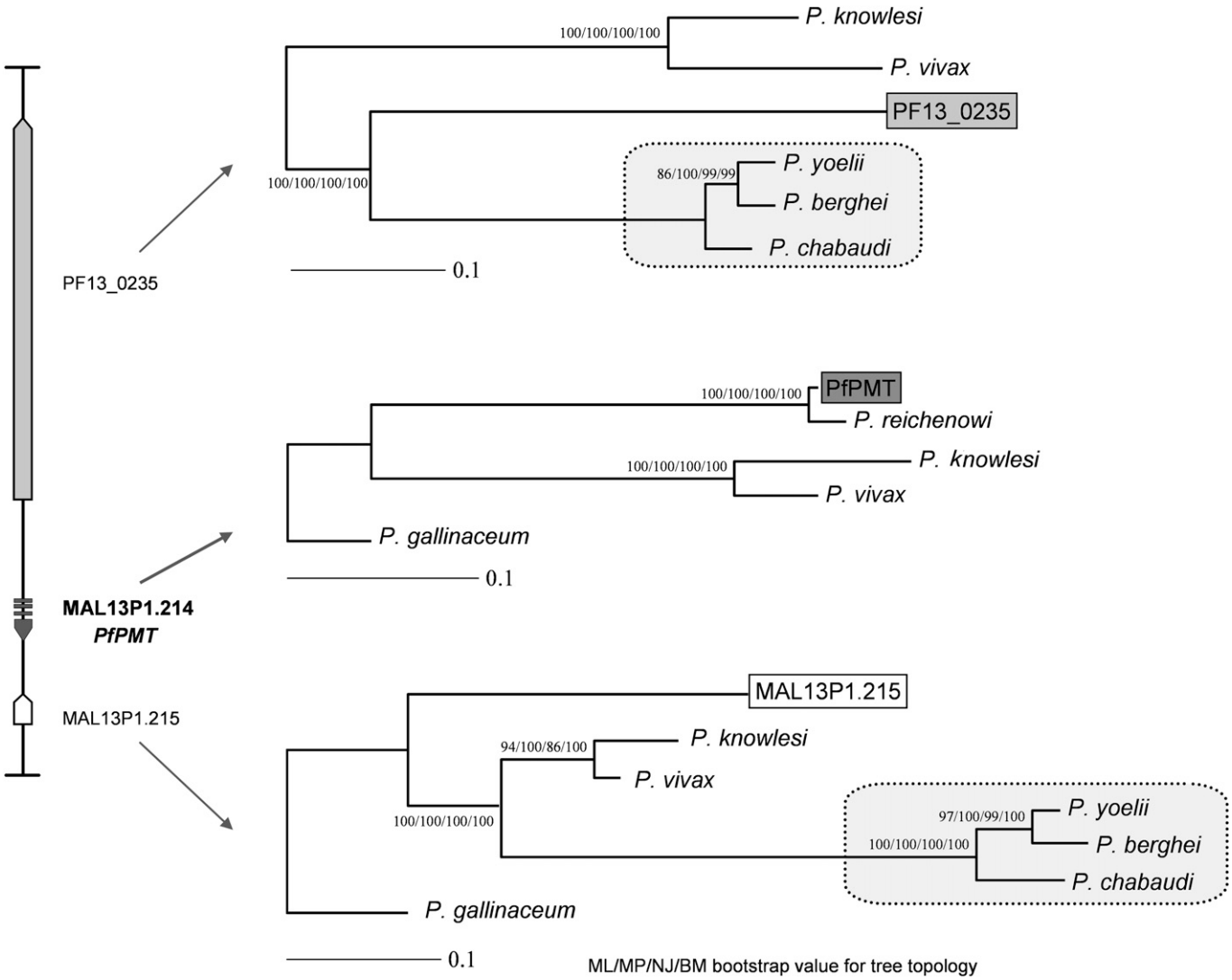

Fig. 3. Phylogenomic analysis of the *Plasmodium* chromosomic region harboring phosphoethanolamine N-methyltransferase and flanking genes. Four phylogenetic methods, including ML, MP, NJ, and BM, were used to infer the gene tree for each set of orthologous genes. ML, MP, NJ, and BM inferences were performed using PHYML, PROTPARS in the PHYLIP package, NEIGHBOR, and MrBayes, respectively. The level of bootstrap support was inferred by 100 resamplings of the alignment. The phylogenetic trees were then first computed using the ML method, and the resulting tree topology was assessed by bootstrap values compared with those obtained with MP, NJ, and BM methods. Rodent sequences are framed in light gray.

be incorporated within their respective PLs and water-soluble metabolic intermediates of normal and infected erythrocytes.

Incorporation of radioactive material into uninfected suspension metabolites was always very low, whereas the infected red blood cells showed a great ability to utilize these three precursors to build up PLs (supplementary Tables II–IV).

Radioactive Cho, Etn, and Ser were present both in uninfected and infected erythrocytes at similar levels. Thus, the precursors recovered within infected erythrocytes could not be precisely quantified and the results are not shown. The other labeled water-soluble metabolites were restricted to infected erythrocytes or were weakly recovered within uninfected erythrocytes and the amounts could then be calculated (see Materials and Methods), particularly for infected erythrocytes.

In both human and mouse uninfected erythrocytes, when using [^{14}C]choline and [^{3}H]ethanolamine, significant amounts of their phosphorylated metabolites (P-Cho and P-Etn, respectively) were detected (supplementary Tables II, III), which was probably due to nonspecific kinase activity. Furthermore, with [^{14}C]choline, some CDP-Cho and PC were only detected in uninfected mouse erythrocytes and not in human erythrocytes (supplementary Table II). Similarly, when using [^{3}H]ethanolamine and [^{14}C]serine, small amounts of CDP-Etn and PS were recovered within unparasitized mouse erythrocytes, respectively (supplementary Tables III, IV). These observations were likely related to the presence in mouse blood of immature erythrocytes (i.e., reticulocytes, stained in light blue by Giemsa), in which residual PL biosynthesis still occurs (45, 46).

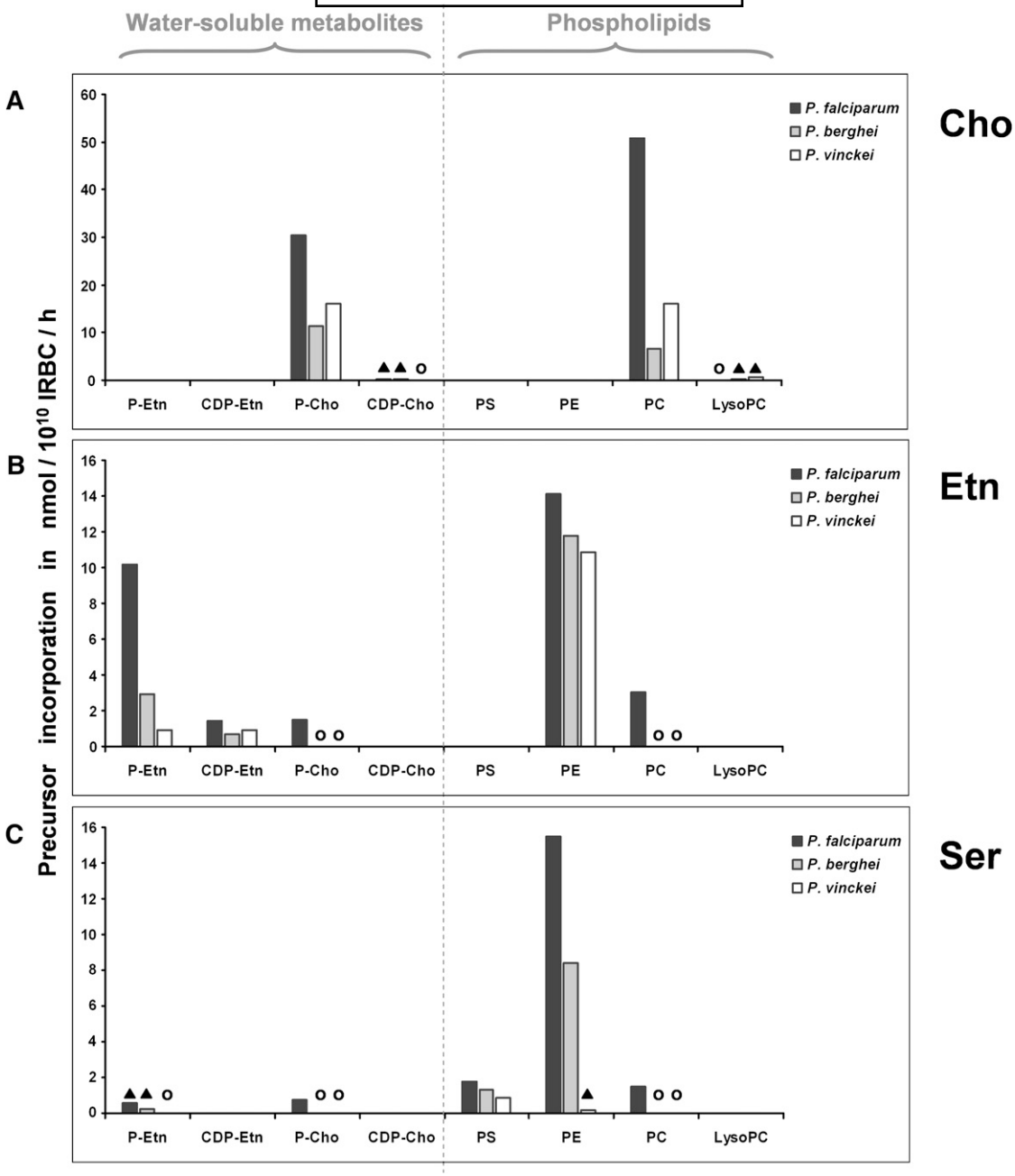

Fig. 4. Differential incorporation of [^{14}C]choline (A), [^{3}H]ethanolamine (B) and [^{14}C]serine (C) into water-soluble metabolites and phospholipids for the three *Plasmodium* species. The incorporation of 50 μM-[^{14}C]choline, 10 μM-[3H]ethanolamine or 38.7 μM-[^{14}C]serine were measured after 4 h (*P. falciparum*) or 3 h (*P. berghei* and *P. vinckei*) incubations at 20% hematocrit. Incorporation into a metabolite was considered as significant only when a radioactive spot for the metabolite was detected on the TLC autoradiogram. Otherwise, incorporation was considered as nonsignificant (nought) and indicated by **o** (see Fig. 5 and supplementary Tables II–IV). Triangles (▲) indicate small but significant radioactive spots. The results are expressed as nmol/10^{10} cells/h and are means of at least two independent experiments conducted in triplicate. Values between independent experiments differed by less than 50%. In the case of infected red blood cells (IRBC), the values were corrected for the activity of uninfected red blood cells (RBC) present in each preparation.

It should be noted that CDP-Cho and CDP-Etn metabolites were hard to detect by TLC throughout this study due to the low levels in infected erythrocytes. Indeed, these metabolic intermediates are produced at limiting steps catalyzed by CTP:phosphocholine cytidylyltransferase (CCT) and CTP:phosphoethanolamine cytidylyltransferase (ECT), respectively, and immediately transformed into their respective PLs (47, 48).

Cho incorporation

Cho was highly incorporated into phospholipid PC, particularly in *P. falciparum* (51 nmol/10^{10} infected cells/h) as compared with the rodent malaria species (7–17 nmol/10^{10} infected cells/h) (Fig. 4A; supplementary Table II). As expected, Cho was recovered into water-soluble metabolic intermediates, P-Cho and CDP-Cho in *P. falciparum*. Cho was less incorporated into these metabolites in the rodent

Plasmodium species. Thus, Cho incorporation into PC metabolic intermediates was qualitatively similar between *P. falciparum* and *P. berghei*/*P. vinckei* species. However, CDP-Cho was not detected for *P. vinckei* by TLC.

Interestingly, we also noted the presence of some LysoPC in both rodent malaria species but the amounts were nonsignificant in *P. falciparum*.

Etn incorporation

Etn was incorporated into PL metabolites via differential biosynthesis pathways in the studied *Plasmodium* species (Fig. 4B; **Fig. 5**; supplementary Table III).

The precursor was highly incorporated into the phospholipid PE in the three malaria species *P. falciparum*, *P. berghei*, and *P. vinckei* with very similar incorporation rates of 14, 12, and 11 nmol/10^{10} infected cells/h, respectively (Fig. 4B). Etn was also clearly incorporated into PC only in *P. falciparum* (3 nmol/10^{10} infected cells/h), whereas incorporation in both rodent malaria species could not be detected (<0.04 nmol/10^{10} infected cells/h) (Fig. 5B). This indicated that both PMT and PEMT activities were not detected with the precursor Etn in the rodent *Plasmodium* species.

As expected, significant incorporations of radioactive Etn were also recovered in the water-soluble PE metabolic intermediates, P-Etn and CDP-Etn in *P. falciparum* and, to a lesser extent, in the rodent *Plasmodium* species (Fig. 4B). As previously described (11, 49), radioactive Etn was also metabolized into the PC metabolic intermediate P-Cho in the single *P. falciparum* species (1.5 nmol/10^{10} infected cells/h), which reflected the *P. falciparum* PMT activity (Fig. 5A). In this species, CDP-Cho was not detected as the low level of this metabolite was below the detection threshold. On the other hand, no radioactive P-Cho could be detected in both rodent malaria species (Fig. 5A), with the incorporation being lower than 0.06 nmol/10^{10} infected cells/h. These results indicate the likely absence of PMT activity that would transform the precursor Etn into Cho metabolites in both rodent *Plasmodium* species.

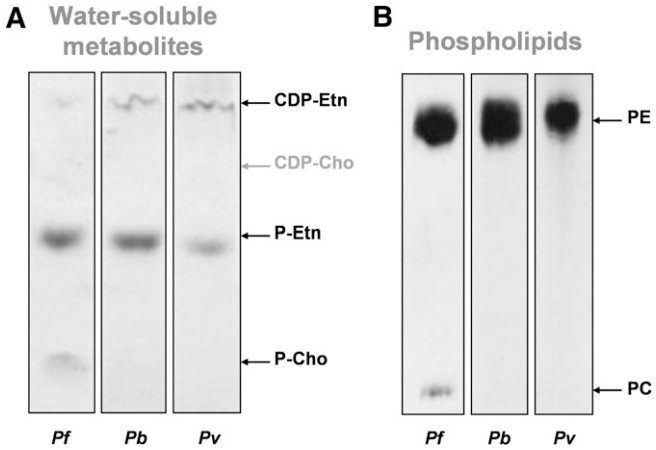

Fig. 5. TLC autoradiographies of water-soluble metabolites (A) and phospholipids (B) from infected erythrocytes labeled with [^{3}H]ethanolamine. Spots were identified by migration and revelation of appropriate standards (marked by arrows) with iodine-vapor or ninhydrin reagent. In some cases, appropriate radioactive standards (Cho, P-Cho, CDP-Cho, Etn, CDP-Etn, and Ser) were also located by autoradiography. Labeled extracts, containing an equal amount of radioactivity (in dpm), were fractionated on the same TLC plate. Chosen autoradiograms are representative of the different TLC performed for metabolite quantifications. *Pf*: *P. falciparum*, *Pb*: *P. berghei*, *Pv*: *P. vinckei*.

Ser incorporation

Incorporation of Ser into cellular lipids of *P. falciparum* was characterized by simultaneous labeling of PS, PE, and PC, with the respective incorporation of 1.8, 15.5, and 1.5 nmol/10^{10} infected cells/h (Fig. 4C; supplementary Table IV). In *P. berghei*, radioactive Ser was also incorporated in high amounts only into the first two phospholipids, PS and PE (1.3 and 8.4 nmol/10^{10} infected cells/h, respectively), whereas in *P. vinckei*, incorporation into PS was lower and the incorporation into PE was barely detectable (0.9 and 0.2 nmol/10^{10} infected cells/h, respectively). It was striking that radioactive Ser was metabolized into PC solely in *P. falciparum* (1.5 nmol/10^{10} infected cells/h) but not in *P. berghei* and *P. vinckei* rodent species (<0.1 and <0.04 nmol/10^{10} infected cells/h, respectively).

Ser was incorporated into the water-soluble PE metabolic intermediates Etn and P-Etn in *P. falciparum*, as previously reported (10). Radioactive Etn could not be precisely quantified and the amounts are, thus, not shown. Indeed, during the washing steps, some leakage of this freely diffusible water-soluble metabolite was observed. The radioactive precursor was also clearly metabolised into P-Etn in *P. berghei* (Fig. 4C). Tiny spots, probably corresponding to Etn, were also visible on the TLC autoradiogram (data not shown). In *P. vinckei,* no PE metabolic intermediates were identified. Ser was also incorporated into the PC metabolic intermediate P-Cho (0.7 nmol/10^{10} infected cells/h) in the single *P. falciparum* species.

DISCUSSION

The malaria parasite requires a considerable amount of PLs at the blood stage. Analysis of the lipid content of purified parasites led to the identification of PC and PE as the two major PLs in the parasite membranes. PC represents 40–50% of the total PL content, whereas PE represents 35–45%, which is unusually high for a eukaryotic organism (48, 50–52). Previous studies on *P. berghei* and *P. vinckei* rodent plasmodia have shown similar drastic changes in PL amounts and composition upon erythrocyte infection (40, 41). Because the exchange of preformed PLs does not account for a substantial part of the entire PLs found in infected erythrocytes, the parasite relies on a productive PL biosynthetic machinery [for a recent review see ref. 8 and for a detailed scheme of lipids and PL metabolic pathways, see the Malaria Parasite Metabolic Pathways database (53)].

This study combined both in silico determinations and biochemical experiments to investigate an essential PL metabolism based on the bewildering variety of biosynthetic pathways already reported in *P. falciparum*.

The pathway scheme appeared to be quite different between *Plasmodium* species (**Fig. 6**). In particular, the human

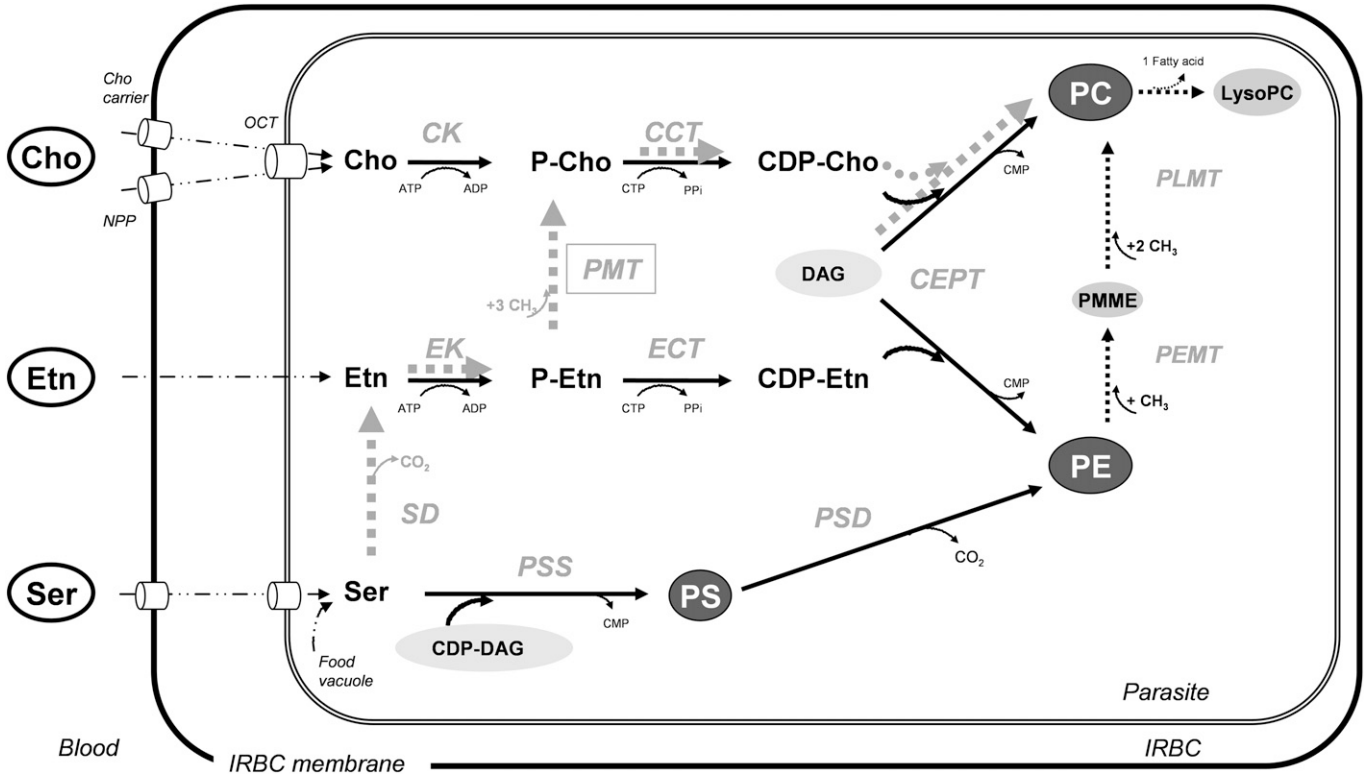

Fig. 6. Scheme of the biosynthesis pathways for PC, PE, and PS in human *P. falciparum*- and rodent *P. vinckei*- and *P. berghei*-infected erythrocytes. Cho entry into the infected erythrocyte implies the erythrocytic choline carrier and the New Permeation Pathway (NPP) (86–88). Cho is provided to the parasite by a characterized Organic-Cation Transporter (OCT) (89) encoded by unknown genes. Etn enters RBC mainly by passive diffusion. Ser is diverted from the host RBC and transported into the parasite, or from hemoglobin degradation in the food vacuole. Pathway steps shared by *P. falciparum* and rodent malaria species are indicated by solid arrows (see text for details). PMT and PEMT (unknown genes) activities were only detected in *P. falciparum*; SD activity was only detected in *P. falciparum* and weakly in *P. berghei*; and LysoPC was recovered only into rodent *Plasmodium* IRBC (see text for details). These steps are marked with dotted arrows. The SDPM pathway is differentiated by gray arrows (dotted). Lipids are shaded in gray. Enzymes are in gray italics. DAG, diacylglycerol; CDP-DAG, cytidine-diphospho-diacylglycerol; PMME, phosphatidyl-*N*-methylethanolamine; CK, choline kinase; CCT, CTP:phosphocholine cytidylyltransferase; EK, ethanolamine kinase; ECT, CTP:phosphoethanolamine cytidylyltransferase; CEPT, choline/ethanolamine-phosphotransferase; PSS, phosphatidylserine synthase; PSD, phosphatidylserine decarboxylase; PEMT, phosphatidylethanolamine *N*-methyltransferase; PLMT, phosphatidyl-*N*-methylethanolamine *N*-methyltransferase; SD, serine decarboxylase; PMT, phosphoethanolamine N-methyltransferase.

parasite possesses a plant-like pathway (SDPM) that relies on SD activity and a *P. falciparum* phosphoethanolamine N-methyltransferase enzyme (PfPMT), which represents an additional way for supplying PC to the intraerythrocytic parasite. Based on both syntenic and labeling studies, the PMT pathway did not appear to exist in *Plasmodium* species infecting rodents.

Orthologous genes of *PfPMT* have been clearly found in other phylogenetically-linked *Plasmodium* species invading humans (*P. vivax*), primates (*P. knowlesi* and *P. reichenowi*), and birds (*P. gallinaceum*), whereas we could not detect any similar sequences in genome shotguns of *P. berghei*, *P. yoelii*, or *P. chabaudi*, i.e., rodent malaria parasites. PfPMT and the four other putative proteins of nonrodent plasmodia are strongly conserved (Fig. 1). The gene is expressed in *P. falciparum* and at least transcribed in *P. vivax*.

Furthermore, syntenic regions in nonrodent malaria parasites (*P. falciparum*, *P. vivax*, and *P. knowlesi*) showed a complete conservation of genes in the *PfPMT* context. Syntenic regions in rodent malaria parasites (*P. berghei*, *P. yoelii*, and *P. chabaudi*) showed conservation of all genes except *PMT* (Fig. 2). The findings of a local phylogenomic analysis of *PfPMT* and upstream/downstream genes indicated a shared evolution for this genomic region (Fig. 3).

Together, the results of these analyses showed that the lack of *PMT* homologous gene at the expected locus in rodent *Plasmodium* species was likely due to an early deletion correlated with the separation of rodent from nonrodent *Plasmodium* branches (1, 54, 55). This gene deletion event is crucial in the current understanding of *Plasmodium* evolution, because it is now sure that *P. falciparum* did not arise as a result of lateral transfer between avian and human hosts as first proposed by Waters et al. (5), in spite of very distinct dipterous vectors. The findings of this study indicated that it is also very unlikely that *P. falciparum* arose as a result of lateral transfer between a rodent and human host, although these *Plasmodium* species are vectored by phylogenetically close *Anopheles* mosquitoes.

Phosphoethanolamine N-methyltransferase activity and, more generally, PL biosynthesis, were investigated in different *Plasmodium* species through labeling studies using the three polar heads Cho, Etn, and Ser as radioactive

precursors. Etn and Ser are more specific for assessing methylations of P-Etn into P-Cho, provided that the water-soluble intermediate metabolites derived from each precursor are simultaneously analyzed.

The experiments were carried out in *P. falciparum* as a positive control of PMT activity and in two rodent malaria parasites, i.e., *P. berghei* and *P. vinckei*. The human parasite has a 48 h blood cycle as compared with the 24 h cycle for rodent malaria species. *P. falciparum* and *P. vinckei* are largely reported to infect mature erythrocytes, whereas *P. berghei* has a clear preference for reticulocytes, i.e., immature blood erythrocytes (56, 57). The *P. berghei* genome has been sequenced (39) whereas no data is available for *P. vinckei*. Experiments were performed in a chemically defined medium in the absence of serum allowing quantification of the incorporated precursors, which were used at physiological concentrations.

Incorporation of the three precursors in uninfected erythrocytes was absent or very low in human erythrocytes but significant and marked in some cases in rodent erythrocyte preparations, probably due to the presence of reticulocytes from mouse blood. In the case of infected cells, the values were corrected for the activity of unparasitized cells present in each preparation. Incorporation of the polar heads into the three main PLs and the water-soluble intermediate metabolites was always substantially higher in infected cells than in control unparasitized erythrocytes, (at least 27-fold increase, except for the 2-fold increase noted in *P. vinckei*-infected erythrocytes incubated with labeled serine) due to the presence of the intracellular parasite.

This study provided evidence that in *P. berghei* and *P. vinckei* rodent malaria parasites both PMT and PEMT pathways were lacking.

Indeed, incorporation of labeled Etn showed that PMT activity was clearly present in *P. falciparum* (Figs. 4B, 5A). In the rodent malaria species, both PMT and PEMT activities were not detected with this precursor. As for the human parasite, no gene encoding for a PEMT was found in the rodent *Plasmodium* genomes.

The incorporation of radioactive Ser indicated the involvement of other PL biosynthesis pathways. In *P. falciparum*, it has been shown that Ser is a precursor of phosphatidylserine (PS), a minor PL (less than 5% of the PL content) (10, 58). This reaction uses CDP-diacylglycerol (CDP-DAG) as a lipid donor and is catalyzed by a phosphatidylserine synthase enzyme (PSS), which is not yet characterized (10) (Fig. 6). A large amount of PS is rapidly decarboxylated into PE (10, 14, 58) by a previously characterized PS decarboxylase enzyme (PSD) (59). Both genes have been identified in the *P. falciparum* genome and are also annotated in the *P. berghei* GeneDB database (supplementary Table V). In *P. falciparum*, as mentioned above, Ser can be decarboxylated into P-Etn (10), and the corresponding gene of SD has not yet been identified.

The results obtained with [^{14}C]serine (Fig. 4, C; 5, B) showed that: *i*) Ser was incorporated into PS in the three species, as expected. *ii*) Ser was also recovered into PE in human and rodent plasmodia. However, in *P. vinckei*, the amount incorporated into PE was very low, representing 2% of the labeled PE amount in the rodent counterpart *P. berghei* (42-fold less). In *P. falciparum*, we clearly detected the biosynthesis of PE that resulted at least partially from decarboxylation of Ser into Etn, which was then integrated into the de novo PE pathway. In *P. berghei*, weak incorporation of labeled Ser into Etn and P-Etn was observed, but additional experiments are needed to confirm the decarboxylation of Ser as in the case of *P. falciparum* (10). In *P. vinckei*, the inability to detect this last step was probably related to the fact that very little Ser was incorporated into PE and, thus, all of the putative metabolic intermediates would be produced in too low amounts to be detected by TLC. PE produced through the decarboxylation of PS (Fig. 6) could not be specifically identified with this labeling even though the presence of the corresponding gene highly indicates the presence of this pathway. *iii*) Ser was recovered into P-Cho and PC in *P. falciparum* only. This confirmed that PMT and PEMT activities were not detected with either Ser or Etn and that these alternative pathways were lacking in *P. berghei* and *P. vinckei* species. Furthermore, we provide, for the first time, evidence that Ser can be incorporated into PC via the so-called SDPM pathway in *P. falciparum* (8). Indeed, previous studies only separately identified the SD pathway with Ser (10, 58) and the PMT pathway with Etn (17, 60, 61).

[^{14}C]choline was incorporated at some level into LysoPC in rodent malaria parasites, particularly in *P. vinckei* (9-fold increase, compared with *P. falciparum*). LysoPC can be produced by a phospholipase (A2) or by a phosphatidylcholine-sterol acyltransferase (LCAT). Both genes are annotated in PlasmoDB (PFB0410c and PFF1420w, respectively), and the orthologous genes were found in rodent and nonrodent *Plasmodium* species. Phospholipase A2 activity was detected in *P. falciparum*-infected erythrocytes but not in uninfected erythrocytes (62). Conversely, cholesterol esters produced by LCAT activity were not detected during intraerythrocytic development of the human parasite (63). However, this activity could take place at some level in gametocytes, as this gene is also transcribed at this stage (PlasmoDB data). It should be noted that some gametocytes were naturally present in *P. vinckei*- and *P. berghei*-infected mouse blood, although it is difficult to give a precise percentage. These particular stages could explain this unusual amount of LysoPC.

Finally, no sphingomyelin was detected in the three *Plasmodium* species as previously shown for *P. falciparum* and *P. knowlesi* (58, 64).

To our knowledge, this is the first study to reveal a crucial difference in phospholipid metabolism between *Plasmodium* species. This feature is one of the very few examples of basic metabolism that distinguish malarial species at the asexual intraerythrocytic stage, such as the mechanisms of hemoglobin degradation (65–67). Differences between species-specific acyl-CoA synthetase gene families have also been studied (68). A list of species-specific genes of *P. falciparum* or rodent plasmodia was published by Kooij et al. (7).

This likely reflects different host-parasite interactions but the difference does not concern the capacity to invade

reticulocytes, which possess residual lipid metabolism, because the rodent *P. vinckei* species preferentially invades mature erythrocytes (69).

Acquisition of the *PMT* gene in the *Plasmodium* genus and its probable deletion specifically in rodent *Plasmodium* species remains unclear.

The *PMT* gene was first identified in higher plants such as *Arabidopsis thaliana* (18) and, until recently, was considered to be a plant-like feature. However, it was then reported in the lower eukaryote *P. falciparum* (11, 16). It is interesting to note that *Plasmodium* species possess a plastid called apicoplast, derived from a secondary endosymbiosis involving a red alga ancestor (for review, see refs. 70–72). Some genes contained in the original alga have been translocated to the nuclear genome of the parasite and we assume that the *PMT* gene was acquired following such horizontal transfer. Since then, two copies of the gene were identified in the worm *Caenorhabditis elegans* (19, 20). Interestingly, some PMT activity was detected in chicken and rat brains but has not been further studied (73–75).

To extend our study and gain some insight into the acquisition of the *PMT* gene in eukaryotes, we performed additional in silico searches for this gene in other organisms, such as in the Apicomplexa phylum, in other protists, in Eubacteria and Archaea. It should be noted that the characterized PMT proteins exhibited the four conserved motifs of the SAM-binding domain (**Fig. 7**), common to the SAM-dependent methyltransferase family, but the features involved in the P-Etn substrate recognition are not known. This absence of specific PMT motifs complicates sequence searches, resulting in the retrieval of methyltransferases, which are not specific to P-Etn substrate. Characterized PMT proteins are members of the *Methyltransf_11* PFAM family (PF08241) (76) and constitute more specifically the SYSTERS (SYSTEmatic Re-Searching) Protein Family (Cluster) 141933 (77), data that allowed us to refine the searches. In sequenced Apicomplexa genomes, beyond the *Plasmodium* genus, no PMT sequence was annotated or could be detected by BLAST searches. Interestingly, putative *PMT* genes were found in several other organisms (Fig. 7, supplementary Fig. I), particularly in species of fishes and frogs, and in the bacterium *Burkholderia pseudomallei*, which is the agent of the human disease melioidosis (Fig. 7, supplementary Fig. I). *B. pseudomallei* PMT protein sequence shares 31% sequence identity with *A. thaliana* PMT (AtPMT, spliced variant 1), and 23% with PfPMT and *C. elegans* PMT 2 (CePMT2).

The plant enzyme contains two methyltransferase domains in N- and C-terminal positions, which were shown to catalyze the first and the two last methylation steps, respectively (18) (Fig. 7). *C. elegans* possesses two distinct PMT enzymes, each one exhibiting only one complete catalytic domain and an incomplete SAM-binding domain. These two enzymes were shown to catalyze the first and the last two methylation steps separately and successfully (19, 20). On the other hand, the *P. falciparum* PMT enzyme contains a single methyltransferase domain, which appears to catalyze the three methylations steps (11). The putative PMT of *B. pseudomallei* also exhibits a single but yet uncharacterized catalytic domain. Interestingly, the catalytic domains of PfPMT and BpPMT are more similar to the C-terminal domain of AtPMT and CePMT2 than to the N-terminal domain (over 23% sequence identity) (Fig. 7, supplementary Fig. I). The monodomain enzyme of *P. falciparum* might, therefore, be closer to the ancestral prokaryotic enzyme, and duplication and specialization might alternatively have occurred and generated either specialized enzymes in *C. elegans* or fusion proteins in higher plants. More complex phylogenies could also account for the present occurrence of PMT enzymes in eukaryotes.

Besides, in higher plants and in *P. falciparum* (this work), the PMT enzyme is associated with SD with both enzymes forming a particular metabolic pathway. The *SD* gene is annotated in several higher plants (15, 78) and also in the single celled green alga *Chlamydomonas reinhardtii* (XP_001689659, NCBI REFSEQ). This novel pathway has long been ignored and the SD gene has only been identified and characterized in plants and, therefore, it should now be considered in other organisms.

Therefore, *PMT* genes seem to have a complex evolutionary history and they are markers of a complete phospholipid metabolic pathway. The search for *PMT* genes and their study in other organisms is of considerable interest and highlights the features of this plant-like pathway unexpectedly recovered in an animal cell.

In plants, PMT activity is an additional way to provide Cho to the different organs. This alternative way provides PC and also contributes, in certain plants, to the synthesis of Cho-derived glycine betaine (i.e., trimethylglycine), an osmoprotectant useful for the resistance to osmotic stresses, such as salt stress and cold, hot, or drought acclimation (79–81). In *A. thaliana*, which do not synthesize betaine, silencing of the gene results in multiple morphological phenotypes, including pale-green leaves, early senescence, temperature-sensitive male sterility, and salt hypersensitivity (82). Disruption of the same gene in *A. thaliana* revealed a critical role of PMT activity in root system development and epidermal cell integrity (83). Thus, in plants, the PMT pathway plays an important role in plant growth and development and also contributes to tolerance to environmental stresses. In *C. elegans*, both genes *pmt-1* and *pmt-2* are essential for growth and development (19, 20).

In *P. knowlesi*, no betaine formation was detected at the erythrocytic stage (47). On the other hand, in *P. falciparum*, the PMT pathway is an alternative route to provide PC to the parasite (49, 60, 61). The *PfPMT* gene is not essential for *P. falciparum* survival at the blood stage even though disruption of the gene results in severe growth defects (61).

The *PMT* gene is, thus, present in *P. falciparum* and other primate and bird plasmodia but absent in *P. berghei*, *P. vinckei*, *P. chabaudi*, and *P. yoelii* rodent malaria parasites. As a result of the loss of the *PMT* gene and activity and the probable absence of PE methylation into PC, the

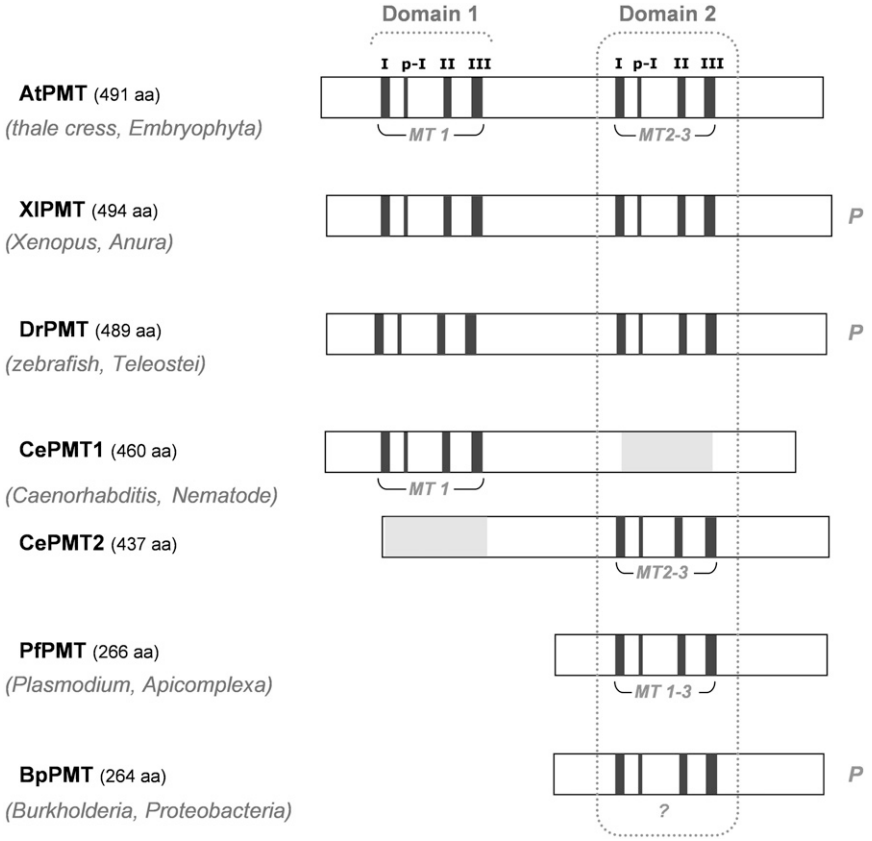

Fig. 7. Schematic comparison of the domains of different annotated PMT proteins. PMT proteins of *A. thaliana* (AtPMT, spliced variant 1, NP_188427, NCBI-REFSEQ), of *C. elegans* (CePMT1: NP_871998, CePMT2: NP_504248) and *P. falciparum* (MAL13P1.214, PlasmoDB) have been previously characterized (11, 18–20) but not the putative (*P*) PMT of the zebrafish *Danio rerio* (DrPMT, NP_001070105), the African clawed frog *Xenopus laevis* (XlPMT, NP_001087172), and the pathogenic bacteria *Burkholderia pseudomallei* K96243 (BpPMT, YP_111122). The four conserved motifs (I, postI, II, III) of the SAM-binding domain are shown in black (11). CePMT1 domain 2 and CePMT2 domain 1 did not exhibit the complete conserved motifs (19) and are consequently indicated by a gray box. The reaction(s) performed by each domain is/are indicated underneath: first (MT1), second (MT2), or third (MT3) methylation, except for DrPMT, XlPMT, and BpPMT (not determined). The highly conserved domain 2 is framed in gray. The size of the proteins in amino acids (aa) is indicated.

de novo PC pathway is the main and probably sole route for PC synthesis in rodent plasmodia. In another study based on genetic modifications, we obtained evidence that the genes involved in this pathway (CK, CCT, and CEPT, Fig. 6) are all essential at the blood stage of *P. berghei* (Déchamps et al., unpublished observations).

Coincidently with PMT, SD activity was detected in *P. falciparum* and weakly in *P. berghei*. The corresponding but yet unidentified gene has probably been conserved in this rodent malaria parasite. This shows that Ser acts as a PE provider for the parasite via the SD pathway and not only via the PSD pathway (Fig. 6) (10, 14, 58). A previous study suggested that PE lipids acquired from both routes form two separate PL pools (10). Moreover, in *P. berghei*, we obtained evidence, by gene disruption experiments, that genes involved in the de novo PE pathway (ECT and CEPT, Fig. 6) are essential for infected-erythrocyte survival (Déchamps et al., unpublished observations).

Our findings relaunch the debate on the existence and enzymatic nature of the PEMT pathway. As introduced above, PEMT activity is clearly identified in *P. knowlesi* and probably present in *P. falciparum*. On the other hand, some experimental results did not confirm the existence of this pathway and questioned its enzymatic nature. Indeed, deletion of the *PfPmt* gene in *P. falciparum* parasites abolishes the incorporation of Etn into PC (61), and, thereby, also the PEMT pathway. Furthermore, the PfPMT protein (recombinant or expressed in mutant yeast cells) lacks the ability to catalyze the transmethylation of PE (11, 17). Because the corresponding genes coding for PEMT have not been found in any *Plasmodium* species, altogether these findings suggested that PfPMT might have PEMT activity in vivo in particular conditions (which are not met in vitro or in yeast). Our study clearly showed that PEMT activity was not detected in either *P. berghei* or *P. vinckei* rodent malaria parasites. The absence of this metabolic pathway could also be explained by the lack of PMT enzyme in these species.

Because *P. vinckei*-infected erythrocytes have a high PE content (41), the weak recovery of labeled serine into PE

suggests differential PE biosynthesis pathways between the two rodent malaria parasites. This particular feature would be interesting to study in further detail.

Besides, our laboratory has identified Cho analogs (bisthiazoliums) that exert potent antimalarial activity both in *P. falciparum* and *P. vinckei* parasites (9). They are now in phase 2 of clinical development in humans. Pharmacological, biochemical, and structure-activity relationships indicate that these analogs exert their antimalarial activity by inhibiting *Plasmodium* PC biosynthesis. The major primary effect is thought to be the inhibition of Cho transport into the parasite, thus, inhibiting parasite PL biosynthesis. We recently showed, by proteomic and metabolic studies, that the T4 choline analog not only blocks de novo synthesis of PC but also prevents PC biosynthesis that occurs through the SDPM pathway. This is due to the inhibition of enzymes downstream of the formation of P-Cho (ref. 49 and Vial et al., unpublished observations). The *PfPMT* gene is not essential at the erythrocytic stage (61). However, we do not know whether, in *P. falciparum*, the SDPM pathway is an alternative pathway for de novo PC biosynthesis, which could allow the parasite to survive in the absence of exogenous Cho, as suggested by previous in vitro growth assays using dialyzed sera (84, 85). This question should now be investigated through appropriate studies. It should be noted that, in *P. berghei*, the PMT pathway is absent and the three enzymes involved in de novo PC synthesis are essential (Déchamps et al., unpublished observations), suggesting that choline is crucial for parasite survival. To conclude, this study focused on the malaria asexual blood stages and it could be interesting to extend these labeling assays to gametocytes from different *Plasmodium* species. The analysis of phospholipid content of malaria liver and mosquito stages could also be performed to shed light on other host-parasite interactions.

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