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Characterization of *Leishmania major* phosphatidylethanolamine methyltransferases *Lmj*PEM1 and *Lmj*PEM2 and their inhibition by choline analogs

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Abstract

Phosphatidylcholine (PC) is the most abundant phospholipid in the membranes of the human parasite *Leishmania*. It is synthesized *via* two metabolic routes, the *de novo* pathway that starts with the uptake of choline, and the threefold methylation of phosphatidylethanolamine. Choline was shown to be dispensable for *Leishmania*; thus, the methylation pathway likely represents the primary route for PC production. Here, we have identified and characterized two phosphatidylethanolamine methyltransferases, *Lmj*PEM1 and *Lmj*PEM2. Both enzymes are expressed in promastigotes as well as in the vertebrate form amastigotes, suggesting that these methyltransferases are important for the development of the parasite throughout its life cycle. These enzymes are maximally expressed during the log phase of growth which correlates with the demand of PC synthesis during cell multiplication. Immunofluorescence studies combined with cell fractionation have shown that both methyltransferases are localized at the endoplasmic reticulum membrane. Heterologous expression in yeast has demonstrated that *LmjPEM1* and *LmjPEM2* complement the choline auxotrophy phenotype of a yeast double null mutant lacking phosphatidylethanolamine methyltransferase activity. *Lmj*PEM1 catalyzes the first, and to a lesser extent, the second methylation reaction. In contrast, *Lmj*PEM2 has the capacity to add the second and third methyl group onto phosphatidylethanolamine to yield (lyso)PC; it can also add the first methyl group, albeit with very low efficiency. Finally, we have demonstrated using inhibition studies with choline analogs that miltefosine and octadecyltrimethylammonium bromide are potent inhibitors of this metabolic pathway.

Keywords

Leishmania major; Phosphatidylethanolamine methyltransferase; Phosphatidylcholine biosynthesis; Choline analogs

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1. Introduction

Leishmania species are protozoan parasites of medical relevance that cause a wide range of important human and animal diseases, collectively named leishmaniasis. They affect at least 10 million patients worldwide, primarily in tropical and sub-tropical areas of the world, where approximately 300 million people are at risk. This parasite cycles between the sand fly vector as a flagellated promastigote and the vertebrate host's macrophages as a non-motile amastigote.

Phosphatidylcholine (PC) is the most abundant lipid of the *Leishmania* biological membrane, representing 30–40% of total cellular lipids [1,2,3]. Structurally, it contains unusually long unsaturated fatty acid chains that are believed to confer a higher degree of resistance towards host-derived oxidants [3]. PC fulfills its structural function as a main constituent of cellular membranes. However, it also serves as a reservoir of secondary messenger metabolites (e.g. PA, DAG, and lysoPC) that play critical roles in signaling pathways controlling key cellular processes including mitogenesis, cell differentiation, and gene transcription (reviewed in [4,5,6,7]). PC seems to be an essential lipid of *Leishmania* membranes because a decrease of PC levels to 25% or below is deleterious to the parasite [8].

Three distinct routes lead to PC biosynthesis (Fig. 1). Firstly, the *de novo* pathway starts with the uptake of extracellular choline which is subsequently phosphorylated to give phosphocholine. The latter is then linked to a diacylglycerol (DAG) moiety to yield PC. Secondly, PC can be produced by the threefold methylation of phosphatidylethanolamine (PE) by one or several PE methyltransferases (PEMT) that utilize *S*-adenosylmethionine (SAM) as the methyl donor (reviewed in [9,10,11,12]). Lastly, in a pathway seemingly restricted to prokaryotes, PC results from the condensation of choline with CDP-DAG carried out by a PC synthase (reviewed in [11,12]). PC biosynthesis in *Leishmania* seems to occur *via* the *de novo* and methylation pathways (Fig. 1; [8]). Based on biochemical studies, *Leishmania* has the capacity to take up choline from the medium or the host [13]. Sequence analysis of the *L. major* genome reveals the presence of orthologs of the choline kinase (Lmjf27.1420 and Lmjf35.1470), CTP:phosphocholine cytidylyltransferase (Lmjf18.1330), and choline phosphotransferase (Lmjf18.0810); however, the enzymatic activities linked to these gene products have yet to be established [13,14]. In addition, *Leishmania* has the ability to convert PE into PC, suggesting that it possesses one or several PE methyltransferases [8].

PEMT enzymes have been characterized from various organisms (reviewed in [9,11,12,15]). Eukaryotic PEMT can be divided into two classes based on substrate specificity and protein structure. In yeasts, such as *Saccharomyces cerevisiae* and *S. pombe*, class II PEMTs have an internal sequence duplication and carry out the first methylation step, forming monomethyl-PE (MMPE) from PE [15,16]. Class I PEMTs usually catalyze the last two methylation reactions to form PC from MMPE with dimethyl-PE (DMPE) as the intermediate. Additionally, yeast class I PEMTs are related to mammalian PEMT which carries out all three methylation steps. Interestingly, in *S. cerevisiae*, *Sc*PEM2, a class I

PEMT, can perform all three methylation reactions as the mammalian counterpart, although it performs the first methylation step with much lower efficacy than the class II PEMT enzyme, *Sc*PEM1 [16].

Phosphocholine analogs, such as miltefosine and edelfosine, have potent anti-microbial activities, including anti-leishmanial activity. Several potential mechanisms responsible for the anti-leishmanial properties of these compounds have been proposed ([17,18,19,20]; reviewed in [21,22,23]). These potential mechanisms include interference with enzymes involved in lipid metabolism, induction of apoptosis, instigation of mitochondrial dysfunction, inhibition of cell signaling, and immunostimulation. However, their exact mechanism of action has yet to be established.

Choline is not essential for *L. major* growth demonstrating that the *de novo* pathway is dispensable for PC production in this parasite [13]. Thus, PE methylation likely represents the primary route for PC biosynthesis. To address this hypothesis, we have initiated a characterization of this metabolic pathway by identifying the PE methyltransferase enzymes and determining their substrate specificity, subcellular localization, and their inhibition by choline analogs.

2. Materials and Methods

2.1. Strains and media

Promastigotes of *L. major* Friedlin strain V1 (MHOM/IL/80/Friedlin) were grown in liquid M199-derived media [24]. Amastigotes were isolated from mouse footpad lesions resulting from inoculation with wild-type *Leishmania* as described in [24] following protocol 1697.0 approved by the Institutional Animal Care and Use Committees (IACUC) at St John's University.

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Standard methods for yeast culture, transformation, and genomic DNA isolation were used [25]. Yeast was cultivated at 30°C in YPD rich medium (1% yeast extract, 2% Bacto peptone, and 2% glucose) or synthetic minimal medium (yeast nitrogen base, 2% glucose). The synthetic minimal medium was supplemented with histidine (30 μg/ml), uracil (30 μg/ml), leucine (100 μg/ml), methionine (100 μg/ml), or choline (10 μM) as required to maintain cell growth.

For growth curves, yeast cultures were grown in synthetic minimal medium supplemented with 1 μM choline, washed twice in sterile water, and resuspended to an OD_{600}=0.1 in minimal medium in the absence or presence of 10 μM choline. The turbidity of the cultures was monitored with a spectrophotometer as a function of time.

2.2. Molecular biology

Genomic DNA from *L. major* and *Trypanosoma brucei* was prepared as described by Acosta-Medina and Cross [26].

pXGHYG2.SS-GFP-MDDL (Ec613) was created as follows. The signal sequence of *T. brucei* BIP was PCR-amplified with primers O281 (5'-CCCGGGATGTCGAGGATGTGGCTGAC-3') and O290 (5'-TCTAGAGTATGTTGTGCCGAGGTCGATG-3') using wild-type genomic DNA as template. The GFP-MDDL encoding region was amplified with O291 (5'-TCTAGAGTGAGCAAGGGCGAGGAG-3') and O280 (5'-GGGCCCTTACAGATCGTCCATCTTGTACAGCTCGTCCATGC-3') using pXG.GFP' as template [27]. Both resulting DNA fragments were cut with XmaI and XbaI, and XbaI and ApaI, respectively, and triple ligated into the XmaI and ApaI sites of pXGHYG2 (derived from pXG.HYG [28] but bears an ApaI site downstream of the XmaI site).

Expression vectors GST-LmjPEM1 (Ec648) and GST-LmjPEM2 (Ec652) were constructed using genomic DNA from *L. major* FV1 as a template. Primers O352 (5'-CGAATTCTCACTGATTCTTGCGACATTC-3') and O353 (5'-GCGGATCCATGCGCAAGCGCTACGGTAAC-3') were applied to amplify the 3'end of *LmjPEM1*, while O350 (5'-CGAATTCTCACTGCTTCTTCACCGAGGC-3') and O351 (5'-GCGGATCCCTGGTGTATCATGTGTCGAC-3') were used to amplify the 3'end of *LmjPEM2*. Both PCR products were digested with BamHI and EcoRI, and ligated into the corresponding sites of pGEX-2T (GE Healthcare Bio-Science AB). The resulting heterologous proteins bear the 46 and 39 C-terminal amino acids of *Lmj*PEM1 and *Lmj*PEM2, respectively, fused to the C-terminus of the glutathione *S*-transferase.

The plasmid pBEVY-L-LmjPEM2 (Ec723) was generated by PCR-amplifying *LmjPEM2* with oligonucleotides O391 (5'-CGGATCCATGACGCAGTTGCCCAC-3') and O390 (5'-CGGATCCTCACTGCTTCTTCACCGAGGCAG-3') using genomic DNA from *L. major* FV1 as a template. The obtained PCR products were digested with BamHI and cloned in sense orientation into the BamHI site of pBEVY-L [29]. pBEVY-U-LmjPEM1 (Ec714) was created as follows. *LmjPEM1* was amplified with the primers O388 (5'-CGGATCCTCACTGATTCTTGCGACATTCCAG-3') and O370 (5'-GGATCCATGTCTCTTGAAAGCGCGTC-3') using genomic DNA from *L. major* FV1 as a template. The resulting PCR products were digested with BamHI and cloned in sense orientation into the BamHI site of pBEVY-U [29].

The episomes pBEVY-U-ScPEM1 (Ec886) and pBEVY-L-ScPEM2 (Ec887) were constructed using wild-type *S. cerevisiae* genomic DNA as a template. Primers O526 (5'-GTCTAGATGTCCAGTTGTAAAACCACTTTGTC-3') and O532 (5'-CCTGCAGTCAAGCAAGACTATCAAGCGTTTG-3') were applied to amplify the *ScPEM1* (YGR157W) gene, while O531 (5'-GTCTAGATGAAGGAGTCAGTCCAAGAG-3') and O533 (5'-CCTGCAGTTACATATTCTTTTTGGCCTTATCACGG-3') were used to amplify the *ScPEM2* (YJR073C) gene. Resulting DNA fragments were digested with PstI and XbaI, and ligated into the respective sites of pBEVY-U and pBEVY-L, respectively [29]. All PCR products were verified by sequencing.

2.3. Antigen production and purification

Recombinant proteins GST-LmjPEM1 and GST-LmjPEM2 were produced in the *Escherichia coli* strain pLysS (F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm (DE3) pLysE (Cam^R); Novagen); their expression was induced in the presence of 0.04 mM IPTG for 5 hr at 37°C. Both recombinant proteins were detected in inclusion bodies that were isolated by lysing the cells in phosphate buffered saline (PBS) containing 1 mM EDTA using sonication. Inclusion bodies were pelleted by centrifugation at 15,000 g and washed three times in PBS. Finally, the inclusion bodies were solubilized in 1× SDS-PAGE loading dye and the recombinant proteins separated by SDS-PAGE. The gel area containing the recombinant protein was used for immunization of two rabbits according to the NIH guidelines. This was accomplished by the company Cocalico Inc. The obtained sera were subsequently purified according to a small scale protocol described in [30]. Briefly, the antibodies were purified by binding to GST-LmjPEM1 or GST-LmjPEM2 recombinant proteins previously electroblotted onto a PVDF membrane, followed by elution with 100 mM glycine, pH 2.5. Purified anti-*Lmj*PEM1 and anti-*Lmj*PEM2 antibodies were used throughout this work.

2.4. Digitonin fractionation, protein quantification, Western blot, and immunofluorescence assay

Digitonin fractionation was carried out as follows. Approximately 2×10^8 late-log phase parasites were washed once with cold PBS. Cell pellets were resuspended in 1 ml TED buffer (20 mM TrisHCl, pH8.0, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitor cocktail (Roche Life Sciences)) and 100 μl aliquots were prepared into seven tubes. Cells were permeabilized by adding digitonin (from a stock solution of 15 mg/ml in PBS) to final concentrations of 0.075, 0.15, 0.30, 0.45, 0.6, and 0.75 mg/ml and incubating at 26°C for 10 min. Supernatants and pellet fractions were separated by centrifugation at 20,800 g for 3 min at 4°C.

Protein concentration was determined according to the bicinchoninic assay with bovine serum albumin as a standard. Western blots and immunofluorescence assays were carried out as described before [24]. The purified anti-*Lmj*PEM1 and anti-*Lmj*PEM2 antibodies were used at 1:500 and 1:250 dilutions, respectively, for Western blots and immunofluorescence assays.

2.5. Enzymatic assay

Yeast cultures were grown to an optical density of OD_{600} =1.0 and washed twice in ice cold water. *Leishmania* parasites were harvested at mid-log (5×10^6/ml), late-log ($2–5\times10^7$ /ml) or stationary phase of growth and washed once in cold PBS. Yeast and *Leishmania* cell pellets were then resuspended in lysis buffer (0.5 M sucrose, 0.1 M TrisHCl, pH7.5, 2 mM EDTA, and protease inhibitor) and lysed using beads by vortexing for 10 min at 4°C. Cell debris was separated by low speed centrifugation (1300 g) and the supernatant was used for the PEMT assay. Reactions were set up using 200 μg protein and 0.1 μCi *S*-[Methyl-^{3}H]adenosyl-L-methionine (specific activity of 15 Ci/mMole; Perkin Elmer) in 0.1 M TrisHCl, pH7.5. Reactions were incubated at 30°C for 50 minutes and stopped by the addition of 2 ml of chloroform/methanol (1:1 (v/v)). Total lipids were then extracted and

quantified by liquid scintillation spectrometry (Perkin-Elmer; [31]). Each experiment was performed at least twice in duplicate.

2.6. Lipid analysis

Yeast strains By4741 (wild type), *scpem1*Δ*scpem2*Δ+*LmjPEM1+L*, *scpem1*Δ*scpem2*Δ +*LmjPEM1+LmjPEM2*, and *scpem1*Δ*scpem2*Δ+*U+LmjPEM2* were grown in minimal media lacking choline and harvested at an OD_{600}=1–1.5. The strain *scpem1*Δ*scpem2*Δ+*U+L* was grown in minimal medium containing 5 μg/ml choline to an OD_{600}=0.3, washed twice in water and further incubated in minimal medium lacking choline for 10 hr. Lipids were purified according to [32] and were profiled by electrospray ionization tandem mass spectrometry using the method described by Zufferey *et al*. [33], except that internal standards were (with some small variation in amounts in different batches of internal standards): 0.6 nmol di12:0-PC, 0.6 nmol di24:1-PC, 0.6 nmol 13:0-lysoPC, 0.6 nmol 19:0-lysoPC, 0.3 nmol di12:0-PE, 0.3 nmol di23:0-PE, 0.3 nmol,14:0-lysoPE, 0.3 nmol 18:0-lysoPE, 0.3 nmol 14:0-lysophosphatidylglycerol (lysoPG), 0.3 nmol 18:0-lysoPG, 0.3 nmol di14:0-phosphatidic acid (PA), 0.3 nmol di20:0(phytanoyl)-PA, 0.2 nmol di14:0-phosphatidylserine (PS), 0.2 nmol di20:0(phytanoyl)-PS, 0.23 nmol 16:0–18:0-phosphatidylinositol (PI), 0.3 nmol di14:0-PG, and 0.3 nmol di20:0(phytanoyl)-PG. In addition to the scans previously described [33], a scan for PG as $[M + NH_4]^+$ in the positive mode with NL 189.0 was performed with collision energy of 20 V, declustering potential of 100 V, and exit potential of 14 V. Scans for MMPE (NL155.0) and DMPE (NL 169.0) were performed with the same instrument parameters as for the PE scans. MMPE and DMPE were determined in comparison to the PE standards without correction for response factor for these compounds as compared to PE.

2.7. Statistical analysis

Two-tailed Student's test was used for statistical analysis which was performed using GraphPad Prism Software (version 5.0). *P*-values ≤0.01 were considered to be statistically significant. Asterisks indicate statistically significant differences: *** $p \leq 0.001$, and ** $p \leq 0.01$.

3. Results and discussion

3.1. Identification of *Lmj*PEM1 and *Lmj*PEM2

Biochemical studies established that PC biosynthesis occurs *via* two pathways in *Leishmania*, the *de novo* pathway that starts with uptake of choline, and the PE methylation pathway (Fig. 1; [8]). Previous studies have shown that choline is dispensable for growth of *L. major* [13]. Thus, to understand the importance of the PE methylation pathway in the biosynthesis of PC in this parasite, we searched for PE methyltransferase (PEMT) gene orthologs in *L. major* genome using the *Saccharomyces cerevisiae* PEMTs *ScPEM1* (gene ID YGR157W) and *ScPEM2* (gene ID YJR073C) as a query. *LmjPEM1* (LmjF31.3120) and *LmjPEM2* (LmjF31.2290) were identified and they code for predicted proteins of 582 and 225 amino acids, respectively (Fig. 2). *Lmj*PEM1 exhibits 32% and 28% identity, and 49% and 42% similarity to *Sc*PEM1 and *Sc*PEM2, respectively. Interestingly, *Lmj*PEM1 does not display any significant similarity to human PEMT. In contrast, *Lmj*PEM2 shows 43% and

41% identity, and 57% and 54% similarity to yeast *Sc*PEM2 and human PEMT, respectively. TMHMM algorithm predicts four putative hydrophobic stretches (amino acids to 13 to 35, 54 to 76, 109 to 131, 167 to 189) in *Lmj*PEM2 while eight nonpolar domains (amino acids 54 to 76, 86 to 105, 187 to 204, 235 to 257, 336 to 358, 403 to 425, 438 to 460, and 496 to 518) could be identified in *Lmj*PEM1. In addition, *Lmj*PEM2 has 3 conserved blocks of amino acids shared by PEMT enzymes. These domains may be involved in substrate recognition or catalysis. Notably, similar to *Sc*PEM1, *Lmj*PEM1 bears two of these domains and thus, may arise from internal gene duplication. Both *Lmj*PEM1 and *Lmj*PEM2 carry a putative C-terminal endoplasmic reticulum retrieval signal (RKNQ and VKKQ, respectively), suggesting that they reside in this organelle [34]. In contrast, mammalian counterparts and the yeast *Sc*PEM1 lack an endoplasmic retrieval signal but not *Sc*PEM2 (KKNM; data not shown).

3.2. *Lmj*PEM1 and *Lmj*PEM2 are expressed throughout the life cycle of *Leishmania*

To address whether PEMT activity is regulated during the growth phase of the parasite, PEMT activity was measured with whole cell extracts derived from promastigotes harvested at different stages of growth (Fig. 3). PEMT activity was highest in mid-log parasites that are actively dividing (Fig. 3A). In contrast, PEMT activity in stationary phase parasites dropped to 25% of that of replicating cells. Western blot analyses performed with *Lmj*PEM1 and *Lmj*PEM2 specific antibodies confirmed these results. *Lmj*PEM1 and *Lmj*PEM2 levels were highest when the cells were actively growing (mid-log phase) and their expression decreased as the cells entered stationary phase (Fig. 3B). Thus, the level of expression of *Lmj*PEM1 and *Lmj*PEM2 seems to correlate with the demand for PC biosynthesis linked to cell growth and multiplication. These enzymes are also expressed in vertebrate cell stage amastigotes, although at lower levels, suggesting that the methylation pathway is functional and may be important for PC production in the vertebrate host.

3.3. PEMT activity in Leishmania is not regulated by choline

Yeast *S. cerevisiae Sc*PEM1 and *Sc*PEM2 have been shown to be regulated by lipid precursors, such as choline [15,35,36]. Thus, we assessed whether PEMT activity in *Leishmania* is regulated by choline. Cells were grown at low (35 μM) or high (1 mM) concentration of choline. Cell extracts derived from these cultures were assayed for PEMT activity and for protein expression by Western blot analysis (Fig. 4). PEMT activity was similar independently of the choline concentration present in the medium (Fig. 4A). Also, *Lmj*PEM1 and *Lmj*PEM2 protein levels were comparable in cells grown in low and high concentration of choline (Fig. 4B). Thus, in contrast to yeast, *Leishmania* does not regulate its PEMT enzymes according to choline availability.

3.4. LmjPEM1 and LmjPEM2 localize to the endoplasmic reticulum membrane

Both *Lmj*PEM1 and *Lmj*PEM2 bear a putative endoplasmic reticulum retention motif at their C-terminus suggesting an endoplasmic reticulum subcellular localization. Immunofluorescence assays carried out in the presence of antibodies specific to *Lmj*PEM1 or *Lmj*PEM2 displayed a punctate signal around the nucleus that overlapped with that of

GFP-MDDL, an endoplasmic reticulum resident ([37]; Fig. 5). This demonstrates that *Lmj*PEM1 and *Lmj*PEM2 reside in the endoplasmic reticulum.

We then assessed whether *Lmj*PEM1 and *Lmj*PEM2 are membrane associated or integral membrane proteins as both proteins bear several hydrophobic stretches (Fig. 2). Whole cells were incubated in the presence of increasing concentration of the mild detergent digitonin, followed by separation of supernatants and pellets by centrifugation. Both fractions we re analyzed by Western blot in the presence of antisera specific to the phosphomannomutase (PMM) used as a cytosolic marker, the luminal endoplasmic reticulum marker BIP, the GPI-anchored protease GP63, *Lmj*PEM1 and *Lmj*PEM2 (Fig. 6A; [38,39,40]). PMM was released in the supernatant fraction at very low digitonin concentration while the luminal BIP stayed in the pellet fraction until higher concentration (0.3 mg/ml) of the detergent. The GPI-anchored integral membrane GP63 remained in the pellet even at very high digitonin concentration (0.75 mg/ml). *Lmj*PEM1 and *Lmj*PEM2 behaved similarly to GP63, suggesting that they are integral membrane proteins. The fact that *Lmj*PEM1 and *Lmj*PEM2 were not released from the pellet membrane fraction by 0.5 M NaCl or the detergent CHAPS further supports the notion that both are integral membrane proteins (Fig. 6B). The endoplasmic reticulum membrane localization of *Lmj*PEM1 and *Lmj*PEM2 is reminiscent of yeast orthologs *Sc*PEM1 and *Sc*PEM2, as well as mammalian PEMT [41,42]. It makes sense that PEMT enzymes are localized in the same organelle where PE is produced [43].

3.5. LmjPEM1 and LmjPEM2 complement the choline auxotrophy phenotype of S. cerevisiae double mutant scpem1Δscpem2Δ

To assess whether *LmjPEM1* and *LmjPEM2* encode PEMT enzymes, they were expressed both individually (*scpem1*Δ*scpem2*Δ*+LmjPEM1+L, scpem1*Δ*scpem2*Δ*+U+LmjPEM2*) or together (*scpem1*Δ*scpem2*Δ*+LmjPEM1+LmjPEM2*) in the *S. cerevisiae* double null mutant *scpem1*Δ*scpem2*Δ that lacks endogenous PEMT activity [44,45]. An empty vector strain (*scpem1*Δ*scpem2*Δ*+U+L*) was generated to serve as a negative control. Additionally, positive control lines expressing *Sc*PEM1 or *Sc*PEM2 alone (*scpem1*Δ*scpem2*Δ *+ScPEM1+L, scpem1*Δ*scpem2*Δ*+U+ScPEM2*) or together (*scpem1*Δ*scpem2*Δ *+ScPEM1+ScPEM2*) were created. The PC biosynthetic pathways in yeast are identical to those of *Leishmania*. Two routes lead to PC production in yeast: i) the *de novo* pathway that starts from uptake of choline and ii) the methylation pathway that involves the threefold methylation of PE catalyzed by *Sc*PEM1 and *Sc*PEM2. Thus, abrogation of both *ScPEM1* and *ScPEM2* renders the double null mutant *scpem1*Δ*scpem2*Δ a choline auxotroph [15,44]. These strains were grown in minimal medium in the absence or presence of 10 μM choline (Fig. 7). Growth curve assays showed that all strains grew equally well in the presence of choline (Fig. 7A). As expected, the double null mutant bearing empty vectors (*scpem1*Δ*scpem2*Δ*+U+L*) remained a choline auxotroph (Fig. 7B). In contrast, both *LmjPEM1* and *LmjPEM2* individually as well as in combination had the capacity to complement the choline auxotrophy of the *scpem1*Δ*scpem2*Δ strain (Fig. 7B). However, similar to the *ScPEM1* transgenic, yeast expressing *Lmj*PEM1 alone grew much slower in the absence of choline and reached a lower cell density during the stationary phase of growth than strains expressing either *Lmj*PEM2 or *Sc*PEM2. Also, the line *scpem1*Δ*scpem2*Δ*+LmjPEM1+LmjPEM2*, as *scpem1*Δ*scpem2*Δ*+ScPEM1+ScPEM2*, grew

better and reached a higher cell density during the stationary growth phase than *scpem1*Δ*scpem2*Δ*+U+ScPEM2* or *scpem1*Δ*scpem2*Δ*+U+LmjPEM2* (Fig. 7B). This assay has demonstrated that both *LmjPEM1* and *LmjPEM2* relieve the choline auxotrophy phenotype of *scpem1*Δ*scpem2*Δ and that this complementation is better achieved with *LmjPEM2* and *LmjPEM1* together, than with *LmjPEM2* or *LmjPEM1* alone.

3.6. LmjPEM1 forms MMPE and DMPE while LmjPEM2 catalyzes all three methylation reactions to form PC

To address whether *Lmj*PEM1 and *Lmj*PEM2 exhibit PEMT activity, PEMT assay was performed with whole cells extract derived from yeast wild type, *scpem1*Δ*scpem2*Δ*+U+L, scpem1*Δ*scpem2*Δ*+LmjPEM1+L, scpem1*Δ*scpem2*Δ*+U+LmjPEM2* and *scpem1*Δ*scpem2*Δ *+LmjPEM1+LmjPEM2* (Fig. 8). As expected, the double null mutant lacked PEMT activity (Fig. 8A). *Lmj*PEM1 showed only 15% of wild-type PEMT activity while *Lmj*PEM2 gave even lower PEMT activity than *Lmj*PEM1 (Fig. 8A). This suggests that PE is a better substrate for *Lmj*PEM1 than *Lmj*PEM2. When *Lmj*PEM1 and *Lmj*PEM2 were co-expressed in the same cell, PEMT activity was higher than the combined individual activities of *Lmj*PEM1 and *Lmj*PEM2. The same effect was observed when equal amounts of protein derived from yeast strains *scpem1*Δ*scpem2*Δ*+LmjPEM1+L* and *scpem1*Δ*scpem2*Δ*+U +LmjPEM2* were mixed (Fig. 8B). This can be explained by a model where the product of either *Lmj*PEM1 or *Lmj*PEM2 serves as a substrate for the other enzyme, a phenomenon also observed with yeast PEMT enzymes [46].

To determine the substrate specificity of *Lmj*PEM1 and *Lmj*PEM2, bulk lipids from yeast transformants *scpem1*Δ*scpem2*Δ*+U+L, scpem1*Δ*scpem2*Δ*+LmjPEM1+L, scpem1*Δ*scpem2*Δ *+U+LmjPEM2, scpem1*Δ*scpem2*Δ*+LmjPEM1+LmjPEM2*, as well as from the wild type were isolated and analyzed by comprehensive electrospray ionization tandem mass spectrometry. Expression of *Lmj*PEM1 lead primarily to the formation of lysoMMPE and MMPE, and to a lower extent to lysoDMPE and DMPE with concomitant decreases in lysoPE and PE levels. No lysoPC or PC was detected (Fig. 9A, B). The best lysoPE substrates for *Lmj*PEM1 were those carrying medium length fatty acyl groups, specifically palmityl, palmitoyl, and a stearyl group (Table 2). The best diacyl-PE substrates for *Lmj*PEM1 were PE(32:2) and PE(32:1) (Table 3). This result demonstrates that *Lmj*PEM1 has the ability to methylate PE and lysoPE as well as MMPE and lysoMMPE, albeit with lower efficiency, and that it cannot catalyze the third methylation reaction (Fig. 9D). Alternatively, lysoMMPE and lysoDMPE may result from *sn*-2 deacylation of MMPE and DMPE by the action of an endogenous phospholipase A2 activity [47,48]. The *scpem1*Δ*scpem2*Δ*+LmjPEM1+L* strain produces large amounts of MMPE and thus, can be used as an excellent source of this lipid.

The strain *scpem1*Δ*scpem2*Δ*+U+LmjPEM2* contained minute amounts of MMPE, lysoMMPE, DMPE, lysoDMPE, a small but significant amount of PC, and similar amounts of PE as the double null mutant (Fig. 9A, B). The lysoPC species produced by *Lmj*PEM2 are lysoPC(16:1) followed by lysoPC(16:0) and lysoPC(18;1), while the wild type generated mainly lysoPC(16:1) followed by lysoPC(18:1) and lysoPC(16:0) (Table 2). These species may be the product of *Lm*PEM2 activity or the action of yeast phospholipase A2 [47,48]. In

addition, *Lmj*PEM2 seemed to be more specific to the length (C16) of than to the presence of double bonds in the fatty acyl group of the lysoPE, lysoMMPE, and lysoDMPE. The most abundant PC species produced by *Lmj*PEM2 were PC(32:2) and PC(32:1) (Table 3). These data demonstrate that *Lmj*PEM2 inefficiently adds the first methyl group to PE, but catalyses the second and third methylation reaction with higher efficacy to give PC (Fig. 9D). This also explains the lower PEMT activity of *Lmj*PEM2 compared to that of *Lmj*PEM1 when expressed in the yeast background *scpem1*Δ*scpem2*Δ when endogenous PE was used as substrate (Fig. 8A). In addition, *Lmj*PEM2 conferred better growth to yeast *scpem1*Δ*scpem2*Δ than *Lmj*PEM1 in medium lacking choline owing to its ability to produce PC in contrast to *Lmj*PEM1, which cannot generate PC but only MMPE and DMPE (Fig. 9B).

Coexpression of *Lmj*PEM1 and *Lmj*PEM2 gave small amounts of MMPE, lysoPE, DMPE, and lysoDMPE, but more PC than the *scpem1*Δ*scpem2*Δ*+U+LmjPEM2* strain but less than the wild type (Fig. 9A, B). Levels of PE in the *scpem1*Δ*scpem2*Δ*+LmjPEM1+LmjPEM2* strain were similar to that of the wild type. It seems that *Lmj*PEM2 does not efficiently convert all MMPE, lysoMMPE, DMPE, and lysoDMPE produced by *Lmj*PEM1 into PC (Fig. 9A, B). This may be due to the fatty acid composition of PE and lysoPE produced by yeast versus *Leishmania*. In fact, yeast PC fatty acids are of medium length while *Leishmania* produces PC species with long to very long fatty acids (Tables 2 and 3; data not shown; [3]). It is unclear as to which subspecies of PC are produced *via* the methylation or the *de novo* pathway in *Leishmania*. Alternatively, the level of expression of *Lmj*PEM2 in yeast was not high enough to efficiently support MMPE and DMPE, and lysoMMPE and lysoDMPE conversion into PC and lysoPC, respectively.

The fact that *LmjPEM1* bears an internal gene duplication, and its gene product is involved in the addition of the first and second methyl groups onto PE, is very reminiscent of *Sc*PEM1. Thus, *Lmj*PEM1 can be classified as a class II PEMT enzyme. In contrast, *Lmj*PEM2 lacks internal gene duplication, and catalyses all three PE methylation reactions similar to mammalian PEMTs and yeast *Sc*PEM2; therefore, it belongs to class I PEMT.

Interestingly, the double null mutant *scpem1* Δ*scpem2*Δ produced six times more PG than the wild type, likely to compensate for the lack of PC (Fig. 9C). This effect was also seen in the *Lmj*PEM1 and *Lmj*PEM2 expressing lines *scpem1*Δ*scpem2*Δ*+LmjPEM1+L* and *scpem1*Δ*scpem2*Δ*+U+LmjPEM2* although to a lower extent. In addition, the double null mutant generated approximately four times more PI than the wild type, and double null mutant expressing *Lmj*PEM1 and *Lmj*PEM2 individually or together.

To our knowledge, this is the first study to identify the PEMT enzymes in a protozoan parasite. The presence of the methylation pathway for PC biosynthesis seems to be unique to *Leishmania*. The related parasites *Trypanosoma brucei* and *T. cruzi*, as well as *Toxoplasma gondii, Giardia lamblia*, and *Trichomonas vaginalis* lack PEMT encoding genes. They produce PC exclusively from the *de novo* pathway, and thus, are choline auxotrophs [14,49]. Alternatively, *Plasmodium falciparum* utilizes a phosphoethanolamine *N*-methyltransferase to produce phosphocholine from phosphoethanolamine for PC synthesis [50,51]. In this parasite, similarly to plants, phosphocholine is then used to generate PC *via* the CDP-choline

pathway [45,51]. Why does *Leishmania* have two pathways for PC biosynthesis? *Leishmania* may encounter a choline poor environment during its life cycle, and thus, relies on the methylation pathway for PC production. In fact, nutrient starvation occurs after digestion of the blood meal in the sand fly and triggers metacyclogenesis. This hypothesis is supported by data showing that purine starvation increased PEMT protein levels [52].

3.7. LmjPEM1 and LmjPEM2 inhibition by choline analogs

Previous studies have demonstrated that miltefosine, a new drug used for the treatment of leishmaniasis, led to decreased PC production that is accompanied by concomitant increases in PE levels, suggesting that enzymes involved in the methylation of PE may be inhibited by this drug [20]. Therefore, we examined whether *Lmj*PEM1 and/or *Lmj*PEM2 are inhibited by miltefosine, while also testing choline and phosphocholine analogs (Fig. 10). *Lmj*PEM1 activity was best inhibited by hexadecyltrimethylammonium bromide, followed by miltefosine, octadecyltrimethylammonium bromide, and octadecylphosphocholine, while choline had a very small effect. Phosphocholine, ethanolamine, and the ethanolamine analog hydroxyethylhydrazine did not affect *Lmj*PEM1 activity at all (Fig. 10A). *Lmj*PEM2 was less sensitive to the compounds tested than *Lmj*PEM1. However, it was efficiently inhibited by hexadecyltrimethylammonium and octadecyltrimethylammonium bromide (Fig. 10B). Phosphocholine and miltefosine impacted *Lmj*PEM2 activity minimally, while choline, octadecylphosphocholine, ethanolamine, and hydroxyethylhydrazine did not diminish its activity. The fact that higher concentrations of drugs are needed to inhibit *Lmj*PEM1 and *Lmj*PEM2 indicates that these PEMTs are likely not the primary target of these drugs.

Surprisingly, hydroxyethylhydrazine, a specific inhibitor of *S. cerevisiae Sc*PEM1 and *Sc*PEM2 enzymes, had no impact on either *Lmj*PEM1 or *Lmj*PEM2 activity; this may reflect structural differences despite the high level of similarity [53]. Octadecyltrimethylammonium bromide was shown to be a very potent anti-leishmanial drug that also inhibits choline transport [13]. Therefore, this drug may kill the parasite by inhibiting PC biosynthesis by blocking both the *de novo* and methylation pathways.

In conclusion, this study established, for the first time in a protozoan parasite, that in *Leishmania*, the PE methylation pathway consists of two enzymes, *Lmj*PEM1 and *Lmj*PEM2 which localize at the endoplasmic reticulum membrane. *Lmj*PEM1 adds the first and second methyl group onto PE and lysoPE, while *Lmj*PEM2 adds the second and third methyl group to give PC and lysoPC, respectively. Future studies should investigate the role of *LmjPEM1* and *LmjPEM2* in parasite biology by creating null mutants. Importantly, our results have demonstrated that *Lmj*PEM1 and *Lmj*PEM2 are inhibited by the choline analogs miltefosine and hexadecyltrimethylammonium bromide, indicating that they could represent novel targets for anti-leishmanial therapies.

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Abbreviations

DAG	diacylglycerol
DMPE	dimethyl-phosphatidylethanolamine
MMPE	monomethyl-phosphatidylethanolamine
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	PE methyltransferase
PA	phosphatidic acid
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SAM	*S*-adenosylmethionine

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Highlights

- *Lmj*PEM1 and *Lmj*PEM2 are expressed throughout the life cycle of *Leishmania*
- *Lmj*PEM1 and *Lmj*PEM2 localize to the endoplasmic reticulum membrane
- *Lmj*PEM1 adds the first and the second methyl group to phosphatidylethanolamine
- *Lmj*PEM2 methylates thrice phosphatidylethanolamine to give phosphatidylcholine
- *Lmj*PEM1 and *Lmj*PEM2 are inhibited by hexadecyl- and octadecyltrimethylammonium

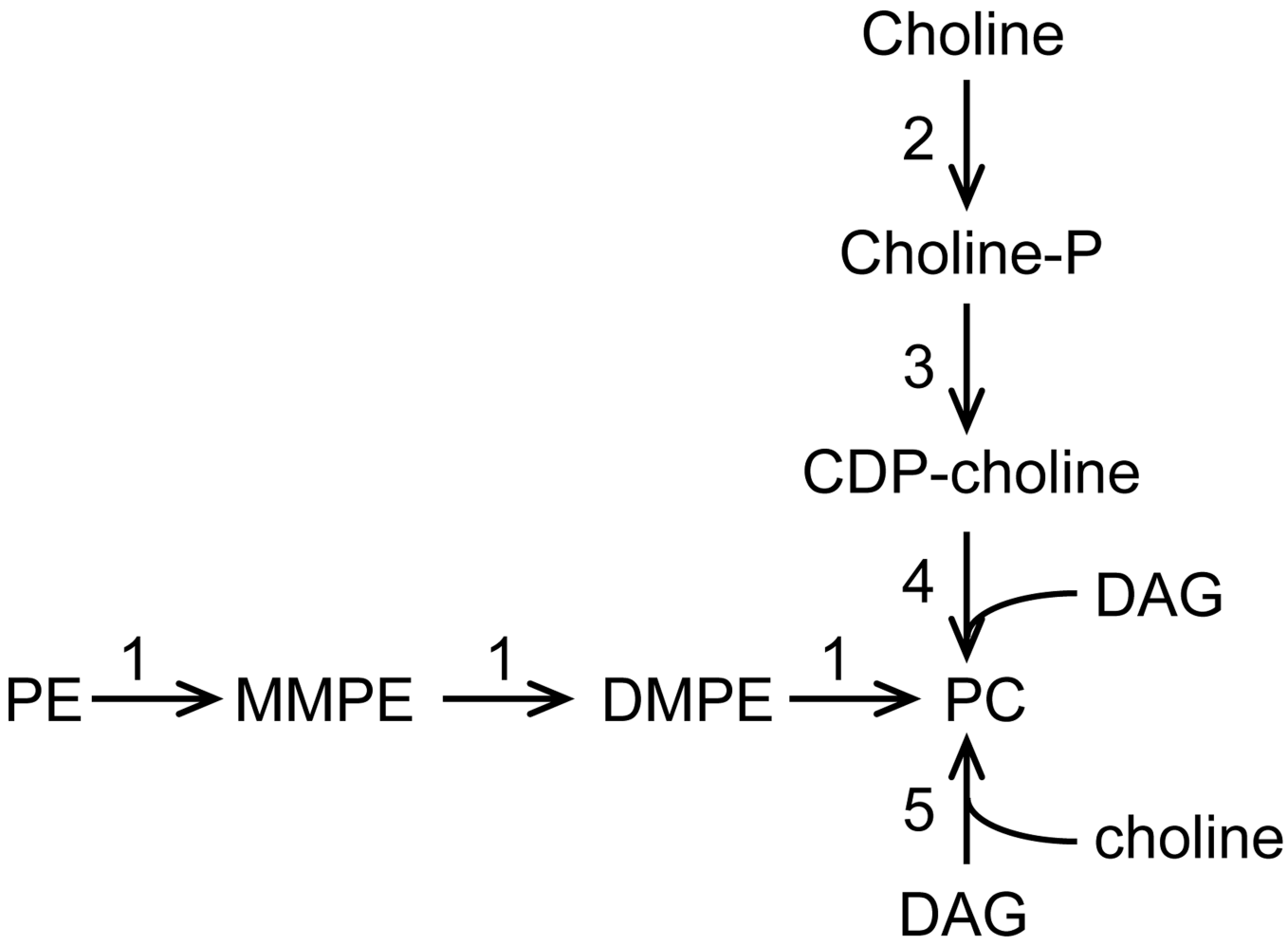

Fig. 1.

General PC biosynthetic pathways. 1. PEMT; 2. choline kinase; 3. phosphocholine cytidylyltransferase; 4. choline phosphotransferase; 5. PC synthase. DAG, diacylglycerol; DM, dimethyl; MM, monomethyl; P, phosphate.

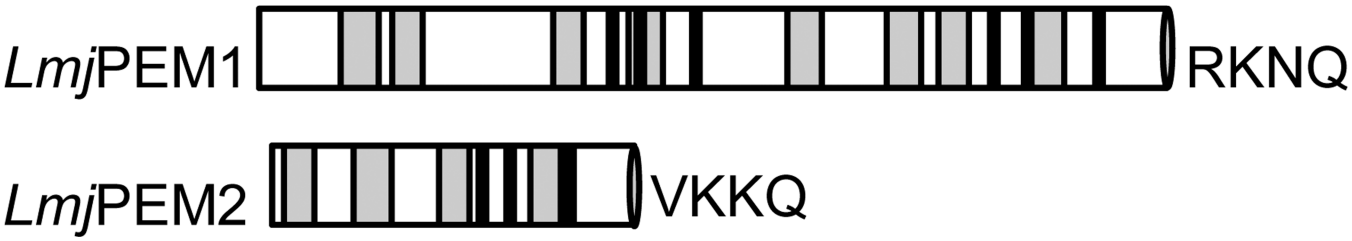

Fig. 2.

Schematic representation of the PE methyltransferases *Lmj*PEM1 and *Lmj*PEM2 of *L. major*. The grey rectangles represent the putative transmembrane spans, the black rectangles depict the conserved amino acids diagnostic of PE methyltransferases, and the grey ovals symbolize the putative endoplasmic reticulum retention motif.

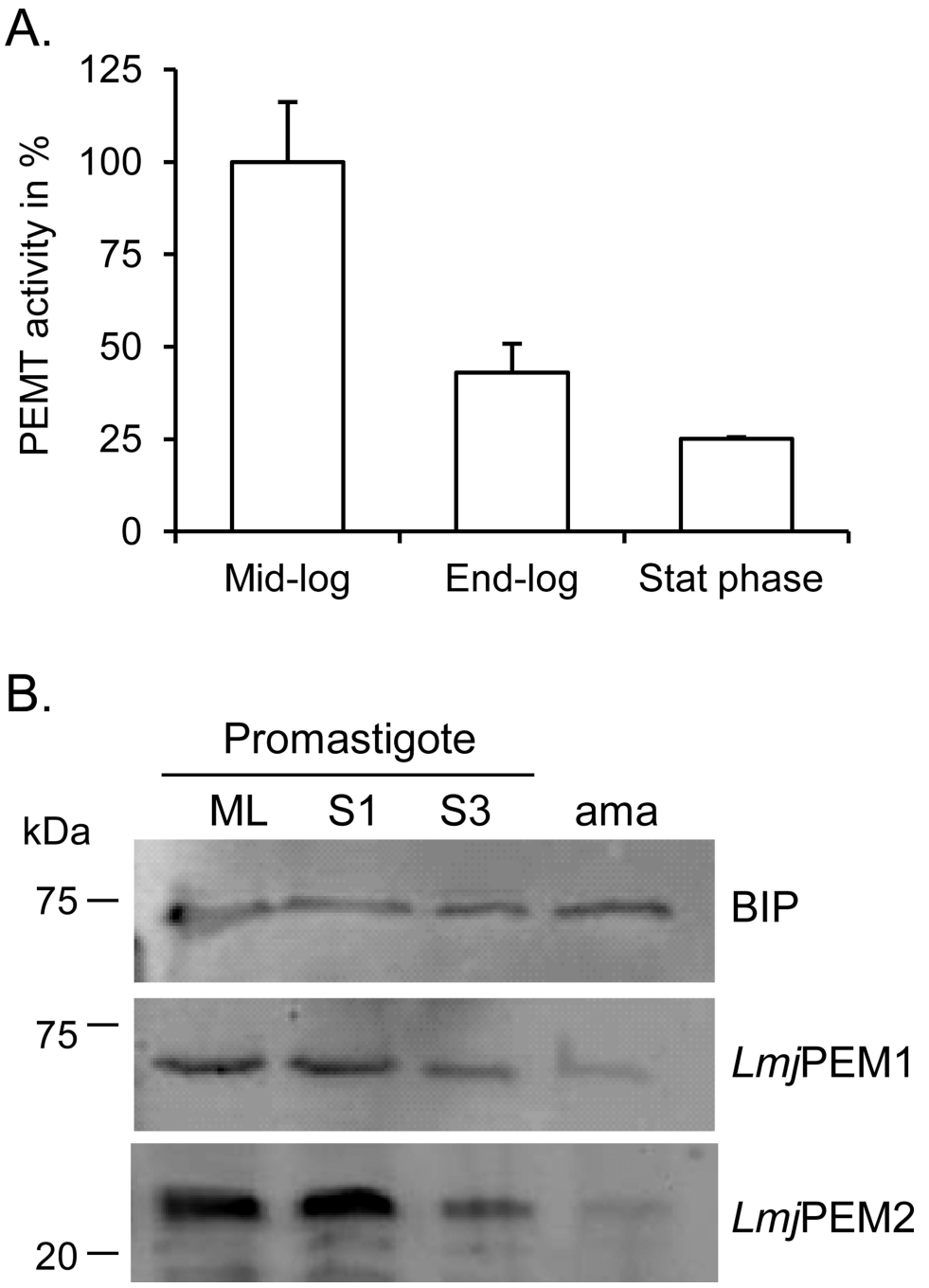

Fig. 3.

*Lmj*PEM1 and *Lmj*PEM2 are expressed throughout the life cycle of *Leishmania*. (A) Growth phase dependent PEMT assay was performed as described in Materials and methods. The enzymatic assay was carried out twice in duplicate and a representative experiment is shown. Standard deviations are depicted. An activity of 100% corresponds to approximately 15 pmol/mgxhr. (B) Western blot with whole cell extract derived from promastigotes harvested in mid log phase [54], 1 day (S1) or 3 day (S3) stationary growth phase, and from amastigotes (ama) in the presence of anti-*Lmj*PEM1, anti-*Lmj*PEM2, and anti-BIP sera [39].

Approximately 2×10^7 cells were loaded in each lane. The protein ladder is shown on the left.

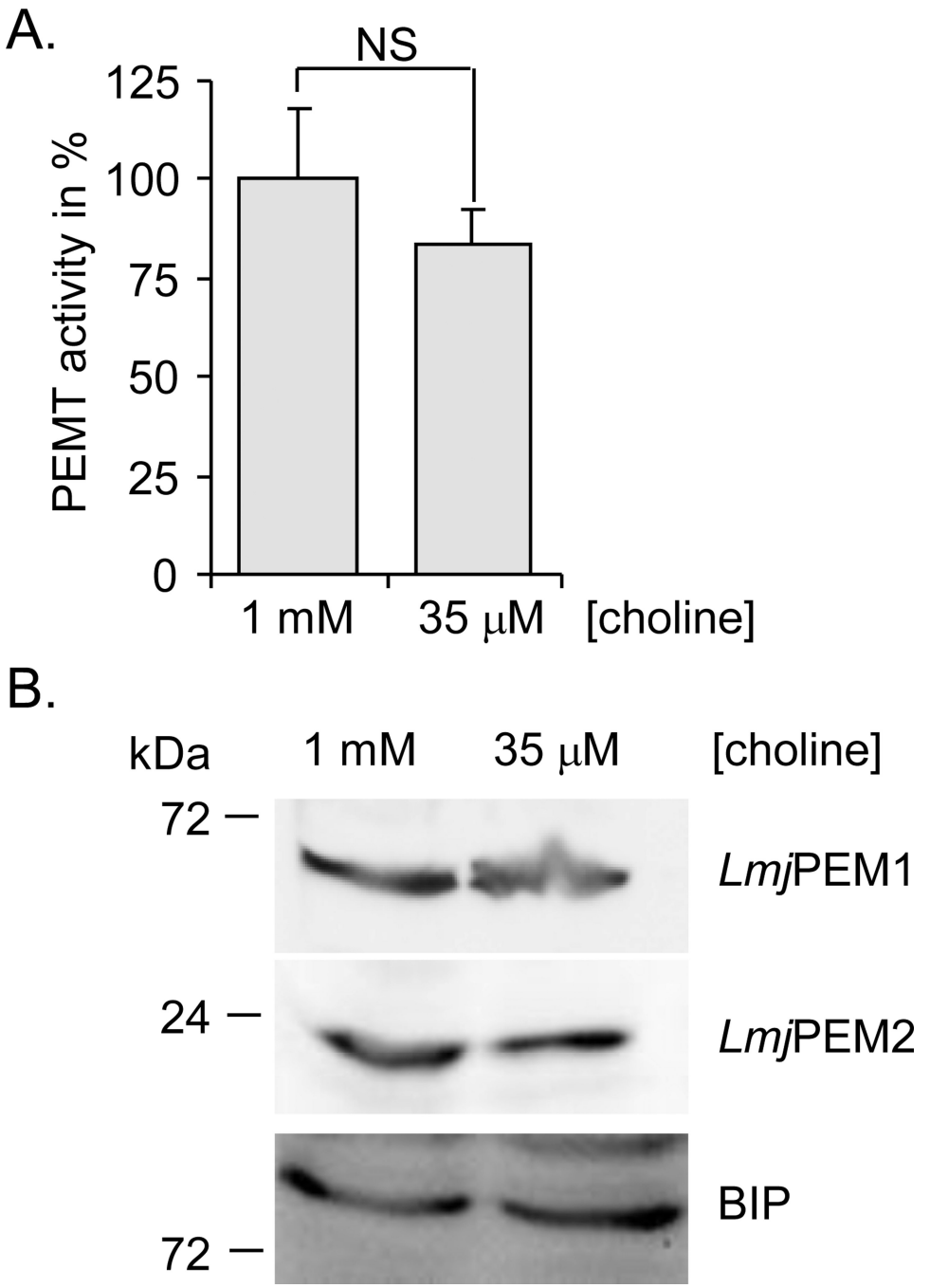

Fig. 4.

Expression of *Lmj*PEM1 and *Lmj*PEM2 is choline independent. (A) PEMT assay was carried out with wild-type cells grown in the presence of low (35 μM) or high (1 mM) concentration of choline and harvested in the mid-log phase of growth. The assay was performed at least twice in duplicate and a representative experiment is shown. Standard deviations are depicted. An activity of 100% corresponds to approximately 15 pmol/mgxhr. NS denotes statistically not significant, with a *p*-value > 0.05. (B) Western blot analysis with cells grown in the presence of low (35 μM) or high (1 mM) concentration of choline using

antibodies specific to *Lmj*PEM1, *Lmj*PEM2 or BIP [39] as a loading control. Approximately 2×10^7 cells were loaded in each lane. The protein ladder is shown on the left.

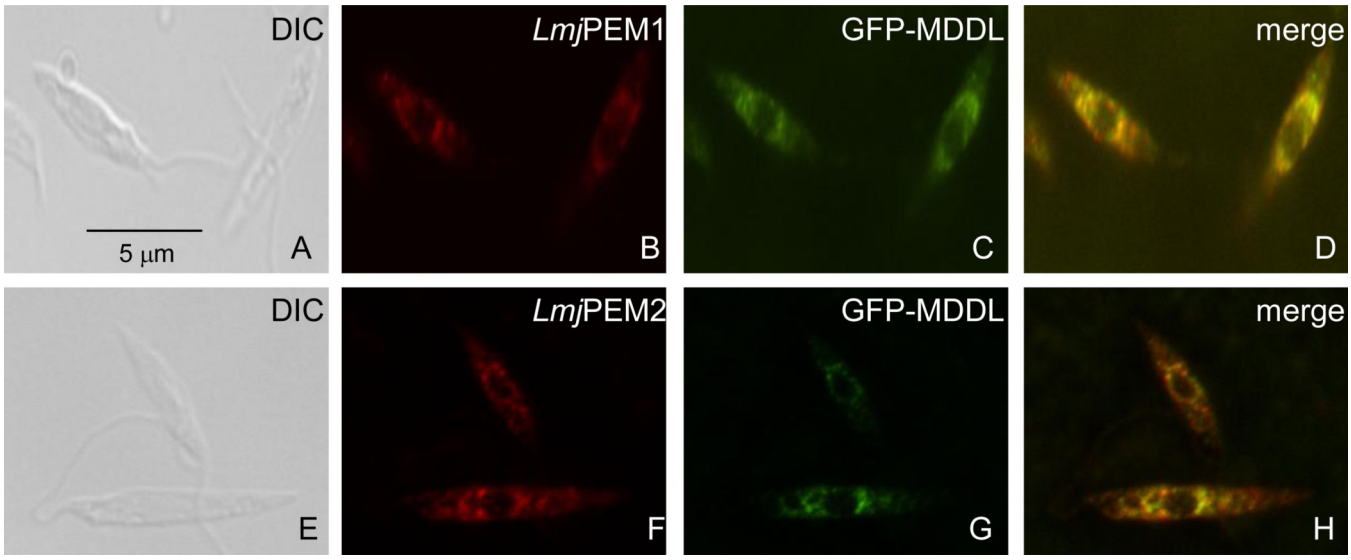

Fig. 5.

*Lmj*PEM1 and *Lmj*PEM2 localize to the endoplasmic reticulum. Immunofluorescence assays were carried out with GFP-MDDL *Leishmania* transformants using anti-*Lmj*PEM1 (B) and anti-*Lmj*PEM2 (F) antibodies. (C), (G), autofluorescence of GFP-MDDL, which is used as an endoplasmic reticulum marker [37]; (D), (H), merge of (B) and (C), and (F) and (G), respectively. The scale bar is shown.

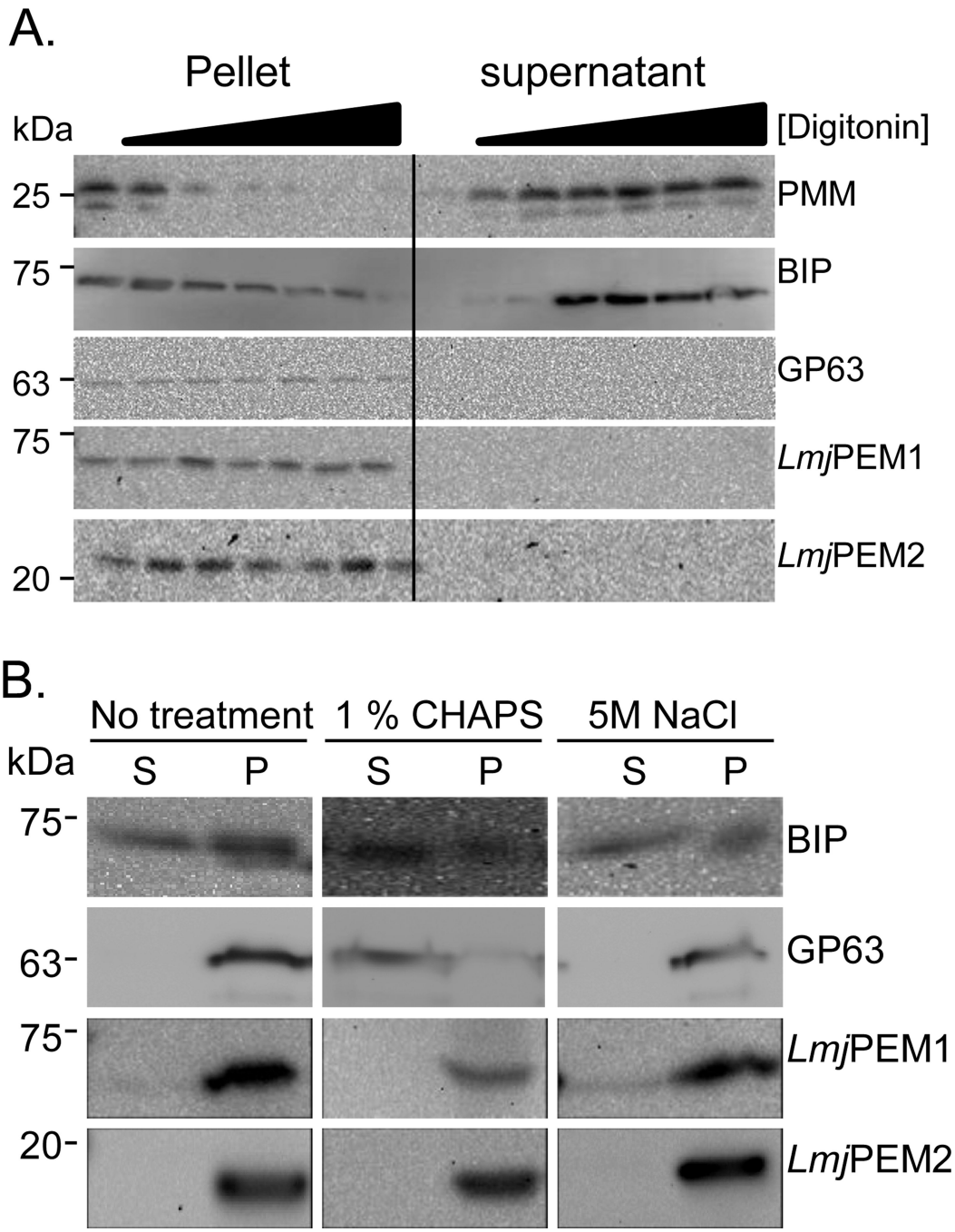

Fig. 6.

*Lmj*PEM1 and *Lmj*PEM2 are integral membrane proteins. (A) Whole cells were subjected to digitonin fractionation followed by Western blot analysis in the presence of antibodies specific to PMM (cytosolic marker), BIP (luminal endoplasmic reticulum marker), GP63 (GPI-anchored integral membrane protein), *Lmj*PEM1, and *Lmj*PEM2 [38,39,40]. (B) Whole cell extracts were incubated in the absence or presence of 1% CHAPS or 0.5 M NaCl followed by ultracentrifugation. The resulting pellets and supernatants were then analyzed by Western blot in the presence of antibodies specific to BIP, GP63, *Lmj*PEM1, and

*Lmj*PEM2 [39,40]. (A), (B), Apparent molecular weight are shown on the left. P, pellet; S, supernatant.

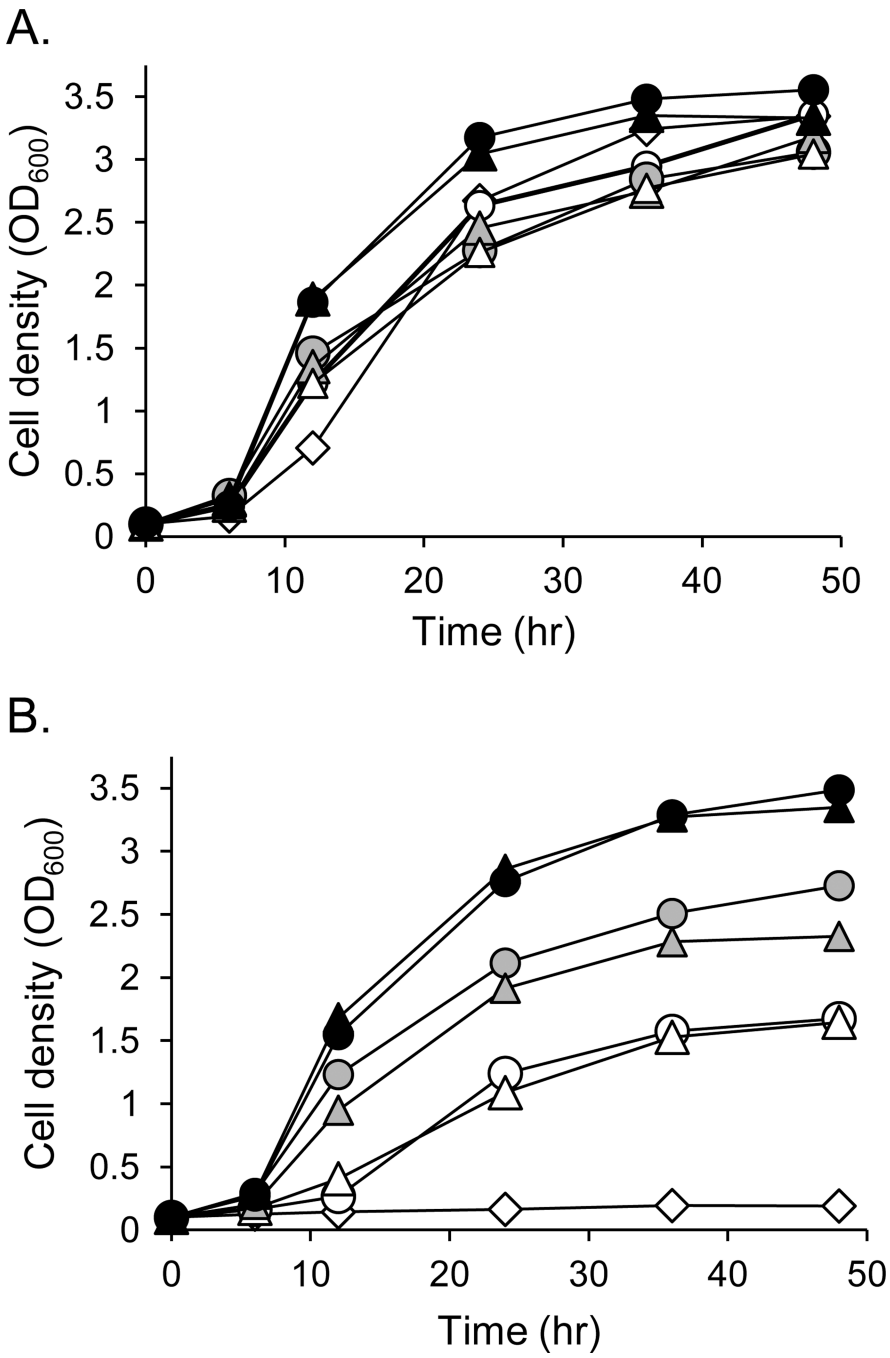

Fig. 7.

LmjPEM1 and *LmjPEM2* complement the choline auxotrophy phenotype of *S. cerevisiae* double null mutant *scpem1*Δ*scpem2*Δ that lacks PEMT activity. Growth curves in minimal medium in the presence of 10 μM (A) or absence of choline (B). (A), (B) Open diamonds, *scpem1*Δ*scpem2*Δ+*U*+*L*; open triangles, *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*L*; grey triangles, *scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2*; black triangles, *scpem1*Δ*scpem2*Δ +*LmjPEM1*+*LmjPEM2*; open circles, *scpem1*Δ*scpem2*Δ+*ScPEM1*+*L*; grey circles, *scpem1*Δ*scpem2*Δ+*U*+*ScPEM2*; black circles, *scpem1*Δ*scpem2*Δ+*ScPEM1*+*ScPEM2*. This

experiment was carried out in duplicate and was repeated at least once. A typical experiment is represented.

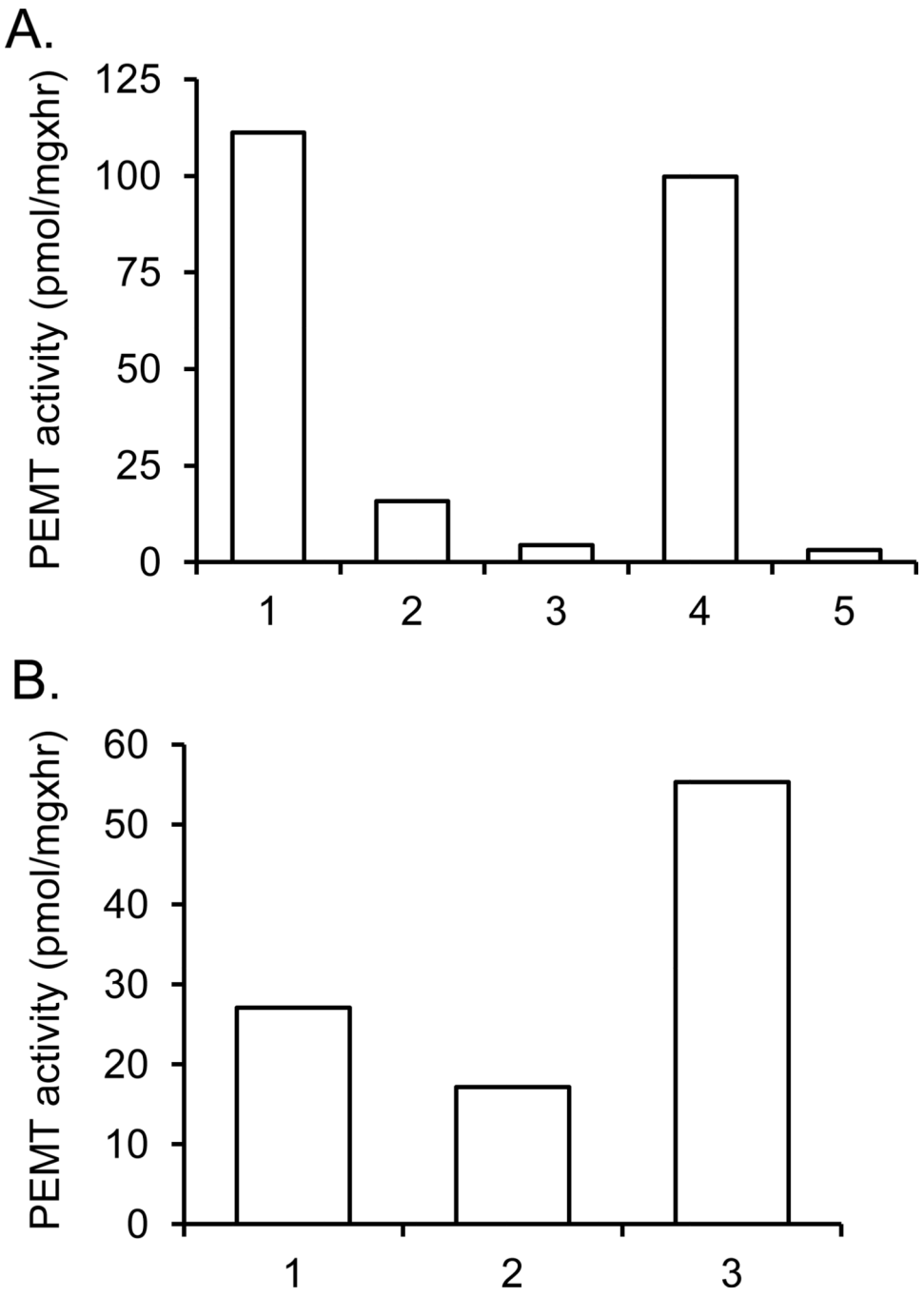

Fig. 8.

*Lmj*PEM1 and *Lmj*PEM2 act as PEMT enzymes. (A), (B) PEMT assay with yeast transformants was carried out at least twice in duplicate as described in Materials and methods. A typical experiment with standard deviations is shown. (A), 1, wild type; 2, *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*L*; 3, *scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2*; 4, *scpem1*Δ*scpem2*Δ +*LmjPEM1*+*LmjPEM2*; 5, *scpem1*Δ*scpem2*Δ+*U*+*L*. (B) 1, *scpem1*Δ*scpem2*Δ +*LmjPEM1*+*L*; 2, *scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2*; 3, *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*L* and *scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2* mixed 1:1 (v:v).

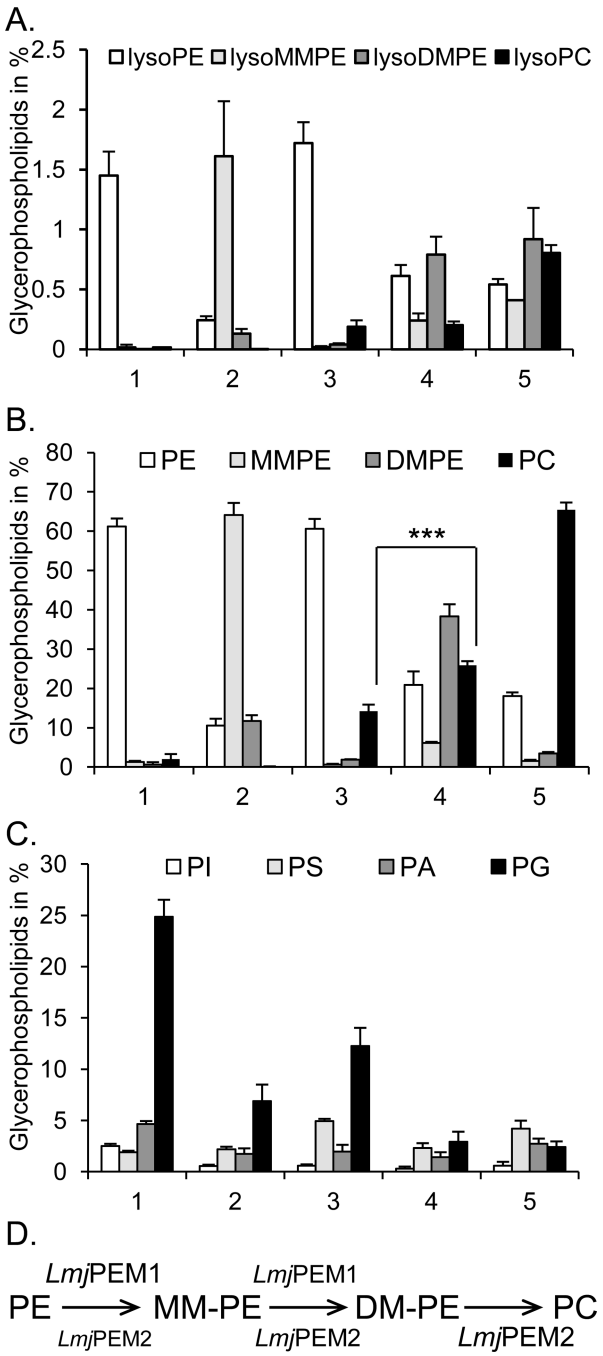

Fig. 9.

*Lmj*PEM1 and *Lmj*PEM2 substrate specificities. Bulk lipids were purified from yeast transgenics and analyzed by electrospray ionization tandem mass spectrometry as described in Materials and method section. (A) Percentages of lysoPE, lysoMMPE, lysoDMPE, and lysoPC. (B) Percentages of PE, MMPE, DMPE, and PC. ***, *P*-value <0.001. (C) Percentages of glycerolipids PI, PS, PA, and PG. (D) Schematic representation of the involvement of *Lmj*PEM1 and *Lmj*PEM2 in PE methylation leading to PC production. (A), (B), (C), 1, *scpem1*Δ*scpem2*Δ+*U*+*L*; 2, *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*L*; 3,

*scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2*; 4, *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*LmjPEM2*; 5, wild type. Standard deviations are shown.

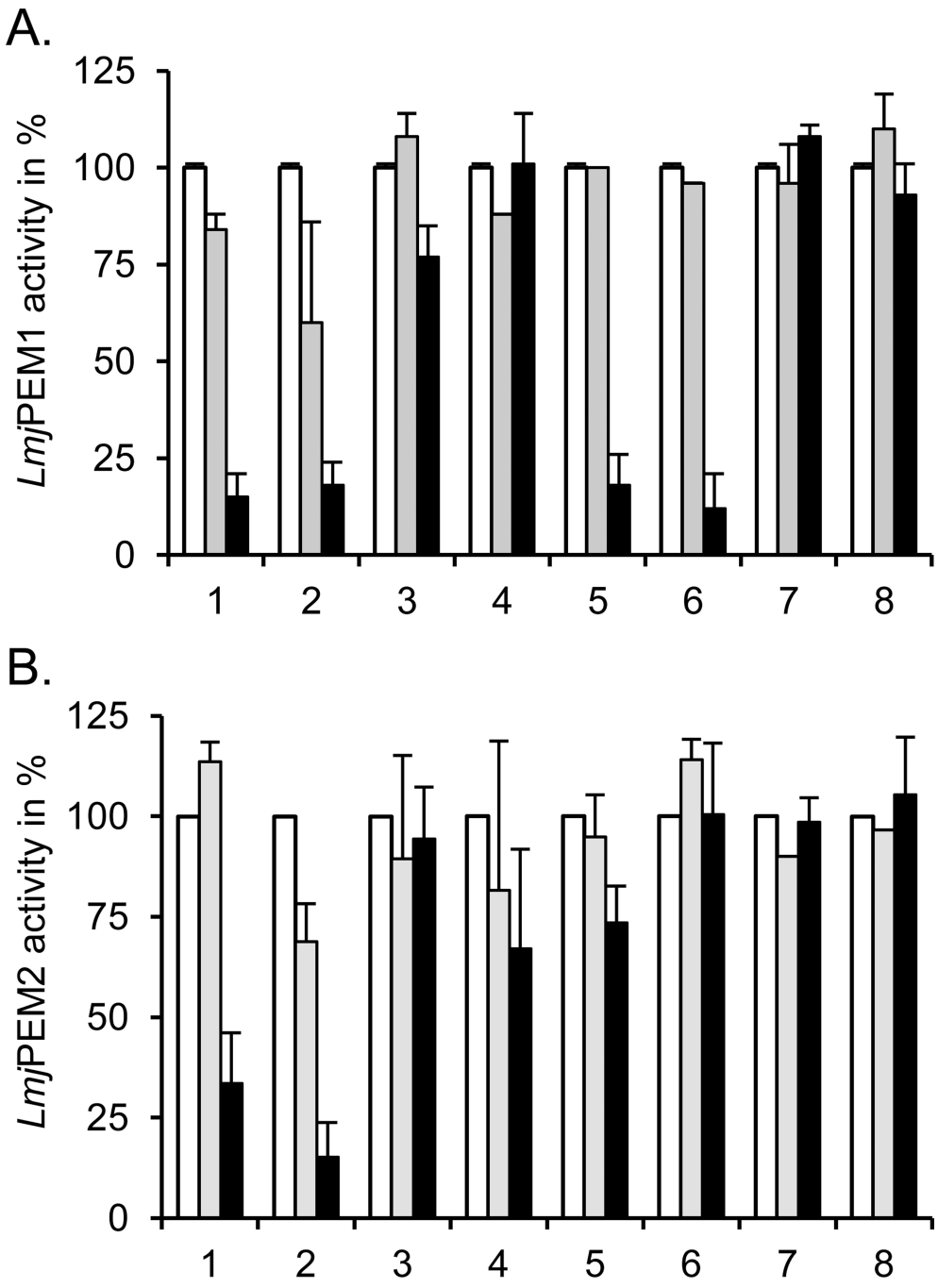

Fig. 10.

*Lmj*PEM1 (A) and *Lmj*PEM2 (B) inhibition by choline analogs. PEMT assay with yeast strains *scpem1*Δ*scpem2*Δ+*LmjPEM1*+*L* (A) and *scpem1*Δ*scpem2*Δ+*U*+*LmjPEM2* (B) was carried out at least twice in duplicate as described in Materials and methods in the absence or presence of 10 or 100 μM of various compounds. An activity of 100% corresponds to approximately 100 pmol/mgxhr and standard deviations are depicted. 1, octadecyltrimethylammonium bromide; 2, hexadecyltrimethylammonium bromide; 3, choline; 4, phosphocholine; 5, miltefosine; 6, octadecylphosphocholine; 7, ethanolamine; 8,

hydroxyethylhydrazine. (A), (B), open bars, no drug; grey bars, 10 μM of compound; black bars, 100 μM of drug.

Table 1

S. cerevisiae strains used in this study.

Strain	Genotype	Source
By4741 (Sc1)	*MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
*scpem1*Δ*scpem2*Δ (Sc55)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r*	[45]
*scpem1*Δ*scpem2*Δ*+U+L* (Sc140)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U][pBEVY-L]*	this work
*scpem1*Δ*scpem2*Δ*+LmjPEM1+L* (Sc118)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U-LmjPEM1] [pBEVY-L]*	this work
*scpem1*Δ*scpem2*Δ*+ScPEM1+L* (Sc50)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U-ScPEM1] [pBEVY-L]*	this work
*scpem1*Δ*scpem2*Δ*+U+LmjPEM2* (Sc82)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U][pBEVY-L-LmjPEM2]*	this work
*scpem1*Δ*scpem2*Δ*+U+ScPEM2* (Sc174)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U][pBEVY-L-ScPEM2]*	this work
*scpem1*Δ*scpem2*Δ*+LmjPEM1+LmjPEM2* (Sc84)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U-LmjPEM1] [pBEVY-L-LmjPEM2]*	this work
*scpem1*Δ*scpem2*Δ*+ScPEM1+ScPEM2* (Sc175)	*MATα his3ΔI leu2Δ0 ura3Δ0 pem1::Kan^r-pem2::Kan^r [pBEVY-U-ScPEM1] [pBEVY-L-ScPEM2]*	this work

Table 2

Fatty acid composition of lysoPE, lysoMMPE, lysoDMPE, and lysoPC present in yeast. Numbers represent the percentage of total cellular glycerolipids.

Glycerolipid	*U+L*	*LmjPEM1+L*	*U+LmjPEM2*	*LmjPEM1+LmjPEM2*	WT
lysoPE(16:1)*	0.623	0.078	0.800	0.336	0.191
lysoPE(16:0)	0.563	0.108	0.674	0.161	0.066
lysoPE(18:1)	0.235	0.050	0.227	0.144	0.172
lysoMMPE(16:1)	0.014	0.621	0.010	0.106	0.019
lysoMMPE(16:0)	0.010	0.601	0.010	0.081	0.012
lysoMMPE(18:2)	0.000	0.196	0.000	0.029	0.011
lysoDMPE(16:1)	0.000	0.058	0.015	0.328	0.038
lysoDMPE(16:0)	0.000	0.029	0.012	0.208	0.022
lysoDMPE(18:1)	0.000	0.016	0.003	0.089	0.027
lysoPC(16:1)	0.010	0.000	0.093	0.108	0.334
lysoPC(16:0)	0.000	0.000	0.050	0.040	0.067
lysoPC(18:1)	0.214	0.000	0.025	0.036	0.183

*, the first number indicates the total carbon atoms and the second number represents the total number of double bonds present in the fatty acids.

U+L, scpem1Δscpem2Δ+U+L; LmjPEM1+L, scpem1Δscpem2Δ+LmjPEM1+L; U+LmjPEM2, scpem1Δscpem2Δ+U+LmjPEM2; LmjPEM1+LmjPEM2, scpem1Δscpem2Δ+LmjPEM1+LmjPEM2; WT, wild type.

Table 3

Fatty acid composition of the most abundant PE, MMPE, DMPE, and PC present in yeast. Numbers represent the percentage of total cellular glycerolipids.

Glycerolipid	*U+L*	*LmjPEM1+L*	*U+LmjPEM2*	*LmjPEM1+LmjPEM2*	WT
PE(30:1)*	1.37	0.224	2.577	0.602	0.268
PE(32:2)	20.626	0.954	22.022	7.731	3.192
PE(32:1)	15.599	0.435	14.068	3.439	2.067
PE(34:2)	9.845	2.291	9.157	5.048	5.205
PE(34:1)	9.911	2.904	8.631	2.706	2.992
MMPE(30:1)	0.041	2.213	0.026	0.277	0.029
MMPE(32:2)	0.429	17.135	0.217	2.237	0.270
MMPE(32:1)	0.541	20.995	0.184	1.685	0.221
MMPE(34:2)	0.199	9.788	0.106	1.018	0.460
MMPE(34:1)	0.172	9.471	0.081	0.585	0.220
DMPE(30:1)	0.000	0.439	0.107	1.586	0.095
DMPE(32:2)	0.046	3.296	0.555	12.227	0.994
DMPE(32:1)	0.000	3.472	0.618	10.711	0.678
DMPE(34:2)	0.014	1.747	0.234	6.996	0.984
DMPE(34:1)	0.014	1.902	0.264	4.615	0.583
PC(30:1)	0.095	0.006	1.061	1.226	1.985
PC(32:2)	0.765	0.037	4.159	8.462	14.927
PC(32:1)	0.882	0.033	3.424	6.170	9.361
PC(34:2)	0.402	0.020	2.301	4.823	13.430
PC(34:1)	0.476	0.024	1.711	2.911	5.880

*, the first number indicates the total carbon atoms and the second number represents the total number of double bonds present in both fatty acyl groups.

U+L, scpem1Δscpem2Δ+U+L; LmjPEM1+L, scpem1Δscpem2Δ+LmjPEM1+L; U+LmjPEM2, scpem1Δscpem2Δ+U+LmjPEM2; LmjPEM1+LmjPEM2, scpem1Δscpem2Δ+LmjPEM1+LmjPEM2; WT, wild type.