Online Data Supplement

Pneumocystis S-Adenosylmethionine Transport: A Potential Drug Target

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Expression of GFP tagged PcPET8 in *Saccharomyces cerevisiae.* To produce PcPET8 tagged at the C-terminus with Green Fluorescent Protein (GFP) for cellular localization studies, we used the shuttle vector PKT128 (1, 2) designed to produce such fusion proteins in yeast. *PcPET8* was amplified by PCR from *P. carinii* DNA using the forward primer 5'AACTGCAGAAAATGGATTTGAA ACTAATTTATGG 3' and reverse primer 5'ACGCGTCGACGCCCACCCTTTAT ATATATATATA 3' (underlined sequences include the restriction sites of PstI and SalI, respectively). The yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter was amplified from *S. cerevisiae* DNA using the forward primer 5'ACGCGTCGACACGCTTTTTCAGT TCGAGTT3' and reverse primer 5'CCAATGCATGTTTGTTTATGTGT GTTTATTCG 3' (underlined sequences include the restriction sites of SalI and NsiI, respectively). After digesting the *PcPET8* product with PstI and the promoter product with NsiI, the digested products were joined by T4 DNA ligase. Another PCR was performed using the ligated product as template, a proof reading DNA polymerase, forward primer as for the promoter above, and the reverse primer as for *PcPET8* above. This last PCR product and the PKT128 vector were digested with SalI and the cut vector dephosphorylated using alkaline phosphatase (Promega). T4 DNA ligase was used to insert *PcPET8* into PKT128 creating plasmid PcPET8-PKT128. Competent cells (TOP10) were transformed, plated, and positive colonies selected by colony PCR. Selected colonies were grown in liquid media for plasmid isolation and the insert was confirmed by sequencing. Following manufacturer's instructions, the BY4741 strain of *S. cerevisiae* (Frozen EZ Yeast Transformation II kit, Zymo Research Corp., Orange, CA) was transformed with PcPET8-PKT128. Because BY4741 lacks histidinol-phosphate aminotransferase (HIS5) activity and requires histidine in the growth medium but PKT128 expresses HIS5, transformants could be selected using plates prepared with histidine dropout medium (1.7 g l^{-1} Yeast Nitrogen Base [Becton Dickinson, Rockville, MD], 2 μg l^{-1} biotin, 1.92 g l^{-1} of Yeast Dropout Amino Acid Mix lacking histidine [Sigma, St. Louis, MO]), and 20 g l^{-1} glucose. To detect co-localization of mitochondria and GFP-labeled PcPET8 by confocal microscopy, a colony was grown in liquid histidine dropout medium to an optical density of 0.6 then the mitochondrial marker MitoTracker Red 580 (Molecular probes) was added at a final concentration of 100nM. Images were captured with a Leica DM IRE2 microscope equipped with a TCS SL confocal system, 100x oil immersion objective and Leica imaging software using excitation lasers of 488 and 561 with emission spectra set at 510 and 600

for GFP and MitoTracker Red 580, respectively.

***Saccharomyces cerevisiae PET8* knockout (PET8Δ) complementation by *PcPET8*.** The *S. cerevisiae* PET8Δ strain was obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). The following steps were used to insert *PcPET8* into pGREG506 to allow expression in *S. cerevisiae* without extra amino acids. The complete *PcPET8* open reading frame was prepared by PCR using the forward primer 5'TCG ATA TCA TGG ATT TGA AAC TAA TTT ATG GA 3' and reverse primer 5'GAG GTC GAC AAC TAT ATA TAT ATA TAT AAG TAA AAA TAA AA 3' (underlined sequences contain restriction sites for EcoRV and SalI, respectively). After cutting both the PCR product and pGREG506 plasmid with EcoRV and SalI, T4 DNA ligase was used to ligate *PcPET8* into the plasmid creating pGREG506-PcPET8. Because BY4741 and PET8Δ yeast lack *Ura3*, the gene coding for uracil-synthesizing gene orotidine-5-monophosphate decarboxylase, but this gene is present in pGREG506 and constitutively expressed, transformed cells could be selected using medium lacking uracil (Yeast Nitrogen Base [1.7 g l^{-1}, Becton Dickinson], biotin [2 μg l^{-1}], yeast amino acid dropout mix lacking uracil [1.92 g l^{-1}, Sigma] and glucose [20 g l^{-1}]). The ability of *PcPET8* to complement PET8Δ was tested as follows. Tubes containing 5 ml of uracil-lacking but biotin-containing medium were inoculated with fresh colonies of BY4741 transformed with empty vector (WT), PET8Δ transformed with empty vector (ΔPET8), and PET8Δ transformed with pGREG506-PcPET8 (ΔPET8-PcPET8) taken from plates lacking uracil that were grown overnight at 30ºC. The liquid cultures were harvested by centrifugation, washed three times with sterile water, and resuspended in 5 ml of medium lacking uracil and containing biotin as above but with galactose substituted for glucose to induce expression of the *PcPET8* insert. After incubation at 30ºC for 12 h to allow recombinant protein expression, the cells were tested for mitochondrial AdoMet transporter function as follows. The cells were washed again 3 times to remove biotin and dilutions of 50, 100, and 200 fold were prepared from each of the 3 cultures and 1 μl aliquots of each dilution were spread onto plates based on the same galactose-containing medium except that 20 μg^{-1} desthiobiotin was substituted for biotin (if mitochondria can import AdoMet, desthiobiotin can be converted into biotin). The plates were incubated at 30ºC for 48h and colony growth evaluated.

1. Sheff MA, Thorn KS. Optimized cassettes for fluorescent protein tagging in saccharomyces cerevisiae. *Yeast* 2004;21(8):661-670.
2. Marobbio CM, Agrimi G, Lasorsa FM, Palmieri F. Identification and functional reconstitution of yeast mitochondrial carrier for s-adenosylmethionine. *EMBO J* 2003;22(22):5975-5982.