

A Novel ADP/ATP Transporter in the Mitosome of the Microaerophilic Human Parasite *Entamoeba histolytica*

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Supplemental Experimental Procedures

Cloning of the Mitosomal Carrier

The mitosomal adenine nucleotide carrier was identified with BlastP on data generated by the *Entamoeba histolytica* Genome Project and amplified by PCR from *E. histolytica* genomic DNA. Rapid amplification of cDNA ends (RACE) was used to amplify the full open reading frame (ORF) from a cDNA library. The gene was modified by PCR to introduce a BspHI site coinciding with the start codon and an NheI site immediately after the stop codon. The product was ligated into vector pNZ8048 [S1]. The resulting plasmid pNZEhis was transformed into *Lactococcus lactis* NZ9000.

Expression and Localization Studies of the *Entamoeba* Carrier in Yeast

The *E. histolytica* gene was amplified by PCR with primers to introduce an in-frame NcoI site, incorporating the start codon, as well as an NheI site after the 3' stop codon. The product was cloned into pYES-Pgal-aac2 [S2], resulting in the expression vector pYES-Ehis, which was transformed into *S. cerevisiae* INVSc1. Yeast mitochondria were prepared [S3], and soluble and peripheral proteins were removed by rupture of the outer mitochondrial membrane to produce mitoplasts and then by sodium carbonate extraction [S4]. For differential solubilization, mitochondria were extracted with digitonin [S5], and proteins in the soluble fraction were precipitated with 10% trichloroacetic acid.

Preparation of *E. histolytica* Cellular Fractions

E. histolytica HM-1:IMSS clone 9 was grown in YI-S medium with 15% adult bovine serum. *E. histolytica* trophozoites were lysed by

freeze-thawing. Crude extracts were fractionated by differential centrifugation as described previously [S6]. The postnuclear supernatant was centrifuged at $100,000 \times g$ for 30 min to generate a high-speed supernatant (the cytosolic fraction) and a high-speed sediment. The latter was resuspended in SMDI (0.25 M sucrose, 10 mM MOPS, and 10 mM DTT at pH 7.2) containing Complete Protease Inhibitors (Roche) and loaded on a 45% Percoll density gradient in SMDI [S7]. Fractions were collected after centrifugation at $68,000 \times g$ for 30 min at 4°C.

Enzyme Assays for Acid Phosphatase

Acid phosphatase activity was determined with *p*-nitrophenyl phosphate (*p*-NPP) as substrate [S8]. Fractions (0.1 ml) were incubated with 0.9 ml of substrate solution (Sigma) for 30 min at 37°C. Reactions were terminated by addition of 0.2 ml 2N NaOH, and the concentration of *p*-nitrophenol was determined with an extinction coefficient of 18.5 mM cm^{-1} . One unit of activity is defined as the amount of enzyme hydrolyzing 1 nmol of substrate per min at 37°C.

Isolation of Membrane Vesicles of *Lactococcus lactis*

Lactococcus lactis strain NZ9000/pNZEhis was grown at 30°C in M17 broth supplemented with 1% glucose and 5 $\mu\text{g/ml}$ chloramphenicol. Expression was induced at A_{600} of 0.6 by the addition of a dilution of 1:1000 of Nisin A stock [S1]. Cultures were incubated for 3 hr at 30°C and left at 20°C for an additional 16 hr. Cells were collected by centrifugation for 10 min at $6,000 \times g$ and 4°C and were washed once in ice-cold Tris-buffered saline (TBS; 20 mM Tris [pH 7.4] and 150 mM NaCl). The final pellet was resuspended in 50 ml TBS, and the cells were lysed with a Constant Systems 2.2 kV disrupter at 30,000 psi and 0.5°C. Lysed cells were left for 10 min on

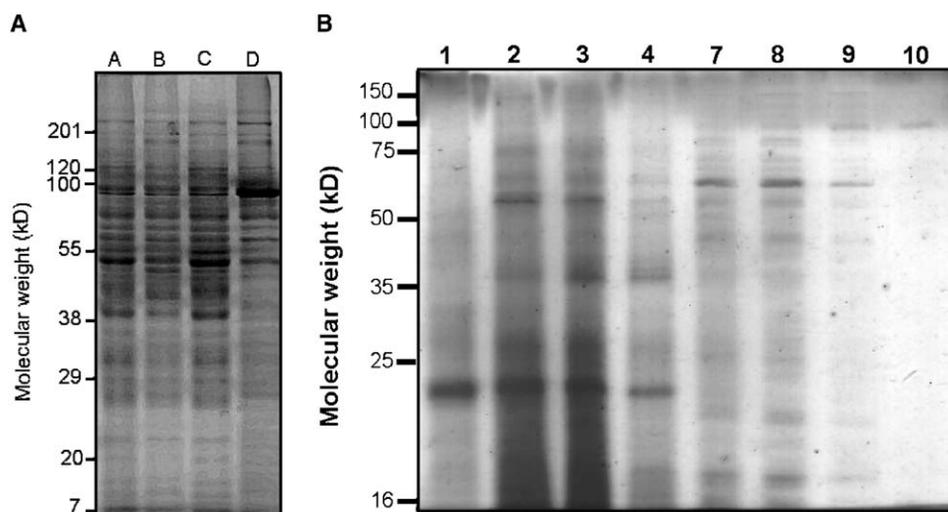


Figure S1. Protein Content of *Entamoeba* Cell Fractions

(A) Representative Coomassie-blue-stained gel of crude cell extracts fractionated by differential centrifugation: (A) crude extract; (B) nuclear fraction; (C) high-speed supernatant; and (D) high-speed sediment. The gel corresponds to the Western blot shown in Figure 2A.

(B) The high-speed sediment was further fractionated on a 45% Percoll density gradient as described in the Supplemental Experimental Procedures. (B) shows a corresponding Coomassie-stained gel showing protein distribution in all fractions; these fractions correspond to lanes 1–10 in Figure 2A.

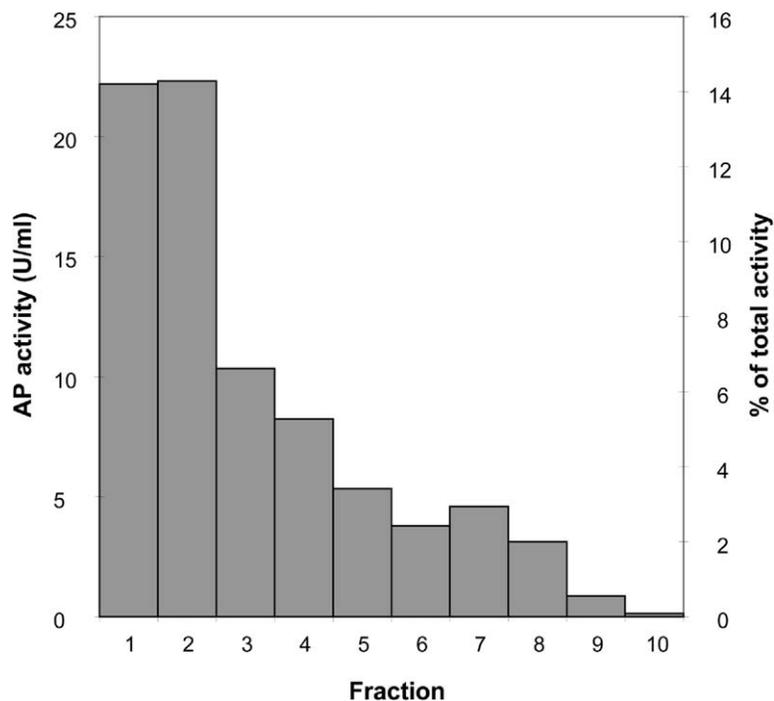


Figure S2. Distribution of the Lysosomal Marker Enzyme Acid Phosphatase Activity in Percoll Gradient Fractions

Acid phosphatase (AP) activity was assayed in fractions 1–10 of the Percoll gradient shown in Figure 2A, as described in the Supplemental Experimental Procedures. The extent of mitosome enrichment in organellar preparations is illustrated by comparing the distribution of the mitochondrial protein Cpn60 (Figure 2), the pattern of Coomassie staining (Figure S1B), and the distribution of contaminating lysosomal vesicles in the Percoll gradient, as indicated by the marker enzyme acid phosphatase.

ice, and whole cells were subsequently removed by centrifugation at $11,000 \times g$ for 10 min at 4°C . The membrane vesicles were collected by ultracentrifugation at $200,000 \times g$ for 30 min at 4°C after one wash step with TBS.

Production and Purification of Antibodies

Antibodies against a synthetic peptide, corresponding to the region 84–97 of the mitochondrial carrier, were produced in hen (Agrisera). The antibodies were affinity purified with mitochondrial carrier protein.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Samples were loaded onto 15% SDS-PAGE gel and separated at 30 mA. For Western analysis, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane at 120 mA for 1 hr. The membrane was blocked with 5% (w/v) skimmed milk in TBS buffer containing 0.1% (v/v) Tween overnight at room temperature with gentle agitation. The purified primary antibody and secondary antibody (anti-chicken HRP; Sigma) were used at titers of 1:250 and 1:20,000, respectively. Antibody-stained protein bands were visualized with ECL (Amersham).

Preparation of Liposomes and Fused Membranes

Escherichia coli total lipid extract and egg yolk phosphatidylcholine (Avanti Polar Lipids) were mixed in a 3:1 w/w ratio in buffer to a final lipid concentration of 20 mg/ml. Membrane vesicles were mixed with liposomes in a 1:5 (w/w) protein to lipid ratio in 800 μl of buffer in the presence of substrates, ionophores, and inhibitors. Membranes and liposomes were fused by freezing in liquid nitrogen and thawing at room temperature seven times.

Transport Assays

The fused membranes were extruded nine times through 400 nm polycarbonate filters and collected by centrifugation for 30 min at $390,000 \times g$ and 4°C . The pellet was resuspended in the appropriate buffer, containing the substrate, inhibitors, or ionophores. Two different transport methods were applied. In one method, the external substrate was removed by passing the fused membrane suspension over a 3.5 ml bed volume Sephadex G-75 gel filtration column equilibrated with buffer without substrate. The fused membranes were eluted in 1 ml buffer, and transport was initiated by 4-fold dilution

in buffer containing radio-labeled substrate, competitors, inhibitors, or ionophores as indicated. In the second method, the substrate-loaded fused membranes were concentrated in 50 μl buffer and directly diluted 200-fold in buffer containing radio-labeled substrate, competitors, inhibitors, or ionophores, depending on the experiment. All uptake experiments were performed at 25°C with constant stirring. Uptake was stopped by addition of 4 ml of ice-cold TBS and then by rapid filtration over cellulose nitrate filters (0.45 μm pore size). The filters were washed once with 2 ml ice-cold TBS and transferred to a vial for scintillation counting.

Phylogenetic Analyses

Alignment of conceptually translated protein sequences of all available MCF sequences was performed with ClustalW [S9], with manual adjustment of the resulting alignment. Phylogenetic analysis of this data set (199 sequences, 232 positions) was performed with MrBayes [S10], with 200,001 generations under the Whelan and Goldman (WAG) amino-acid substitution model, with one invariable and eight invariable substitution-rate categories. 501 trees were discarded as “burn-in.”

On the basis of this analysis, a second data set of 39 sequences most closely related to the *Entamoeba histolytica* carrier was analyzed further. Bootstrap replicate data sets (100) were each analyzed with MrBayes as before. Branch lengths for the bootstrap consensus tree (Figure S4) were estimated with Tree-Puzzle version 5. Constraint trees, consistent with different hypotheses of relationship for the *Entamoeba* carrier, were constructed with MrBayes and evaluated against the best unconstrained tree we found with the approximately unbiased (AU) test [S11]. Using this test, we were able to reject at $p = 0.05$ the hypothesis that the *Entamoeba* MCF was a member of the classical ADP/ATP carrier clade.

Supplemental References

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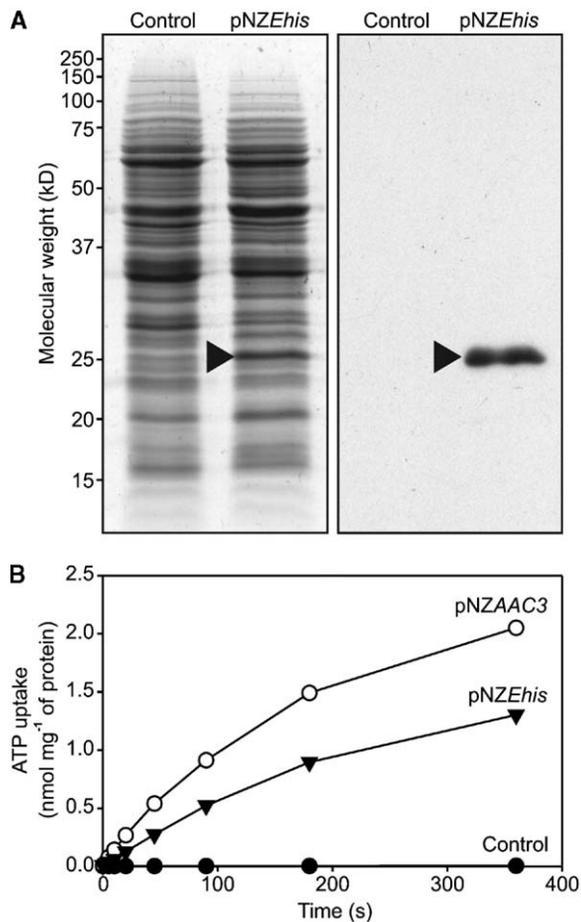


Figure S3. Expression and Functional Analysis of the Mitosomal Carrier in Lactococcal Membranes

(A) Coomassie-stained SDS-polyacrylamide gel and Western-blot analysis of membrane vesicles of the *L. lactis* control (lane 1) and expression strain NZ9000/pNZEhis (lane 2), with purified antibodies raised against peptide 84–97 of the mitosomal carrier. Approximately 15 μ g total membrane protein was loaded per lane. The triangle indicates the band of the expressed mitosomal carrier.

(B) [¹⁴C]-ATP uptake by membrane vesicles of the expression NZ9000/pNZEhis (closed triangles) and control strain (closed circles) preloaded with 5 mM ADP. Membranes of a strain expressing the yeast ADP/ATP carrier 3 were used as a positive control (open circles). After removal of external ADP by gel filtration, the exchange was initiated by diluting the membrane vesicles 3-fold in buffer containing 1 μ M valinomycin, 1 μ M nigericin, and 0.7 μ M [¹⁴C]-ATP. The accumulated radiolabeled ATP was determined by scintillation counting after removal of external radio-labeled substrate by filtration.

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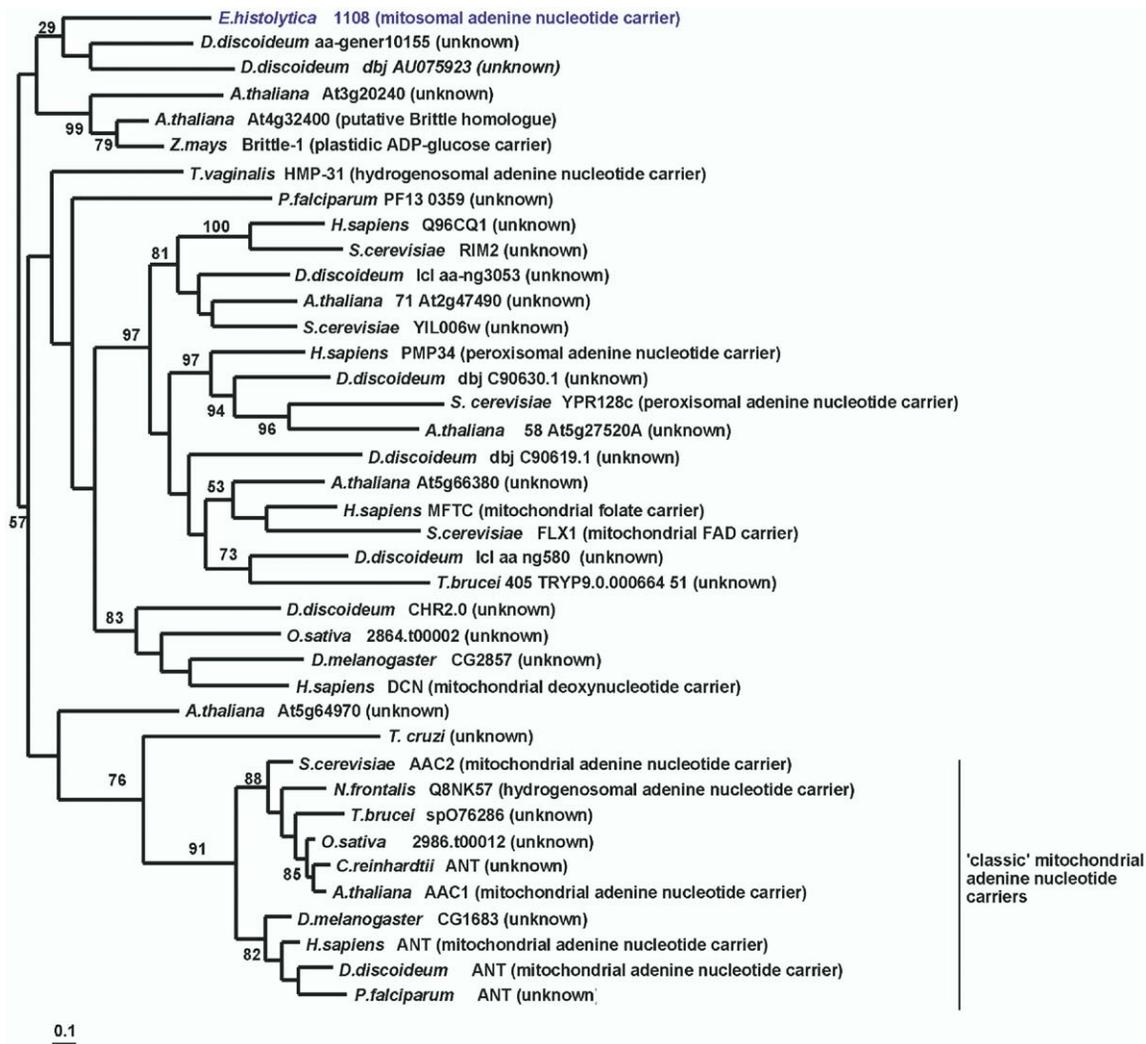


Figure S4. Phylogenetic Analysis of the Mitosomal ADP/ATP Carrier

The tree shown is a Bayesian consensus tree from 100 bootstrap replicate data sets. Bootstrap values over 50% are given at relevant nodes. The scale bar represents 0.1 substitutions per site.