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Construction of a *Saccharomyces cerevisiae* strain expressing the *Leishmania major* nucleoside hydrolase gene

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Abstract

Nucleoside hydrolase (NH) (EC. 3.2.2.3) is an essential enzyme in the purine–pyrimidine salvage pathway utilised by many protozoan parasites and may be a useful drug target. However, the search for NH inhibitors has been hampered by the lack of suitable in vitro screens. We have constructed a *Saccharomyces cerevisiae* strain that requires expression of the *Leishmania major* nucleoside hydrolase (LmNH) enzyme for growth and that may be suitable as a screen for NH inhibitors. The gene encoding LmNH was amplified using polymerase chain reaction with *L. major* genomic DNA as the template, cloned into an expression vector and used to transform a yeast mutant unable to grow on uridine as the sole source of uracil. Expression of LmNH yielded an ca. 35.6 kDa protein, which was shown to be functional as the mutant strain was able to grow on medium containing uridine as the sole source of uracil. Importantly, this work has resulted in a strain that can be used to screen compounds as potential inhibitors of this essential *Leishmania* enzyme.

Keywords

Leishmania major; Nucleoside hydrolase; Drug screening; Leishmaniasis

1. Introduction

Similar to many tropical parasites, *Leishmania* spp. are introduced into mammalian hosts via an insect vector; for example, malaria parasites are transmitted by mosquitoes, most trypanosomes by tsetse flies, *Trypanosoma cruzi* by triatomids, and *Leishmania* spp. by sand flies. Leishmaniasis is endemic throughout Africa, India, the Middle East and southern Europe where it is transmitted by female phlebotomine sand flies, and in Central and South America where it is transmitted by *Lutzomyia* sand flies [1].

There are five principal species of *Leishmania*: *L. tropica* and *L. major* cause ulcer-like lesions at the site of the bite; *L. braziliensis* and *L. mexicana* can cause destruction and malformations of the nose, pharynx, palate and lips; and *L. donovani* causes the visceral form of the disease, in which fever and swelling of the spleen and liver occur. If left untreated, visceral leishmaniasis has a 100% mortality rate [2].

The primary treatment of leishmaniasis [3] is pentavalent antimony complexed to carbohydrate; sodium stibogluconate (pentostam) [4]; or meglumine antimoniate (glucantime)

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[5]. These compounds have been in use since 1940 but their mode of action is still not known. They are expensive and drug resistance is a significant problem (e.g. 70% of parasites from infected patients in India are resistant). Severe reactions, including death, occur in 10% of those treated [6].

The *Leishmania* non-specific nucleoside hydrolase (NH) is an essential enzyme in the purine–pyrimidine salvage pathway utilised for DNA and RNA synthesis. *Leishmania* parasites are purine auxotrophs [7–11] that must salvage pre-formed nucleosides from their hosts. Therefore, NH could be an excellent target for novel anti-*Leishmania* drugs.

We have exploited the availability of a yeast mutant defective in NH (*urh1*) and uridine kinase (*urk1*) that cannot grow on uridine as the sole source of uracil [12] and have expressed the *L. major* NH (LmNH) in this strain. The recombinant strain was able to grow on uridine, indicating that the expressed LmNH enzyme is active. This strain is an experimental tool that paves the way for screening for inhibitors of LmNH activity.

2. Materials and methods

2.1. Chemicals and antibodies

Restriction and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). Primers were synthesised by Invitrogen Corp. (Carlsbad, CA). The *c-myc* antibody was purchased from Novus Biologicals (Littleton, CO). All other chemicals were of reagent grade from Sigma-Aldrich, Inc. (St Louis, MO).

2.2. Gene cloning

The LmNH gene was amplified by polymerase chain reaction (PCR) from genomic DNA of the Neal Strain of *L. major* (LV39/MRHO/Sv/59/P), generously donated by Dr Richard Titus (Colorado State University, Fort Collins, CO). Forward and reverse primers were designed based on the DNA sequence of the NH of *L. donovani* (LdNH) [8]: forward primer, 5'-GGA TCC ATG CCG CGC AAG ATT ATT CTC GAT TGT GAT CCC GGG-3'; reverse primer, 5'-GAA TTC TTA TCA CTC GAG GTC TTC TTC GGA AAT CAA CTT CTG TTC TTG AGG ATC GCC GAT GCG CTT GAG TGC GTC AAT CAC GAG GCA CCA AAA CTT GTC G-3'. *Bam*H1 and *Eco*R1 restriction sites (underlined) were incorporated into the 5' and 3' primers, respectively, to facilitate cloning into the pYEX-BX protein expression vector (see Table 1 for the plasmids and strain used). A *c-myc*-encoding DNA sequence (bold) and two STOP codons, TGA and TAA (italicised), were also incorporated into the 3' primer. The DNA sequence encoding a *c-myc* peptide (EQKLISEEDLE) was included as a reporter to detect the protein using an antibody directed against the *c-myc* peptide.

PCR amplification was performed with an Idaho Technologies RapidCycler (Salt Lake City, UT) using the following conditions: 94 °C for 30 s with 30 cycles of 94 °C for 0 s, 40 °C for 7 s and 72 °C for 70 s, followed by a final extension at 72 °C for 30 s. PCR products were separated and the band corresponding to the DNA fragment of the expected sized was extracted.

The DNA band was sequenced by the University of Colorado Health Sciences Center DNA Core Facility (Aurora, CO) and found to contain the 942 bp LmNH ORF, the two restriction sites, two stop codons and 33 bp encoding the *c-myc* peptide. The PCR product was then cloned into the pCR2.1 TA cloning vector as per the manufacturer's instructions. The vector was subsequently amplified by transforming DH5α *Escherichia coli* cells and growing colonies using standard conditions. Individual colonies were screened for the presence of the LmNH gene by isolating plasmid DNA and determining the DNA sequence of the insert using M13 universal primers and gene-specific primers.

2.3. Expression plasmid construction

The LmNH-encoding gene containing the *c-myc* epitope was excised from pCR2.1 by digestion with *Bam*H1 and *Eco*R1. The appropriate DNA band was isolated and ligated into the copper-inducible pYEX-BX protein expression vector [13]. The resulting plasmid was amplified in *E. coli* DH5 α cells, isolated and digested with *Hind*III and *Kpn*I to isolate the DNA encoding the LmNH-*c-myc* fusion protein and the entire copper promoter. The digest was subsequently treated with DNA polymerase I, Klenow fragment, whilst pRS425 was digested with *Sma*I and treated with Calf intestinal alkaline phosphatase. After ligating the two DNA fragments together with T4 DNA ligase, the resulting plasmid (pML426; Table 1), which contained the copper-inducible promoter from pYEX-BX and the LmNH ORF fused to the *c-myc* tag, was used to transform *E. coli* DH5 α cells. Several ampicillin-resistant colonies were isolated and cultured. DNA was isolated and screened for the presence of pML426 by PCR using LmNH gene-specific primers. Two isolates were subsequently sequenced and one clone that contained the copper promoter as well as DNA encoding the LmNH-*c-myc* fusion protein and stop codons was identified.

2.4. Transformation of *Saccharomyces cerevisiae* YZRM18

The expression plasmid constructed above (pML426 and the empty vector pRS425) was used to transform the *S. cerevisiae* YZRM18 strain [12] using a lithium acetate method [14]. After 30 min incubation at 30 °C and 20 min heat shock at 42 °C, cells were plated onto solid yeast nitrogen base (YNB) medium in the absence of leucine (YNB-leu) and plates were incubated for 3–4 days at 30 °C.

Approximately 200 resulting yeast colonies were streaked onto each of the following media: YNB-leu plus uracil, plus or minus copper sulphate (CuSO₄); and YNB-leu minus uracil, plus uridine, plus or minus CuSO₄. Plates were incubated for 3 days at 30 °C and five transformants containing the empty vector (transformed with the empty vector and grown on medium plus uracil only) and five transformants containing the pML426 construct that grew on medium containing uridine were isolated.

The ten individual yeast transformants were grown at 30 °C with shaking (180 rpm) in 50 mL of liquid YNB-leu plus uracil medium. LmNH protein expression was induced by addition of 0.4 mM CuSO₄ (final concentration) during mid log phase and the cultures were incubated for an additional 16 h. Cells were harvested by centrifugation (1940 $\times$ g, 10 min), washed twice with ice cold water and lysed by the addition of 100 μ L of glass beads (710–1180 μ m) and 200 μ L of 2 $\times$ sample lysis buffer (0.1 M Tris (pH 6.8), 20% (v/v) glycerol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.5% (v/v) β -mercaptoethanol). Tubes were mixed by vortexing for 1 min and mixtures were boiled for 3 min. Debris was removed by centrifugation at 550 $\times$ g for 5 min. The cleared cell lysates were separated by 15% (w/v) Tris-HCl SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to poly(vinylidene fluoride) (PVDF) membranes using a mini-genie transfer apparatus (Idea Scientific, Minneapolis, MN).

2.5. Western blots

Yeast proteins were screened by Western blot to determine the presence of the LmNH-*c-myc* fusion protein as follows. Membranes were blocked in TBST (100 mM Tris, 1.5 M NaCl and 0.1% (v/v) Tween-20) containing 5% (w/v) powdered milk for 1 h at room temperature. Blocked membranes were then incubated with primary anti-*c-myc* antibody or serum raised against LdNH in TBST containing 1% (w/v) milk overnight at 4 °C and membranes washed five times for 15 min each in TBST. Secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce Chemical Company, Rockford, IL) was added in TBST/milk and membranes were incubated for 1 h at room temperature. The membranes were washed five times for 5 min each in TBST and were exposed to SuperSignal West Femto ECL reagent

(Pierce, Rockford, IL) for 1 min then developed using an Alpha Innotech digital system (San Leandro, CA).

2.6. Growth experiments

One yeast transformant that grew both on uracil- and uridine-containing medium, presumably containing the LmNH-*c-myc* fusion protein, and one yeast transformant that grew on uracil-containing medium only, transformed with the empty vector, were grown overnight in liquid YNB plus uracil at 30 °C with shaking (180 rpm). Cell numbers were determined using a haemocytometer and each strain was diluted to 1×10^6 cells/mL. Tenfold serial dilutions were prepared and 2 µL of each dilution was spotted onto solid YNB-leu plus uracil medium and YNB-leu minus uracil, plus uridine medium. The plates were incubated for 3 days at 30 °C and the amount of growth was recorded using an Alpha Innotech digital system.

3. Results

3.1. DNA and protein sequence comparisons

The DNA sequence of the cloned LmNH (GenBank accession no. AY603045) revealed a 942 bp ORF with a 60% G+C content, containing no introns, as well as an additional 33 bp that corresponded to the 3' *c-myc* epitope. This DNA sequence predicted a fusion protein of 325 amino acids with a predicted molecular weight of ~35.6 kDa (results not shown).

DNA sequence alignment was performed with the NH from *L. major* strain LM247 [10], *L. major* strain MHOM/SU/73/5ASKH (Banuls et al., unpublished data) and *L. donovani* clone WR0130 [8] and revealed a highly conserved sequence of 314 amino acid residues (results not shown). A comparison of DNA and protein sequences with the recently completed genome of the *L. major* Friedlin strain [15] revealed 99% identity to a portion of chromosome 18 encoding the non-specific NH (results not shown). Further database analysis revealed 79% homology to an inosine-uridine-preferring NH from *Crithidia fasciculata* [10], 67% homology to inosine-uridine-preferring NH from *Photorhabdus luminescens* and 64% homology to *Corynebacterium diphtheriae* (results not shown). This high degree of conservation between and among pathogens and parasites presents a powerful strategy for drug discovery against diverse microbiological parasites that require a non-specific NH for growth.

3.2. LmNH is expressed and functional in yeast

The *S. cerevisiae* YZRM18 strain was transformed with the pML426 vector containing the LmNH-*c-myc* gene as described in Section 2.4. All of the ca. 200 yeast isolates transformed with the pML426 vector grew on medium containing uridine as the sole source of uracil, whilst none of the colonies transformed with the pRS425 empty vector grew on the same medium. This is shown in Fig. 1 for one pair of isolates. These results demonstrate that the expressed LmNH is functional and is essential for growth of this yeast strain on medium containing uridine.

3.3. Expression of LmNH in *S. cerevisiae* YZRM18

The above results showed that the LmNH enzyme was functional in YZRM18 yeast transformants. To confirm the physical presence of the LmNH protein in transformants and to determine whether expression could be increased by inducing the copper promoter, five yeast isolates transformed with the LmNH-*c-myc* gene and five isolates transformed with the empty vector were selected. Each transformant was grown and induced to express the LmNH-*c-myc* protein. Cells were then lysed and lysates were separated by SDS-PAGE. Proteins were transferred to membranes and the membranes were probed with the *c-myc* antibody as

described in Section 2.5. Controls included each transformant incubated in 0.4 mM NaSO₄ (uninduced) rather than 0.4 mM CuSO₄. The results are shown in Fig. 2.

Note that the *c-myc*-specific antibody bound primarily to a protein of ~35 kDa in both copper-induced (Fig. 2A (arrow), lanes 1–5 indicated by [+]) as well as in uninduced (Fig. 2A, lanes 1–5 indicated by [–]) isolates containing the LmNH-*c-myc* gene. However, the amount of *c-myc* antibody-reactive protein was greater in induced cells compared with uninduced cells. As expected, no LmNH-*c-myc* protein was detected by the *c-myc* antibody in either induced or uninduced cells containing the empty vector (Fig. 2A, lanes 6–10).

A duplicate membrane was treated with polyclonal serum directed against the LdNH protein (Fig. 2B). Note that the polyclonal serum detected a single protein of ~35 kDa in both copper-induced (Fig. 2B (arrow), lanes 1–5 [+]) and in uninduced (Fig. 2B, lanes 1–5 [–]) cells containing the LmNH-*c-myc* gene. In accordance with results described above, no LmNH-*c-myc* fusion protein was revealed by the polyclonal serum directed against the LdNH in either induced or uninduced cells transformed with the empty vector (Fig. 2B, lanes 6–10).

Importantly, the polyclonal serum detected a single protein band, identical in molecular mass to that detected using the *c-myc*-specific antibody.

4. Discussion

In this work, we have isolated and cloned the gene encoding the NH from *L. major*. We have formed a yeast DNA expression vector containing an inducible copper promoter followed by the LmNH-encoding gene fused in frame with a *c-myc* epitope-encoding DNA fragment. The resulting vector was used to transform a yeast mutant that requires NH activity for growth on uridine as the sole source of uracil. Our results clearly demonstrate that yeast cells expressed the LmNH enzyme. Expression of the LmNH-encoding gene occurred in the absence of copper ions, indicating that the promoter is ‘leaky’; however, expression was elevated when copper ions were added to the growth medium. Notably, not only did this strain of yeast express the LmNH protein, but this protein was also active, even with fusion of the C-terminal *c-myc* peptide. This was clearly indicated by the ability of this strain to grow on uridine as the sole source of uracil.

Several observations suggest that NH is a promising candidate as a target for pan-*Leishmania* drugs [8,11], including the absence of NH activity in mammals, its intracellular location and its constitutive expression in promastigotes [8]. Moreover, NH homologues are present in *Trypanosoma brucei gambiense*, *T. cruzi* and *T. brucei brucei* as well as in *L. donovani*, *L. mexicana* and *L. tropica*. Other human pathogenic protozoa with a purine salvage pathway include *Plasmodium*, *Entamoeba histolytica*, *Giardia lamblia* and *Toxoplasma gondii* [8].

However, developing an in vitro assay for inhibitors for NH that is suitable for screening has not been feasible owing to a number of intrinsic difficulties, which include growing large numbers of *L. major* promastigotes, isolating and purifying LmNH activity and devising a simple assay for enzyme activity. We have constructed a yeast strain expressing the LmNH enzyme to overcome these obstacles. This strain can now be used to screen chemical libraries for compounds that inhibit growth of the yeast mutant on medium containing uridine as the sole source of uracil but have no effect on growth on uracil-containing medium. Using this extremely simple screen, compounds that are inhibitors of LmNH activity should be able to be quickly and easily identified.

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Fig. 1. *Saccharomyces cerevisiae* YZRM18 cells transformed with either pML426 or the pRS425 empty vector, isolated and grown on medium containing (A) uracil or (B) uridine as the sole sources of uracil, as described in Section 2.4. (See Table 1 for definition the plasmids and strain used.)

A

Fig. 2.

Expression of *Leishmania major* nucleoside hydrolase (LmNH) in *Saccharomyces cerevisiae* YZRM18 cells. YZRM18 cells were transformed with either pML426 or the pRS425 empty vector and transformants were isolated as described in Section 2.4. Resulting isolates were grown in liquid medium containing uracil and incubated with either 0.4 mM NaSO₄ or 0.4 mM CuSO₄, then harvested and lysed. Proteins were separated by SDS-PAGE. Proteins were transferred to poly(vinylidene fluoride) (PVDF) membranes and treated with either (A) an antibody directed against *c-myc* or (B) serum against *Leishmania donovani* nucleoside hydrolase. Lanes 1–5, isolates transformed with pML426; lanes 6–10, isolates transformed with pRS425 (empty vector). + indicates isolate grown in the presence of CuSO₄; – indicates isolate grown in the presence of NaSO₄. The numbers indicate the positions of molecular mass markers in kDa. (See Table 1 for definition the plasmids and strain used.)

Table 1

Strains and plasmids used in the study

	Relevant genotype	Source/reference
<i>Saccharomyces cerevisiae</i> YZRM18	MATa ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::P _{3xERE} -URA3 Δpdr5::hisG Δsnq2 hisG (2 μm, HIS3-P _{PGK1} -hER) Δurk1::TRP1 Δurh1::kanMX	[12]
Plasmids		
pCR2.1	lac promoter, lacZα fragment, T7 promoter and priming site, M13(-20)F priming site, M13R priming site, ori(pUC), amp ^R , kan ^R , ori(f1)	Invitrogen
pYEX-BX	Amp ^R , ori(pUC), CUP1 promoter, leu2-d, URA3, ori(2μ), MCS(<i>Bam</i> H1, <i>Sal</i> I, <i>Pst</i> I, <i>Eco</i> R1)	Amrad
pRS425	ori(f1), lacZ, T7 promoter, MCS (KpnI-SacI) T3 promoter, lacI, ori(pMB1), amp ^R , ori(2μ) LEU2	ATCC 77106
pML426	pRS425 with CUP1 promoter; LmNH- <i>c-myc</i>	This study