Research Article

Identification of the *Leishmania major* Proteins LmjF07.0430, LmjF07.0440, and LmjF27.2440 as Components of Fatty Acid Synthase II

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Leishmania major causes leishmaniasis and is grouped within the Trypanosomatidae family, which also includes the etiologic agent for African sleeping sickness, *Trypanosoma brucei*. Previous studies on *T. brucei* showed that acyl carrier protein (ACP) of mitochondrial fatty acid synthase type 2 (FASII) plays a crucial role in parasite survival. Additionally, 3-oxoacyl-ACP synthase *Tb*KASIII as well as *Tb*HTD2 representing 3-hydroxyacyl-ACP dehydratase were also identified; however, 3-oxoacyl-ACP reductase *Tb*KAR1 has hitherto evaded positive identification. Here, potential *Leishmania* FASII components LmjF07.0440 and LmjF07.0430 were revealed as 3-hydroxyacyl-ACP dehydratases *Lm*HTD2-1 and *Lm*HTD2-2, respectively, whereas LmjF27.2440 was identified as *Lm*KAR1. These *Leishmania* proteins were ectopically expressed in *Saccharomyces cerevisiae htd2Δ* or *oar1Δ* respiratory deficient cells lacking the corresponding mitochondrial FASII enzymes Htd2p and Oar1p. Yeast mutants producing mitochondrially targeted versions of the parasite proteins resembled the self-complemented cells for respiratory growth. This is the first identification of a FASII-like 3-oxoacyl-ACP reductase from a kinetoplastid parasite.

1. Introduction

Leishmania species represent a group of parasitic protozoa that cause widespread morbidity and even mortality (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm), which are transmitted to humans through the bites of sandflies (for reviews on visceral, cutaneous, and mucosal leishmaniases, see [1, 2]). *Leishmania* spp. are clustered within the Trypanosomatidae family that additionally contains *Trypanosoma* spp. and *Crithidia fasciculata*. The World Health Organisation estimates that approximately 12 million people suffer from various forms of leishmaniasis, some of which are lethal if left untreated. It is estimated that about 350 million people are at risk worldwide, and human infections are found in 16 countries in Europe (http://www.who.int/tdrold/diseases/leish/files/leish-poster. pdf). As a genus, *Leishmania* represents a major global human disease agent. Apparently, there is a rise in the rate of new *Leishmania* infections, and *Leishmania*/HIV coinfection has been termed as an emerging disease in southern Europe.

Moreover, drug resistance has also been reported recently. Since most available drugs are expensive, and treatment is long and accompanied by severe side effects but at the same time becoming less effective, there is a renewed interest and urgency in developing new antileishmanial drugs.

An attractive target process for novel drug design is represented by the type 2 fatty acid synthase (FASII) system [3, 4], which occurs in the mitochondria of trypanosomatids [5]. Despite the fact that the *L. major* genome was completely sequenced in 2005 [6], there is currently no information available on mitochondrial FASII in this particular kinetoplastid parasite. Pioneering studies on *T. brucei* revealed that it has an essential mitochondrial FASII process, which it employs for the production of octanoate and palmitate [7]. It was found that RNAi depletion of the mitochondrial acyl carrier protein (ACP) significantly reduced cytochrome-mediated respiration in the procyclic form of trypanosomes, and effected changes in mitochondrial morphology and alterations in the membrane potential [7, 8]. In addition to ACP, the *T. brucei* FASII enzymes

TABLE 1: Plasmids and oligonucleotides used.

Plasmids or oligonucleotides	Description	Sources or references
plasmid[a]		
1] pBluescript II KS	pKS cloning vector	Stratagene
pKS:LmjF07.0440[1]	LmjF07.0440 in pBluescript (with primers for *Lm*HTD2-1)	This study
pKS:LmjF07.0430[1]	LmjF07.0430 in pBluescript (with primers for *Lm*HTD2-2)	This study
pKS:LmjF27.2440[1]	LmjF27.2440 in pBluescript (with primers for *Lm*OAR1-1)	This study
pKS:LmjF24.2030[1]	LmjF24.2030 in pBluescript (with primers for *Lm*OAR1-2)	This study
2] YEp352	*URA3*-marked multicopy plasmid	[17]
3] YEp:CTA1[2]	*CTA1* behind its promoter (pYE352:*CTA1*)	[18]
YEp:LmjF07.0440[3]	LmjF07.0440 behind the *CTA1* promoter	This study
YEp:LmjF07.0430[3]	LmjF07.0430 behind the *CTA1* promoter	This study
YEp:LmjF27.2440[3]	LmjF27.2440 behind the *CTA1* promoter	This study
YEp:LmjF24.2030[3]	LmjF24.2030 behind the *CTA1* promoter	This study
YEp:OAR1[3]	*OAR1* behind the *CTA1* promoter	[14]
YEp:YHR067w[3]	*HTD2* behind the *CTA1* promoter	[12]
oligonucleotide[b]		
Lm HTD2-1 5′ NcoI	TTATCCATGGAAGCGGCAGCACGCCGCATCATC	This study
Lm HTD2-1 3′ HindIII	TATTAAGCTTAAAGCCGACGCTCCACTCGCTC	This study
Lm HTD2-2 5′ NcoI	TTATCCATGGCCAGCGAGACTGCACCACTTGTC	This study
Lm HTD2-2 3′ HindIII	TATTAAGCTTACTTCGTCACAGACCACTCACTC	This study
Lm OAR1-1 5′ NcoI	TTATCCATGGGTGCCCCTAACGCACACCCAC	This study
Lm OAR1-1 3′ HindIII	TATTAAGCTTACGATAAGCCGCCATCGACGGCG	This study
Lm OAR1-2 5′ NcoI	TTATCCATGGACGCCAAGTCCATCCAGTCG	This study
Lm OAR1-2 3′ HindIII	TATTAAGCTTACTTGGCTGCCATACGGGTTGCC	This study

[a]The numbers in superscript following the plasmids' designation refer to their parental vectors, for example, pKS:LmjF07.0440[1] was derived from 1] pBluescript II KS.
[b]Lm HTD2-1 refers to LmjF07.0440, Lm HTD2-2 to LmjF07.0430, Lm OAR1-1 to LmjF27.2440, and Lm OAR1-2 to LmjF24.2030.

3-oxoacyl-ACP synthase KASIII [7] and 3-hydroxyacyl-ACP dehydratase HTD2 [9] have also been identified. Furthermore, mitochondrial compartmentalisation of three FabG candidates of *T. brucei* FASII, annotated as KAR1, KAR2, and KAR3, has been convincingly demonstrated using fusions with green fluorescent protein GFP [7]; however, their physiological function as 3-oxoacyl-ACP reductases of mitochondrial FASII has hitherto not been reported. Thus, it is important to identify *Leishmania* KAR1 so as to complete our picture of mitochondrial FASII in trypanosomatids, and in doing so to gain a better understanding of the enzymes undertaking this process, thereby refining our appreciation of their potential suitability for novel drug design.

A convenient and safe method for revealing the identity of parasite proteins is to demonstrate their physiological function in *Saccharomyces cerevisiae* mutant cells missing the corresponding yeast enzymes. In fungi, FASII is a mitochondrial process that is critical for organellar function and morphology, and a lesion in any one of the genes encoding FASII proteins results in a respiratory growth phenotype, underdeveloped mitochondria lacking cytochromes, and, in some cases, loss of mitochondrial DNA (reviewed in [10, 11]). For instance, yeast *htd2*Δ mutants devoid of the mitochondrial FASII enzyme 3-hydroxyacyl-ACP dehydratase Htd2p [12] were used previously to identify *T. brucei*

HTD2 [9]. Another more recent example of how yeast FASII mutants were employed as a surrogate host for functional complementation studies is represented by the manner in which *oar1*Δ mutant cells lacking the mitochondrial 3-oxoacyl-ACP reductase Oar1p [13] had helped to identify the human and nematode counterpart reductases [14, 15].

Here, *S. cerevisiae* was exploited further as a heterologous system for determining whether candidate *L. major* HTD2 and KAR1 proteins might represent physiologically functional FASII enzymes. This was done by monitoring the potential interchangeability of ectopically expressed *Leishmania* proteins that were preceded by a mitochondrial leader sequence (MLS) with the analogous fungal enzymes Htd2p or Oar1p in the aforementioned respiratory deficient strains (*htd2*Δ and *oar1*Δ mutant cells) during growth on glycerol medium. The results are viewed from the perspective of the utility of yeast FASII mutants for identifying *Leishmania* FASII enzymes.

2. Materials and Methods

2.1. S. cerevisiae Strains, Plasmids, and Oligonucleotides. Plasmids and oligonucleotides used are catalogued in Table 1. The *Escherichia coli* strain TOP10 F′ (Invitrogen, Carlsbad, CA, USA) was exploited for all plasmid multiplications
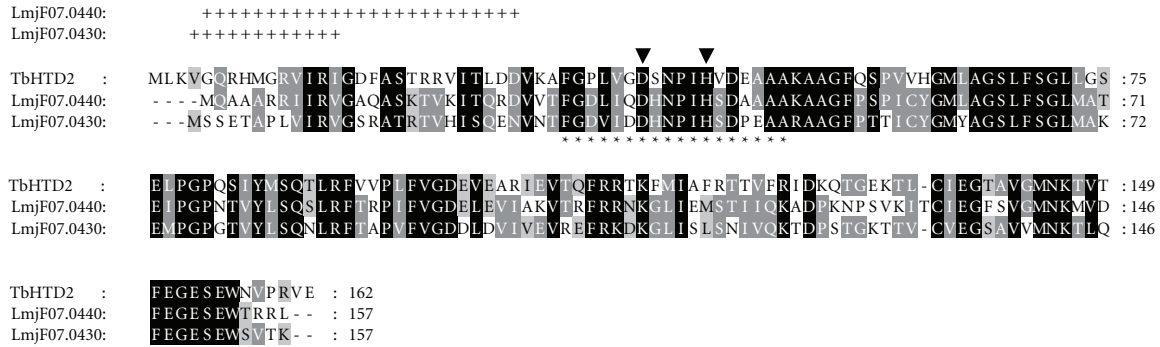
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LmjF07.0440:       ++++++++++++++++++++++++++++
LmjF07.0430:       ++++++++++++

TbHTD2     :  MLKVGQRHMGRVIRIGDFASTRRVITLDDVKAFGPLVGDSNPIHVDEAAAKAAGFQSPVVHGMLAGSLFSGLLGS  : 75
LmjF07.0440: ----MQAAARRIIRVGAQASKTVKITQRDVVTFGDLIQDHNPIHSDAAAAKAAGFPSPICYGMLAGSLFSGLMAT  : 71
LmjF07.0430: ---MSSETAPLVIRVGSRATRTVHISQENVNIFGDVIDDHNPIHSDPEAARAAGFPTTICYGMYAGSLFSGLMAK  : 72
                                               *****************

TbHTD2     :  ELPGPQSIYMSQTLRFVVPLFVGDEVEARIEVTQFRRTKFVIAFRTTVFRIDKQTGEKTL-CIIEGTAVGMNKTVT  : 149
LmjF07.0440: EIPGPNTVYLSQSLRFTRPLFVGDELEVIAKVTRFRRNKGLIEMSTIIQKADPKNPSVKITCIEGFSVGMNKMVD  : 146
LmjF07.0430: EMPGPGTVYLSQNLRFTAPVFVGDDLDVIVEVREFRKDKGLISLSNIVQKTDPSTGKTTV-CVEGSAVVMNKTLQ  : 146

TbHTD2     :  FEGESEWNVPRVE  : 162
LmjF07.0440: FEGESEWTRRL--  : 157
LmjF07.0430: FEGESEWSVTK--  : 157
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FIGURE 1: Similarity of *L. major* LmjF07.0440 and LmjF07.0430 to their *T. brucei* homologue, *Tb*HTD2. Explanations for this and Figure 3 are given. Multalin- and Genedoc-based comparison of the deduced amino acid sequences is provided. Dashes indicate the arrangement of the sequences for best fit. Black shadings refer to conserved amino acid residues among all three sequences whereas the darker and lighter grey shadings denote regions with more relaxed residue similarities not necessarily shared by the full set of sequences. MitoProt predictions of cleaved mitochondrial leader sequences are indicated (+). Active site residues of human mitochondrial dehydratase D62 and H67 are denoted (▼), whereas the hydratase 2 domain is underscored (∗). The sequences used for the *Leishmania* proteins throughout this work were retrieved from GeneDB of the Sanger Institute, whereas the accession number for *Tb*HTD2 was Q580H9_9TRYP.

and purifications. The mutant yeast strains BY4741*oar1*Δ (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ykl055c::kanMX*) and BY4741*htd2*Δ (*yhr067w::kanMX*) were acquired from EUROSCARF (http://www.uni-frankfurt.de/). Expression plasmids were incorporated into yeast cells using a reported procedure [16], and transformants were kept on SD-Ura medium, outlined below.

2.2. Plasmid Constructions. DNA inserts were assembled within plasmids according to standard methods [19]. The LmjF07.0430, LmjF07.0440, LmjF27.2440, and LmjF24.2030 genes were amplified from genomic *L. major* DNA by polymerase chain reaction (PCR) and the corresponding oligonucleotide pairs (Table 1). Sequential electrophoresis, processing, and tethering of amplicons to an *Eco*RV-digested pBluescript II plasmid vector (Stratagene, La Jolla, CA) are described [20]. The inserts were purified following digestion of the recombinant pBluescript plasmids with *Nco*I and *Hin*dIII restriction enzymes and fitted within a previously published plasmid YEp352:CTA1 [18] that is based on a *URA3*-marked YEp352 multicopy vector [17]. These inserts were placed behind the *CTA1* promoter as gene fusions with the nucleotides for a cleavable N-terminal yeast MLS directing Coq3p to the organelle [21], as described in [20]. Nucleotide sequencing of the pBluescript inserts ensured that no mutations were introduced during the PCR amplification step. In addition, this procedure was also applied to the YEp352-based expression plasmids, verifying that the reading frame across the fusion boundary between the *L. major* genes and the yeast *COQ3* nucleotides encoding the MLS was intact. Plasmid YEp352:OAR1 expressing yeast *OAR1* as well as plasmid YEp352:YHR067w producing fungal Hdt2p are both described [12, 14].

2.3. Media, Enzyme Assays, Respiration Competence, and Lipoic Acid Measurements. Standard media were made for yeast [22] and *E. coli* [23]. *URA3*-marked multicopy plasmids

were kept in transformed *S. cerevisiae* strains by bearing upon the cells selective pressure using solid SD-Ura medium. This medium consisted of 0.67% (wt/vol) yeast nitrogen base without amino acids, 2% (wt/vol) D-glucose, 3% (wt/vol) agar, and with all necessary supplements added with the exception of uracil (Sigma-Aldrich Inc., St. Louis, MO, USA). Solid synthetic complete glycerol medium (SCglycerol) was processed essentially as the previous SD-Ura medium, with the only two differences being the addition of uracil and replacement of glucose with 3% (wt/vol) glycerol as the exclusive carbon source. Respiration competence was tested using an overlay made with 0.1% (wt/vol) 2,3,5-triphenyltetrazolium chloride, (TTC) [24]. Lipoic acid was determined using a biological method that followed the growth of a lipoic acid-deficient *E. coli* strain in response to the presence of lipoic acid in yeast cells, with each yeast strain being propagated in triplicate cultures [25, 26].

2.4. Mitochondrial Localisation Predictions and Sequence Comparisons. MitoProt (http://ihg2.helmholtz-muenchen. de/ihg/mitoprot.html), PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html), and TargetP (http://cbs.dtu.dk/services/TargetP/) were used to identify N-terminal mitochondrial targeting sequences [27, 28]. Multalin (npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalin.html) and Genedoc (http://www.nrbsc.org/gfx/genedoc/index.html) were used to compare the sequences in Figures 1 and 3.

3. Results

3.1. LmjF07.440 and LmjF07.430 Are Functional 3-hydroxy-acyl-ACP Dehydratases in Yeast. The *L. major* database (http://www.genedb.org/) contains two sequences with a high degree of similarity to *T. brucei* HTD2 (Q580H9_9TRYP) representing mitochondrial 3-hydroxy-acyl-ACP dehydratase (Figure 1). Both LmjF07.0440 and

LmjF07.0430 are annotated in this database as maoc family dehydratase-like proteins with very high probability values for being identical to TbHTD2 (BlastP E-scores 1.6e-30 and 5.0e-29, resp.). To predict whether the two Leishmania proteins might reside in mitochondria, three separate algorithms were used to analyze their deduced amino acid sequences: MitoProt (MP), PSORT II (PS), and TargetP (TP). The probability (in brackets) that each of the Leishmania proteins would represent a mitochondrial enzyme was calculated and compared to the values obtained for TbHTD2: LmjF07.0440 (MP, 0.9974; PS, 78.3%; TP, 0.739), LmjF07.0430 (0.7896; 30.4%; 0.577), and TbHTD2 (0.8086, 43.5%, 0.699). In addition, both Leishmania proteins also contained the hydratase 2 motif [29] as well as amino acids germane with the active site residues of human mitochondrial dehydratase [30]. Hence, LmjF07.0440 and LmjF07.0430 were promising HTD2 candidates.

To investigate whether LmjF07.0440 and LmjF07.0430 might function as 3-hydroxyacyl-ACP dehydratases, their respective genes were expressed from the promoter of the yeast CTA1 gene for peroxisomal catalase A (Cta1p), which is only slightly derepressed under the glycerol medium conditions used here [31, 32]. Due to the lowered PS values for LmjF07.0430 compartmentalisation (compared to LmjF07.0440 and TbHTD2), a fungal MLS was fused to the two Leishmania proteins. Yeast htd2Δ mutant cells were transformed with plasmids for expressing native Htd2p (positive control), Cta1p (negative control), or the mitochondrially targeted Leishmania proteins LmjF07.0440 and LmjF07.0430. Transformants were grown on SD-Ura medium, and after successively diluting the yeast cells by a factor of ten, a volume of 2.5 μL of the dilutions was applied to SD-Ura or SCglycerol media, and the plates were kept at 30°C until single colonies were discernible. The results in Figure 2 revealed that mutant cells expressing LmjF07.0440 or LmjF07.0430 were proficient for growth on glycerol, much like the situation with the self-complemented strain, whereas those mutant cells producing excess Cta1p could not grow or divide on glycerol. Hence, LmjF07.0440 and LmjF07.0430 were adequate at substituting for Htd2p within the htd2Δ mutant strain and are referred to hereafter as HTD2-1 and HTD2-2, respectively. Unlike T. brucei, which contains only a single 3-hydroxyacyl-ACP dehydratase HTD2, the related species L. major contains two functional dehydratases, and this finding is briefly discussed.

3.2. Choosing LmjF27.2440 and LmjF24.2030 as Candidates for Mitochondrial KAR1.

The present study is additionally concerned with identifying Leishmania KAR enzymes with physiological 3-oxoacyl-ACP reductase activities. Three candidate T. brucei KAR proteins have been described previously [7], including TbKAR1; however, their physiological function has hitherto not been chronicled. A blast search of the L. major database for proteins with similarity to TbKAR1 revealed the existence of several candidates with a significant probability of representing mitochondrial KAR1. The two top scorers, LmjF27.2440 (BlastP E-score 1.4e-73) and LmjF24.2030 (2.1e-05), are
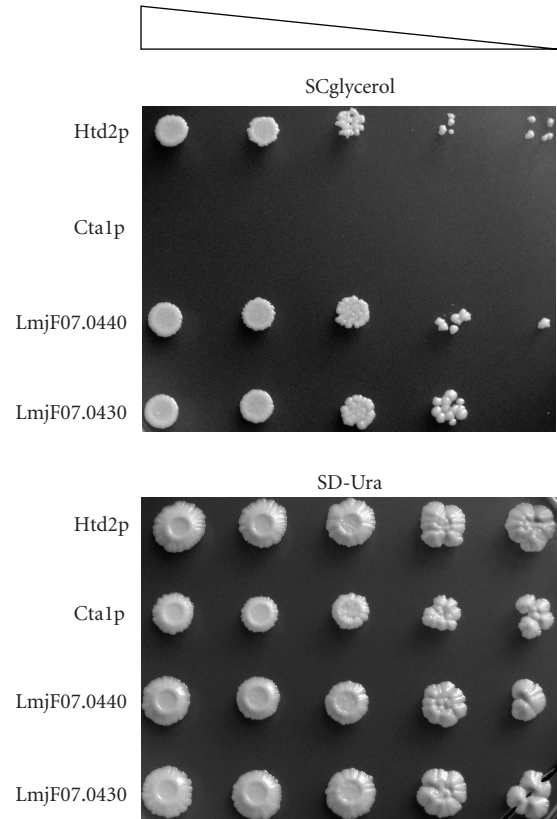

FIGURE 2: Phenotype rescue of a yeast htd2Δ strain expressing LmjF07.0440 or LmjF07.0430. Mutant S. cerevisiae BY4741htd2Δ cells producing native mitochondrial Htd2p, peroxisomal catalase A Cta1p, or each of the mitochondrially targeted Leishmania proteins LmjF07.0440 and LmjF07.0430 were grown on SD-Ura medium selecting for plasmid presence, and following tenfold serial dilution (triangle), the cells were applied to the indicated solid media.

already annotated in the database as putative 3-oxoacyl-ACP reductases. As expected, both proteins were found to be similar to their T. brucei counterpart (Figure 3) and to contain amino acid residues matching those in the catalytic triad of bacterial FabG [33]. The probability that each of the Leishmania proteins would represent a mitochondrial enzyme was similarly estimated on the basis of the algorithms used in the previous section, and the values were compared to those obtained for TbKAR1: LmjF27.2440 (MP, 0.0909; PS, 11.1%; TP, 0.133), LmjF24.2030 (0.7445; 21.7%; 0.640), and TbKAR1 (0.8434; 13.0%; 0.732). These data led to the assessment that LmjF27.2440 and LmjF24.2030 were potential KAR candidates, albeit the former protein, LmjF27.2440, did not appear to be preceded by a recognisable MLS.

3.3. Expression of LmjF27.2440 Rescues the Respiratory Phenotype of oar1Δ Cells.

To disclose the putative functions of LmjF27.2440 and LmjF24.2030 as 3-oxoacyl-ACP reductases, the corresponding genes were similarly ligated behind the CTA1 promoter. The low prediction values for LmjF27.2440 being a mitochondrial protein meant that a fungal MLS

FIGURE 3: Similarity of *L. major* LmjF24.2440 and LmjF24.2030 to their *T. brucei* homologue, *Tb*KAR1. MitoProt predictions of cleaved mitochondrial leader sequences are indicated (+). Catalytic amino acid residues occurring in *E. coli* FabG are marked (▼). The accession number for *Tb*KAR1 was AAX79999.

was appended to the N-terminus of each of the *Leishmania* candidates. In the present case, yeast *oar1Δ* mutant cells harbouring plasmids for expressing fungal Oar1p, Cta1p, or mitochondrially targeted *Leishmania* LmjF27.2440 and LmjF24.2030 were streaked on SCglycerol medium, and this revealed that mutants expressing LmjF27.2440 or Oar1p could grow on glycerol but not those with plasmids for LmjF24.2030 or Cta1p (data not shown).

In a similar strategy to that used in the previous section, the sufficiency of *Leishmania* LmjF27.2440 at replacing yeast Oar1p was demonstrated using serial dilutions that were applied to SD-Ura or SCglycerol media. The results in Figure 4 revealed that mutant cells expressing LmjF27.2440 were competent for growth on glycerol, to about one tenth of the situation with the self-complemented strain, whereas those mutant cells producing excess Cta1p could not grow or divide on glycerol. Hence, the respiratory growth phenotype of the *oar1Δ* mutant could be rescued, at least in part, by a mitochondrially targeted LmjF27.2440.

3.4. The Electron Transfer Chain in oar1Δ Mitochondria is Restored by LmjF27.2440. *S. cerevisiae* cells devoid of Oar1p lack assembled cytochrome complexes [13], and so their return to respiratory growth was presumed to coincide with restored cytochrome complexes and a rehabilitated electron transport chain. To demonstrate that the electron transfer chain of mutant cells expressing LmjF27.2440 had been regenerated, respiration competence was monitored with an overlay consisting of 2,3,5-triphenyltetrazolium chloride (TTC) that was applied to the aforementioned SD-Ura plate.

The results revealed that only mutant *oar1Δ* cells expressing Oar1p or LmjF27.2440 could metabolise TTC to generate the red colour, whereas high concentrations of *oar1Δ* cells expressing Cta1p remained white (lower panel; Figure 4). This indicated that within the LmjF27.2440-expressing mutant, at least a partial recovery of the mitochondrial electron transfer chain had been achieved.

3.5. Expression of LmjF27.2440 Resumes Lipoic Acid Production in the oar1Δ Mutant. As mentioned in the introduction, trypanosome FASII is involved in the synthesis of the octanoate precursor of lipoic acid [7]. Hence, to couple the expression of LmjF27.2440 with lipoic acid biosynthesis in yeast mitochondria, a biological assay relying on mutant bacterial cells was employed to measure the levels of lipoic acid in the previous three strains (each grown in triplicates). The results showed that extracts produced from the negative control yeast strain (*oar1Δ* cells expressing Cta1p) gave rise to a growth level of these lipoic-acid deficient bacterial cells that was equivalent to 23.3 ± 2.8 ng lipoic acid per gram wet weight yeast cells (values are mean $\pm$ S.D., $n = 3$), whereas the lipoic acid present in mutant yeast cells expressing fungal Oar1p or *Leishmania* LmjF27.2440 promoted bacterial growth to levels corresponding to 294.3 ± 54.5 and 59.0 ± 15.4 ng lipoic acid per gram wet weight, respectively. The combined results for LmjF27.2440, referred to hereafter as *Lm*KAR1, were commensurate with the functions of a FASII-like 3-oxoacyl-ACP reductase, and the implications of this finding are discussed.
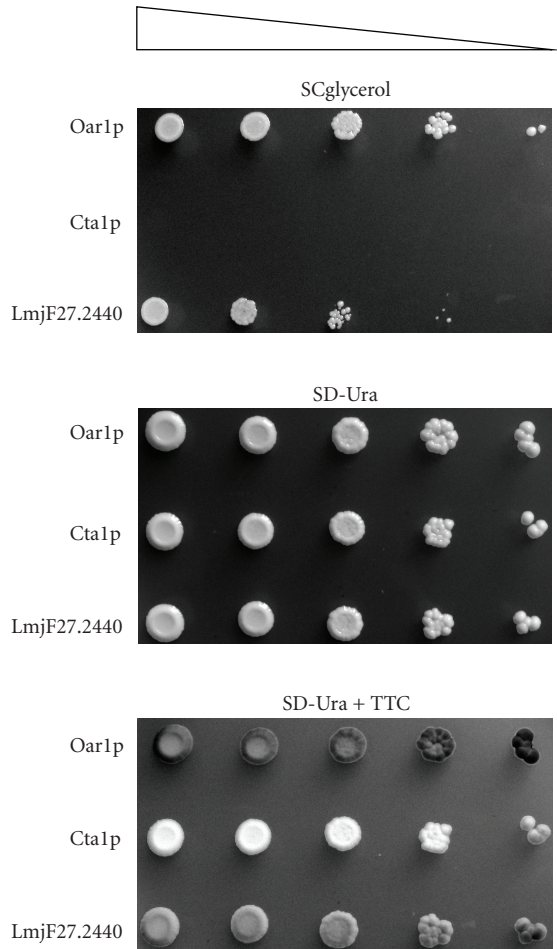
FIGURE 4: Phenotype rescue of a yeast *oar1Δ* strain expressing LmjF27.2440. *S. cerevisiae* BY4741*oar1Δ* mutants producing native mitochondrial Oar1p, Cta1p, or mitochondrially targeted LmjF27.2440 were propagated on SD-Ura medium that applied selective pressure for plasmid maintenance, and following sequential tenfold dilution (triangle), the cells were spotted onto the indicated solid media. Respiration competence was examined by applying 2,3,5-triphenyltetrazolium chloride (TTC) as an overlay to the SD-Ura medium.

4. Discussion

Although several components of *T. brucei* FASII have been revealed before, *Trypanosoma* and *Leishmania* are sufficiently different to warrant a study dedicated to identifying FASII also in *L. major*. In the present work, two novel *L. major* proteins, LmjF07.0440 (HTD2-1) and LmjF07.0430 (HTD2-2), were shown to rescue the mutant phenotype of *S. cerevisiae htd2Δ* cells with respect to respiratory growth, thereby acting as physiologically functional 3-hydroxyacyl-ACP dehydratases. The use of yeast *htd2Δ* cells for identifying Trypanosomatidae HTD2 has been established previously [9], affirming that functional complementation studies entail a major experimental advantage over more conventional methods for characterising enzymatic activities. In the aforementioned case of *Tb*HTD2, difficulties in measuring enzyme activities could only be overcome by using a bacterially expressed fusion protein [9]. In order to obviate the lengthy and laborious task of generating soluble *Leishmania* proteins, complementation of the fungal *HTD2* gene was used here instead, and this method provided a very effective alternative for demonstrating the mitochondrial dehydratase function of *Lm*HTD2-1 and *Lm*HTD2-2. Unlike the situation with *T. brucei*, the observation that *L. major* contains two *HTD2* genes is surprising. However, since there are no data presently available in the *Leishmania* databases regarding the potential expression of the two genes at different life-cycle stages, the selective advantage behind this gene duplication remains enigmatic.

A further important result to emerge here was the identification of LmjF27.2440 as a physiologically functional 3-oxoacyl-ACP reductase *Lm*KAR1, potentially of mitochondrial FASII. In *T. brucei*, three different proteins have been chronicled as representing this enzyme, and although they were all experimentally shown to be directed to the parasite's mitochondria [7], no data were presented for their enzyme activities or physiological functions. Here, the ability of LmjF27.2440 to replace Oar1p in *oar1Δ* mutant cells was determined, albeit the extent of the phenotype rescue achieved by ectopically producing LmjF27.2440 was limited. Judging from the difference in respiratory growth of the *Lm*KAR1-rescued mutants compared with the self-complemented strain (upper panel; Figure 4), it can be surmised that the former strain grew to a level that was approximately five- to tenfold less than the latter. The reduced respiratory growth seen here was additionally mirrored by a comparatively lower efficiency at metabolising TTC (lower panel; Figure 4). Moreover, a fivefold difference in levels of lipoic acid production could also be discerned between the two strains. The reason for this apparent discrepancy remains unclear, since by using an identical approach with the *htd2Δ* mutant, its phenotype could be rescued by *Lm*HTD2-1 or *Lm*HTD2-2 essentially to the level achieved by native Htd2p. One possible explanation leans on the efficacy of mitochondrial targeting.

The poor predictability regarding the potential mitochondrial targeting of LmjF27.2440 prompted the addition of a fungal MLS to the N-terminus of the protein. In this respect it should be noted that although the process of importing proteins into the mitochondria is well conserved across eukaryotes, some differences do exist, especially between yeast and mammals [34]. Since an exhaustive discourse on these differences is beyond the scope of this discussion, it can at least be speculated that in the situation of a lack of a formal MLS, LmjF27.2440 might actually contain a cryptic signal for compartmentalisation (beginning with the amino acid residue at position 53; Figure 3), which may or may not be recognised by yeast. The potentially deleterious effect of adding a fungal MLS on top of such a cryptic signal cannot be presently assessed. While the ultimate confirmation for mitochondrial localisation of *Lm*KAR1, but also *Lm*HTD2-1 and *Lm*HTD2-2, will require dedicated studies on *Leishmania* using specific antibodies or GFP fusion proteins, within the context of yeast it is not

uncommon to submit to the temptation of ensuring that heterologous mitochondrial proteins be correctly targeted by artificially appending an additional fungal MLS [14]. Nevertheless, when considering the situation in *T. brucei*, whereby the KAR1 [7] and HTD2 [9] proteins have been convincingly localised to the mitochondria, it is tempting to speculate that the analogous *L. major* components will also be similarly compartmentalised.

In reference to the number of KAR enzymes present in FASII of *Leishmania*, the second protein tested, LmjF24.2030, which was predicted to be a mitochondrial protein, nevertheless, failed to rescue the mutant phenotype. In the context of a yeast system, this observation alone is not sufficient to discount completely the possibility that LmjF24.2030 might still represent a functional KAR in *Leishmania*. However, it was no longer attractive to pursue LmjF24.2030 any further once it failed to rescue the yeast mutant phenotype, since it was conspicuously less similar to *Tb*KAR1 compared with LmjF27.2440 (Figure 3). To conclude, the present identification of three probable FASII components in *L. major* will enrich our knowledge of the molecular biology of an organism threatening the health of hundreds of millions of people worldwide and may help expose its mitochondrial FASII system to novel drug design.

Acknowledgments

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