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Generation and Characterization of a *Leishmania tarentolae* Strain for Site-Directed *in Vivo* Biotinylation of Recombinant Proteins

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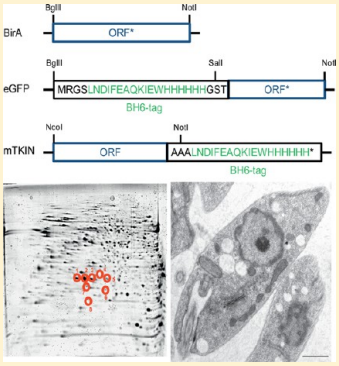
Supporting Information

ABSTRACT: *Leishmania tarentolae* is a non-human-pathogenic Leishmania species of growing interest in biotechnology, as it is well-suited for the expression of human recombinant proteins. For many applications it is desirable to express recombinant proteins with a tag allowing easy purification and detection. Hence, we adopted a scheme to express recombinant proteins with a His_6-tag and, additionally, to site-specifically *in vivo* biotinylate them for detection. Biotinylation is a relatively rare modification of endogenous proteins that allows easy detection with negligible cross-reactivity. Here, we established a genetically engineered *L. tarentolae* strain constitutively expressing the codon-optimized biotin-protein ligase from *Escherichia coli* (BirA). We thoroughly analyzed the strain for functionality using 2-D polyacrylamide-gel electrophoresis (PAGE), mass spectrometry, and transmission electron microscopy (TEM). We could demonstrate that neither metabolic changes (growth rate) nor structural abnormalities (TEM) occurred. To our knowledge, we show the first 2-D PAGE analyses of *L. tarentolae*. Our results demonstrate the great benefit of the established *L. tarentolae in vivo* biotinylation strain for production of dual-tagged recombinant proteins. Additionally, 2-D PAGE and TEM results give insights into the biology of *L. tarentolae*, helping to better understand Leishmania species. Finally, we envisage that the system is transferable to human-pathogenic species.



KEYWORDS: *Leishmania tarentolae, in vivo biotinylation, biotin ligase, recombinant protein expression, AVI tag, 2-D PAGE, LC−MS/MS, transmission electron microscopy*

INTRODUCTION

The eukaryotic protozoan parasite *Leishmania tarentolae* has become a model organism for many different medical and biotechnological applications. For instance, it has been used as vaccine candidate,[1,2] to study RNA editing[3] and gene amplification,[4,5] as well as for the expression of eukaryotic recombinant proteins. In particular, recombinant protein production is a strongly growing application area.[6,7] Advantages of *L. tarentolae* are, for example, its easy bacteria-like handling,[8,9] satisfying yields of recombinantly expressed proteins (0.1−30 mg/L),[6] and the high similarities of posttranslational protein modifications to human-derived counterparts.[10,11] Worldwide, more than 150 laboratories in academia and industry are working with *L. tarentolae* as protein expression host (R. Breitling, personal communication). We expect that the community is continuously growing, especially since the genome of *L. tarentolae* (Parrot-TarII strain) was successfully sequenced in 2012, showing that >90% of the gene contents are shared with human-pathogenic Leishmania species.[12] In this study, we broaden the application area of *L. tarentolae* (strain P10, Jena Bioscience) by establishing a powerful *in vivo* biotinylation system allowing site-specific biotinylation of a desired target protein.

Only plants, most bacteria, and some fungi can synthetize biotin (vitamin H),[13] which is essential for many metabolic pathways in higher eukaryotes. These include amino acid catabolism and fatty acid synthesis as well as gluconeogenesis.[14] Biotin is attached to specific proteins in an ATP-dependent reaction by a highly conserved family of biotin-protein ligases.[15] The best characterized biotin-protein ligase is the BirA enzyme from *E. coli*,[16] which exclusively biotinylates the biotin carboxyl carrier protein (BCCP). In 1993, Schatz used a phage-display approach to generate a synthetic biotin acceptor peptide, which in contrast to natural biotin acceptor domains (~75 amino acids[13]) has only a length of 13 amino acids (AA): LNDIFEAQKIEWH.[17] BirA covalently attaches a single biotin molecule to the only lysine residue within this sequence.

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Biotinylation is a relatively rare posttranslational protein modification.[13,18,19]

Since its discovery, this synthetic biotin acceptor peptide has been biotechnologically exploited. Its incorporation into genetically engineered vector systems allows site-directed biotinylation of target proteins in *E. coli*.[20,21] However, this motif is not recognized by eukaryotic biotin ligases, in contrast to the natural biotin acceptor domain. Therefore, the use of the short biotin acceptor peptide in eukaryotes requires co-expression of the *E. coli* biotin ligase BirA, preferably in a mammalian codon-optimized fashion.[22]

In our system, *in vivo* biotinylation requires the co-expression of a biotin ligase from *E. coli* together with a target protein carrying the described specific biotin acceptor peptide. Therefore, we have established a genetically engineered *L. tarentolae* strain (P10-BirA), which constitutively expresses the biotin ligase in the cytosol, and used it in combination with a novel expression vector harboring a biotin acceptor site. Screening the genome of *L. tarentolae* revealed an endogenous biotin/lipoate ligase-like protein without any further characterization. To exclude cross-reactivity between the endogenous putative and the recombinant biotin ligase, we searched the genome for the used biotin-acceptor sequence (AVI tag) and did not find similar motifs. Comparative analysis of P10-BirA with the parental P10 strain by 2-D PAGE revealed around 30 endogenous biotinylated proteins with 2 additional proteins from the recombinant biotin ligase. Hence, the enzyme is showing minimal off-target effects. Growth rate analyses showed no significant differences between the parental strain and eGFP-co-transfectants. Furthermore, results obtained from transmission electron microscopy demonstrated no structural differences between all strains and clones. To address the functionality of the *E. coli* biotin ligase in *L. tarentolae*, we expressed eGFP as a model protein. Possible eGFP protein spots from co-transfectants were excised from 2-D gels and analyzed by an LC−MS/MS. Eight protein spots could be assigned to eGFP. Additionally, we successfully co-expressed p53, polyubiquitin, FDFT1, and mTKIN in the P10-BirA strain and could show that all proteins were biotinylated. Moreover, the heterologous expression of human p53 in *Leishmania* produced a protein with biological activity: the cells displayed autophagosome-like structures by TEM, a known role of p53 in mammalian cells.

MATERIAL AND METHODS

Cloning of Constructs into *L. tarentolae* Expression Vectors

Mammalian codon-optimized biotin ligase from *E. coli* (hBirA) was amplified from vector pIRES-hBirA by PCR using primer pair 5_Lexsy_hBirA (5′-GAAGATCTGCCATGAAGGAC-AACACCGTGC-3′) and 3_Lexsy_hBirA (3′-ATAAGAAT-GCGGCCGCCTACTTCTCTGCGCTTCTCAGGG-5′). For cloning, amplicon and vector pLEXSY_hyg2 (Jena Bioscience) were digested with *Bgl*II and NotI, followed by ligation. Ligated DNA was purified and transformed into electrocompetent DH10B cells forming pLEXSY_hyg2-hBirA. The gene cassette of enhanced green fluorescent protein (eGFP), including biotin acceptor peptide and His_6 tag (BH6), was PCR-amplified from vector pIRES-BH6-EGFP-hBirA using primer pair 5_Lexsy_MRGS-Avi (5′-GAAGATCTGCCATGAGAGGCTCCC-TGAACG-3′) and GFP-stop_NotI (3′-TTTTCCTTTTGC-GGCCGCTTACTTGTACAGCTCGTCCATGCCG-5′). GFP-stop_NotI introduces a NotI restriction enzyme site with a stop codon. The N-terminal sequence, including the BH6-tag, reads as follows: MRGSLNDIFEAQKIEWHHHHHH. For cloning into pLTEX-1,[23] amplicon and vector were digested with *Bgl*II and NotI, and fragments were ligated forming pLTEX-6_eGFP. The construct contains the BH6-tag at the N-terminus of eGFP. Further, the coding sequences of cellular tumor antigen p53, polyubiquitin (Ubi8), and farnesyl-diphosphate farnesyltransferase (FDFT1) were isolated by restriction enzyme digest with *Sal*I + NotI from vectors pRSET-BH6-p53, pRSET-BH6-Ubi8 and pQE30NST_FDFT1, and cloned into vector pLTEX-6-eGFP by exchanging eGFP with respective gene fragments forming pLTEX-6-p53, pLTEX-6-Ubi8, pLTEX-6-FDFT1.

Next, we cloned a cysteine-free mutant of kinesin from *Thermomyces lanuginosus* (mTKIN) into pLTEX-1 introducing the BH6-tag at the C-terminus. For this, mTKIN was PCR-amplified from vector pQDKG2-mTKIN using primer pair mTKIN-NcoI-5′ (CATGCCATGGCGGGAGGCGGTAAC) and mTKIN-AVI-PSP-3′ (AATATGGGCCCTTAATGGTG-ATGGTG). Primer mTKIN-NcoI-5′ introduces an NcoI restriction enzyme site, and primer mTKIN-AVI-PSP-3′ introduces the biotin acceptor peptide and a PspOMI restriction enzyme site. PspOMI and NotI have compatible overhangs. For cloning into pLTEX-1, the amplicon was digested with NcoI + PspOMI, whereas the vector was digested with NcoI + NotI. The construct was purified and transformed into electrocompetent DH10B cells forming pLTEX-7-mTKIN (Supplementary Figure 1 contains main constructs and vector maps).

For all constructs, successful cloning was verified by restriction enzyme digests and by Sanger sequencing of single transformants. Positively evaluated clones were used in subsequent experiments to generate expression clones in *L. tarentolae* as described.[23]

Production and Purification of Recombinant Proteins

Positive transfectants were used for recombinant protein production. To verify protein expression, cleared cell lysate corresponding to 10 mL culture volume (OD_{600} > 1.5; >7.5 × 10^7 cells/mL) was tested. For this, cultures were centrifuged for 5 min, 1811*g*, and 20 °C followed by two washing steps with 1 mL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4·$2H_2O$; pH 7.4). Next, 60 μL of lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 1 mM EDTA, 2.4 μL Protease inhibitor and 0.1 μL/mL Benzonase, pH 8.8) was added, and the mixture was incubated for 30 min on ice. Finally, 20 μL 4× SDS sample loading buffer (0.2 M Tris-HCl, 8% SDS, 0.1% bromophenol blue, 40% glycerol, 10% β-mercaptoethanol, pH 6.8) was added, the mixture was incubated for 5 min at 95 °C, and 2−8 μL was loaded onto 4−12% Bis-Tris SDS-PAGE (Invitrogen).

For recombinant protein production, a 100−150 mL portion of LEXSY BHI medium containing porcine Hemin, Pen/Strep, Nourseothricin (NTC), and Hygromycin (Hygro) was inoculated 1:40 with a densely grown culture of a clone and cultivated in agitated suspension culture (125 rpm) for 2−3 days at 26 °C until OD_{600} = 2.5−4.5 (1.25 × 10^8 cell/mL−2.25 × 10^8 cells/mL) was reached. Cells were harvested for 20 min, 1811*g* at 4 °C. Pellet and supernatant were separated, and 10 mL of NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 400 μL protease inhibitor, 200 μL PMSF [0.1 M; phenylmethanesulfonyl fluoride, Sigma-Aldrich] and 1 μL Benzonase; pH 8.0) were added to the pellet followed by 30

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min of incubation on ice. Next, the mixtures were sonicated on ice (3 × 20 s, each with 20 s break) and centrifuged for 12 min, 12857*g* at 4 °C. Cleared cell lysates were loaded onto 1-mL Ni-NTA superflow cartridges (Qiagen) for fast protein liquid chromatography (FPLC)-based purification on an Åkta Explorer system. Washing and elution were carried out using a segmented concentration gradient of imidazole in buffers NPI10 and NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Segment 1: washing step 1 with 10 column volumes (CV) of 20% NPI-250 (50 mM imidazole). Segment 2: washing step 2 with 6 CV of 20−32% NPI-250 (50−80 mM imidazole). Segment 3: elution step 1 with 5CV of 50% NPI-250. Segment 4: elution step 2 with 5CV of 100% NPI-250. Here, proteins normally elute from the Ni-NTA column during elution step 1 (fractions B5−B12, C1−C2; data not shown).

Protein Expression and Growth-Rate Evaluation

Elution fractions were analyzed on 4−12% Bis-Tris SDS-PAGE (Invitrogen) and Western blot after electrophoretical transfer to PVDF membrane. Detection was carried out using Protein L-HRP (Pierce) diluted 1:5000 in 2% milk powder in PBS with 0.1% Tween 20 and subsequent incubation with CN/DAB substrate (Pierce). BirA was detected by chicken anti-BirA (1st antibody) and mouse anti-chicken IgY, AP-conjugated (2nd antibody). eGFP was detected by chicken anti-eGFP (1st antibody), followed by HRP-conjugated anti-chicken (2nd antibody). Additionally, eGFP, p53, and mTKIN were visualized by incubation with HRP-conjugated streptavidin.

The cell growth of P10 and P10-BirA was monitored by measuring the cell density of an agitated suspension culture at different time points with a photometer at a wavelength of 600 nm. A starting OD_{600} = 0.2 (~1.0 × 10^7 cells/mL) was determined, and measurements were stopped once the culture had reached the stationary growth phase. Additionally, in a second agitated culture the growth medium was supplemented with 10 mM D-biotin (final concentration) to investigate the influence of biotin supplementation on the growth behavior.

Two-Dimensional Gel Electrophoresis (2-D PAGE)

For protein extraction, shock-frozen cell pellets with an equal volume of sample buffer (50 mM TRIZMA Base (Sigma-Aldrich), 50 mM KCl, and 20% w/v glycerol at pH 7.5) and proteinase inhibitors (Roche Diagnostics) were ground to fine powder in a mortar cooled by liquid nitrogen. Subsequently, samples were thawed to 4 °C and sonicated on ice. DNase (Roche Diagnostics), urea (6.5 M), and thiourea (2 M) were added, and protein extracts were supplied with 70 mM dithiothreitol (Biorad) and 2% v/w of ampholyte mixture (Servalyte pH 2−4, Serva) and stored at −80 °C until further use.[24]

Protein extracts were separated by 2-D polyacrylamide-gel electrophoresis as described previously.[25] The gel format was 5 cm (isoelectric focusing) × 10 cm (SDS-PAGE) × 1.0 mm (gel width), and gels were silver-stained as described.[25]

For identification by mass spectrometry, protein spots corresponding to signals observed on 2-D Western blots (same location on the gel) were excised from silver-stained 2-D gels and analyzed by mass spectrometry.

Mass Spectrometry Analysis of Protein Spots from 2-D PAGE

For protein identification by mass spectrometry, 640 μg of protein extract was separated on 2-D gels and stained with a MS-compatible silver staining protocol.[24] Protein spots of interest were excised from 2-D gels and subjected to in-gel tryptic digestion. Peptides were analyzed by LC−MS/MS on a LCQ Deca XP ion trap instrument (Thermo Finnigan, Waltham, MA) coupled to nano-LC (Proxxeon).

ESI-MS data acquisition was performed throughout the LC run. Three scan events, (1) full scan with mass range 350−2000, (2) zoom scan of most intense ions in (1), and (3) MS/MS scan of most intense ions in (1), were applied sequentially. No MS/MS scan was performed on single charged ions. The isolation width of precursor ions was set to 4.50 *m*/*z*, normalized collision energy at 35%, minimum signal required at 10 × 10^4 (MS) and 0.5 × 10^4 (MSn), zoom scan mass width low/high at 5.00 *m*/*z*. Dynamic exclusion was enabled using the following parameters: (i) repeat count 3, (ii) repeat duration 1 min, (iii) exclusion list size 50, (iv) exclusion duration 3 min, (v) exclusion mass width was set to low/high 3.00 *m*/*z*.

The raw data were extracted by TurboSEQUEST algorithm; trypsin autolytic fragments and known keratin peptides were filtered out. All DTA files generated by Sequest were merged and converted to Mascot Generic Format Files (MGF). Mass spectra were analyzed using in-house Mascot Server (Matrix science; version 2.1) automatically searching the SwissProt database (version 51.8/513877 sequences). MS/MS ion search was performed with the following set of parameters: (i) taxonomy: Metazoa, (ii) proteolytic enzyme: trypsin, (iii) maximum of accepted missed cleavages: 1, (iv) mass value: monoisotopic, (v) peptide mass tolerance: 0.8 Da, (vi) fragment mass tolerance: 0.8 Da, and (vii) variable modifications: oxidation of methionine and acrylamide adducts (propionamide) on cysteine. No fixed modifications were considered. Only proteins with scores corresponding to $p < 0.05$, with at least two independent peptides identified were considered. The cutoff score for individual peptides was equivalent to $p < 0.05$ for each peptide as calculated by Mascot.

Autophagy Induction Assay

A densely grown culture of P10 promastigotes was transferred (1:10) from static to agitated suspension culture (110 rpm). Approximately 4 h later, medium was exchanged with RPMI nutrient-deficient medium (RMPI 1640, Gibco, Lot 3103095) to starve cells. Alternatively, cells were inoculated with 100−250 nM rapamycin (Life Technologies, PHZ1233). After 2 h and 12 h, samples were taken and washed 2× with PBS. Cells were fixed for 1 h in 2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.4. Additionally, samples from densely grown cultures of untreated P10, P10-BirA, and P10-BirA-p53 were washed and fixed as described above. Starvation and rapamycin are known autophagy inducers in Leishmania; overexpression of tumor suppressor p53 is a known autophagy inducing factor in mammals.[26] Samples were investigated for development of autophago(lyso)somes by TEM.

Transmission Electron Microscopy (TEM)

Cells were pelleted, embedded in 2% LMP-agarose, cut into small pieces, and washed overnight in 50 mM cacodylate buffer with 50 mM NaCl. Following, agarose-Leishmania pellets were postfixed in cacodylate buffer including 0.5% OsO_4 for 2.5 h. After washing with water, probes were incubated in 100 mM Hepes (pH7.4) with 0.1% tannic acid for 1 h, followed by incubation in 2% uranyl acetate for 90 min. Next, probes were dehydrated in a graded series of ethanol and embedded in Spurr's resin (Low Viscosity Spurr Kit, Ted Pella, CA, USA). Ultrathin sections (70 nm) were prepared with an ultra-

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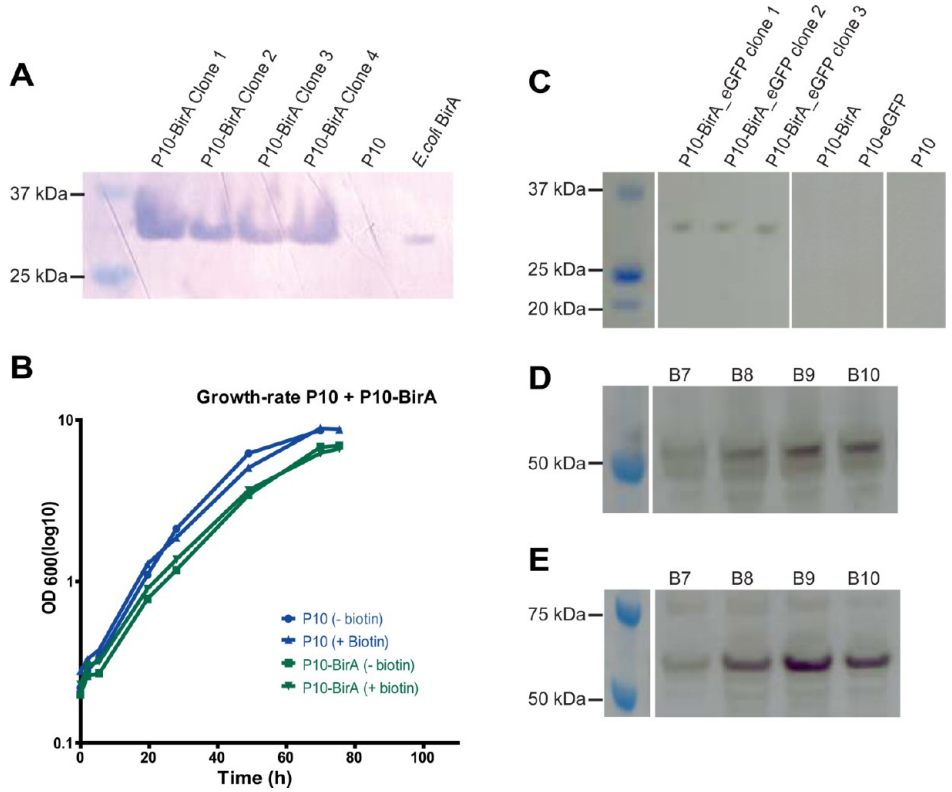

Figure 1. Analyses of the novel P10-BirA protein expression strain. (A) Western-blot evaluation of biotin ligase (BirA, 35.3 kDa) expression clones including wildtype P10 and *E. coli* as negative and positive controls, respectively. First antibody: chicken anti-BirA. Second antibody: mouse anti-chicken IgY, AP-conjugated. (B) Growth-rate comparison between P10 (blue) and P10-BirA (green) in agitated suspension culture, with and without addition of 10 mM D-biotin (blue triangle for P10, green triangle for P10-BirA). (C) WB of P10-BirA_eGFP (29.8 kDa) expression clones (pLTEX-6_eGFP) with negative controls (P10-BirA, P10-eGFP, P10). (D, E) WB detection of recombinant p53 (P10-BirA_p53; D) and mTKIN (P10-BirA_mTKIN; E) in elution fractions (B7−B10) after Ni-NTA-FPLC-purification. (C−E) Detection of biotinylated proteins was carried out with HRP-conjugated streptavidin (1:5000), followed by incubation with CN/DAB as substrate.

microtome (Reichert Ultracut E, Leica) and mounted on pioloform-coated electron microscopy copper grids (200 mesh). Sections were counterstained with uranyl acetate and lead citrate for 20 s. Image acquisition was performed on a FEI Tecnai Spirit transmission electron microscope operated at 120 kV and on a Philips CM100 TEM.

RESULTS AND DISCUSSION

Expression Analysis of BirA in *L. tarentolae* P10

In the first step, four individual transformants were analyzed for cloning and expression success of BirA in *L. tarentolae* strain P10. Transfection and integration of the BirA expression cassette into the ssu locus of the genome was verified by analytical PCR as described (data not shown).[10] To investigate BirA protein expression, cell lysates were separated on SDS-PAGE and electrophoretically transferred onto PVDF membrane to perform an immuno Western-blot. Expression of the 35.3 kDa biotin ligase was visualized by specific antibody detection. All four clones were shown to express BirA (Figure 1A). Clone 1 was chosen as new working strain P10-BirA and utilized in the following experiments. To confirm the usefulness of the newly developed strain P10-BirA as expression host as well as to investigate possible negative effects of BirA, its overall growth performance was compared with the parental strain P10 in agitated suspension culture under two conditions: with and without addition of D-biotin (Figure 1B). The observed cell density of P10-BirA (+ D-biotin: OD_{600} = 6.53, − D-biotin: OD_{600} = 6.80) remained slightly below the one of P10 (+ D-biotin: OD_{600} = 8.88, − D-biotin: OD_{600} = 8.62). Supplementing culture medium with D-biotin had no obvious influence. Similarly, little to no differences in cell density has been observed when growing P10 and P10-BirA in static culture (data not shown). Note, the number of harvested cells range between 3×10^8 and 4.5×10^8 cells/mL, which correspond to OD_{600} = 6.0 and OD_{600} = 9.0, respectively.

Expression Analysis of eGFP and Other Proteins

The eGFP protein consists of a 238 AA sequence, which is translated into a protein of 26.9 kDa. In this study, eGFP is linked to the N-terminal BH6-tag increasing the size to 29.8 kDa (263 AA). After transfection of pLTEX-6_EGFP in P10-BirA, individual clones were analyzed by fluorescence microscopy for eGFP expression (data not shown). Subsequently, three fluorescent clones were compared to a series of controls for the expression of biotinylated eGFP. Biotinylation was confirmed by applying streptavidin-HRP to a Western blot of cell lysates (Figure 1C). The interaction between biotin and streptavidin is known as the strongest noncovalent binding of two biomolecules. In all three P10-BirA_eGFP clones, the expected band could be observed. There was no band observed in the control lanes from P10 or P10-BirA as well as pLTEX-6-eGFP transfected into the parental strain (P10-eGFP). Next to eGFP, we have also expressed and Ni-NTA purified several other proteins in pLTEX-6 via the specific interaction with the His_6-tag, such as human tumor suppressor protein p53,

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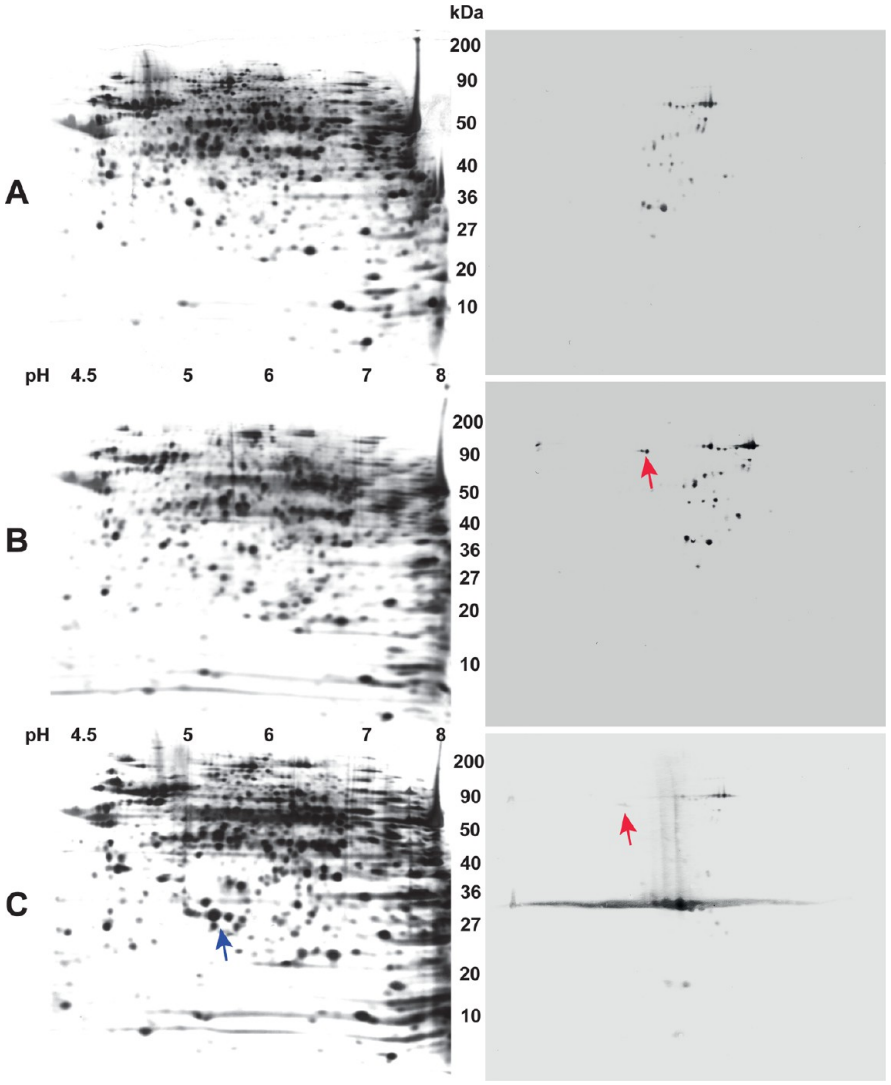

Figure 2. Two-dimensional gel electrophoresis of wild type (A), P10-BirA (B), and P10-BirA-eGFP (C) *L. tarentolae* proteomes and corresponding Western blots showing biotinylated proteins. 2-DE gels (left panel) were silver stained. WBs (right panel) were incubated with HRP-conjugated streptavidin to visualize endogenous biotinylated proteins and to uncover off-target effects of the recombinant biotin ligase. Expression of the biotin ligase resulted in biotinylation of two endogenous protein spots (red arrows in B and C, right panel). Overexpression of eGFP is highlighted in C (blue arrow, left panel).

polyubiquitin (Ubi8), and farnesyl-diphosphate farnesyltransferase (FDFT1) as well as a cysteine-free mutant of kinesin from *Thermomyces lanuginosus* (mTKIN) in pLTEX-7. Figure 1D and E shows detection of biotinylated p53 and mTKIN in a Western blot. In the mTKIN construct, the BH6-tag is incorporated at the C-terminus (Supplementary Figure 1). This shows that the biotin acceptor peptide is efficiently recognized by BirA in *L. tarentolae* independent of its position.

2-D PAGE and Mass Spectrometry Analysis of P10-BirA

Several studies demonstrated functionality of BirA and the biotinylation success of the biotin acceptor peptide in, for example, mammalian and insect cells.[22,27−29] To date, this enzyme has not been expressed in a eukaryotic protozoan parasite, such as Leishmania. Therefore, possible off-target effects, e.g., the *in vivo* biotinylation of endogenous parasitic proteins, had to be analyzed. For this, the number of endogenous biotinylated proteins and the possible existence of endogenous biotin acceptor sites recognized by BirA needed to be investigated to exclude enzymatic cross-reactivity. We separated protein extracts by 2-D PAGE from *L. tarentolae* strains P10 and P10-BirA as well as from eGFP-co-transfectants of the same strains. Furthermore, Western blots of 2-D gels were prepared to analyze biotinylation of proteins.

2-D Western blot analysis of biotinylated proteins (using Strep-HRP antisera) revealed around 30 biotinylated protein spots in parental strain P10 (Figure 2A, right panel). 2-D Western blots of P10-eGFP (data not shown) revealed the same set of biotinylated proteins as observed in the parental strain, indicating that BH6-tagged eGFP is not recognized by endogenous biotin ligases. According to TriTrypDB, *L. tarentolae* possesses an endogenous biotin/lipoate protein ligase-like protein (www.tritrypdb.org; ***LtaP31.1020***).

Two additional biotinylated protein spots were observed in P10-BirA and P10-BirA_eGFP (Figure 2B and C, right panels, indicated by red arrows). Screening the *L. tarentolae* genome for the synthetic biotin acceptor peptide sequence verified the absence of the exact motif. However, seven proteins with sequence homologies were found (data not shown). Whether the two additional biotinylated protein spots correspond to any

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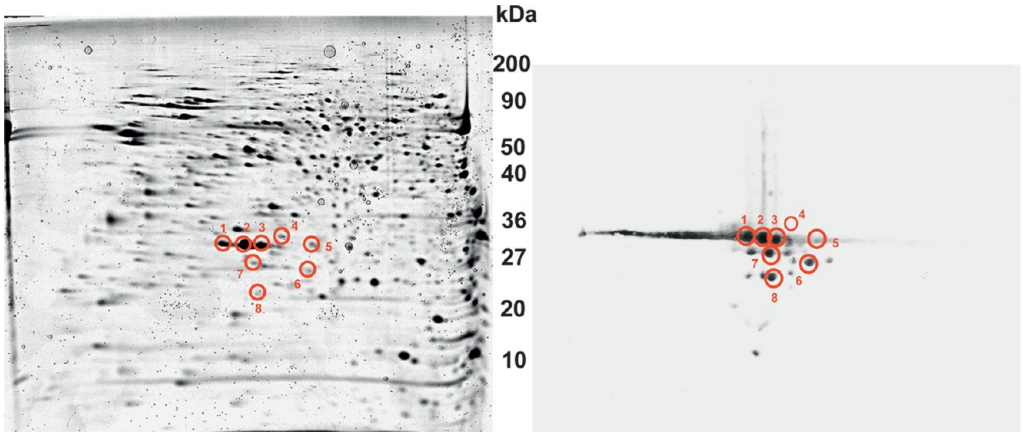

Figure 3. Two-dimensional gel electrophoresis of P10-BirA-eGFP *L. tarentolae* proteome and corresponding Western blot showing eGFP. (Left panel) 2-D PAGE. (Right panel) WB incubated with chicken anti-eGFP (1st antibody), followed by HRP-conjugated anti-chicken (2nd antibody) to visualize eGFP. Red circles show selected protein spots for analysis via LC−MS/MS on a LCQ Deca XP ion trap instrument, which were picked from 2-D PAGE.

of these identified proteins could not be further clarified. Major protein spots in Figure 2C correspond to eGFP, as verified with anti-eGFP antibody shown in Figure 3, right panel. Interestingly, eGFP is already visible in 2-D PAGE (Figure 2C, left panel) as indicated by a blue arrow. Further verification of protein spots labeled in Figure 3 (left panel) was conducted by mass spectrometry analysis. Protein spots corresponding to eGFP signals observed on 2-D Western blots were excised from 2-D gels and analyzed by mass spectrometry. Mass spectrometry verified that these protein spots contained eGFP (Table 1).

Table 1. Mass Spectrometry Results of Excised Protein Spots from 2D PAGE of P10-BirA_eGFP

sample	protein name	MOWSE score	peptides	sequence coverage (%)	species
1	green fluorescent protein	473	6	33	*Aequorea victoria*
2	green fluorescent protein	563	6	48	*Aequorea victoria*
3	green fluorescent protein	463	7	31	*Aequorea victoria*
4	green fluorescent protein	152	2	11	*Aequorea victoria*
5	green fluorescent protein	441	2	11	*Aequorea victoria*
6	green fluorescent protein	144	2	11	*Aequorea victoria*
7	green fluorescent protein	274	4	28	*Aequorea victoria*
8	green fluorescent protein	243	4	21	*Aequorea victoria*

Microscopic Analysis of P10, P10-BirA, and P10-BirA_eGFP

Expression of recombinant proteins, especially biologically active enzymes such as BirA, can have a direct influence on the shape, inner structure, and metabolism (growth rate) of the host cell. To uncover possible negative consequences of BirA expression on cell shape or inner structure of the parasite, we analyzed the parental strain P10 (reference control) as well as the new strains P10-BirA and P10-BirA_eGFP via light microscopy, confocal-laser-scanning microscopy, and TEM.

In cell culture, *L. tarentolae* parasites occur as flagellated, extracellular, and motile promastigotes. During exponential growth, their shape ranges from round-like to lance-like structures with a length of 6−12 μm and a width of 1−3 μm. Light microscopy observations of promastigotes within the logarithmic as well as the stationary growth phase revealed no differences between P10, P10-BirA, and P10-BirA_eGFP (data not shown). Next, analyses of intracellular structures by TEM were conducted. Commonly shared organelles of Leishmania parasites are the kinetoplast, glycosomes, or acidocalcisomes. Figure 4A shows a detailed TEM image of *L. tarentolae* P10. Comparison of P10-BirA and P10-BirA_eGFP to images of P10 revealed no differences (data not shown). To be able to analyze how *L. tarentolae* looks when the metabolism is disturbed, we chose to induce autophagy, which is a catabolic process every eukaryotic cell undergoes to recycle long-lived proteins and to eliminate damaged macromolecules and organelles, thus maintaining cellular homeostasis. Among the different mechanisms of autophagy, macroautophagy (here referred to as autophagy) is characterized morphologically by the engulfment of cytoplasmic constituents, wrapped by double or multiple membrane sacs called autophagosomes. Lysosomes fuse to the autophagosomes to degrade the cytoplasm-derived contents and the inner membrane. Autophagy is induced by limitation of nutrients and growth factors, allowing the cell to survive for longer periods by recycling components[30] and is negatively regulated by the target of rapamycin (TOR) complex. Hence, rapamycin has been widely used to induce autophagy.[31] Leishmania is also sensitive to rapamycin, having three genes for TOR.[32] Therefore, in this study we induced autophagy in P10 either by exposure to rapamycin (Figure 4B) or by starvation (nutrient limitation; Figure 4C). In both cases, P10 showed different structures rich in membranes that could be autophago(lyso)somes. Furthermore, promastigotes grown in RPMI medium were extremely thin and elongated. The basic molecular machinery for the formation of autophagic vesicles is conserved in evolution. In *Saccharomyces cerevisiae*, around 20 genes have been identified as essential for autophagy (coined Atg genes), and functional orthologous genes have been demonstrated in several species, including Leishmania. Notably, mammalian or leishmanial orthologous genes complement Atg yeast mutants.[33,34] In mammals, several proteins regulate autophagy, among others, the tumor suppressor protein p53.[26] Dysregulations of p53 is associated with human diseases such as cancer and metabolic disorders. In order to determine whether the heterologous expression of a mammalian gene in

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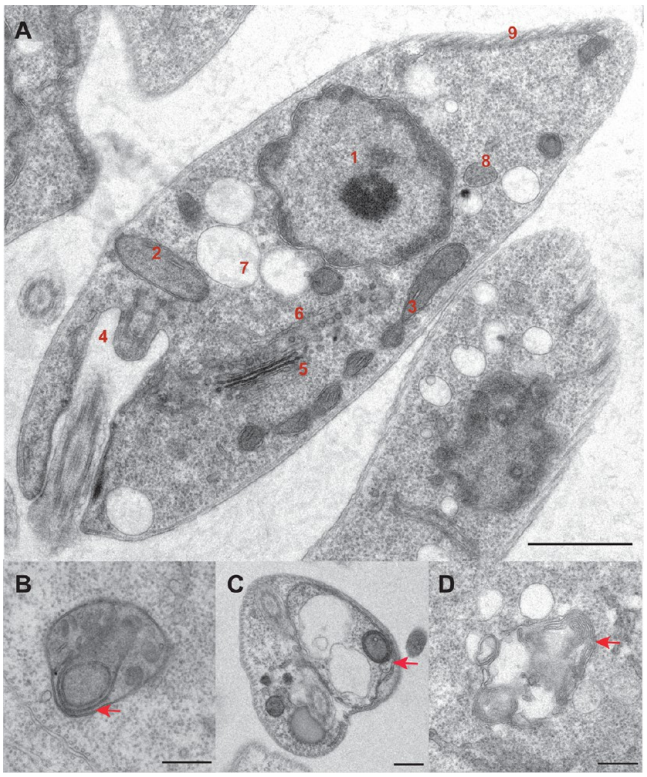

Figure 4. Transmission electron microscopy (TEM) images of *L. tarentolae*. (A) Overview of *L. tarentolae* promastigote from P10-BirA in longitudinal section; scale bar corresponds to 1000 nm. The following organelles are visible: (1) nucleus with nucleolus, (2) mitochondrion with kinetoplast, (3) more parts of the single mitochondrion, (4) flagellar pocket with flagella, (5) golgi apparatus, (6) endoplasmic reticulum, (7) acidocalcisome, (8) glycosome, and (9) subpellicular microtubules. (B–D) Images are showing autophago-(lyso)somes, indicated by red arrows. Scale bar corresponds to 400 nm. (B) P10 incubated for 2 h with 250nM rapamycin. (C) P10 starved for 12 h in RPMI nutrient-deficient medium. (D) P10-BirA-p53 expression clone.

Leishmania would produce a protein with preserved biological activity, we expressed human p53 in P10-BirA. Accordingly, clone P10-BirA-p53 (Figure 4D) displayed autophagosome-like structures observed by TEM, strongly suggesting that p53 is able to induce autophagy in a heterologous system.

CONCLUSION

Here, we have established a novel *L. tarentolae* expression strain that allows efficient production of *in vivo* biotinylated recombinant proteins. Integration of the *E. coli* BirA gene into *L. tarentolae* P10 was successfully demonstrated, and detailed characterization revealed neither metabolic changes nor structural abnormalities and also no significant off-target effects. We have developed expression vectors allowing N- or C-terminal BH6-tagging of recombinant cytosolic proteins and have shown that the position of the biotin acceptor peptide has no influence on biotinylation. On a number of proteins, we could demonstrate that the functionality of recombinant biotinylated proteins can be preserved. For instance, human p53 expression induced autophagy as shown by TEM. Noteworthy, birA in *E. coli* is expressed and active in the cytoplasm. Here we have expressed birA in the cytosol of *L. tarentolae*. Therefore, we envisage that only recombinant proteins targeted to the cytosol of the host will be biotinylated. For secretory proteins, where expression occurs at the endoplasmic reticulum, the biotin ligase should be equally targeted to be in the ER. Barat and Wu have shown that in mammalian cells, birA is active even when targeted to the ER and can efficiently biotinylate secretory proteins in said cellular compartment.[35] As a consequence, we envisage a whole series of *L. tarentolae* strains capable of *in vivo* biotinylating recombinant proteins in different cellular compartments of the host. Alternatively, in cases where *in vivo* biotinylation does not work, the recombinant proteins containing the BH6-tag can be biotinylated in a consecutive step using the birA enzyme *in vitro*. Therefore, our current system not only enables production and simple detection of recombinantly expressed proteins but should also allow *in vitro* and *in vivo* interaction studies to be performed. As the *in vivo* biotinylation system should also be valid for human-pathogenic Leishmania species, it can be applied in future to better understand the biology of all Leishmania species.

ASSOCIATED CONTENT

Supporting Information

Schematic representation of vector constructs and vector maps. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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