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ScienceDirect
Molecular & Biochemical Parasitology 155 (2007) 71–83
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Construction and analysis of *Leishmania tarentolae* transgenic strains free of selection markers

Sergei Mureev [a], Susanna Kushnir [a], Alexander A. Kolesnikov [b], Reinhard Breitling [c], Kirill Alexandrov [a,*]

[a] *Department of Physical Biochemistry, Max-Planck-Institute for molecular Physiology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany*
[b] *Department of Molecular Biology, Moscow State University, Vorobjevy Gory 1, Build. 12, 119992 Moscow, Russia*
[c] *Jena Bioscience GmbH, Löbstedter Str. 80, 07749 Jena, Germany*

Received 27 December 2006; received in revised form 12 May 2007; accepted 17 May 2007
Available online 24 May 2007

Abstract

The trypanosomatid protozoan *Leishmania tarentolae* has been extensively used as a model system for studying causative agents of several tropical diseases and more recently as a host for recombinant protein production. Here we analyze the rates of partial or complete deletions of expression cassettes integrated into small ribosomal RNA and tubulin gene clusters as well as into ornithine decarboxylase gene of *L. tarentolae*. In approximately 60% of cases gene conversion was responsible for the deletion while in the rest of the cases deletion occurred within the expression cassette. We used this observation to design constitutive and inducible expression vectors that could be stably integrated into the genome and subsequently depleted of the antibiotic resistance genes using thymidine kinase or bleomycin resistance genes as negative selection markers. This enabled us to obtain *L. tarentolae* strains containing constitutive or inducible markerless expression cassettes. Analysis of the markerless strains demonstrated that although stability varied among clones some were stable for as many as 200 generations. We expect that this approach will be useful for the construction of strains carrying multiple expression cassettes for analysis of trypanosomatid pathogenicity mechanisms and overexpression of multi-subunit protein complexes for biochemical and structural studies.

Keywords: *Leishmania tarentolae*; Homologous recombination; Thymidine kinase; Antibiotic resistance

1. Introduction

The family Trypanosomatidae (*Eugelenozoa*, *Kinetoplastida*) which includes genera *Leishmania* and *Trypanosoma* is one of the oldest groups of eukaryotes and has attracted researcher's attention for two interconnected reasons. Firstly, they are notorious for finding unique solutions to general processes of the eukaryotic cell. Among the unique features of trypanosomatids are RNA editing, arrangement of genes in tandem arrays, polycistronic transcription followed by trans-splicing and regulation of gene expression almost exclusively at the post-transcriptional level [1]. Secondly, a number of Leishmania and Trypanosoma species are extra- and intracellular parasites of humans and livestock and cause a range of debilitating or fatal diseases [2]. Due to its public health significance, the Trypanosomatidae group is one of the best-studied eukaryotic groups after yeast. The development of methods of trypanosomatid cultivation *in vitro* and genetic manipulation has allowed the dissection of the mechanism of non-conventional gene expression [3]. Trypanosomatids can be efficiently transformed by various types of genetic vectors. In both *Trypanosomes* and *Leishmania* expression cassettes can be stably integrated in different parts of the genome and their expression can be driven by read-through of endogenous RNA polymerase I or II [3]. Alternatively, expression of genes can be driven by co-integrated promoters for RNA polymerase I or heterologous RNA polymerases such as T7

Abbreviations: APRT, adenine phosphoribosyltransferase; *BLE*, gene encoding for phleomycin binding protein; *CAM A*, calmodulin A gene; *CAM* IR, calmodulin intergenic region; *EGFP*, gene encoding for enhanced green fluorescent protein; *HYG*, hygromycin resistance gene; IR, intergenic region; *NEO*, neomycin resistance gene; NLS, nuclear localization signal; NTC, nourseothricin; *ODC*, ornithine decarbocsilase locus; Pol II, RNA polymerase II; rfu, relative fluorescent units; *SAT*, streptothricin acetyltransferase; *SSU*, small subunit RNA gene in rDNA locus; T7 Pol, T7 RNA Polymerase; Tet, tetracycline; TMP, thymidine monophosphate; TR, tetracycline repressor; *TUB*, β-tubulin gene; UTR, untranslated region

* Corresponding author. Tel.: +49 231 1332356; fax: +49 231 1331651.
E-mail address: kirill.alexandrov@mpi-dortmund.mpg.de (K. Alexandrov).

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doi:10.1016/j.molbiopara.2007.05.007

or T3 [4]. The latter approach was used in a number of trypanosomatid species to provide inducible protein expression for functional studies or for recombinant protein production [3,5]. With the exception of transposon mediated transformation or random mutagenesis/selection procedures the addition to, or deletion of, chromosomal material is achieved by homologous recombination coupled to drug selection [6]. A considerable difference exists between *Leishmania* and *Trypanosoma* in regard to the genome integration efficiency. This distinction may be the consequence of the different usage of recombination within the mechanisms of pathogenicity in the two genera. In trypanosomes equal frequencies of gene targeting were observed independently of target gene copy number [7]. Remarkably, stable integration was observed even with the homologous sequences as short as 30 nucleotides [8]. In the case of *Leishmania* a decrease in the length of homologous sequences below 1 kb on one arm of the vector linearly decreases the targeting frequency with no homologous recombination occurring when the targeting regions were <180 bp [9]. Targeted recombination is strongly affected by base pair mismatches and different chromosomal locations are targeted with significantly variable levels of efficiency [10]. Finally, different strains of the same species show differences in gene targeting frequency [7]. Overall, gene targeting mediated by homologous recombination in *Leishmania* shows similarities to that of yeast and mammals [11,12].

The traditional scheme of DNA sequence delivery into the trypanosomatid genome has one substantial drawback. Since the selection marker is required for each integration round, the procedure can be repeated only a number of times equal to the number of selection markers. In principle, up to six antibiotic selection genes are currently used for trypanosomatid manipulation [3], however, more than four marker genes are rarely employed simultaneously. Saturation of the genome with the selection marker genes precludes further genetic manipulation of the strain. To an extent this problem can be alleviated since the marker gene(s) can be purged from the genome when a negative selection pressure is applied. The selection typically involves use of a conditionally toxic marker gene or a fusion gene composed of two markers where one is used for positive and one for negative selection. Examples of negative selection markers used include nucleoside modifying enzymes, e.g. thymidine kinase (*TK*), 3′ nucleotidase/nuclease, dihydrofolate reductase-thymidylate synthase and cytidine deaminase [13,14]. In this configuration, addition of cell permeable nucleotide analogs (for instance gancyclovir, acyclovir) results in broad toxicity leading to cell death. Selection of cells capable of growth on medium containing such analogues allows the isolation of clones that have lost the toxic gene as a consequence of genomic rearrangement or gene inactivation. An elegant application of this approach to *Leishmania*, called a hit-and-run strategy, involves the fusion of an antibiotic selection marker with thymidine kinase that is first integrated into the locus of interest and then replaced by a null targeting fragment containing no exogenous DNA [15]. However, in many cases it is desirable to construct transgenic strains of *Kinetoplastida* that express multiple heterologous genes either constitutively or, preferably, inducibly. This would be useful in the construction of genetically modified trypanosomatid strains expressing multiple siRNA constructs or protein overproduction hosts carrying the heterologous protein glycosylation pathways or expressing multi-subunit enzymes. This would require a procedure that would allow integration of a di-cistronic expression cassette comprising the gene of interest and a selection marker where the latter can be removed in a targeted fashion so that the procedure could be repeated iteratively. This objective is not trypanosome specific and has been dealt with in other organisms, particularly in yeast. In this case a vector comprising a Bleo-selectable marker flanked by *loxP* sequences is genomicaly integrated and the marker is excised by the activity of episomaly delivered CRE-recombinase [16–18]. Other approaches rely on the activity of highly selective endonucleases such as FLAP endonuclease [19] or homing endonuclease I-SceI [20].

In this work we analyze the frequency of rearrangements in heterologous gene expression cassettes integrated into the genome of *Leishmania tarentolae* and observe their complete or partial deletion. We use this information to design a system that allows integration of an inducible expression cassette into the genome of *L. tarentolae* using standard selection procedures followed by subsequent removal of the selection marker.

2. Experimental procedures

2.1. Construction of the expression vectors for stable integration into SSU locus

Plasmid pF9efp1.4sat1.4 (Fig. 1B) containing the EGFP expression cassette linked to the *SAT* marker gene which is flanked by identical 1.4 kb intergenic regions (*IR*) of calmodulin CB genes of tricistronic calmodulin (*CAM*) *gene* cluster of *L. tarentolae* was derived from plasmid pEGFP1.4camCB (Breitling & Klingner unpublished). The 0.9 kb (F9) PCR product containing 5′ ornithine decarboxylase (accession AF159564) UTR was amplified using the specific primers A1013 and A1014 (see Table S2 for primer sequences) and genomic DNA of *L. tarentolae* as a template. The fragment was digested with SalI and NcoI and inserted into the pEGFP1.4camCB digested by the same enzymes. The resulting plasmid was opened with FseI and BcuI and ligated to a FseI and BcuI digested PCR fragment containing 1.4 kb calmodulin CB intergenic region obtained with primers A1501 and A1502 using plasmid pEGFP1.4camCB as a template.

Plasmid pF4ble_egfp1.4sat1.4 (Fig. 1C) was constructed by the insertion of a 1.6 kb SalI and NotI fragment from plasmid pF4blegfpB (Breitling & Ehrlich unpublished) containing a 0.4 kb fragment (F4) from the 5′ *CAM CB* UTR and a *BLE-EGFP* gene fusion into the SalI and NotI digested pF9egfp1.4*SAT*1.4 vector thereby replacing the 5′UTR and *egfp* gene.

Plasmid pF9hyg1.4*SAT*1.4 (Fig. 1D) was constructed by PCR amplifying a 1 kb fragment containing the open reading frame of the *HYG* gene with primers P1132 and P1133 and using plasmid pF4X1.4hyg as a template (JenaBioscience). The fragment was digested with NcoI and NotI and inserted into pF9egfp1.4*SAT*1.4 digested with the same enzymes, thereby replacing the *EGFP* gene with the *HYG* gene.

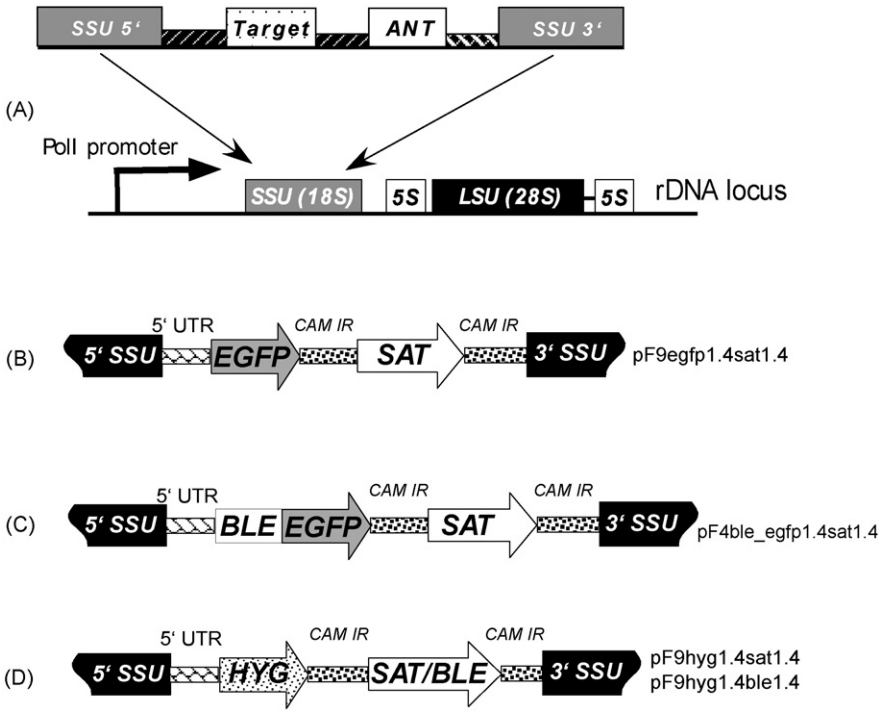


Fig. 1. Principle scheme for the construction of the stably transfected *L. tarentolae* strains used in this investigation. (A) Construction of constitutively expressing strains by integrating the expression cassette into the *SSU* gene cluster. *5S*, small (5S) ribosomal RNA gene; *LSU*, large (28S) ribosomal RNA gene; Target, denotes gene intended for expression while *ANT*, denotes a coupled antibiotic selection marker. (B and C) Expression cassettes containing repeated intergenic regions flanking the marker gene. *CAM IR*-denotes the intergenic region of the calmodulin gene forming a repeated UTR around the selection marker.

Plasmid pF9hyg1.4ble1.4 (Fig. 1D) was obtained by digesting plasmid pF9hyg1.4*SAT*1.4 with NotI-SpeI resulting in the removal of *CAM* IR and the *SAT* gene. This fragment was replaced with a NotI-SpeI fragment excised from plasmid pF4mcsX11.4dBble that contained the calmodulin CB intergenic region used as the 5′ UTR of the *BLE* gene.

Plasmid pF9hyg1.4TKmyc1.4 (Fig. 3D) was constructed by PCR amplification of the thymidine kinase (*TK*, GeneBank accession AY437640) gene using pGEX2T_TK as template. The plasmid was generously donated by Manfred Conrad. In a two-step PCR reaction, primers TK_BamHI_for and Tka_myc_rev were used in the first amplification step and the primers TK_BamHI_for and Tkb_SpeI_myc_rev in the second step resulting in a PCR product encoding the *TK* gene C-terminally tagged with the myc-tag sequence EQKLISEEDL. The PCR product was digested with BamHI and SpeI enzymes and cloned into vector pF9hyg1.4ble1.4 digested with the same enzymes, replacing the *BLE* gene with the myc-tagged *TK* gene.

Plasmid pF9hyg1.4mycTKble1.4 was constructed by PCR amplification of the *TK* gene as described above. The PCR product was digested with BamHI and cloned into the F9hyg1.4ble1.4 vector digested by the same enzyme. This resulted in a 5′ myc-tagged *TK_BLE* fusion gene connected by a linker coding for sequence GGGGGLGI.

For construction of plasmid pF9hyg1.4bleTKmyc1.4 the *ble* gene was PCR amplified using primers A678_18 and Ble_SpeI_TGAdef_rev and plasmid pF9hyg1.4ble1.4 as a template. The *TK* gene was amplified using the same template with two step PCR using the following primer pairs: TK_SpeI_TKlinker_for, TKa_myc_rev and TK_SpeI_TKlinker_for, Tkb_SpeI_myc_rev. The PCR products were treated with either SpeI or BamHI, respectively, and cloned together into pF9hyg1.4ble1.4 digested by the same enzymes.

2.2. Plasmids for stable integration into the host β-tubulin locus and inducible expression under the T7 RNA polymerase promoter

Plasmid pTUB_F4egfp1.4ble1.4 was constructed by cutting the pF9hyg1.4ble1.4 FseI HindIII1.4 vector (derivative of pF9hyg1.4ble1.4) with HindIII and blunting the linearized vector by T4-polymerase treatment. Next, the fragment containing the *BLE* gene was excised by treating the vector with NotI. The isolated fragment was subcloned into pTUB_F4egfp1.8ble subjected to ApaI digestion, T4 treatment and subsequent digestion with NotI.

Plasmid pTUB_APegfp1.4ble1.4 was constructed as described above but using pTUB_APegfp1.4neo as a backbone.

For construction of the plasmid pTUB_F4egfp1.4bleTK1.4 a fragment containing the open reading frame for a fusion *BLE-TK* gene was excised from the plasmid pF9hyg1.4ble_tkΔFseI_HindIII1.4 (a derivative of pF9hyg1.4bleTKmyc1.4) by the procedure described above. The fragment was cloned into the vector pTUB_F4egfp1.8ble [5] subjected to ApaI digestion, T4 treatment and subsequently partially digested with NotI. The partially digested products, where only the NotI site at the 3′ end of target gene was cleaved, were isolated by gel purification and used as a backbone.

Vector pTUB_APegfp1.4bleTK1.4#5 was constructed as described above for pTUB_F4egfp1.4bleTK1.4 but using pTUB_APegfp1.4neo as a backbone.

2.3. Transfection and cultivation of Leishmania tarentolae

L. tarentolae Parrot laboratory strain P10 and strain T7/TR (JenaBioscience) were cultivated as static suspension cultures in LEXSY BHI (Jena Bioscience) at 26 °C. In the case of T7/TR strain appropriate antibiotics were added to the culture medium. Transfections were carried out by electroporation of *in vitro* cultivated promastigotes essentially as described [21] except that electroporation was performed directly in growth medium at cell density of ca. 10^8 cells/ml. Recombinant clones were selected as single colonies on solidified growth medium containing 10% fetal calf serum (FCS) (Bio Whittacker) and 40 mM HEPES pH 7.4 and the appropriate antibiotics. As selective antibiotics we used LEXSY NTC (Jena Bioscience) at 100 μg/ml, LEXSY Hygro (Jena Bioscience) at 100 μg/ml, LEXSY Bleo (Jena Bioscience) at 100 μg/ml and LEXSY Neo (Jena Bioscience) at 50 μg/ml. Recombinant cell lines were selected in liquid static suspension cultures with the indicated drug concentrations. For electroporation approximately 10 μg of the expression plasmids were digested either with SwaI (for stable integration into the *SSU* locus) or with PacI and PmeI (for stable integration into the *TUB* or the *ODC* locus). Integration of the expression constructs into the *SSU*, *TUB* or *ODC* loci was confirmed by PCR on genomic DNA with specific primers (Table S1).

2.4. Ganciclovir treatment and selection of recombinant clones

Ganciclovir (9-((1, 3-dihydroxy-2-propoxy) methyl) guanine) (Sigma) was added to the liquid and solidified media at concentrations from 0.1 to 1.5 mM. All experiments with ganciclovir were conducted in dim light. To select the ganciclovir-resistant variants, cells were passaged twice by 1:100 dilution in 10 ml LEXSY BHI supplemented with the relevant antibiotics and grown up to $OD_{600} = 2$. After the second passage the cells were plated out on solidified media containing 1 mM ganciclovir, appropriate antibiotics and tetracycline. A 100 ml of plate media contained 50 ml of 2% Bacto-Agar (Difco), 35 ml of 2× LEXSY BHI, 10 ml inactivated FCS, 1 ml Penicilline/Streptomycine mix (GIBCO), 0.2 ml of Hemin (0.25% in 50% Triethanolamin) and 40 mM HEPES pH 7.4. The individual clones became visible after 3–5 days and were transferred to 96 well plate containing LEXSY BHI with appropriate antibiotics. After expansion the antibiotic resistance and/or EGFP fluorescence were determined and the nature of recombination events was confirmed by genomic PCR with specific primers (Table S1).

2.5. EGFP and dsRED expression analysis

Fluorimetric assays of EGFP and dsRED expression were performed in 96 well plates with 0.2 ml of cell lysates obtained from of 2×10^7 cells in 10 mM Tris pH 8.0, 1% Triton. The plates were measured using the Fluoroscan Ascent FL fluorescent plate reader (Labsystems). The excitation/emission wavelengths were set to 485/510 nm for EGFP and 544/590 nm for dsRED.

2.6. Total DNA isolation and Sothern blot analysis, SDS-PAGE and Western blotting

See Supplementary materials.

3. Results and discussion

3.1. Construction of vectors for excision of selection markers

The development of a system for selection marker mediated integration of a heterologous gene expression cassette into the genome of *Leishmania* followed by subsequent removal of the marker, poses several technical problems. First, one needs to ascertain that only the marker gene is removed and the functionality of the expression cassette is not affected. This requires the integrity of the open reading frame and the flanking untranslated regions (UTRs) which are essential for the proper processing of the pre-mRNA transcripts. Second, a high degree of sequence identity over an extended DNA stretch is required for efficient homologous recombination. Since the marker gene is typically placed either upstream or downstream of the gene of interest, and shares an intergenic region with it, the latter requirement is difficult to fulfill. We conjectured that this problem might be avoided if the marker gene lies downstream of gene of interest and is flanked by UTRs made of identical sequence (Fig. 1B). In this case homologous recombination should lead to the removal of the marker and result in the restoration of a functional 3′ UTR for the gene of interest. However, the practicability of this approach depends on the frequency of recombination events in *L. tarentolae* genome. To experimentally measure this, we created a set of constructs that could be stably integrated into the *SSU* locus of *L. tarenolae* where they are transcribed by RNA polymerase I as described earlier [22,23] (Fig. 1A; S1). The constructs carried a combination of genes coding for antibiotic resistance markers such as *HYG*, *SAT* or *BLE* and enhanced green fluorescent protein (*EGFP*) or its fusion with *BLE* (Fig. 1B–D). The 3′ gene was flanked by two identical 1.4 kb sequences derived from the intergenic region of successive B and C calmodulin genes from the *CAM* locus of *L. tarentolae* [22]. The resulting constructs were transformed into P10 strain of *L. tarentolae* and the drug resistant clones were selected. The resulting strains P10#F9egfp1.4*SAT*1.4, P10#F4blegfp1.4*SAT*1.4, P10#F9hyg1.4*SAT*1.4 and P10#F9hyg1.4ble1.4 were cultivated for 10–30 passages with and without all possible combinations of antibiotics. The cells were then plated on the agar plates in the absence of antibiotics and 96 clones derived from each plate were picked and grown in suspension in multi-well plates without antibiotic selection. The cultures were analyzed for possible recombination events by either measuring EGFP fluorescence or by supplying the growth medium with the relevant antibiotics to analyze the drug sensitivity of the clones. When a gene deletion was suspected it was confirmed by a PCR on the total genomic

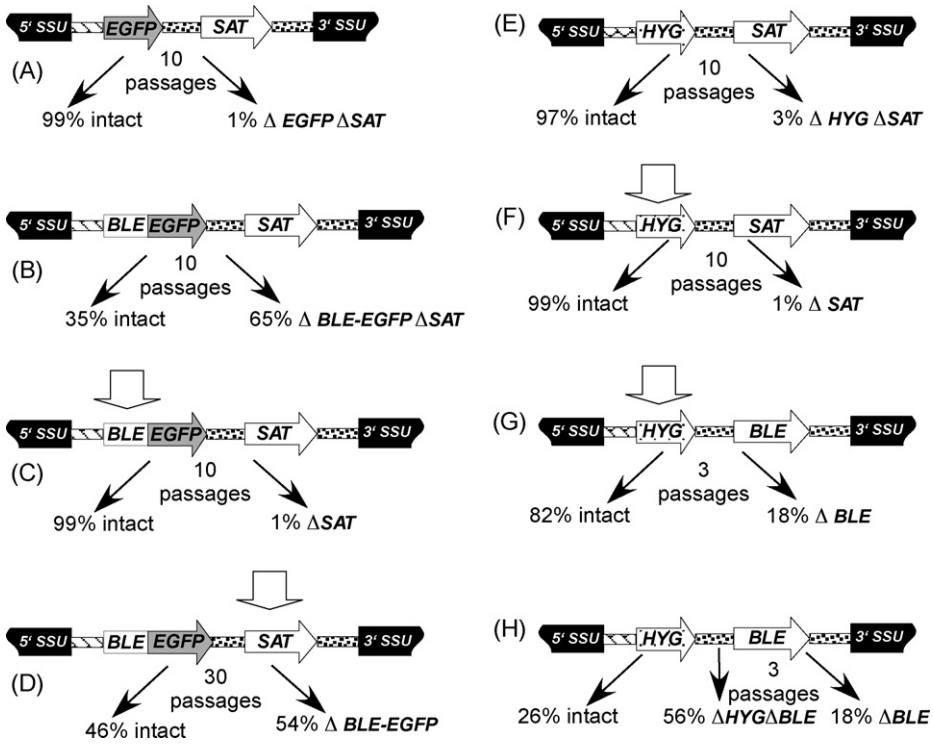


Fig. 2. Analysis of recombination events in the *SSU* gene of *L. tarentolae* during prolonged cultivation. Ninety-six clones of each strain were analyzed. The constructs are presented graphically and the white block arrow indicates the presence of the respective drug pressure. The type and frequency of the observed rearrangements and the number of passages are indicated under each construct.

DNA using a combination of *SSU* and construct specific primers (Fig. S1C).

The results of these experiments are summarized in the Fig. 2 and show that after prolonged passaging without antibiotic pressure the integrated gene cassette can be deleted either partially or completely. The strains that were maintained on two antibiotics or on an antibiotic for the second marker surrounded by the direct repeats did not display an appreciable loss of expression cassette (not shown). The only exception to the latter case is the F4blegfp1.4SAT1.4 construct where pressure on the second selection marker resulted in the loss of *BLE-EGFP* fusion gene while retaining the *SAT* marker. PCR amplification and sequencing of the integrated construct showed that a recombination occurred between short homology stretches (25–76 bp) in 5′ UTRs of *BLE-EGFP* and *SAT* genes (Fig. S2). Most of the recombination events in the absence of the selection pressure led to a deletion of the entire cassette, presumably due to gene conversion or to recombination between two *SSU* genes of neighbouring clusters. Selection pressure on the first marker led to deletion of the second marker via recombination within direct repeats.

It became apparent from these experiments that in the absence of Bleo, excision of the *BLE* gene was found ca. 20 times more frequently than excision of the *SAT* or *HYG* genes. This was also the case when *BLE* was in fusion with *EGFP* indicating that *BLE* gene product exerted a significant negative selection pressure on the cells (Fig. 2B, D, G and H). In contrast, both *SAT* and *HYG* selection markers have no selective effect on the cells and therefore allow a first estimation of the deletion frequency as 10^{-4} cells with a deletion per cell division (Supplementary materials). This number should be taken with a degree of caution since it is based on the analysis of a small experimental set. An additional potential pitfall in this interpretation of the data relates to the possible rapid expansion of an early emerging deletion mutant within growth suppressed population as is clearly the case when a *BLE* gene is used as a marker. Therefore, determination of a true recombination rate across the number of possible integration sites requires fluctuation analysis [24]. However, the data allow discrimination between recombination events occurring via the Campbell-out mechanism (deletion of the repeat flanked gene) and deletion by gene conversion (deletion of the entire integrated cassette). Under the chosen experimental conditions the latter mechanism is used by *L. tarentolae* three times more frequently (Fig. 2H).

3.2. Use of herpes simplex virus thymidine kinase as a negative selection marker for L. tarentoale

The observed rates of spontaneous recombination between the duplicated intergenic regions are sufficiently high to be employed for the removal of the selection marker genes from transgenic *Leishmania.* The negative selection pressure exerted by the *BLE* gene could potentially be utilized for this purpose but it does not offer a way to select clones with the *BLE* deletion. Hence we chose thymidine kinase (*TK*) from herpes simplex virus which is expected to become toxic by the addition of the guanosine analogue known as ganciclovir [25]. Increasing the concentration of ganciclovir in the suspension

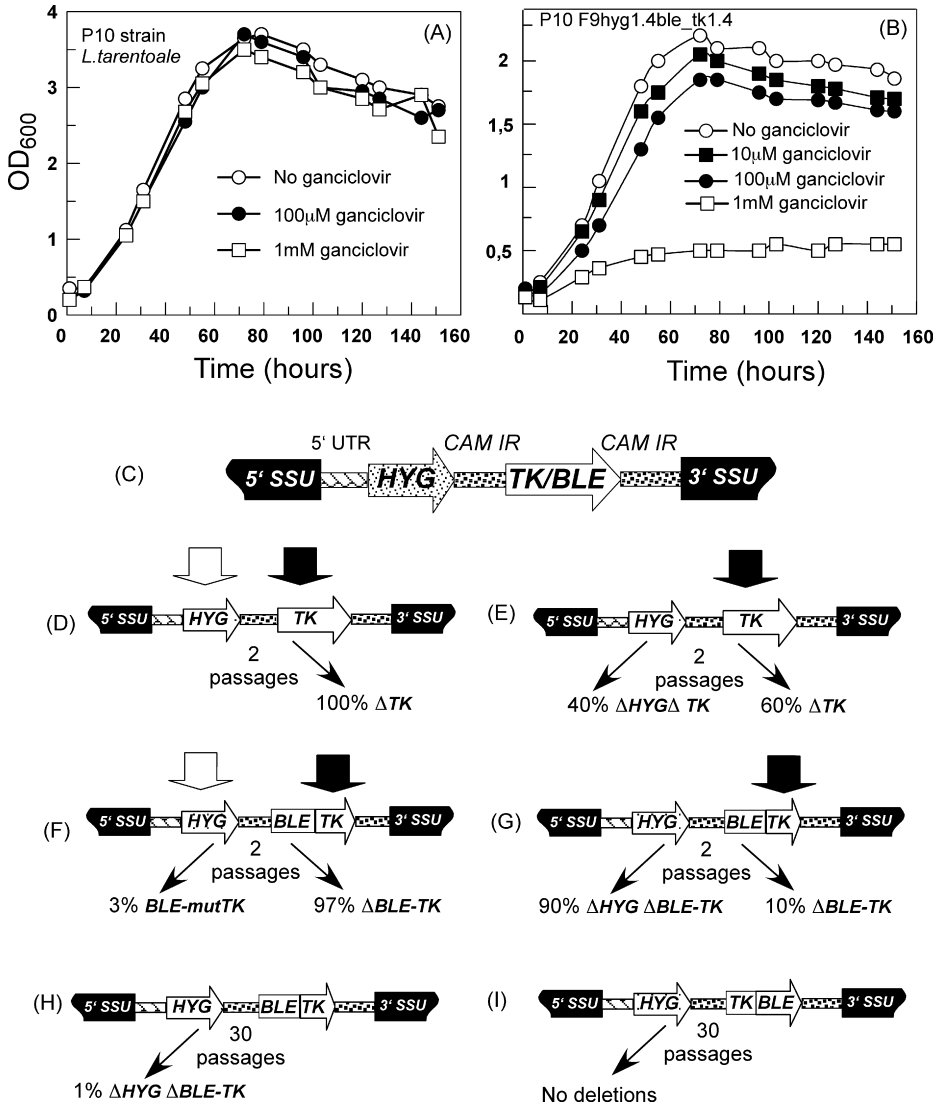


Fig. 3. Influence of ganciclovir on the growth rates of wild type and bleomycin-thymidine kinase fusion expressing *L. tarentolae* cells. (A) Changes in the optical density over time of the suspension cultures of wild type *L. tarentolae* incubated with various concentrations of ganciclovir. (B) As in A but with an *L. tarentolae* strain containing F9hyg1.4ble_tk1.4 expression cassette integrated into the *SSU* locus. (C) Schematic drawing of F9hyg1.4ble_tk1.4 expression cassette targeted into the *SSU* locus. (D–H) Analysis of recombination events in F9hyg1.4ble_tk1.4 expression cassette integrated into the *SSU* gene under different selection conditions. The black arrow denotes the presence of 150 μM of ganciclovir (negative selection pressure) in the culture medium while the white arrow indicates the presence of 100 μM of hygromycin (positive selection pressure). (I) Stability analysis of F9hyg1.4tk_ble1.4 expression cassette targeted into the *SSU* locus.

culture of wild type *L. tarentolae* up to 1 mM did not affect cell growth, indicating that the nucleoside was not itself toxic to the cells (Fig. 3A). To test the possible effect of products of ganciclovir phosphorylation on the growth of *L. tarentoale* we constructed vectors F9hyg1.4tk1.4, F9hyg1.4tk_ble1.4 and F9hyg1.4ble_tk1.4 where the *TK* gene was expressed either alone or in fusion with *BLE* (Fig. 3C). Interestingly, in contrast to the earlier characterized *BLE* or *BLE-EGFP* expressing constructs vectors expressing *BLE-TK* or *TK-BLE* fusion proteins did not exert detectable negative selection pressure on the transformed cells (Fig. 3H and I). The nature of *BLE* gene product toxicity and the reason for its absence in the fusion with thymidine kinase remain unclear.

Addition of ganciclovir to the growth medium of the resulting transgenic cells led to a dose dependent reduction in growth rates while growth of the wild type cells was unaffected (Fig. 3A and B). We determined IC_{50} values of ca. 250 μM for the P10#*F9hyg1.4tk_ble1.4*, P10#*F9hyg1.4ble_tk1.4* and P10#*F9hyg1.4tk1.4* strains (Fig. 3B and data not shown). Next we wanted to select for strains that gained resistance to ganciclovir due to the loss of the thymidine kinase gene. To this end the cell lines described above were grown in the presence of ganciclovir at a concentration below the IC_{50} at 150 μM or at a saturating concentration of 500 μM. After two passages cells were plated out on solid medium containing 1 mM ganciclovir and the resulting clones were expanded in liquid culture and analyzed as described above (Fig. 3D–G). When saturating concentrations of ganciclovir were used the suspension cultures failed to grow and their subsequent plating yielded no colonies indicating that the cells were killed before the *TK* gene could be eliminated.

Selection on 150 μM ganciclovir led to deletion of the negative marker in 10–60% of the cases (Fig. 3E and G). The rest of the clones lost the entire expression cassette. If cells expressing *HYG* and *BLE-TK* genes were cultured in the presence of both antibiotics and 150 μM ganciclovir 97% lost the negative selection marker while 3% of the surviving clones retained the entire expression cassette. PCR amplification and sequencing analysis of expression cassettes in these clones uncovered inactivating mutations in *TK* gene (not shown). Based on the described observations we concluded that the toxic effect of ganciclovir allows rapid selection of recombinant clones that are generated by the homologous recombination between direct *CAM BC* intergenic repeats flanking the *TK* gene or via gene conversion.

3.3. Use of ganciclovir mediated gene excision to construct marker gene-free inducible *L. tarentolae* expression clones

Inducible expression of homologous and heterologous genes is a very important tool in the study of all organisms including *Kinetoplastidae*. To study whether the marker removal strategy outlined above could also be applied to inducible expression systems we employed the *L. tarentolae* strain T7/TR that constitutively expresses both the T7 RNA polymerase and the TET repressor which can mediate tetracycline regulated protein expression from expression cassettes bearing a T7 promoter [5].

We constructed two derivatives of the previously described pTUB expression vectors that can be integrated into the β-tubulin locus by homologous recombination [5]. In these vectors the gene of interest and the following selection marker are coupled in a bi-cistronic assembly. We modified these vectors by replacing the section marker with *BLE-TK* gene fusion surrounded by repeated UTRs (Fig. 4A). The vectors differ by UTRs upstream of the *EGFP* gene and the presence of the T7 terminator sequences downstream of the 3′ UTR following the marker gene in TUB_F4egfp1.4bleTK1.4. The plasmids were transfected into the T7/TR strain of *L. tarentolae* and 10 resulting clones were expanded in suspension culture and EGFP expression was induced with tetracycline. Under induction the

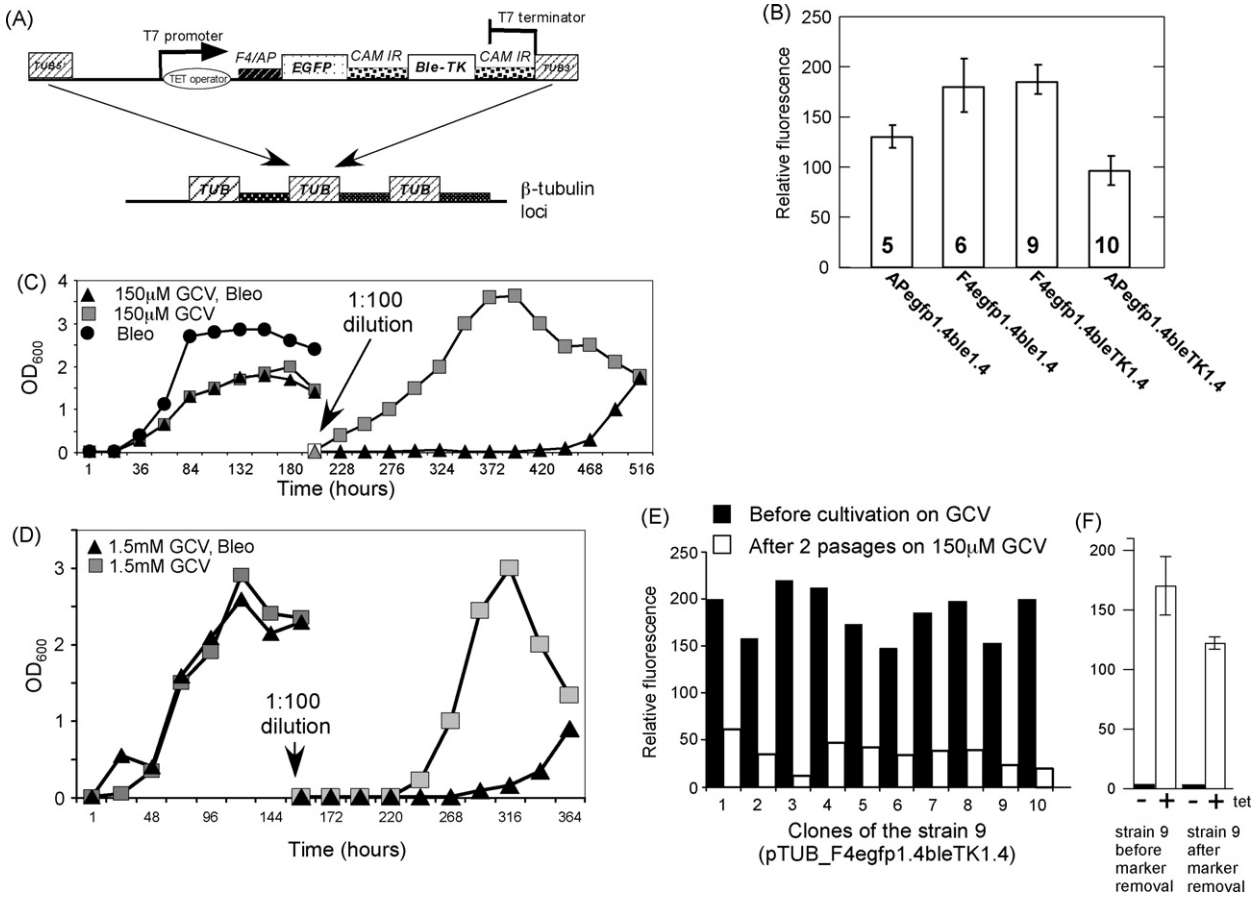


Fig. 4. Construction of the *L. tarentolae* strains inducibly co-expressing EGFP and a bleomycin-thymidine kinase fusion protein and removal of selection marker gene using ganciclovir selection. (A) Principle scheme for construction of *L. tarentolae* strains inducibly co-expressing *EGFP* and *BLE-TK* genes under control of the T7 polymerase and the Tet repressor. (B) Normalized fluorescence of several *L. tarentolae* strains transformed with inducible EGFP expression constructs and induced with tetracycline for 24 h. Genotypes are indicated and the corresponding strain numbers are shown at the bottom of the bars. Fluorescence was measured as described in Section 2. (C) Changes in the optical density of the *L. tarentolae* strain transformed with pTUB_F4egfp1.4bleTK1.4 and cultivated in the presence of tetracycline with either ganciclovir, Bleo or both. After reaching stationary phase the cells were diluted as indicated and grown under the same conditions for an additional 9 days. (D) As in C but the cells were cultivated without induction and at a 10-fold higher concentration of ganciclovir. (E) Normalized fluorescence of independent clones of an *L. tarentolae* strain transformed with pTUB_F4egfp1.4bleTK1.4 before and after cultivation in the presence of ganciclovir. (F) Comparison of EGFP fluorescence of clones of pTUB_F4egfp1.4bleTK1.4 strain before and after the deletion of the *BLE-TK* gene.

pTUB_F4egfp1.4bleTK1.4 transformed clones gave ca. 40% higher fluorescence than pTUB_APegfp1.4bleTK1.4 in contrast to the previous report which reported the reverse relationship of these 5′ UTRs and expression yields [5]. Moreover, variation in the fluorescence levels of individual clones was observed with several clones displaying fluorescence levels comparable to non-induced cultures (not shown). The fluorescent clones were analyzed by genomic PCR to confirm correct integration of the intact expression cassettes (not shown). Expression of the bleomycin-thymidine kinase fusion protein was confirmed by Western blotting demonstrating, in accord with previous observations, that the protein expression levels were comparable to the levels observed with Pol I driven constructs (Fig. S3) [26]. No signal was observed in uninduced cells indicating that the basal expression level was at least 20 times lower than in the induced cells. The absence of detectable leakage is a beneficial feature of an inducible expression system. However, it implies that for ganciclovir selection the *TK* expression must be induced to obtain the amounts of kinase necessary for efficient phosphorylation of the compound and the ensuing toxicity. Therefore, we supplied the medium with tetracycline to ensure sufficient levels of thymidine kinase expression. We also included the uninduced control strains that were incubated with the same or with a 10 times higher concentration of ganciclovir in order to test whether the background levels of *TK* expression would be sufficient to create negative selection pressure. As an additional control we supplied culture medium with both ganciclovir and Bleo expecting that under these conditions the cells would not be viable. Initially in the presence of ganciclovir, cells grew with and without Bleo at a comparable rate (Fig. 4C). The difference became apparent in the subsequent passage where cells grown in the presence of Bleo showed a dramatic growth inhibition (Fig. 4C). Selection could also be carried out with uninduced cultures if the concentration of ganciclovir was increased to 1 mM. Such suspension cultures showed a growth pattern very similar to that of induced cells in the presence of 150 μM of ganciclovir (Fig. 4D). We compared the EGFP fluorescence of the cultures before and after the selection procedure and observed that the total fluorescence of the cultures decreased significantly indicating that either the expression cassette was lost as a result of ganciclovir treatment or it was no longer efficiently expressed (Fig. 4E). In order to analyze the resulting mutants the cultures that displayed the smallest fluorescence reduction upon ganciclovir treatment were plated on the solid medium containing ganciclovir in the absence and presence of tetracycline. The individual clones were picked, expanded in suspension culture and tested for their ability to grow on Bleo and ganciclovir and to produce EGFP in the presence of tetracycline. The deletions in the expression cassette were diagnosed by genomic PCR and confirmed by sequencing of the PCR products (not shown). The results are summarized in Table S3. It transpires that the majority of recombination events led to the loss of the entire expression cassette yielding non-fluorescent clones. In a small number of cases the recovered clones were weakly fluorescent and retained the *BLE-TK* fusion gene. We presume that in these cases deleterious mutations occurred in the T7 RNA polymerase gene. The proportion of recombinant clones where only the marker gene was removed did not exceed 8%. The combination of induction with low concentrations of ganciclovir showed a recombination efficiency similar to that with 1.5 mM ganciclovir treatment. Strain 9 (transformed with pTUB_F4egfp1.4bleTK1.4) which produced more EGFP underwent marker excision with significantly higher frequency than a less well expressing strain transformed with pTUB_APegfp1.4bleTK1.4. Finally, we analyzed the clones that contained the entire expression cassette but were able to grow in 1.5 mM of ganciclovir. Sequencing of two of these clones revealed a, presumably deleterious, Q125L mutation in the thymidine kinase gene.

To verify the functionality of the clones that had lost the marker we compared the EGFP fluorescence of the *EGFP_ΔBLE-TK* clones cultured with and without tetracycline with that of the parental strain. As can be seen in Fig. 4F the levels of induced fluorescence were ca. 30% lower in markerless clones. This implies that deletion of the downstream marker gene influences the expression levels of the target gene perhaps due to alterations in RNA processing [27].

3.4. Construction of an *L. tarenolae* strain containing two markerless *egfp* expression cassettes

In the next step, we decided to test whether the procedure developed could be applied sequentially to obtain cell lines containing several markerless inducible expression cassettes. To this end we chose markerless clones with the genotype *EGFP_ΔBLE-TK* and transfected them with pTUB_F4egfp1.4bleTK1.4. The fluorescence of the resulting clones was analyzed in the presence and absence of tetracycline. As can be seen in Fig. 5A the resulting clones were approximately twice as fluorescent as the progenitor clone 9 (see Supplementary materials). This indicates that the second expression cassette is integrated into a different copy of the *TUB* gene and did not replace the first expression cassette. In order to purge the second selection marker from the genome of the resulting clones we performed a second round of selection on 150 μM ganciclovir in the presence of tetracycline. As in the previous case the selection cultures were analyzed by fluorescence and the cultures displaying the highest fluorescence were plated and the resulting clones analyzed. Fluorescence analysis of the chosen clones showed heterogeneity where roughly four categories could be identified: cells that fluoresce twice as strongly (>200 rfu) as the parental clone and hence represented the desired markerless organisms with two *EGFP* expression cassettes (10%), cells with fluorescence identical to the parental clones in which presumably one of the two expression cassettes was deleted (20%), clones that displayed no fluorescence and presumably had lost both expression cassettes (66%). Finally, we recovered 4% of clones that displayed intermediate fluorescence and may represent either mixture of two clones or contain some other types of genetic rearrangements leading to a reduction in EGFP expression levels.

To confirm that the highly fluorescent clones indeed represented a result of two independent integrations and two independent marker excision events, we performed Southern blot analysis of total genomic DNA of parental and markerless

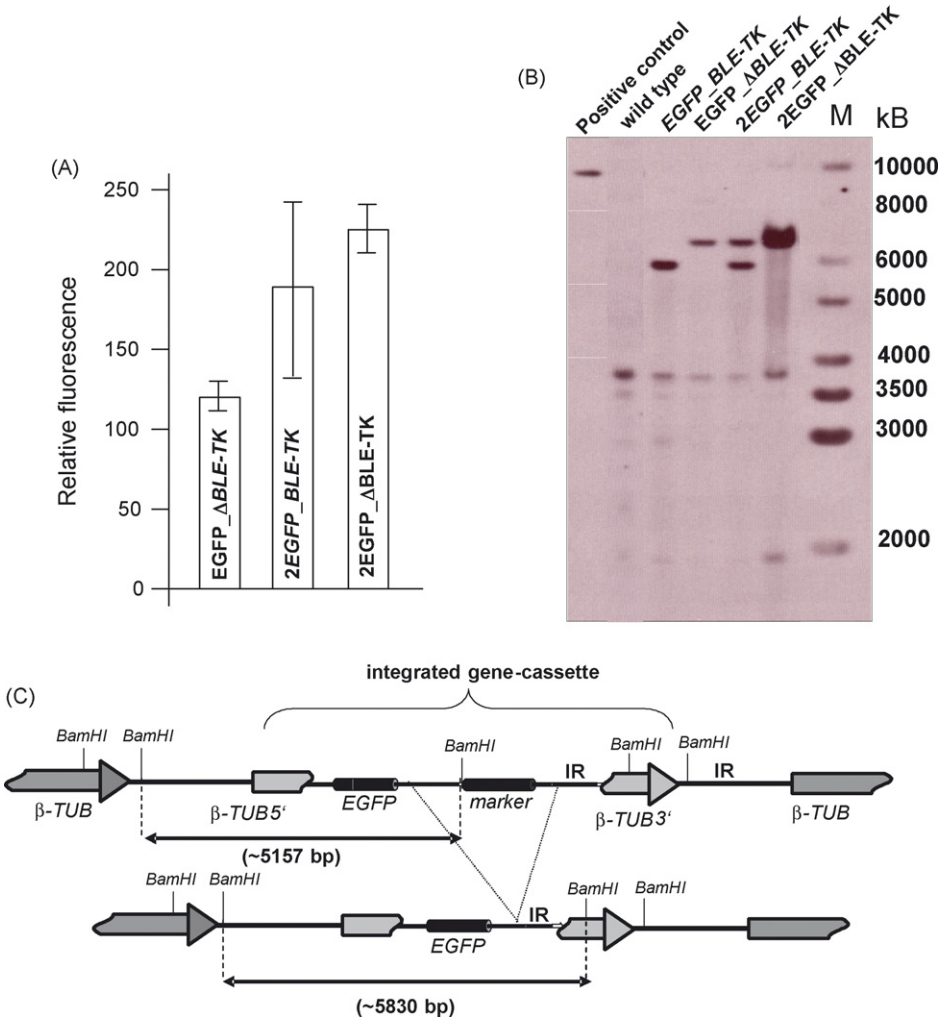


Fig. 5. Construction of *L. tarentoale* strains containing two markerless cassettes. (A) Relative fluorescence of clones containing 1 markerless *EGFP* expression cassette, 2 *EGFP* expression cassette and one *BLE-TK* gene or clones containing 2 markerless *EGFP* expression cassettes. (B) The Southern blot analysis with *EGFP* probe of BamHI digested total DNA of *L. tarentoale* strains with one or two *EGFP_BLE-TK* expression cassettes before and after ganciclovir treatment. (C) Inferred organization of *L. tarentolae* tubulin cluster targeted by *EGFP_BLE-TK* expression cassettes.

clones digested with the BamHI enzyme and using the *EGFP* gene as a probe. As can be seen in Fig. 5B and C integration of F4egfp1.4bleTK1.4 expression cassette into β-tubulin resulted in the emergence of a ca. 5.9 kB band. In those cells that underwent deletion of the *BLE-TK* part of the cassette, the fragment migrates at a position corresponding to ca. 7 kB (Fig. 5C) indicating that an internal BamHI site has been lost during the recombination event in accord with the available sequence of *L. tarentolae* β-tubulin (Fig. 5C). Integration and deletion of the second expression cassette resulted in the identical behavior of the specifically hybridizing bands in all clones analyzed, indicating that the targeted β-tubulin genes were highly conserved and arranged in a similar fashion to that of *Leishmania major* [28].

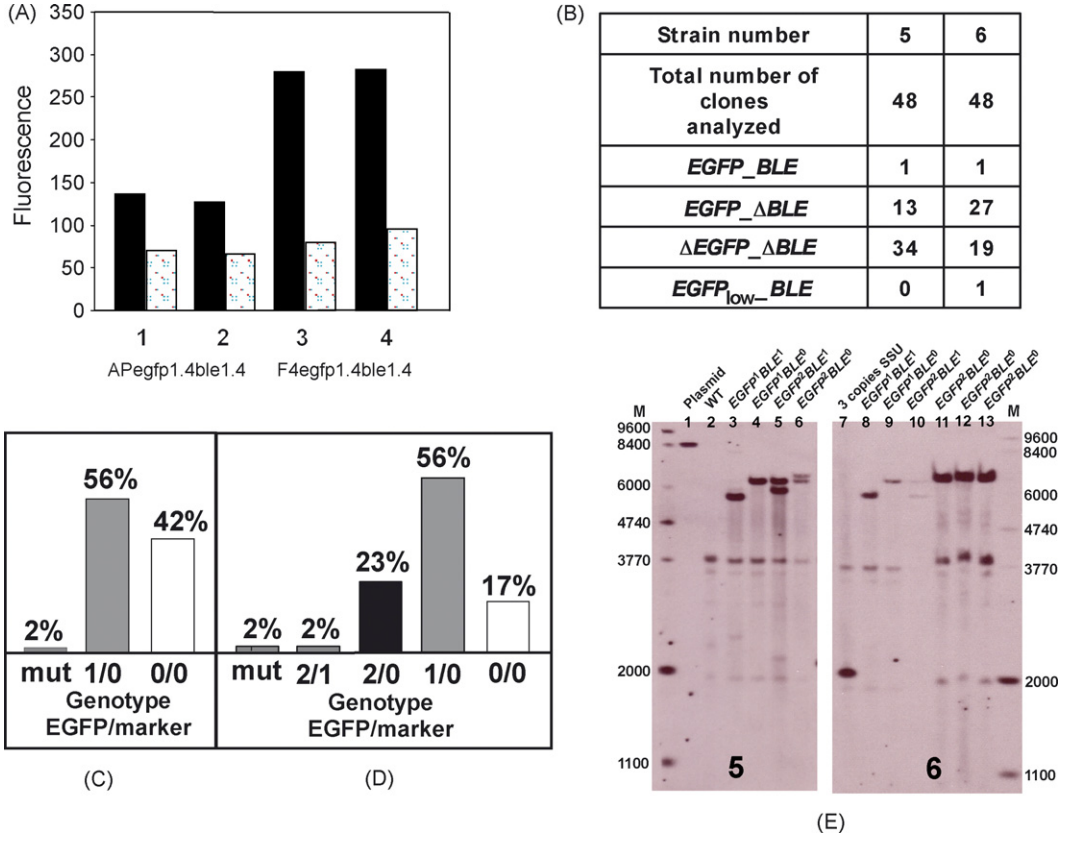
3.5. Construction of *L. tarentolae* markerless strains inducibly co-expressing EGFP and dsRED proteins

The number of recovered markerless clones with two EGFP expression cassettes obtained by the procedure described in the previous section was disappointingly low. We conjectured that primary selection on the basis of fluorescence resulted in elimination of the clones that contained two expression cassettes but, for unknown reasons, did not produce the double fluorescent yield. To test this possibility directly we constructed an expression vector pTUB_F4dsRed1.4bleTK1.4 which inducibly expresses a rapidly maturing variant of red fluorescent protein dsRed [29]. Expression of dsRed was clearly detectable in the induced cells even in the visible light due to the bright red color of the pelleted cells (not shown). We than transformed the dsRed expression construct into derivatives of the *L. tarentolae* strains 5, 6, 9 containing one markerless EGFP expression cassette (not shown). The resulting clones, inducibly co-expressing both fluorescent proteins, were subjected to a round of selection on ganciclovir and the resulting clones were analyzed for Bleo resistance and for fluorescence. However, we were unable to recover clones that contained both EGFP and dsRed markerless expression cassettes. In all clones analyzed either the entire dsRed expression cassette or both expression cassettes were deleted. This implies

that the low number of markerless clones carrying two EGFP expression cassettes reflects the high rate of recombination between β-tubulin genes leading to elimination of expression cassettes. This may be promoted by the repetitive nature of the β-tubulin gene cluster and/or the DNA repair mechanisms activated by the ganciclovir treatment. Treatment of *Leishmania* organisms with DNA damaging agents was previously shown to significantly increase the rates of recombination [14].

3.6. Use of the bleomycin resistance gene as a negative selection marker for construction of markerless expression strains

To test whether activation of DNA repair induced by ganciclovir was responsible for the high rate of expression cassette deletion in the β-tubulin gene cluster we decided to make use of the observed toxicity of bleomycin resistance gene product. We deleted the thymidine-kinase gene from the pTUB_F4egfp1.4bleTK1.4 and pTUB_APegfp1.4bleTK1.4 expression vectors and transformed these plasmids into the T7/TR strain of *L. tarentolae*. The resulting clones were analyzed for inducible EGFP expression, and it was found to be comparable to that of parental BLE_TK plasmids (Fig. 4B). The clones were passaged in the presence of tetracycline for 20 generations and were subsequently plated on agar. The fluorescence of the cultures was monitored during the cultivation and showed reduction of fluorescence in all cases, albeit the degree of reduction varied among clones (Fig. 6A). The resulting clones were picked, expanded in suspension cultures and analyzed for bleomycin resistance and for inducible EGFP fluorescence (Fig. 6B). The clones that appeared to be recombinant were analyzed by genomic PCR and sequencing of the PCR products. Surprisingly we found that in this case up to 56% of the clones that lost the *BLE* gene did so due to recombination between flanking UTRs (Fig. 6B). To independently confirm this we performed Southern blotting analysis of their genomic DNA (Supplementary materials). As can be seen in Fig. 6E a single specific band hybridized with the *EGFP* probe in the transformed clones. In the clones that underwent deletion of the selection marker the specifically hybridized band migrated ca. 1 kB higher than in the parental strain due to deletion of fragment containing the BamHI site, in accord with the expected rearrangement of target locus (Fig. 5C).



(B)

Strain number	5	6
Total number of clones analyzed	48	48
EGFP_BLE	1	1
EGFP_ΔBLE	13	27
ΔEGFP_ΔBLE	34	19
$EGFP_{low}$_*BLE*	0	1

Fig. 6. Use of *BLE* gene as a negative selection marker. (A) Fluorescence of the clones derived from strains 6 (1 and 2) and 5 (3 and 4) after induction with tetracycline (black bars) or after 3 passaging under induction (dotted bars). (B) Analysis of the progeny of strains 5 and 6 cultivated under induction for 20 generations. (C) Distribution of genotypes in the progeny of strain 6 following cultivation under induction. (D) Distribution of genotypes among progenitors of the strain 6 that underwent marker removal and were subsequently transformed with another APegfp1.4ble1.4 expression cassette and cultivated for 30 generations under induction. Mut, denotes the category of clones where the genotype could not be established. (E) Southern blot analysis using a fragment of the *EGFP* gene as a probe on the BamHI digested total DNA of *L. tarentoale* strains that underwent sequential rounds of expression cassette integration and marker removal. Strain 5 (left panel) was transformed with APegfp1.4ble1.4 and the strain 6 (right panel) with F4egfp1.4ble1 expression cassettes. Lane 7 was loaded with the genomic DNA of an *L. tarentolae* strain containing three constitutive expression cassettes integrated into the *SSU* locus.

To test whether the self-induced *BLE* removal can be performed repeatedly we transformed the obtained clones containing one markerless EGFP expression cassette with either pTUB_APegfp1.4ble1.4 or pTUB_F4egfp1.4ble1.4 plasmids. As judged by the levels of EGFP fluorescence, cultivation of the resulting strains under induction followed by subsequent cloning of the cultures, yielded markerless clones containing two expression cassettes with frequency of over 20% in addition to twice as many clones containing only one markerless expression cassette (Fig. 6D). We analyzed the most fluorescent clones by Southern blotting and confirmed that they indeed contained two EGFP expression cassettes per genome (Fig. 6E). We concluded that the toxicity of the *BLE* gene product presumably does not activate the mechanisms of DNA repair and may thereby result in a smaller number of complete deletions compared to the selection procedure based on toxicity of thymidine kinase.

3.7. Analysis of long term retention of the markerless expression cassettes in the genome of L. tarentolae

The usefulness and general applicability of the described markerless *L. tarentolae* strains depends on the long term stability of the expression cassettes in the genome of the parasite. To test this we chose two markerless strains containing one *EGFP* expression cassette (derivatives of strains 6 and 9) and one strain containing two *EGFP* expression cassettes (derivative of strain 9) in the tubulin gene locus. Considering the fact that the repetitive nature of this locus promotes recombination events we expected that the rates of markerless expression cassette deletion would represent the low limit of stability compared to other parts of genome. The strains were cultured in suspension, with and without induction for 200 generations. The strains cultured in the absence of tetracycline were then induced and their fluorescence measured. Fig. 7A–C summarizes the fluorescent yields for several representative clones from each genotype. It becomes apparent from these data that continuous induction of EGFP expression results in the expansion of the non-fluorescent population. Although the exact reason for loss of fluorescence cannot be extracted from these experiments, the difference among clones in regard to phenotypic stability under both cultivation protocols is obvious. One of the most plausible interpretations is a variation in the stability of different parts of the tubulin gene cluster which is in accord with previous studies showing that the gene position in tandem arrays affects its expression and stability [30,31]. An alternative, and less likely, explanation for the observed differences in long term stability may be related to sporadic deactivation of the T7 transcription system that would lead to a similar phenotype. Interestingly, we recovered two clones that retained the same levels of EGFP expression throughout the cultivation under induction (Fig. 7A). We conjecture that in these transformants the expression cassettes may be integrated on the edge of the tubulin gene cluster thus reducing the frequency of non-allelic gene conversion. Alternatively, and less likely, these clones could have an overall lower rate of recombination possibly due to a mutation introduced by the gancyclovir treatment.

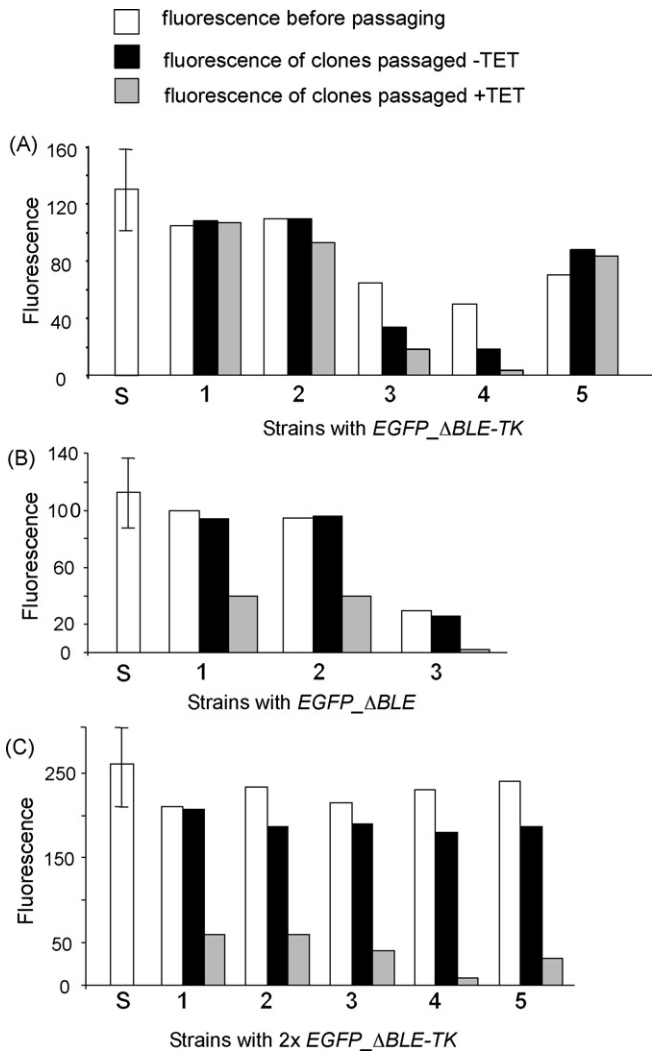


Fig. 7. Analysis of long term stability of transgenic *L. tarentolae* strains bearing markerless expression cassettes. (A) Fluorescence of the suspension cultures of markerless strains bearing *EGFP_ΔBLE-TK* expression cassette before and after passaging for 200 generations in the presence (grey bars) or absence the tetracycline (black bars). Numbers denote individual clones cultivated under different protocols. S denotes average fluorescence of induced suspension cultures of 10 clones from the parental strain. (B) As in A but with markerless strains carrying the *EGFP_ΔBLE* expression cassette. (C) As in A but with strains containing two *EGFP_ΔBLE-TK* expression cassettes.

3.8. Retention of the markerless expression cassettes in a non-repetitive gene locus

Next, we decided to test whether integration of the expression cassette into a non-repetitive gene locus could increase their stability by eliminating non-allelic gene conversion. To test this approach we have chosen the ornithine decarboxylase (*ODC*) gene locus reported to be unique in *Leishmania* [32]. We integrated an *egfp*1.4*ble_tk*1.4 cassette into one *ODC* locus and confirmed that resulting clones inducibly expressed EGFP (Fig. S5). We subsequently carried out the procedure for marker removal using ganciclovir and analyzed 48 of the resulting clones. We found that 57% of the clones underwent recombination within the expression cassette yielding the geno-

type *EGFP_ΔBLE-TK* while 42% of the clones lost the entire cassette, presumably by gene conversion with the allelic copy. The genotype of the remaining clones was not easily interpretable (not shown). The observed high percentage of the markerless clones compares very favorably with just 8% which was achieved when the same cassette was integrated into the repetitive (*TUB*) gene locus. These data support the idea that the high frequency of elimination of the expression cassette from the *TUB* gene cluster is a consequence of a repetitive nature of that gene locus. This is consistent with the idea of the existence of the mechanisms that maintain homogeneity among members of repetitive gene cluster [33].

4. Conclusions

Here we describe approaches for the construction of markerless *L. tarentotae* strains carrying constitutive or inducible expression cassettes. We show that thymidine-kinase fused to a bleomycin resistance gene can be used as conditional positive-negative selection marker that can be evicted from the integrated expression cassette if surrounded by repetitive UTRs. However, the repetitive gene clusters such as 18S RNA and tubulin genes traditionally used for integration of expression cassettes appear to be suboptimal in terms of genetic stability. As a result the efficiency of precise deletion of the undesired marker, while retaining other inserted elements, is often low (∼10%) in comparison to wider rearrangements. This implies that optimization of selection conditions and efficient screening protocols may be required when applying this method. Interestingly, once established, clones containing markerless expression cassettes were stable for as many as 200 generations, suggesting that this approach is potentially useful for the construction of transgenic organisms. We see the use of non-repetitive parts of the genome for integration of expression cassettes as the most promising approach. Here the true markerless clones are recovered in ca. 50% of the clones. Since the majority of those clones that lost the entire cassette appear to have done so via gene conversion it appears that invasion of both alleles bears the promise of long term selection-free stability of heterologous expression cassettes. Moreover, use of non-coding regions of chromosomes may be useful in this case since they are less likely to be essential.

Since our marker removal strategy based on the endogenous recombination machinery inevitably includes some undesired recombination events an alternative strategy using heterologous site-specific recombination systems such as Cre-Lox or Flp-FRT would be worth considering [16,34]. However, several technical issues such as, compatibility of the recombination sequences with UTR function and methods of recombinase delivery may turn out to be challenging.

Acknowledgements

This work was in part supported by grant AL 484/5-4 to KA and grant B309-98020 from the Thuringian Ministry of Sciences, Research and Culture to Jena Bioscience GmbH. S.M. was supported by a fellowship of Max-Planck Society. We are very grateful to C. Delon for critical reading of the manuscript and stimulating discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.05.007.

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