

Short communication

Blasticidin resistance: a new independent marker for stable transfection of *Leishmania*

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Protozoan parasites of the genus *Leishmania* are the etiologic agents of a spectrum of human diseases referred to as leishmaniasis. *Leishmania* alternates life stage between an intracellular amastigote form residing within the phagolysosome of vertebrate macrophage and an extracellular promastigote form living in the gut of sand flies. Molecular characterization of the genome of *Leishmania* has been greatly facilitated by the development of stable transfection methods, expression vectors and gene knockouts [1,2]. Stable transfection of *Leishmania* and trypanosomes requires the use of dominant selectable markers, and a number of independent markers have been used successfully. These include neomycin phosphotransferase (*NEO*) [3]; hygromycin phosphotransferase (*HYG*) [4], streptothricin acetyltransferase (*SAT*) [5], puromycin acetyl-

transferase (*PAC*) [6] and the bleomycin binding protein from *Streptoalloteichus hindustanus* (*PHLEO*) [6], which confer resistance to G418, hygromycin B, nourseothricin, puromycin and phleomycin respectively. In addition, overexpression of N-acetylglucosamine-1-phosphate transferase (*NAGT*) or dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) can confer resistance to tunicamycin and methotrexate respectively, allowing their use as selectable markers on episomal vectors [7,8].

Since *Leishmania* is diploid and lacks an experimentally manipulable sexual cycle, generation of null mutants requires the inactivation of two alleles [4]. Typically this is accomplished by two rounds of targeting with independent selectable markers, with a third marker needed to generate an ‘add-back’ construct restoring gene expression; this is an essential control for biological function [9]. While the use of loss of heterozygosity approaches can reduce the number of markers needed for null mutant construction [10], with the current repertoire of markers one can manipulate

Abbreviations: BSD, gene encoding blasticidin deaminase.

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only two or three loci simultaneously within a given cell. Thus the development of new independent selectable markers would improve our ability to genetically manipulate *Leishmania* and related trypanosomatids.

Here we show that blasticidin S and its associated resistance gene *BSD* [11] can be used as an effective selectable marker in *Leishmania*. Blasticidin S is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes*, which inhibits protein synthesis in both prokaryotes and eukaryotes [12,13]. Recently, it has been shown that *BSD* can be used as a selectable marker for transfection of mammalian cells and *Plasmodium falciparum* [11,14]. Since the activities of blasticidin S/*BSD* differ considerably from that of other drug/marker combinations used in *Leishmania*, we expected they would function independently.

We tested the sensitivity of two different species of *Leishmania* to blasticidin S. One was *L. major* (MHOM/LL/80/FRIEDLIN, strain V1; this is the reference strain for the *Leishmania* genome project [15]), which causes cutaneous disease, and the other was *L. donovani* (MHOM//SD/00/1S-2D), which causes visceral disease. Blasticidin S was a potent growth inhibitor for both species, with an EC_{50} of $0.07 \mu\text{g ml}^{-1}$ for *L. major* and $2 \mu\text{g ml}^{-1}$ for *L. donovani* (Table 1). Differences in drug sensitivity amongst strains and/or species have been seen previously, and are evident for other selective drugs tested in Table 1.

To test whether expression of *BSD* in *Leishmania* could confer resistance to blasticidin S, we created the vector pXG-*BSD* (Fig. 1) which substitutes *BSD* for the *NEO* marker present in pXG [16]. Five micrograms of pXG-*BSD* DNA was transfected into *L. major* and *L. donovani* by electroporation as described [3], and clonal lines were obtained by plating on semisolid M199 media containing blasticidin S. Preliminary tests showed that $5 \mu\text{g ml}^{-1}$ for *L. major* or $25 \mu\text{g ml}^{-1}$ for *L. donovani* yielded no survivors in control platings, and these concentrations were used for the plating of pXG-*BSD* transfections. For *L. donovani*, 1000–1500 colonies were obtained, and for *L. major* 75–105 colonies were obtained. Higher concentrations of blasticidin S in the plating media resulted in the recovery of fewer colonies; for *L. donovani*, at $50 \mu\text{g ml}^{-1}$ no colonies were obtained, while for *L. major* at $10 \mu\text{g ml}^{-1}$ only 15–35 colonies were obtained. For each species, several colonies were picked and grown in media containing blasticidin S (2 and $6 \mu\text{g ml}^{-1}$ for *L. major* and *L. donovani*, respectively). Each of these colonies contained episomal pXG-*BSD*, as determined by recovery and analysis following transformation of *E. coli* [17]. *L. major* pXG-*BSD* transfectants showed an EC_{50} of $3 \mu\text{g ml}^{-1}$ (42-fold wild-type), while *L. donovani* pXG-*BSD* transfectants showed an EC_{50} of $8 \mu\text{g ml}^{-1}$ (four-fold wild-type; Table 1).

To test the independence of the *BSD* marker with other drugs commonly used in *Leishmania*

Table 1

Sensitivity of *Leishmania* and pXG-*BSD* transfectants to drugs commonly used in transfections^a

Drugs	EC_{50} ($\mu\text{g ml}^{-1}$)			
	<i>L. major</i>	<i>L. major</i> /pXG- <i>BSD</i>	<i>L. donovani</i>	<i>L. donovani</i> /pXG- <i>BSD</i>
Blasticidin	0.07	3	2	8
G418	0.2	0.2	3	3
Hygromycin B	1	1	9	9
Puromycin	7.5	7.5	12	12
Nourseothricin	2	3	4	6
Phleomycin	0.4	0.4	0.7	0.7

^a Late-log cells were inoculated at $10^5 \text{ cells ml}^{-1}$ into drug-containing media and incubated until controls were in late log phase ($10^7 \text{ cells ml}^{-1}$) at which time the cells were counted. The EC_{50} is defined as the concentration of drugs, which causes a 50% reduction in cell density relative to the controls [23]. The values represent the average of at least two independent experiments; these differed by no more than 10%.

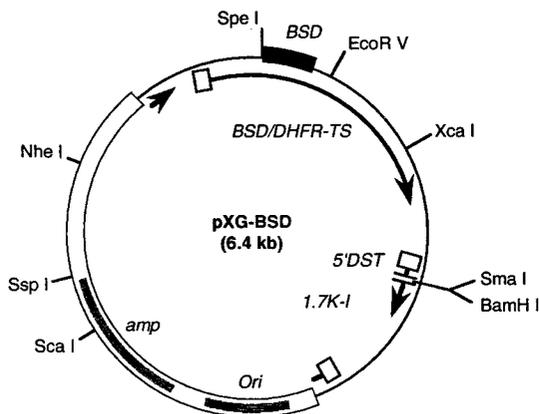


Fig. 1. Structure and features of pXG-BSD. pXG-BSD, like other pX series vectors, can be used as an expression vector by inserting the ORFs for genes of interest into the *Bam*HI or *Sma*I sites, or as a shuttle vector using any site compatible with function [16,20]. The *Aspergillus terreus* *BSD* gene was amplified by the PCR using the primers 5'-GGA AGA TGC ATG CCA AGC CTT TGT CTC AAG AAG AAT CCA CCC TC and 5'-GAC GGG AAG CTTTGC TCC TCG GCC ACG AAG TGC, and the template DNA pcDNA6/V5-His (Invitrogen); the product was inserted into pCRII (Invitrogen), yielding pBSD-TA (strain B4106). pXG-BSD (Beverley lab strain B4098) was made by removing the *NEO* gene from pXG1a (strain B1459; pXG1a is pXG [16] in which a polylinker *Spe*I site has been removed by partial digestion, filling in with T4 DNA polymerase, and self-ligation; S. Ha and S.M. Beverley, unpublished) by digestion with *Spe*I, and insertion of the 517 bp *Spe*I/*Xba*I fragment from pBSD-TA. Bold lines represent known or predicted mRNAs directed by the processing signals present in *L. major* intergenic regions. Small rectangles and arrows bounding the mRNAs denote the sites for the addition of the miniexon or poly(A) tail, respectively. *BSD/DHFR-TS* depicts the *BSD* ORF (black box) which is flanked by regions surrounding *DHFR-TS* [21,22]. *5'DST* denotes the miniexon addition site while *1.7K-I* denotes the poly(A) site for the *1.7K-I* transcript [22]. The open region represents the bacterial vector (pSP6-T3; Gibco-BRL) which contains the ampicillin resistance marker and origin of replication. All sites for the indicated restriction sites are shown; the size of the plasmid is 6434 bp.

transfection, we determined the EC_{50} for the pXG-BSD transfectants against G418, hygromycin B, puromycin, nourseothricin and phleomycin (Table 1). For most drugs, the EC_{50} s were identical to that of wild-type. For nourseothricin, a 50% increase in the EC_{50} was seen for both the *L. donovani* and *L. major* pXG-BSD transfectants (Table 1). Practically, such a

low level of cross-resistance can be overcome by adjusting the concentration of selective drugs, as shown previously with the *PAC* marker and the low level of cross-resistance seen with G418 and hygromycin [6]. Lastly, we showed that *L. donovani* transfected with a plasmid expressing the *NEO* marker (pXG1a) were as sensitive to blasticidin S as the wild type, with an EC_{50} of $2 \mu\text{g ml}^{-1}$.

In summary, these data establish the suitability of the *BSD* gene as a dominant selectable marker for *Leishmania*. As noted above, the EC_{50} and plating characteristics with blasticidin S varies amongst different *Leishmania* strains and species, making it necessary to determine the appropriate concentrations of selective drug in culture media and platings empirically, as shown previously with other markers [18]. Since *BSD*-mediated blasticidin S resistance is independent from other selective agents used in *Leishmania* transfections, the *BSD* marker can be used in conjunction with the markers encoding resistance to these compounds. Given the success of the blasticidin marker in *Leishmania* and other eukaryotes, it seems likely that it may find success in transfections of other protozoa. Recently, *BSD* has been utilized as an independent marker for chromosomal gene replacement in both *Leishmania* and trypanosomes [19]. The availability of a new marker will thus expand our ability to create genetically modified parasites.

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