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Stable expression of a GFP-BSD fusion protein in *Babesia bovis* merozoites ☆

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ABSTRACT

Transfection has been a valuable technique for elucidating gene function in many pathogens. While transient transfection of *Babesia* spp. has been reported previously, stable integration of exogenous genes in *Babesia* has proven difficult. In this study, a plasmid was designed to target integration of a *gfp-bsd* gene into the *Babesia bovis ef-1α* locus. *Babesia bovis*-infected erythrocytes of the biologically cloned Mo7 strain were transfected by electroporation with either circular or linear plasmids and selected in cultures with varying amounts of blasticidin 24 h after electroporation. Several blasticidin-resistant *B. bovis* transfected cell lines emerged at different rates, ranging from 5 to 26 days after the start of selection. One transfected parasite line (1-2-124) was selected for further analysis based on a rapid growth rate and bright GFP fluorescence in the presence of a lethal concentration of blasticidin. Continued expression of the *gfp-bsd* fusion gene was confirmed by reverse transcriptase-PCR, Western blot analysis and fluorescence microscopy for longer than 9 months after electroporation. No plasmid or episomal DNA could be detected in this line, and plasmid recovery in *Escherichia coli* was unsuccessful. Southern blot results and sequencing of PCR amplicons flanking the putative insertion site are consistent with integration of at least one *gfp-bsd* cassette into the targeted *ef-1α* locus in the transfected parasite line. Overall the results demonstrate, we believe for the first time, chromosomal integration and stable expression of a foreign gene in *B. bovis*. With the availability of the *B. bovis* genome, targeted stable transfection will provide a means to determine the role of specific genes in the biology, clinical disease and immunity of *B. bovis*, one of the three major tick-borne parasites that limit global livestock production.

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1. Introduction

Babesia bovis is a tick-borne apicomplexan parasite that causes an acute, often fatal, disease in cattle resulting in significant economic losses worldwide. Recovery from clinical disease results in a persistent infection and infected cattle serve as a continuous source for transmission through vector ticks. Genetically defined attenuated and effective inactivated or subunit vaccines are a major goal. With the recent availability of the *B. bovis* genome sequence (Brayton et al., 2007), the ability to stably transform *B. bovis* parasites would provide a valuable means to better understand the biology of the parasite and to investigate parasite-associated determinants of virulence, tick transmission and immunity. While several studies have demonstrated transient expression of foreign genes in of *B. bovis* after transfection by electroporation or nucleofection (Suarez et al., 2004, 2006; Suarez and McElwain, 2008), stable transformation of *B. bovis* has been difficult to achieve.

In addition to finding effective techniques for delivering plasmid DNA inside the parasite nucleus, a stable transformation system requires a construct containing an optimal combination of signals for expression, one or more selectable markers and target insertion sequences whose disruption does not lead to a loss of viability (van Dijk et al., 1996; Ménard and Janse, 1997; Waterkeyn et al., 1999; de Koning-Ward et al., 2000a,b; Gardiner et al., 2003; Carvalho and Ménard, 2005; Janse et al., 2006). Ideally, promoters used in transfection constructs should be able to drive high level expression of selection genes. We previously tested *B. bovis* regions flanking the rhoptry associated protein-1 and elongation factor-*1α* (*ef-1α*) genes for their ability to promote gene expression after transient transfection. Consistent with observations in related parasites, the strongest *B. bovis* promoter tested thus far drives *ef-1α* transcription (Fernandez-Becerra et al., 2003; Suarez et al., 2006). In all constructs used previously for *B. bovis* transient transfection studies, selection has relied on resistance to pyrimethamine or WR99210 conferred by the *hdhfr* gene. This gene has been successfully used in transfection of *Plasmodium* (Fidock and Wellems, 1997; de Koning-Ward et al., 2000a). However, it has been shown that selection in *B. bovis* using the *hdhfr* gene leads to the rapid

☆ *Note:* Nucleotide sequence data reported in this paper is available in the GenBank™ database under the Accession No. EU880350.

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evolution of parasites with natural resistance to the drug (Suarez et al., unpublished data; Gaffar et al., 2004), likely due to the presence in wild type *B. bovis* dihydrofolate reductase (DHFR) of all but one amino acid associated with drug resistance in *Plasmodium*. In *Plasmodium*, selection using blasticidin/blasticidin-deaminase (BSD) has also been effective (Mamoun et al., 1999; Wang et al., 2002). Unlike selection with WR99210 or pyrimethamine, which relies on the *dhfr* gene, there are no *bsd*-related genes in the genome of apicomplexan parasites, including *B. bovis* (Brayton et al., 2007). Thus, in *B. bovis* blasticidin selection would have an advantage over pyrimethamine or WR99210. Finally, the feasibility of targeting one of the *ef-1α* genes in malaria parasites as a site of integration without greatly affecting parasite viability has been demonstrated (Janse et al., 2003). The *B. bovis* genome sequence has confirmed preliminary studies that, similar to *Plasmodium*, the *ef-1α* locus contains two identical gene copies separated by a ~1 kb regulatory region (Vinkenoog et al., 1998; Suarez et al., 2006). The structural and putative functional conservation of this locus suggests it may also be a good target for exogenous gene insertion in *B. bovis*.

Thus, in this study, we investigated whether stable transfection of *B. bovis* could be achieved using *bsd* as a selectable marker under the control of the *ef-1α* promoter, with *ef-1α* open reading frame (ORF) flanking regions targeting integration into the *ef-1α* locus.

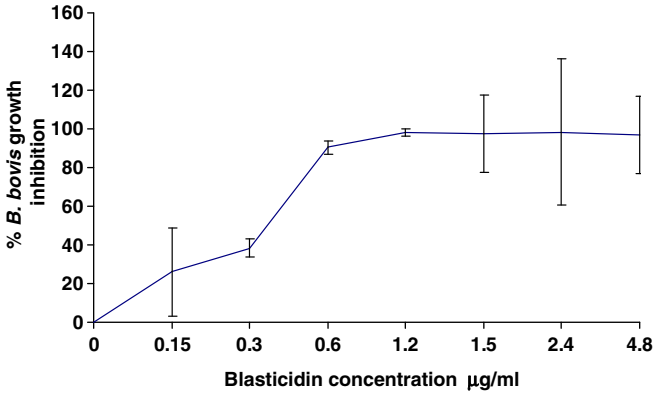

Fig. 1. Growth inhibition of *Babesia bovis* with blasticidin. Error bars indicate mean and SD percentage inhibition obtained from triplicate cultures compared with mean percentage of parasitised erythrocytes (ppe) of control cultures without blasticidin.

2. Materials and methods

2.1. Parasites

The Mo7 biological clone of *B. bovis* was derived by limiting dilution of the Mexico strain as previously described (Rodriguez et al., 1983; Hines et al., 1989) and was maintained as a cryopreserved stabilate in liquid nitrogen (Palmer et al., 1982). Parasites were grown in long term microaerophilous stationary-phase culture using previously described techniques (Levy and Ristic, 1980; Hines et al., 1989).

2.2. Blasticidin inhibition

Babesia bovis parasites of the Mo7 strain were cultured in ~1 ml cultures in 24-well plates containing 10% bovine red blood cells. Triplicate wells were cultured with or without increasing amounts of blasticidin added to the culture media at 0.15, 0.3, 0.6, 1.2, 1.5, 2.4 and 4.8 µg/ml, prepared from a 5 mg/ml stock solution of blasticidin (Invitrogen) in sterile water, for 3 consecutive days. Culture media was replaced daily. The percentage of parasitised erythrocytes (ppe) was estimated by microscopic counting of Diff-Quik (Dade Behring) stained slides as described (Suarez and McElwain, 2008). To calculate the percentage of *B. bovis* growth inhibition we used the following formula:

$$[\text{Mean ppe in control wells} - \text{mean ppe in blasticidin treated wells}/\text{mean ppe in control wells}] \times 100.$$

2.3. Plasmid constructs

Plasmid *pgfp-bsd-ef* (see Fig. 2 for diagrammatic representation) was derived from previously described plasmid *p40-15-luc* (Suarez et al., 2006). The first 673 bps of the 5′ region of the *B. bovis ef-1α* orf were amplified from *B. bovis* Mo7 genomic DNA using primers *Xho-EF-orf-F1* (5′-ctg acg ctc gag atg ccg aag gag aag act cac-3′) and *Xho-EF-orf-R1* (5′-cag ctg ctc gag atc tga tca agg gcc tcg acc-3′). The

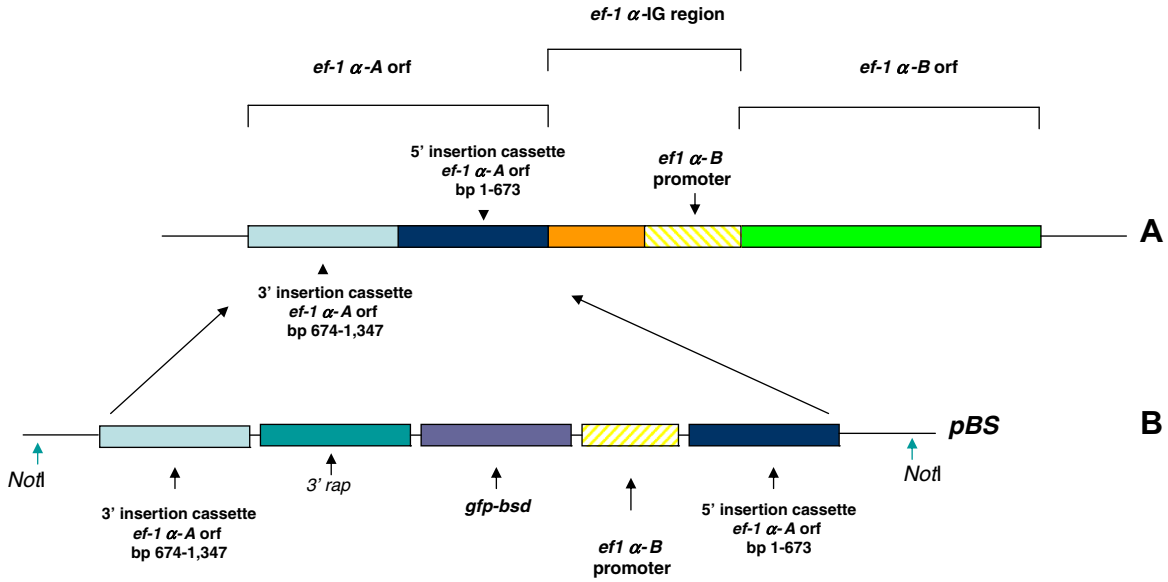

Fig. 2. Structure of the *ef-1α* locus of *Babesia bovis* (A) and plasmid *pgfp-bsd-ef* (B). The restriction sites used for linearisation (NotI) are indicated with arrows. 3′ rap: flanking region and control elements of the *B. bovis rhoptry associated protein-1 (rap-1)* gene.